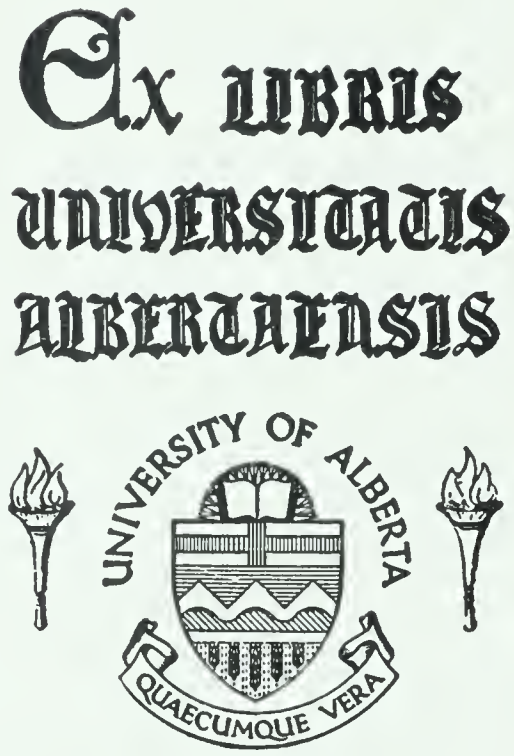

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THE UNIVERSITY OF ALBERTA

SOIL AS A HABITAT FOR SOME PLANT PATHOGENIC MICROORGANISMS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PLANT SCIENCE

by

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Soil as a habitat for some plant pathogenic microorganisms" submitted by Robert William Cromarty in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Twenty-four samples of soils representing the four major soil zones of Alberta were tested for toxicity towards several plant pathogenic fungi, bacteria and actinomycetes and one saprophyte in each group. All microorganisms tested were found to be inhibited by the natural soils. No consistent differences in fungitoxicity of soils collected at different seasons of the year were detected by the methods used.

Natural soils when treated with hot water, diethyl ether, or when air dried were found temporarily to have lost their inhibitory properties towards the organism Helminthosporium sativum. Steam sterilization of soils investigated appeared to remove their toxicity to all of the organisms used. Ultraviolet light appeared to destroy toxicity. Water and diethyl ether extracts of the soil were not toxic to Erwinia carotovora and H. sativum. Lysis and abnormalities of H. sativum were observed. Antimicrobial activity of living soil micro-organisms appeared to be the principal basis of soil toxicity.

ACKNOWLEDGEMENTS

The writer wishes to express his sincere thanks and appreciation to Dr. A. W. Henry, under whose supervision this investigation was carried out, for invaluable discussion, criticism and guidance.

The financial assistance received from the National Research Council of Canada during these studies is gratefully acknowledged.

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INTRODUCTION

Diseases of plants and animals have been observed for a long time and as investigators learned that many of the causal agents were microbes the relationship of these microorganisms to soil was considered since many must enter the soil upon the death and decay of the plants and animals.

It was not until the latter part of the nineteenth and the early part of the twentieth century that evidence was produced to indicate that soil is not a suitable environment for many microorganisms entering it. Frost (1904) made a careful study of the survival of the human pathogen, (Bacillus typhosus (Salmonella typhosa (Zopf) White) in soil and water and concluded that there was an inhibitory factor present in both which not only prevented the growth but also brought about the "extinction" of the pathogen.

In an attempt to explain this unfavourable condition, investigators have attributed it to various chemical, physical and biological factors or combinations of them, but no general agreement has been reached as to the nature of it.

The toxicity of soils has been studied and demonstrated in several countries (Dobbs and Hinson, 1953; Jackson, 1958a; Melin, 1946; Pochon and Barjac, 1952; Rybalkina, 1938; Waksman and Woodruff, 1942; Winter, 1955) and is claimed by some people to occur generally (Dobbs and Hinson, 1953; Jackson, 1958a). It has been indicated that toxicity may vary at different seasons of the year (Dobbs and Bywater, 1959;

Dobbs, Hinson and Bywater, 1957; Greig-Smith, 1915; Hutchison and Thayson, 1918; Jackson, 1958a, b; Stover, 1958), and in different horizons of the same soil (Dobbs and Hinson, 1953; Dobbs, Hinson and Bywater, 1957; Jackson, 1958a; Newman and Norman, 1943).

In a study of soil toxicity towards microorganisms various test organisms have been used. Plant pathogens (Chinn, 1953; Jackson, 1958a; Jefferys and Hemming, 1953; Stover, 1958; Winter, 1940), soil saprophytes (Dobbs and Hinson, 1953; Hessayon, 1953a; Jackson, 1958a, b), and some human pathogens (Frost, 1904; Melin and Wiken, 1946; Waksman and Woodruff, 1942) have all been used. Here we are concerned chiefly, though not entirely, with plant pathogens.

A knowledge of soil toxicity and other factors which create unfavourable conditions for the survival and development of plant pathogens in the soil may have considerable agricultural importance, especially in relation to the control of seed and root-rot diseases. As early as 1908 attempts were made to control root-rot diseases by treating the seeds and plants with toxins which were produced by the causal agents (Potter, 1908). Other attempts have been made to control such diseases by treating the seed with pathogen antagonists or by introducing the antagonists into the soil (Novogrudskii, 1936; Waksman, 1941). A third method, which has been the most successful in the control of seed and root-rot diseases is the cultural method. Here the pathogens may be starved out by summerfallowing or they may be eliminated by favouring the growth of soil saprophytic microorganisms by crop rotation, and by incorporating organic and inorganic substances into the soil (Garrett, 1955; Garrett, et al., 1959).

No direct investigation has been reported on the toxicity of Canadian soils towards microbes, and the purpose of the present study has been to determine if Alberta soils possess this property. The chief aim has been to ascertain to what extent plant pathogenic microorganisms are affected and, if possible, to find the basis or bases of such soil toxicity.

PURPOSES OF INVESTIGATION

The main purposes of this investigation were to determine:

A. (1) to what extent Alberta soils possess toxicity to plant pathogenic fungi,

(2) whether there are seasonal differences in soil toxicity, and

(3) whether toxic properties of soils affecting plant pathogenic fungi also affect plant pathogenic bacteria and actinomycetes.

B. the possible basis or bases of soil toxicity toward plant pathogenic microorganisms.

GENERAL MATERIALS STUDIED

Soil Samples Studied

Collection and storage of soil samples

Samples of virgin and summerfallow soil were collected from the four main soil zones of Alberta, namely: Brown, Dark Brown, Black, and Grey Wooded, at different seasons of the year - spring, summer, fall, and winter. Each consisted of about ten pounds of the top four inches of soil sieved through a 3/16" mesh. They were placed in plastic bags and stored in a cold room (about 4° C.) until used.

Table 1 gives the soil type, location, cultural state, pH value, percent moisture, date obtained of each sample studied.

Microorganisms Studied

The test microorganisms studied in this investigation were certain plant pathogenic and soil saprophytic fungi, bacteria, and actinomycetes. Soil saprophytes were included in the investigation to provide a comparison with the plant pathogens.

Most of the microorganisms studied were from stock cultures and were previously isolated in the laboratory, except where otherwise stated. All of them were grown on potato-sucrose agar. They are listed below.

Table 1. Table of soils studied

Soil type	Location	Cultural state	pH	Per cent* moisture	Date obtained
Brown	Taber	virgin	7.6	11.0	May, 1959
Brown	Taber	virgin	7.5	14.7	July, 1959
Brown	Taber	virgin	7.8	12.6	Oct., 1959
Brown	Taber	virgin	7.6	18.5	Jan., 1960
Brown	Taber	summerfallow	7.7	7.0	May, 1959
Brown	Taber	summerfallow	7.6	9.0	July, 1959
Brown	Taber	summerfallow	7.5	10.7	Oct., 1959
Brown	Taber	summerfallow	7.6	16.4	Jan., 1960
Dark brown	Provost	virgin	5.7	6.4	July, 1959
Dark brown	Provost	virgin	5.4	40.0	Jan., 1960
Dark brown	Provost	summerfallow	5.7	9.7	July, 1959
Dark brown	Provost	summerfallow	5.3	15.2	Jan., 1960
Black	Edmonton	virgin	5.7	31.1	May, 1959
Black	Edmonton	virgin	6.0	49.6	Jan., 1960
Black	Edmonton	summerfallow	5.4	24.5	May, 1959
Black	Edmonton	summerfallow	5.3	28.1	Jan., 1960
Grey wooded	Cooking Lake	virgin	6.8	47.1	May, 1959
Grey wooded	Cooking Lake	virgin	6.5	20.8	July, 1959
Grey wooded	Cooking Lake	virgin	6.3	20.0	Oct., 1959
Grey wooded	Cooking Lake	virgin	6.9	17.2	Jan., 1960
Grey wooded	Cooking Lake	summerfallow	5.9	13.8	May, 1959
Grey wooded	Cooking Lake	summerfallow	6.3	12.5	July, 1959
Grey wooded	Cooking Lake	summerfallow	5.5	16.1	Oct., 1959
Grey wooded	Cooking Lake	summerfallow	6.3	27.1	Jan., 1960

* The per cent moisture was that of the soil when stored and was based on oven dry weight.

Fungi

Claviceps purpurea (Fr.) Tul.

C. purpurea is the causal agent of ergot of cereals and grasses.

Fusarium oxysporum f. cubense (E.F. Sm.) Sny. and Hans.*

F. oxysporum f. cubense is responsible for inciting the banana wilt or Panama disease. The yellow race was used (Stover, 1957).

Helminthosporium sativum Pamm., King, and Bakke. (Bipolaris sorokiniana (Sacc. in Sorok.) comb. nov. (Shoemaker, 1959)).

H. sativum incites root-rot and seedling blight of cereals and other grasses.

Trichoderma viride Pers. ex Fries.

T. viride is one of the commonly isolated soil saprophytes (Bisby, et al., 1933). It is a known producer of the antibiotics viridin and gliotoxin which are particularly active against fungi and bacteria (Brian, et al., 1945; Waksman, 1947).

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* Obtained by Dr. A. W. Henry from Dr. Otto A. Reinking and Mr. G. L. Poland. The United Fruit Company, Pier 3, North River, New York 6, N.Y., U.S.A. Nov. 25, 1957.

Bacteria

Agrobacterium tumefaciens (E.M. Sm. and Towns.) Conn.*

A. tumefaciens causes crown gall in a great variety of plants.

Corynebacterium sepedonicum (Spieck. and Kotth.) Skapt. and Burkh.**

C. sepedonicum is the causal agent of bacterial ring-rot of potatoes.

Erwinia carotovora (L.R. Jones) Holland.**

E. carotovora incites the bacterial soft-rot of vegetables and blackleg of potatoes.

Bacillus subtilis (Cohn) Prazm.

B. subtilis is a common soil saprophyte and produces the antibiotic subtilin and other antibiotics. Subtilin affects various bacteria (Jansen and Hirschman, 1944; Waksman, 1947).

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* Obtained by Dr. A. W. Henry from Dr. A. J. Riker, Professor of Plant Pathology, College of Agriculture, University of Wisconsin, Madison 6, Wisconsin, U.S.A. May 29, 1959.

** Obtained from Dr. C. A. Mitchell, Division of Bacteriology and Dairy Research, Science Service, Dept. of Agriculture, Ottawa. Dec. 12, 1958.

Actinomycetes

Streptomyces scabies (Thaxt.) Waksman and Henrici.

