PHYSIOLOGICAL STUDIES AND TRIAL APPLICATION OF ANTI-FUNGAL ANTIBIOTICS FOR THE CONTROL OF PLANT DISEASES

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INTRODUCTION

Introduction of in vitro section.

During the past decade extensive efforts have been directed toward the development of antibacterial compounds to control human and other animal diseases. Until very recently little attention has been directed toward the development of a good antifungal substance from an antibiotic source to control plant diseases due to fungi. The same situation is true for human diseases caused by fungi.

Recently Anderson and Gottlieb (2) published a survey of the literature concerning antibiotics that may be of value in controlling plant diseases. Last year, Klomparens and Vaughn* reported the results of their studies on the effects of three antibiotics, actidione, endomycin, and streptomycin, on Bonnie Best Tomato variety inoculated with Agrobacterium tumefaciens. Actidione in the soil did reduce the amount of infectivity of the bacteria although in all cases gall formation continued to develop. The effect of fungicidal activity with different carbon sources, dextrose, lactose and maltose, was found to vary when Fusarium lycopersici was grown in liquid medium. Actidione was found to be more fungicidal when the medium contained lactose as the carbon source.

During the current year's studies of antifungal substances active against plant pathogens, there are two distinct phases--in vitro and in vivo studies. In the first phase, a number of antibiotics were tested in agar and liquid media to determine the effectiveness of the compound

* Unpublished report on "Studies of the properties of antibiotics--translocation and physiological studies with cycloheximide (Acti-dione), endomycin, and streptomycin sulfate." submitted for the grant from the Horace H. Rackham Research Endowment in 1953, Department of Botany and Plant Pathology, Michigan State College.

against the test organisms. The stability of the compounds were also tested under varied environmental conditions. After these preliminary procedures were done, physiological studies were undertaken to determine what effect the antifungal substance has under various controlled conditions.

Introduction of in vivo section

The testing of antibiotic materials of possible antifungal activity increases in importance as new and perhaps better materials are dicovered. To date actidione is the only antibiotic material applied to plants for the control of fungal plant diseases on a commercial basis. There are many other antibiotics which are currently being investigated for their commercial applicability against fungal plant diseases. Sometimes these compounds are specific against the causal agent of a plant disease which is poorly controlled. However, in vivo tests must be made to determine the interaction of pathogen, host, environment, and antibiotic. Surface and systemic applications are run against the different types of plant parasites. Both protective and eradicant foliar applications are carried out.

The use of antibiotic materials in plant protection programs raises important questions. In addition to tests on the activity, stability, availability, and sticking qualities, tests must be made to determine host reaction to one or more applications of the antibiotic material. Another important question is whether these materials are fungistatic or fungicidal. Any fungal pathogen used in connection with these antifungal antibiotics might produce lines resistant to these materials. In one hundred years of spraying and dusting with the wide spectrum organic and inorganic fungicides, no resistant fungal lines have arisen. Therefore, checks must be made to determine if and how fast such lines might appear if antibiotics are used. Such antibiotic resistant organisms are already known in bacterial pathogens of animals.

Because of the nature of in vivo tests in the greenhouse, certain limitations of space, suitable hosts, pathogens, and time exist.

Therefore, hosts such as common garden vegetables and small grain plants are used; and the test organisms are those which cause foliar disorders. However, as new tests are made, more organisms are tested since some antibiotics are specific in their action against certain fungi.

Not all antibiotics which appeared desirable could be used. Some of the compounds were not available in quantities large enough to allow spray tests to be made. Others were discontinued and were no longer available. Certain antibiotics were not soluble in water or dilute ethyl alcohol. Therefore, unless they could be suspended or supplied as a soluble salt, they were not used.

The in vivo section of this report shows the progress which has been made in one year. The data presented is not conclusive, but rather indicates some very interesting lines of work to be investigated. Among these problems are the tocicity tests on some of the dicots and the investigation of lines of causal agents after they were sprayed with antibiotics. With more information on the in vitro stability and temperature effects, further refinements and interpretations may be made of some of the spraying results. Eradicant properties and run off of spray materials need additional study.

In Vitro Literature Review

An extensive literature review was conducted up to September, 1953. No published information was available concerning the possible role that nutritive substances might have in affecting the efficiency of antifungal compounds. Klomparens and Vaughn* found that acti-dione had increased antifungal activity when Fusarium lycopersici was grown in liquid medium containing lactose as the carbohydrate source. In addition, they also observed the same to be the case for endomycin when maltose was the carbohydrate source. Environmental factors other than nutrition may influence the activity rate of an antibiotic. Leben (26) in 1954 found that pH alone plays a part in retarding phytopathogenic fungi when acidic buffers are employed in in vitro tests.

Hillborn (24) found Rhizoctonia and Verticillium infections in tomato plants to be considerably reduced by using rimocidin or thiolutin. Davisson et al (12) reported that rimocidin inhibits many of the human pathogenic fungi in vitro at concentrations of 1 to 5 ppm.

Candicidin, a new antibiotic discovered by Lechevalier and his co-workers (31), is reported by them to have an activity spectrum against yeasts but it is only poorly active against yeasts but it is only poorly active against the filamentous fungi and no activity against the bacteria. The authors report this compound to be very soluble in water but it is thermolabile and shows a marked loss of potency after being kept in an aqueous solution for 24 hours at room temperature. The minimal inhibition concentrations for the antibiotic M4575, nystatin and pyridinethione are 6-12 ppm, 6-12 ppm, and 0.3-1.0 ppm respectively for many fungi**. Diaphine hydrochloride is effective against many of the human pathogenic fungi in vitro at concentrations of 100 - 1000 ppm.***

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*Unpublished report on "Studies of the properties of antibiotics - translocation and physiological studies with cycloheximide (Acti-dione), endomycin, and

streptomycin sulfate", submitted for the grant from the Horace H. Rackham Research Endowment in 1953, Department of Botany and Plant Pathology, Michigan State College.

**Information kindly furnished by E. R. Squibb and Sons.
***Information kindly supplied by Chas. Pfizer and Co.

Literature review of in vivo section

The vast majority of antibiotics are derived from bacterial and fungal activity (4,8,9,27,30,43). Other antibiotics are derived from various sources, but these as a group are not important in plant disease control programs (4,5,6,15,35,37,40). In the control of plant diseases by antibiotics, four general methods of application are used. These are: soil, systemic, seed treatment, and foliar applications.

In soil applications, antibiotic materials are either applied to the soil as a drench or mixed in as a dust. However, for most soils, the diverse microflora and colloidal complexes such as clays and soil organic matter absorb or effectively inactivate most antibiotics that have been tested (16,18,36,42). Numerous strains of antibiotic producing fungi and bacteria were added to soils, but these have proven to be ineffective in plant disease control (16,38).

Antibiotics are also tested as systemic fungicides or chemotherapeutants (8,10,13,20,25,44). A chemotherapeutant is defined as a substance which is translocated throughout a plant to combat the disease organism from within. Davis and Dimond, (11), state that there is no consistant relation between fungitoxicity and chemotherapeutic activity. Thus, an antibiotic may have excellent antifungal properties but little or no chemotherapeutic activity. This makes it necessary to screen antibiotics for both fungistatic and chemotherapeutic activity. Although chemotherapeutants hold promise in future disease control programs, they are not widely used to date.

Antibiotics are tested as seed treatment compounds. Results demonstrate that field control of plant diseases caused by seed-borne fungi is possible through the use of antibiotics (22,29). Helixin was included

in the United States Department of Agriculture iniform seed treatment nursery on oats and spring wheat in 1953 (32,45).

Many antibiotics are screened for use as foliar sprays. Several fungal diseases, which formerly were poorly controlled or not controlled at all, are now controlled by antibiotics. Actidione at 2 ppm applied as a foliage spray controls cherry leaf spot (21,39). This antibiotic is very widely used against a number of turf diseases such as dollar spot, pink patch, fading out, copper spot, and brown patch (45). Powdery mildew on brambles is also controlled by spraying with actidione at a concentration of 2-5 ppm (47). Actidione gives both a protectant and an erradicant action against many plant pathogens (17,21,39), but may cause severe plant injury on susceptible hosts and tissues. Helixin controls early blight of tomato, and also proved rather effective as a small grain seed treatment compound (28,29,32). Although this compound shows high activity against certain fungi, it is not being produced commercially. Helixin has been found to contain several different active fractions. Griseofulvin shows promise of controlling early blight in England (41).

Some plant diseases caused by bacteria, such as fire blight of pome fruits, are now controlled by the applications of terramycin and streptomycin (1,23,46). Streptomycin is used at several different concentrations, and critical work needs to be done to check this point.

The reaction of the antibiotic sprays is important, and antifungal activity of a spray material is increased by lowering the pH. However, Leben (26) in work with early blight of tomato showed that this was not increased antifungal activity on the part of the antibiotic, but rather the result of the lowered pH alone. He reports that from 53 to 79 percent

control of early blight was obtained by using acid buffers alone.

Most of the tests with the various antibiotics and organisms to date are *in vitro* and relatively few are *in vivo* tests.

METHODS AND RESULTS IN VITRO

A. Assay testing of antifungal materials

Methods

Several compounds were screened to determine their minimal inhibition concentrations. The following is a list of the compounds assayed and the methods for preparing the stock solutions:

*Sodium pyridinethione (MC2113): very soluble in water.

*Nystatin: soluble in acid methanol.

*M4575: raise pH of suspension to pH 11 with NaOH, solution then occurs.

Back titrate immediately with acid.

Rimocidin sulfate: soluble in water. Stock solutions of 1 mg/ml will be slightly cloudy.

Thiolutin: sparingly soluble in water, in magnitude of 50ppm. Solubility can be increased to 150ppm by using 70% methanol.

Diaphine HCL: very soluble in water.

Candicidin A: very soluble in water (31).

With the exception of Diaphine and pyridinethione, the compounds listed above are all antibiotics. For the initial screenings, such high concentrations were employed, that sterilization of the test fungicides weren't deemed necessary. It is recommended however, that the experimental compounds be sterilized via Seitz filtration to prevent bacterial infection of the cultures. This procedure is followed until thermostability tests are completed in order to determine if some of the compounds can be sterilized by autoclaving. In all cases unless otherwise indicated, the test solutions were freshly prepared prior to use. Stability tests are discussed in another portion of this report.

The following fungi were used as test organisms in the physiological studies:

Collectotrichum phomoides C80A: isolated from tomato fruits.

Colletotrichum phomoides 101: isolated from tomato fruits.

Helminthosporium sativum 925: isolated from barley in the thumb area, Michigan.

Lilly-Barnett synthetic glucose asparagine medium (33) was employed as a basis for physiological studies. The following constituents were of C.P. grade:

D-glucose	10 gm
L-asparagine	2 gm
$MgSO_4.7H_2O$	0.5 gm
KH_2PO_4	1.0 gm
Fe^{+++}	0.02 mg
Zn^{++}	0.02 mg
Mn^{++}	0.01 mg
thiamine	100 ug
biotin	5 ug

pH to 6.0 add distilled H_2O to make one liter.

In some instances agar was added to the medium at a concentration of 2% in order to have both solid and liquid media for the assays. The medium was autoclaved at 15 lbs. pressure and at 240°F. for 20 minutes.

In the beginning, mycelial inoculum was used. This was accomplished by placing a small portion of mycelium from the desired organism in the center of a petri plate to which the medium and the test compound had previously been added. Currently however, spore suspensions prepared in

sterile water are used as inocula. The spore suspensions are prepared from heavily sporulating cultures. The spore suspensions are adjusted so that a concentration of 400-500 spores per ml of medium will result when 1 ml is used as inoculum for 20 ml of medium. Spore counts were determined with the aid of the Levy Hemacytometer.

Aseptic techniques are maintained throughout the procedure. One ml of inoculum is pipetted into a petri plate followed by the medium and then by the test compound. The latter is serially diluted so as the amount used per 20 ml total volume in the petri plate gives the desired concentration for the test. Each plate is gently swirled after the addition of each constituent. All assay plates and controls were run in duplicate and incubated at room temperature.

