A Study of Brown Spot Disease of Lupinus spp. caused by Pleiochaeta setosa (Kirchner) Hughes.

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Kenneth Stuart Milne
Massey University of Manawatu
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INTRODUCTION

Lupins (Lupinus) are leguminous plants grown in most countries, from the hot equatorial lands of Egypt, North Africa, and Brazil, to the cooler climates of New Zealand and Chile. Over three hundred species have been described, including arborescent, herbaceous and ornamental forms.

Lupins are grown in different countries for various reasons, but their ability as a 'nitrogen-fixer', which enables them to grow in poor light soils and to increase fertility, is perhaps their main attribute.

In New Zealand Lupinus arboreus L., L.angustifolius L., and L.polyphyllus Lindhl., are the three most common species grown. The perennial arborescent L.arboreus is the form so prevalent on much of New Zealand's coastal sand country and along such rivers as the Manawatu and Rangitikei. In many places it is regarded as a weed and is treated accordingly. However, the New Zealand Forestry Department do make use of its tenacious capacity to grow on inhospitable exposed sand dunes where it is sown as an intermediary between marram grass (Ammophila arenaria) and pine trees. The consolidation of moving sand dunes by the use of marram grass and tree lupin has proved quite adequate to allow the establishment of large tracts of pine forests along areas of the west coast of the lower North Island.

Blue lupin (L.angustifolius) is an annual herbaceous form grown for stock food and/or seed, and for a green cover crop by the home gardner. As a fodder crop the popularity of blue lupin has risen and fallen. Inch (1947) writes in a New Zealand Department of Agriculture Bulletin:- "Over the past ten years, with the stimulus given by the development of a 'sweet' variety for lamb fattening,

they [blue lupins] have now become a crop of considerable economic importance in Canterbury, and their use is gradually spreading to other parts of New Zealand. They have a high nutritive quality, and are an excellent crop for raising the fertility of the soil."

Just on twelve years later Whatman (1959) writes: "Blue lupins are grown principally on the medium to light soils of Canterbury, particularly in those districts of less than average rainfall, either as a replacement for the turnip crop or to supplement turnips or other winter feeds." He then goes to point out that over the last ten or twelve years various factors have meant much less dependence on the lupin crop. These factors include: the improved fertility of light lands; the increase in lucerne area and in hay feeding; the greater use of other greenfeed crops such as 'Grasslands Paroa' Italian ryegrass and saved pasture. Personal correspondence with Whatman (1963) indicated a still further decline in the acreage of 'pure' stands of blue lupin grown from the approximately 5,000 acres grown in 1959. He now estimates that 1,000-2,000 acres are grown annually in the Ashburton district and that a further 5,000 acres occur in which lupins are sown at a light rate with other seeds, mainly turnips, and quite frequently with new grass in the autumn. Lupins can be extremely valuable when used in this manner and this constitutes one of their main uses today. They may be sown as a seed crop during the development of light land in order to obtain seed supplies for the grower's use as well as surplus for sale.

With more cereal cropping occurring now on light land, lupins can also fill an important role as a fertility restorative crop. White (1961), a lecturer in Plant Science at Canterbury Agricultural

College, considers that a crop of lupins following wheat on lighter land is desirable. Wheat causes a considerable drain on the soil fertility but a subsequent lupin crop helps considerably in ensuring a reasonable establishment of grass or lucerne.

Soil Conservation officers of the Department of Agriculture have demonstrated that bitter blue lupins, drilled with fertiliser, will grow vigorously and reseed freely on loose, windblown sand of any depth. Work in the Manawatu and Rangitikei districts (where there are about 150,000 acres of sand country) has shown that of the many plants tried for quick establishment on bare sand, blue lupin was the most promising. One to two years after the establishment of such a crop, a ryegrass-cocksfoot-white clover pasture can be established (Pearse 1958 and 1964). Despite the promising results obtained by this Department with lupins, farmers have, for various reasons, been slow to utilise them and only about 550 acres of moving sand has been reclaimed to pastures.

One of the main factors limiting increased use of blue lupins is the scarcity of seed in some years, and its high price compared with other forage crops. This is especially so with the sweet Borre variety which is generally more expensive than the bitter blue. Two factors cause the high seed prices—

(1) low seed yield, and

(2) difficulty of harvesting.

For blue lupins to increase in importance, a non-shattering, high yielding variety of lupin is required.

Russell lupin (L.polyphyllus) is the most common type of ornamental lupin grown in New Zealand, although the annual L.hartwegii Lindhl., and L.mutabilis Sweet, are also available.

Yates's Garden Guide (1961) notes that the perennial type is very beautiful and more suited to the colder climates such as the South Island or the cooler central districts of the North Island and that the annual varieties grow better in Auckland and other northern districts.

Particularly in Europe, lupins are extensively grown and a number of diseases have been recorded in the literature. A disease commonly referred to as 'Brown Spot' (a rather nebulous title for a fungus disease), appears to be of considerable importance. Brown Spot disease of lupins (Lupinus spp.) was not officially recorded in New Zealand until July 1958 (Dingley 1959) when it was found on leaflets of seedling Lupinus angustifolius. No record can be found of the disease on any other species of lupin in New Zealand and any literature dealing with fungus diseases of lupins in New Zealand makes no mention of Brown Spot caused by Pleiochaeta setosa (Kirchner) Hughes.

Numerous records of this fungus occur in overseas literature, particularly its occurrence in European countries. According to Germar (1940), the Brown Spot disease was first described on lupins in Germany in 1898 by Wagner and Sorauer as a disease caused by the fungus Pestalozzia lupini. Subsequent investigations revealed this parasite to be the same as that described by Kirchner in 1892 on Cytisus plants.

That the fungus attacks a wide range of lupin species is certain from a cursory glance at the literature pertaining to it. Richter (1938) found the disease on a dozen lupin species including L.angustifolius, L.albus L., and L.luteus L., and these observations were later confirmed by another German, Germar (1940), who found 22

lupin species to be susceptible. These included L.albus, L.polyphyllus, and L.luteus. According to Pape (1927) the yellow lupin (L.luteus) and the white lupin (L.albus) are relatively resistant to the disease. Pulselli (1928) also notes that L.albus shows a certain degree of resistance to Brown Spot disease. However, work by du Plessis and Truter (1953) in South Africa fails to confirm these views on resistance.

In the literature, premature defoliation of the infected plants appears to be a common characteristic of the disease, for several writers make specific mention of this, in particular Pape (1927) on L.cruikshankii Hook and L.mutabilis Sweet, and Richter (1938) on L.albus. Hogetop (1938) in Brazil states that on young white lupin (L.albus) affected leaves are prematurely shed. He also notes that young white lupin plants are frequently killed after complete defoliation, and a reduction in yield invariably follows. Weimer (1952) in the United States makes similar observations on blue lupin (L.angustifolius).

A fungus disease that has the capability of becoming seed-borne often provides an ideal method of carry-over from crop to crop or from season to season. P.setosa has quite frequently been recorded as capable of this and du Plessis and Truter (1953) developed a technique which proved very satisfactory for isolation of the fungus from diseased white lupin seed. They suggest this infected seed offers ample opportunity for the transmission of the disease from infected areas to disease free areas, and further suggest that the disease was imported into the Union of South Africa by this means. Weimer (1952) opens his account of Brown Spot disease of lupins thus:- "The brown spot disease was probably brought here [the United States

of America], from Europe in the seed. It is now widespread in this country."

Preliminary investigations carried out on the three lupin species grown in the Manawatu have enabled ready isolation of P.setosa in all cases. Although recognising that lupins are not an important plant in New Zealand and thus diseases on them do not warrant study from this viewpoint, the following relative facts were noted:

(i) Despite the large number of records which have been made of this fungus overseas, few detailed studies have been made and these pay little, if any attention to the disease cycle, i.e. how the fungus is disseminated, carries out infection, and survives away from the host plant.

(ii) The fungus has only been officially recorded in New Zealand on blue lupins but is known to be prevalent on Russell and tree lupin also.

(iii) Overseas the fungus has been shown to be seed-borne and knowledge on this facet of lupins in New Zealand would possibly prevent us importing diseased seed and would at least lead to the possibility of either healthy seed being sown, or some treatment being devised to eliminate the fungus from the seed.

Consideration of these facts led to the following topic being chosen: "A Study of Brown Spot Disease of Lupinus spp. caused by Pleiochaeta setosa (Kirchner) Hughes", with the following objects of study:

(1) To isolate, identify, and prove pathogenicity of the fungal pathogen causing Brown Spot symptoms on tree, Russell and blue lupin.

(2) To study the fungus in pure culture.

(3) To investigate the disease on Russell, tree and blue lupin in the field.

(4) To study the disease cycle with particular reference to survival and spread.

(5) To investigate the possibility that the disease is seed-borne, and if this is verified, to develop satisfactory techniques to identify the fungus and determine the importance and form of the seed-borne inoculum.

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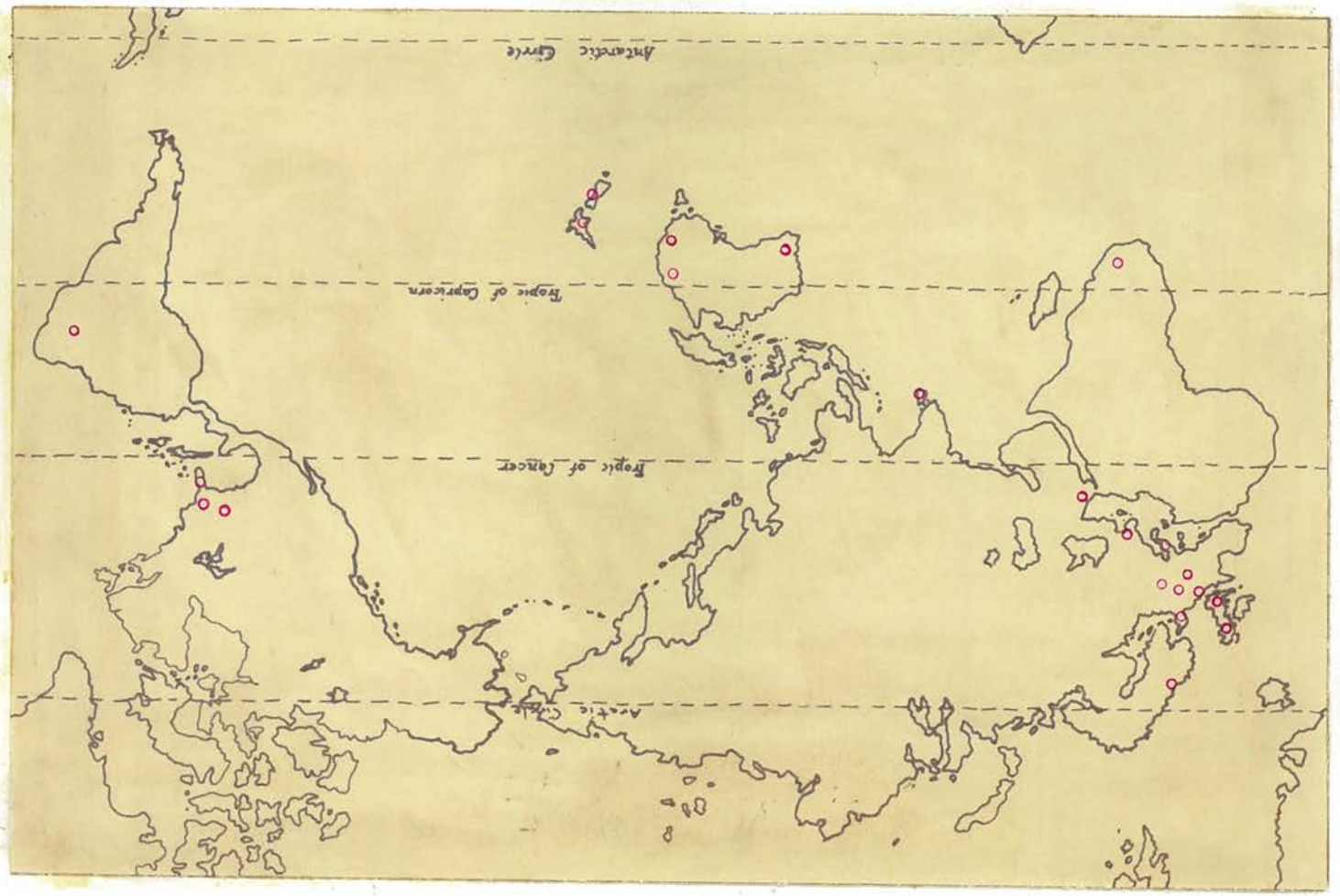
(R.A.M.—Review of Applied Mycology)

MAP SHOWING WORLD DISTRIBUTION OF PLEIOCHAETA SETOSA (KIRCHN.) HUGHES ON SPECIES OF LUPINUS, CYTISUS, LABURNUM, CROTOLARIA, AND PHASEOLUS.



o Countries where P.setosa has been recorded:

Asia: Ceylon, Palestine.
Australia (Western Australia, New South Wales, and Queensland).
Europe: Bulgaria, Czechoslavakia, Denmark, England, Estonia, Germany, Holland, Italy, Latvia, Lithuania, Norway, Poland, Scotland, Switzerland.
New Zealand: North and South Island.
North America: United States (Alabama, Florida, Georgia). South America: Brazil.

Illustration 1

CHAPTER I

MATERIALS AND METHODS

General materials and methods used commonly in the study are given in this chapter. Those specific to particular aspects of the work are detailed in the appropriate chapters.

A. MATERIALS AND METHODS PERTAINING TO LABORATORY STUDIES

1. Collection of diseased material from the field and subsequent treatment

(a) Collection and Laboratory Examination

Specimens of leaf spots on Russell, tree and blue lupin were frequently collected from the field and placed in small 9" x 4" plastic bags. These were labelled according to the variety of lupin, place collected, suspected pathogen, and the date of collection.

In the laboratory, the specimens were arranged on the bench in groups according to the macroscopic symptoms, and a brief written description was made of each type. Examination of lesions under a binocular microscope (x50 magnification) was made to ascertain presence of spores or other fruiting bodies, and details were recorded.

(b) Isolation techniques

In conducting pathogenicity experiments it is necessary to isolate the suspected pathogen to pure culture, assuming the organism concerned is capable of saprophytic growth. In preliminary experimentation two different methods were tried.

The first method involved a SURFACE STERILISATION technique. Small pieces of tissue approximately 10 mm x 15 mm were cut from the margin of lesions and placed in a square of butter muslin approximately 3" x 4" in size. The four corners of this square were gathered and secured with a rubber band thus forming a small muslin bag. Different coloured bands or combinations thereof were used when a number of samples were being washed at one time to ensure correct identification. The bag was then placed in a 500 ml beaker with a quarter-inch wire

mesh basket inverted over the beaker mouth. A rubber hose from the tap was placed so that the tube on the water outlet from a vacuum pump was passed right into the beaker. The taps were turned on at a pressure sufficient to cause vigorous agitation of the bag thus permitting thorough surface washing of the enclosed tissue pieces. After two to four hours the tissue pieces were removed to sterilised filter paper and dried. The pieces were then transferred to media with a sterilised needle. Prune agar, potato-dextrose agar (PDA) and potato-carrot agar (PCA) were all tried and each had advantages as a plating medium. However, in most instances prune agar was preferred as it tended to suppress bacterial growth and P.setosa formed typical chlamydospores, thus enabling ready identification. PCA was also useful in that P.setosa formed conidia abundantly on it but at times bacterial contamination was a problem.

When bacterial contamination did occur it was still possible to obtain the fungus in pure culture utilising the van Tieghem cell method as used by McKeen (1949). This technique proved particularly useful when isolating root-rotting or damping-off fungi where bacteria were almost invariably present. Incubation of plated tissue pieces for three to five days at 24°C was usually sufficient to enable satisfactory isolation of the fungus involved.

Although this surface sterilisation technique proved quite satisfactory, the following HIGH HUMIDITY method was generally used as results were obtained more rapidly and less time was involved in preparing material.

Leaflets or portions of leaflets exhibiting lesions were given high humidity for 36-60 hours at 24°C. The method involved utilised a Petri plate as a high humidity chamber. Water was used in

the base of the Petri plate and small triangles were formed with quarter-inch glass-tubing and placed in the water. One 3" x 1½" glass slide was placed on the triangle and the tissue pieces or leaflets arranged on this. The slide was removed for examination at different intervals and isolation of spores was made using the binocular microscope. Because of their size, individual spores could be readily removed with a sterilised needle and transferred to a Petri plate containing PCA. Five inoculations per plate proved quite satisfactory. After four or five days subcultures could be prepared from the resultant colonies.

2. Preparation of Media

The details of media preparation are given in Appendix I. Laboratory potato dextrose agar (lab PDA) was initially used for all culturing and spore production in the laboratory, but after experimentation PCA replaced lab PDA for spore production. Prune agar was used for production of chlamydospores. Prepared media was held in partially filled 250 ml flasks 'bunged' with cotton wool, or in 5oz or 10oz McCartney bottles.

3. Pouring of Plates

Contamination of plate cultures by Penicillium and Mucor proved a problem during the study but was minimised by pouring plates at night when the air was still, and spraying the laboratory and incubators with a dilute solution of "Zephiran"*.

Petri plates, 90 mm in diameter, were used for culturing the

* Zephiran - 10% aqueous solution of alkyldimethylbenzylammonium chlorides.

fungus in the laboratory. Five or six plates were normally poured from 100 ml of media, as use of less than 15-20 ml per plate tended to cause irregular growth of the fungus and certainly led to quicker drying out of the media at higher temperatures e.g. 28°-37°C.

At times mites were troublesome, being inadvertently introduced into the incubators with plant material from the field. These were overcome by clearing the incubator of all contents, wiping out with kerosene and then placing a little of this in a Petri plate on the floor of the incubator. This method proved more convenient than the raising of the temperature.

4. Inoculation of Media

Except when isolating from host material to media, a 5 mm diameter circle of inoculum obtained from an actively growing culture of the fungus was placed in the centre of the Petri plate. Experimentation showed that placing the inoculum upright or mycelium side down had no influence on colony diameter. These 5 mm circles were obtained by the use of a metal cork-borer.

5. Criteria used to determine the effect of specific environmental factors on growth in pure culture

Experimentation showed that growth rate was linear in respect to time. Accordingly, the diameter of colonies was measured at the end of a given time interval and not each day. Brancato and Golding (1953) established that colony diameter is a valid measure of the effects of environmental factors such as pH, temperature, and medium constituents. Accordingly this criterion was used throughout the study when factors affecting the fungus in plate culture were being

studied.

Five or six cultures were measured for each treatment, two measurements per plate at right angles to each other being made, and the results expressed as an average figure. Measurements were usually made nine or ten days after inoculation.

6. Production and Preparation of Inoculum

(a) Conidia

When conducting pathogenicity studies it is essential to have a medium on which the pathogen sporulates readily. On PCA *P.setosa* produced a large number of conidia within ten days at 24°C.

The following standard technique was evolved for the production of spore suspensions: 10 ml of distilled water was pipetted over the surface of a ten day old culture; a small sterile paint brush was used to lightly brush the surface of the culture to encourage dislodgement of the spores. At first, attempts to determine the specific concentration were made using a Neubauer haemocytometer. However, results were not repeatable, possibly because the large spore size and protruding setae did not allow an uninhibited passage of spores beneath the cover slip. The method finally adopted involved a relative comparison of spore numbers and this was done by making several counts (usually eight), of the number of spores in the area of the Petri plate suspension as seen in the field of a x50 binocular microscope. Unless otherwise stated, the same level of inoculum was used in all inoculation experiments (a spore concentration of 30 conidia per field).

Depending on the purpose for which the inoculum was required, so the number of plates used varied. Where a box of plants was to be

inoculated the spore suspension was transferred to a conical flask and an 'Atomic Mist Sprayer'* used to atomise the plants. With lupins, particularly blue lupins, there was considerable wasteage of inoculum because of the smallness of leaflets. Consequently it was found to be more satisfactory, when plants were small and in pots, to use a fine paint brush to 'paint' the spores on whilst the individual leaflets were held between the fingers. Although tedious, this method proved quite satisfactory for it also overcame the difficulty of wetting the leaflets. Incorporation in the suspension of a few drops of a wetting agent such as 'Teepol' proved unsuccessful as it inhibited germination of the conidia.

(b) Chlamydospores

A sterile filter paper was placed in a Petri plate and moistened with sterile water. Five mm circles of P.setosa from prune agar cultures were used to inoculate these filter papers and the plates incubated at 24°C. The fungus grew out from the inoculum into and on the filter paper forming large numbers of chlamydospores. These were readily removed with a needle for examination under a microscope for the purposes of drawing.

7. Preparation of Permanent Slides

Because conidia of P.setosa are lightly coloured, a stain is not essential for microscopic examination. However, lactophenol acid fuchsin does make the spores easier to measure, photograph, and draw. Spores or chlamydospores were mounted in a drop of lactophenol acid fuchsin and a cover-slip applied. Clear nail varnish was

* Commercial name for a small atomizer.

applied lightly around the edge of the coverslip and on the slide. For the nail varnish to seal satisfactorily, it was essential that very little mounting fluid should be used. Permanent slides of spores on cleared leaflets were also prepared in this way.

8. Design of Experiments

The number of replications used in an experiment varied according to incubator space available or the availability of other equipment. Where colony diameter was to be measured, 5 or 6 colonies were used.

In an experiment such as the effect of pH on colony diameter, random numbers were used to label the plates so no bias would be introduced. Similarly, plates were arranged in a random fashion within the incubator.

B. MATERIALS AND METHODS PERTAINING TO THE GLASSHOUSE

1. Production of Plants

A standard John Innes soil mix was used for growing all plants. This was supplemented with fertiliser, and in addition, plants were watered once weekly with a foliar nutrient solution, 'Maxicrop'*.

Pots and boxes were soaked in a 2% solution of formalin after washing and then put aside to dry for two days before use. This sterilisation of pots and boxes together with the use of sterilised soil was a necessary precaution taken to obviate the possibility of seedling damping-off.

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* A commercial preparation derived from seaweed.

Early in the work it was established that P.setosa isolated from Russell, tree and blue lupins had similar cultural characteristics, spore dimensions and ready cross inoculation. As blue lupin seed was cheap, readily obtainable and capable of quick germination and subsequent growth, it was chosen as the most suitable species for disease cycle studies. It was found that soaking the seed in water prior to sowing resulted in more rapid germination. Small seed boxes (18" x 12" x 3") were most commonly used for production of seedlings. After the first two compound leaves had fully emerged the seedlings were transferred to clay pots. Three blue lupins were planted per pot but with Russell and one or two of the other species grown for host inoculation studies, one per pot was more satisfactory.

Dormancy due to hard seed is quite a common feature of legumes and many of the lupin species and the laburnum grown for host studies of P.setosa were no exception.

The recommendation offered by the New Zealand Department of Agriculture for breaking dormancy in lupin seed is to chill seed at 5°C for two days (Lithgow, 1956). In the present study this method was found to be ineffective whereas scarification enabled rapid germination of all species sown. Lupinus digitatus Forsk. was the most difficult to germinate and it was necessary to rub the seed on an emery wheel before water was absorbed. L.albus and L.mutabilis did not require scarifying.

Plants were watered twice daily during the period November to March as the blue lupins were particularly prone to wilting. In cooler weather plants were watered every second day. During summer the floor and glass of the house was kept wet in an attempt to keep temperatures down but despite this, maximum temperatures of 95°F were

recorded. Through the winter an average temperature of approximately 65°F was maintained by means of thermostatically controlled heaters situated under the benches.

To ensure availability of plants of the required age at all times for disease cycle studies, a box of blue lupin was sown each week. The application of fertiliser as a side dressing proved essential in cases where plants were grown through until maturity.

A plot of blue lupins was sown to enable studies to be made on the spread of the fungus in the field.

2. Control of Pests

White Butterfly caterpillar and aphids were the two insect pests causing most concern in the glasshouse. DDT or Lindane dust gave good control of caterpillar and Malathion spray controlled green aphid.

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CHAPTER II

THE FUNGUS

Morphology and Taxonomy of *Pleiochaeta setosa*, together with studies of the fungus on culture media.

A. MORPHOLOGY OF PLEIOCHAETA SETOSA

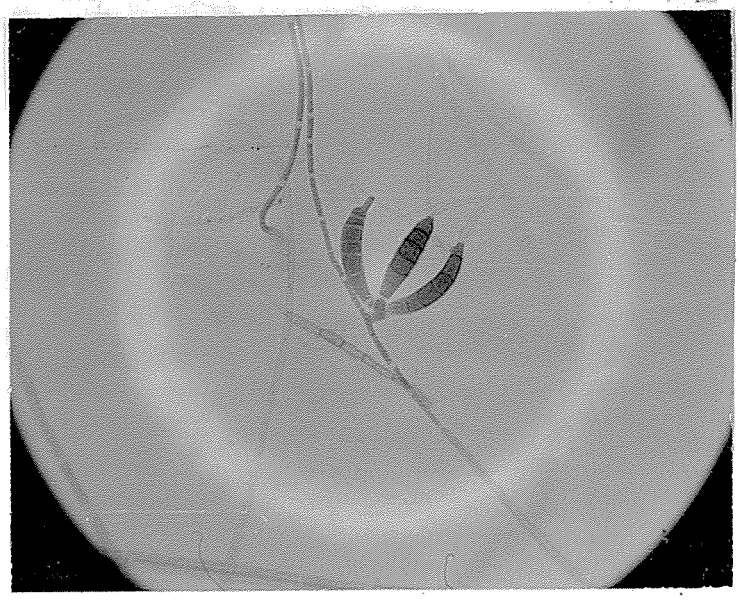
The mycelium of P.setosa spread extensively within host tissues, often initially progressing in a cobweb formation, lesioned areas coalesce, and host cells become unrecognisable. The young hyphae are hyaline but, as they age, darken, become closely septate, and assume a brown colouration. Hyphae range from 5-17μ in width.

Chlamydospores in chains or groups may be found irregularly scattered in the invaded tissues. The chlamydospores range from 10-26μ in diameter and appear most frequently as 'knots' of swollen heavy-walled cells which are dark in colour (Illustration 2).

The fungus produces conidia from conidiophores which arise from the mycelium, but which are not arranged in a definite fruiting body (Illustration 3). Conidiophores are more often solitary, but occasionally occur in small groups. Normally a narrow hypha penetrates the cuticle of a leaflet and forms a swelling on the outside. A prolongation of this basal swelling arises and a conidiophore is differentiated; sometimes two conidiophores develop from a single swollen cell. In most instances conidiophores are hyaline and range in length from 20-132μ and are approximately 8.5μ wide. Conidia arise as a swelling developing from the end of the conidiophore and when one is thus formed a new growing point can develop below it (Illustration 4). A succession of similar growing points results in the production of a bent conidiophore which may assume a regular zigzag formation if it continues to develop (Illustration 4(b)). Occasionally conidia may arise directly from the vegetative mycelium (Illustration 4(a)).

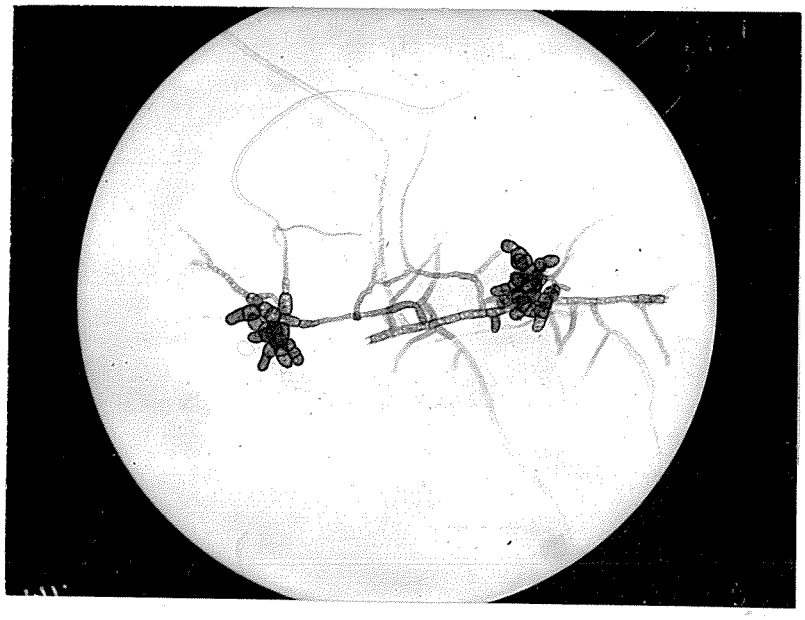
The conidia of P.setosa are roughly spindle-shaped,

Illustration 2 (x320)



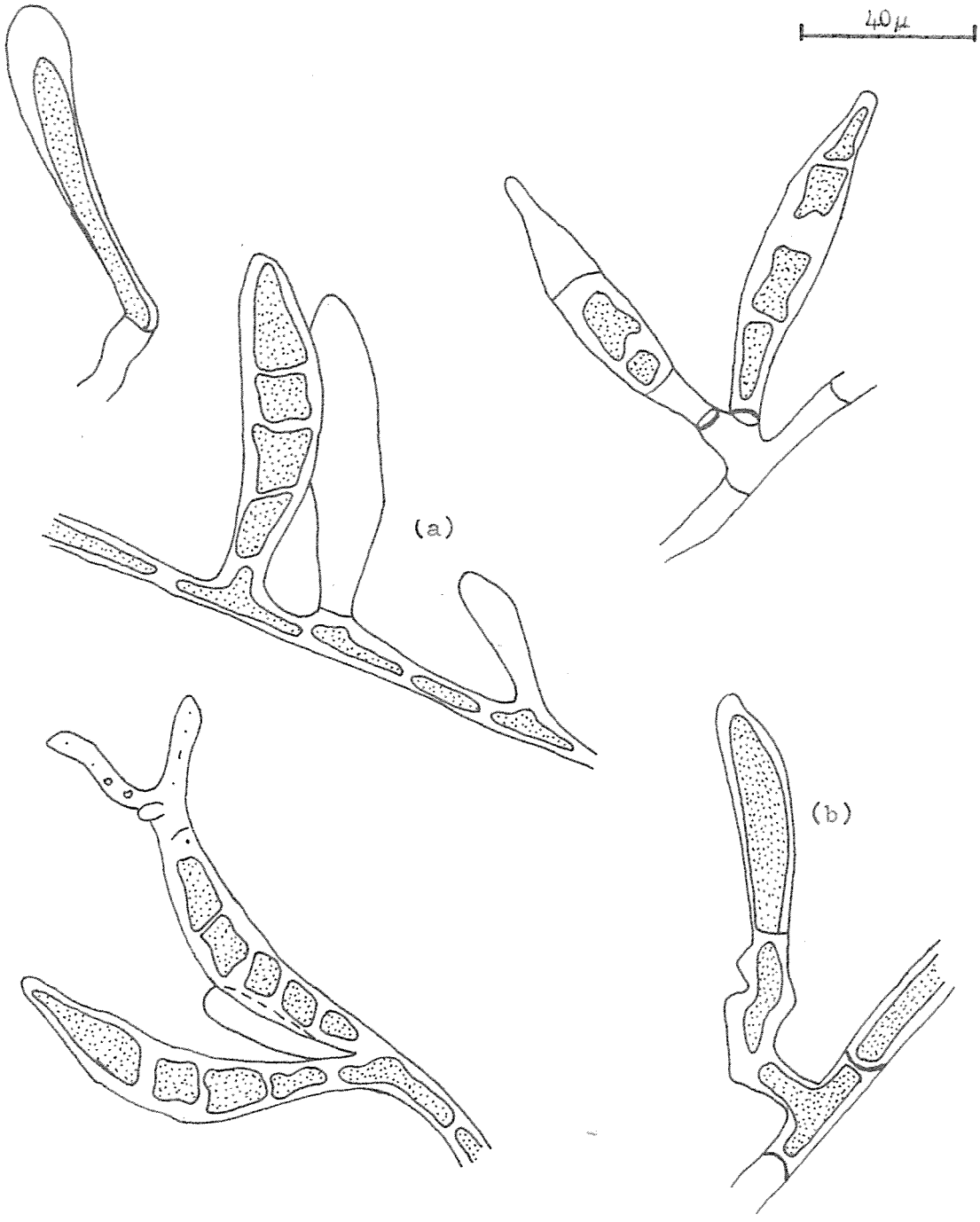
Chlamydospores of Pleiochaeta setosa
(produced on prune agar)

Illustration 3 (x320)



Arrangement of a group of conidia of
Pleiochaeta setosa on conidiophore
(produced on water agar)

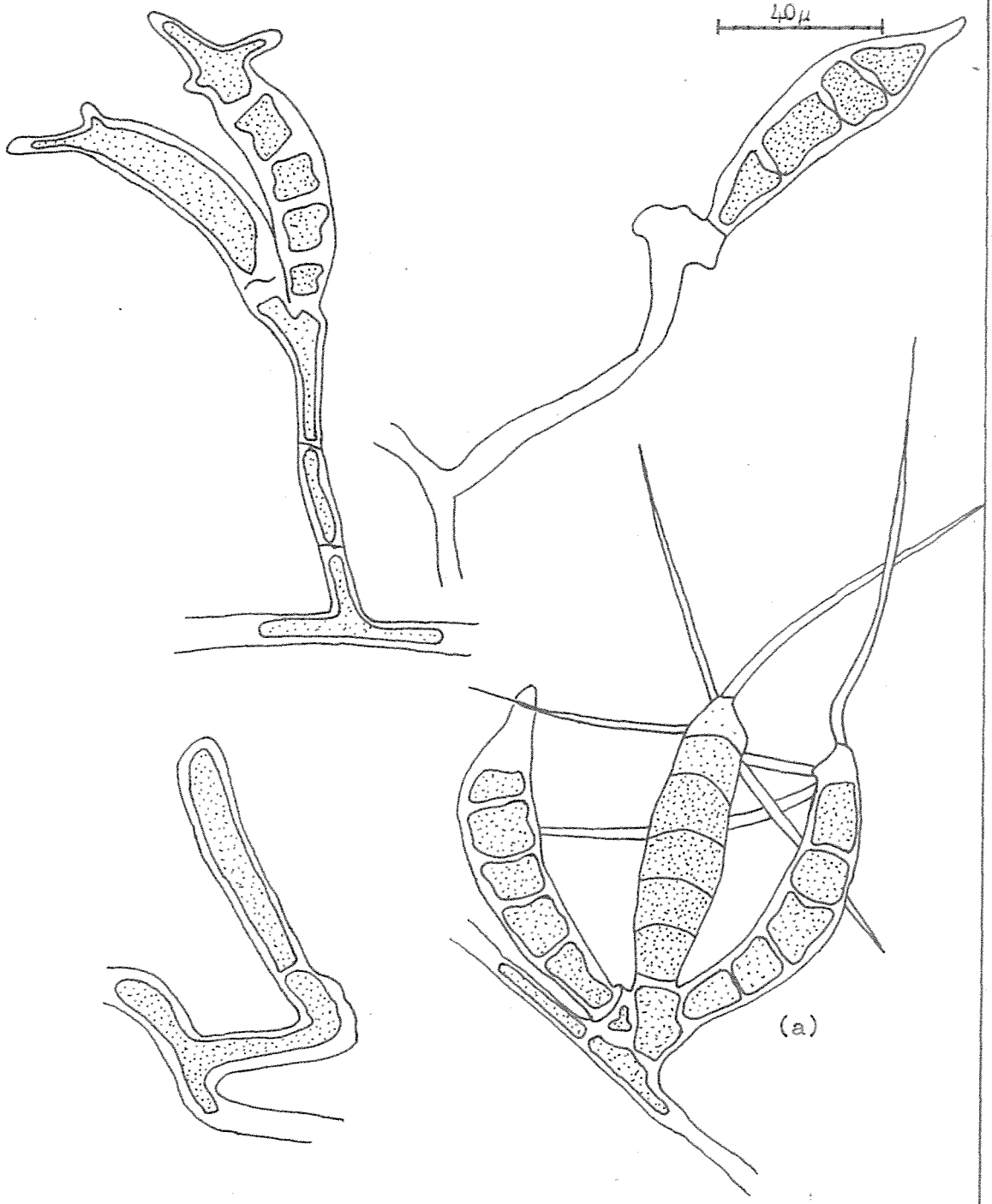
Illustration 4



Stages in the development of conidia of Pleiochaeta setosa

(a) conidia arising directly from the mycelium
(b) zigzag-shaped conidiophore

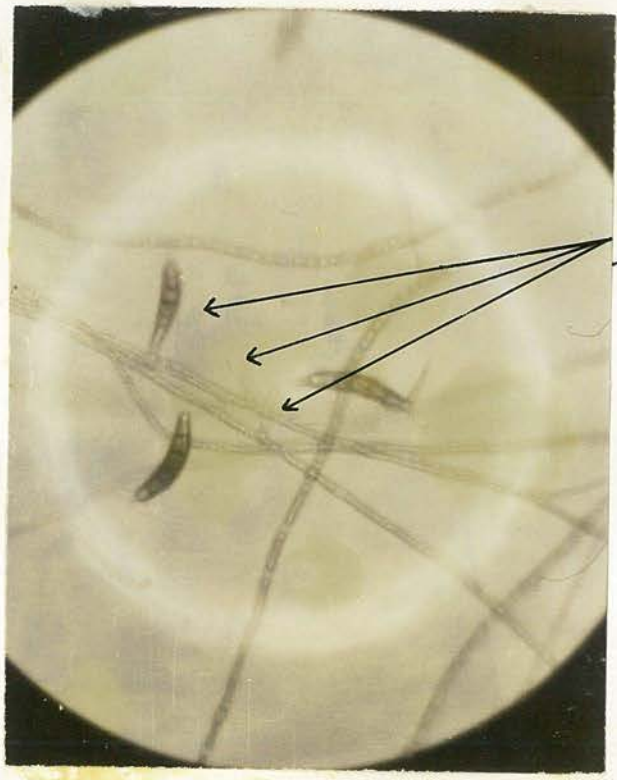
Illustration 5



Stages in the development of conidia of Pleiochaeta setosa

(a) mature conidium showing long terminal setae

Illustration 6 (x320)

