STUDIES ON STEMPHYLIUM LEAF SPOT AND LEPTOSPHAERULINA PEPPER SPOT, TWO FOLIAGE DISEASES OF LUCERNE.

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Phillip Donald Whitwell

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SUMMARY

STEMPHYLIUM LEAF SPOT

1. The symptoms of Stemphylium leaf spot of lucerne in the Manawatu are described.

2. The morphological features of the imperfect and perfect states of the pathogen on the host conformed closely to those recorded overseas for Stemphylium botryosum Wallr. on lucerne. Conidia were sub-spherical to ovoid or oblong, light brown, echinulate, 17.6 x 29.4u, muriform, and with a major constriction at the median transverse septum. The globose, black pseudothecia contained several large (30.2 x 184.6u), cylindrical to clavate asci, with an obtuse apex tapering to a swollen or claw-like base. Ascospores were ellipsoid to clavate, yellow-brown, 16.3 x 37.2u, muriform, and with slight constrictions at all 7 transverse septa.

3. The cardinal temperatures for vegetative growth on PDA_L at 10 days were 4, 24 and 36°C. Of 9 media tested greatest growth at 24°C occurred on 20% V-8 juice agar. Gross colony characteristics changed with temperature and media.

4. Maximum conidial production occurred on PDA_L and 20% V-8 juice agar cultures exposed to continuous NUV light for 12 days at 23-27°C.

5. Greatest production of protopseudothecia occurred following exposure of actively growing colonies to NUV radiation for a minimum of 5 days at 23-27°C. Protopseudothecia matured when such cultures were incubated at 8-12°C for a further 6 weeks.

6. Germ-tubes produced from conidia streaked on PDA_L slides and incubated at 24°C were first evident within 2 hours, emerging initially from the lateral cells and later from the terminal cells of the muriform conidia. Within 4 hours 90% of the conidia had germinated, each producing between 4 and 10 germ-tubes.

7. Conidium ontogeny and morphology of mature conidia of an isolate initially identified as Stemphylium vesicarium (Wallr.) Simmons and a typical isolate of S. botryosum were compared; both isolates were considered identical and typical of the latter species.

8. Incubation temperature of isolates grown on 20% V-8 juice agar was shown to considerably affect morphology of mature conidia.

LEPTOSPHAERULINA PEPPER SPOT

1. The symptoms of Leptosphaerulina pepper spot of lucerne and red clover in the Manawatu are described.

2. Isolates from natural infections on lucerne and red clover were most readily obtained when infected tissue pieces were water-washed for 4-6 hours, plated to antibiotic PDA_L and incubated at 24°C.

3. Isolates from either host could not be differentiated on the basis of pathogenicity since in reciprocal cross-inoculations identical symptoms were produced. A species of Leptosphaerulina has not previously been recorded as a pathogen of red clover in New Zealand.

4. Viable inoculum was associated with two of 10 lucerne seed-lines screened, at levels of 0.4% and 1.2%. Following germination of seed of these two lines in a Copenhagen germinator, protopseudothecia of the pathogen were located on ungerminated seed and infected seedlings.

5. Protopseudothecia were not located in field infections, but they were readily produced in 20% V-8 juice agar or PDA_L cultures incubated at 24°C in the dark for 10 days. Protopseudothecia matured only after exposure to light for 5-7 days. Maturation was most intense under cyclic fluorescent light (12 hour light/12 hour dark) and least under natural/ diurnal light.

6. Germ-tubes produced from ascospores naturally ejected from lucerne agar isolates onto PDA_L slides and incubated at 24°C were first evident within 1 hour, emerging from either the lateral or terminal cells of the muriform ascospores. After 3 hours all ascospores had germinated, each producing between 3 and 7 germ-tubes.

7. The morphological features of the perfect state of isolates on artificially inoculated excised lucerne leaves and on agar were essentially similar, and conformed with overseas descriptions of the pathogen on lucerne. On host tissue pseudothecia were black, globose, erumpent with several large (140.1 x 78.7u), saccate, thick-walled bitunicate asci. Ascospores were oblong, ellipsoid or clavate, hyaline, 14.2 x 35.3u, phragmosporous or muriform, and surrounded by a thin gelatinous sheath.

8. Lucerne and red clover isolates on agar could not be separated on the basis of gross colony characteristics or dimensions of pseudothecia, asci and ascospores.

9. The majority of ascospores from lucerne isolates were transversely 4-septate; those of red clover isolates were predominantly 3-septate. However, this distinction in itself was considered insufficient to warrant recognition of the two series of isolates as separate species.

10. *Leptosphaerulina trifolii* (Rost.) Petr., published in 1959, has priority over *L. briosiana* (Poll.) Graham & Luttrell and is proposed as the correct binomial for the species pathogenic to lucerne and red clover.

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INTRODUCTION

Over recent years the area of lucerne (*Medicago sativa* L.) grown in New Zealand has rapidly increased (Table 1) as farmers and commercial firms have realised the many and varied virtues of this crop in farming enterprises.

TABLE 1. Areas of lucerne grown in New Zealand used for hay, silage and seed production.

Year	Area (acres)	Authority
1958-59	144,516	N.Z. Year Book, 1963
1963-64	164,860	N.Z. Year Book, 1969
1968-69	202,770	N.Z. Year Book, 1971
1970-71	300,000	Meeklah & Allen, 1971

Lynch (1967) estimated that if the present trend continued a lucerne area of at least 300,000 acres could be expected by 1977. However, this area was attained by 1970, and the increased rate of expansion can be accounted for in several ways:

1. Due to its agronomic features lucerne is primarily grown in free-draining soils subject to an unreliable summer rainfall, so ensuring a continued feed supply over this period.

2. Lucerne has a greater versatility than most crops within a farming enterprise in that it can be utilised in several ways; for example, hay and silage, grazing, seed production, and more recently for lucerne meal or pellets, and protein extraction.

3. A gross margin analysis of lucerne as a cash crop indicates a return comparable with, or better than, such other popular crops as wheat or peas (Lamb, 1969; Tocker, 1970; Anon, 1970; Anon, 1972).

Because lucerne must be treated as a cash crop, factors causing a decrease in foliage yield and quality must be minimised to ensure the greatest gross returns. Factors implicated are:

1. Agronomic. In a survey of farmer practice in relation to lucerne, Blair (1965) found the average duration of stands was nine years and that deterioration was mainly due to grass and weed invasion. This can be encouraged by a soil pH divergent from the optimum of 6.2, inefficient inoculation of seed with Rhizobium meliloti Dangeard, unsatisfactory seed-bed conditions and poor crop management.

2. Disease. This factor appears to operate in varying degrees in the decline of older stands (Close, 1967). Diseases can affect lucerne at all stages of development and so influence establishment, herbage yields, seed quantity and quality, and the longevity of stands.

Several soil and seed-borne fungi can cause pre- and post-emergence damping-off of seedlings, such as Phoma medicaginis Malbr. and Roum., Thanatephorus cucumeris (Frank) Donk, and species of Fusarium and Pythium (Close, 1967). Foliage diseases primarily cause a reduction in herbage quality and premature leaf fall, especially when infections are severe, thereby reducing the potential yield of a crop. In a study in Canada to determine the effect of leaf and stem diseases on yield, defoliation, protein and carotene content, Willis, Stuteville and Sorensen (1969) found that plots sprayed with mancozeb (Dithane M-45) yielded up to 27% more hay than unsprayed plots, increased carotene content up to 45% and decreased stem defoliation up to 27%. A similar increase in yield (up to 42%) due to leaf disease control was also demonstrated by Wilcoxson and Bielenberg (1972). Recent work with lucerne (Loper and Hanson, 1964; Loper, Hanson and Graham, 1967) and with white clover (Wong and Latch, 1971) indicated that an accumulation of coumestrol was more likely to occur in plants infected with foliar pathogens, with possible oestrogenic effects when fed to livestock. All these reports serve to emphasise the potential detrimental effects of foliar pathogens on both herbage yield and herbage quality.

Dingley (1969) records eight foliar diseases of lucerne in New Zealand (Table 2), of which common leaf spot (Pseudopeziza medicaginis (Lib) Sacc.) and Stemphylium leaf spot (Pleospora herbarum (Fr.) Rab.) are the most prevalent (Close, 1967).

The latter disease is world wide in distribution on lucerne (Benedict, 1954; Nelson, 1955; Focke, 1966; Perisic and Stojanovic, 1967) and the imperfect stage is invariably referred to as Stemphylium botryosum Wallroth. In the course of preliminary studies an isolate considered by the author to be S. botryosum was tentatively identified by Laundon (pers. comm.) as Stemphylium vesicarium (Wallr.) Simmons. This information stimulated studies on the Stemphylium disease in New Zealand, and mycological studies of the causal organism.

TABLE 2. A list of foliage pathogens recorded on lucerne in New Zealand.

Pathogen	Authority
Colletotrichum trifolii Bain & Essary	Dingley, 1965
Leptosphaeria pratensis Sacc. & Briard	Smith, 1955
Peronospora trifoliorum de Bary	Cunningham, 1922b
Phoma medicaginis Malbr. & Roum.	Cunningham, 1956
Pleospora herbarum (Fr.) Rab.	Brien & Dingley, 1959
Pseudopeziza jonesii Nannf.	Cunningham, 1922b
Pseudopeziza medicaginis (Lib.) Sacc.	Cunningham, 1922b
Uromyces striatus Schroet.	Cunningham, 1922b

In reviewing the diseases of lucerne in New Zealand, Close (1972) included a previously unreported disease first located at Putaruru in 1969 and subsequently found at Murupara, Hastings and in Mid-Canterbury. The causal organism he cited as Leptosphaerulina trifolii (Rost.) Petr., and commented that it is also common on white clover. What is apparently the same pathogen was located by the author in late 1971 in an eight acre lucerne stand in the Manawatu. This disease, commonly known overseas as pepper spot, is one of the major diseases of lucerne in the humid, temperate areas of the United States (Martinez and Hanson, 1963). Graham and Luttrell (1961) in their study of Leptosphaerulina species on forage plants considered that two distinct species existed on the Leguminosae, namely L. briosiana (Poll.) Graham and Luttrell and L. trifolii, both of which were pathogenic to Medicago and Trifolium species. Subsequently Booth and Pirozynski (1967a) listed L. briosiana as a synonym of L. trifolii

with the resulting confusion amongst plant pathologists as to the correct epithet to apply to the pathogen on lucerne. This raises the question as to whether in fact the diseases as present on lucerne and clover species in New Zealand are caused by the one morphological species.

STUDIES ON STEMPHYLIUM LEAFSPOT OF LUCERNE

CHAPTER I

INTRODUCTION

Neergaard (1945) in summarising knowledge on the pathogenicity of *Stemphylium botryosum* Wallr. stated that it had been reported from nearly every part of the world on hundreds of plant species. He further commented that "*S. botryosum* is a pronounced ubiquitous facultative parasite which most often is encountered on dead or greatly weakened plant elements; less frequently it may be demonstrated as the direct cause of morbid phenomena". The species has been recorded as a primary pathogen on a diverse range of hosts (Table 3), and reports indicate the existence of both host specific and non-host specific strains (Smith, 1940; Neergaard, 1945; Rotem, Cohen and Wahl, 1966; Close, 1967).

The first record of Stemphylium leafspot of lucerne was by Gentner (1918) in Germany. He cited *Macrosporium sarcinaeforme* Cav. as the causal organism but subsequent taxonomic studies by Wiltshire (1938) and Smith (1940) have shown the correct binomial of the pathogen to be *Stemphylium botryosum*, a fungus characterised by sarcinaeform, echinulate conidia. The perfect state of this species is *Pleospora herbarum* (Fr.) Rab.

Stemphylium leaf spot of lucerne was first recorded in New Zealand by Brien and Dingley (1959). The disease is regarded by Close (1967) as being common and generally of minor importance, but when severe, is capable of causing considerable damage by inducing premature defoliation. Observations in the Manawatu indicated the disease to be very common during the spring and autumn, and capable of causing most severe defoliation when the crop was held longer than the usual 4-6 weeks between grazings or cuts.

In the U.S.A., Nelson (1955) found that the fungus overwintered as protopseudothecia in plant debris, with ascospores functioning as the major source of primary inoculum. In the present study the ascigerous stage was not located in the field, although in the laboratory pseudothecia developed on excised leaves spray inoculated with a conidial suspension. Close (1967) comments that since in New Zealand the pathogen also occurs on a wide range of pasture legumes and other hosts, inoculum is available throughout the year.

This suggestion of alternate hosts being a source of primary inoculum pre-supposes that the pathogen on lucerne is not host specific.

TABLE 3. A partial list of hosts on which *S. botryosum* has been recorded as a primary pathogen.

Host	Authority
Allium cepa L. (onion)	Cunningham, 1922a
Chrysanthemum indicum L. (chrysanthemum)	Dingley, 1969
Cucumis melo L. (muskmelon)	Petzer, 1958
Dianthus caryophyllus L. (carnation)	Dingley, 1969
Lactuca sativa L. (lettuce)	Padhi and Snyder, 1954
Lupinus angustifolius L. (blue lupin)	Wells, Forbes, Webb and Edwardson, 1956; Tate, 1968
Lupinus arboreus L. (tree lupin)	
Lycopersicon esculentum Mill (tomato)	Dingley, 1960; Rotem, Cohen and Wahl, 1966
Medicago sativa L. (lucerne)	Smith, 1940; Nelson, 1955; Brien and Dingley 1959; Focke, 1966
Trifolium pratense L. (red clover)	Smith, 1940

A seed-borne phase of the disease also exists. In the U.S.A. Nelson (1955) frequently isolated the pathogen from naturally-infected seed and showed that the disease depressed both seed yield and seed quality. In screening 86 New Zealand produced lucerne seed-lines, Percival and Wenham (1972) found that the incidence and level of infection of *S. botryosum* was low, and that only a small percentage of the isolates were pathogenic to seedlings, which in turn suggests that the seed-borne phase is relatively unimportant in this country.

In New Zealand, apart from a review by Close (1967) wherein he describes symptoms and comments on some aspects of the disease cycle no detailed study of the disease or the causal organism has been reported. In the present study specific topics investigated were the pathogenicity of S. botryosum to lucerne, symptomatology, factors affecting inoculum production and establishment of infection, and the mycological features of the pathogen on the host and on agar.