Results

The results as presented in Table I indicate that pyridinethione and Rimocidin are most effective against the organisms tested. The minimal inhibition concentration for C. phomoides C80A, C. phomoides 101, and H. sativum 925 using pyridinethione was less than 1 ppm and with Rimocidin, less than 10 ppm. It was on this basis that the aforementioned compounds were selected for pysiological studies. In some cases, duplicate runs were also made on agar. For the most part, the results were the same as in liquid. The data in Table II indicate that agar may tie up the compound in some cases so that a higher concentration than the liquid assay is required to inhibit the organism. When nystatin is used, the minimal inhibition concentration against

TABLE I

Activities of antifungal agents
Minimal inactivation concentration in ppm in liquid

Compound	C phomoides C80A	C. phomoides 10I	H. sativum 925
Pyridinethione	<1.0	<1.0	<0.1
Nystatin	>10.0	50.0	>50.0
M4575	>200.0	>50.0	>200.0
Rimocidin	<10.0	<10.0	<10.0
Thiolutin	>15.0	>15.0	>15.0
Diaphine	-	100.0-200.0	50.0
Candicidin A	-	200.0	200.0

TABLE II

Growth comparison on agar and liquid media

Fungicide	Organism	Conc. of fungicide in agar in ppm				Conc. of fungicide in liquid in ppm			
		10	50	100	200	10	50	100	200
Nystatin	C phomoides C80A	++	+	±	-	++	-	-	-
	C phomoides 10I	++	+	+	-	++	±	-	-
	H. sativum 925	+++	-	-	-	+++	+	-	-
Rimocidin	C. phomoides C80A	+	-	-	-	±	-	-	-
	C. phomoides 10I	+	-	-	-	±	-	-	-
	H. sativum 925	-	-	-	-	-	-	-	-

C. phomoides C80A on agar is 100 ppm whereas in liquid, it is 10-50 ppm. Plates (I, II, IV) show concentration effects on C. phomoides and H. sativum when fungicidal compounds were put in both liquid and agar media.

B. Stability tests on pyridinethione, rimocidin and diaphine

Methods

Stability tests were made on the above listed compounds with regard to thermostability and "shelf life" of the material in solution under varying conditions. In order to determine whether or not the fungicides could be autoclaved together with the medium, a series was set up for comparison of the minimal inhibition concentration of fungicide sterilized by Seitz filtration with those of the fungicide autoclaved together with the media. In another series, the "shelf life" of two compounds, pyridinethione and rimocidin, were investigated by placing 10 ppm of the Seitz sterilized materials in either a water solution or combined with the liquid medium at 5°C., room temperature, and at 30°C. The 30°C tests were made in shake flasks on the Kershaw Rotary Shaker (see plate III) in order to duplicate conditions for the subsequent physiological studies and to find out if there might be a loss of potency during an incubation run.

Results

The data in Table III show a definite potency loss when pyridinethione, rimocidin or diaphine are autoclaved with the medium. This might be due to a breakdown of the compounds. Plate IV is a typical example,

depicting potency loss when diaphine was autoclaved together with the medium. In the case of pyridinethione, the media darkened upon autoclaving. The other compounds screened were not tested for stability as the minimal inhibition concentrations were too high and did not merit further consideration at this time.

The synthetic compound pyridinethione shows complete "shelf life" stability under the conditions tested, whereas the antibiotic rimocidin holds up only under refrigeration. In addition, it is of interest to report here that an aqueous solution of pyridinethione showed no potency loss after being refrigerated for 10 weeks. The "shelf life" results are for 15 days and are being carried further but the information will not be ready in time for this report.

TABLE III

Thermostability of 2 Fungicides
Minimal Inhibition Concentration in ppm

Compound	Sterilized by Seitz Filtration		Sterilized by Autoclaving With The Media *	
	C. phomoides C80A	H. sativum 925	C. phomoides C80A	H. sativum 925
Pyridinethione	1.0	0.1	10.0	5-10
Rimocidin	10.0	10.0	15.0	15.0
Diaphine	100-200	50	200	200

* 15 lbs. pressure and 240 degrees F. for 20 minutes.

C. Physiological studies with colletotrichum phomoides C80A

1. Determination of harvest time optimum

Methods

The organisms C. phomoides C80A, C. phomoides 101 and H. sativum 925 were grown in liquid medium to ascertain the optimum harvest time. The standard medium used is the Lilly-Barnett synthetic glucose-asparagine medium. Fifty ml of medium were dispensed into 250 ml erlenmeyer flasks which was then plugged with cotton and autoclaved 20 minutes. The inoculum was prepared from potato dextrose slants containing a 3 to 4 weeks old organism. A spore suspension was prepared in sterile distilled water and diluted so that 1 ml of inoculum per flask yielded a concentration of 6-7,000 spores /ml medium. Spore counts were made with the aid of a Levy Hemacytometer. Cultures prepared in triplicate were incubated in a stationary flask series as well as a shake flask series. The latter series was on a Kershaw Rotary Shaker at 140 rpm. All cultures were maintained at 28-30°C. in the dark. At harvest time, the best two out of three flasks were processed. The mold was filtered via suction on a Buchner funnel through a nylon filter. The mycelium was then washed three times with distilled water to remove any adhering nutrient substances after which it was placed in a numbered tared aluminum weighing cup and dried overnight at 60°C. The cups with the dried mucelium were placed in a dessicator until weighed.

Results

As indicated in Table IV, it appears that the optimum harvest time for shake cultures is 4 days after which time autolysis seems to set in. The stationary cultures were harvested at a 9 day optimum. Although

maximum growth was not achieved at the end of 9 days, this time period was chosen for the sake of expediency. The typical growth pattern in stationary cultures is a mat-like mass with a sub-surface gelatinous consistency. On the other hand, shake cultures produce a pellet and glob-like growth.

TABLE IV

Growth Pattern
On Lilly-Barnett Synthetic Glucose-Asparagine Media
28-30°C. in the dark
recorded as mg dry mold/50 ml medium

Organism	4 Days	6 Days	9 Days
C. phomoides C80A			
Shake	198	173	154
Stationary	154	155	176
C. phomoides 10I			
Shake	200	189	146
Stationary	76	171	177
H. sativum 925			
Shake	134	120	200
Stationary	98	148	204

2. Carbohydrate variation effect on fungicidal activity.

Methods

Four different carbohydrate sources were investigated separately in conjunction with varying concentrations of fungicides to determine how nutritive environmental conditions might influence fungicidal activity towards a pathogenic fungus. The basal medium used was Lilly-Barnett synthetic glucose-asparagine medium complete with the exception of the carbon source.* The standard medium contains glucose equivalent to

* It should be noted that the sugars employed did not supply the only carbon in the medium but that the L-asparagine might also enter the carbohydrate metabolic cycle. It was observed that the standard medium minus the carbohydrate (glucose) supported limited growth when tested on agar slants. It is therefore advisable that an inorganic nitrogen source be utilized. It has been found that C. phomoides and H. sativum grow well on the standard media modified to contain sodium nitrate in place of the asparagine.

4 gm of carbon per liter; hence the following sugars were used in the quantities specified so as to give 4 gm of carbon per liter: D-glucose 10 gm, sucrose 9.5 gm, D-xylose 10 gm and alpha-lactose H_2O 10 gm. The organism employed for this study was C. phomoides C80A. Runs were made under shake and stationary conditions according to the specifications set up under the methods of the previous section. The fungicides selected for these studies on the basis of greatest activity in the initial screenings were pyridinethione and rimocidin.

Results

The compound pyridinethione caused inhibition on shake and stationary cultures at less than 1 ppm when D-glucose, sucrose and alpha-lactose were used. In the case of D-xylose, inhibition was between 1 and 3 ppm. (See plate V.) No mycelium or at best very scanty amounts were obtainable under the range of fingicidal concentrations set up for these studies (1, 3, 6 and 10 ppm). Consequently, tabulated data on dry mold weights are lacking for this report.

The results with rimocidin are tabulated in Tables V and VI wherein varying amounts of the antibiotic were used in conjunction with four different sugars; the pentose D-xylose, hexose D-glucose and the disaccharides sucrose and alpa-lactose. In addition, the data also include results under both stationary and shake conditions. The pH readings of the filtrates made at the time of harvest are included in the above mentioned tables.

Sucrose appears to render the fungus more susceptible to the rimocidin at lower concentrations whereas the antibiotic is effective only at higher

concentrations (10 ppm) when D-xylose or alpha-lactose is used as the carbohydrate source. The data of Tables V and VI have been plotted on the following graphs:

Graph I Varying amounts of rimocidin in combination with different carbohydrate sources - shake cultures

Graph II Varying amounts of rimocidin in combination with different carbohydrate sources - stationary cultures

Graph III Disparity for Xylose and Glucose under stationary conditions when rimocidin reaches critical point.

Referring to the plotted curves the following observations can be made:

1. In graph I, the antibiotic activity rate is markedly increased when using D-glucose or sucrose in comparison to D-xylose or alpha-lactose.

2. The curves do not drop as sharply in the stationary cultures as compared to the shake cultures.

3. Xylose in stationary culture seems to show a stimulatory effect when rimocidin is present up to 3 ppm. In addition, the xylose control in the stationary culture has a dry mold weight of 173 mg in 11 days as compared to 85 mg in 6 days for the shake culture. C. phomoides C80A is not inhibited at 10 ppm rimocidin when grown in xylose under stationary conditions.

4. Graph III indicates that there is a disparity for xylose and glucose when the antibiotic approaches the critical range of activation. This is indicated by the dotted lines, the fact that no replicates agree. In the case of glucose, there seems to be a tendency for the divergent lines to rejoin again at higher antibiotic concentrations. This is not evident for xylose as apparently the concentrations used were not high enough.

It is interesting to note that rimocidin is more effective in inhibiting C. phomoides C80A when it is incorporated into sucrose medium than when in glucose medium. Shake flask cultures on these two sugars at 6 and 10 ppm of the antibiotic were maintained over a 5 week period. From the data presented in Table VII, it is apparent that when the organism

was grown in a glucose medium, growth started at 9 days when the concentration or rimocidin was 6 ppm and at 15 days when at 10 ppm. In the sucrose media, highly retarded growth was apparent in 27 days at 6 ppm and total inhibition was still maintained at 10 ppm at the end of 38 days at which time the experiment was terminated.

TABLE V

Dry mycelial weight of Colletotrichum phomoides C80A grown in liquid medium containing varying amounts of rimocidin in conjunction with different C sources expressed as mg from the average of the best 2 of 3 replicates. Incubated at 20-30°C; D-glucose, sucrose.

Ppm of Fungicide	Shake 4 days				Stationary 9 days			
	D-glucose		Sucrose		D-glucose		Sucrose	
	mg	pH*	mg	pH	mg	pH	mg	pH
0	211.0	7.5	163.0	7.6	176.0	8.1	184.0	7.6
1	180.0	7.5	160.0	7.0	171.0	8.1	180.0	7.9
3	1.5	6.0	0.0	-	161.0 90.0 } 126**	5.2 6.7	5.0	5.0
6	0.0	-	0.0	-	12.0 7.0 } 10**	6.2 6.1	0.0	-
10	0.0	-	0.0	-	5.0 3.0 } 4	6.1 6.1	0.0	-

TABLE VI

Same conditions as TABLE V except incubated at 30-33°C: D-xylose, and lactose.

Ppm of Fungicide	Shake 6 days				Stationary 11 days			
	D-xylose		Alpha-lactose		D-xylose		Alpha-lactose	
	mg	pH*	mg	pH	mg	pH	mg	pH
0	85.0	5.9	20.0	8.3	172.0	7.5	37.0	8.3
1	51.0	5.5	6.0	7.3	182.0	8.2	37.0	8.4
3	46.0	5.8	7.0	7.7	181.0	8.2	25.0	8.2
6	20.0	5.6	2.0	6.7	113.0 150.0 } 132**	7.2 7.4	11.0	8.0
10	5.0	5.6	1.0	7.8	86.0 41.0 } 64**	7.0 6.4	0.0	-

*pH of filtrate at time of mycelial recovery.