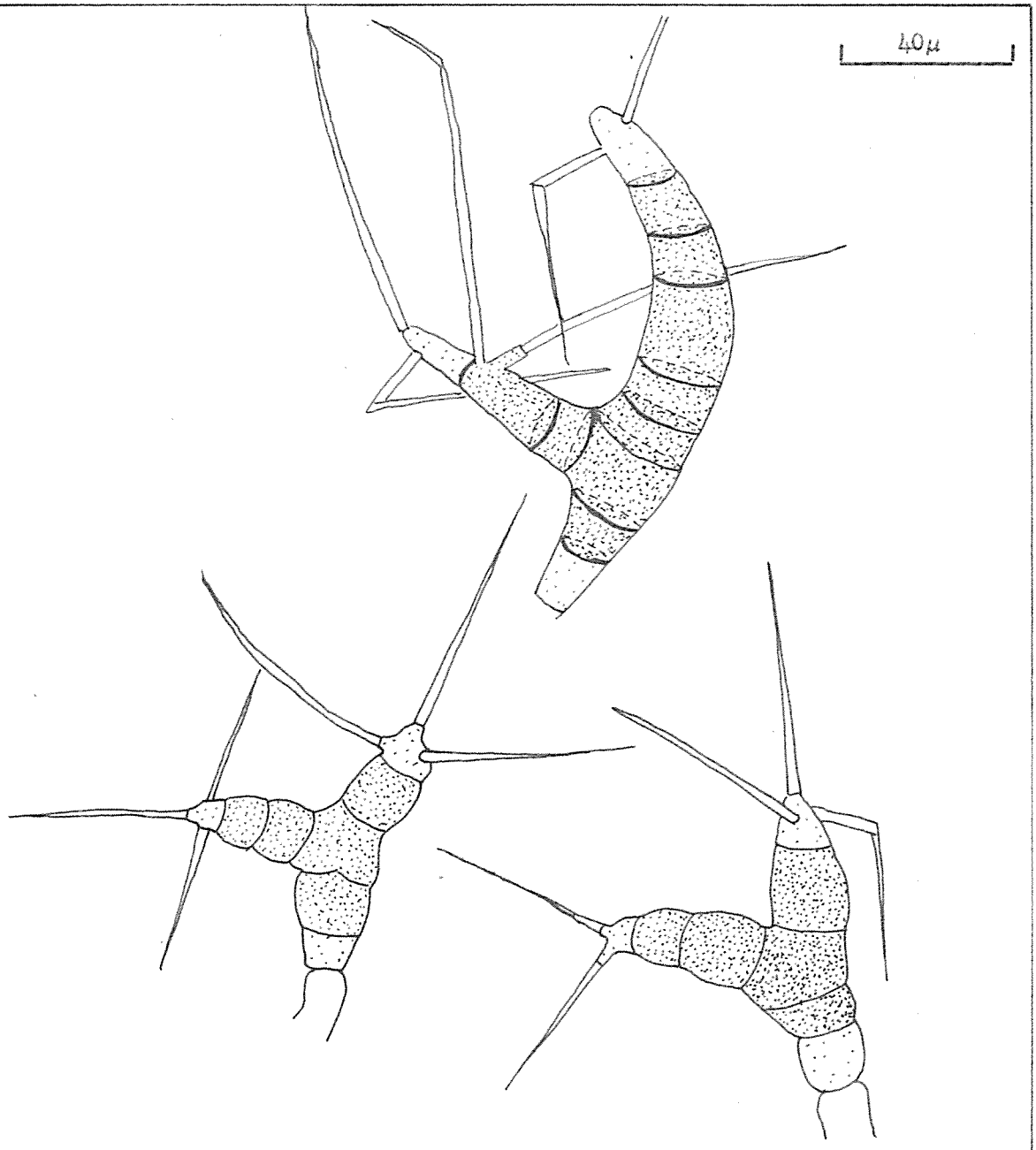


Development of conidia
of Pleiochaeta setosa
(on water agar)

Illustration 7 (x320)

Mature conidia of Pleiochaeta setosa
(produced on diseased blue lupin
leaflets after 60 hours
at 24°C and 100% RH)

Illustration 8



Abnormal conidia of Pleiochaeta setosa
produced on diseased blue lupin leaflets

multi-cellular in plane, and have bristle-like appendages at the terminal end. The bodies of conidia range from 67-86.4μ in length, and from 12.3-18.5μ in width; the number of septa vary from four to eight; the basal and apical cells are hyaline whilst the central cells are pale brown. The apical cell is subconical and bears a terminal, hyaline, thin-walled and tapering appendage, and is up to 115μ long and approximately 2.5μ wide. The apical cell also bears one to four similar lateral appendages, but these are generally shorter than the terminal one (Illustration 5(a)). These appendages may be branched but in most instances are simple (Illustration 14(a)). Occasionally a single conidium may have one base but divide into two part way up the body, both theselatter portions then producing their own apical appendages (Illustration 8).

The figures quoted and the measurements used in this section were obtained from specimens of P.setosa taken from infected leaflets of blue lupin (L.angustifolius) held at 100% RH (24^{o}C) for 36 hours.

Measurements of conidia were also taken from specimens of P.setosa on several other species of lupin which had been subjected to a similar period of high humidity.

The figures in the following table show that there is quite a variation in conidial size at a given time, both within populations on one host, and between populations on different host species. Similar variations in conidial measurements are obtained if samples are taken from conidia produced on different artificial media, even if isolates being cultured are taken from the same host. Conidia with a body length of up to 108.6μ were measured from potato agar and down to 53.3μ from tobacco extract agar.

That the figures obtained for conidial measurements in this

Table 1

Conidial Measurements (in μ for 100 samples per species) of *P.setosa* ex Host after 36 hours at 100% RH (24°C)

Host Species	Ave.Body Length	Range	Ave.Body width	Range	Ave.no. of septa	Range
L.angustifolius	77.4	67.0-86.4	15.7	12.3-18.4	4.9	4-8
L.polyphyllus	78.3	69.7-86.1	15.2	12.3-16.4	4.9	4-6
L.arboreus	79.5	69.0-88.1	14.4	12.1-16.2	5.2	6-8
L.hartwegii	59.4	47.1-71.7	14.2	11.9-15.3	4.0	3-6
L.albus	73.8	63.5-84.0	13.4	12.3-14.2	4.6	4-6
L.pilosus	75.8	65.6-88.1	14.6	12.7-16.2	4.2	3-6
Laburnum anagyroides	76.7	55.3-87.0	14.8	12.4-16.5	4.9	4-7

study fall in a similar range to those quoted by workers overseas is borne out by the following information:

1. Kirchner (1892) originally described the fungus on Cytisus capitatus and gave the following conidial measurements: 40-80μ x 15-19μ; 3-8 cells; 3-4 spikes at apical end, occasionally more—these may exceed the body length and are about 2.5μ at the base.
2. Cavara (1924) on L.albus: 70-80μ x 14-16μ; 3-5 septa and polar flagellum up to 90μ.
3. Green and Hewlett (1949) on Cytisus spp. 46.5-93.0μ x 15.5-23.3μ (average 75μ x 17μ); 2-7 septa.
4. Hughes (1951) on Lupinus spp. in Herb. I.M.I. (as 7691) 63-98μ x 13-19μ; 4-8 septa and main apical appendage up to 108μ x 3μ.

The genus Pleiochaeta includes only one other species, namely Pleiochaeta albizziae Petch which is pathogenic to species of Albizzia. This latter fungus has so far only been recorded in tropical countries and differs from P.setosa in two ways; (i) the conidia are colourless, and (ii) it is pathogenic only to Albizzia spp. P.setosa shows considerable variability in detailed morphological characteristics, but the basic morphology and the results of host range trials, recorded later in the study, confirm the generally accepted view that only one species in involved in causing Brown Spot disease of Lupinus species, namely P.setosa.

B. TAXONOMY OF PLEIOCHAETA SETOSA

Pleiochaeta setosa (Kirchn.) Hughes is a member of the form-class Deuteromycetes, a large group of fungi for which sexual stages have not been discovered or no longer exist, but in which a parasexual cycle may be present. The characters used for classification within the Deuteromycetes are: the type of fructification; shape, colour, and septation of the conidia. Types of fructification form the basis for separation of form-orders and Pleiochaeta setosa is included in the Moniliales as it produces conidia on conidiophores not arranged in a definite fruiting body. On the basis of conidial colour, P.setosa is placed in the form-family Dematiaciae. Form-genera of the Deuteromycetes are delimited by such characters as shape, septation, and colour of conidia, and the type of conidiophores. Because of the large number of form-genera included in the Deuteromycetes, Saccardo (1899) proposed a further breakdown into groupings (sections). Using this scheme P.setosa would be placed in the Phaeophragmiae, as the conidia are coloured and have two to many transverse septations. Although this system is very convenient for mycologists, the 'section' is not recognised as an official category in the classification of the Deuteromycetes.

Research in recent years has repeatedly demonstrated that characters such as spore colour, shape, size, and septation, lack stability under varying environmental conditions. Accordingly the very basis for classification within the Deuteromycetes is suspect and the current trend is therefore towards the development of a scheme based on alternative characters not subject to variation with changes in environment. Hughes (1953) proposed one such scheme

using structure of conidiophores and method of conidial production to delimit eight sections. Tubaki (1958) elaborated further on Hughes's work adding one section and subdividing others. To date, only species included in the Hyphomycetes* have been studied by Hughes and Tubaki with a view to reclassification. Commenting on this scheme Alexopoulos (1962) states:- "This seems to be a much more natural foundation for a classification system, and it is probable that it will eventually replace the Saccardian system when all the known form-genera have been studied and their probable affinities have been determined." Under this new system P.setosa is classified in 'Section II', where conidia are produced singly from successively formed new growing points.

History of the Systematic Position of Pleiochaeta setosa

1880. Saccardo proposed the generic name Ceratophorum Sacc. and presented a Latin diagnosis, a translation of which is:- "Conidia forming on leaves are spindle shaped to cylindrical, fusoid, and pale in colour. Example C.helicosporium Sacc. (sub Sporid.)."

1892. Kirchner described a fungus on seedlings of Cytisus laburnum L. (= Laburnum anagyroides Medic), and C.alpinus L. Consideration of the fungus in relation to the systematic classification of fungi caused Kirchner to place it in "the family Ceratophorum Sacc. (Sylloge Fungorum IV, p.495)" Kirchner (1892). A photocopy of the first drawings of conidia, made by Kirchner in 1892, are shown in illustration 9.

1895. Saccardo made Ceratophorum setosum Kirchner the type

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* Hyphomycetes = Moniliales + Mycelia Sterilia (Ainsworth & Bisby 1961)

species of the subgenus Pleiochaeta Sacc. The following is a translation of the Latin description:- "Ceratophorum Sacc. Subgen. PLEIOCHAETA Sacc. in O. Kirchn. Conidia bearing at the apex two to several setae—Here also you find Cer. Tripartitum and C.Hypodermium. Ceratophorum setosum O. Kirchner
Located on leaves and stems of plants of Cytisi capitati near Hohenheim Germany—Spots on stems are brownish. Hyphae are abundant, hyaline, septate and branched, and are 7-8μ in width. Conidia arising from such hyphae are sessile, smooth, cylindrical and fusoid, and are composed of 3-8 cells (average 6-7), gradually tapering towards the apices. Spore dimensions are recorded as being 40-80μ x 15-19μ. Spores are dark brown and furnished with apical setae which are terminally pointed and hyaline."

1898. Sorauer (Ref. Wagner and Sorauer, 1898) described the species Pestalotia lupini on the cotyledons and leaves of Lupinus mutabilis Lindl. in Germany. Conidia of the type species of Pestalotia are shown in Illustration 10.

1905. Rostrup (cited from Hughes, 1951) expressed doubt as to whether the fungus Ceratophorum setosum was correctly included in the genus Ceratophorum and suggested it might be more correctly placed in the genus Camposporium Hark. Hughes (1951) mentions the morphological differences between these two genera (Illustrations 11 and 13) and adds that the development and shape of the conidia of the type species Camposporium antennatum and Ceratophorum setosum are sufficiently divergent as to justify these two species being placed in distinct genera.

1924. Cavara isolated a fungus causing a disease of Lupinus albus L., and recognised it "by its large flagellate spores, as a species of Mastigosporium, differing considerably from the only known species of the genus, M.album Riess." Cavara (1924). Cavara compared the Mastigosporium from white lupin with Sorauer's Pestalotia lupini on L.mutabilis, decided the two fungi were the same and he accordingly proposed a new combination, namely, Mastigosporium lupini (Sorauer) Cavara. syn. Pestalotia lupini Sor. Illustration 12 shows conidia of the type species for Mastigosporium.

1926. Doyer. The fungus described by Sorauer as P.lupini was found by Doyer on Lupinus polyphyllus Lindl. She asserted that P.lupini was more properly Ceratophorum setosum (the spores of Pestalotia being borne in pycnidia or acervuli). She found both to be identifical in culture and also in cross inoculations on Cytisus and Lupinus species. Doyer stated that Cavara saw only young hyaline conidia to influence him to name the fungus Mastigosporium lupini (Sor.) Cavara, and Doyer accordingly reduced this to synonymy with C.setosum. Hughes (1951) notes that C.setosum differs from the type species of Mastigosporium in method of conidial production and in being a dry-spored fungus.

1928. Pulselli illustrated and described the development and structure of C.setosum on L.albus L. He amended Saccardo's diagnosis of Ceratophorum and then proposed the subdivision of species in the genus according to the following:

1. Subgenus Monochaeta: apical cell with one appendage
2. Subgenus Pleiochaeta: apical cell with more than one appendage.

Saccardo had already proposed the subgenus Pleiochaeta but Pulselli was responsible for Monochaeta, not to be confused with Pestalotia de Not., subgenus Monochaetia Sacc.

(NOTE: Pestalotia is the correct spelling for the genus sometimes referred to as Pestalozzia. The original spelling—Pestalotia—was changed by Saccardo but this violates Article 72 of the International Code of Nomenclature (Guba, 1961).)

1951. Hughes regarded Ceratophorum setosum and C.albizziae Petch. as congeneric, for apart from colour, branching of setae, and conidial size, the two species are very similar.

Because C.setosum and C.albizziae produce mostly internal mycelium on the plant (whereas the type species of Ceratophorum produces mostly superficial mycelium), and since C.setosum and C.albizziae produce conidia with a number of apical appendages (whereas C.helicosporum does not produce a true apical appendage but rather a subulate (owl-shaped) terminally hooked prolongation of the conidium, Illustration 13), Hughes considers these two fungi to be completely out of place in the genus Ceratophorum. Being unable to find a suitable genus for C.setosum and C.albizziae, he raised Saccardo's subgenus Pleiochaeta to generic rank, with C.setosum as type species and C.albizziae congeneric. Thus the currently accepted systematic classification of the fungus originally named by Kirchner Ceratophorum setosum, is that defined by Hughes (1951):- "Pleiochaeta (Saccardo) Hughes comb. nov. ≡ Subgenus Pleiochaeta Saccardo in Kirchner (1892) and later in Saccardo (1895).

Conidia produced singly at the apex of conidiophores and their successive growing points which arise singly and successively below

Illustration 9

Fig. 2.
Junge Spore von *Ceratophorum setosum n. sp.* Vergr. 300 fach.

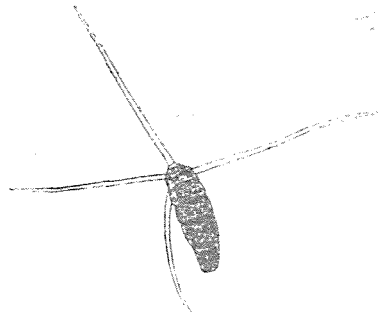


Fig. 4.

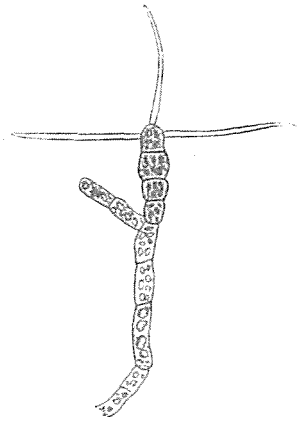


Fig. 3.
Ceratophorum setosum n. sp.
Spore auf dem sie erzeugenden Myceiast. Vergr. 300 fach.

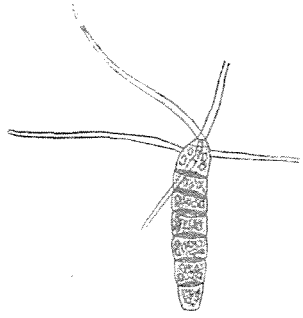
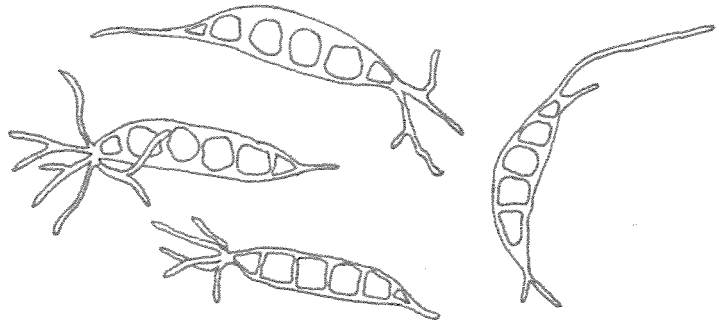


Fig. 5.
Fig. 4 u. 5. *Ceratophorum setosum n. sp.*
Reife Sporen. Vergr. 300 fach.

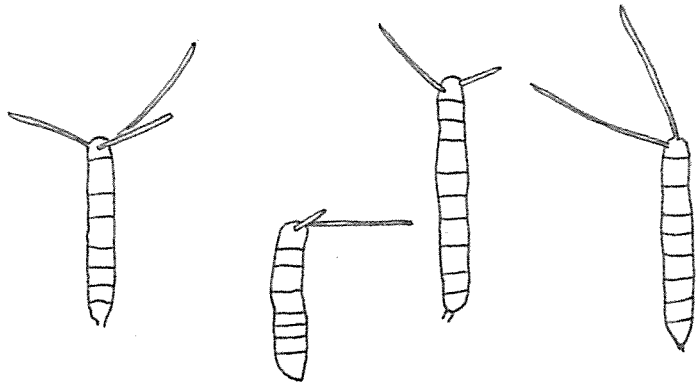
First drawings of conidia of Pleiochaeta setosa (Kirchn.) Hughes
[photocopy from Kirchner 1892]

Illustration 10



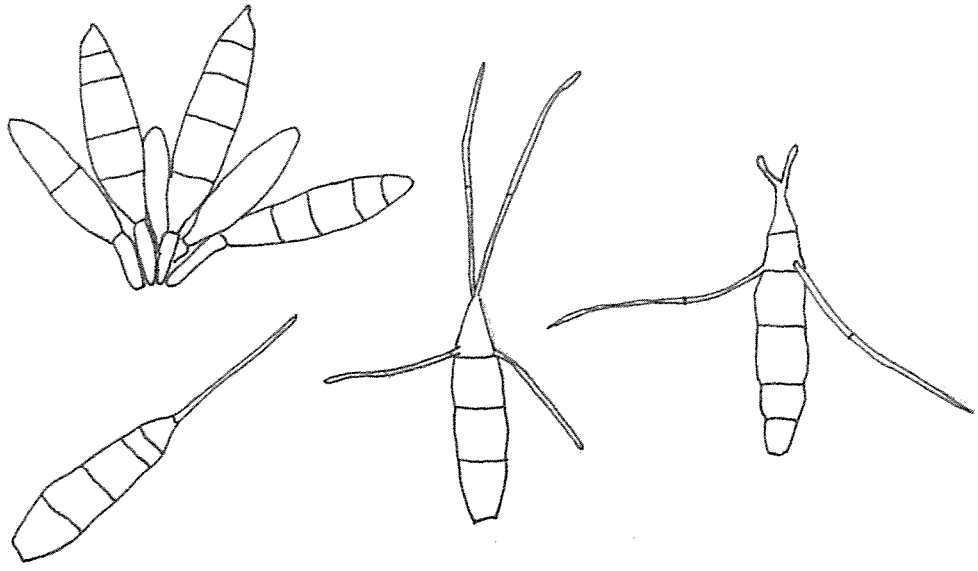
Conidia of Pestalotia pezizoides
P.pezizoides is the type species of the genus Pestalotia, in which Sorauer 1898 placed Pleiochaeta setosa (Kirchn.) Hughes.
[redrawn from Guba, 1961]

Illustration 11 (x500)



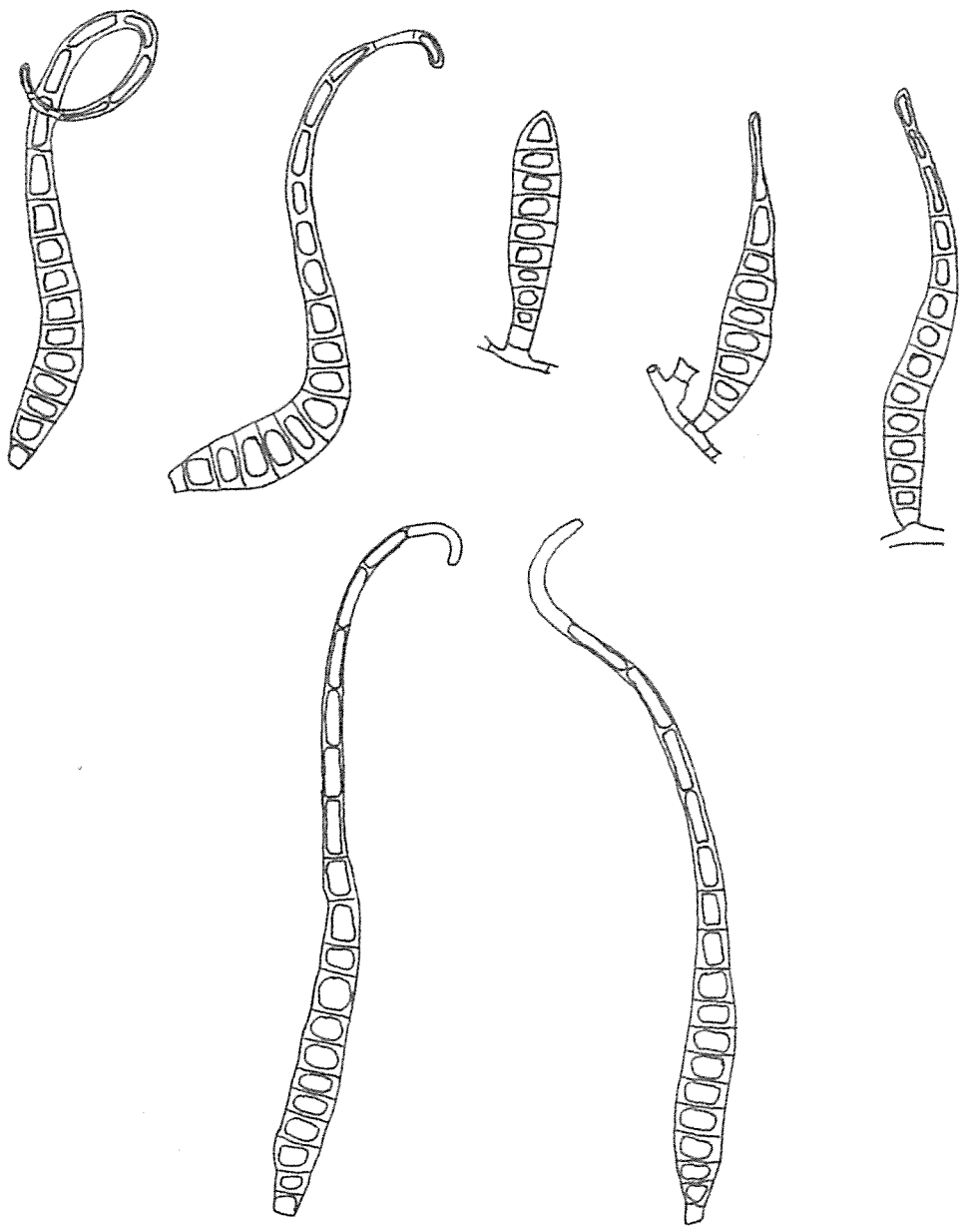
Conidia of Camposporium antennatum, the type species of the genus in which Rostrup (1905) placed Pleiochaeta setosa (Kirchn.) Hughes [redrawn from Hughes 1951]

Illustration 12 (x500)



Conidia of Mastigosporium album, the type species of the genus in which Cavara (1924) placed Pleiochaeta setosa (Kirchn.) Hughes [Redrawn from Hughes, 1951]

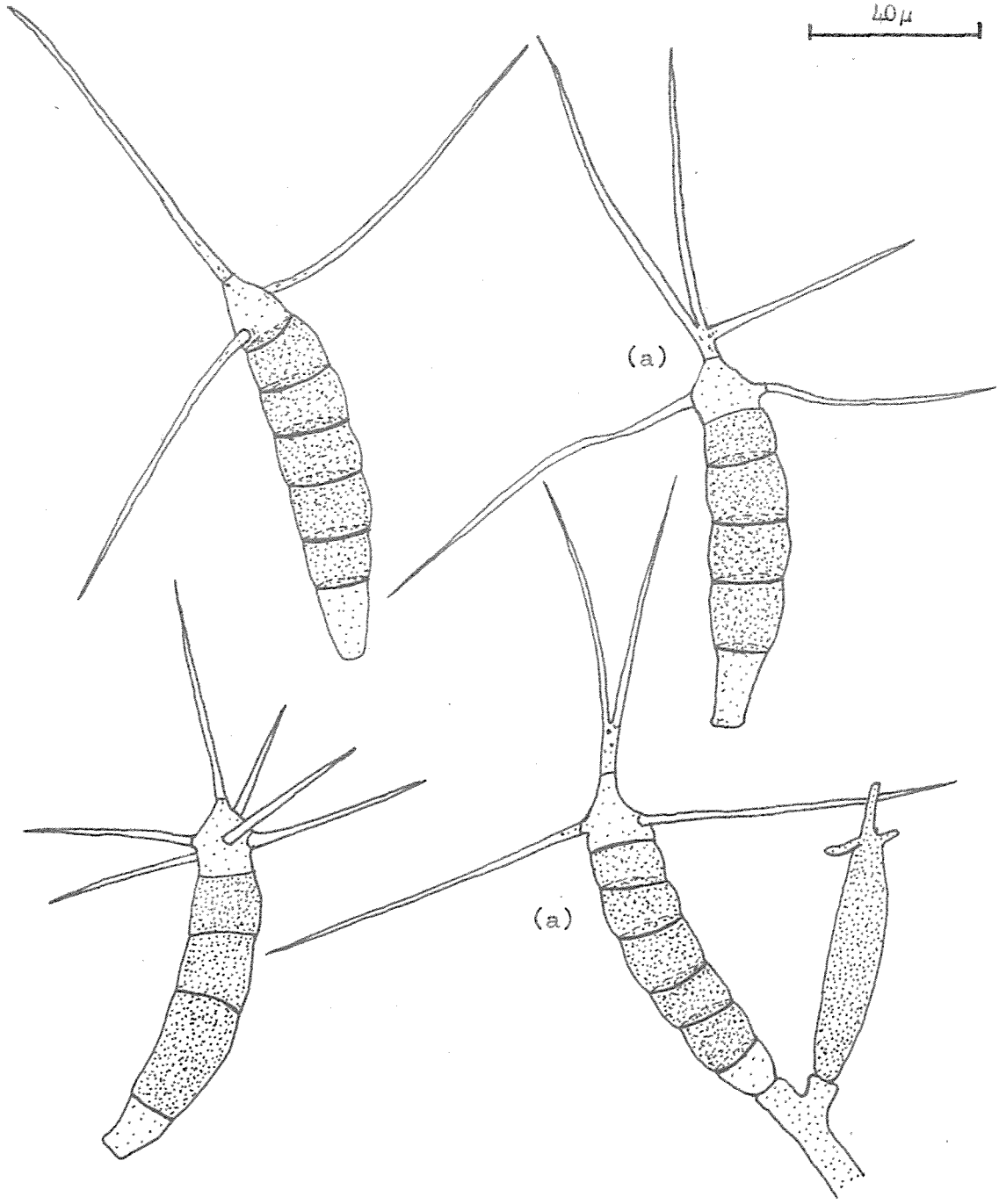
Illustration 13 (x500)



Conidia of Ceratophorum helicosporum, the type species of the genus in which most workers have placed the Brown Spot fungus until recent reclassification by Hughes

[Redrawn from Hughes, 1951]

Illustration 14



Conidia of Pleiochaeta setosa (Kirchn.) Hughes
(a) conidia showing branched terminal setae.

the youngest conidial scar. Conidia elliptical to fusoid, 3-8 septa, hyaline or brown with simple or branched apical appendages.

A. Type species: Pleiochaeta setosa (Kirchner) Hughes

B. Pleiochaeta albizziae (Petch) Hughes."

C. STUDIES ON CULTURE MEDIA

1. The influence of media on the cultural features of P.setosa

In order to prove the relationship between a species of fungus or bacterium and a particular diseased condition, Plant Pathologists have adopted a set of rules which must be satisfied before any organism is accepted as a specific cause of a disease. These conditions were first stated by Robert Koch in 1882 and are known as 'Koch's Postulates'. Applied to fungi they are as follows:

(i) The specific fungus must always be associated with the disease.

(ii) It must be isolated from the diseased area and cultivated in pure culture.

(iii) The pure culture when inoculated back to a susceptible host must produce the same disease.

(iv) The specific fungus must be reisolated in pure culture from the artificially diseased plants and proved identical to the original cultures.

Growth in pure culture is a basic requirement of Koch's Postulates and fortunately P.setosa is not a difficult fungus to grow away from the host, so that the second requirement in the rules can be met. A fairly comprehensive empirical study has been made here to find, not only a medium for optimum growth but also one on which

abundant conidia and chlamydospore production occurs. Rapid growth and fulfilment of the latter mentioned attributes of P.setosa on media would provide a very useful tool in the study of this organism. Wolf and Wolf (1947) state:- "It is not unusual to find now that a particular medium, compounded according to a certain formula, is a favourite with a given mycologist and that he attempts to cultivate all species in which he may be interested on this particular medium."

In the literature relating to studies of P.setosa scant reference is made to cultural characters. Two workers do, however, mention the suitability of various media. Germar (1940) found potato decoction agar most suitable for conidial production, followed by oatflakes, and oatflake yeast agar, while chlamydospores were abundant on carrot decoction, oatflake-glucose-saccharose and yeast-glucose-saccharose agars. du Plessis and Truter (1953) found that by far the best growth and sporulation occurred on lupin extract agar. Numerous chlamydospores but few conidia were formed on nutrient agar.

When the present study was commenced most of the culturing of P.setosa was carried out on lab PDA because this was the standard culturing medium used in the laboratory. Sporulation was not abundant on this, and was irregular in occurrence. Eventually, a treatment of these PDA cultures, detailed later under sporulation in culture, overcame this comparative inhibition of spore production. However, as a result of a preliminary trial carried out in which 21 media were tested, a medium capable of giving quick growth and rapid sporulation and which was cheap and easy to prepare, was chosen.

Method

The morphology of the fungal colonies, characteristic of each

type of medium, was recorded and relative comparisons of conidia and chlamydospore production made.

Details of the preparation of the media used are presented in Appendix I. Five Petri plates were used for each medium and labelled with the corresponding code number. Pouring and inoculation of plates was by the standard method outlined in Materials and Methods p. 12. Following inoculation the plates were incubated at 24°C and all results recorded after ten days. Where conidia were formed, permanent slides were prepared.

Results and Discussion

Table 2 gives details of the influence of media type on cultural characteristics. The wide diversity of results is readily apparent and the implications of such information in the study of any fungus in the laboratory have already been stressed.

Practically all colonies were divided into a large central area and a fringe which were distinguished from each other by a definite difference in colour. The fringe was predominantly white and invariably lighter than the centre, with colonies on five media lacking a fringe altogether. The central portion varied in colour from dark red to complete absence of colour and 50% of the media gave translucent aerial mycelium.

The amount of aerial mycelium formed varied greatly. Water agar produced the least and V-8 juice agar the most aerial mycelium, the latter resulting in a particularly raised colony centre.

Some media had a greater propensity for producing mycelium within the substrate than others, in particular carrot agar and prune agar.

