A STUDY OF THE LEAFSPOT DISEASE OF LETTUCE CAUSED BY *STEMPHYLIUM BOTRYOSUM* WALLR.

A Thesis Presented in Partial Fulfilment
of the Requirements for the Degree of
Master of Agricultural Science
in the University of New Zealand.

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November 1961

CONTENTS.

contd.....

LIST OF ILLUSTRATIONS.

contd.....

Illustration.		After page.
18	Host penetration.	120
19	Typical lesions caused by Stemphylium botryosum (Naturally infected leaf.)	150
20.	Apparatus used for spore dispersal studies.	170
21	Infections due to aerial dispersal of conidia.	201
22	Discharge of ascospores from Peritheciun	201
23) 24)	Infections of Stemphylium botryosum on plants of various ages	211

LIST OF FIGURES

LIST OF TABLES.

contd.....

ACKNOWLEDGEMENTS

Grateful acknowledgement is made to Mr. H. T. Wenham for his encouragement and guidance in the supervision of this study, and for helpful criticisms in the preparation of this thesis.

Thanks are also due to Mr. K. C. Hockey and the Horticultural Staff for loan of the coolstore for environmental studies and the use of equipment; the Horticulture Division Officers, Department of Agriculture, at Palmerston North, Hastings and Levin for assistance with field survey work; the Staff of the College Library for their assistance in obtaining numerous journals, and to Mrs. J. Little for typing this thesis.

For the duration of this study the author was assisted financially by a Public Service Study Award and the 1960 Shell Scholarship.

INTRODUCTION

Lettuce (Lactuca sativa L) is the most important salad crop and a principal vegetable of New Zealand where in many districts it can be grown out of doors all the year round. Winter lettuce growers commonly plant from ½ - 5 acres of lettuce, and a good crop will cut 400 cases an acre. In late winter and early spring when other vegetables are scarce, returns to the grower from lettuce may be as high as 20/- to 60/- a case. Returns per acre from winter lettuce are therefore relatively high compared with other winter crops. The success of this crop is of major importance to the grower who commonly depends on it to cover the high winter and spring labour expenses which usually coincide with a period of low production.

From season to season, and even from week to week in the same season, the quality and quantity of lettuce available is a direct reflection of the prevailing weather conditions. Weather may act directly on the lettuce plant affecting its rate of maturation, appearance and plant size. Weather may also have a profound indirect effect by providing conditions conducive to disease development. It is well recognised that a disease may reach epiphytotic proportions only if weather conditions are favourable to the causual organism.

Several diseases of lettuce have been recorded in New Zealand (Appendix I). Of these the disease variously known as ring-spot, rust or anthracnose and caused by Marssonina panattoniana (Bert)

Magn., has long been considered the major foliage disorder affecting winter lettuce production in this country. This disease is world wide in occurrence and has been the subject of considerable research. (Chittenden 1912, Salmon and Wormald 1923, Stevenson 1939, Taylor and Li 1944).

Recently however Brooks (1958)* recorded Stemphylium botryosum Wallr. in its sexual phase on lettuce crops in the Auckland area, where large individual losses have occurred during the wet and cool winter months. This organism is known to be pathogenic to a wide range of unrelated hosts (Neergaard 1945) but comparatively little has been published on this fungus as the causual organism of a leafspot of lettuce.

Weber and Foster (1928) in their bulletin on 'Disease of Lettuce, Romaine, Excarole and Endive' state that a leaf spot of Escarole, (Cichorium intybus, which is closely related to lettuce), is caused by an Alternaria spp. They state, "There is a possibility that with further study it will be found that this organism is the same as the one that causes a similar leaf spot on lettuce." The description of the leaf spot on Escarole

* See Dingley, Joan M. N.Z. Journ.Agric.Res. 3(3) 461-467 1960.

and the photograph of an infected leaf appear to be very similar to the symptoms of S. botryosum on lettuce. No further reports appear to have been published on subsequent work to determine the causual organism of this or the leafspot on lettuce.

Ogilvie and Mulligan (1931) were the first to describe a leaf spot disease of lettuce, from which they isolated Macrosporium sarcinula Berk. amend Bolle. Identification was corroborated at that time by S. P. Wiltshire. The genus Macrosporium was later reduced to synonomy with Stemphylium by Wiltshire (1938), and M. sarcinula was thus synonymous with Stemphylium botryosum.

Ogilvie and Mulligan state that this previously undescribed leaf spot, from which they isolated and proved the pathogenicity to lettuce of the above fungus, was first noticed in April 1930. They later found the disease to be common on lettuce planted in greenhouses and out of doors in the West of England. The symptoms they describe appear to be similar to those caused by S. botryosum on lettuce in New Zealand.

While the disease was evidently not sufficiently serious to warrant control measures, they considered that under favourable environmental conditions it may become important.

Dippenaar (1939) reports that in 1935 he discovered lettuce grown on the Cape Flats, South Africa to be affected with a serious leaf spot disease previously unrecorded from South Africa. Further investigation showed that this was one

of the major diseases, if not the most important, affecting winter lettuce grown on the Cape Flats. Dippenaar considered the disease and the causual organism to be similar to that reported by Ogilvie and Mulligan (1931). He also successfully proved pathogenicity of the fungus isolated.

Neergaard (1945) states that spores of S. botryosum not infrequently occur on lettuce seed. In further infection experiments with seed borne fungi Neergaard found that this fungus attacked both sterile seedlings and wounded and unwounded six-week-old lettuce plants.

Gossens (1951) has reported a leaf spot disease of young lettuce caused by P. herbarum Rabenh (perfect stage of S. botryosum). Young lettuce plants in Dutch nurseries, expecially about Rotterdam, were attacked during January-February 1950. Gossens states that infection was generally mild but in one case 70% of the crop was involved.

Pettinari (1951) obtained the P. herbarum stage of S. botryosum from leaves of lettuce growing at Apulia, Italy, but in the review of his article no mention is made of the extent of the infection, or how he obtained the perfect stage from infected leaves.

Padhi and Snyder (1954) state that a leaf spot of lettuce had been observed for at least twenty years prior to 1951 on lettuce crops in or near Colma, a district on the coast of

California, U.S.A. They considered the incitant to be a form of Stemphylium botryosum Wallr. an imperfect stage of P. herbarum.

Although the above mentioned report by Brooks (1958) is the only reference to Stemphylium botryosum causing a foliage disease of lettuce in New Zealand, it would appear that this disease has been present for many years in this country. Local instructors in the Department of Agriculture have been aware of the existence of two types of leaf spots on winter lettuce. One spot, characteristically small, caused by Marssonina panattoniana, was first recorded in New Zealand by Taylor and Li (1944). The other leaf spot disease, which under favourable conditions may develop larger lesions, is often present on winter lettuce and is commonly confused with ring spot. It would appear that this latter disease is in fact caused by Stemphylium botryosum.

The shothole effect, which occurs when the centre of a lesion collapses is characteristic of both diseases, but it is commonly associated with lesions caused by M. panattoniana. Many of the more observant growers in the Manawatu and Horowhenua district distinguish between the two diseases by calling one 'ring spot' and the other 'leaf spot'. One Chinese grower, who has observed the difference in the type of lesions, recalled that in the early years of World War II, probably 1942, he suffered substantial losses from the 'leaf spot' type disease, and in several seasons since has lost much of his late winter lettuce crops from this disorder. However, the majority of

growers consider that both diseases are similar.

Thus from discussions with the growers of winter lettuce in the Manawatu and Horowhenua district, local Instructors, and records of the Department of Agriculture on diseases of lettuce since 1946, it has not been possible to cite any definite period when the leaf spot of lettuce caused by S. botryosum was first observed. It seems probable, however, that the disease has been attacking winter lettuce for at least twenty years in this country.

Objects of the present Study.

In view of the lack of detailed research on leaf spot of lettuce caused by S. botryosum and its undoubted importance in many districts, the following study was undertaken, the principle objects of which were:-

1. To assess by way of field surveys the incidence and relative importance of the two foliage disorders of winter lettuce caused by Marssonina panattoniana (Bert) Magn., and Stemphylium botryosum Wallr.
2. To study in detail the morphology and physiology of S. botryosum.
3. To study the taxonomy and nomenclature of the New Zealand form of S. botryosum pathogenic to lettuce.
4. To study the influence of environmental factors on the disease cycle.
5. To investigate control of the disease by use of protective fungicides.

MATERIALS AND METHODS

The materials and methods which have been used for each experiment are described in detail in the relevant chapters of this study. Materials and methods which are applicable to several sections of this study are here outlined before this experimental work is described in detail.

1. MATERIALS AND METHODS USED IN THE LABORATORY

(a) Preparation of media

The details of the preparation of media are given in Appendix II. Stocks of potato dextrose agar (P.D.A.), prune agar, and water agar were prepared in 1 or 2 litre quantities. These were stored after sterilization in partly filled 250 ml. flasks stopped with cotton wool plugs. Other media were prepared as required.

(b) Pouring Plates

Petri plates, 90mm. in diameter, were used for culturing the fungus in the laboratory. Successful sporulation occurred when 15-20ml. of medium was used in each plate. Less media resulted in poor sporulation from 10-14 day colonies. Consequently, six petri plates were normally poured from 100ml. of media.

(c) Inoculation of Media

Difficulty was experienced in obtaining a form of inoculum which had no influence on the subsequent diameter of colonies. It was determined that as the size of the square of agar inoculum (containing mycelium) became smaller, the resulting

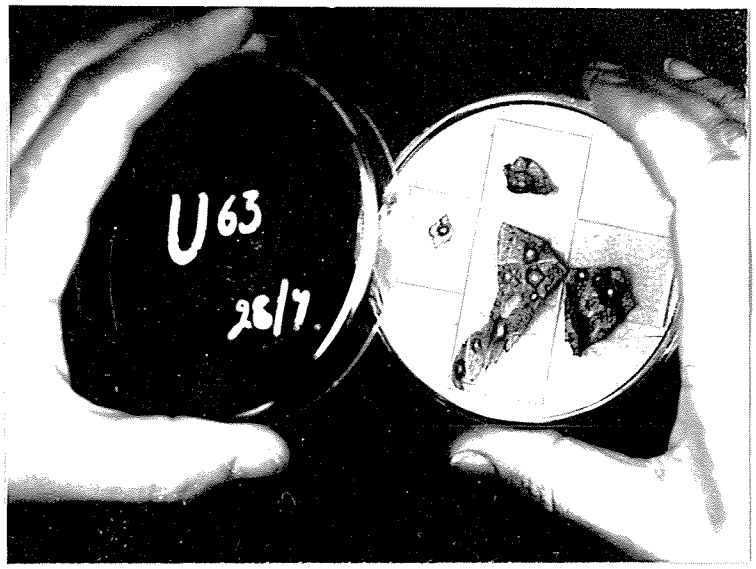


ILLUSTRATION 1.

Laboratory identification of lesions: High humidity treatment.

ILLUSTRATION 2.
The glasshouse unit used during these studies.

ILLUSTRATION 4.

The relative humidity cabinet.

colony sizes became increasingly variable. If squares of agar containing mycelium measured 6 x 6 mml variation due to the size of inoculum did not occur. Therefore, during all experiments the inoculum consisted of a square of agar measuring 6 x 6 mm. taken from the fringe of an actively growing colony on P.D.A. This agar square was placed upside down on the media being inoculated. Growth rate was linear in respect to time. Therefore 6 mm. was subtracted from the diameter of colonies recorded. These are the diameters reported in this study.

(d) Design of Experiments

Where the diameter of colonies were to be measured, 3 - 5 colonies were used, according to the incubator space available and the type of experiment. Measurements were taken of two diameters at right angles and results were expressed as an average. Unless contaminants were present, the colony diameter for similar treatments were generally very uniform often agreeing to within 1 - 2 mm. If much variation in diameters for similar treatments was encountered the experiment was repeated until uniform results were obtained.

In experiments where a bias may have been introduced due to a previous knowledge of the treatments, the Petri plates were marked with a number to indicate the treatment. Records were kept of the code used which was selected from random tables available in the standard texts. After subjecting the colonies to required treatments the plates were removed from the incubator

and, prior to recording, arranged in the random order indicated by the numbers.

(e) Measurement of growth rate on Artificial Media

Brancdto and Golding (1953) after testing and evaluating the effect of several environmental factors on colony diameter concluded that it may be used for measuring the rate of growth of fungus, comparing the influence of variously altered media on their growth, and investigating the effects of other environmental factors.

With the exception of the first twenty-four hours of growth, colony diameters increased at a constant and linear rate. Records of colony diameters were therefore made only once, normally ten days after inoculation.

(f) Incubators

Refrigerated incubators which operated at 5^{o}, 10^{o}, 12.5^{o}, 15^{o} and 20^{o}C. were used for temperature studies of the fungus on culture media. Unfortunately the 5^{o}C incubator failed to function correctly. Incubators were also available operating at intervals from 24^{o}C - 50^{o}C. The normal growth temperature of 24^{o}C was selected because it was the lowest temperature at which the incubators would function without fluctuations occurring due to changes in room temperatures. All normal cultural work was undertaken in incubators operating at this temperature.

Artificial light was not provided in the incubators.

At times mite became a problem, frequently causing considerable

contamination. It was found that they were readily controlled if the shelves and walls of the incubator were occasionally wiped with a cloth soaked with kerosene.

(g) Production and Preparation of Inoculum

(i) Conidia

Isolates were selected for their ability to produce abundant conidia on culture media. This ability was usually related to the length of time the isolate had been maintained on culture media. Petri plates containing approximately twenty milli litres of P.D.A. were inoculated with squares of P.D.A. and mycelium obtained from other cultures, or mycelial fragments taken from stock cultures maintained on petri plates held in a refrigerator. The mycelial fragments proved most satisfactory. A sterile needle bent to form a small L was used to lift tufts of mycelium from the cultures. The needle was moved lightly across the surface of the P.D.A. to facilitate dislodging the mycelium fragments.

(Other methods of inoculation tested, but which failed to induce prolific production of conidia included streaking with suspensions of conidia or mycelial fragments, and incorporation of conidia or mycelial fragments into the medium while it was still liquid.)

After 10-14 days growth at 24°C. conidial suspensions were prepared by flooding the culture with sterile water and scraping off the superficial mycelium. This was placed in a flask with a dozen small glass beads and shaken vigorously for several

minutes. Usually six cultures were used to every 100 mls. of sterile water. The mycelial fragments and P.D.A. were removed by filtering the suspension through two layers of cheese cloth.

The concentration of conidia was estimated using a haemocytyometer and was then adjusted, usually to contain 5000 conidia per ml.

(ii) Ascospores:

Selected isolates were grown at 24°C. on P.D.A., prune agar or lettuce decoction agar for periods of up to 12 weeks. At this stage ascospore discharge commenced if the colonies were removed from the incubators. During the first ten days incubation colonies were frequently removed from the incubator and exposed to sunlight or ultra violet light to stimulate production of perithecia.

Perithecia were obtained by scraping off the aerial mycelium and perithecia on the surface of the P.D.A. Colonies were then washed with sterile water and the perithecia which remained partly imbedded in the media were scraped off. These were washed several times to remove as much agar as possible and finally shaken with glass beads until all mycelium attached to them was dislodged. After several further washes the perithecia were transferred with a minimum amount of water into a thick-walled test tube, where they were crushed with a close-fitting glass rod. The resulting fragments were suspended in water, filtered through cheese cloth and the

suspensions examined under the microscope. If conidia were present, the suspension was discarded and another prepared, taking further precautions to elimate all conidia before perithecia were crushed.

(h) Identification of Organisms Causing Leaf Spot Symptoms

The method used to isolate fungi from leaf spots is shown in Illust. I. The petri dish, lined with moist filter paper and containing the lesions supported on glass slides, wase incubated for 24 hours at 24°C. After this period of time S. botryosum produced numerous conidia. Identity was confirmed by microscopic examination of conidia and isolation of single spores on to P.D.A. with subsequent culturing and identification of the colonies which developed.

This method, used for rapid isolation and identification of specific organisms, was described by Stevenson (1939) for identifying Marssonina panattoniana on lettuce. The symptoms of these two diseases were sometimes similar, but once the lesions were subjected to this high humidity treatment, differentiation between the two types of spores produced was readily made and indicated with certainty which pathogen was involved.

2. FIELD SURVEY: RECORDS AND METHODS.

A card was prepared for each property visited during the survey and details of stage of growth of the crop, crop variety, and other relevant information was entered during each visit. An estimate of the infection and tentative identification of the foliar disorders present were also made. Provision was left for subsequent confirmation or amendment. If the crop was about to be harvested the percentage of heads rendered unsaleable due to leaf spot disease was estimated.

Specimens of leaf spots from lettuce or other suspected hosts were collected and placed individually in labelled plastic bags.

In the laboratory each leaf spot selected for identification was numbered consecutively and details entered in a register. This series of lesions were prefixed by "U" for "unknown" series (e.g. lesion U25). After successful isolation on to P.D.A. had occurred, selected colonies from the same lesion were designated where necessary with an appended letter (e.g. U23C). Identification was often possible at this stage. To ensure that future work on the isolate would not be complicated by contaminants, single spore isolates were prepared using a method similar to that described by Keitt (1915). These isolates were distinguished by a numeral preceding the U (e.g. 3U23C) to indicate which single spore isolate had been selected. This system of numbering and recording isolates

proved to be very satisfactory. (Two isolates were used in preliminary work before this system was established. These were the "A" and "P" isolates).

Stock cultures of most isolates of S. botryosum were prepared by inoculating P.D.A. slants in ⅝" test tubes with mycelial fragments from the single spores colonies. These slants were fitted with cotton wool plugs, labelled and incubated at 24°C until growth commenced. They were then removed from the incubator sealed with "Parafilm" (see p. 103) before storing at room temperature in the laboratory.

Kocks postulates were performed on several isolates from each district and inall cases the fungus S. botryosum isolated from lettuce was proved the incitant of the leaf spot disease.

3. PRODUCTION OF LETTUCE PLANTS

The glasshouse and workroom used for plant production is shown in Illustration 2. This same glasshouse unit was used for handling plants during the disease cycle studies.

(a) Soil Composts

Loam was obtained when possible from turf which had been stacked for eighteen months. At other times a clay loam from the College soil heap was used. This clay loam was heat sterilized (by electrode method) before use. The loam obtained from the turf was treated with methyl bromide prior to the preparation of seedling composts. It was not treated in this way when it was to be used in potting composts.

Seedling composts, based on John Innes mix, contained 2 parts of loam, 1 part of peat and 1½ parts of sand. To every bushel of this mixture was added 1½ ozs superphosphate and ¾ ozs of ground limestone.

Pricking out and growing on composts finally adopted consisted of three parts of turf-loam passed through a ½ inch sieve, and one part of sand. To every bushel of this mixture was added 1½ ounces of ground limestone and four ounces of a mixture consisting of ½ part sulphate of ammonia, 1½ parts superphosphate and ¾ part of sulphate of potash.

(b) Seed:

The variety of lettuce used during all studies of the disease cycle was Black Seeded Triumph (ex Coopers, Lot 745/1960). This was sown in small seed trays at weekly intervals to ensure a continuing supply of plants.

Seed of other varieties of lettuce and that used for host range studies, if not available from lines of seed held in the Micro-biology Department, were purchased from chain stores.

(c) Pricking Out and Potting.

When two true leaves had formed and expanded the young plants were pricked out into 3" clay pots or seedling trays. Approximately 100 plants were potted each week, the actual numbers depending on the requirements of the experiments. A 10% surplus of plants was provided above the requirements of the experiment to allow for plant losses and discarding atypical plants.

(d) Supplementary Nutrients:

During periods when water loss from the pots was high, constant watering leached soluble nitrogen from the potted and boxed plants. Plant growth became slow and hard. The base fertilizers were supplemented during these periods by applying a liquid fertilizer to the plants. This was prepared by dissolving one rounded tablespoon of sulphate of ammonia or a similar quantity of "Zest" plant food in one gallon of water. This was applied to the foliage of the plants at weekly intervals.

4. GLASSHOUSE MANAGEMENT

(a) Temperature

Minimum temperatures were maintained at or above 50°F. by electrical radiators fixed under the glasshouse benches. During winter the glasshouse minimum and maximum were usually between 50°F. and 70°F. In summer the maximum temperature at times rose to above 90°F. By heavy shading and maximum ventilation consistent with the weather conditions prevailing, it was usually possible to hold the temperatures down to 80°F.

(b) Relative Humidity and Watering

The benches in the glasshouse were originally fitted with 2½" slats. When the heaters operated the pots became overheated and the relative humidity about the plants dropped, causing excessive moisture loss and plant damage. The benches were then fitted with asbestos sheeting and the amount of water required by the potted plants was considerably reduced. Thus during winter it was usually necessary to water the potted

plants at weekly intervals. In summer the plants were watered every morning and if temperatures were high and a wind blowing it was found advisable to damp the house down during the afternoon.

(c) Control of Miscellaneous Pests and Diseases

(i) Insects.

Aphis were the only insects which attacked the lettuce plants. The glasshouse was fumigated with Lindane at weekly intervals by burning one "Nexa" paper (commercial size) in each glasshouse.

This was done in the evenings so that the house could be shut down without undue temperature rises occurring.

(ii) Fungi.

Considerable plant losses were experienced due to Botrytis cinerea Pers. ex Fr. attacking the young potted plants. Losses were reduced or eliminated by replacing the dried blood in the base fertilizer with sulphate of ammonia; by improving glasshouse hygiene and removing of all dead plant parts in an effort to eliminate the source of primary inoculum; and by eliminating root damage which occurred when the heaters were operating for any length of time by covering the slat benches with sheets of asbestos.

(iii) Bacteria

During early investigations bacterial infection of fungal lesions caused a masking of symptoms of disease incited by S. botryosum, particularly at the higher temperatures used in the growth cabinets.

It was believed that the bacteria were multiplying in

the hoses used for watering the glasshouse. Samples of water taken from the hose had a very high count of bacteria. Once the water had been running for a short time samples taken had either very low counts or were free from bacteria.

Extra precautions were taken to ensure that no practice was facilitating the introduction of bacteria on to the lesions. Before watering or damping down the house, the water in the hose was run off and the hose flushed for several minutes before use. The high humidity cabinet was washed out at weekly intervals using a 1:120 dilution of "Savlon"*. The plastic sheets used for covering the plants were treated in a similar manner prior to use. The seed trays and flower pots were also sprayed with "Savlon" 1:120. After these precautions had been taken no further trouble was experienced with bacterial contamination.

5. PRELIMINARY EXPERIMENTAL WORK

Before any detailed studies of the effect of disease incited by S. botryosum on lettuce was undertaken, preliminary experimental work was necessary to determine a method suitable for recording and measuring the effect of disease on the lettuce plants.

It was essential that symptoms of disease should be immediately recognised. The preliminary work provided an

* I.C.I. Trade Name for Disinfectant.

opportunity for observation of the lesions from first appearance until death of the leaf occurred. The importance of post recording identification of the incitant of the disease was demonstrated when bacterial infection of the leaf occurred following penetration by S. botryosum. Consequently it was considered necessary to take samples of the leaf spots from each experiment and subject them to 100% relative humidity. If conidia of S. botryosum developed from these lesions, it was considered that similar infections had resulted on the remainder of the plants. Where bacterial infection occurred, sporulation was prevented. It was necessary to repeat those experiments where bacterial infection occurred.

Inoculum prepared as described previously was most satisfactorily applied to the lettuce plants by evenly spraying all plants to the point of run-off with the spore suspension, using a small hand atomiser.

The type and age of plant introduced considerable variation in expression of the symptoms of disease. It became apparent early in the studies that the desirable type of plant was one which had made rapid growth after being potted or pricked out and had expanded a total of five true leaves, in addition to the two lower leaves and the cotyledons. The type of plant used is shown diagrammatically in Illustration 3.

Two criteria were used to record the effect of the disease on the plants. Where a measure of the number of lesions

produced was required, actual counts were made of lesions occurring on the lower five leaves (No. 1 - 5, Illustration 3) excluding the cotyledons,and first two true leaves. During normal growth conditions in the glasshouse these recordings were made between the seventh and tenth days from inoculation.

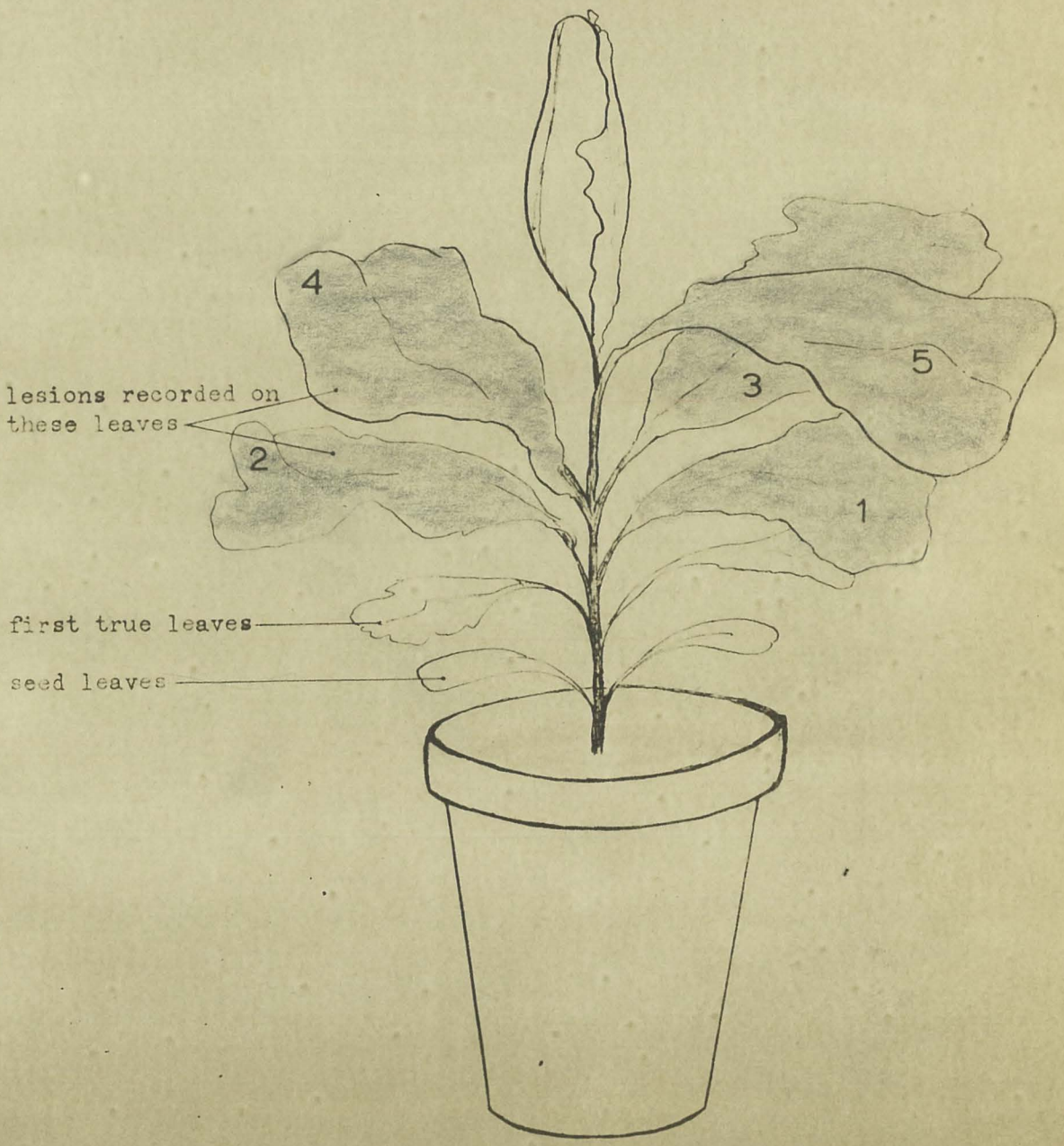
When a measure of the effect of environment on the expression of symptoms was required the individual leaves were removed from the plants and placed into one of ten classes indicated by photographs of diseased leaves which were ranked into ten classes. Leaves free from lesions were rated 0, and those leaves which were dead were rated 10. Therefore a score for a healthy plant was 0 and the maximum score for a dead plant was 50, there being five leaves recorded on each plant.

The sum of leaf totals were termed plant totals. The most successful method of recording investigated was where four plants were used for each plot, and plot totals subjected to statistical analyses.

Most experiments involved four or five treatments. Four of five replications of these treatments applied to plots of four plants enabled differences likely to be important in nature to be measured by the normal statistical procedures (Snedecor. 1956) Cochran and Cox (1957) and Duncan (1953)) and permitted maximum use of glasshouse facilities. Missing plants or plots were estimated using the methods described by Cochran and Cox (1957).

ILLUSTRATION 3

DIAGRAMATIC REPRESENTATION OF TYPE OF LETTUCE PLANT USED FOR DISEASE CYCLE STUDIES



During preliminary experimentation, it was determined that the optimum temperature for growth was above 40°F and below 60°F, the minimum temperature was below 40°F and the maximum approximately 80°F. Therefore the temperature range investigated during the studies of the disease cycle was between 40° - 70°F.

Temperatures were controlled by growing the plants in temperature controlled cabinets. The construction of the cabinets used in this study has been described by Latch (1957). They are a modified form of those used in the Department of Plant Pathology at the University of Wisconsin. One larger cabinet measured 3 x 2 x 2½ feet. Due to development of local "hot spots" in this cabinet it was operated at temperatures closest to the surrounding air temperature. The other cabinets measured 2½" x 2'2" x 2' and were more efficient. A fan was installed in the cabinet which operated at temperatures 20°F. or more above the surrounding air temperatures.

By placing the cabinets in a cool store operating at 40°F, temperatures ranging from 40° - 80°F. were obtained.

The temperatures in the cabinets were controlled by TS2 Sunvic thermostats connected in series with three 100-watt bulbs. Providing the contacts in the thermostats were kept clean and adjusted correctly, temperatures were controlled to within 2°F. of the required temperature.

Cabinets were operated for twelve hours before the plants were introduced with the thermographs in place to check on the operation of the thermostats. Thermographs were also placed in the cabinets when space was available. As soon as an increase in the temperature differential was noticed, the contact points were cleaned or replaced, and re-adjusted.

If the plants were to remain in the cabinets for several days light was provided for twelve hours a day. One 150 Watt reflector type floodlight was suspended 12" above each cabinet. To minimise the heating effect of the lamp, a sheet of glass was placed 2" above the cabinet. The lights were operated during the night by a time switch.

After inoculation, prior to placing the plants in the incubator they were held for 24 hours in 100% relative humidity.

A glass cabinet measuring approximately 2'3" x 3' x 2'6" was fitted with a rack to support two shallow metal trays. Following inoculation the plants were placed on the metal trays inside the cabinet and the trays filled with 1½" of water. The door was tight fitting and when closed it enabled a relative humidity of 100% to be maintained.

When larger numbers of plants were involved, they were placed in trays on a bench and covered with polythene sheeting which was kept in contact with the wet bench by weighted timber.

CHAPTER X

THE FUNGUS

CHAPTER I THE FUNGUS

Introduction

The morphology and physiology of *S. botryosum* isolated from lettuce in New Zealand was studied on artificial media in the laboratory to enable comparisons to be made with other forms of this organism reported by overseas authors.

During these studies the most suitable type of media for production of conidia and perithicia received special consideration, due to the need for a reliable medium to support abundant conidial production for Disease Cycle studies.

The taxonomy and nomenclature of the fungus causing the leaf spot of lettuce was also considered, utilizing the knowledge obtained from the artificial media studies, and by investigating the host range of the organism and comparing it with that reported by overseas authors.

A. ARTIFICIAL MEDIA STUDIES

1. The Influence of Media on Cultural Features of *Stemphylium botryosum* Wallr.

While species of the genus Stemphylium usually grow well on culture media there are many examples where great difficulty has been experienced to induce sporulation (Andrus et al. (1942), Hendrix (1946), Neergaard (1945), Diener (1952),(1953), McDonald 1958).

Difficulty was experienced in maintaining sporulation of some isolates from lettuce after several weeks growth on artificial media. Accordingly studies were undertaken to determine what medium was most suitable for conidia and ascospore production. A total of 25 media were investigated and the morphology of the fungal colonies characteristic of each type of medium were recorded. Comparisons of the description of colonies reported by other authors on the various media were made with those recorded for colonies of the New Zealand isolates.

Padhi and Snyder (1954) have reported that *S. botryosum f.lactucum* would grow well on potato dextrose agar (P.D.A.), prune agar, oatmeal agar, cornmeal agar, yeast extract agar and straw agar. The straw agar favoured rapid and abundant formation of conidia.

Snyder and Hansen (1947 (a)) describe the preparation and use of natural media, and subsequently (1947 (b)) outlined the

advantages of using this type of media. In order to avoid altering the natural material by subjecting it to high temperatures sterilization, ethylene or propylene oxides were used as fumigants. Snyder and Hansen outlined various ways of using the natural host material as media for culture of fungi. Padhi and Snyder (1954) used one of these methods to prepare the straw agar which they found stimulated spore production.

Bollard (1950) and Kilpatrick & Johnson (1956) have described the preparation and use of plant agar media.

Similar media to those described by these authors were used during the study of S. botryosum on culture media.

Materials and Methods

Details of the preparation of the 11 artificial media and 14 natural media used in these studies are presented in Appendix II. Due to the fumigants used by Snyder & Hansen (1947 (a) & (b)) being unavailable in New Zealand, the natural host material was fumigated with Chloropicrin.

While heat sterilization was employed during the preparation of the media described by Bollard (1950) & Kilpatrick & Johnsnn (1956), this media has been included under the general heading of 'natural media' because it was prepared directly from living plant parts.

The advantages to be gained from the use of natural media may

be due to small amounts of essential growth substances present in the organic material used. However, the major nutrients supplied by Snyder & Hansen (1947 (a)), may be insufficient to support growth equivalent to that obtained on the artificial media. Comparisons between natural media and artificial media may not give as much information as comparisons amongst artificial media, natural media, and natural media with added nutrients. From results of a preliminary trial it was obvious that insufficient nutrients were present in the natural soil and straw agars. It was also apparent that dextrose, when incorporated in agar supported abundant mycelial growth, thus fulfilling the requirements of a major nutrient.

A second type of natural media was therefore prepared by supplementing the natural nutrients in the media (prepared using the methods of Snyder and Hansen, Bollard, and Kilpatrick and Johnson,) with dextrose. This type of media was called 'Natural Nutrient Media'.

All artificial media were prepared sometime before they were required and stored in flasks held at room temperature. Natural media were prepared during the three days prior to their use. When required the prepared media or agar were heated in a boiling water bath until they became liquid. Before pouring, the media were cooled to 50°C. in a holding bath.

Five petri plates were labelled with the number corresponding to each media and 15-20ml. of the appropriate liquid medium was asceptically poured into each plate and evenly distributed by

rocking the plate.

After the agar had solidified and cooled to room temperatures the plates were inoculated with 6 x 6mm squares of P.D.A. and mycelium (See Materials & Methods. P 8).

Following inoculation the plates were incubated at 24°C for 10 days. The five plates were inspected and unless contaminants were present, the fifth plate was discarded.

The diameter of the colonies were measured (as described previously) and the average for the four colonies on each media calculated. Experiments were repeated until uniform results were obtained.

Results and Discussion

Average colony diameters, and characteristics are presented in Table I and discussed below.

The colour of the colonies on most media was grey. The various shades or tones of grey which occurred have been designated light grey, grey and dark grey. Four types of media supported colonies which were greenish-gray, and one media induced a bluish-grey coloured colony. The fringe of the colonies were lighter coloured than the major part of the colony and were often white. Colonies on dextrose agar had a pinkish fringe.

Most colonies growing on artificial media were circular. However, colonies growing on the natural media which contained organic material were often irregular in shape.

Some media supported abundant aerial mycelium, while others, particularly the natural media, appeared to favour growth of the mycelium within the substrate.

Production of conidia and perithecia varied widely from media to media. On the media containing natural material conidial production was often highest near the plant or organic material embedded in the agar. However colonies suitable for a source of conidia should have a uniformity high production of conidia over the entire mature colony.

TABLE: I

DESCRIPTION OF COLONIES OF STEMPHYLIUM BOTRYOSUM ON MEDIA

Media Number	Type of Media	(a) Average colony Diameter	(b) Colour of Colony			(c) Shape of Colony	(d) Density of Mycelium **	(e) Conidial Production ***	(f) Relative Conidial Production (a)x(e)/(c)	(g) Production of Perithecia ***	(h) Relative Perithecial Production (a)x(g)/(d)	Remarks
		mm.	Fringe	Central	Aerial Mycelium							
	Artificial Media											
1(a)	Cornmeal Agar	70	LGG	GG	GG	I	1	3	210	3	210	High production conidia
(b)	Czapek-Dox "	25	LG	DG	DG	I	1	3	75	1	25	
(c)	Dextrose "	59	P-G	G	W	R	1	4	236	1	59	High production conidia
(d)	Malt "	59	W	DGG	W	R	1	1	59	1	59	
(e)	Oatmeal "	80	W	DGG	G	R	2	Nil	0	1	80	
(f)	Peameal "	50	W-LG	LG	LG	R	2	1	25	Nil	0	
(g)	Potato Dextrose " (Lab)	68	W	G-DG	G,W	R	1	4	272	2	136	High production conidia
(h)	Potato Dextrose " (Oxoid)	65	W	G-DG	G,W	R	1	4	260	4	260	High production conidia
(i)	Prune Agar	61	DG	DG	DG	R	2	2	61	4	122	
(j)	Tomato Juice Agar	61	W	GG	W	R	1	3	122	Nil	0	
(k)	Water Agar 1.2%	47	T	T	T	I	3	Nil	0	Nil	0	
	Natural Media											
2(a)	Autoclaved Lettuce Leaf	65	DG	DG	DG	R	3	4	87	5	108*	
(b)	Fumigated Lettuce Leaf	68	DG	DG	DG	I	3	4	91	5	113*	
(c)	Lettuce Decoction Agar	54	LG	DG	DG	I	1	4	216	4	216	High production conidia
(d)	Autoclaved soil "	75	T	T	A	R	3-	1-	19*	Nil	0	Few spores
(e)	Fumigated soil "	32	T	T	A	I	3-	Nil	0	Nil	0	Some bacterial contamination
(f)	Autoclaved straw "	73	T	G-T	A	I	3-	4	73*	1	18*	(Perithecia on or
(g)	Fumigated straw "	22	T	G-T	A	I	3	5	37	2	11	(near straw
	Natural Nutrient Media											
3(a)	Autoclaved Lettuce Leaf	76	W	DG	G,W	R	2	5	190	Nil	0	
(b)	Fumigated Lettuce Leaf	50	W	DG	DG	I	2	4	100	Nil	0	Bacterial contaminants
(c)	Lettuce Decoction Agar	73	LG	GB	G	R	1	5	365	Nil	0	High production conidia
(d)	Autoclaved soil Agar	67	LG	G	G	I	2	3	100	3	201	
(e)	Fumigated soil Agar	12	LG	G	G	I	2	2	12	Nil	0	Bacterial contaminants
(f)	Autoclaved straw Agar	42	LG	DG	DG	I	2	5	105	Nil	0	
(g)	Fumigated straw Agar	20	LG	LG	G	I	2	1	10	Nil	0	Bacterial contaminants

Abbreviations:

Colours:
- D Dark
- G Grey
- GB Grey-blue
- GG Grey-green
- L Light
- P Pink
- T Translucent
- W White
- A Absent

Shape:
- I Irregular
- R Regular

** Density of Mycelium
- 1 Dense
- 2 Medium
- 3 Sparse

* on portion of colony only
(-)less than

*** 1 Very low
2 Below average
3 Average
4 Above average
5 Very high

Factors which influenced the total number of conidia produced from each colony appeared to be the area of the colony, the relative density of the mycelium and the relative average density of conidia per unit area. In the Table I an expression for the average production of conidia has been obtained by multiplying the average diameter of the colony by the average relative number of conidia produced per unit area, and dividing this figure by the relative density of the mycelium. Because production of perithecia was influenced by similar factors an expression for the total number of perithecia was also calculated.

Visual comparison of the colonies arranged in descending order, as indicated by these expressions demonstrated that they indicated the relative ability of the various media to induce sporulation or perithecial production.

Lettuce decoction nutrient agar, P.D.A. (Laboratory & Oxoid), dextrose agar, cornmeal agar, lettuce decoction agar and Autoclaved lettuce leaf in nutrient agar all had high scores expressing production of conidia. After visual examination the first three media listed above were considered to produce the highest numbers of conidia per colony. P. D. A. was selected as being most suitable for use in later portions of this study. Production of conidia on P.D.A. was very uniform and could be readily stimulated by exposure to ultra violet or sun light. The constituents of P.D.A. were always available and the media was simple to prepare. The prepared media, when sealed to prevent desication would store indefinitely at room temperatures. During

some studies where uniformity and repeatability were necessary P.D.A., was available as a standard medium.

Perithecia were produced abundantly on Oxoid P.D.A., cornmeal agar, lettuce decoction agar, and autoclaved soil nutrient agar. While production of perithecia on natural media appeared to be very dense on parts of the plate, the total area involved was generally very small.

In later studies, media used for production of perithecia included P.D.A. lettuce decoction agar, and autoclaved soil nutrient agar.

In general the addition of nutrients to the natural media induced increased colony size and spore or perithecial production. However, contaminants present in the fumigated natural material were encouraged to commence activegrowth by these nutrients. Competition from these organisms reduced the colony size on the natural host material treated with Chloropicrim.

2. The Effect of Medium pH on Growth

These studies were undertaken to ascertain the effect of medium pH on the growth features of colonies of S. botryosum, using P.D.A. as the standard medium. Observations were also made on the effect of medium pH on production of conidia and perithecia.

Materials and Methods

The P.D.A. was buffered and the pH altered by adding 2N hydrochloric acid or sodium hydroxide. The buffer consisted of:-

3.8g glycine

2.6g citric adid

3.8g potassium dihydrogen phosphate dissolved in 100 ml. of distilled water.

Two litres of Oxoid P.D.A. were prepared and 100ml. placed in 19 flasks. The media was autoclaved at the same time as the buffer, acid and alkali.

On removal from the autoclave 5ml. of the buffer were immediately added aseptically to each flask of media. Drops of sodium hydroxide or hydrochloric acid were then added with a standard pippette in accordance with the quantities set out in Table 2.

The flasks of liquid media were placed in a 50°C holding bath.

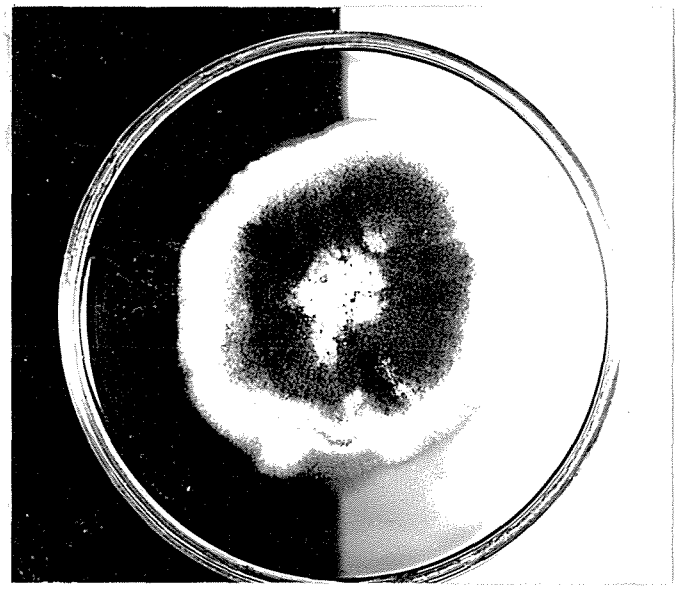


ILLUSTRATION 5.

A 10 day old colony on
P. D. A. 24°C

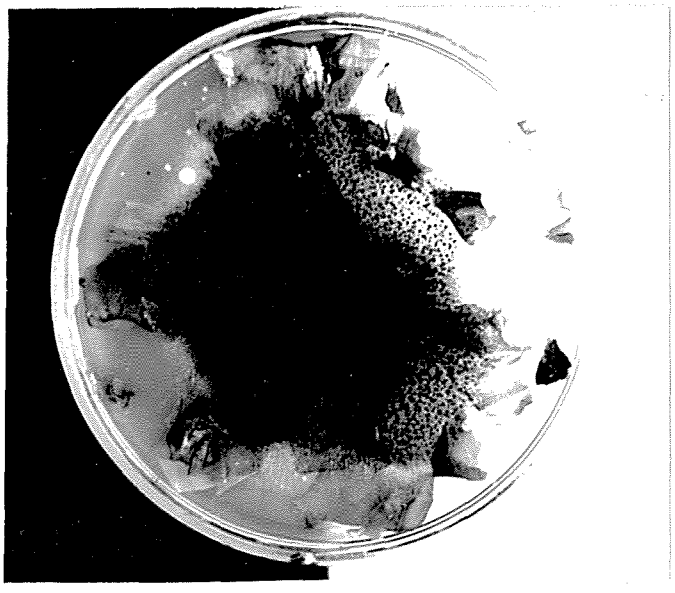


ILLUSTRATION 6.

A 14 day old colony grown
on Natural Nutrient (lettuce)
Medium at 24°C

Table 2 Quantities of Acid and Alkali Added to Buffered P.D.A. to obtain a range of Media pH.

2N HCl	No. of drops	Approx. pH.
Flask 1	20	2.8
2	15	3.0
3	10	3.3
4	Buffer Only	4.0
2N NaOH	No. of drops	
5	5	4.5
6	10	4.9
7	12	5.3
8	15	5.4
9	18	5.7
10	21	6.0
11	24	6.2
12	27	6.6
13	33	7.0
14	36	7.3
15	40	7.5
16	45	7.8
17	50	8.1
18	60	8.5
19	70	9.0

Each media was poured into four petri plates using approximately 20ml. per plate. One ml. of medium was withdrawn from that remaining in the flask and mixed while molten with 9ml. of distilled water. The flask was returned to the holding bath and the next medium poured.

The pH of the diluted media was determined using a glass electrode pH meter. Previous experimentation had indicated agreement between the pH of the solid and diluted media

The pH was plotted against the number of drops of alkali or acid used. If any pH deviated from the general curve it was rechecked and if found to be above or below the expected pH, the medium was discarded. This method eliminated any errors introduced during the adjustment of media pH, or by contaminated glassware. (Particular care was necessary when washing and rinsing glassware used during these experiments.)

When solidified the media were inoculated with 6 x 6 mm squares from the fringe of actively growing colonies. The plates were placed in the 24°C incubator. Three isolates, 4A, 4P, and 1U29A were used during three runs of this experiment.

On the fifth day from inoculation the diameter of the colonies were recorded.

After recording the pH of the media in the region of the colony was determined by melting the agar, and dilution as

previously described. Only small changes in pH of the media were recorded after five days incubation. If the colonies were recorded on the tenth day or later, the pH of the highly acid or alkaline media had become considerably altered towards the optimum pH range.

