A STUDY OF A HELMINTHOSPORIUM DISEASE OF PORTULACA OLERACEA L. AND PORTULACA GRANDIFLORA L.

A

thesis

by

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INTRODUCTION

The Helminthosporium disease of Portulaca oleracea L. (purslane) was first discovered and described as it occurred on purslane by Rader (39) in 1948. He proved that the disease was due to an unknown pathogenic fungus which he described briefly and named Helminthosporium portulacae nov. sp.

In 1940, a diseased rose moss specimen from a private garden in Gananoque, Ontario, was sent to the Central Laboratory of the Division of Botany and Plant Pathology, at Ottawa, Ontario, for diagnosis. Various fungi were found fruiting on the specimen, but an unfamiliar one seemed to predominate. This organism was not isolated, but it is now believed that it probably was H. portulacae.

In 1949, when the writer first became attached to the Division, another diseased rose moss specimen, apparently from the same garden at Gananoque, was again sent to Ottawa, The same unfamiliar organism was found fruiting on the seed capsules of the diseased plant, and numerous isolations yielded a fungus which resembled H. portulacae as described by Rader (39). Artificial inoculation tests on purslane proved it to be pathogenic to that species. That same year, Reeder (40) isolated H. portulacae from diseased purslane plants growing in field plots and in the greenhouse at the University of Saskatchewan, Saskatoon, Saskatchewan, and the writer isolated the fungus from both hosts in field plots and in a public

park at Ste. Anne de Bellevue, Quebec.

Purslane, a prolific, fleshy, annual herb, is a persistent nuisance in rich fields and gives much trouble in gardens despite the use of every known precautionary measure. It is well distributed throughout Canada and occurs in every province but Newfoundland (41). The rapidity with which it re-establishes itself from pieces of stem and leaves, and the large quantities of seeds it produces each year make it difficult and costly to control.

Rose moss is also a fleshy, annual herb. It produces colourful blooms continuously throughout the season and is used as an ornamental in borders and rock gardens. Although not of great economic worth, it is cultivated to some extent for its seed.

AIM AND SCOPE OF WORK

This disease appeared to be worthy of study for the two following reasons:

1) It is a very destructive disease and conceivably might become a problem to those growing rose moss. The destructive nature of its pathogen suggests that it might be utilized in biological control to kill out purslane.

2) The apparent capacity of the pathogen to kill host tissue so extensively and rapidly suggested that it might be a suitable organism to use in investigating the physiology of parasitism.

To thoroughly understand a parasitic plant disease it is essential to have a comprehensive knowledge of the parasite and the factors which affect the development of the disease it causes. Further, since the disease itself expresses the interacting behaviours of both organisms when they are closely associated, a study of the factors affecting this relationship is also essential.

Two dominant factors affecting the development and behaviour of host and parasite are temperature and moisture. They determine the success or failure of a parasite to infect the host, and influence the expression and course of development of the disease which it causes (13) (32) (33) (38).

These studies, which were attempted with the above considerations in mind, are the first to be conducted on this disease.

However, they are of a preliminary nature because of the limited amount of time spent in investigating the disease. The investigation was begun in the fall of 1949 and was concluded in the spring of 1951.

Since the studies include observations on the fungus and the disease itself, they fall naturally into two parts, the pathogen and the disease. PART I - THE PATHOGEN is a study of the fungus and of its development under various conditions, and PART II - THE DISEASE is a detailed study of the effects of the pathogen on the host, its parasitism, and the effects of various conditions on infection and development of the pathogen in the host.

REVIEW OF LITERATURE

Due to the fact that Helminthosporium portulacae and the disease it causes on purslane were described very recently, the original report of Rader (39), which was published in 1948, is the only literature dealing with the fungus and the disease. Reeder's report (40) of the fungus in 1949 was merely a note reporting the isolation of the fungus from diseased purslane. He made no mention of the disease itself. However, in the course of planning the experiments pertaining to the investigation of this Helminthosporium disease, literature concerning various techniques commonly employed in the study of a disease was constantly consulted. Some of the most useful literature on the methods employed follows.

Techniques commonly used to study germination of conidia, and the limitations of the techniques, are reviewed by Wolfe and Wolfe (50). These authors also discuss in detail the various factors affecting the process of conidial germination and germ tube development. Matters dealing with the relation of temperature and humidity to infection by certain fungi are discussed by Lauritzen (29). Papers by Brown (3) (4), Brown and Harvey (6), Dey (14) (15), Leach (30), and others (1) (12), relating to the various aspects of the physiology of parasitism of fungi were found invaluable for providing techniques to study the mode of parasitism of a fungus.

In this connection, the physiology of parasitism of that group of fungi classed as facultative parasites is comprehensively dealt with by Brown (5). Numerous other references on phytopathological methods, including staining techniques, were read, but no mention of them will be made at this point. Suitable reference to all the literature reviewed during the course of this investigation is made in the text.

PART I - THE PATHOGEN

MORPHOLOGY OF THE PATHOGEN

Conidia of the fungus were scraped off diseased purslane and rose moss plants collected at Ste. Anne de Bellevue, Quebec, and a diseased rose moss specimen sent from Gananoque, Ontario, into drops of water on a glass slide, and compared under the microscope. Although the general shape of the conidia from the three sources was the same, the number of septa in the conidia from purslane and rose moss showed considerable variability. However, there were no differences in septation, or very little at any rate, between the conidia from rose moss collected at Ste. Anne de Bellevue, and those on the same host from Gananoque. This is brought out by Table I.

Single conidia from each of the above plants were planted on potato dextrose agar in test tubes and incubated for 14 days at room temperature. After this period of time, the subsequent growth was examined macroscopically and with a dissecting microscope. The cultures of the three isolates were identical (see Figure 1), and were fruiting abundantly. For easy reference, the cultures from purslane and rose moss plants from Ste. Anne de Bellevue were designated A201 and A101 respectively, while that from the rose moss specimen from Gananoque was labelled G102.

TABLE I - Percentage Distribution* of Conidial Types of *Helminthosporium portulacae* Occurring on *P. oleracea* and *P. grandiflora* Collected at Ste. Anne de Bellevue, Quebec, and *P. grandiflora* from Gananoque, Ontario.

Conidial class	Source of Conidia		
	Ste. Anne de Bellevue		Gananoque
	P. oleracea	*P. grandiflora*	*P. grandiflora*
1-celled	0.5	-	-
2 "	2.0	2.0	1.0
3 "	2.5	2.5	1.0
4 "	3.5	3.0	1.5
5 "	3.5	4.5	3.0
6	8.0	15.0	17.5
7 "	13.0	19.5	25.0
8 "	14.0	29.0	26.5
9 "	15.5	15.5	14.0
10 "	13.5	7.0	8.0
11 "	11.5	0.5	2.5
12 "	6.5	1.5	-
13 "	3.0	9.0	-
14 "	2.0	-	-
15 "	0.5	-	-

*Based on the measurements of 200 conidia from each isolate.

Figure I - 14-day old cultures of H. portulacae isolates A101, G102, and A201 on potato dextrose agar showing identical growth. Each culture originated from a single conidium.

Conidia from each isolate were then examined and the characters noted are given in Tables II and III. The data show that on the same medium each of the isolates produces practically identical conidia. The minor differences which do exist between them might

TABLE II - Percentage Distribution* of Conidial Types from Isolates A101, G102, and A201 Occurring on Potato Dextrose Agar and Incubated at from 25°- 31°C.

Conidial class	Isolate A101	Isolate G102	Isolate A201
1-celled**	7.0	7.0	8.5
2 "	6.0	5.0	4.0
3 "	4.0	6.0	7.0
4 "	5.5	6.5	6.0
5 "	5.0	6.5	6.0
6 "	6.0	8.0	6.5
7 "	14.5	13.0	12.0
8 "	27.5	14.0	17.5
9 "	16.5	18.0	5.5
10 "	22.5	19.0	20.5
11 "	3.0	4.0	2.5
12 "	1.0	2.0	2.0
13 "	0.3	0.5	0.5
14 "	1.0	-	0.5
15 "	0.5	0.5	1.0

* Based on the measurements of 200 conidia from each isolate.

**The high percentage of non-septate spores was later found to be due to the effect of the high temperature of incubation employed in this trial.

TABLE III - Size of Conidia* of Helminthosporium portulacae Isolates A101, G102, and A201 in Microns.

Conidial class	Length			Width		
	A101	G102	A201	A101	G102	A201
1-celled	19.8	22.2	24.4	9.9	10.3	10.1
2 "	22.7	31.7	26.3	10.2	10.7	12.1
3 "	33.9	30.4	36.8	10.3	10.2	11.3
4 "	41.5	44.8	39.7	10.2	10.2	10.1
5 "	49.1	52.1	55.0	10.3	10.1	10.4
6 "	66.0	69.2	67.3	10.7	11.1	11.0
7 "	84.4	92.4	86.5	11.1	11.3	11.5
8 "	100.7	103.9	107.2	10.8	11.3	10.4
9 "	114.9	111.3	121.0	12.1	10.8	10.1
10 "	131.2	126.0	128.0	12.4	12.3	11.8
11 "	145.4	126.7	148.4	12.9	12.7	12.2
12 "	154.7	142.6	153.9	13.1	12.7	12.6
13 "	170.6	167.3	162.6	13.4	13.0	12.8
14 "	179.8	182.1	176.3	14.0	14.0	13.6
15 "	184.3	180.4	188.6	14.6	15.1	15.3

*Based on the measurements of at least 10 conidia in each class.

be due to the numbers examined being too small. Similar lesions were caused on both hosts when inoculated with the three isolates.

The results of all these comparative tests satisfactorily prove that isolates A201, A101, and G102 are identical. As this was found to be true, isolate A 201 was used in all subsequent experiments involving the fungus, unless otherwise stated.

The hyphae of the pathogen are septate and in culture are usually 5 - 7.3 μ in diameter, and vary from olive brown to brown in colour. In some cases, especially in old cultures, hyphae may measure as much as 10.4 μ. These larger hyphae have barrel-shaped cells and anstomose freely. Young hyphae anastomose infrequently. Anastomosing seems to be more prevalent in the mycelium which is at a considerable depth below the surface of the agar. Various types of hyphae are shown in Plate I, figs. 1 and 2.

A striking feature is the formation of knotty masses of swollen mycelium. These structures, which may be formed from a single coiling hyphal strand or from cells close to or involved in hyphal fusions, continue to increase in size and eventually become irregularly shaped to round sclerotia-like bodies or bulbils (see Plate I, figs. 3 - 5, and Fig. 2). The bulbils are hyaline to brown at first, but soon become black and measure 50 - 310 μ in diameter. The outer portion is composed of one or two layers of cells and when crushed the bulbils exude masses of oil droplets and other materials.

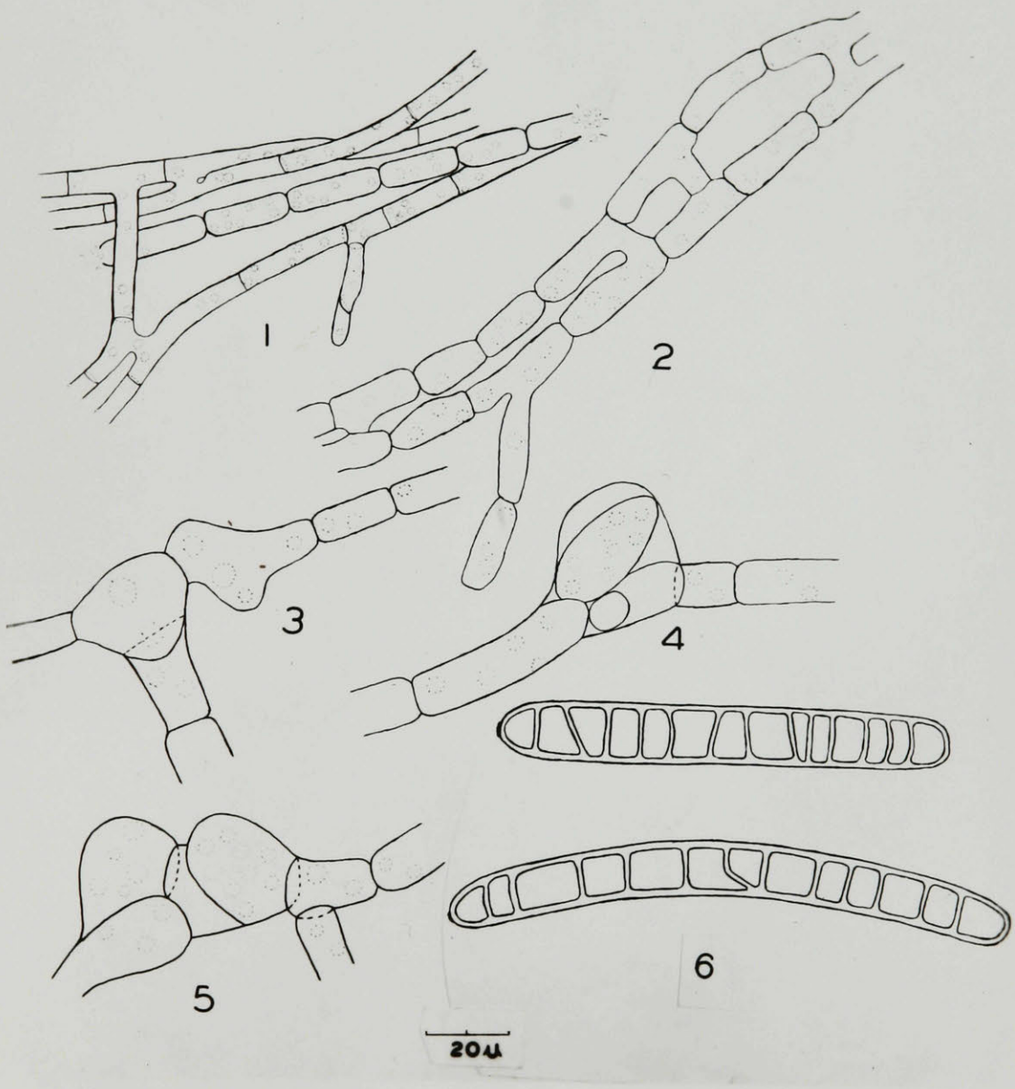
Plate I

Camera lucida drawings showing different features of H. portulacae.

figs. 1 and 2 - Different types of hyphae produced in culture.

figs. 3, 4, and 5 - Swollen hyphae in the process of bulbil formation.

fig. 6 - Two mature conidia.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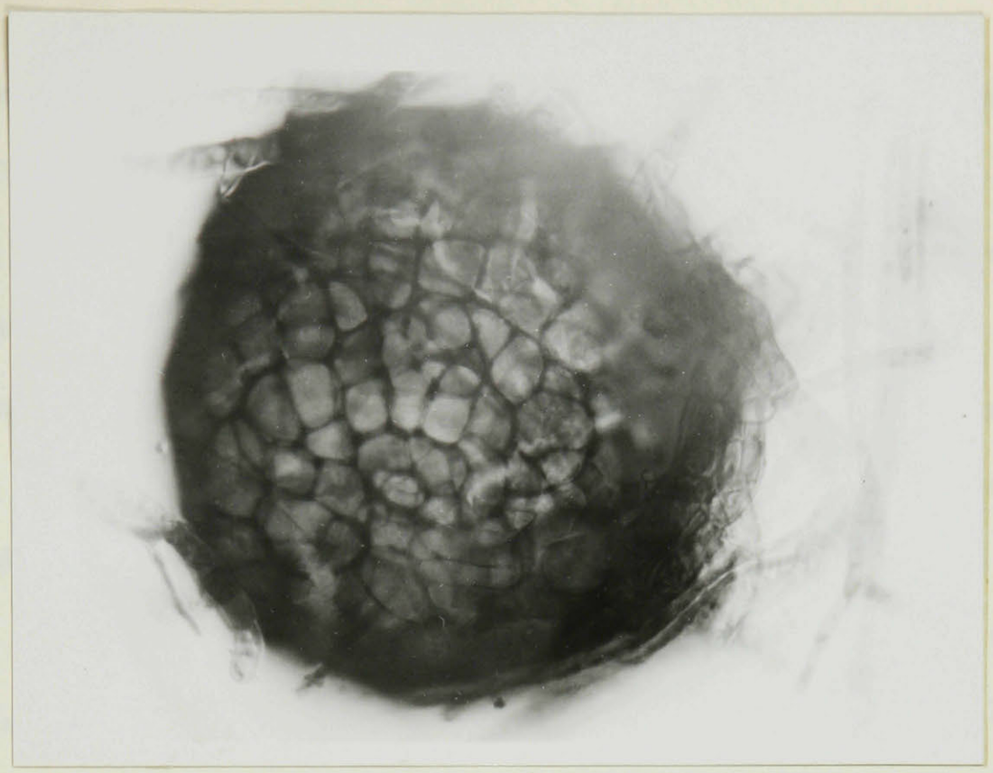


Figure 2 - Photomicrograph of a bulbil. Approximately x 300.

Bulbils also form abundantly on all diseased parts of the host plants. Although the exact function of the bulbils is not known, it would appear that they represent immature perithecia. These structures are not peculiar to this fungus alone as they have been described in other Helminthosporium spp.

Concerning the function of these structures in the case of other Helminthosporium spp., Drechsler (18) wrote:

> "..... in whatever species such sclerotia or abundant anastomoses (resulting in the production of complexes of lobulate segments) are found to occur, perithecia may be sought with considerable prospect of success."

Details of attempts made to discover the nature of these structures will be discussed later.

The conidia are elongate, straight or slightly curved, and rounded at both ends. They are brown in colour, smooth, and vary in size from 19.5 - 190 μ x 9.2 - 17 μ, averaging 117.4 x 13.2 μ (see Plate I, fig. 6). The number of cells in a conidium varies and seems to depend upon the medium on which the fungus was grown. On purslane they are 1 - 15-celled (mostly 7 - 10), on rose moss 9 - 12 (mostly 7 and 8), and on potato dextrose agar 2% 1 - 15-celled (mostly 10). The basal cell of a conidium is most characteristic because it bears the hilum or a scar region which marks the point of attachment of the conidium to the conidiophore. The hilum is visible as a dark protrusion situated on the outer wall at the base of the cell.

The conidiophores are septate, simple, sometimes branched, geniculate, and vary in size from 41.4 -208.4 μ x 5.2 - 7.8 μ. The point of attachment of the conidium is marked by a sunken black spot. The number of conidia on a conidiophore varies from 1 - 12, but in most cases four or five conidia are produced on a single conidiophore. The colour of the conidiophore is brown to dark brown and is easily distinguished from the mycelium by its shape and darker colour. Conidiophores are produced singly or in clusters on both hosts and on artificial media.

Newly formed conidia develop at the apex of the conidiophore, and seem to remain non-septate until their full size has been attained.

Cross walls then form and at first appear as thin lines extending right across the wall of the conidium. The conidium thus becomes divided into a number of segments, each of which deposits a wall of its own. A mature conidium is therefore composed of an exterior wall inclosing a number of individual cells.

Conidiophores continue to grow, causing the first formed conidia to be pushed aside and produce other conidia farther on. The process continues until a number of conidia are produced which eventually fall or are blown off.

Plate II (figs. 1 - 9) shows conidiophores and conidia at different stages of development.

Plate II

Camera lucida drawings of conidia and conidiophores at different stages of development. Arrows point to scars which mark the points of attachment of conidia previously attached to the conidiophore.

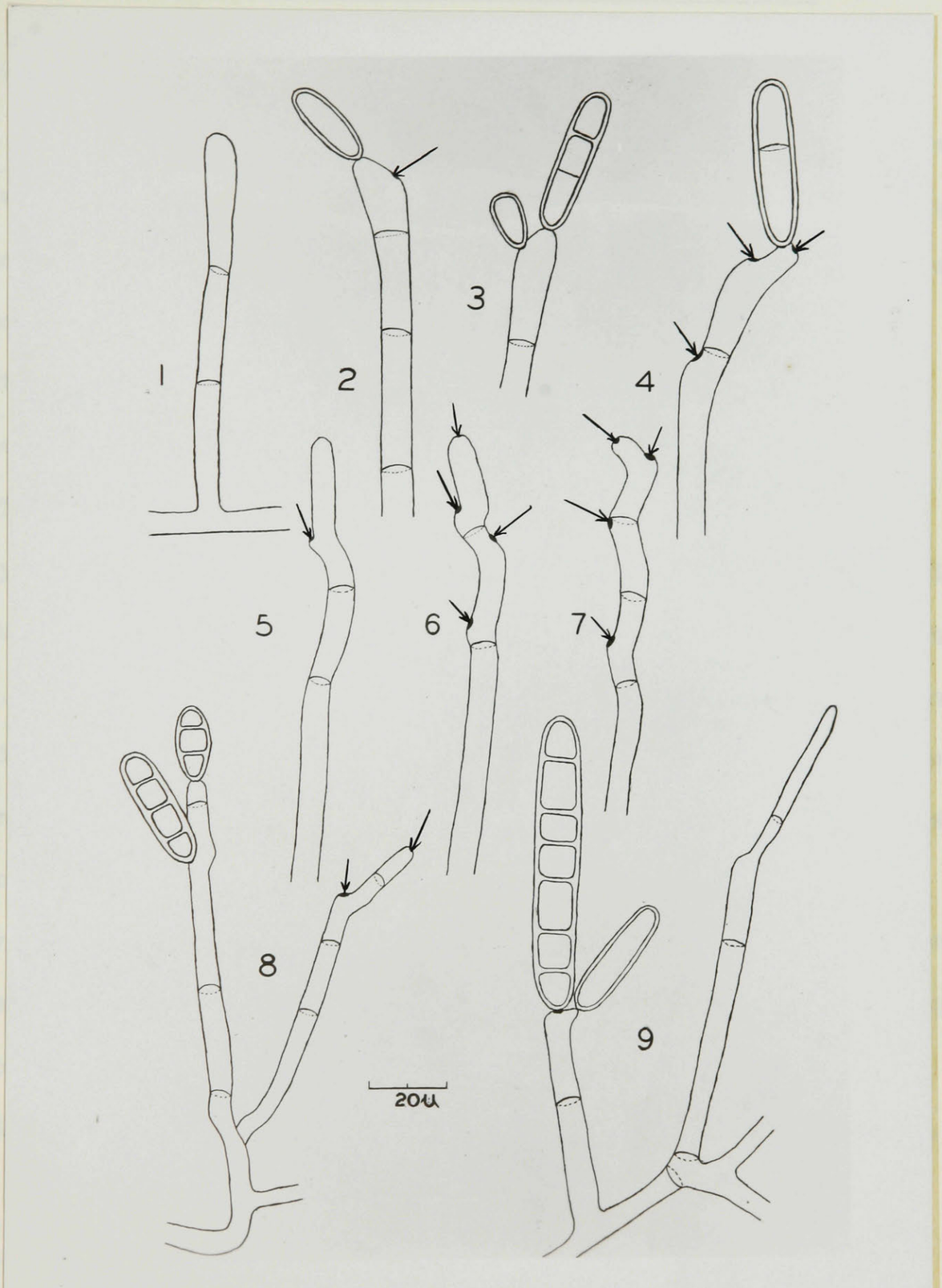
fig. 1 - A developing conidiophore.

figs. 2, 3, and 4 - Conidiophores bearing conidia at different stages of development. Note: conidiophores in figs. 3 and 4 each bear a conidium which had attained full size and is in the process of forming septa.

figs. 5, 6, and 7 - Conidiophores of different ages showing geniculations.

fig. 8 - A branched conidiophore. The one on the left bears two mature conidia.

fig. 9 - Two conidiophores. The one on the left bears one mature conidium and one young aseptate conidium. The one on the right has germinated to form a germ tube.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GERMINATION OF CONIDIA AND GERM TUBE DEVELOPMENT

Germination tests on conidia were made with two purposes in mind: (1) to determine the mechanism of germination and (2) to determine whether the optimum conditions for this process and the subsequent development of the germ tube correspond to the optimum for growth of the fungus. Experiments undertaken to determine the optimum conditions for the growth of the fungus on artificial media are discussed in the next section.