S. scabies causes the common scab of potatoes.

Streptomyces antibioticus (Waksman and Woodruff) Waksman and Henrici.*

S. antibioticus is a soil saprophyte and produces the antibiotic actinomycin. Actinomycin affects many bacteria, especially the Gram-positive type, and fungi (Waksman and Woodruff, 1941).

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* Obtained from Dr. C. A. Mitchell, Division of Bacteriology and Dairy Research, Science Service, Dept. of Agriculture, Ottawa. Dec. 12, 1958.

PART A. TOXICITY OF ALBERTA SOILS

Introduction

In studies of soil toxicity towards microorganisms, two general approaches (indirect and direct) have been used by investigators. In the indirect approaches, extracts from the soil are tested for their toxicity against selected fungi and bacteria (Frost, 1904; Greig-Smith, 1915; Melin and Wiken, 1946; Stover, 1958; Waksman and Woodruff, 1942), or microorganisms of the soil are isolated and their antagonistic properties determined (Frost, 1904; Lockwood, 1959; Patrick, 1954; Stevenson, 1956). In the direct approach, test organisms are placed in contact with the soil and after an interval of time, removed and observed for injurious effects (Caldwell, 1958; Chinn, 1953; Dobbs and Hinson, 1953; Jackson, 1958a; Legge, 1952; Stover, 1958). The latter has been used in this investigation.

One of the greatest difficulties in the direct approach is the finding of the test organism after it has been added to the soil. However, a few methods have been developed in studies of soil toxicity effects on fungi (fungitoxicity) which permit observation of the test organism when desired. All of these methods have faults but give approximate values. The principal ones that have been used are briefly described below.

Blair (1943) used an adaptation of the Rossi-Cholodny glass slide technique by placing an agar disc bearing the test fungus against the slide and inserting the slide into the soil. Legge (1952) buried

glass fibres inoculated with the test fungus in the soil. Chinn (1953) dipped glass slides into melted agar containing a spore suspension of the test fungus and after setting, pressed soil firmly against them. Dobbs and Hinson (1953) sprayed spores of the test fungus inside folded cellulose film and inserted it into the soil. Stover (1958) dipped 6-inch dialyzing bags, 1.2" in diameter, filled with soil, into a spore suspension of the test fungus in melted agar. Jackson (1958a, b) inoculated agar blocks on soil plates with spores of the test fungus.

Very limited studies have been made with actinomycetes and bacteria, using the direct approach. Chinn (1953) made some studies on actinomycetes using the glass slide technique. Frost (1904) studied the effect of soil on the typhoid organism by inoculating broth contained in a collodion sac with Bacillus typhosus (Salmonella typhosa) and placing the sac in a broth suspension of soil.

Since all three groups of microorganisms, fungi, bacteria, and actinomycetes, were to be studied in this investigation, Jackson's (1958 a, b) method appeared the most suitable. It was therefore followed in essential details. However, a few modifications were made in it. These are included in the description of the method which follows.

Materials and Methods

Media used

Peptone agar

Peptone agar was used for the preparation of the agar blocks in the fungitoxicity studies. It included the following constituents and was autoclaved twenty minutes:

5 grams of Difco "Bacto-peptone"

25 grams of Difco "Bacto-agar"

1000 ml. of distilled water.

Potato-sucrose agar

Potato-sucrose agar was used for the culturing of the test microorganisms and for the preparation of agar blocks for bacteria and actinomycetes toxicity tests. It consisted of the following ingredients and was autoclaved twenty minutes:

17 grams of Difco "Bacto-agar"

20 grams of sucrose

1000 ml. of potato extract*

Sterilization

Sterilization was accomplished by using a Barnstead sterilizer set at 15 lbs. (241° F.) for the length of time specified in the methods of items requiring sterilization.

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* Prepared by adding 200 grams of sliced, peeled potatoes to 1000 ml. of distilled water and cooking for 20 minutes in an autoclave. After straining the extract was made up to volume, if necessary, by the addition of distilled water.

The inoculum

Inoculum of fungi

Since the conidia of certain fungi have been reported to be more sensitive to fungitoxins than the mycelium (Jackson, 1958a; Stover, 1958), a conidia of test fungi were used chiefly in this investigation.

A suspension of conidia was prepared by adding about five ml. of sterile-distilled water to a 7-day-old culture slant of the test microorganisms and shaking. It was necessary to scrape the surface of certain fungal cultures with a sterile needle to free the conidia.

Bacterial inoculum

A bacterial suspension was prepared by transferring two or three loopfuls of bacterial cells, from a week-old culture of the test bacterium, to a test tube containing about five ml. of sterile-distilled water and shaking until the suspension was uniform.

Inoculum of actinomycetes

A suspension of spores of actinomycetes was prepared in a similar manner to that used in producing inoculum of the fungi, except that three to four week-old cultures were used.

Preparation and use of agar blocks

Agar blocks were prepared by pouring sufficient melted agar into Petri dishes to give a layer approximately 1.5 mm. thick. After setting, blocks were cut out with a flamed 12 mm. diameter cork borer and transferred to soil plates.

Inoculation* procedure

Agar blocks were inoculated on the surface by transferring to each with a flamed needle a loopful of the inoculum suspension.

Agar-block soil-plate technique

Soil plates were prepared by first placing approximately 50 ml. of soil in each deep Petri dish (100 mm. diameter x 20 mm.) required. The soil was then levelled, lightly packed, and moistened with distilled water almost to the saturation** point. One-half of the soil plates were then sterilized in the autoclave for one hour. A sterilized Whatman No. 1 filter paper disk (9 cm. diameter) was placed on the soil of all soil plates. Agar blocks were then placed on top of the filter paper (Figure 1). Sterile-distilled water was added, when necessary, at this point of the procedure to ensure a moisture contact between the soil and agar blocks through the filter paper. No additions of water were made later.

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* The word "inoculation" is used in this investigation to mean the placing of microorganisms in or on a substratum not necessarily on a living one.

** Jackson used a soil at 60% water-holding capacity.

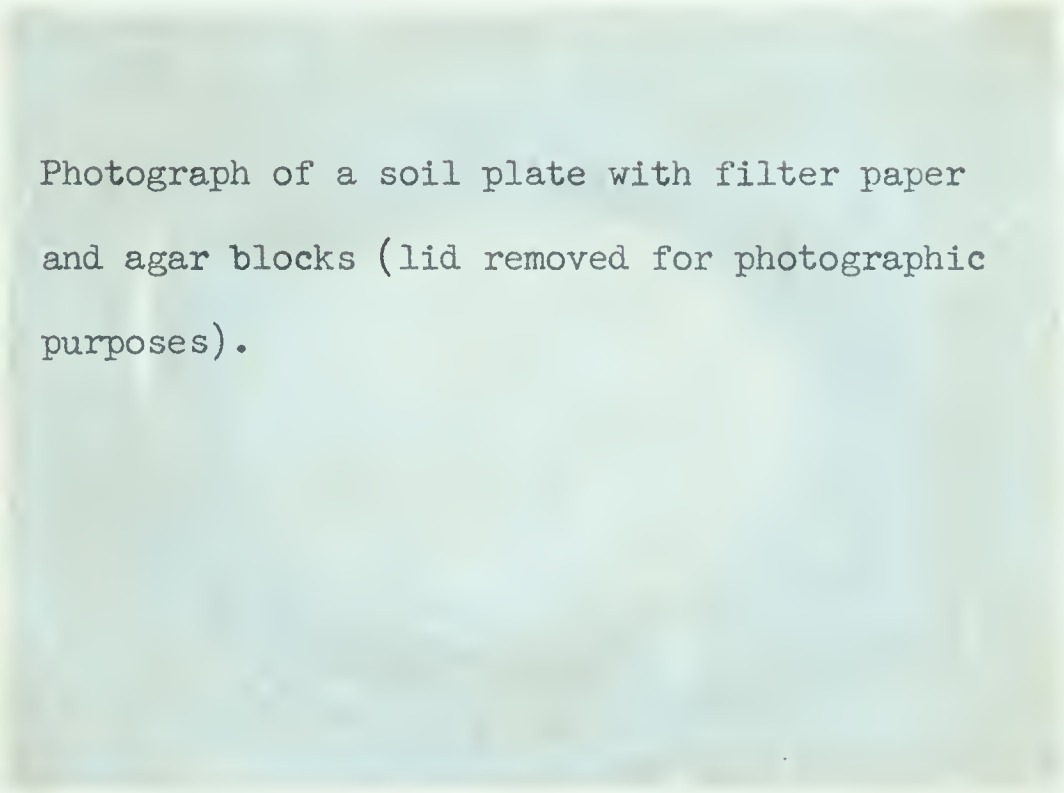
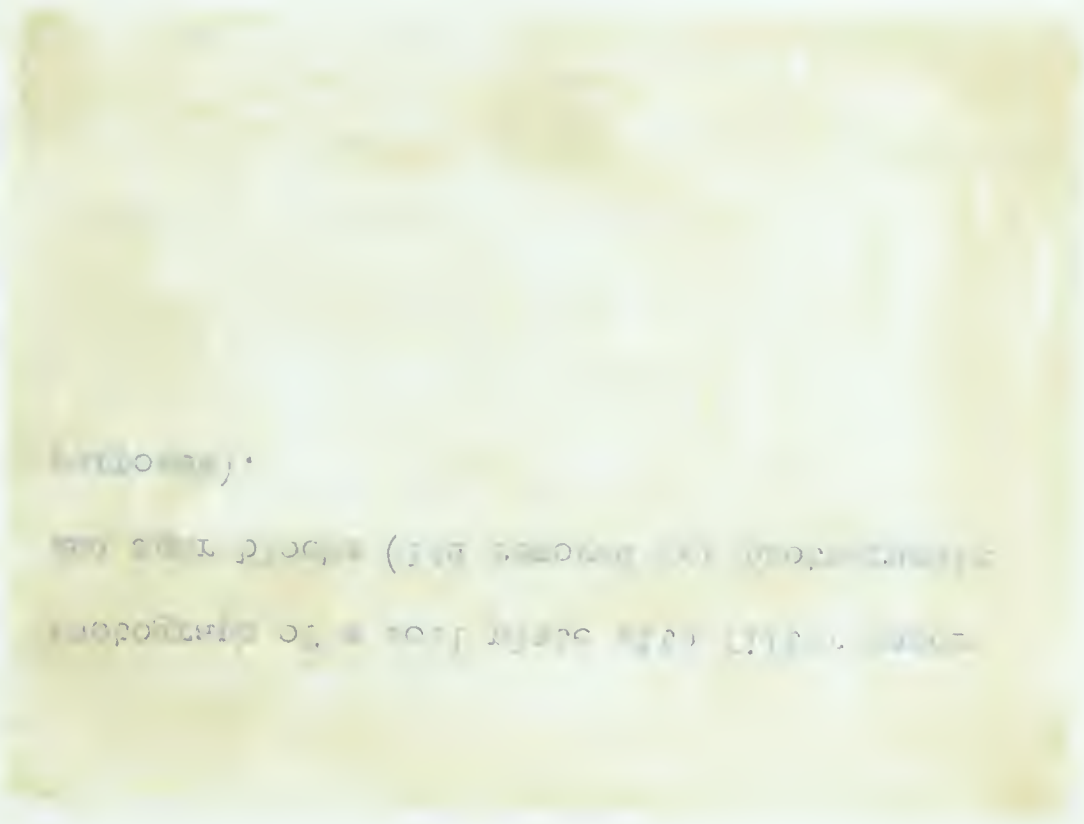
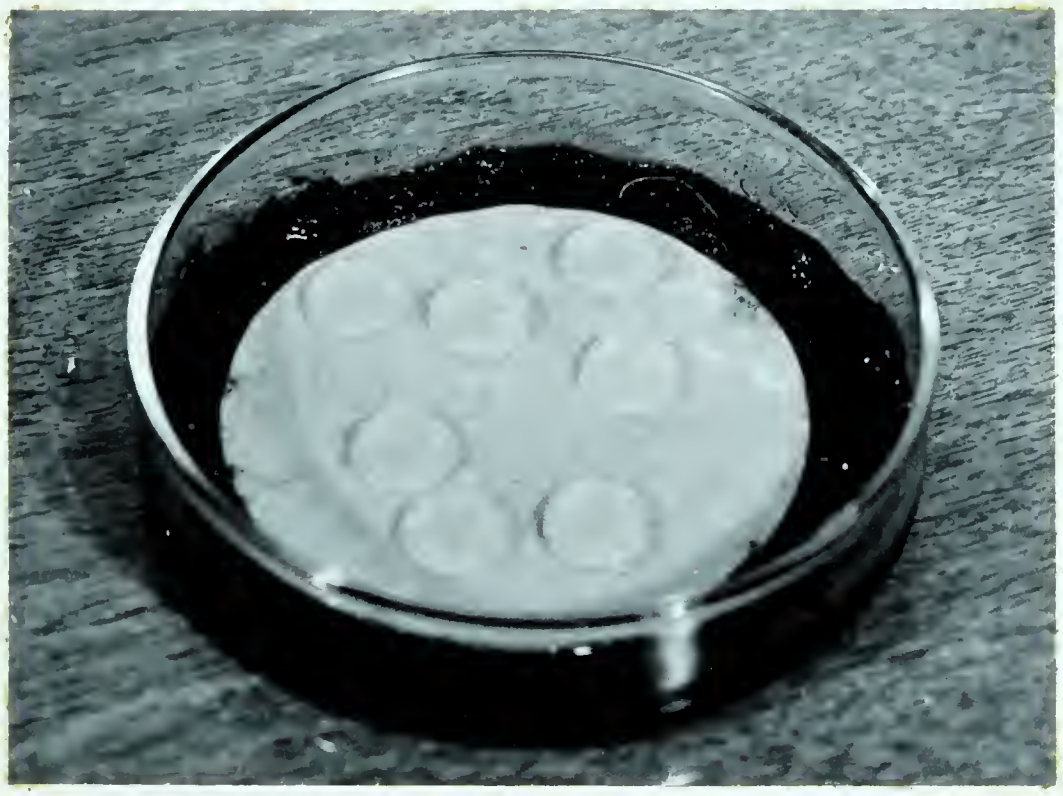