Results.

The average colony diameters of isolates 4P, 4A and 3U29A are recorded graphically in Figure 2. The optimum pH for colony growth was between pH 4.0 and pH 8.5 with maximum growth occurring between pH 5.3 and pH 6.3.

Colony characteristics were similar between pH 4.0 and pH 7.5 and typical of those reported elsewhere (p 32). On media below pH 4.0 the colonies became progressively lighter coloured and developed a mass of white aerial mycelium. Below pH 3.8 the media became progressivly liquid due to hydrolysis of the agar. Colonies developing on media of pH 8 or higher were small and usually darkly pigmented with abundant white aerial mycelium.

Conidia were produced abundantly on media in the range pH 4.0 - 6.5 with highest production occurring between pH 5.0 - 6.0. No conidia were recorded from colonies on media with a pH of 3.8 or less or an alkaline media with pH above 7.8.

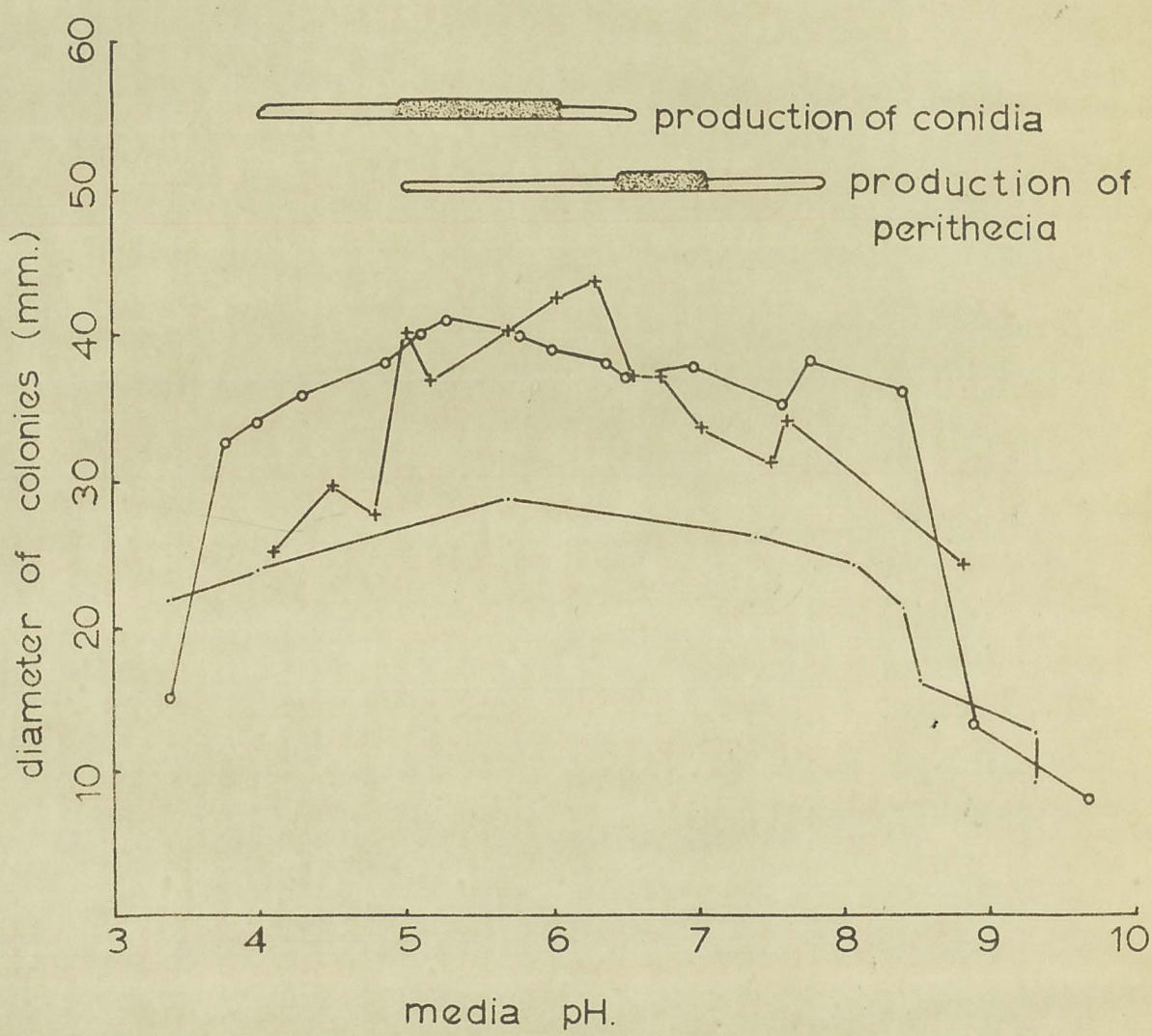
Perithecial production occurred between pH 5.0 - 7.8 with maximum production between pH 6.5 - 7.0.

The pH favouring production of conidia and perithecia are presented in Figure I, the thicker portion of the line represenving the optimum pH range.

FIGURE I. EFFECT OF MEDIA pH ON COLONY GROWTH

+ isolate 1U29A
o - - 4A
· - - 4P

colonies five days old on P.D.A.



Discussion

The optimum pH range for growth of *S. botryosum* on P.D.A. occurred on unmodified Oxoid, Difco, and Laboratory P.D.A. which had a pH of 5.6. The unmodified medium was therefore used for maximum production of conidia. Maximum production of perithecia occurred on neutral or slightly acid media.

3. The Effect of Temperature on Growth

A study of the effect of temperature on colony size was undertaken and observations made on the growth features of the colonies including the production of conidia and perithecia, to determine what the optimum temperature range for growth on culture media was.

Materials and Methods

Incubators were set to run at 5°, 10°, 12.5°, 15°, 20°; 24°, 28°, 32° and 36°C. Difficulty was experienced in maintaining 5°C due to the incubator functioning incorrectly.

Two isolates, 4A and 4P were used for inoculating Difco P.D.A. and three plates of each isolate were incubated at each temperature. The inoculum consisted of the normal agar squares measuring 6 x 6mm. These squares were placed face down in the centre of the media. Prior to placing the plates at the various temperatures they were held at 24°C for four hours until some growth of the mycelium had occurred.

The diameter of the colonies were measured after ten days and the average for each temperature calculated.

Results.

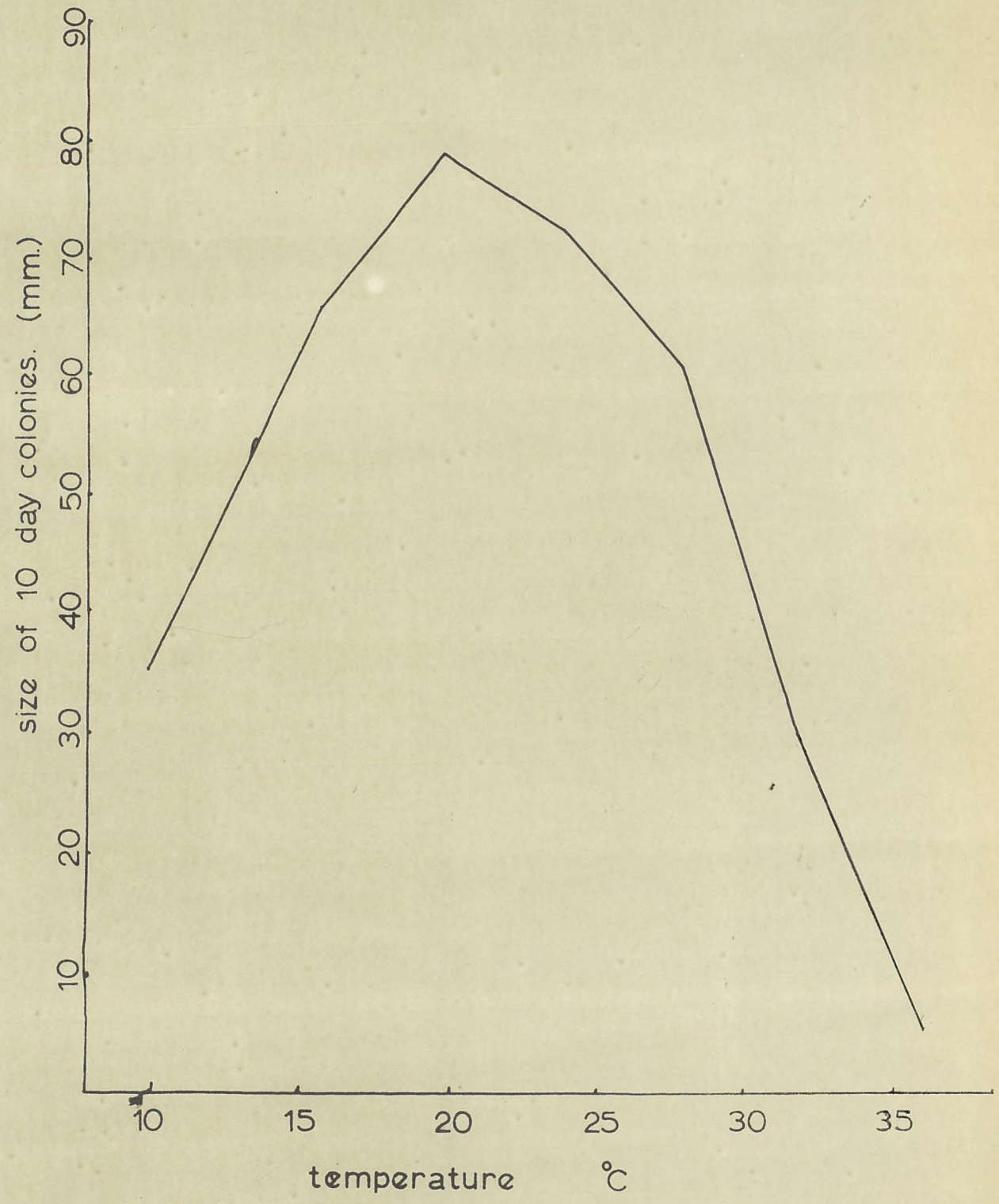
The differences in growth rate between Isolates 4A and 4P were not significant. The average size of ten day old colonies

are recorded in Fig.II. The cardinal temperatures for growth on P.D.A. were below 10°C, 20°C and between 32° - 36°C.

Conidia were produced abundantly at temperatures between 15° and 28°C., but only sparsely at other temperatures. Immature perithecia were produced on the majority of the larger colonies, but maximum production occurred at temperatures between 20° and 24°C.

At optimum temperatures for growth colonies were grey with a darker grey central area and a white fringe. Higher or lower temperatures induced production of lighter coloured colonies which often possessed a wider white fringe than is typical at optimum temperatures.

FIGURE II EFFECT OF TEMPERATURE ON COLONY GROWTH ON P.D.A. AFTER 10 DAYS.



Discussion.

Petzer (1958), Padhi & Snyder (1954) and Dippenaar (1939) consider that the optimum temperature for growth of S. botryosum on P.D.A. was in the range 22°C - 28°C which is several degrees higher than the optimum temperature for growth of the New Zealand isolates on similar media. These authors have reported a similar maximum and minimum temperatures to those recorded for New Zealand isolates.

At 20°C colonies on Difco P.D.A. had a constant average radial growth rate of 3.9 mm. per day. (The actual range observed varied from 3.5 - 5.0). According to Brancato and Golding (1953) this constant growth rate is due to the multiplication of the apical cells of the mycelium filaments. O'Rahn (1948) has concluded that three factors related to the morphology and physiology of the fungus determine the growth rate of fungal colonies, viz:

(a) The rate of division of the apical cell

(b) The rate of branching i.e. the increase of apical cells

(c) Rate of enlargement of the cells.

The difference observed between optimum temperatures for the New Zealand and overseas isolates may therefore be due to slight morphological or physiological differences altering one or more of the factors described by O'Rahn.

While the optimum rate of growth occurred at 20°C. no

incubators in the laboratory normally used would operate at this temperature. The 24°C incubator was therefore selected because it operated at the lowest temperature which was unaffected by fluctuating room temperatures. This resulted in an average decrease in radial growth rate of 0.3 mm. per day. Because production of conidia at this temperature was similar to that at 20°C. the slight reduction in colony size at 10-14 days was not of practical importance.

4. The Effect of Light on Colonies

Colonies in covered petri plates were subjected to light by removing them from the 24°C incubator at various intervals from inoculation until they were 14 days old, and placing them on a bench in front of windows. The colonies were thus exposed to sunlight which passed through the window glass and Petri plate cover, for periods of up to 3 hours during the eight hours they were on the laboratory bench. The plates were returned to the incubator overnight.

This treatment stimulated the production of conidia, especially from isolates which had been cultured for some period of time. On cultures under 10 days old the area which had been exposed to light was readily discernible due to the increased production of conidia. If the plates were exposed to light every second day the colony developed concentric pigmented areas associated with dense production of conidia. If cultures between 10-14 days old were subjected to light treatment sporulation was improved over the entire colony.

Production of perithecia was also improved by light treatment.

Diener (1950) has described the use of ultra violet light for inducing sporulation. If cultures with lids of the petri dishes in place were placed six inches below a WL30 ultra violet tube for twenty minutes a similar effect on production of conidia and perithecia was induced as that obtained by exposure to sunlight for a considerably longer period.

5. The Effect of Refrigeration on Colonies

Refrigeration of cultures at temperatures of from -5°C to -10°C stopped the growth of the colonies but failed to induce any morphological changes. Once the colonies were returned to favourable temperatures normal growth continued. In some cases conidial and perithecial production was improved by refrigeration, but results were inconclusive.

6. Pigmentation

Some isolates produced colour changes in the P.D.A. Usually this involved a change from the slightly yellow or muddy P.D.A. to a brown or redish brown colour. Isolates which caused pigmentation of the media were always prolific producers of perithecia. Unpigmented colonies occassionally produced perithecia but no pigmented colony was ever observed without perithecia.

7. Production of Conidia on Artificial Media

Materials and Methods.

Liquid P.D.A. was run on to sterile microscope slides placed on filter paper within a petri dish. The filter paper was moistened with sterile water. A spore suspension was prepared and one loopfull was streaked across the solidified agar on the slide. The petri dishes containing the slides were incubated at 24°C. Every twentyfour hours for ten days slides were removed and examined under the microscope to determine the method of conidial production. Observations were also made on the early formation of perithecia. Before the slides were returned to the petri plates after observation, sterile distilled water was ddded to keep the filter paper moist.

Results.

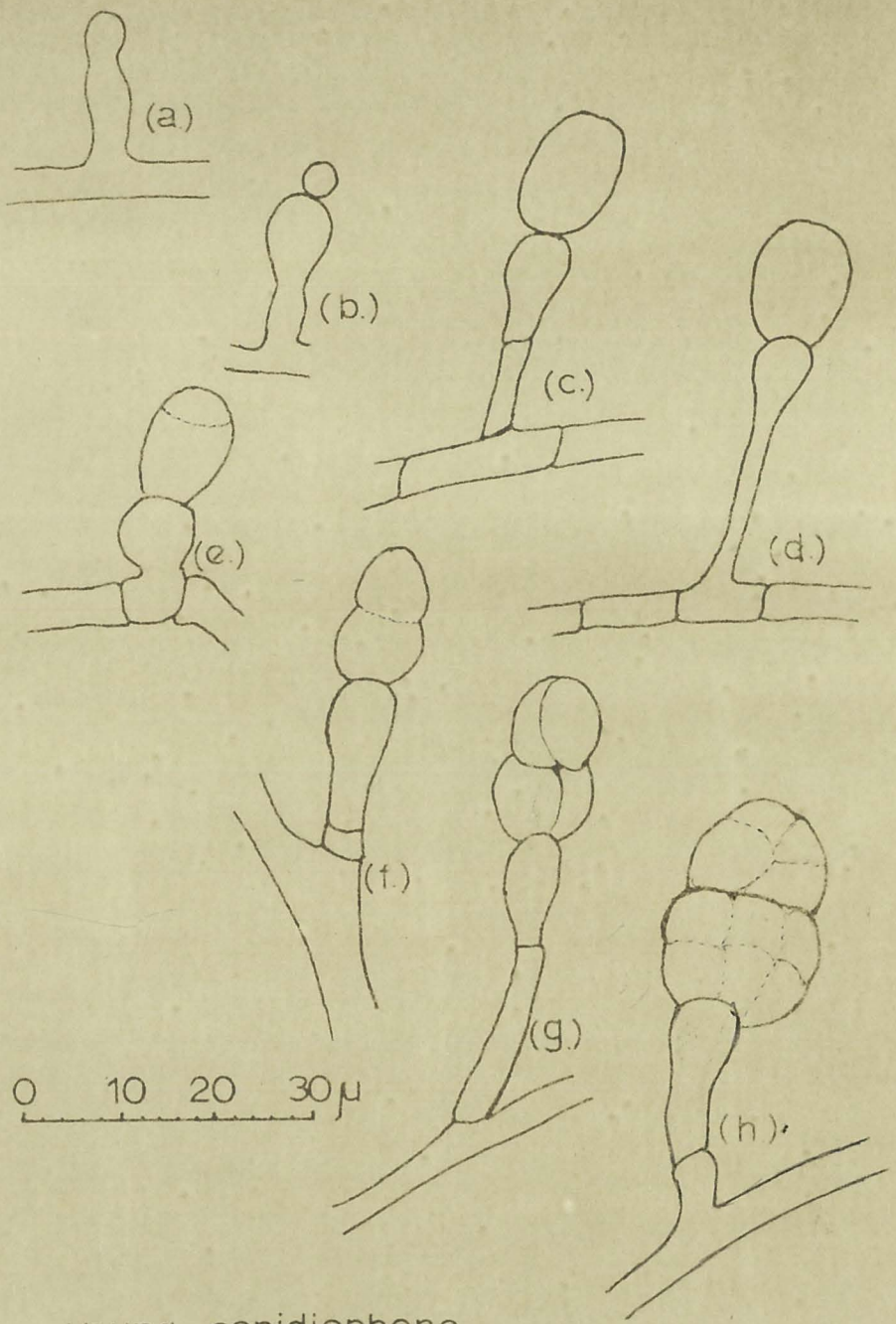
During the first forty-eight hours spore germination and production of mycelium occurred. Between forty-eight and seventy-

two hours from when the spore suspension was prepared hyphal tips or tips of lateral branches began to swell slightly to form young conidiophores (See Illustration 7a). At 72 hours small conidia appeared on the young conidiophores (Illustration 7b). At first these were spherical, translucent bodies up to 2 µ in diameter. The swollen tip of the conidiophore measured approximately 8 - 10 µ long and from 5 - 8 µ wide at this stage. The young conidia elongated slightly during the next twentyfour hours and often became two celled when a transverse cell wall was laid down. The conidiophores' swollen tips remained approximately the same width but they often became elongated during this period. (Illustration 7 c,d, & e.) From the fifth day of observation all stages of conidial production were present. The first formed conidia had continued to grow and a constriction had developed at the median transverse septa. Vertical cell walls were laid down and three or more cells were present (Illustration 7f & g). By further division in both planes conidia with nine to twelve or more cells were formed. These last divisions appeared to occur at random and seldom followed any well defined pattern. On the tenth day conidia were present with darkly pigmented sculptured walls. At this stage they were readily shed and nodular conidiophores similar to those shown in Illustration 9 were producing their second or third conidia.

On the fifth day it was apparent that two types of hyphae were present. One was hyaline and approximately 2 - 8 µ wide. Individual cells measured 10 µ or more in length. The other was from 4 µ up to 30 µ wide, and possessed short cells of from 5-10 µ

long. The hyphae of this mycelium was frequently associated in bundles of five or more hyphae and was yellowish brown in colour. On this latter type of mycelium knotts or fusion bodies frequently occurred, which by the tenth day measured up to 2mm. x 0.75mm. Subsequent observations confirmed that these bodies developed into immature perithecia. Conidia were observed developing on both types of mycelium.

ILLUSTRATION 7 DEVELOPMENT OF CONIDIA.



(a) young conidiophore
(b) conidiophore with small conidium.
(c) (d) conidium enlarging
(e) (f) two celled stage
(g) four celled stage
(h) eight to twelve celled stage

B. THE TAXONOMY AND NOMENCLATURE OF STEMPHYLIUM BOTRYOSUM. WALLR.

Stemphylium botryosum Wallr., a member of the Fungi Imperfeci, is placed in the order Moniliales, and the Family Dematiaceae (Clements & Shearer 1931). The original and modern concepts of the Stemphylium genus have been discussed by Wiltshire (1938).

1. History of the Genus Stemphylium with Special Reference to S. Botryosum

Wallroth (1833)* founded the Genus Stemphylium on a single species Stemphylium botryosum from the type host asparagus. This species described by Wallroth was essentially characterised by the conidiophores which were swollen at the apex with short nodular segments and bearing ovate, sub angular muriform spores. Wiltshire (1938) examined the original specimen and described the species as having conidiophores which were fasciculate (in groups ranging from three or four up to fifty) apparently arising from an immersed stromatic cushion and sometimes sparsely on superficial mycelium. They were typically dark in colour, occasionally branched, septate at intervals of 9 - 10 μ, mostly 30 - 70 μ long and swollen at the apex to a diameter of 7 - 10 μ. The spical swelling was darker below than in the upper half and was thickened by an inner wall. The growth of the conidiophore through the apex gave rise to a succession of "nodular segments" involving up to four swellings.

Conidia were dark, verrucose, roughly oval or subangular, slightly constricted at the medium transverse septum and to a lesser extent at other cross septa. These spores were muriform

with transverse verticle and oblique septa dividing the conidium into many cells, and possessed a prominent basal scar.

Wiltshire (1938) considered that the organisation of the conidiophore corresponded to that described for Macrosporium scarcinula (Berk), and M. parasiticum, Thum. by Berkely (1838)*. From Berkeley's illustrations, it was apparent that Alternaria type spores were also present as contaminants. These were considered by Berkeley to be immature conidial stage of Pleospora herbarum Rabenh. Wallroth (1833)* had mentioned the association of S. botryosum with Sphaeria complanata Tode. The perithecia of Pleospora species are similar to those of Sphaeria species and may have been readily confused by Wallroth.

The early studies on the Stemphylium species left a very donfused field which was unravelled by Wiltshire (1938), and is discussed briefly below. Elliot (1917)* considered that the genus Macrosporium Berk. and Thyrospora Tehon and Daniels. were synonymous with Stemphylium Wallr. In 1924 Bolle based her identification of S. Botryosum on an isolate considered by Oudeman* to be S. botryosum. Bolle reported that this S. botryosum isolate was similar to Wallroth's but considered the genus Macrosporium differed from this and should be retained for species with sarcinaeform spores (viz. those for which Tehon and Daniels made the Thyrospora genus). Before this, Harz (1871)* had described a species, Stemphylium lanuginosum, which corresponded to Oudeman's S. botryosum. Wiltshire (1938) considered that the isolates of Stemphylium sp. described by Harz and Oudeman

were identical and differed from S. botryosum Wallr. To avoid transferring Harz's S. botryosum to another genus, Wiltshire proposed that both types by included in the genus Stemphylium, but types similar to Wallroth's be termed Eustemphylium, and those allied to S. lanuginosum Harz should be placed in the Pseudostemphylium group. Wiltshire considered that the Pseudostemphylium would ultimately be transferred to a new genus.

Neergaard (1945) has agreed with Wiltshire and followed his system of classification.

Padhi and Snyder (1954) have considered that specialization within the S. botryosum species may be common and some populations have developed as pathogens. They have suggested that it would seem desirable to indicate this specialigation, which they consider has occurred in the case of lettuce, in the nomenclature of the pathogen. Padhi and Snyder (1954) have therefore designated the incitant of leaf spot of lettuce "Stemphylium botryosum f. lactucum forma nova, the imperfect stage of Pleospora herbarum f. lactucum forma nova. pathogenic to above-ground parts of the species Lactuca sativa."

Graham and Zeiders (1960) have reported a wide variation in the host range of various isolates of S. botryosum from leguminicolous hosts, and name two types of isolates that were host specific. They have not, however, designated this in the

nomenclature of the organism.

The perfect stage of Stemphylium botryosum Wallr. is considered to be Pleospora herbarium Rabenh., an ascomycete, of the order Spaeriales and the Phaeodictyae group (Clements & Shear 1931).

Wehemeyer (1953) has stated that while the genus Pleospora is generally referred to Rabenhorst, it is based on a series of exsiccati which in themselves do not constitute a specific or generic description. He adds that the first published description of the name Pleospora was by Cersati and de Notaris in 1861, but that their description excludes P. herbarum Rabenh.

Owing to the uncertain taxonomic position of Pleospora herbarum and the minor significance of the perfect stage in the disease cycle of leafspot of lettuce, the incitant has been taxonomically considered as Stemphylium botryosum.

2. Studies on the Taxonomy and Nomenclature of New Zealand Isolates of S. Botryosum from lettuce.

Neergaard (1945) discussed the diagnostic criteria of the Alternaria and Stemphylium genera. These are listed below in order of relative importance.

* As cited by Wiltshire (1938) and Neergaard (1945)

(a) Morphological Criteria:

Form of Conidia

Septation of conidia

Size of conidia

Colour of conidia

Sculpture of conidia

Arrangement of conidia

Characteristics of the conidiophore

Characteristics of the hyphae

Cultural characteristics

(b) Physiological-pathogenic criteria:

Natural media i.e. hosts

Symptoms

Temperature requirements

Other physiological properties

In determining the identity of the New Zealand isolates and comparing the form of the fungus present on lettuce in the Hawkes Bay, Manawatu and Horowhenua districts with that described by authors from other countries, the above criteria were used. The isolates obtained from lettuce are desdribed in detail, using these headings as an aid to determining the correct taxonomy and nomenclature of the causual organism.

(a) Morphological Criteria:

(i) Form of Conidia

Typical conidia have been drawn with the aid of a camera lucida and are presented in Illustration 8, While polymorphism is very common in the Alternaria and Stemphylium genera,

S. botryosum does not have the large number of spore types commonly found in many other species. The spore shape may vary from oblong, ends obtuse, to ovate (Ainsworth and Bisby 1953) and is sometimes irregular. With age the spore wall becomes dark and thickened. The spores were never beaked.

(ii) Septation of Conidia:

Mature conidia generally possess from 5 to 8 transverse septs with 1 to 5 longitudinal or oblique septa irregularly located across the conidium. The conidia are markedly constricted at the transverse median septum and to a lesser extent at other transverse septa.

(iii) Size of Conidia:

Williams (1959) studied spore size in relation to cultural conditions and considered that almost any change in the nutrition, or in other environmental conditions, leads to a significant and often quite large change in spore size. The fact that this character is so readily altered has considerable bearing on taxonomy since it is frequently used to separate taxa (Neergaard 1945, Williams 1959). Therefore in reporting spore sizes, the conditions prevailing during spore production and development have been defined.

Dimensions of Conidia:

(i) Host material: Lactuca sativa L. var. Black Seeded Triumph.

Incubated in 100% relative humidity at 24°C for 48 hours with complete darkness. (50 conidia measured.)

Length:	Mean	26.0 µ	S.D.	4.2 µ
	Mode	27.1 µ		
	Range	14.8 - 32.8 µ		
Width:	Mean	14.5 µ	S.D.	2.2 µ
	Mode	14.7 µ		
	Range	9.8 - 18.0 µ		

(ii) Artificial Media (Oxoid P.D.A.)

Media in petri plates inoculated with 6 x 6 mm. square of mycelium in P.D.A. and placed in dark incubators at 20°C. and 24°C for 14 days.

		20°C.	24°C.
Length:	Mean	25.1 µ S.D. 3.7 µ	19.0 µ S.D. 4.2 µ
	Mode	24.7 µ	17.1 µ
	Range	19.0 - 30.4 µ	13.4 - 25.7 µ.
Width:	Mean	16.5 µ S.D. 1.5 µ	15.4 µ S.D. 2.8 µ
	Mode	17.1 µ	15.2 µ
	Range	13.3 - 19 µ	11.4 - 20.9µ

NOTE:

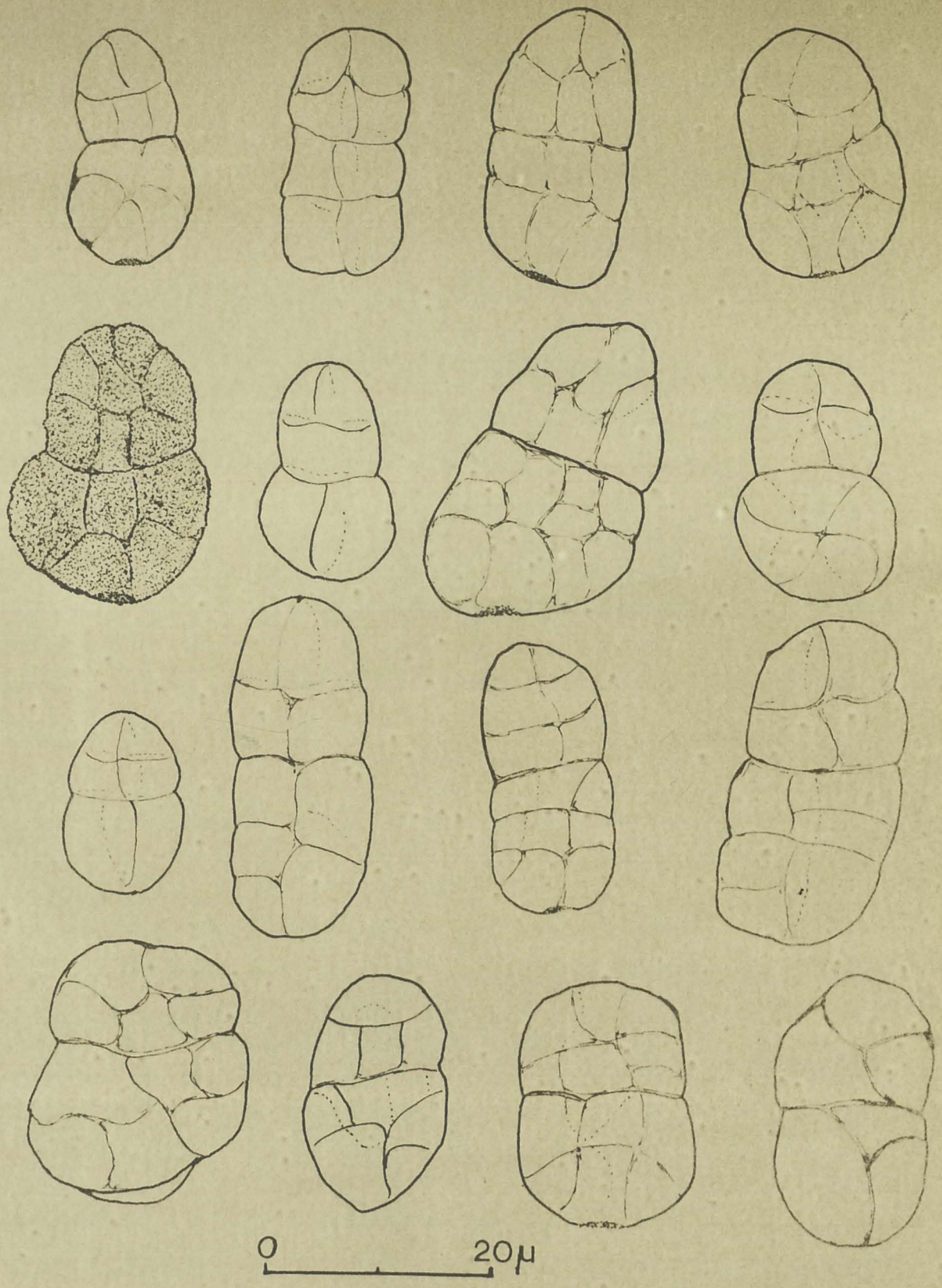
1. The mean lengths and mean widths of conidia grown at the two incubation temperatures differ significantly ($p = 0.01$).
2. The mean length of conidia produced on P.D.A. and host plant material at 24°C, differ significantly ($p = 0.01$).
3. The mean widths of conidia produced on P.D.A. and host plant material at 24°C. do not differ significantly. ($p = 0.05$)

(iv) Colour of Conidia:

The colour of conidia varied with age. After 48 hours' incubation at 24°C. in 100% relative humidity, conidia were a light olive brown. As they aged they became considerably darker.

ILLUSTRATION 8

CONIDIA OF Stemphylium botryosum WALLR



(v) Sculpture of Conidial Epispore:

Conidia were mainly echinulate or verrucose although smooth spores were not infrequently observed. Occasionally spores were observed which were partly smooth and partly sculptured.

(vi)Arrangement of Conidia:

Conidia were born singlely on the apex of conidiophores. Continued growth of the hyphae at the base of the conidiophore and production of further conidiophores and conidia at close proximity to the older nodular conidiophores resulted in the prolific porduction of conidia from central areas of lesions. Conidia were not arranged in 'bunches'.

(vii) Characteristics of Conidiophores:

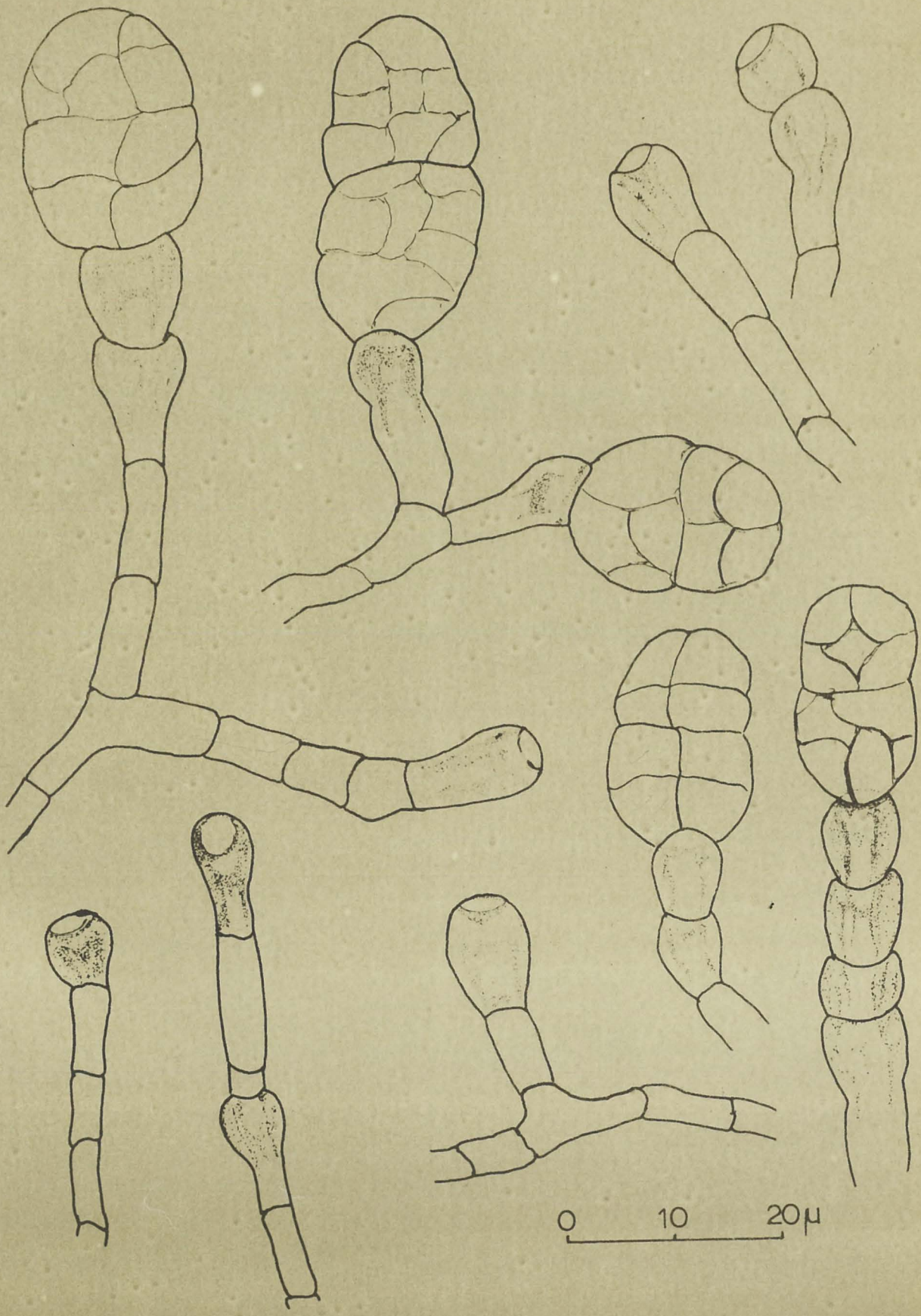
Conidiophores are very characteristic for this species. They are hyaline on artificial media or olive brown to dark brown, depending on age, on host material. After incubation at 24°C. meas-urements of twentyfive conidiophores indicated that they ranged in length from 12.3 µ - 115 µ, possessed cross septs at intervals of approximately 10 µ, but varying from 5 µ - 30 µ, and were from 2.9 - 4.1 µ wide.

The apical cell of the conidiophore was swollen to from 5.7 - 8.2 µ, possessed a dark area on the lower part of the swelling due to the presence of an internal cell wall, and was from 8.2 - 13.1 µ long.

Continued growth from the original conidiophore through the terminal swollen cell may give rise to another conidiophore.

ILLUSTRATION 9

CONIDIOPHORES OF
Stemphylium botryosum WALLR



Such swellings in close succession give rise to the "nodular" type conidiophore characteristic of this species. Up to four swellings have been observed on the one conidiophore. (See Illustration 9). Conidiophores bear the conidia terminally. In most instances conidiophores are formed singly as side branches of the hyphae, and were seldom seen to branch.

Occasionally on host material groups of five or more conidiophores were produced in bundles from the stroma formed on the dead plant tissues.

(vii) Hyphal Characteristics:

Hyphae were variable in size and septation but two types were present on both artificial media and host plant material. The common type of hyphae was hyaline to slightly brown, from 2 - 8 µ wide, but mainly 4 - 5µ with individual cells measuring from 8.2 µ - 41 µ long. The other type was from 4 µ - 30 µ wide, septate at intervals of from 5 - 10 µ, occasionally longer, yellowish brown, and often associated with several similar hyphae to form bundles of mycelium. Conidia were produced from both types of hyphae but perithecia were associated with the latter type.

(ix) Cultural Characters:

Stemphylium botryosum causing leaf spot of lettuce produced identical colonies from isolate to isolate. Similarly isolates from other hosts were usually indistinguishable from the lettuce isolates. The exception was an isolate from Sonchus oleraceus L. where colonies on P.D.A. sporulated

abundantly and were characterised by numerous white tufts of mycelium.

Characteristics of the colonies growing on various media are described in Table I. Growth of S. botryosum on P.D.A. is identical to that described by Neergaard (1945) for growth on "Standard nutrient agar".

Saltants occasionally occurred. Sectors of the colonies at times lost pigmentation or became darkly pigmented, varied in their ability to produce spores, or in their ability to produce perithecia. Growth rate was not altered.

Exposure to ultra violet light or sunlight usually stimulated production of spores and perithecia. Pigmentation was usually increased by exposure to light.

(b) Physiologic - Pathogenic Criteria:

Neergaard (1945) states:

"Fundamentally the taxonomy of fungous species and varieties has to be based on morphological criteria while the physiologic pathogenic critera merely may supplement the morphological - and be of decisive significance only in the establishment of physiological races (formae speciales)".

Considerable attention was therefore given to the host range of isolates from lettuce.

(i) The Host Range:

The host range for S. botryosum (and Pleospora herbarum) is very wide (Neergaard 1945), Wehmeyer 1953).

Neergaard states that S. botryosum is a faculative organism, and that even parasitic species with a narrow host range may be encountered as saprophytes on widely different media. This observation has been confirmed in these studies.

To determine the host range for S. botryosum as a parasite, a representative number of recorded hosts and other potential hosts were artificially inoculated with spore suspensions derived from lettuce isolates of the fungus, and the resulting infection was recorded. Isolates from onion, tomato, lupin, sow thistle, and Black Nightshade (Solanum spp.) were also tested for pathogenicity to lettuce.

Materials & Methods

Pathogenicity tests were performed either on the seedlings, or on older plants. Growth was kept succulent to predispose the

plants to infection.

Seedlings were grown in 6" clay pots or small seed trays and later transplanted into small seed trays or 3 - 4" clay pots.

One dozen plants were provided for each test. Eight plants were inoculated and four were retained as controls. Four lettuce were also inoculated as controls for infection. Inoculum consisted of a suspension of 3,000 conidia per ml.

Following inoculation, the plants were placed in 100% relative humidity for 24 hours before they were returned to the glasshouse bench.

Experimentation:

The hosts inoculated with isolates from lettuce at various periods during these studies, are tabulated in Table 3. If a host proved susceptible after completing Kocks Postulates with the idolate, pathogenicity tests were repeated on lettuce to ensure that a similar strain of the fungus was causing infection.

Isolates from crops other than lettuce were inoculated on to lettuce using a similar method to that described above.

RESULTS

The results are tabulated in Tables 3 and 4.

Lupinus spp. were only "infected" with S. botryosum when the plant was wounded. Because lesions failed to enlarge, it was considered that S. botryosum was present as a saprophyte on the

damaged tissue.

Plants of Pastinaca sativa in one instance produced yellow-white blotches on the inoculated leaves. These areas remained chlorotic but no necrosis developed. When plated on to P.D.A. after surface sterilization, S. botryosum grew out on to the media. During subsequent experiments, the flecking could not be induced again.

Solanum nigrum and S. nodiflorum were not susceptible to S. botryosum in the seedling stage. Older leaves on flowering plants became readily infected.

Sonchus oleraceus was infected only when wounded and although the fungus could be reisolated from the host, lesions remained the same size. S. botryosum may have been existing as a saprophyte on the injured tissue.

Zinnia elegans appeared to be moderately susceptible to S. botryosum isolated from lettuce. Lesions failed to develop beyond 2 - 3 mm, but re-isolation and inoculation indicated that it was definitely pathogenic to Zinnia.

Only isolates from naturally infected Amaranthus retroflexus, Sonchus oleraceus Solanum nigrum and S. nodiflorum would infect lettuce.

Table 3.	PATHOGENICITY OF STEMPHYLIUM BOTRYOSUM ISOLATED FROM LETTUCE					
Potential Host:	Type	Pathogenicity Recorded by:	Expts	Results	Re-isolation and Inoculated on to	
					Host	Lettuce
Aster sp	P	Anon (1958)	2	-		
Beta vulgaris	S	*	1	-		
Brassica oleracea	S	Neergaard (1945)				
		*	1	-		
Clarkia elegans	P	"	2	-		
Cucumus sativus	S	* "	1	-		
Caucus Carota (Taranaki) strong top)	P	* "	1	-		
Dianthus caryophyllus	S	"	1	-		
Lupinus sp.	S	Anon (1958)	2	-		
	P	"	4	-		
	P	"	W 1	+	-	+++
Lycopersicum esculentum	F	Brooks(1958)	1	-		
Nicotiana tobacum	P	Neergaard (1945)	1	-		
Pastinaca sativa	S	*	1	-		
	P	*	1	+	-	+++
	P	*	3	-		
Phaseolus vulgaris	S	Anon (1958)	1	-		
Pisum sativum	S	"	1	-		
	P	"	1	-		
Pyrus malus	F	Elliott (1917)	3	-		
Solanum nigrum	S	*	1	-		
	P	*	4	+++	+++	+++
Solanum nodiflorum	P	*	1	+++	+++	+++
	S	*	2	-		
Sonchus oleraceus	P	*	?	-		

Table 3 contd.

Potential Host:	Type	Pathogenicity Recorded by:	Expts.	Results	Re-isolation and Inoculated on to	
					Host	Lettuce
Sonchus oleraceus	P	*	W 1	+	-	+++
Trifolium spp.	P	Anon (1958)	2	-		
Zinnia elegans	P	Neergaard (1945)	2	++	++	+++

KEY:

S = Seedling
P = Plant
F = Fruit

* Possible alternative Host
/ Recorded from seed
W Wounded

\+ Weakly pathogenic
++ Moderately pathogenic
+++ Pathogenicity similar to that on lettuce.

References:

Anon 1958

"Host Fungus Index for the Pacific North west. I. "Hosts". Washington Agric. Expt. Stat. Inst. of Agric. Sciences, State College of Washington. Circ. 335. 1958.

Elliott 1917
See Neergaard 1945.

Neergaard 1945
Refer Bibliography

Table 4 PATHOGENICITY OF VARIOUS ISOLATES OF STEMPHYLIUM BOTRYOSUM TO LETTUCE

Host	Type	District	Expts.	Results	Re-isolation and Inoculated on to	
					Host	Lettuce
Allium cepa	P	Palm. North	2	-		
Amaranthus retroflexus	P	Palm. North	1	+++	-	+++
Lupinus Spp.	P	Palm. North	4	-		
			3 W	-		
Lycopersicum esculentum	F	Otaki	2	-	?	-
Senecio vulgaris	P	Bay View	1	-		
Sonchus sp.	P	Palm. North	2	+	+	+
Solanum nigrum	P	Otaki	4	+++	+++	+++
S. nodiflorum		Otaki	1	+++	+++	++++

? Not performed

Abbreviations : See Table 3

It was assumed that the lesion obtained from a single infected A. retroflexus plant was abnormal. No A. retroflexus plants grown artificially became infected following artificial inoculation. It was considered that the one infected plant may have been damaged and S. botryosum from the lettuce in the same block may have infected the damaged tissue. Alternatively, this plant may have been genetically different from the normal population.

(ii) Symptoms:

While symptoms were typical of the disease incited by this organism, they varied considerably due to the influence of the environment. While they corresponded to symptoms described by other authors, no detailed comparisons could be made due to the variations which occurred.

(iii) Temperature Requirements:

These have been considered during the study of the disease cycle and are believed to be important when comparing the form of fungus present on lettuce in New Zealand with that recorded by overseas authors.

(iv) Other Physiologic Properties:

During studies of the disease cycle, both stomatal and direct penetration were recorded. This may be due to a difference between the New Zealand isolates and those studied by Padhi and Snyder (1954).

Discussion

The definition and limitation of the concept of species has always been a fundamental problem to the taxonomist. This problem in relation to the causual organism of the leaf spot of lettuce, has been studied by Wiltshire (1938) and Neergaard (1945) with the result that the taxonomy of the genus Stemphylium Wallr. has been considerably elucidated.

Butler (1929) and Ciferri (1932) consider that morphological criteria may be applied with few exceptions to define a species, and only in special instances should biological differences be stressed. Yerkes and Shaw (1959) have utilized host specialisation at the host family level to delimit species of the smut fungi. Specialization at the host genus level has been used in the rust fungi to delimit varieties (Stackman & Hamar 1957). However, these latter fungi are obligate parasites.

Purdy (1955) when studying the genus Sclerotinia, concluded that host specialization could not be used at any level to delimit species of this genus, which are saprophytic.