In the course of the study it was found that isolates from field infected plants varied considerably in conidial characters. The majority broadly conformed to S. botryosum, but one cultural isolate in the opinion of Laundon (pers. comm.) was more typical of S. vesicarium, suggesting that both species may be present as pathogens of lucerne in New Zealand. However, since this particular isolate was incubated at 8°C it was possible the low temperature had influenced the morphology of the conidia in a manner similar to that reported by Leach and Aragaki (1970).

Accordingly, a comparison was made of the effect of temperature on conidial morphology of a typical S. botryosum isolate and the atypical isolate. Further, since S. botryosum and S. vesicarium differ in the shape of juvenile conidia and in the morphology of mature conidia (Simmons, 1969), these features of the two isolates were also studied.

CHAPTER II

PROOF OF PATHOGENICITY

A. INTRODUCTION

Before a specific organism can be cited as the cause of a previously defined disease condition the connection between the two must be established, thereby guaranteeing that the organism is the pathogen and not an associated saprophyte. This requirement is met by adhering to the Rules of Pathogenicity, first enunciated by Robert Koch in 1882. Briefly stated these are:

1. the constant association of the organism with the disease must be demonstrated,
2. the organism must be isolated and grown in pure culture,
3. the isolated organism when used to inoculate healthy host plants must produce symptoms typical of the disease,
4. the organism re-isolated from the inoculated plants must be identical in all characters to the original culture.

In the present study, the Rules of Pathogenicity were completed as follows.

B. ISOLATION TO PURE CULTURE

Asexual reproduction was induced by subjecting infected leaflets to high humidity in petri dishes lined with moistened filter paper. Using a sterile needle seeker, single conidia were aseptically transferred to media in either petri dishes or test tubes, and incubated at 24°C.

The pathogen was also readily isolated to agar by the tissue plating method. Infected tissue pieces (approximately 2mm square) transferred to a muslin bag were surface-sterilised by immersion in a 1:7 dilution of commercial Janola (approximately 1.3% available chlorine) for 30 to 90 seconds, rinsed in sterile distilled water, and aseptically transferred to potato-dextrose agar (PDA) plates to which the two antibiotics penicillin (as crystalline benzyl penicillin) and streptomycin sulphate had been incorporated, each at a concentration of 50 ppm. Ten tissue pieces were plated to each petri dish, and incubated at 24°C.

On the basis of conidial and conidiophore morphology the isolates were identified as Stemphylium botryosum Wallr.

Stemphylium botryosum is also known to have a seed-borne phase (Nelson, 1955; Leach, 1960; Noble and Richardson, 1968). Isolates were prepared from infected lucerne seed following the method of Percival (1972), which involved plating of seed to antibiotic malt agar.

C. ARTIFICIAL INOCULATIONS

Stemphylium botryosum is known to exist in the field as both pathogenic and non-pathogenic strains (Dingley, 1969; Percival and Wenham, 1972). This fact required that isolates from both lesioned leaflets and seed be tested for pathogenicity before stock cultures were prepared.

Inoculations were conducted by spraying healthy potted plants (var. Wairau) with a conidial suspension (37,000-65,000 conidia/ml) prepared from PDA cultures incubated at 25-27°C for 10 days under continuous near-ultraviolet light (NUV). Following inoculation the plants were placed in a high humidity cabinet (90-100% R.H.) for 72 hours at 20°C, after which they were transferred to a glasshouse bench.

Controls were provided by plants atomised with distilled water only, but otherwise given identical treatment.

Symptoms were first apparent within 48 hours of inoculation as dark pin-head sized necrotic spots which gradually increased in size, became irregular in shape and tan in colour, and often developed a darker margin. This latter lesion type was characteristic of those observed locally in the field and was typical of overseas descriptions, where the causal organism is considered to be Stemphylium botryosum (Smith, 1910; Nelson, 1955).

D. RE-ISOLATION OF THE CAUSAL ORGANISM AND COMPARISON WITH THE ORIGINAL CULTURE

Using both of the above described methods, the causal organism was readily re-isolated to agar. The colonies were macroscopically similar to the original isolates, and on microscopic examination were found to be morphologically identical.

By fulfilling the requirements of Koch's Postulates as described above, it was established that Stemphylium botryosum was the causal organism of a foliage leaf spot disease prevalent on lucerne in the Manawatu, and further, that the pathogen was associated with New Zealand produced lucerne seed.

CHAPTER III

THE DISEASE

A. SYMPTOMATOLOGY

1. INTRODUCTION

Specific diseases are usually identified on the basis of symptoms expressed by infected plants. However, in field practice this can be extremely difficult, particularly when several pathogens causing similar symptoms are co-existent, and where environmental variables influence the type of symptom induced. This situation is well illustrated by the work of Renfro and Kernkamp (1963) who compared symptoms on lucerne stems caused by six fungi. They found that only slight differences occurred and with so much overlapping as to prevent ready identification of the specific diseases.

The author experienced similar difficulties in studying the foliage diseases of lucerne present in the Manawatu. Besides Stemphylium leaf spot (*Stemphylium botryosum*) and pepper spot (*Leptosphaerulina trifolii*), black stem (*Phoma medicaginis*) and common leaf spot (*Pseudopeziza medicaginis*) were located in local crops, and particularly in the initial stages of symptom expression accurate diagnosis was only possible following isolation of the causal fungi to agar. Accordingly detailed field and glasshouse studies were made of the symptomatology of both Stemphylium leaf spot and pepper spot. The following account relates to the former disease.

2. FIELD OBSERVATIONS

In all crops the disease was widespread and most severe during late spring/early summer, and again in the autumn.

Symptoms were observed on leaves (Figure 1) and occasionally on petioles, and were essentially similar to those reported overseas (Table 4). On leaves first evidence was the development of black, circular, punctate spots with a distinct margin which were randomly located over the upper leaflet surface. It was at this stage that the disease could not be differentiated from others of the complex.

FIGURE 1.
Symptoms on lucerne leaves naturally infected with S. botryosum.

FIGURE 2.
Lucerne leaves naturally infected with S. botryosum showing atypical symptoms (left) and typical symptoms (right).

TABLE 4. A summary of overseas descriptions of symptoms caused by S. botryosum on lucerne (Tehon and Daniels, 1925; Smith, 1940; Benedict, 1954; Nelson, 1955).

1.	Parts infected	Leaf, stem, petiole, peduncle, flower and pod.
2.	Leaf symptoms	Numerous; initially punctate, 1-2 mm diameter, with a tan-coloured centre and reddish-brown margin. Become larger, irregular in shape, the reddish-brown margin becoming less distinct and with numerous, minute black pseudothecia randomly located over the greyish-tan centre; the total lesion surrounded by a straw-coloured halo. Under wet conditions enlarge rapidly and coalesce to involve most of leaf; become blackened due to intense production of conidia; leaves may shrivel and abscise.
3.	Stem and petiole symptoms	Small, black and linear; under wet conditions will elongate and coalesce to girdle and kill stem and petiole.
4.	Flower and pod symptoms	Symptoms not obvious or common. From floral infections can get subsequent pod and seed infection; pods become deformed; infected seed shrivelled and dark coloured.

However, further development produced the characteristic mature field symptoms, namely, large (1-2 mm in diameter), irregular lesions, with tan centres and distinct reddish-brown margins, in turn surrounded by a straw-coloured halo, and located on both leaflet surfaces. These latter symptoms were so characteristic as to enable ready identification of the disease.

A further lesion type was occasionally observed differing from the typical situation in not having the characteristic reddish-brown margin (Figure 2). Both lesion types were found randomly located on the same leaflets and the causal organisms when isolated from each lesion type proved to be morphologically identical. Further, when lucerne plants were inoculated with isolates of each fungus similar symptoms developed and with equal severity.

Although an overseas report (Nelson, 1955) indicated that protopseudothecia * and pseudothecia were commonly found within the tan centres of mature lesions and on infected leaf debris, they were not located during this study. However, in the laboratory protopseudothecia were readily produced on artificially inoculated leaves (Chapter IV A 1), but only in one experiment did they mature, to produce asci and ascospores that conformed to the perfect state, Pleospora herbarum (Fr.) Rab.

Petiole symptoms were rarely observed and only appeared as small, black, elongate lesions.

As the pathogen is associated with some New Zealand seed-lines (Percival and Wenham, 1972), floral infections must also occur in this country. However, in the present study symptoms on the reproductive structures of the host were not observed.

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* A protopseudothecium is a well developed ascostromatic ascocarp in which asci and ascospores have not differentiated. On maturity they develop bitunicate asci in clusters in one or more unwalled locules in a large stroma (pseudothecium) (Luttrell, 1965).

3. GLASSHOUSE OBSERVATIONS

Symptom development was observed in conjunction with studies conducted to fulfil the requirements of Koch's Postulates (Chapter II).

Symptoms first appeared on leaves within 48 hours of inoculation as small, black, circular necrotic areas, surrounded by a yellow halo, and randomly located on the upper leaf surface. By 10 days after inoculation lesions were numerous and ranged in size from pin-point up to 1.5mm in diameter (Figure 3), but with the majority remaining small (up to $\frac{1}{2}$ mm diameter). These lesions were circular to irregular in shape, black to brown in colour, slightly sunken, with a distinct margin and were often surrounded by a yellow halo. Lesions initially observed on the upper leaf surface were now also visible on the lower surface.

Only a few spots developed into the typical, mature symptom type as observed in the field and reported overseas, and these were more often located at the leaflet margin. In general, the glasshouse symptoms were similar to the initial stages of field infections, and only occasionally developed beyond this stage into the mature lesion type. As with field infections, lesioned leaflets failed to produce protopseudothecia when subjected to conditions of high humidity.

Symptoms on petioles similar to those found in the field were occasionally observed.

B. DISEASE CYCLE STUDIES

1. FACTORS AFFECTING INOCULUM PRODUCTION

A problem encountered while completing the requirements of Koch's Postulates was the reluctance of isolates to sporulate readily on cultural media. Before further inoculation studies could be conducted there was need for an investigation into those factors that would ensure ready and intense sporulation on agar media. A survey of the literature revealed that restricted conidial production on agar is a common feature of Stemphylium species, and that several techniques have been used to overcome this problem.

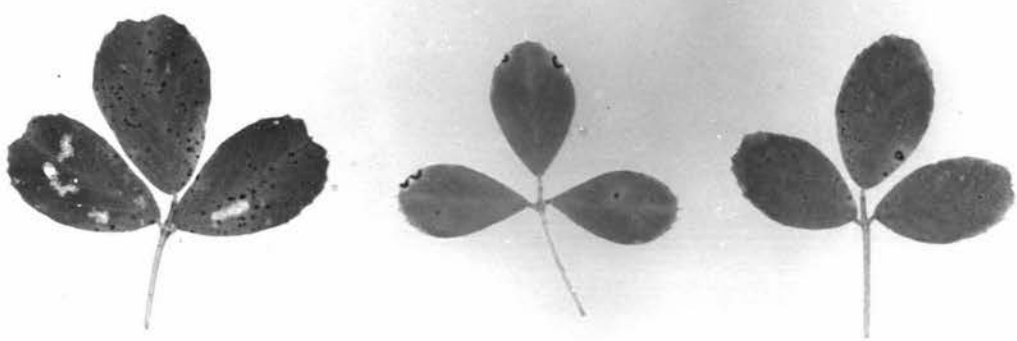


FIGURE 3.
Symptoms on lucerne leaves artificially inoculated with S. botryosum.

Several environmental factors are known to influence the intensity of conidial production of fungi on culture media, including substrate, incubation temperature, duration of incubation and light (Lilly and Barnett, 1951). These factors were investigated as follows.

In all experiments the plates were centrally inoculated with an agar plug (4 mm diameter) taken from a ten day old monosporous isolate growing on lab. PDA at 24°C.

The intensity of sporulation was determined using the method described by Tammen (1963) and Tate (1968). This involved flooding the plates with a known volume of distilled water, dislodging the conidia, passing the suspension of conidia and mycelial fragments through cheese-cloth and then ascertaining the conidial concentration using a Neubauer haemocytometer.

(a) Effect of substrate

The intensity of sporulation was determined following growth on the following media:

Lab. potato-dextrose agar	PDA_L
Maknur potato-dextrose agar	PDA_M
V-8 juice agar	V-8
Cornmeal agar	CMA
Oatmeal agar	OMA
Malt agar	MA
Prune agar	PrA
Water agar	WA
Lucerne decoction agar	LDA

The media were prepared as indicated in Appendix I. The plates were incubated for ten days in the dark at 24°C. There were three replicates of each treatment.

The results (Table 5) show that with the exception of WA some sporulation occurred on all media, with V-8 and PDA_L clearly the most satisfactory. However, the spore concentration on these latter two media was insufficient for critical glasshouse inoculations, thus requiring further studies to improve spore production. Lab. PDA was used in subsequent work due to the regular availability of its constituents and ease of preparation.

TABLE 5. Effect of substrate on the intensity of conidial production.

Medium	Conidia/ml ($\times 10^4$)			
	Replicate			
	1	2	3	Average
V-8	13	10	15	12.7
PDA_L	9	8	8	8.3
OMA	3	5	4	4.0
LDA	5	3	3	3.7
CMA	2	2	3	2.3
MA	2	3	1	2.0
PrA	0	2	1	1.0
PDA_M	1	0	1	0.7
WA	0	0	0	0

(b) Effect of temperature

The influence of temperature on sporulation was determined by incubating plates of PDA_L for ten days in the dark at the following temperatures:

4, 8, 12, 16, 20, 24, 28, 32 and 36 $\pm$ 1°C

There were three replicates per treatment.

The results (Table 6) show that sporulation was most intense following incubation at 24°C. At 12, 16 and 20°C sporulation plateaued at a constant level of approximately 8 x 10^4 conidia/ml, with a gradual decrease below 12°C and above 24°C. Sporulation did not occur at either 4°C or 36°C. The greater sporulation at 24°C may be partly attributable to the more frequent use of this incubator, and hence exposure of the cultures to irregular, short periods of natural light, assuming of course that light has a beneficial effect on sporulation.