Two commonly used techniques for germinating conidia were employed in the first tests. These consisted of (1) placing conidia in drops of water on cover slips, and inverting them over Van Tieghem cells, and (2) placing conidia in drops of water on glass slides and covering them with cover slips. Although the percentage germination of conidia obtained with both these methods was high, the techniques had to be abandoned. With the first technique, the conidia in the hanging drops were difficult to count and draw because most of them settled to the bottom of the drop of water. With the other technique, some of the germ tubes did not develop normally and became swollen and distorted. This was no doubt due to lack of sufficient oxygen, since conidia near the edges of the cover slips developed normal germ tubes. In subsequent tests, conidia were mounted in drops of water on glass slides with no cover slips, and incubated in Petri dishes containing a little water, which served as moist chambers.

Prior to germination, the contents of the cells of the conidia are olive brown in colour and of a homogeneous structure. During the process of germination, the cytoplasm becomes granular and numerous round bodies form therein. These spherical bodies stain a brilliant red with Sudan III, indicating that they are globules of fat. These alterations appear first in the germinating cells.

Under favourable conditions, conidia of H. portulacae germinate within 15 or 30 minutes. Normal germination takes place from the apical end or from the hilum end, but more often from both ends. The intermediate cells never develop lateral germ tubes. However, if a developing germ tube perishes for some reason or other, the adjoining cell sends out a germ tube. This is not formed as a lateral projection, but thrusts its way through the dead end cell and out through the pore already formed by the first germ tube. The contents of this dead cell disappear, and the remains of the dead germ tube are sloughed off in the process (see Plate III, fig. 3).

In the very early stages of conidial germination, the cytoplasm at the tips of the terminal cells directly associated with the process takes on a clear shiny appearance and develops a round protrusion which presses against the rounded top of the cell wall (see Plate III, figure 1B). This portion elongates and gradually pushes its way through a pore which forms in the rounded distal end of the wall. The ragged appearance of the cell wall about the base

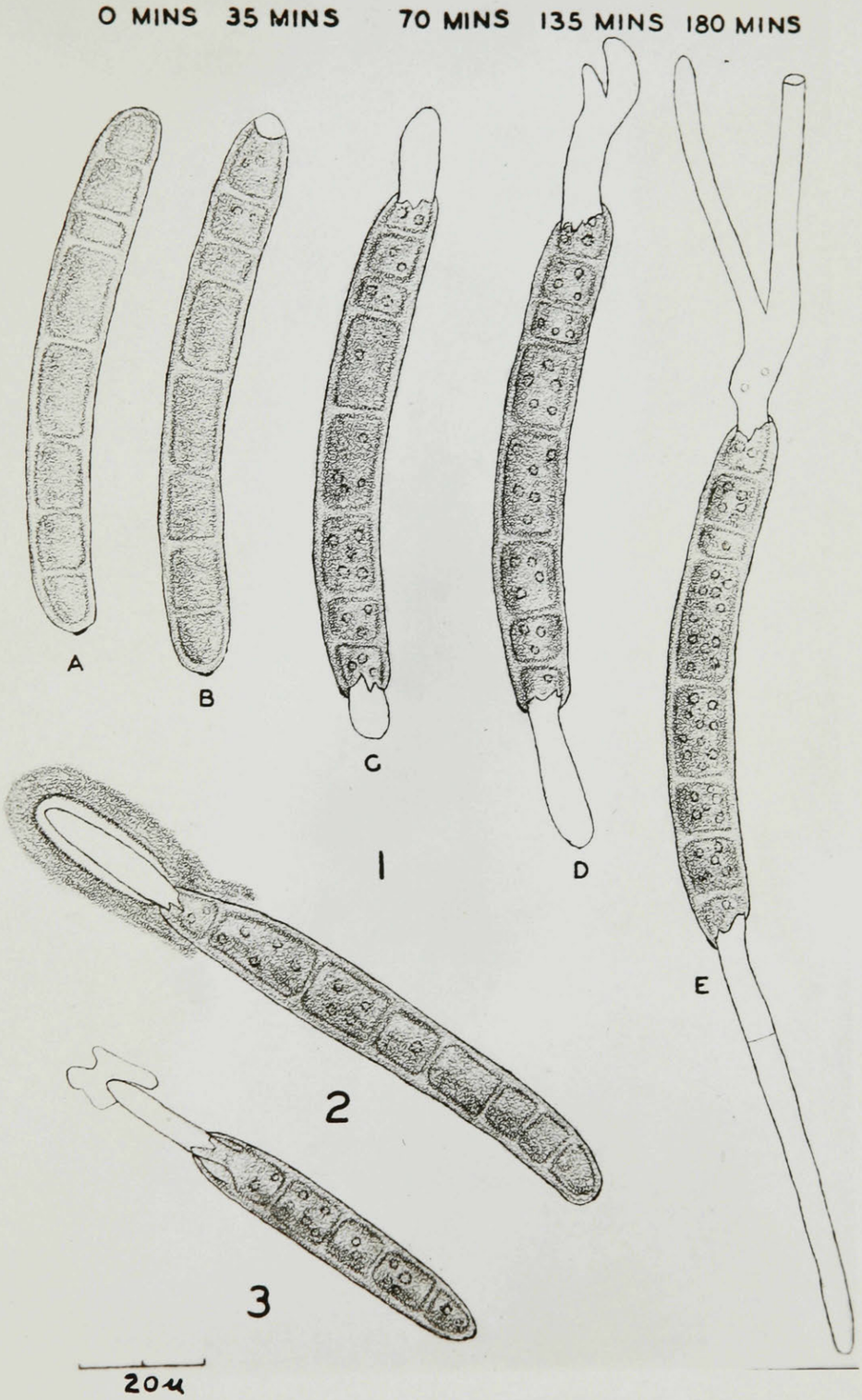
Plate III

Camera lucida drawings of germinating conidia.

figs. 1A to 1E - Various stages in the germination of a conidium in relation to time.

fig. 2 - Muscilagenous sheath surrounding a germ tube revealed by staining with Gentian violet.

fig. 3 - Germination of cell adjacent to an apical cell whose germ tube perished. Note dead remains of first germ tube.


0 MINS
35 MINS
70 MINS
135 MINS
180 MINS
A
B
C
D
E
1
2
3
20μ

of a germ tube suggests that the germ tube pushes its way through the pore in the cell wall by mechanical pressure. When the germ tube becomes one to one and a half times as long as the conidium, the first septum appears at some distance from the base of the germ tube. Various stages of germination of conidia are shown in Plate III, figs. 1A, 1B, 1C, 1D, and 1E. It was found that the length of the germ tube varied a great deal in the same length of time after setting up a germination test, depending upon the conditions during the test.

The developing germ tubes in contact with the hard glass surface were never seen to form appressoria. Young (52) also reported that Alternaria and Helminthosporium conidia failed to form these organs when they were germinated in water under cover glasses on glass slides. However, he was able to demonstrate gelatinous sheaths about the germ tubes in each case. These sheaths held the tubes firmly to the glass slides. Such sheaths about the germ tubes of Botrytis cinerea were also demonstrated by Blackman and Welsford (1).

Attempts were made to dislodge the germ tubes of H. portulacae from the glass surface by the force of the water from a tap of a laboratory sink. Since these failed, a sheath cementing the germ tube to the slide was indicated. The same technique employed by the above mentioned workers to demonstrate sheaths about germ tubes was therefore applied. The slides carrying the germ tubes were flooded with a very dilute aqueous solution of Gentian violet for 30 seconds and covered with a glass cover slip for observation. The slide and

germ tube stained a light purple colour, while a narrow zone around the germ tube remained unstained, indicating the presence of a sheath surrounding the tube. The sheath probably held the germ tube firmly to the slide (see Plate III, fig. 2).

In an attempt to discover whether nutrient substances influence appressorial formation, conidia were mounted in water and dry on glass slides coated with dried and fresh films of agar agar alone, and two nutrient agars composed of the juice of potatoes and dextrose, and the juice of purslane tissue. These were incubated in Petri dishes to which a little water was added to function as moist chambers. In all cases, the conidia germinated but no appressoria formed, which indicates that nutrient substances do not affect their formation.

Germination tests to determine the effect of temperature and free moisture on germination and germ tube development were made by mounting wet and dry conidia on glass slides held in the moisture saturated atmosphere of Petri dishes. Since only three temperature control chambers were available at the time, it was decided to operate them at 25°, 30°, and 35°C. The middle temperature corresponds closely to the optimum for fungal growth (see Plates V, VI, VII, and VIII). The results of the test are given in Table IV and are graphically illustrated in Plate IV.

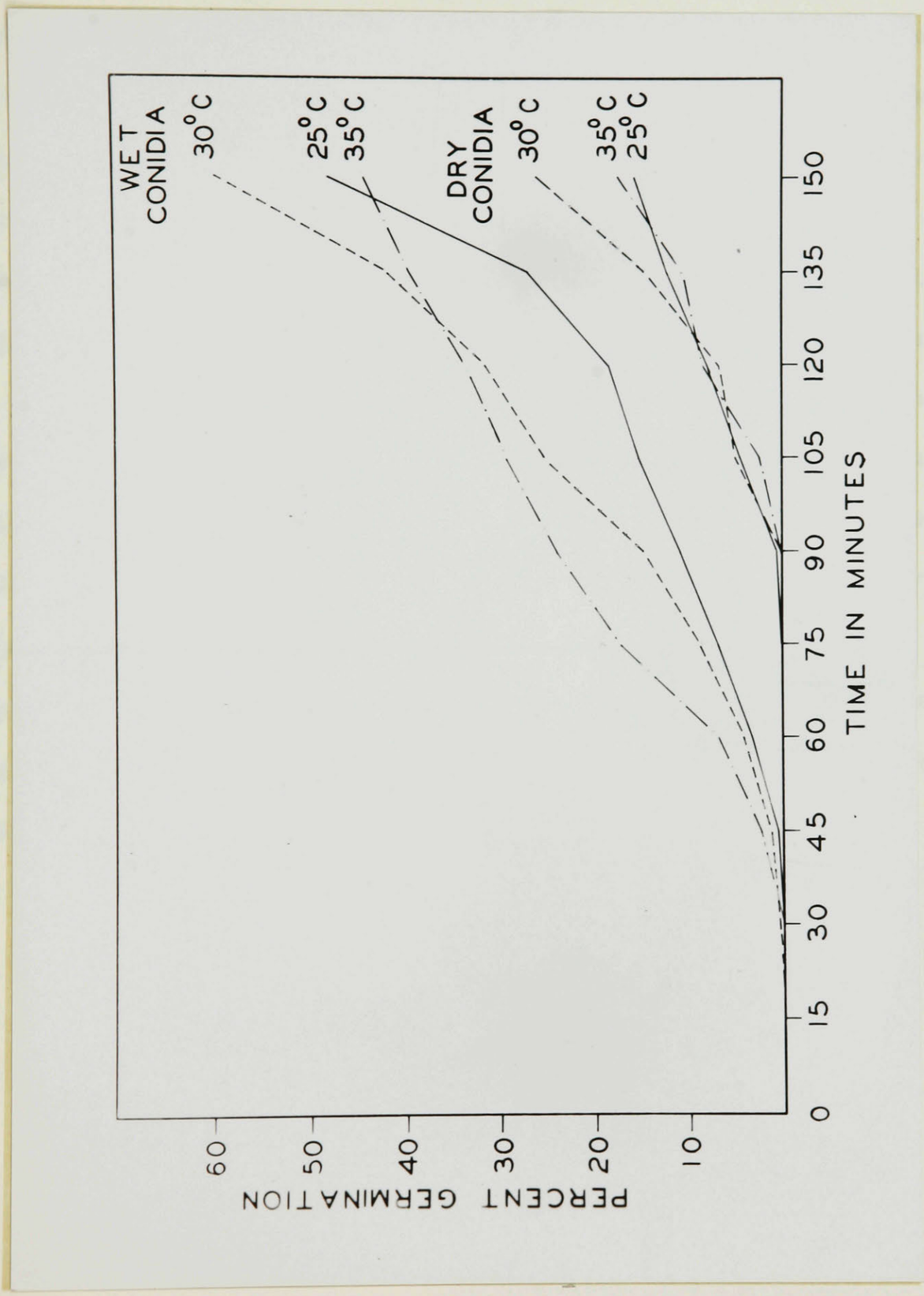
It is interesting to note that germination of wet conidia takes place more rapidly at 35°C. than at the temperature optimum

TABLE IV - Effect of Time, Temperature, and the Presence and Absence of Free Water on the Percentage Germination of Conidia of Helminthosporium portulacae in a Moisture Saturated Atmosphere.

Time in minutes	Spores mounted wet			Spores mounted dry		
	25°C.	30°C.	35°C.	25°C.	30°C.	35°C.
15	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.54	0.00	0.00	0.00	0.00
45	0.61	1.27	2.19	0.00	0.00	0.00
60	2.88	3.65	6.58	0.00	0.00	0.00
75	6.80	8.95	17.36	0.00	0.00	0.00
90	10.92	14.62	24.15	0.57	0.00	0.00
105	15.66	25.59	29.34	4.02	4.25	2.04
120	18.96	31.99	33.73	7.74	6.38	8.16
135	27.21	41.86	39.52	12.06	14.89	10.20
150	47.83	59.96	44.31	15.51	26.24	17.34
Average %	13.08	18.84	19.71	3.96	5.17	3.77
% After 24 hrs.	98.14	98.90	92.20	96.54	97.16	95.91

Plate IV

Effect of temperature and time on the per cent germination of wet and dry conidia in a moisture saturated atmosphere.


WET CONIDIA
30°C
25°C
35°C
DRY CONIDIA
30°C
35°C
25°C
PERCENT GERMINATION
10
20
30
40
50
60
0
15
30
45
60
75
90
105
120
135
150
TIME IN MINUTES

for fungal growth. However, over a long period of time, considerably more conidia germinated at 25° and 30°C. In the case of dry mounted conidia, most rapid germination occurred at about the temperature optimum for growth. After 24 hours, the percentage germination of conidia in the presence of free moisture was higher than that of the conidia mounted dry, excepting at the highest temperature, which gave a slightly lower percentage of germination of the wet conidia.

Although dry conidia in a saturated atmosphere took three times as long to germinate as conidia in the presence of free moisture, the percentage germination after 24 hours was only slightly less. Under all conditions the germ tubes developed normally. The rate of growth of germ tubes developing from dry and wet conidia was practically the same, and at 30°C. averaged 11 and 13 μ respectively at the end of three hours. Appressoria did not form in the absence of free water.

EFFECT OF TEMPERATURE AND MEDIA ON THE GROWTH RATE OF THE PATHOGEN

In this study, H. portulacae was grown on different solid and liquid media in a series of temperature control chambers set to operate at the temperatures shown in Table V. The solid media employed were three potato dextrose and three potato sucrose agars containing different amounts of sugar, corn meal agar (Difco), and P. oleracea agar (containing the juice from 300 gm. of plant material per litre of water). The liquid media used had the same composition as the solid media excepting for the omission of the agar. The corn meal agar, however, was used only in the solid form. The purpose of the liquid media was to take into account weight of fungal growth, which on agar would be difficult to determine. All media were filtered.

The solid media were poured into ten centimeter Petri dishes. Each dish contained 20 cubic centimeters of medium. By means of a cork borer, $\frac{1}{4}$-inch in diameter, uniform disks were cut from plate cultures of the fungus and used as inoculum. One disk was placed in the centre of each dish. Cultures used as a source of inoculum were of the same age and of monoconidial origin. Only the advancing margin of the fungus was used for inoculum.

The liquid media were contained in flasks of 125 cubic centimeter capacity. Each flask contained 25 cubic centimeters of media and was inoculated with $\frac{1}{2}$ cubic centimeter of a dilute conidial suspension.

However, due to the fact that the cabinets were not sufficiently large to accommodate the dishes and flasks together, the tests with the solid and liquid media were conducted separately. Each test was run for the same length of time and under the same conditions.

Spread of growth was measured five days after inoculation in three of the five dishes in each cabinet. Contaminated dishes and those showing abnormal growth were discarded. Diameter measurement, as a measure of growth of the colonies, was not suitable because the margins of the colonies on media containing added sugar were irregular, especially at the high temperatures. Therefore, the growth of the colonies was determined by tracing the outline of each colony on paper of equal weight. The tracings were then cut out, weighed, and recorded. The use of this method as a measure of growth is justified, because it is not the exact amount of growth, but the relative amount, that is important.

Growth of the fungus was determined by weighing the fungus matter in each flask. The liquid contents of each flask were filtered off through a filter paper of known weight. The filter papers containing the fungal matter were then spread out to dry at room temperature, weighed, and the exact weight of the fungus was obtained by subtraction. The average results of the tests are graphically illustrated in Plates V, VI, VII, and VIII. Tables V

and VI show the average growth over a five-day period.

The data was also analyzed statistically and the analyses of variance are given in Tables VII and VIII. An examination of these tables reveals that there is no significant difference between replicates. Advantage was taken of this fact in constructing the graphs and tables, which are based on the average of the three replicates.

Plate V

Effect of temperature on the relative growth of H. portulacae on indicated media. Measurements were made after a five-day growth period.
Note: The percentage figures indicate the amount of dextrose in the medium.

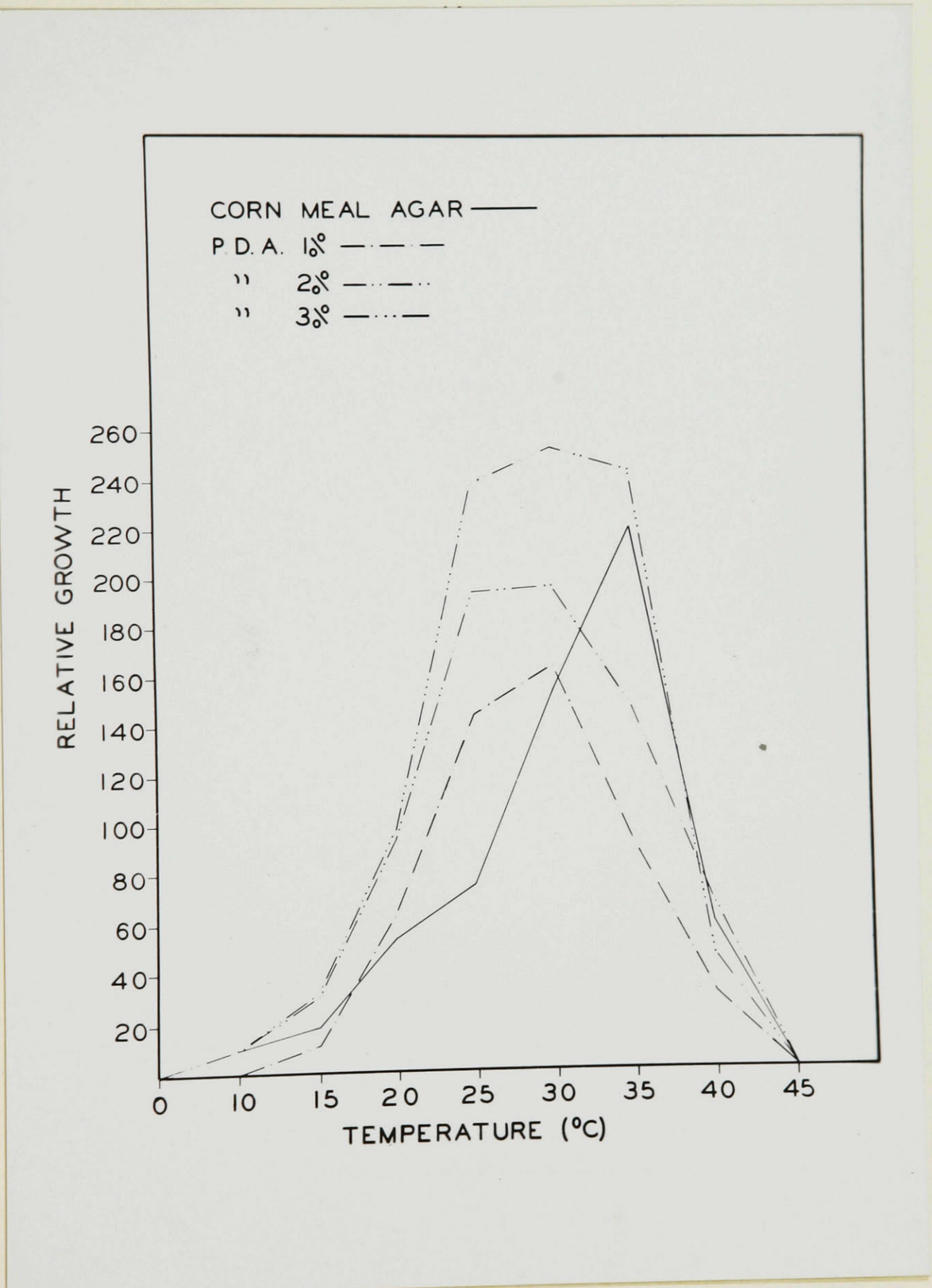

CORN MEAL AGAR
P.D.A. 1%
" 2%
" 3%
RELATIVE GROWTH
260
240
220
200
180
160
140
120
100
80
60
40
20
0
10
15
20
25
30
35
40
45
TEMPERATURE (°C)

Plate VI

Effect of temperature on the relative growth of H. portulacae on indicated media. Measurements were made after a five-day growth period.
Note: P.S.A. refers to potato sucrose agar and the percentage figures indicate the amount of sucrose in the medium.