Table 2

Influence of media type on cultural characteristics of P.setosa

Type of Medium	Average Colony Diameter (mm)	Colour of Colony			Shape of Colony	Density of Mycelium	Production per unit area*		Relative production of		pH of Medium	Remarks
		Fringe	Central	Aerial			Conidia	Chlamyd.	Conidia	Chlamyd		
Tryptone glucose yeast extract agar	57	white	absent	grey	reg	medium/flat	5	0	286	0	7.0	High conidial production
Tomato juice agar	70	white	grey	translucent	reg	dense/raised	3	4	210	225	6.1	Relatively high conidial and chlamydospore production
Tryptone yeast extract agar	49	white	white	translucent	reg	dense/flat	0	1	0	42	7.0	
Cornmeal agar	77	white	absent	translucent	reg	medium/raised	2	1	153	77	5.4	
Soya Peptone agar	42	fawn	fawn	white	reg	dense/raised	0	1	0	43	7.3	
Carrot agar	65	translucent	white	black	irreg	dense/raised	3	1	196	65	6.45	
Potato carrot agar	80	absent	black	white	reg	sparse/flat	5	1	402	80	6.3	Cheapest media for abundant conidia. Highest radial growth.
lab potato dextrose agar	73	white	black	translucent	reg	medium/flat	2	3	146	218	5.7	High chlamydospore production.
Oxoid potato dextrose agar	52	white	grey/black	grey	irreg	medium/raised	0	3	0	157	5.6	
24D potato dextrose agar**	55	translucent	grey	grey/black	irreg	medium/raised	3	2	166	110	6.0	Reasonable growth—suitable for seed plating
Malt agar	33	light brown	brown	grey	irreg	medium/raised	0	3	0	96	5.4	
Prune agar	63	absent	grey/green	translucent	irreg	sparse/flat	0	4	0	252	5.6	Highest production of chlamydospores.
Nutrient agar	47	light brown	brown	white	reg	medium/raised	2	3	95	0	7.4	
Kligler Iron agar	53	white	dark red	translucent	reg	sparse/raised	4	0	210	0	7.4	
Lupin extract agar	69	white	black	translucent	irreg	dense/raised	0	2	0	137	5.3	
Lupin seed agar	77	translucent	black	translucent	reg	medium/raised	5	0	420	0	5.9	Highest conidial production.
V-8 juice agar	38	absent	white	grey	irreg	dense/raised	0	5	0	191	4.3	High chlamydospore production.
Starch agar	41	white	light brown	translucent	irreg	sparse/flat	0	2	81	0	5.2	
Milk agar	41	white	grey	translucent	reg	sparse/flat	5	0	205	0	7.2	
Water agar	54	absent	grey	translucent	reg	sparse/flat	1	0	54	0	5.8	Media clear—ideal for observation on conidial development.
Tobacco extract agar	55	absent	grey	translucent	reg	medium/raised	5	0	273	0	6.4	High conidial production.

* Conidia and Chlamydospore Production

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

** 2,4, dichlorophenoxy acetic acid.

With six exceptions media promoting the production of conidia did not produce chlamydospores. Tomato juice agar was the only medium which gave a relatively high production of both conidia and chlamydospores, and all media were capable of the formation of either one or the other.

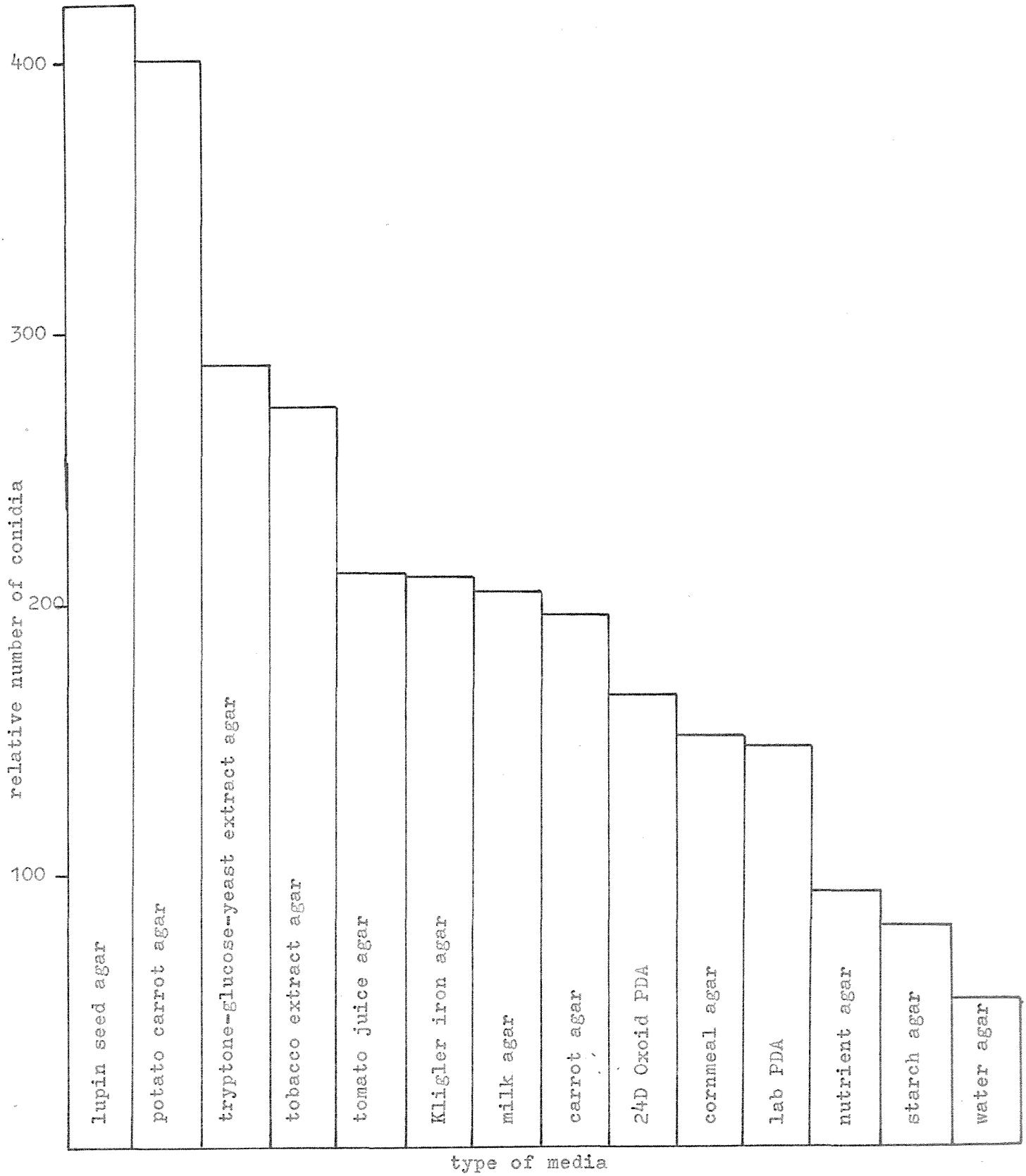
Two factors which must influence the total number of conidia and chlamydospores produced by a colony are the total area of the colony and the number per unit area. To enable comparison of different media as sources of spores the rating for conidia or chlamydospore production per unit area was multiplied by the diameter of the colony, to give a figure of relative production of conidia or chlamydospores as illustrated in Figures 1 and 2.

Lupin seed agar and PCA gave by far the highest production of conidia. This high score was partly due to the fact that on these two media P.setosa had a high growth rate thus giving a large colony diameter in the ten days. Tryptone-glucose-yeast extract, tobacco extract, tomato juice agar, Kligler iron agar and milk agar also gave good sporulation. PCA was selected as being the most suitable medium for general use in the later part of the study. P.setosa grew rapidly and evenly on PCA, produced abundant conidia and, because of the sparseness of constituents used in it, the cost of materials was negligible.

Prune agar, tomato juice agar and lab PDA all gave satisfactory chlamydospore production. Where chlamydospores were required for later work prune agar was used.

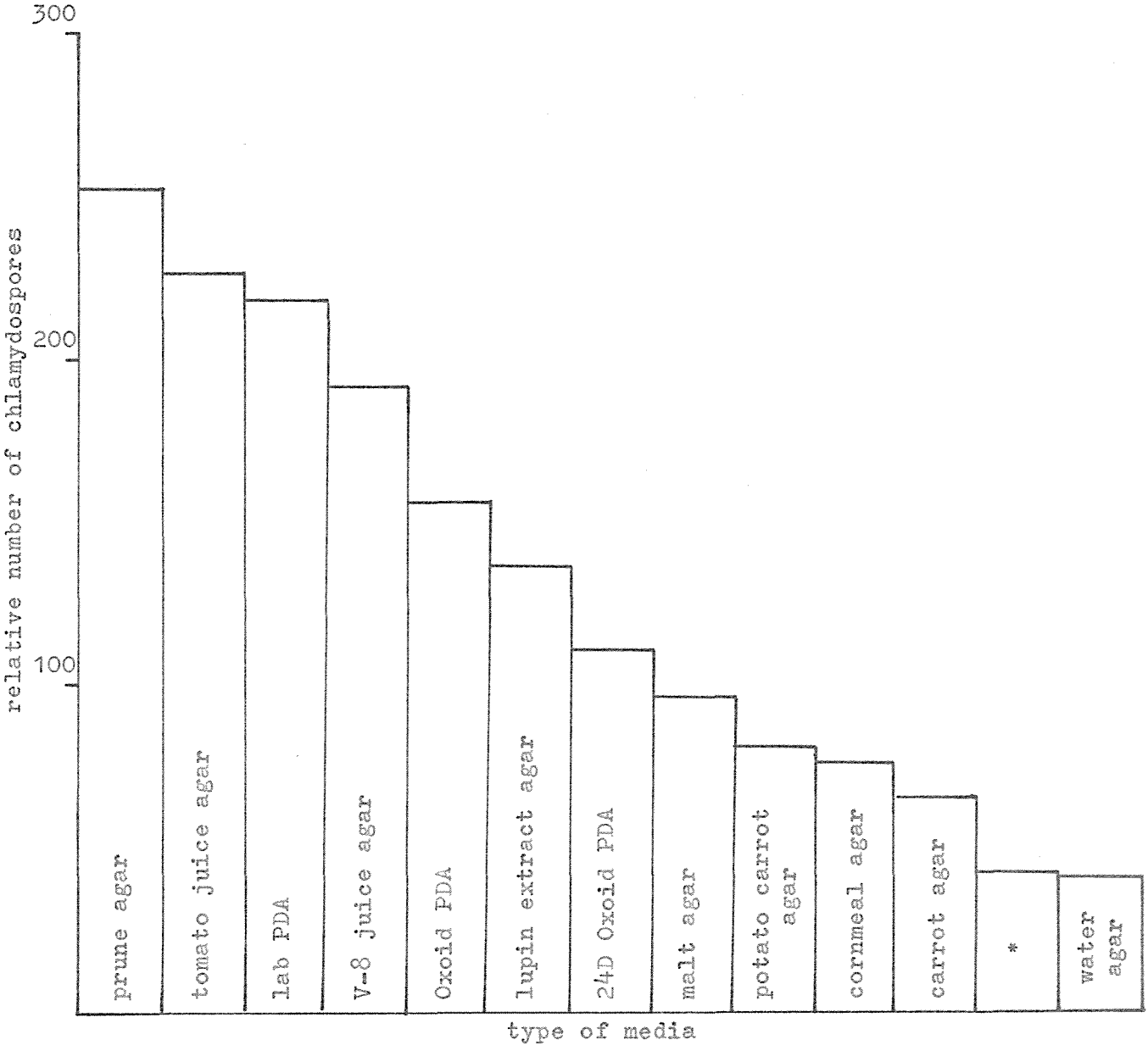
The clarity of water agar and the sparse production of mycelium and conidia on the culture surface made this medium the most suitable for a study of conidial development.

Figure 1



Relative number of conidia produced by Pleiochaeta setosa on various media (after 10 days at 24°C)

Figure 2



Relative number of chlamydospores produced by Pleiochaeta setosa on various media (after 10 days at 24°C)

* tryptone-glucose-yeast extract agar

PCA contains 20 gm of potato and 20 gm of carrot per litre whereas lab PDA, a commonly used laboratory media for culture of fungi, contains 200 gm of potato and 10 gm of dextrose per litre. The success of PCA as a medium capable of inducing abundant sporulation of P.setosa, suggested that concentration of nutrients may be an important factor in determining the type of growth a fungus adopts on an artificial medium. For this reason the following trial was carried out using differing amounts of carrot and potato in media.

Method

Media were prepared containing 20 and 40 gm of carrot per litre; 20, 40, and 60 gm of potato per litre; 20, 50, 100, 150, and 200 gm of both potato and carrot per litre. Colony diameter and relative conidial production were recorded after ten and a half days.

Results and Discussion

On the carrot agars chlamydospore production was sparse—the mycelium was coarse with a dark brown pigmentation and en masse the colonies had a green/black appearance. The colonies on the potato media were pale coloured, assuming slightly darker intensities as the nutrient concentration increased. As the level of nutrients used in PCA increased, the level of sporulation increased markedly, together with a change in colour of the colony. The 20/20 dilution gave a colony which was almost translucent except for spore clumps, but the others were a green/black colour with the surface of cultures on 200/200 a wet mass of conidia.

The trial showed conclusively that inducement of sporulation of P.setosa on PCA in the earlier media trial was not due to the low

Table 3

Effect of different media concentrations of Potato, Carrot, and Potato/Carrot on the growth and sporulation of P.setosa.

Amount of nutrient per litre of Media (grams)	Colony Diameter after 10½ days (mm)	Relative Production of Conidia per unit area
CARROT		
20	78.5	very low
40	78.5	very low
POTATO		
20	79.0	very low
40	78.0	below average
60	71.0	below average
POTATO/CARROT (equal quantities of each)		
20	82.0	below average
50	78.5	average
100	78.0	above average
150	78.5	above average
200	79.0	very high

level of nutrients stimulating conidial formation, but rather to the possession of some specific factor (or factors) in the potato and carrot. As the quantity of potato and carrot used in the media was increased so too was the factor(s) and in due accordance the amount of conidial production increased to a high level. Both potato and carrot contained the factor(s) inducing sporulation, but potato contained a higher quantity. An interesting observation was made when some 20/20

PCA media was prepared using washed but unpeeled potatoes. This medium gave a grey/black colony with considerable aerial mycelium and also greater spore production than the normal PCA used.

2. Medium pH and Growth

Under given conditions maximum fungal growth occurs over a certain range of initial pH values of the medium, and does not occur at high and low extremes. However, it is important to realise that at different parts of a pH growth curve the pH may be affecting different metabolic activities. For example, acidity may affect the entry of essential vitamins (Wyss et al 1944), entry into the cell of organic acids, or the uptake of minerals (Overstreet and Jacobsen 1952).

Cochrane (1958) points out that in view of the above facts it is not surprising to find that almost any factor in the environment—including temperature, growth factor supply, and supply of calcium and magnesium—may change the shape of a pH growth curve. Furthermore, pH is affected during growth by metabolic activities and Cochrane notes that these effects of growth on pH complicate results, particularly in poorly buffered media.

A review of the literature relating to the pH optima of some fungi would indicate that many of the broad optima recorded reflect the ability of a fungus to raise or lower the pH of an initially unfavourable medium.

In contrast to bacteria and actinomycetes, fungi are relatively more able to invade acid environments and use of this fact is often made in the laboratory when trying to obtain a fungus culture

free of bacterial contamination. Most plant pathogens grow best in a media with an initial pH of 5.0 to 6.5. Cochrane concludes that the pH growth curve has a limited usefulness and poses the question as to whether the information to be gained is worth the effort required.

In this study it was felt that a pH run would help as a rough guide to choice of media and a possible indication of ecological preferences. With P.setosa a tolerance of high alkalinity would be suspected because of the high alkaloid content in bitter blue lupins at certain stages of their growth. Consequently, experiments were carried out using (i) Oxoid PDA with different pH values to determine pH growth effects, and (ii) PCA with different pH values in an attempt to determine the effect of pH on conidia and chlamydospore production.

Materials and Method

In most physiological studies it is necessary that the pH be controlled, at least within limits. From the literature it is evident that numerous buffers have been employed and the most common and useful is the phosphate buffer. Even with this, usefulness may be dependent on tolerance of the fungus to the phosphate ion.

(a) Oxoid PDA

This was used as the standard medium in the first trial and was buffered using the following system:

2.6 gm citric acid)
3.8 gm glycine) dissolved in 100 ml of distilled water.
3.8 gm potassium dihydrogen phosphate)

The pH of the media was altered by the addition of 2N sodium hydroxide or hydrochloric acid.

Table 4

Quantities of acid and alkali added to buffered Oxoid PDA to obtain a range of pH

Number of drops	Approximate pH
2N HCl	
30	2.9
20	3.2
10	3.6
Buffer only	4.0
2N NaOH	
10	4.4
30	5.4
Oxoid PDA only	5.6
35	5.9
40	6.3
43	6.5
50	6.8
55	7.0

One and a half litres of Oxoid PDA were prepared and 100 ml quantities accurately measured into 12 flasks. A further flask of 200 ml was prepared as the stock supply. The media was autoclaved at the same time as dropper pipettes, buffer, acid and alkali.

On the completion of autoclaving, a 5 ml quantity of buffer was added to all except one of the twelve 100 ml quantities of media, and 10 ml added to the 200 ml stock supply. All flasks were then transferred to a water bath held at 50°C to prevent solidification.

Ten-ml samples from the stock supply were withdrawn using a

10 ml pipette and the pH adjusted by the addition of a number of drops of 2N NaOH or 2N HCl until a suitable range of pH values had been attained. These samples were then discarded.

The amount of acid or alkali necessary to approximately reach these pH values for the 100-ml samples was then calculated and the required quantities added using an aseptic technique.

Five replications for each pH were used, plates being poured immediately upon completion of pH adjustment. Enough media remained in the bottom of each flask to enable a further sample to be taken (the volume was of no consequence), and the exact pH determined on the meter.

Upon solidification of media in the plates the standard inoculum disc technique of inoculation was used and the plates incubated for ten days at 24°C. The control consisted of Oxoid PDA without the addition of buffer, acid, or alkali, and a range of pH from 2.9 to 7.0 was used.

(b) PCA

This method was repeated using PCA as the medium. Here, 19 samples were used giving a range of pH from 2.3 to 8.75 and a greater number of samples around the suspected optimum pH growth range. PCA without buffer, acid, or alkali addition provided the control.

All pH values were determined using a Beckman glass electrode pH meter. Care was taken with all glassware during the experiment to see that it was thoroughly washed and then rinsed with distilled water to prevent pH discrepancies.

Table 5

Quantities of acid and alkali added to buffered PCA to obtain a range of pH

Number of drops	Approximate pH
2N HCl	
40	2.30
30	2.60
20	2.90
10	3.20
Buffer only	3.70
2N NaOH	
10	4.10
20	5.00
23	5.30
25	5.60
27	5.90
PCA only	5.95
30	6.20
33	6.40
35	6.50
40	7.10
41	7.15
45	7.50
50	8.00
60	8.75

Results and Discussion

(a) Oxoid PDA

The graph of growth of P.setosa (Figure 3) on Oxoid PDA adjusted to different pH levels, shows that P.setosa tolerates a wide range from pH 4.4 to at least pH 7.0, with pH 4.35 the optimum for media plus buffer. From the results it would appear that the buffer itself has a depressing effect on colony growth either because of the acid pH of the medium with buffer alone (pH 4.0), or because of the effect of phosphate ion on the fungus (Darby and Mandels 1954; Jermyn 1953). The control gave nearly twice the growth of any of the other media used in the trial.

It is evident that a more alkaline pH than 7.0 is needed to discern the point where P.setosa growth declines at this end of the pH scale.

(b) PCA

A more comprehensive range of pH levels was used in this trial but again similar growth optima to that on Oxoid PDA were obtained, with a maximum for buffered media of approximately pH 4.3 and good growth between pH 4.3 and pH 7.15. Below pH 4 there was a very rapid fall off with finally no growth at pH 2.6. There was a less marked decline above pH 7 and still more than 50% of maximum growth at pH 8.75 (Figure 4).

The difference between control and the broad plateau of good growth was not as great as in Oxoid PDA but the buffer plus 2N NaOH still exhibited a marked effect on growth.

The control was the only medium on which sporulation was abundant although at pH 2.9 and pH 3.2 a few conidia were formed.

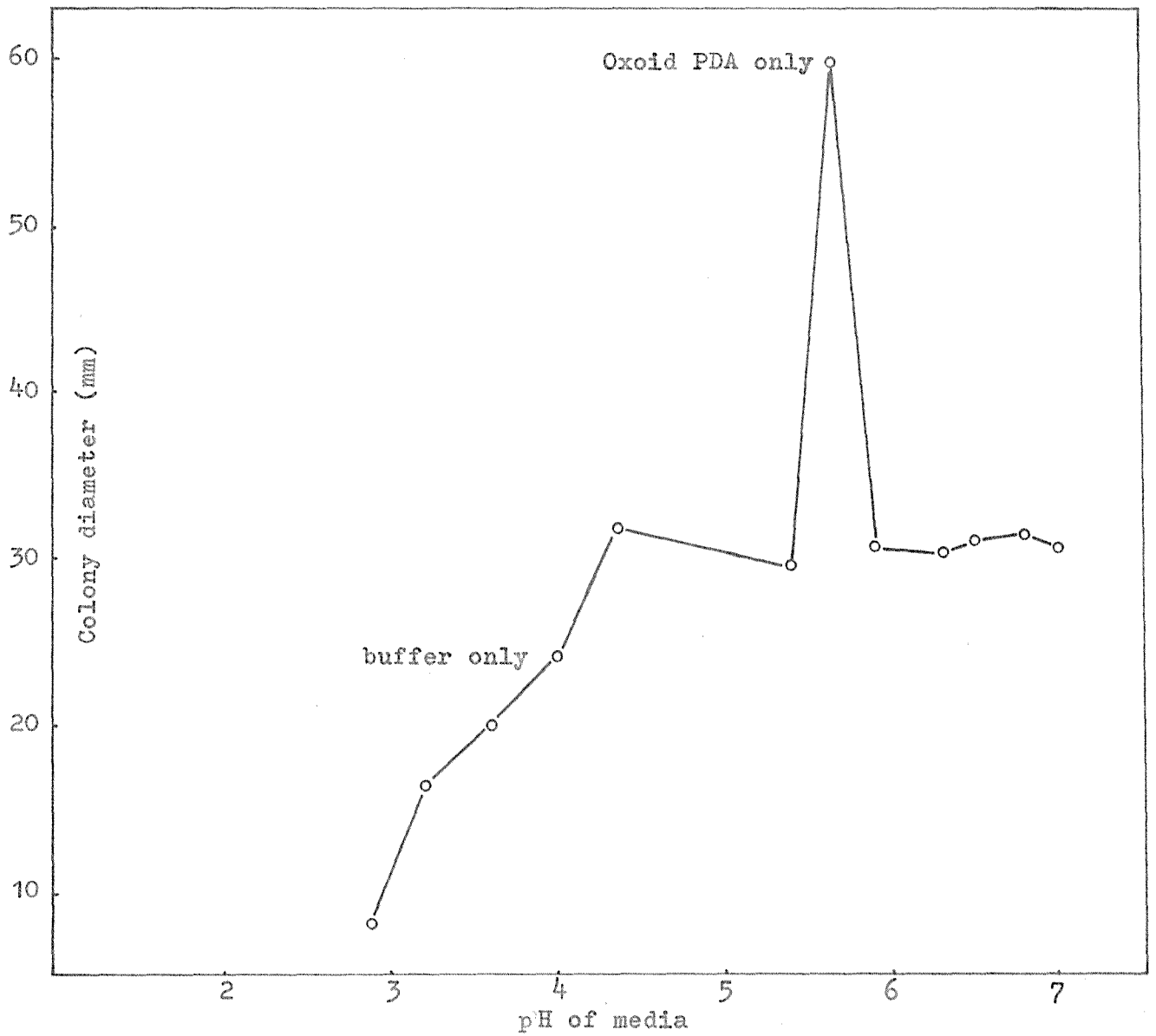
A small number of aerial chlamydospores were produced at pH 2.9 and between pH 3.2-pH 8.0 they were abundant; at pH 8.75 chlamydospore production was sparse.

Conclusion

Unfortunately, the desired results of effect of media pH on sporulation were not obtained from this trial, but it was considered that the time involved in testing different buffer solutions would not compensate for the value of data obtained. Barnett and Lilly state that as a rule the pH range for sporulation is narrower than for vegetative growth. Robbins and Schmitt (1945) have shown that within a physiological range an acid reaction is often less favourable than a neutral or mildly alkaline reaction but exceptions to this rule have been reported by Hopkins (1922). In the trial conducted with P.setosa on different media it was noted that several of the media which gave excellent conidial production were in the 6.0-7.4 pH range, whereas media favouring chlamydospore production tended to have a more acid reaction. However, no definite conclusion can be drawn from these observations because of the interaction of nutrient availability and pH.

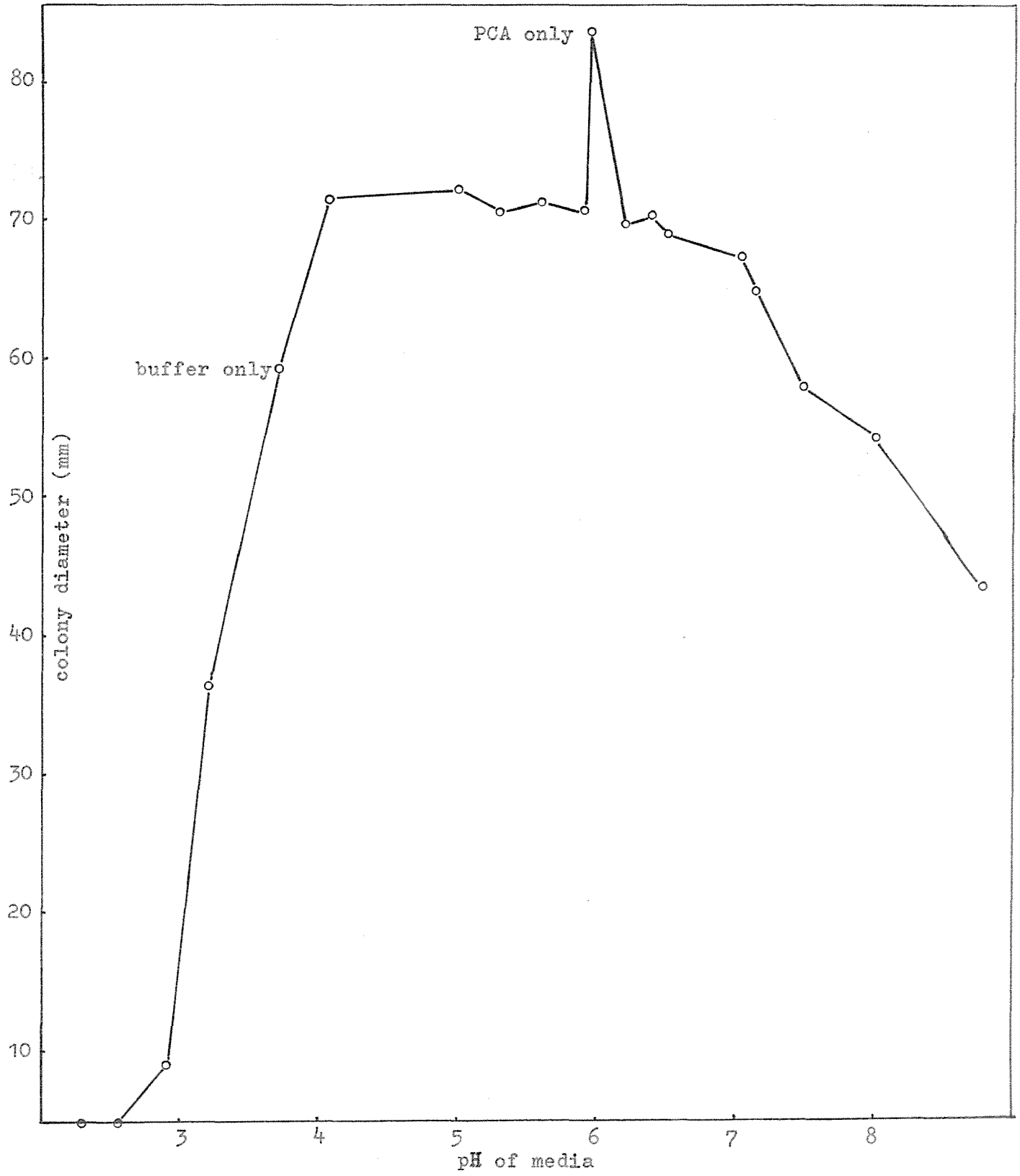
One can conclude from the results of the pH trial with Oxoid PDA and PCA that P.setosa can tolerate a wide range of pH of medium. Inhibition of growth is rather sharply defined at the limits of this range but the optimum is less clearly defined, hence giving a curve similar to that described by Hawker (1950) as typical for fungi, namely, a curve steep at both ends and tending to flatten out over the central area.

Figure 3



Growth of Pleiochaeta setosa on Oxoid PDA adjusted to different pH values (after 10 days at 24°C)

Figure 4



Growth of Pleiochaeta setosa on PCA adjusted to different pH values (after 10 days at 24°C)

3. Effects of temperature on P.setosa in artificial culture

Temperature is one of the most important environmental factors affecting the metabolic activities of fungi, influencing the rate of growth, spore germination, reproduction, and indeed all activities of the organism. Undoubtedly this is due to its intrinsic effect on the chemical and physical processes involved in life. Respiration rate and subsequent release of energy essential for growth, the uptake of water and solutes, the processes involved in the synthesis of organic compounds, are all processes which are individually influenced by temperature, so that one would expect the collective process of growth to be profoundly influenced.

According to Cochrane (1958) typical curves of growth, as a function of temperature, have a linear portion in which growth increases directly with temperature, an optimum range which may be narrow or rather broad, and a descending limb as the temperature becomes too high for growth.

Under experimental conditions temperatures are either maintained continuously or else fluctuate to only a small degree, whereas in nature they vary continually. Whether all metabolic activities can be maintained at a constant optimal level over an indefinite period, or whether one activity is favoured by a given temperature whereas another is adversely affected by this temperature, does not appear to be clear from a study of relevant literature. However, it would appear unlikely that a temperature which is optimum for mycelial development would also be optimum for germination, reproduction, and chlamydospore formation.

Fungi have a characteristic sigmoid growth curve typical of all organisms, with a lag at the initiation of growth followed by a

period of acceleration and eventually terminated by a period of deceleration.

Brancato and Golding (1953) established that colony diameter is a valid measure of the effects of environmental factors (such as pH, temperature and medium constituents) and this criterion has been used throughout this study. However, it is noted that other workers have raised fundamental objections. Chaudhuri (1923) found no response of colony diameter to even four-fold dilution of the nutrient medium. Nevertheless, because measurement of linear growth is the least laborious method of estimating growth it is correspondingly the most popular method in use.

The relationship between temperature and habitat of fungi does not appear to be entirely clear, but by taking into account the world wide distribution of P.setosa it would be suspected that this organism possesses a fairly wide temperature tolerance. Cavara (1924) reports it capable of withstanding temperatures of 5°-6° below zero and Hogetop (1938) reported the fungus in Brazil severely damaging L.albus. du Plessis and Truter (1953) report the optimum for growth to be near 25°C with a rapid decrease at higher temperatures and no visible growth at 35°C. However the fungus still grew fairly well even at 5°C.

At the commencement of this study of P.setosa, isolations were made from Russell, tree and blue lupin. There is no evidence in the literature pertaining to P.setosa, that isolates from various hosts differ in strain factor, variety or even species. However, it was decided in view of (i) P.setosa has only been officially recorded on blue lupin in New Zealand, and (ii) this is the only detailed study made of this fungus in New Zealand, that a comparison of these isolates

from the three different lupin species would be desirable. One of the comparisons made was the growth on Oxoid PDA at different temperatures. The influence of temperature on conidial formation on PCA and on chlamydospore production on prune agar was also studied.

Materials and Methods

Linear growth rate on the respective agar plates was measured after ten days at the different temperatures. In all cases inoculum was once-removed from the host material. Five replications were used per treatment.

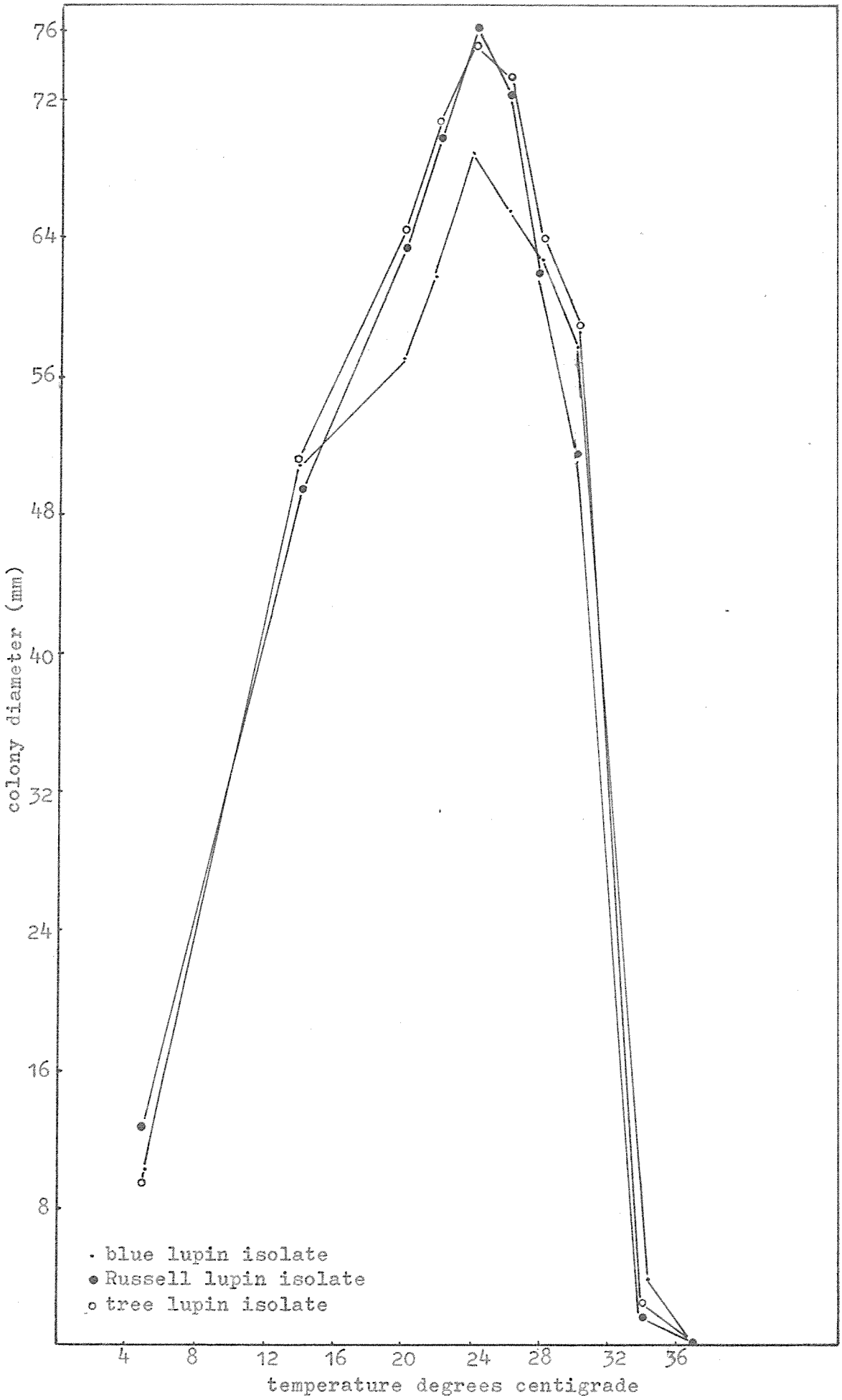
Spore production was measured by counting the number of spores per binocular microscope field, as outlined in the chapter on Materials and Methods.

Results and Discussion

(a) Isolates from Russell, tree, and blue lupin grown on Oxoid PDA at different temperatures.