Figure 1. Photograph of a soil plate with filter paper and agar blocks (lid removed for photographic purposes).





The soil plates were then incubated at room temperature for 2 days* to permit inhibitory substances to move up into the agar blocks.

After the incubation period the agar blocks were inoculated with the test organism and incubated at room temperature for a specified time depending on the organisms being tested.

It may be noted that modifications of the agar block soil plate technique were tested but not adopted since with H. sativum as the test organism they gave results essentially similar to those obtained with the procedure followed. They were as follows:

(a) Soil was moistened to only 60% water holding capacity instead of being saturated.

(b) Water agar (2.0%) and potato-sucrose agar blocks were substituted for the peptone-agar blocks.

(c) Peptone-agar blocks were placed directly on the soil instead of on the filter paper.

(d) Filter paper (on the soil) was inoculated directly instead of the agar blocks.

Collecting and recording of data

Fungi

To observe conidial germination, or its absence, the inoculated blocks, after an incubation period of 15 - 20 hours, were transferred to glass microscope slides. A drop of water and a cover slip were placed

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* Jackson used a 1 - 4 hour incubation period. Increasing the incubation period to 2 days greatly increased the toxic effect during the initial stages of conidial germination and gave more uniform results and lower germination.

on top of each block and observed under a microscope. Usually 100 or more conidia were observed on each agar block.

The following symbols appeared adequate to record the results and were therefore used:

- no germination or very poor germination (below 5%)

+ complete germination or very good germination (above 85%)

Bacteria and actinomycetes

Data on bacteria and actinomycetes were based on colony growth, visible to the naked eye. Records of colony growth were taken 4 - 8 days after inoculation and were recorded by means of the following symbols:

- no colony growth

+ colony growth

Results

Conidia of *Helminthosporium sativum* did not germinate on the peptone-agar blocks placed on unsterilized virgin and summerfallow soils of the brown, dark brown, black, and grey wooded soil types, but did germinate well on such blocks placed on the same soils after they were steam sterilized (Table 2). (Figures 2 and 3, respectively, show the effect of unsterilized and sterilized virgin brown soil on conidia of *H. sativum*.) These same soils had a similar effect on the germination of conidia of *Claviceps purpurea*, *Fusarium oxysporum* f. *cubense*, and *Trichoderma viride* (Table 3).

Table 2. Toxic effect of different soil types on the conidia of Helminthosporium sativum

Soil type	Cultural state	Unsterilized	Sterilized
Brown	virgin	-*	+**
	summerfallow	-	+
Dark brown	virgin	-	+
	summerfallow	-	+
Black	virgin	-	+
	summerfallow	-	+
Grey wooded	virgin	-	+
	summerfallow	-	+

* Almost no germination
** Almost complete germination

Table 3. Toxic effect of different soil types on the conidia of four fungi

Soil type	Cultural state	Treatment	H. sativum	C. purpurea	F. oxysporum	T. viride
Brown	virgin	unsterilized	-*	-	-	-
		sterilized	+**	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Dark brown	virgin	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Black	virgin	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Grey wooded	virgin	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+

* Almost no germination
** Almost complete germination

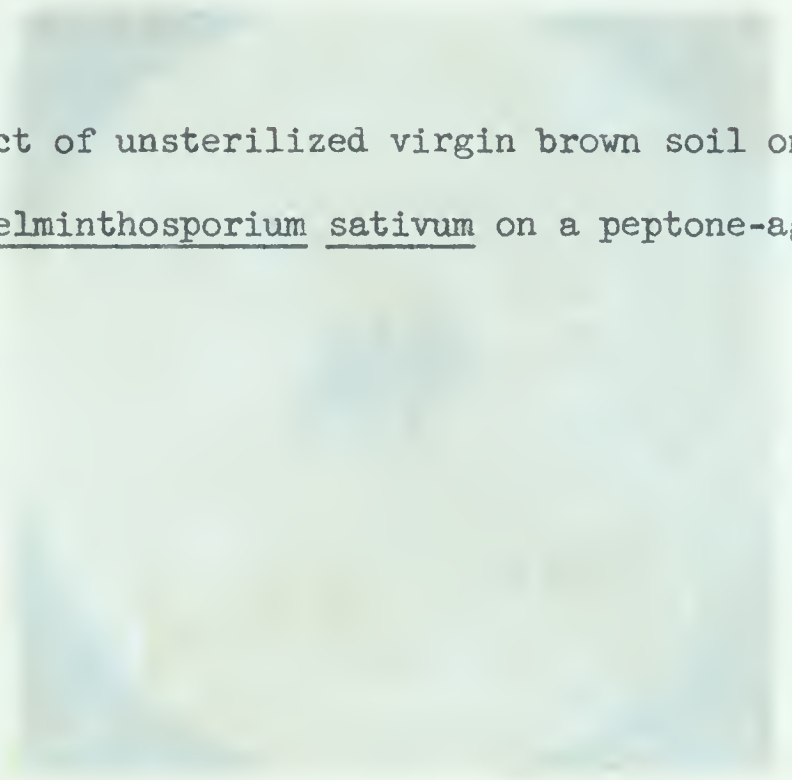


Figure 2. Effect of unsterilized virgin brown soil on conidia of Helminthosporium sativum on a peptone-agar block.

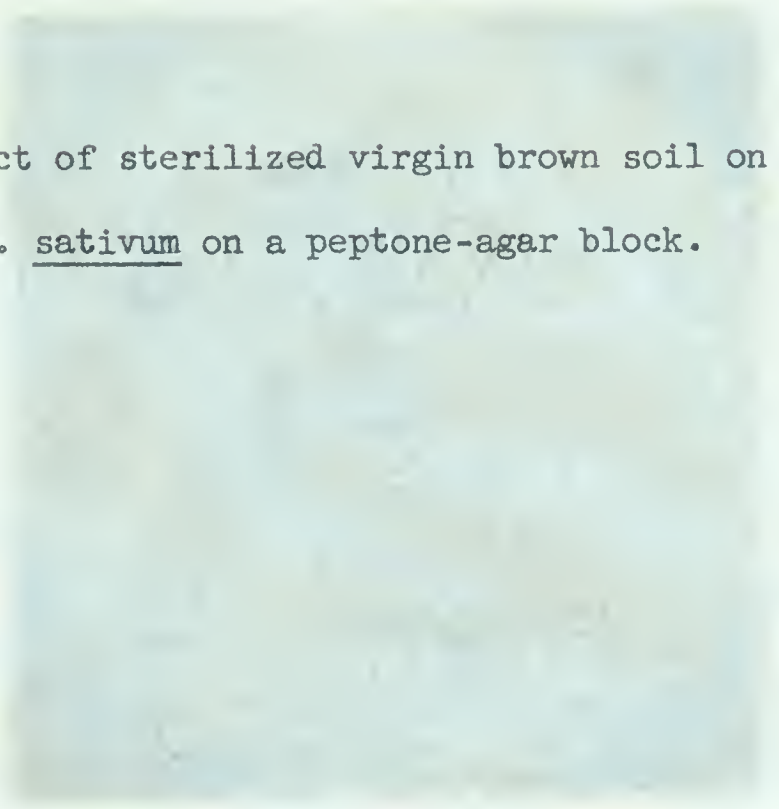
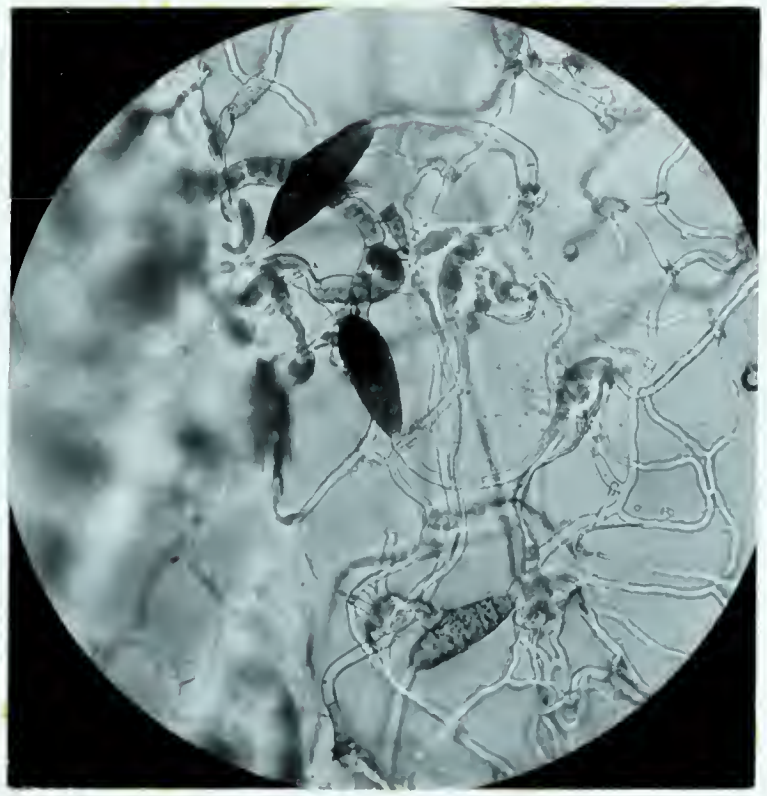


Figure 3. Effect of sterilized virgin brown soil on conidia of H. sativum on a peptone-agar block.