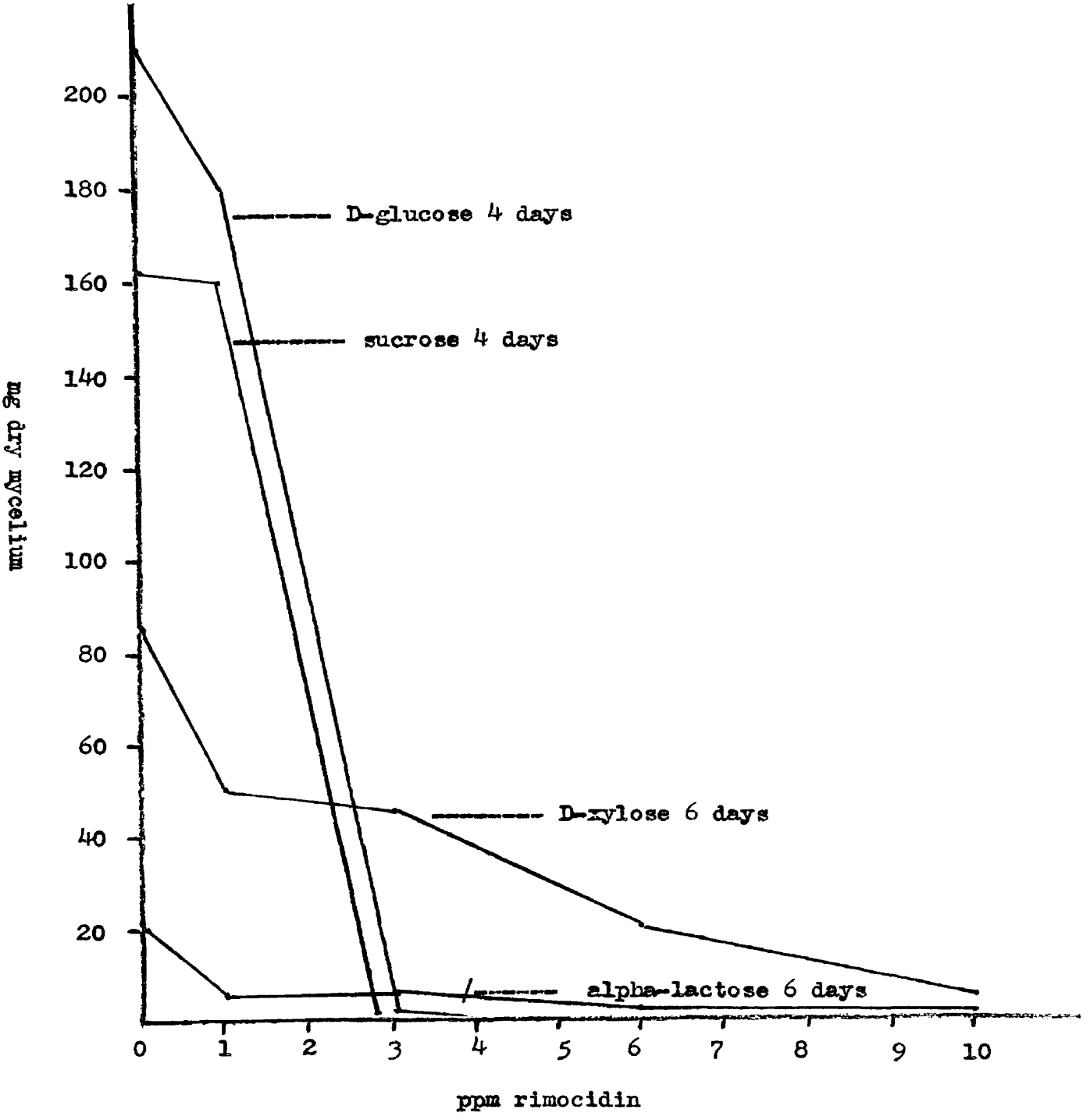
**Greater deviation occurred in these cases when rimocidin was used.

TABLE VII

Protective effect of 2 different sugars incorporated into the liquid media with rimocidin. Shake cultures at 30°C. Recorded as the incubation time required for the appearance of growth.

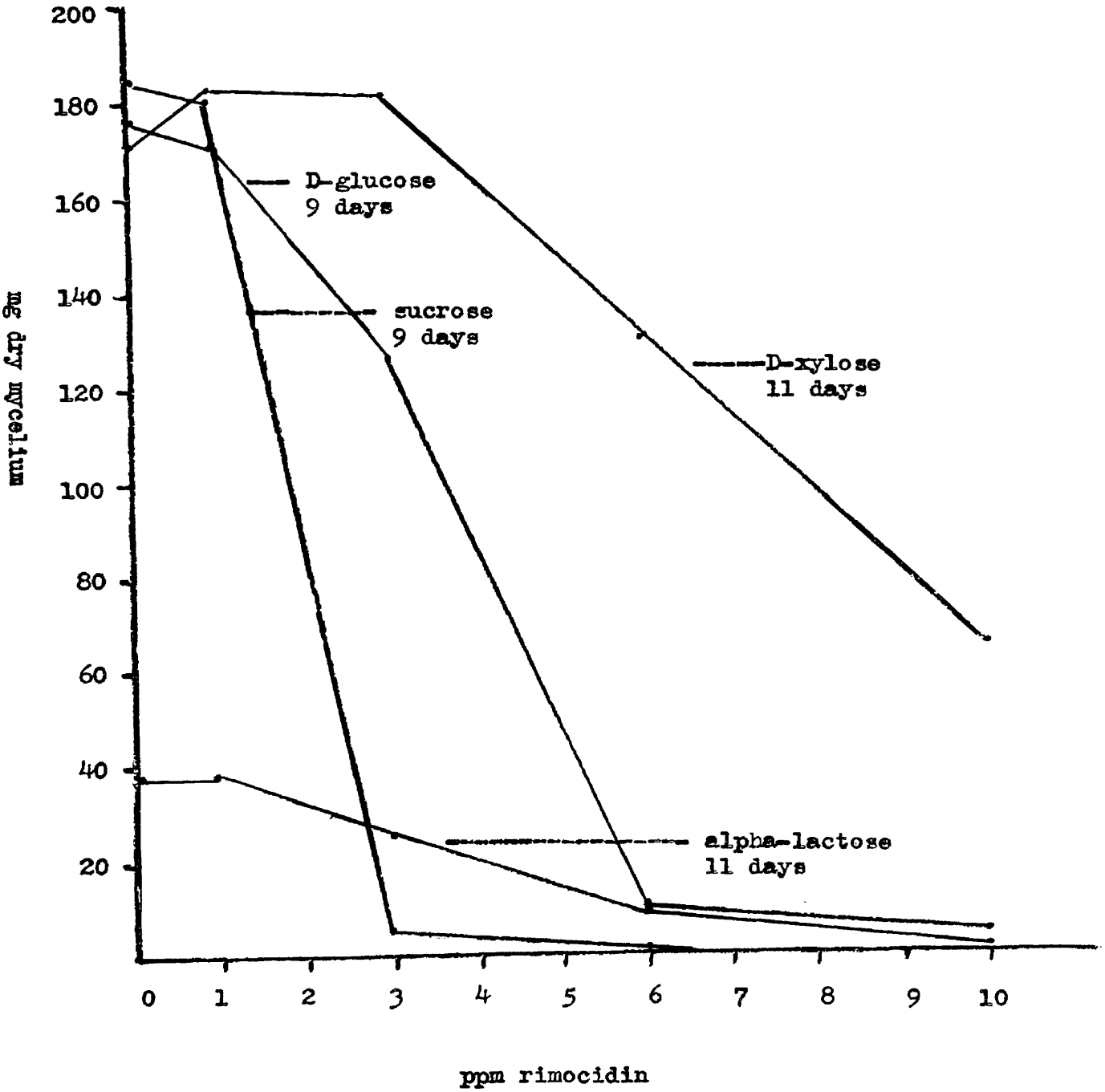
Rimocidin in ppm	Sugar	Incubation in days				
		9	15	22	27	38
6	D-glucose	growth				
10	D-glucose	-	growth			
6	Sucrose	-	-	-	growth	
10	Sucrose	-	-	-	-	-

Shake Culture. Growth of C. phomoides C80A
In Liquid Medium Containing Varying Amounts of Rimocidin Sulfate
With Different Carbohydrate Sources.

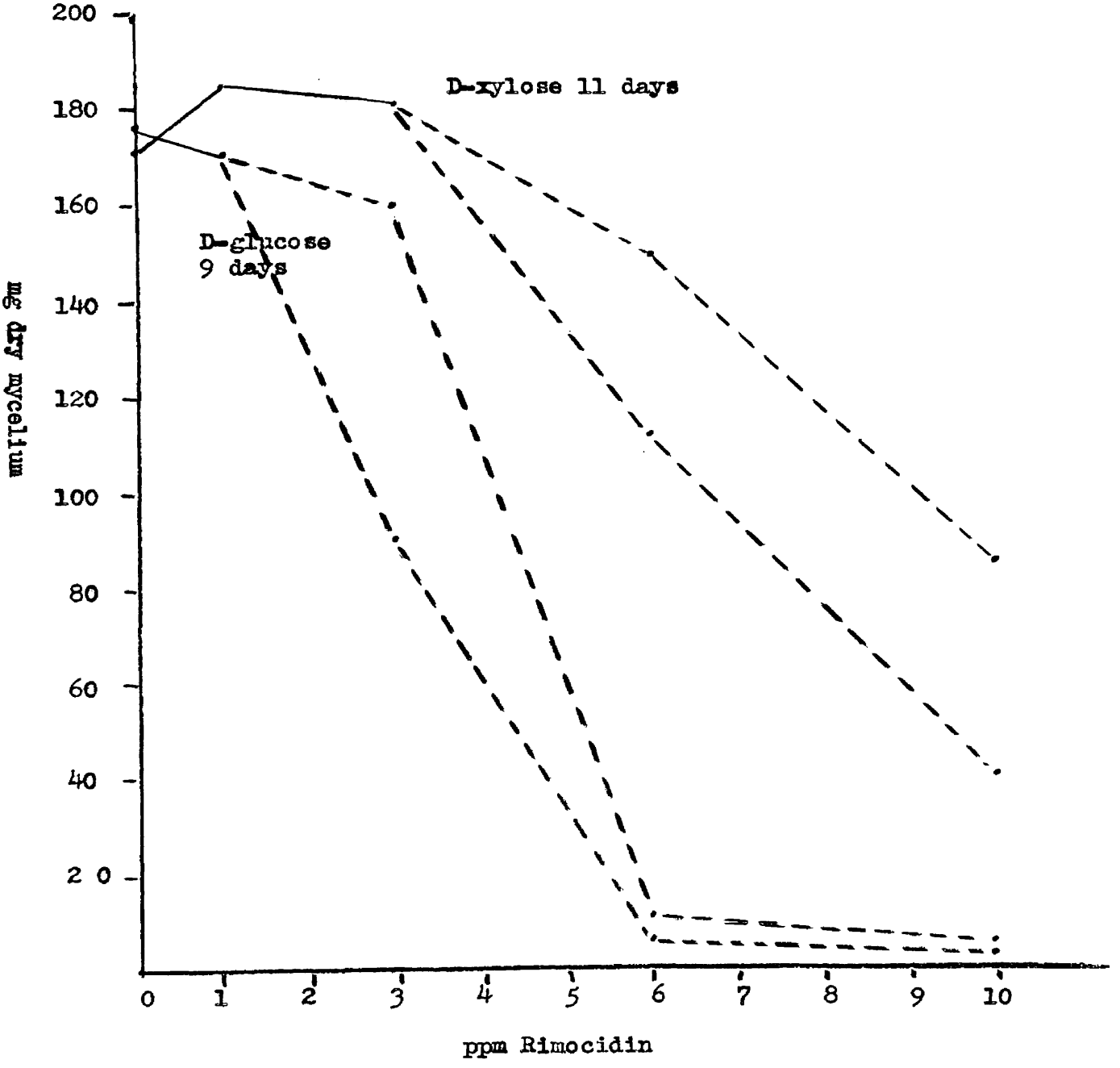


GRAPH II

Stationary Culture. Growth of C. phomoides C80A
In Liquid Medium Containing Varying Amounts of Rimocidin Sulfate
With Different Carbohydrate Sources.



Stationary Culture. Growth of C. phomoides C80A Showing Divergencies Between Replicate Flasks When The Concentration of Rimocidin Sulfate Reaches The Critical Point.



In vivo methods and results

Four antibiotic materials, thiolutin, fungistatin XG, nystatin, and rimocidin sulfate, were tested along with two synthetics, diaphine hydochloride, and sodium pyridinethione. The solubility and stability of thiolutin, rimocidin, diaphine hydrochloride, and sodium pyridinethione are discussed in the in vitro section of this report. Fungistatin XG, supplied by Charles Pfizer and Co., Inc., was tested for antifungal properties in vivo. Further communications with the Pfizer Company revealed that this antibiotic was not in production nor were any plans made for future production. Therefore, nystatin, an antibiotic produced and supplied by E. R. Squibb and Sons, was substituted in the testing program for fungistatin XG. The compound was listed by the company as soluble in methanol, stable at neutral pH, and unstable at pH2 and pH9. A partial spectrum of antifungal activity was furnished which indicated a fair degree of activity at high concentrations in the in vivo tests. Actidione produced by Upjohn Co., and panogen, an organic mercury compound, produced by Panogen Inc., New York, were added later to the testing program as treated checks.