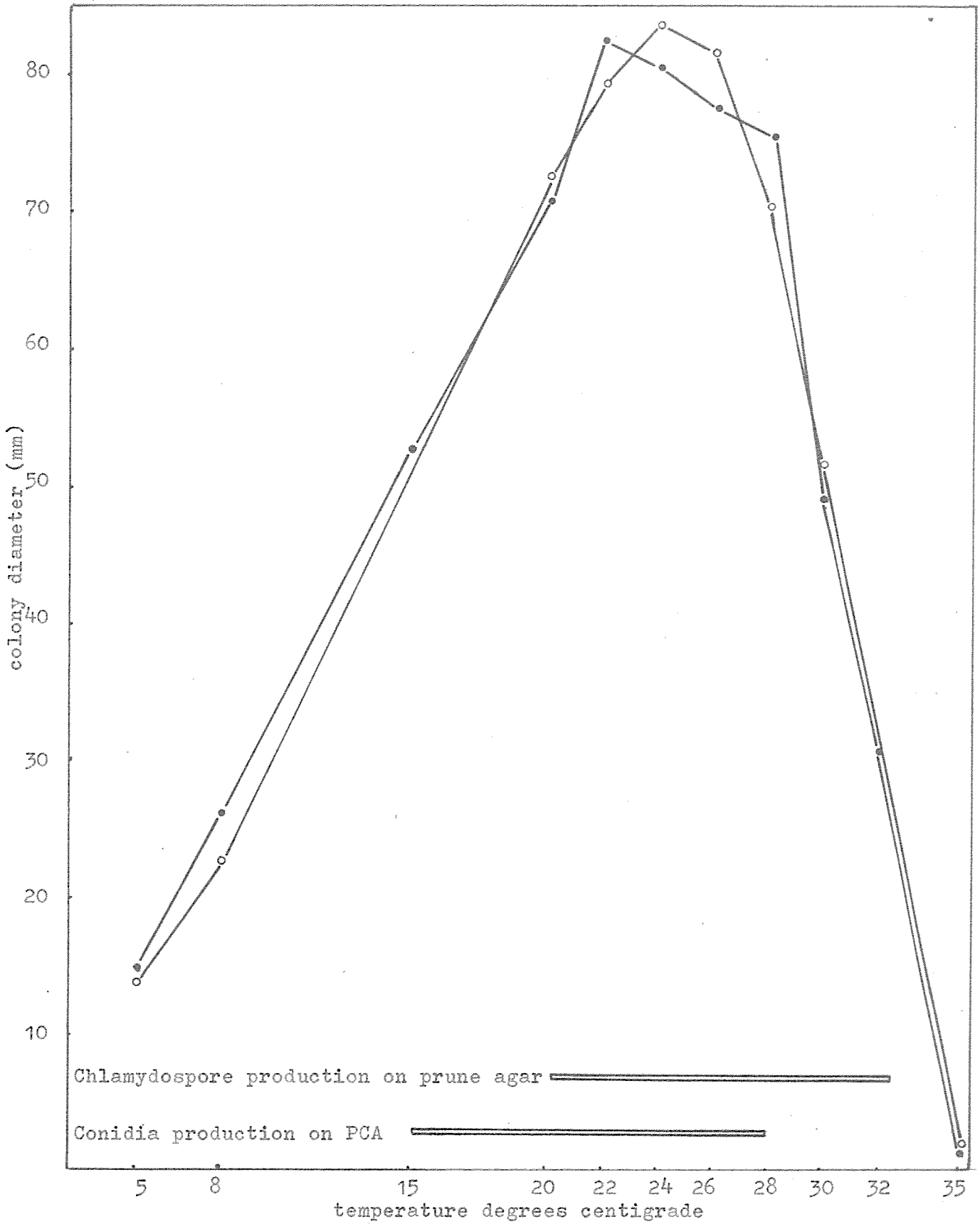
The temperature/growth curve for these three isolates follows a very similar pattern, as can be seen from the graph of results (Figure 5). The blue lupin isolate has a lower optimum growth rate than the other two which are very similar. The blue lupin isolate also has a slightly greater rate of growth at higher temperatures. Although there is a difference in growth rate between the isolates, all three give optimum growth at approximately 24°C and the three curves as a whole are skewed to the right. This skew is not marked but is in accordance with that found by other workers. Lauritzen (1932), Cartwright and Findley (1934), and Fisher (1939), all found that fungi with optima of 22°-24°C or less, often have a much less skewed curve than those with a higher temperature optima.

Figure 5



Temperature/growth curves of isolates of Pleiochaeta setosa on Oxoid PDA (after 10 days)

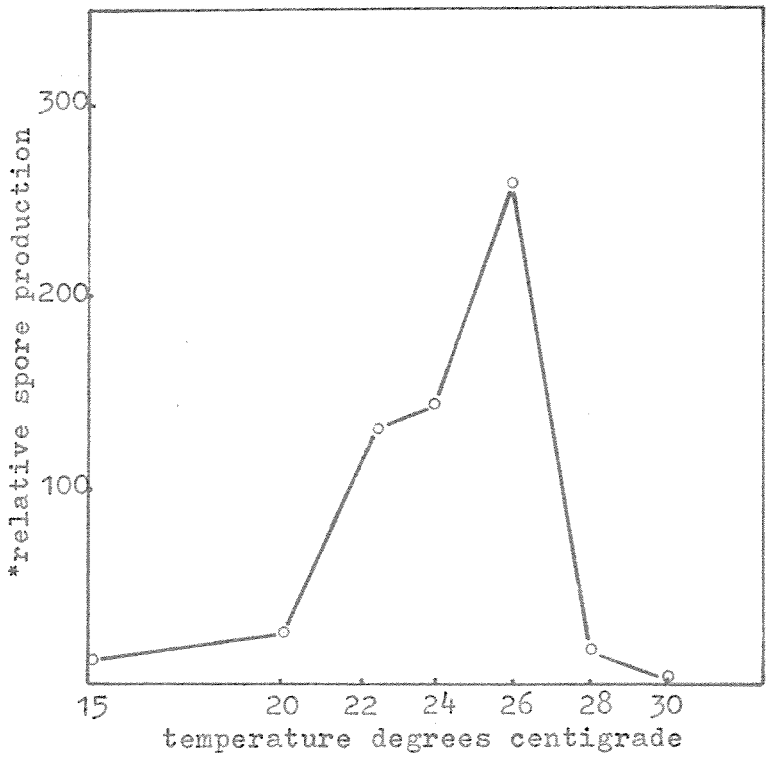
Figure 6



Growth/temperature curves of Pleiochaeta setosa after 10 days on:

- ∘ potato carrot agar
- • prune agar

Figure 7



*Average number of spores as counted in five fields of a x50 binocular microscope.

Effect of temperature on abundance of conidial production of Pleiochaeta setosa (on PCA)

Reasonable growth occurred at 5°C but was almost non-existent at 34°C and cultures held at 37°C would not grow when placed on the laboratory bench after the trial.

As one is sampling different populations of the fungus it is not unexpected that the three curves fail to be identical, but the overall similarity is readily evident. Spore measurements from host material (p.26) and later work involving cross inoculation studies verify this.

As infected blue lupin leaflets were generally the most readily available, fresh isolations for laboratory studies were obtained from this host for later species.

(b) Influence of temperature on growth and conidial formation on PCA.

The optimum temperature for growth on PCA is 24°C and a curve similar to that of Oxoid PDA results (Figure 6).

The optimum temperature for sporulation on PCA is 26°C (Figure 7). Sporulation at 15°C was sparse and irregular with one plate producing no spores, indicating that the lower range for sporulation on PCA lies in this region. Sporulation was sparse at 28°C and absent at 30°C. It is interesting to note here that in an experiment involving sporulation of P.setosa on blue lupin leaflets conidial formation occurred over a range of temperatures from 5°-30°C.

(c) Influence of temperature on growth and chlamydospore formation on prune agar.

On prune agar the optimum growth occurred at 22°C and chlamydospore production was abundant between 20°-28°C. Chlamydospores were produced at 30°C and 32°C but were not so numerous. At 5°C and 8°C

chlamydospores were not formed but the mycelium was coarse and closely septate.

The cardinal temperatures for P.setosa cannot be fixed exactly, nor in fact, can they for any fungus because of inherent variability, but as a result of this study it is apparent that the minimum temperature for growth is below 5°C, the optimum 22°-24°C, and the maximum approximately 35°-36°C. These figures are similar to those given by Germar (1940) and du Plessis and Truter (1953).

Germar (1940) found chlamydospore development began at 18°C and increased in abundance up to 32°C, but no reference to the effect of different temperatures on production of conidia on media can be found. du Plessis and Truter state—"sporulation was good on suitable media if the cultures were incubated at 25°C for a few days and thereafter kept at room temperature and in diffused light."

Finally, it should be noted that although this information on temperature/growth relations of P.setosa on media is valuable as an aid to laboratory studies of the fungus, the effect of temperature on the fungus as a field pathogen may be quite different for it will also reflect the effect of temperature on the host, causing possible interactions between the two.

4. Sporulation in culture

In any study of a phytopathogenic fungus it is important that an abundant supply of spores be readily available from cultures. Information is presented in this section on the influence of several factors as they affect reproduction of P.setosa in culture.

P.setosa belongs to the form-class Deuteromycetes and as such no sexual spore stage is known for it. It is further classified in

the form-order Moniliales, the characteristic feature of which is the production of asexual spores on more or less cottony hyphae. Most fungi in the Moniliales, including P.setosa, produce asexual spores known as conidia but a few reproduce by oidia, or by budding.

Of the many factors influencing intensity of spore production and the methods used to induce sporulation in culture, the type of medium and the temperature of incubation were found to be two of the most important with P.setosa. The influence of light was also considered and a culture washing technique investigated.

Factors influencing sporulation of P.setosa in artificial culture

Nutrition

In the preceding section on growth of P.setosa on artificial media it was shown that media type has a profound influence on the intensity of sporulation. Further, the experiment involving relative concentration of nutrients in one medium demonstrated the effect of nutrient concentration on intensity of reproduction. The work of Klebs (1899), cited by Lilly and Barnett (1951), indicates that most of the fungi he worked with were apparently induced to sporulate by an exhaustion of food supply. Experiments in the present study using PCA gave findings contrary to those of Klebs (1899), in that sporulation of P.setosa was restricted by a decrease in the amount of potato and carrot used in the medium.

The availability of an artificial medium which met the nutritional requirements of P.setosa for sporulation obviated the need for experimentation with natural media (here the term artificial medium includes all media which has been prepared in such a way that the chemical properties of the original constituents have been altered).

However, natural media can be of considerable value in inducing sporulation in some fungi (Snyder and Hansen, 1947). Heat is the chief means by which composition of media is altered, in particular the alteration of the physical-chemical nature of the substance being sterilised. Hansen and Snyder (1945) advocate gaseous sterilisation of biological materials for culture media and they found the use of ethylene oxide or propylene oxide as sterilants in the place of heat to be quite satisfactory. These were not available for this study but one attempt was made to ascertain the influence of natural media on P.setosa (here referring to natural media as material not subjected to temperatures above 50°C and which is extracted from living material).

Method used to prepare natural lupin extract agar

One hundred and fifty gms of Russell lupin leaflets and 150 gms of blue lupin leaflets were taken and macerated in a Waring blendor run at top speed, adding distilled water as necessary until a volume of 1500 mls was attained.

The mixture was then transferred to a 2-litre flask and held in a water bath at 50°C for two hours to facilitate the dissolving of soluble material. Following this, the mixture was filtered through a double layer of cheesecloth to remove all coarse and fibrous material.

The mixture was filtered through a Buchner funnel and then twice through a Seitz-Werke filter, the volume of the lupin extract having been previously adjusted to 1500 mls. The resulting extract was held in sterile 100-ml McCartney bottles.

A range of media incorporating this lupin juice extract was prepared as follows: water agar (1.5%) was prepared in the normal way, autoclaved, and held at 50°C; dilutions of lupin juice extract agar

ranging from 1%-10% were prepared by the addition of an appropriate amount of extract to the required water agar volume.

The plates were then poured and inoculated and the amount of sporulation and mycelial production recorded after incubation at 24°C for ten days. Six plates per treatment were used with PCA as a control .

Results and Discussion

Relative spore production was recorded after examination of cultures with a binocular microscope using an arbitrary classification involving five categories; very low, below average, average, above average, and very high. Mycelial production was similarly rated as either sparse, medium, or intense, and these results were compared with PCA.

The amount of mycelium increased in intensity as the concentration of the media became stronger, but it could not be classed as intense at any stage. With this increase in mycelial production the colonies became progressively darker in colour, the colour change being partly due to the greater amount of mycelium and partly to cells of hyphae showing signs of swelling and the walls thickening, finally leading to the production of chlamydospores.

The use of natural media did not result in the production of as many spores as PCA, neither was it as convenient to prepare. Consequently no further use was made of it.

Table 6

The influence of concentration of natural lupin extract agar on mycelial production and sporulation of P.setosa.

Dilution	Sporulation	Mycelial Production
1%	very low	sparse
2%	below average	sparse
3%	average	sparse
4%	average	medium
5%	average	medium
6%	average	medium
7%	average	medium
8%	average	medium
9%	average	medium
Control	very high	medium

Temperature

Earlier in this study it was determined that the optimum temperature for formation of conidia on PCA was 26°C and for growth on the same medium 24°C. du Plessis and Truter (1953) obtained good sporulation on lupin extract agar if cultures were incubated at 25°C "for a few days and thereafter kept at room temperature in diffuse light."

Results of trials conducted on PCA indicated that P.setosa will sporulate at temperatures between 15°C and 28°C.

Light

As already mentioned in this section, du Plessis and Truter

(1953) placed cultures of P.setosa, after they had been grown a few days on lupin extract agar at 25°C, in diffused light and obtained abundant sporulation. No more specific details of their method were available.

Method

A trial was carried out to ascertain the importance of light to sporulation of P.setosa on PCA. Twelve plates were inoculated and six were placed in aluminium foil bags. These, together with the remainder, were placed in the laboratory so that the uncovered plates would receive only diffuse daylight. Ten days later they were examined.

Results and Discussion

Macroscopic examination showed that colonies grown in the dark had more aerial mycelium and fewer spore clumps (seen as black dots on colony surface). Measurements of average colony diameters were: light/dark, 72.4 mm.; dark, 72.5 mm. Relative spore numbers were counted and the averages were: light/dark, 56 spores per field; dark, 30.5 spores per field. A comparison of the ability of these spores to germinate on PCA at 24°C resulted in no obvious differences.

The results indicate that light has little effect on radial growth of P.setosa on artificial media but that almost a two-fold increase in spore production can be attained by exposure to alternating dark and diffuse light. This ability to sporulate in the dark is not found in all fungi for Barnett and Lilly (1940) showed with Choanephora cucurbitarum (a member of the Mucorales causing blossom blight and fruit rot of cucurbits and other plants), that under conditions of continuous darkness no conidia were formed.

Ultraviolet Light (U.V.L.)

The destructive action of sunlight upon micro-organisms, especially bacteria, has been recognised for many years. The lethal action of U.V.L. is conditioned by the wavelength of the irradiation, by time of exposure, and by whether bacteria or fungi are involved. A considerable number of workers have studied the effect of U.V.L. upon sporulation of different species of fungi and both favourable and unfavourable results have been obtained.

Stevens (1928) found that U.V.L. induced the formation of perithecia by various isolates of Glomerella cingulata a few days after irradiation. Young cultures were found to give best results and one effect of the irradiation was the killing of the aerial mycelium. Diener (1955) achieved abundant sporulation with Stemphylium solani by use of U.V.L. Ramsey and Bailey (1930) increased sporulation in Macrosporium tomato, Fusarium cepae and Alternaria spp. by suitable periods of exposure to U.V.L.

McCallan and Chan (1944) showed in their work on factors influencing sporulation of Alternaria solani that exposure of a scraped culture of the fungus on PDA, to U.V.L. of 250 mm wavelength for 20 seconds at a distance of 10 cm from the lamp gave excellent sporulation. Factors of secondary importance were optimum temperature ($20^{\circ}C$ for their strain) and optimum humidity.

In view of this work, and the fact that early in this study with P.setosa difficulty was experienced in obtaining abundant and consistent sporulation, a trial was performed to determine whether U.V.L. would induce sporulation of P.setosa.

Method

Each treatment consisted of nine plates, three each of isolates from Russell, tree, and blue lupin. These cultures were on PDA grown at 24°C and twelve days old.

Plates were placed colony face down and exposed to the lamp at a distance of two inches. In all cases after treatments, plates had lids replaced and were held on laboratory benches and plates were examined each day for five days after treatment.

Treatments, Results and Discussion

(a) Exposure of 60 seconds and 120 seconds with lids on: sporulation not induced.

(b) Exposure of 10, 20, 30 seconds, 1, 2, 10, 20 minutes with lids off: sporulation not induced

(c) Exposure of 10, 20, 30 minutes after first scraping the colony surface: after treatment a moist filter paper was placed in the lid of each dish to maintain high humidity. All had a few spores, with the combination involving 20 minutes U.V.L. the best.

(d) Colony scraped, no U.V.L. but with filter paper: trace sporulation.

(e) Colony scraped, no U.V.L., no filter paper: trace sporulation.

From the results obtained, it can be concluded that U.V.L. does not stimulate sporulation of P.setosa. In treatments outlined in (c) U.V.L. appeared to stimulate the formation of aerial mycelium.

Although eagerly sought, no sign of a perfect stage could be found.

Other Factors

Many and varied are other factors which various workers have

considered in their attempts to induce fungi to reproduce either asexually and/or sexually. While many factors may influence reproduction, there is little evidence that the fungi need specific chemical substances to induce reproduction. Two factors considered in this study were method of inoculation of cultures and culture wounding and washing.

Method of inoculation

The method of inoculation and type of inoculum used have been shown to be important factors affecting sporulation in culture. One method quite frequently used, involving culture flooding, is described by Berger and Hanson (1963) for obtaining sporulation of Cercospora species.

Three different forms of inoculum of P.setosa were investigated to find the method producing the largest number of viable conidia, viz., conidia, mycelium, and cork-bored agar plus mycelium, in each instance grown originally from a single spore isolate ex host. No differences in the intensity of sporulation could be discerned.

Wounding and washing

Injury to fungal mycelium is known to stimulate sporulation in certain fungi. McCallan and Chan (1944) and Venkata Ram (1961) demonstrated that by scraping the colonies of certain fungi sporulation could be induced.

A different approach to induction of sporulation was incorporated in a technique developed by Ludwig, Richardson and Unwin (1962). These workers obtained abundant sporulation after washing scraped V-8 juice agar cultures of the fungus for 24 hours and then

stacking the plates in an inverted slanted position under laboratory conditions of light and temperature.

Experiment to determine the effects of wounding and washing on the sporulation of P.setosa

Method

Eighteen ten-day-old cultures of P.setosa on Oxoid PDA were used with three treatments being applied.

Treatments and Results

(a) scraping and no washing resulted in practically no sporulation

(b) scraping and washing resulted in abundant sporulation as did—

(c) washing without prior scraping.

Results were taken 48 hours following treatments. Spores were washed off colonies, by means of a jet of water from a plastic wash-bottle, and used for inoculation experiments in which they showed normal pathogenicity. Using this washing technique, a second crop of spores could be obtained three days later.

Discussion

The results obtained indicate that the wounding achieved by scraping the colony surface with a glass slide is not necessary to induce sporulation of P.setosa, and as colonies on Oxoid PDA do not have excess aerial mycelium, removal of spores after washing is not difficult. Various hypothesis have been put forth by workers using the washing technique to explain the resultant sporulation. Ludwig, Richardson and Unwin (1962) suggest the washing eliminates an anti-sporulating factor which may be produced by the fungus in culture.

However, the washing experiment was repeated with P.setosa on prune agar (upon which abundant chlamydospore production occurs but no conidia form), without success. This does not invalidate the hypothesis of Ludwig et al (1962) but it does show that the method is not universal in its application to different media.

The above technique was not used for spore production once the media trial results became available.

From the results of the above experiments it can be concluded that sporulation of P.setosa can be readily achieved using PCA inoculated with cork-bored agar circles of the fungus placed at 24°C for ten days in incubators which, except for the occasional opening of the door, would provide total darkness.

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CHAPTER III

STUDIES TO DETERMINE WHETHER *PLEIOCHAETA SETOSA* IS SEED-BORNE.

These include the determination of a suitable technique for isolation of *P.setosa* from inoculated seed; examination of samples of commercial lines of lupin seed for infection with *P.setosa*; and examination of flowers, pods and seed taken from a naturally infected blue lupin crop.

A seed-borne disease may be defined as one which is potentially capable of becoming established in a crop as a result of the use of seed carrying the causal organism of that disease. That is, the seed may provide the source of primary inoculum.

The nature of the association between fungus pathogen and host seed varies, but two broad categories are recognised, namely:

1. Seed Contamination—where crop debris infected with the fungus, or fungal structures such as spores or sclerotia are loosely mixed with the seed. In such instances, the pathogen is effectively distributed with the seed, and if viable at the time of seed sowing, may resume growth and cause seedling infection.
2. Seed Infection—where the fungus is established within seed tissues, usually as dormant vegetative mycelium.

The fact of a pathogenic fungus being transmitted by way of seed creates a situation likely to ensure successful establishment and development of disease in the resultant crop. To begin with, the association means that the strain of fungus present is pathogenic, and in addition, that the host variety is susceptible to that strain. For infection to follow, environmental conditions must be satisfactory. Seed is usually spring or autumn sown, at which time temperature and humidity are likely to be suitable for the initiation of infection. Seed is frequently sown so thickly as to produce dense stands of drawn, sappy and highly susceptible seedlings, which in turn favour successful infection. Further, the presence of such a dense host population means that an occasional infected seedling can provide sufficient inoculum to ensure effective secondary spread either by air currents, or overhead splashing during watering or rainfall. That is, in such situations even trace infection within a seed line must be viewed as

being of considerable economic importance.

A study of relevant literature reveals that numerous workers have recorded P.setosa as capable of attacking the pods and seeds of lupins, and by implication at least, that seed may provide a source of primary inoculum.

1. Pape (1927) discusses the disease on several lupin varieties and states—"the fungus may also attack the pods and penetrate the seeds."

2. Siemaszko (1929) reported P.setosa attacking the leaves, pods and seeds of lupins in Lublin and Warsaw districts in Poland.

3. Hogetop (1938) mentions that in Brazil the fungus is found on the pods of L.luteus before ripening.

4. Richter (1938) recorded spotting on the pods of L.angustifolius in Germany and noted seed weight was affected.

5. Germar (1940) described two types of pod infection on L.albus, each of which yielded diseased seed characterised by a brown discolouration of the testa from whence infection could pass to cotyledons or embryo. The latter impeded germination of the seed.

6. Weimer (1952) postulates Brown Spot disease was probably brought to the United States from Europe in the seed. The fungus has been found attacking the blossoms, pods, and seeds of L.angustifolius in the United States.

7. du Plessis and Truter (1953) describe Brown Spot on the pods and seeds of lupins but in their account of the disease they discuss three host species, L.angustifolius, L.mutabilis, and L.albus, without stating specifically whether they have found the disease to be seed-borne in all of these species in South Africa. Their detailed work appears to have been conducted with L.albus.

8. Noble, de Tempe and Neergaard (1958) have recorded P.setosa as a seed-borne disease of serious economic importance on Lupinus spp. noting seed infection is partly internal.

9. Ostazeski (1960) obtained up to 20% diseased plants from infected L.albus seed, and he developed a method to kill the fungus in white lupin seed without impairing the germination of healthy seed controls.

In New Zealand, three species of Lupinus are frequently found. The blue lupin (L.angustifolius) is grown as stock food, Russell lupin (L.polyphyllus) is a popular ornamental species, and the tree lupin (L.arboreus) is extensively used for sand stabilisation, and is also widespread throughout the country as a weed. Preliminary work in this present study revealed Brown Spot disease to be commonly present on all three lupin species in the Manawatu. In the case of Russell lupin, the disease was found not only in garden plantings, but was also common in nursery seedlings raised in sterilised soil.

In view of Brown Spot disease being prevalent and widespread in New Zealand, and the fact that overseas work has established the pathogen to be transmitted by way of seed, the hypothesis that seed could well be an important source of primary inoculum in this country was considered. Accordingly, studies were initially aimed at determining the extent to which commercial lines of lupin seed in New Zealand carried viable inoculum, and whether the seed was contaminated or infected. This information was required as a prerequisite for further studies on control by way of seed treatment.

Materials and Methods

At the commencement of this study a circularised letter was

sent to leading seed firms in areas where blue lupins are grown for seed purposes. A half-pound sample of seed was requested (preferably non-dressed) together with information relating to the climate of the district of harvest, and the acreage. Comments on whether diseases are normally prevalent or important in lupin crops in that particular district were also invited.

The response to the letter was very poor, only eight firms replied, and of these three forwarded samples. A personal visit to the South Island enabled eleven further samples to be obtained and these, together with the six from the firms and fifteen from Seed Testing Station Palmerston North, gave a reasonable range of blue lupin seed to be examined.

Three lines of Russell lupin seed, four of L.mutabilis and two of L.hartwegii were purchased from wholesale seed firms, and a further line of Russell lupin used in a local nursery was obtained. At the nursery, diseased seedlings were found in boxes sown with the seed.

As only one method is described in the literature for isolation of P.setosa from lupin seed it was deemed advisable to first ascertain which method of seed screening was in fact most suitable for indicating the presence of viable inoculum in a given seed line. In order to obtain such a method it was necessary to have a line of seed with a known percentage of infection. From preliminary work with lupin seed no such line was available when this work was proceeding. A method was therefore used to artificially infect a line of blue lupin seed.

Production of diseased seed

Method

Seed was softened, without splitting, by soaking in a solution containing 62.5 mgm of the sodium salt of 24D* in 1000 ml of distilled water. A solution of this strength retarded seed germination but eventually on sowing in soil or on blotters the seeds were capable of germination. Pre-softening of the seed in this manner was found necessary to ensure penetration of the testa by the pathogen.

After soaking, the seed was inoculated with a 30-ml spore suspension of P.setosa obtained from three Petri plates of ten-day-old cultures of the fungus on PCA. For inoculation, seed was spread on newspaper which had been dampened. After inoculation further sheets of dampened paper were placed over the seed and this was moistened at frequent intervals to ensure high relative humidity to facilitate infection of the seed. These conditions were maintained for two days.

Two facts are used to support the contention that inoculated seed was infected and not just contaminated with conidia and mycelium. The first one is that binocular examination of the seed after inoculation and high humidity indicated mycelial growth emanating from the testa. The second fact supporting infection of the seed is that using a running-water washing technique lasting two hours, any material contaminating the surface of blue lupin seed (which are comparatively smooth) would be removed.

Screening of lupin seed for P.setosa

Methods

(a) surface sterilisation of seed for two minutes in 0.1% mercuric

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* 24D—2, 4, Dichlorophenoxy acetic acid

chloride solution, followed by three rinses in sterile distilled water and then plating to Oxoid PDA. The method used was that found to be satisfactory by du Plessis and Truter (1953) in South Africa for the isolation of P.setosa from L.albus seed;

(b) seed sterilised as in (a) but plated to PCA;

(c) seed sterilised as in (a) but plated to prune agar;

(d) Ulster Method (Muskett and Malone 1941)—no surface sterilisation is used in this technique and malt agar was the plating medium;

(e) New Zealand method (Newhook 1947)—seed is washed in running tap water and then plated to malt agar; in this method the seed was washed for one hour, embedded in malt agar and then a further layer was poured over the seed;

(f) New Zealand method—as in (e) with the modification that seed was plated on the surface of the malt agar.

One hundred seeds were used for each treatment with five seeds to one plate. All plates were incubated at 24°C and examined after three days.

Results and Discussion

(a) No growth of P.setosa occurred indicating that the surface sterilisation with 0.1% mercuric chloride for two minutes was too severe. Four colonies of Penicillium were recorded.

(b) As for (a).

(c) As for (a).

(d) The Ulster method with no sterilisation of seed resulted in P.setosa growing out from all seed. Thirty per cent of the seed gave Penicillium species; 58% bacteria; and 2% mucor.

These results would indicate that although P.setosa was

identifiable, despite the high contamination, a method where contaminants were controlled would be more desirable.

(e) Results of the New Zealand method where the seed was covered with malt agar. Again 100% colonies of P.setosa were recognisable but bacteria were spread all over the plate so this technique was discarded as being unsatisfactory.

(f) The New Zealand method with surface plating of seed resulted in 100% P.setosa, 6% Penicillium, 10% bacteria and 2% mucor. Although contamination was still present this method appeared to offer possibilities. As P.setosa grows slowly on malt agar (33 mm in ten days) and does not produce conidia on it, it was decided to use PCA because of its ability to promote quick growth and sporulation.

Technique adopted for seed screening work

Seed was placed in a small muslin bag, sealed with a rubber band, and washed for two hours in running tap water. Seed was removed from the bag to sterile filter paper to aid drying, and then plated to PCA and incubated at 24°C for three days.

Where field samples of seed were being screened for infection, PCA containing 62.5 mgm of the 80% sodium salt of 24D was incorporated into the medium to retard germination of the seed (Hagborg et al 1950). Earlier tests with P.setosa inoculated to this 24D-PCA medium indicated that the fungus would grow satisfactorily upon it.

The results of the trial to determine a suitable method for screening lupin seed established the need to determine why a recognised technique for obtaining P.setosa from the seed had failed—namely the failure of du Plessis and Truter's (1953) mecuric chloride method.

On comparing the method used in South Africa and that used in this work, two differences were immediately apparent: (a) du Plessis and Truter (1953) were working with diseased L.albus seed whereas blue lupin (L.angustifolius) was being used in this study, and (b) the blue lupin seed being used to compare methods was artificially inoculated.

It was decided that it was this second factor which could possibly provide the explanation for apparently contradictory results. Mercuric chloride is used in certain instances as a means of controlling seed-borne diseases (Hill, 1964). However, it is to be noted that in such instances where this method is used as a successful control, the infection is superficial or only in the testa. In view of this information the following trial was carried out.

Determination of site of infection in inoculated blue lupin seed

Method

Fifty seeds were soaked in water to soften the testa and then the testa was split off from the embryo. The component parts were washed separately in running water for two hours, plated to PCA and incubated at 24°C for three days.

Results and Discussion

Colonies of P.setosa grew out from every testa but no growth developed from the embryos.

These results indicate that in the inoculated seed infection does not reach the embryo. This fact could account for the failure of du Plessis and Truter's (1953) method in this preliminary work, and if this is the correct reason, then figures obtained by their method

would not give as close an approximation to total infection as would the washing method used in this study. Even the washing method does not give the chance of obtaining a 100% infection figure from field seed as it would not enable surface contamination to be determined. However, this difficulty could be overcome by shaking the seed sample in sterile water, centrifuging the wash, and inoculating plants. If infection occurred it would be known the seed was contaminated with viable inoculum.

In an attempt to verify the thought that du Plessis and Truter's (1953) method failed because infection was only in the surface layers of the seed, a further trial was performed with varying periods of time for the seed in 0.1% mercuric chloride.

Determination of whether a reduced time in 0.1% mercuric chloride would enable P.setosa to emerge from infected seed on media.

Method

Seed was soaked in 0.1% mercuric chloride for $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 2 minutes with seed washed for two hours in running tap water as the control.

Results and Discussion

From Table 7 below it can be seen that 0.1% mercuric chloride allows a higher percentage of P.setosa to emerge as the sterilisation time is reduced. However, even with $\frac{1}{2}$ minute in 0.1% mercuric chloride the washing technique is superior giving 12% contamination as compared with 23%, and 100% P.setosa. Another disadvantage of the mercuric chloride method is that P.setosa does not grow out as quickly from the seed and few if any conidia are visible after three days.

Table 7

Percentage of infection obtained from plating inoculated seed after different periods of surface sterilisation with 0.1% mercuric chloride.

Time in $HgCl_2$	Percentage of seeds producing P.setosa after:		Contaminants after 5 days
	3 days	5 days	
½ min	35%	75%	23%
1 min	15%	60%	17%
1½ min	5%	40%	19%
2 min	-	1%	15%
Control	100%		12%

Thus at three days colony characteristics have to act as the sole criterion for identification of P.setosa, and this is not as fool-proof as being able to identify the colony by the typical conidia formed.

It is convenient to reiterate at this juncture that it was felt necessary to try and explain the failure of du Plessis and Truter's (1953) technique for isolation of P.setosa from seed, before proceeding to apply the 'washing technique' decided on for the remainder of this study. It is believed that the results obtained from the trials performed to do this not only offer a reasonable explanation for the failure of this proven technique, but that they go further by showing that only seed containing deep seated infection would result in the fungus growing out in a plating experiment. On the other hand the 'washing technique' allows both surface and internal infection to be obtained.

Examination of seed samples of blue lupin, Russell lupin, L.Hartwegii and L.Mutabilis for infection with Pleiochaeta setosa.

Method

Because of the number of lines involved and the demand on Petri plates, only 100 seeds per sample were plated to PCA. However, to ensure that a reasonable number of seeds were examined, 200 seeds per sample were sown in sterilised soil in the glasshouse and a further 200 seeds were washed in sterile water, the water centrifuged, and samples examined under a microscope for spores of P.setosa. Two host plants per sample were 'painted' with some of the sludge as a further test.

Use of these three methods provides the opportunity for surface contamination alone to be found by use of the centrifuging of wash water. The plating method provides opportunity for percentage infection to be found, and the sowing in soil should provide figures giving the percentage of effective or viable inoculum, together with proof of pathogenicity.

Results and Discussion

In no instance was Pleiochaeta setosa found in any of the tests. The seed from blue lupin crops was obtained within six to eight weeks of harvesting and that from Seed Testing Station of Palmerston North a little later. The seed of the ornamental varieties was purchased as soon as the new season's stocks became available so in all cases if P.setosa was in the seed and capable of causing infection the fungus would have to be still viable. The only conclusion that can be drawn from these results is that the lupin seed examined in this work from the 1963 harvesting season was not infected with P.setosa.

No information was available as to whether P.setosa is important as a foliage pathogen in lupin crops in New Zealand apart from the knowledge gained in this study of the disease in the Manawatu. In the circularised letter, the few replies mentioning the question of diseases in blue lupin crops all stated that (in that particular season anyway) disease was of little importance. Eventually a visit to the South Island in May 1963, during which seven crops were examined in the Christchurch district, confirmed this view, although some quite large areas of plants approaching maturity were showing disease symptoms due to Ascochyta/Phoma. The plants were becoming dark-grey-black on the stems and stem collapse, wilting, and death was a feature of the disease. Pycnidia could be discerned with the naked eye on the stems and that the disease was caused by an Ascochyta/Phoma species was confirmed by collecting specimens, examining and culturing the fungus.

Specimens of diseased leaflets were collected during the trip and on giving high humidity, P.setosa was isolated from only one sample. Ascochyta/Phoma and Stemphylium species were predominant from the isolation.

Conversation with an Agricultural Officer from Christchurch provided the information that the east coast of the South Island had experienced a dry summer/early autumn and that this could have had a bearing on the amount of disease seen in the crops that season and also on the cleanliness of the seed.

Examination of pods, flowers, and seed collected from a diseased blue lupin crop

In February 1964 a small patch of blue lupin grown on a steep

bank at Massey, by the Soil Conservation Department, provided an ideal crop for field observations. Until the 27th February only 0.14" of rain had fallen and 4.09" had been recorded for the year (meterological details as supplied by Grasslands Division, D.S.I.R.). A few plants showed symptoms of disease caused by P.setosa. On February 28th, 0.66" and on February 29th, 1.33" of rain fell. The spread of the disease in this small crop was quite spectacular and within three days, spotting on leaflets and pods (which were well developed) was abundant. Besides P.setosa, a leaf-spot caused by a Stemphylium spp. was prevalent and this fungus was isolated and proven, by artificial inoculation, to be the pathogen (see Appendix III).