No germination of the conidia of the four fungi occurred when tested on agar blocks on unsterilized samples of the four soil types collected at different seasons of the year. In contrast, very good germination of conidia of these fungi was obtained on all these soil samples when they were steam sterilized (Table 4). There were, however, a few of the macroconidia of F. oxysporum f. cubense which germinated on some of the replicates of the agar blocks on unsterilized soil. Macroconidia represented less than five per cent of the total conidia present in the microscopic field.

There was no observable growth of the four bacteria, Erwinia carotovora, Agrobacterium tumefaciens, Corynebacterium sepedonicum, and Bacillus subtilis, and the two actinomycetes, Streptomyces scabies and S. antibioticus on potato-sucrose agar blocks tested on unsterilized virgin and summerfallow soils of the four soil types. However, growth of these test organisms was evident on agar blocks kept on sterilized samples of these soils (Tables 5 and 6). Figure 4 shows results obtained with the two actinomycetes. The bacteria appeared as follows on agar blocks on the sterilized soils. A. tumefaciens produced large conspicuous convex colonies. Colonies of E. carotovora and C. sepedonicum were much smaller, the latter growing rather poorly. B. subtilis developed flat colonies, which were somewhat dry in appearance.

Table 4. Seasonal effect on the toxicity of different virgin soils indicated by the germination of conidia of four fungi

Soil type	Season	Treatment	H. sativum	C. purpurea	F. oxysporum	T. viride
Brown	spring	unsterilized	-*	-	-	-
		sterilized	+**	+	+	+
	summer	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	fall	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	winter	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Dark brown	summer	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	winter	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Black	spring	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	winter	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Grey wooded	spring	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summer	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	fall	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	winter	unsterilized	-	-	-	-
		sterilized	+	+	+	+

* Almost no germination
** Almost complete germination

Table 5. Toxic effect of different soil types on four bacteria

Soil type	Cultural state	Treatment	E. carotovora	A. tumefaciens	C. sepedonicum	B. subtilis
Brown	virgin	unsterilized	-*	-	-	-
		sterilized	+**	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Dark brown	virgin	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Black	virgin	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+
Grey wooded	virgin	unsterilized	-	-	-	-
		sterilized	+	+	+	+
	summerfallow	unsterilized	-	-	-	-
		sterilized	+	+	+	+

* No colony growth
** Colony growth

Table 6. Toxic effect of different soil types on two actinomycetes

Soil type	Cultural state	Treatment	Streptomyces scabies	Streptomyces antibioticus
Brown	virgin	unsterilized	-*	-
		sterilized	+**	+
	summerfallow	unsterilized	-	-
		sterilized	+	+
Dark brown	virgin	unsterilized	-	-
		sterilized	+	+
	summerfallow	unsterilized	-	-
		sterilized	+	+
Black	virgin	unsterilized	-	-
		sterilized	+	+
	summerfallow	unsterilized	-	-
		sterilized	+	+
Grey wooded	virgin	unsterilized	-	-
		sterilized	+	+
	summerfallow	unsterilized	-	-
		sterilized	+	+

* No colony growth
** Colony growth

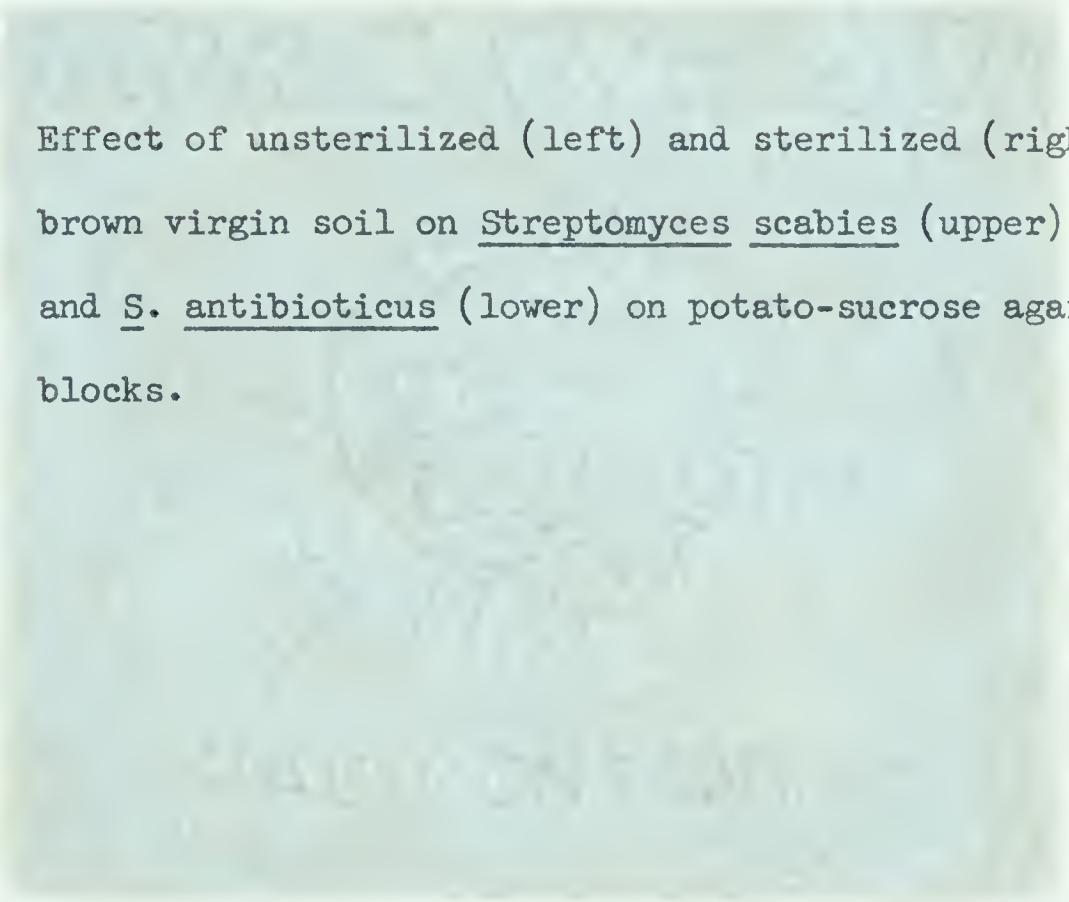


Figure 4. Effect of unsterilized (left) and sterilized (right) brown virgin soil on Streptomyces scabies (upper) and S. antibioticus (lower) on potato-sucrose agar blocks.

FIGURE 4. Effect of sucrose (left) and sorbitol (right) on chromatophore (above) and epidermal (below) expansion in Loligo pealei. Note the multiplicative (left) vs. additive (right) effects.

Discussion

In this study of Alberta soils, all of them examined in their natural state were found to exhibit fungitoxic properties. These same soils also proved toxic toward bacteria and actinomycetes. Whether the ultimate basis of this toxicity is the same for fungi, bacteria, and actinomycetes remains to be determined but it is important if, as the present studies suggest, it can be traced to soil micropopulations as the source. The fact that in all cases this was lost when the soils were steam sterilized indicates it originates from soil organisms and probably from microorganisms.

It is rather interesting that not only plant pathogens were inhibited by the natural soils but, also, saprophytic soil fungi, bacteria, actinomycetes. Each of those tested, T. viride, B. subtilis, and S. antibioticus, are producers of antibiotics in culture. The inhibition of them by natural soils indicates the complexity of the soil toxicity phenomenon.

There were no distinguishable seasonal effects on toxicity of the various soils studied to the fungi tested. It is possible that the methods used here for studying soil toxicity may not be sufficiently sensitive to indicate minute differences that may have characterized these seasonal samples but this does not seem likely.

PART B. THE POSSIBLE BASIS OR BASES OF SOIL TOXICITY TOWARD PLANT PATHOGENIC MICROORGANISMS

Introduction

In an attempt to explain microbial inhibition by the soil, many factors have been considered. These may be grouped into four main divisions, nutritional, physical, chemical, and biological.

It is known that water extracts of the soil will not support the growth of a wide range of microorganisms and they would be less suitable when many organisms were in competition for the same nutrient supplies. Moisture and temperature are physical factors that are often unfavourable for the development of microorganisms in the soil. The presence of certain acids, gases, and other chemicals in the soil may also account for the inhibition of microorganisms entering it. Many soil organisms and especially microorganisms are known to inhibit the development of other microorganisms. Their presence in soil may, therefore, partly account for the biological basis of its inhibitory properties.

Since the results of Part A of this investigation suggest that the microorganisms of the soil are a major cause of its antimicrobial properties, they have received exclusive attention in the remainder of this paper.

Many fungi (Dubos, 1942; Garrard and Lochhead, 1938; Hessayon, 1953a; Jefferys, et al., 1953; Park, 1955; Patrick, 1954; Waksman and Woodruff, 1942; Waksman and Horning, 1943), bacteria (Dubos, 1939, 1942;

Hessayon, 1953a; Hoogerheide, 1944; Khudiakoff, 1935; Patrick, 1954; Stokes and Woodward, 1941; Vasudeva, et al., 1952; Waksman, 1941; Waksman and Woodruff, 1940, 1941; Wilkins, 1945; Wilkins and Harris, 1943), and actinomycetes (Carter and Lockwood, 1957a, b; Dubos, 1942; Hessayon, 1953a; Johnson, 1952; Lockwood, 1959; Saltan, 1955; Stevenson, 1954; Vanek, et al., 1958; Waksman, 1941, 1947; Waksman and Woodruff, 1940) isolated from soils produce antibiotics in culture and are antagonistic to many other microorganisms. Some of the microorganisms, isolated from the soil, produce antibiotics in sterilized soils, especially when organic matter is added (Gottlieb and Siminoff, 1952; Gregory, et al., 1952; Grossbard, 1952; Lewis, 1929; Wright, 1955). A few investigators have shown that antibiotics may be produced in amended unsterilized soils (Gregory, et al., 1952; Wright, 1955). Hessayon (1956) reported that trichothecin may be produced in natural unamended soils.

Effect of Water and Diethyl Ether Extracts of Natural Soil on Erwinia carotovora and Helminthosporium sativum

Previous investigations

Previous investigators have attempted to extract toxic substances from the soil with varying results. Pochon and Barjac (1952), Rybalkins (1938), and Winter (1940, 1955) reported that bacteria and fungi were inhibited by soil extracts; Dobbs and Hinson (1953), Nutman (1959) and Stevenson (1954) were not able to extract any inhibitory substances from the soil. Some investigators reported toxic properties in water extracts (Greig-Smith, 1915; Melin and Wiken, 1946; Stover, 1958),

ether extracts (Hessayon, 1953a, b; Jefferys and Hemming, 1953; Waksman and Woodruff, 1941), and alcohol extracts (Jefferys and Hemming, 1953; Stover, 1958) of natural soils. On the contrary, certain investigators have failed to find any toxic properties in water (Dobbs and Hinson, 1953; Frost, 1904; Hessayon, 1953a; Waksman and Woodruff, 1941) and alcohol (Waksman and Woodruff, 1941) extracts. These differences may be accounted for by differences in test organisms, soils, and methods used.