TABLE 6. Effect of temperature on the intensity of conidial production.

Temperature (°C)	Conidia/ml (x10⁴)			
	Replicate			
	1	2	3	Average
4	0	0	0	0
8	3	4	3	3.3
12	7	10	8	8.3
16	9	6	6	7.0
20	8	8	9	8.3
24	16	15	18	16.3
28	5	7	7	6.3
32	3	5	5	4.3
36	0	0	0	0

(c) Effect of duration of incubation

Twenty-one inoculated PDA_L plates were incubated in the dark at 24°C. At two day intervals three plates were removed and the intensity of sporulation determined.

The results (Table 7) show that between the fourth and twelfth day there was a gradual increase in sporulation with a pronounced intensification over the next two days. It would seem that these results are related to colony size. Twelve days were required for colonies to completely cover the agar surface, and since conidia production lags behind vegetative growth, high sporulation levels in each plate were not attained until after this time; that is, on the fourteenth day. In subsequent work when requiring a bulk supply of conidia plates were incubated at 24°C for 12 days.

TABLE 7. Effect of duration of incubation on the intensity of conidial production.

Days after inoculation	Conidia/ml ($\times 10^4$)				Average colony diameter (mm)
	Replicate				
	1	2	3	Average	
2	0	0	0	0	27.0
4	3	2	3	2.7	37.5
6	2	4	3	3.0	48.0
8	4	5	5	4.7	57.2
10	6	5	6	5.7	72.0
12	6	6	7	6.3	82.5
14	11	10	11	10.7	85.0

(d) Effect of light

In recent years considerable research has been conducted on the significance of light in inducing spore production on culture media (Leach, 1961, 1962 ; Carlile, 1965). Graham (1957) and Leach (1962) found that when they exposed Stemphylium trifolii Graham to diffuse light and continuous near-ultraviolet radiation, respectively, it readily formed conidia and sclerotial bodies on common laboratory media, but would not do so in darkness. Hannon and Weber (1955) reported that sporulation of S. floridanum Hannon and Weber could be stimulated by normal or artificial daylight. Diener (1955) concluded from his experiments with S. solani Weber that the optimum wavelengths of light that stimulated conidial formation was between 312-546 nm.

The effect of light on sporulation was investigated by exposing PDA_L plates for ten days to the following treatments:

(i) Total darkness, provided by enclosing the plates in a metal cylinder.

(ii) Continuous near-ultraviolet (NUV) light provided by a Phillips 40 watt black light blue tube held 40 cm above the plates.

(iii) Cyclic (12 hour cycle) fluorescent light provided by a Phillips 80 watt cool white fluorescent tube held 40 cm above the plates.

(iv) Natural diurnal periods of diffuse light provided by placing the plates on a laboratory bench out of direct sunlight.

There were four replicates per treatment.

The results are summarised in Table 8. The fact that sporulation in all treatments exposed to a light source was significantly higher than in total darkness clearly demonstrated the beneficial effect of light on sporulation. Greatest sporulation occurred under NUV light and least under natural diurnal light. These findings were in accord with those of Diener (1955) working with S. solani, and Leach (1962, 1963, 1968) working with S. botryosum.

It must be noted that although the culture medium and source of inoculum were identical for all treatments, comparisons between treatments were not strictly valid since the plates of each series were not subjected to the same incubation temperature. However, the fact of sporulation in the NUV treatment being substantially greater than from the other treatments would suggest that this light source was of considerable significance. Accordingly, a further experiment was conducted to determine the effect on intensity of conidial production of different exposure durations to NUV light.

Inoculated PDA_L plates were initially incubated in the dark at 22-24°C for five days. The plates were then exposed to NUV light (at 22-24°C) with four plates being returned to the dark in the 22-24°C incubators after each of the following exposure periods:

0, 1, 6, 12, 24, 72, 120 and 168 hours.

At the completion of the last treatment period (168 hours = 7 days) the conidial concentration for all treatments was determined.

TABLE 8. Effect of light conditions on the intensity of conidial production.

Light treatment	Conidia/ml (x10⁴)				
	Replicate				Average
	1	2	3	4	
Darkness	10	8	7	5	7.5
Continuous NUV	78	62	68	65	28.2
Cyclic fluorescent	27	30	32	33	30.5
Natural/diurnal	10	21	35	19	21.2

The results (Table 9) clearly show the positive effect of time of exposure to NUV light in stimulating conidial production in cultures incubated at 22-24°C. In continuous darkness only sterile cultures were produced. Contrary to the findings of Leach (1967, 1968) there was no further increase in sporulation following return of the plates to the dark after exposure to NUV light for 12 hours.

TABLE 9. Effect of different exposure durations to NUV light on intensity of conidial production.

Exposure time (hrs)	Conidia/ml (x10⁴)				
	Replicate				Average
	1	2	3	4	
0	0	0	0	0	0
1	0	1	0	0	0.25
6	1	0	0	1	0.5
12	0	0	1	1	0.5
24	1	2	1	1	1.25
72	1	2	3	2	2.0
120	5	6	5	6	5.5
168	8	7	8	8	7.75

In view of the results from the above experiments the following procedure was adopted for obtaining large numbers of conidia:

Plates of PDA_L or V-8 juice agar (when available) were centrally inoculated and exposed to a continuous NUV light source for 12 days at 23-27°C. The conidia were harvested as previously described and the conidial concentration determined. Frequently concentrations of 150,000 conidia/ml were obtained and occasionally up to 200,000 conidia/ml. Higher concentrations were obtained by centrifugation followed by a haemocytometer estimation of conidial numbers and then simple dilution to the concentration required.

2. FACTORS AFFECTING THE ESTABLISHMENT OF INFECTION

During preliminary pathogenicity studies there was considerable variation in the extent to which plants became infected. It was considered that this was due to variation in such environmental variables as the inoculum medium and spore concentration used, the temperature prevailing during the establishment of infection, and the length of time plants were subject to a saturated environment following inoculation. Experiments were conducted to determine the best combination of these factors required to produce consistent and typical foliage lesioning. The experiments were conducted using either excised leaves in the laboratory (Yarwood, 1934), or potted plants in the glasshouse. In both experiments the variety was Wairau.

In the former method, whole leaves of the same age were removed from lucerne plants. These were set out in high humidity petri dishes (Tate, 1968) and spray inoculated to the point of surface saturation with a conidial suspension. Controls were provided by one replicate of leaves treated identically to the inoculated leaves (several replicates), but inoculated only with the inoculum medium. The control was essential to prevent any incorrect interpretation of symptoms caused by conditions not related to the experiment (for example, high temperatures). The results were recorded at or before leaflet abscission occurred in the controls, usually after 4-6 days at 20-24°C.

Inoculated potted plants were initially placed in a high humidity cabinet under the required environmental conditions and then transferred to a glasshouse bench where the temperature (18-22°C) was thermostatically controlled.

(a) Inoculum medium

The value of adding nutrients or other substances to the inoculum suspension to increase the amount of infection is well known (Martinez and Hanson, 1963; Renfro and Wilcoxson, 1963). Such nutrients are considered to increase the inoculum potential of the pathogen (Banttari and Wilcoxson, 1964). By adding a sticker-spreader (unspecified) to his inoculum suspension, Benedict (1954) obtained typical symptoms of Stemphylium leaf spot on lucerne.

In the present study the effect on the establishment of infection of adding nutrients and stickers to the inoculum was determined. The nutrients and stickers used were as follows:

1% dextrose
1% lactose
1% gelatin (Difco)
0.5% gelatin (Difco)
1% agar (Davis)
distilled water

The inoculum suspension was obtained by flooding two plate cultures per treatment with the inoculum medium and then harvesting the conidia. After adjusting the conidial concentration of each treatment to approximately 90,000-110,000 conidia/ml, two pots per treatment were inoculated. At the time of inoculation the plants were 8-10 cm high and eight weeks old. The inoculated plants were held in a high humidity cabinet at 20°C for 72 hours, and then transferred to a glasshouse bench. Control plants were inoculated with distilled water only, but otherwise given identical conditions as the test plants. The extent to which each treatment affected the establishment of infection was determined by recording the number of lesions produced (expressed as a disease intensity rating) seven days after inoculation. The type of lesion produced was also recorded.

The results (Table 10; Figure 4) show that all the treatments, when compared with distilled water, gave an increased number of infections, with 1% gelatin and 1% lactose the most effective.

In all treatments the majority of lesions were not typical of those found in the field, being more typical of pepper spot caused by L. trifolii. That is, lesions were small, black, slightly sunken, occasionally surrounded by a yellow halo and more rarely with a light-coloured centre.

Although the addition of 1% gelatin provided the greatest increase in infection, use of this additive had the distinct disadvantage of blocking the atomiser and causing leaflets to adhere together. Accordingly, in subsequent experiments 1% lactose was used.

(b) Inoculum concentration

The effect on disease intensity and symptom expression was determined by using the following inoculum concentrations:

190,000 conidia/ml
155,000 "
100,000 "
58,000 "
32,000 "
13,000 "

The inoculum concentrations were obtained by serial dilution of a conidial suspension harvested from ten plate cultures flooded with a 1% lactose solution. Two pots of young plants (approximately 12 cm high) per treatment were atomised to the point of run-off with each respective conidial suspension, held in the high humidity cabinet for 72 hours at 20°C, and then transferred to a glasshouse bench. Control plants were inoculated with 1% lactose only. The effect of each treatment was assessed seven days after inoculation.

TABLE 10. Effect of inoculum media on disease intensity and symptom expression.

Inoculum medium	Inoculum concentration (conidia/ml)	Disease intensity *	Symptom expression
1% dextrose	90,000	2	Lesions on leaflets and petioles; pin-point to ½ mm dia; majority brown to black, with indefinite margin and yellow halo; typical lesions present. Some leaflets chlorotic.
1% lactose	116,000	3	Lesions on leaflets and petioles; pin-point to ½ mm dia; majority brown to black, slightly sunken with indefinite margin and yellow halo; in general, larger lesions more typical.
1% gelatin	95,000	4	Lesions on leaflets and petioles; on leaflets pin-point to 1 mm dia; majority black, irregular, slightly sunken, with indefinite margin and little local chlorosis; typical lesions abundant. Many leaflets chlorotic, due to lesion coalescence. On petioles lesions small, light-coloured, elongate.
0.5% gelatin	105,000	2	Lesions on leaflets and petioles; on leaflets wide variation in size (pin-point to 1-1.5 mm dia), irregular, black with indefinite margin and little local chlorosis; larger lesions more typical.
1% agar	112,000	1	Lesions only on leaflets; pin-point to 1 mm dia; majority large, irregular, with tan centre and dark, definite margin.
distilled water	113,000	1	Lesions only on leaflets; pin-point to 1 mm dia; majority small, irregular, black, with a definite margin; larger lesions more typical.

Continued over....

TABLE 10. (contd)

Inoculum medium	Inoculum concentration (conidia/ml)	Disease * intensity	Symptom expression
Control (distilled water)	-	0	Symptoms not produced.

* 0 = no lesions
1 = slight lesioning
2 = moderate lesioning
3 = severe lesioning
4 = very severe lesioning

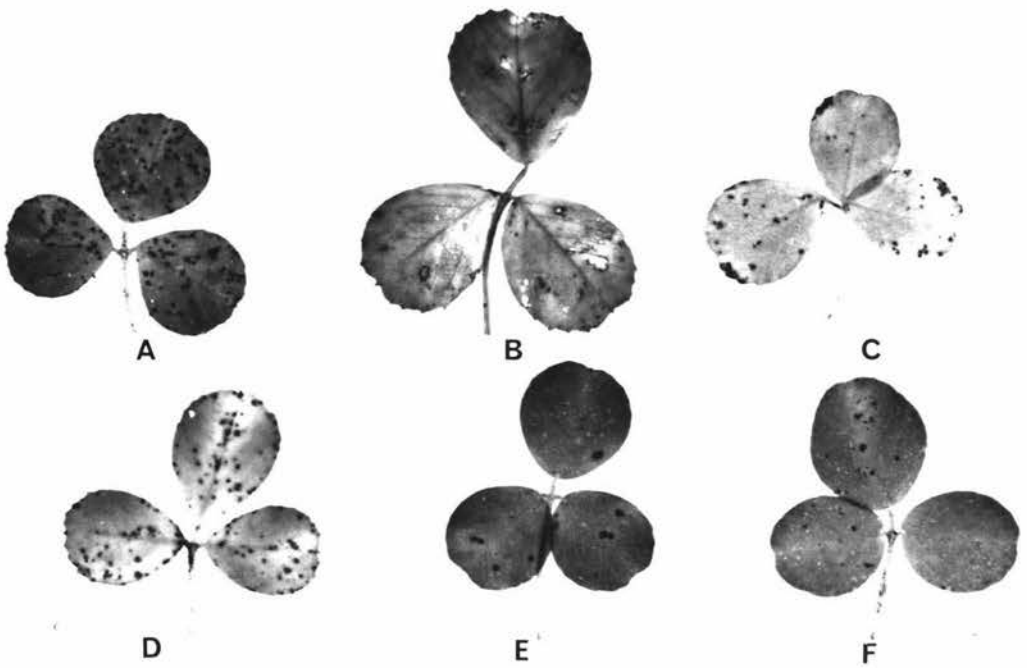


FIGURE 4.

Comparison of leaf symptoms after inoculation with S. botryosum using different inoculum media.

A. 1% gelatin
B. 0.5% gelatin
C. 1% dextrose
D. 1% lactose
E. 1% agar
F. distilled water

The results (Table 11; Figure 5) show that a moderate infection was obtained using conidial concentrations of 58,000 conidia/ml and above. However, in each instance symptom expression was not typical of the disease in that lesions were devoid of the characteristic tan centre and reddish-brown margin. The heaviest concentration (190,000 conidia/ml) resulted in some lesion coalescence and chlorosis of leaflets not normally found in the field, whereas the conidial concentrations of 58,000, 100,000, and 155,000 conidia/ml produced degrees of foliage lesioning commonly present under natural situations. Accordingly, in subsequent inoculations a conidial concentration between 60,000 and 150,000 conidia/ml was used.