Stemphylium botryosum is a faculative organism, (Neergaard 1945) and its host range in the saprophytic state is very wide compared with its range as a parasite. With probably the majority of fungi, genera and species have been defined while the organism is still in the state of evolution, and they are thus not stable taxa. Consideration was given to the taxonomy

of the isolates from lettuce in New Zealand in an attempt to ascertain whether or not the fungus, which is parasitic to lettuce, has reached that stage of evolutionary development where this form of the fungus should be designated by a variety or form epithet or considered as a distinct species.

Studies of the morphology of the fungus were undertaken and consideration was given to the important criteria described by Neergaard (1945).

Following Wiltshire's (1938) taxonomy, it is considered that the incitant of leaf spot of lettuce is Stemphylium botryosum Wallr. belonging to the Eustemphylium group of the genus Stemphylium.

The form, septation, sculpture, colour and size of conidia were in agreement with those described by Wiltshire (1938) and Neergaard (1945) for Stemphylium botryosum, and are similar to those reported by other authors for this species (for example Brooks (1953), Hughes (1948), Petzer (1958), Olgilvie and Muligan (1931), Dippenaar (1939)). Likewise the characteristics of the conidiophore and the arrangement of conidia confirmed that the species isolated from lettuce in New Zealand was S. botryosum.

The growth relationship with temperature on P.D.A. for the New Zealand isolates from lettuce are identical to those described for isolates from Phlox, Zinnia and Onion growing

on Standard Nutrient Agar. (Neergaard 1945). The morphology of the New Zealand isolate therefore appears to be identical to that described by other authors for this species isolated from lettuce.

The only exception however is to be found in the description of growth on culture media by Padhi & Snyder (1954). To quote

"Under cultural conditions the conidia not infrequently arose in short chains. Two and three, rarely four conidia have been observed in chains on the isolates from lettuce..... The same phenomenon has been reported for the species of Stemphylium pathogenic to carrot (Grogan and Synder 1952)".

At no time were conidia observed to be formed in chains during these studies. S. radicinum (M., Dr. & E.) Neerg. is grouped by Wiltshire (1938) by definition, and placed by Neergaard (1945), in the Pseudostemphylium group where formation of conidia in chains is not uncommon (Neergaard 1945). It was Wiltshire's belief that the Pseudostemphylium would eventually be transferred to another genus. If this is so, Padhi and Snyder's comparison of the spore production of S. botryosum with S. radicinum is not valid. Neergaard (1945) and other authors cited during these studies have not recorded the formation of conidia of S. botryosum in chains.

Neergaard (1945) does not report host specificity in the case of Stemphylium botryosum although he has acknowledged that it may occur in the Alternaria and Stemphylium genera. Host specificity of some degree has been recorded for S. botryosum by Padhi & Snyder (1954), Petzner (1958), Graham & Zeiders (1960) and has been observed during these studies. Padhi and Snyder

alone, have indicated this host specialization in the nomenclature of the pathogen.

Thus the morphological criteria indicated that the incitant of leaf spot of lettuce was Stemphylium botryosum Wallr. but failed to indicate any specialization of form. Neergaard (1945) considered that physiologico- pathogenic criteria may be of decisive significance in the establishment of physiological races. Host range studies were therefore initiated.

In the saprophytic state the host range of S. botryosum appeared to be unrestricted, as indicated by studies on the survival of this organism and the range of artificial media which support its growth. Padhi Snyder (1954) however while stating that this organism isolated from lettuce would grow readily on several artificial media considered that it survived from season to season only on dead lettuce leaves.

As a pathogen S. botryosum isolated from lettuce in New Zealand would readily reinfect lettuce and the older leaves of Solanum nigrum and S. nodiflorum. Zinnia elegans was also moderately susceptible to infection. Hosts reported by several other authors and the potential hosts which were tested (weeds and vegetables), failed to become infected by the lettuce isolates at the inoculum rates which caused infection of lettuce, and the additional hosts recorded above. Neergaard (1945) using agar squares of mycelium has recorded a wide host range for this species.

The isolates from lettuce in New Zealand have a wider host range in both the saprophytic and parasitic state than that reported for S. botryosum f. lactucum by Padhi and Snyder (1954).

Other comparisons were also made of characters reported by these and other authors for isolates of S. botryosum from lettuce.

Spore germination is reported by Padhi and Snyder (1954) to occur over a similar temperature range to that observed for isolates from lettuce in New Zealand. However the optimum temperature for germination was approximately 5°C below that recorded for New Zealand isolates. Relative humidity requirements also varied. Padhi and Snyder found germination would only occur at relative humidities above 95%. These studies have indicated that germination of conidia from New Zealand isolates will occur at a relative humidity of approximately 90%. Penetration of the lettuce leaf has been studied by Padhi and Snyder who report only stomatal penetration. In this study stomatal penetration was predominant but approximately one-third of the infections recorded had originated from direct penetration.

Dippenaar (1939) has recorded an incubation temperature 10°C above that which favours this portion of the disease cycle investigated during these studies. While some variation can be expected from the different experimental techniques, it is possible that the form of the fungus studied by Dippenaar is

responsible for a large part of this temperature variation.

Previous authors have not made detailed studies of the environmental conditions which favour growth and development of subsequent phases of the host parasite relationship.

Due to the differences discussed above it appears doubtful that all isolates of S. botryosum from lettuce, in particular Padhi & Snyder's (1954) S. botryosum f. lactucum, are identical to isolates from lettuce in New Zealand. It would therefore be unwise to designate the form pathogenic to lettuce in New Zealand with a similar epithet to that used by Padhi & Snyder.

It is the present authors considered opinion that the existing genetical understanding of the genera Stemphylium and Pleospora is insufficient to enable forms or varieties of the species in question to be defined with accuracy.

While cultures derived from single spores have been used throughout this study the resulting colonies are not necessarily composed of the one genotype because commonly more than one cell of the spore may produce a germ tube and mycelium. No information is available on the mutation rate of this species, although visible mutant forms were often observed on artificial media.

It is considered that a detailed study of the genetics of this species in relation to its host range and an assessment of

the variation which may be expected due to the combination or segregation of different genotypes, (or mutants), is required. The stability of the forms showing host specificity could then be ascertained and specialization indicated in the nomenclature of the pathogen if it was considered to be a stable entity within the species.

Conclusions

The identity of the incitant of leaf spot of lettuce is *Stemphylium botryosum* Wallr. While some host specificity was apparent, the present genetical understanding of this genus is considered to be too meagre to permit the recognition of this specialization with a form or varietal epithet. It is also not certain that the isolates from New Zealand lettuce are identical to *S. botryosum f. laetucum* described by Padhi & Snyder (1954). The Wide host range that this organism has in the saprophytic state indicates that the evolutionary development of this fungus has not resulted in a marked degree of host specialization.

No attempt therefore has been made to indicate host specialization of the fungus pathogenic to lettuce in this country in the nomenclature of the pathogen.

CHAPTER II

FIELD OBSERVATIONS

ON THE OCCURRENCE

OF LEAF SPOT OF LETTUCE

CHAPTER I I

FIELD OBSERVATION ON THE OCCURRENCE OF LEAF SPOT OF LETTUCE

During 1960 crops of lettuce in Hawkes Bay, Manawatu and Horowhenua were kept under observation from autumn until spring. From October 1960 until August 1961 lettuce were observed in the Manawatu and Horowhenua areas. From visits to market gardens and discussions with the growers it was apparent that the foliage disorder of lettuce are frequently confused. These surveys were undertaken to observe the occurrence of leaf spot on lettuce and to ascertain its importance relative to other disorders.

Materials and Methods

During March and April 1961 commercial growers of lettuce in the Napier-Hastings areas of the Heretaunga Plains, in the Manawatu about Palmerston North, and at Ohau, Manakau and Otaki in the Horowhenua were visited to ascertain which growers produced winter and spring lettuce. Those growers who had appreciable areas were visited at intervals of from 6 - 8 weeks and the crop inspected for foliar disorders. Records of visits and details of observations made on each property were entered on a card. A questionaire was prepared and details were completed during discussions with the growers. The questions which were asked are listed below.

List of Questions put to Growers During Discussion of Winter Lettuce

1. What variety of winter lettuce do you grow?
2. How many successive crops of winter lettuce have you had on the area you are using this season?

3. Have you experienced difficulties in producing winter lettuce?

4. What do you consider are the most important diseases of spring or winter lettuce?

5. Do you have a regular spray programme during winter and spring?

6. What therapeutants do you use?

7. What type of spray equipment do you use to apply these sprays?

Samples of plants infected with ringspot and leaf spot were shown to growers who, during discussion, were asked:

8. Do you normally distinguish between these two types of disorders?

9. If so, which occurs most frequently on your property?

10. What months of the year are these diseases prevalent?

11. Can you estimate what loss you have from leaf spot, ring spot or both disorders?

During each visit samples of lesions were collected from the lettuce crops and placed in labelled plastic bags. On return to the laboratory they were subjected to high humidity which induce sporulation and facilitated identification of the casual organism. Lesions from weeds and other vegetable crops were also collected and examined in a similar way. Records were kept of the date of collection of the host, the property and the district from which the lesions were obtained. The identity of the organism was later entered. Stock cultures of S. botryosum were prepared from isolates collected at each property by placing individual conidia on P. D. A. slants. After incubating at 24°C for 5 days the tubes were sealed with 'parafilm' and kept at room temperature.

A. Discussion of Observations made in each District

1. Hawkes Bay

The properties of twelve growers were observed on the Heretaunga Plains. Five of these properties were located in Bay View, three near Taradale, two in the 'Lomas Block' near Hastings, and one at Havelock North. Bay View, being on the coast of Hawkes Bay, has a milder climate than the other districts.

Leaf spot caused by S. botryosum appeared on crops nearing maturity at Bay View during April. The lesions were mainly confined to the older leaves of the plants and although 50% of a crop of Great Lakes were infected on the property most severly affected losses were not high. Some wrapper leaves were heavily spotted and this caused a down grading of a small percentage of heads. On most properties an estimated 5% of the plants were infected with S. botryosum but this had no affect on the total yield marketable heads.

Once the last crops of the Great Lakes were harvested in May the disease did not appear to cause further down grading of heads. It made its appearance on occasional blocks of lettuce during winter but was never serious.

In the other areas on the Heretaunga Plains leaf spot was not recorded throughout the period of the survey. However from July until September loss of plants due to ring spot was very high. It was not possible, during the milder conditions to distinguish visually between lesions caused by Stemphylium

botryosum and Marssonina panattoniana.

The weather conditions for this area were abnormal during the period of the survey. The temperatures were above normal, there being fewer frosts, and rainfall for the winter months was above that of previous seasons.

2. Manawatu

Six growers properties were kept under observation from late March 1960 until February 1961. Four of these properties were in the Whakaronga area to the North of Palmerston North and two were in the market garden area to the South of the city. Leaf spot on heads of lettuce first occurred during late March. On one property losses of Great Lakes in May due to severe infections with S. botryosum represented up to 50% of the crop, while the remaining plants harvested had a very unattractive appearance on the market floor.

Lesions caused by S. botryosum were present on all winter lettuce crops, and if the crop had been planted in the same area for three years or more the incidence of lesions was very high. Few lettuce crops matured during winter, but rapid development of heads occurred during late August and September. On crops where the incidence of lesions on the outer leaves was high during winter, up to 75% of the heads at harvest exhibited lesions caused by S. botrycsum. Some crop losses did occur, but due to the spread of harvest and ploughing of the areas

once the crop was harvested no accurate estimation of losses could be made.

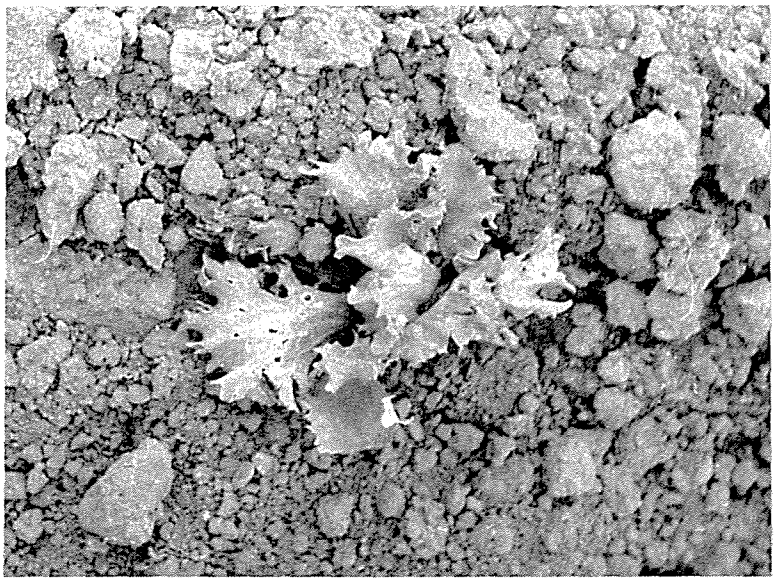
On the market floor buyers avoided the lines which were badly spotted. Often lettuce which were heavily infected were picked before they were properly hearted to avoid further losses. These practices all contributed to loss of revenue to the growers who considered leaf spot of lettuce one of the most devastating diseases of lettuce.

Ring spot caused by M. panattoniana appeared in one garden in July. This disease was most serious during September but apart from losses of a group of twenty or more plants in several localised regions of lettuce blocks, crop losses from ring spot were never severe.

It was generally possible to distinguish visually between ring spot and leaf spot lesions from Manawatu. The ring spot lesions were smaller and often contained a central area which produced a profusion of pink spores. Some Chinese distinguished between the two types of lesions and were able to recall seasons when they had lost crops due to one or other disorder.

Observations were continued at intervals through the summer and on most crops after some searching it was possible to locate lesions caused by S. botryosum. Invarably, these lesions were present on leaves next to the soil.

ILLUSTRATION 10. Lesions caused by Stemphylium botryosum on lettuce growing at Otaki, March-April. 1961.



3. Horowhenua.

Ten properties were observed during winter 1960 in the Horowhenua. Six of these properties were at Otaki, two at Ohau West and two at Manakau. Detailed observations were made on the spread and occurance of the disease during 1960 and 1961 on one Otaki property.

In Manakau a few lesions were collected during April but no further leaf spot was observed during the season. This may have been due to the relatively small size of these crops and their isolation.

At Ohau West lesions caused by S. botryosum were abundant on one of the properties during late March. These lesions were confimd to the lower leaves. In late August, just prior to harvest of the crop it was estimated that 80% of the crop would not be marketable. Between July and the end of August the spread of the disease through the crop proceeded at a phenomenal rate.

Of the six winter crops observed in the Otaki area, four were not seriously affected with either leaf spot or ring spot, although occassional lesions of one or other disease were usually located. Portion of one other crop was lost with leaf spot and ring spot attacking the same plants, while in another Block on the same property losses from ring spot during the July and August did occur. However these losses were low compared with an estimated 90% loss of marketable heads due to leaf spot

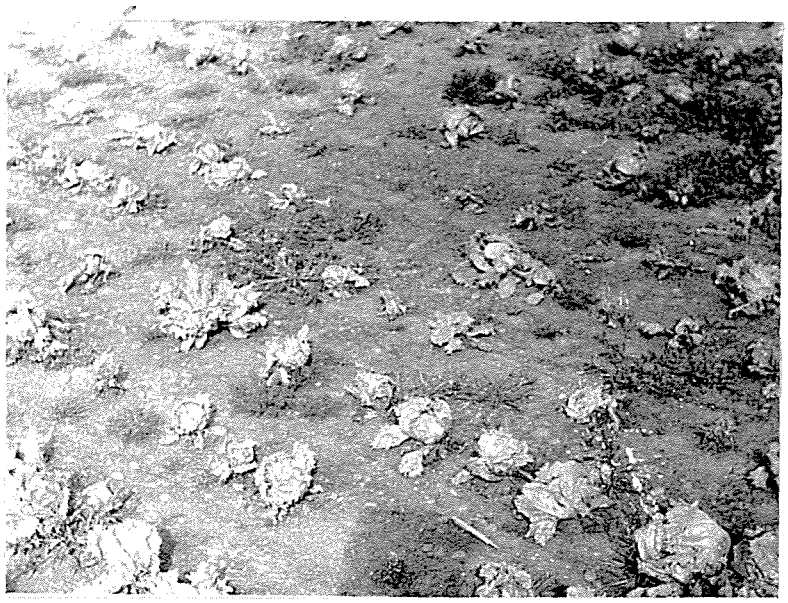
caused by S. botryosum from a nearby property during August. Due to the incidence of the disease on this property it was kept under observation for a total of fifteen months.

The first visit to this garden was made during late March 1960. At that stage Great Lakes were being harvested and an estimated 2% of the plants were infected with S. botryosum, but this was mainly confined to the outer leaves which were discarded during harvesting. All Great Lakes were harvested by the end of April and the same ground replanted with Black Seeded Triumph. In June 80% of the Black Seeded Triumph were infected with S. botryosum. Where hedges shaded the lettuce beds, the disease was more serious than in the central areas of the blocks.

The first heads matured in August, but at this time the leaf spot had also reached its maximum activity due to favourable environmental conditions. Twenty instead of two hundred cases of lettuce were cut from each block approximately twice a week. This represented a total loss of 2000 cases to this grower. He was obtaining at the time from 20/- to 50/- a case for the lettuce he was able to harvest. A regular Thiram spray programme was applied when the weather permitted, and the grower considered that the returns from the crop would barely pay for the cost of spraying and harvesting.

The grower stated that the season was not normal due to a very high rainfall in July followed by a relatively mild August.

ILLUSTRATION 11. Lettuce remaining after harvest due to infection with *Stemphylium botryosum*. September 1961.



(a) Whakaronga.

(b) Otaki.

average rainfall - inches
5"
4"
3"
2"
1"
0
PALMERSTON NORTH
J
F
M
A
M
J
J
A
S
O
N
D
month.
80°
70°
60°
50°
40°
30°
temperature °F

3. Discussion on the weather conditions observed which favoured leaf spot of Lettuce.

S. botryosum leaf spot disease of lettuce first became apparent in autumn when the average temperature dropped from about 55°F. in early April to 50°F. at the end of May. No apparent increase in the disease then was observed until the period when the average minimum temperatures commenced to rise in early spring. At this stage frosts became fewer and lighter, and the maximum screen temperatures had started to rise slowly.

Epidemics occurred on rising average temperatures when the average minimum screen temperature was between 40°F. and 45°F, the average maximum screen temperatures below 60°F, and the average of the maximum and minimum screen temperatures was between approximately 45 - 55°F. These conditions had been preceeded by a period of heavy rainfall.

The Meterological Records for 1960 from Napier, Palmerston North and Levin are presented graphically in Figures III, IV , & V . The shaded areas represent the periods in which epidemics occurred. Because Otaki is slightly milder than the Levin district, the epidemic conditions illustrated in Figure III occurred at Levin approximately fourteen days after they were noted at Otaki. Unfortunately there are no detailed meterological records for Otaki.

Observations made on the effect of environment on epidemics in the field agree with those determined during the study of the disease cycle presented in Chapter III.

Conclusions

Field observations revealed that leaf spot caused by Stemphylium botryosum was unimportant in Bay View, and was not found in other areas of the Heretaunga Plains during the 1960 winter period. Leaf spot however caused a larger crop loss in areas of the Manawatu and Horowhenua than ring spot, caused by Marssonina panattoniana.

Growers indicated that the control of these two foliage disorders was one of the major problems associated with production of winter lettuce. Considerable confusion existed concerning the identity of the actual foliar disorder as most growers attributed both diseases to the one causal organism and commonly called the leaf spot, caused by S. botryosum, ring spot. Both diseases appeared in epidemic levels during late winter and spring.

Due to inefficient spray machinery and wet weather conditions, most growers considered little benefit was to be gained from the application of various therapeutants during winter to protect foliage from infection. However the efficacy of Thiram plus Sticker was demonstrated on one property during the 1961 winter period.

Observations on the period at which epiphytotics occurred indicated that rising average temperatures of between 45-55°F. favoured the disease. These periods were preceeded by heavy rainfalls. Disease cycle studies presented in the Chapter III confirm observations reported in this Chapter.

CHAPTER III

THE DISEASED

PLANT.

INTRODUCTION

A modern concept of the diseased plant has been presented and discussed by Horsfall & Dimond (1959). These authors emphasise the importance of the diseased plant, and consider that two phases are involved; the first "the science of learning and understanding disease," and the second "the art of applying the knowledge to real life problems."

The first two sections of this chapter have been devoted to a study of the diseased plant. The last section discusses the conditions which are required for an epiphytotic by applying the knowledge obtained during the preceding studies.

Because disease results from the continuous irritation of the host by the pathogen (Horsfall & Dimmond 1959), it was convenient to study the effect of enviromental conditions on each phase of the host-parasite relationship prior to, during, and after the process of disease had resulted. In this way a complete study of the factors involved with each stage of the host-parasite relationship was made. The chain of events which lead up to the development of the disease process have been termed the "Disease Cycle" by Walker (1950). This is distinct from the life cycle of the pathogen. In this study, to enable a continuity of the disease cycle from one season to another to be considered, it has been necessary to include that portion of the pathogen's life cycle concerned with the survival of the fungus from season to season.

Many attempts have been made to define the disease cycle. (Heald 1933, Brown 1936, Butler & Jones 1949, Gaumann 1950, Walker 1950, Lilly & Barnett 1951, and Yarwood 1959). Since the potential combination of pathogens and host are innumerable it is not surprising that many differing definitions exist. It was considered that the study of Stemphylium botryosum infecting lettuce was facilitated by dividing the disease cycle into the phases defined below.

1. Spore Germination. Germination is the process involved when the spore imbibes water swells and produces a germ tube which has been recognised in these studies by two criteria, viz: (a) when the germ tube sides become parallel or (b) when the length of the germ tube equals the average diameter of the spore.

2. Penetration. This process, the first stage of infection, occurred when a germ tube or hypha produced by the germinated spore entered the host tissue.

3. Infection. When the fungus had penetrated the host tissue and become independent of the spore for nutrition and survival, infection was considered to be complete. When studying a sample of the infected population it was not possible to distinguish between Penetration and Infection, although two distinct processes were involved.

4. Incubation. The period of Incubation in the host tissues commenced when infection occurred and was terminated by the expression of macroscopic symptoms of disease.

5. Manifestation of Disease. Manifestation occurred at the completion of the Incubation Period when symptoms on the host became macroscopically discernable. This period was considered to have reached its climax when the death of the entire host leaf or plant had resulted due to the activities of the pathogen.

6. Production of Inoculum. During the later stages of manifestation and after exposure to favourable enviromental conditions production of conidia occurred. Ascospores, another form of inoculum were produced from perithecia containing asci which arose from dead host tissue or other organic matter.

7. Liberation of Inoculum. The process of Liberation of Inoculum was concerned with the release of the spores from the conidiophores or from perithecia, and was closely associated with the dispersal of these spores to potential hosts to initiate further development of disease.

8. Survival During the Summer Months. Consideration was given to the methods available for the pathogen to survive from one season to another to initiate development of disease the following season.

A. THE DISEASE CYCLE

1. Spore Germination.

Spore germination is the initial stage in the production of the fungal mycelium. It may be considered as the first step in the development of the disease cycle. Factors which influence spore germination are therefore important and an understanding of them would indicate under what environmental conditions germination is likely to occur. It is generally accepted that the individual fungal spore is capable of causing infection. (Garrett 1960). Studies were therefore undertaken to observe how environmental conditions affected the germination of individual conidia of S. botryosum.

A study of literature relevant to the effect of environmental factors on spore germination revealed that temperature and relative humidity were the two most important factors involved. A series of experiments were conducted to determine the effect of these two factors on the germination of S. botryosum conidia. However, some consideration has been given to the effect of other environmental factors, such as sunlight and frost.

(a) The Effect of Temperature on Spore Germination.

In reviewing literature relating to spore germination it quickly became apparent that there was considerable variation in details of the methods used by various workers, dependent apparently on the fungus species being studied. Further there was divergence of opinion as to when in fact a spore had begun germination. A series of preliminary experiments were therefore conducted with

the object of developing a method most satisfactory for studying the germination of spores of S. botryosum.

(i) The Criteria for Measurement of Spore Germination.

The first process of spore germination is observed when the spore imbibes water and starts to swell (Gottlieb 1950, Hawker 1950). This process is not readily measured when large samples of spores are to be observed.

Gottlieb (1950) cites other criteria used to estimate spore germination. He considers the percentage of spores which produce germ tubes is the criteria most commonly used. This was readily observable and in the early studies was assumed to have occurred when the sides of the developing protrudence became parallel. (Bollard 1959). This however did not give a true measure of spore germination at higher temperatures since above 35°C spores produced germ tubes which failed to continue growth. In the later experiments with higher temperatures and also when studying the effects of relative humidity, germination was considered to have occurred when the length of germ tube equalled the average diameter of the spore. The criteria used is stated in each instance.

Bonner (1948) suggested that because wide differences occur in the percentage and rate of spore germination it is more important to know when the first spores germinate, since these spores could initiate infection. In his studies the time required for at least ten spores to germinate in the sample was recorded. The importance of Bonner's observation was recognised

and times are given here for the germination of definite percentages of spores. Where the percentages to be recorded did not occur at an observation period, the time required for a definite percentage of spores to germinate was extrapolated by means of a graph.

The effect of temperature was measured by observing the percentage germination of spores. In most cases approximately 100 spores were observed on each slide and there were three petri dishes with inoculated P. D. A. slides to each treatment,(see page 96) except in a few instances where incubator space prevented this number of replications. Using this method, spores taken from 10-14 day old colonies during the course of these studies, showed remarkably little variation in germination percentage or time.

(ii) The Suitability of Various Substrates.

Laboratory studies on spore germination will provide information of practical value only if such studies are conducted under circumstances likely to be encountered in the field. A prime requirement in this investigation therefore was to locate a substrate on which spore germination would occur in a manner similar to that occurring on lettuce plants in the field. The following types of substrate were compared:

1. Drops of sterile distilled water on a microscope slide.
2. Drops of tap water on a microscope slide.
3. Drops of sterile distilled water as hanging drop slides.
4. A thin film of P. D. A. on microscope slides.

5. Two centimetre squares cut from the outer leaves of six week old lettuce plants, set out in microscope slides.

In each series the inoculum used was a spore suspension containing approximately 3,000 spores per. ml, prepared from 14 day old colonies grown on P. D. A. at 24°C.

All slides were placed in petri dishes lined with moist filter paper and incubated for 3 hours at 24°C. Counts were then made of the spores which had germinated. Approximately 100 spores were observed in five or six random low power fields. Four replications of each substrate were used. Observations on the length of the germ tubes in relation to spore diameter were also made.

Results are tabulated in Table 5 .

Conidia of S. botryosum, germinated readily at 24°C. in sterile distilled water. No external nutrients appeared to be necessary and water as a medium appeared to satisfy the requirements of the spores. However, it was very difficult to shift the slides with water drops containing spores without altering the position of the spores on the slide. If the drops ran, spores were dispersed over a wide area and observations became difficult. Hanging drops appeared to overcome this disadvantage, but the spores tended to collect in a group at the bottom of the drop and accurate observation became impossible.

The thin film of P. D. A. on the slide "fixed" the spores in

position and closely approximated germination observed on the lettuce leaf sections. P. D. A. stimulated the growth of the germ tubes slightly more than lettuce leaves but had no effect on the percentage of the spores which germinated. The other media had comparable germination percentages also but growth rate on them was considerably less.

TABLE 5.

COMPARISON OF MEDIA FOR SPORE GERMINATION STUDIES

Media	% Germination; 3 Hours at 24°C. (Averages from 4 ovservations) Average ± S.E.	Average length of Germ Tube (Spore Diameters)
Water:		
Hanging Drop (Sterile Distilled)	85% ± 9.7%	x 1 - 1.5
Sterile Distilled (on glass)	76% ± 2.9%	x 0.5 - 1.5
Tap water (on glass)	76% ± 6.1%	x 1 - 1.5
Sterile Distilled (on lettuce leaf)	82% ± 2.5%	x 1 - 2.5
Sterile Distilled (on Potato Dextrose Agar)	88% ± 8.3%	x 1 - 3.0
Note: Germination criteria; when the walls of the germ tube became parallel.		

While observations of germination made on lettuce leaves would closely approximate field conditions, this method proved impractical on account of the opaqueness of the leaf sections causing difficulty in locating and observing the germinating spores. This method also suffered from the disadvantage that the water drops containing the spores became dispersed over a wide area when the preparations were moved.

In view of the above results the method using P. D. A. films on microscope slides was considered the most practical, while at the same time it gave results very similar to those obtained on the host plant material. Nutman and Roberts (1960) when studying the percentage and rate of germination and the cardinal temperatures for spore germination of Collectotrichum coffeanum, Noak. in water, on P.D.A. and host leaves, likewise concluded that the P. D. A. film method gave results very similar to those recorded from host material.

(iii) The pH of the Substrate and its Effect on Spore Germination.

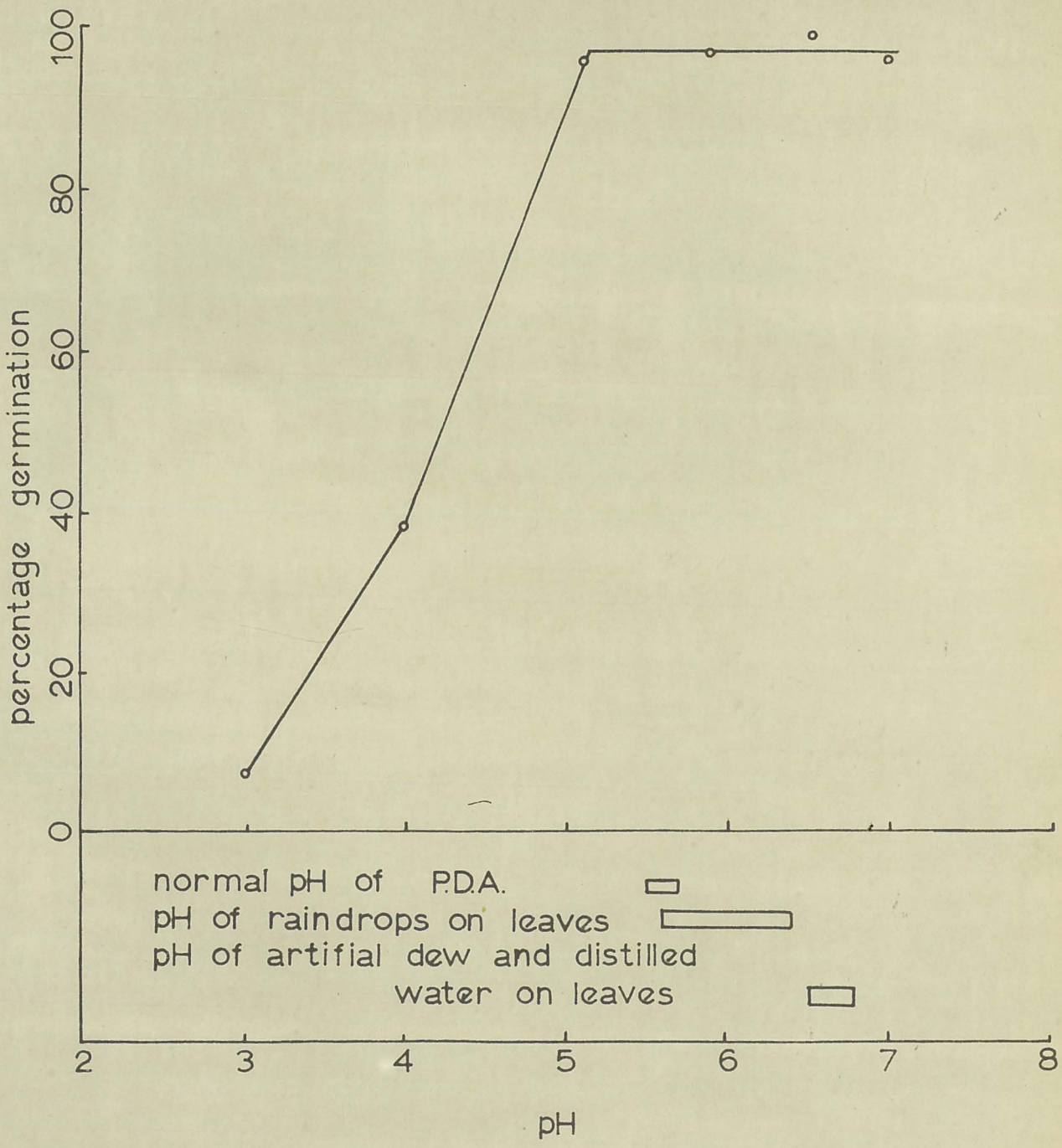
To ensure that the pH of the P. D. A. media, compared with that of the plant surface, would not be limiting factor observations were made of spore germination on P. D. A. at pH 3.0, 4.0, 5.1, 5.9, 6.5 and 7.0. The effect of the pH of this medium on spore germination is presented in graphical form (Figure VI). The normal pH of the P. D. A. used was 5.6. Dew collected from lettuce leaves and distilled water sprayed on the leaves had a pH of 6.5 - 6.8. Rain drops collected from lettuce leaves in the field varied from pH 5.6 - 6.4. These figures fall well within the range of pH values at which spores of S. botryosum readily germinate

(iv) Details of Method Finally adopted.

In view of the above results the following method using P. D. A. films on microscope slides was finally adopted.

Well rinsed microscope slides measuring 25 x 75 m.m. were

FIGURE VI EFFECT OF P.D.A. pH ON SPORE GERMINATION.



placed on a 90 mm. filter paper lining the bottom of a sterile petri dish. By means of a sterile 1 ml. pippette a small amount of molten P. D. A. was run along the slide. This spread out evenly over the surface of the slide forming a uniform layer of agar suitable for microscopic observation. Finally, using a 25 ml. pippette sufficient sterile distilled water was added to the dish to saturate the filter paper beneath the slide. Usually 2 - 3 ml. of water were required. Doran (1919) considered that when studying any one physiological agent, other conditions should be as near their optimum as possible to prevent them from becoming limiting factors. For this reason the filter paper was at all times kept saturated to maintain 100% R. H. within the dish. At temperatures above 25°C it was found necessary to add distilled water every 24 hours to compensate for loss from the petri dish.

Slides prepared as described and placed inside a sterile petri dish were incubated at the required temperature for one hour prior to inoculation with a spore suspension, to enable their temperature to reach an equilibrium with that of the incubator.

Spore suspensions were prepared by adding about 10 ml. of sterile distilled water to approximately six petri dish cultures, scraping off the aerial mycelium with a sterile scalpel and adding this to a 250 ml. flask containing a dozen small glass beads. After shaking vigorously for a few minutes the mycelium suspension was filtered through two layers of cheese cloth. The filtrate, a spore suspension, was used immediately, or if more than a few minutes was to elapse after preparation of the suspension the

sterile distilled water used was chilled to 5°C in a refrigerator.

If the position of individual spores was to be recorded the lower right hand corner of the slide was marked and the reading of the vernier of the microscope stage noted.

Experimentation.

When conducting this experimental work on spore germination in relation to temperature it was not possible to observe all temperature series concurrently because of limitations of time and incubator space. The observations were divided into five parts as follows.

Part 1

Observations on spore germination at 5°, 10°, 15° and 17°C were made at four hourly intervals. Four replications were used, and the germination percentage of twenty spores was observed on each slide.

It was found that except at 5°C the four hourly observation period was too long. Further, because of the variation in germination of the small number of spores sampled it was apparent that a larger number of spores should be recorded.

Part II.

Observations were made in 10°, 15°, and 17°C incubators at intervals down to half an hour during the early stages of germination, and later at the intervals necessitated by the rate of spore germination. With these frequent observations it was not possible to use the same slides repeatedly because

the frequent exposure to room temperatures would alter the germination rate. Consequently, a set of slides were prepared for each observation and discarded after use.

Two 'Weeder' counters were used to record the number of germinated and ungerminated spores in approximately five to six random low power fields. In this way approximately one hundred spores were recorded from each slide, and the number germinated was expressed as a percentage of the total number of spores observed. Note. About 5 - 10% of the spores at each temperature were considerably slower germinating than the remainder. Therefore obeervations were discontinued after 90% of the spores had germinated.

Part III.

Observations were made at 25°, 30°, and 35°C every half hour, for four hours. Details of the method used are similar to Part II.

Part IV.

Observations were made at 40°, 45°and 50°C every half hour until four hours, and then at four hourly intervals until 24 hours. Slides incubated at 45° and 50°C were also observed at 48 and 96 hours.

When observations were completed the petri dishes containing the slides were placed in the 25°C incubator to observe the effect of exposure to the higher temperatures on growth at optimum temperatures.

Part V.

To ovserve the effect of high temperatures on spore longevity, suspensions containing 10,000 spores per ml. were

prepared using a sterile 1% dextrose solution. Reports by Nutman & Roberts (1960) suggest that at higher temperatures nutrients such as dextrose produce a similar effect on spore germination as the host plant viz. a marked increase in temperatures at which germination occurs. A pilot trial using distilled water, 1% dextrose and lettuce decoction confirmed this fact.

Water baths running at 30°, 35°, 40°, 50°, 60°, 70° and 80°C. contained test tubes with 9 ml. of sterile 1% Dextrose at equilibrium with the temperature of the baths. 1 ml. of the concentrate 10,000 spores/ml. spore suspension was placed in each test tube, there being three tubes per water bath. Immediately one ml. of suspension was removed and diluted with 9 ml. of sterile water. Similarly at ½ hour intervals up to 6 hours, one ml. samples were withdrawn and diluted with 9 ml. of sterile water. This dilute spore suspension contained 100 spores/ml, and one ml. was transferred from these tubes on to the surface of a P. D. A. plate. Thus Three plates were used per temperature for each observation.

The plates were incubated at 24°C. for 10-16 hours. Germination of the spores was observed visually and confirmed where necessary with a 50x binocular microscope.

Results.

Part I.

The percentage of conidia germinated out of 20 observed and the average percentage of conidia germinated are presented

in Appendix III and Figure VII . Observations were made at 5, 10, 15 and 17°C. at intervals of four hours.

Parts II - IV.

Records of the percentages and average percentage of spores germinating at the times recorded, covering temperatures from 25°C - 50°C. are presented in Appendix IV.

Results are extrapolated and summarised in Table 6 have also been recorded graphically in Figure VII.

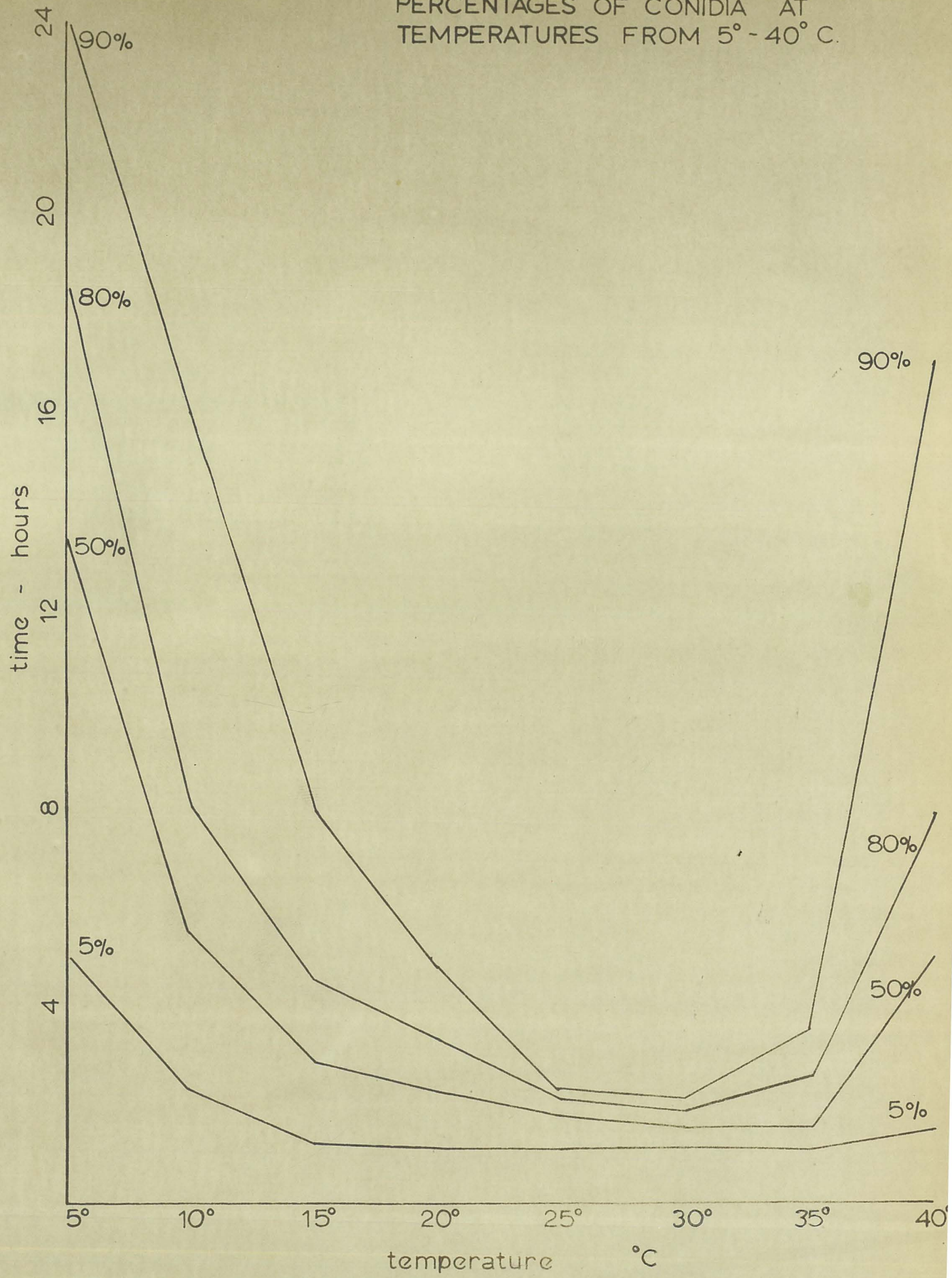
TABLE 6 Time (hours) required for Germination of 5, 10, 50, 80 and 90% of conidia of S. botryosum 100% RH.

Temperature		5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C
Average % Germination	5%	5.0	2.3	1.2	1.1	1.1	1.1	1.1	1.5
	10%	6.9	3.2	1.1	1.5	1.3	1.3	1.3	4.0
	50%	13.5	5.5	2.8	2.2	1.7	1.4	1.4	5.0
	80%	19.0	8.0	4.5	3.3	2.0	1.8	2.5	8.0
	90%	24.0	16.0	8.0	4.8	2.3	2.1	3.5	17.0

Between 35°C and 40°C a proportion of germinating spores produced germ tubes which failed to continue growing although observations were continued for 96 hours. After observation the slides were transferred to 25°C but growth of germ tubes failed to recommence on those slides originally held at 40°C and above for periods of 96 hours. About 2% of the spores originally held at 40°C for this period of time developed germ tubes twice as long as the spore diameter, but growth did not

FIGURE VII

RATE OF GERMINATION OF VARIOUS PERCENTAGES OF CONIDIA AT TEMPERATURES FROM 5° - 40° C.



recommence from these spores. At 45°C spores failed to recommence growth after 20-24 hours or more exposure. After 4 hours in the 50°C incubator all spores had lost their viability.

In defining the cardinal temperatures for conidial germination on P. D. A. slides at 100% relative humidity, the criteria used must be redefined. In this case germination is assumed to have occurred when the length of the germ tubes of the conidia equals or exceeds the diameter of the speres. Using this criteria the cardinal temperatures appear to be below 5°C, 25-35°C and between 35-40°C. The redefinition of germination does not alter the minimum and optimum temperatures but lowers the maximum temperatures by approximately 10°C, thus replacing a theoretical temperature with one at which germination occurs and continued growth is possible. This temperature is the important one in nature.

Part V.

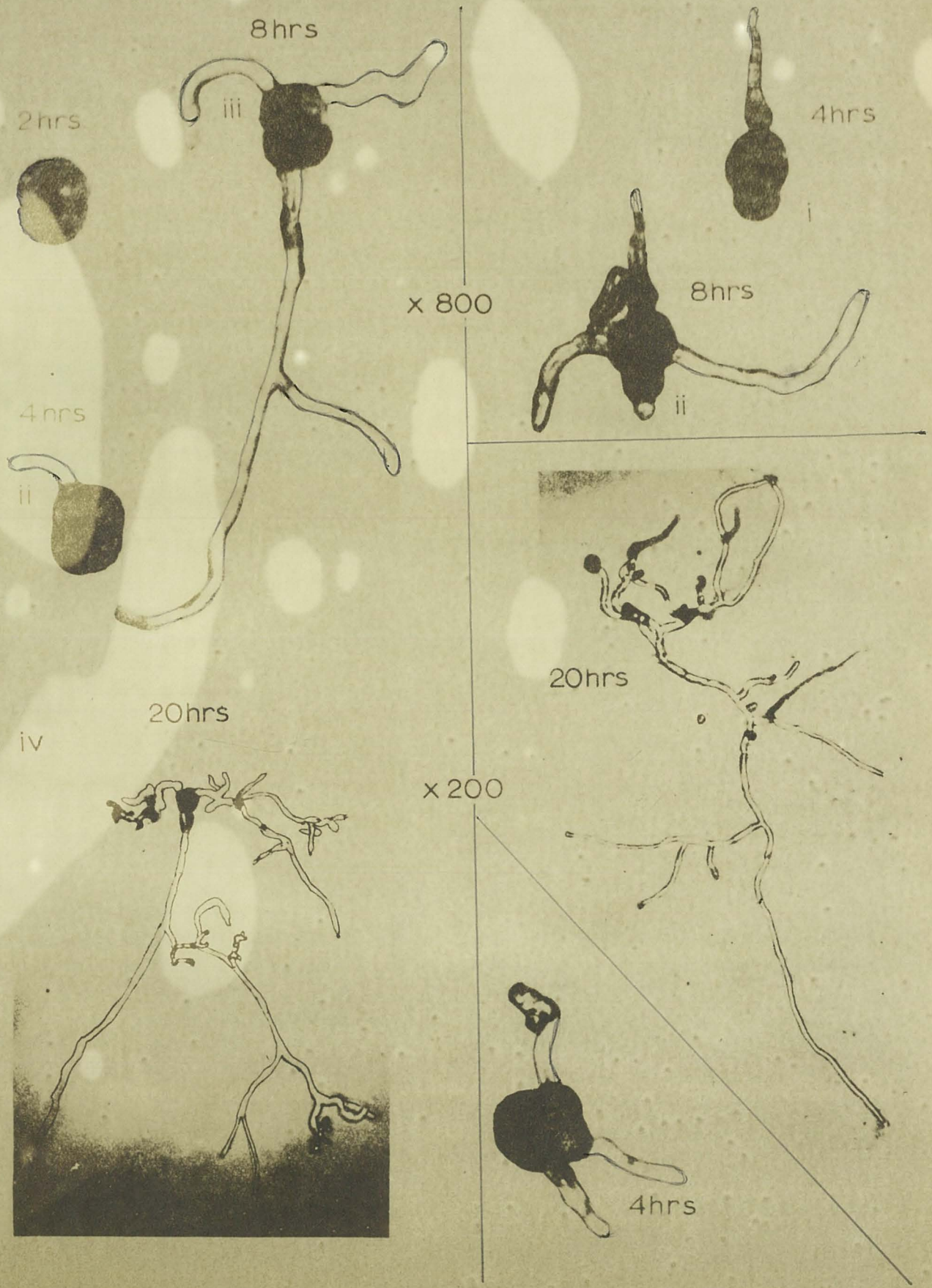
The Thermal death point for conidia of S.botryosum appeared to lie between ½ - 1 hour at 50°C. while at temperatures below 40°C. it was above 6 hours (see Table 7). Temperature and time combinations above 50°C. are not encountered in nature and were not investigated further. The effect of temperatures and spore viability below 50°C. has been reported elsewhere.