Materials and methods

An extraction apparatus (Figure 5) was made by fitting a glass column (1" x 12" long) on a 500 ml. side-tube filtering flask with a rubber stopper. A test tube (18 mm. x 150 mm.) was placed inside of the flask to collect the extract when small volumes were required. A piece of glass tubing (8 mm. diameter) was pushed through a hole in the rubber stopper until flush with the upper surface and protruding below the bottom surface about two inches. By this means the extract could be directed into the test tube. The lower end of the glass tube was slightly constricted and the tube partially filled with non-absorbent cotton to prevent soil from falling into the extract. Sufficient extracting agent was added to the top of the soil column to provide approximately ten ml. of extract. Larger volumes of extract (200 ml.) were also experimented with. The larger volume was either left as it was or concentrated by vacuum and gentle heating to ten ml.

Figure 5. Extraction apparatus.

Sterile filter paper disks (1/2" diameter) were then dipped into the extract and placed on potato-sucrose agar in Petri dishes which had been seeded with a test organisms. The filter paper disks were deposited just after the seeding of the agar. Test organisms used were Erwinia carotovora and Helminthosporium sativum. The agar plates were seeded by spraying the surface of the hardened agar with water suspensions of conidia of H. sativum or of cells of E. carotovora. A sterilized atomizer (DeVilbiss Nebulizer No. 40) was used to do the spraying. Observations were made when abundant growth was clearly visible to the naked eye. Toxicity was determined by the presence or absence of a halo about the filter paper disks and recorded in the following manner:

- = no inhibition (no halo)

+ = inhibition (halo present).

Results

Water extracts showed no apparent toxic properties to Erwinia carotovora and to Helminthosporium sativum in any of the extracts tested (Table 7).

Table 7. Effect of water extracts of natural soil on Erwinia carotovora and Helminthosporium sativum

Treatment of filter paper disks	E. carotovora	H. sativum
10 ml. extract	-	-
200 ml. extract	-	-
200 ml. extract concentrated to 10 ml.	-	-

There was no inhibition of the test organisms in the presence of the small and large volume diethyl ether extracts, but when the large volume of extract was concentrated to ten ml. a halo 1/2 inch in diameter was produced (Table 8).

Table 8. Effect of diethyl ether extracts of natural soil on Erwinia carotovora and Helminthosporium sativum

Treatment of filter paper disks	E. carotovora	H. sativum
10 ml. extract	-	-
200 ml. extract	-	-
200 ml. extract concentrated to 10 ml.	+*	+

* Approximately 1/2" inhibition

At this point the extracting agent was tested for inhibitory impurities. Two hundred ml. of diethyl ether were concentrated to ten ml. and when tested against E. carotovora was found to give a similar degree of inhibition as the concentrated diethyl ether extract of the soil. Upon triple distilling the diethyl ether, the inhibitory impurities* were apparently lost as the tester, E. carotovora, grew uninhibited. At the same time, however, there was no inhibition of the testers, E. carotovora and H. sativum, when extracts of the soil were made using triple distilled diethyl ether (Table 9).

* Probably sulfuric acid.

Table 9. Effect of triple distilled diethyl ether extracts of natural soil on Erwinia carotovora and Helminthosporium sativum

Treatment of filter paper disks	E. carotovora	H. sativum
10 ml. extract	-	-
200 ml. extract concentrated to 10 ml.	-	-

Discussion

It is probable that if toxic substances are present in the soil they occur in such small quantities they are not detected by the methods used.

Effect of Diethyl Ether on the Fungitoxicity of Soil

Previous investigations

Exposing soils to such chemical vapours as those of ethane oxide, ether, propylene oxide, and chloroform has been reported to greatly reduce toxicity (Dobbs and Hinson, 1953; Greig-Smith, 1915; Neilson-Jones, 1941; Park, 1956; Stover, 1958). It was noted by Park (1956) in one investigation that fourteen days after the treatment with either propylene oxide or ether the toxicity was restored in the soil.

Materials and methods

A glass column (1" x 12" long) was filled with brown soil and lightly packed. Sufficient triple distilled diethyl ether was added to moisten the column of soil. Both ends of the column were then corked with rubber stoppers and left for two days. Fifty ml. portions of the

soil were removed from the column and placed in each of two Petri dishes and most of the diethyl ether vapours were permitted to dissipate. The soil plates were then saturated with water and one was steam sterilized for one hour. A third plate was prepared by placing a fifty ml. volume of untreated soil in a Petri dish and saturating it with water. A filter paper disk and four peptone-agar blocks were placed on top of each soil plate. The agar blocks were inoculated immediately with conidia of Helminthosporium sativum. Five days later all three soil plates were again tested for toxic properties by inoculating fresh agar blocks with freshly prepared inoculum of H. sativum.

Results

The diethyl ether treated soil did not inhibit the germination of conidia of Helminthosporium sativum on agar blocks (Table 10). The sterilized soil, also did not inhibit germination of conidia of the tester, but, as was expected, the natural soil did.

Table 10. Effect of diethyl ether treatment of soil on its toxicity towards Helminthosporium sativum

Treatment of soil	Germination of conidia
Diethyl ether soaked	++++*
Diethyl ether soaked then sterilized	++++
Untreated	+**

* 85 - 100% germinated
** 15 - 25% germinated

Five days later, however, the diethyl ether treated soil had regained its toxicity as determined by the degree of inhibition of conidial germination of H. sativum, which was similar to that of the untreated soil. The sterilized soil was not toxic to the test organism.

Discussion

It would seem that the diethyl ether treatment of the soil did not effect complete sterilization but simply inactivated the microorganisms in it for a short time. This assumption seems probable since toxicity was restored in the diethyl ether treated soil after it was remoistened and allowed to stand in the covered Petri dish five days.

Effect of Desiccation and Steam Sterilization on Fungitoxicity of Soil

Previous investigations

The loss of fungitoxic properties by steam sterilization and temporary loss by desiccation of soils has been reported by Dobbs and Hinson (1953), Greig-Smith (1915), Neilson-Jones (1941), and Stover (1958). Fulton (1920) reported the long persistence of Pseudomonas citri Hasse (Xanthomonas citri (Hasse) Dowson) in very small numbers in soils held in an air-dry condition, but the organism "seemingly suffered prompt extinction" when water was again added.

Materials and methods

Eight soil plates, using brown soil, were prepared in the usual manner (described in Part A) except that no water was added to four of the soil plates until after a three-week desiccation period at room temperature. The other four plates were kept moist for the same period. At the end of the period all plates were moistened to the saturation point and two of each set were sterilized in an autoclave for one hour. A filter paper disk and four peptone-agar blocks were placed on the soil of each soil plate and inoculated immediately with conidia of Helminthosporium sativum. One week later the same soil plates were tested again by inoculating fresh agar blocks with the same tester.

Results

The desiccated and steam sterilized soils were similar in their effect in that they showed no apparent inhibitory properties as measured by the germination of conidia of Helminthosporium sativum on agar blocks (Table 11). One week later, however, the apparent loss of fungitoxicity was restored in the desiccated remoistened soil plates but not in the steam sterilized soils (Table 12).

Table 11. Effect of desiccation and steam sterilization of soil on its toxicity towards Helminthosporium sativum

Treatment of soil	Unsterilized	Sterilized
Desiccated for three weeks	++++*	++++
Kept moist for three weeks	+**	++++

* 85 - 100% germination
** 15 - 25% germination

Table 12. Effect of remoistened desiccated soil on its toxicity towards Helminthosporium sativum

Treatment of soil	Unsterilized	Sterilized
Remoistened desiccated soil	+**	++++*
No further treatment	+	++++

* 85 - 100% germination
** 15 - 25% germination

Discussion

Air drying of the soils brought about a temporary loss of fungitoxicity as measured by the tester H. sativum. This would seem to be best explained by assuming that air drying reduced, temporarily, the activity of the soil microorganisms. The restoration of toxicity of the soil was demonstrated a week after the first test probably resulting from a renewal of activity of the soil microorganisms.

Effect of Hot Water Treatment of Natural Soils on Fungitoxicity and Microbial Populations

Effect of hot water treatment of soils on fungitoxicity

Previous investigations

Some investigations on the effect of hot water treatment of soils on fungitoxicity have been made by Dobbs and Hinson (1953), Neilson-Jones (1941), and Park (1956). They reported that treating soils in a water bath, using temperatures between 50 and 60° C. for one hour, greatly reduced the fungitoxicity. Park (1956) noted that toxicity was restored in soil fourteen days after treatment with hot water.

Materials and methods

Sixteen soil plates, using shallow Petri dish lids and black soil, were prepared in the usual manner. After saturating the soil with water the soil plates were placed in about 3/8" of water in a water bath set at 60° C. for varying periods of time ranging from 0 - 120 minutes. Immediately after the hot water treatment sterilized filter paper disks and peptone-agar blocks were placed on the soils and inoculated with conidia of Helminthosporium sativum.

Results

There was a rise in the germination of H. sativum conidia on agar blocks from 17.5 - 39.8% as the time of the hot water treatment of the soil increased from 0 - 120 minutes (Table 13).

Table 13. Effect of duration of hot water treatment of natural soil on the germination of Helminthosporium sativum conidia

Time of hot water treatment of soil in minutes	Replicates				
	1	2	3	4	Ave.
0	20*	15	20	15	17.5
10	42	23	20	18	25.8
20	38	40	22	15	28.8
120	40	40	37	42	39.8

* No. of conidia germinated out of 100

Effect of hot water treatment on microbial populations of the soil

Materials and methods

Immediately after the hot water treatment of the soil plates, a ten gram (air dry basis) sample of soil from one replicate of each treatment was placed in each of four flasks (300 ml.) containing one hundred ml. of sterile distilled water and shaken five minutes on a Ross-Kershaw shaker apparatus. One ml. of this suspension was then transferred with a one ml. sterile pipette to a second flask containing one hundred ml. of sterile distilled water and shaken by the same method for five minutes. Immediately after the shaking a one ml. of the final dilution (1:1000 of soil to water) was added to each of four sterile test tubes containing previously melted and cooled (to 45° C.) water agar (1.5%). These soil dilution water agar suspensions were shaken by hand for a few moments and poured individually into separate sterile

Petri dishes previously prepared by pouring ten ml. of sterile water agar (1.5%) into each and allowing the agar to set thus providing a smooth surface for the second layer. The microbial count was taken four days later and was based on the number of colonies which developed. After the colony counts were taken five ml. of nutrient (Czapek's) agar were poured into each plate and the number of colonies were observed five days later.

Results

The number of colonies which developed on water agar greatly decreased with increased length of time the soil plates were treated with hot water (Table 14).

Table 14. Effect of duration of hot water treatments of natural soil on microbial populations

Time of hot water treatment of soil in minutes	Replicates				
	1	2	3	4	Ave.
0	3840*	2400	3000	2600	2960
10	520	360	250	400	383
30	176	125	75	180	139
120	20	10	6	14	13

* Figures in thousands

Five days after the nutrient agar was added, the same dilution plates which previously had low colony counts were examined and the number of colonies appeared to have increased to a level similar to that on dilution plates of untreated soil.