The control plants remained healthy throughout the experiment.

(c) Temperature

Temperature has a considerable effect on most phases of the disease cycle, especially the establishment of infection and the incubation period. The present experiment was conducted to determine the optimum temperature for the completion of the disease cycle up until first evidence of infection.

Excised leaves were subjected to an environment of approximately 100% relative humidity by placing in petri dishes lined with moistened filter paper. The leaves were spray inoculated with a spore suspension (60,000 conidia/ml) and the petri dishes incubated at a range of constant temperatures (15, 20, 25 and 30°C) for 72 hours. There were four replicates per temperature. At 24, 48 and 72 hours after inoculation the mean number of lesions per treatment was recorded. This gave an indication of disease intensity for each temperature at each point in time.

TABLE 11. Effect of inoculum concentration on disease intensity and symptom expression.

Inoculum concentration (conidia/ml)	Disease intensity *	Symptom expression
190,000	4	Lesions atypical; are large (up to ½ mm dia.), black, randomly scattered over the leaflets; some lesion coalescence and chlorosis of leaflets.
155,000	3	Lesions atypical; are pin-point, black, with a definite margin and little chlorosis; some lesion coalescence.
100,000	2-3	Few typical lesions, majority atypical; small (¼ mm dia.), black and slightly sunken with little chlorosis.
58,000	3	Few typical lesions, majority atypical; small (¼ mm dia.), black and slightly sunken with little chlorosis.
32,000	2	Lesions atypical; very small, dark-brown, with a definite margin and slightly sunken; no chlorosis.
13,000	0-1	Six leaves with lesions; atypical; very small, black, with an indefinite margin; no chlorosis.
0	0	Lesions not produced.

* See Table 10

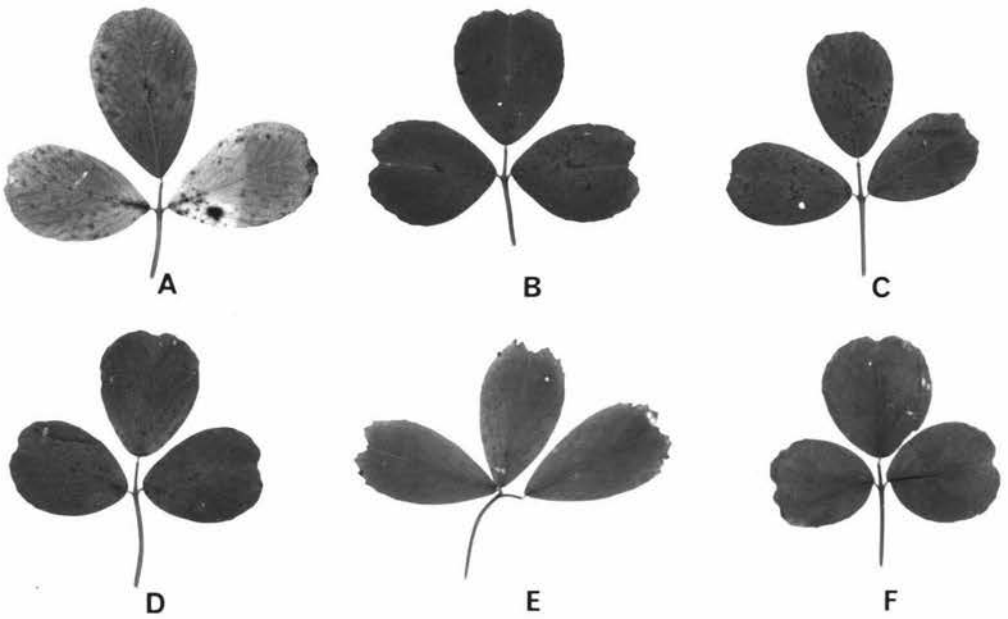


FIGURE 5.
Comparison of leaf symptoms and disease intensity after inoculation with S. botryosum using six conidial concentrations.

A. 190,000 conidia/ml
B. 155,000 "
C. 100,000 "
D. 58,000 "
E. 32,000 "
F. 13,000 "

The results (Table 12) show that at 72 hours from the time of inoculation the greatest number of lesions were produced at 25°C, and least at 15°C. At 20, 25 and 30°C lesions were visible within 24 hours, indicating the rapidity at which the disease cycle up until symptom expression can be completed. At 15°C this stage was reached within 48 hours. The control leaves remained healthy throughout the experiment.

These results are similar to those of Nelson (1955) and McDonald (1958), but differ from those of Renfro and Kernkamp (1963) who found S. botryosum on lucerne to be most pathogenic at 30°C.

In subsequent inoculations temperatures within the inoculation cabinet were maintained between 20 and 25°C.

TABLE 12. Effect of temperature on disease intensity 24, 48 and 72 hours after inoculation.

Temperature (°C)	Mean number of lesions/treatment		
	24 hours	48 hours	72 hours
15	0	2.0	4.5
20	1.5	15.2	21.2
25	2.7	18.0	22.7
30	1.5	12.0	20.0
control	0	0	0

(d) Humidity

Following inoculation plants must be held under conditions of high humidity for a period sufficient to ensure spore germination, host penetration and the establishment of infection. The following experiment was aimed at determining the length of time that both excised leaves and potted plants need be held under high humidity conditions following inoculation to produce moderate infection levels.

(i) Excised leaf method

Excised leaves in petri dishes were spray inoculated with a spore suspension (60,000 conidia/ml) and exposed to 100% R.H. for 0, 24, 36, 48, 60, 72 and 96 hours at 24°C. After each exposure period the plate lids were removed to reduce the relative humidity around the leaves. The filter paper liners were kept moistened to prevent dehydration and the effectiveness of the treatments was measured after 96 hours by recording the number of lesions per leaf.

The results (Table 13) show that some infection occurred without the provision of high humidity conditions. However, for a moderate intensity of lesioning to result, at least 36 hours was required.

The control leaves were healthy at the time of assessment.

TABLE 13. Effect of high humidity duration following inoculation on disease intensity.

High humidity duration (hours)	Number lesions/leaf				Treatment mean
	Replicate				
	1	2	3	4	
0	-	5	1	-	1.5
24	1	4	3	14	5.5
36	7	7	31	21	16.5
48	15	15	22	56	27.0
60	16	21	18	17	18.0
72	28	10	31	29	24.5
96	17	18	9	24	17.0
control	-	-	-	-	-

(ii) Pot plant method

Eight week old plants were inoculated with a spore suspension in 1% lactose (90,000 conidia/ml) to the point of run-off and then placed in a high humidity cabinet. At 0, 24, 36, 48, 60, 72 and 96 hours two pots were removed to a glasshouse bench. Disease intensity and symptom expression of all treatments was recorded 96 hours from the time of inoculation; that is, after removal of the last treatment from the high humidity cabinet. Two pots inoculated with 1% lactose and maintained under high humidity for the longest duration served as controls.

The results (Table 14) were essentially similar to those of the previous experiment and show that to obtain moderate disease intensity plus the expression of typical symptoms, 24 hours high humidity duration is required. Nelson (1955) found that at 22-27°C, 12 to 24 hours high humidity was needed for maximum infection. However, in the present study as lesions were still small in size, 36 hours was considered to be the minimum to give consistent symptom expression.

The control plants were healthy at the time of assessment.

As a result of the above experiments the following procedure was adopted for use in later inoculation studies:

> Conidial suspensions of approximately 60,000 - 150,000 conidia/ml in a 1% lactose nutrient medium were atomised on to foliage to the point of run-off. The potted plants were then held in a high humidity cabinet for at least 36 hours at 20-25°C, and then transferred to a glasshouse bench.

TABLE 14. Effect of high humidity duration following inoculation on disease intensity and symptom expression.

High humidity duration (hours)	Disease intensity *	Symptom expression
0	1	Typical symptoms produced.
24	2	Majority symptoms typical, but small.
36	3	Symptoms from small, black spots to typical lesion type, i.e. tan centre, reddish-brown margin, and surrounded by a yellow halo.
48	2	Symptoms similar in range to those observed at 36 hours.
60	3	Symptoms similar in range to those observed at 36 hours.
72	4	Symptoms from black, moderate size lesions, with an indefinite margin and chlorotic halo to typical lesion type; severe leaflet defoliation and chlorosis.
96	4	Symptoms very similar in range to those observed at 72 hours.
control	0	Symptoms not produced.

* See Table 10.

CHAPTER IV

THE PATHOGEN

A. MYCOLOGICAL STUDIES

Mycological features of the causal fungus were studied both on the host and on agar to determine whether the pathogen as present in the Manawatu conformed with overseas descriptions. Although the Stemphylium leaf spot disease of lucerne was first reported fifty-five years ago (Gentner, 1918) and has since been recorded in many parts of the world (Nelson, 1955; Brien and Dingley, 1959; Focke, 1966; Perisic and Stojanovic, 1967), the only account of the mycology of the pathogen is that by Smith (1940). Isolates from lucerne have not previously been studied in New Zealand.

1. MYCOLOGICAL FEATURES ON THE HOST

The causal organism is characterised by both an imperfect state (Stemphylium botryosum Wallr.) and a perfect state (Pleospora herbarum (Fr.) Rab.). Whereas asexual reproduction was readily produced in the field, pseudothecia were not encountered. However, mature sexual fruiting bodies were formed on excised leaflets in the laboratory enabling then the study of the morphology of pseudothecia, asci and ascospores.

(a) Materials and Methods

(i) Morphological features of the asexual state.

Abundant production of conidiophores and conidia occurred within 2-3 days when infected leaflets were subjected to conditions of high humidity in petri dishes lined with moistened filter paper. Slides were prepared by transferring conidiophores and conidia to a drop of Shear's mounting fluid on a glass slide using a flamed needle. The morphology of the conidiophores and conidia were microscopically determined and measurements made using an eye-piece micrometer.

(ii) Production of the perfect state on autoclaved excised leaflets

Pseudothecia were produced on autoclaved excised leaflets in petri dishes, following the method of McDonald (1958). This involved spray inoculating leaflets in petri dishes with a conidial suspension and incubating for four days at 24°C, after which the petri dishes were

placed outside the laboratory on a wall facing east. The leaves were examined after a further 7 and 17 days for the presence of pseudothecia. The experiment was repeated several times between April and August when air temperatures and daylength were low.

At 7 days protopseudothecia were in evidence, and by 17 days mature fruiting bodies were formed. Slides were prepared of pseudothecia, asci and ascospores and their morphology determined under the high power (x 400) of a compound microscope.

(b) Results and Discussion

(i) Morphological features of the asexual state

The morphology of conidiophores and conidia of the fungus are summarised and compared with those recorded overseas in Table 15.

The morphological features as described correspond closely with those recorded overseas for the species in general (Booth and Pirozynski, 1967b; Simmons, 1967), and for the species on lucerne (Smith, 1940). That is, the conidiophores were brown, upright, with a darker, swollen apical sporogenous cell which was slightly roughened at its apex, and were 1-9 septate and 5-64u long. The light brown, muriform conidia (Figure 6) were variously subspherical to ovoid or oblong, echinulate, and with a major constriction at the median transverse septum. Their average dimensions were 17.6 x 29.4u with a length/breadth ratio of 1.67.

Simmons (1969) described the diagnostic features of the type material of S. botryosum to include the maximum conidial dimensions (23 x 33u), a length/breadth ratio in the range of 1.0-1.5, and the conspicuous constriction at the median transverse septum. These features conform very closely with the measurements and observations made in the present study and help give confirmation of the identity of the causal organism as Stemphylium botryosum.

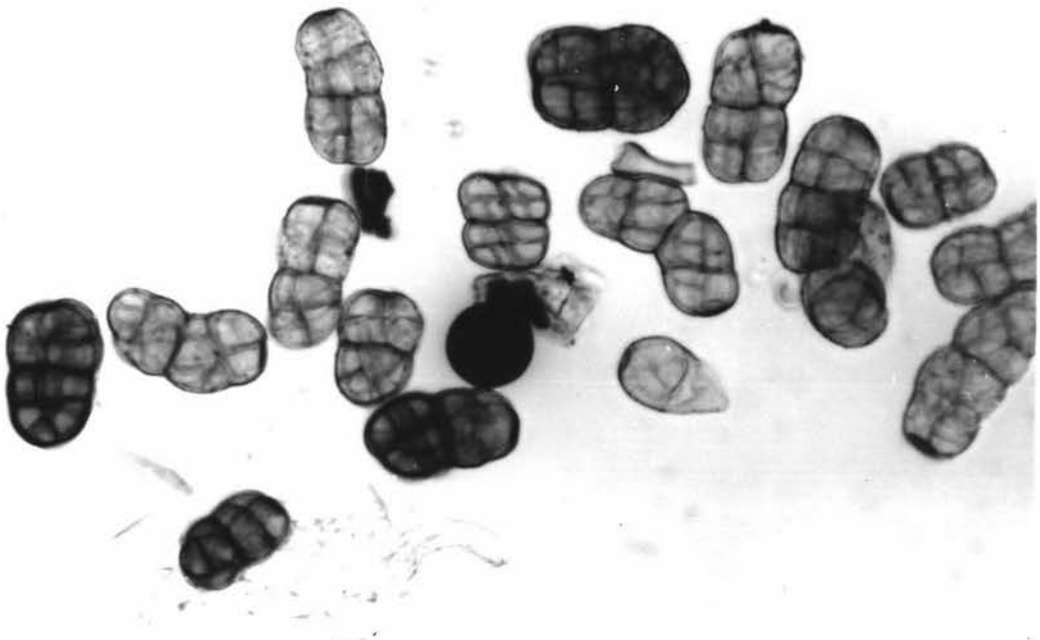
TABLE 15. Comparison of the morphological features of conidiophores and conidia of S. botryosum from naturally infected host tissue.