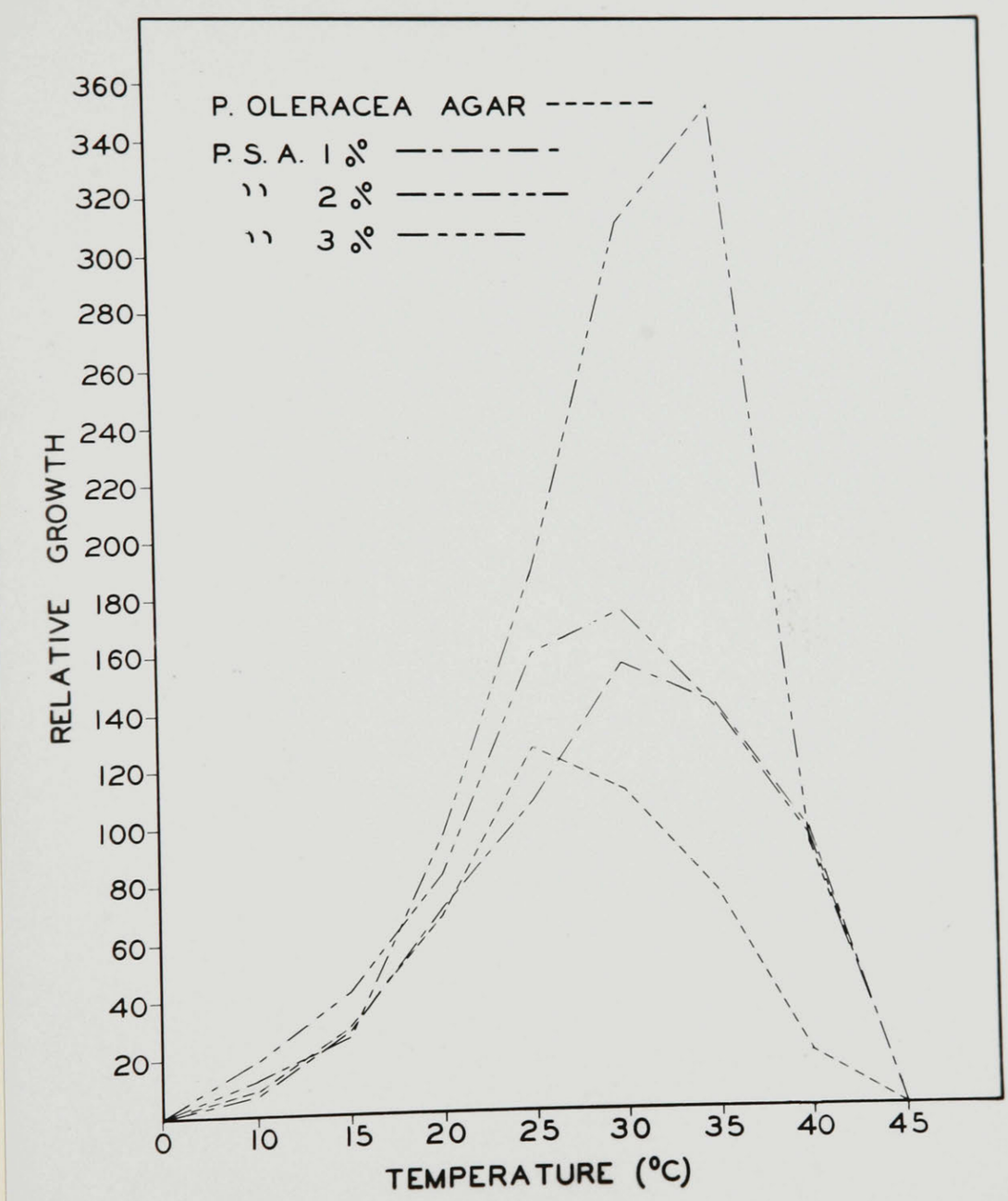

P. OLERACEA AGAR ------
P. S. A. 1 % —-—-—-
" 2 % —--—--—
" 3 % —----—
RELATIVE GROWTH
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
10
15
20
25
30
35
40
45
TEMPERATURE (°C)

Plate VII

Effect of temperature on the growth in mg. of H. portulacae on liquid media. Weight measurements were made after a five-day growth period. Note: P.D. refers to the liquid form of potato dextrose agar. The percentage figures indicate the amount of dextrose in the medium.

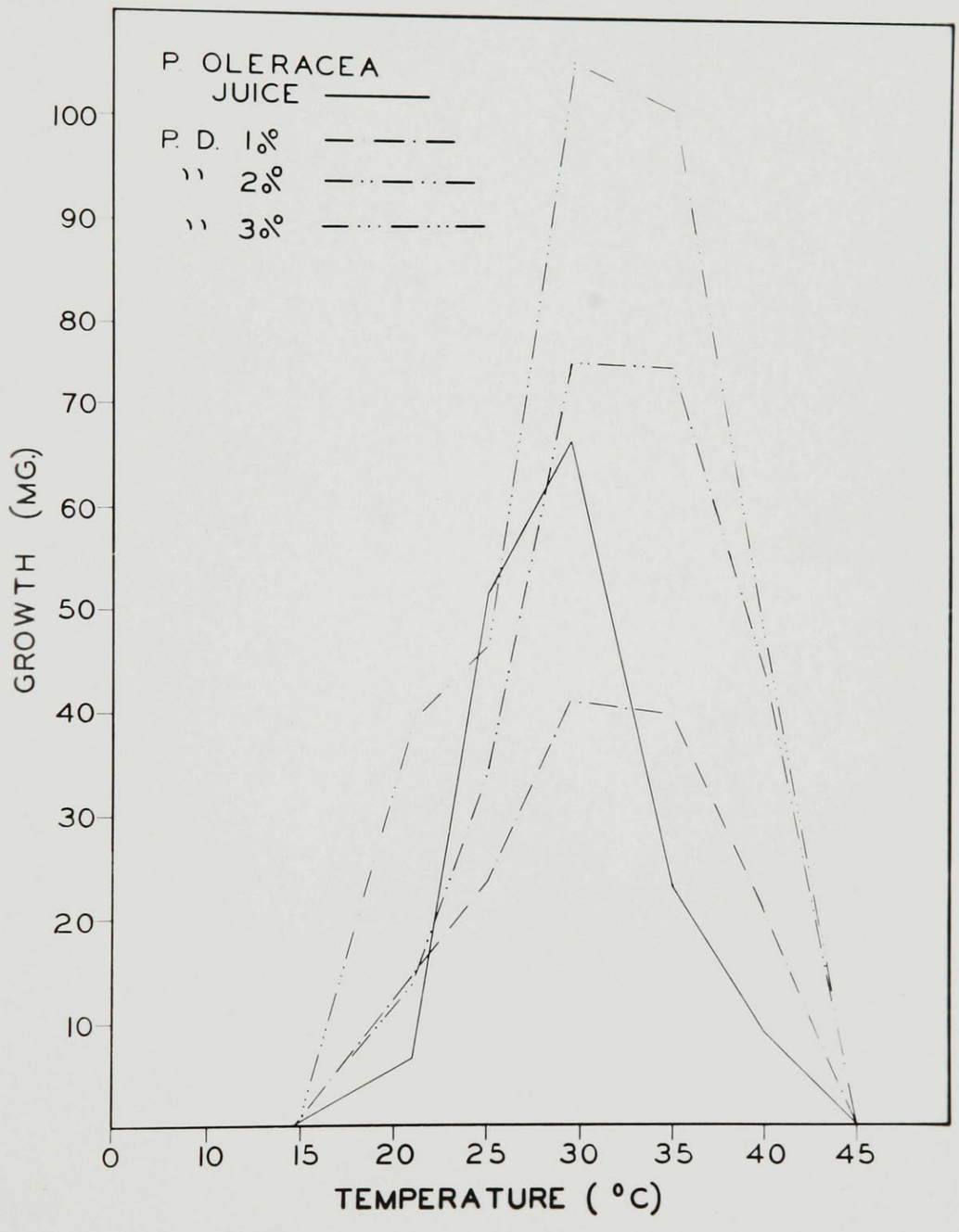

P OLERACEA
JUICE
P. D. 1%
" 2%
" 3%
GROWTH (MG.)
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
35
40
45
TEMPERATURE (°C)

Plate VIII

Effect of temperature on the growth in mg. of H. portulacae on liquid media. Weight measurements were made after a five-day growth period. Note: P.S. refers to the liquid form of potato sucrose agar. The percentage figures indicate the amount of sucrose in the medium.

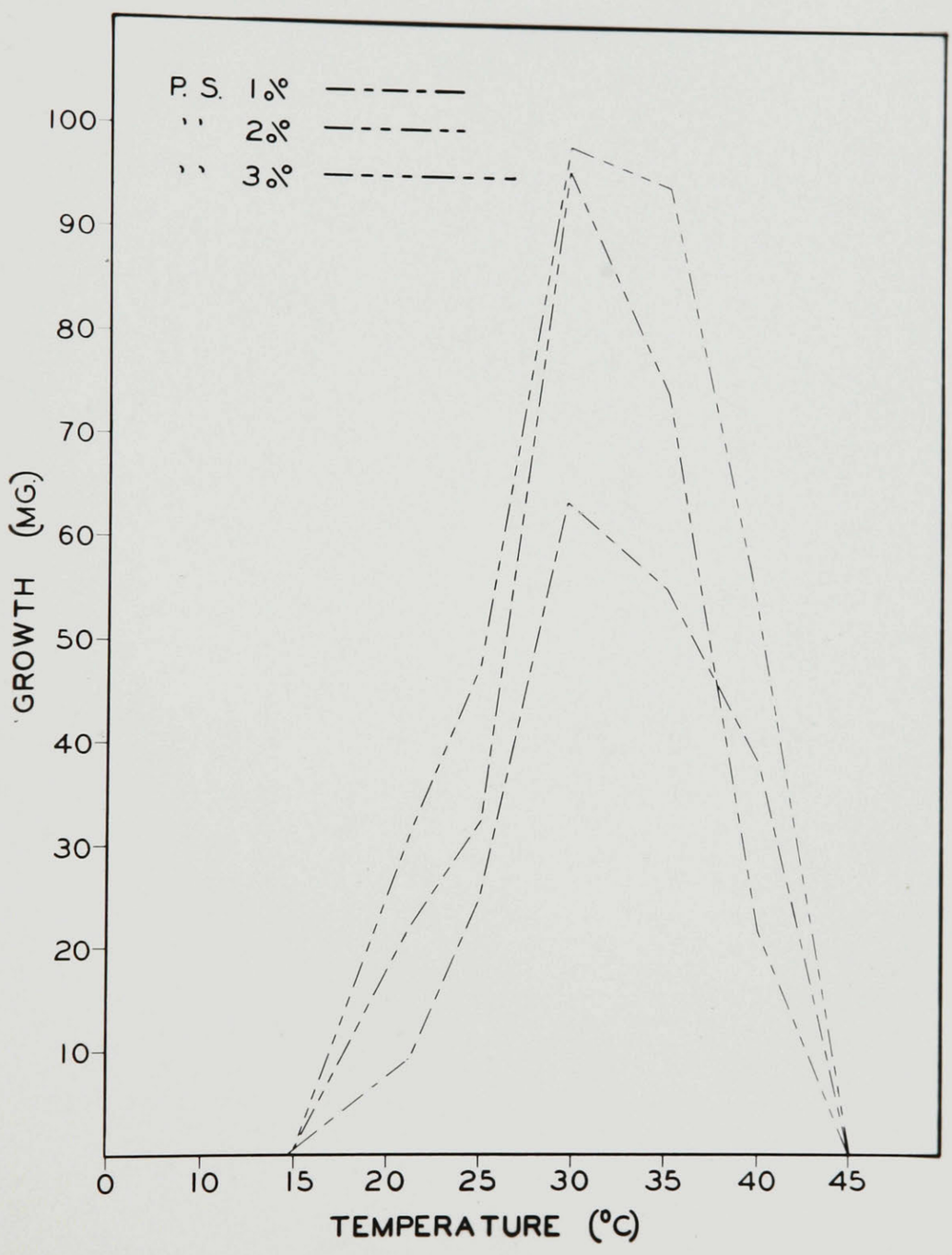

P. S. 1%
" 2%
" 3%
GROWTH (MG.)
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
35
40
45
TEMPERATURE (°C)

TABLE V - Effect of Temperature on the Spread of H. portulace when Grown on Different Solid Media in Petri Dishes During a Five-day Period. Each Reading is the Average of Three Dishes.

Medium* used	Relative amounts of spread** during a five-day period at indicated temperatures as given in degrees Centigrade.							
	10°	15°	20°	25°	30°	35°	40°	45°
P.D.A. 1%	0	10.6	64.0	144.6	164.0	92.0	30.0	0
2%	10.0	30.3	93.0	194.0	197.0	147.0	67.6	0
3%	10.0	30.6	96.3	238.6	253.3	244.0	55.6	0
P.S.A. 1%	7.3	28.6	70.6	108.6	156.0	142.6	97.0	0
2%	19.6	42.0	83.0	160.0	174.0	142.0	95.6	0
3%	12.6	25.6	93.6	189.0	312.6	353.6	93.6	0
P.O.A.	9.0	31.0	69.0	125.3	112.0	76.0	19.0	0
C.M.A.	10.0	18.3	53.3	75.0	154.6	220.6	59.3	0

*P.D.A. - potato dextrose agar; P.S.A. - potato sucrose agar; P.O.A. - P. oleracea agar (juice from 300 gm. of plant tissue per litre of water); C.M.A. - corn meal agar (Difco). The percentage figures indicate the amount of sugar in the medium.

**The spread figures are the weights in mg. of paper within outlines of the colonies drawn on paper of the same weight. See context page 27.

TABLE VI - Effect of Temperature on the Growth in mg. of Cultures of H. portulacae When Grown on Different Liquid Media in Flasks During a Five-day Period. Each Reading is the Average of Three Flasks.

Medium* used	Average amount of growth during a five-day period in milligrams at indicated temperatures given in degrees Centigrade.								
	1.5°	10°	15°	21°	25°	29.5°	35°	40°	45°
P.D. 1%	0	0	T**	14.6	23.6	41.6	40.0	21.0	0
2%	0	0	T	13.3	34.0	75.6	75.3	44.3	0
3%	0	0	T	39.6	47.0	107.0	102.6	47.0	0
P.S. 1%	0	0	T	9.6	25.6	63.3	55.3	38.6	0
2%	0	0	T	22.3	32.6	96.0	74.6	22.0	0
3%	0	0	T	31.3	47.6	98.6	94.6	53.6	0
P.O.J.	0	0	T	6.6	52.3	67.3	23.3	9.3	0

* P.D. - potato water and dextrose; P.S. - potato water and sucrose; P.O.J. - purslane juice (juice of 300 gm. of plant tissue per litre of water). The percentage figures indicate the amount of sugar in the medium.

**T - trace of growth.

TABLE VII - Analysis of Variance* of Data Obtained by Growing H. portulacae on Different Solid Media at Various Temperatures.

Source	D. of F.	S. of S.	M. S.	F
Replicates	2	319.90	159.95	
Temperature	8	1,150,544.70	143,818.08	802.42**
Error "a"	16	2,878.77	179.23	
Media	7	107,786.03	15,398.00	267.88**
Media x temperature	56	236,864.60	4,229.72	73.58**
Error "B"	126	7,243.00	57.48	
Total	215	1,505,637.00		

* analysis was carried out using total figures.
**level of significance, P.01

TABLE VIII - Analysis of Variance* of Data Obtained by Growing H. portulacae on Different Liquid Media at Various Temperatures.

Source	D. of F.	S. of S.	M.S.	F
Replicates	2	172.26	83.13	3.27
Temperature	8	154,178.74	19,272.34	723.87**
Error "a"	16	430.49	26.90	
Media	6	12,210.92	2,035.15	77.52**
Media x temperature	48	22,121.56	460.86	17.55**
Error "B"	108	2,835.25	26.25	
Total	188	191,949.22		

* analysis was carried out using total figures.
**level of significance, P.01

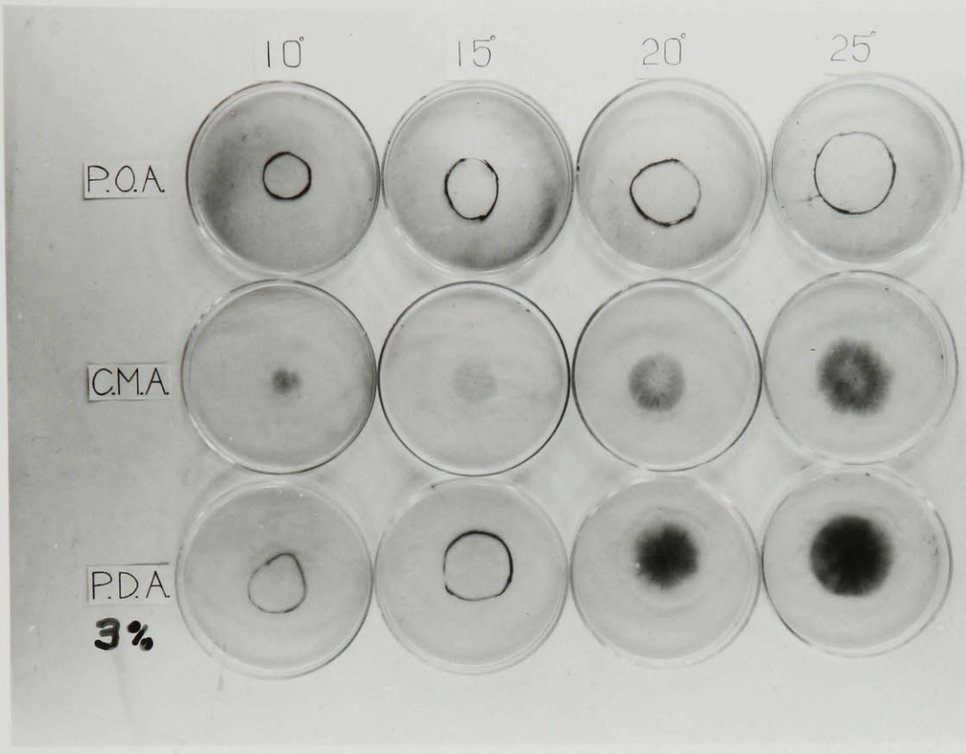
It is very obvious that the growth behaviour of H. portulacae is greatly influenced by temperature and medium. Further, it is also obvious that the growth of the fungus on liquid media closely paralleled its relative spread on solid media. The growth behaviour of the fungus on the solid and liquid media was, for the most part, the same. On media containing sugar as the only carbon source, growth increased with increasing sugar concentration, and on these media, the optimum temperature for growth was slightly above 30°C. The optimum temperature for growth of the fungus on corn meal agar was nearer 35°C., and the optimum temperature for its growth on P. oleracea agar and juice was nearer 25°C. The exact optimum temperature for growth was difficult to determine because the temperature intervals were 5°C., from 10°C. upwards.

The minimum temperature for growth on the solid media tested was below 10°C., but no growth occurred at 1.5°C. The maximum temperature for growth of the fungus was above 40°C. The minimum temperature for growth of the fungus on liquid media was about 15°C., the maximum above 40°C. Although no growth occurred on both types of media at the lowest and highest temperatures tested, the fungus remained viable. Plate IX, figs. 1 and 2 show the effect of temperature on the spread of the fungus on three of the media used.

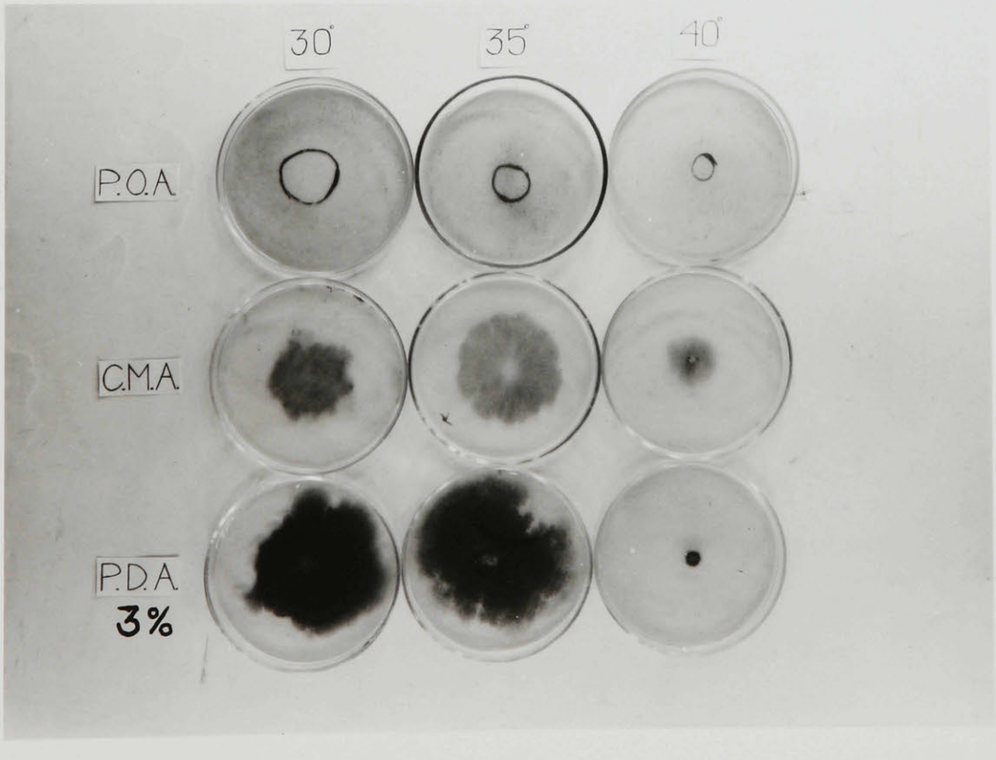
Differences in the appearance of colonies and in their growth habits were evident in addition to differences in the amount of growth at different temperatures and on different media. These differences are discussed under the next heading.