As diseased pods were in abundance, the task remained to determine the causal agent. Pods were brought into the laboratory and examined under the binocular microscope. Because of the very hairy nature of the pod surface, any signs of fungal growth were difficult to detect. The most predominant symptom on the pods was a small right angled brown-black flecking (Illustration 15) but later, areas of this flecking could be seen to have coalesced forming larger lesions. On slicing these lesions off with a scalpel it was evident that the lesion area was fairly superficial and did not pass through into the hard cellulose layer lining the inside of the pod wall.

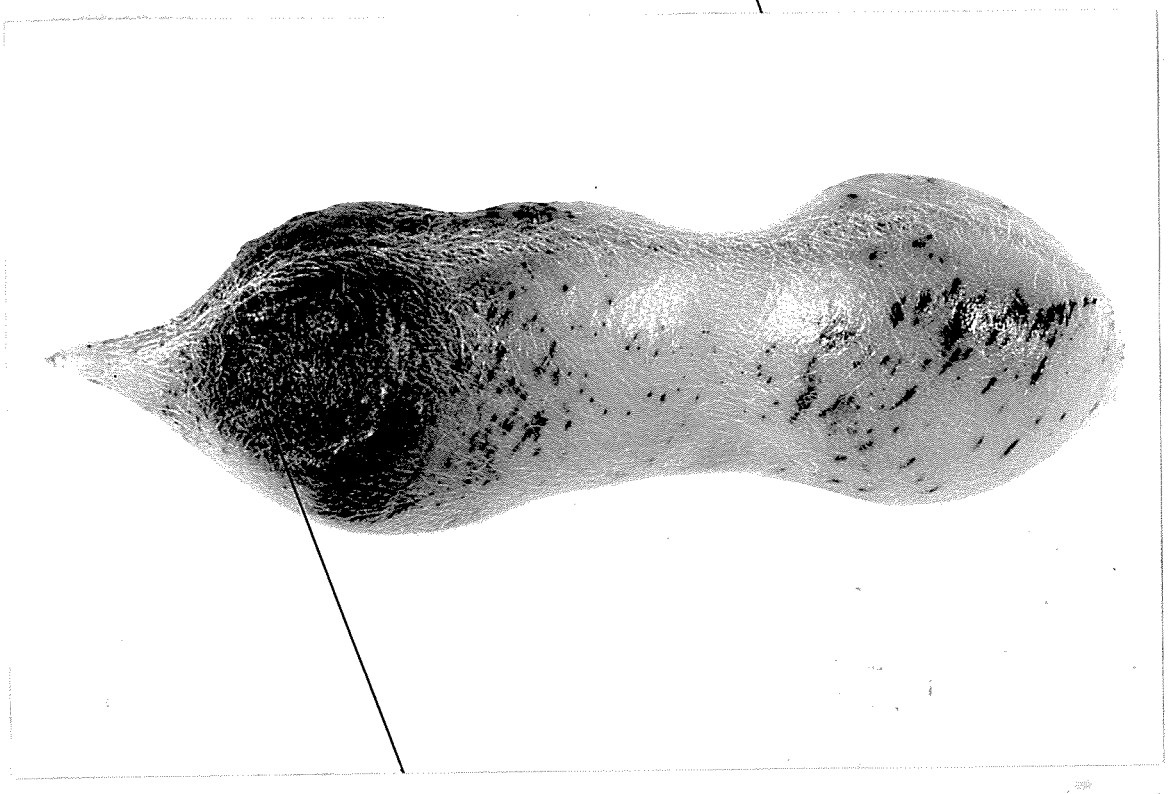
Isolation of the causal agent of pod-spotting of blue lupin

Methods

(a) Lesioned areas were sliced off the pod and given high humidity.

(b) Small areas at the margin of the lesion were cut off the pod, placed in muslin, and washed in running tap water for two hours, and then plated to PCA.

Illustration 15 (x2½)



Disease symptoms caused by
Pleiochaeta setosa on blue lupin pod

Results and Discussion

(a) At no time, either using whole pods or portions thereof, could sporulation of a fungus be induced.

(b) Varying results were obtained with tissue plating, with P.setosa being isolated in several instances. A Stemphylium species was also isolated and this proved pathogenic to blue lupin plants as did the P.setosa isolate.

Having isolated P.setosa from pods it was then necessary to examine the seed in the pods. Numerous seeds were examined and although at this stage they were still green and comparatively soft, no lesioning was observed. To confirm that the seed from diseased pods collected from the crop was not infected, the following experiment was conducted.

Determination of whether seed from pods showing infection was also infected

Method

Pods showing symptoms which could be associated with infection by P.setosa were selected. Small tissue pieces were cut from the margin of pod lesions, washed, and plated to PCA.

Seed. (a) Pods were sliced open aseptically and seeds removed with sterile forceps and transferred directly to PCA containing 24D. (b) Seeds were washed beflore plating.

Results and Discussion

Pods. Of the 50 tissue pieces plated out, 32 produced colonies of P.setosa, and 15 colonies of Stemphylium. Six tissue pieces gave such fungi as Alternaria tenuis, Penicillium and a

Cercospora-like saprophyte. (Note that here there is a total of 53 colonies from 50 tissue pieces, the reason being that in a few instances, more than one fungus emanated from the tissue piece.)

Seed. (a) Forty seeds which had been transferred aseptically to media gave no growth at all. (b) Forty seeds which had been washed, similarly failed to produce colonies.

From these results it can be concluded that at the stage at which pod infection had occurred in this crop, the seed was not infected.

Screening of seed collected from diseased blue lupin crop

Method

When the seed in this crop had matured, several pounds were collected and treated in a manner similar to that used for seed in the main seed-screening experiment, with the addition that two pounds of seed was sown in a garden plot.

Results and Discussion

No spores of P.setosa were observed in the sludge from seed washing, and no infection resulted when plants were inoculated.

From plating seed, six colonies of a Stemphylium spp. resulted, three of a Fusarium spp., two of Alternaria tenuis and two of a Cercospora-like saprophyte. In the trial where seed was sown in sterilised soil, a pathogenic Stemphylium species was isolated from one seedling. No seedlings from which P.setosa could be isolated were found in the garden plot.

As a result of this work with seed collected from a diseased crop it can be concluded that in this set of circumstances (in

particular late infection of the pods), diseased seed did not result. Late in the season when one or two old plants started shooting again, two lupin flowers were noticed showing a brown spotting on the petals. The causal organism was isolated by the tissue plating method and found to be P.setosa but this was the only time the fungus was isolated from a flower in the field.

Diseased seedlings of tree lupin and blue lupin, including those with lesions on the cotyledons, have been found in the field. In this diseased blue lupin crop, much of the seed was shed and after an elapse of time many young seedlings appeared in the debris. However, infection of these could have resulted from the soil or from air-born spores originating from infected crop debris. Later work in Chapter IV shows that P.setosa can survive in the soil in the form of conidia and that seedling contact with debris may result in infection.

The results of the investigation described above, to determine whether P.setosa is seed-borne, would appear to justify the view that lupin seed plays no role in the spread of Brown Spot Disease of lupins in New Zealand.

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CHAPTER IV

THE ETIOLOGY AND EPIPHYTOLOGY OF THE BROWN SPOT FUNGUS

This chapter includes a phase-by-phase consideration of the disease cycle, commencing with spore germination and culminating with the production of primary inoculum. Miscellaneous factors influencing the disease cycle, including host susceptibility, predisposition, and inoculum potential, are then discussed. Finally the individual results of this work and their implications in regard to the importance of Pleiochaeta setosa as a pathogen of Lupinus spp. in New Zealand are considered in their entirety in the section on epiphytology.

A. THE DISEASE CYCLE

Walker (1950) has defined the disease cycle as the chain of events which lead to the development of the disease process. In studying the disease cycle one is in fact considering the continuous relationship between pathogen and host, which begins with spore germination and culminates in the production of the diseased plant, as evidenced by the development of symptoms. However, in order that there may be continuity of the disease cycle from season to season, the pathogen must also effectively survive away from the living host. Thus, in a study of all the events relating to disease production by a fungus, one must also consider the means whereby the pathogen 'over-winters', and further, determine the method of production and dispersal of the primary inoculum from the 'overwintered' material.

For convenience, in the present study the disease cycle has been viewed in this expanded form. As such, this section of the work becomes a study of the etiology (defined as "the study of the causes of disease", Ainsworth and Bisby 1961) and epiphytology (the study of the factors affecting outbreak and spread of infectious disease among plants) of Brown Spot disease of lupins.

To facilitate the study of the etiology of Pleiochaeta setosa on lupins, the disease cycle was divided into a number of phases.

1. Spore germination. Germination is the initial stage in the development of fungal mycelium from a spore and commences when the spore swells after imbibing water. For the purposes of this study a spore was said to have germinated when the length of any one germ-tube exceeded its own width, for it was impossible to determine when swelling first took place.

2. Penetration and Infection. Penetration occurs when a germ tube or hyphae from the germinated spore enters the host tissue. Infection is only said to have taken place when, following penetration, the fungus becomes dependent on the host (and not the spore) for its nutrition. In many cases fungi penetrate a host but are unable to infect it. Although there is, in fact, a distinct difference between penetration and infection in terms of the nutrition of the fungus, the close integration of these two physiological phenomena in parasitism make separate study of the changes and processes involved, both in relation to pathogen and host, difficult.

3. Incubation. This is the period in the disease cycle which commences with host infection and terminates with the expression of macroscopic disease symptoms.

4. Manifestation of Disease. Manifestation by the production of macroscopic disease symptoms is the first apparent indication that plant metabolism is abnormal. The symptoms produced are often typical of a particular pathogen and may lead to its early identification and subsequent control before the fungus becomes too well established.

5. Production of Inoculum. Inoculum may be defined as the propagules from which the fungus infects a plant. Numerous forms of inoculum are produced by fungi, but in general it may be said that some form of inoculum will be produced by the pathogen in the later stages of manifestation, providing environmental conditions are suitable.

6. Dispersal of Inoculum. Having produced inoculum it is necessary that a mechanism, or external agency is available to ensure liberation and adequate dispersal of the propagules. Here, as in production of inoculum, environmental conditions may play an important role and it is noted that the most important plant pathogens are those

which produce abundant and readily dispersible inoculum.

7. Survival of the Fungus. A parasitic fungus is not always in contact with a suitable living host and hence there is a need for some form of existence or survival to carry the organism through this period.

8. Primary Inoculum. Primary inoculum is inoculum which initiates new season's infection and because of this it is closely associated with the survival phase of the disease cycle.

The above eight phases will now be discussed in detail.

1. Spore Germination

The process of spore germination is generally regarded as belonging to growth phenomena and hence is subject to modification by all those factors influencing growth. Spore germination has much in common with seed germination, and the factors affecting germination of spores, as of seeds, are of two types: hereditary or internal; environmental or external. Hereditary factors include: maturity, longevity, dormancy, and vitality of spores. The environmental factors include: influence of temperature, moisture, pH, kind and concentration of nutrients, light, and the presence of oxygen and carbon dioxide.

Germination is the initial stage in the development of fungus mycelium from a spore and differs from the growth of hyphae primarily in the ability of the spore to utilise its stored food reserves for metabolism, i.e. germination implies a change from an INACTIVE to an ACTIVE state. Morphologically, germination is usually characterised by a swelling of the spore and production of a germ tube.

It is generally accepted, that the individual fungal spore is capable of causing infection (Garrett 1960) and, as spore germination

precedes penetration and infection in most instances, it is convenient to consider this as the first stage in the disease cycle.

Spore germination can be a vulnerable phase in the life cycle of a fungal pathogen and a study of the literature revealed that in many cases critical requirements must be met before this process can proceed. It was therefore considered necessary to ascertain from the literature the most important factors in nature affecting spore germination, and then investigate these in relation to P.setosa.

A study of the literature relevant to spore germination indicated that environmental conditions were most critical in germination, in particular, temperature and relative humidity. A series of trials was planned and carried out to determine the effect of these two factors on the germination of conidia of P.setosa. Before consideration is given to this work it is necessary to discuss several factors which are basic in any spore germination study.

Assessment of Germination

(a) When a spore has germinated.

The imbibing of water and subsequent swelling of the spore is the first process in germination (Gottlieb, 1950; Hawker, 1950), but this is difficult to discern. In germination studies several different arbitrary criteria have been chosen as a suitable basis to ascertain when a spore can be considered to have germinated (Bonner 1948, Naqvi and Good 1957, Manners and Hossain 1963). From preliminary studies on the germination of P.setosa spores it was decided that for the purposes of this study the criterion chosen by Manners and Hossain (1963)—"when the length of any one germ-tube exceeded its own width"—

would provide a satisfactory basis for deciding 'when a spore has germinated'.

(b) Expression of germination results

In disease development the abundance, speed, and type of germination of inoculum are important factors. With P.setosa, abundance or percentage, and speed of germination are the two factors to be considered when choosing criteria for measurement of germination. Gottlieb (1950) considers that—"the percentage spores producing germ tubes"—is the most common criterion used, and Cochrane (1958)—"the fraction of spores that in a given time form a germ tube, the time chosen usually being sufficient to enable all viable spores to germinate." Use of this final percentage as the only measurement of spore germination is not satisfactory, for where different environmental factors are being compared the result will not show possible differences in interim germination, i.e., it could be possible for two samples of spores held under different temperatures to give a similar final germination after a set time, but to differ quite markedly in how soon germination occurred, or when, for instance, the first 30% had germinated. Hawker (1950) in a discussion on this point mentions that if measurements are delayed too long a real difference in germination may fail to show up. Bonner (1948) suggests that because wide differences in rate and percentage of germination occur it is more important to know when the first spores germinate, since these spores could initiate infection. A similar criterion is suggested by Hawker (1950).

Average length of germ tube is proposed for a measurement of germination rate by Brown (1936) but here the measurement would be of

vegetative growth rather than germination. Although the rate of germ tube elongation can conceivably be of considerable importance in determining whether or not penetration of a plant occurs, it is difficult to measure because of the often tortuous path a germ tube follows in its growth.

There is no doubt that spore germination figures expressed in the literature vary greatly in their value because of the lack of standardisation of basic criteria. However, although it would doubtless be simpler if one figure of germination could be expressed, this is not possible. As far back as 1932 Tomkins stated—"many ambiguities might be avoided if three criteria were employed: (1) the latent period of germination; i.e., the average time for germ tubes to appear (2) the percentage germination (not after so many hours but the final percentage of spores germinating) (3) the rate of elongation of the germ tubes." Manners and Hossain (1963) have shown that speed of germination, and to a lesser extent germ tube growth, are more sensitive to environmental factors than ultimate germination percentages.

In view of these facts as revealed by a study of the relevant literature, and preliminary experiments with P.setosa, it was decided to express germination in terms of:-

(i) Percentage germination at various intervals, thus measuring rate or speed of emergence.

(ii) The final germination percentage which will give a measure of the effective inoculum potential of the fungus under the particular environmental conditions studied.

The suitability of various substrates

In all cases where a study of this nature is being undertaken it is essential that the experiments should be performed under conditions simulating those in the field, otherwise the results will be of theoretical interest only.

Initially a trial was carried out using Russell lupin and blue lupin leaflets as a substrate for germination and using a range of temperatures from 20°-30°C. The technique was successful and germination percentages could be recorded, but, when large numbers of observations were made every half hour, examination proved to be a strain on the eyes because of the dark background provided by the leaflets. Even with staining of the spores and judicious use of the microscope condenser, this problem could not be obviated.

Consequently it was decided to experiment with different substrates in order to find one which closely simulated results obtained on leaflets. As 98% germination could be obtained within three hours on Russell lupin leaflets held at 100% RH (24°C), this was used as a standard for comparison of the different substrates.

Spores were placed in drops of tap, rain, and distilled water, on glass slides and in no case did germination percentage exceed 75% after three hours. Using the cavity slide technique, 15% germination was recorded after three hours. Finally a PCA smear on a glass slide was found to give results most closely approximating those obtained on the detached leaflet and this was chosen as the substrate to determine the influence of temperature on germination.

Trial to determine the effects of temperature on germination

Materials and Methods

A comprehensive range of temperatures from 5°-37°C was used for the trial. Spores were obtained from ten-day-old cultures of P.setosa grown on PCA. A 30-ml spore suspension was prepared and a portion of this was brushed onto an agar smear. The slides were placed in Petri plate humidity chambers which had been held at the respective temperatures for 24 hours prior to commencement of the trial to ensure 100% RH. From preliminary experimentation six plates per treatment were found to be sufficient, except at temperature extremes, enabling one plate to be examined every half hour and then discarded. Because of the number of plates to be examined and counts to be made of germinated spores, spores were killed and stained with lactophenol acid fuchsin immediately on removal from the incubator. This enabled rapid and efficient counting.

Two 'Veeder' counters were used in recording germination, one for counting total number of spores, and the other for counting germinated spores. Two hundred spores were counted at each examination and the trial was performed twice, the average figure being given in the results (Table 8).

Results and Discussion

In the table showing the influence of temperature on the germination of spores of P.setosa, figures for germination on the leaflets of Russell lupin are given where determined.

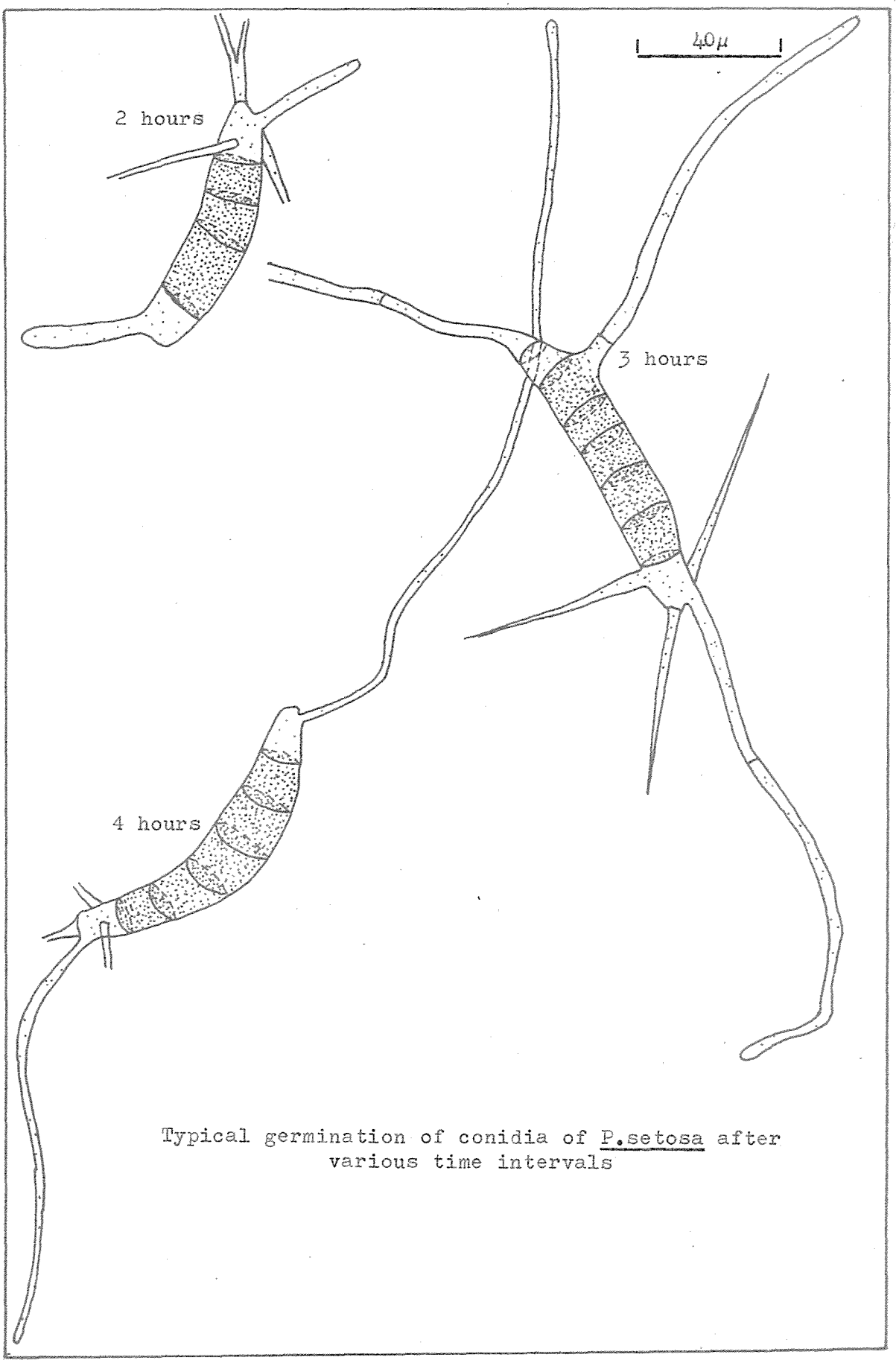
The results indicate that P.setosa is capable of very rapid germination from 20°-30°C and even at 10°C and 34°C germination figures close to 100% are obtained. However, at 37°C no germination

Table 8

Percentage germination of conidia of P.setosa at temperatures ranging from 5°C-37°C (on PCA smears). (Figures for germination on Russell lupin leaves in parenthesis.)

Temp.	Percentage Germination after—												
	1 hour	1½ hrs	2 hrs	2½ hrs	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	8 hrs	9 hrs	10 hrs	11 hrs
5°C	-	-	-	-	-	-	-	5	14	47	81	94	98
10°C	-	7	33	67	96								
15°C	-	12	65	96									
20°C	61.2 (62.5)	99 (97)											
22°C	66.0 (68.5)	95 (96)											
24°C	75.5 (79.2)	86 (87)	98 (96)										
26°C	85.0 (88.0)	96.9 (97.0)											
28°C	81.5 (86.0)	97 (100)											
30°C	63.0 (72.0)	98.0 (98.5)											
32°C	3.0	63	91	96									
34°C	-	10	60	92	97								
37°C	-	-	-	-	-								

Illustration 16



Typical germination of conidia of P.setosa after various time intervals

was recorded, the conidia in fact dried up very quickly. At 5°C it was six hours before any germination was recorded. These results are in full accord with those obtained by Germar (1940) and du Plessis and Truter (1953). Further, in growth studies of P.setosa on PCA and Oxoid PDA, no growth occurred at 37°C showing that 36°-37°C is the upper temperature limit for this organism.

Germination most commonly commenced from either of the colourless cells at the ends of the conidial body. The setae, or appendages, appear to be sterile as they were never seen to produce a germ tube and no record can be found of such an occurrence. In many instances the production of a germ-tube from both ends of the conidial body typified the germination of P.setosa (Illustration 16 (a)), although all body cells were capable of producing a germ tube. Even conidia with part of the body missing could germinate. Appressoria were never formed on PCA but on Russell lupin leaflets they could be found within $2\frac{1}{2}$ hours at temperatures between 20°-30°C.

With the ability to germinate and extend germ tubes so rapidly over a considerable temperature range, it can be concluded that temperature is very unlikely to be a limiting factor in conidial germination of P.setosa on lupins in New Zealand.

Trial to determine the effects of relative humidity on germination

Relative humidity has often proved to be a limiting factor in spore germination, and many fungi will not germinate unless they are in contact with liquid water, for example, Sclerotinia fructicola and Phyllosticta antirrhini. Others will germinate on dry surfaces in an atmosphere of high RH, usually exceeding 95%, and a third group,

represented by some of the Powdery Mildew fungi, can produce short germ tubes at or near 0% RH.

Materials and Methods

Several methods are available for maintaining an atmosphere within a closed vessel at a constant relative humidity (Galloway 1935, Bonner 1948, and Wexler and Brombacher 1951). Spencer-Gregory and O'Rourke (1957) 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. Spencer-Gregory and O'Rourke (1957) state that for convenience, only saturated salt solutions should be considered in order to obtain appropriate humidities.

Saturated salt solutions and glycerine plus water were used to obtain the relative humidities required for this study. For spore germination a range of humidities from 80%-100% was used. The solubility of the salts was determined from the International Critical Tables (1928) and saturated solutions prepared by allowing approximately 10% excess salt in 50 ml of distilled water (see Appendix II for composition of Constant Humidity Solutions).

The problem to be overcome was to find a suitable method by which spores could be suspended over, or held in, a constant humidity chamber. A method used by MacNeill (1950), which involved the use of Petri dishes with agar in the lid and a solution in the bottom, was tested. The dish was sealed with vaseline, left to reach constant humidity and spores were then placed on the agar. This technique proved unsuitable because of problems with contamination of the agar whilst constant humidities were being attained, and also because

the method of sealing the plates was impracticable.

Lens tissue was used by Slade (1961) but when this was tried it was found to be unsatisfactory due to the difficulty of distinguishing germ tubes from the cellulose fibres in the tissue. Wax paper similarly proved unsuitable.

Finally, a method was used where spores were suspended above the solutions on pieces of cellophane (3 x 2 cm). Jars with rubber stoppers were used and the cellophane suspended on a piece of cotton stapled to the stopper. To ensure the solutions had reached an equilibrium for that temperature, the jars were placed in the appropriate incubator overnight. Spores were collected on the cellophane by lightly brushing the tissue pieces over a ten-day-old PCA colony. The experiment was conducted at 25°C and results recorded after 24 hours incubation.

Results and Discussion

Table 9

Percentage germination of conidia of P.setosa at relative humidities ranging from 80%-100% (25°C) after 24 hours

Relative humidity	Germination
100.0%	98%
97.0%	83%
94.9%	38%
93.0%	-
90.0%	-
88.0%	-
80.0%	-

This experiment was carried out at only one temperature, namely 25°C, because of the difficulty of obtaining a satisfactory range of constant humidity solutions at other temperatures. As the optimum temperature for germination was not definite, but was found to be in the 24°-28°C region, 25°C was chosen as being representative of the interaction between temperature and relative humidity. The range of relative humidity at which germination occurs is greatest at the optimum temperature (Hawker 1950, Manners and Hossain 1963). In view of this it could be concluded from the results in Table 9 that germination of conidia of P.setosa falls off markedly below 97% RH with no germination at all at 93% RH. The significance of this requirement of a high relative humidity for spore germination will be discussed in the section of the disease cycle relating to Penetration and Infection.

An interesting observation made in this study of relative humidity in relation to spore germination, was made when some blue lupin leaflets inoculated with conidia were exposed to different relative humidities—conidia of a Stemphylium spp. were observed on the leaflets and seen to germinate at humidities down to 87%. Later work proves the pathogenicity of a Stemphylium spp. isolated from blue lupin (Appendix III).

Other factors influencing germination

Conidia of P.setosa germinated equally well in light or dark and there was no evidence of an inherent dormancy in spores once they were removed from the conidiophore.

2. Penetration and Infection

Penetration includes the method by which the fungus enters host tissue but not the subsequent development within the host (Flentje, 1959). Penetration does not necessarily lead to infection and Wolf and Wolf (1947) note that fungi are capable of penetrating a wide range of plants, but are unable to establish pathogenic relationships in every case.

When the fungus becomes established within the host following penetration and is independent of external food reserves for continued development, infection has occurred (Walker 1950).

These two process are inseparable in a study of this nature as it is difficult to determine when the fungus becomes parasitic. In fact, Gaumann (1951), cited by Eide (1955) defines infection as— "that phase of an infectious disease which extends from germination of the pathogen to the time of its entry into a stable parasitic parasitic relationship with its host." The study of this phase of the disease cycle becomes basically a study of the method of penetration and effect of environmental factors on penetration and infection.

(a) Method of host penetration.

A study of the literature reveals that a pathogen may enter a host by one or more of three ways, namely (i) direct penetration through the intact surface of the host; (ii) through natural openings such as stomata and lenticels; and (iii) through wounds.

In order to determine the method by which P.setosa penetrates lupins, leaflets of Russell and blue lupin were sprayed with a conidial suspension and placed under conditions favourable for infection.

Materials and Methods

Freshly detached leaflets from Russell and blue lupin plants were inoculated with P.setosa and placed on glass slides in Petri plates in high humidity chambers at 25°C.

Samples of these inoculated leaflets were examined at two-hourly intervals. Preliminary inoculation studies with detached leaflets revealed that symptom expression occurred within 18 hours of the time of inoculation. By examination of inoculated leaflets at two-hourly intervals, information on the time required for penetration as well as the method of penetration, would be achieved. Furthermore, examination of the leaflets after penetration until the first symptoms appeared, would enable determination of the incubation time required by the fungus under those conditions.

As already mentioned, spore germination and hyphal extension across the host surface can be regarded as the first stage in the disease cycle. In fungi there are typically further preliminary reactions before penetration of the host. These consist of cessation of hyphal elongation, appressorium formation, and attachment of the fungus to the host surface.

In order to determine the exact site and method of penetration it was necessary to find a satisfactory procedure for clearing host tissue and for staining the fungus.

Several methods of clearing host tissue were tried (MacNeill 1950, Minderman 1956, Couch and Grogan 1955, and Latch and Hanson 1962). The lactophenol* clearing solution used by Couch and Grogan (1955) gave quite good results but took 30 minutes or more at a temperature just

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*Formula for lactophenol clearing solution used: 20 ml distilled water, 20 ml lactic acid, 20 gm phenol and 40 ml glycerine.

below boiling for reasonable chlorophyll removal from the leaflets. The method used by Latch and Hansen (1962) involved the use of a hot solution of 1:1 glacial acetic acid and 95% ethanol. The solution was boiled gently for a few minutes on a sand bath until the leaflets lost their chlorophyll and became opaque. Five minutes was found to be sufficient time for clearance. The use of a strong chloral hydrate solution, as used by Latch and Hanson (1962) for final clearance of leaves on Melilotus, was not found necessary with lupin leaflets. Instead of using lactophenol cotton blue for staining the fungus, 0.2% trypan blue in lactophenol was used. (Minderman, 1956). Trypan blue was superior to cotton blue in that it stained the fungal tissue but not the leaflet tissue.

Using the glacial acetic/ethanol for clearing and trypan blue for staining, all stages of penetration could be readily observed microscopically both on the leaf surfaces and within the leaf tissues.

Results and Discussion

The majority of spores produced a germ tube, many of which were seen to form a swelling at the tip of the hypha after varying amounts of hyphal extension. This swelling undoubtedly is an appressorium, and in a few instances, a fine infection peg could be seen emanating from the appressorium. The infection peg penetrated directly through the cuticle of either surface of the leaflet. Five per cent of the germ tubes had produced appressoria on Russell lupin leaflets within $2\frac{1}{2}$ hours and 61% within four hours. The earliest penetration observed was four hours after inoculation, and the first expression of symptoms occurred after 18 hours, giving a possible minimum incubation period for P.setosa, under the above conditions, of 14 hours.

Stomatal penetration was observed only once and in this particular instance an appressorium was formed and an infection peg was readily visible entering through the closed stomata (Illustration 17(a)). No definite pattern of germ tube behaviour could be discerned, and in many cases germ tubes grew right across stomatal pores.

Once the fungus had penetrated the leaflet by an infection peg normal size hyphae emanated from this peg and from here all growth was intracellular. It was impossible to distinguish individual hyphae in advanced lesions except at the margins of healthy and diseased tissue.

The first discernible effect on the host was the production of minute pock marks on the leaflet surface. Examination of these under the microscope indicated that these were due to cell collapse brought about by the action of the fungus.

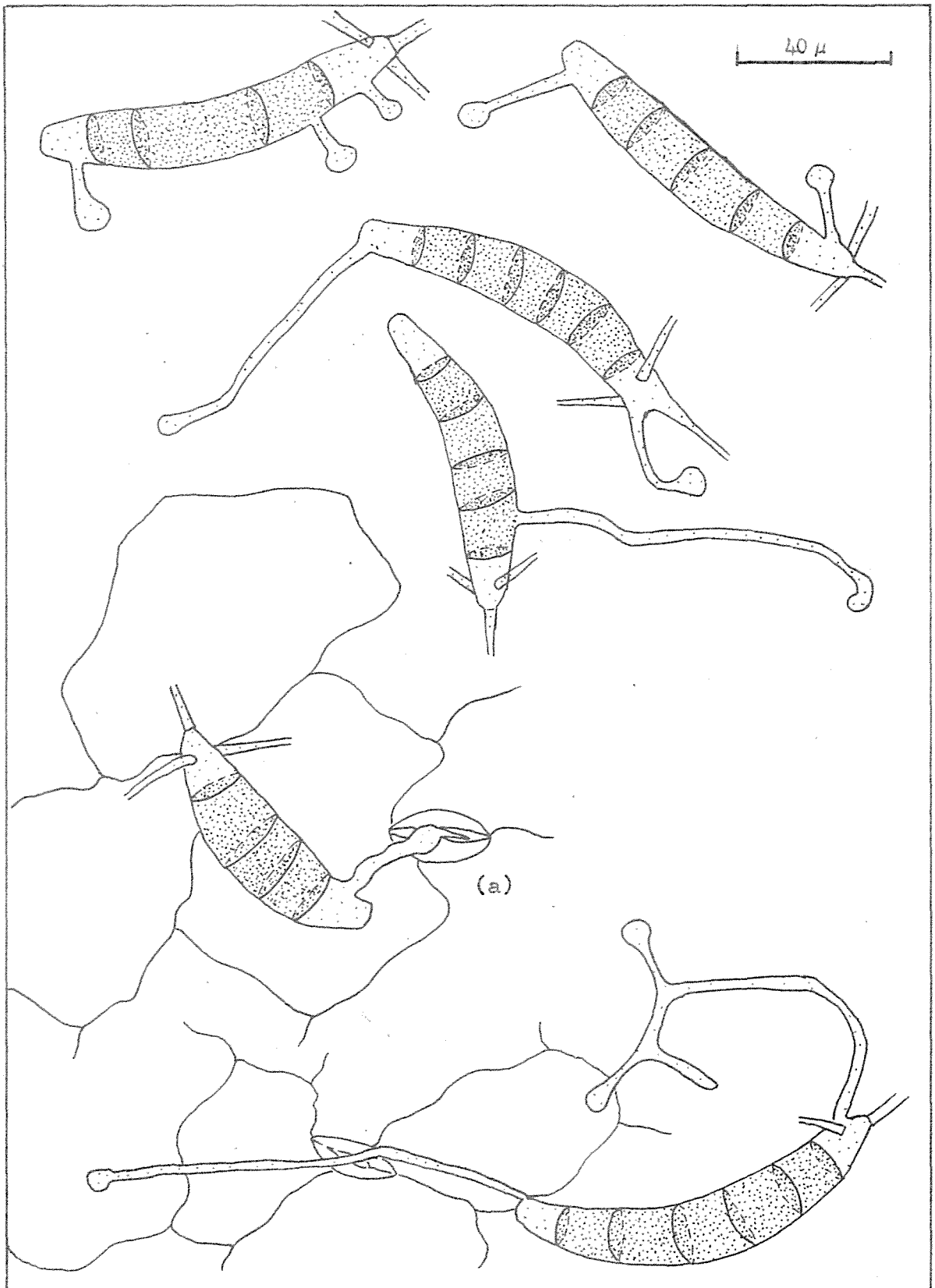
(b) Time a leaflet must be wet for penetration and infection.