Fungal Structure	Author	Smith (1940)	Booth and Pirozynski (1967b)	Simmons (1969)
Conidiophores				
(i) Morphology	Brown, upright, single with slightly darker, swollen apical sporogenous cell; slightly roughened toward the apex. Nodulose appearance due to proliferation through apical pore or branching at or near apex; 1-9 septate.	Brown, upright, either single or grouped in fascicles (rarely 4 hyphae); bulbous at base and apex. Nodulose appearance due to continued apical growth.	Pale brown to brown, erect, with swollen apical sporogenous cell; slightly roughened toward the apex. Several successive sporogenous cells may form by proliferation through the apical pore; 1-7 septate.	Dilute to medium olive-brown, straight to variously bent or curved, simple or occasionally 1-branched, with swollen apical cell; slightly roughened toward the apex. Up to 4 successive sporogenous cells produced by renewal of conidiophore growth through previously formed apical pores; 1-7 septate.
(ii) Dimensions:				
a) Length	5 - 64u	-	20 - 72u	20 - 72u
b) Diameter	-	3.5 - 5.5u	4 - 6u	4 - 6u

Continued over....

TABLE 15. (contd)

Fungal Structure	Author	Smith (1940)	Booth and Pirozynski (1967b)	Simmons (1969)
Conidia				
(i) Morphology	Variously subspherical to broadly ovoid or oblong, light brown, echinulate; 3(-5) transverse, 1-2 complete or near complete series of longitudinal septa, with major constriction at median transverse septum.	Oblong, olive-brown, echinulate, and muriform; constricted at median transverse septum.	Oblong to ovoid or subdoliiform, olive to brown, with a roughened outer wall; occasionally constricted at 1-3 transverse septa and at 1-3 longitudinal septa if complete.	Oblong or broadly ovoid to subdoliiform, dilute to deep olive-brown, echinulate; slightly to conspicuously constricted at 1-3(-4) complete or nearly complete series of longitudinal septa.
(ii) Dimensions:				
a) Length-range	22.3 - 33.4u	24 - 40u	-	24 - 33u
mean	29.4 ± 0.4u	31 ± 0.4u	28.5u	28.3u
b) Breadth-range	14.1 - 24.2u	14 - 23u	-	15 - 24u
mean	17.6 ± 0.3u	19 ± 0.2u	19.5u	19.5u
c) L/B ratio	1.67	1.63	1.46	1.50



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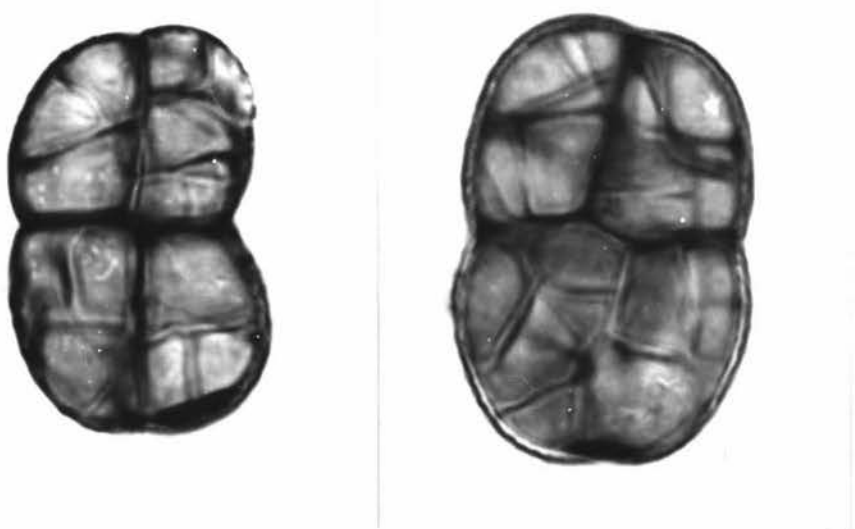

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FIGURE 6.

Conidia of S. botryosum produced on a naturally infected leaf subjected to high humidity conditions for three days.

(ii) Production of the perfect state on autoclaved excised leaflets

Within 21 days after inoculation pseudothecia had been produced over the surface of the excised autoclaved leaflets. Five to twenty asci per pseudothecium in all stages of maturation were observed.

The morphological features of the perfect state of the fungus are summarised and compared with those recorded overseas in Table 16.

The black, globose pseudothecia were erumpent in the host tissue and contained several bitunicate, narrowly cylindrical to clavate asci. These asci were obtuse at their apex, tapering to a swollen or claw-like base and with average dimensions of 30.2 x 184.6u (Figure 7). The yellow-brown, muriform ascospores were ellipsoid to clavate in shape with slight constrictions at all 7 transverse septa. Their average dimensions were 16.3 x 37.2u with a length/breadth ratio of 2.28.

Allowing for variability in environmental conditions and between isolates of the pathogen, this description of pseudothecia, asci and ascospores conforms closely to that described by Smith (1940) and Booth and Pirozynski (1967b) for Pleospora herbarum.

2. MYCOLOGICAL FEATURES ON AGAR

Cultural studies involving the observation of the macroscopic and microscopic features of the fungus, its physiological behaviour with respect to temperature and media, and the production of the perfect state were carried out. The cultural characteristics studied were gross colony features such as shape, colour and growth rate (macroscopic features) and the morphology of conidiophores, conidia, pseudothecia, asci, and ascospores (microscopic features). In the physiological studies the effect of temperature and media on gross colony features and growth were also examined.

TABLE 16. Comparison of the morphological features of the perfect state of S. botryosum from infected host tissue.

Fungal Structure	Author	Smith (1940)	Booth and Pirozynski (1967b)
Pseudothecia			
(i) Morphology	Globose, black, randomly scattered, erumpent in tissue.	Globose, black, sometimes possess a slender neck.	Globose or somewhat flattened, scattered, immersed to erumpent in host tissue.
(ii) Diameter	185.0 - 421.0u	-	100 - 500u
Asci			
(i) Morphology	Bitunicate, cylindrical to clavate, obtuse apex and tapering to swollen or claw-like base, with 8 ascospores.	Elongate, cylindrical and typically 8-spored.	Bitunicate, cylindrical to clavate, with 8 irregularly distichous ascospores.
(ii) Dimensions:			
a) Length-range	125.0 - 229.0u	183 - 267u	90 - 250u
-mean	184.6 ± 1.36u	239 ± 0.2u	-
b) Breadth-range	22.4 - 38.6u	27 - 37u	20 - 50u
-mean	30.2 ± 0.4u	34 ± 0.3u	-

Continued over....

TABLE 16. (contd)

Fungal Structure	Author	Smith (1910)	Booth and Pirozynski (1967b)
Ascospores			
(i) Morphology	Ellipsoid to clavate, light to dark yellow-brown, ends obtuse, sub-apical quarter more swollen, 7 transverse septa, 1-4 complete or near complete series of longitudinal septa, slight constrictions at all transverse septa.	Rounded at both ends, yellow to brown, muriform, 7 cross septa and 3-5 longitudinal septa.	Ellipsoid to clavate, light to dark yellow-brown, 7-septate, slightly constricted at the 3 primary transverse septa, finally muriform.
(ii) Dimensions:			
a) Length-range	29.6 - 44.2u	32 - 48u	26 - 50u
-mean	37.2 ± 0.4u	39 ± 0.4u	-
b) Breadth-range	11.6 - 19.4u	14 - 21u	10 - 20u
-mean	16.3 ± 0.3u	17 ± 0.2u	-
c) L/B ratio	2.28	2.30	-

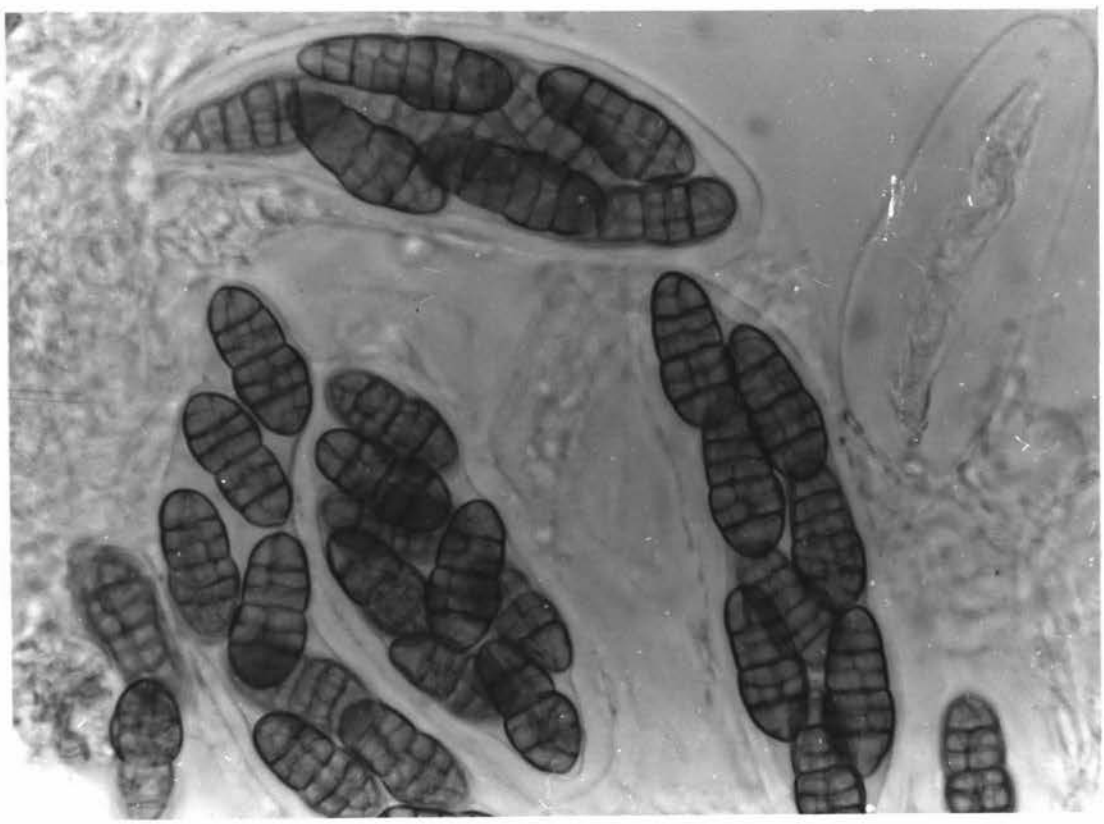

FIGURE 7.
Mature asci of P. herbarum produced on artificially inoculated lucerne leaflets. (x 400).

The origin of the isolates utilised in this section of the study were as follows:

Isolate	Source	Locality
1	Lucerne leaves	Kairanga
2	Lucerne leaves	D.S.I.R., Palmerston North
3	Lucerne seed (var. Wairau)	Christchurch

(a) Materials and Methods

(i) Cultural characteristics

In the following investigation isolates 2 and 3 were grown on PDA_L at 24°C, with each isolate replicated on three plates. The colony diameter was measured on the fourth, seventh and tenth day by taking the average of two measurements at right angles to each other. The macroscopic features of the isolates were also observed at these times.

To obtain sufficient production of conidia ten day old cultures were placed under NUV light for four days. The production of pseudothecia was induced by transferring some cultures to an 8°C incubator. Leach (1971) reported that protopseudothecia readily matured at this temperature.

In the microscopic studies small portions of the colonies were mounted in Shear's mounting fluid on glass slides and observed under a compound microscope. Using an eye-piece micrometer the dimensions of conidia were determined on the fourteenth day, and pseudothecia, asci and ascospores on the sixth week following transfer to the 8°C incubator.

(ii) Physiological features

The reaction of the fungus to temperature and media was examined by recording its radial growth after four, seven and ten days incubation and its gross colony characteristics after ten days.

a. The effect of temperature

The effect of temperature on the gross colony characteristics and growth on PDA_L of two isolates (isolates 1 and 2) was examined. Each isolate was replicated on four plates at each of the following nine temperatures:

4, 8, 12, 16, 20, 24, 28, 32 and 36 ± 1°C.

b. The effect of media

The effect of media on the gross colony characteristics and radial growth at 24°C of two isolates (isolates 1 and 2) was examined. Each isolate was replicated on three plates for each of the following media: Laboratory potato-dextrose agar (PDA_L), Maknur potato-dextrose agar (PDA_M), oatmeal agar (OMA), cornmeal agar (CMA), malt agar (MA), prune agar (PrA), V-8 juice agar (V-8), water agar (WA), and lucerne decoction agar (LDA). These were prepared as described in Appendix I. The pH of a molten sample of each medium (at 45°C) was also determined.

(b) Results and Discussion

(i) Cultural characteristics

a. Macroscopic features

The essential macroscopic features of the fungus on PDA_L after four, seven and ten days incubation at 24°C are summarised in Table 17, and its gross colony characteristics on V-8 juice agar after ten days incubation at 24°C is illustrated in Figure 8. The average growth rate of each isolate is illustrated graphically in Figure 9.

After ten days incubation at 24°C on PDA_L, colonies were 50 mm in diameter, and were circular, flat, devoid of aerial mycelium and had an entire margin. Viewed from above the colonies were olive-green at the centre and surrounded by a 3-4 mm wide white margin. In the older central area of colonies abundant conidia and a few black protopseudothecia were in evidence.

The macroscopic features on culture media of *S. botryosum* from lucerne have not been previously recorded, but they essentially agree with descriptions given for the species from other hosts (Petzer, 1958; Rotem, Cohen and Wahl, 1966).

b. Microscopic features

The microscopic features of conidiophores, conidia, pseudothecia, asci and ascospores are summarised in Table 18 and illustrated in Figures 10 - 12.

TABLE 17. The macroscopic colony characteristics of *S. botryosum* on PDA_I after four, seven and ten days at 24°C.

Macroscopic features	Description
Fourth day	
1. Colony shape	Circular, flat with entire margin.
2. Colony colour	Centre and middle olive-green, margin white, some slight sectoring.
3. Aerial mycelium	Absent.
4. Spore body production	From centre/middle moderate conidia production. No protopseudothecia.
Seventh day	
1. Colony shape	Circular, flat with entire margin.
2. Colony colour	Centre and middle olive-green, small margin (3-4 mm) white, sectoring more prominent.
3. Aerial mycelium	Absent.
4. Spore body production	From centre/middle greater conidia production than at fourth day. Protopseudothecia being formed, few in number, black, carbonaceous, scattered around colony centre.
Tenth day	
1. Colony shape	Circular, flat with entire margin.
2. Colony colour	As on seventh day.
3. Aerial mycelium	As on seventh day.
4. Spore body production	Abundant conidia production from centre/middle, giving black tinge to colony. A few protopseudothecia scattered randomly over centre/middle of colony.