Table 7	Effect of high temperature on Viability of Conidia		
Temperature	°C 30° 35° 40° °F 86° 95° 104°	50° 122°	60° 70° 80° 140° 158° 176°
Thermal Death Point.	more than six hours.	Between ½-1 hour.	less than ½ an hour.

ILLUSTRATION 14

GERMINATINATION OF CONIDIA

24°C



ILLUSTRATIONS 15 & 16.

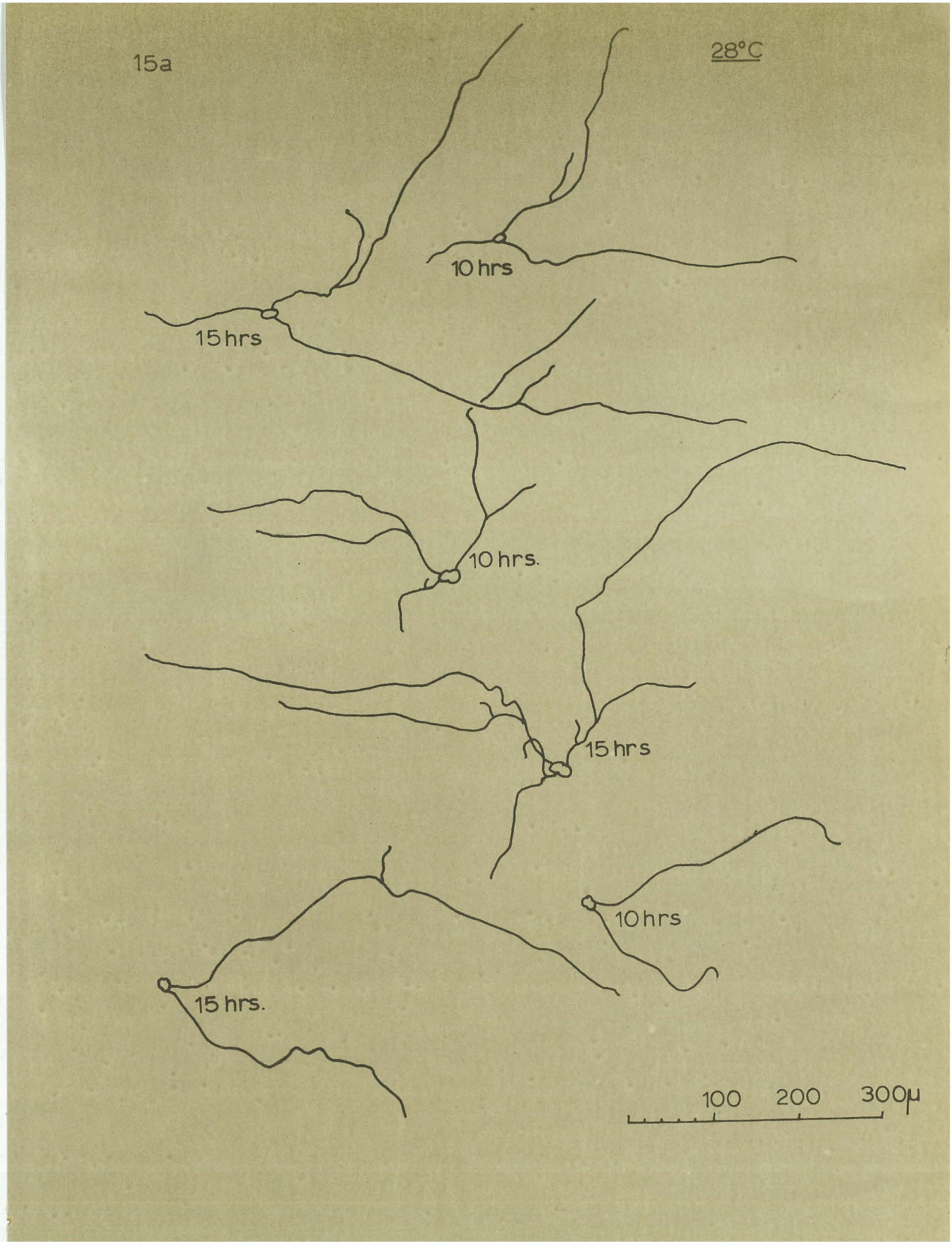
GROWTH OF YOUNG HYPHAE FROM GERMINATED CONIDIA

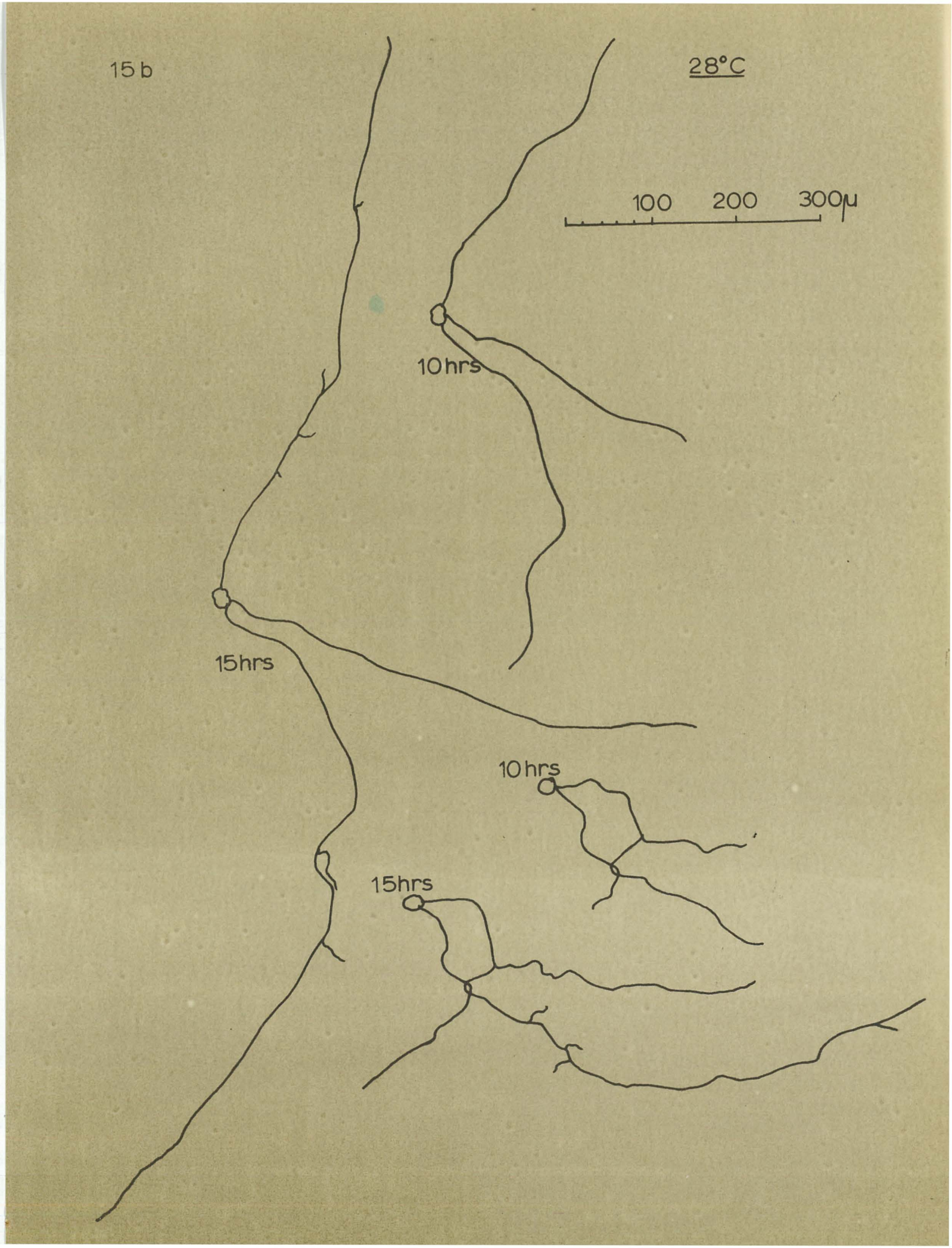
15a } 28°C observed after 10 hours
15b } and 15 hours incubation:

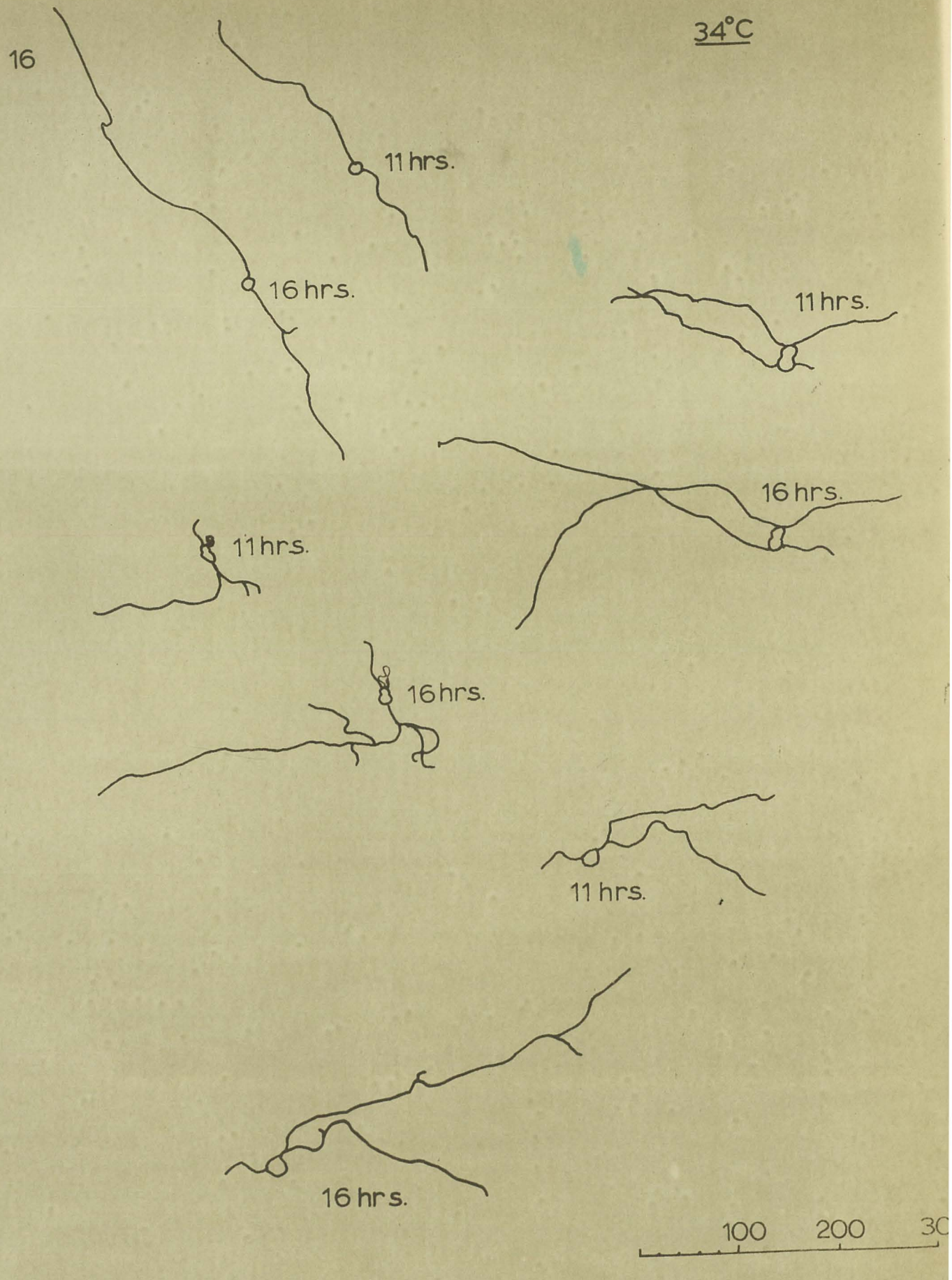
16 34°C observed after 11 hours and 16 hours incubation:

on P.D.A., relative humidity 100%

drawings made with the aid of camera lucida.


15a
28°C
10 hrs
15 hrs
10 hrs.
15 hrs
10 hrs
15 hrs.
100 200 300μ


15 b
28°C
100
200
300μ
10hrs
15hrs
10hrs
15hrs


34°C
16
11 hrs.
16 hrs.
11 hrs.
16 hrs.
11 hrs.
16 hrs.
11 hrs.
16 hrs.
100
200
30

(b) The Effect of Relative Humidity on Spore Germination.

Materials & Methods:

Several methods are available for maintaining an atmosphere within a closed vessel at a constant relative humidity. Wilson (1921), Balloway (1935), Bonner (1948) and Smith (1954) describe the use of various concentrations of acids and other solutes to maintain a known humidity. These concentrations are critical and are difficult to prepare, check and maintain. Spenser - Gregory and O'Rourke (1957) state that for convenience, only saturated salt solutions should be considered in order to obtain appropriate working ranges of humidities. O'Brien (1948) examined the literature exhaustively and has compiled a table of saturated salt colutions giving the relative humidities expected above them at various temperatures, with notes on their use and suitability for specific purposes.

From O'Brien's tables suitable saturated solutions were selected. The solubility of the salts were determined from the International Critical Tables (1927) and saturated solutions prepared by allowing about 10% excess salt in 50 ml. of distilled water. The solutions were made up in 600 ml. flasks which had previously been cleansed with saturated chromic acid followed by several rinses in tap water and a final rinse in distilled water. The flasks containing the predetermined amount of salt and distilled water were sealed with Parafilm* and put aside for 24 hours to ensure complete saturation of the solution.

Ideally spores used in these studies should not have been exposed to free moisture at any stage. Various methods as

* Parafilm - A plastic sealing film manufactured by A. Gallenkamp & Co. Ltd., London.

described in literature were followed whereby spores were collected and held above the various solutions without the use of water, but in each case they were found to be unsatisfactory. Bonner (1948) described the use of cellophane squares dipped in nutrient agar, on to which the spores from cultures would readily adhere. These squares were suspended in sealed bottles containing the appropriate solute. Bonner's method appeared simple, but when used the cellophane proved most difficult to handle at all stages. After three types of cellophane had been tried without any improvement in handling tests were made with other materials.

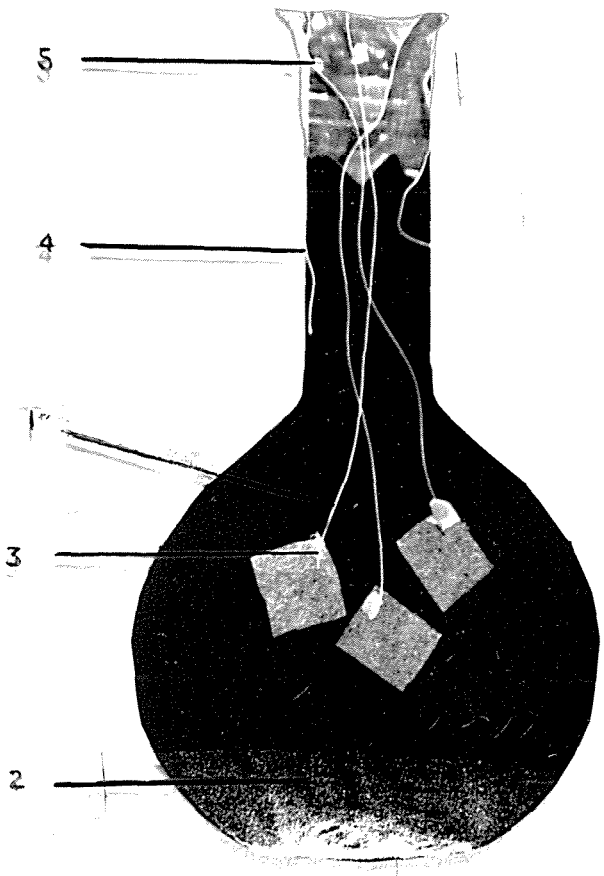
Finally, spores were suspended above the saturated salt solutions on 15 mm. squares of Lens Tissue.* A cotton threat about six inches long was attached to the bottom left hand corner of the square with a drop of parafin wax. To facilitate handling, three tissue squares were placed on a microscope slide and the wax drop fixing the cotton thread to the squares also fixed them on to the slides. When required the tissue squares were removed by passing the slides over a flame.

To ensure that the flasks had reached an equilibrium with the surrounding temperature they were placed in the appropriate incubator overnight.

Spores were collected on the tissue pieces by lightly brushing the surface of a sporulating culture holding the tissue

* Green's Lens Tissue No. B105

ILLUSTRATION 17. Method of Maintaining Constant Relative Humidities.



1. Cotton threads.
2. Saturated salt solutions.
3. Wax attaching Lens Tissue squares to cotton.
4. 'Cellotape' attaching cotton to neck of flask.
5. Flask sealed with 'Parafilm'.

square by the left hand corner, wax drop uppermost. These inoculated squares were suspended over the appropriate solution, three to a flask, by temporarily fixing the cotton to the neck of the flask with a small piece of "sellotape". The flask was sealed with "parafilm" and quickly returned to the incubator.

After twentyfour hours the lens tissue squares were removed from the flasks and the wax drop cut off with a pair of scissors. To record germination a tissue square was placed on a microscope slide with the cut off corner to the top left hand so that the side of the tissue square coated with spores would be uppermost. A drop of lactophenol fuschin was applied to the lens tissue and a cover slide dropped on and pressed into position to facilitate the expulsion of air bubbles. Slides were set aside for 15 minutes before recording to allow the germ tubes to become well stained. Meanwhile, the flasks required for further work were resealed with "parafilm".

By careful adjustment of the iris diaphram and condenser of the microscope a position was obtained where the matrix of the lens tissue, which stained only slightly became quite translucent and the dark spores with brightly stained germ tubes were readily observable.

The percentage of germinated spores was recorded in the first part of the trial by counting approximately one hundred spores on each tissue square, using two "Veeder" counters. In the second part an estimated one hundred or more spores were

observed and if germination had occurred this was recorded as positive, i.e. (+). This second recording acted as a check on the humidities at which germination had occurred.

Germination was considered to have taken place when the length of the germ tube equalled the mean diameter of the spore.

Germination was recorded at 25°, and 30°C. The saturated salt solutions used with their respective relative humidities are recorded in Appendix V .

Results

Observations after twenty four hours showed that spores were capable of germinating at relative humidities between 87 - 90%.

The numbers of germinating spores were reduced as the relative humidity decreased. Similarly there was an obvious reduction in the length of the germ tube or the amount of mycelium produced, as the relative humidity decreased. The results of these studies are recorded in Table 8 .

TABLE 8 EFFECT OF RELATIVE HUMIDITY ON SPORE GERMINATION

Temperature 30°C.	R.H.%	PART I*	PART II
	100	92%	+++
	98	84	+++
	96.6-97.4	67	+++
	94.9	75	+++
	92.9	10	+++
	90	41	+--
	87	0	---
	Below 87%	0	---
Temperature 25°C.			
	100	95	+++
	98	82	+++
	97	75	+++
	93	30	+++
	88	0	---
	Below 88%	0	---

* Percentage of spores germinating. Criteria: Germ tubes equalled or exceeded average spore diameter.

(c) The Effect of Miscellaneous Factors on Spore Germination.

To ensure that conidia are capable of remaining viable after exposure to the extremes which are encountered in nature during spore production and dispersal, the effect of sunlight and frost on spore germination were measured.

(i) Sunlight

During February six uncovered plates of ten day old sporulating colonies growing on P. D. A. were placed on the outer ledge of a window facing North. In the position chosen the colonies received approximately seven hours of direct sunlight during the first day, nine hours of darkness then eight hours of daylight, the latter three hours of which was again direct sunlight. The days were clear with temperatures above 70°F.

Control plates were incubated at 24°C.

After the above exposure a spore suspension was prepared and the percentage of germinated spores estimated using the agar slide technique.

(ii) Frost.

Two sporulating cultures in covered petri plates were placed in the freezing compartment of a refrigerator for twenty four hours. The temperature inside the cabinet was between -10° and -12°C. Thick glass plates were used so that cooling and reheating of the cultures would be gradual. Controls were incubated at 24°C.

After twenty four hours in the freezing compartment the petri plates were removed and left at room temperature for four hours. The agar slide technique was used to estimate spore germination.

Results

(i) Sunlight.

Exposure to the atmosphere for twenty four hours, including strong direct summer sun for a total of ten hours had no effect on spore germination. (Table 9). It is also interesting to note that S. botryosum spores germinated readily amongst spores of other contaminants which were collected by the uncovered plates. Most of the aerial contaminants with sizeable spores were species of Alternaria. Numerous smaller spored and unidentified contaminants were also present.

(ii) Frost.

No difference in the average percentage of spores germinating after exposure to the low temperatures were apparent. (Table 9).

TABLE 9 AVERAGE PERCENTAGE OF SPORES GERMINATING AFTER EXPOSURE TO EXTREMES OF LIGHT AND LOW TEMPERATURE

Treatment	Results
(i) Sunlight	95.7% (Average for six samples)
(ii) Frost	94.5% (" " four ")
(iii) Control	98.9% (" " six ")

Discussion

The germination requirements of conidia of S. botryosum have been considered in the main since, as will be seen from later studies (Page 159), ascospores appear to be unimportant in the epidemiology of this fungus on lettuce. Conidia appear to be responsible for the establishment and spread of this disease in the field, and for the large number of infections which preceed an epidemic. Ascospores undoubtedly do in some cases perpetuate the disease from one year to another or cause infections in the field. However they do not entitiate disease of an epidemic proportion. Because of the seasonal nature of this disease on lettuce, temperature appears to be a major factor involved and it has therefore received particular consideration. The minimum temperature at which germination of conidia of S. botryosum occurred was below 5°C. The optimum temperatures for maximum spore germination occurred between 25°C. and 31°C. However, if consideration is given to the germination of the first 50% of the conidia, the optimum lies between 24°C - 36°C. Again, if the first 5% of the spores to germinate are considered, the optimum range for germination is very wide and lies between 15°C and 36°C. Each temperature range has been measured by adding 20 minutes to the observed minimum germination time for the appropriate percentage, (See Fig. VII) and includes all temperatures at which germination occurred within this period.

The maximum temperature at which germination and continued growth of the germ tube occurred was between 35° - 40°C. Above

35° - 40°C. conidial germination was inhibited.

The cardinal temperatures for conidia of S. botryosum fall within the range of Ascomycete examples selected from literature by Hawker (1950) but are slightly higher than examples which she selected from the Fungi Imperfecti. Padhi & Snyder (1954) report that spore germination of S. botryosum f. lactucum occurred from 4°C - 37°C, beginning after 3 hours between 22°C - 25°C., but was delayed increasingly by lower and higher temperatures. The range of temperatures at which they observed germination is similar to the range at which conidia of N. A. isolates germinated. Padhi and Snyder do not, however, state what criteria was used to determine when germination occurred. In this study germ tubes had appeared after three hours on the majority of germinating conidia between 24°C - 34°C, a temperature somewhat above that recorded by Padhi & Snyder.

Hawker (1950) considered that temperatures which are lethal to spores are much higher than those inhibiting germination. However, during spore germination studies at higher temperatures few conidia resumed growth after being held at 40°C for a period of 96 hours or more. This is only 5°C above the optimum temperature range. Similarly 24 hours exposure to 45°C proved lethal to the majority of germinating conidia despite their production of germ tubes. Subjecting conidia to 50°C. in a 1% glucose solution for one hour was lethal. At 60°C. and above, exposures of less than half an hour proved lethal to the

conidia.

It is interesting to note that at temperatures between 35°C - 50°C conidia produced protrudences often with parallel sides, and these spores were thus recorded as having germinated. However, later examinations revealed that these germ tubes rarely grew to equal the diameter of the spore unless they were removed to more favourable conditions before the lethal effect of the high temperature occurred. This may have been due to the high temperatures favouring the metabolic processes of germination, but also blocking or slowly destroying some essential enzyme system concerned with continued development of the germ tube. The effect of temperature on the balance of these two systems may determine how the higher temperatures are to affect the germinating spore.

Low temperatures appear to have little effect on the viability of conidia of S. botryosum. Hawker (1950) stated that low temperatures are rarely lethal, but merely slow down the metabolic reaction rate of the spores.

The Study of germination requirements of conidia and the rate of germ tube elongation indicated that these processes closely followed or paralleled the effect of temperatures on colony growth. Similar relationships for other fungi have been recorded. (Crossan 1954, Mutman & Roberts 1960).

The effect of temperature on germination of ascospores

was not studied in detail because of its relative unimportance. Ascospores germinated readily between 15°C and 25°C, but no determination of cardinal temperatures was made.

The humidity requirements of germinating conidia are also of considerable importance. Conidia germinated readily in water vapour as observed by Padhi & Snyder (1954). However, germination of conidia of the N.Z. isolates was recorded at relative humidities at and above 90%. Padhi & Snyder report that ascospores and conidia required humidities above 95% to support spore germination. An increase in humidity up to 100% increased the number of spores which germinated in a given time, the rate of growth, and final length of the germ tube. The germination percentage of spores in water vapour was similar to that occurring in free water, but the time required for the same stage of spore germination to be reached was longer. These findings are in general agreement with results reported by Gottlieb (1950).

Conidia of S. botryosum isolated from lettuce germinated readily once removed from the conidiophore. While attached to the conidiophore, and even when dislodged and resting on the P. D. A. of the parent colony conidia failed to germinate even though other requirements for spore germination appeared to be fulfilled. There was however no evidence of dormancy once conidia were removed from the culture on which they were produced with a volume of sterile water or nutrient solution. (Conidia of S. botryosum isolated from onion however germinated readily,

producing short nodular septate germ tubes, while still attached to the conidiopheres). Dormancy of conidia has not been recorded by Neergaard (1945) or other authors cited working with S. botryosum (e.g. Olgilvie & Milligan 1935, Dippenaar 1938, Petzer 1958, Padhi & Snyder 1954, McKay 1959).

Ascospores obtained by crushing the perithecia often failed to germinate, but because septation of some spores still within the asci was incomplete these were considered immature. When ascospores were obtained from the material exuding from the ostiole of the perithecium, or from crushed perithecia which were exuding spores, germination occurred readily once the spores were removed from the vicinity of the perithecia in a suitable quantity of water. Spores remaining in close association with the perithecium or its contents usually failed to germinate. These results are contrary to those reported by McKay (1959) who observed germination of ascospores of S. botryosum isolated from onion even while they were still inside the perithecia.

Shear & Dodge (1927) and Ryan (1948, 1949) report that ascospores of fungi whose conidia germinate readily, often remain inactive for long periods. It is unknown if the conditions which initiate ascospore discharge also are responsible for breaking dormancy of ascospores, or whether ascospores once mature germinate when given a suitable environment.

Perithecia containing asci were held at room temperatures for periods of up to 3 months. Providing desication was

prevented in this period germination of the ascospores occurred when they were removed from the perithecium and placed in suitable conditions.

The age of the conidia of S. botryosum had a considerable effect on their rate of germination. Conidia on colonies 21 days old required about twice as long to germinate as those from 10-14 day old colonies. The spores were usually very heavily pigmented at this age and the delay in germination may have resulted from the effect of the secondary thickening of the spore wall as reported by Neergaard (1945).

Conidia present on 9 month old colonies germinated with a considerable but highly variable reduction in the percentage of conidia compared with germination of conidia from young cultures. Studies on the longevity of conidia were discontinued after mite contaminated the cultures. However, after 14 months a very small percentage of spores from the contaminated cultures appeared to be still viable. Padhi & Snyder record that approx. 10% of the conidia from 12 month old colonies were viable.

Light appeared to be unnecessary for spore germination since all observations were carried out in unlit incubators. Gottlieb (1950) states that if any generalisation can be made, it is that the germination of spores is not materially altered by diffuse or low light intensities.

2. Penetration and Infection

Penetration has been used in these studies to include the method by which the fungus enters the host tissue but does not include subsequent development within the host. (Flentje 1959). Successful penetration does not necessarily constitute infection (Johnson 1932). It is generally assumed that fungi are capable of penetrating a wide range of plants, but are unable to establish pathogenic relationships in every case (Wolf & Wolf 1947). Similarly, penetration of a potential host does not always constitute successful infection. (Johnson 1932).

Infection is said to have occurred when the fungus becomes established within the host following penetration and is independent of external food reserved for continued development (Walker 1950).

Because these processes are continuous, it may be impossible to determine when penetration has been completed and infection occurred. When observing the host-parasite relationship there was no method available to distinguish between these two stages because the rate at which germination and penetration occurred was highly variable within the spore samples used.

In this study therefore, it was necessary firstly to determine by what method host penetration occurred, and secondly to observe the effect of environmental factors on penetration and infection.

(a) The Method of Penetration of the Host

From literature on methods of penetration by germinating spores of pathogenic fungi reported by various authors, it was apparent that a pathogen may enter a potential host by one or more of the following methods:

(a) by way of the intact surface of the host, commonly referred to as direct penetration,

(b) by way of wounds,

(c) by way of natural openings.

To determine what type of penetration was involved in the disease cycle of S. botryosum on lettuce, a large number of spores were observed germinating on lettuce leaves under conditions favourable for infection.

Materials and Method

Leaves of lettuce plants, previously placed in a high humidity cabinet for a period of twelve to eighteen hours were marked with an "L" in several places. A "Biro" type pen proved most satisfactory for this purpose. Different coloured inks were used when marking upper and lower leaf surfaces.

A spore suspension containing 10,000 spores per ml. was prepared from ten day old sporulating colonies, and drops of this suspension were pippetted on to the areas indicated by the "L" ink marks.

The inoculated plants were then returned to the high humidity cabinets for periods, 8, 16, 24, 36, 48, 60 and 72 hours, calculated from the time the spore suspension was prepared.

After the appropriate time interval the plants were removed and the marked leaves detached and dropped into labelled beakers containing 70% ethyl alcohol.

In the laboratory leaves were removed from the alcohol and, using a $\frac{3}{4}$" cork borer, discs were cut out in positions indicated by the ink marks. These discs were then cleared and stained using one of the methods described.

Nelson (1955) described a technique for staining fungi during the penetration phase. This method was followed in the early observations. Discs were cleared overnight in a mixture of 3 parts 95% ethyl alcohol and 2 parts glacial acetic acid. The cleared discs were then stained for 24 hours in 3% methylene blue, followed by 48 hours in a 2:1 mixture of lactophenol and methylene blue. Finally they were cleared fixed and mounted in lactophenol. This staining procedure produced satisfactory preparations but was time consuming. Since few spores were found still adhering to the regions of inoculation it was considered to be too severe on the delicate germ tubes and infection pegs.

A method was adopted where the number of processes involved were reduced and the materials used were less severe in action. The discs were cleared in 70% ethyl alcohol warmed on a sand bath for 2 hours. After cooling they were stained for approximately half an hour in 3% methylene blue, until the desired contrast between leaf tissue, fungal spores and hyphae resulted. More spores were retained using this method, which also permitted a

more rapid assessment of penetration.

The leaf surface was examined under the low power objective of the microscope to locate penetrating spores which were then examined under higher power. Penetration was confirmed by rocking the cover-slips violently with a mounted needle. If penetration had occurred the spore remained attached and the site of the infection peg was generally revealed.

Numbers of spores successfully penetrating the host and the type of penetration was recorded. In the case of stomatal penetration hyphae appeared to free themselves more readily from the cells during the early stages of penetration. If it appeared that the fungal germ tube actually had commenced stomatal penetration but had broken free due to the treatment described above, its penetration was recorded as "uncertain". Selected spores were drawn with the aid of a camera lucida.

Several attempts to section leaf material failed to reveal details of penetration.

Results

Sixty-nine spores were recorded penetrating the leaves in several dozen discs examined, and twenty spore were classified as uncertain. Results of the methods of penetration recorded are set out in Table 10 .

Table 10 The Method of Host Penetration

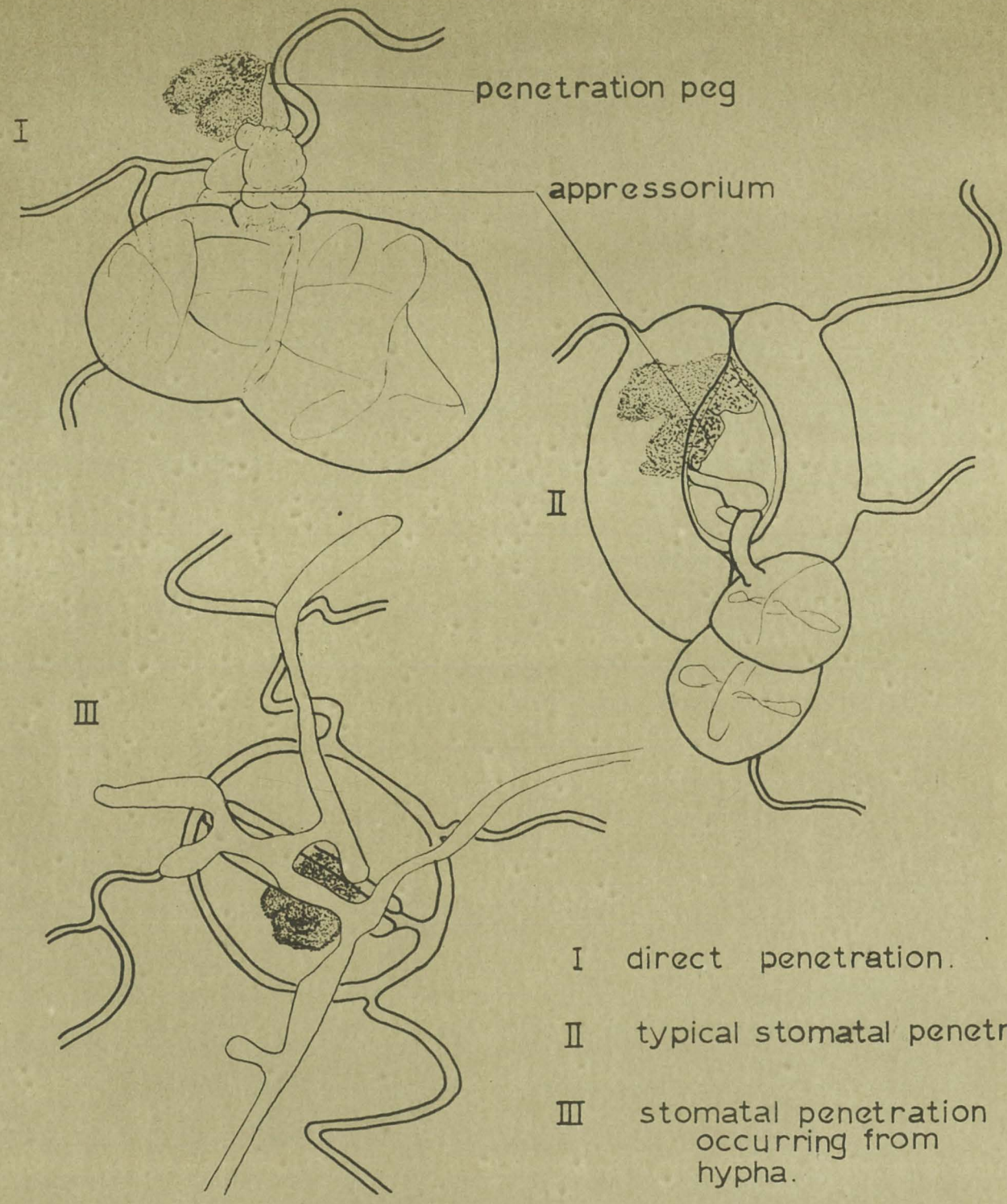
Observation, Hours	8	16	24	36	48	60	72	Totals*
Direct Penetration	3	5	1	4	4	1	5	23
Stomatal Penetration:								
Certain	2	8	7	1	5	9	14	46
Uncertain	5	0	1	3	2	4	5	20
Totals:	10	13	9	8	11	14	24	89

*Note: It must be emphasised that not all points where penetration occurred have been recorded in this table. Only those where the method of penetration was visible have been recorded. During the later half of the periods at which observations were made many lesions were present but at this stage it was impossible to determine the method of penetration.

Description of the Method of Penetration

Following germination of conidia, several hyphae developed from different cells of the spore and spread apparently in any direction over the leaf surface. The majority of conidia failed to penetrate the host. A very small percentage of conidia produced germ tubes which did not ramify over the leaf surface but became slightly thickened, producing an appressorium varying but little in morphological characteristics from the hyphae. Direct penetration of the cuticle and epidermal cells occurred when a slender penetration peg pierced the cuticle and entered the epidermal cell generally close to a vertical cell wall in the region of dense cytoplasm. Stomatal penetration was accomplished by hyphae which, soon after formation had grown directly towards a stomatal pore. They became swollen on the tip once they had entered the stomatal cavity. Entry was not

ILLUSTRATION 18 HOST PENETRATION



I direct penetration.

II typical stomatal penetration.

III stomatal penetration occurring from hypha.

0 50μ.

proceeded by the formation of an appressorium on the surface of the epidermis, but within the stomatal cavity the swollen hyphal tip subsequently developed into a convoluted body considered to be an appressorium. The wall of this body remained thin and ill defined compared with the appresoria which formed on the surface of the leaf. Fungal development continued inside the cells adjacent to the stomatal cavity but the method of penetration was not discernible. Inside the cells the fungus produced a thick, ill-defined body which later became obscured due to the disorganised cellular contents becoming densely stained.(See Illustration 18)

Disorganisation of the penetrated cells occurred during both direct and indirect penetration. Adjacent cells became disorganised apparently before the fungus had penetrated them. However, because fine fungal threads may not have been detected by the staining techniques used, the possibility of prior penetration occurring must not be excluded.

(b) <u>The Effect of Environment on Penetration and Infection</u>

(i) Temperature and Relative Humidity:

Temperature and humidity may be expected to have an important influence on the rate of penetration and the degree of resulting infection. During investigations on the effect of temperature on penetration and infection it was apparent the humidity about the plant after penetration also influences infection.

<u>Materials and Methods</u>

Spore germination occurs for a small percentage of spores

after 1½ hours at favourable temperatures. It is important that these first spores germinating are considered because they will initiate infection under marginal conditions. To measure the effect of temperature on penetration and infection, the conditions occurring during spore germination must be identical for each temperature used during penetration and infection studies. Consequently, a spore suspension containing approximately 3000 spores per ml. was prepared using a 1% solution of glucose to simulate conditions which occur naturally on host plants (Nutman & Roberts 1960, also p. 96). This spore suspension was incubated for 1½ hours at 25°C by which time germination of 5-10% of the spores had occurred.

At this stage the suspension was sprayed on to lettuce plants which were immediately covered with plastic bags and placed in artificially illuminated cabinets running at 40°, 50°, 60° and 70°F.

The effect of temperature on penetration and infection was observed by removing the plastic bags from the plants at various intervals and drying them in front of a fan. This prevented further penetration occurring. If penetration and infection had been successful lesions would later occur and these were recorded after a period of 10 days.

It was assumed that penetration of the first spores would occur after two hours or more at favourable temperatures, timed from the entry of the plant into the cabinets. However, it soon

became apparent that infection could occur after short periods even at the lower temperatures. Three experiments were conducted on different lines of plants. Larger plant numbers were used for the first of these experiments but due to limitations of space and the advantage of rapid handling, the numbers of plants in the last two experiments were reduced to two plants per observation for each temperature.

Cabinets were provided operating at each temperature, and all treatments were arranged at random during inoculation and recording.

Results:

Results are summarised in Table 11.

Penetration and infection occurred after 6 - 8 hours at 40°F, 4 hours at 50°F, 2 - 4 hours at 60°F. and after 2 hours at 70°F. Favourable temperatures for penetration and infection therefore must lie about 70°F. Times refer to the period from when the first 5 - 10% of the spores had germinated until infection occurred.

The effect of relative humidity was demonstrated in the difference between infection resulting from the first two and last experiment. The fans normally operated for 20 minutes by a time clock were allowed to run for the full two hours between observations in the last experiment. Resulting infection was less than the former experiments, despite standardised conditions.

Table 11 Infections Occurring on Plants inoculated and exposed to various temperatures, at 100% RH.

Temperature		40°F	50°F	60°F	70°F	
Expt. I						
Time	0	Nil	Nil	Nil	Nil	Totals from
Hours from	2	N.R*	Nil	28	11	16 plants
arbitrary	4	N.R.	40	214	33	
germination	6	N.R.	N.R.	192	109	
(2 hours from	8	N.R.	258	447	142	Fans: 20 mins.
preparation of	12	337	429	287	124	
suspension of	16	269	559	-	-	
conidia)	24	536	628	494	368	
	32	629	-	-	-	
Controls		0	0	0	0	
Expt. II						
Time	0	Nil	Nil	Nil	Nil	Totals from
(Hours)	2	Nil	2	Nil	126	2 plants
	4	Nil	Nil	155	126	
	6	Nil	102	-	126	
	8	200	102	155	126	Fans: 20 mins.
	12	200	102	155	126	
Controls	0	0	0	0	0	
Expt. III						
Time	0	Nil	Nil	Nil	Nil	Totals from
(Hours)	2	0	0	0	13	2 plants
	4	0	74	58	39	
	6	2	89	56	47	
	8	13	99	86	76	Fans: 2 hrs.
	10	10	120	75	81	
	12	28	106	78	84	
Controls	0	0	0	0	0	

* N.R. - Not recorded

It was concluded that the constant air movement reduced the humidity about the plant and lowered the number of spores successfully infecting the plant. At a later stage ten plants were inoculated with a spore suspension containing 3000 spores per ml. and placed in a humibity cabinet for two hours. Five plants were placed under a fan for 20 minutes until they were dry, while the remaining five were left in position for a full two hours. The treated plants were arranged in random order on the glasshouse bench. Ten days later it was possible to identify the five plants which had received the twenty minute exposure to the fan from this group of plants. They were severly diseased, while those receiving the longer exposure had comparatively few lesions. The penetrating spore must therefore still be dependent to some extent on a favourable humidity during the early stages of penetration and infection.

(ii) Light

Light is an important factor to be considered in host parasite reactions (Wolf & Wolf 1947). Since light governs the matabolic behaviour of the higher plants and controls the stimuli which are responsible for the opening and closing of stomata, and many other processes, its effect on the relationship of the parasite to the host may be considerable. (Yarwood 1959.) Light itself may also have a direct effect on the fungus (Wolf & Wolf 1947, Diener 1955, Yarwood 1959).

An attempt was made therefore to study the effect of light

on host penetration and infection. Investigations were made firstly to determine if exposure to light prior to inoculation would predispose the plant to penetration or infection, or if light after inoculation increased or decreased the resulting infection. This was followed with a more critical trial measuring the effect of light after inoculation.

Materials & Methods

In the first part of this study plants were exposed to twelve hourly periods of light (L) and dark (D) as follows;

Treatment (a) L prior to inoculation, followed by D
(b) D prior " " " " L
(c) D prior " " " " D
(d) L prior " " " " L

During these periods 100% relative humidity was maintained by placing the plants over trays filled with water and covering them with plastic sheeting.

There were four plants per plot, and four replications of the four treatments arranged in randomised blocks. Inoculation was carried out in these randomised blocks arranged on a bench under low light intensities. This operation occupied twenty minutes from the time the plants were assembled until they were returned to the appropriate light or dark treatment.

Illumination was provided by four 150 watt flood lamps set on stands, with one pair directed down on to the plants from a height of three feet, while the other pair were set at an angle of 45° and were six feet from the centre of the blocks of plants. This arrangement of light was checked

with a photo-electric exposure meter and the position of the lamps altered until an even distribution of light resulted. Air circulation was provided by two electric fans which directed air towards the plastic sheeting covering the plants.

The exposure to the dark period was accomplished by placing the plants in a cabinet covered with black plastic. During the day this cabinet, being near a window, was indirectly heated by the sun. Temperatures approximated those recorded from the illuminated plants under the clear plastic sheeting. Unfortunately, at night this cabinet ran from 10-14° below that recorded from the plants receiving the light treatment. Temperatures varied from 56°F to 70°F. between these two treatments at the maximum stage of divergence. Consideration must be given to this when results are interpreted because the lower temperatures recorded during the night from the dark treatment, which occurred during the post-inoculation stage, would introduce a bias to the results.

In later trials, attempts were made to eliminate the variable introduced with different temperatures between light and dark treatments. After several unsuccessful attempts to provide artificial illumination for one group of plants and maintain the other group at a similar controlled temperature in the dark, a method utilizing larger units and natural light was devised. The first trial indicated that it was unlikely that predisposition to penetration and infection was involved. Therefore considerationwwas only given to the post-inoculation treatments.

A trial was designed using a randomised block layout, there being two treatments and five replications with four plants per plot.

A large wooden box 4' x 3' x 2' was placed in the glasshouse and fitted with a series of light traps made from galvanised iron trays and cardboard. Prior to sunrise the plants were inoculated in a predetermined randomised block with a spore suspension containing 3000 spores per ml. Those plants which were to remain in the dark were covered by the box with its system of light traps, and those plants remaining in the light were removed to a predetermined position where temperature fluctuations were minimised. The temperatures inside the plastic which covered both groups of plants were checked at hourly intervals, or closer if necessary, and temperature differences between treatments exceeding 2^{o}F. were promptly corrected.

At 12 hours from sunrise all plants were removed, arranged again in the random blocks and dried with an electric fan.

The effect of light on the penetration and infection was assessed by recording the number of lesions which were present after ten days.

Results:

Preliminary trial results are presented in Appendix VI and are summarised in Table 12 . (Explanation of abbreviations given in Materials and Methods, p 126)

The Effect of Light on Penetration and Infection

Table 12	Number of lesions per plot of four plants				
	Tmt. (a) L + D	Tmt. (b) D + L	Tmt. (c) D + D	Tmt. (d) L + L	Totals
Rep. I	69	446	204	309	1028
II	177	432	346	474	1429
III	28	506	238	562	1334
IV	195	264	216	560	1235
Totals:	469	1648	1004	1905	5026
Av.lesions per plant.	29.3	103.0	64.8	119.0	
Results F. test	N.S.	N.S.	N.S.	N. S.	