All of the antibiotics were prepared in water solutions or suspensions for application to the test plants. An emulsifying agent, tergitol, was used to keep thiolutin in suspension at concentrations above 50 ppm Nystatin and rimocidin were first disolved in two milliliters of 95 percent methanol and then diluted with distilled water to the desired concentration. Stock solutions of all the materials were prepared at a dilution of 1/1000. Such stock solutions were then stored under refrigeration at 5° F.

Five fungal test organisms were used. These are listed below with the common name of the disease which they cause.

Scientific name of organism	Common name of the disease
Alternaria solani (Ell. and G. Martin) L. R. Jones & Grout	Early blight of tomato
Colletotrichum phomoides (Sacc.) C80A Chester	Anthracnose of tomato
Phoma lingam (Tode es. Fr.) Desm.	Blackleg of crucifers
Helminthosporium sativum (Pam.) King and Bakke	Spot blotch of barley
Puccinia graminis tritici Eriks, and E. Henn.	Stem rust of wheat.

A. solani, P. lingam, and H. sativum are included in several in vitro testing programs, (26,27,28). They were used in these tests because of their ease of application to foliage spray programs as well as their foliar type of infections. C. phomoides on tomato is included in numerous spray testing programs and is used in these tests because of its difficult control, importance as a ripe fruit rot, and as a representative of an important group of fungi attacking plants.

Two monocotyledonous hosts, wheat and barley, and two dicotyledonous hosts, tomato and cabbage, were used in the testing program. In addition to these, corn, beans, cucumber, and tobacco were used in phytotoxicity tests. These species were used to provide a range of host material in testing host reaction to antibiotic applications. Phytotoxicity tests were run for all substances tested at 200 ppm. Diaphine hydrochloride was tested further at 100, 50, and 25 ppm.

The materials were sprayed directly on the foliage by means of air pressure and atomizers or by use of hand spray guns. In the case of cabbage, the wax, or bloom, on the leaves was rubbed off by hand as the sprays were applied. This was done to insure contact between host,

sprays, and inoculum and to reduce run-off. Although some run-off was present on the other plants, by care in application it was not a serious problem.

A single isolate of each of the test organisms, except stem rust, was maintained on potato dextrose agar, and used throughout the experiments. The fungi growing on agar were then ground in a blender, and the suspension was used as inoculum. The rust spores were collected from inoculated hosts which were used to build up the amount of inoculum. The suspension of ground mycelium or spores was sprayed on the plant with atomizers or hand spray guns.

The test plants were first sprayed with the test organism and allowed to dry for several minutes. The antibiotic materials were then applied, and shields of cardboard were used to prevent drift from one test row to another. After the antibiotic materials were applied, the plants were placed in a humidity chamber which was kept moist by a fine spray of water and air under pressure. The plants were removed from the moist chamber after three or four days, and the disease was allowed to develop. Readings were taken when the lesions on the checks appeared to be well developed. In most cases the readings were based on the number of lesions per plant. Where this could not be used, readings were based on the numerical gradiations of 0--5, where 0 indicated no disease symptoms on all plants and 5 represented each plant having heavy infection.

The data presented in this section deals with only two phases of the problem. The first of these is the phytotoxic reactions caused by spraying antibiotic materials on the foliage of the test plants. The second is the actual control of the pathogens by spraying shortly after the host plants were inoculated. In one preliminary run against Sclerotinia sclerotiorum (Lib.)Bdy. on bean the antibiotic materials were applied to the soil as drenches.

The initial phytotoxicity tests were made over a wide range of test plants (Table I) to determine as quickly as possible the host reactions to antibiotic sprays. A concentration of 200 ppm was used as a starting point since it approached the highest concentraion at which control work is generally feasible from a cost standpoint. The monocotyledonous plants as a group were seemingly more tolerant of antibiotic sprays than were the dicotyledonous plants. In no case was any damage noticed on the graminaceous plants under test.

Several types of injury were found on the dicotyledonous plants depending on the plant and the antibiotic used. Thiolutin produced a downward bending of the petioles and bronzing of the leaves of tomato plants but on bean it cuased only a slight mottling. Fungistatin XG caused a downward bending of tomato leaf petioles, leaf crinkle on cabbage, slight leaf puckering on beans, and chlorotic spots on cucumber leaves (Plate VI). Diaphine gave a toxic reaction on all dicotyledonous plants tested (Table I, Plate VI). In most cases this consisted of chlorotic spots or chlorotic leaf margins on the leaves which were immature at the time of application. On cabbage diaphine produced chlorotic rings which were similar in appearance to certain virus symptoms. In most cases, however, the host plant grew out of the damage, and any newgrowth that

appeared was either free of injury or only slightly injured. Rimocidin caused some slight puckering on bean and cucumber leaves, but the damage was not severe. Sodium pyridinothione caused leaf puckering on bean and marginal necrosis on cucumber leaves (Plate VI). Actidione caused necrotic spots on the leaves of most test plants, but such damage is known to occur at higher concentrations of this material. Panogen which is a very volatile organic mercury gave no pnytotoxic reactions on the plants which were sprayed with it. In the only soil application made diaphine was found to cause injury. Again this injury was associated with the leaf which was immature at the time of the application of the material to the soil.

Dilution tests were run using diaphine at 200, 100, 50, and 25 ppm (Table II). Tobacco, cucumber, and beans exhibited chlorosis at all dilutions tested although the chlorosis became less pronounced at the lower concentrations. Cabbage and tomato showed a marked reduction in phytotoxicity at the lower concentrations although some chlorosis was still present.

The results of the spray tests against pathogens varies with host and pathogen. None of the antibiotics tested to date have exhibited high levels of antifungal activity in these in vivo tests. H. sativum, Erysiphe graminis, A. solani, and C. phomoides were all poorly controlled (Tables III, V, VI, VII). In a number of cases slight to rather strong stimulation of infection was found to occur with the use of thiolutin (Table IV). The results for P. lingam are as yet inconclusive with good control occurring in at least one run (Table IV). In the trials with P. graminis tritici (Table VII) tiolutin, diaphine, and sodium pyridinothione gave perfect control while rimocidin and nystatin allowed some lesioning.

In the two trials in which panogen and actidione were tried they were better than any of the other materials. This indicates that the other compounds are not as antifungal as might be desired for a spray compounds.

Table I. Summary of the phytotoxicty tests of several antibiotic materials applied to several different test plants as sprays in concentrations of 200 ppm. 0 no damage---5 severe damage.

Test Plant	Spray Materials				
	Thiolutin	Fungistatin	Diaphine	Rimocidin Na	Pyridinethione
Old tomato	1	1+	2	0	1
Old cabbage	0	1+	0	0	0
Old tobacco	0	0	1	0	0
Bean	1	1	3+	1	-1
Cucumber	0	1+	2+	-1	1
Corn	0	0	0	0	0
Wheat	0	0	0	0	0
Barley	0	0	0	0	0
Young tomato	-1	-1	3	-1	1
Young Cabbage	0	0	3	1	0
		Nystatin			
Barley	0	0	0	0	0
Young tomato	0	0	3	0	0
Wheat	0	0	0	0	0

Table II. Phytotoxicty tests using diaphine hydrochloride at four concentrations.

Test plant	Number of plants out of 8 showing chlorosis at: 200ppm.	100ppm.	50ppm.	25ppm.
Cabbage	8	8	6	2
Tobacco	8	8	8	8
Tomato	8	5	5	4
Beans	8	8	8	8
Cucumber	8	8	8	8

Table III. Results of several antibiotic materials sprayed on plants for the control of plant pathogens.

Pathogen and Host	Disease ratings (0-no infection, 5 severe infection) for the six antibiotic materials tested. Control	Thiolutin	Fungistatin	Diaphine	Rimocidin	Na Pyridinethione
Erysiphe graminis Powdery mildew on barley. Trial #1	1	3	2	2	3	1
Trial #2	3	2	1	3	4	3
Helminthosporium sativum Spot blotch on Barley	4	3	3	4	4	3
Alternaria solani Early blight on tomato	2	2	1	1	3	1
Colletotrichum phomoides Anthracnose on tomato	3	2	-2	2	1	2

Table IV. Results of several antibiotic materials applied to Cabbage for the control of Phoma lingam (blackleg)

Table A

Treatment	Total number of lesions on eight plants.
1. Control	6
2. Thiolutin	18**
3. Fungistatin	18**
4. Diaphine	3
5. Rimocidin	1
6. Na. Pyridinethione	1

*L.S.D. at the .05 level---5.01
**L.S.D. at the .01 level--6.72

Table B

Treatment	Total number of lesions on eight plants.
1. Control	630
2. Tholutin	118**
3. Diaphine	234**
4. Nystatin	93**
5. Rimicidin	82**
6. Na Pyradinethione	34**
7. Panogen	5**
8. Actidione	1**

*L.S.D. at the .05 level----67.50
**L.S.D. at the .01 level----86.20

Table V. Results of several antibiotic materials applied to tomato leaves for the control of Colletotrichum phomoides (anthracnose), C80A.

Treatment	Total number of lesions on eight plants.
1. Control	7
2. Thiolutin	8
3. Diaphine	6
4. Nystatin	5
5. Rimocidin	4
6. Na Pyridinethione	3
7. Panogen	1
8. Actidione	1

Table VI. Results of several antibiotic materials applied to tomato leaves for the control of Alternaria solani (early blight)

Treatment	Total number of lesions on twelve plants.
1. Control	122
2. Thiolutin	131
3. Fungistatin	83
4. Diaphine	105
5. Rimocidin	76
6. Na Pyridinethione	171

Table VII. Results of several antibiotic materials applied to wheat leaves for the control of Puccinia graminis tritici (wheat stem rust).

Treatment	Average number of lesions per plant per treatment
1. Control	5.9
2. Thiolutin	0
3. Nystatin	.3
4. Diaphine	0
5. Rimocidin	.4
6. Na pyridinethione	0

Discussion and conclusions of the in vitro section.

Preliminary screenings were conducted on compounds reported to have anti-fungal properties. Seven anti-fungal compounds were tested against two strains of the tomato fruit anthracnose organism, Colletotrichum phomoides and the leaf spot and root rot organism of wheat, Helminthosporium sativum. Of these, three compounds were found to possess fairly good inhibitory properties. The minimal inhibitory concentration for thiolutin was 15-30 ppm, for rimocidin, less than 10 ppm, and for pyridinethione, 0.1-1.0 ppm. When tested in both agar and liquid media, the concentration of nystatin required for the inhibition of C. phomoides C80A was found to be 100 ppm on agar and 10 ppm in liquid. This data seems to indicate that the antibiotic may be tied up with the agar in such a way that its activity rate is reduced.

Thermostability tests showed the two most promising compounds, pyridinethione and rimocidin, are inactivated in the medium when subjected to high temperatures such as autoclaving. This of course has some depreciation on the value that can be placed on the compounds. So far as solubility is concerned for the active compounds, pyridinethione alone is readily soluble in water. This feature is particularly desirable when undertaking physiological studies. Although rimocidin is not as soluble as pyridinethione, stock concentrations of 1 mg/ml can be prepared by adjusting the pH of the suspension so that almost all of the material will go into solution. As mentioned in the literature review, no published information is available on pyridinethione and very little concerning rimocidin.

The results on the "shelf life" studies again point towards pyridinethione as the compound of choice. By way of comparison, pyridinethione showed complete stability with no potency loss when maintained at temperatures of 5°C., room temperature and at 30°C. in aqueous solution as well as when

combined with the standard nutrient medium for a period of 15 days. Rimocidin on the other hand, retained its full potency only when maintained at 5°C.

It was deemed advisable at this time to carry on the preliminary work regarding the ways in which environmental modifications may affect the activity of an anti-fungal agent towards a fungus. On the basis of the previous Rackham report, the carbohydrate studies were continued using the following sugars: D-xylose, D-glucose, sucrose and alpha-lactose. The anti-fungal materials to be used in conjunction with these sugars selected from the initial screening program, were pyridinethione and rimocidin.