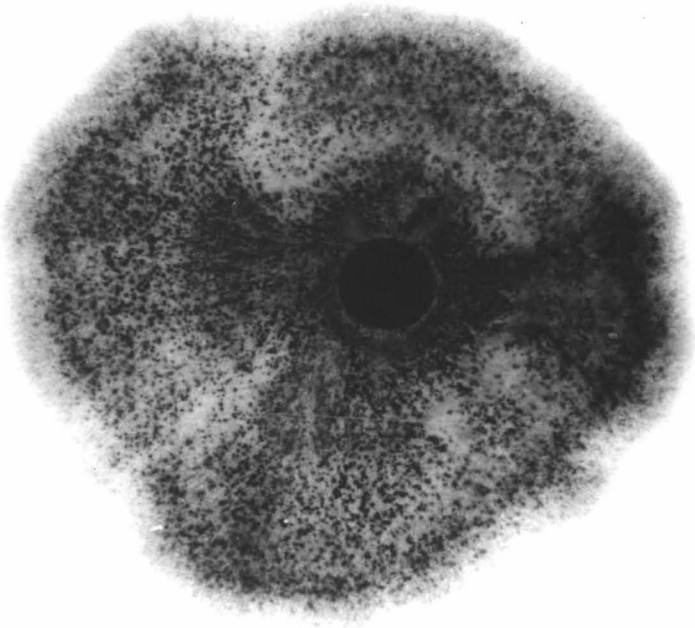

FIGURE 8.
Colony of S. botryosum (isolate 3) after ten days growth on V-8 juice agar at 24°C in the dark.
Note the intensity of protopseudothecia production.

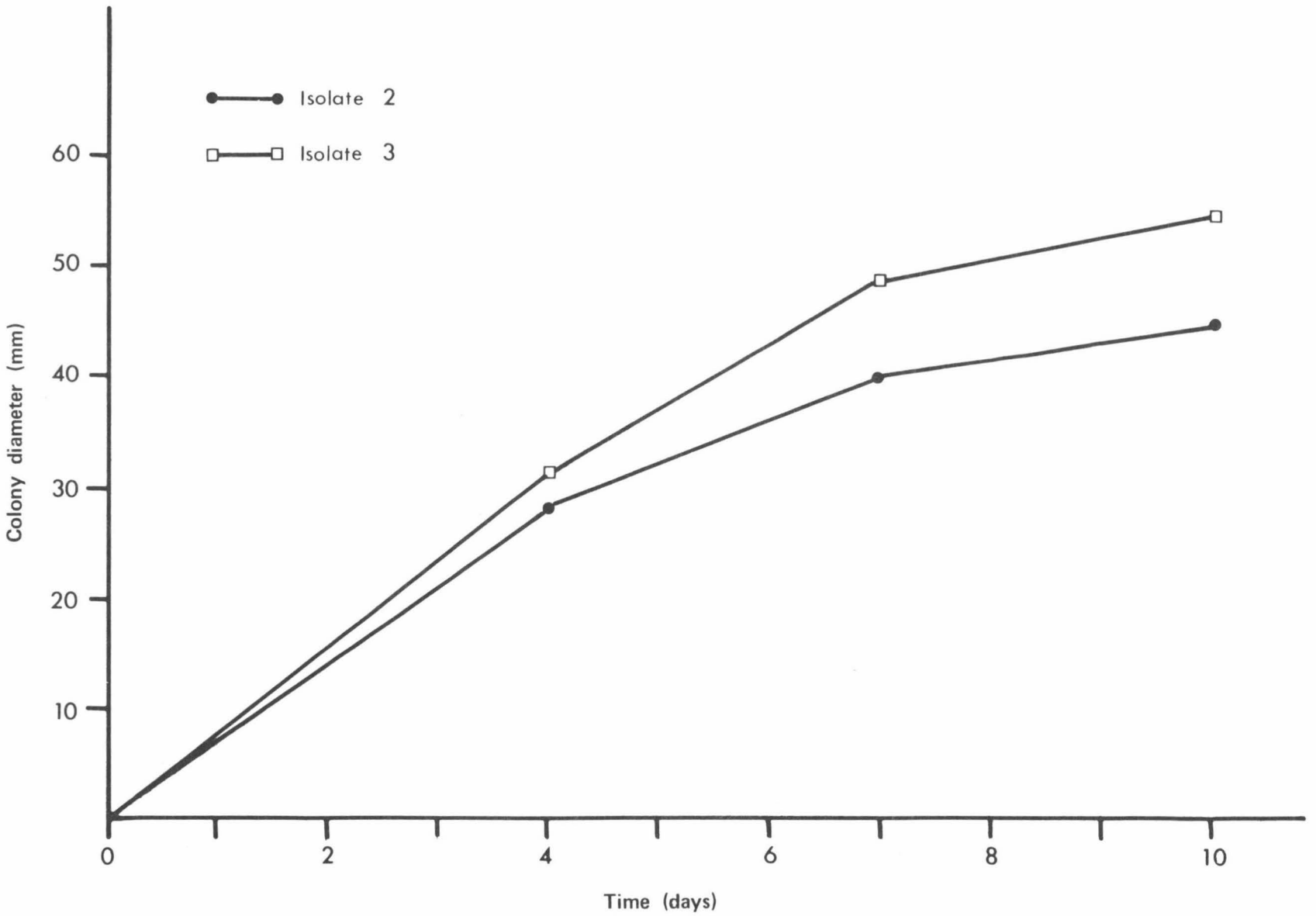

FIGURE 9. Growth rate of S. botryosum (isolates 2 and 3) on PDA_L at 24 C.

TABLE 18. Description of the microscopic structures produced by S. botryosum on culture media.

Fungal Structure	Description
1. Conidiophores	
(a) Morphology	Branching, erect, 0-5 septate, with swollen apical cell which is slightly roughened toward the apex; sometimes nodulose due to apical proliferation.
(b) Colour	Brown, darker towards apex.
(c) Dimensions:	
Length	12.6 - 94.2u
Diameter apical cell	5.9 - 7.6u Av. 6.6u
2. Conidia	
(a) Production	Borne on apex of swollen apical cell; production stimulated by NUV light.
(b) Shape	Variously subspherical to oblong or broadly ovoid, constricted at medium transverse septum and occasionally at other transverse septa; when immature spherical to oval.
(c) Colour	Light to dark olive-brown; when immature translucent yellow-brown to olive-brown.
(d) Septation	3(-5) transverse and 1-3 complete or incomplete series of longitudinal septa.
(e) Epispore sculpture	Distinctly roughened or echinulate.
(f) Dimensions:	
Length	21.8 - 41.7u Av. 33.72 ± 0.52u
Breadth	18.3 - 35.3u Av. 24.05 ± 0.41u
L/B ratio	1.40

Continued over....

TABLE 18. (contd)

Fungal Structure	Description
3. Pseudothecia	
(a) Production	Variable; induced by exposure to NUV light for 7 days followed by incubation at 8°C for 4-6 weeks.
(b) Disposition	Immersed, becoming erumpent as mature.
(c) Morphology	Globose, black, ostiolated with a short apical beak being produced as mature.
(d) Diameter:	
Range	246.0 - 389.0u
Average	304.18 ± 5.56u
4. Asci	
(a) Morphology	Narrowly cylindrical to clavate, bitunicate, with obtuse apical end and tapering to a swollen or claw-like base. Contain 8 ascospores arranged randomly in one oblique series.
(b) Dimensions	
Length	140.0 - 238.0u Av. 188.24 ± 2.96u
Breadth	21.2 - 35.2u Av. 28.08 ± 0.41u
5. Ascospores	
(a) Shape	Ellipsoid to clavate with ends obtusely rounded; slight constrictions at all transverse septa, sub-apical quarter usually more swollen.
(b) Colour	Initially pale yellow, deepening to translucent yellow-brown.
(c) Septation	7 transverse septa, 1 complete longitudinal septum and incomplete series across 1-4 of the transverse septa.
(d) Dimensions	
Length	28.2 - 38.9u Av. 34.14 ± 0.38u
Breadth	12.7 - 17.6u Av. 14.90 ± 0.15u
L/B ratio	2.29

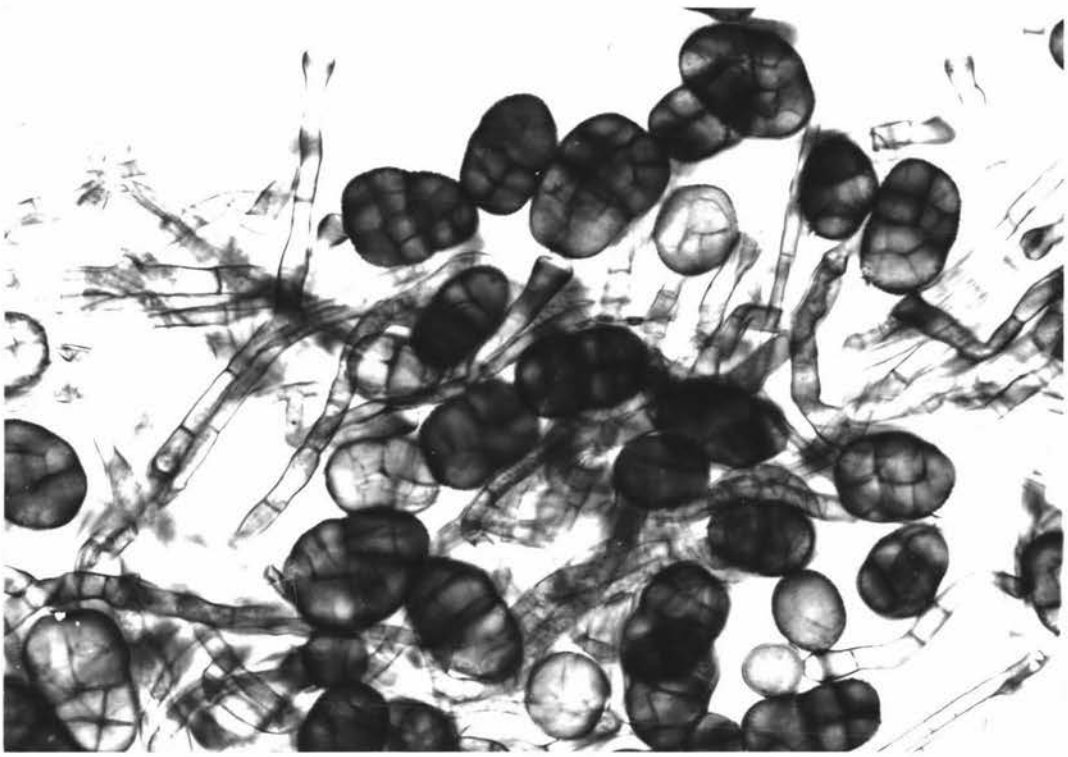

FIGURE 10.
Conidia produced by S. botryosum (isolate 2) after ten days incubation on PDA_L at 24°C in the dark. (x400)

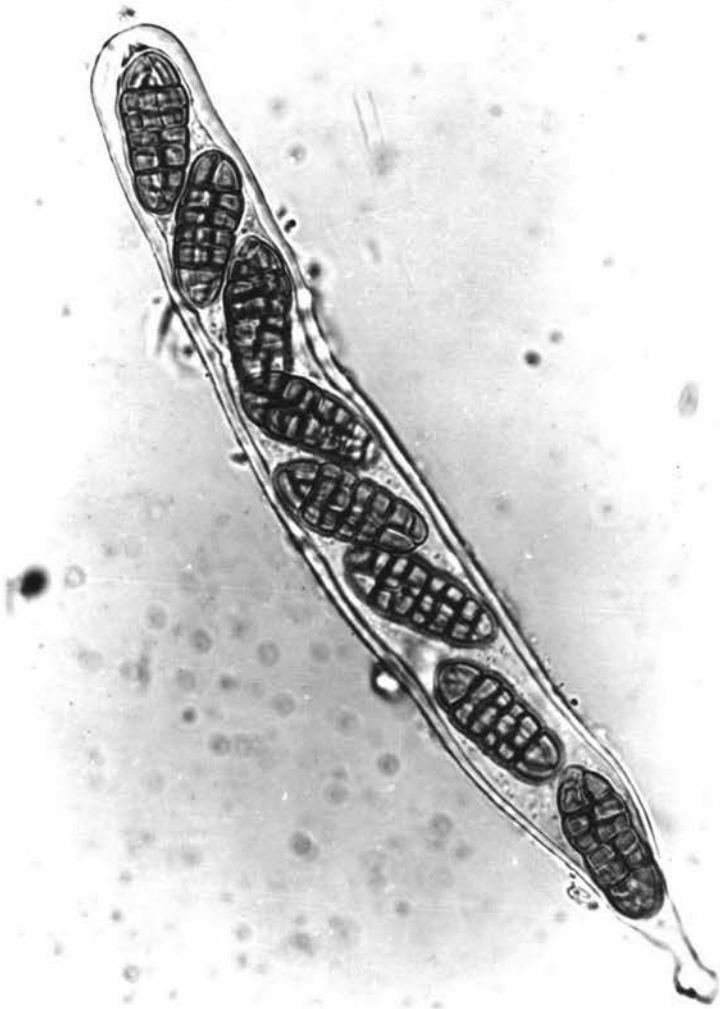

FIGURE 11.
Ascus containing eight ascospores from a P. herbarum colony on V-8 juice agar after six weeks incubation at 8°C in the dark. (x500)

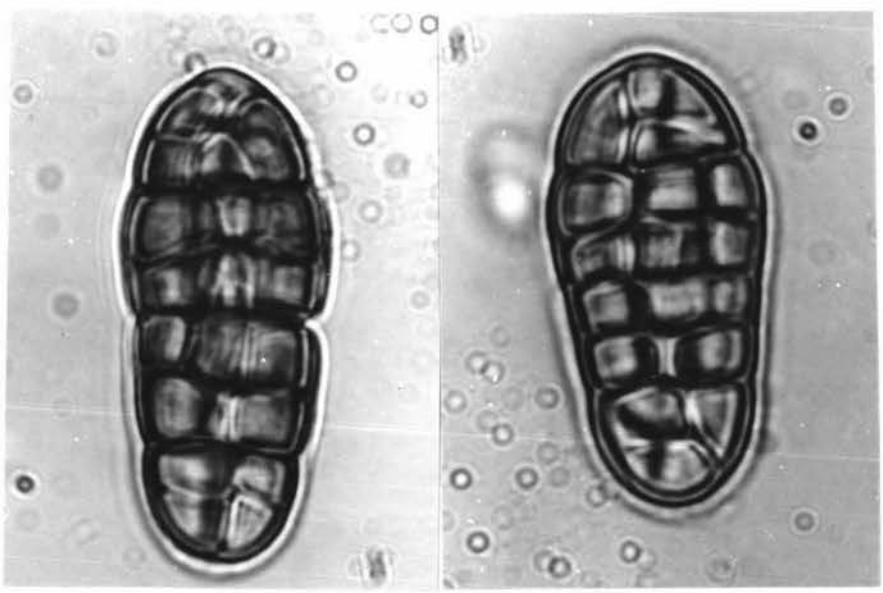

FIGURE 12.
Mature ascospores from a P. herbarum colony on V-8 juice agar after six weeks incubation at 8°C in the dark. (x900)

These observations are essentially the same as those described overseas for the fungus on lucerne (Smith, 1910; Graham and Zeiders, 1960), and on other host species (Groves and Skolko, 1944; Padhi and Snyder, 1954; Graham and Zeiders, 1960; Rotem et al, 1966). That is, the conidiophores were erect, 0-5 septate, brown, each with a swollen apical sporogenous cell bearing a single conidium which was subspherical to oblong, olive to brown, echinulate, muriform and constricted at the median transverse septum. The black, globose pseudothecia contained bitunicate asci which were narrowly cylindrical to clavate, each with eight ascospores arranged in an oblique series. When mature the ascospores were ellipsoid to clavate, yellow-brown, muriform, with seven transverse septa.