(Explanation of abbreviations given in Materials and Methods, P 126)

Table 12a		
Treatment	Av. lesions per plant	F. test
(a) + (d) Light prior to inoculation	66.6	N.S.
(b) + (c) Dark prior to inoculation	91.1	N.S.

By combining the results of Treatments (a) + (d) and comparing these with treatments (b) + (c) some measure of the predisposing effect of light on the plant is obtained (See Table 12a).

If Treatments (a) + (c) are compared with (b) + (d) a measure of the effect of exposure to light after inoculation is obtained. (see Table 12b)

Table 12b		
Treatment	Av. lesions per plant	F. test
(a) + (c) Dark following inoculation	46.2	* *(1% level
(b) + (d) Light following inoculation	111.0	* *(1% level

It appears therefore that post-inoculation treatments have a modifying influence on the number of infections, but pre-inoculation treatments do not appear to alter the subsequent number of infections. Because the temperature differential which occurred between the light and dark treatments of the latter trial, some bias has been introduced. It was surmised from later work that the effect of lower temperatures would retard spore germination and infection. The degree of bias introduced would therefore depend at what stage the temperatures acted on the developing spores. During the later stage of development, temperature differentials were at their maximum and one would expect them therefore to have a smaller influence on infection than if they had occurred earlier. Because the stage at which low temperatures hindered infection was not known this section of the trial was repeated under uniform temperatures. Results are presented in Appendix VII and these confirm the results suggested by the previous trial, viz that exposure to light after inoculation, compared with exposure to dark for an equal period of 12 hours, results in a higher infection.

Discussion

Both direct penetration via the intact host leaf surface and stomatal penetration were recorded from lettuce using conidia of Stemphylium botryosum.

Direct penetration is reported to be a mechanical process. The germ tube or appressorium of the germinated spore may become attached to the host by a matrix of mucilagenous material which provides an anchor against the force of thrust required by the infection peg when it pierces the outside of the host. (Flentje 1959, Wolf and Wolf 1947). Appresoria observed on the surface of the leaf during this study were small compared with descriptions of other fungi recorded in literature. They seldom exceeded the size of the spore either in width or length and were often not immediately recognised or present.

The initial stages of penetration via wounds or stomata appears to be a passive process often influenced by chemotropic responses due to substances diffusing from the openings (Bald 1952). Howeger, the stimulus may result from a response to higher water vapour gradiants found near such openings. (Brown 1936, Bald 1952, Bide 1955.) Within these openings the fungus may penetrate live tissues following the formation of an appressorium, or alternatively kill additional host cells by excretion of toxic enzymes which weaken or dissolve part of the cell wall. (Brown 1922, 1936, Kerr & Flentje 1957). Entry of hyphae into a stomatal cavity is considered by Pristau and Gallegly (1954), and Flentje (1959) to be usually preceeded by the formation of an appressorium on the leaf surface in a manner similar to

that occurring during direct penetration. In these studies appressoria were not located which had formed on the leaf surface in the case of stomatal penetration. Appressoria like, convoluted bodies were confined to the substomatal cavity. Penetration of adjacent cells occurred but it was not possible to determine whether penetration of these cells was similar to that involved with direct penetration or if toxic enzymes were excreted by the appressoria, resulting in the cell walls becoming weakened or dissolved.

Stomatal penetration was recorded more frequently than direct penetration, although the proportion of direct penetration recorded was higher than that reported by other authors for S. botryosum. Smith (1940), Nelson (1955) and McDonald (1958) have all observed both stomatal and direct penetration of S. botryosum on various hosts. Padhi & Snyder (1954) report that only stomatal penetration was observed during their studies of S. botryosum f. lactucum on lettuce. This may indicate that the form of S. botryosum isolated from New Zealand lettuce may be distinct from the forms isolated from Colma, California, U. S. A. by Padhi and Snyder.

Because stomata are involved and are more numerous on the underside of lettuce leaves one may expect more frequent penetration and infection of this leaf surface (Diener 1952). The number of spores recorded actually penetrating the host were too small to indicate if penetration occurred more frequently on the upper or lower leaf surface. There was no suggestion however, that

any particular side of the leaf favoured penetration. While Gloyer (1931), Baker & Davis (1950), and Padhi & Snyder (1954), reported only stomatal penetration of S. botryosum penetration was not restricted to this method of entry with the N. Z. isolates. Thus in this country the presence or absence of a favourable microclimate about or on a particular leaf surface, rather than an abundance of stomata, may have a larger influence on resulting infection. Further, during these studies it was most apparent that while the majority of spores germinated readily on the host, there were only a small percentage which finally infected the host. The spacing of stomata would therefore not become a limiting factor and is of lesser importance than if the percentage of spores causing infections were higher.

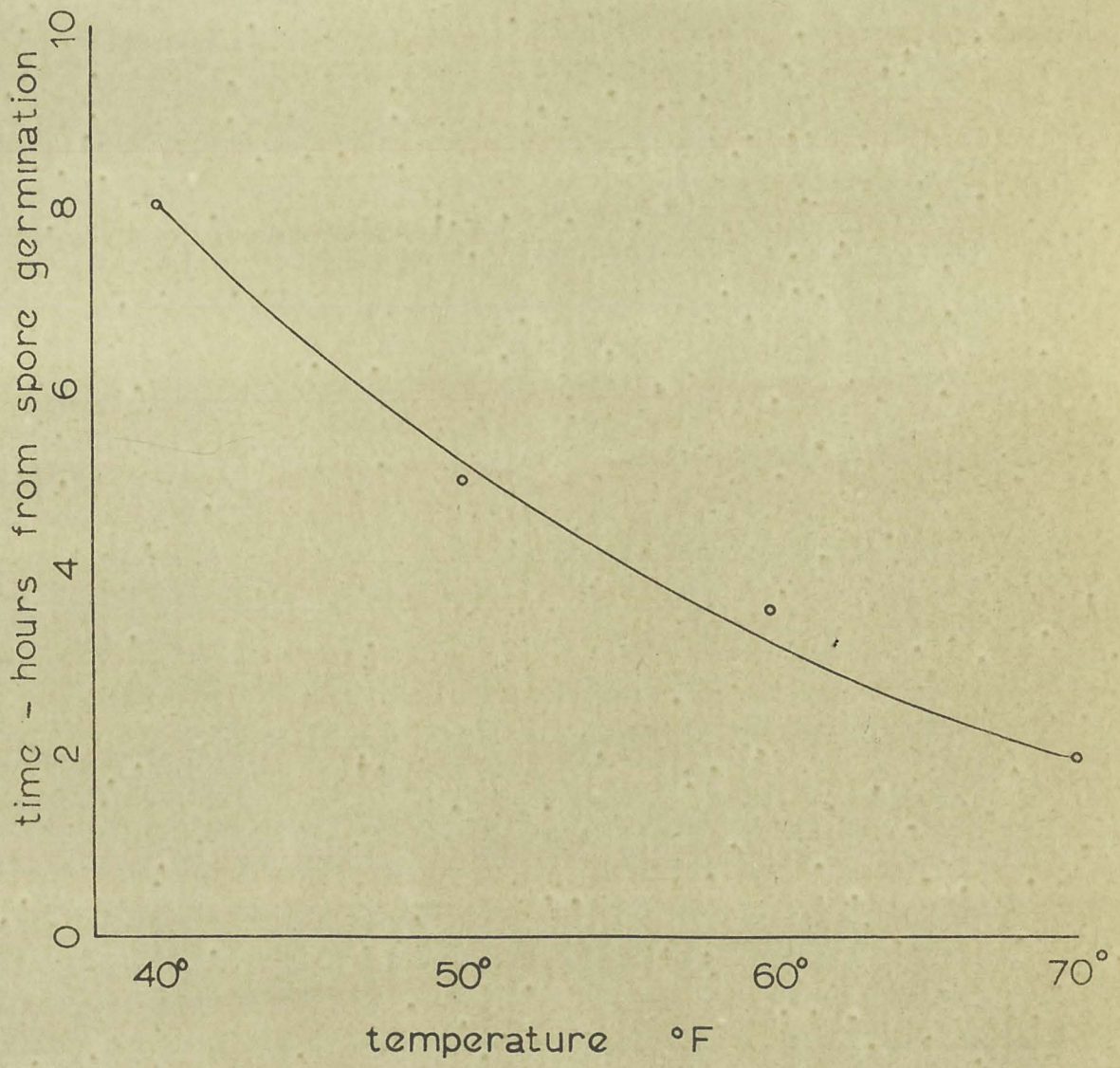
In Padhi & Snyder's(1954) illustration of penetration fungal hyphae are visible ramifying through the leaf tissue. Disorganisation and dark staining properties of the contents, which occurred in advance of the fungus, noted also by both Padhi & Snyder (1954) and McDonald (1958) prevented similar observations being made here. The former authors also state that fungal hyphae were both inter and intracellular, but during observations in the course of the present study hyphae were found only within living cells or ramifying through dead tissue. McDonald (1958) also has recorded only intracellular hyphae. Intracellular existance is often associated with obligate parasites. However, this group of organisms usually have a gentle non-necrotic or stimulating effect on the host

which is in contrast to the necrotic effect observed here immediately preceeding or at penetration. This reaction by the host is normally associated with faculative organisms (Yarwood 1956).

During these studies on the method of penetration some spores were still found which had apparently only recently penetrated the host after periods of up to 72 hours from inoculation. At optimum temperatures it was therefore thought that penetration and infection may be a comparitively slow process. However, it was apparent from later studies this was not so. If sufficient time was allowed to permit the first 5-10% of the spores to germinate, infection would result from the exposure of leaves to suspensions of these germinated spores for periods of two hours at temperatures between 60°-70°F. At lower temperatures penetration and infection required a longer period as illustrated in Figure VII . Peltier (1925) found that the minimum time required for uredospores of Puccinia graminus tritici to penetrate the stomata of wheat and cause infection was between 2-3 hours at optimum temperatures. Hawkens and Harvey (1919) when studying a species of Pythium on potato tubers found the actual time required for direct mechanical penetration of the epidermal cell by the infection peg was within approximately five minutes. Therefore it is obvious that when conditions are optimum, penetration and infection of a small percentage of spores will occur after comparatively short periods, but if conditions permit penetration and infection may result at up to three days from inoculation, even when optimum temperatures have prevailed

FIGURE VIII

THE EFFECT OF TEMPERATURE ON THE PERIOD REQUIRED FOR PENETRATION AND INFECTION.



during this period.

Low relative humidities after penetration appear to retard the number of spores causing infection. While spores are not as dependant on favourable relative humidities for continued development during infection compared with during germination and penetration they are not yet completely independent of the microclimate about the surface of the leaf.

Light did not appear to predispose the plant to penetration and infection, if Yarwood's (1959) definition of predisposition is accepted (see p. 207). However, it would seem to be an important factor involved in the actual penetration and infection of the plant. Thus the plant may be considered *in sensu* Gaumann(1950) to be predisposed to infection by light. Stomata open when exposed to light (Hart 1929) and stomatal penetration is the main method of entering the host tissues used by this fungus. The logical conclusion, as noted by other authors (Hart 1929, Yarwood 1936, Allington & Feaster 1946, Pool & McKay 1946) would be therefore that the barrier resulting from the closed guard cells is removed when the stomata open, and penetration is then able to occur more readily. However, the possibility of light acting directly on the process of penetration or infection and thereby increasing infection, must not be dismissed. Other authors for example, Caldwell & Stone (1936) do not regard closed stomata as effective barriers to penetration.

Wolf and Wolf (1947) consider light to be of such

importance that they recommend due consideration should be given to it when pathogenicity studies are undertaken. Consequently during these studies plants were always inoculated and placed in a humidity cabinet for a period of twenty four hours to ensure that there was an adequate period of light to permit penetration and infection to occur under optimum conditions.

3. INCUBATION

Walker (1950) defines the incubation period as the interval between infection (establishment of the pathogen within the host following penetration) and the appearance of symptoms of disease. By use of a microscope and suitable staining techniques it was apparent that disease symptoms were visible once penetration and infection had occurred. However at this stage the infection cites were not mascroscopically visible, or apparent when the untreated host material was examined under the microscope. The incubation period was therefore considered to extend until macroscopic symptoms of disease occurred.

The lesions which marked the completion of the incubation period developed in an area determined by the host-parasite relationship during and after the incubation period. At this stage the pathogen had become relatively independant of all external influences except temperature. Observations were made of the effect of temperature(during the incubation period on the number and size of lesions appearing after infection had occurred). The effect of the type of plant on the number of lesions was also recorded.

Materials and Methods.

(a) Length of the Incubation Period

Observations of the period which elapsed from inoculation until manifestation of symptoms were recorded during experiments in this and other sections of the disease cycle study.

Penetration continues during high relative humidities but ceases once plants are removed to low relative humidities and dried. Because plants were normally inoculated and placed in the high humidity cabinet for 24 hours before removal, penetration may occur during 22 hours from the germination of the first spores. In some studies (see p 121) the first spores to germinate were permitted to penetrate the host and the plants were then dried to stop further penetration. During this type of experiment a more exact measure of the length of the incubation was obtained.

By marking lesions as they appeared on leaves it was possible to determine when further lesions developed. In this way the length of the incubation period for any lesion appearing on the leaf was determined.

(b) <u>The effect of Temperature on the size of lesions</u>.

(i) Preliminary Experiments.

Observations on incubation at 50°F, 60°F, and 80°F indicated that 50°F was above the minimum temperature for expression of the required range of symptoms. During these preliminary experiments observations were made on plants held at 80°F during the incubation period.

(ii) Experiments to determine the optimum temperature for growth during incubation.

To overcome the difficulties associated with low temperature studies, the temperature controlled growth

cabinets were transferred to a coolstore and adjusted to run at 40°, 50°, 60°, and 70°F. Because the coolstore completely eliminated all light, artificial light was provided for twelve hours during each twenty four hour period. Provision was also made for a group of plants to remain in the glasshouse where natural light was present and temperatures ranged from 50°-70°F. This group of plants was similar to groups held in the temperature controlled cabinets.

Seventy five lettuce plants were divided into two types according to their vigour. Type A plants (see Appendix VIII and IX) were large and soft, while Type B plants were small and hard leafed. Four control plants, two of each type, were allocated to each treatment. Of the remaining fifty five plants, thirty were classified as Type A and twenty five as Type B, and these were allocated (at random within each Type) to the various treatment temperatures. The plants were inoculated with a spore suspension containing approximately 1000 spores per ml. and held in a humidity cabinet for 24 hours. After this period they were placed into the temperature controlled cabinets or on the glasshouse bench, for forty-eight hours. To allow further development of lesions during similar conditions the plants after removal from the cabinets were placed on the glasshouse bench for 24 hours.

The effect of temperature on the development of the host-parasite relationship during the incubation period was recorded by measuring the diameter of the lesions. A low power binocular microscope was used to permit accurate measurement

of the small lesions. Each leaf was removed from the plant and placed on the microscope stage, and every lesion was measured. Because the plants were more advanced that those normally used, some having up to ten leaves, and since the inoculum load was one third of that used for other work this method of recording was feasible. The size of the lesions were classified into six groups, viz (1) below 0.25 mm - 0.75 mm, (ii) 0.76 - 1.5 mm, (iii) 1.6 mm - 2.5 mm, (iv) 2.6 - 3.5 mm, (v) 3.6 - 4.5 mm and (vi) 4.6 - 5.5 mm, and their frequency recorded. The above groups were given a class mark to facilitate recording of (i) 0.5 mm, (ii) 1.0 mm, (iii) 2.0 mm, (iv) 3.0 mm, (v) 4.0 mm, and (vi) 5.0 mm respectively.

(iii) The effect of alternating temperatures.

The effect of alternating temperatures during the incubation period was also explored. Pairs of plants were moved alternately from an original temperature to another and visa versa for periods of twelve hours during the 60 hour incubation period. The following two groups of temperature combinations were used: 50°, 70°, and 80°F, and 40°, 50°, 60° and 70°F. Plants were moved in pairs, one pair remaining at the original temperature while the remaining pairs were moved to or returned from each of the other temperatures.

Results

(a) Length of Incubation period.

Expression of symptoms at optimum temperatures generally occurred after 72 hours from inoculation with the spore

suspension. Thus the normal period for incubation at temperatures between 40° - 60°F was approximately 68 hours. More critical trials revealed that the period of incubation in some cases was as short as 32 hours. At 70°F complete expression of symptoms occurred about 24 hours later than at 60°F, while the incubation period lasted for approximately five days when plants were held at 80°F.

Soft plants or young leaves on plants were usually the first to show symptoms, especially if the local inoculum load was high. Shortest incubation times were from soft leaves where heavy local inoculum rates occurred.

(b) <u>The Effect of Temperature on Size of Lesions</u>

(i) Preliminary Experiments

These experiments indicated that 80°F was above the normal growth range for the fungus, and 50°F was not sufficiently low to approach the other extreme of growth. Observations were made on the effect of 80°F treatment on developing lesions and these are recorded in Table 13 .

(ii) Experiments to determine the optimum temperature for growth.

The size of lesions recorded from plants subjected to temperatures from 40°F - 70°F during the incubation period are presented in Appendix VIII and summarised in Table 14 and Figure IX . It is apparent that the temperatures between 40°F - 60°F favoured the development of larger lesions than the

FIGURE IX. EFFECT OF INCUBATION TEMPERATURE ON SIZE OF LESIONS.

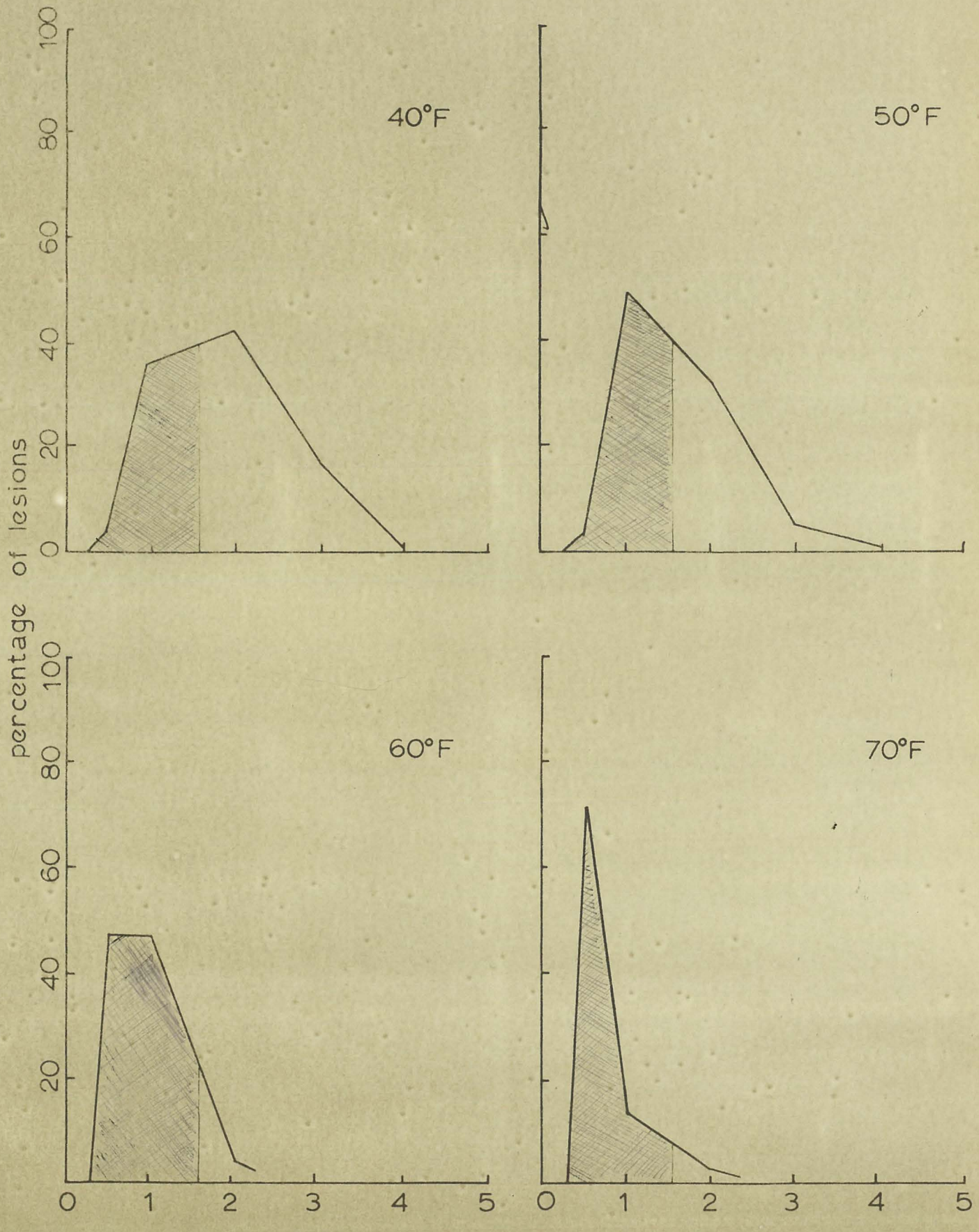


Table 13 Description of lesions at completion of the Incubation period at various temperatures

Incubation Temperature	Colour	Leaf surfaces on which present	Margins	Remarks
40°F*	Light brown	Sunken area on lower surface Upper leaf surface darker coloured but not sunken	Indefinite	Difficult to see on lower leaf surface since lesions often of similar colour to the leaf.
50°F*	Light brown	Both surfaces Upper leaf surface only slightly sunken	Definite	Lesions on upper leaf surface slightly concave
60°F*	Greenish brown	Depressions on both leaf surfaces	Definite	Lesion on upper side of leaf often darker than on lower side of leaf.
70°F*	Dark brown	Both surfaces, occasionally only on upper side	Definite	Small
80°F **	Dark brown	Usually only upper leaf surface	Definite	Very small
50°- 70°F ***	Light brown to greenish brown	Both surfaces with major depresion on under side of leaf	Definite	Similar to 40°F and 50°F groups above

Notes
Upper leaf surface inoculated
* Plants from temperature controlled cabinets in coolstore
** Plants from temperature controlled cabinet in glasshouse (preliminary experimentation)
*** Plants held on glasshouse bench during incubation period.

Table 14 Effect of temperature during the Incubation Period on size of lesions.

Range	Class	Frequency of lesions at various temperatures				
		*40°F	*50°F	*60°F	*70°F	**50° - 70°F
0.25mm - 0.75mm	0.5mm	12	46	204	129	142
0.76mm - 1.5 mm	1.0mm	139	216	203	41	299
1.6 mm - 2.5 mm	2.0mm	162	140	20	6	38
2.6 mm - 3.5 mm	3.0mm	65	23	6	1	2
3.6 mm - 4.5 mm	4.0mm	3	6	2	0	0
4.6 mm - 5.5 mm	5.0mm	1	1	0	0	0
	Totals	382	433	435	179	481

* From temperature controlled cabinets in coolstore.

** From plants remaining on glasshouse bench.

higher temperatures. The shaded area represents the percentage of lesions produced which are below the average lesion size for the whole experiment. It was also apparent that the lesions expressed on the group of plants held in the glasshouse during the incubation period were very similar in size to the 60°F group, but they resembled those from the groups of plants held at 40°and 50°F during the incubation period. The type of lesions produced were characteristic from each temperature and are described in Table 13 .

The number of lesions recorded on each type of plant have been presented in Appendix II and show the effect of the plant on infection, the harder plants having significantly fewer lesions than the soft plants. However, the type of plant did not appear to affect the size of lesions developing after incubation at the various temperatures. (See Table 14 , and Appendix VIII) The fewer lesions recorded from the 70°F treatment is a result of the longer incubation time required at this temperature.

(iii) The effect of alternating temperatures on growth.

The effect of alternating the temperatures during the incubation period did not appear to result in an alteration of the length of the incubation period except where 80°F treatment was involved. There was no apparent stimulation of the size of lesions except that noticed when plants were moved from 70° - 80°F to lower temperatures. Such results are readily explained when the favourable effects of the lower temperatures, recorded by previous trials, are considered.

Discussion

The descriptions apply to the lesions at the completion of the incubation period and expresses the effect of the conditions prevailing during this period. They do not apply to subsequent development of the lesions.

Results suggest that as temperature of 40°F is approached during the incubation period the size of lesions increases. Optimum temperatures appear to be between 40°- 60°F. Above 60°F, the size of lesions progressively decreases, until at 80°F. lesions remain small pin points in size. Artificial illumination provided a total of 24 hours light during the 48 hours the plants remained in the cabinet. This may have raised the temperature of the plant leaves above that of the surrounding atmosphere. While this would also be expected to occur in nature the actual temperature at which the fungus developes within the leaf during that period may be slightly above that of the surrounding air as suggested by Curtis & Clark (1950). A similar experiment was carried out by Dippenaar (1939) to find the effect of temperature on this period of the disease cycle. He discovered that the optimum temperature for development within the lettuce leaf was between 59°F and 72.8°F which is higher than that recorded for the New Zealand isolates. This may be due to different experimental techniques, a different strain of the fungus, or a combination of both of these conditions.

Lesions marking the end of the incubation period were darker and better defined at the higher temperatures. At 80°F lesions were confined to the upper inoculated leaf surface. At 40°F lesions were generally most noticeable on the lower leaf surface, the upper leaf surface being stained in the region of the lesion. Between these two temperature extremes the lesioned area was approximately evenly distributed on both sides of the leaf.

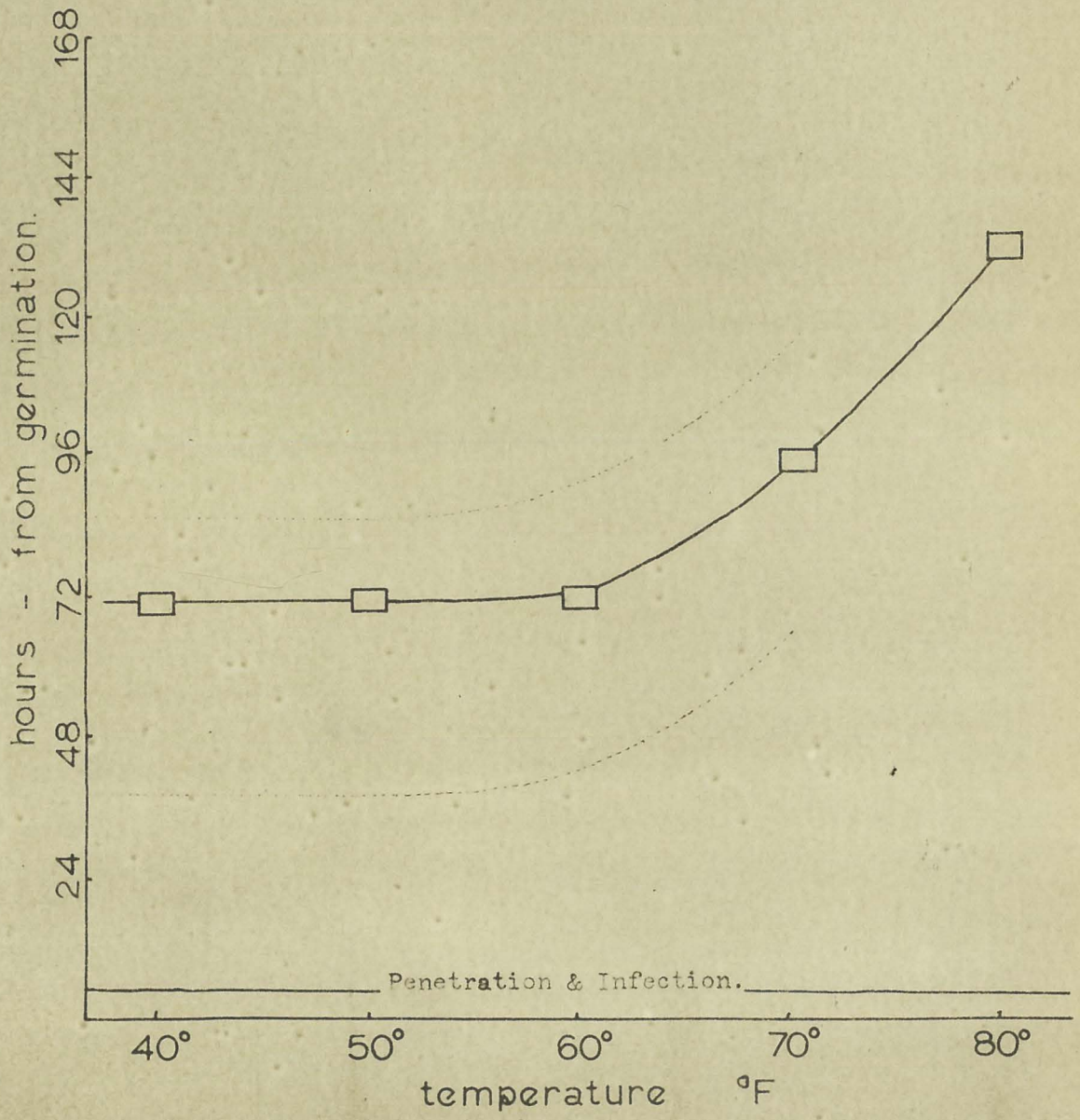
Lesion sizes recorded from the two groups of plants of different vigour recorded in Appendix VIII do not differ significantly. However during recording it was noticed repeatedly that lesions on young soft leaves were invariably larger and visible sooner than lesions on older mature leaves.

The length of the incubation period, observed many times during various experiments appeared to be similar at temperatures between 50-60°F. At temperatures above 60°F the length of the incubation period was increased. It is believed that a relationship may exist between temperature and length of the incubation period similar to that expressed in Figure X The dotted lines mark the boundaries of the observed minimum and maximum length of this period and the blocked area represents the approximate period at which maximum expression of lesions was observed at the various temperatures.

The length of the incubation period recorded from the plants placed in the glasshouse was similar to that recorded from the plants incubated at 40-60°F. However the lesions

FIGURE X

RELATION OF TEMPERATURE TO LENGTH OF INCUBATION



appeared to correspond to those in the 40-50°F range. This may have been due to the fluctuating temperatures and natural light present in the glasshouse. Because the house was well shaded from direct sunlight leaf temperatures during periods of light may also have been lower than those occurring on the plants subjected to artificial light in the temperature controlled cabinet.

It is therefore possible to give only an optimum range for temperature in relation to its effect on the size of lesions. However a similar range of optimum temperatures were observed when observations on the effect of temperature on the length of the incubation period were made.

The lower numbers of lesions recorded from 70°F treatment in Table 14 reflect the longer period required for expression of symptoms at this temperature. Observations showed that the number of lesions eventually expressed were similar to the number expressed by the lower temperature groups. At 80°F the number of lesions appearing after seven days exposure to this temperature were below numbers expressed at the lower temperatures and moving the plants to a more favourable temperature did not increase the number of lesions which eventually appeared. Thus is appears that temperatures of 70°F may lengthen the incubation period by temporarily retarding expression of symptoms. However, at 80°F the plants defences may be able to withstand the onslaught of the fungus during the incubation period, so that the actual number of infections surviving the incubation period are

noticeably reduced. It can be assumed that infection was successful; if it were not expression would not have occurred at the favourable temperatures. These temperatures acted after successful infection was known to have occurred and thus affected the host-parasite relationship during incubation.

The local inoculum load appeared to influence the length of the incubation period. Those leaves with high numbers of lesions recorded in Appendix IX were usually the first to express symptoms. Microscopic examination revealed the higher inoculum load on this type of leaf.

4. Manifestation of Symptoms of Disease

Manifestation of symptoms of disease, or simply 'manifestation' has been the term applied to the section of the disease cycle which commences when the first lesions appear at the conclusion of the incubation period, and continues until death of the leaf or plant occurs as a result of this disease.

The effect of the environment during the incubation period on the type of lesions which follow was considered in some detail in the previous section on that period. In this section the effect of environment is studied in its relation to the disease during the manifestation period, i.e. from the stage at which lesions appear.

When conditions are suitable production of conidia may occur during the period of manifestation, Because the factors which favour production of inoculum are distinct from those favouring manifestation, the former process is considered separately. However, before considering in detail the environmental conditions favouring manifestation, some attention was given to the type of lesions which occurred during artificial growth conditions, as well as lesions from naturally infected plants from the field.

(a) Description of Lesions

At favourable temperatures the first symptoms of disease to appear were small water-soaked depressions present on both sides

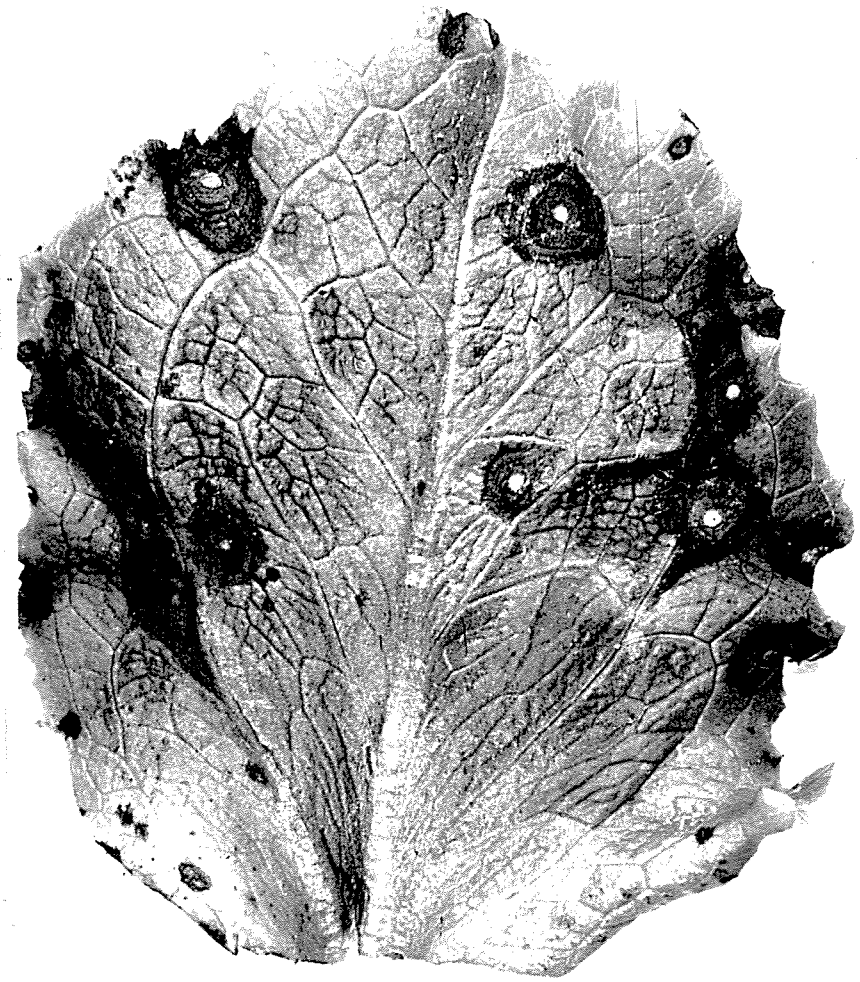
of the leaf. Occasionally these lesions were present on the veins or midrib. Lesions were brown and often confined to the inoculated leaf surface at temperatures of 40°F, or above 70°F. After one to two days the central area of the lesions became slightly darkened and often the surrounding zone became partially bleached, particularly during favourable conditions. Lesions continued to increase in diameter and retained their identity until they reached a maximum size of about 1 c.m. Although the lesions were typically round, the shape of the larger diseased areas was often influenced by the veination of the lettuce leaf.

During conditions favourable for continued growth of the fungus individual lesions often coalesced with other lesions or dead areas to form aggregate lesions. When veins were engulfed by these lesions sections of the leaf supported by the vein would die and become quickly colonised with S. botryosum or secondary organisms. Often, during optimum conditions, the death of a large part of the leaf would result after seven days from the first manifestation of symptoms. Despite this lesions remained visible on the dead leaf due usually to concentric zonation about the infection site.

Fluctuating temperatures, or water regimes, normally occurring from day to day appeared to induce zonate lesions. (Those lesions on plants maintained at constant temperatures and

ILLUSTRATION 19. Typical lesions caused Stemphylium botryosum (Naturally infected leaf.)

X1.



relative humidities failed to become zonate.) This characteristic, typical of symptoms present on plants in the field, has led to the general description of this type of lesion as a "target spot."

Often the central area of zonate lesions fell out or split radially in several directions. This gave rise to a shot hole effect. Generally lesions were 0.75 cm. in diameter or larger, before the central area started to disintegrate. This normally occurred also when conditions did not appear to favour continued growth of the fungus within the tissue of the leaf and was characteristic of lesions present during late spring, summer and autumn. Occasionally during autumn it was observed that the smaller lesions caused by S. botryosum would disintegrate after the return of prolonged periods of dry weather. In the field this type of lesion was often incorrectly attributed to the ringspot disease caused by Marssonina panattoniana. (See Illustration 12)

During periods of high humidity, or in situations where the leaves were frequently wet, the fungus was observed developing on the surface of the host, originating at first from the central area of the lesion. Such growth was visible to the naked eye as light fluffy white mycelium, and under continued favourable conditions this frequently spread over the leaf in advance of the lesion.

(b) The Diseased plant

In the field the larger lesions and dead areas were

usually on the lower older leaves of the plants, where little harm was done to the lettuce plant or to its appearance at market. However, during favourable conditions when abundant inoculum was present, the wrapper and surrounding leaves become heavily infected and the lesions occurring here caused an unsightly appearance, and provided entry points for secondary decays. Lettuce plants whose wrapper leaves were infected were not suitable for packing for market. The lettuce which were presented on the market floor with this disease failed to bring a reasonable return to the seller, even though wrapper leaves may have been only slightly infected.

(i) The effect of Temperature and Size of Plant on Manifestation.

Materials and Methods

Plants growing in 3" pots were grouped into four equal classes according to their vigour and size, viz;-

(i) Hard and small

(ii) Hard and medium

(iii) Soft and medium

(iv) Soft and large.

These classes were subdivided into five groups of four, labelled, arranged at random, and inoculated with a spore suspension containing 3,000 conidia per ml. The plants were transferred to the humidity cabinet for 24 hours, and from there placed on the glasshouse bench until manifestation occurred. On the fifth day from inoculation the plants were transferred to cabinets operating at 40°, 50°, 60°, and 70°F, one group of four plants from each of the above classes

being included in each treatment. Similarly another group from each of the above classes was placed in the glasshouse. The plants in the cabinets were provided with artificial light for twelve hours every day. To ensure the heat radiated from the globes and falling on the plants would be minimised a sheet of glass was placed between the globes and the polythene sheathing of the cabinets.

After the plants had been in the above temperatures for a period of six days manifestation of symptoms was recorded on the four leaves immediately above the cotyledons and the first two true leaves. These 4 leaves were removed and grouped into classes according to the severity of the manifestation. A group of ten photographs of symptoms of 0 for no infection to 10 for complete death of the leaf were used to ensure similar standards were maintained during the recording of results.

The results were subjected to Duncans Multiple Range test (Duncan 1953) and valid comparisons between treatments made.

During a previous trial of this type individual lesions were measured, but due to the nature of the lesions, and sampling difficulties this method proved unsuitable.

Results

Tabulation of results and analyses are given in Appendix X and are summarised below.

TABLE 45

Score of Diseased Leaves: Effect of Temperature and Type of Plant on Manifestation (Scores per plot of 4 plants. Maximum possible 160.)

Temperature	40°	50°	60°	70°	50-75°	Totals	Mean	Result*
Type of plant								
Hard-small	70	94	49	47	64	324	64.8	A
Hard-medium	60	98	90	75	71	394	78.8	B
Soft-medium	81	107	75	60	66	389	77.8	B
Soft-large	107	107	92	91	94	491	98.2	C
Totals	318	406	306	273	295	1598		
Mean	79.5	101.5	76.5	68.2	73.7			
Result *	A	B	C	C	C			

* Treatments with the same letter do not differ significantly at the 1% level of significance. C = 6.6%

Conditions for optimum manifestation of symptoms occurred at 50°F. At the 1% level of significance the average score per plant at 50°F. was significantly above that obtained from plants placed in the 40°and 60°F. temperature cabinets during the period of manifestation. Neither the score from plants held at 60° and 70°F, nor the score from plants held at 50°-75°F. on the glass-house bench, differ significatnly at the 1% level.

These results indicate that the optimum temperature for manifestation is approximately 50°F and that conditions prevailing in the glasshouse, where the average temperature was approximately 63°F, gave similar results to those observed from the temperature cabinets in the 60-70°F. temperature range.

Host vigour also affected the degree to which symptom were manifested. The smaller hard type plants failed to be as severely affected during this period as the medium sized plants. Manifestation occurred to a significantly greater extent when the plants were large and soft. (See Table 15)

Symptoms varied between those plants held in the glasshouse and those confined in the cabinets. The plants from the glasshouse developed zonate lesions, while these were absent from the plants held in the cabinets. This was believed to be due to the fluctuating temperatures encountered in the glasshouse.

(ii) The Effect of Miscellaneous Factors on Manifestation.

During early observations when a trial run on the effect of temperatures on manifestation was being observed at 60°, 70° and 80°F., observations were also made to see what effect alternating temperatures had on manifestation. It appeared that plants growing at lower temperatures and then subjected to 90°F for two days or more, manifested synptoms in excess of plants remaining at the lower temperatures. Subsequently it was observed that at 80°F. the compost in the pots became very dry, sooner than that in pots held at the lower temperatures, due to the heating globes being on for a longer period and the presence of forced air circulation. If the pots at 80°F. were kept moist by frequent watering, symptoms did not develop noticeably at this temperature. Thus it appeared that wilting may favour the growth of the lesions during the manifestation period.

This was later confirmed by letting two seed trays containing 32 diseased plants, become very dry. (Another pair were kept moist for this period.) Sufficient water was applied to allow these plants, which all wilted during the day, to recover during night. These plants were held at temperatures favourable for manifestation in the glasshouse. After ten days of this treatment the symptoms expressed by the plants which were allowed to wilt were noticeably more severe than symptoms expressed by the other group.

Discussion

The description of lesions observed and recorded here is in general agreement with those described by other authors for species of Stemphylium. (Dippenaar 1939, Neergaard 1945, Nelson 1955, Padhi & Snyder 1957, Wells et al. 1957).

While lesions of S. botryosum appear to very similar to those caused by species of Alternaria the temperatures favouring the development of Alternaria spp. on their hosts are above those recorded for S. botryosum (McDonald 1958, Neergaard 1945, Jackson 1959, Riley 1949).

Most authors, however, state that the favourable conditions for manifestation of the symptoms of disease occur during a certain season. Dippenaar (1939) made an attempt to study the effect of temperature on the development of lesions but confined these studies to a period equivalent to the incubation period previously discussed.

It is interesting to observe that the fungus will grow readily at 80°F (27°C) on culture media but is favoured by lower temperatures when growing on plants. Dippenaar (1939) and Padhi and Snyder (1954) have observed a similar relationship with S. botryosum.

Host vigour is a very nebulous term as Yarwood (1959) has shown. In this study, since sowing dates and pricking out

dates were all similar the differences in the size of the plants at the time of experimentation were considered to be due to different degrees of host vigour. Yarwood considered host vigour may be negatively or positively corelated with infection. Stackmann and Harrar (1957) consider that lush or succulent growth, resulting in weak cell walls being readily penetrated or destroyed by pathogens may aggravate the disease. This study has confirmed that a vigorous host plant with large soft leaves is more seriously affected by S. botryosum than the less vigorous plants.

The complete implication of this portion of the study in relation to the environment is discussed later.

5. Production of Inoculum.

Because both conidia and ascospores may serve as inoculum for disease development, consideration must be given to the production of both types of spores. It was therefore necessary to demonstrate the pathogenicity of both spore types before a study of the disease cycle was undertaken. In this way, further studies could be correctly developed and related to field observations.

Kocks Postulates were carried out using both conidia and ascospores. The methods used have been detailed previously. Both types of spores produced similar infections and lesions following inoculation. This was also observed by Padhi and Snyder (1954). They state that both conidia and ascospores may serve as inoculum. Dippenaar (1939) observed that conidia were only responsible for the spread of the disease in the field.

During this study it has been observed that conidia are the only inoculum of importance acting as both primary and secondary inoculum. Ascospores have not been found in nature, but experimental evidence suggests that these spores may provide primary inoculum and perhaps in some cases secondary inoculum. The importance of the latter is however insignificant when the extent of the production of conidia is considered.

Conidia may be produced during the Manifestation period,

or at a later stage, from the conidiophores originating from the dead host tissues or superficial mycelium. Perithecia producing ascospores appear only after prolonged periods of suitable environmental conditions on dead host tissues or other organic material.

Because of the relative unimportance of ascospores as secondary inoculum in the field, and since the development of perithecia with the subsequent production of ascospores is concerned with the survival of the pathogen from season to season, this type of inoculum is considered in the section on survival of this organism.

Production of Conidia.

The description of the production of conidia on culture media is applicable also to the production of conidia on host plant material. There are one or two notable differences however. The conidiophores produced from the central area of lesions supporting sporulation were very short and usually unbranched. The length of these conidiophores was frequently little different from the length of the conidia. Where conidia were produced from surface mycelium, which at times ramified over the surface of the host, the method of production of conidia was identical to that found on culture media. During favourable conditions, the density of production of conidia on the host plant appeared to greatly exceed the normal density of conidial production on artificial media. The conditions which favour production of conidia are often discussed

in relation to sporulation on artificial media. (For example Wolf and Wolf (1947), Stackman and Harrar (1959), McDonald (1958), Miller (1955), Diener (1955)) The factors which appeared to be most important in influencing production included the type of media, temperature, humidity, age of colony and light. Investigations of the effect of similar factors on sporulation upon the host tissues were therefore undertaken.

(a) Age of the Infection:

Method:

During trial work observations were made to find the minimum time from inoculation or manifestation until production of conidia. At normal glasshouse temperatures it was apparent that conidia were only produced during periods of high humidities. Consequently observations were made on plants held in high himidities obtained by covering them with polythene, placing them in the high humidity cabinet, or by keeping the glasshouse atmosphere very humid by frequently damping down.

Results:

Production of conidia occurred on the central portion of the lesions, other conditions being favourable, during the fifth day from the first signs of manifestation. Abundant production of conidia occurred whenever favourable conditions were present if portion of the leaf had been killed. The most active region of conidial production was at the site of the original lesion and the density of the production in other regions appeared to depend on whether the mycelium bearing the

conidiophores was superficial or within the host tissue. In the latter case, conidial production always appeared to be higher than that from the mycelium ramifying across the surface of the host.

Thus during favourable temperatures (50° - 60°F.) production of inoculum may occur five days after first appearance of lesions or seven to eight days from inoculation, and was found to be most abundant when dead leaf areas were present. Usually during favourable conditions portion of the leaves dies ten days after inoculation.

(b) Relative Humidity.