It is conceivable that an organism might be rendered more susceptible to an inhibitory compound if its growth (hence its metabolic rate) can be accelerated. In order to investigate this possibility, the organisms involved were grown simultaneously in shake and stationary cultures, using C. phomoides C80A and 10I , as well as on Helminthosporium sativum 925 to determine the optimum incubation period prior to the physiological studies. Shake cultures grown in the standard glucose-asparagine media for 4 days at 28 to 38°C. was considered to be the optimum incubation period for harvesting. If allowed to continue longer, autolysis sets in (see Table VI). In stationary cultures, the growth curves for the above listed organisms continued to increase over a longer period of time, but the rates are much slower than those of the shake cultures. The harvest time for the stationary cultures in the physiological studies was taken as nine days although the organisms involved had not attained their maxiumu growth. The nine day incubation period was chosen from the expediency viewpoint. The mycelial dry weights from the stationary cultures agree quite well with the results obtained by Lilly and Barnett (34) in their work on the utilization of sugars by fungi in which the same standard medium with glucose as the carbohydrate source was used. Colletotrichum phomoides has a dry weight of 176-177 mg

at the end of 9 days as compared to Lilly's results of 155 mg for 5 days and 225 mg for 12 days. For Helminthosporium sativum the dry weight at the end of 9 days was found to be 204 mg as compared to Lily's results of 217 mg for 12 days.

While it would have been advisable to conduct the physiological studies with 2 organisms, it was felt that a greater range of variations could be accomplished if only one organism was chosen, hence the studies were made by arbitrarily choosing the fungus Colletotrichum phomoides C80A as the test organism.

It was found that the working range for pyridinethione, 1.0 - 10.0 ppm, was too high. Inhibition occurred at 1.0 ppm for all the sugars tested with the exception of D-xylose under shake conditions, in which case inhibition was between 1 and 3 ppm. It is suggested therefore, that the working range for pyridinethione be modified to 0.0 - 1.0 ppm.

The data concerning the rimocidin studies contain some interesting features for discussion. The organism grows best on D-glucose or sucrose and rather poorly on D-xylose or alpha-lactose. The validity of true correlations may be questioned because of the difference in incubation time and temperature. The glucose and sucrose phase was conducted at the same time and the preset conditions for the experiment were followed. In the case of the xylose and the lactose run, also conducted simultaneously, the temperature in the incubating room caused some difficulty. Instead of maintaining a 28 to 30°C. range, it rose to 33°C. This was high enough to somewhat retard the growth of the organism and hence required a longer incubation period. The harvest time in this last instance was based upon the glucose control which was run at the same time. When the latter showed a growth comparable to that of previous runs, all the flasks were harvested. For the shake cultures, this was 6 days whereas for the stationary cultures, it took 11 days.

Taking the above into consideration, some trends can be evaluated. The activity rate for the rimocidin is quite rapid when combined with glucose or sucrose while an attenuation is evidenced when combined with xylose or lactose. This can be readily ascertained by the corresponding slopes depicting activity rate as presented in Graphs I and II. The difference between shake and stationary cultures does not appear to markedly influence the organism's susceptibility to the rimocidin. The fact that the controls grew much better when cultured with glucose or sucrose than when cultured with xylose or lactose indicates a high metabolic rate for the former sugars. This seems to correlate well with fungal toxicity. Highly active tissue such as meristematic growing points on plants are usually more susceptible to phytotoxicity effects when sprayed with these test compounds. This is discussed in the section on in vivo studies. Thus the analogy seems to hold for C. phomoides C80A. Conversely, when this fungus shows a low or moderate metabolic rate such as when grown in xylose or lactose, correspondingly, the susceptibility to the compound is not as marked and required a greater concentration for inhibition. This last point is particularly indicated in the case with xylose. The organism apparently can utilize this sugar only slowly at first, perhaps requiring an adaptive enzyme system. Adaptation is gradual but after a lag period of about 6 days, estimated on the basis of xylose controls, this sugar can ultimately be utilized as well as glucose or sucrose. Our results with the xylose controls agree with those of Lilly and Barnett (34). They found the dry mycelial weight of C. phomoides in stationary culture to be 33 mg in 5 days and 210 mg at the end of 12 days whereas our weight in shake culture was 85 mg in 6 days and 172 mg at the end of 11 days in stationary culture. A slight stimulatory effect was also noticed when C. phomoides was grown in xylose together with rimocidin at 1 to 3 ppm. This may not be considered too unusual for small amounts of antibiotics have been known to have a stimulatory

effect on organisms.

Graph III shows a disparity between replicate flasks containing those concentrations of rimocidin having retarding effects on the organism. However, replicate flasks agree when either no antibiotic is present or is present in those amounts which have no noticeable effect on the fungus. The data and the curve seem to indicate that when the anti-fungal substance is present at those concentrations that start to affect the organism adversely, one flask will not agree with another in the same series. For the purpose of this report, this range is called the "critical range". Biological variations from flask to flask might account for this disparity in this critical range. In the case of glucose, however, mycelial weights of replicate flasks containing 6 ppm of rimocidin are not too far apart and are still closer together when at 10 ppm. At these two concentrations, the fungus is retarded almost to the point of total inhibition. That the disparity curve for xylose does not seem to come together as does the curve for glucose indicates that the critical range with this sugar has not been surpassed and higher concentrations of rimocidin approaching the inhibition range are needed to show the joining tendency of the curve.

When flasks containing glucose and rimocidin at a concentration of 10 ppm are inoculated with a spore suspension of C. phomoides C80A, a fungistatic effect is observed for a period of 15 days after which time growth is apparent. If sucrose is substituted, all other conditions being equal, the fungistatic effect is prolonged considerably, in this case for a period of 38 days, with no visible growth at the time the experiment was terminated. A possible explanation for this might be attributed to the reducing property of glucose which slowly breaks down the antibiotic rimocidin over a period of time. After its potency is so altered, the spores that were held in check are allowed to germinate. Sucrose on the other hand, apparently does not contribute to the rimocidin breakdown.

The disparity occurring between replicate flasks when concentrations of rimocidin show a retarding effect needs further investigation to determine, if possible, the cause of this spread in the curves before inhibition occurs. This may also be found to be the case for other organisms when glucose and xylose are used as a carbon source and rimocidin as the antifungal substance.

Continued *in vitro* studies of the test compounds and any new ones in connection with effects on metabolism of the pathogens will give a better picture of what takes place. This includes all of the various combinations and variations in the nitrogen sources, growth factors such as vitamins, pH studies, and determination of whether the compounds are fungicidal or fungistatic.

It is proposed that arrangements could be made with the Abbott Laboratories for a tagged compound with radio isotops so that it will be possible to trace the fate of the compound in the organism, if it is absorbed. It would also be possible to trace what takes place when this material is put on the surface or in the internal parts of a plant.

Discussion and conclusions of the in vivo section.

Because of the relatively short time that these experiments have been carried out, emphasis can not be made on these conclusions. However, there are certain interesting phenomena which have been observed and recorded.

There was a general tendency for the test plants to grow out of the injury caused by the applications of these antibiotic materials. The new growth on the plants which had exhibited injury was frequently less damaged than the growth that had been immature at the time of antibiotic application. The amount of injury on the new growth in the case of diaphine was proportional to the concentrations of the material in the spray. This phytotoxic effect of diaphine was translocated through the bean stem to the leaves that were immature at the time of application when the material was applied to the soil as a drench. However, the leaves which developed after this initial injury were nearly free of injury. These observations seem to indicate that the action of this antibiotic was against some part of the maturation process and that the mature cells were much more resistant to this type of injury. This idea is further developed when it is learned that bean plants which were at the primary leaf stage had to be sprayed with heavy application of diaphine at 200 ppm to cause necrosis of these mature leaves. These results would seem to indicate that the activity of this material decreases in contact with living tissue and that it is not translocated to the meristematic tissues in any large amounts. The rather interesting chlorotic rings which were found on cabbage need further investigation as to their association with the types of injury caused by diaphine.

The rather inconsistent results in the spray trials may be caused by several factors. The temperatures of the greenhouse could be controlled

only at night. No daytime controls existed. This resulted in temperature changes on sunny days of from 25°F. to 30°F.. On cloudy days the temperature stayed within 8°F. of the night temperature. This temperature effect became more important after the *in vitro* tests showed rimocidin to be very unstable at high room temperatures. Another important factor affecting the results of the control trials is run-off. Run-off is the amount of active material which either runs off at the time of application or is washed off by the subsiquent mist applications. Care in applying the materials was used to avoid as much run-off as possible. If the mist particles in the humidity chamber are not fine enough, then the amount of run-off is greatly increased. Also the more soluble the compounds;the poorer the sticking qualities;and therefore, the greater the run-off.

The type of lesion produced on the leaves of the host were also important in the final analysis of the results. *C. phomoides* will attack the leaves of tomato, but the lesions are small and difficult to read. Therefore, in future test programs and studies anthracnose of bean may be substituted. Probably the most significant factor was the low *in vivo* fungicidal or fungistatic activity of these materials. Although some of these materials exhibited high activity *in vitro*, such activity may not be exhibitied in association with living plants (9). This is the primary reason for *in vivo* testing programs.

The trials against the stem rust organism were the only ones that gave total control in some cases. However, lesions appeared on some of the sprayed plants. These lesions may represent areas that escaped coverage. However, it would be interesting to reisolate from such lesions to determine if any of them contained fungi which were more resistant to the materials under test. Such isolates have been collected in the case of rust on corn.

SUMMARY

Summary of in vitro section

1. The antibiotics, M4575, rimocidin, thiolutin, diaphine, candicidin and sodium pyridinethione were tested by the use of agar and liquid media with varied concentrations as a preliminary *in vitro* test against strains of *Colletotrichum phomoides* and *Helminthosporium sativum*. All test organisms were inhibited at less than 10 ppm by sodium pyridinethione. The other antifungal substances were less effective.

2. Nystatin inhibited *C. phomoides* C80A at 100 ppm on agar and 10 ppm in liquid. This data seems to indicate that the antibiotic may be tied up with the agar.

3. In the thermostability tests all the compounds showed a definite loss of potency after autoclaving. However, sodium pyridinethione was the only compound that showed no potency loss in an aqueous solution in various temperature ranges through 30°C. which is a desirable characteristic. This compound was the only one of those tested that is readily soluble in water.

4. The growth pattern of *C. phomoides* C80A, *C. phomoides* 10I, and *Helminthosporium sativum* 925 was determined for both stationary and shake cultures. The maximum growth for shake cultures was 4 days before autolysis occurred and 9 days for stationary cultures.

5. *C. phomoides* C80A was found to grow best on D-glucose or sucrose and rather poorly on D-xylose or alpha-lactose.

6. In both shake and stationary cultures the antibiotic activity rate against *C. phomoides* C80A for rimocidin was markedly increased when using D-glucose or sucrose in comparison to D-xylose or alpha-lactose.

7. The rate of growth of the test organism does not decrease as rapidly in the stationary cultures as it does in the shake cultures as the amount of rimocidin is increased.

8. A stimulatory effect occurred in stationary culture when rimocidin is present up to 3 ppm.

9. There was a marked disparity for xylose and glucose when rimocidin approached the critical range of retardation of C. phomoides C80A for different replicates. As the concentration of the antibiotic increased, the rate of growth for the replicates became about equal near the point of complete inhibition.

10. The fungistatic activity of rimocidin was maintained for 15 days when spores of C. phomoides C80A were put in a glucose medium with 10 ppm of the antibiotic. If sucrose is substituted under the same conditions, the fungistatic effect was prolonged for at least 38 days.

Summary of the in vivo section

Eight antifungal materials obtained from various sources were tested on eight plants to determine their phytotoxicity. Certain of these materials tested were antibiotics in the strict sense of the word, but others were synthetic organic compounds. One of these materials, panogen, was an organic mercury. Of all the materials tested, diaphine exhibited the most consistent phytotoxic reaction at dilutions as low as 25ppm. Some of these reactions appeared similar to the chlorotic ring spot reactions found associated with certain viruses in plants. Phytotoxic reactions were associated with the parts of the plant which were immature at the time of applications. The gramineous plants tested exhibited no phytotoxicity when sprayed with antibiotics at 200 ppm.