Discussion

Soil toxicity and microbial activity decreased as the length of the hot water treatment was increased. The reduction in microbial activity was much greater than would be expected from the loss in soil toxicity. There are several possible explanations for this. For example, soil toxicity may not be directly correlated with microbial numbers or it may be dependent on a small proportion of microorganisms relatively more resistant to the hot water treatments.

The effect of nutrient addition in causing a relatively greater increase in colony numbers on the treated plates is difficult to explain. Water agar may be selective, favouring microorganisms generally more sensitive to the hot water treatments. It is also possible that these treatments merely caused a temporary inactivation of a portion of the soil microflora. Soil toxicity may be dependent, therefore, on active soil microorganisms rather than on accumulated toxic substances.

Effect of Ultraviolet Light Treatment of Soil on its Toxic Properties

Previous investigations

Nutman (1959) reported that when soil extracts were treated with ultraviolet light their fungitoxic properties were lost.

Introduction

In preliminary investigations it was noted that peptone-agar blocks after being in contact with natural soil for a day or more possessed toxic properties towards conidia of Helminthosporium sativum, and that these properties were retained in the blocks at least twenty-four hours after transferring them to sterile glass slides.

To determine whether the presence of microbes on or in the agar blocks referred to above was responsible for their toxic properties, the following experiment was designed in which ultra-violet light was employed to sterilize the blocks after they had been in contact with natural soil.

Materials and methods

Soil plates, prepared and moistened as described previously, were incubated for four days. Sterile peptone-agar blocks were then placed on sterile filter paper circles on the soil surface. After incubation for 1 day the agar blocks were treated as indicated in Table 15. The blocks were then inoculated with conidia of H. sativum and, following incubation for a further 20 hours, the per cent germination recorded. Irradiation was carried out by exposing the blocks to a 15 watt, 18" germicidal ultraviolet lamp for 10 minutes at a distance of 2-1/2". Previous studies had indicated that sterilization was sometimes incomplete with less than 5 minutes irradiation and that there was no detrimental effect on germination of H. sativum conidia from periods of irradiation up to 15 minutes.

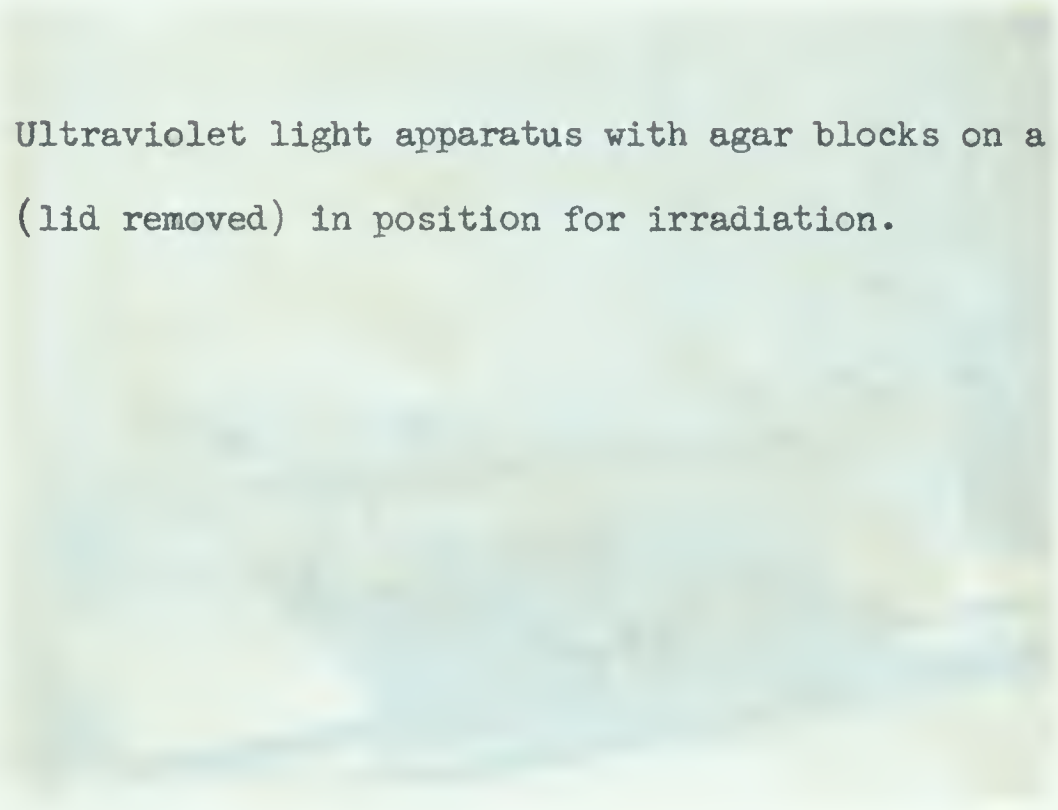


Figure 6. Ultraviolet light apparatus with agar blocks on a soil plate (lid removed) in position for irradiation.

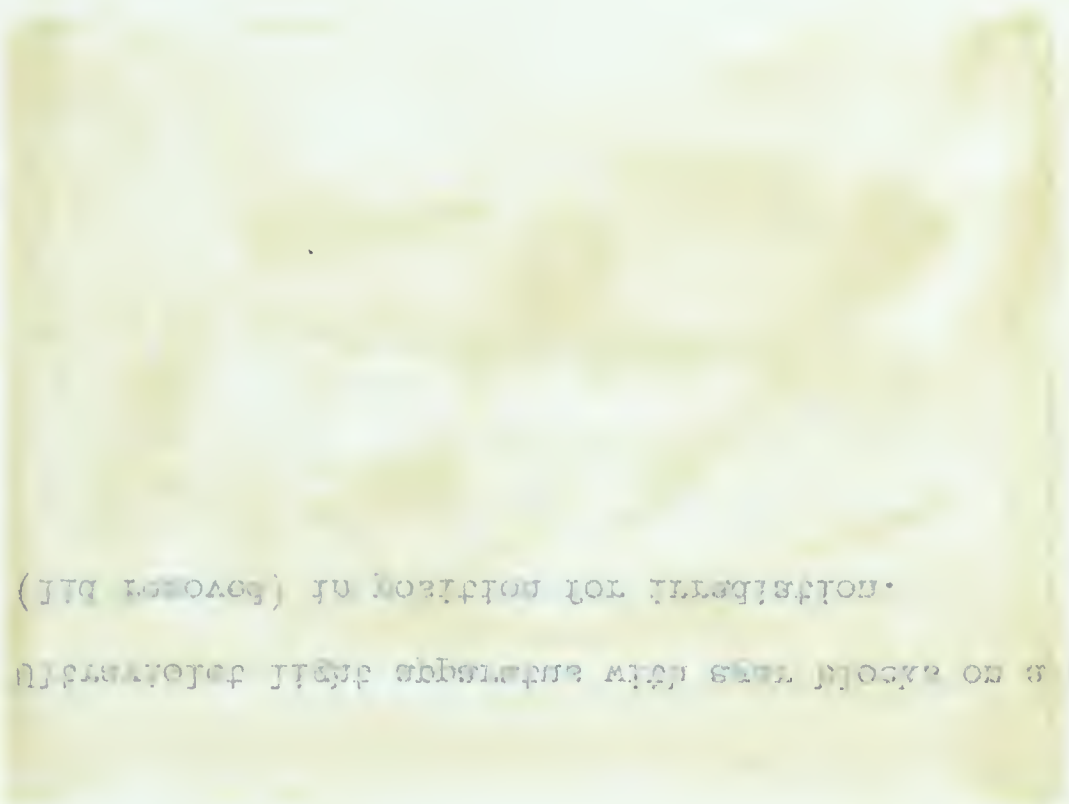
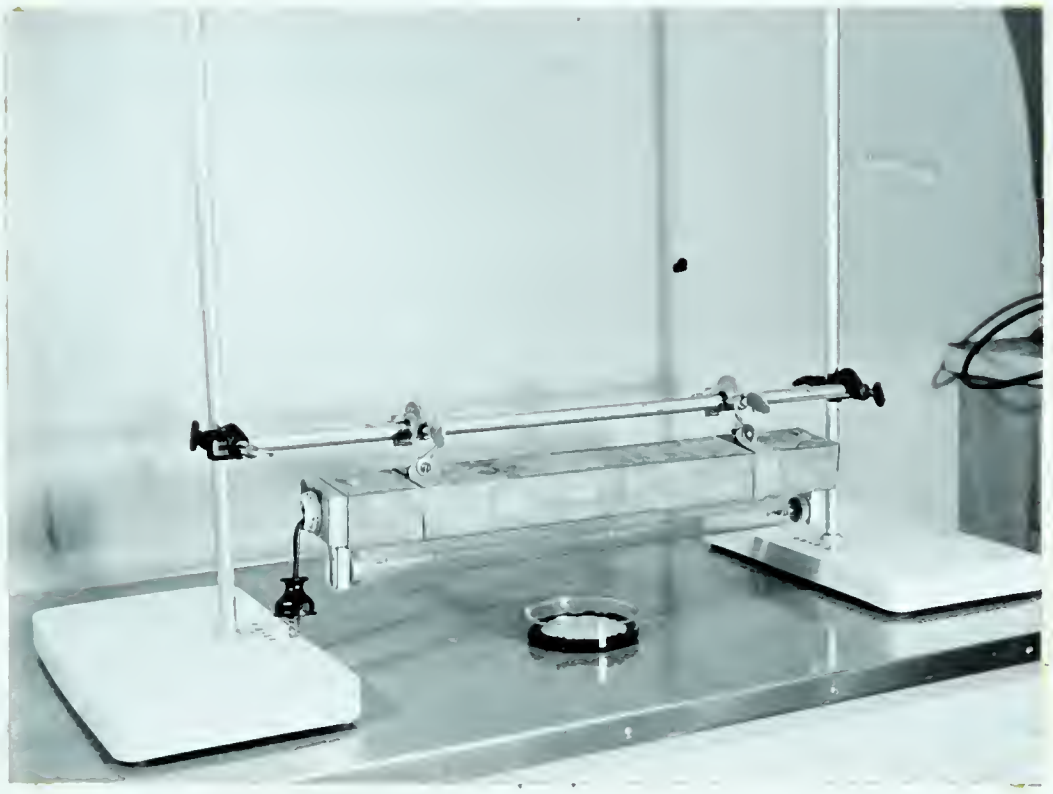


Figure 5. Illustration of right angle antenna with open block on a coil lead.
(Not to scale) Position for termination.



Results

Of the two sets of agar blocks on glass slides, the set not irradiated retained most of its toxic properties, whereas the set irradiated apparently lost its toxic properties as tested by the germination of conidia of H. sativum. There was some loss of toxicity when the agar blocks on the soil were irradiated and left on the soil as compared with the non-irradiated, but when the agar blocks were irradiated and immediately transferred to a glass slide there appeared to be some toxicity either retained or developed in the blocks (Table 15).

Table 15. Effect of ultraviolet light on fungitoxicity of peptone-agar blocks on natural soil and on glass slides transferred from the soil using Helminthosporium sativum as a tester

	Replicates				
Treatment of agar blocks	1	2	3	4	Ave.
Not irradiated (on glass slides)	25*	23	28	18	23.5
Irradiated (on glass slides)	90	80	85	80	83.8
Not irradiated (left on soil)	0	3	0	1	1
Irradiated (left on soil)	8	20	15	15	14.5
Irradiated on soil and transferred to glass slide	80	75	80	78	78

* % germination

N.B. Previous studies of ultraviolet light treatment of agar blocks in contact with sterile soil and on water agar had shown that such treatments had no apparent effect on the germination of conidia of the test organism, sown on the irradiated agar blocks (see p. 42, l. 10).