Normal relative humidities encountered in the glasshouse were not high enough to support conidial production, If the plants were covered with plastic sheeting or individual plants covered by plastic bags, sporulation occurred after 24 hours from sizeable lesions at normal temperatures. Similarly plants held in the glass humidity cabinet developed conidia within 24 hours. Production of conidia from plants in the glasshouse could be encouraged by reducing ventilation and damping down to keep the plants and floor of the house damp during the day.

It appears therefore that relative humidities approaching 100% are necessary for production of conidia, and that such periods must be longer than those normally occurring in the glasshouse during the night when dew was often observed on the plants.

(c) Temperature.

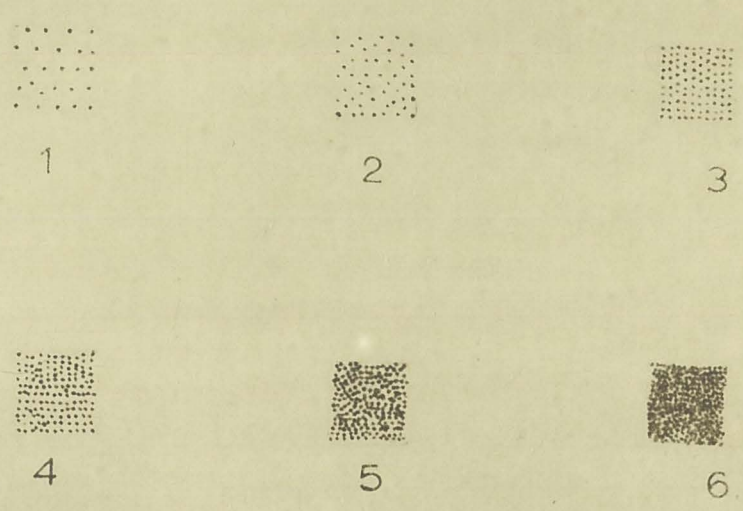
Materials and Methods:

Forty-eight plants inoculated with a suspension of conidia containing 1000 conidia per ml. were held in the humidity cabinet for a period of 24 hours before returning them to the glasshouse bench. Six days from when manifestation appeared, the plants were divided into four groups of twelve. Eight plants were covered with plastic bags and four were placed, uncovered, on the glasshouse bench to act as controls. Each group of eight plants were removed to the respective temperature cabinets in the cool store, operating at 40°, 50°, 60° and 70°F.

Lesions on pairs of these plants were observed every six hours and the density of conidial production was recorded after thirtysix hours. In the final recording five lesions of 1 cm. or over were selected at random from each but the two lowest true leaves of every plant. The density of conidial production was compared using a 50X binocular microscope, with a standard set of centimeter squares marked with from 25-250 dots per square centimeter. (See figure XI). The individual plants which had been subjected to the high humidity were assembled at random to ensure that no bias was introduced during recording. Lesions on leaves of control plants which had been held on the glasshouse bench had failed to sporulate. To hasten recording these plants were not included in this randomisation.

FIGURE XI

STANDARD CENTIMETER SQUARES FOR ESTIMATION OF CONIDIA DENSITIES



Results:

(a) Summary of observations made at 6-hourly periods.

Temperature:	Sporulation commenced after the following period in high relative humidities:	Notes:
40°F.	36 hours	No young perithecia present.
50°F	18 - 25 hours	A few young perithecia present after 36 hours
60°F	12 - 18 hours	
70°F	12 - 18 hours	Abundant young perithecia present after 36 hours.

(b) Summary of observations on spore densities after 36 hours' exposure to high relative humidities.*

Temperature:	Average Spore Density ⁄
40°	1.12 ± 0.56
50°	2.16 ± 0.62
60°	1.40 ± 0.36
70°	0.88 ± 0.34
* Controls:	Normal temperature and RH of glasshouse - no conidia produced
⁄ Figures refer to densities illustrated in figure XII .	

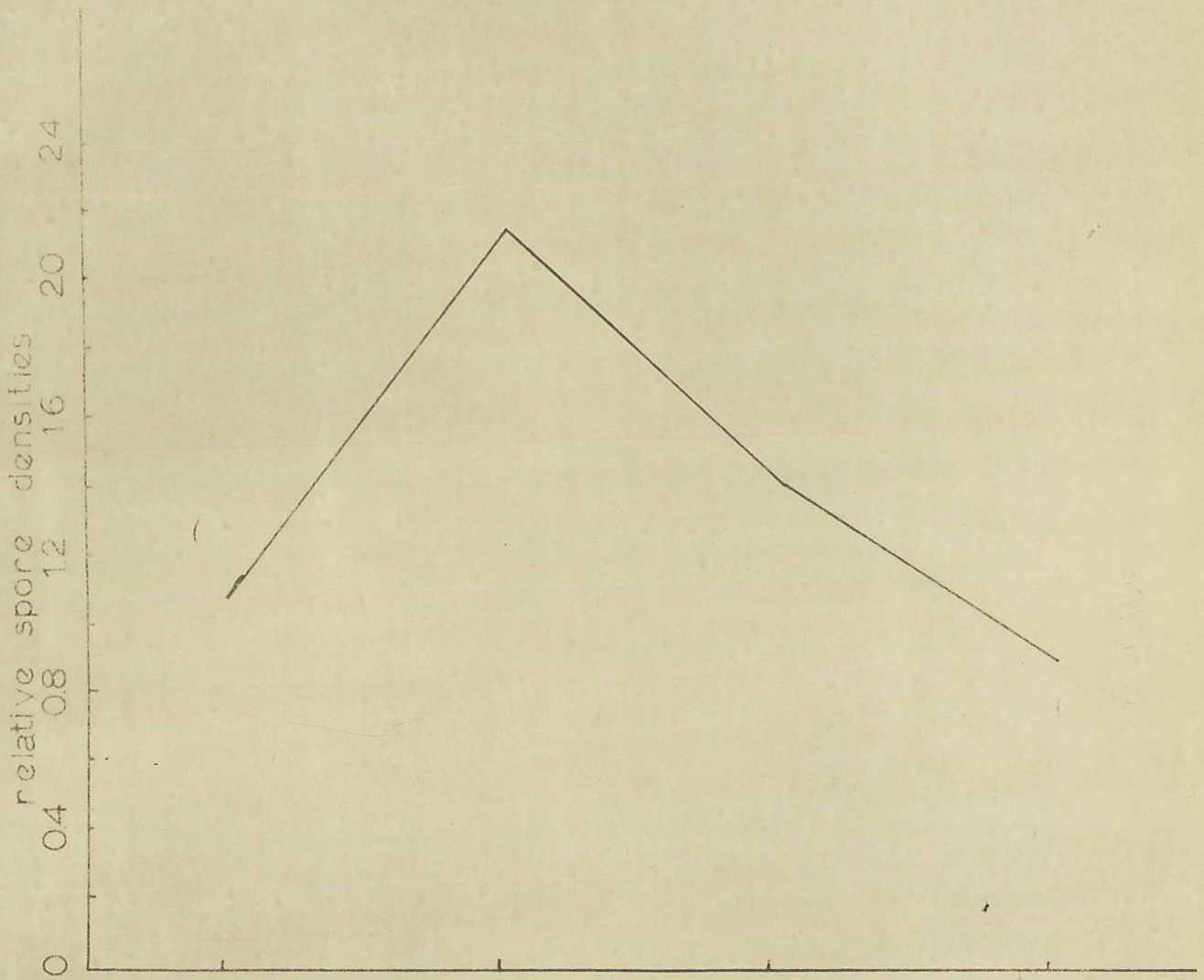
These results are also presented graphically in Fig.XII , and in Appendix XI.

The temperature at which maximum production of conidia occurred was between 40°F and 60°F, probably in the vicinity of 50°F.

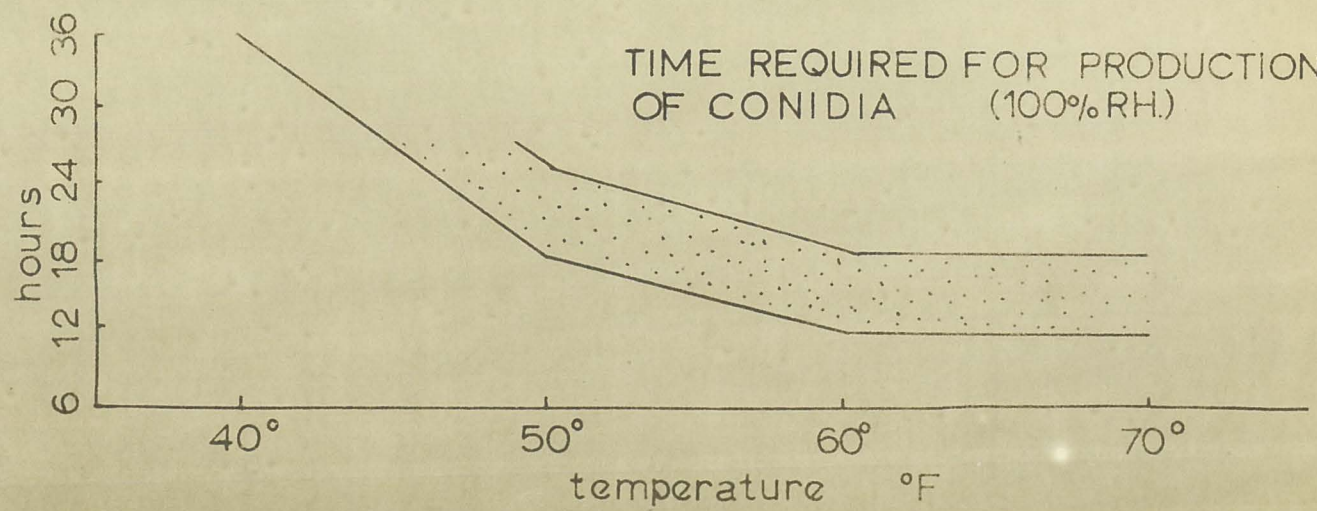
Conidia were produced mainly from areas of dead tissue or the larger lesions used for recording this trial. A large area of dead tissue was required before sporulation occurred.

FIGURE XII

DENSITY OF PRODUCTION
OF CONIDIA. (100% RH.)



TIME REQUIRED FOR PRODUCTION
OF CONIDIA (100% RH.)



This was not in agreement with previous observations made in the glasshouse, and on detached leaves, where sporulation occurred freely under conditions of high humidities from lesions of about 0.5 cm. even though these lesions were surrounded with live host tissue.

The only variable which seemed to account for these results was light. The observations and recordings made above were carried out on plants which had been held in temperature cabinets in complete darkness except for light used to make observations every six hours. In the glasshouse the sporulating plants previously observed were subject to approximately ten hours of daylight every twenty-four hours.

(d) Light.

The effect of light on sporulation was observed by using the box fitted with light traps previously described (see p.128). Eight plants manifesting disease were covered with plastic bags and placed in the light tight box for a period of 36 hours. A similar group of eight plants were covered with plastic bags and placed on the glasshouse bench. During the daytime temperatures within the box and glasshouse were checked at hourly intervals to keep temperature variations at a minimum. At night the temperatures within the box were similar to those on the portion of the glasshouse bench used during this experiment. After 36 hours, when the plants outside the box had been exposed to daylight for two periods of approximately ten hours, the plastic bags were removed and the plants labelled and arranged

at random. A start was made to record the results using the method of the previous trial, but this was modified. Plants were arranged according to whether conidia had been produced on the smaller entire lesions, or not. On decoding the labels, with one exception all plants which had been exposed to daylight were producing abundant conidia on all types of lesions, while those plants not exposed to daylight had produced conidia only on the older lesions which involved dead tissue, or on the dead tissue itself. This was considered sufficient proof to show that light was essential for the production of conidia from the smaller entire lesions.

Discussion

During this series of investigations on the production of conidia from the host plant, it was apparent that a high relative humidity was required before conidia were produced from lesions of any type. Lesions became potential producers of conidia at temperatures between 50° - 60°F. about one week after inoculation and infection, and appeared to reach a maximum when the lesions caused death of areas of the lettuce leaves. The minimum time required for the appearance of conidia from established lesions occurred during the higher temperatures (60° - 70°F.) when conidia appeared after 12 - 18 hours exposure to these temperatures. However, it is interesting to note that the most abundant production of conidia resulted at temperatures of 50°F. The different temperature requirements for these two processes is probably a reflection of the effect of temperature firstly on the growth of the fungal mycelium and the production of conidiophores, and secondly, on the development of the conidia. The higher temperatures favoured growth of the fungus on culture media (p. 42) and also apparently on the dead host material. However, the lower temperature still appears to favour the production of conidia. Hutchison and Ashton (1930) observed within certain limits, that the time of sporulation is an inverse expression of the rate of growth. It appears that this is also applicable to the production of conidia of S. botryosum on host plant material.

Light has a marked effect on sporulation of colonies

growing on artificial media, particularly if inoculum is used from cultures several times removed from the original isolate by successive subculturing. The effect of light increasing sporulation on artificial media was considered to be due to it supplementing a requirement not present in the media, but provided by isolates not far removed from the host. However, because light is also required for the production of conidia on the smaller entire lesions on host material, it may have a direct effect on the fungus itself, inducing it to produce and store some essential factor necessary for sporulation.

6. Liberation and Dispersal of Inoculum

Conidia constitute the main type of field inoculum and are considered here in this section. Because ascospores are formed within the perithecia which are the bodies concerned with the survival of the host from season to season, their liberation is considered separately in that section on survival. (Page 177).

It is very important to know what conditions cause liberation of the numerous conidia produced on sporulating host plant material. The presence of suitable conditions for liberation and dispersal of conidia largely determines to what extent the disease will spread in a particular crop or district. Gregory (1952) states that "most fungi show characters which heavily bias the spores future, favouring one dispersal route and restricting others".

Dye and Vernon (1952) have reported that the majority of spores collected during aeroplane flights at various altitudes in New Zealand belonged to six species, two of which were Stemphylium and Alternaria. Both these species have comparatively large spores. Because air dispersal had been observed by these authors and was readily demonstrated in the laboratory, observations were made on the effect of differing temperature and humidities on liberation of spores into a gentle air stream.

(a) The Effect of Temperature and Relative Humidity on Liberation of Inoculum:

Materials and Methods.

Sporulating plant material or cultures were placed in a gentle air stream produced by a "Robot"* type fan enclosed in a tube of polythene. Microscope slides thinly smeared with vaseline were placed in the air stream at an angle of 60° from the horizontal. The apparatus used is illustrated diagramatically in Illustration 20.

Very few spores were found on the slides when the sporulating plant material was used. This method was discarded in favour of sporulating colonies growing on artificial media in 90mm. Petri plates. The density of spore production on colonies of a similar type and age, and their liberation was found to be very similar. Using these colonies it was possible to expose a larger sporulating surface to the air stream, and the curved sides of the Petri plates probably helped to direct the liberated conidia towards the prepared slides.

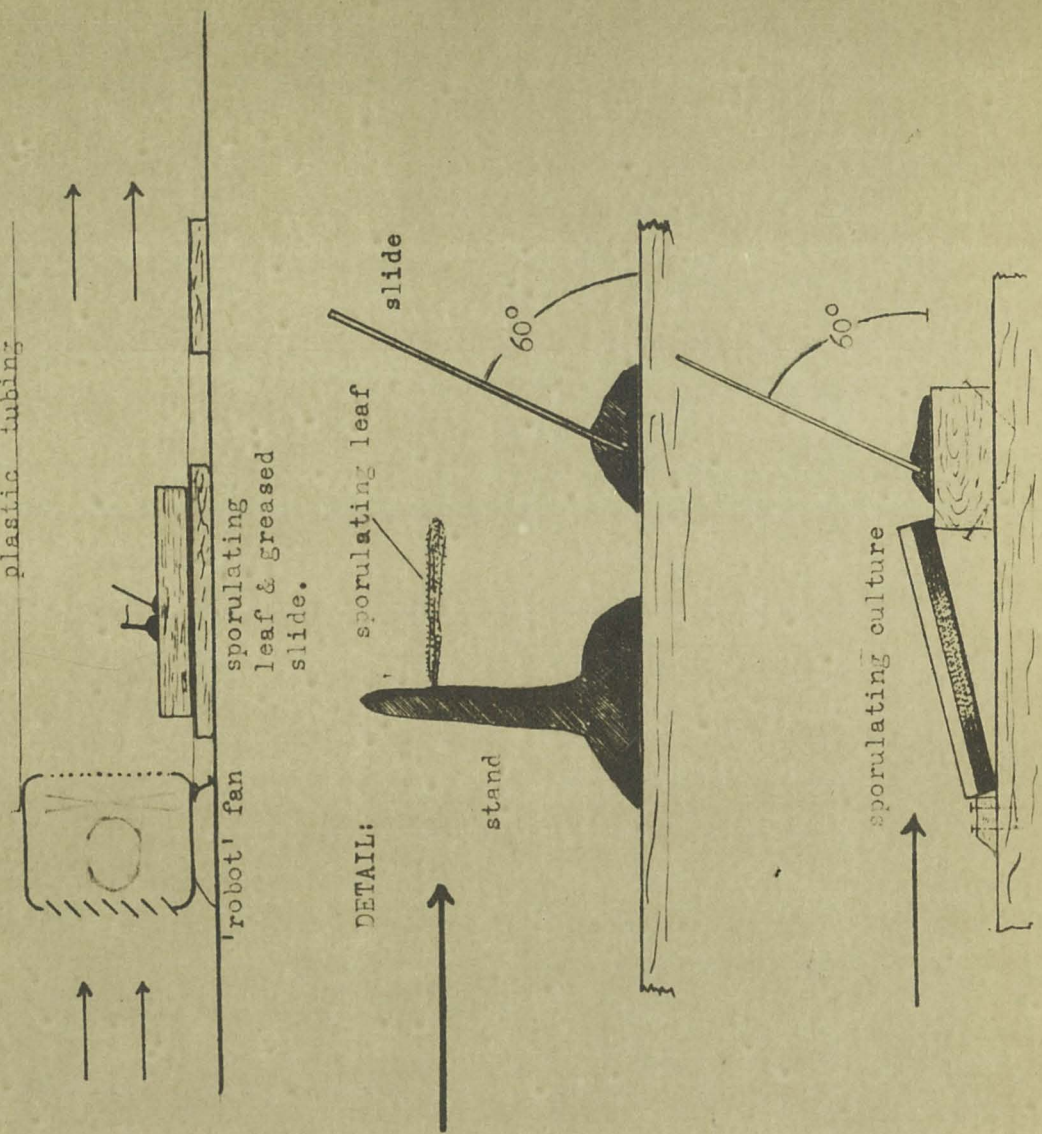
In the apparatus described above, it was possible to regulate the temperature of the air stream using the heating coils of the "Robot" fan type heater. By regulating the period the coils were heated for, the temperature within the plastic tubing could be maintained at between 75° - 85°F.

Humidity was increased by drawing the air passing through the fan over a series of water troughs in which inverted wet

* Trade Name for a domestic type combination fan heater manufactured in Christchurch, New Zealand.

ILLUSTRATION 20

APPARATUS FOR STUDY OF SPORE DISPERSAL



flower pots stood. This arrangement was built inside the humidity cabinet and air used by the fan moved over an area of approx. 11 sq. feet of water trays in which the damp inverted flower pots were standing.

All observations on liberation of spores were conducted during the morning in the glasshouse building so that temperature rises and fluctations, encountered in this building during the afternoon, were avoided. Air temperature was controlled by means of a heater in the room.

Using a similar group of sporulating cultures for each set of observations, one plate was placed in a marked position in front of a treated slide for the required period of time and treatment. The culture was then discarded, the slide labelled using a code to ensure later anonymity during recording, and another slide and culture placed in position for the next treatment.

To record the effect of the increase of temperature on spore liberation, pairs of plates were selected and the first exposed to the airstream at the air temperature of the room for the required period of time. The other plate was then placed in the air stream and the temperature regulated to 75^{o} - 85^{o}F. for the same period of time. Observations were made after 10, 20, 30 and 60 minute intervals and were repeated on four different occasions.

A similar procedure was followed when the effect of the increased humidity was observed.

The labelled slides were arranged according to a random design and the results assessed by observing the density of spores present on the central area of the slide under a 50X binocular microscope, and comparing these with the standard centimeter squares illustrated in Fig.XI .

Results

Conidia were released immediately heat was applied to the airstream moving across the cultures. Results indicate that this liberation occurred during the first 10 minutes of the heat treatment, but it was subsequently established that 1 minute was sufficient to release the majority of conidia found on the slide.

The effect of the high humidity on spore liberation was not measurable with the apparatus or method of recording used.

Results are presented in Appendix XII .

(b) Dispersal of Conidia.

The various agents available for dispersal of conidia of Stemphylium botryosum may include wind, rain, insects, man and cultural procedures. Because of the results of dispersal observed in the field, the latter two agents are considered to be unimportant. Dispersal was not consistent with that caused by insects due to the rapid spread in localised areas in the absence of any pests capable of carrying conidia in the manner

indicated by the area infected. Wind and rain are considered to be the two main factors concerned with dispersal of conidia.

Water drops running off leaves of infected plants with sporulating lesions were caught on filter paper. Spores were often found in these water drops, indicating that the spread over a particular leaf or plant may be facilitated by water movement.

Two plants, one infected, and one healthy, were placed side by side on the glasshouse bench with the infected plant slightly below the level of the healthy plant. The glasshouse was kept very humid during a period of wet weather and sporulation occurred. After a period of a week, the leaves of the healthy plant above these infected leaves became diseased. These plants were placed in such a way that air movement would not make their leaves touch and care was taken to ensure that no water was splashed on them. The only way by which the conidia could leave the sporulating lesions and cause infection on the healthy leaves above them, was by moving through the air. (See illustration 21).

Spread of the disease through a crop of lettuce was consistent with dispersal of conidia by air movement.

Discussion.

Of the various agencies which may cause dispersal of conidia of S. botryosum, water in the form of dew or rain-drops may spread conidia over the plant or to nearby plants. Air movement appears to account for the dispersal of the majority of spores in the field. However before a spore can be dispersed it must be released from the conidiophore. Hirst (1959) considers that this demands the removal of some restraining influence.

Observations indicated that the violent action of simulated rain may be sufficient to dislodge spores from conidiophores. However, investigations have also shown that an increase in temperature results in the removal of the restraining influence attaching the spores to the conidiophores. The changes in relative humidity also investigated in these trials failed to indicate the effect of this factor on spore liberation. The effect of the rise in temperature on spore liberation may be due either to a direct temperature effect, or to the combined increase in temperature accompanied by a reduction in the relative humidity. Further investigations on the liberation of inoculum in relation to changes in relative humibity were not pursued because of the practical difficulties involved and the amount ofwork that would be required to accurately measure the effect of this factor. Such work would be in excess of the practical importance of this problem. It was considered sufficient to observe that conidia were readily and quickly released during short exposures to high

temperatures.

Similar observations were made during the examination of sporulating lesions on the microscope stage. If the illuminating lamp was brought too close to the stage, the heat from the bulb would cause rapid loss of conidia which either fell off or were carried away in air currents. However, changes in relative humidity from 100%, used to induce sporulation, to normal room humidities, or vice versa, failed to cause a similar loss of conidia from the conidiophores. Gregory (1952) and Ingold (1953) consider that, particularly with spores borne on short conidiophores, a rising temperature and fall in humidity are important causes of spore liberation.

The layer of calm air about the leaf surface is smallest in the daytime (Ingold 1953). This author considers that conidia born on short conidiophores (as those produced by S. botryosum and affected by the above factors,) are liberated mainly during the day. Conidia therefore may be abundant in the air during the daytime but during calm nights they may be deposited on susceptible hosts. Gregory(1952) considers that during rain 80-90% of the spores the size of those produced by S. botryosum would be trapped and carried to the earth by the rain drops. Therefore, rain may remove spores of S. botryosum very efficiently from the air and deposit them on susceptible hosts.

Gregory's statement (p. 169) concerning the method of

dispersal of spores appears to be applicable to the conidia produced by S. botryosum on lettuce, where the conditions which favour spore liberation and dispersal are such that conidia are given a fair chance of continued development should they reach a suitable host plant.

7. Survival During The Summer Months

During field surveys growers repeatedly stated that if they grew winter lettuce in the same area for a period of three years, they would normally get three good crops. If they used the same area for winter lettuce production again during the following season, or successive seasons, leaf spot became troublesome and would often cause serious crop losses. Examples of this were often seen during field surveys.

These observations all indicated that the fungus must survive from one winter to another in the same area to reinfect subsequent winter crops, producing eventually a very high level of potential primary inoculum in that area. It would appear that this occurred after the third season to such an extent that subsequent winter lettuce crops were often a financial loss to the grower. Many methods are available for the survival of pathogens from one season to another in the soil or on plant debris. (Waksman 1944, Stover 1959, Garrett 1959). Thus,it could be speculated that one of several methods (viz. survival on crop or other organic matter of the soil,survival as an active soil saprophyte, or as dormant mycelium within the soil) may be available for the survival of S. botryosum from one season to another. This organism also forms perithecia which have been considered by some authors, for example Petzer (1958), to be the bodies responsible for the survival of Stemphylium botryosum from

season to season. Alternatively survival may be by means of dormant asexual spores present in the soil, or conidia produced from lesions on other hosts. These methods of survival would all be consistent with field observations on the occurrence and first appearance of S. botryosum on lettuce.

Neergard (1945) has recorded spores of S. botryosum from lettuce seed. Thus survival and introduction into a particular area may be by seed-borne spores, but normal survival by this method is not consistent with the observations made during field surveys.

Investigations were undertaken to determine by what methods S. botryosum may survive from season to season to reinfect crops during the late autumn.

Investigations

Due to the manner of infection observed on winter crops grown in the same area for several seasons the soil was suspected as being the main source of primary infections. Investigations were therefore first directed to determine whether or not S. botryosum survived from season to season within the soil and, if so, in what form.

During investigations on the host range of isolation from lettuce it became apparent that survival may occur on various weeds if the fungus could exist during the summer months on these hosts. A study of this method of survival was also undertaken.

In summer S. botryosum is rarely seen on harvested lettuce heads. However, during the summer period 1960-61, leaf spots caused by S. botryosum were often located within lettuce crops. Thus a third type of survival, viz. on the host plant, was investigated.

Perithecial productions occurred freely on straw placed in artificially inoculated soil. It was considered that survival in this form may also occur from season to season. Consequently development of perithecia also received some consideration.

Materials and Methods:

(a) Investigations on survival within the soil:

(i) Direct infection from soil in situ.

To determine if plants growing in artificially inoculated soil and soil collected from a heavily diseased block of lettuce would develop infections if the leaves made contact with the soil surface, a series of seed boxes measuring 7" x 12" x 1½" were half-filled with three types of soil:-

(a) Sterilized soil

(b) Sterilized Soil inoculated with diseased leaf tissue

(c) Soil collected from a diseased block of lettuce.

One dozen seedling lettuce plants were planted in each box. It was observed by chance that where soil from (b) or (c) was scattered on the leaves, infection by S.botryosum or areas later colonised by S. botryosum would occasionally occur. This was investigated further.

(ii) Direct Infection from Soil Applied to Leaves:

Samples of soil were collected from the cultivated zone

of several lettuce crops in the Otaki district. Samples from one particular crop heavily infected with S. botryosum were collected on 7 and 14 September during harvesting, on 21 September after harvesting, and on 4 October after the area had been deeply ploughed. A sample was also collected on 21 April the following autumn.

Samples of the soil were sieved through a fine wire mesh sieve and held in a plastic bag to prevent loss of moisture. Similar quantities of sterilized soil were prepared using the same method.

Prior to inoculating with the soil, the plants were sprayed to the point of run-off with sterile water. Plants in one pair of boxes were then lightly dusted with the soil from the field, and the other pair with the sterilized soil. After 24 hours in the high humidity cabinet, the boxes were labelled using a code and placed in random order on the glasshouse bench. After one week in the glasshouse the plants were washed and any leaves with lesions were removed and placed in a petri dish lined with moist filter paper. This induced the formation of conidia if S. botryosum was present. Individual spores were removed and cultured on P. D. A. to confirm the identity of the fungus. Some of the cultures so produced were used for production of conidia which were later inoculated on to lettuce.

This series of experiments did not give conclusive

evidence of the method of survival in the soil and further attempts were made to ascertain by what method survival occurred.

(iii) Dilution Plate Method:

This method, described by Garrett (1951), was used in an endeavour to isolate S. botryosum from various types of soil. One gram samples of air dry soil were shaken with 9 ml. of sterile water. These suspensions were diluted serially five times and 1 ml. alaquates were plated on to P. D. A. and Prune Agar. Duplicate series were made on to each media and eight samples of soil were used. Four samples were taken from the soil obtained from the block of lettuce infected with S. botryosum at Otaki, andfour from artificially inoculated soil maintained in the glasshouse. Plates with 20 - 30 colonies were selected for further culturing and identification work.

(iv) Soil Plate Method:

Warcup (1950) devised this method for isolation of soil fungi. It is said to record more species than the previous method and favours the medium to fast growing fungi (Warcup 1959). 0.1 - 0.2 grams of soil were distributed evenly over the surface of P. D. A. and Prune Agar in 90 mm. petri plates. These were then incubated at 24°C. Twenty samples of the soil from the Otaki property and twenty samples from the artificially inoculated soil were used. As cultures similar to Stemphylium developed, they were removed on to P. D. A. for continued growth and identification.

(v) Immersion Techniques:

A modified method of Mueller and Durrell (1957) using

individual capillary tubes was investigated. Capillary tubes were prepared by drawing out ¼" glass tubing over a bunsen burner. When cool these tubes were filled with P. D. A., using a vacuum pump, and plugged with cotton wool. 24 tubes were inserted into artificially inoculated soil at depths of from 0.5 - 10.0 c.m. A similar set of tubes were inserted at varying depths into the soil sample from the Otaki property. Both soil samples were held in deep trays on the glasshouse bench. The capillary tubes were broken immediately before insertion into the soil to ensure that the P. D. A. was sterile.

On every second day for one week, eight tubes were removed from the soil. The tips of these tubes were broken off and placed on Prune Agar. As fungi grew out from the tubes hyphal tips were removed and transferred to P. D. A. for subsequent growth and identification.

(vi) Hyphal Isolation Technique:

1. From Soil Particles:

Warcup (1955) developed this technique after observing that fungal hyphae remain in association with the larger soil particles after shaking a soil and water suspension together and removing the finer suspended material. Using a binocular microscope and 90X magnification, soil particles were broken apart and hyphae removed using a mounted needle and forceps with a very finely ground point. These hyphae were plated on to

prune agar until growth commenced. Hyphal tips of colonies considered to be similar to S. botryosum were removed and plated on to P.D.A. for subsequent growth and identification. Artificially inoculated soil was used.

2. From organic matter:

This was a modification of Warcup's method. It was observed that much of the organic material was lost when the suspended material was poured off from the larger soil particles. The organic material was removed from the soil by shaking the soil with water and removing the material which floated to the surface. This material, after several washes in sterile water, was examined in a similar way to the soil particles described above. Both artificially inoculated soil and soil from the Otaki property sample were used.

(vii) Isolation of Spores from Soil Samples from the Field:

The method described by Ledingham and Chim (1955) was used to determine if the soils causing infection when dusted on to lettuce plants contained appreciable numbers of spores of the Stemphylium type. A thick paste of the air-dry soil was prepared using a mineral oil of medium viscosity. This was shaken with water until an emulsion formed and then stood aside for several minutes. The larger spores present in the soil separated out with the oil as it rose to the top of the test tubes. Samples were pippetted on to slides and microscopically examined for spores of the Stemphylium type.

(viii) Straw Burial Technique:

This method, originally devised by Sadasivan (1939) and adapted for laboratory or field work by Garrett (1956) was used on soil samples from the field and also in artificially inoculated soil seven months after inoculation. Barley straw, autoclaved for 10 minutes at 15 p.s.i., was buried in the various samples of soil for a period of six weeks, during which time they were kept moist on the glasshouse bench. After this period, the decomposing straw was removed to the laboratory for examination. Some samples were subjected to 100% relative humidity in petri plates lined with filter paper soaked in 1% glucose solution. Other samples were examined under the microscope for typical fungal structures, particularly in the central cavity of the straw. Any perithecia which developed were removed, and subsequently used to inoculate plates. Ascospores likewise often helped identify the perithecia and were used as inoculum for plates.

(ix) Lettuce Burial Technique:

This method was a modification of that described above, using lettuce leaves in place of straw to determine if any selection of fungi occurred. Unfortunately decomposition of the leaf was too rapid to permit it to be of much value for subsequent production and isolation of perithecia. If after burial the leaf was subjected to high relative humidities to induce conidial production, bacterial rots quickly developed and usually prevented sporulation of fungi.

(x) Survival on Dried Host Material:

To induce dormancy and observe how long the dormant

mycelium remained viable, diseased lettuce leaves were collected from artificially inoculated plants on 12 February 1960. At regular intervals for fourteen months samples of these dried leaves were removed from the envelopes in which they had been stored in the room adjoining the glasshouse and placed on glass slides in petri plates lined with moist filter paper. Observations were made after twentyfour hours, andlater, if necessary, to determine the extent of sporulation, and again after seven days to determine if perithecia were forming.

(b) Investigations on Survival on other Hosts:

(i) Field Surveys:

During visits to properties where lettuce were grown, a collection of leafspots typical of Stemphylium species was made from weeds and other vegetables. The lesions were cut out from the leaf and subjected to 100% relative humidity as described elsewhere. After 24-36 hours, the lesions were examined under the binocular microscope for spores similar to Stemphylium Spp. Samples of these spores were removed individually on to P. D. A. for culturing and subsequent identification. Conidia from the cultures identified as S. botryosum were inoculated on to a group of six lettuce plants, and three plants were inoculated with conidia known to be pathogenic. The plants were labelled, held in a high humidity cabinet for 24 hours then removed to the glasshouse bench until expression of symptoms occurred on the three plants used as "infection controlls". A similar group of three control plants were sprayed with sterile water and moved with the other

inoculated plants. Similar pathogenicity tests were used on the original host if the isolate was pathogenic to lettuce. This method was also used in the later investigation on headland weeds.

(ii) Host Range Studies:

Conidia from cultures of S. botryosum isolated from lettuce were inoculated on to a wide range of plants reported as being a host of this fungus. Details of this investigation concerned with the taxonomy of the New Zealand isolate from lettuce, are given in the Chapter of this study devoted to "The Fungus" (see p. 59). Consideration is also given to this here because of the possibility of survival on other types of hosts.

(iii)Headland Weeds:

A thorough collection of all types of leafspots found about the headlands of market gardens in the region of the previous season's winter lettuce were made during summer 1960/61. Lesions collected were subjected to a high humidity and conidia obtained as described previously. Cultures of S. botryosym were grown and conidia used to inoculate lettuce plants in the glasshouse. Details of inoculations are similar to those used in Method B 1 above.

(c) Investigations of Survival on Lettuce:

Lesions caused by S. botryosym on lettuce were often found to be present in late spring and early summer. Frequent visits during December, January, February and March, were made to

lettuce crops about Palmerston North and Otaki to determine the incidence of the disease during summer.

(d) Investigations on Survival as Perithecia and Ascospores:

During investigations on the isolation of S. botryosum from soil using straw, it was observed that perithecia of S. botryosum were frequently present. Observations were therefore made on the development of perithecia and on the production and discharge of ascospores.

(i) Development of Perithecia:

The development of perithecia was observed by incubating infected dried leaf material at 24°C. and following the formation of perithecia from first appearance until ascospores were formed.

(ii) Discharge of Ascospores:

During the previous experiments already discussed, the effect of temperature on the formation of perithecia was observed (See P. 41).

Observations on the effect of various environmental conditions on discharge of ascospores were made using artificial cultures on lettuce decoction agar. On this medium it was observed that the majority of perithecia produced mature ascospores after 8 weeks' incubation at 24°C. Pairs of cultures of this age in petri plates were subjected to the various treatments outlined below for one week.

1. Laboratory temperature (58°F - 84°F.) indirect light with lids remaining in place.
2. As for 1, but plates were not exposed to light.
3. Exposed, on a window ledge facing north, to direct

sunlight. Petri plate lids were not removed.

4. Exposed to alternating periods of cold and warmth by subjecting the cultures to 0°C. for 24 hours followed by at 24-hour period at 24°C. No light was provided. Plates were subjected to this treatment for seven days.
5. The plates were buried in the soil so that the lids were flush with the soil surface. Minimum temperatures on the soil surface were as low as 40°F. during the week the plates were in position, and during the day they were subjected to light and heat from the sun.
6. One pair of control plates remained at 24°C for a period of one week.

To determine what type of spore discharge occurred, the lids of these petri plates were coated with a thin layer of P.D.A. If spores had been liberated by expulsion, colonies would develop on the P.D.A. above the perithecia. A similar technique illustrated by McDonald (1958) suggested this method.

Pathogenicity tests on lettuce were carried out with ascospores, and with conidia derived from cultures originating from ascospores.

Longevity tests on ascospores from perithecia produced on artificial cultures and held at laboratory temperatures were performed at regular intervals for five months. After this period of time observations were discontinued owing to mite infestation of the colonies.

Results:

(a) Results of Investigations on Survival Within the Soil:

(i) Direct infection from the soil in situ:

Results obtained from this experiment showed conclusively that both naturally and artificially inoculated soil are capable of transmitting the fungus to healthy plants growing in the soil. Observations led to the development of the next technique.

(ii) Direct infection from the soil applied to leaves:

Typical results obtained with this method are recorded in Table 16 , using a naturally infected soil. Soil samples taken from the same area on 4.10.60 after the diseased crop had been ploughed in failed to cause any infections yielding S. botryosum. Details of the other results of other investigations using this method are recorded in Table 17 . Conidia from cultures originating from lesions developed in this experiment were pathogenic to lettuce in all instances tested.

(iii) Dilution Plate Technique:

No culture of S. botryosum were isolated from dilution plates using P.D.A or Prune Agar when either naturally infected soil or the artificially inoculated soil of the glasshouse was tested. The main disadvantage of this method was that spreaders quickly over-ran any other fungi present.

(iv) Soil Plate Method:

Several cultures were obtained from various plates which possessed Stemphylium like characters but it was later found that no S. botryosum had been isolated using this method.

(v) Immersion Techniques:

Fungi similar to S. botryosum were isolated but subsequent

Table 16	Infection Resulting When Naturally Infected Soil Was Dusted on to Leaves of Healthy Lettuce Plants.											
Date Soil Collected												
Plant No.	Box A	Box B	Control A	Control B	Box A	Box B	Control A	Control B	Box A	Box B	Control A	Control B
1	0	0	0	0	1(-)	2	0	0	(7-)	1	0	0
2	(-)	0	0	0	0	0	0	0	1	1	0	0
2	0	0	(-)	0	1	1	0	0	2	3	0	0
4	2(-)	0	0	0	0	(2-)	0	0	2	3	0	0
5	0	1	0	0	0	2	0	0	0	0	0	0
6	2	0	0	0	2	0	1	0	0	3	0	0
7	(-)	(-)	0	(-)	(-)	0	0	0	0	1	1	0
8	0	0	(-)	0	1(-)	1	0	0	3	0	0	0
9	0	1	0	0	2	1	0	0	1	1	0	0
10	(-)	0	0	*	0	1	0	0	0	0	0	0
11	0	0	0	0	(-)	(-)	0	0	3	0	0	1
12	0	0	0	0	0	1	0	0	0	1	0	1
Total *S. botryosum* infected lesions	4	2	0	0	7	9	1	0	12	14	1	2

* Plant missing.

(-) Lesions from which *S. botryosum* was not isolated.

TABLE 17 Results of testing Various Soil Samples for Presence of S. Botryosum Using Soil Dusting Technique

Description of Soil Sample:	Date Collected*	Date Observed	Results
1.Wing Property,Block I, Lettuce heavily infected	7.9.60	17.9.60	Few infections occurred.
2. "	14.9.60	22.9.60	Infections present.
3. "	21.9.60	29.9.60	Infections present.
4.Wing Property Block I, Lettuce heavily infected during previous winter	21.9.60	29.9.60	No infection present
5. Wing Property,BlockIII, No lettuce for seven years	21.9.60	29.9.60	No infection present
6.Young Property. Lettuce heavily infected	21.9.60	29.9.60	Infection present
7.Wing Property Block I Lettuce ploughed under	4.10.60	16.10.60	No infection present
8.Soil artificially inoculated 22.9.60			
(a) After 6 weeks	2.11.60	14.11.60	Infection present
(b) After 12 weeks	15.12.60	2.12.60	Infection present
(c) After 6 months	20.3. 61	30.3.60	Infection present
9.Wing Property Block I Autumn following winter crop	21.4.61	1.5.61	No infection present

* Soil samples were collected and inoculated on to plants on the same day.

identification proved these were not S. botryosum. They were not pathogenic to lettuce. Spreading type colonies usually developed from the capillary tubes. These fungi tended to mask other types and very soon swamped the entire plate.

(vi) Hyphal Isolation Techniques:

1. From soil particles:

Of the thirty four plates of prune agar, all inoculated with five fungal hyphae, four Stemphylium spp. were isolated of which three were considered to be S. botryosum. All three isolates proved to be pathogenic to lettuce. The soil particles were from the artificially inoculated soil.

2. From Organic Matter:

Fifty samples of hyphae were obtained from the organic matter of both artificially inoculated soil and soil from the Wing property, Otaki, and several Stemphylium-like cultures were obtained. Only one isolate was pathogenic to lettuce. This isolate obtained from the soil from the Otaki property and later identified as S. botryosum was only weakly pathogenic to lettuce. It varied from other isolates of S. botryosum, producing abundant conidia on a sparse mycelium, a property retained after re-isolation from the lesions of the host.

The hyphal isolation methods, though successful, were very tedious and hard on the eyes. Because S. botryosum was isolated in very small numbers, this

method was not considered suitable for routine detection of S. botryosum in soils.

(vii) Isolation of Spores from Soil Samples:

Very few S. botryosum type spores appeared, although other types of large spores were commonly present. There was no way of proving the identity of the spores observed. However, S. botryosum type spores were present so infrequently that this method of survival in the soil samples used does not appear to be of importance.

(viii) Straw Burial Technique:

1. Sporulation from Straw:

Fungi similar to Stemphylium species were frequently found sporulating on the straw after high relative humidity treatment. Some species sporulated sufficiently to enable collection of spores and preparation of slides to be made for microscopic observation. However, S. botryosum was not a prolific spore producer on the straw. Cultures of S. botryosum pathogenic to lettuce were obtained by spore isolations, and culture on P.D.A. only from straw buried in artificially inoculated soil. Four of the cultures were selected and the conidia used to inoculate lettuce. All isolates proved pathogenic. No cultures of S. botryosum from the soil sample obtained from Otaki were pathogenic.

2. Perithecial Production:

Perithecia were frequently found within the cavity of the straw. By removing these and placing them

on P. D. A. colonies developed and sporulated. Individual conidia were removed and used to establish pure cultures. Mature perithecia were squashed on a microscope slide and ascospore suspensions were placed on prune agar. The small colonies developing on prune agar were transferred to P. D. A. for culture and identification. Those colonies identified as S. botryosum were sub-cultured and conidia from four selected isolates were inoculated on to lettuce. One of these isolates proved pathogenic. Of another group of four isolates, all proved to be pathogenic to lettuce. No perithecia from the straw buried in the Otaki soil samples produced S. botryosum.

(ix) Lettuce Burial Technique:

Due to rapid decomposition in the soil and bacterial decay during incubation in the laboratory, it was found that this method was impracticable.

(x) Survival on dried host material:

The fungus in the dormant state remained viable for a period of fourteen months on the dried host material. Results of observations are recorded in Table 18 . During the early period of these observations sporulation was prolific when dried leaves were held for 24 hours at 24°C. in 100% relative humidity. After nine months sporulation from the dried leaves started to decline and was confined chiefly to small areas on the leaf. Perithecial production likewise decreased, but after fourteen months some spores and immature perithecia were produced from the dormant mycelium indicating that the fungus was capable

Table 18 Survival of Stemphylium botryosum as Dormant Mycelium in Dried Leaf Material.

Date:	Remarks:
12.2.60	Diseased leaves removed from artificially inoculated plants and placed on glasshouse bench until dry. Held in envelopes in shed attached to glasshouse.
20.4.60 20.6.60	Prolific production of conidia after 24 hours' incubation in 100% relative humidity at 24°C., followed after 7 days by immature perithecia
10.8.60	Spore production variable on different leaves.
22.9.60 12.10.60	Prolific production of conidia and perithecia.
17.11.60 22.11.60 8.12.60	Prolific sporulation from some lesions; other sporulation sparse. Only one quarter of the samples now produced conidia over complete leaf surface.
7.1.61 14.2.61 21.4.60	Variable production of conidia on different leaves and on different regions of the same leaf. Production of perithecia not as prolific as formerly. Observations discontinued.

of surviving on the dried host material for this period. Observations were discontinued after fourteen months.

Conidia were used to produce cultures which sporulated freely after exposure to sunlight of ultra violet. All isolates tested were pathogenic to lettuce and the conidia so produced were often used to obtain new cultures of S. botryosum for pathogenicity tests.

(b) Results of Investigations of Survival on Other Hosts:

(i) Field Surveys:

The following crops and weeds were found to possess lesions from which S. botryosum was isolated. Each isolate was inoculated on to lettuce after a pure culture was obtained from single conidia.

Table 19 Pathogenicity of S. Botryosum Isolated From Hosts During Field Surveys

Suspected Host:	Pathogenic to lettuce:	Pathogenic to Host:	Isolate Number
Silver Beet (Beta Vulgaris L.)	No	-	U23C
Sow thistle (Sonchus oleraceus L.)	Yes (Weakly)	Yes (Weakly)	U29B
Prince of Wales Feather (Amaranthus retroflexus L.)	Yes	No	U37B
Onion (Allium cepa L.)	No	-	U65A
Tomato (Lycopersicum esculentum Mill)	No	-	U87B

Isolate U23C from silver beet was not pathogenic to lettuce, although morphologically similar during artificial culture on P. D. A.

After inoculating the sow thistle isolate, U29B on to lettuce, small lesions occurred which failed to continue developing. Pricking the lettuce prior to inoculation did not induce any better manifestation. An isolate from the same area on lettuce, U28A, while pathogenic to lettuce,

infected the sow thistle only if the leaves were pricked. Lesions then developed to a maximum size of 3 mm. Isolates normally pathogenic to lettuce did not infect sow thistle plants which were not wounded. Isolate U29B was less vigorous on P. D. A. media than normal lettuce isolates, appeared to be darkly pigmented, and produced abundant perithecia.

Isolate U37B was identical to isolates from lettuce in all respects. Reinfection of A. retroflexus was not successful despite several attempts.

Isolates U65A from onion was not pathogenic to lettuce. It produced identical colonies on P. D. A. to isolates from lettuce butconidia at first similar to those found on lettuce isolates, when subjected to high humidities proliferated or "budded" in one or more directions to give a complex shaped spore.

Isolates U87B from tomatoes was identical to isolates from lettuce on culture media, but failed to be pathogenic to lettuce.