It should be noted that depending on the isolates under study so morphological features of fungi, in particular spore dimensions, may vary under the one set of environmental conditions. Further, spore dimensions of any one isolate may vary with changes in environmental conditions (Williams, 1959; Leach and Aragaki, 1970). Thus it must be recognised that the morphological features as described above relate to isolates 2 and 3, and under the cultural conditions as defined in the experiment.

(ii) Physiological features

a. The effect of temperature

The effect of temperature on radial growth of S. botryosum is illustrated in Figure 13 and shown graphically in Figure 15.

The cardinal temperatures for vegetative growth on PDA_L at ten days were 4, 24 and 36°C, these closely agreeing with the temperatures reported by other investigators working with S. botryosum (Padhi and Snyder, 1954; Graham, 1957; Rotem et al, 1966). After four days incubation greatest growth had occurred at 28°C, but by the tenth day the radial growth at both 20 and 24°C had exceeded that at 28°C. It should be noted that these results only refer to the average growth of two isolates, and that individual isolates differed slightly from the above.

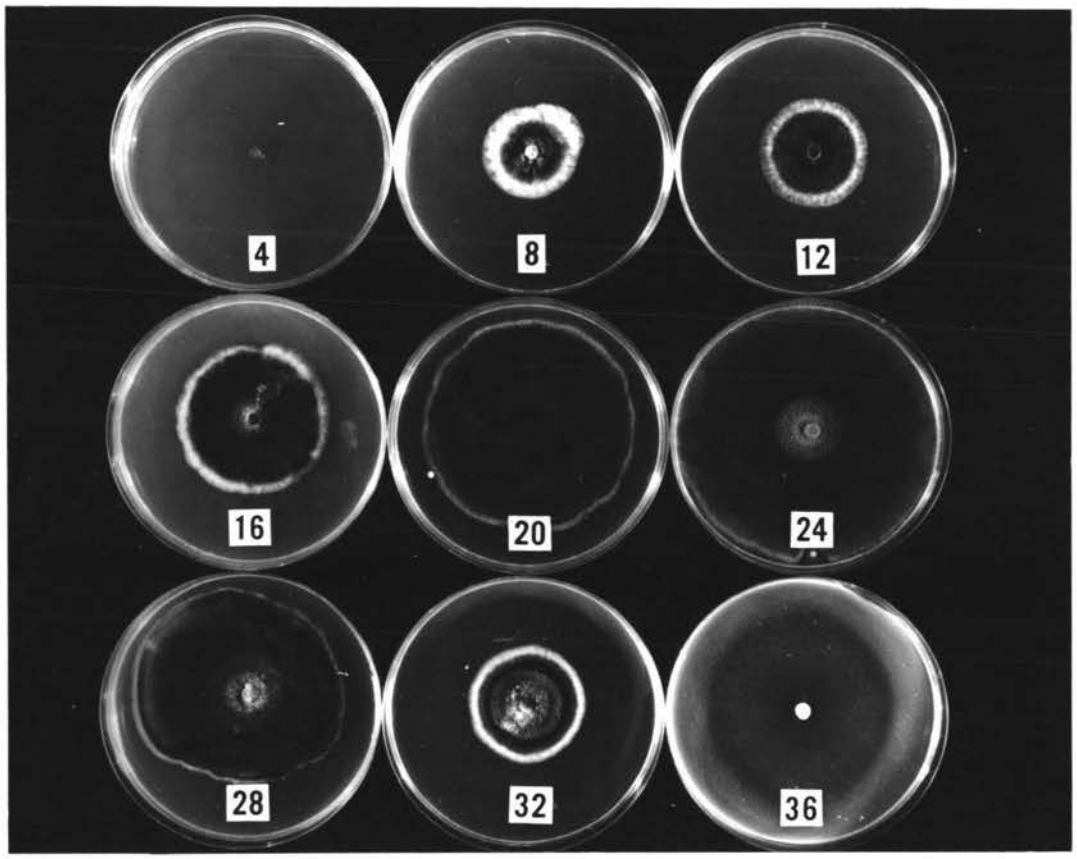

FIGURE 13.
Effect of temperature on the growth of S. botryosum (isolate 2) on PDA_L after ten days incubation in the dark.

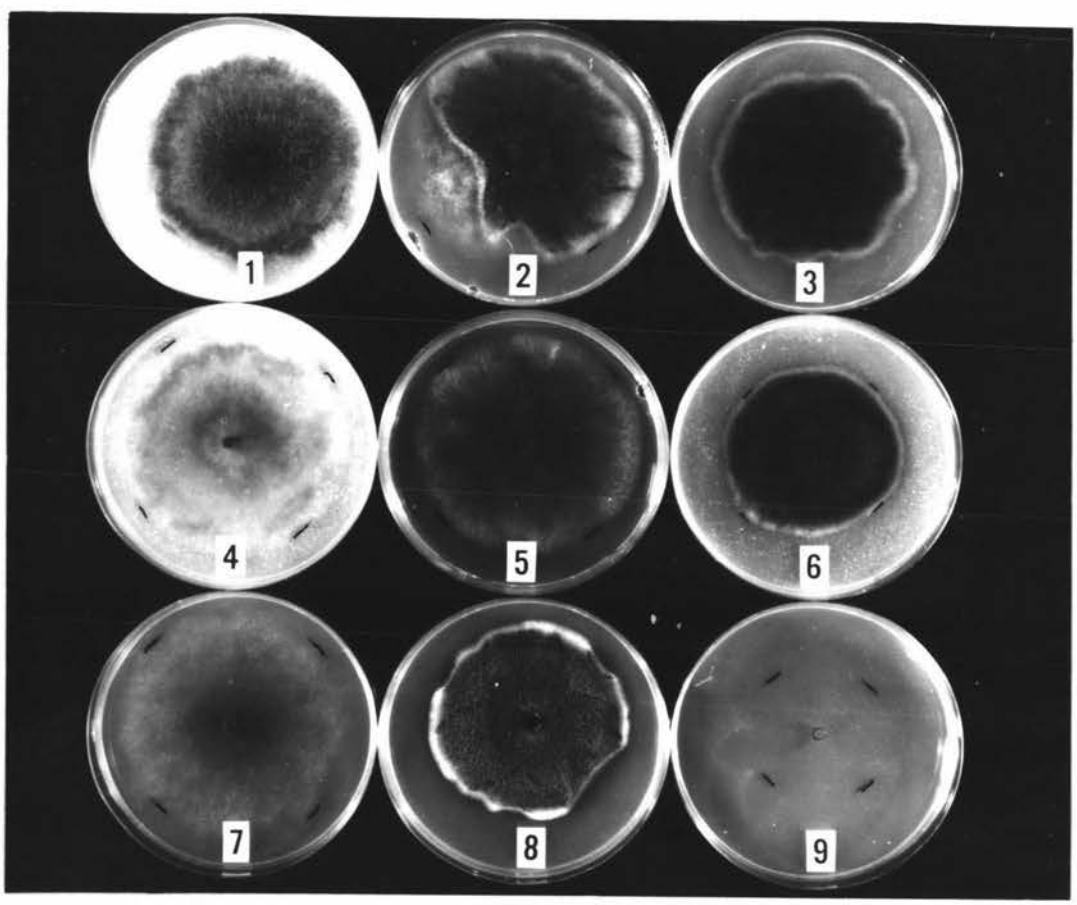

FIGURE 14.
Effect of media type on the growth and gross colony characteristics of S. botryosum (isolate 2) after ten days at 24°C in the dark.

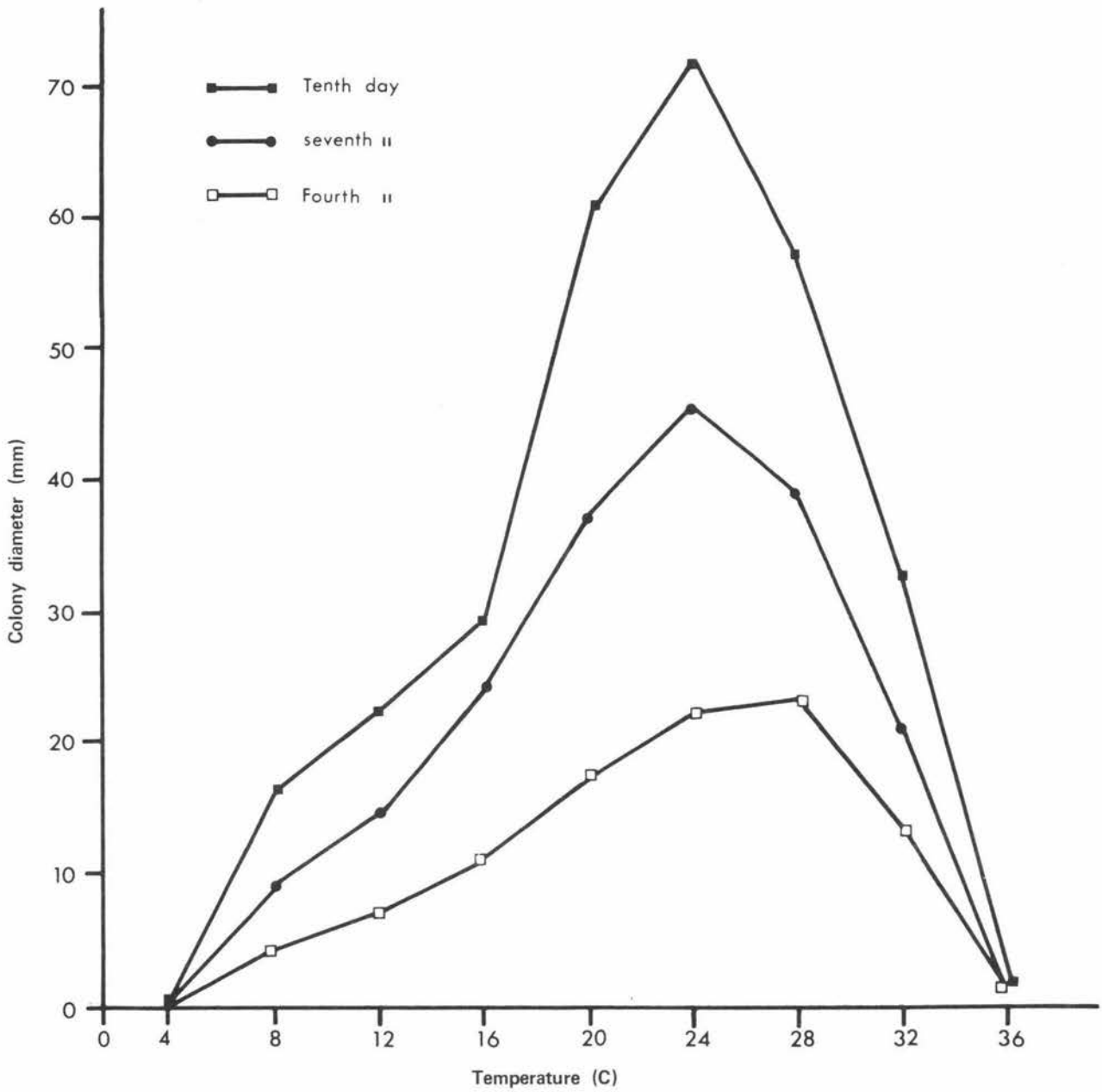

FIGURE 15. Effect of temperature on radial growth of S. botryosum on PDA_L after four, seven and ten days incubation.

Gross colony characteristics changed with temperature, with colonies being generally darker below 20°C, and completely white at 4 and 36°C. Aerial mycelium was sparse between 28 and 36°C, and absent at all other temperatures. Where present it was white and fluffy and confined to the colony centre. Protopseudothecia were produced in the cultures only between 16 and 24°C, whereas conidial production occurred between 8 and 32°C, with the maximum production at 24°C.

There were also changes in the morphology of conidia at the different temperatures. In general, there was greater pigmentation and echinulation with an increase in temperature, but spore size remained constant. Similar gross changes in the morphology of conidia associated with different temperatures have been reported by Leach and Aragaki (1970) for S. floridanum Hannon and Weber.

b. The effect of media

The effect of media type on the gross colony characteristics is illustrated in Figure 14 and summarised in Table 19.