Plate IX

Effect of temperature and medium on the growth of *H. portulacae*. Note: The spread of colonies has been outlined in those plates in which the growth was so thin or so closely appressed to the medium that it was not possible to bring it out clearly in the photograph.



1



2

EFFECT OF TEMPERATURE AND MEDIA ON THE GROWTH HABIT OF THE PATHOGEN

The differences observed in the growth habit of the fungus on the different solid media at the various temperatures, are listed in Table IX.

In general, pigmentation of all cultures was least at the lower and highest temperatures employed. However, there was no great variation in the intensity of the colour of the colonies on the same medium at the different temperatures. Variation in intensity of colour was least on colonies on media offering only sugar as a carbon source.

The growth characters of the fungus showed much variation. However, it appears that temperature had more effect on the fungus than did media. Up to 20°C., the margins of the colonies were for the most part regular in outline, but at the higher temperatures growth at the margins was irregular.

The production of bulbils on the surface and within the media, and the production of conidia, increased with increasing temperatures up to 30°C., and then decreased. Among the media tested, potato dextrose and sucrose media produced the greatest number of these bulbils and conidia. Aerial bulbils were formed in abundance at 30°C. and at all higher temperatures on all media, but did not appear at the lower temperatures. These bulbils were not included in determining the relative amounts of bulbils produced on each medium at the different temperatures, since they were only formed at the higher temperatures.

High temperatures seemed to reduce the formation of septa in conidia, and also caused many of the conidiophores to germinate instead of developing conidia. The aseptate conidia germinated normally so they must have been mature.

Table IX - The Effect of Temperature and Media on the Growth Habit of the Pathogen

Medium	Temp.	Colour of Colony	Character of Growth	Production [X] of bulbils [XX]	Production [X] of conidia
P.D.A. 1%	10°C.	white	thin aerial mycelium; margin regular	nil	nil
P.D.A. 2%		"	"	"	"
P.D.A. 3%		"	"	"	"
P.S.A. 1%		"	"	"	"
P.S.A. 2%		"	"	"	"
P.S.A. 3%		"	"	"	"
C.M.A.		gray	"	"	"
P.O.A.		white	"	"	"
P.D.A. 1%	15°C.	white	mycelium mostly appressed; margin regular	+	+
P.D.A. 2%		"	"	+	+
P.D.A. 3%		"	"	nil	nil
P.S.A. 1%		"	"	+	nil
P.S.A. 2%		"	"	+	"
P.S.A. 3%		"	"	+	"

Table IX - Continued

Medium	Temp.	Colour of Colony	Character of Growth	Production[X] of bulbils[XX]	Production[X] of conidia
C.M.A.	15°C.	gray	mycelium mostly appressed; margin regular	+	nil
P.O.A.		white	"	nil	nil
P.D.A. 1%	20°C.	black with white margin	mycelium mostly appressed; margin regular	++	++
P.D.A. 2%		dark gray with white margin	"	+++	+++
P.D.A. 3%		light gray with white margin	mycelium mostly appressed; margin irregular	+++	++
P.S.A. 1%		dark gray with white margin	"	+++	+++
P.S.A. 2%		"	"	++	++
P.S.A. 3%		gray with white margin	"	+++	+++
C.M.A.		"	mycelium mostly submerged; margin regular	+++	++
P.O.A.		light brown	mycelium mostly appressed; margin regular	nil	+
P.D.A. 1%	25°C.	black with white margin	mycelium mostly appressed; margin irregular	++++	+++

Table IX - Continued

Medium	Temp.	Colour of Colony	Character of Growth	Production X of bulbils XX	Production X of conidia
P.D.A. 2%	25°C.	black with narrow white margin	mycelium mostly appressed; margin irregular	++++	+++
P.D.A. 3%		"	"	++++	++++
P.S.A. 1%		"	mycelium appressed; margin very irregular	++++	+++
P.S.A. 2%		"	"	+++	+++
P.S.A. 3%		"	"	+++	+++
C.M.A.		dark gray	mycelium mostly submerged; margin slightly irregular	++++	++
P.O.A.		brown	mycelium appressed; margin regular	+	++
P.D.A. 1%	30°C.	black	mycelium appressed; margin very irregular; aerial bulbils	++++	++++#
P.D.A. 2%		"	"	++++	++++#
P.D.A. 3%		"	"	++++	+++ #
P.S.A. 1%		"	"	++++	++++#

Table IX - Continued

Medium	Temp.	Colour of Colony	Character of Growth	Production[X] of bulbils[XX]	Production[X] of conidia
P.S.A. 2%	30°C.	black	mycelium appressed; margin very irregular; aerial bulbils	++++	+++ #
P.S.A. 3%		"	"	++++	+++ #
C.M.A.		dark gray	mycelium mostly submerged; margin irregular	++++	+++ #
P.O.A.		orange brown	mycelium appressed; margin regular	++	+++ #
P.D.A. 1%	35°C.	black with gray tinge	mycelium appressed; margin very irregular; aerial bulbils	++	++++ #
P.D.A. 2%		"	"	+++	++++ #
P.D.A. 3%		"	"	+	++++ #
P.S.A. 1%		dark gray	"	+++	+++ #
P.S.A. 2%		"	"	+++	+++ #
P.S.A. 3%		dark brown	"	nil	+++ #
C.M.A.		brown with white margin	mycelium mostly submerged; margin regular	nil	+++ #
P.O.A.		orange brown	mycelium appressed; margin regular	nil	++ #

Table IX - Continued

Medium	Temp.	Colour of Colony	Character of Growth	Production[X] of bulbils[XX]	Production[X] of conidia
P.D.A. 1%	40°C.	black with white margin	mycelium mostly appressed; margin regular; aerial bulbils	+	+ #
P.D.A. 2%		"	"	+	+ #
P.D.A. 3%		gray	"	+	nil
P.S.A. 1%		brown	"	++	nil
P.S.A. 2%		"	"	++	nil
P.S.A. 3%		"	"	++	nil
C.M.A.		"	mycelium submerged	nil	++ #
P.O.A.		light brown	mycelium appressed	nil	++ #

X +, very slight; ++, slight; +++, moderate; ++++, abundant.
XX Only the bulbils produced on or within the medium are considered in this table.
Many of the conidia were aseptate; many of the conidiophores germinated instead of producing conidia.

VARIATION IN THE GROWTH OF THE PATHOGEN

During the course of investigating the effects of temperature and media on the rate and habit of growth of H. portulacae on artificial media, saltants developed in some of the cultures. These appeared to be of three types. This phenomenon occurred only in colonies which were growing on solid media rich in sugar, and within the temperature range of 25° to 45°C. Saltations were not observed in liquid media nor on corn meal and purslane agars.

The variants did not appear as sectors or patches within the colony, as is commonly the case in colonies of many fungi, including other Helminthosporium spp., but rather as fan-shaped outgrowths from the margin of the colony, which because of their more rapid rate of growth, soon outgrew the original colony. In order of decreasing frequency of occurrence, the variants were designated H1, H2, and H3. These are shown in Figure 3, colony B, C, and D. Colony A is a typical non-salting colony. All dishes were incubated at 30°C. The kind of growth exhibited by each variant was compared to the growth of a typical non-salting colony, macroscopically and with the aid of a dissecting microscope. The results of the comparison are given in Table X.

The nature of variation in fungi, or the mechanism by which variants arise in culture, is not too well known. Much work has been done to attempt to explain the phenomenon (9) (11) (16) (21) (23) (46), and in general it has been attributed to either (a) genetic changes

(heritable changes) which may result from hybridization, aggregation of nuclei, mutation, and possibly heterokaryosis, and (b) non-heritable changes due to the environment.

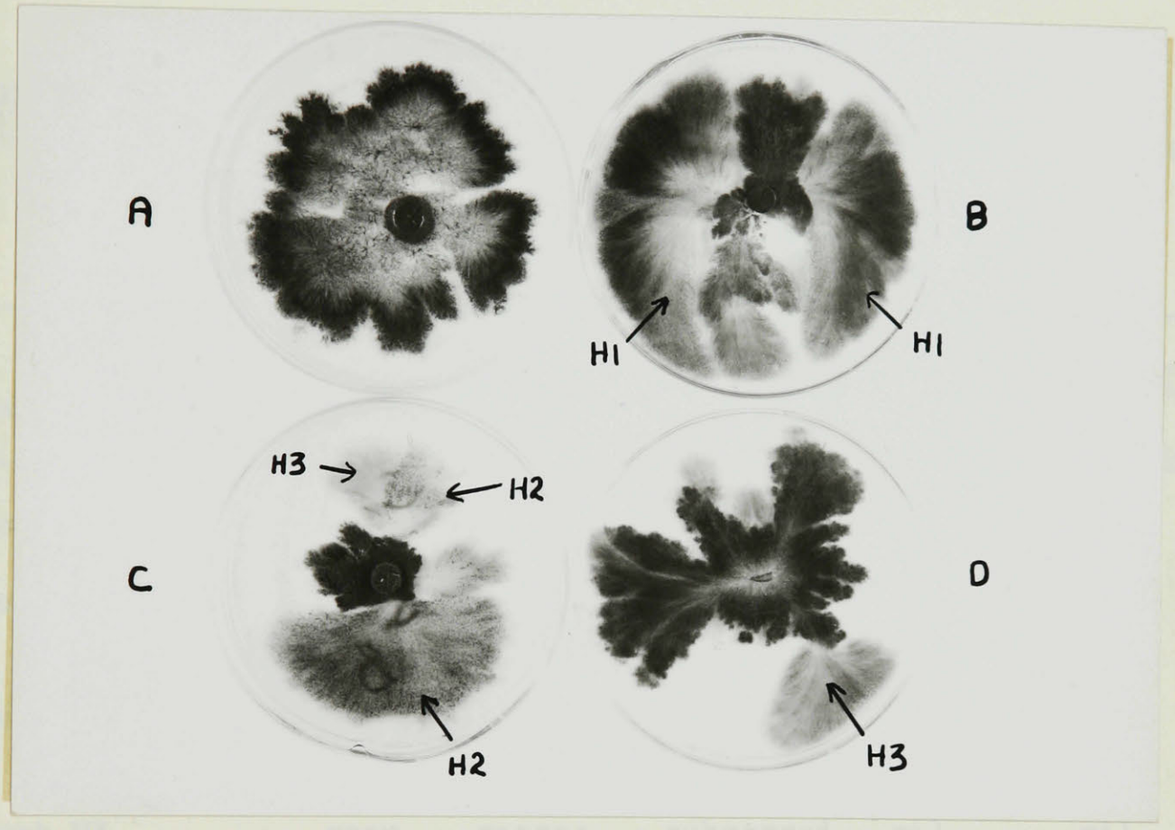


Figure 3 - Saltation in H. portulacae. Colony A is a typical non-salting one. Colonies B, C, and D show what appear to be three types of saltants labelled H1, H2, and H3. Only variant H3 remained stable throughout successive transfers. All cultures were incubated at 30° C.

Of the three H. portulacae variants H1, H2, and H3, only variant H3 remained stable throughout successive transfers to fresh medium. Variants H1 and H2 reverted immediately upon being transferred (see Plate X). Obviously then, only the kind of growth exhibited by variant H3 was heritable, whereas alterations in the latter variants

were non-heritable or phenotypical, and probably due to unfavourable environmental conditions within the Petri dish, and not to genetic changes.

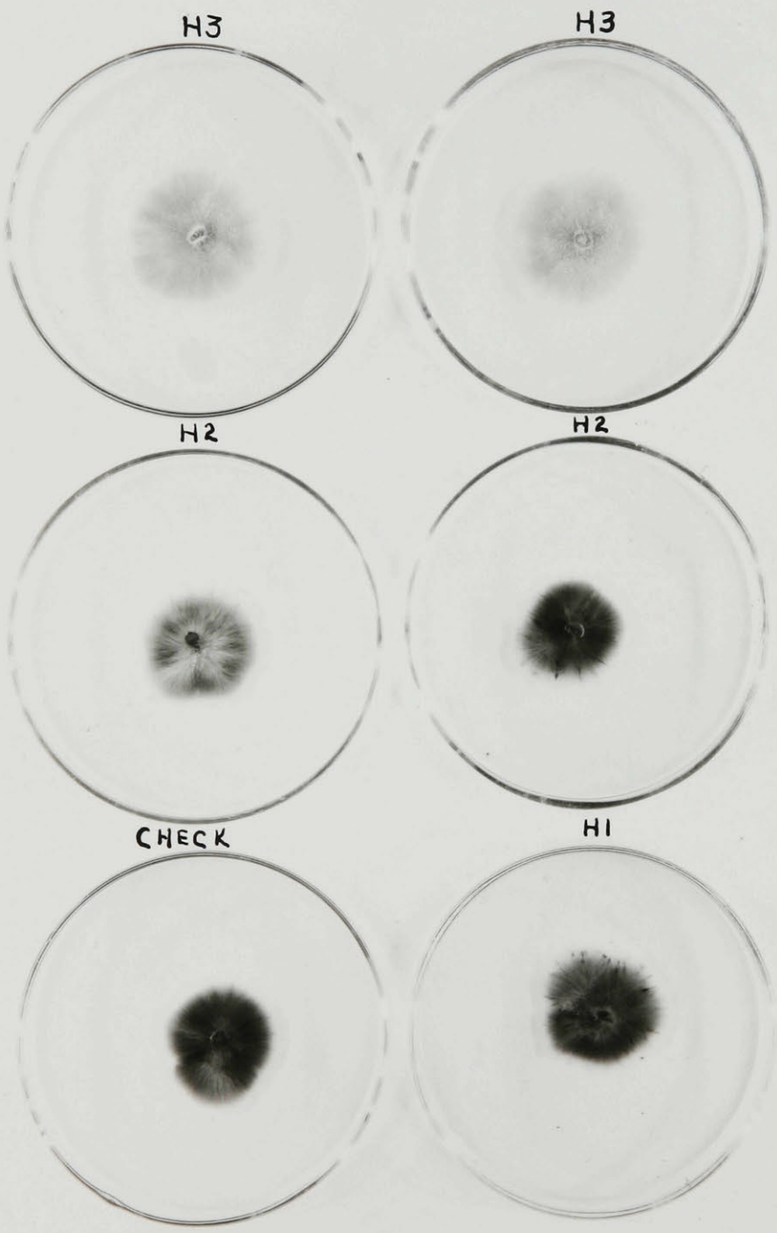
TABLE X - The Kind of Growth Exhibited by Variants H1, H2, and H3 as Compared to the Growth of a Typical Non-salting Colony. All cultures were Incubated at 30°C.

Variants	Growth characteristics			Production* of	
	Colour	Density	Type	bulbils	conidia
Non-salting colony	black	dense	appressed	+++	++++
Variant H1	black	fairly dense	very appressed	+++	+
Variant H2	dark gray	sparse	submerged	++++	nil
Variant H3	gray	sparse	submerged	nil	nil

* +, very slight; +++, abundant; ++++, very abundant.

Plate X

Transfers of variants H1, H2, and H3 on nutrient agar. Only variant H3 remained stable through successive transfers. Variants H1 and H2 reverted to normal.


H3
H3
H2
H2
CHECK
H1

NUCLEAR STUDIES

Work by Hansen and Smith (23) has suggested that heterokaryosis might account for variation in Botrytis cinerea cultures because the nuclei in the conidia of this fungus were found to be genetically different. Genetically different nuclei can become associated through anastomosis and segregate during the process of conidial germination or branch formation. Graham (21) found that individual cells in hyphae, conidiophores, and conidia of H. gramineum were multi-nucleate and genetically different, and he therefore concluded that variation in this fungus might also be due in part to heterokaryosis. However, Christensen and Davies (11), working with H. sativum, and Dickinson (16), with other Helminthosporium spp. obtained evidence that although the conidia of these Helminthosporium spp. were multi-nucleate, the nuclei were derived from a single nucleus. Christensen and Davies (11) and Dickinson (16) therefore concluded that changes within a nucleus, rather than heterokaryosis, were responsible for variation in the Helminthosporium spp. they studied.

With the above findings in mind, nuclear studies on H. portulacae were undertaken for the purposes of determining (a) the number of nuclei in the individual cells in hyphae, conidiophores, and conidia, and (b) the origin of the nuclei in the conidia. Once these facts are known, it might be possible to draw certain conclusions regarding the nature of the genetic change that had occurred in those parent

colonies which gave rise to the H3 type of variants.

The fungus material was prepared and stained as follows: Single conidia of H. portulacae from a fruiting culture were placed on thin films of dried nutrient agar on glass slides, and were incubated in Petri dishes to which a little water had been added. At various intervals, the stage of development of the conidia was determined by examining them with a dissecting microscope. Germinated conidia, young colonies, and colonies just beginning to fruit were fixed in Flemings weak fixing solution for about 15 minutes and washed in running water for 30 minutes. The slides were then immersed for 24 hours in equal parts of hydrogen peroxide and 95 per cent alcohol contained in Coplin staining jars for bleaching, and washed for about 30 minutes in running water. They were then stained in iron-alum haematoxylin, cleared in clove oil, mounted in balsam, and examined with an oil lens. Table XI summarizes the observations on the number of nuclei found in the individual cells of the fungus.

TABLE XI - The Number of Nuclei Present in Individual Cells of Different Organs of H. portulacae.

	Organs observed					
	Conidia*	Young aseptate germ tubes	Older aseptate germ tubes	Hyphae	Conidiophores	Apical cell of conidiophores
No. of Nuclei	3 - 4	3 - 4	3 - 8	2 - 7	2	1

* Only the nuclei in apical or basal cells just about to germinate were visible.

The fact that the apical cells of all young conidiophores examined contained only one nucleus, strongly suggests that all the nuclei in a conidium are genetically alike assuming normal mitosis. Therefore, all the growth resulting from a single conidium would contain genetically similar nuclei, and since there were no indications of nuclear fusions in any of the hyphal cells examined, it would appear that variation in H. portulacae is due to a genetic change in a nucleus (mutation), and not to heterokaryosis.

ATTEMPTS TO DEVELOP THE PERFECT STAGE

Tests were conducted to determine whether H. portulacae could be induced to form a perfect stage. It seems likely that the small black sclerotial bodies or bulbils, which this fungus produces abundantly in culture and on infected host tissue, are immature perithecia. This assumption is based on the following facts: The ascigerous stage of many Helminthosporium spp. has been found in nature or has been developed on artificial media. Further, it was demonstrated that bulbils of H. teres, H. tritici-repentis, and H. bromi, which are produced on infected host tissue and in culture, represent immature perithecia; and as mentioned previously, according to Drechsler (18) the ascigerous stage of any Helminthosporium sp. which forms these structures in culture or on host tissue might be sought with considerable prospect of success.

The sexual stage of H. teres was found on old barley straws by Johnson (27), but he made no mention of the occurrence of this stage in culture. Chamberlain and Allison (7) found the sexual stage of H. bromi in nature, and also succeeded in inducing it to form in culture by subjecting bulbil producing cultures of the fungus to the low temperatures of late fall and winter. The perfect stage of H. sativum was first discovered by Kuribayashi (28). Small black sclerotia-like bodies were always formed on all sides of host tissue plated on a starch rich medium composed of rice culms. However,

Tinline (48) showed that H. sativum produced perithecia in culture only when isolates belonging to different compatibility groups were paired or mated on a starch rich agar medium.

The tests conducted to induce the ascigerous stage of H. portulacae were as follows: Infected, dry, dead stems and branches of purslane, bearing bulbils and conidia were placed outdoors in Petri dishes, in winter, for a period of five weeks. The temperature during this period ranged from about 6° to -19°C. (average-4°C.). Each week a piece of stem or branch tissue was brought into the laboratory, examined to see if the bulbils had shown any development, and planted on potato dextrose agar to see if they would develop into perithecia. Although the fungus was recovered from the infected dead tissue, the bulbils showed no tendency to develop into perithecia.