Relative humidity has already been shown to be an important factor in spore germination, the minimum RH at which conidial germination occurred being shown to be 94.9% (25°C). Leaflet wetness will obviously be closely related to the relative humidity at the leaflet surface. In view of the importance of this factor before infection by certain fungi can occur (e.g. Venturia inequalis), an experiment was conducted to determine how long blue lupin plants must be kept wet before infected resulted.

Materials and Methods

Healthy one-month-old blue lupin plants were sprayed with a spore suspension of P.setosa, and placed in a humidity chamber at 22°C and 100% RH. Plants were removed every six hours up to 24 hours.

Illustration 17



Appressorial production by Pleiochaeta setosa

(a) the only example of stomatal penetration observed during this study

On removal plants were dried with an electric fan and placed in a room at 18°-23°C and approximately 72% RH. Infection occurred in plants that had been held in a saturated atmosphere for six hours or more.

In view of these results, 36 blue lupin plants were treated as in the above experiment except that in this case six plants were removed and dried at hourly intervals up to six hours and results taken 48 hours after the commencement of the trial.

Results and Discussion

Plants which had been exposed to four hours or more of saturated atmosphere showed infection i.e. it was necessary for a leaflet of blue lupin to be wet for at least four hours before the fungus could develop independently of free leaflet surface moisture. These results are of considerable interest for they were obtained with complete plants. Earlier in this section of the disease cycle relating to penetration and infection it was noted that the earliest penetration of detached lupin leaflets was observed four hours after inoculations.

Although only a hypothesis, it would appear that it is after approximately four hours in a saturated atmosphere at 22°C P.setosa actually first infects a lupin leaflet.

(c) Influence of light on penetration and infection

Light has been shown to have a considerable influence in some host parasite reactions (Wolf and Wolf 1947). Because light plays a major part in the metabolic activity of a plant, including its influence through photosynthesis on stomatal behaviour, numerous workers have given consideration to the importance of light in relation to the

disease cycle, and in particular to its influence on penetration. Besides the influence of light on the host it may also have a direct effect on the fungus (Wolf and Wolf 1947, Diener 1955, Yarwood 1959).

Materials and Methods

A trial was performed to determine the influence, if any, of light on penetration and early infection stages of P.setosa on blue lupin. As manifestation can occur after 18 hours at 22°C it was decided to use only two post-inoculation treatments:

(i) plants exposed to eight hours of light

(ii) plants exposed to eight hours of darkness

and then all plants received twelve hours of darkness before results were taken.

Twelve one-month-old plants were used for each treatment. Illumination was provided by two 150-watt lamps mounted 3ft 6ins above the plants on stands in such a manner as to provide an even light distribution. Plants receiving the dark treatment were covered with black plastic bags sealed around the base of the pots with rubber bands. These latter pots were randomly distributed amongst the plants receiving light so that all plants would receive similar temperatures of approximately 24°-26°C. Plants were misted with water at regular intervals to maintain 100% RH whilst treatments were being applied.

The number of plants expressing symptoms after 20 hours was recorded, and leaflets were taken from plants receiving the two treatments, cleared, stained, and examined microscopically for mode of fungal penetration.

Results and Discussion

Plants from both treatments showed symptoms after 20 hours and no difference was evidenced between the dark and light exposure treatments either in terms of symptom type or number of lesions.

Microscopic examination of leaflets revealed only direct penetration by the fungus and, in view of earlier work in this study on method of penetration, it was concluded that direct epidermal penetration is the most important way in which P.setosa enters blue lupin leaflets. Despite its influence on plant metabolism, light does not affect penetration or the early stages of infection. The fungus appeared to have an equal facility for penetration through upper and lower epidermis and this, coupled with the ability to penetrate directly through the epidermis rather than the stomata, would substantiate the results obtained in the trial to determine the influence of light on penetration.

(d) Influence of temperature on penetration and infection

Having established that light does not affect this stage of the disease cycle, use was made of the laboratory incubators to obtain information on the influence of temperatures ranging from 15°-32°C on penetration and infection of blue lupin plants.

Materials and Methods

An experiment was conducted with two different exposure periods to the temperatures used, namely (a) five hours, and (b) seven hours, in all cases 100% RH being maintained in the incubator by regular misting.

Plants were inoculated at the four leaf stage of growth and six

plants used for each treatment. At the conclusion of treatments plants were held in the laboratory at 22°-24°C and 68%-74% RH. Samples of leaflets were taken from plants after treatments (i.e. after five and seven hours respectively) and these were cleared, stained, and examined microscopically.

Results and Discussion

Microscopic examination of the leaflets after five and seven hours at the respective temperatures revealed no differences in the percentage germination of the spores. This would be expected in view of the results obtained in spore germination studies for at all these temperatures 96% germination had been obtained after 2½ hours. However, at temperatures from 20°-26°C germ tubes had made greater growth than at the other temperatures, and at 24°C, 71% of the germ tubes had produced appressoria after seven hours whereas at 15°C and 32°C only 8% and 3% respectively had produced appressoria after the same time lapse.

Table 10, besides providing information on the results of the influence of temperature on penetration, goes further in that through recording the number of plants diseased and giving these plants a disease severity rating, the influence of temperature on infection, incubation, and manifestation is also obtained.

All plants were diseased at 22°C and 24°C but under the conditions provided in the trial and in terms of the intensity of disease expression, 24°C appears to be the optimum temperature for the early stages of the disease cycle of P.setosa on blue lupins.

When lesion areas from leaflets at 20°-26°C are examined under a binocular microscope after one or two days the irregular lesion outline

Table 10

Effects of exposure of inoculated blue lupin plants to temperatures ranging from 15°-32°C and 100% RH (for five and seven hours respectively), and then to a common environment. Results were recorded up to one week after treatment.

Incubation Temperature	Time at given temp. (hours)	Number of plants showing infection after 24 hours (6 plants per treatment)	Disease Severity rating**	Remarks after 24 hours	Remarks after 48 hours	Remarks after 1 week
15°C	5	3	1	Distinct spotting, almost like pepper grains, but not prevalent	No further development of lesions	Most of leaflets (60%-70%) fallen
	7	4	1			
20°C	5	5	1	Like 15°C except lesions developing with blurred or cobweb outline		
	7	5	1			
22°C	5	6	4	Cobweb-like spots. Lesions on plants at these first 3 temperatures a pale sepia brown and 1-2mm in size	Spotting still small but very distinct	Defoliation almost complete (80%-100%) and plants withered
	7	6	3			
24°C	5	6	5	Lesions have indistinct cob-web outline, dark in colour and may reach up to 5 mm in size.	Two leaflets starting to wither	
	7	6	5			
26°C	5	4	2	Cobwebbing again apparent but colour of lesions pale		Most of leaflets fallen
	7	4	4			
28°C	5	0	0	Where symptoms are present there is a small depression slightly lighter in colour than the rest of the leaflet. These could be likened to minute pock marks in the leaflet surface	Brown colour in pock marks becoming apparent	Defoliation least severe (20% or less) and the plants starting to grow through the disease producing young healthy leaflets.
	7	(3)*	1			
30°C	5	0	0			
	7	(6)*	1			
32°C	5	0	0		No brown colour in pock marks but several leaf-lets withering	
	7	(5)*	1			

* plants showing no lesion in terms of necrosis due to cell death, but rather a visible depression in the leaflet. These depression areas are slightly lighter in colour than surrounding tissue

** Disease severity rating based on scale 0-5; 0 = no disease; 5 = all leaflets showing numerous lesions. (Recorded after 24 hours)

can be clearly seen. From a small spot, wavy thread-like lines of dead cells radiate from this focal point. These are responsible for giving the lesions, even at this early stage, a cobweb appearance.

At temperatures ranging from 28°-32°C infection still occurs, but does not appear to progress the same as at the lower temperatures and certainly the short exposure times to these higher temperatures influences manifestation of the disease. It is interesting to note that the plants are able to overcome or grow through the disease at these latter temperatures, after one week, despite some defoliation.

This defoliation appears to be quite characteristic of the disease for numerous workers make specific mention of it as a feature of the disease. (Cavara 1924, Hogetop 1938, Richter 1938, Germar 1940, Green and Hewlett 1949, and Weimer 1952) Only two of these reports refer to blue lupin. Weimer (1952) mentions in a U.S.D.A. Farmers Bulletin that "brown spot of blue lupin causes a great deal of defoliation, often leaving most of the stem bare except for a few younger leaves at the top of the plant", and Richter (1938) also refers to premature defoliation of blue lupin. Table 10 indicates that after one week defoliation was almost complete at 24°C but it was only slight at temperatures ranging from 28°-32°C.

Finally it was noted during this trial that many of the leaflets which had fallen on the soil in the pot were covered with conidia, and in some cases mycelial growth was visible to the unaided eye. Further comment will be made on this production of conidia on fallen leaflets in the sections of disease cycle relating to production and dispersal of inoculum.

3. Incubation period

No detailed work was done in the study on this interval between infection and the appearance of macroscopic symptoms of the disease. It was noted in the trial on method of host penetration that at 25°C on detached Russell lupin leaflets the earliest penetration observed was four hours after inoculation, and the first expression of symptoms occurred after 18 hours. This would indicate a possible minimum incubation period, in this instance, of 14 hours.

Numerous trials performed during this study have indicated considerable symptom expression after 24 hours. These trials have generally been performed at 22°C or 24°C and 100% RH but the rapidity of manifestation does indicate that given optimum conditions P.setosa quickly becomes established, and the incubation period is short.

4. Manifestation of disease

Manifestation is the term used to denote the expression of disease symptoms in terms of the disease cycle as used in this study. It marks the end of incubation and continues until the death of the plant occurs, or perhaps until a plant recovers from the particular disease in question.

Reference has already been made to the influence of temperature on the manifestation of disease symptoms in the section pertaining to penetration and infection. A description of the disease symptoms, in the field, commonly expressed on species of Russell, tree, and blue lupins respectively, now follow.

Russell lupin (Lupinus polyphyllus)

P.setosa causes a greater range of symptoms on Russell lupin

than either of the other two lupin species commonly grown in New Zealand. The disease spots occur on leaflets, stems, and flowering parts and although pod lesions were found, at no time could P.setosa be isolated.

The early phase of the disease occurs on the young leaflets as small blackish-brown, almost circular, spots 1-3mm in diameter and often surrounded by a bright green zone. On the lower surface these spots appear to be limited by the venation of the leaflet during the early stages of development. The spots enlarge as the leaflets mature until large, greyish-brown necrotic lesions ultimately coalesce. Severely affected leaflets turn yellow and drop prematurely. On the more mature leaflet, spots are sharply demarcated, usually circular to irregularly circular (Illustration 18), and often bear one or more concentric rings. The outer part of these circular lesions is generally a greyish-brown with a more chestnut-brown centre. On giving high humidity to this latter type of lesion, sporulation more commonly occurred on the central portion.

Where lesions are numerous, as in Illustration 19, severe necrosis occurs together with subsequent withering and browning of the leaflet(s). The mottled colouration of the leaflets as depicted in Illustration 19 is very typical of a severely diseased Russell lupin plant.

The manifestation on petioles and stems of the plant is often quite prominent. The lesions are blackish-brown, oval to elongated, and may extend for a considerable distance along and around the stem. They can eventually cause stem collapse which hastens the withering of leaves. On mature plants which may be two or three years old, manifestation was most common on the leaflets nearest the ground and often the young foliage in the centre of the plant was 'clean'.

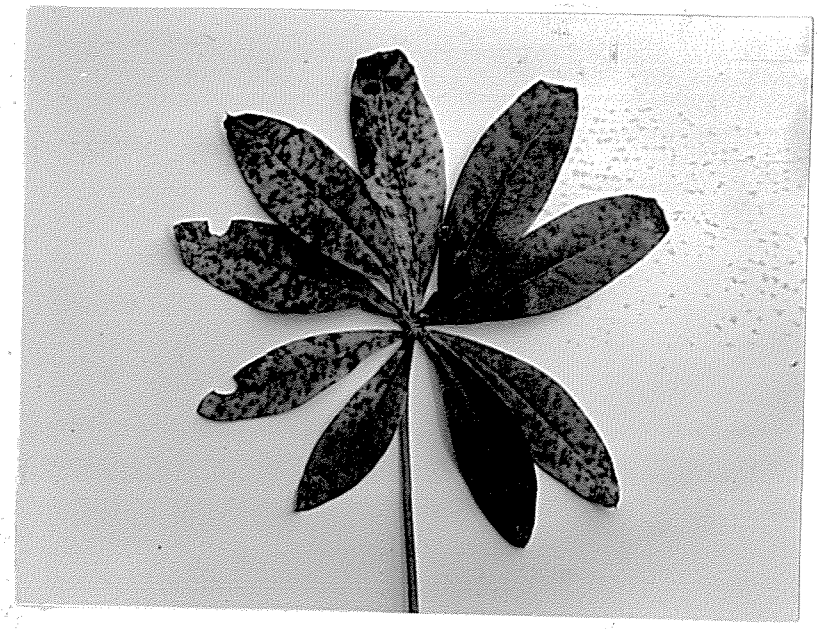
Illustration 18

Typical sharply demarcated lesions, caused by Pleiochaeta setosa, on mature Russell lupin leaflets

Illustration 19

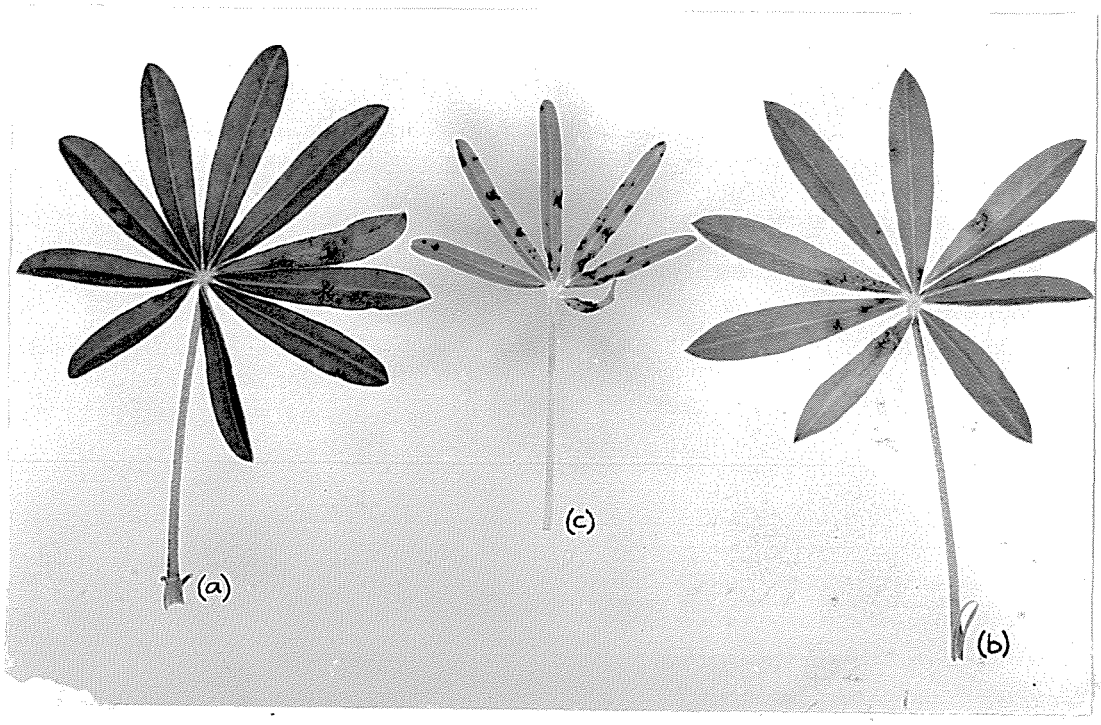
Mottled colouration of Russell lupin leaflets severely diseased with Pleiochaeta setosa

Illustration 20



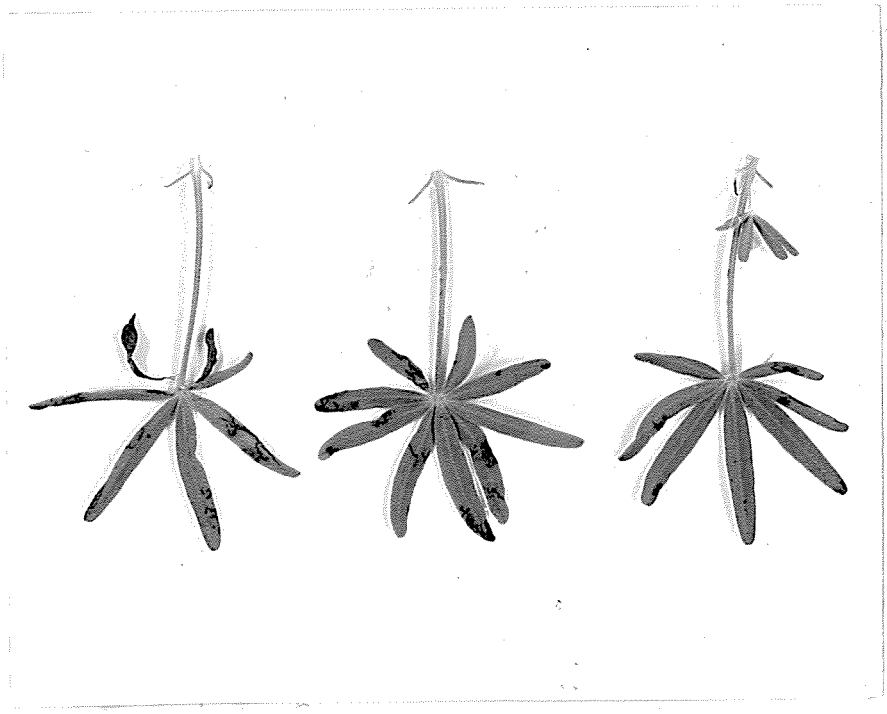
Colour and form of 'cobweb' lesions, caused by Pleiochaeta setosa, on blue lupin leaflets

Illustration 21



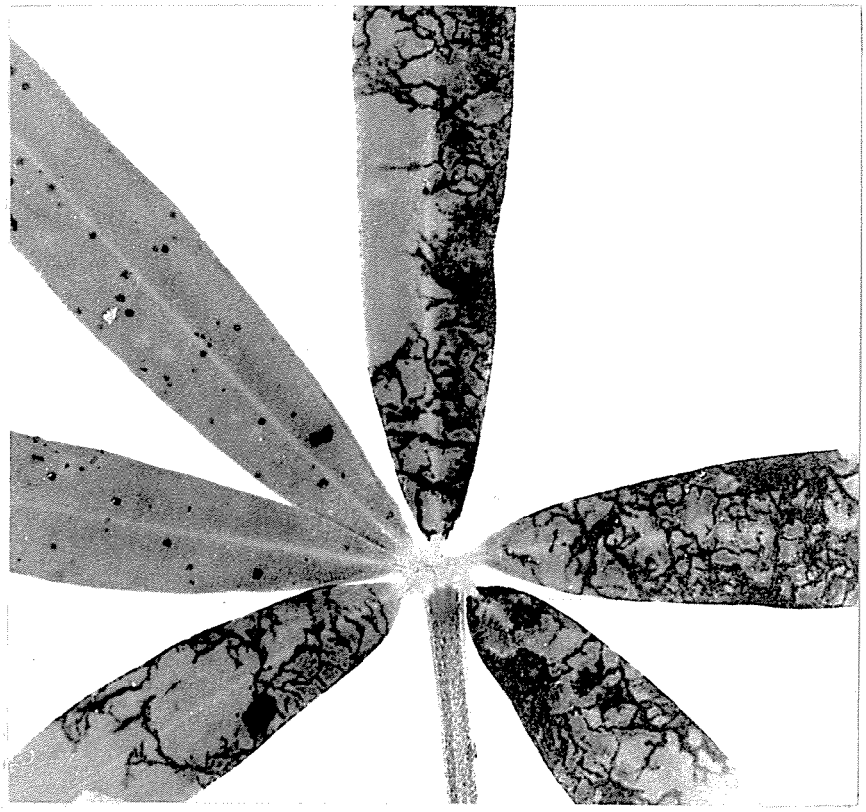
Early stages of symptoms manifested by Pleiochaeta setosa on tree lupin leaflets (a and b) contrasted with those on blue lupin (c)

Illustration 22



Typical 'cobweb' lesions caused by Pleiochaeta setosa on blue lupin leaflets

Illustration 23



Enlargement of 'cobweb' lesions shown in Illustration 22

Besides being found on adult plants in the field, P.setosa was found to cause disease on Russell lupin seedlings in a nursery.

Tree lupin (Lupinus arboreus)

Disease symptoms are never as severe, in terms of prevalence, as they can be in Russell lupins. This is due to the fact that the diseased leaflets of tree lupin are shed before extensive symptom expression occurs. The first evidence of the disease is a small necrotic area 1-2mm in size and surrounded by a green-yellow zone. The lesion normally does not assume a regular shape but instead thread-like areas of dead tissue extend haphazardly from the original infection site (Illustration 21 (a) and (b)). These necrotic areas are dark blackish-brown, and in more advanced cases may form a dark network with the enclosed tissue a light brown colour. Once this stage is reached, leaflets quickly yellow and fall.

A few lesions were found on petioles and these were small, elongated, and blackish-brown. Manifestation was not found on stems of adult tree lupin plants.

Seedlings of tree lupin diseased with P.setosa were found in the field, and besides the typical symptoms on the leaflets, dark scab-like lesions up to 5mm in diameter were present on the cotyledons. However, because of the prevalence of diseased plants in the vicinity of these seedlings, the infected seedlings need not necessarily have arisen from diseased seed. In fact, the fungus was never isolated from the reproductive structures of tree lupin.

Besides the expression of disease through cell necrosis, as evidenced by abnormal colourations of the various parts of the plant and other structural or physical defects, P.setosa manifests itself in

another way--this is physiological and results in premature defoliation. In the early autumn 1964 during a period of warm wet weather when this disease was prevalent in the field, diseased tree lupins had a carpet of fallen leaflets beneath them. In an inoculation experiment with four-month-old potted tree lupins a count of fallen leaflets, expressed as a percentage of total leaflets on the plant, revealed an average leaflet-fall for twelve plants of 35.2%. This figure was attained after inoculated plants had been given twelve hours at 22°C (100% RH). They were then transferred to the glasshouse bench. Premature defoliation may be described as a typical manifestation of the disease caused by P.setosa on tree lupins and, for that matter, also in blue lupins. Reference has already been made to various workers who have noted this as a feature of the disease. In diseased Russell lupin, leaflets reach the ground by stem collapse rather than by defoliation.

Blue lupin (Lupinus angustifolius)

The symptoms, as manifested on young plants under controlled laboratory conditions, have already been described in penetration and infection trials (page 116). In the field the symptoms caused by P.setosa are very similar to those in tree lupins and in both cases the combination of physical and physiological expression provides a manifestation which can be said to be typical of the disease. Illustrations 22 and 23 clearly show the physical expression of the disease on adult blue lupin leaflets.

The leaflet lesions are variable in size, shape, and colour. They may be either just visible to the unaided eye or have a diameter equal to the width of the leaflets. The spots may be circular but

they are more often irregular, especially when two or more lesions have merged or where they have reached the margin of the leaflet. Many of the lesions consist of fine dark lines or streaks that form an irregular netlike pattern (Illustration 23). The spots and streaks are blackish-brown to almost black. Where circular lesions are present the outer portion of the lesion is dark with a pale brown interior. In advanced cobweb-type lesions, all the tissue in the network is eventually killed and the lesions assume a rusty brown colouration. It was on advanced lesions of this type that the small amount of sporulation was found on leaflets still attached to the plant. Diseased leaflet abscission is very common, the disease often almost completely defoliating the plant, whilst other plants may lose only their lower leaflets.

The Brown Spot fungus has also been found attacking the petioles, stems, blossoms, and pods of blue lupin. On the petioles the lesions are dark and elongated in shape. Where they are numerous they may coalesce and involve the whole petiole diameter causing it to collapse, thus hastening defoliation. In severely infected plants the stem may be almost entirely covered with large, brownish-black lesions or cankers with slightly lighter borders. Stems thus affected usually lose their leaflets and die before maturing seed.

Blossom lesions were found in the field on rare occasions, and are brownish-black. No sporulation could be obtained by giving high humidity to these lesions, but the fungus was isolated by tissue plating. A similar problem occurred with isolation from pod lesions. In the autumn of 1964 when there was a severe outbreak of Brown Spot disease in the blue lupin crop under observation, infected pods were present in abundance. The spots usually remained small and were typified

by a small, almost rectangle shaped, dark brown-black fleck. These spots may merge and involve a large part of the pod. Despite examination of pods and seeds at all stages of maturity, at no stage could pods be found where the fungus had grown through the pod wall and infected the seed. Details of this work have already been discussed in Chapter III relating to the seed-borne phase of P.setosa. Weimer (1952) recorded that the causal fungus of Brown Spot disease may grow through the pod wall into the seeds and produce reddish-brown areas, similar to some of the lesions produced on seeds by the anthracnose fungus (Glomerella cingulata).

Seedlings can be severely affected and in some instances killed by the fungus. In the glasshouse, boxes of blue lupin seedlings (two weeks old) could be killed, within four days of inoculation, with P.setosa. Here, besides a withering of leaflets, there was total stem collapse resulting in the rapid death of plants.

5. Production of Inoculum

In most instances the sexual and/or asexual spores produced by phytopathogenic fungi play an integral part in the disease cycle. Spores commonly provide the means whereby new season infections are initiated and are also the means whereby secondary spread is achieved. Thus one of the factors which must be fulfilled before an epiphytotic can occur is the production of abundant viable inoculum. Consequently factors which control, modify, or inhibit sporulation will obviously play an important part in the development of any epiphytotic, and for this reason, warrant close study.

As conidia are the only dispersible form of inoculum produced

by P.setosa, this section has been devoted to factors influencing sporulation, in particular, sporulation on the host. The actual mode of conidial production is described in Chapter II, A. THE MORPHOLOGY OF THE FUNGUS, and accompanying illustrations in that section illustrate the stages in the development of conidiophores and conidia.

No one set of external environmental conditions will promote sporulation of all fungi. Lilly and Barnett (1951) point out the necessity of realising that even when all the physical and chemical conditions are optimum, no reproduction can occur without the presence of favourable genetic factors.

Any critical investigation of factors influencing reproduction requires that the fungus be studied in the laboratory or greenhouse where external environmental conditions can be controlled. It is a great advantage in physiological studies to be able to grow the fungus in pure culture on synthetic or semi-synthetic media. However, a study of the literature indicates that the behaviour of the fungus in nature cannot always be duplicated in the laboratory. For the purposes of disease cycle work, studies have been made of factors influencing sporulation of P.setosa on excised diseased blue lupin leaflets or portions thereof. Only one variable was studied at a time, all other factors being controlled.

Environmental factors appear to have the greatest influence on spore production on the host and of these, temperature and humidity are of major importance.

Environmental Factors

Temperature

Bisby (1943) recognised that temperature was an important factor

governing the geographical distribution of the fungal species. Further, it is recognised that temperature plays a significant part in the life cycle of any one species, by its influence on growth, and also on the production and germination of distributed spores.

Various workers have shown that the temperature range which allows sporulation is narrower than the range for growth, and that in general, the temperature limits for sexual reproduction are narrower than the limits for asexual reproduction. Further, it has been established that there is an optimum temperature for growth as well as sporulation and these two optima may be different.

Materials and Methods

A trial was conducted to determine what effect temperatures ranging from 5°-37°C had on sporulation of P.setosa on excised diseased leaflets. Whole diseased leaflets of blue lupin were arranged on glass slides placed on glass triangles in Petri plates. To ensure 100% RH, a free water surface was provided in each plate. Earlier work had shown that sporulation occurred first on the more advanced type of lesion (where cobwebbing has coalesced), and where possible this type of lesion was used in the experiment. Inevitably, a variation existed in the size of lesion used. Consequently, it was decided that rather than try to obtain a measure of spore numbers at various temperatures, the main object of this trial would be to determine whether sporulation could occur at a particular temperature (given conditions of 100% RH, assumed at this stage to be optimum for spore production).

Six tissue pieces per Petri plate were used, with three replicates for each temperature. Tissue pieces were added only after plates had been held eight hours at their respective temperatures, thus

ensuring 100% RH at the commencement of each run.

After the various time intervals, results were taken of the number of tissue pieces producing (a) mycelium, and (b) conidia. The intensity of spore production at any one temperature is a factor of great importance in ensuring the availability of dispersible propagules. Accordingly, a record was made to ascertain whether sporulation was intense or sparse, at each temperature, after an elapse of 60 hours. This arbitary assessment was considered the most convenient way of recording intensity of spore production.

Results and Discussion

Table 11

Sporulation and Growth of P.setosa (on excised blue lupin leaflets) after the elapse of various time intervals, and at temperatures ranging from 5°-37°C

Temperature	Percentage of leaflets producing— (a) mycelium, and (b) conidia											
	26 hr		20 hr		24 hr		40 hr		48 hr		60 hr	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
5°C	0	0	11	0	11	11	28	11	28	17	44	17
13°C	0	0	6	0	22	11	33	11	33	22	50	28
15°C	11	6	22	6	33	11	33	22	39	28	39	28
22°C	0	0	22	6	28	6	33	11	44	17	66	55
24°C	0	0	11	6	33	55	50	6	61	22	72	66
28°C	0	0	22	0	28	22	66	28	55	28	88	28
30°C	0	0	22	0	22	6	44	6	44	6	61	22
37°C	0	0	6	0	17	0	28	0	28	0	44	0

These results indicate that P.setosa can produce vegetative mycelium on host tissue over temperatures ranging from 5°-37°C, and that sporulation can occur at temperatures between 5°C and 30°C. This is greater than the range over which sporulation occurred on artificial media (namely between 15°C and 28°C on PCA). This ability to sporulate over a wide temperature range has obvious advantages for a plant pathogen in nature. In the case of P.setosa one may conclude that given 100% RH, temperature would not be a factor preventing spore production under New Zealand climatic conditions.

Because of the unavoidable variation in the stage of development

Table 12

Relative amounts of sporulation and growth of P.setosa on blue lupin leaflets after 60 hours at various temperatures and 100% RH

Temperature	Comparative levels of sporulation and growth used			
	Percentage of leaflets giving—		Percentage of leaflets giving—	
	sparse sporulation	intense sporulation	sparse mycelial growth	intense mycelial growth
5°C	0	11	44	0
13°C	22	6	39	11
15°C	11	17	28	11
22°C	22	33	33	33
24°C	39	28	39	33
28°C	11	17	55	33
30°C	17	6	28	33
37°C	0	0	44	0

of the lesions used in this experiment the optimum temperature for sporulation in nature could not be determined. However, in this particular trial it was apparent that temperature may have a considerable effect on the intensity of sporulation. From Table 12 it can be seen that more intense spore production occurred at temperatures of 22°C and 24°C.

Humidity

Early in this work it was observed that sporulation occurred on infected leaflets, only after a period of continued leaf wetness or extreme high humidity. This suggested that in the field relative humidity would play an important part in governing availability of inoculum for secondary spread of the pathogen. Accordingly, experiments were designed to determine the part relative humidity plays in determining the inoculum potential.

Materials and Methods

To determine the broad range at which sporulation could occur, a series of solutions were prepared giving relative humidities increasing by 10% up to 100%. (For composition of solutions see Appendix II).

Diseased leaflets were removed from blue lupin plants and where possible leaflets with advanced symptoms were chosen. Six leaflets were threaded onto a piece of cotton knotted at one end, stapled onto the rubber bung sealing the jar, and suspended over the humidity solutions. The jars were held at 25°C for three days prior to the addition of leaflets so that constant relative humidities could be

obtained. Each treatment was duplicated, and all preparations held at 25°C. To gain a more accurate picture of the effects of relative humidity on the sporulation of P.setosa a second trial was carried out using 80%, 88%, 90%, 93%, 94.9%, 97%, and 100% RH solutions.

Results and Discussion

The initial run indicated that at a temperature of 25°C sporulation of P.setosa did not occur below 90% RH. At 80% RH sparse mycelial growth was observed under a binocular microscope, but even after five days no sporulation was evident.

In the second trial sporulation was apparent on tissue pieces held at humidities of 94.9% and 97% and was abundant at 100%. After two days mycelium appeared at humidities ranging from 88% to 93% but even after five days no sporulation had occurred.

When infected leaves, which had been kept for several days in humidities at which no sporulation occurred, were placed in an atmosphere of 100% RH, abundant sporulation occurred in 24-28 hours. This indicated that treatments over the different solutions were not harmful to the initiation and formation of conidia.

It is interesting to note at this point that Berger and Hanson (1963) working with Cercospora zebrina found that sporulation did not occur on leaves of Trifolium pratense held at 95.6% RH, or at lower relative humidities.

In the case of P.setosa, at temperatures of 25°C a relative humidity of at least 94.9% is required for sporulation and this would indicate that in the field, humidity rather than temperature is more likely to be the factor limiting sporulation.

Light

The effect of light on spore production varies greatly between fungal species. Hawker (1950) divides fungi, according to their light requirements for sporulation, into the following four groups:

(1) Many forms able to produce spores in complete darkness and not showing increased spore production in light.

(2) Some fungi produce spores in the absence of light but do so more freely when illuminated.

(3) Some fungi must have light at some particular stage of spore production.

(4) Those few fungi where light has an inhibitory effect on sporulation.

The influence of light on the growth and sporulation of P.setosa on culture media has been discussed (Chapter II) but one may not assume that this factor will affect spore production on host tissues in a similar manner. Accordingly, an attempt was made to ascertain what part light plays in sporulation on the host.

Materials and Methods

Lesioned areas of blue lupin leaflets were cut into pieces approximately 4mm x 3mm and added to Petri plate humidity chambers. Twelve tissue pieces were used in each plate, with three plates enclosed in aluminium foil bags and three left uncovered. All six plates were arranged on a laboratory bench near a window facing north.

Results and Discussion

Examination of both series after 24 hours revealed greatest sporulation on those tissue pieces subjected to complete darkness. However, within 48 hours there was also considerable sporulation on

tissue pieces in Petri plates receiving alternating day and night conditions.

These results would place P.setosa in category (1) of Hawker's (1950) scheme, and this would appear to be the group in which the largest number of fungi are found, including most species of common moulds such as Mucor, Penicillium, and Aspergillus. The perithecia of many Pyrenomycete fungi are also formed equally well in the presence or absence of light.

An unusual feature associated with spore production of this organism is the rarity with which active sporulation occurs on lesioned leaflets still adhering to the growing plant. That is, studies have shown that the majority of sporulation occurs on fallen diseased leaflets where one can presume the conditions of relative humidity encourage this process.

In a dense crop, intensity of light reaching these leaflets would be reduced and the above experiment indicates that even under these conditions sporulation would not be adversely affected by this factor. Consequently a plentiful supply of inoculum would be available for secondary spread.

Other Factors

One other factor was found in this study to have a profound effect on spore production, namely the particular plant species serving as host.

Not only have species of lupin differed in susceptibility to P.setosa, but they have also varied in ability to promote or inhibit sporulation of the fungus on their tissues. Early in the study it was found that sporulation was more readily achieved on blue lupin than

tree lupin tissues and that Russell lupin tissues required a longer period of high humidity before sporulation of P.setosa could be induced.

When species of lupin, Cytisus, and Laburnum were placed in the field to observe natural infection, attempts to reisolate from diseased plants were made in due course. Leaflets were brought into the laboratory and symptoms described and recorded. They were then given high humidity at 24°C to induce sporulation.