The materials tested except for actidione and panogen, which were introduced as checks in the later experiments, were not of high general antifungal activity. The in vivo level of activity was lower than that found in some of the in vitro tests. Some of the compounds reduced the number of lesions, but did not totally prevent infection. The pathogen should be reisolated from such lesions and its pathogenicity studied for possible antibiotic resistance. Good controls with certain materials were found in blackleg of cabbage and stem rust of wheat. No significant amount of control was found for early blight of tomato, spot blotch of barley, anthracnose of tomato, and powdery mildew of barley. Fluctuating greenhouse temperatures and run-off of the spray materials were factors affecting the results.

These findings represent a statement of progress and are subject to results of further investigations.

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A P P E N D I X

(PLATES)

Plate I

A. Concentration Series of Rimocidin in p.p.m., against Colletotrichum phomoides C80A, Incubated for 6 Days at Room Temperature.

Upper left: Control on liquid.

Upper right: Control on agar.

Lower, from left to right:

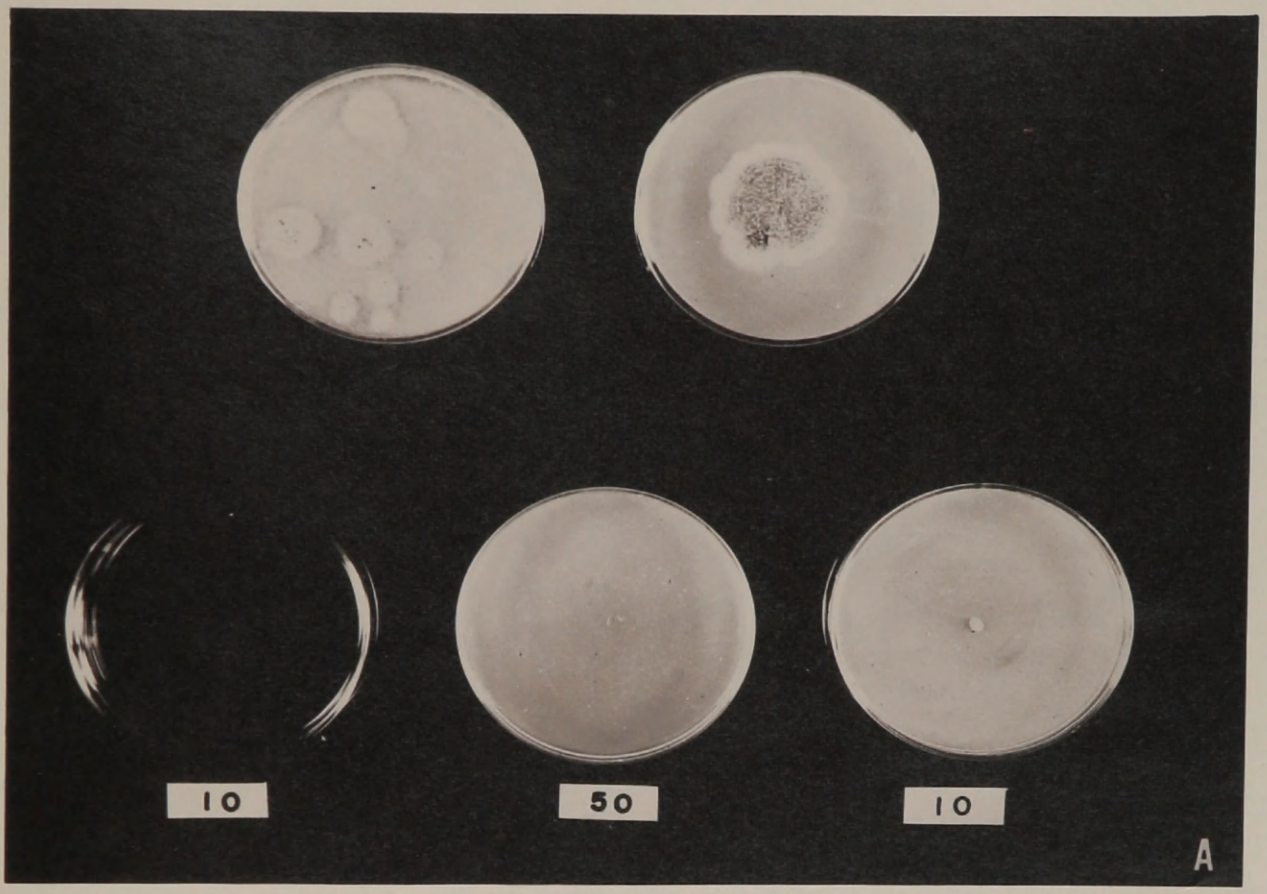
liquid 10 p.p.m.

agar 50 p.p.m.

B. Concentration Series of Nystatin in p.p.m., against Colletotrichum phomoides 101, Incubated for 6 Days at Room Temperature.

Upper: On agar media.

Lower: In liquid media.


10
50
10
A

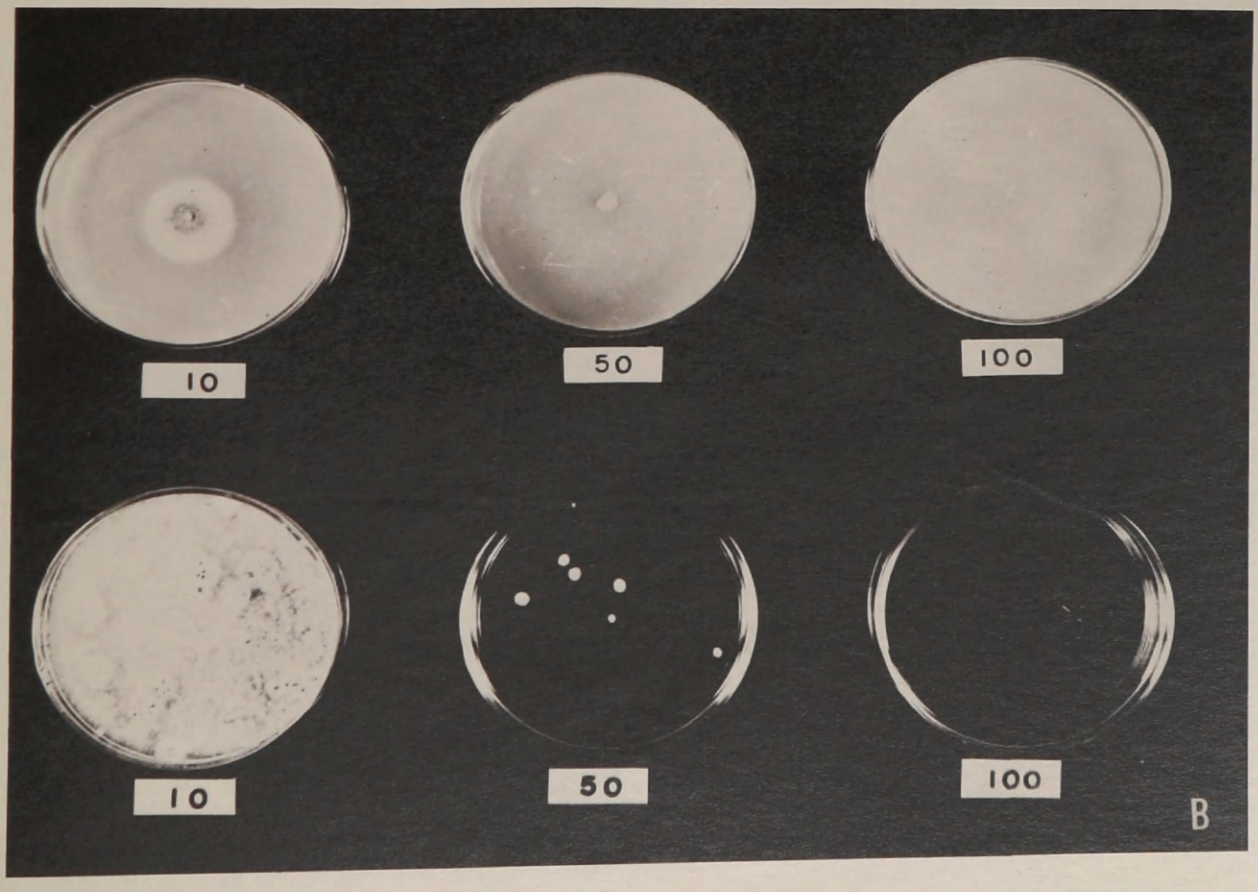

10
50
100
10
50
100
B

Plate II

A. Concentration Series of Thiolutin in p.p.m., against *Colletotrichum phomoides* C8OA, Incubated for 6 Days at Room Temperature.

Upper: on agar media.

Lower: on liquid media.

B. Concentration Series of Compound M4575 in p.p.m., in Liquid Media, Incubated for 7 Days at Room Temperature.

Upper: *Colletotrichum phomoides* C8OA.

Lower: *Helminthosporium sativum* 925.

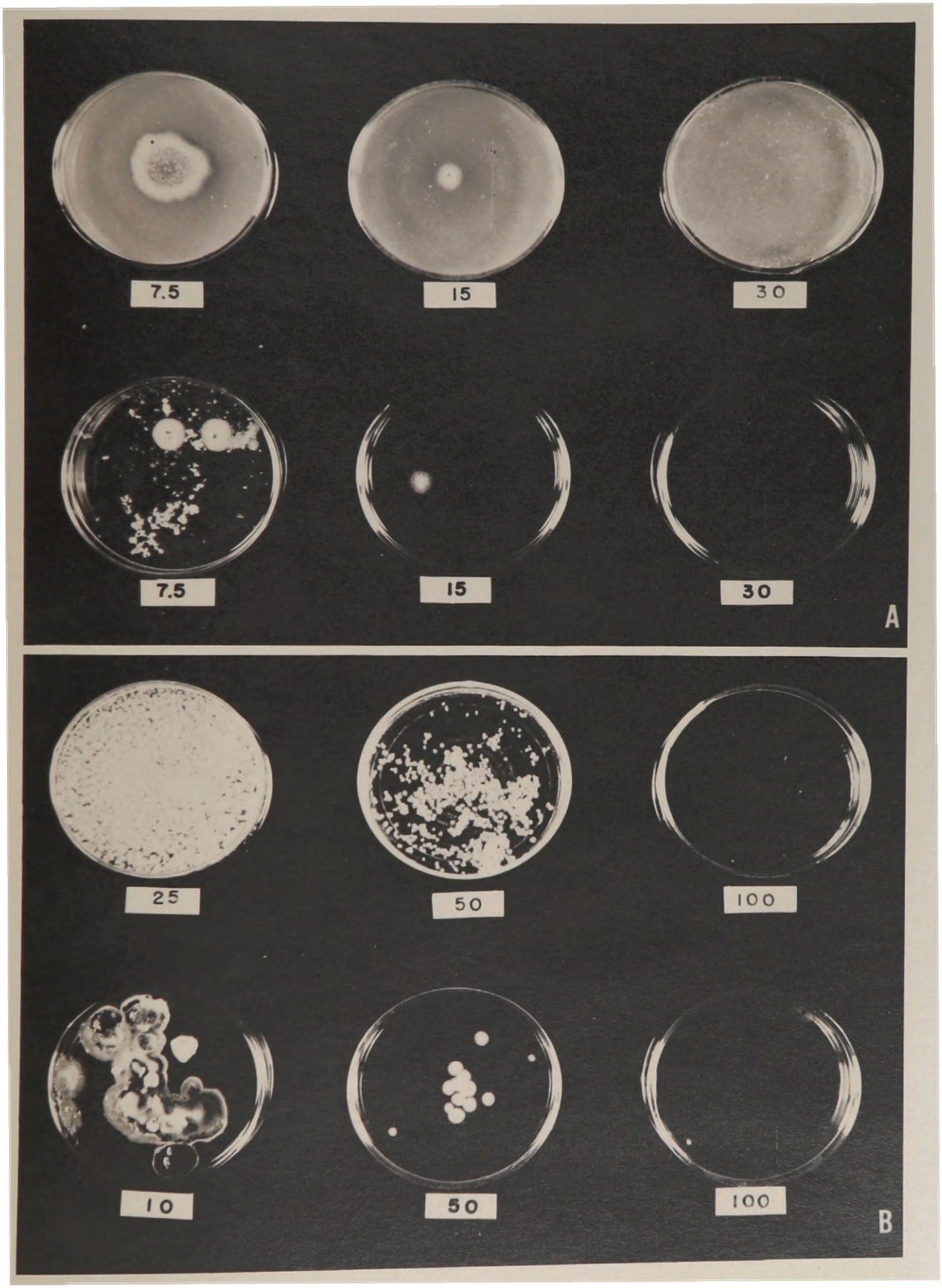

7.5
15
30
7.5
15
30
A
25
50
100
10
50
100
B

Plate III

View of Kershaw Rotary Shaker. This machine is standardly used in this project for the physiological studies involving liquid shake cultures.