Following Tinline's lead (48), five Petri dishes containing potato dextrose agar were inoculated with three conidia from a fruiting colony of isolate A201. In each dish, the three conidia were planted in close proximity at the corners of an equilateral triangle. The results of these tests (see Figure 4) show that some colonies grew together or were compatible, and that others were incompatible. However, when single conidia from any one colony, or from two compatible or incompatible colonies were plated together, variable results were always obtained. Similar results were also obtained when a single conidium from isolate A201, A101, and G102 were plated together. The

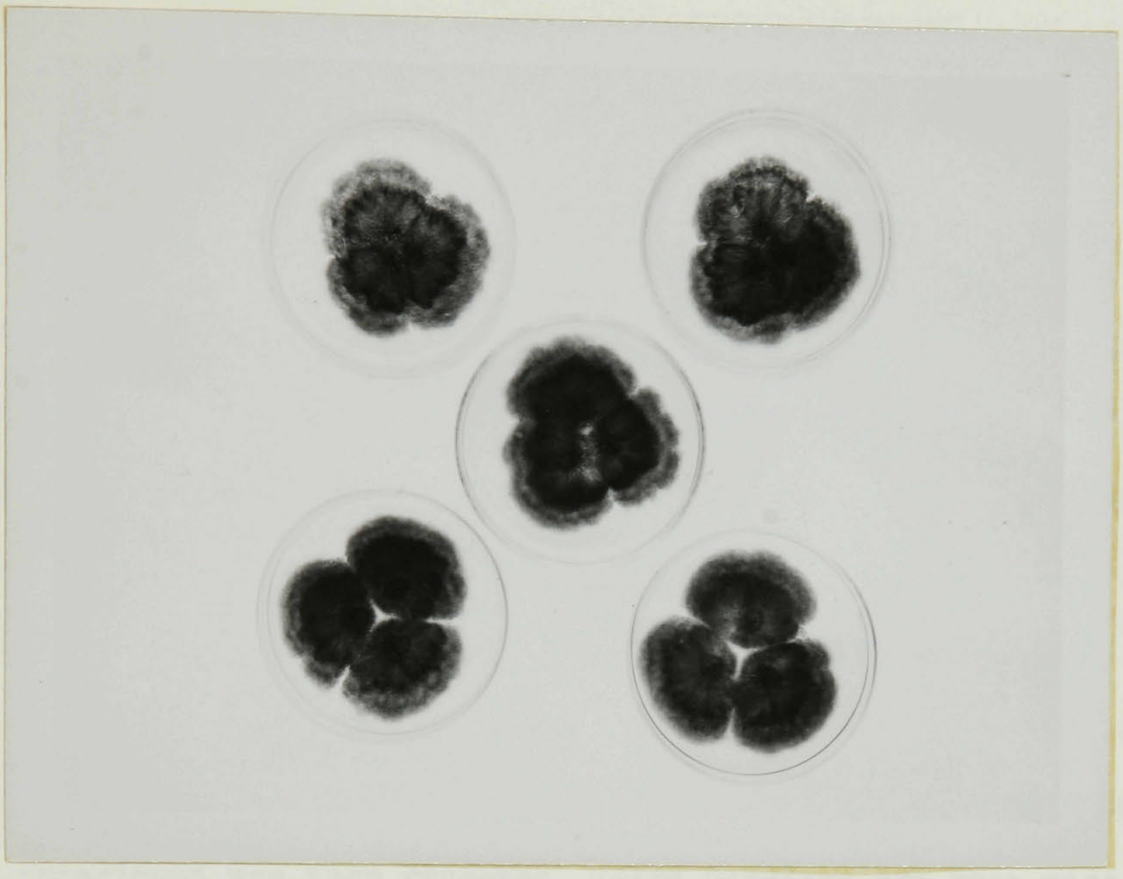


Figure 4 - Colonies from single conidia of H. portulacae isolate A201, showing results of close association.

inconsistent results are difficult to explain. It should be mentioned that more abundant bulbils were always formed along the margins of colonies which did not grow together than were normally produced when colonies grew together.

The starch rich agar medium used by Tinline (48) to develop the perfect stage of H. sativum was then prepared, sterilized, and poured into seven Petri dishes. When the medium solidified, the dishes were inoculated by pouring conidial suspensions of isolates A101, A201, and G102 separately, two isolates mixed together in all possible combin-

ations, and all three mixed together, over the medium. The inoculated dishes were then incubated at room temperature for six weeks.

The medium just referred to was prepared as follows: Kernels of barley were surface sterilized with mercuric chloride (1:1,000), washed in several changes of sterile water and then boiled for a few minutes to kill the embryos. Numerous kernels were spread over the surface of the agar in each of several Petri plates each of which contained 30 cubic centimeters of Sach's nutrient agar, adjusted with calcium carbonate to a pH of approximately 6.5. The dishes were inoculated and then incubated at 23°C. for six weeks.

Numerous bulbils and conidia formed in the nutrient agar and on the dead barley kernels, but the bulbils did not develop into perithecia. This experiment was then repeated, using purslane and rose moss seeds instead of barley kernels. Again, numerous bulbils formed in the agar and on the seeds, but they did not develop into perithecia.

These negative results seem to indicate that either different techniques must be sought which will provide conditions more favourable for the development of the sexual stage of the fungus; or that H. portulacae is a heterothallic organism, only one strain of which has so far been found.

The fact that conidia of H. portulacae show bipolar germination makes it possible to predict, with a high degree of certainty, that if the perfect stage of this fungus exists it is most likely to belong

to the genus Cochliobolus. This genus was erected by Drechsler (19) in 1934 to separate the Ophiobolus spp., which have a helicoid arrangement of ascospores in the asci of mature perithecia, from all other Ophiobolus spp. which not only do not show this character, but have never been known to give rise to Helminthosporium forms with bipolar germination in pure culture. Conversely, the known ascigerous stage of individual species of these Helminthosporium spp. with bipolar germination have never been found connected with Ophiobolus spp. other than those which have their ascospores spirally arranged in the asci.

PART II - THE DISEASE

SYMPTOMS AND SIGNS

Symptoms of the Helminthosporium disease of purslane and rose moss were observed on greenhouse grown plants which had been artificially inoculated with conidial suspensions, on diseased plants in the field, and on infected field plants which were lifted from the field at various stages of development, potted, and placed in a greenhouse for further observation.

On Portulaca oleracea

All parts of purslane, except the seeds and roots, are susceptible to attack by Helminthosporium portulacae. Attacks on the above ground parts of the plant are first evident as small, pale, water-soaked spots with necrotic centres. This symptom is visible 40 to 48 hours after inoculation with conidial suspensions on plants kept in moist chambers. However, infections can be seen much sooner with the aid of a pocket lens. At this earlier stage, necrotic centres are usually lacking.

The spots enlarge rapidly, remaining round on leaves and becoming elongated on stems and branches. Just before the diseased tissues become necrotic, they take on a brownish watersoaked appearance. The advancing margins of the lesions are a paler colour than the healthy tissues. Individual spots, if close together, coalesce to form

larger irregularly shaped lesions. At this stage in the development of the disease, affected leaves are soft and flaccid and are easily knocked off, suggesting that an abscission forms at the base of the reduced petiole. Those which still adhere to the branches shrivel up and turn black.

The watersoaked lesions, which appear on the branches and stems, develop rapidly in all directions and soon girdle the affected parts. The diseased tissue behind the advancing margin dries out and the girdled stem becomes wiry. This symptom is followed by a collapse of the affected stem or branch, and a wilting of the leaves on that portion of the stem or branch above the point of collapse. The colour of the diseased tissue changes to a red brown or dark brown and finally turns black. On examining diseased stem or branch tissue more closely, it is seen that an active girdling lesion is composed of three distinct zones or regions. The most conspicuous zone is in the centre of the lesion. It is black in colour and composed of dead tissue. The edge of this zone blends into a brown watersoaked region which is outlined by a paler, yellow, watersoaked margin, which in turn blends into the healthy stem tissue. The significance of these zones will be discussed more fully later.

When an infection arises on a branch, the disease spreads downwards into the main stem, and under unusually favourable conditions, the entire plant so affected dies in from ten to 14 days. When an infection arises on the main stem (see Figure 5), the affected plant

succumbs much quicker. As soon as the main stem is girdled, the affected

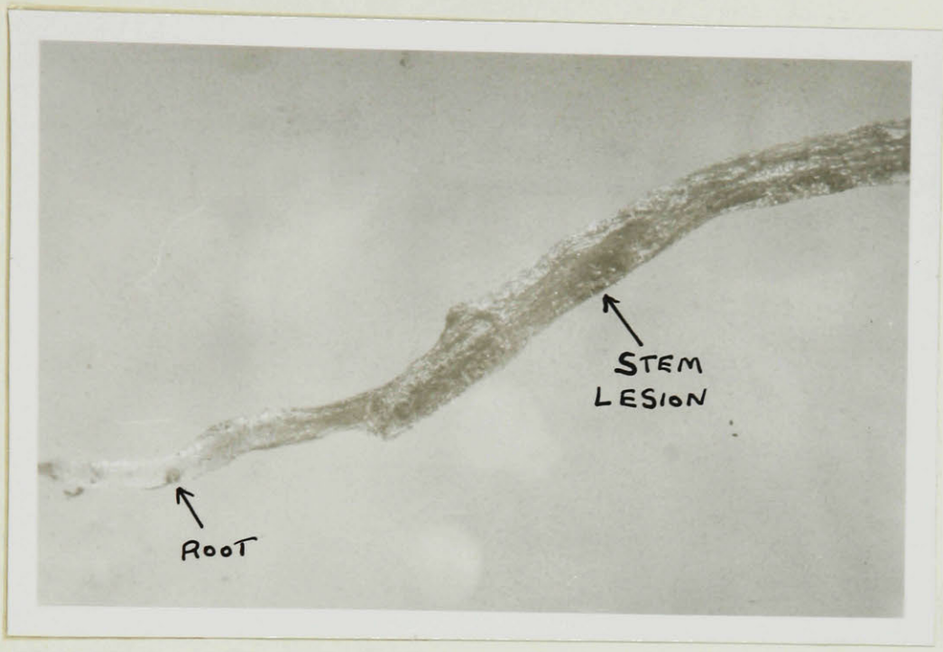


Figure 5 - A girdling lesion on the main stem of a diseased purslane plant. Approximately x 7.

plant topples over then wilts, and all the secondary branches and stems are rotted by the fungus, and possibly secondary organisms. The occurrence of conditions checking disease development after the plant has fallen over and before wilting becomes irreversible, often permits healthy portions of the stem to develop roots at the nodes in contact with the soil. Infected seedling plants are rapidly killed.

Bulbils form abundantly on the blackened areas on the leaves, stems, branches, and capsules (see Figures 6 and 7). The organism also fruits over the dead infected areas, but near moisture saturated atmospheres are required for fruiting.

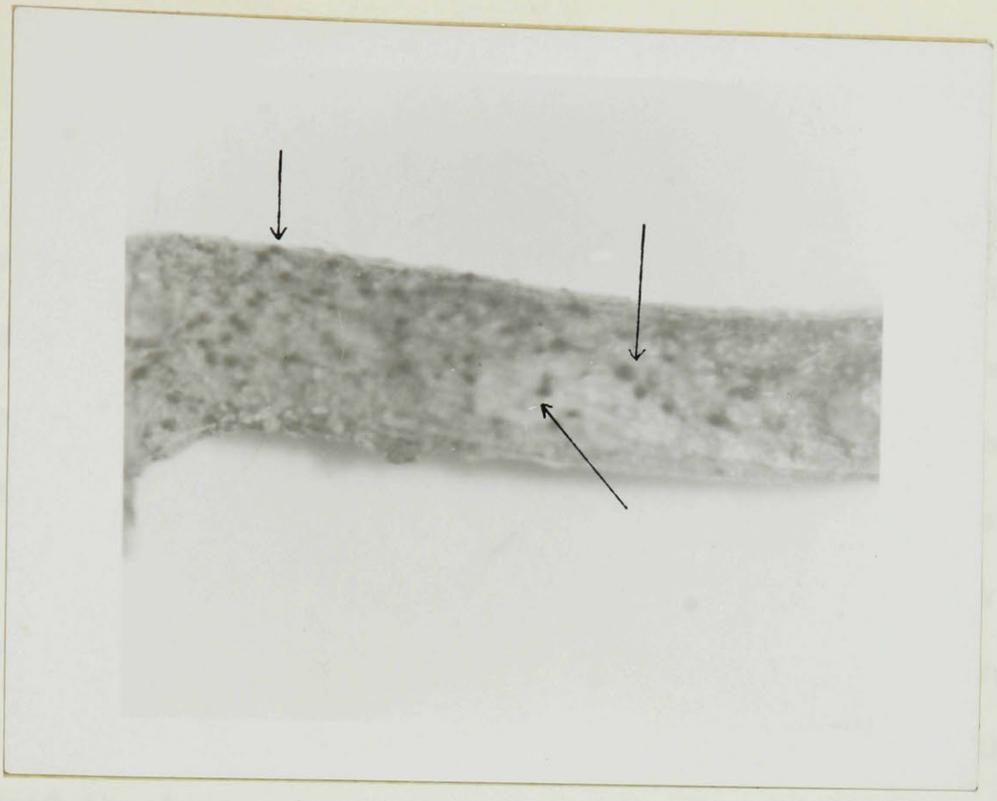


Figure 6 - A portion of a naturally diseased purslane stem bearing bulbils. Approximately x 12. Arrows point to bulbils.

Figure 7 - Inside view of a diseased purslane seed capsule. Approximately x 9. Note the bulbils embedded in the inner wall of the capsule. b - bulbil; c - inner wall of the capsule.

On Portulacae grandiflora

H. portulacae attacks only stems, flowers, and capsules of the rose moss plant. The symptoms of the early stages of the disease on susceptible rose moss parts are similar to those previously described on purslane, but because of the woody nature of the stems and branches of rose moss plants, dead areas do not collapse and girdled stems do not become wire-like. Consequently, in the later stages of the disease, affected branches and stems remain erect. Newly diseased tissue is brown to red brown in colour, but the older portions of the lesion turn gray to dark gray, never black. Dead stems and branches are brittle.

The leaves and more tender branch and stem tips above the girdled parts wilt. The leaves abscise, and the stem tips bend over, droop, and dry up. In warm moist conditions, the organism advances rapidly and individual branches on the main stem may be killed in three or four days. Heavily infected plants die in about ten to 14 days time (see Figure 8). An infection arising on a branch rapidly spreads downwards into the main stem, and under favourable conditions, the entire plant becomes involved in a few days. When an infection arises on the main stem, the wilting soon appears throughout the whole plant. As before, conidia and bulbils are formed on dead invaded tissue (see Figures 9, 10, and 11).

Figure 8 - Left and right: rose moss plants killed by H. portulacae; centre: a healthy rose moss plant. Note the erect woody stems and branches of the diseased plants.

Figure 9 - Lower portion of a main stem of rose moss bearing numerous bulbils. Approximately x 10. Arrows point to bulbils.

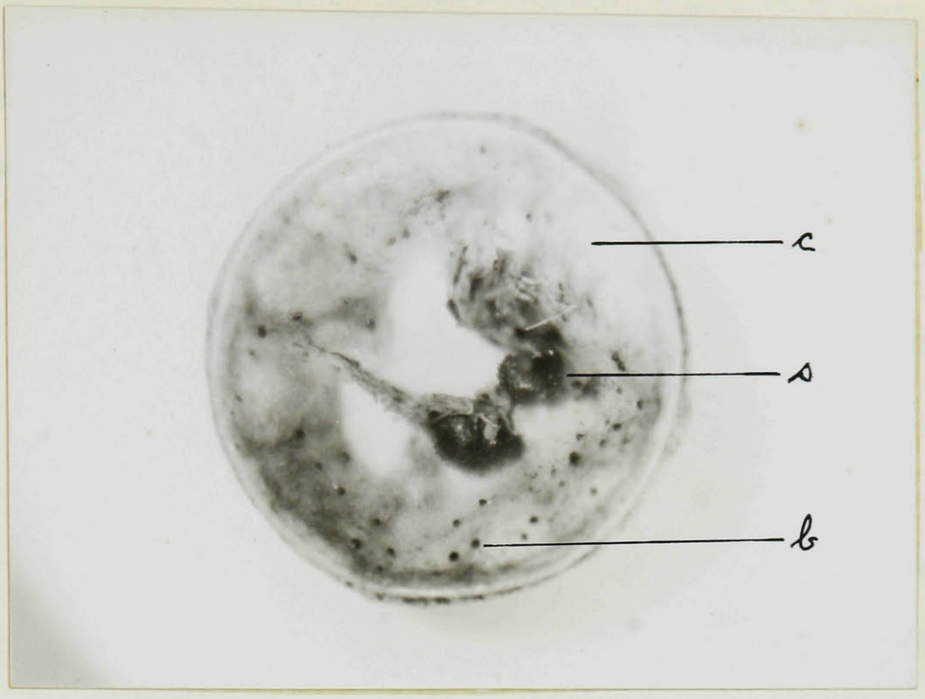


Figure 10 - Inside view of a diseased rose moss seed capsule. Approximately x 12. Note the numerous bulbils embedded in the inner wall of the capsule. Mycelium and conidia are also present, but these are not visible. b - bulbils; c - inner wall of capsule; s - seed.

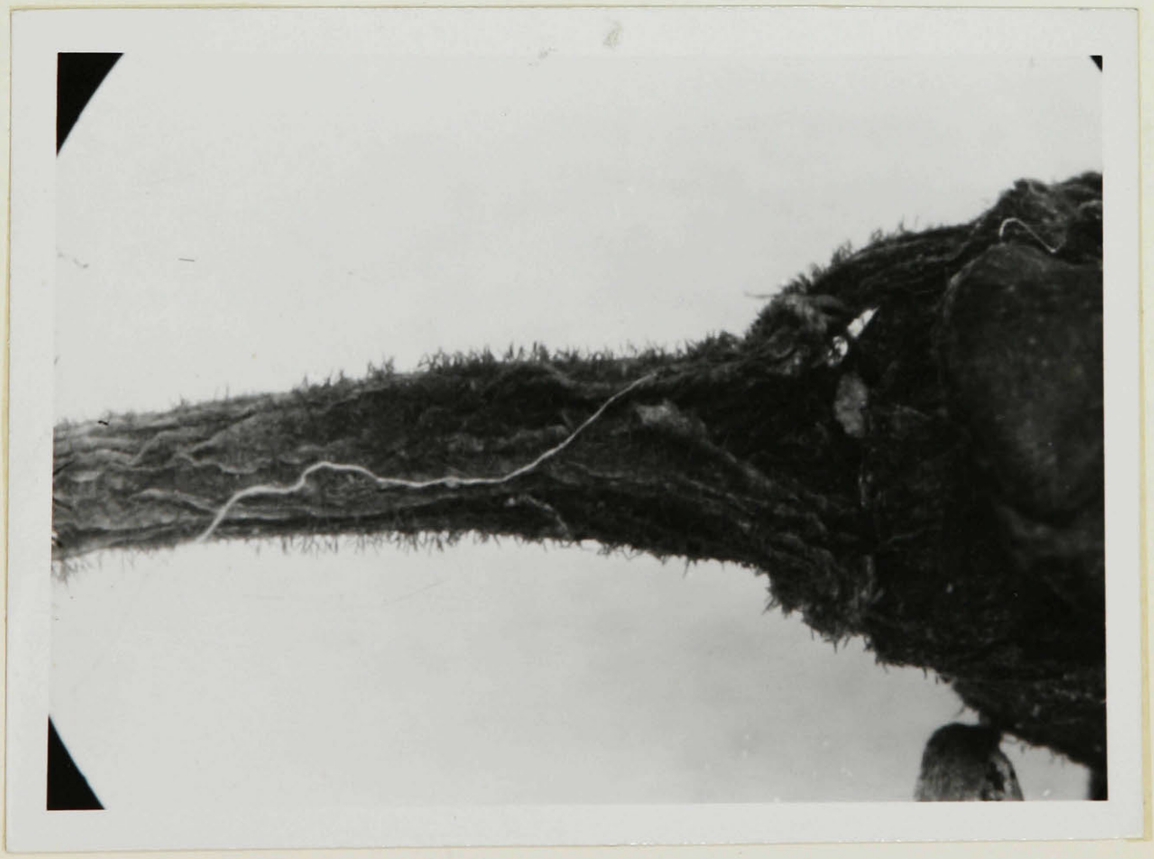


Figure 11 - A photograph of a diseased portion of a naturally infected rose moss stem. Approximately x 6. Conidia of H. portulacae can be seen along the upper and lower edges of the stem.

Although seeds of purslane and rose moss do not become infected, diseased seed capsules and seed capsules from diseased plants usually contain large numbers of underdeveloped, shrivelled seeds. The severity of this shrivelling depends upon the stage of development reached by the plant before infection occurs. In general, so long as the capsules are nearing maturity, seed development is not disturbed regardless of where infection occurs. However, if stems become infected during the early stages of capsule formation, seeds will either not develop at all, or will be shrivelled. If at this time the climate is unfavourable for the organism, some viable seeds will be formed (see Figure 12). If the capsules themselves become infected at an early stage in their development, they are destroyed together with the developing seeds.

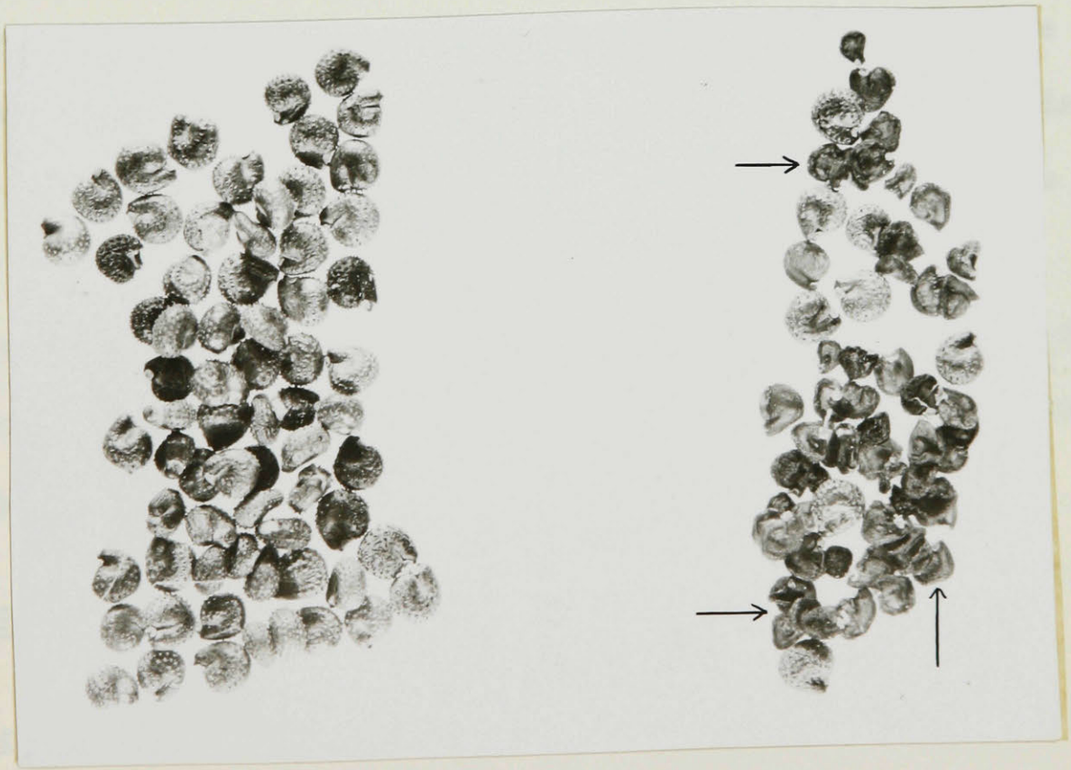


Figure 12 - Left: seeds from a healthy seed capsule which developed on a healthy rose moss plant; right: seeds from a seed capsule which developed on a branch that became infected at an early stage in the development of the capsule. Approximately x 8. Arrows point to shrivelled seeds.