(ii) Host Range Studies:

Of the widely different types of hosts inoculated with isolates during host range studies Zinnia elegans and Solanum nigrum were the only hosts which S. botryosum isolates pathogenic to lettuce repeatedly infected. Zinnia plants did not support vigorous sevelopment of lesions. However the lettuce isolates readily infected the older leaves of S. nigrum but no infections were recorded from the younger leaves. Reisolation of the fungus from the S. nigrum plants and

culturing to produce conidia demonstrated that this fungus was morphologically similar to the lettuce isolates in every way. It would also reinfect either lettuce or S. nigrum plants.

This observation led to a thorough examination of weeds occurring on the headlands, as a separate project from the field surveys.

(iii) Headland Weeds:

S. botryosum was isolated from lesions occurring on the weeds indicated in Table 20 which were found growing on headlands during January 1961 and April 1961.

Table 20 Pathogenicity of S. botryosum Isolated from Headland Weeds

Host:	Pathogenic to Lettuce:	Pathogenic to Host:	Isolate No.
Solanum nigrum L Solanum nodiflorum Jacq (Black nightshades)	Yes	Yes	U79 U83 U92 U93 U94
Polygonum persicaria L (Lady's thumb)	No	-	U89
Sonchus oleraceus L (Sow thistle)	No	-	U84

During January, isolates of S. botryosum were only obtained from S. nigrum. Lesions were present on the older leaves close to the ground and were small and zonate, often without centres. During April, S. botryosum was recorded from two other weeds but

these isolates were not pathogenic to lettuce. S. botryosum also occurred on S. nodiflorum Jacq. and isolates from this species were pathogenic to lettuce. During the latter survey, the majority of the mature leaves of both species had large lesions producing conidia of S. botryosum. In culture, the isolate from Sonchus oleraceus was similar to the previous isolate from this species. Isolate U89 (from Polygonum sp) produced cultures typical of the lettuce isolates but with rather more grey aerial mycelium. All cultures from Solanum nigrum and S. nodiflorum, were indistinguishable from the lettuce isolates.

(c) Results of Investigations on Survival on Lettuce:

During summer occasional lesions caused by S. botryosum were present in most lettuce crops, particularly those which were sheltered with weed growth. Lesions were seldom seen on the marketable heads, but were confined to the outer leaves in contact with the soil. Normally lesions in this position are overlooked during observations of lettuce crops. All isolates which were tested after subculturing in the laboratory on P. D. A. proved pathogenic to lettuce.

(d) Results of Investigations on Survival as Perithecia and Ascospores:

(i) Development of Perithecia:

The first indication of perithecial formation was when knots appeared in the mycelium ramifying over the surface of the leaf. After 72 hours incubation at 24°C. these bodies, first observed at 24 hours, had developed into translucent spheres, apparently

composed of aggregated hyphae. After seven days, the young bodies had become pigmented and measured up to 320 µ in diameter. Growth continued at a reduced rate and after 14 days the young perithecia, now possessing a short neck, were completely black and beginning to harden.

Asci were observed in crushed or sectioned perithecia from the time pigmentation commenced. One or two celled ascospores were present in some perithecia from the second week. After incubation at 24°C. for three weeks some ascospores were observed which appeared mature, but the majority of perithecia contained asci with apparently mature ascospores during the sixth week. At this stage, perithecia measured from 320 µ - 2000 µ in diameter. Of 50 perithecia measured at this stage, the majority were between 375 - 450 u in diameter.

(ii) Discharge of Ascospores.

Discharge of the spores occured during fluctuating environmental conditions. When plates were exposed to extremes of temperature for one week, (Treatments 3, 4 and 5) an estimated 80% of the perithecia from artificial cultures discharges spores. With smaller temperature variations, (Treatments 1 and 2) spores were discharged from between 30 - 50% of the perithecia. Where the cultures were held at 24°C (Treatment 6) a very small number of perithecia were induced to discharge spores.

The manner of spore discharge appeared to be comparatively passive. No ascospores were ejected on to the agar placed

above the perithecia. Masses of spores merely extruded from the ostiole of the perithecia and remained bound together in a mucilagenous material, forming round shiny black balls on the tip of the necks of the perithecia. (See illustration 22). These disintegrated if they were allowed to dry out or when they were splashed with simulated rain.

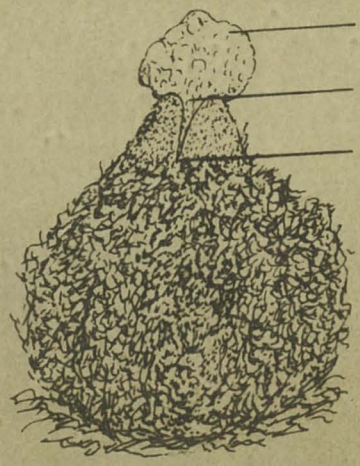
All suspensions of ascospores obtained from isolates from lettuce were pathogenic to lettuce. Conidia produced from cultures derived from single ascospores also produced typical lesions on lettuce plants.

Perithecia held at laboratory temperatures or at 24°C for periods of up to five months contained viable ascospores, providing no desiccation of the colonies had occurred. If the culture became dehydrated, perithecia collapsed and ascospores were not viable, or in many cases present.

ILLUSTRATION 21

Infection due to aerial dispersal of conidia
from smaller leaf.

ILLUSTRATION 22 DISCHARGE OF ASCOSPORES FROM PERITHECIUM



Mature Perithecium.

1mm.

Discussion.

Detailed comparative studies of methods used for isolation of Stemphylium botryosum from the soil, where it was suspected of spending the summer months in the absence of crop plants, were not undertaken. However, it has been possible to make some evaluation of the methods used.

The soil dusting technique was successful in the isolation of the organism from artificially inoculated soil at all times, but results of its use on naturally infected soils have been inconclusive. This may have been due to insufficient sampling or loss of the organism in a virulent form due to its low competitive saprophytic ability (Garrett 1950).

Dilution plate, soil plate, and immersion tube methods failed to isolate S. botryosum from the soil. Samples used may have been too small, but the main disadvantage of these methods was that spreaders were invariably present which swamped any slower growing fungi.

If sufficient samples were taken the hyphal isolation technique on either soil or organic matter particles may be a reliable method for isolation of hyphae of S. botryosum from the soil. Unfortunately the microscope work involved is very tedious and is a distinct disadvantage if this method were to be used for routine isolation.

Isolation of spores from soils is only of advantage if the spores are very distinctive. Unfortunately individual S. botryosum spores are indistinguishable from many other species. This method did not permit subsequent culture of the spores observed.

The straw burial technique was considered to be the most suitable for isolation of this organism from the soil. Fortunately S.botryosum conidia are produced after 24 hours' exposure to 100% relative humidity and providing observations were made during this period isolation of this organism was not difficult. However, after this period many types of spores similar to Stemphylium species developed and identification of S. botryosum then involved considerable numbers of isolations and much culturing.

Perithecia in the cavity of the straw were readily discernable and easily cultured. Identification of perithecia with mature ascospores was rapid. Because several other types of perithecia were present and very similar in their immature stage to those of Pleospora, identification at this stage was carried out by culturing. Otherwise preliminary identification was made using ascospores, and single spore cultures established.

Survival appears to occur from season to season in the soil. Lesions in the field were usually first observed on the leaves of the plants in contact with the soil. Due to infection resulting after artificial inoculation with infected soil, it

is possible that cultivation processes which throw soil over the plants may help to provide primary inoculum. Due to the absence of appreciable numbers of spores in the soil samples used to inoculate the plants, there was little possibility of soil born spores acting as inoculum. Graham and Zeiders (1960) illustrated the ability of the Stemphylium species to survive in the soil by maintaining their stock cultures on sterile soil.

In the glasshouse the presence of S. botryosum could be detected on artificially inoculated soil samples after six months, but detection in field soil was not possible six months after a heavily infected crop was ploughed under. This may have been due to insufficient samples of the field soil being taken or perhaps to the low saprophytic ability (Garret 1950) of this organism. Soil sterilization may have allowed this organism to become well established in the absence of other saprophytes and may explain why some methods were more successful in isolating S. botryosum from the artificially inoculated soil, compared with soil from the field.

Successful isolation of fungal hyphae present in the larger soil aggregates suggests that S. botryosum may exist either as an active or dormant soil saprophyte, during summer. Such an existence would be in agreement with findings of Petzer (1958) and Graham & Zeiders (1960). The former author was able to isolate S. botryosum from the soil after a period of three years. Due to the ability of this organism to survive in the soil, Petzer has concluded that mycelium in the soil is the most

important survival method. Mycelium may retain its viability for some time when it is dormant. These studies have shown that dormant mycelium may survive for periods in excess of 14 months.

S. botryosum rapidly colonised decaying straw. It appears that this organism may readily colonise organic matter during the early stages of decomposition. It was on this material that perithecia were observed. Temperatures above 60-70°F. favour the production of perithecia and fluctuating temperatures favour the discharge of ascospores. Petzer (1958) has concluded that ascospores as well as mycelium in the soil may be responsible for primary infections. It appears that this may also be so in New Zealand.

Padhi and Snyder (1954) consider that survival occurs on decaying host material. They also present evidence that perithecia, ascospores, and conidia are capable, under laboratory conditions of survival for periods in excess of those between two seasons. Similar evidence of the period of survival has been obtained during this study.

In New Zealand lesions caused by S. botryosum have been recorded on lettuce throughout the year. Such lesions, during favourable conditions produce conidia which may serve as potential sources of primary inoculum.

Host range studies and field observations led to a

thorough investigation of weeds on headlands about areas previously containing infected crops. Conclusive evidence has been obtained to demonstrate that S. botryosum may survive during summer on weeds about headlands. The black nightshades (Solanum nigrum L and S. nodifolium Jacq) were the only type of weed which was consistently involved. Although S. botryosum was isolated from lesions on other plants, some isolates were not pathogenic to the host from which they were isolated suggesting that the S. botryosum infection may have been secondary to some other damage.

Conclusions.

Several methods for survival of S. botryosum from season to season appear to be available in this country. This organism may survive on crop debris or other material in the soil, later to infect lettuce crops due to release of ascospores, conidia, or by growth of mycelium from the soil on to the plant's leaves.

It may also survive from season to season as lesions on the lower leaves of summer lettuce crops, or on weeds, in particular the black nightshades. This is the first record of survival of the fungus which is pathogenic to lettuce on another host and it is considered to be an important means of survival in the districts in which these studies were made.

B. MISCELLANEOUS FACTORS & THEIR INFLUENCE ON THE DISEASE CYCLE

1. Predisposition

Yarwood (1959) has considerably clarified the concepts of predisposition. He defines predisposition as "the tendancy of nongenetic conditions, acting before infection, to affect the susceptibility of plant to disease". Environment affects disease through its direct effect on the pathogen, through its effect on the potential host, or through its effect on the interaction of the host and pathogen. (Yarwood 1959). The first category has already received consideration in the previous sections. The second constitutes predisposition, while the third is sometimes difficult to separate from this. Thus the effect of light on infection is considered to be excluded by definition from predisposition, due to this factor operating during infection and penetration. The effect of light has been discussed previously in the relevant section of the disease cycle. Other factors which have been considered by various authors to predispose the plant to infection include temperature and frost injury (Moore 1944, Kerling 1952), relative humidity (Allen 1957), and age of plant (Padhi & Snyder 1954). These factors all appeared to be potentially important. Due consideration has been given to these factors which may predispose the plants to infection.

Materials and Methods.

To Quote

"Experimentally, predisposition can be clearly demonstrated only by exposing similar healthy plants to contrasting conditions.

then placing them all in the same environment, and inoculating them..... . If the experimental preinoculation treatment causes differences in disease, predisposition may be said to have occurred. While predisposition has usually been demonstrated by this type of experiment, it can also be logically inferred, although not so safely, from less exacting evidence." (Yarwood 1959)

(a) Temperature predisposition.

A group of 80 plants, excluding controls, were arranged in four groups of twenty plants per group according to the type of plant, viz. two groups made up of small hard plants, one group of larger soft plants and an intermediate group. Each block of twenty plants was subdivided into five groups of four plants at random, there being five temperature treatments and four plants per treatment in each block. All plants were a similar age.

After labelling the plants were subjected to -10°C in an icebox of a refrigerator, 40°, 50°, 60°, & 70°F for twenty-four hours, all plants remaining in the dark. On removal the plants were pladed on the glasshouse bench for 30 minutes to regain a similar temperature before inoculation.

Unfortunately the -10°C was too severe for most of the plants and although inoculated, this group of plants were not recorded along with the others due to plant damage occuring and possibly introducing a bias when results were recorded.

This experiment was then repeated at 30°, and 50-80°F using two groups of twenty plants. The 50-80°F treatment was

carried out on the glasshouse bench in the box with light traps. This second experiment was designed to find out if frost conditions which the plants could withstand would predispose them to infection.

Results were recorded by counting the number of lesions per plant in the normal manner.

TABLE 21 Effect of Temperature on Predisposition to Infection. (No. of lesions)

Part I	Temperature * 40°	50°	60°	70°	Total
Block I	93	307	164	102	666
II	47	95	70	28	240
III	112	104	58	97	371
IV	65	101	96	115	377
	317	607	388	342	1654

Results of Analyses (F. Test.) Differences between treatments not significant, Block II (Plants hard and small) almost significantly different, P = 0.05 C = 47.5%

Part II	Temperature -2°C	10°-27°C.
Average no. lesions per plant	74.8 ± 50.6	114.5 ± 50.8
Results of Analyses	(T test) Differences not significant p = 0.05	

* -10°C Plant damage too severe to record infections.

(b) Relative humidity predisposition.

Groups of six plants were pricked out into 16 small seed trays and grown on for 3 weeks when they were suitable for inoculation and recording. Four trays were exposed to 100% relative humidity for twenty-four hours. The design used was a 4 x 4 latin square and plants were arranged in this manner during all the treatment procedures and finally on the glasshouse bench while manifestation was awaited.

Observations were made on the time when the first lesions appeared and counts were made of lesions per plant.

(c) Age of plant.

Plants were inoculated at 1, 2, 4, 6, 7 and 10 weeks from germination, and at 3 and 6 months. Groups of twelve plants were used but due to the variation in size of the plants no comparative evaluation of predisposition could be made.

Results of these inoculations were photographed and are recorded in Illustrations 23 & 24

Results.

(a) Temperature Predisposition.

Results tabulated in the Appendix are summarised in Table 21 S. botryosum did not readily colonise the leaves of the -10°C treatment. This may have been due to prior bacterial or fungal infections. Differences between the various temperatures were not significant, but the larger soft plants had a higher

number of infections which barely reach significance at the 5% level. This indicated that the type of plant had a larger effect on predisposing the plant to infection than the temperatures.

(b) Relative Humidity Predisposition.

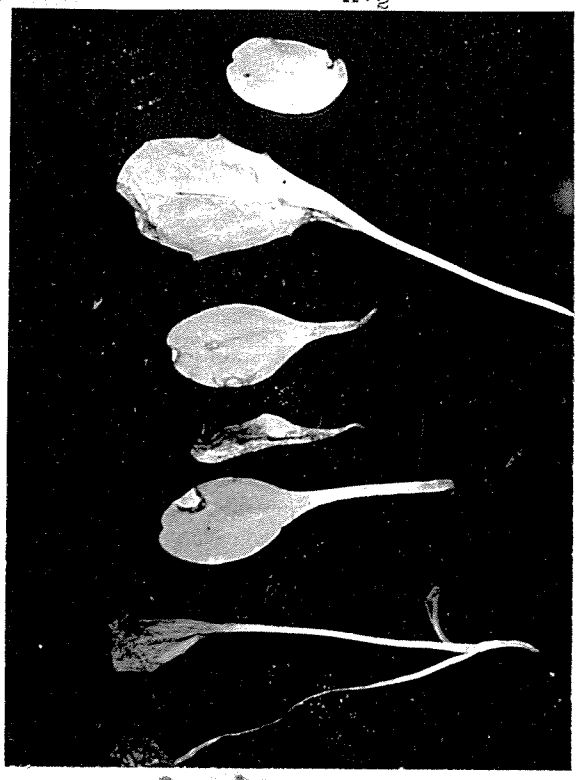
Holding plants in 100% relative humidity failed to significantly increase the plants predisposition to disease. After the results of this trial were analysed the practice of holding plants in 100% humidity was discontinued without any apparent loss or reduction in the incidence of infection.

(c) Age of Plant.

Lettuce plants proved to be susceptible to infection by Sl botryosum from seedling stage until flowering. Results of these studies are recorded in Illustrations. 23 & 24.

ILLUSTRATION 23. Infection of Stemphylium botryosum on plants of various ages. X1½

1 week

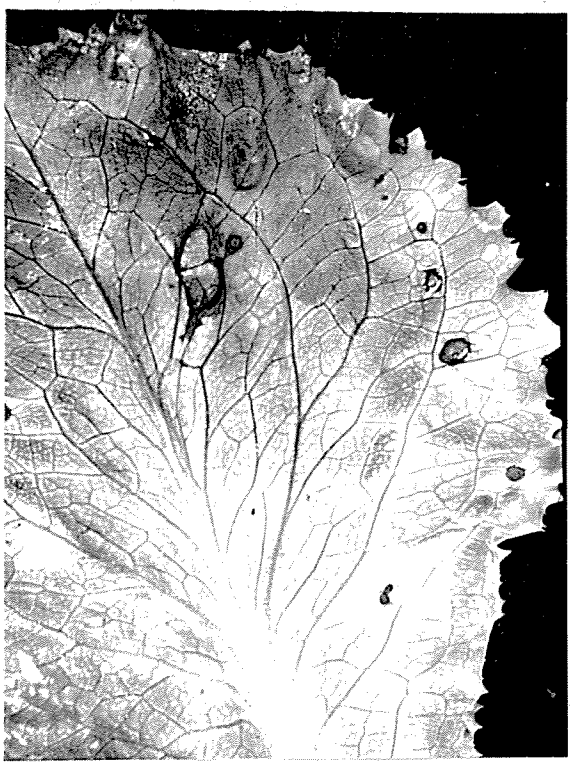


2 weeks

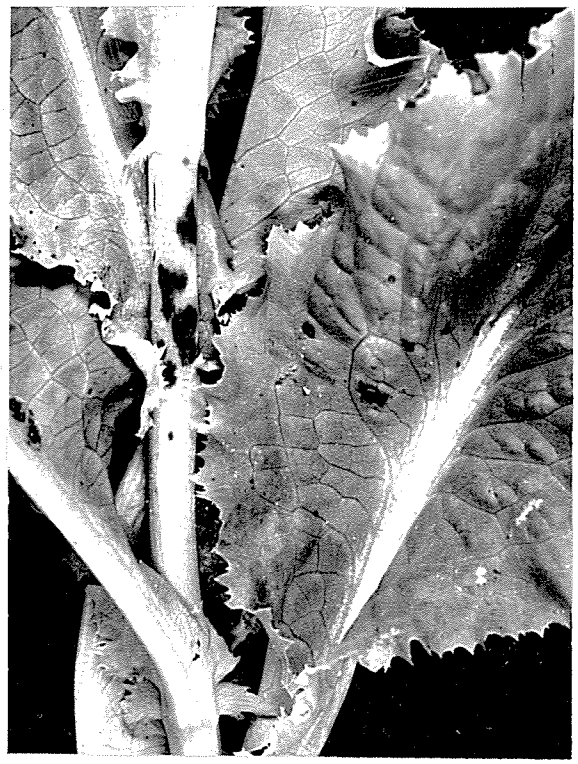
6 weeks

7 weeks

ILLUSTRATION 24. Infection of *Stemphylium botryosum* on plants of various ages. X1½

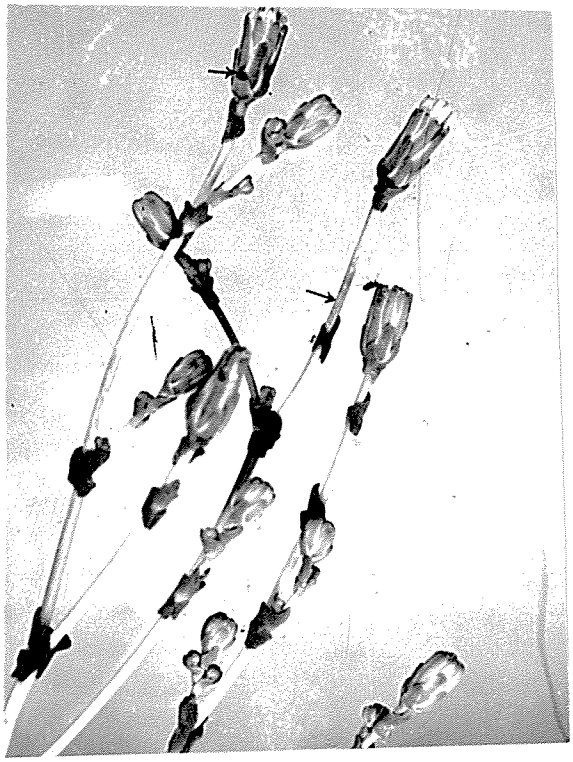


3 months



6 months

6 months



6 months

Discussion

Differences between the low and higher temperatures investigated failed to reach significance but there appeared to be a trend towards the softer type of plant favouring predisposition to infection and subsequent development of disease. Frost failed to predispose the plant to infection.

Prior exposure to high relative humidities was not a pre-requisite for successful infection. Padhi and Snyder (1954) came to similar conclusion.

Susceptibility of the plant does not appear to alter markedly with age. Therefore the plant is not predisposed to infection or non-infection at any period during its life. Padhi & Snyder (1954) state that infection established readily on plants of different ages, but younger leaves were difficult to infect. They concluded that the stage of development of a leaf may have an important bearing on its susceptability. A similar observation was made during these studies. However, investigations revealed that during inoculation very little inocolum remained on these leaves. This was considered to be due to their upright position. Infection would readily develop if one drop of "Teepol" detergent was included with every 250ml. of spore suspension, or if the leaves were turned out flat.

2. Varietal Susceptability

Seedlings, and plants with their lower six leaves at the 'mature' stage, were inoculated with the conidia of S. botryosum. Because small differences were not important this experiment was not treated by statistical methods. The varieties were allocated code numbers and the boxes arranged at random. It was considered that if any winter variety of lettuce was resistant to S. botryosum to an extent likely to be of value to growers, the resistance would be demonstrated on the seedlings in the pairs of trays of each variety or on the eighteen plants inoculated with conidia.

During these investigations the following varieties of lettuce were tested for susceptibility to S. botryosum

1. Great Lakes (ex Ferry Morse)
2. Yatesdale (ex Yates)
3. Imperial 615 (ex Yates)
4. Imperial 847 (ex Yates)
5. Neapolitan (ex Yates)
6. Black Seeded Triumph (ex Coopers)

These were the most important varieties of lettuce grown in the districts surveyed. Black Seeded Triumph and Imperial 615 accounted for all the majority of winter lettuce varieties encountered during field surveys except for one grower who was trying a new Winter Great Lakes variety.

Results

All varieties tested were susceptible to S. botryosum. There was no indication that there was any difference in their susceptibility.

3. Pathogenicity of Isolates

Isolates from lettuce, used to inoculate lettuce plants did not consistently show that any one isolate was more pathogenic than others. Three experiments were carried out on groups of four plants but variations which occurred between these experiments exceeded the variation between isolates. Therefore during the pathogenicity and disease cycle studies it was considered sufficient if one isolate only was used, and when its ability to sporulate diminished it was replaced with another isolate. Records were kept of the isolates used, and within any one series of experiments the same isolate was always used even though it was necessary at times to induce sporulation with ultra-violet light.

4. Inoculum Potential

There is considerable evidence that for some organisms there may be a critical level of inoculum strength which is required before infection will take place. (Gooding & Lucas 1959, Cole and Couch 1958, Garrett 1959) In defining 'inoculum potential', Garrett (1959) states that it is the energy of growth of a pathogen available at the surface of the host for infection, and that there are two ways of altering this. Firstly by increasing the number of units of the pathogen per unit per area, and secondly by increasing the nutrient states of each unit. Because of the nature of the units of infection, occurring in the field and concerned with the build up of the disease to epidemic levels (that is the conidia) the first category only is applicable to S. botryosum.

Experiments were therefore conducted to determine if there was a critical level of inoculum potential.

Methods and Materials.

Ten mls. of concentrated spore suspension was prepared from sporulating colonies on P. D. A. by centrifuging down the spores from suspensions prepared in the normal manner, combining them and adjusting to contain 1×10^{5} spores per ml. This was then serially diluted to 1×10^{-1} spores per ml. During the course of these studies this experiment was repeated on three occassions under similar conditions.

The concentrations of spores from 1 x 10^5 to 1 x 10^3 were checked with the haemacytometer before applying the spore suspensions to groups of ten plants. The lower counts were assumed to be correct if these counts corresponded to the required concentrations.

Results.

See Table 22

Table 22 The Level of Inoculum Required for Infection

Spore Conc. per ml.	Expt. 1	2	3
1 x 10^5	3	3	3
1 x 10^4	2	3	3
1 x 10^3	1	3	1
1 x 10^2	1	1*	0
1 x 10^1	1*	0	1*
1 x 10^0	0	0	0
1 x 10^{-1}	0	0	0

* 1 - 3 spots only

Note: Infection Resulting: 3 heavy, 2 intermediate, 1 low

Lesions from the plants inoculated at the higher two levels consistently were the first to produce signs of manifestation of disease.

Discussion

When inoculum consisted of ten spores per plant on an average, low infection resulted in two out of the three experiments. Therefore as Garrett (1959) concluded in his review of 'inoculum potential', the infectivity of the average air borne conidia appears adequate for infection of the natural host.

These experiments indicate that about 3% of the conidia may cause infection during favourable conditions. During critical conditions no spores may be able to germinate and cause infection if spore numbers are low. However if the inoculum is high it would be expected that some spores would be present which were able to cause infection during marginal conditions. The normal spore concentrations used in experimental work were between 1×10^3 and 3×10^3 spore / ml. and this proved adequate for infection in all experiments.

Gooding and Lucas (1959) found that the level of inoculum greatly influenced the rate of disease infection and the severity of its development. They also reported along with others (e.g. Garnett 1959, Cole and Couch 1958, Bush & Walker 1958) that the actual level of inoculum causing infection varies with the particular conditions prevailing at the time. Both these observations were also made in these studies, indicating that while a single spore may act as potential inoculum, a high level of potential inoculum may cause a more rapid onset of disease. However one would not find that this level of inoculum is

present in nature but a similar effect could be expected with several lower doses providing conditions were favourable for continual disease development. In the field it was observed that once a lettuce crop became diseased, if conditions were favourable for spread of inoculum and disease development for seven days, it was possible during this time to loose the entire crop. This may be due to a process similar to that suggested and observed at high inoculum levels.

C. EPIDEMIOLOGY

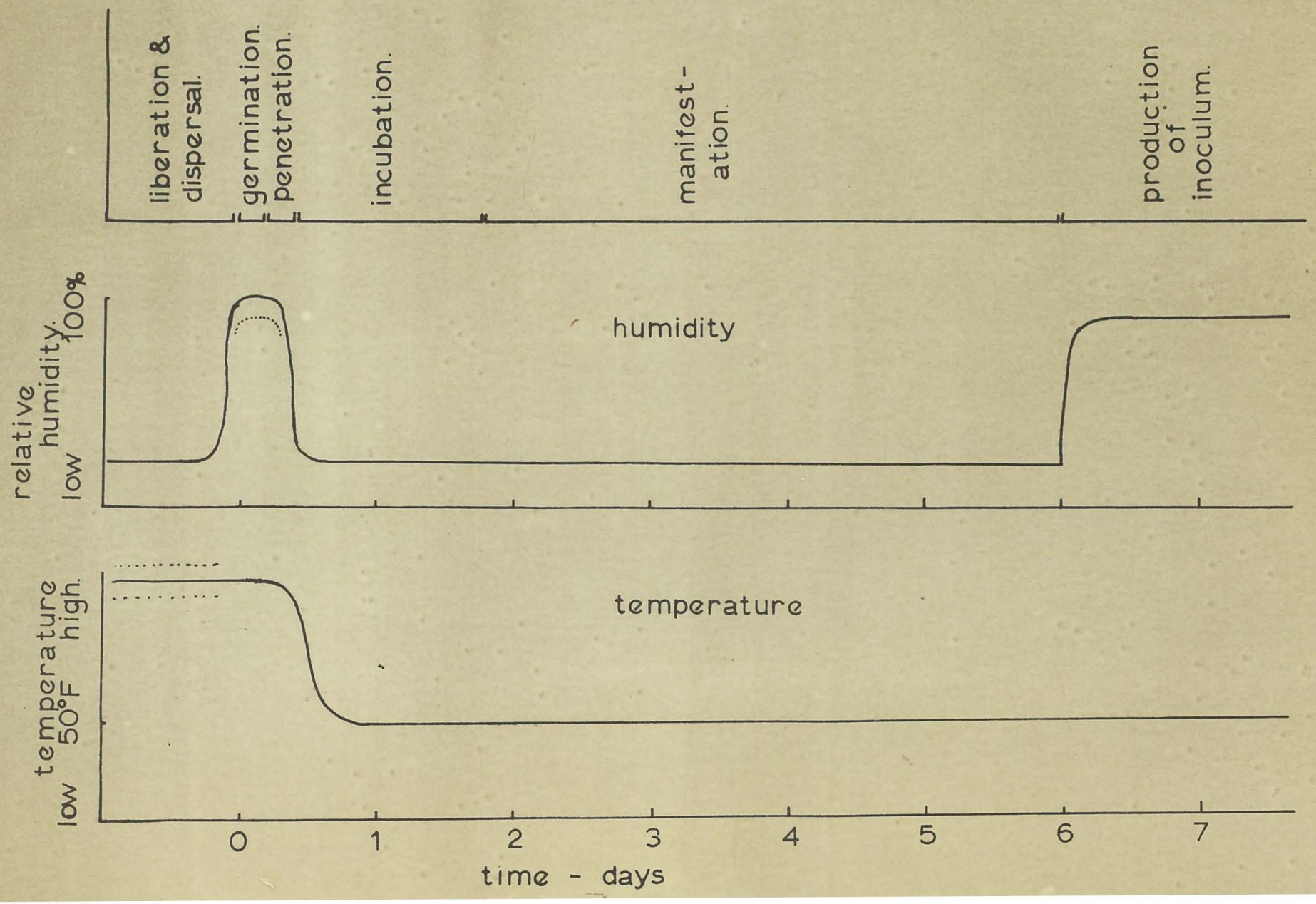
In the preceding pages the effect of the environment on the various phases constituting the disease cycle have been individually considered. In this, the concluding section of this chapter, consideration is given to the practical implications of the results of these studies. Conditions which may cause an epidemic are discussed so that the various portions of the disease cycle previously considered separately may now be treated as a continuing process commencing with the germination of conidia.

Yarwood (1956) has studied the relative humidity requirements of foliage pathogens and has been able to place fungi into four groups depending on their requirements during each particular portion of the disease cycle. S. botryosum clearly falls into Class II considered by Yarwood to be typical of the downy mildew group of diseases characterised by a low relative humidity requirement during dispersal, incubation and manifestation, and a high relative humidity requirement during penetration, infection and sporulation (See Figure XIII). The present study indicated that foliage pathogens possibly may also be grouped according to their temperature requirements of each particular portion of the disease cycle. The optimum humidity and temperature requirements for S. botryosum are graphically presented in Figure XIII.

During optimum conditions, the length of each particular

FIGURE XIII

THE OPTIMUM HUMIDITY AND TEMPERATURE REQUIREMENTS OF THE DISEASE CYCLE.



period of the disease cycle will be at a minimum and these periods are expressed graphically in Figure XIII.

Germination of conidia of S. botryosum isolated from lettuce in New Zealand occurs at the range of temperatures normally found during the day time in any season. Humidity must be above 90% however. If germination is considered to have occurred after the sides of the germ tube become parallel a small percentage of spores may germinate between 15-36°C (59°-98.8°F) after 1½ hours. About the same time is again required for the growth of the germ tube to the stage where penetration may occur. Therefore even during short periods of high humidities and the above temperatures a small number of spores may be successful in penetrating the host leaf. It is possible to predict from Figure what percentage of spores may germinate at a given temperature. It is logical to assume that the infection which occurs will be related to the percentage of spores which germinate in a given time. Therefore potentially high infection may be expected at temperatures between 25-31°C (77-88°F) when maximum spore germination occurs. This disease is present and troublesome during the winter and spring months. It appears therefore that the reason for the seasonal appearance of this disease is not directly related to the spore germination requirements for S. botryosum.

Plants appear to be predisposed to infection when they are soft and succulent. During the flush of spring growth

lettuce plants develop rapidly and during this period of their growth they are more susceptible to disease than at other stages. Also during this period of the year there is a reduction in the number and severity of frosts which may predispose the plant towards resisting infection by checking growth, thus producing small plants with hard leaves.

Penetration and infection are also favoured by temperatures at and above 21°C (70°F). At these temperatures penetration and infection may occur after a period of two hours. However at the lower day temperatures normally present in the field during spring and winter, say 5°C (41°F) between 6 - 8 hours are required for the same processes. Therefore the seasonal appearance of the disease cannot be directly related to the environmental requirements of these periods.

It should be noted however, that even during low temperatures germination, penetration and infection will occur within 24-36 hours. It is not uncommon for plants to remain wet for this period of time in winter and spring. During the later stages of penetration and after infection occurs, the developing spore relies less and less on the relative humidity about the plant until at some time after infection the fungus becomes established within the plant and is entirely independent of external humidities.

The incubation period is favoured by lower air temperatures than the preceding phases of the disease cycle. Experimentation

has indicated that at 5°C (40°F) growth of the fungus within the tissues is higher than at the temperatures which favoured spore germination, penetration and infection. Due to certain limitations in the experimentation actual leaf temperatures may have been above 5°C. However it is safe to assume that temperatures between 4.5°C (40°F) and 15.5°C (60°F) are favourable for the establishment of the fungus within the host tissues. At these temperatures the incubation period occupied 36-60 hours. At temperatures of 26.7°C (80°F) manifestation was restricted. Similar observations on Stemphylium species have been made by Dippenaar (1939) and Nelson (1955).

Manifestation of symptoms of disease and the continued development of the disease cycle occupies a longer period of time than the previous processes. Manifestation reaches its climax with the death of the entire host leaf. Experimentation has shown conclusively that temperatures of 10.0°C (50°F) are most favourable for the rapid growth of the lesions. Larger soft plants are affected more by the disease than the smaller hard plants.

It therefore appears that since incubation and manifestation occupy a longer period of time than previous periods of the disease cycle, and are favoured by the lower temperatures, it is these stages which are responsible for the seasonal development of the disease. However other factors contribute to this also. After a reduction in the number and severity of frosts the lettuce plants tend to make very soft growth as

previously noted. This growth is affected more by the disease than the harder type growth typical of that occuring during winter.

At first sight it appears strange that the optimum growth temperature on culture media, and the optimum for spore germination, penetration and infection are fairly high all being in the region of 25-30°C (77°-86°F). However it is interesting to note that at temperatures of 26.7°C (80°F) and higher, disease development following successful inoculation, germination, penetration and infection is limited. Incubation is the first stage where the fungus comes into direct contact with host reactions it has stimulated. At high temperatures host reactions to infection may be well developped. Lower temperatures may cause a reduction in the host's defensive reaction rate and thus allow the fungus to grow effectively at the lower temperatures normally encountered in winter and spring. Dickson (1923) and Stackman & Harrar (1957) have noted similar phenonema. They have considered that the host becomes weakened so much by the adverse temperatures that the pathogen, even though it is also weakened at these lower temperatures, can cause maximum damage. Padhi & Snyder (1954) however consider the evidence that the temperature of leaves may be above air temperatures is relevant. They have concluded that the fungus may find temperatures in the leaf are more favourable than might be indicated by the prevailing air temperatures, suggesting that the growth within the leaf tissues may also

be favoured by higher temperatures. This theory has not been substantiated by these studies.

During conditions of high humidities and at temperatures above 15.6°C (60°F) conidia may be produced from diseased tissue after 12-18 hours. Abundant production of conidia may occur 6-7 days after infection during optimum conditions. Production of conidia may occur therefore during the period of manifestation which is considered here to reach a climax with the death of the leaf. Dead leaf tissues infected with S. botryosum appear to have an unlimited capacity to produce successive crops of conidia whenever the temperature and humidity are favourable for the required length of time. Thus following rain or during periods of heavy dews which fail to dry during one day a very high number of conidia may be produced from diseased leaves. With continued periods of wet weather, conidia may be dislodged and scattered by raindrops over the same or adjacent plants. The most spectacular release of conidia occurs during high temperatures when the humidity is lower than that required for their production. During these conditions the smaller air currents may readily dislodge the conidia and carry them considerable distances through the same or adjacent crops of lettuce.

Thus in spring when there are frequent periods of damp or wet days, followed by warm sunny days, ideal conditions prevail for the production and spread of inoculum. After fine warm days nights are generally calm with heavy dews or light frosts which keep the plant wet often until the late morning.

These conditions favour the deposition of air born spores, their subsequent germination, and rapid penetration and infection.

However epidemics do not occur after one successful revolution of the disease cycle unless the supply of inoculum is very high. Several successive revolutions of the disease cycle are necessary before sufficient inoculum is produced and liberated. Winter and spring lettuce remain in the soil for a long period, often from as early as April until September or October. During winter, conditions do not favour rapid development of the early processes of the disease cycle. Despite this there is ample opportunity for the slow build up of disease tissue. Once the milder conditions occur which favour the first process of the disease cycle there is an abundant development and release of inoculum followed by a rapid infection of the plant leaves. It has been observed that where the source of inoculum is high, at the first period of milder weather infection may occur which within fourteen days renders previously saleable plants unmarketable. During this period of time only two revolutions of the disease cycle could occur. It is considered that the presence of a potentially high source of inoculum determines whether conditions which favour the early stages of the cycle will permit disease build up to an epiphytotic level. If the crop has been relatively free of infection during winter there will be insufficient inoculum present during these periods favouring infection to permit anything but a light infection to occur.

These observations have been made giving consideration to the results of studies of the various processes of the disease cycle and have been repeatedly confirmed during two seasons of field observation.

Survival during the summer months.

Towards late spring and early summer temperatures rise and eventually will reach the point where the rate of manifestation and production of inoculum becomes very much reduced. Generally by this time the crops which came through the winter period have been harvested and the diseased plants and debri may be ploughed or incorporated into the soil often to a depth of approximately nine inches. Any infections on other lettuce crops planted during late winter or spring appear to be restricted in size and are of little importance as far as the crop is concerned. The higher temperatures do not favour the host-pathogen relationship. However temperatures between 20-25°C (68-77°F) are optimum for the saprophytic development of S. botryosum on culture media and probably a similar temperature range is favourable for the saprophytic existance of this organism on natural organic material. This temperature is not exceeded in the first few inches of soil where this organism is likely to be active during summer. S. botryosum has been isolated from artificially infected soil as hyphae. It has not been possible to say whether these were dormant or active, but survival of this organism as mycelium in the soil occurs during the summer months when temperatures which favour the saprophytic existence of this organism are present. During

studies on survival it was evident that in the saprophytic stage this organism has a relatively poor competitive saprophytic ability. However on some types of soil organic matter, for example decomposing straw, perithecia of Pleospora herbarum may develop. Temperatures favouring the development of perithecia were similar to the optimum conditions for growth on artificial media viz, between 20-25°C. Survival therefore may occur as dormant or active mycelium or as perithecia on organic matter within the soil. Perithecia will not form on lettuce tissue until it is dead and colonised with S. botryosum. In nature the breakdown of lettuce crop debris is considered to be too rapid for diseased plant material to harbour this fungus from one season to the next.

While high temperatures do not favour the pathogen-host relationship, lesions of S. botryosum were often located during summer on Solanum nigrum and S. nodiflorum in areas where winter lettuce had previously been heavily affected by the disorder. In autumn when cooler temperatures favour spore production the diseased weeds may produce sufficient inoculum to infect nearby lettuce crops.

The main methods of survival during the summer appear to be dormant or active mycelium in the soil, lesions on weeds or on other lettuce crops and perithecia on organic material.

D. CONCLUSIONS

High temperatures favour the saprophytic existence of Stemphylium botryosum. The fungus may survive during the summer as a soil saprophyte, or on hosts. Primary infection of lettuce may occur during autumn or winter directly from the soil mycelium, from conidia produced on weeds or other lettuce crops, or from ascospores released from perithecia which may develop on organic matter during summer. High temperatures do not favour manifestation of disease or production of conidia, but permit rapid germination of conidia to penetrate and infect the host.

Lower temperatures favour incubation, manifestation and production of conidia. These processes occupy the major part of the disease cycle and their importance is indicated by the seasonal appearance of the leaf spot disorder of lettuce. The conditions which are believed to cause an epiphytotic involve the slow but steady infection of lettuce with S. botryosum which occurs during the low temperatures of winter. These conditions favour incubation, manifestation and production of conidia. With rising temperatures in spring conidia are released, germination, penetration and infection occur very readily, but low average temperatures still favour manifestation and production of inoculum. This means that there is a rapid increase of infection. Whether or not an epiphytotic occurs depends on the level of the inoculum which is available within a lettuce crop when conditions in spring favour dispersal of the spores and rapid germination and infection of the lettuce plants.

CHAPTER IV

THERAPEUTIC CONTROL OF

LEAF SPOT OF LETTUCE

CHAPTER IV THE THERAPEUTIC CONTROL OF LEAF SPOT OF LETTUCE.

Growers visited during the field surveys considered that leaf spot of lettuce was one of the most difficult foliage diseases they had to control. Most considered that it was impossible to control in the wet weather of a mild spring. Dippenaar (1939) however, considered that copper oxychloride and other copper based sprays or dusts would control this leaf spot disease. Unfortunately these materials leave an unsightly deposit on the foliage. Today there are available a wide range of fungicides which may be suitable for leaf spot control which do not leave conspicuous deposits. Experiments were conducted to test the efficacy of some of these new fungicides.

Materials and Methods.

1. Laboratory Evaluation

A glass slide assay described by Durbin et al. (1955), was used to find an effective fungicide.

Microscope slides measuring 25 x 75 mm. were thoroughly cleaned in concentrated chromic acid, washed for an hour in running tap water, and finally rinsed four times in distilled water. This procedure completely elimated any chromic acid as indicated by trial germination counts on slides cleaned in this manner.

Pairs of slides were coated with a similar even film of

fungicide under uniform conditions, using a small syringe type sprayer, and set aside to dry. The concentration of fungicide used corresponded to the normal commercial rates for the material concerned. The fungicides tested, details of their composition and mate of use are given in Table 23 .

Table 23 Details of Fungicides used During Experimentation on Control.

Common Name	Percentage Active Ingredient.	Active Ingredient.	Rate per 100 gal.
Zineb (Dithane Z78)	65%	Zinc ethylene bis dithio carbamate	2 lb.
Cuprox	50% Cu	Copper oxychloride	5 lb.
Thiram (Thirospray)	80%	Tetra methyl thiuram disulphide	2lb.
Bordeaux			6.8.100
Ziram	75%	Zinc dimethyl dithiocarbamate	2 lb.
Dichlone	50%	2:3 dichloro-1:4 naphtho-quinone	1 lb.
Copox MM.	50% Cu. 1% P.M.	Copper oxychloride * phenyl mercury	3 lb.
Captan (Flit 406)	50%	N-trichloromethyl-mercapto-4-cyclohexene-1:2 dicarboximide.	3 lb.
Ialine	13% Cu.	Colloidial copper in oil	1 gal.
Control		Distilled Water	

Spore suspensions were prepared from 10-14 day old colonies grown on P.D.A. at 24°C by suspending the conidia obtained in a 1% dextrose solution. The conidial suspensions were concentrated by centifuging and adjusted with 1% dextrose solution to obtain a concentration of 50,000 conidia/ml. The concentration of conidia was determined with a haemocytometer.

A pipette drawn out from 3/16" glass tubing was used to apply drops of the conidial suspension to the slides coated with fungicide. This pipette was held upright and moved steadily over the slides placed on the bench. It delivered 0.014 ml. per drop, and two drops were placed on each microscope slide. These slides were transferred into a petri dish lined with moist filter paper, and incubated at 24°C.

The effect of the fungicide on spore germination was measured by observing the number of spores which had germinated in a random sample of approximately 100 spores. This was expressed as a percentage. Counts were made after 24 hours and slides were observed again after 50 hours to determine if further development had occurred.

McCallan et al. (1959) has recommended that tests should be repeated at different places or times rather than relying on replications within a test. Therefore each series was repeated at a later date with a different isolate of S. botryosum.

Experimentation

(a) Evaluation of Fungicides using Glass Slide Method:

Slides coated with the fungicides were placed on the laboratory bench to dry. Drops of the spore suspensions were placed on the pairs of slides from each treatment after 4, 24, 72, & 168 hours exposure to the sun and air on the laboratory bench. From the information gained from these tests fungicides were selected for evaluation of their tenacity during wet conditions.

(b) Tenacity Studies:

Copper oxychloride, thiram, bordeaux, ziram, phygon, and captan were selected from the previous group of fungicides for these tests. Each material was sprayed on to two pairs of glass slides at the rates given in Table 23.

(a) without additives

(b) with Tenac 1:800 by volume

(c) with a casien/lime sticker at 2 lb/100.

Controls consisted of water, water + Tenac and water + casien/lime sticker.

The treated slides were placed on the laboratory bench for a period of 24 hours. One of the two pairs of glass slides were then dipped in an agitated water bath running at 22.5°C for 60 minutes. These slides were then removed, drained and placed on the laboratory bench to dry.

Twelve hours after removing the slides from the water bath they were inoculated along with the undipped slides, using the standard pipette and spore suspension. Each series were repeated twice.

2. Glasshouse Experiment.

From the groups of fungicides evaluated in the laboratory four were selected for experimentation on lettuce plants growing in the glasshouse. These were zineb, cuprox, thiram and ziram.

The experiment was designed with five treatments, including the control, four replications, and four plants per plot. Records were made by counting the number of lesions on the lower five leaves of the plant.

A total of four applications of the various fungicides were made at 10 day intervals. All fungicides were applied to wet the plants to the point of run off. Tenac 1:800 by volume was added to all treatments. Lettuce plants used for this experiment were at the 'four-leaf' stage when the first application of fungicide was made.

Seven days after spraying with the fungicide the plants were inoculated with a spore suspension containing 500 spores/ml. The low spore count was used to simulate natural conditions. Four inoculations were made on the 7th, 17th, 27th. and 38th days. Results were recorded 45 days after the first application

of fungicide.

During this period the plants were watered frequently with overhead sprinkling to simulate the action of rain.

Results

1. Laboratory Evaluation.

Results are summarised in Table 24 .