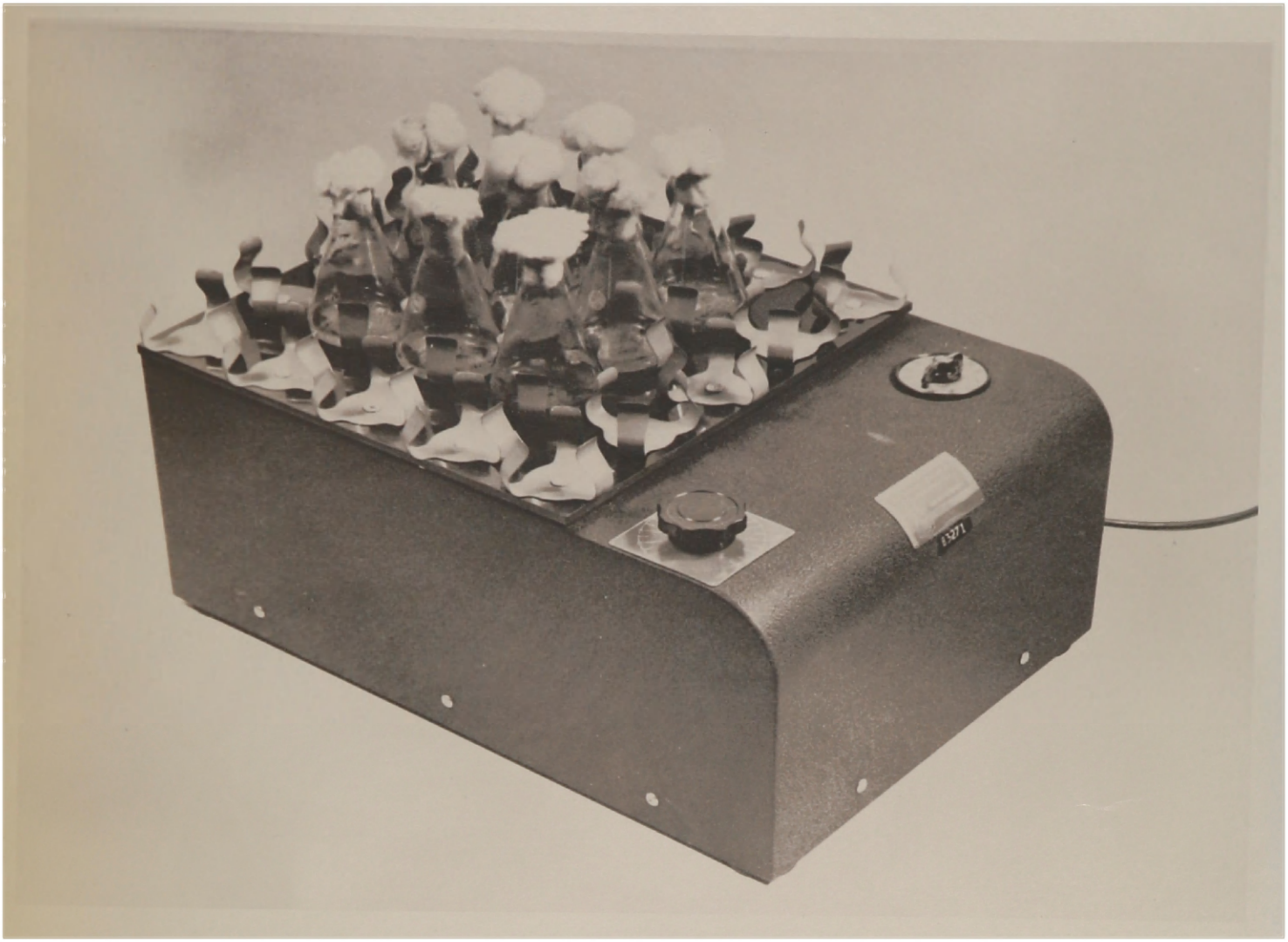


Plate IV

A. Concentration Series of Diaphine against *Helminthosporium sativum* 925.

Upper: diaphine sterilized via Seitz filtration.

Lower: diaphine sterilized by autoclaving directly with the media.

B. Concentration Series of Diaphine against *Colletotrichum phomoides* 101.

Upper: diaphine sterilized via Seitz filtration.

Lower: diaphine sterilized by autoclaving with the media.

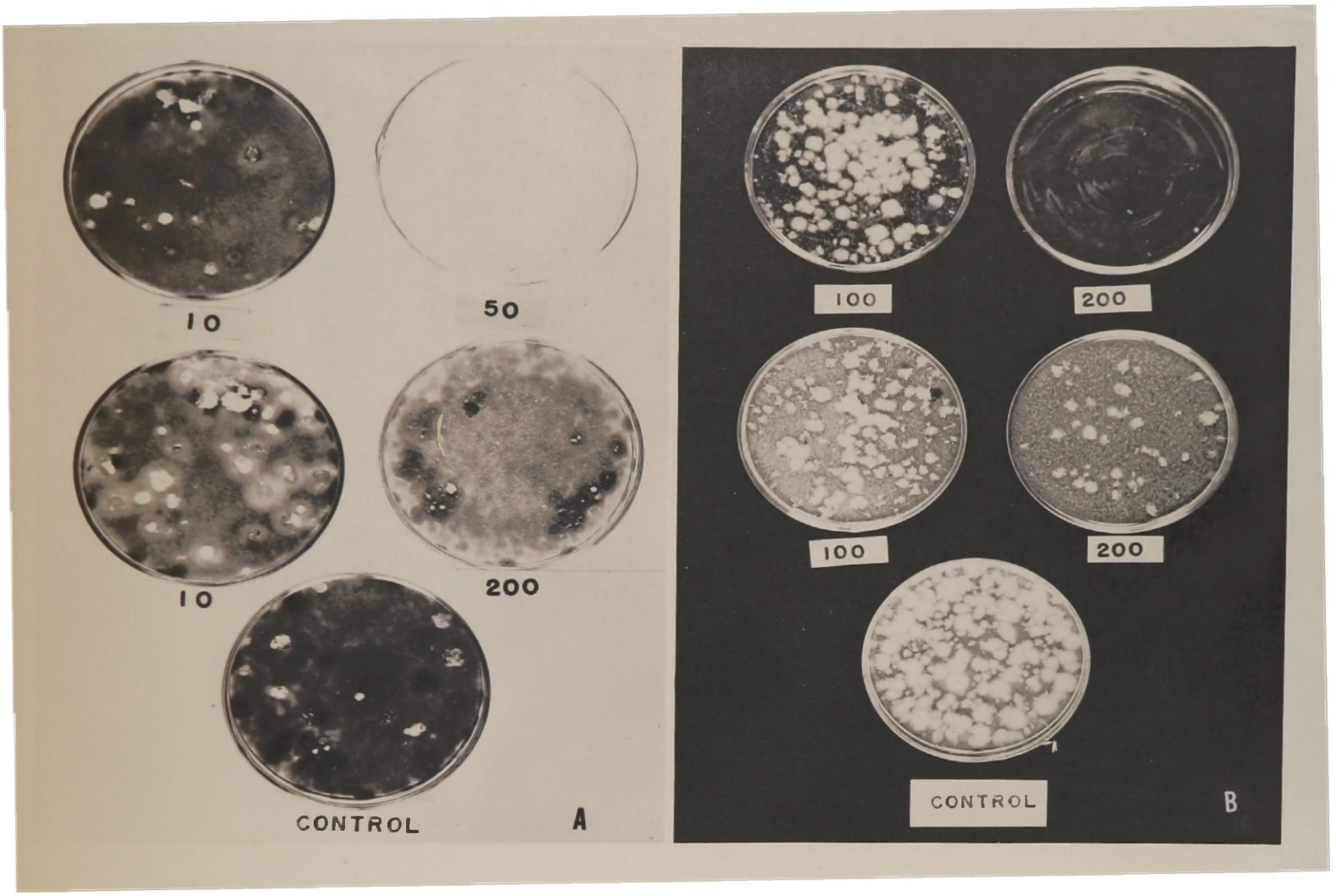

10
50
10
200
CONTROL
A
100
200
100
200
CONTROL
B

Plate V

A. Stationary Cultures of Colletotrichum phomoides C80A. General appearance when grown on different sugars, without antibiotic.

B. Concentration Series of Pyridinethione incorporated with D-xylose. Stationary cultures of Colletotrichum phomoides C80A.

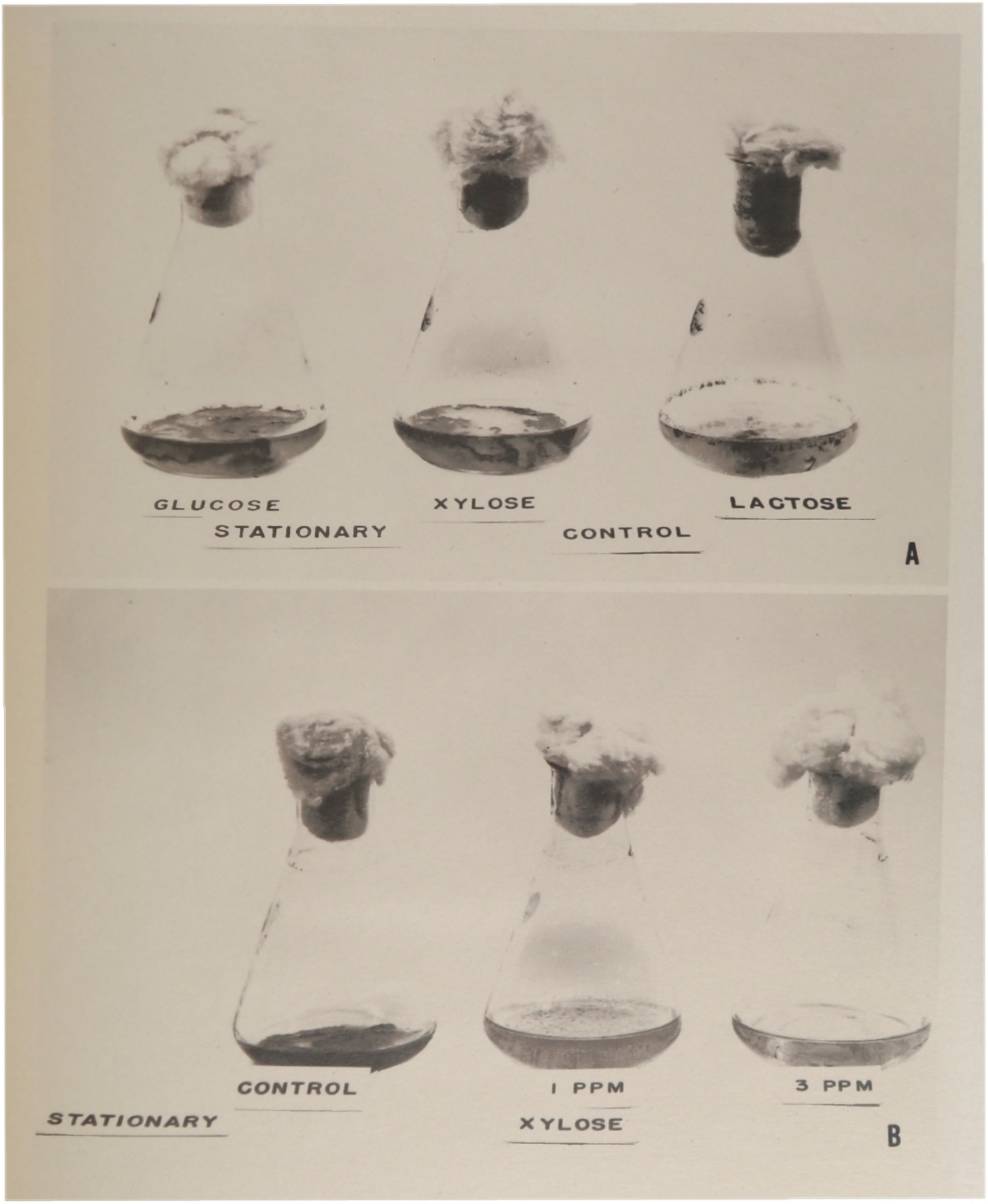

GLUCOSE
STATIONARY
XYLOSE
CONTROL
LACTOSE
A
CONTROL
STATIONARY
1 PPM
XYLOSE
3 PPM
B

Plate VI

A. Phytotoxicity caused by Diaphine at 200 p.p.m., on Cucumber.

Right: treated plant, showing chlorosis.