STUDIES ON THE PHYSIOLOGY OF THE PARASITISM OF THE PATHOGEN

Certain aspects of the physiology of the parasitism of H. portulacae were explored with the hope of discovering the manner in which this pathogen parasitizes its host plants. Information on its method of attack and action once host tissue is penetrated, was obtained by carrying out a series of infection experiments and investigating separately each phase of the parasitism of the fungus.

Unless otherwise indicated, the isolate used for these studies was obtained from the original diseased purslane material collected at Ste. Anne de Bellevue, Que. A fresh stock of cultures derived from a single conidium was used for each experiment. Each stock of cultures was prepared as follows: With the use of a sharp needle and a dissecting microscope, a single conidium was picked off the specimen on which the fungus was fruiting and planted on potato dextrose agar in a Petri dish. The resultant growth was then used as a source of inoculum to obtain the supply of stock cultures. All cultures were grown on potato dextrose agar in Petri dishes kept at room temperature. Cultures were carefully examined before use, and those showing any abnormal features were discarded.

Penetration and Development of the Fungus in the Host

Young excised purslane leaves were used to study the process of penetration and to follow the further development of the organism in

the host tissue.

The upper surface of leaves, previously washed in water to remove as many micro-organisms as possible without damage to the leaves, and dried between two pieces of filter paper, was inoculated with dry conidia. The conidia were removed from a Petri dish culture by brushing the surface of the agar with a dry camel's hair brush. Then, by gently stroking the leaf with the contaminated brush, a number of conidia were evenly distributed over its surface. The leaves were examined with a dissecting microscope and massed spores and clumps were removed with the brush. The inoculated leaves were floated on water in a covered Petri dish, and the leaves were incubated at room temperature which varied from 23° to 31.5°C., and averaged about 27°C. At intervals of seven, 12, 20, 24, and 36 hours, one leaf was killed and fixed in a Nawaschin type killing and fixing fluid (Craf III) (43), and another leaf was killed and partially cleared in a saturated solution of chloral hydrate for 48 hours. The latter was rinsed in water and transferred to a five per cent solution of potassium hydrate for a similar length of time for further clearing (37). It was then stored in 70 per cent ethyl alcohol until required.

The fixed material was embedded in paraffin by the usual procedure, and sections ten to 15 microns in thickness were cut. Heidenhains iron-alum haematoxylin and Sudan III (Grubler's) were used for staining. In making up the latter stain, ½-gram of Sudan III was mixed with 100 cubic centimeters of 80 per cent alcohol. Sections were mounted in

Canada balsam. Haematoxylin stains hyphae dark purple to black, and Sudan III stains the cuticle orange red.

The cleared material was washed and stained with a solution of lactophenol cotton blue for 15 minutes, washed in water to remove the excess stain, and treated with Sudan III for ten minutes. This schedule stains hyphae blue and cuticle orange red. The specimen was then rinsed in water and mounted in lactophenol for observation.

Penetration by the germ tubes of H. portulacae was effected in two ways, (a) through the stomata and (b) by direct penetration through the cuticle. Stomata were entered regardless of whether they were open or closed, and in direct penetration, the cuticle was penetrated at any point along the juncture of two or more cells. In 50 observations of infection after the 12 hour incubation period, 23 penetrations were accomplished through open or closed stomata, 19 between a guard cell and accessory cell or between an accessory cell and an adjacent epidermal one, and eight between other epidermal cells. Open stomata were entered by the germ tubes immediately, but the entrance through closed ones and penetration of the cuticle was always gained with the help of an appressorium which developed at the tip of the germ tube.

Single germ tubes, on occasions, formed more than one appressorium. In such cases, the second organ developed at the tip of a branch segment which grew out of the primary tube a short distance behind the first formed appressorium. In one instance, three successive

appressoria were seen to have formed from one germ tube (see Plate XI, figs. 1A, 1B, and 1C).

The appressorium itself is a more or less rounded structure which averages 9.7 microns in diameter. It is separated from the body of the germ tube by a septum. The upper surface is curved and the lower one, which is pressed close to the leaf surface, is flattened and follows the contours of the cuticle. A gelatinous sheath attaches the appressorium to the cuticle, and in stained sections it appears as threads or strands (see Plate XI, figs. 4 and 5). Older appressoria do not show this feature.

Germ tubes are also pressed close to the cuticle, but whether they are attached to its surface by a gelatinous envelope, as was the case with germ tubes developing on dry glass slides, is not definitely known. This appears likely, since it was not possible to dislodge them intact with a sharp needle from the leaf surface.

Most investigators are of the opinion that the stimulus for the formation of appressoria is one of contact (6) (15) (17) (25). However, contact alone cannot account for the formation of appressoria by the germ tubes of this fungus, since these organs were not formed by conidia germinating on dry glass slides. This was shown in a previous experiment on conidial germination. It is obvious then, that either an entirely different stimulus, or a combination of stimuli, which may include contact, must be involved.

Plate XI

Camera lucida drawings of appressoria and method of penetration by H. portulacae.

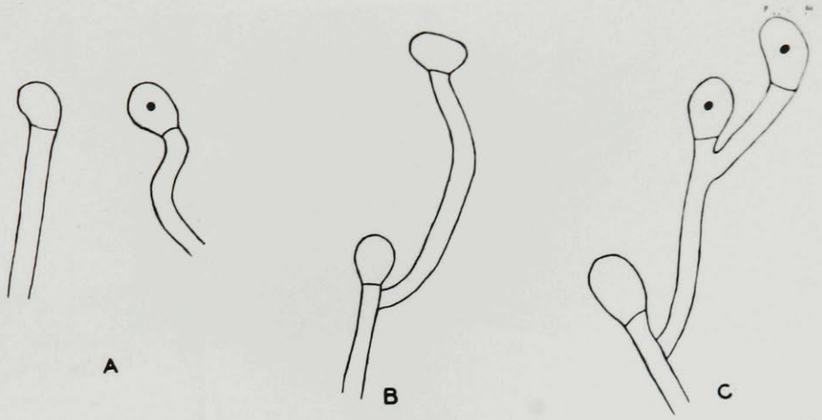
figs. 1A, 1B, and 1C - Surface view of the appressoria. Note the dark spot which might be the base of an infection hypha in the centres of appressoria in figs. 1A and 1C.

fig. 2 - Surface view of an appressorium formed over the guard cells of a closed stoma. The dark centre spot seems to indicate successful penetration.

fig. 3 - Surface view of a germ tube entering an open stoma.

fig. 4 - An early stage of penetration through a closed stoma. Note the constricted infection hypha and the thready appearance of the gelatinous sheath cementing the appressorium to the guard cells.

fig. 5 - A very early stage of cuticular penetration. Note that the infection hypha, (a) which has just emerged from the cuticle, is expanding and is surrounded by a clearing (b) of the softer subcuticular wall tissue.



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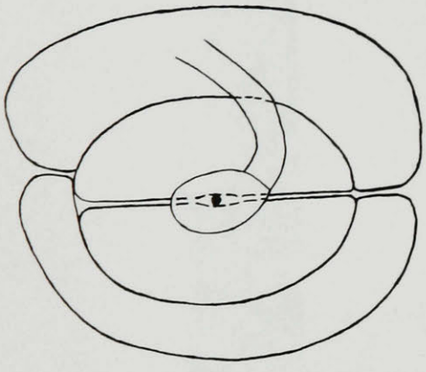

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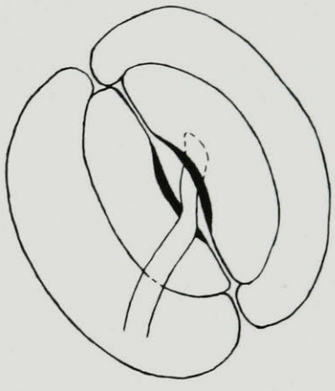

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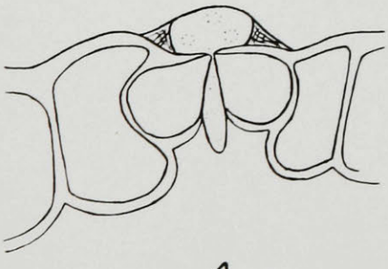

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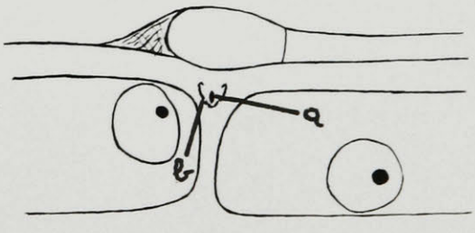

5

10.4 μ

The stimulus which causes infection hyphae to grow out of appressoria attached to leaf surfaces, may or may not be the same as the stimulus which causes them to form. Brown and Harvey (6) and Dickinson (17) believe that the stimulus to penetration in Botrytis cinerea and in rusts is purely one of contact, but Brown (5) hinted that chemical factors might well influence the nature and degree of this response. In the case of Colletotrichum gloesporioides, Dey (15) showed that appressoria on young lemon leaves send out infection hyphae as a result of a stimulus derived from substances diffusing outwards through the cuticle. He suggests that the failure of appressoria to germinate on older, more mature leaves is due to the inability of nutrient substances to pass through the thicker cuticle of these older leaves. The stimulus for the formation of these organs on lemon leaves is one of contact.

In H. portulacae the stimulus for infection also seems to be supplied by substances which exude from the stomatal pore, and probably from the guard and accessory cells which are closely associated with it. This is evident from the fact that 84 per cent of the observed leaf infections after a 12 hour incubation period, were initiated either through the pore or between the cells in close proximity. The other 16 per cent were initiated between cells farther removed from the stomata. The cuticle covering the cells adjacent to guard cells measured one micron or less in thickness. It gradually became thicker with increasing distance of the epidermal cells from the stomata

and measured as much as 4 to 4.2 microns over the farthest removed cells. This thick portion of the cuticle was penetrated less frequently than thinner portions. According to Dey's theory (15), these results can be interpreted as meaning that the thicker portions of the cuticle probably hinder exosmosis and thus would not permit substances stimulating germ tube penetration to exert their full influence. In comparative tests with mature purslane leaves, the percentage infection through the thicker portions of the cuticle averaged only 6.4 per cent of the total, however, measurements showed that there was no significant difference between cuticle thicknesses in old and young leaves. This undoubtedly means that the physical or chemical nature of the cuticle of older leaves undergoes some change which makes it still less permeable to the stimulating substances, or makes it more resistant to penetration by the infection hyphae.

That moisture may play an important role in stimulating the formation of appressoria and infection by H. portulacae appears to be ruled out from the results noted in leaf inoculation tests. These tests, which are discussed fully later on, showed that free moisture seemed to hinder infection rather than induce it. As for appressorial formation, it was found not to occur when conidia were allowed to germinate in water drops on glass slides and on very thin layers of fresh nutrient agar. Although these findings do not warrant definite conclusions, they certainly seem to indicate that the probable stimulus

for appressorial formation and penetration is derived from a substance or substances which emanate from the leaf itself.

Stomatal penetration

Entrance through closed stomata is accomplished by means of an exceedingly fine thread which develops from the flattened portion of the appressorium in contact with the guard cells. This fine thread, or infection hyphae, forces its way in between the outer edges of the closed guard cells and then immediately enlarges in the inverted V-shaped space between the closed guard cells (see Plate XI, fig. 4 on page 70). On entering the substomatal chamber, the hypha is of normal size. As mentioned earlier, the germ tube passes through open stomata without appressorial formation or constriction (see Plate XI, fig. 3 on page 70), and regardless of whether stomatal entrance was forced or not, the further development of the penetrating portion follows one of two patterns.

In the majority of cases, it continues to elongate until it makes contact with the spongy parenchyma cells directly below the stomatal chamber. As soon as they are reached, the hypha branches and the intercellular mycelium starts to advance between the cells. At other times, the penetrating portion branches into a number of segments as soon as the stomatal chamber is entered. This results in the upper part of the chamber becoming filled with mycelium, which then sends out a large number of branches, thus making a mass attack on the host tissues (see Figure 13).

Figure 13 - Photomicrograph showing a substomatal cavity. The upper portion of the cavity is filled with hyphae which originated from an infecting germ tube after it entered an open stoma. Stoma is not visible due to the angle of cut through tissue. Approximately x 450.

Cuticular penetration

The details of the process of cuticular penetration cannot be given, as it was not possible to observe the actual ingress. Nevertheless, there is reason to suppose that the penetration was affected by mechanical pressure, in much the same way as was forced entry into closed stomata. A thorough study of the cuticle did not show any signs

of disorganization or swelling anywhere along its line of contact with the appressorium, which might suggest solvent action on the cuticle. Further, in all cases of successful infection initiated through the cuticle and closed stomata, as observed with cleared material, a minute black point was distinctly visible in the appressorium (see Plate XI, figs. 1A, 1C, and 2 on page 70). By carefully focusing the microscope, the point was seen to be located in the lower wall of the organ pressed close to the epidermis, and appeared as though it might be the base of an infection hypha. Stevens (47), working with a Helminthosporium sp. that attacks wheat, found such points in the appressoria too. He observed them as bright points, and suggested that they were holes in the cell wall.

The penetrating portion of the infection hypha is undoubtedly extremely fine, since it is not visible in the cuticle even with the highest magnification.

Invasion

Penetration of the cuticle being accomplished, the infection hypha becomes visible only after it has passed for a short distance into the softer walls of the epidermal tissue. In sectioned material, the tip of the primary hypha is slightly swollen and stains a dense black colour. Before the tips of the primary hyphae can be detected, a definite change can be seen in the epidermal cell wall between two cells at the point where later the hyphal tip develops. The wall, at

this point, becomes clear. Later on, when the hyphal tip makes its appearance, it is surrounded by a similar clearing of the wall material. This is well seen in Plate XI, fig. 5 on page 70 . As the infection hypha continues to expand and elongate, it penetrates the common wall of the epidermal cells directly beneath it, thus assuming an intercellular position. Soon it is of normal size, and the cell wall changes, as just described, become evident well in advance of it.

The action of the fungus in advance of the hyphal tip is undoubtedly upon the middle lamella and must be due to a substance which is secreted by the fungal tip. Furthermore, the substance is apparently quite diffusible and seemingly selective in its action since even after the middle lamella is destroyed, there are no signs of swelling or disorganization of the secondary cellulose materials of the affected cells (see Figure 14).

If the initial infection is initiated through the thicker portions of the cuticle of the upper surface, the infection hypha, as it thrusts through the epidermal layer, enters the palisade region. The hypha branches and intercellular mycelium gradually invade all of the living parenchyma of the leaf. On the other hand, if infection is initiated through the thinner cuticle portions near the stomata, the fungus, on passing through the epidermis, enters a substomatal cavity. In this way, the palisade cells are by-passed, and invasion of the host tissue begins in the spongy parenchyma region. Later,

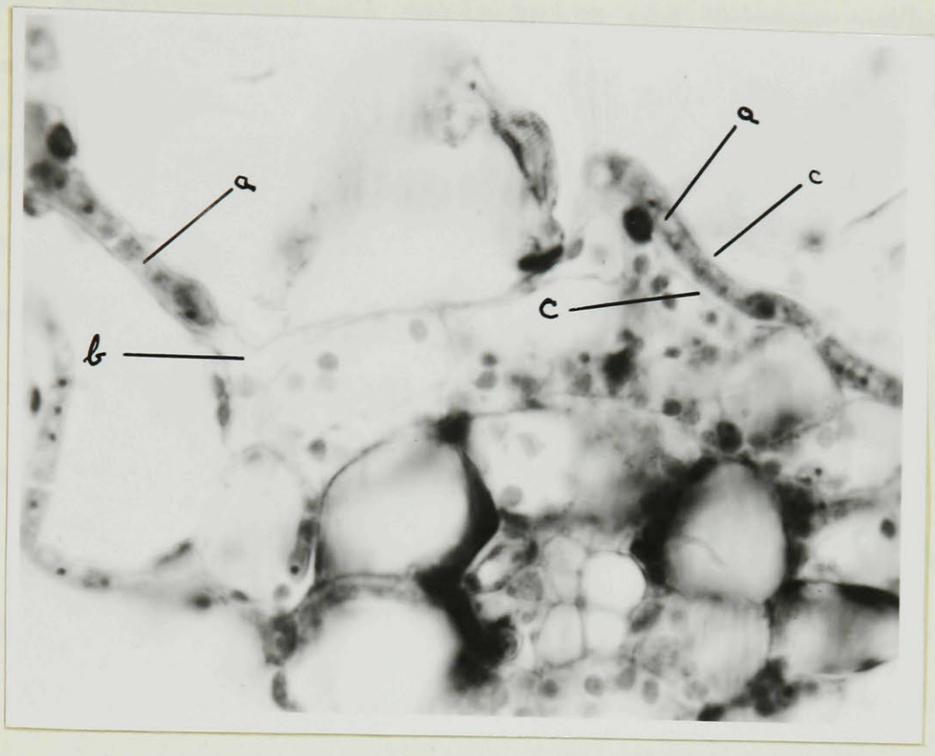

Figure 14 - Photomicrograph of a cross section through living and yellowed purslane leaf tissue 12 hours after inoculation. Approximately x 450. Note: a - the intercellular position of hyphal tip advancing through live tissue; b - separation of cells in advance of the hyphal tip ; c - the absence of any swelling or distortion of the cell walls in direct contact with the hyphae.

when the tissue becomes necrotic, the fungus invades it both intercellularly and intracellularly.

After the seven-hour incubation period, infections through the stomata, whether open or closed, and through thinner cuticle portions about the stomatal pore,were well established. It is obvious then that in these cases penetration into the host occurred previous to seven hours.

In 12-hour material, the fungus appeared merely as swollen hyphal

tips in the subcuticular cell walls below the thicker cuticle portions, while the organism had advanced well into the leaf, sometimes half-way through the thickness of the leaf when it had entered through the stomata or through a thinner portion of the cuticle near a stomata. When the hyphal threads had grown half-way through the leaf thickness, action on the middle lamellae had extended to several layers of cells in advance of the hyphal tips.

Dark discolouration of cells, a characteristic of death, was not observed in inoculated leaves incubated less than 20 hours. Observations on cleared leaves indicated that the first cells to become brown were those adjacent to and directly below the point where the organism had originally penetrated the epidermal layer. Observations on sectioned material revealed that the first cells to become affected, actually were those between which the intercellular hyphae had first advanced in the initial stages of invasion. For example, in direct penetration through the thicker portions of the cuticle, the first cells to turn brown were the epidermal and palisade cells, between which the infection hypha had advanced in establishing infection. However, in the case of stomatal entrance, the first cells to die were those about the substomatal cavity between which the organism had advanced. These cells died while the guard cells were still alive.

The discolouration, which was at first limited to a few cells, gradually spread to involve others, and in the meantime the first necrotic cells were rapidly invaded by intracellular mycelium.

Penetration of the cell walls was accomplished by a fine hyphal thread, which developed from mycelium in contact with the wall. This thread was just visible with an oil immersion lens.

In leaves incubated for 36 hours, the necrotic spots ran together to form irregular dead areas while the leaves were completely water-soaked (see Figure 15), and the pathogen was fruiting abundantly on the dead tissue.

Figure 15 - A healthy leaf above, and a diseased leaf below, photographed at approximately 9 x with transmitted light. The lower leaf is completely diseased through the coalescence of a number of separate infections. The dark areas, which are necrotic, represent the older portions of the separate infections. The semi-transparency of the intervening tissues, is due to watersoaking of the tissues in the early stages of degeneration.

In cleared leaves stained with lactophenol, hyphal strands were seen to have reached all portions of the leaf tissue, and invasion was therefore considered to be complete. When a leaf was removed and allowed to dry at this stage, the organism formed bulbils in great abundance.

The symptom picture in naturally and artificially inoculated plants and plant parts, and the observations on the development of the fungus in host tissue just described, reveal clearly an important aspect of the mode of parasitism of H. portulacae. The outstanding feature of the parasitism of this organism is the fact that it secretes a substance which causes tissue to lose its coherence in advance of its growth through the tissue by dissolving the middle lamellae. In this way, resistance to invasion by the intercellular hyphae is removed, and the organism can permeate the affected tissue with ease. The next stage in its parasitism is marked by death of cells behind the hyphal tips which begins about 14 hours after the organism has established infection.

The sequence of events just described outlining the manner in which the fungus advances through infected tissue, satisfactorily explains the occurrence and nature of the three distinct zones. These zones are visible in old lesions previously described on page 59, that girdle the infected stems and branches of host plants. The black centre zone of the girdling lesion, the brown watersoaked zone next to it, and the inner part of the paler yellow margin immediately

bordering the brown zone contain the fungus. The outer part of the yellow coloured margin marks the extent of stem tissue being acted upon by the secretions of the advancing fungal tips. To test this theory, thin cross sections of diseased stem tissue cut through the three different zones, and healthy tissue were plated in order on potato dextrose agar. The fungus was recovered from the sections of tissue cut from the black and brown zones and from the inner part of the yellow margin bordering the brown zone, but it was not recovered from the rest of the yellow margin or from the healthy tissue (see Figure 16).