Reisolations were readily made from Lupinus angustifolius, L.polyphyllus, L.arboreus and Laburnum anagyroides by picking off individual spores. However, sporulation on Lupinus hartwegii and L.albus was sparse, and after four days there was no sporulation on L.rothmali or L.digitatus. Eventually after chilling tissue pieces of L.rothmali for one day at 5°C and then placing plates on a laboratory bench, sporulation was induced. P.setosa was reisolated from L.digitatus by washing and tissue plating to agar. Abundant sporulation of this isolate occurred on PCA media.

This work indicates that different plant species, although showing varying degrees of susceptibility to P.setosa, modify the extent to which sporulation can occur. This fact is of obvious importance in determining whether this disease could be a serious problem in a stand of any one of these species, assuming they were all grown in New Zealand. With the apparent lack of a sexual stage the production of an abundant supply of conidia must play a key part in the disease cycle of P.setosa. It was interesting to note that ease of sporulation was not necessarily correlated with degree of susceptibility of a host species to the fungus. For example, under field conditions, L.rothmali was one of the most susceptible species

but it was difficult to induce P.setosa to sporulate on it, and Laburnum anagyroides was both susceptible and easy to promote sporulation on.

Without detailed work on chemical constitution of these different species it would appear that a nutritional factor influenced sporulation. This could be analagous to the variation in sporulation obtained on different culture media in the laboratory.

6. Dispersal of Inoculum

P.setosa is a Hyphomycete and conidia constitute the main, if not the only, form of field inoculum. Because chlamydospores are produced within the hyphae as an integral part of the cellular structure it is unlikely that they are of any importance in dispersal of P.setosa.

In most fungi spore dispersal can be conveniently considered in two parts. First, spore liberation—the escape of spores from immediate contact with the parent body—and secondly the actual dispersal of the liberated spores by wind, rain splash or running water, insects, and larger animals, particularly man.

(a) Liberation of Conidia

Many different forms of conidial apparatus are found in the Hyphomycetes and during the numerous attempts to establish a 'natural' classification of this group of fungi, Mason (1937) proposed a system based on a fundamental biological distinction, namely, whether spores were 'slimy' or 'dry' (Gloisporae and Xerosporae respectively). A large number of species do not fit adequately into either of these

two groups and, although the distinction as outlined has not received wide recognition as a basis for classification of the Hyphomycetes, the characters chosen by Mason are of considerable importance when considering the method of liberation of conidia. In most instances conidia are dry and can be readily blown off their conidiophores and dispersed by wind. Slime spores, however, normally cannot be wind dispersed, at least in the first instance, water and insects being important dispersal agents of these spores (Ingold 1953).

Observation of the production and type of conidia of P.setosa reveals that they are of the dry spore type. No reference is made in the literature to mode of liberation and dispersal of the conidia although Germar (1940) makes one negative observation in his work relating to range of conidia, namely that they failed to cover distances of 200 metres and 400 metres.

Because the presence of suitable conditions for liberation and dispersal of conidia largely determines the extent to which a disease will spread in a particular crop, trials were performed in order to determine these conditions for P.setosa.

The influence of wind and moisture on liberation of conidia

Materials and Methods

Blue lupin leaflets were held for 60 hours at 24°C (100% RH) and at the termination of this time an abundant supply of conidia was present. These leaflets were placed in a shallow tray in front of a two-speed electric fan. Glas slides (4¼" x 3¼"), thinly smeared with vaseline, were mounted vertically on wooden stands and placed in the air stream. The slides were arranged in a semi-circle in order to catch spores deflected out to the side of the air stream and in such a manner that the centre of the semi-circle was 2ft 6in from the fan.

When moist air was used, an Atomic Mist Spray enabled a fine spray to be directed into the air stream. For each treatment an exposure time of five minutes was used and spore numbers averaged over all the slides. Note that conidia were taken from an atmosphere of 100% RH for the trial.

Treatments and Results

(i) Dry air. No conidia were found on the slides.

(ii) Moist air. An average of 3.2 conidia per slide were present after treatment.

Discussion

From the results obtained it is evident that dry air moved by the fan was insufficient to dislodge the conidia. However, when a fine spray was incorporated in the air stream a small number of conidia were dislodged.

When the leaflets used in the treatment were observed under a binocular microscope, whilst the air stream was being passed over them, the conidia could be seen wavering slightly, but it appeared that the maximum air speed of the fan was insufficient to dislodge them. However, continued observation during the moist air treatments indicated that some conidia were dislodged when hit by moisture droplets, either because of the impact of the droplet, or moistening of the conidia.

A further observation made during this trial was that under the conditions used to obtain sporulation of P.setosa on the leaflets, considerable aerial mycelium was produced and in treatment (ii) some conidia were observed to be dislodged but caught by the setae in the aerial mycelium. Two further experiments were performed in view of the

results obtained.

The influence of drying, and of moistening conidia before exposure to moist and dry air

Materials and Methods

One sample of diseased leaflets producing conidia was placed in a dry Petri plate in the 24° incubator for two hours, whilst another sample was moistened and maintained at 100% RH.

Treatments and Results

(i) Dry air over dry conidia. No conidia were found on the slides.

(ii) Dry air over moist conidia. No conidia were found on the slides.

(iii) Moist air over dry conidia. An average of 6.3 conidia per slide were present after treatment.

(iv) Moist air over moist conidia. An average of 3.7 conidia per slide were present after treatment.

Discussion

This trial shows that it is the impact of water droplets which causes liberation when the spores have been kept moist (compare with treatment (ii) in previous trial). However, when the spores have been dried, the results indicate that the impact of moisture droplets in the air, together with the wetting of the conidia, causes greater conidial liberation than moist air over conidia which have been kept at 100% RH.

The influence of natural wind on liberation of conidia

As a fan is only capable of providing air movement which at the most could only be described as a mild breeze, it was resolved to

expose some conidia on leaflets to strong wind.

Materials and Methods

A sample of diseased leaflets producing conidia was placed near an open laboratory window through which a strong wind was blowing. Slides were arranged as in earlier experiments. By arranging the experiment near the window rather than outside, an almost unidirectional air stream was obtained. Individual diseased leaflets had to be cellotaped onto the tray to prevent movement.

Results and Discussion

The trial resulted in an average of 5.2 conidia per slide, indicating that a strong wind could cause liberation of conidia of P.setosa. Although no trial was performed it could be assumed that in view of the laboratory experiments, strong wind plus rain could cause even greater liberation of conidia than strong wind alone.

Because the number of spores present on the leaflets used in each treatment would not be the same, the figures given are of limited value. The results do indicate that conidial liberation in P.setosa, as with the majority of Hyphomycetes, is passive, i.e. the fungus is reliant on some external force to liberate the conidia.

(b) Dispersal of Conidia

It is obvious that in the case where conidia are liberated by some external force dispersal will be by means of this same force. With P.setosa laboratory studies indicated that strong winds, either with or without rain, could cause conidial liberation and dispersal.

In attempting field studies to determine dispersal of conidia

one is confronted with the practical difficulties of measurement. Hirst (1958) notes that it would be more logical to measure dispersal after deposition but this is impractical and instead it is usually assessed by trapping spores during transport.

Numerous methods for trapping particles have been devised for use in fields apart from plant pathology, including traps for air pollution work, chemical warfare, and studies of hygiene and respiratory allegeries. In view of the fact that no spore trap was available for this study, a freely exposed surface was used. Hirst (1958) notes that these are almost valueless for small spores, but that they "estimate reasonably reliably the relative frequency of large spores over long periods." Elsewhere in the same article it is noted that slides or cylinders usually need 24 hours exposure to accumulate enough spores to justify scanning.

P.setosa produces a large readily identified spore and consequently does not require the use of a trap designed for cultural recognition.

Determination of conditions suitable for dispersal of P.setosa in the field

Materials and Methods

Glass slides ($4\frac{1}{4}$" x $3\frac{1}{4}$") were mounted on wooden stakes in a manner similar to that used by Van Der Zwet and Lewis (1963), so that the slides were facing in four different directions. Van Der Zwet and Lewis used nitrocellulose on the slides but in this study vaseline was found to be quite satisfactory for obtaining adherence of conidia. Four stakes were placed in a rectangular patch of blue lupin approaching maturity and showing isolated diseased plants. The crop was

approximately 200 square yards in area and was situated on a bank sloping in a westerly direction.

Figures for rainfall and wind were taken from the 'Manawatu Evening Stand' as recorded by the Grasslands Division of the Department of Scientific and Industrial Research, Palmerston North. Figures are for the 24 hours preceding 9 a.m. on the date of recording. Slides were changed each day at this time and then examined under a binocular microscope for conidia of P.setosa.

During the period recorded, 400 slides were examined. Slides had been placed in the crop for one week prior to the first results recorded in Table 13, but during this time no conidia were found on the slides.

Results and Discussion

By the 27th February only 0.14" of rain had fallen during the month. Laboratory studies have shown high humidity to be a major requirement for conidial production and the lack of conidia found on slides prior to the 27th indicate that shortage of inoculum was the basic factor which limited spread of infection during this period. Although 0.66" of rain fell on the 28th February, less than 20 conidia per slide were present but with the continued heavy rain, more than 80 conidia per slide were counted the following day indicating a rapid inoculum build-up.

Because of the interaction between the availability and dispersal of inoculum, the conidial rating figures given in Table 13 must be interpreted cautiously. Besides being dependent on suitable conditions for dispersal, the number of conidia on each slide on any one day will also be influenced by the number of conidia present in the crop.

Table 13

Influence of rain and wind on the dispersal of conidia of P.setosa in a diseased blue lupin crop

Date	Rainfall (inches)	Wind (average air movement m.p.h.)	Conidia rating*	Remarks
FEB.27	0.00	2.6	0	
28	0.66	4.1	1	Low inoculum available
29	1.33	2.3	5	Rapid inoculum build-up, rain splash important
MAR. 1	0.26	2.3	4	
2	0.01	1.8	0	Wide disease manifestation
3	0.00	2.0	0	
4	0.38	4.5	3	
5	0.01	6.8	3	
6	0.01	2.8	1	
7	0.00	3.8	0	Low inoculum level
8	0.00	7.5	1	
9	0.00	8.5	1	
10	0.10	5.0	0	
11	1.26	4.2	2	Rainsplash again evident on slides but low inoculum level
12	0.28	7.3	5	Rapid inoculum build-up
13	0.22	11.2	5	
14	0.00	12.2	3	
15	0.43	9.0	3	Severe leaf fall occurring by this time
16	0.03	6.0	1	
17	0.38	3.0	2	
18	0.20	7.2	2	Strong winds but inoculum level relatively low
19	0.24	11.6	2	
20	0.01	8.0	1	
21	0.01	14.2	1	
22	0.02	6.0	0	

*Conidial rating obtained from 16 slides examined each day and expressed as an average of conidia per slide: 1 = 1-20; 2 = 20-40; 3 = 40-60; 4 = 6-80; 5 = 80 +.

However, the results obtained do confirm laboratory work showing that both wind and rain play a part in the dispersal of conidia on P.setosa.

Slides were mounted on stakes 6" long but the distance of slides from the ground has not been given due to the fact that the crop was on a steep bank and thus the distance conidia may have travelled from the ground could not be assessed. During heavy rain soil was splashed on the slides and it was generally during such periods that the greatest number of conidia were observed.

Mention has already been made, in the section of disease cycle relating to production of inoculum, that the bulk of sporulation of P.setosa occurred on fallen leaflets. The scarcity of sporulation of P.setosa on the living host must complicate dispersal of the fungus for it means that as the plant grows, spores have further to travel from the site of production to infect new growth. Because of the situation of this crop on a steep bank, this would not be such a problem, but in a dense crop on flat land the effectiveness of spread could be impaired through the sheltering of inoculum at the base of the crop. Russell lupin and tree lupin are often found growing in a more dispersed manner than blue lupins and consequently the site of inoculum production would be less likely to impair effective dispersal of the fungus. In the autumn of 1963 it was observed that in a blue lupin crop, grown at the University, the disease did not spread noticeably from a small diseased area (present from the early stages of crop growth) near the edge of the crop.

During the trial to determine the mode of dispersal of conidia of P.setosa in the field, slides were placed at 20 ft intervals up to 180 ft from the blue lupin crop. Conidia of P.setosa were found up to 80 feet from the diseased crop. Taking into account the observed

dilution of spores with increasing distance from the source, together with the diminishing probability of trapping the spores on isolated glass slides, this confirms that wind can play a significant part in dispersal.

Tree lupins may grow into bushes several feet high, and during this study infected leaflets have been observed on the upper portions of these plants indicating again that only wind or wind-borne rain could carry inoculum from the ground to the higher leaflets.

While studying the possibility of P.setosa being seed-borne in New Zealand the disease was observed and the fungus isolated from Russell lupin seedlings grown in a local nursery. The plants were being grown in sterilised soil and boxes, and this, coupled with the failure of attempts to obtain the fungus with seed plating or the growing of seed at the University, suggested an external source of infection. Work in this section on dispersal has shown that conidia of P.setosa can be wind-borne and in view of the close proximity of the nursery to the Manawatu River, along which tree lupin grows wild, wind-borne infection from this source appears most likely. Certainly diseased tree lupins were common in the area and the observation of the owner of the Nursery that the disease was not expressed until boxes of seedlings were moved outside the glasshouse for hardening-off, provides further confirmation that wind plays a role in dispersal of conidia of P.setosa.

Late in the study a box of healthy Russell lupin seedlings from the glasshouse of this nurseryman was transferred to the University. The plants were grown-on, and at the time of potting-out were perfectly healthy. Boxes of the same line of plants left at the nursery and

later transferred outside for hardening-off before sale, became diseased.

7. Survival of the Fungus

A parasite is an organism which obtains its food from a living host, as contrasted with a saprophyte which is an organism obtaining its food from dead organic material. Many parasites are not always in contact with or able to infect the host to which they are specific and P.setosa is no exception to this. Consequently, there often arises in the life cycle of a parasite, a need for some form of survival or alternative existence apart from parasitism. Resistant structures are often produced by fungi by means of which they can survive apart from the host; sclerotia are produced by species of Sclerotinia and Rhizoctonia for example, and chlamydospores are important in the survival of Fusarium solani f. phaseoli. Spore dormancy is another means by which fungi can survive and Helminthosporium sativum is a commonly cited example of a fungus able to survive in the soil for months as dormant conidia. Other fungi may persist as sexual spores, either loose or more commonly contained within a resistant fruiting body. Some fungi, besides having the ability to attack and obtain nutrition from a living host, also have the facility to live saprophytically. This gives them an excellent mode of survival when suitable hosts are absent, for very often they can build up their inoculum potential while existing in the saprophytic state.

Investigations were carried out during this study to determine the survival value of chlamydospores and conidia produced by P.setosa. Consideration was also given to seed and perennial hosts as a means of survival.

Chlamydospores

P.setosa has not been found capable of sexual reproduction and does not produce sclerotia but it has been recorded as being capable of producing chlamydospores. These are described in Chapter II in the section on MORPHOLOGY OF THE FUNGUS. Pulselli (1928) and du Plessis and Truter (1953) both make reference to the production of chlamydospores on various artificial media but there is no reference to chlamydospores being produced by the fungus in nature, nor for that matter, to their possible function.

In view of these facts, before survival of chlamydospores was considered, preliminary trials were necessary to provide information on the following: (a) production in nature (b) germination, and (c) pathogenicity.

(a) Production in nature

Before any study of their function could be made it was necessary to establish that P.setosa does produce chlamydospores in nature. Microscopic examination of numerous lupin leaflets after they had been cleared and stained revealed chlamydospores to be present in leaflets of Russell, tree and blue lupin. Chlamydospores were not numerous and were generally found in association with advanced lesions.

Examination of collapsed stems of blue lupin present at the base of the crop indicated abundant conidial production in some cases. Mycelium was often connected to clusters of conidia picked off with a needle and transferred to a slide. Examination of these slides after staining and teasing out of the mycelium, again revealed a few chlamydospores to be present.

(b) Germination

Chlamydospores were produced in abundance on prune agar but before the germination could be studied it was necessary to first separate them from the mycelium. Chlamydospores near the surface of the agar were selected and under a binocular microscope the mycelium on either side of the chlamydospore was severed with a needle. Fifty spores were freed in this way and then transferred to a PCA smear in a Petri plate humidity chamber and held at 24°C. Examination eight hours later revealed that several of the constituent cells in the chlamydospores had produced germ tubes. When PCA plates inoculated with chlamydospores were compared for growth rates with those obtained with conidia or mycelia as inoculum, similar results were found after ten days incubation at 24°C.

(c) Pathogenicity

Detached chlamydospores were used as inoculum on blue lupin plants and this resulted in the production of disease symptoms typical of P.setosa.

Survival by Chlamydospores

Having obtained positive results in the preliminary trials the ability of chlamydospores to survive was now tested.

Materials and Methods

As chlamydospores were not produced abundantly in the field, or at least could not be readily found, prune agar was used as a source. Three ten-day-old cultures of P.setosa on prune agar were placed in a Waring Blendor together with 50 ml of water to provide

the fluid to keep the agar in contact with the cutters. The machine was run at low speed until a gelatinous emulsion was obtained and this was strained through a piece of doubled cheesecloth. The bulk of the chlamydospores were strained out from the agar in this way and these were used in two survival trials, described below.

(i) Survival in soil. Chlamydospores from the cheesecloth were washed into a beaker and a 50-ml chlamydospore suspension prepared. Half of this was mixed with 300 gm of field soil and the remainder with 300 gm of heat-sterilised soil. The soil in both instances was held in glass screw top jars in the laboratory. A modification of a method used by Ledingham and Chinn (1955) was utilised for removal of chlamydospores from the soil. This is a flotation technique but instead of using mineral oil (as used by these two workers) or glycerine (as initially used in this study), water proved more satisfactory. This was because the oil and glycerine impaired germination of the spores. Spores were floated out from the soil once a week and a sample transferred to a PCA smear where germination was observed.

(ii) Survival on glass slides. Four glass slides were smeared with chlamydospores, pieces of mycelium and some emulsified agar. These slides were transferred to four Petri plates and held at 5°C, 15°C, 24°C and outside at winter temperatures in Palmerston North. Five chlamydospores from each treatment were transferred to PCA plates once a week and incubated at 24°C.

Results

(i) Survival in soil. This experiment proved unsatisfactory owing to the fact that the small amount of agar, which was still present in the solution added to the soil, stimulated chlamydospore growth.

In the field soil, numerous other fungi took advantage of the sudden increase in nutrient supply.

(ii) Survival on glass slides. Chlamydospores survived equally well at all four temperatures for three weeks, but there failed to be any growth at 5°C, 24°C, and at outside temperatures, after four weeks. Growth ceased after four weeks at 15°C. These results indicate that on glass slides under the conditions outlined, chlamydospores would not be of value as a means of survival between crops. It was interesting to note that pieces of mycelium also present on these slides and plated to PCA were found incapable of surviving one week. On repeating this at daily intervals mycelial fragments were found to survive only two days on glass slides, indicating that the chlamydospore did have a greater resistance to dessication than mycelium.

Discussion

The value of this information in terms of the survival of the fungus may be limited for in nature the chlamydospores are produced in host tissue and are an integral part of the mycelium. Furthermore, the buffeting the chlamydospores received in the Waring Blendor would possibly not enhance their ability to survive. Because of the necessity to clear and stain leaflets to find chlamydospores, it was not possible to do a trial which would provide conclusive proof that the fungus could survive in diseased leaflets as chlamydospores for a period long enough to carry the fungus over any period away from a host.

Conidia

Several references are made to the ability of P.setosa to

retain its viability in its conidial form. No specific details are given in the literature on this but Hogetop (1938) mentions that conidia may "remain viable for a long time in the soil." Germar (1940) found conidia to be capable of 'overwintering' on infected white lupin pods held indoors, and du Plessis and Truter (1953) state:- "It has also been found that the fungus may retain its viability in its conidial form especially on infected plant material for a whole year."

Observations of a practical nature were made in Australia by Valder (1952) when it was found that the disease was prevalent on land which had been planted in lupins for green manure the previous season, whereas on land which had not been planted in lupins before, the crop usually developed well. Valder concludes that the fungus P.setosa "apparently persists in crop debris."

In view of this work a number of trials were performed in an attempt to establish whether in fact conidia can provide a means of survival of P.setosa during periods away from living hosts.

Methods and Results

(i) Conidia on glass slides. Conidial survival was investigated at 5°C, 15°C, 24°C and winter temperatures outside. A fine dry paint brush was used to transfer conidia from a ten-day-old PCA culture of the fungus to glass slides. The slides were then placed in Petri plates and incubated at the respective temperatures. To determine survival, spores to be removed for testing were moistened and then transferred with a needle to PCA plates. Formation of a visible colony of P.setosa was the criterion for conidial viability. Conidia survived for only three days on glass slides.

(ii) Conidia on infected leaflets. Infected blue lupin leaflets

were given 60 hours high humidity until there was abundant sporulation and mycelial growth over large areas of the leaflets. The leaflets were then placed in Petri plates and held in the laboratory at 18°-23°C. Conidia were removed periodically and placed on a PCA smear where germination was observed. At 30-day intervals pathogenicity of conidia on detached lupin leaflets was recorded. Conidia were found to remain viable for 184 days and pathogenic for 180 days.

(iii) Survival of the fungus in diseased leaflets in the field. Infected blue lupin leaflets were placed between two pieces of wire gauze wired together to form a container or bag. This was placed at the base of dying blue lupin crop in May 1963. The wire gauze was to keep the leaflets being used in the trial together.

The trial basically became one of conidial survival under field conditions, for after one week leaflets were found to be covered with a mass of conidia. Samples of these were germinated on PCA smears at weekly intervals. Unfortunately, this trial only proceeded for five weeks; after this the leaflets rotted beyond recognition and were overgrown with saprophytes. Conidia were still viable at five weeks.

(iv) Survival of conidia in soil. In order to confirm work by Hogetop (1938) the following trial was performed. Samples of sterilised soil (autoclaved for ½ hour at 20 psi) and unsterilised clay loam, were inoculated with concentrated spore suspensions of P.setosa (1,200,000 spores in each sample). The soil was adjusted to a moist consistency and then placed in airtight containers.

The modification of Ledingham and Chinn's spore flotation technique (1955) mentioned earlier in this section was used to obtain conidia from the soil. Five grams of soil and 15 ml of water was used for each test. The conidia of P.setosa are difficult to get into

true suspension, the majority floating on the surface of the water. Thus it was easy to remove the spores on the water surface with a paint brush and transfer them to PCA smears or lupin leaflets.

Initially samples were tested at weekly intervals but after a month the frequency of sampling was lessened in order to conserve the supply of inoculated soil.

Conidia from unsterilised soil retained their pathogenicity for 327 days and those from sterilised soil 341 days.

At the commencement of this conidial survival trial in soil, a method used by Couch and Grogan (1955) was tried for testing pathogenicity of the conidia. Samples were taken of the inoculated soil, wetted to a pasty consistency, and applied to lupin leaflets. After treatment the plants were held at 22°C and 100% RH for 24 hours and then transferred to the glasshouse. As infection did not consistently result the method used by Ledingham and Chinn (1955) was tried and found to be more satisfactory.

(v) Survival in field soil and plant debris. Soil containing some crop debris obtained from the vicinity of a severely diseased blue lupin crop was placed in seedling trays. Blue lupin seed, which had been shown to be free of P.setosa was sown in this soil. As a control, seed from the same source was sown in sterilised soil. Both treatments were liberally watered and when seedlings emerged they were periodically watered in such a manner as to splash soil on the growing plants.

After four weeks, 4% of the plants growing in the unsterilised soil were showing symptoms of P.setosa but even after six weeks there was no infection of plants grown in the sterilised soil.

Discussion

The fact that conidia are unable to survive on glass slides, whereas on lupin leaflets they survive 180 days, may seem contradictory at first glance. However, it would appear that the reason for this difference is that on leaflets the conidia were present in a thick mass or strata. The observation has often been made that fungi which produce their spores in a fruiting body often 'overwinter' in this spore form, the spores not actually being released until the beginning of a new season. Although P.setosa does not produce spores in a fruiting body the results of these two trials indicate that the spores on leaflets did not lose their viability as rapidly as those on the slides, possibly due to slower rate of dessication. Due to the failure of the trial performed under field conditions, it is not known whether the changes in moisture content of conidia, as experienced in nature, would have reduced their ability to survive. However, under controlled conditions the ability of conidia to survive in field soil was demonstrated, and the infection of seedlings in soil plus debris from an infected crop indicate that the soil plays a significant part in the 'overwintering' or survival of P.setosa away from its host. The observation that numerous diseased seedlings appeared as regrowth in this crop (the seed from the parent crop was found to be free of P.setosa) would, in the light of survival trials performed, confirm that P.setosa can overwinter in soil either in a free form as conidia, or in infected debris.

Seed

du Plessis and Truter (1953) established that in South Africa P.setosa could survive from season to season in the seed and numerous

other workers made similar findings. No details are given of the form of P.setosa in the seed but the seriousness of fungus survival in seed is evidenced by the need for work performed by Ostazeski (1961) in the United States to find a control for the fungus in seed of L.albus. Work outlined in Chapter III shows that no infection was present in lines of lupin seed screened in this study during 1963 and 1964 in New Zealand and therefore that the seed had no part in carrying P.setosa over from one crop to another.

Perennial Hosts

Where perennial plants are included in the host range of a fungus the survival problem is often less difficult than in the case where only annuals are infected. Tree lupins are evergreen perennial shrubs and field observations have indicated the presence throughout the year of disease symptoms typical of P.setosa. This indicates that the fungus is capable of existing entirely as active infection where suitable hosts are available. Although Russell lupin is also a perennial the availability of host material throughout the year does not always occur. In a well managed garden Russell lupins often have all foliage removed, with the stems being cut back almost to the ground. New foliage is produced from the crown of the plant the following spring. However, pruning does not always take place and in such instances diseased foliage has been found during winter.

Goats rue is a leguminous weed often found growing with tree lupins in the Manawatu and in view of the prevalence of disease symptoms on these plants it was investigated as a possible means of survival for P.setosa. A species of Phoma was isolated from Goats rue and this proved pathogenic but at no time was P.setosa isolated and

similarly isolations of P.setosa from tree and blue lupins proved non-pathogenic to goats rue.

8. Primary Inoculum

Because primary inoculum initiates new seasons' infection it is logical that it must arise from the survival form of the fungus. To understand the source of primary inoculum it is necessary to consider the seasonal growth pattern or availability of the host plant over the course of the year and this can best be done by considering independently the three lupin species used in this study.

Blue lupin (Lupinus angustifolius)

Blue lupin is an annual plant, but, depending on the locality in which the crop is grown and the purpose for which the crop is to be used, so the time of the year when plants are present varies. In the South Island, where blue lupin is more commonly grown, the versatility of the crop's uses for lamb-fattening, winter feed, and as a seed crop results in plants being present throughout the year. In the Manawatu the disease has been found to be more prevalent in the spring and autumn, but nevertheless, disease plants have been found in summer and winter. Thus primary inoculum for a newly sown crop could conceivably originate from conidia disseminated from diseased crops in the locality.

In the Manawatu and Rangitikei, in areas where blue lupins are grown, tree lupins are often widespread as a weed or sand stabiliser and theproven ability of inoculum from tree lupin to readily infect blue lupin would make this an ever present source of conidia for

primary inoculum.

This study has shown that another source of primary inoculum could exist, namely conidia in soil or infected debris in the soil. This would only be of importance where a blue lupin crop is being grown in the same soil for consecutive years as may occur, for instance, in trial plots.

Russell lupin (Lupinus polyphyllus)

Russell lupin is a perennial garden plant. Where the plant is cut back after flowering the diseased stems and foliage are all removed and should be burnt. Russell lupin plants present in the Esplanade Palmerston North, were kept under observation for nearly two years. These plants were all diseased with P.setosa but were heavily pruned each year. Although the resulting new spring foliage was observed to be healthy in a short time disease symptoms typical of P.setosa appeared on the older leaflets near the ground. Trials conducted during this study to determine whether age of leaflets predisposed plants to attack revealed negative results. However, in view of the results of trials determining conidial survival in soil and the fact that new season's infection occurred first on leaflets near the soil, samples of surface soil around the plants were taken. These samples were examined using the same technique as in conidial survival in soil studies (p.153). This revealed the presence of viable conidia in the soil indicating them to be a good source of primary inoculum.

Where plants are not pruned and the debris is left around the plant this could also provide inoculum capable of initiating new season's infection.

Tree lupin (Lupinus arboreus)

Like Russell lupin this species is a perennial. As mentioned under blue lupins, tree lupins have a widespread occurrence and this coupled with the frequent existence of diseased plants means a source of primary inoculum is invariably present for infection of young tree lupin seedlings.

B. MISCELLANEOUS FACTORS INFLUENCING THE DISEASE CYCLE

1. Host Susceptibility

P.setosa has been recorded as pathogenic to a large range of Lupinus spp. but beyond this its recorded range of hosts is small. The fungus has been isolated from species of Cytisus, Laburnum, Crotolaria, and in one instance Simmonds (1960) isolated it from Phaseolus vulgaris (beans).

In the literature considerable divergence of opinion exists as to the relative susceptibility of certain species of lupin. Pape (1927) records L.mutabilis and L.cruickshanksii as most susceptible, with L.albus and L.luteus comparatively resistant. (Gladstones (1958) lists L.cruickshanksii as a synonym of L.mutabilis). Siemaszko (1929) cites "L.albus as most susceptible, L.mutabilis and L.polyphyllus less liable to infection, and L.angustifolius and L.luteus resistant." Germar (1940) worked with a host range including 22 lupin species and found that L.angustifolius showed the mildest symptoms and that L.pilosus was resistant.

In South Africa, du Plessis and Truter (1953) state that the Brown Spot fungus is abundant on L.albus and L.mutabilis and causes distinct lesions but that it is rare and less distinct on L.angustifolius.

They incoulated L.angustifolius, L.albus, L.luteus, L.hartwegii, and L.mutabilis and found that after five days infection was so severe that they could not distinguish differences in susceptibility.

Numerous other workers record P.setosa on lupin species but the examples cited suffice to show the varrying host records over the years. It is helpful at this point to consider possible reasons for apparently conflicting results in varietal susceptibility, before discussing work carried out in this study.

Three requirements must be met before production of a disease by a pathogenic fungus is possible, namely:

(1) presence of a pathogenic form of inoculum

(2) presence of a susceptible host, and

(3) suitable environmental conditions for development of the disease cycle.

Genetical variation in (1) and (2) may be an important factor in the divergent results obtained. Strains of a fungus species may differ in pathogenicity just as strains of one plant species may differ in susceptibility. The latter may well be the case here for lupins have a strange breeding background and coupled with this is the existence of some confusion in the taxonomy of certain lupin species.

Many of the records of susceptibility do not include information on whether the record is for field susceptibility, whether artificial inoculations have been used, or of the environmental conditions to which the inoculated plant has been subjected. Finally, the form of inoculum, the amount of inoculum (the inoculum potential), and the method of inoculation are three factors which can have a considerable bearing on the results obtained. For instance, it may be argued that the wound inoculations used by Richter (1938) do not give a true

indication of the pathogenic ability of a fungus.

Two approaches have been used in this study of the hosts attacked by P.setosa. The first involved the determination of susceptibility of plants in the field to natural inoculum and the second the susceptibility of plants to artificial inoculation under controlled conditions.

Host susceptibility under field conditions

Materials and Methods

One-month-old plants of ten different lupin species, Laburnum anagyroides, Cytisus scoparius and Spanish Broom were planted out in a crop of blue lupin (L.angustifolius) which was near maturity and severely diseased with Brown Spot.

Vaselined slides were placed on stakes at various heights and angles in the crop to determine whether air-borne inoculum was available. Because blue lupin seedlings have been shown to be very susceptible to P.setosa they were placed in the crop to provide a control. If viable inoculum was available and environmental conditions were suitable for disease development, then these blue lupin seedlings should have developed Brown Spot disease. The experiment was carried out in May 1964 when the weather following planting out was cold, and rain and strong winds were prevalent.

Results and Discussion

Examination of the vaselined slides each day under a binocular microscope indicated a plentiful supply of inoculum of P.setosa, with as many as 15 conidia per slide. After one week prominent disease symptoms were visible on a number of plants, including the control

Table 14

Relative susceptibility to P.setosa (under field conditions) of species of Lupinus, Laburnum anagyroides, Cytisus scoparius, and Spanish Broom (results recorded after one week)

Species	Disease rating
Lupinus angustifolius	severe
L.hartwegii	severe
L.mutabilis	severe
L.rothmali	severe
L.albus	moderate
L.luteus	moderate
L.arboreus	moderate
L.polyphyllus	severe
L.digitatus	mild
L.pilosus	*
Laburnum anagyroides	severe
Spanish Broom	*
Cytisus scoparius	mild

* After one week L.pilosus and Spanish Broom showed only a pock-marking on the leaflets. However, after 13 days mild lesions were apparent on the leaflets of both these species.

series. Leaflet infection was more striking than stem infection but closer examination of plants revealed stem lesions in many cases. Illustration 24 shows symptoms on L.mutabilis.

The relative susceptibility of the different species was recorded using a visual comparative rating involving three different disease levels, namely, mild, moderate, and severe.

In all cases reisolation of P.setosa was made from the diseased

Illustration 24

Lesions caused by *Pleiochaeta setosa* on leaflets of *Lupinus mutabilis*

plants and the fungus cultured on PCA. Spores from these cultures were used to inoculate blue lupin seedlings and successful infection was achieved in all cases after plants were exposed to 100% RH at 22°C for 24 hours.

These results show that a difference in susceptibility between Lupinus species to P.setosa does exist but, under the field conditions detailed, no species of Lupinus is completely resistant to the fungus. L.pilosus, a variety of lupin grown in Western Australia, showed the greatest degree of resistance.

Infection of Laburnum anagyroides confirms the work of Weimer (1948), and Cytisus scoparius the work of Raabe (1938). (Kirchner (1892) originally described the disease caused by P.setosa on year-old seedlings of Cytisus capitatus.) The infection of Spanish Broom is a new host record.

Host susceptibility to artificial inoculation

The results of the infection of these different species under field conditions was confirmed in artificial inoculation studies. Here inoculum from PCA cultures of P.setosa isolated from blue lupin was used. The spore suspension was prepared and inoculations performed as outlined in Chapter I, MATERIALS AND METHODS. The inoculum concentration was 30 spores per ml. The one-month-old plants were subjected to 100% RH (22°C) for 24 hours and were then returned to the glasshouse bench. Results were recorded after one week.

In New Zealand, three different species of lupin are commonly grown, namely, Russell, tree, and blue lupin. The ability of P.setosa to cross-infect from one lupin species to another could be important under New Zealand conditions where the different species are often

grown in close proximity to each other. Early in this study, an experiment was performed to see whether isolations made from the three main species grown in New Zealand would cross-infect.

Determination of the ability of P.setosa isolated from Russell, tree, and blue lupins to cross-infect

Materials and Methods

Conidial isolations of P.setosa were made from diseased leaflets of the above named species. The three isolates were grown on PCA for ten days and at this stage abundant conidia were available for inoculation. At the time of inoculation all plants were six weeks old. Four treatments were used, namely, inoculation with conidia from isolates of: (a) tree lupin (b) Russell lupin (c) blue lupin, and (d) spraying with water (control). Nine plants of a species were used for each treatment giving a total of 36 replicates of each species for the trial. Plants were held at 100% RH (22°C) for 24 hours following inoculation and then transferred to the glasshouse. Plants were selected by random numbers for the particular treatment they were to receive. The inoculum consisted of a 30 ml spore suspension prepared from four Petri plates containing ten-day-old colonies.