Discussion

When the agar blocks were transferred from the soil to glass slides and sterilized by ultraviolet light no appreciable toxicity to

the tester could be demonstrated. This loss of toxicity parallels the loss of microbial life in the agar blocks. The probable explanation for the slight inhibition of the tester on the blocks irradiated on the soil and then transferred to the glass slide is that some microorganisms of the soil probably contaminated the blocks before the transfer was made. The non-irradiated blocks left on the soil, as expected, almost totally inhibited the tester, but when similar blocks were transferred to glass slides there was some loss in toxicity. This may be accounted for by the limited nutrient in the blocks with the resultant decrease in microbial activity.

It appears that living microorganisms rather than non-living substances present in the natural soils studied are responsible for inhibition of the tester, H. sativum.

Miscellaneous Notes

Observation of Abnormalities of Germ Tubes and Lysis of Mycelium of Helminthosporium sativum on Peptone-Agar Blocks on Natural Soil

Previous observations

Lysis by soil microorganisms of fungal parts on nutrient agar plates has been observed by several investigators (Carter and Lockwood, 1957b; Meredith, 1943; Park, 1956; Skinner, 1953). Lysis of mycelium and sometimes of conidia of various fungi has also been observed when the test fungi have been in contact with natural soil (Chinn, 1953; Lockwood, 1959; Park, 1955, 1956; Stevenson, 1956).

Present observations

When peptone-agar blocks were inoculated with H. sativum immediately after placing the blocks on natural brown soil, usually about twenty-five per cent of the conidia germinated. Of the germinated conidia some germ tubes were found to be abnormally formed (Figures 7, 8), but the majority appeared to develop normally for a limited time. After five days, however, considerable lysis of germ tubes and mycelium was observed (Figure 9). After ten days, germination counts appeared to be almost negative as many of the germ tubes were completely lysed.

Discussion

From observations made in these investigations it would seem that lytic action plays a very important role in preventing microbial development in natural soil. What factors bring about the disintegration of fungal parts in the soil are largely not known. The present studies indicate the need for further study in this field.

Figure 7. Abnormal germination of a conidium of Helminthosporium sativum on an agar block on natural soil.

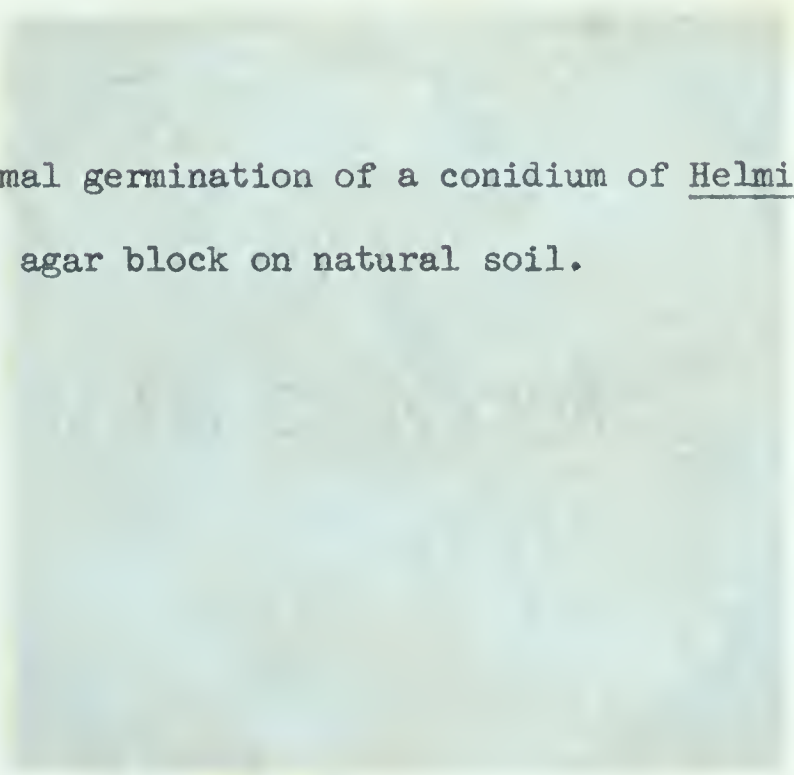


Figure 8. Abnormal germination of a conidium of Helminthosporium sativum on an agar block on natural soil.

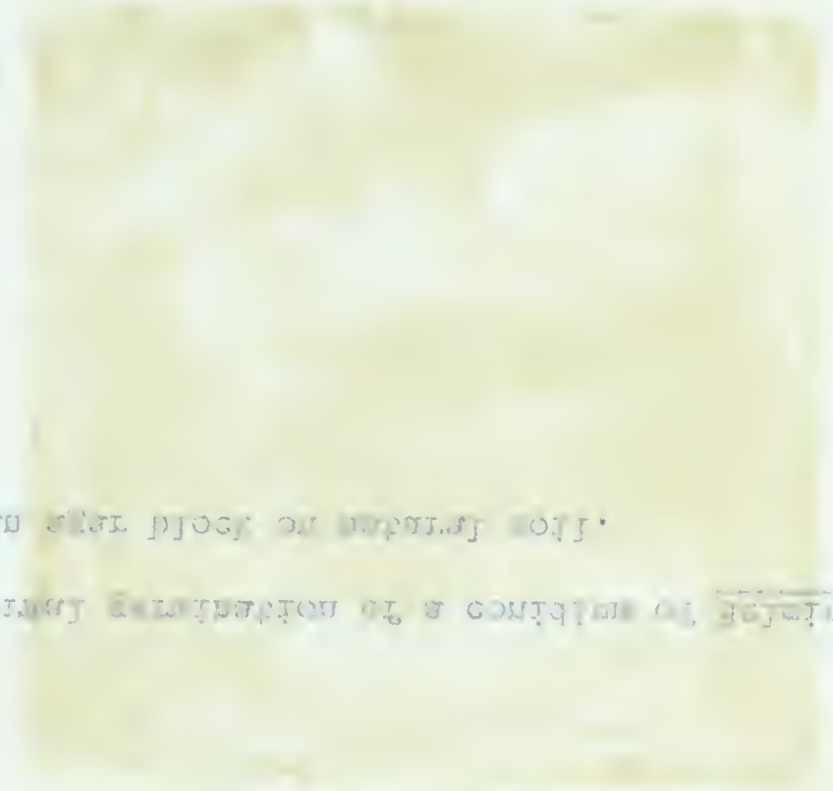


Figure 6. Abnormal segmentation of a colonic polyp [illegible] multiple

.flow between adjacent tissue as no

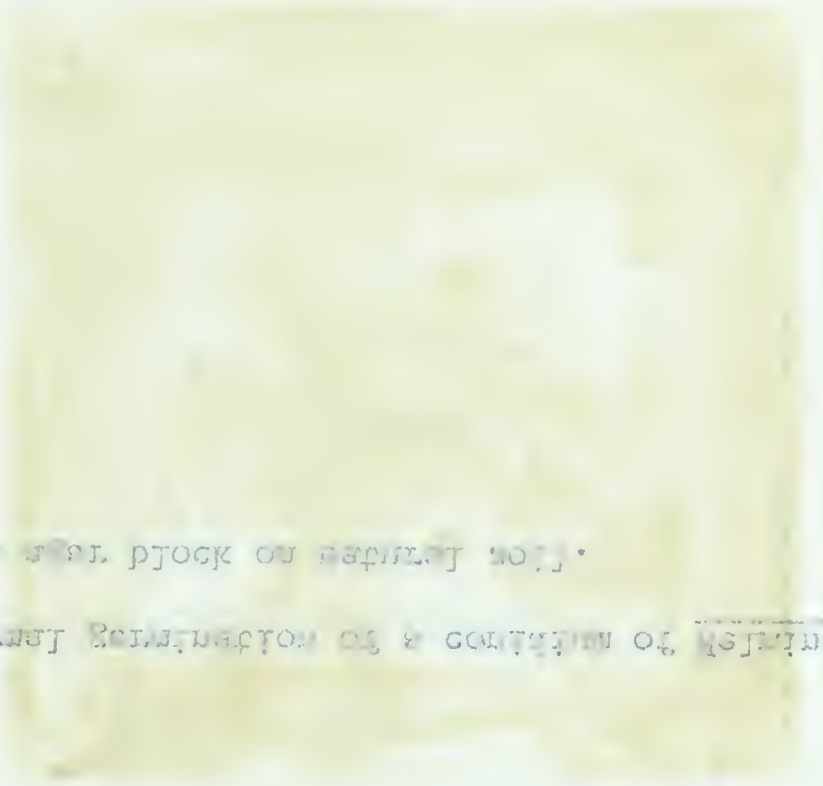
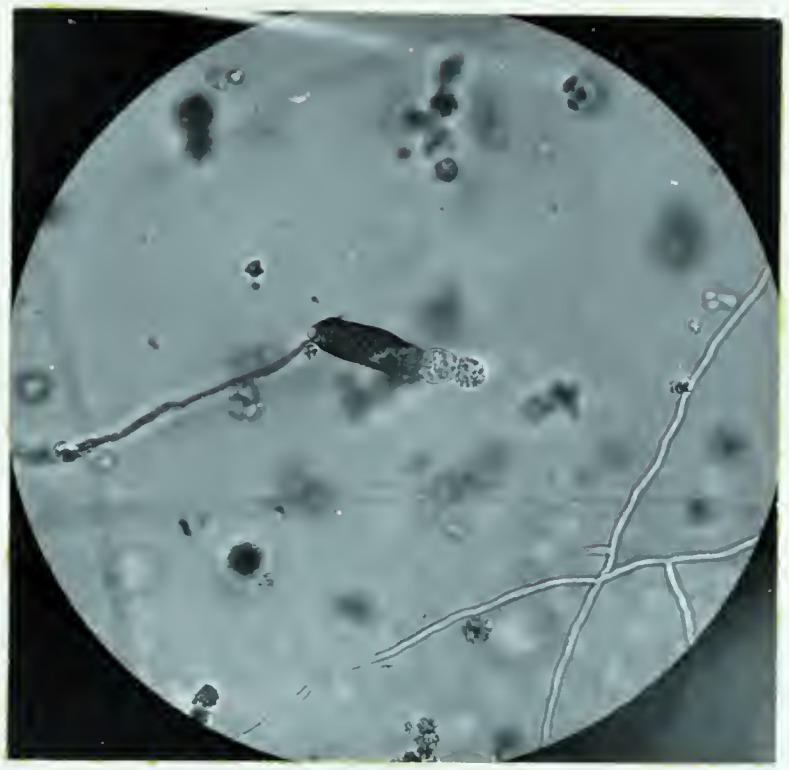
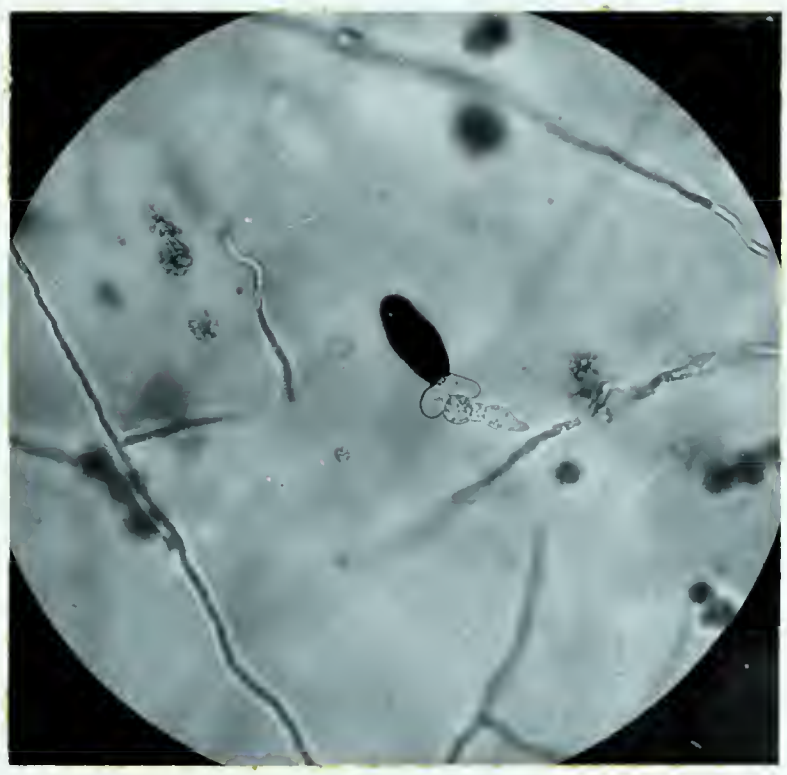


Figure 7. Abnormal segmentation of a colonic polyp [illegible] multiple

on as well block on segment wall.