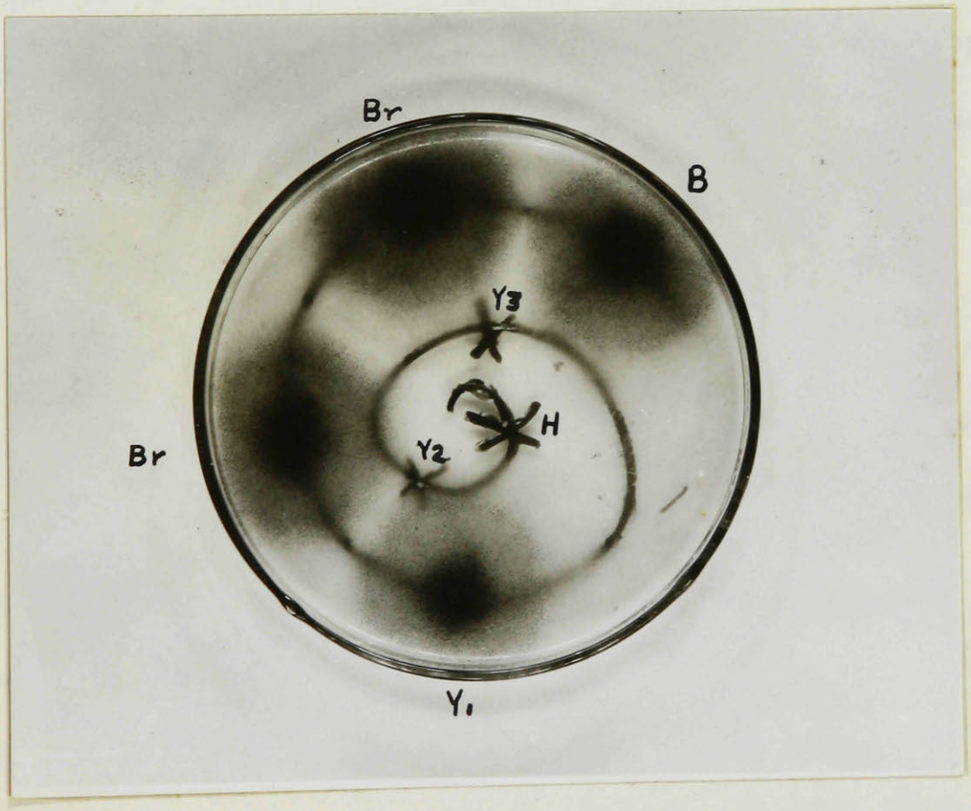

Figure 16 - Results of planting on agar cross sections of stems cut through the three distinct zones of a typical girdling stem lesion and through the healthy tissue just beyond the lesion. B- black zone; Br - brown watersoaked zone; Y- yellow zone; H- healthy tissue. The spiral figure and cross markings visible in the photograph were made on the under-side of the Petri dish to act as guides in positioning the cut stem tissue. Note Y_1 indicates inner part of yellow zone, Y_2 central part, and Y_3 the outer part.

Enzyme and Toxin Secretion Studies

The nature of the substance responsible for 'action in advance' by plant pathogens has intrigued many investigators, and although much work has been done to attempt to answer the question, disagreement still exists. Much of the controversy seems to centre about the question of whether disorganization and death of tissue are due to the same substance or to different substances.

Among the earlier workers on maceration of cells by fungi, Marshall (35) showed that loss of coherence of lily tissue by Botrytis was due to an enzyme which attacked the cell walls as well as the middle lamellae. However, he did not determine whether death of cells was due to the same substance.

Smith (45) compared the actions of a mycelial extract of Botrytis cinerea and weak solutions of oxalic acid on the stems of lettuce, and from the results of the tests he concluded that 'action in advance' was due to secretion of this acid.

Brown (3) obtained from young hyphae of Botrytis cinerea a powerful extract which disorganized and killed tissues of different plants when they were immersed in it, and he secured evidence that both effects were due to an enzyme. He then states that if such is the case, the lethal effect if due to either the direct action of the macerating substance upon the protoplasmic membrane of the cell (toxic enzyme), or to an indirect

action upon the cell wall through some special relationship which exists between the cell wall and the protoplasm. This appears to be the case, since Brown (3) found that if the cell wall was disintegrated, death of the cell ensued; if the cell wall was not affected, neither were the living contents of the cell.

Some time later, Harter and Weimer (24) and Weimer and Harter (49) published reports that Rhizopus tritici, when grown on a sweet potato decoction, produced both an intercellular and intracellular pectinase capable of dissolving the middle lamellae of raw sweet potato, and also, that pectinase was contained in the conidia of R. tritici and R. nigricans. No mention of a toxic substance was made by them.

In the case of Pythium deBaryanum, Hawkins and Harvey (26) found that this organism secretes both an enzyme and a toxin. Further, the enzyme appeared to be a specific one, since only the middle lamellae of potato tissue was affected. The enzyme had little or no effect on the secondary walls of the cells.

Based on experiments in which the juice of apples rotted by certain fungi was used to macerate potato, carrot, and apple tissue, Lin (31) suggested that saprophytic plant pathogens fall into two groups as far as their mode of parasitism is concerned. The first group secretes large quantities of protopectinase and little or no toxins, and may be represented by Penicillium expansum and Sclerotinia fructicola. The other group, represented by Lambertella corni-maris,

Physalospora malorum, and Glomerella cingulata, produces powerful thermostable toxic substances, but little or no protopectinase.

With these findings in mind, enzyme experiments were carried out to determine:

1) whether 'action in advance' by H. portulacae is due to an enzyme

2) whether the lethal effect, which occurs much later and behind the advancing fungus, is independent of any such enzyme

3) whether this lethal action is due to a distinct toxic substance secreted by older hyphae.

The method used for this enzyme experiment is a modified version of that used by Brown (3), and was as follows:

Preparation of material for extraction

Ten cubic centimeters of sterile distilled water were poured into each of six ten-day old Petri dish cultures of H. portulacae chosen from a stock of cultures for their abundant sporulation, and a suspension of conidia was obtained by rubbing the surface of the agar with the finger. To get rid of coarse extraneous material which was loosened in the process, the fluid contents of each dish were poured through two layers of muslin cloth stretched over the mouth of a funnel, the stem of which was inserted into a large test tube. The level of the suspension in the test tube was marked. Finer debris, which passed through the crude filter, was removed from the suspension by centrifuging at the rate of 2200 r.p.m., for a few minutes. This caused the

heavier conidia to be thrown to the bottom of the centrifuge tube, and one half or more of the liquid was carefully decanted. The remaining liquid was agitated to resuspend the conidia, and the suspension was then returned to the original test tube which had been washed and dried. The centrifuge tube was rinsed with a small quantity of distilled water which was added to the large tube, and the volume was restored to 60 cubic centimeters by the addition of the necessary amount of distilled water.

Five cubic centimeters of this conidial suspension were then added to each of 12 sterile flasks of 500 cubic centimeter capacity, containing five cubic centimeters of a potent sterile potato dextrose medium containing 400 grams of potatoes and 40 grams of dextrose per litre of water. The addition of the suspension reduced the concentrated medium by half, and gave a total of ten cubic centimeters of medium in each flask. Ten cubic centimeters was just enough to cover the bottom of a 500 cubic centimeter flask.

After 18 hours at room temperature, the surface of the medium was completely overgrown with mycelium, and gave the appearance of a thin, delicate lace netting. Microscopic examination of teased material showed that apart from clumped conidia, germination was well advanced and normal. These cultures provided an abundance of young active hyphae, and were considered to be, in the light of Brown's findings (3), in an ideal condition for maximum enzyme production.

Each flask was agitated to loosen parts of the mycelial mat

adhering to the sides, and the contents were filtered through one-ply muslin cloth stretched over the mouth of a funnel. The young interlaced mycelium was of course trapped on the cloth, the fluid contents passing through. The whole mass of fungal material was then washed in running tap water for ten minutes. Ungerminated conidia and nutrient medium were by this means washed away, while the net-like nature of the mycelium prevented it from passing through the cloth. The washing was then followed with successive rinsings in large quantities of distilled water. Excess water was allowed to drain off the fungal mat, after which it was spread evenly over a glass plate and dried in a dessicator. When sufficiently dry for grinding, the material, now of a brittle texture, was scraped off the glass plate, weighed, and then mixed with an equal weight of clean quartz sand. The total mass from all 12 flasks plus the added sand weighed 1.72 grams; it was transferred to a mortar and ground until the mycelium was completely disintegrated.

Half the amount of the fine powder was suspended in ten cubic centimeters of distilled water in a test tube, and extracted for one hour. The material, which settled fairly rapidly, was kept in suspension by rotating the tube between the hands at frequent intervals. The liquid was centrifuged for three minutes at the rate of 3400 r.p.m., decanted into a fresh tube, and the process was repeated. The final liquid was olive green in colour, translucent, and had a slight, musty odour.

Action of extract on tissue

The extract was tested in the following manner: Fresh young purslane leaves and pieces of stem about one centimeter long were washed in running tap water and rinsed in three changes of sterile distilled water. This pre-treatment of plant tissue was for the purpose of removing surface micro-organisms, which might possibly interfere with the test. The leaves were then cut into small squares, the stems were quartered lengthwise, and eight pieces of each tissue were immersed in the following, contained in test tubes: (a) extract, (b) extract which had previously been heated to 70°C. and cooled, and (c) distilled water. The tubes were evacuated for a few minutes to ensure penetration of the liquid, and the tissue was examined at intervals.

Resistance of the treated tissue to mechanical pressure and pull was taken as a measure of enzymatic activity, and the loss of coherence of tissue was regarded as complete when the material offered no resistance.

For the sake of convenience and clarity, the results of these tests are recorded in table form and are given in Table XII.

These tests prove conclusively that disorganization of tissue is caused by an enzyme, since heated extract has little or no effect. A turbidity, due to developing bacteria, appeared after 20 hours in both extracts. It was these organisms which caused the stem tissue in heated extract to become soft after 24 hours.

TABLE XII - Effect of Extract and Heated Extract of 18-hour Old Mycelium of H. portulacae and Distilled Water on Purslane Leaf and Stem Tissue in Relation to Time.

Time in extract in hrs.	Treat.*	Condition of tissue		Resistance# to Mechanical Pressure and Pull	
		Stem	Leaf	Stem	Leaf
1	E	dull pink	dull green	++++	++++
	HE	dull pink	dull green	++++	++++
	W	shiny pink	shiny green	++++	++++
3	E	dull pink; curling of cuticle; watersoaking	dull green; watersoaking	+++	++
	HE	dull pink	dull green	++++	++++
	W	shiny pink	shiny green	++++	++++
7	E	loose cells in extract	watersoaked	nil	+
	HE	watersoaking	dull green	+++	++++
	W	shiny pink	shiny green	++++	++++
20**	E	cuticle curled; many cells loose; few cells dead	epidermis curling; few cells loose; few loose cells dead	nil	nil
	HE	watersoaking	watersoaking	+++	+++
	W	outer cells	outer cells	++++	++++
24**	E	groups of cells loose; few dead cells	groups of cells loose; few dead cells	nil	nil
	HE	watersoaked	watersoaked	++	+++
	W	mostly watersoaked	mostly watersoaked	+++	+++

*E - untreated extract; HE - heated extract; W - distilled water (checks)
#+, very little resistance; ++, little resistance; +++, resistant
++++, very resistant
**E and HE - turbid

An examination of the macerated host tissue revealed that the separated cells were still alive. This clearly shows that neither the enzyme nor any other substances in the extract were lethal. Further proof of this fact is demonstrated in Figure 14 on page 77 which shows that cells separated in advance of invading hyphal tips by secretions of an enzyme from these tips, are still alive. Moreover, cells in direct contact with the young invading hyphae are also alive. Therefore, the lethal action is not due to the middle lamellae dissolving enzyme, and suggests that it might possibly be caused by some secretion of older mycelium. In a preliminary test for such a toxic secretion, a one per cent potato dextrose medium, in which H. portulacae had been growing for ten days, was sterilized through a Seitz filter and heated to 70° C. to inactivate enzymes. Cut stems of purslane, rose moss, and bean plants were immersed in this preparation. Ordinary potato dextrose medium and water served as checks.

All plants immersed in the medium in which the fungus had been growing wilted, while the check plants remained turgid (see Figures 17, 18, and 19). Bean plants wilted in 30 minutes, but purslane and rose moss plants took almost three hours. In all cases, browning of the inner tissue of the cut stems was apparent, but only purslane leaves showed necrotic spots. In order to remove doubts that these effects may have been caused by staling products, 36-hour old mycelium was grown on the same medium under the same conditions, extracted, and the extract tested in the same way as was described previously

for 18-hour old mycelium. In this test, cells of tissue immersed in both heated and unheated extract started to turn brown after one hour. The outside cells were affected first, but before long this condition became general. As before, disorganization of cells occurred in the unheated extract only, but this action was considerably slower than the lethal one.

Thus, the extract of a young culture attacks essentially the middle lamella, while that of a comparatively old culture is lethal to host cells and attacks the middle lamella less vigorously.

Figure 17 - Left: a cut stem of a young purslane plant immersed in a Seitz-filtered enzyme inactivated medium in which H. portulacae had been grown for ten days. Note the necrotic condition of the lower leaves; centre:- a cut stem of a young purslane plant immersed in water; right: a cut stem of a young purslane plant immersed in medium alone. The plants were photographed nine hours after immersion in the various liquids.

Figure 18 - Left: a cut stem of a young rose moss plant immersed in water; centre: a cut stem of young rose moss plant immersed in a Seitz-filtered enzyme inactivated medium in which H. portulacae had been grown for ten days. Note the wilted and shrivelled condition of the leaves; right: a cut stem of a young rose moss plant immersed in medium alone. The plants were photographed nine hours after immersion in the various liquids.

Figure 19 - Left: a cut stem of a bean plant immersed in a Seitz-filtered enzyme inactivated medium in which H. portulacae had been grown for ten days. Note the wilted condition of the leaves; centre: the cut stem of a bean plant immersed in water; right: the cut stem of a bean plant immersed in medium alone. The plants were photographed 40 minutes after immersion in the various liquids.

Influence of Air Conditions on Amount of Leaf Infection

This experiment was carried out with the purpose of obtaining information on the optimum air conditions necessary for infection and disease development. It required the use of air conditioned chambers. As only three chambers were available, it was decided to operate them at 25°, 30°, and 35°C. for the following reasons: The optimum temperature for the growth of H. portulacae in artificial culture was previously shown to be close to 30°C. for most media tested (see Plates V, VI, VII, and VIII on pages 29, 30, 31, and 32). The results of preliminary greenhouse tests with P. oleracea and P. grandiflora had shown that there was no apparent difference in the growth habit of these plants at temperatures of 26°, 29°, and 33°C. The pathogen, however, showed marked differences in growth at these temperatures. It seemed reasonable to assume, therefore, that a five degree leeway each side of the optimum for fungal growth would allow for proper interpretation of the infection results which might be obtained at the selected temperatures. Further, since these temperatures are the same as those used for the conidial germination tests, any relationship which exists between infection and the rate of germination of conidia might be revealed.

The detached leaf Petri dish method (2) (20) (36) (51) was used in these trials. Unblemished purslane leaves of the same age and occupying the same stem position were cut from 31-day old plants

with short lengths of stem adhering to the petioles. This was done to minimize the effects of shock and to ensure that the leaves would be able to absorb water. The leaves were surface washed with water in an attempt to have them as free as possible of micro-organisms, and they were then dried between pices of filter paper. Four washed and dried leaves were floated in each of 72 Petri dishes containing water a few millimeters deep. Care was taken to see that the bits of stem tissue were immersed in the water, and 24 Petri dishes were then placed in each chamber in which the temperature was accurately controlled. After a waiting period of eight to ten hours, which should have permitted the leaves to come to the temperatures of the respective chambers, the leaves were inoculated. In 12 dishes, conidia of H. portulacae were mounted wet by brushing the leaf surface with a camel's hair brush dipped into a conidial suspension. The leaves in the other dishes were inoculated by brushing them with a dry brush which had been passed lightly over a conidial bearing colony. One uninoculated leaf in each dish served as a check.

At intervals of three, six, 12, and 24 hours, three dishes were removed from each chamber, and the leaves were immediately killed and partially cleared in a saturated solution of chloral hydrate. Each leaf was then examined with the aid of a dissecting microscope by means of transmitted light, and the number of infection points were counted. By this method, even very young infections were visible as tiny,

translucent, watersoaked spots. The amount of infection arising on the inoculated leaves is given in Table XIII. The uninoculated checks remained healthy in all dishes.

TABLE XIII - Effect of Temperature, Time, and Absence and Presence of Free Water on the Infection of Portulaca oleracea Leaves in a Moisture Saturated Atmosphere.

	Total number of visible infections in nine inoculated leaves					
Time in hours	Conidia mounted wet			Conidia mounted dry		
	25°C.	30°C.	35°C.	25°C.	30°C.	35°C.
3	0	0	0	0	0	0
6*	0	6	0	0	0	0
12*	24	35	33	31	42	51
24**	67	96	89	81	111	99

* infection visible as watersoaked spots only
**first appearance of necrosis.

Of the three temperatures tested, 30°C. was the most suitable temperature for infection with both wet and dry conidia. From the results of the germination tests on conidia, more infections were expected with wet conidia, since after a 24-hour incubation period the percentage germination of wet conidia was seen to be higher than that of dry ones. However, contrary to expectations, more infections were initiated with dry conidia. This held true for all temperatures. The average number of infections in leaves inoculated with wet and dry

conidia was 25°C. - 7.4 and 9; 30°C. - 10.6 and 12.3; 35°C. - 8.8 and 11 respectively. These results might possibly be explained by the fact that germ tubes developing in free water probably take longer to establish close contact with the leaf surface, thus delaying infection.

The shortest incubation period determined microscopically was six hours at 30°C. with wet conidia. At this early stage of infection, watersoaking, the first sign of infection, involved only three or four cells. In leaves incubated for 24 hours, watersoaking was quite extensive, and in many cases the spots ran together to form irregular blanched areas. This made it difficult to count the individual infections. However, it was possible to distinguish individual infections by the brown necrotic spot which developed in the centre of each watersoaked area.

In order to find out how soon after infection necrosis actually occurs, the experiment was repeated, and after 12 hours the leaves were examined at hourly intervals for the first signs of necrosis. The time of appearance of this symptom seemed to vary with conditions. With leaves inoculated with wet conidia and incubated at 30°C., the first signs of necrosis appeared after 20 hours, while those incubated at 25°C. and 35°C. developed brown spots about two hours later. With dry conidia, necrotic spots developed after 21, 19, and 21 hours at 25°, 30°, and 35°C. respectively.

Influence of Soil Conditions on Amount of Stem Infection

The following experiment was designed to determine whether or not the fungus, if introduced into the soil, can attack the main stems of purslane and rose moss plants. This is a very important aspect which must be considered because if the stems of the host plants prove to be susceptible to the fungus when it is introduced into the soil, artificial inoculation of soil with H. portulacae might be a useful method for eradicating this weed.

Main stem infections are more important than branch infections because the former interfere with the supply of nutrients and water to the whole plant, while infections on branches affect only the parts above the branch lesions. Further, as soon as the stem infections have developed into girdling lesions, the entire supply of these essential materials is cut off. Consequently, even if environmental conditions unfavourable for the subsequent development of the disease occur, affected plants or plant parts would eventually die through lack of water and nutrients. This, of course, assumes that the living branches of fallen purslane plants do not develop new roots at points of contact with the soil. This latter eventuality would not apply to rose moss, because as already mentioned, girdled rose moss stems remain erect; therefore, rose moss plants so affected never recover.

Older, more mature purslane plants, which usually grow prostrate, are not likely to die of starvation if the main stem is girdled,

because they are extremely succulent and can apparently stand long periods of drought. Hence, as soon as sufficient soil moisture is available, roots will form from the prostrate branches.

In contrast, to what happens when infection arises on the main stem, an infection on a branch does not cause distress in other branches unless the infection spreads downwards into the main stem. Without doubt, the whole pattern of events depends upon the conditions of the environment.

The soil inoculation experiment was conducted as follows: Twelve new flats, measuring 18 ins. x 9½ ins. x 3 ins., were divided crosswise into two equally sized compartments by means of tight-fitting wooden slats. Half the flats were filled with steam sterilized greenhouse soil, and the remainder with unsterilized soil. The soil in one half of each flat was removed and inoculated with 150 cubic centimeters of a heavy conidial suspension of H. portulacae. The soil was then thoroughly mixed by hand to distribute the inoculum evenly throughout the soil. The contaminated soil was then replaced in its proper flat.

Two flats of sterilized, and two of unsterilized soil, were then placed in the temperature control chambers operating at 25°, 30°, and 35°C., and watered. Each chamber was illuminated by thirty 40-watt fluorescent bulbs placed about 4½ feet above the flats.

When the temperature of the soil reached that of the chamber in which it was contained, 24, 35-day old plants of purslane, and 24 of rose moss were transplanted in separate blocks into each half of every

flat. Extreme care was taken to avoid injuring the roots or stems. One flat of sterilized soil and one of unsterilized soil in each chamber were well watered daily with tap water (a supply was kept in each chamber), in order to keep the soil wet, while the other two flats were watered much less so as to keep the soil only moist.

The plants were checked for disease at 12-hour intervals. The results of this test after 60 and 144 hours are shown in Tables XIV and XV respectively.

In all cases, unless otherwise indicated, the disease was definitely identified as that caused by H. portulacae. Unless the organism could be seen on the affected parts, diseased tissue was planted on agar to determine whether the organism was present.

The results show that the main stems of purslane and rose moss were successfully infected with H. portulacae when the fungus was introduced into the soil. It is evident too, that the process of infection was rapid. This was demonstrated when many of the plants became diseased only 60 hours after being transplanted into the contaminated soil. After 144 hours from time of transplanting, infection was greatly increased, and in a few cases more than one half of the plants in a block were involved.