Results and Discussion

Results were recorded after three days. The results, shown in Table 15, indicate that P.setosa isolated from the lupin species most commonly found growing in New Zealand, readily cross-inoculates. This is important because in many instances in the Manawatu, wild tree lupins with Brown Spot disease are growing in the vicinity of stands of blue lupin. In some cases there is as little as 30 yards between the

Table 15

Pathogenicity of isolates of P.setosa from Russell, tree, and blue lupin on each of these three species. (Number of Diseased Plants is recorded)

Plants Inoculated	Inoculum from—		
	Russell lupin	tree lupin	blue lupin
Russell lupin	8	9	9
tree lupin	8	9	9
blue lupin	9	9	9
(controls sprayed with water remained healthy)			

two varieties. By the same token, one nursery in particular in Palmerston North, from which diseased Russell lupin seedlings were obtained, was in close proximity to tree lupins. (The significance of this information has already been discussed in the section of disease cycle relating to survival of the fungus and source of primary inoculum.)

2. Predisposition

Walker (1950) defines predisposition as "the effect of one or more environal factors which makes a plant vulnerable to attack by a pathogen." Many definitions are similar to this in that they only use the term to describe changes toward great susceptibility but Yarwood (1959) ascribes a meaning to predisposition which includes induced changes in disease proneness toward greater or lesser susceptibility— predisposition is "the tendency of non-genetic conditions, acting before infection, to affect the susceptibility of plants to disease." Genetic

susceptibility could be included by arbitrary definition but it is more frequently considered independently as it is of major importance in modern plant pathology. Environment affects disease in three ways: (i) direct effect on the pathogen; (ii) effect on host susceptibility; (iii) influences on interaction of host and pathogen. The first has been considered in Part A of the Disease Cycle whilst the second comprises the subject matter of predisposition. The third effect of environment on disease is sometimes difficult to separate from predisposition effects.

In this study the definition as used by Yarwood (1959) will be followed for it is felt that predisposition includes more than just the influence of environal factors. For instance, the age of the host or stage of development has been shown to be important in such diseases as Peach Leaf Curl (Taphrina deformans), Brown Rot of stone fruits (Monilinia fructicola), and Late Blight of potatoes (Phytophthora infestans). Work done by Germar (1940) with P.setosa on Lupinus albus indicated the existence of strong defensive mechanisms in the young foliage. A trial was carried out to determine whether this was the case with P.setosa on Russell and blue lupin.

Ontogenetic predisposition

Plants of different ages and consequently at different stages of development but grown under similar conditions were used for this trial.

Materials and Methods

Seeds of Russell and blue lupins were planted at intervals so that plants of the following ages were available for the trial: two,

four, eight, and thirteen weeks. Russell lupin was included in the trial because of the observation in the field that the disease is more prevalent on the older, lower leaflets of plants. Six plants of each species, at the required age, were inoculated with a standard inoculum concentration (30 conidia per binoc. field), and the plants placed at 22°C and 100% RH for 24 hours.

Because of the difference in size of plants between treatments, care was taken when comparing the relative susceptibility of the different age groups. It was decided to express infection as a percentage of the number of diseased leaflets over the total number of leaflets. Results were recorded after three days and remarks made after one week.

Results and Discussion

The percentage infection figures obtained show little definite trend or predisposing effect of age on initial susceptibility of either Russell or blue lupin to P.setosa. The lower infection levels obtained with Russell lupin and in particular the two-weeks-old plants, as compared with blue lupin, were due to two factors: (i) Russell lupin plants at all ages had considerably less leaflets than blue lupin, and (ii) unfolding leaflets in the centre of Russell lupins were not readily infected; this appeared to be due to the protected way in which these young leaflets were unfolded and also to the density of hair per unit area of leaflet at this particular stage of leaflet development.

When these two factors are combined together it can be seen that only one or two leaflets need to remain healthy for the percentage to be reduced considerably and this did occur with the two-weeks-old Russell lupins.

Table 16

The influence of Ontogenesis of Russell and blue lupins on infection by P.setosa, and subsequent disease manifestation

Age (Weeks)	Percentage of infection Russell lupin	blue lupin	Remarks (after one week)
2	68.2	92.6	All blue lupins killed
4	81.7	94.1	Leaf-fall in blue lupins most conspicuous, but new healthy foliage unfolding.
8	84.0	91.9	
13	86.3	92.8	Older Russell lupin foliage shrivelled but new leaflets healthy.

It may be argued that the lack of infection of the unfolding leaflets in Russell lupin is a form of predisposition due to the physical nature of the young leaflets. Certainly, a leaflet at this stage of development has no inherent chemical resistance to infection. This was proven at the conclusion of the trial when the unfolding leaflets of Russell lupin were carefully inoculated by opening out the leaflets and brushing conidia against the lie of the leaflet hairs. Resultant infection was severe. Leaflets of L.albus are also very hairy, particularly in their early stages of development, and again in this case it was found that careful inoculation of young leaflets was needed before infection occurred. This protective function of the hairs on the young leaflets could possibly be the reason for Germar (1940) deciding that L.albus plants had a 'strong defensive mechanism' in the young foliage. (Note: during the growth of the leaflet there does not appear to be any increase in number of hairs, but as the leaflet expands they become more sparsely arranged. This results

in less protection being afforded to the leaflet.)

In terms of the definition proposed by Yarwood (1950) the developing or unfolding leaflets of all Russell lupins used in the trial were less susceptible to P.setosa at certain stages of growth, i.e., they exhibited ontogenetic predisposition.

An important point arising from this trial on the influence of stage of development on infection is not evidenced by the figures giving percentage infection after three days (Table 16). Examination of the plants after one week revealed a difference in manifestation and ultimate reaction of the blue lupin plants of different ages to P.setosa. Blue lupin plants which were two weeks old when inoculated, were dead a week later. The stem and petioles of these plants completely collapsed, the stem collapse being typical of that generally associated with post-emergence damping-off. All other ages of blue lupin, and all Russell lupin plants were commencing to put forth new growth by the end of the week.

It may be concluded from this work that despite the lack of difference shown between infection percentages recorded for different ages of blue lupins, the two-week-old plants were more susceptible to P.setosa in that the infection resulted in the death of the plants; that is, these plants were predisposed as a result of their age and ultimately this predisposition is due to stage of development.

As it was only the very young unfolding leaflets of Russell lupin which showed any resistance to infection, the field observation made regarding the prevalence of infection on the older lower leaflets would appear to be due to their positioning rather than age. Here it could be said the micro-climate, together with proximity to inoculum was predisposing these leaflets toward greater susceptibility to P.setosa.

Temperature predisposition

Yarwood (1959) states:- "Experimentally, predisposition can be clearly demonstrated 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."

When plants are exposed to high or low temperatures before inoculation, their susceptibility may be altered and an examination of the literature reveals that an increase in susceptibility is the most common result. For instance, frost injury increases the susceptibility of some plants to Botrytis. A trial was performed to determine whether temperature predisposition to P.setosa occurred with blue lupin plants.

Materials and Methods

One-month-old blue lupin plants were exposed to 5°C, 15°C, 22°C, 28°C, and 32°C for 24 hours and then placed on the laboratory bench for 30 minutes to enable plants to regain a similar temperature before inoculation. Following inoculation plants were held at 22°C (100% RH) for 24 hours and then returned to the laboratory bench. Plants which were held at 22°C prior to inoculation were regarded as controls. Twelve plants were used for each treatment and results recorded in terms of disease severity. In order to get a comparison between treatments the disease rating was multiplied by the number of plants with that rating and the total for each treatment was divided by 12 to give the average disease rating for that temperature.

Results and Discussion

From Table 17 it can be determined that 88% of the plants received a similar moderate (2) disease severity rating indicating that the pre-inoculation temperatures used in this experiment did not predispose these blue lupin plants to infection.

Table 17

Effect of temperature on predisposition to infection

Pre-inoculation temperature	Number of plants showing disease severity			Av.Disease severity rating
	Low (1)	Moderate (2)	Severe(3)	
5°C	1	11	0	1.93
15°C	0	11	1	2.08
22°C	1	10	1	2.00
28°C	0	11	1	2.08
32°C	2	10	0	1.80

3. Inoculum potential

Horsfall (1932) first used the term 'inoculum potential' and as initially definied it is "the number of infective particles present in the environment of the uninfected host." According to Horsfall such a concept embodies two ideas:

(i) mass action—i.e. the greater the number of organisms present the greater the disease, or the more severe it will be, and

(ii) virulence—i.e. the more virulent the organism the more severe will be the disease.

A number of other definitions occur in the literature (Zentmeyer

et al 1944, Wilhelm 1950, Garrett 1956 and 1960, and Dimond and Horsfall 1959) and these all embody a similar basic idea. Gaumann (1950) discusses the density of spore inoculum and states that there is a minimum density known as the numerical threshold of infection— "the number of individual parasites which, under favourable conditions is necessary to establish infection." Gaumann's numerical threshold of infection is a valuable concept and in fact relates to a specific inoculum potential, namely the minimum required to establish infection.

The concept of inoculum potential is important not only under field conditions, where a minimum number of propagules will obviously be required before any infection occurs, but also in laboratory studies of a particular disease incited by a pathogenic organism. The statement 'hordes of soldiers will overrun almost any defense' is very appropriate when related to infection of a plant by a fungus. It would appear that all too frequently in laboratory studies, pathogenicity of a particular fungus is proven under conditions not even remotely resembling those in the field. Two instances are the use of wound inoculations and the use of very high inoculum concentrations. Never can it be hoped to exactly simulate conditions in the field but at least a realistic inoculum potential can be used in studies if in the first instance trials are conducted to determine the influence of inoculum potential on infection.

It is generally accepted that an individual spore is capable of causing infection but in most instances the infectivity of a single propagule is inadequate (Garrett 1960). To the contrary there is considerable evidence that for some organisms there may be a critical level of inoculum strength which is required before infection can occur (Cole and Couch 1958, Garrett 1959, Gooding and Lucas 1959). It

has already been shown that conidia are the propagules responsible for the spread and initiation of new infections of P.setosa. Experiments were therefore conducted to determine the numerical threshold of infection for P.setosa and also to establish whether or not inoculum potential influences disease severity and manifestation by P.setosa on lupins.

Materials and Methods

A conidial suspension was prepared from eight ten-day-old PCA cultures of P.setosa. This was found to have a concentration of 180 conidia per binocular field. This was diluted until eight conidial suspensions were obtained. These were 10 mls in volume and the concentration of spores ranged from 180 down to 3 conidia per binocular field. Six blue and six Russell lupin plants were inoculated per treatment (one-month-old plants). Following inoculation all plants were held at 22°C (100% RH) for 24 hours. The experiment was repeated.

Results and Discussion

No difference in disease severity between blue and Russell lupin could be observed at a particular inoculum level and as a consequence the results from the two species were not kept distinct.

Table 18 indicates that inoculum levels above 60 spores per binocular field resulted in severe disease expression and in the case of blue lupins in the death of the plants within a week. Gooding and Lucas (1959) found that level of inoculum greatly influenced the rate of disease infection and the severity of its development. These workers, together with Cole and Couch (1958) and Garrett (1959),

reported that the actual level of inoculum causing infection varies with the particular conditions prevailing at the time. During adverse conditions for infection, and low inoculum, it would be expected that few if any spores would germinate and infect but with high inoculum there would be a greater possibility that during marginal conditions at least a few spores would breach the host defenses.

Infection in the field was never observed to be as heavy as that rated as severe in this trial but moderate infection was quite common during wet weather. The normal inoculum level used throughout this study was 30 conidia per binocular field in a 10 ml suspension

Table 18

Influence of inoculum potential on infection by P.setosa, and subsequent disease manifestation

Conidia concentration per binoc. field	Disease severity rating* Expt. 1	Expt. 2	Remarks
180	3	3	With heavy infection lesions only remained distinct for 36 hours after treatment. After 3 days leaflets became dried and shrivelled. Blue lupins killed. Only Russell lupins alive.
140	3	3	
100	3	3	
60	2	3	
30	2	2	Cobweb symptoms appearing after 1 week. Manifestation did not occur until 60 hours after treatment.
15	2	1	
5	0	0	
5	0	0	

* 1 = low: 2 = moderate: 3 = severe.

as this proved an inoculum potential suitable for infection in inoculation trials but which still provided a reasonable comparison with the disease in the field.

Under the particular set of conditions used in this experiment the numerical threshold of infection would appear to be in the vicinity of 15 conidia per binocular field. Examination of inoculated leaflets receiving this treatment revealed approximately eight conidia per leaflet. 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.

C. EPIPHYTOLOGY

There is no exact criterion as to what constitutes an epiphytotic, but the general characteristics are unusual prevalence and destructiveness of a disease within a certain area. Prevalence is the primary requisite—to be considered epiphytotic a disease must affect a high percentage of individual plants in a population. Intensity of attack is also implied, although a disease may be epiphytotic even if it is not very destructive by nature.

In the preceding sections of this Chapter, consideration has been given to the development of disease—a series of events commencing with inoculation and culminating in symptom expression. The development of an epiphytotic comprises a series of similar events which, more or less simultaneously, affect many plants, generally in an extensive area. Each series of events is termed a disease cycle, and the intensity of each series is its quantitative effect on the development of an epiphytotic. Stakman and Harrar (1957) note that for an epiphytotic,

speed, intensity, and continuity are essential in each series. These writers express succintly the interrelationship of all the events constituting the disease process in the following: "In simplest terms, the length of a disease cycle is the total time required for dissemination, inoculation, infection, growth, and multiplication or sporulation of the pathogen. The intensity depends on the amount of initial inoculum, the percentage germination and infection, the extent of growth, and the abundance of sporulation."

Consideration will now be given to the practical implications of the results obtained from the study of the etiology of P.setosa on Lupinus spp., commencing with spore germination of the fungus.

Germination of conidia of P.setosa on lupin leaflets occurs rapidly over a wide temperature range (10°-34°C). Under New Zealand conditions germination is not likely to be limited by lack of suitable temperatures. Humidity requirements were found to be more critical however, with no germination occurring below 93% RH (25°C). Coupled with this high humidity requirement for germination, there is the necessity for leaflets to be continuously wet for a minimum of four hours before the fungus becomes independent of leaf surface moisture.

Although the fungus has a broad temperature tolerance for germination it was found that at temperatures from 20°-26°C germ tubes made more rapid growth and at 24°C, 71% of the germ tubes produced appressoria after seven hours compared with 8% and 3% at 15°C and 30°C respectively. Inoculum potential studies revealed that comparatively low levels of spores can cause infection under optimum conditions. Therefore, despite the falling-off in appressorium production at temperatures above and below the optimum, a high inoculum would in all probability ensure that at least a few spores would penetrate host defences.

The production of disease symptoms on lupins by P.setosa within 24 hours of inoculation indicates that the initial stages of the disease cycle can proceed rapidly under optimum conditions.

P.setosa was found to be capable of producing conidia at temperatures ranging from 5°-30°C (100% RH), with most abundant sporulation occurring in the region of 22°-24°C. As with spore germination, humidity requirements for sporulation were more critical than those of temperature, with 94.9% RH (25°C) the minimum. Besides the effects of environment on spore production, host species have a considerable influence on both speed and abundance of conidial production. Conidia were produced more rapidly and abundantly in the laboratory on detached diseased blue lupin leaflets, than on those of either tree or Russell lupin. On diseased leaflets of Lupinus digitatus (a species of lupin used in host susceptibility trials), the fungus could not be induced to sporulate.

Blue lupin plants which had been inoculated with a conidial suspension of P.setosa, given 24 hours at 22°C (100% RH) and then returned to the glasshouse, showed leaflet abscission within three days of inoculation and abundant sporulation on these fallen leaflets within five days. This series of events occurred under conditions which were optimum for the fungus. They do indiate that under these conditions on blue lupins, P.setosa has the genetic capabilities—up to this stage in the disease cycle—of becoming epiphytotic. However, as pointed out by Stakman and Harrar (1957), most pathogens could cause epiphytotics if given favourable conditions for a sufficient length of time.

A feature of Brown Spot disease on blue and tree lupins was the sparsity of sporulation of the fungus on infected areas of the host plants, and instead the ability of the fungus to cause defoliation of

infected plants and subsequent sporulation of P.setosa on these fallen leaflets.

Although conditions near or on the ground are more likely to be conducive to rapid conidial production as a result of high humidity, the spores have to be effectively disseminated from this site. Studies revealed that wind-borne rain was most effective in disseminating P.setosa but that wind alone can disseminate the pathogen. With blue lupins especially, fallen leaflets will often be sheltered at the base of the crop in weeds or other growth, making effective dissemination difficult. Effective dissemination is stressed here for rain splash will still disperse conidia in a blue lupin crop but this dispersal will be at a level near to the ground. Leaflet abscission is common on the lower part of the plant but it has been observed that blue and tree lupins have the ability to grow through the disease, i.e. to produce new healthy foliage near the top of the plant. Infection of this foliage in older plants was not observed nearly as frequently as that on the lower parts or on young plants. This general failure to attack to any extent the upper regions of blue and tree lupins reflects the difficulty of achieving sufficient effective dispersal to enable the disease to become epiphytotic.

In the blue lupin crop in which spore trapping was performed, the disease did reach epiphytotic proportions, and the reasons for this are considered in the section of the disease cycle on dispersal of inoculum.

With Russell lupin, dissemination does not present the same problem as with blue and tree lupin for sporulation occurs more frequently on shrivelled leaflets and stems still attached to the plant. Two

important factors occur in relation to the disease on this species of lupin, namely:

(i) Young foliage is protected in the early stages due to the way in which leaflets unfold and also on account of their hairy nature, i.e., the young foliage of Russell lupin exhibits ontogenetic predisposition.

(ii) Owing to the use of Russell lupin as a garden plant and the general tendency to scatter these plants around an area rather than aggregate them in beds, there is a lack of readily available susceptible hosts for the pathogen.

This latter factor in particularly makes the chances of an epiphytotic in this species remote.

Finally the amount of initial inoculum is important in determining whether an epiphytotic occurs. This initial inoculum often relates directly to survival of the fungus—with P.setosa it has been shown that infection of perennial tree lupin means that the fungus is invariably available from this source. The survival of conidia in the soil for nearly a year indicates that the disease can carry-over between successive lupin crops grown in the same ground. If a fungus has a ready means of survival the chances are high than an abundant viable inoculum will be available early in the season near host plants.

Conclusion

As a result of studies carried out on the disease cycle of the Brown Spot fungus Pleiochaeta setosa (Kirchn.) Hughes on Lupinus spp., it may be concluded that lack of effective dissemination in many instances prevents the disease reaching epiphytotic proportions. The various stages in the disease cycle leading up to dissemination all

require specific environmental conditions. The most likely pre-dissemination limiting factor in the New Zealand climate would be the high relative humidity required for spore production and subsequent germination, together with the period of leaf wetness necessary for successful infection of plants. The apparent freedom from Brown Spot disease in blue lupin crops inspected in the South Island during the late autumn 1963 could conceivably be due to lack of moisture, for the Canterbury area visited had experienced a particularly dry season (this visit and the implications of the weather are discussed in Chapter III in relation to the seed-borne phase of P.setosa).

However, owing to the relatively small number of lupin crops available for observation during this study and the limited area involved in the lupins which were examined, the above conclusions cannot be applied in a general way. Before this could be done a larger area of lupins would need to be kept under surveillance for several seasons in order to obtain a more accurate picture of the pattern of outbreaks of the disease in relation to environment.

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APPENDICES

APPENDIX I

COMPOSITION AND PREPARATION OF CULTURE MEDIA

Recipes are given for media used in this study other than that prepared with Oxoid. Distilled water was used to prepare all media and the volume of preparations adjusted as required before autoclaving.

Unless otherwise stated, media were autoclaved at 15 p.s.i. for 20 minutes immediately after preparation and then stored in 250-ml flasks or 5oz and 10oz McCartney bottles.

Laboratory Media

(a) Cornmeal Agar

agar	17 gm
cornmeal	20 gm
water	1000 ml

The cornmeal was cooked for one hour by placing a flask containing the cornmeal and suspended in 500 ml of water, in a simmering water bath. The cooked cornmeal was filtered through cheesecloth and the filtrate added to 500 ml of water in which the agar was dissolved.

(b) Potato-Dextrose Agar (lab PDA)

agar	12 gm
potatoes (sliced and peeled)	200 gm
dextrose	10 gm
water	1000 ml

The sliced potatoes were cooked gently for one hour in 500 ml of water and the filtrate retained after straining the mixture through cheesecloth. Agar was melted in 500 ml of water, dextrose added and then the two solutions combined.

(c) Potato-Carrot Agar (PCA)

agar	12 gm
carrot	20 gm
potato	20 gm
water	1000 ml

The potato and carrot were cooked gently for an hour in 500 ml water and the procedure for lab PDA followed except that no dextrose was added.

(d) Carrot Agar

agar	12 gm
carrot root juice	200 ml
water	800 ml

Four large carrots were put through a Braun juice extractor and a filtered extract of carrot obtained. This, plus dissolved agar, was added to the water.

(e) Lupin Seed Agar

agar	12 gm
blue lupin seed	100 gm
water	1000 ml

The seed was chopped up in a Waring Blendor until of a fine consistency. It was then added to water, cooked for half an hour, and filtered through cheesecloth. The filtrate was made up to the required volume with water containing dissolved agar.

(f) Tobacco Extract Agar

agar	4 gm
tobacco leaves	3 oz
water	200 ml

The leaves and 50 ml of water were macerated in a Waring Blendor for 15 minutes. The filtrate was collected after straining the solution through cheesecloth and added to 150 ml water plus 4 gm of agar.

(g) Water Agar

agar	12 gm
water	1000 ml

The agar was dissolved in hot water before autoclaving.

(h) 24D Potato-Dextrose Agar

Oxoid potato dextrose agar	30 gm
2,4, dichlorophenoxy acetic acid (80% sodium salt)	62.5 mg
water	1000 ml

62.5 mg of 24D was dissolved in the 100 ml solution of Oxoid PDA before autoclaving.

(i) V-8 Juice Agar

agar	10 gm
V-8 juice	500 ml
water	250 ml

The agar was dissolved in a 750 ml solution of V-8 juice before autoclaving.

(j) Starch Agar

Oxoid nutrient agar	28 gm
starch	20 gm
water	1000 ml

The starch was added to the Oxoid nutrient agar before autoclaving.

(k) Lupin Extract Agar

Recipe on Page 64.

Oxoid Media

Potato Dextrose Agar (Oxoid PDA)

Kligler Iron Agar

Tomato Juice Agar

Milk Agar

Nutrient Agar

Prune Agar

Malt Agar

Soya Peptone Agar

Tryptone Yeast Extract Agar

Tryptone Glucose Yeast Extract Agar

Reference

The Oxoid Mannual (1962) reprint second edition.

APPENDIX II

CONSTANT HUMIDITY SOLUTIONS

Relative humidity(%) at 25° C	Glycerine (ml)	Water (ml)
10	38.0	12.0
20	36.8	13.2
30	35.2	14.8
40	33.6	16.4
50	31.3	18.7
60	28.8	21.2
70	24.3	25.7
80	20.4	29.6
90	13.2	36.8
100	-	50.0

Relative humidity(%) at 25°C	Saturated Salt Solutions
88.0	$BaCl_2.2H_2O$
93.0	KNO_3
94.9	$CaHPO_4.2H_2O$ (at 30°C)
97.0	$CaHPO_4.2H_2O$
98.0	$KClO_3$

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APPENDIX III

OTHER FUNGUS DISEASES ISOLATED FROM LUPINS DURING THIS STUDY

As mentioned in the Introduction to this study, lupins are not of major importance in New Zealand and no doubt because of this only a small amount of literature can be found relating to them. Articles by Inch (1947), Whatman (1959), and White (1961) all mention that lupins, as a crop, are relatively free from disease, although Whatman (1959) concedes that "several fungus diseases can be prevalent under moist, warm conditions, though they rarely cause material reduction of the yield of feed or seed." These diseases include Ascochyta spp., Botrytis cinerea, Sclerotinia sclerotiorum and Phytophthora cinnamomi. All except the latter disease were found and isolated to pure culture during the course of work on P.setosa.

Ascochyta/Phoma

In Chapter III relating to the seed-borne phase of P.setosa it is noted that an Ascochyta/Phoma species was prevalent in quite large areas in blue lupin crops approaching maturity in the South Island. (Note: because of the lack of an adequate basis for distinction between species of Ascochyta and Phoma, together with the fact that there was wide variation in the number of spores having a cross wall as compared with those that there were unicellular, no specific name was given to the fungus. Ascochyta pisi, Ascochyta lupinicola, Phoma lupinicola and Phoma lupini have all been recorded overseas as foliage pathogens of Lupinus spp. The unsatisfactory nature of the nomenclature of Phoma and Ascochyta species is highlighted by the controversy which still rages in the United States on the name of the incitant of Spring Black Stem Disease of Red Clover and Alfalfa—recent literature relating to

this includes: Schenk and Gerdemann (1956), Ellingboe (1959), and Edmunds and Hanson (1960).

In New Zealand the disease caused by Ascochyta/Phoma is commonly and justly referred to as stem canker because of the large black lesions on the stems which ultimately can cause collapse of the plant. Defoliation of the plant prior to total collapse is common, no doubt due to the disease making water supply insufficient for the plants needs. Pycnidia can be discerned with the unaided eye and during wet weather pink masses of conidia are produced. The fungus was isolated from seed of Russell and blue lupin and from the foliage or stems of these two species as well as from tree lupin.

Sclerotinia Sclerotiorum

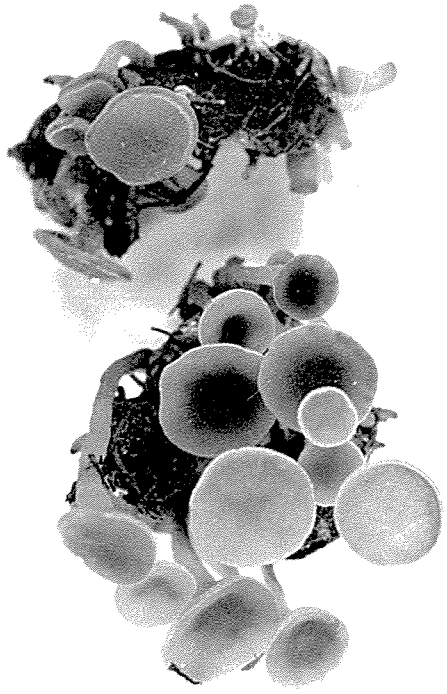
This is a fungus which attacks a large range of plants. It was very prevalent in an area of autumn-sown blue lupins at the university, particularly during the winter months. It caused a stem rot which commenced at or above ground level. The infected area becomes a light brown colour and rather soft and slimy; during prolonged wet weather the decayed portion of the stem becomes overgrown with a mass of fluffy white mycelium. This mycelium and the production of hard black bodies (sclerotia) amongst it or within the stem tissues, is a characteristic feature of the disease and can be clearly seen in Illustration 25. The disease results in premature defoliation and eventual collapse of infected plants.

The disease is soil-borne but unlike many soil-borne diseases it also has a facility for aerial spread. This aerial spread occurs as a result of the sexual phase of the fungus. Ascospores are produced in cup-like fruiting bodies known as apothecia. During this study

Illustration 25

Stem rot on blue lupins caused by Sclerotinia sclerotiorum. The production of sclerotia and aerial mycelium are typical of the disease

Illustration 26 (x12)



Apothecia of Sclerotinia sclerotiorum
produced from sclerotia

sclerotia from cultures of the fungus on PDA were taken and thoroughly washed in running water for two hours. They were then transferred to test tubes containing approximately 15 ml of sterile water. After shaking thoroughly in this, sclerotia and wash water were placed in sterile Petri plates and held at 15°C until apothecial initials appeared. This occurred after approximately four weeks. Plates were then placed near a window in the laboratory, care being taken to ensure that sterile water was always present in the Petri plate. The technique as outlined here for the production of sclerotia is a modification of that used by Purdy (1955) and proved most satisfactory for production of the sexual phase of the fungus. Illustration 26 shows an enlarged view of sclerotia producing numerous apothecia.

Botrytis Cinerea

Like Sclerotinia this fungus is ubiquitous in its host range but it is most common on plants that have been damaged in some way. Although not as common as the other diseases found during the study, this disease was responsible for causing some loss of blue lupins at the University in 1963. The disease was most prevalent in the late winter/early spring and this increased prevalence would very likely be due to frost damage.

The fungus causes a pale brown lesions area on the stem (Illustration 27) which quickly becomes covered with a grey, feathery growth. This growth is due to the production of conidia. Botrytis cinerea also produces sclerotia but at no time was production of these observed in the field. However, on PDA the fungus produced numerous sclerotia, all of which were much smaller than those produced by Sclerotinia. Inoculation attempts with mycelium of Botrytis cinerea

Illustration 27

Typical manifestation of Botrytis cinerea
on blue lupins

were unsuccessful unless a wounding technique was used. Sterile tooth picks were placed in cultures of the fungus on PDA and infection resulted when these were inserted into the stems of plants. This indicates that this strain of B.cinerea is really only a facultative parasite dependent on wounded tissue in lupins for establishment.

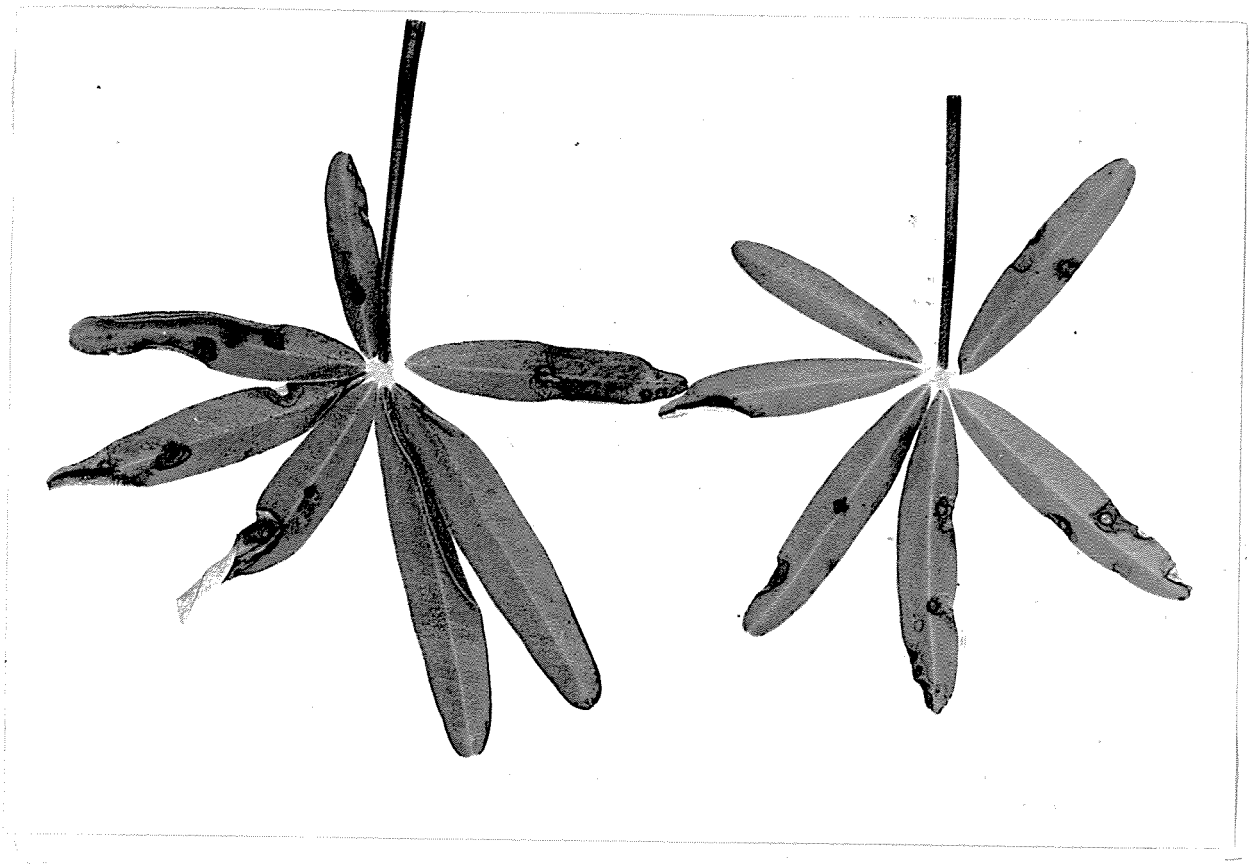
Stemphylium spp.

During the study of the Brown Spot disease of lupins a species of Stemphylium was frequently found associated with leaflets of Russell, tree, and blue lupins, becoming most evident as a result of abundant sporulation after high humidity. This fungus was most conspicuous during the early stages of the study when familiarity with symptoms produced by P.setosa was limited. Similarly in seed plating work the fungus was found growing out from sterilised blue lupin seeds on agar.

Numerous saprophytic species of Stemphylium are known to exist but no record in published literature could be found of this fungus on lupins in New Zealand. However, Slade (1961) obtained an isolate from Lupinus spp. and he called this S.botryosum. In pathogenicity tests he found Lupinus spp. were only 'infected' with S.botryosum when the plant was wounded. Because lesions failed to enlarge, he considered that S.botryosum was present as a saprophyte on damaged tissue.

Examination of records of overseas work reveals that Neergaard (1945) isolated S.botryosum from lupin seed. In 1956 in the United States two Stemphylium diseases of blue lupins were recorded for the first time (Wells et al 1956). One of these diseases was called 'little leaf spot' and was found to be due to Stemphylium botryosum. The second disease was called 'grey leaf spot' of lupins and was attributed to S.solani. According to the symptoms as described for these two

Illustration 28



Leaflet lesions on blue lupins caused by a species of Stemphylium

diseases the disease found in the Manawatu is similar to that caused by S.solani i.e. grey leaf spot. The symptoms of this disease are typical of those evidenced in Illustration 28. The leaf spots are circular to oblong in outline and range from 2mm-6mm in diameter. In the early stages the spots are mid-brown on both surfaces. Later the centre portion of the leafspot becomes a bleached grey-brown colour with a well defined mid-brown margin. As with the American work it was found that disease was capable of causing rapid defoliation. Stem and pod lesions were present, the lesions being a chestnut or cinnamon colour and ranging from a few millimetres to several inches.

Isolations of this fungus were made to PDA and inoculations of blue lupin and lettuce plants was carried out. Infection of blue lupin resulted but lettuce was not infected.

Although no more detailed work has been carried out on this Stemphylium isolate, available information on the causal agent appears rather contradictory. Symptoms observed in New Zealand, which have been proved to be caused by a species of Stemphylium, are almost identical to those described by Wells et al (1956) for grey leaf spot. However, work by Graham and Zeiders (1960) on some leguminicolous and related species of Stemphylium complicates the question. Conidia of Stemphylium isolated from lupins in New Zealand had echinulate walls and were in the vicinity of 20μ x 30μ in size. They produced perithecia in culture. These specifications are similar to those for S.botryosum isolates from blue lupin (Graham and Zeiders, 1960). On the other hand, S.solani from blue lupin has smooth conidial walls, does not produce perithecia and has an average conidial size of 53μ x 17μ.

There is obviously scope for work on this Stemphylium disease present in New Zealand. Cross inoculations of the isolate with tomato

and alfalfa would possibly help. According to American work, grey leaf spot has advanced from an unknown disease prior to 1956, to a disease of sufficient importance to warrant searching for resistant varieties of blue lupin. Certainly during late February/early March 1964, when spore trapping of P.setosa was proceeding, this disease was as equally conspicuous in its spread as P.setosa and numerous Stemphylium conidia were observed on vaselined slides.

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