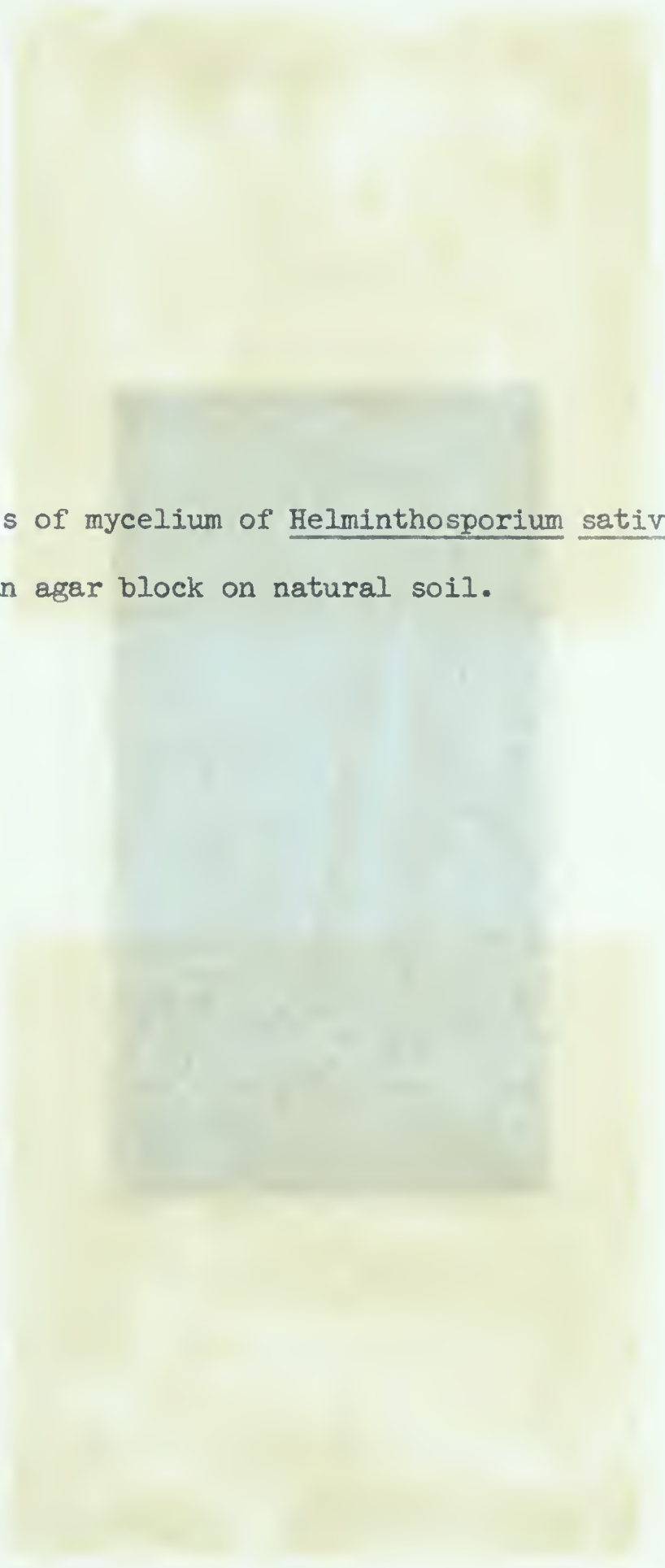
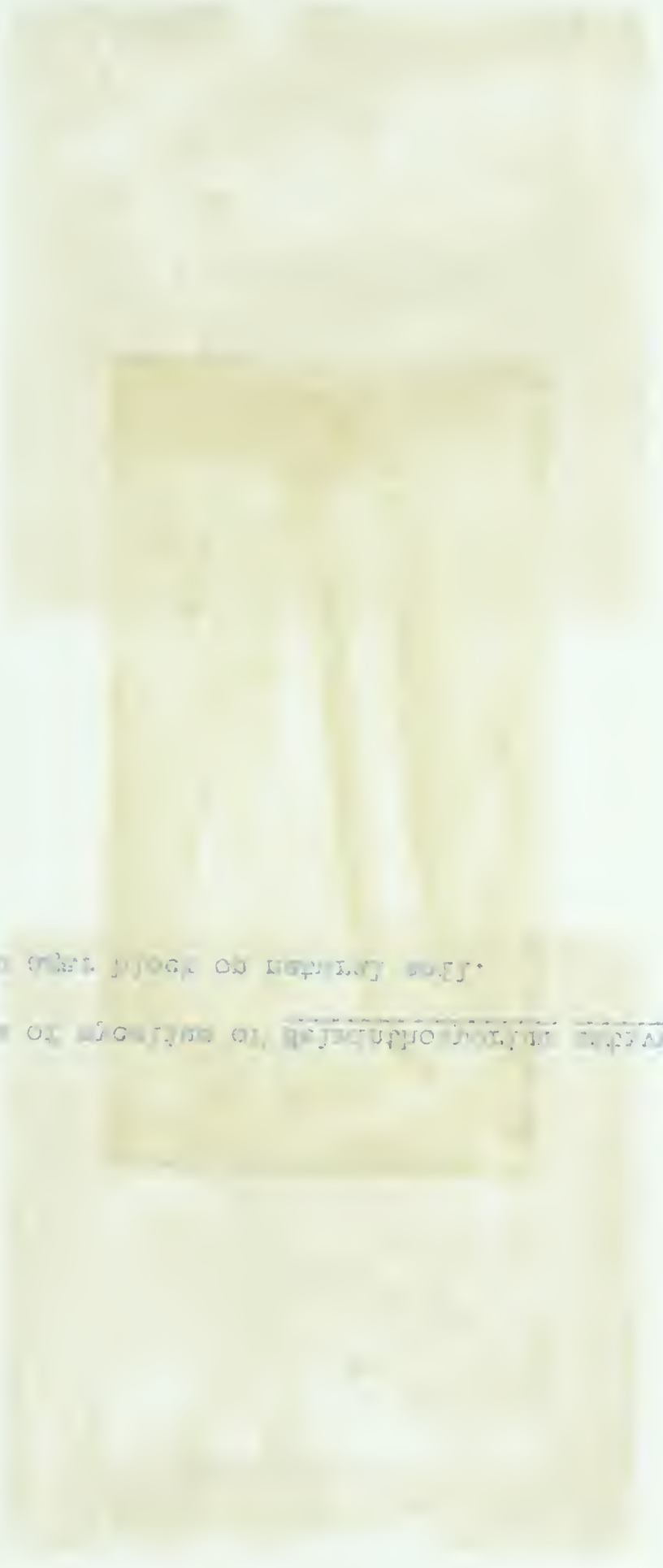


Figure 9. Lysis of mycelium of Helminthosporium sativum on an agar block on natural soil.



Discussion

Toxicity, as encountered in these studies, appears to be due to the antagonistic activity of living soil microorganisms. This conclusion is based on the inability to separate soil toxicity from soil microorganisms. Any factor which affected the activity of the soil microorganisms also affected toxicity of the soil. It does not seem likely that if toxic substances are present in the soil they are accumulated or remain effective for any length of time.

Jefferys (1952) has shown that several antibiotics produced in culture by certain soil microorganisms are quickly inactivated when introduced into the soil. Several explanations have been offered to account for this inactivation. Two of them are the adsorption of the antibiotics to soil particles and the inactivation of antibiotics by soil microorganisms. This may account for the failure of antibiotics, if produced, to accumulate in the soil. However, another interesting aspect is the apparent absence of toxicity in agar blocks which had been in the presence of natural soil and then made sterile by a germicidal lamp. There were no living soil microorganisms present to break toxic substances down and not likely any soil particles present to adsorb them.

The observance of lysis on some of the mycelium and the complete disappearance of most germ tubes and mycelium a short time later suggests that the factor or factors which bring about lyses

may be very important, at least under some conditions, in causing microbial inhibition.

Garrard and Lochhead (1938) state that, "The idea that toxins are responsible for antagonism of different organisms is probably the most frequently advanced theory." Many investigators have used the term "soil toxicity" and more specifically "fungi-toxins" and "bacteriotoxins" to explain the inhibition of micro-organisms entering the soil and in certain extracts made from the soil. In this investigation it appears that microbial inhibition is caused in the main by the activities of living soil micro-organisms and not by toxic substances present in the soil. If the term "toxins" is used to mean those substances produced by one or more microorganisms which adversely affect other microorganisms, then the term "soil toxicity" does not properly describe the type of inhibition encountered.

GENERAL SUMMARY

1. Twenty-four samples of soils representing the four major soil zones of Alberta, namely, Brown, Dark Brown, Black, and Grey Wooded, were tested for toxicity towards several plant pathogenic fungi, bacteria and actinomycetes, and one saprophyte in each group.

2. All of the plant pathogens tested, namely, Helminthosporium sativum, Claviceps purpurea, Fusarium oxysporum f. cubense, Erwinia carotovora, Agrobacterium tumefaciens, Corynebacterium sepedonicum, and Streptomyces scabies, and three soil saprophytes, namely, Trichoderma viride, Bacillus subtilis, and Streptomyces antibioticus were inhibited on agar blocks on natural soil.

3. It is noteworthy that not only fungi but bacteria and actinomycetes tested were inhibited by the natural soil.

4. No consistent differences in fungitoxicity of soils collected at different seasons of the year were detected by the methods employed.

5. It was found that when natural soils were treated with hot water, diethyl ether, or air dried they temporarily lost their toxic properties as measured by the tester H. sativum.

6. Steam sterilized soils did not appear to be toxic to any of the testers used.

7. Ultraviolet light appeared to destroy toxicity.

8. Water and diethyl ether extracts of the soil were not toxic to H. sativum and E. carotovora.

9. Lysis of mycelium and germ tubes of H. sativum was observed and was thought to play an important role in the microbial inhibition of that fungus in natural soil.

10. Antimicrobial activity of living soil microorganisms appeared to be the principal basis of soil toxicity.

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