(a) Glass Slide Method

Zineb was the only material which gave consistently poor control of spore germination at all observations. Of the copper sprays 'Ialine' appeared to give superior control to the other two materials, although results were inconclusive. Cuprox and bordeaux definitely retarded spore germination and development, but after 50 hours incubation under favourable conditions often over 10% of the spores had continued to germinate. The 1% phenyl mercury added to copper oxychloride, and formulated as 'Copox MM,' did not appear to improve the action of the copper at the rate used. Dichlone gave some control of spore germination but often over 10% of the spores had germinated after 24 hours incubation at 100% relative humidity. Similarly Captan appeared to give variable results. However, it was frequently observed that spores on the surface of the droplet containing the spores, would germinate freely, while those in closer contact with the film of captan on the slide showed no signs of germination. Thiram and ziram both gave very good control of spore germination.

Table 24 Summary of results of Experiments on Age of Deposit of Fungicide, and its Efficacy, and Tenacity, as measured by Glass Slide Technique

Time of application after coating slide	4 hrs	24 hrs	72 hrs	168 hrs	Assessment
Bordeaux	?	-	?	-	Some control
Captan	?	?	+	?	Doubtful
Copper oxychloride	+	-	+	-	Some control
'Copx[illegible] MM'	?	-	?	?	Some c ontrol
Dichlone	?	-	?	?	Some control
Ialine	-	+	-	-	Good control
Thiram	-	-	-	-	Very good control
Zineb	+	?	+	+	No control
Ziram	-	-	-	-	Very good control
Control	+	+	+	+	No control

Note. Control of spore germination obtained, expressed:-

(+) More than 10% of spores germinated.

(-) Less than 10% of spores germinated.

? Variable Results.

Tenacity Tests	No Sticker		Tenac		Casien/Lime	
	Undipped	dipped	Undipped	dipped	Undipped	dipped
Bordeaux	-	-	-	-	-	-
Captan	-	+	-	-	-	-
Copper oxychloride	-	+	-	-	+	+
Thiram	-	-	-	-	-	-
Ziram	-	-	-	-	+	-
Control	+	+	+	+	+	+

Notes: Bordeaux was as effective with or without stickers. Captan tenacity improved with sticker, Copper Oxychloride improved with Tenac, impaired by Casien/Lime. Thiram effective with or without sticker, as was ziram except it was not compatible with casien/lime sticker.

The fungicides applied to glass slides did not appear to lose their efficacy after up to 7 days exposure on the laboratory bench.

(b) Tenacity Studies:

Copper oxychloride and captan were both readily removed from the glass slides during the washing procedure. Tenac improved the tenacity of these deposits.

Results suggested that the casien/lime sticker may reduce the efficacy of copper oxychloride and ziram. Washing the slides treated with ziram plus casien/lime sticker, improved the control of spore germination presumably due to the removal of the alkaline portion of the sticker. Washing did not improve the action of copper oxychloride - casien/lime combination. The casien/lime sticker was not compatible with phygon or captan and was not tried in combination with these materials.

Results of this and previous experiments indicated that copper oxychloride + Tenac sticker, thiram, bordeaux, ziram, phygon and captan excluding combinations with casien/lime sticker gave promising control of spore germination.

2. Glasshouse Experiment.

Results are recorded in Appendix XV and summarised below in Table 25 . Copper oxychloride, thiram and captan, all applied with Tenac 1:800 controlled the development of leaf

spot on the artificially inoculated lettuce plants in the glass house. The zineb treatment did not differ from the untreated control at the 1% level of probability. However, at the 5% level ~~of infection~~, zineb was significantly superior to the control, but inferior to the other three materials which gave effective control under glasshouse conditions.

Table 25 Summary of Results: Glasshouse Control Experiment.

Materials*		Mean Number of Lesions per plot	Result+ p=.05	p=.01
Captan	3 lb/100 gal.	42.5	c	B
Copperoxychloride	5 lb/100 gal.	27.3	c	B
Thiram	2 lb/100 gal.	40.7	c	B
Zineb	2 lb/100 gal.	212.0	b	A
Control	Sterile Water	307.2	a	A

* all plus 'Tenac) (Shell Co. Sticker) 1:800

\+ Treatments with same letter do not differ significantly. Those with different letters differ significantly. (Analyses Duncans (1953) Multiple Range Test.)

Discussion.

McCallan (1959) believed that laboratory methods of evaluation of fungicides gives a fair prediction of the field performance of the fungicide, particularly if they are used in conjunction with weathering tests. In this study some materials, e.g. the coppers failed to give conclusive results using the slide assay technique. However, copper oxychloride gave good control of leaf spot on the lettuce plants in the glasshouse experiment.

Other inconclusive results were probably due to the spraying techniques used to coat the slides. The control of actual amounts of fungicides applied to the slides was not critical.

This technique of glass slide assay of fungicides appears to be suitable for evaluation of the organic group of fungicides. Effective fungicides were indicated by this method and later experiments proved that these results are applicable to the control of leaf spot on the lettuce plants growing under glasshouse conditions. Copper oxychloride, however, gave better control on the plants than the glass slide techniques indicated.

Glasshouse pot trials confirmed observations made on various materials in the field. It was anticipated that control trials would also be run in the field on crops of lettuce near Otaki where control of this disease has proved very difficult. However this proposed experiment had to be abonded because

of the loss of spring lettuce in this district due to suspected lettuce mosaic virus and brown blight.

Conclusions

Thiram 80% w.p. at the rate of 2 lbs/100 gals, captan 50% w.p. at 3 lb/100 gals and ziram at 2 lbs/100 gals when applied to glass slides prevented the germination of conidia of S. botryosum. 'Tenac' Sticker imporved the tenacity of copper oxychloride and captan on the glass slides. Copper oxychloride, thiram and captan, all with 'Tenac' sticker gave satisfactory control of leaf spot in the glasshouse experiment. Zineb gave inferior control of spore germination.

Summary.

A range of fungicides were evaluated for the control of germination of S. botryosum conidia, using a glass slide technique. These experiments were followed by a test of a selected group of the fungicides on lettuce in the glasshouse.

It is believed that the techniques used for laboratory evaluation of the fungicides gave a good indication of which fungicides were effective. It would be desirable to improve the method used for coating the slides with the fungicides, since it is considered some of the doubtful results were due to uneven rates of applications of the fungicides to the slides.

Copper oxychloride, thiram, captan and ziram at normal commercial rates appeared to control spore germination and resulting infection. Zineb was not effective.

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A P P E N D I C E S

APPENDIX I Diseases Recorded on Lettuce in New Zealand		
Causual Agent	Common Name	Authority
Botrytis cinerea. Pers.	Grey mould Red Leg	Brien 1959
Bremia lactucae Reget	Downy Mildew	Cunningham 1922
Cucumber Mosaic Virus		Brien et al. 1957
Erwinia carotovora(Jones) Holland	Soft Rot.	Brien et al. 1957
Lactuca Virus I	Lettuce Mosaic Virum	Chamberlain 1948
Marssonina panattoniana (Bert) Magn	Ring Spot Rust	Taylor & Li 1944
Olpidium brassicae (Woron.) Dang	Big Vein	Fry 1958
Pellicularia fillamentosa (Pat.) Roger	Damping off	Brien et al. 1957
Pleospora herbarum Robenh.	Leaf spot Target spot	Brooks 1958.
Pseudomonas marginalis (Brown) Stapp.	Marginal leaf spot	Brien et al. 1957
Sclerotinia sclerotiorum (Lib) de Barry	Soft Rot Lettuce Drop	Cunningham 1927
Septoria lactucae. Pers.	Septoria leaf spot	Dingley 1957
Spotted Wilt Virus Disease		Chamberlain 1954

APPENDIX II

COMPOSITION AND PREPARATION OF MEDIA.

1. ARTIFICIAL MEDIA.
2. NATURAL MEDIA.
3. NATURAL NUTRIENT MEDIA.

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1. ARTIFICIAL MEDIA

(a) Cornmeal Agar (Riker & Riker 1936)

Agar	17g.
Cornmeal	20g.
Water (distilled)	1000ml.

Preparation: The cornmeal was cooked for 1 hour by placing a flask containing the cornmeal suspended in 500ml. of water in a simmering water bath. The cooked cornmeal was filtered through cheesecloth and the filtrate added to the 500ml. of water in which the agar had been dissolved. After the volume was adjusted the media was placed into several smaller flasks.

Sterilization: All media unless otherwise stated were sterilized in an autoclave at 15 p.s.i. for 20 mins. immediately after preparation.

(b) Czapek-Dox Agar (Thom & Raper 1945)

Agar	15.00g.
$NaNO_3$	3.00g.
K_2HPO_4	1.00g.
$MgSO_4 \cdot 7H_2O$	0.50g.
K Cl	0.50g.
$FeSO_4 \cdot 7H_2O$	0.01g.
Sucrose	30.00g.
Water (distilled)	1000.00ml.

Preparation: The nutrients were dissolved in 500cc. of hot water in the above order and mixed with the agar which had been dissolved in 500cc. of water. The mixture was placed in ten 250cc. flasks and autoclaved.

(c) Dextrose Agar (Difco Manual 1953)

Agar	15g.
Beef extract	3g.
Tryptose	10g.
Dextrose	10g.
NaCl	5g.
Water (distilled)	1000ml.

Preparation: The agar and nutrients were dissolved in hot water. The mixture was placed in ten 250cc. flasks and autoclaved.

(d) Malt Extract Agar (Raper & Thom 1949)

Agar	25g.
Malt extract (Difco)	20g.
Dextrose	20g. (omitted)
Peptone	1g. (Omitted)
Water (distilled)	1000ml.

Preparation: Proceeded as for 1(c).

(e) Oatmeal Agar Johnson et al 1959)

Agar	17g.
Oatmeal	75g.
Yeast extract	1g.
Water (distilled)	1000ml.

Preparation: Proceeded as for 1(a).

(f) Peameal Agar: *

Agar	15g.
Peameal	42g.
Water	1000ml.

Preparation: Proceeded as for 1(a).

(g) Potato Dextrose Agar (Laboratory) (Riker and Riker 1936 - slightly modified)

Agar	12g.
Potatoes (sliced and peeled)	200g.
Dextrose	10g.
Water	1000ml.

Preparation: The sliced potatoes were cooked gently for an hour in 500 ml. of water after which the liquid was separated from the cooked potato by straining through cheesecloth. Agar was melted in 500ml. of water and added to the filtrate. Dextrose was finally added and the volume adjusted to 1000ml. before it was poured into ten 250cc. flasks.

(h) Potato Dextrose Agar (Difco or Oxoid)

Potato infusion	200g.
Bacto Dextrose	20g.
Bacto Agar	15g.

Preparation: 39 grms of the above mixture were suspended in 1000mls. of distilled water and boiled to dissolve the medium. After placing this media into smaller flasks it was autoclaved.

(i) Prune Agar (Difco)

Prune infusion	36g.
Bacto-agar	15g.
Distilled water	1000ml.

Preparation: 24 grams of this mixture were suspended in 1000 mls. of distilled water. Method as for 1(h).

(j) Tomato Juice Agar (Oxoid)

The tablet preparationwwas used, one tablet being dissolved per 5ml. of water. This media was prepared and autoclaved immediately prior to use.

(k) Water Agar: *

Agar	12g.
Water (distilled)	1000ml.

Agar dissolved in hot water before autoclaving.

2. NATURAL MEDIA

(a) Autoclaved Lettuce Leaf Material:

Lettuce leaves were collected from six weeks old plants and cut into pieces approximately 10mm. square. These were placed in a flask and autoclaved for 20 mins. at 15 p.s.i. Sufficient of this material was transferred to petri dishes, with flamed forceps, to cover the bottom of the dish evenly. A 2% solution of agar was prepared and autoclaved for 20 mins. at 15 p.s.i. Approx. 15ml. of this agar was poured into the dishes and the sterile leaf pieces were evenly redistributed if necessary by gentle rocking of the dish or by use of flamed forceps.

(b) Lettuce Leaf Material Sterilized with Chloropicrin (Snyder & Hansen 1947 Modified).

Lettuce leaves prepared as described above were placed into 250cc. bottles fitted with tight lids and gaskets. A moist filter paper was placed down the side of each bottle.

Because propylene and ethylene oxides (as used by Snyder & Hansen 1947 (a)) were unobtainable in New Zealand Chloropicrin was used as the fumigant for this natural material. To facilitate the handling of this unpleasant volatile liquid the bottle of chloropicim was placed in the cooling compartment of the refrigerator some hours prior to use. A pippette connected to a suction pump, by way of a flask fitted with a variable air intake control, was used to measure o.5ml. of chloropicrin into each bottle. The caps of the bottles were screwed down securely. This operation took about two minutes, very little loss of chloropicrin occurring.

The bottles were incubated overnight at 30°C. to ensure volatilization of the chloropicrin. After this time the lids of the jars were loosened and the bottles stood on a bench near an open window. After six hours the material was incorporated into molten agar as described in 2(a) above. The warm agar expelled any chloropicrin which remained in the material.

(c) Lettuce Decoction Agar (After Kilpatrick & Johnson 1956)

Lettuce leaves	300g.
Agar	12g.
Water (distilled)	1000ml.

Six week old lettuce plant leaves were removed, cut into small pieces and added to a flask containing 500ml. of distilled water. The flask was placed in a boiling water bath for one hour. The liquid was strained off through two layers of cheesecloth, and added to 500mls. of distilled water in which the agar had been dissolved, and the volume made up to 1000ml.

(d) Soil Agar - Autoclaved.

Garden soil, a sandy loam with a high organic matter content, was moistened and autoclaved for 20 mins. at 15 p.s.i. Approx. 5 grms of autoclaved soil was transferred into petri plates using a flamed spatula. This soil was mixed with approximately 15cc. of 2% agar in distilled water by gently rocking the dish.

(e) Soil Agar - Sterilized with Chloropicrin:

Approx. 50g. of moist garden soil of the same sample as that used in 2(d) above was placed in 250ml. bottles, moistened and 0.5ml. of chloropicrin added as described previously. After overnight exposure to the chloropicrin at 30°C, the lids were loosened and the bottles placed near a window for six hours. The soil was then incorporated into a 2% agar solution as described in 2(d) above.

(f) Straw Agar - Autoclaved.

Barley straw was cut into approx. ½" lengths, moistened and autoclaved in flasks at 15 p.s.i. for 20 mins. This straw was then incorporated into 2% agar solution as described in 2(a) above.

(g) Straw Agar - Sterilized with Chloropicrin:

Barley straw was cut into pieces, moistened, and sterilized with chloropicrin in 250ml. jars as described previously.

The sterilized straw was incorporated into a 2% agar solution.

3. NATURAL NUTRIENT MEDIA

Natural media was prepared as described in "Natural media 2(a) - (g)" above. However, instead of incorporating this into 2% agar the following nutrient agar was prepared.

Dextrose	20g.
Agar	20g.
Water	1000ml.

Preparation: The agar and dextrose were dissolved in 500mls. of distilled water by placing the mixture in a flask held in a boiling water bath. When the media was completely dissolved the volume was made up to 1000ml. by adding distilled water. This quantity was transferred to several 250ml. flasks prior to autoclaving.

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* Laboratory Recipe

Appendix III

SPORE GERMINATION: Percentage of spores of S. botryosum germinated at 4 - hourly intervals 0 - 24 hours

Time in hours	INCUBATION TEMPERATURE PART I *			
	5°C	10°C	15°C	17°C
4 hours	0	60	60	100
	0	50	60	100
	0	70	50	95
	0	60	65	100
Av. %	0	60	59	99
8 hours	30	80	100	95
	10	70	90	100
	0	75	90	100
	15	50	85	90
Av. %	14	69	91	96
12 hours	70	85	95	95
	40	80	100	90
	50	70	100	100
	45	90	90	100
Av. %	51	81	96	96
16 hours	55	90	80	100
	50	90	95	100
	60	95	100	95
	50	100	100	100
Av. %	54	94	94	99
20 hours	90	85		
	90	95		
	60	80		
	95	100		
Av. %	84	90	N.R.	N.R.
24 hours	95			
	100			
	95			
	95			
Av. %	96	N.R.	N.R.	N.R.

* PARTS II - IV See Appendix IV

N.R. Not recorded.

APPENDIX IV

SPORE GERMINATION: Percentage of spores of S. Botryosum germinated at time intervals indicated, 10°-50°C

(N.R. = Not Recorded)

Time in hours	INCUBATION TEMPERATURE.								
	PART II			PART III			PART IV		
	10°	15°	17°	25°	30°	35°	40°	45°	50°
½hr Av.%	0	0	0	0	0	0	N.R.	N.R.	N.R.
	0	0	0	2	2	0	0	0	0
	0	0	0	1	3	0	0	0	0
	-	-	-	6	6	0	0	0	0
1hr.Av.%	0	0	0	3	4	0	0	0	0
	2	9	4	8	58	11	0	0	0
	1	11	5	28	45	43	0	0	0
	-	-	-	38	-	8	0	0	0
1½ hr. Av. %	1.5	10	4.5	25	51	21	0	0	0
	6	42	27	80	85	65	2	1	0
	3	17	10	68	91	84	2	0	0
	-	-	-	82	92	73	2	0	0
2hr.Av.%	7	29.5	18.5	77	89	74	2	0	0
	3	43	48	93	90	48			
	5	40	46	97	96	73			
	-	-		95	95	77			
2½hr.Av.%	4.0	41.5	47	95	96	75	N.R.	N.R.	N.R.
	9	67	57			87	3	6	0
	10	57	57			80	8	1	0
	-	-				91	4	4	0
3 hr.Av.%	9.5	62.0	57	N.R.		86	4.5	4	0
	4	64	72			92			
	15	60	72			92			
	-	-	-			88			
3½hr.Av.%	9.5	62.0	72	N.R.	N.R.	91	N.R.	N.R.	N.R.
	23	87	74	99	98	96	0	6	0
	17	83	83	98	98	100	14	9	0
	-	-	-	100	96	99	7	5	0
4hr.Av.%	20	85	78.5	99	97	98	10	7	0
	43	79	83						
	35	85	84						
	-	-	-						
4½hr.Av.%	39	82	83.5	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.

Contd.

APPENDIX IV CONTD.

Time in Hours	INCUBATION TEMPERATURE								
	PART II			PART III			PART IV		
	10°	15°	17°	25°	30°	35°	40°	45°	50°
	67	85	93				49	6	2
	43	86	90				52	9	0
							64	12	1
6hr.Av.%	55	85	91.5	N.R.	N.R.	N.R.	55	9	1
	82	92	84				79	16	4
	80	90	96				78	12	16
							80	6	12
8hr.Av.%	81	91	90	N.R.	N.R.	N.R.	79	11	11
	87						70	24	12
	92						85	21	4
	88						83	20	2
12hr.Av.%	89	N.R.	N.R.	N.R.	N.R.	N.R.	79	22	6
							80	22	6
							94	27	4
							90	22	6
16hr.Av.%	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	88	23	5
							93	22	8
							95	24	1
							84	17	10
20hr.Av.%	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	91	22	6
								23	1
								40	3
								36	4
24hr.Av.%	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	33	3
								30	10
								39	8
								36	9
48hr.Av.%	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	35	9

APPENDIX V CONSTANT HUMIDITY SALT SOLUTIONS

30°C

Relative Humidity	Saturated Salt Solution
87%	$Na_2CO_3 \cdot 10H_2O$
90%	KOH. 16.0g/100g H_2O
92.9%	$NH_4H_2PO_4$
94.9%	$CaHPO_4 \cdot 2H_2O$
96.6 - 97.4%	K_2SO_4
98%	KOH 3.5g/100g H_2O
100%	Distilled Water.

25°C

75 - 76.5%	NaCl
79%	KBr
85%	Sucrose
88%	$BaCl_2 \cdot 2H_2O$
93%	$NH_4H_2PO_4$
97%	$CaHPO_4 \cdot 2H_2O$
98%	$KClO_3$
100%	Distilled Water

References

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"The control of humidity by saturated salt solutions"
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APPENDIX VI

The Effect of Light on Penetration and Infection

Part I

(Abbreviations given on p129)

Treatment	(a)					(b)					(c)					(d)				
Plant	1	2	3	4	T	1	2	3	4	T	1	2	3	4	T	1	2	3	4	T
									BLOCK I											
Leaf 1	4	3	0	3		8	10	3	1		7	1	22	5		4	2	18	1	
2	5	5	2	3		8	74	5	9		2	0	7	32		9	10	0	0	
3	1	6	4	6		6	23	14	27		6	4	71	6		20	47	4	1	
4	3	2	19	0		76	3	85	21		28	10	0	0		27	41	6	75	
5	0	0	0	3		23	0	0	2		2	0	1	0		20	3	2	17	
Total	13	16	25	15	69	121	110	155	60	446	45	15	101	45	204	80	103	32	94	309
									BLOCK II											
1	0	1	3	4		7	6	11	100		1	0	5	0		45	17	8	16	
2	0	0	0	2		16	9	12	22		2	4	32	9		44	6	8	11	
3	0	3	3	28		27	3	5	11		1	12	19	120		9	2	94	4	
4	7	0	19	15		7	4	14	12		4	21	95	3		39	3	143	5	
5	0	6	26	60		15	15	44	92		0	12	0	6		4	6	8	2	
Total	7	10	41	109	177	72	37	86	237	432	8	49	151	138	346	141	34	261	38	474
									BLOCK III											
1	0	0	3	0		5	2	16	2		6	2	5	8		15	12	11	12	
2	1	0	0	0		2	4	4	5		46	2	1	118		2	19	11	22	
3	3	1	3	5		4	101	116	8		1	0	6	0		22	24	53	68	
4	6	0	0	0		9	56	11	18		8	2	18	0		20	67	83	29	
5	4	0	2	0		7	2	127	7		0	10	4	1		52	15	22	1	
Total	14	1	8	5	28	27	165	274	40	506	61	16	34	127	238	111	137	182	132	562
									BLOCK IV											
1	4	3	4	2		10	8	7	93		3	13	7	11		0	8	5	6	
2	7	11	5	5		1	40	0	3		0	8	9	81		0	7	12	13	
3	4	32	42	15		41	13	1	4		4	7	5	2		11	14	193	4	
4	7	1	3	24		6	7	8	0		0	2	1	13		19	21	11	70	
5	14	5	7	0		7	7	1	7		29	0	39	2		6	67	1	93	
Total	36	52	61	46	195	65	75	17	107	264	36	30	41	109	216	36	116	222	186	560
Treatment Totals					469					1648					1004					1905
Result F Tests treatment Differences Not Significant. C.V. = 53.4%																Grand Total				5026
Analyses for effect of light after inoculation																				
Light after inoculation	69	177	28	195	204	346	238	216								1473				
Dark after inoculation	446	432	506	264	309	474	562	560								3553				
F. Test Result: Differences significant 1% level. C.V. = 33.0%																** 5026				
Analyses for effect of light as predisposing factor.																				
Light before inoculation	69	177	28	195	309	474	562	560								2374				
Dark before inoculation	446	432	506	264	204	346	238	216								2652				
F. Test Result: N.S. C.V. = 29.4%																5026				

APPENDIX VII.

PART II

Dark treatment following Inoculation

Plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	T
Leaf 1	117	4	2	0	5	2	14	0	14	3	45	20	4	3	16	2	
2	8	8	6	7	23	2	0	2	2	2	5	3	35	35	0	3	
3	4	2	13	0	71	1	67	34	7	4	7	10	7	26	1	3	
4	2	4	3	3	19	49	0	5	3	10	0	3	0	4	4	16	
5	79	25	6	0	25	35	4	13	4	10	0	0	101	4	6	4	
Plant Total	210	43	30	10	143	89	85	54	30	29	57	36	147	72	27	28	1090
					Light treatment following Inoculation												
Leaf 1	28	56	32	109	19	9	142	79	8	93	20	13	6	2	105	38	
2	5	17	72	100	54	104	161	59	30	10	5	86	14	96	19	71	
3	32	14	87	144	138	141	19	71	4	47	9	154	150	35	127	91	
4	39	141	5	6	2	190	28	145	78	4	7	90	43	124	17	106	
5	7	55	15	0	21	0	47	60	32	38	80	2	0	73	16	32	
Plant Total	111	283	211	359	234	444	397	414	152	192	121	345	213	330	284	338	4428

Results:

Treatment Averages: Dark treatment following Inoculation 68.1 ± 37.9
Light " " " 276.7 ± 26.1

Result of T test Differences between treatments significant at p = 0.01

APPENDIX VIII

INCUBATION PERIOD. The effect of Temperature during incubation on lesion size.

* mm.

Range*	Class*	Plant	1	2	3	4	5	6	T	1	2	3	4	5	T'	G.T.
							40°F.									
<0.25-0.75	0.5		3	1	0	0	0	4	8	4	0	0	0	0	4	12
0.76-1.5	1.0		9	11	27	27	24	17	115	7	3	1	3	10	24	139
1.6 - 2.5	2.0		29	30	27	20	20	14	130	10	3	7	3	9	32	162
2.6 - 3.5	3.0		10	9	13	18	4	0	54	1	1	1	1	7	11	65
3.6 - 4.5	4.0		0	0	0	1	0	0	1	0	1	1	0	0	2	3
4.6 - 5.5	5.0		0	0	0	0	0	0	0	0	0	0	0	1	1	1
									308						74	382
							50°F.									
<0.25-0.75	0.5		0	0	0	0	14	3	17	3	9	7	4	6	29	46
0.76- 1.5	1.0		47	19	6	31	39	10	152	12	29	11	12	0	64	216
1.6 - 2.5	2.0		31	18	18	18	9	17	111	13	8	5	1	2	29	140
2.6 - 3.5	3.0		6	0	3	4	2	5	20	2	0	1	0	0	3	23
3.6 - 4.5	4.0		0	1	5	0	0	0	6	1	0	0	0	0	1	7
4.6 - 5.5	5.0		0	0	1	0	0	0	1	0	0	0	0	0	0	1
									307						126	433
							60°F.									
<0.25-0.75	0.5		16	38	29	15	33	37	168	13	8	8	3	4	36	204
0.76- 1.5	1.0		16	20	34	21	43	38	172	8	3	9	5	6	31	203
1.6 - 2.5	2.0		3	4	3	0	1	3	14	0	0	1	0	5	6	20
2.6 - 3.5	3.0		2	1	2	0	0	0	5	0	1	0	0	0	1	6
3.6 - 4.5	4.0		1	0	0	0	0	1	2	0	0	0	0	0	0	2
4.6 - 5.5	5.0		0	0	0	0	0	0	0	0	0	0	0	0	0	0
									361						74	435

APPENDIX VIII Contd.

mm. Range	Class*	Plant	1	2	3	4	5	6	T	1	2	3	4	5	T	G.T.
			70°F													
<0.25-0.75	0.5		19	5	12	14	37	28	115	1	6	2	1	4	14	129
0.76- 1.5	1.0		8	10	1	0	5	12	36	2	2	0	0	3	7	41
1.6 - 2.5	2.0		0	3	1	0	0	2	6	0	0	0	0	0	0	6
2.6 - 3.5	3.0		0	1	0	0	0	0	1	0	0	0	0	0	0	1
3.6 - 4.5	4.0		0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.6 - 5.5	5.0		0	0	0	0	0	0	0	0	0	0	0	0	0	0
									158						21	179
		Plants on Glasshouse Bench During Incubation 50° - 70°F.														
<0.25-0.75	0.5		13	39	27	5	13	7	104	5	13	8	3	9	38	142
0.76- 1.5	1.0		32	115	34	16	23	12	232	13	16	13	6	19	67	299
1.6 - 2.5	2.0		6	13	1	5	4	0	29	3	2	0	1	3	9	38
2.6 - 3.5	3.0		1	1	0	0	0	0	2	0	0	0	0	0	0	2
3.6 - 4.5	4.0		0	0	0	0	0	0	0	0	0	0	0	0	0	0
4.6 - 5.5	5.0		0	0	0	0	0	0	0	0	0	0	0	0	00	0
									367						114	481

APPENDIX IX

Incubation Period

Number of Lesions Visible after exposing plants to various temperatures during the Incubation period

40°F.	A*							B*						
Plant	1	2	3	4	5	6	T	1	2	3	4	5	T	G.T.
Leaf 1	2	0	0	0	0	0		0	1	0	1	0		
2	5	1	1	1	7	0		2	1	0	0	1		
3	3	1	5	2	99	2		0	2	0	1	0		
4	1	2	3	4	5	1		11	1	1	0	0		
5	6	4	9	6	0	16		2	2	0	0	2		
6	22	4	24	18	27	4		4	1	2	1	4		
7	12	8	23	15	0	6		3	0	5	4	7		
8		19	2	20	-	6				2		1		
9		1										12		
10		1												
T	51	41	67	66	48	35	308	22	8	10	7	27	74	382
50°F														
1	0	6	0	0	0	1		0	0	2	0	1		
2	0	3	4	2	2	5		3	0	7	2	1		
3	1	1	0	0	1	2		2	2	3	2	4		
4	6	2	4	8	4	2		2	16	4	1	2		
5	9	7	4	1	9	0		3	4	6	1			
6	29	8	18	13	11	10		6	18	2	6			
7	30	6	3	9	19	10		15	3		5			
8	4	5		20	15	5			3					
9	3				3									
10	2													
T	84	38	33	53	64	35	307	31	46	24	17	8	126	433
60°F														
1	0	1	1	0	0	1		3	1	0	1	0		
2	1	8	2	0	2	0		1	0	0	2	0		
3	0	1	0	0	0	1		1	0	1	0	0		
4	1	11	6	3	2	0		2	2	0	0	0		
5	12	13	10	0	1	4		4	0	8	0	3		
6	12	10	5	0	30	21		5	1	3	4	6		
7	11	6	18	3	22	27		5	3	6	1	1		
8	1	13	15	17	20	19			4	0	0	5		
9			11	13					1					
10														
T	38	63	68	36	77	79	361	21	12	18	8	15	74	435

Appendix IX Contd.

70°F.	A*							B*						
Plant	1	2	3	4	5	6	T	1	2	3	4	5	T	G.T.
Leaf 1	2	0	0	0	0	0		0	4	0	0	0		
2	0	0	0	0	0	0		0	0	0	0	0		
3	1	0	0	0	8	2		0	4	0	0	1		
4	9	0	1	0	16	15		0	0	2	1	0		
5	9	4	0	0	0	22		0	0	0	0	0		
6	6	5	2	0	10	3		1	0	0	0	4		
7	0	1	5	9	4	0		1	0		0	0		
8		9	6	5	4			1			0	2		
9											0			
10											0			
T	27	19	14	14	42	42	158	3	8	2	1	7	21	179
50°-70°F	Plants on Glasshouse Bench During Incubation													
1	0	0	2	0	0	2		0	0	3	0	0		
2	4	0	5	1	0	1		1	1	4	0	2		
3	7	5	21	8	0	6		0	0	5	0	5		
4	6	8	15	0	0	8		2	19	0	2	6		
5	12	26	13	2	1	1		1	0	0	7	6		
6	22	68	6	5	11	0		2	3	5	1	8		
7	1	6		9	12	1		4	8	1	0	4		
8		55		1	13			10	0	3				
9					3			1						
10														
T	52	168	62	26	40	19	367	21	31	21	10	31	114	481

* Note: Group A Large soft plants. Group B Smaller hard plants.

Summary of analysis for effect of plant vigour on number of lesions

Temp	Average number of Lesions per plant		Result of t-test	P
40°F	51.3 ± 9.7	14.8 ± 6.1	* * *	<.001
50°F	51.2 ± 14.9	25.2 ± 9.6	*	.02-.05
60°F	60.2 ± 14.1	14.8 ± 3.4	* * *	<.001
70°F	26.3 ± 9.7	4.2 ± 2.1	* *	.001-.01
50-70°F	61.2 ± 40.7	28.8 ± 5.8	n.s.	.2-.3

APPENDIX X

Effect of Temperature and Type of Plant on Manifestation

Temperature		(1) 40°F					(2) 50°F					(3) 60°F					(4) 70°F					(5) 50-75°F					Rep.Totals
	Leaf	1	2	3	4	T	1	2	3	4	T	1	2	3	4	T	1	2	3	4	T	1	2	3	4	T	
Rep. I	Plant 1	8	8	7	4	27	7	6	6	10	20	3	2	4	2	11	3	2	2	6	13	2	2	4	3	11	
	2	8	4	5	3	20	6	5	6	2	19	4	3	2	2	11	2	2	2	2	8	7	4	2	3	16	
	3	3	2	3	2	10	5	6	6	5	22	3	2	2	2	9	3	1	2	2	8	5	2	3	7	17	
	4	3	2	3	5	13	8	8	8	9	33	6	5	3	4	18	6	3	3	6	18	3	6	6	5	20	
						70					94					49					47					64	324
II	5	3	5	7	4	19	6	7	6	7	26	8	4	6	6	24	3	3	6	3	15	4	2	6	3	15	
	6	2	3	5	4	14	7	9	4	6	26	8	4	3	9	24	1	3	4	5	13	7	6	8	8	29	
	7	6	3	2	2	13	7	5	9	5	26	2	3	6	8	19	10	10	3	1	24	3	2	6	3	14	
	8	3	2	6	3	14	5	5	6	4	20	6	3	9	5	24	5	1	10	7	23	3	2	2	5	13	
						60					98					90					75					71	394
III	9	1	4	2	2	9	8	4	3	4	19	2	3	3	5	13	6	4	5	4	19	9	7	2	4	22	
	10	6	8	6	9	29	10	10	4	7	31	2	10	10	5	27	2	4	2	6	14	6	6	5	4	21	
	11	6	4	7	5	22	7	8	6	6	27	8	10	2	4	24	2	3	3	8	16	2	4	2	5	13	
	12	5	5	6	5	21	9	9	5	7	30	2	3	2	4	11	2	4	2	3	11	2	3	3	2	10	
						81					107					75					60					66	389
IV	13	2	5	3	7	17	7	8	7	7	29	3	7	8	9	27	6	2	2	7	17	7	7	6	4	24	
	14	7	7	9	6	29	7	6	6	8	27	2	2	2	3	9	7	5	5	6	23	6	5	2	3	16	
	15	6	8	7	8	29	6	7	6	7	26	7	8	6	9	30	7	7	5	3	22	7	6	9	8	30	
	16	9	6	8	9	32	5	6	7	7	25	2	5	10	9	26	7	6	8	8	29	6	6	7	5	24	
						107					107					92					91					94	491
Tmt. Totals:						318					406					306					275					295	1598

Analyses (Duncan's (1955) Multiple Range Test)

(a) Analyses for Score of Diseased Leaves.
Valid Comparisons, Temp. 1 and 2, Temp. 2 and 3, Temp. 3 and 4, Temp. 5 with Temps. 2 to 4, Temp 2 and 4,

S.E. of mean = 5.315 x 0.500 = 2.657

	p	2	3	
1%	Rp	11.5	12.1	
5%	Rp	8.2	8.6	

Means	T1	T2	T3	T4	T5
	79.5	101.5	76.5	68.3	73.7
*Result					
1%	A	B	C	C	C
5%	a	b	c	d	c

(b) Analyses for Effect of Plant type on Manifestation.

S.E. of Mean = 5.315 x .447 = 2.38

	p	2	3	4
1%	Rp	10.3	10.8	11.1
5%	Rp	7.3	7.7	7.9

Means	BI	BIII	BII	BIV
	64.9	77.8	78.8	98.2
*Results				
1%	A	B	B	C
5%	a	b	b	c

* Treatments with same letter do not differ significantly, treatments with different letters differ significantly.

APPENDIX: XI

OBSERVATIONS ON SPORE DENSITIES AFTER 36 HOURS EXPOSURE TO HIGH RELATIVE HUMIDITIES

Temp. 40°F.		1	2	3	4	5	Total
Plant	1	2	1	1	0	0	4
	2	2	1	3	2	2	10
	3	0	0	0	0	0	0
	4	0	0	2	1	1	4
	5	0	0	0	0	0	0
	6	3	4	2	3	1	13
	7	2	2	1	1	3	9
	8	2	1	1	1	0	3
Totals							45
Averages/Plant. 5.6 ± 2.8							
Averages/Lesion 1.12 ± 0.56							

Temp.50°F.		1	2	3	4	5	Total
		5	4	0	2	3	14
		4	1	2	1	2	10
		3	2	1	3	2	11
		1	1	2	0	1	5
		4	2	1	2	5	14
		4	3	0	2	4	13
		2	2	3	2	3	12
		1	2	1	3	1	9
							87
10.8 ± 3.1							
2.16 ± 0.62							

Temp. 60°F.							Total.
Plant	1	1	2	1	3	3	10
	2	2	3	0	1	1	7
	3	0	1	2	1	2	6
	4	2	2	1	0	0	5
	5	1	1	3	2	1	8
	6	2	2	1	1	0	6
	7	0	0	4	3	2	9
	8	0	2	0	0	3	5
Totals							56
Averages/Plant 7.0 ± 1.8							
Averages/Lesion 1.40 ± 0.36							

Temp.70°F						Total
		1	0	1	1	0	3
		0	0	1	3	2	6
		2	1	2	1	0	6
		-	-	-	-	-	*
		1	1	1	0	3	6
		0	1	1	2	1	5
		1	1	0	0	0	2
		1	0	1	0	1	3
							31
4.4 ± 1.7							
0.88 ± 0.34							

* Plant missing.

APPENDIX XII

OBSERVATIONS ON LIBERATION OF CONIDIA

Room Temperature	58°F.	62°F.	65°F.	59°F.	Total	Average Spore Density
Time Interval 10 min.	0	0	0	0	0	0.0
20 min.	1	0	1	0	2	0.5
30 min.	0	1	0	2	3	0.8
60 min.	0	1	2	1	4	1.0
Air Temperature	70 - 80°F.					
Time Interval 10 min.	2	2	1	1	6	1.3
10 min.	1	1	0	2	4	1.0
30 min.	2	2	2	2	8	2.0
60 min.	2	1	2	1	6	1.3

NORMAL RELATIVE HUMIDITY

Air Temperature	63°F.	56°F.	64°F.	61°F.	Total	Average Spore Density
Time Interval 10 min.	0	1	0	0	1	0.3
20 min.	0	2	1	0	3	0.8
30 min.	0	1	0	1	2	0.5
60 min.	1	0	2	2	5	1.3

HIGH RELATIVE HUMIDITY

Time Invertal 10 min.	0	0	1	0	1	0.3
20 min.	2	1	2	2	7	1.8
30 min.	1	1	0	0	2	0.5
60 min.	0	2	0	1	3	0.8

Part I

Temperature Predisposition 40° - 70°C.
(40°C not recorded due to plants freezing.)

Temperatures		40°						50°						60°						70°						Totals
Block	Plant / Leaf	1	2	3	4	5	T	1	2	3	4	5	T	1	2	3	4	5	T	1	2	3	4	5	T	
I	a	0	4	0	12	15	31	10	77	62	7	0	156	6	9	100	0	0	115	0	1	0	2	0	3	
	b	8	4	7	8	5	32	3	2	7	7	0	19	0	0	10	3	0	13	10	6	10	9	0	35	
	c	15	0	1	3	0	19	8	2	5	5	6	26	0	3	12	5	6	28	0	26	18	0	0	44	
	d	4	4	2	0	1	11	5	11	81	3	6	106	0	0	0	1	7	8	1	0	17	2	0	20	
	T						93						307						164						102	666
II	a	0	0	5	6	0	11	4	4	1	14	11	34	0	0	0	0	2	2	2	0	2	0	0	4	
	b	4	0	2	9	4	19	11	17	1	5	2	36	0	15	22	12	5	24	2	0	1	0	2	5	
	c	0	2	1	2	0	5	5	4	0	4	0	13	14	3	3	6	4	30	3	3	5	2	1	14	
	d	4	2	3	0	3	12	1	2	0	4	5	12	6	3	2	1	2	14	0	0	1	1	3	5	
	T						47						95						70						28	240
III	a	0	4	2	8	9	23	2	0	5	2	0	9	0	0	2	0	0	2	6	2	9	5	2	24	
	b	0	3	4	18	4	29	3	3	2	14	6	28	7	30	5	0	1	43	0	2	1	5	15	33	
	c	1	0	0	0	15	16	13	14	9	2	0	38	3	2	0	3	0	8	6	0	11	4	12	33	
	d	2	1	7	14	20	44	10	1	4	2	12	29	3	0	0	0	2	5	2	0	2	3	0	7	
	T						112						104						58						97	371
IV	a	0	0	0	1	0	1	5	3	5	2	7	22	4	4	3	2	10	23	2	4	10	12	9	37	
	b	0	0	2	2	5	9	2	1	4	21	16	44	2	14	7	7	8	38	3	5	2	5	2	17	
	c	15	2	3	1	4	25	9	2	2	3	0	16	4	2	5	2	4	17	3	3	2	4	2	14	
	d	9	1	7	9	4	30	2	4	9	2	2	19	8	3	1	2	4	18	14	6	4	3	20	47	
	T						65						101						96						115	377
Total							317						607						388						342	1654

See Page 209

APPENDIX XIII Contd.

Part II

Number of Lesions per plant				(20 plants per treatment)			
-2°C				10° - 27°C			
Plant 1	8	11	195	Plant 1	21	11	96
2	23	12	101	2	74	12	47
3	123	13	49	3	91	13	62
4	18	14	226	4	160	14	71
5	280	15	74	5	116	15	122
6	103	16	114	6	94	16	186
7	91	17	44	7	351	17	157
8	51	18	82	8	170	18	113
9	51	19	32	9	29	19	149
10	39	20	93	10	151	20	150
Total			1497	Total			2390

See Page 209

APPENDIX XIV Predisposition:
Relative Humidity Treatment Totals

Row Treatments: Four positions during high humidity treatment.

Col. I & III Plants large and Soft
Col. IV Smaller hard plants
Col. II Intermediate type plants

	I	II	III	IV	Totals
A	Tmt* 12 226	Tmt 0 144	Tmt 9 413	Tmt 24 247	1030
B	9 238	24 204	12 234	0 131	807
C	24 253	12 386	0 122	9 221	1082
D	0 171	9 114	24 138	12 57	480
Totals	988	848	907	656	3399

* Tmts: Hours exposure to 100% relative humidity.

Results F. Test.

Differences not significant $p = 0.05$
C.V. = 49.3 S.E. Mean = 52.4

Note 1. Treatment Totals.

Time exposed to 100% R.H.	Number of Lesions per Treatment
0	568
9	936
12	903
24	942

Note 2. There appears to be a trend towards exposures of from 0 - 9 hours influencing the number of lesions appearing, but this failed to be significant and high infection always resulted even though plants were not exposed to high relative humidities before inoculation.

APPENDIX XV

CONTROL OF LEAFSPOT OF LETTUCE: GLASSHOUSE EXPERIMENT

Number of lesions per leaf:

		Rep. I					Rep. II					Rep. III					Rep. IV					T
Leaf		1	2	3	4	T	1	2	3	4	T	1	2	3	4	T	1	2	3	4	T	
Plant	1	10	1	17	24	52	5	4	1	4	14	37	22	33	5	97	5	6	2	17	32	
Tmt.	2	32	5	10	2	49	9	7	0	2	18	22	8	10	24	64	19	2	37	13	71	
1.	3	24	4	42	2	72	8	1	0	4	13	0	0	22	5	27	3	21	6	35	65	
	4	1	43	2	92	138	25	22	1	0	47	23	11	20	0	54	2	2	30	1	35	
	T					311					92					242					205	848
	1	0	2	3	1	6	0	0	3	2	5	0	5	0	3	8	0	0	0	0	0	
	2	1	0	0	0	1	4	0	3	6	13	4	0	4	0	8	2	1	1	6	10	
Tmt.	3	2	6	3	0	11	3	6	2	1	12	0	0	0	0	0	0	0	0	0	0	
2.	4	2	0	0	0	2	1	4	4	0	9	3	7	1	0	11	7	0	6	10	13	
	T					20					59					27					23	109
	1	0	0	0	0	0	16	0	3	0	19	0	0	0	0	0	23	0	1	0	24	
	2	0	1	0	0	1	0	2	4	6	12	10	0	0	1	11	1	10	2	11	24	
Tmt.	3	1	1	0	0	2	0	0	2	3	5	1	0	0	5	6	2	20	0	0	22	
3.	4	0	0	7	0	7	5	0	0	0	5	10	0	0	0	10	3	1	10	1	15	
	T.					10					41					27					85	163
	1	3	0	3	0	6	0	0	0	0	0	16	0	0	0	16	11	23	0	0	34	
	2	2	0	0	2	4	7	0	0	0	7	0	3	0	0	3	1	10	7	0	18	
Tmt.	3	0	3	1	0	4	8	2	3	0	13	1	0	0	0	1	1	1	0	1	3	
4.	4	0	0	0	5	5	12	11	0	0	23	0	1	0	0	1	4	0	18	0	32	
	T.					19					43					21					87	170
	1	4	14	15	24	55	8	48	25	3	84	12	57	64	43	176	5	41	19	36	101	
	2	17	28	19	27	71	33	29	10	36	108	44	18	38	17	117	9	30	38	3	80	
Tmt.	3	10	28	23	18	79	34	3	19	9	65	4	3	30	0	37	49	13	19	38	119	
5.	4	6	35	7	7	55	12	15	14	20	61	3	50	15	17	85	11	2	3	5	21	
	T.					260					318					330					321	1229
Total.						620					553					647					719	2519

Treatment 1 = Zineb 2 lb/100 gal. + sticker. Treatment 2 = Cuprox 5 lb/100 gal. + sticker
Treatment 3 = Thiram 2 lb/100 gal.+ sticker. Treatment 4 = Captan 3 lb/100 gal. + sticker
Treatment 5 = Control (Sterile Water + sticker

APPENDIX XV

APPENDIX XV Contd.

Results of Analyses (Duncan Multiple Range Test).

S.E. of Treatment Mean = 25.4

Shortest Significant Ranges:

p:	(2)	(3)	(4)	(5)
1% Rp	109.7	115.6	118.9	120.9
5% Rp	78.2	82.1	84.6	85.4

Treatment	5	1	4	3	2
Treatment Mean	307.2	212.0	42.5	40.7	27.3
* 1%	A	A	B	B	B
5%	a	b	c	c	c

* Treatments with same letter do not differ significantly.
Treatments with different letters differ significantly.

Analysis: Control of Leafspot: Glasshouse pot Experiment

	Block					
TMT	I	II	III	IV	T	AV.
T1	311	92	242	203	848	212.0
T2	20	39	27	23	109	27.3
T3	10	41	27	85	163	40.7
T4	19	43	21	87	170	42.5
T10	260	318	330	321	1229	307.2
	620	533	647	719	2519	629.7

CF = 317,268

T.SS = 608,757 - CF = 291,489

B.SS = 1604,059/5 - CF = 320812 - CF = 3,544

Tmt.SS= 2295,895/4 - CF = 574,224- CF = 256,956

Source	SS.	d.f.	M.S.	F.	Freq.	Result
Tmts.	256,956	4	64,239	24.879	3.26(5.41)	* * *
Blocks	3,544	3	1,181	0.457		n.s.
Error	30,989	12	2,582			
Total	291,489	19				

S.E. of a Tmt Mean = $\sqrt{\frac{2,582}{4}}$

= $\sqrt{2582} \times .5$

= 508.14 x 5

= 25.407