The type of stem lesion exhibited by diseased plants was similar in all respects to those on field infected plants. Diseased plants examined after 60 hours showed that the stem lesions extended only a slight distance above the soil line. Infection apparently took place

Table XIV - Effect of Soil Temperature and Moisture on the Amount of Stem Infection on P. oleracea and P. grandiflora Resulting from Inoculation of Sterilized and Unsterilized Soil with H. portulacae

Soil treatment	Number of plants infected out of a total of 24, 60 hours from transplanting					
	Temperature					
	25°C.		30°C.		35°C.	
	P. oleracea	P. grandiflora	P. oleracea	P. grandiflora	P. oleracea	P. grandiflora
Wet sterilized soil						
inoculated	5	6	7	8(1)#	9(2)	6
uninoculated	0	0	0	1(1)	0	1(1)
Moist sterilized soil						
inoculated	2	3	2	2	2	6
uninoculated	0	0	0	0	0	1(1)
Wet unsterilized soil						
inoculated	5(1)	7(2)	7	13(3)	8(1)	6(1)
uninoculated	0	1(1)	3(3)	4(4)	6(6)	2(2)
Moist unsterilized soil						
inoculated	1	3(1)	2	1	4(2)	4(1)
uninoculated	0	0	2(2)	0	3(3)	0

The figure in brackets indicates the number of diseased plants found to be infected with organisms other than H. portulacae.

Table XV - Effect of Soil Temperature and Moisture on the Amount of Stem Infection on P. oleracea and P. grandiflora Resulting from Inoculation of Sterilized and Unsterilized Soil with H. portulacae

Soil treatment	Number of plants infected out of 24, 144 hours from transplanting					
	Temperature					
	25°C.		30°C.		35°C.	
	P. oleracea	P. grandiflora	P. oleracea	P. grandiflora	P. oleracea	P. grandiflora
Wet sterilized soil						
inoculated	9	11(1)#	16(3)	14(2)	13(3)	11(2)
uninoculated	0	0	0	2(2)	2(2)	3(3)
Moist sterilized soil						
inoculated	8(2)	5	8	10(1)	12(3)	8
uninoculated	0	0	1(1)	0	0	0
Wet unsterilized soil						
inoculated	14(3)	11(5)	19(4)	13(2)	18(5)	14(5)
uninoculated soil	2(2)	1(1)	4(4)	5(5)	6(6)	4(4)
Moist unsterilized soil						
inoculated	3	5(1)	5	6	9(3)	7(1)
uninoculated	0	2(2)	4(4)	2(2)	3(3)	1(1)

The figure in brackets indicates the number of diseased plants found to be infected with other organisms other than H. portulacae.

below the soil line. In most cases, the roots were still white and unaffected, and the damage was largely confined to that portion of the stem between the roots and the soil line, or slightly above the soil line. After 144 hours, all the below ground parts of the plant were diseased, and in many cases the whole seedling appeared water-soaked and soft. Bulbils were formed abundantly on the dead portions of the stem. Conidia developed when the dead stem portions were placed in a moist chamber.

Infection occurred at all the temperatures tested, but more plants were infected at 30° and 35°C. than at 25°C. This was to be expected, since it was demonstrated earlier that H. portulacae grows better at the two higher temperatures than it does at 25°C., and similar results were also obtained in the leaf inoculation test. Wet soil was considerably more favourable for infection and disease development than moist soil. Some check plants in both sterilized and unsterilized soil, especially those at the higher temperatures, became diseased, but the trouble was not due to H. portulacae. Check plants grew equally well at all temperatures.

The results in Tables XIV and XV also show that soil sterilization had little affect on the number of plants that became attacked by H. portulacae. This is surprising because a certain amount of natural biological control over H. portulacae might be expected to occur in unsterilized soils. However, such antagonistic effects do not always occur according to reports in the literature. For example, while Greaney and

Machacek (22) and Sandford and Cormack (42) have demonstrated that Cephalothecium roseum and soil isolates of Penicillium and Actinomycetes suppressed H. sativum, other investigators (10) (34) have reported that antagonism and antibioses were of little importance in respect to H. sativum. Further experimental work is necessary before any definite conclusions can be made concerning associative effects of H. portulacae with other soil organisms.

SOURCE OF PRIMARY INOCULUM

A number of tests were run to determine the source of primary inoculum. The results of these, together with some incidental observations, seem to establish that the pathogen has several ways of over-wintering.

In a previous test to attempt to develop the bulbils of the pathogen, which are thought to be immature perithecia, the fungus always grew out of pieces of infected dry dead stems and branches when they were planted on agar after the infected material had been subjected to temperatures ranging from about 14.8° to -22°C. (average -0.5°C.) for varying intervals up to five weeks. Therefore the mycelium is resistant to cold. Further, most of the conidia picked off fruiting material and planted on agar germinated and produced normal colonies, even after the last interval. Of 43 conidia planted on agar, 37 germinated. Obviously, the conidia of the fungus are also resistant to cold.

Some information on the capacity of the fungus to withstand dry conditions was obtained by pouring a conidial suspension of H. portulacae over unsterilized moist soil in a four inch pot. The conidia were taken from a culture on potato dextrose agar. The pot was covered with a Petri dish cover to allow the fungus to become established, and the soil was allowed to dry out slowly. This test was started in June, and in December, six months later, conidia and

bulbils could readily be seen on the surface of the dry soil with a dissecting microscope. The bulbils must have developed from mycelium arising from the conidia during that period of time from when the conidial suspension was poured over the soil until the soil became dry.

When moisture was added to the dry soil to provide moisture conditions favourable for the development of the fungus, there was a marked increase in bulbils, and conidiophores and conidia formed to some extent. The latter seemed to develop mainly on bits of organic matter present on the soil surface. Single conidia, removed from the soil before the addition of water, germinated well on dry glass slides in a moist atmosphere. This is not surprising, since a supply of conidia collected in the summer of 1949 and kept in a dry condition in a small box, still showed about 100 per cent germination during the winter of 1950-51. These results are similar to those obtained by Christensen (8) and Simmonds et al (44) who found that conidia of H. sativum remain viable for long periods of time under adverse conditions.

It was shown previously (see Figures 7 and 10) that the fungus can enter the capsule, suggesting that the fungus might be seed borne. To determine if this does occur, equal lots of viable unsterilized and surface sterilized purslane and rose moss seeds from diseased and healthy capsules were planted on acidified agar to discourage the growth of bacteria. The seeds were sterilized by immersion for three

minutes in a two per cent solution of chlorine. The results of the test are given in Table XVI.

It can be seen from the data that although H. portulacae can be carried in a viable condition on the seed surface, the seed itself does not become infected. In other words, the fungus is surface borne, but not internally borne by the seed.

All these facts, together with the results of the soil infection test (see page 97), which showed that the stems of purslane and rose moss at or below the surface of the soil are easily infected by conidia in the soil, provide excellent proof that the sources of primary inoculum are contaminated plant litter, contaminated seeds, and soil borne conidia, mycelium, and bulbils.

TABLE XVI - Contamination of Seeds from Diseased and Healthy Capsules.

Type of seed	Number plated	Source of seed	Organisms recovered	
			H. portulacae	Others
Purslane (unsterilized)	20	diseased capsules	9	10
Rose moss (unsterilized)	20	"	7	13
Purslane (sterilized)	20	"	0	0
Rose moss (sterilized)	20	"	0	0
Purslane (unsterilized)	20	healthy capsules	0	20
Rose moss (unsterilized)	20	"	0	18

LIFE HISTORY AND ENVIRONMENTAL RELATIONS

The results of tests conducted to determine the source of primary inoculum, and information gained on the effect of temperature and moisture on certain aspects of the development of the pathogen, make it possible to outline quite clearly the life history of the fungus and to discuss its environmental relations.

The primary inoculum is almost certain to be mycelium, conidia, and bulbils present in or on the surface of the soil, and on infected plant litter; and mycelium, and possibly conidia, on the surface of seed from diseased capsules. Since purslane and rose moss plants do not appear in the field until about the middle of June, it would seem that the fungus might build up through saprophytic growth in the soil before host plants were available; but, although sufficient moisture might be present in the soil during May to induce the growth of the fungus, the mean temperature in that month (which in 1949 and 1950 averaged 12.7° and 12.8°C. respectively) might well be the factor limiting its development. It was demonstrated previously that at 15°C. the pathogen grows very poorly and fruits sparingly (see Plates V, VI, VII, and VIII, pages 29, 30, 31, and 32 respectively). However, in June before the host plants appear, the temperature is more favourable for the growth of the fungus, and some build-up of inoculum would be expected.

With the appearance of the plants, temperature is favourable for

the development of both the host and parasite. Because only a few hours of free moisture or very high humidities are required for successful infection, short periods of rain or even dew should be sufficient to induce the disease. Although most infections would be expected to occur on the stem at or below the surface of the soil, it is reasonable to assume that infections in the upper parts of the plants would also be initiated by conidia which are splashed upward. However, most of these infections would probably be initiated from conidia which were originally produced on the primary stem lesions because the fungus fruits sparingly in the soil.

Once the pathogen becomes established, the possible lack of continuous moisture and high temperatures might well be limiting factors in the development of an epidemic. This point is discussed in more detail later.

Since the fungus depends upon wind and rain for dissemination, it is unlikely that conidia can be spread any considerable distance. In the first place, the conidia of H. portulacae are extremely large and are produced on short conidiophores; therefore, they are not well adapted for dissemination by either wind or rain. Secondly, purslane and rose moss plants are comparatively short and grow in dense groups; consequently, conidia would become trapped amongst the foliage parts, particularly those conidia which have developed on the lower parts of affected stems and branches.

Survival tests suggested that the fungus in all its stages is able to over-winter on affected plant debris and in or on the soil. The perfect stage was never discovered in nature, and attempts to develop the sexual stage in culture have failed.

DISCUSSION

The Helminthosporium disease of purslane and rose moss studied in this investigation was proven to be due to H. portulacae as described in 1948 by Rader (39). A detailed description of the disease has been presented for the first time. A detailed study of the disease has revealed that it is very destructive on purslane and rose moss. Affected plants become partially to completely involved in a short time. A single infection on a purslane leaf soon leads to its death. A stem or branch infection on either host soon develops into an elongated girdling lesion, causing all the healthy portions above the lesion to wilt. In the case of purslane, healthy portions above a girdling lesion collapse before wilting occurs, but in rose moss affected stems remain erect because of the woody nature of the plant. In either case, wilted portions are soon overrun by the fungus. With the occurrence of conditions checking the development of the disease in its early stages, affected host plants may recover somewhat by developing new branches. The rapid progress of the pathogen in infected plants indicates that host plants are entirely passive, and therefore unable to alter the course of development of the disease.

The fungus gains entrance into the host through open and closed stomata and by direct penetration through the cuticle. Closed stomata and penetration through the cuticle is apparently mechanical, and penetration is always effected with the aid of an appressorium. Open

stomata are entered by the germ tube directly. Appressoria were never seen to form over open stomata.

H. portulacae, the causal fungus of the disease, shows the behaviour of a primitive parasite, for it rapidly invades and kills the host tissues and continues to thrive on them. The fungus was found to form readily in culture a pectinase able to macerate rapidly pieces of stem and leaves of purslane; and histological studies revealed that the middle lamellae between cells becomes dissolved, causing a separation of cells in advance of its growth. Hence, once the pathogen enters host tissue it dissolves the middle lamellae of cells. Thus, any mechanical resistance which might be offered by host tissue to the advancing hyphae is removed.

The fungus also formed in culture a powerful toxin which caused wilting of cut stems of hosts and bean plants when stood in an enzyme inactivated filtrate of the used medium. From these findings it appears possible to suggest the mode of action of the fungus in host tissues.

The activities of the pathogen are marked by zonation in an enlarging lesion. In the centre of the lesion is a dead area killed by the toxin formed by the fungus. Outside is a brown, watersoaked zone in which the cells are rapidly dying. The brown zone shades into a yellow, watersoaked zone in which the chlorophyll is breaking down. The zonation could conceivably be due to diminishing concentrations of toxin from the centre to the outside of the lesion. The mycelium of the pathogen is found in the inner part of the yellow zone. It causes

a separation of cell walls and some breaking down of chlorophyll in advance of its growth. The wall separation can be explained by its capacity to secrete pectinase.

This behaviour is quite different from the pathogenic activity of some plant pathogens which kill the host in advance of their invading hyphae, and hence only live in dead tissue which they have killed by their own secretions. Hawkins and Harvey (26) found that Pythium deBaryanum secretes both an enzyme and a toxin, and that the enzyme had little or no effect on the cell wall. Smith (45) argued that disorganization and killing of tissue in advance by Botrytis cinerea, a destructive facultative parasite, were due to a toxin, possibly oxalic acid, while Brown (3) claimed that this fungus produced only a pectinase and that death of cells was due to a direct toxic action on the protoplasm by the enzyme, or to an indirect action by a toxic product resulting from the dissolution of the cell walls.

The fact that the enzyme extract prepared from cultures of H. portulacae macerated freely the host tissue without killing it, coupled with the finding that this fungus produces a powerful toxin in culture, seems to justify the conclusion that its action on the host tissue is due to at least two substances secreted by the pathogen, a middle lamellae splitting enzyme and a toxin.

The trials on the influence of environmental factors on the pathogen, host, and disease development are limited, but they form a basis for some speculation on the epidemiology of the disease.

Evidence was obtained that the fungus can over-winter in plant litter and in or on the surface of the soil as conidia, mycelium, and bulbils, and that the pathogen can attack the stems of its hosts when inoculated into the soil as conidia. In this area, host plants are usually not available until about the second or third week in June, which means that the fungus must live saprophytically for several weeks in the spring. Thus, a certain build up of inoculum would be expected to occur each spring before host plants are available.

Experimental work has shown that the lowest temperature at which the pathogen grew in culture from mycelium transfers was 10°C. The mean temperature for May of the year the disease was first observed in this area was 12.7°C; the average maximum temperature was only 18.5°C. At 15°C. fruiting was scarce, and at 20°C. it was not very abundant. The lowest temperature for growth of the pathogen from conidia, as indicated by temperature tests in liquid media inoculated with conidia, was slightly below 15°C. At 15°C. only a trace of growth occurred, indicating that conidia germinated, but that the temperature was unfavourable for growth of their germ tubes. Therefore, since the mean temperature for the month of May in the area where the disease was observed is too low for mycelium to develop to any extent and for conidia to germinate, it seems that the fungus must have remained in a semi-dormant condition in the soil during most of the month.

The temperature during the month of June before the host plants appear would be more favourable for germination of over-wintered

conidia and growth of over-wintered mycelium; therefore some build up of inoculum would be expected to occur. With the appearance of the plants and thereafter, temperatures would gradually rise and become increasingly more favourable for germination of conidia, growth of the fungus, infection and subsequent disease development. Temperatures reach a peak in July when they are nearer the optimum for all the vital processes of the pathogen, which experimentally were determined to occur at about 30°C. Therefore, in respect to temperature, some infection by the over-wintered fungus could be expected to take place in June when host plants are becoming available. As the summer advances, temperature conditions would gradually become more favourable for the development of the disease resulting from these infections by the over-wintered fungus. It would appear, however, that only temperatures during the daylight hours would be favourable for rapid infection and rapid disease development, since temperatures during the evening hours usually drop too low. For example, the mean maximum and minimum temperatures for July of 1949 and 1950 were about 27°C. and 15.5°C. respectively. For about 18 days in July of both years the minimum temperature was never higher than 15.5°C. It seems then that temperatures even during the hottest month of the growing season are not favourable for continuous rapid disease development.

Moisture conditions are also important and must be considered in conjunction with temperature in determining whether the disease would

ever become epidemic on host plants. It was shown that at a temperature of about 27°C. in a saturated atmosphere the fungus completed a full cycle of development in 36 hours. It was noticed during this investigation that near moisture saturated conditions are required for fruiting. Thus, it follows that fairly long and successive or continuous periods of hot moist weather are necessary for the disease to develop in epidemic proportions. Further, the fungus after fruiting must have its conidia disseminated.

Such a combination of moisture and temperature does not normally occur during the growing season. It was mentioned previously that night temperatures are usually unfavourable for the rapid development of the fungus and fruiting. One such continuous hot and moist period did occur at the end of July of 1949. For three successive days and nights it rained continuously,and the temperature during the three wet days never dropped below about 20°C. Therefore, it would be expected that under these unusually favourable conditions a severe outbreak of the disease should have occurred. This appeared to be the case, since results of field observations in this area several weeks after these conditions had occurred showed that the disease had been severe in a large rose moss planting and in a field infested with purslane. However, the disease seemed to have been severe only in patches which were not more than a few feet in diameter. This observation would seem to indicate that the conidia had not been effectively disseminated. It is not surprising, because the conidia

of this pathogen due to their large size and short conidiophores are not well adapted for dissemination either by wind or rain, and even with strong winds most of the conidia would become trapped in the dense foliage of the crowded plants.

It appears that epidemics of the Helminthosporium disease on purslane and rose moss are not likely to occur in nature because of two important facts. First, periods of high temperatures and wet conditions do not occur frequently enough to permit the fungus to fruit and build up sufficient inoculum, and secondly, dissemination of the conidia appears to be very restricted.

The above observations seem to suggest that wholesale destruction of purslane might be brought about by artificially disseminating conidia over all of an area infested with this weed during the times when hot moist conditions prevail. However, further observations on the disease in the field are necessary before definite conclusions can be made concerning the use of H. portulacae for the biological control of purslane.

Features of the life cycle and mode of dissemination of the pathogen indicate certain control measures which might be applied in the event that the Helminthosporium disease becomes a threat to those growing rose moss plants either for seed or as an ornamental. In fields or garden plots where the disease is already established, removal of plant debris in the spring before plants appear, spraying with a suitable fungicide at various intervals after they appear, and

roguing of all diseased plants as soon as noticed, should be practised.

SUMMARY

1. A detailed description of a disease of purslane due to Helminthosporium portulacae has been presented for the first time.

2. It was shown that H. portulacae also attacks rose moss, and the disease symptoms it causes on this host have been described.

3. Morphological comparisons were made of conidia of the pathogen produced on purslane and rose moss. Septation of conidia varied with the host on which they were produced, but isolates from purslane and rose moss, when grown on potato dextrose agar, produced practically identical conidia.

4. At temperatures of 25°, 30°, and 35°C. wet and dry conidia of H. portulacae germinated well. Most rapid germination was obtained at 30°C. with wet conidia in a saturated atmosphere. However, over a period of 24 hours, the percentage germination of wet and dry conidia was almost the same.

5. H. portulacae grew on artificial solid media from below 10° to above 40°C. with an optimum of about 30°C. No growth took place at 15° and at 45°C. On liquid media inoculated with conidia, the fungus showed a trace of growth at 15°C. This indicates that conidia of H. portulacae will not germinate at temperatures much below 15°C. The growth by weight on liquid media closely paralleled the spread of growth on agar. Growth increased with increasing concentration of sugar.

6. The production of conidia by H. portulacae was greatly affected by temperature. Sporulation was abundant at temperatures of 25°, 30°, and 35°C., but the middle temperature was the optimum for sporulation. The maximum was between 40° and 45°C., and the minimum about 15°C. Conidia produced at high temperatures had fewer septa than those produced at low temperatures. Many conidiophores germinated at high temperatures instead of producing conidia.

7. Cultures incubated at a temperature of 25°C. or higher, gave rise to what appeared to be three types of saltants. These saltants appeared as fan-shaped outgrowths from the parent colony. Two saltants reverted immediately on fresh agar, and the other remained stable through successive transfers.

8. Although the cells of most organs in H. portulacae were multinucleate, nuclear studies indicated that variation in H. portulacae was probably due to a genetic change in a nucleus (mutation), and not to heterokaryosis, because the apical cell of all conidiophores examined contained only one nucleus.

9. Recognized standard techniques were employed in attempts to develop the ascigerous stage of H. portulacae, but negative results were always obtained.

10. Penetration of host tissue by germ tubes of H. portulacae was

effected by direct penetration through the cuticle and through open and closed stomata. Entrance into closed stomata and direct penetration through the cuticle were accomplished with the aid of an appressorium, but open stomata were entered by the germ tube without the aid of an appressorium and without constriction.

11. Appressorial formation seemed to result from the combined effects of several stimuli including contact and a substance or substances emanating from the host tissue itself.

12. H. portulacae formed readily in culture a pectinase able to macerate rapidly pieces of stems and leaves of purslane. 'Action in advance' by the fungus could be explained by secretion of this enzyme by the advancing hyphal tips.

13. H. portulacae also formed in culture a powerful toxin which was able to wilt cut purslane, rose moss, and bean plants when the stems were stood in an enzyme inactivated filtrate of a used culture.

14. The mode of action of H. portulacae was described and it was thought that zonation, a characteristic of enlarging lesions, could be due to diminishing concentrations of toxin from the centre to the outside of the lesion.

15. The optimum temperature for infection of purslane leaves by H. portulacae with wet and dry conidia was 30°C. More infections were

obtained with dry conidia in a saturated atmosphere than with wet conidia at all temperatures tested. However, the shortest incubation period was six hours at 30°C. with wet conidia in a saturated atmosphere.

16. Soil inoculation tests with H. portulacae revealed that the stems of purslane and rose moss plants are very susceptible to attack by the fungus when it is in the soil. As much infection was obtained with unsterilized inoculated soil as with sterilized inoculated soil.

17. The epidemiology of the disease was discussed. It appears that natural epidemics of the disease are not likely to develop among host plants in this area because of two important facts: (1) Hot temperatures together with near moisture saturated atmospheres, which are necessary for abundant sporulation and rapid disease development, do not occur frequently enough or continuously during the hot summer months and (2) dissemination of conidia is too greatly interfered with by the crowded host plants themselves.

18. It might be possible to induce epidemics of the disease if conidia of the fungus were artificially disseminated over all of an area infested with purslane during the times when hot moist conditions prevail.

19. Possible control measures were given to apply in cases when the disease is already established in rose moss plantings. These are:

the removal of plant debris in spring before plants appear, spraying with a suitable fungicide at various intervals after plants emerge, and roguing of diseased plants as soon as they are noticed.

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