Left: untreated check.

B. Phytotoxicity caused by Diaphine and Pyridinethione at 200 p.p.m., on Bean.

Right: diaphine treated plant showing chlorosis.

Middle: pyridinethione treated plant showing puckering.

Left: untreated check.

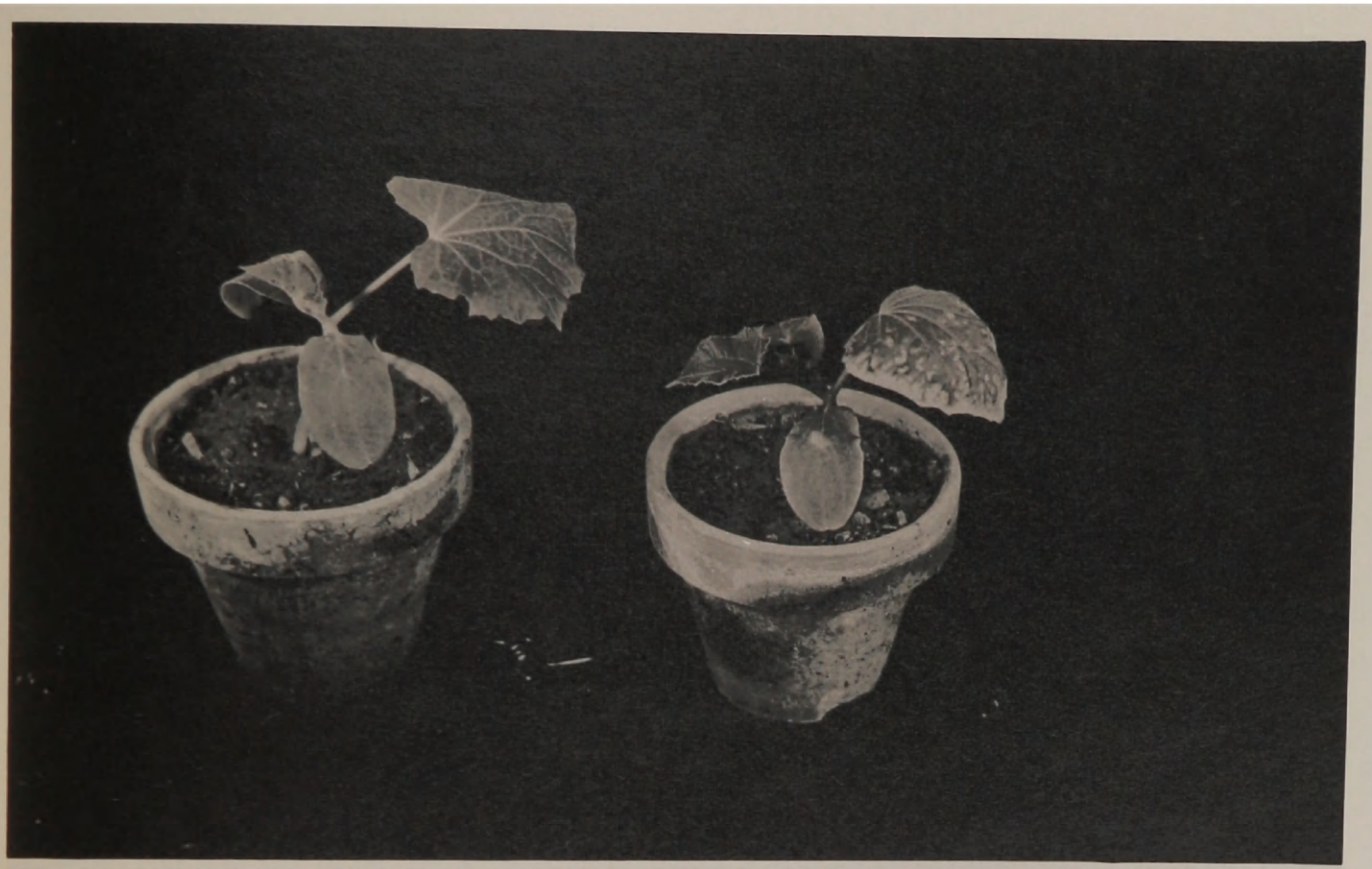



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Plate VII

A. Spray Trials for Control of *Phoma lingam* on Cabbage.

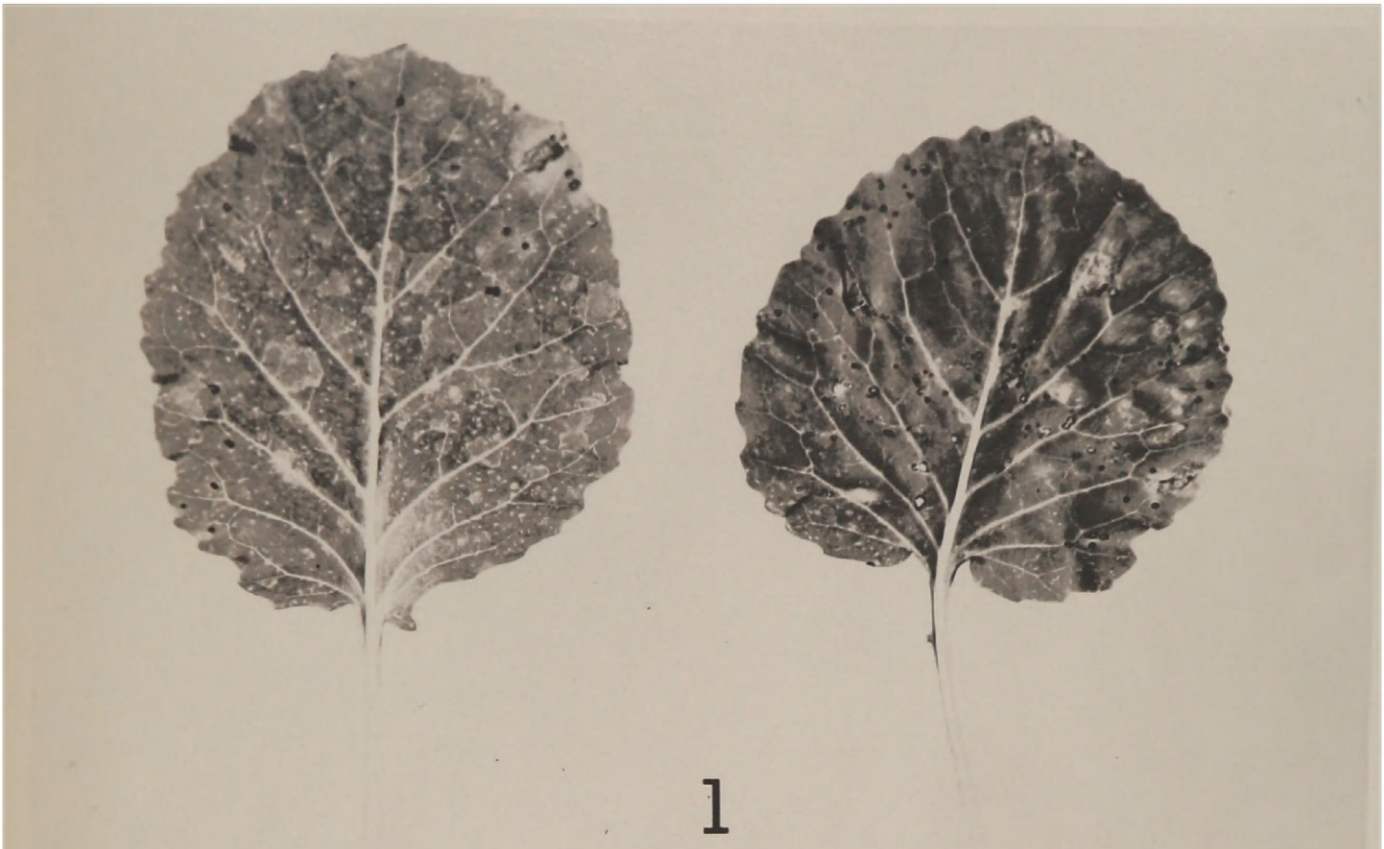
Right: untreated check.

Left: treated with thiolutin at 200 p.p.m.

B. Spray Trials for Control of *Phoma lingam* on Cabbage.

Right: untreated check.

Left: treated with diaphine at 200 p.p.m.



1

2

Plate VIII

A. Spray Trials for Control of *Phoma lingam* on Cabbage.

Right: untreated check.

Left: treated with pyridinethione at 200 p.p.m.

B. Spray Trials for Control of *Phoma lingam* on Cabbage.

Right: untreated check.

Left: treated with panogen at 200 p.p.m.

3

4

Plate IX

A. Spray Trials for Control of Phoma lingam on Cabbage.

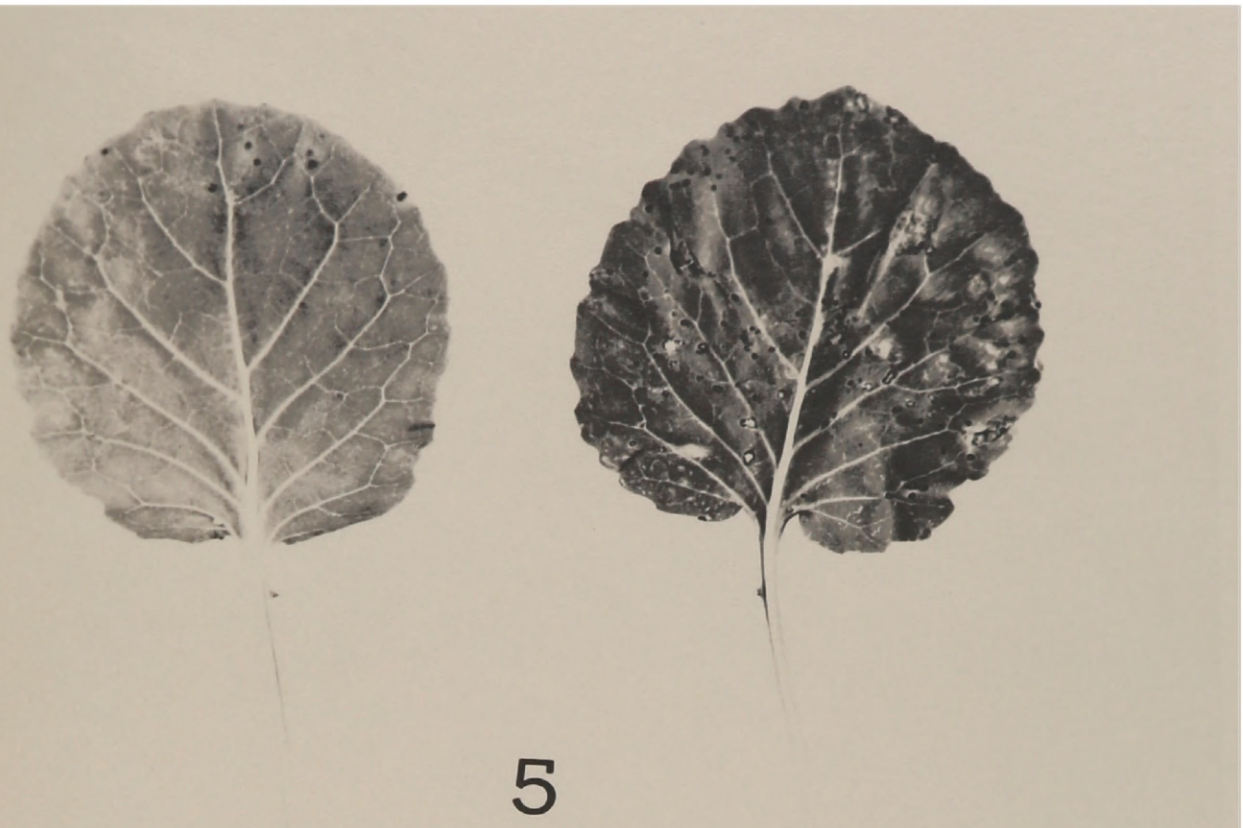
Right: untreated check.

Left: treated with nystatin at 200 p.p.m.

B. Spray Trials for Control of Phoma lingam on Cabbage.

Right: untreated check.

Left: treated with actidione at 200 p.p.m.



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