Growth was dense on all media except PDA_M and WA, where it was very sparse. Surface topography and colour of colonies were essentially the same on all nine media. In general the cultures were circular, flat and when viewed from above light olive to dark brown in colour. On PrA and PDA_M cultures were light olive to white, while on WA they were completely white. Aerial mycelium was lacking on all media except MA, where it was sparsely produced at the colony centre.

On PDA_M, PrA and WA conidial production was either poor or lacking, whereas on all the other media moderate to very dense production occurred. On those media where conidia were observed production was over the total colony surface, with greatest intensity at the centre. The production of protopseudothecia occurred only on PDA_M, PrA and LDA, and in each case was not intense.

The effect of media type on radial growth is illustrated graphically in Figure 16. After ten days incubation at 24°C growth was greatest on V-8, PrA and PDA_M, and least on MA. It should be noted that the histogram only records the average growth of two isolates, and that there was some variation between isolates.

TABLE 19. Effect of media type on the gross colony characteristics of S. botryosum incubated at 24°C for ten days.

	OMA	LDA	V-8	CMA	PrA	MA	PDA_M	PDA_L	WA
Medium pH	6.30	5.44	6.22	5.50	5.75	5.70	6.41	5.80	6.90
Surface Topography									
Shape	C	C	C	C	C	C	C	C	Ir
Elevation	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft
Zonation	NZ	Z	Z	Z	NZ	Z	NZ	Z	NZ
Colony Colour									
Fringe	L Ol	W	L Br	W	W	W	W	W	W
Middle	L Ol	Gn	D Br	Br	W	D Br	W	D Gn	W
Centre	L Ol	Gn	D Br	Br	L Ol	D Br	L Ol	D Gn	W
Aerial Mycelium									
Quantity	0	0	0	0	0	1	0	0	0
Location	-	-	-	-	-	C-M	-	-	-
Morphology	-	-	-	-	-	Ct	-	-	-
Colour	-	-	-	-	-	W	-	-	-
Conidial Features									
Quantity	2	2-3	4	2	1	2	1	3	0
Location	Gen	Gen	Gen	Gen	Gen	C-M	Gen	C-M	-
Colour	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	-
Protopseudothecia Features									
Quantity	0	1-2	0	0	2	0	2	0	0
Location	-	C-M/I	-	-	C/I	-	C/I	-	-
Colour	-	Bl	-	-	Bl	-	Bl	-	-

Key: Shape: C - circular
Ir- irregular
Elevation: Ft - flat
Zonation: NZ - no zonation
Z - zonation

Quantity 0 - absent
1 - sparse
2 - moderate
3 - dense
4 - very dense

Colour: L - light
D - dark
Ol - olive
Gn - green
Br - brown
W - white
Bl = black

Location: C - centre
M - middle
Gen - general
I- immersed

Morphology:
Ct - cottony

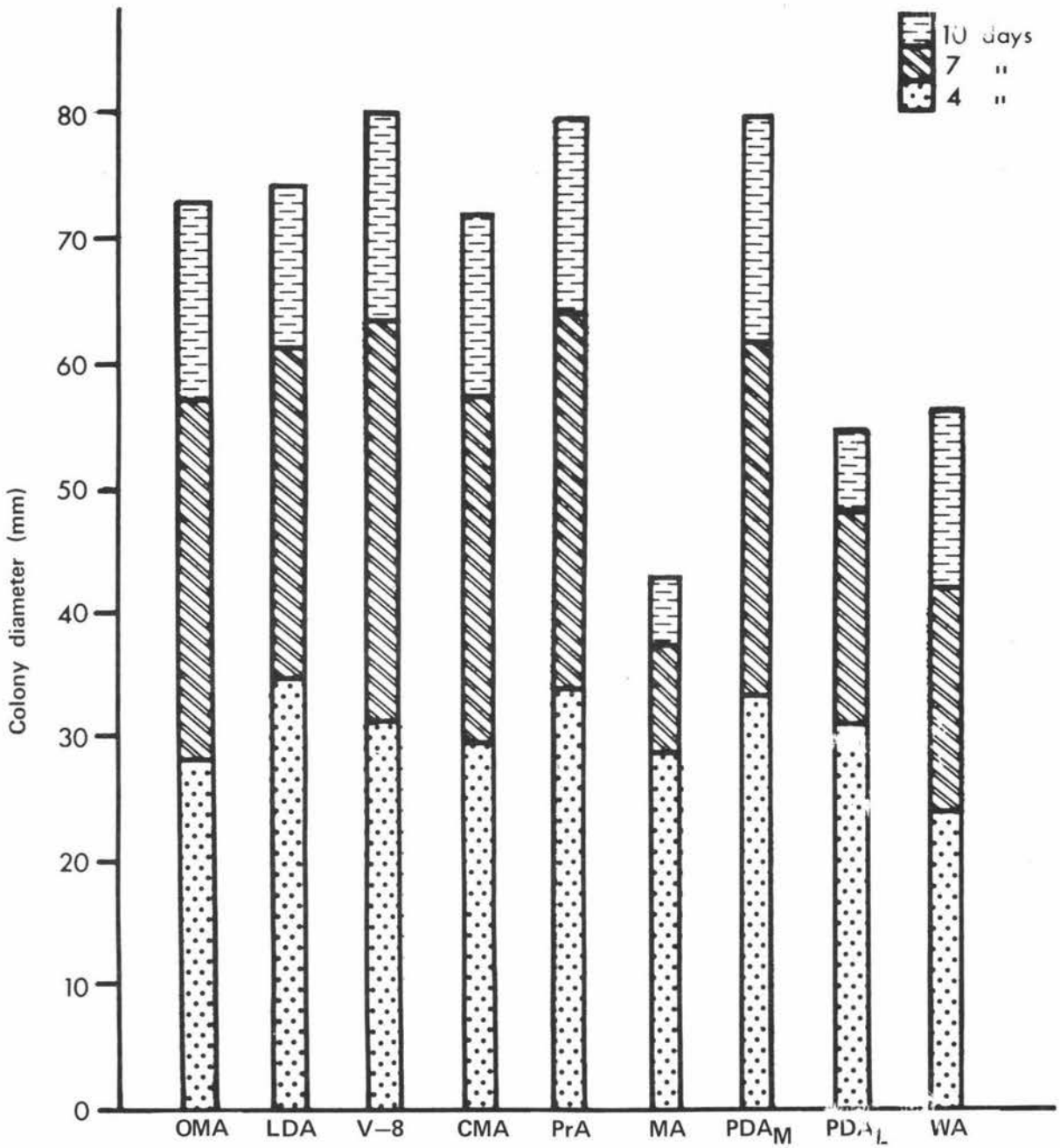

FIGURE 16. Effect of media type on radial growth of S. botryosum after four, seven and ten days incubation at 24 C.

3. PRODUCTION OF THE PERFECT STATE ON AGAR

Evidence to confirm that the isolates causing Stemphylium leaf spot in the Manawatu were in fact Pleospora herbarum required that the perfect stage be produced on culture media. In preliminary cultural studies protopseudothecia were sparingly produced by isolates incubated in the dark at 24°C, but in no instance did they mature. The following experiments were conducted to determine means of inducing firstly, abundant production of protopseudothecia, and secondly, their maturation.

(a) Production of protopseudothecia on agar

The method found by Leach (1971) to effectively stimulate production of protopseudothecia was investigated.

Forty-eight PDA_L plates were inoculated, half with isolate 2 and half with isolate 3. They were incubated initially in the dark at 22-24°C for five days and then exposed to a continuous NUV light source at 22-24°C for the following durations:
0, 1, 6, 12, 24, 72, 120 and 168 hours.
After each exposure duration six plates were returned to the dark incubator (22-24°C). The number of protopseudothecia produced, expressed as an intensity rating, was determined at the completion of the last treatment (168 hours = 7 days).

The results (Table 20) show that for both isolates at least 12 hours exposure to NUV light was required to induce production of a moderate number of protopseudothecia, and that production was more intense as the duration of treatment increased. However, there was a difference in the degree of response by the two isolates to the NUV treatment. With isolate 2 a few protopseudothecia were produced in the absence of NUV radiation, whereas isolate 3 required at least one hour of treatment for a similar number to be produced. Further, compared with isolate 2, production of protopseudothecia by isolate 3 was considerably more intense after 168 hours treatment.

TABLE 20. Effect of exposure duration to NUV light on the intensity of protopseudothecia development.

Isolate	Exposure duration to NUV light (hours)							
	0	1	6	12	24	72	120	168
2	1*	1	1	2	2	3	3	3
3	0	1	1	2	2	3	4	4

* 0 = no protopseudothecia
1 = few protopseudothecia
2 = moderate number of protopseudothecia
3 = large number of protopseudothecia
4 = very large number of protopseudothecia

(Average of three replicates)

(b) Maturation of protopseudothecia on agar

Two methods have been reported that induce the maturation of protopseudothecia of Pleospora herbarum on culture media. Using an isolate from tomatoes Rotem, Cohen and Wahl (1966) found that cultures containing protopseudothecia maintained for two weeks at 10, 15 or 20°C produced ascospores when exposed for an additional two weeks to 25, 20 and 25°C, respectively. Simmons (1969) also found that maturation of P. herbarum isolates occurred at 5°C within periods of time ranging from 2-12 months. This work was in effect confirmed by Leach (1971) who found that protopseudothecia matured when subjected to long uninterrupted periods at low temperatures (e.g. 21 days at 5-10°C).

The relative effectiveness of the above two methods was investigated.

(i) Effect of bi-weekly temperature alterations

Thirty-two PDA_L and V-8 juice agar plates were inoculated, half with isolate 1 and the remainder with isolate 2 and exposed for six days to continuous NUV light (1 x 40w Phillips NUV lamp held 40 cm above the plates) at 22-24°C.

They were then subjected to the following temperature treatments:

> Half the plates (16 plates) were given a temperature modification (two weeks at 20°C followed by two weeks at 25°C) and the other half no temperature modification (four weeks at 20°C).

The effect of bi-weekly temperature alterations on protopseudothecia maturation was assessed 30 days after the plates were subjected to their respective temperature regimes.

At the time of assessment all treatments had failed to induce the production of pseudothecia. A characteristic sign of protopseudothecia maturation in culture is that they become erumpent and form a short apical beak; neither of these two features were observed. The protopseudothecia remained immersed and only contained a mucous-granular material. No sign of differentiation of the protopseudothecial contents into asci or paraphyse primordia was observed.

Hence the results show that bi-weekly temperature alterations were totally ineffective in inducing protopseudothecia maturation. These results are in agreement with those of Gourley (1971) who found that similar bi-weekly temperature alterations, either upward or downward, failed to induce protopseudothecia maturation of an isolate of P. herbarum from tomatoes.

(ii) Effect of constant low temperatures

Twenty-four PDA_L plates were inoculated, half with isolate 2 and the remainder with isolate 3, and exposed to the NUV light at 22-24°C to induce protopseudothecia formation. After five days of this treatment three plates each of isolates 2 and 3 were transferred to incubators held at 8, 12, 16 and 20°C, respectively. Six weeks later all cultures were examined for the presence of pseudothecia.

The results (Table 21) show that maturation of protopseudothecia only occurred in isolate 3. Exposure to low temperatures favoured maturation of protopseudothecia, more pseudothecia being produced at 8 and 12°C than at 16°C.

Pseudothecia were not produced in the 20°C series.

TABLE 21. Effect of constant temperatures on maturation of protopseudothecia.

Temperature (°C)	Presence of pseudothecia in 48 day old colonies	
	Isolate 2	Isolate 3
8	0 *	2
12	0	3
16	0	1
20	0	0

* 0 = no pseudothecia
1 = very few pseudothecia with asci
2 = moderate number pseudothecia with asci
3 = most pseudothecia with asci
(Average of 3 replicates)

On the basis of the above experiments the following procedure was adopted to induce the development and maturation of protopseudothecia of P. herbarum on agar:

> Actively growing colonies were initially exposed to NUV radiation for a minimum of five days at 23-27°C to stimulate the formation of protopseudothecia. The colonies were then subjected to long uninterrupted periods at low temperatures (8-12°C for six weeks) to induce their maturation. It must be noted that not all isolates of P. herbarum from lucerne will form pseudothecia following exposure to the above treatment.

4. SPORE GERMINATION

The period between spore dispersal and the establishment of a new parasitic relationship is a most vulnerable phase in the life cycle of a fungal pathogen, with spore germination being one of the most critical stages over this period.

In the present study the rapidity and pattern of germination of conidia was examined on agar at 24°C, and photographically recorded using a growth chamber similar to that described by Harvey and Wenham (1971). A sterilised coverslip coated with a thin layer of PDA$_L$ was inoculated by streaking with a conidial suspension. The inoculated coverslip was then inverted over the plastic growth chamber and the edges sealed with paraffin wax. Moist air was pumped through the chamber to facilitate spore germination and prevent dehydration of the thin layer of PDA. Germination patterns of one or more conidia were periodically observed and photomicrographically recorded under the high power (x 400) of a compound microscope. Conidia were considered to have germinated when the length of a germ-tube was equal to the breadth of the conidium.

Generally 5-10% of the conidia had germinated after two hours, and by the fourth hour over 90% of the spores had well developed germ-tubes.

The pattern of germination is shown in Figure 17. In all cases there was an initial slight expansion of the conidium, presumably due to absorption of water from the substrate. Germ-tubes were in evidence within two hours, usually originating initially from the lateral cells of the muriform conidium, and followed soon after by emergence from the terminal cells. Within four hours of first evidence of germination individual conidia had produced between 4 and 10 germ-tubes. Once emerged germ-tubes elongated rapidly, becoming septate within 12-14 hours and branched within 13-18 hours.

B. STEMPHYLIUM VESICARIUM AS A POSSIBLE FOLIAGE PATHOGEN OF LUCERNE

During cultural studies of isolates from field diseased plants one isolate (isolate 3) was observed to produce conidia which in septation and length/breadth ratio (L/B ratio) were considered not to be typical of *S. botryosum*. A culture was forwarded to Mr G.F. Laundon, Plant Health and Diagnostic Station, Levin, who considered the isolate to be more typical of *S. vesicarium* (Wallr.) Simmons (Laundon, pers. comm.).

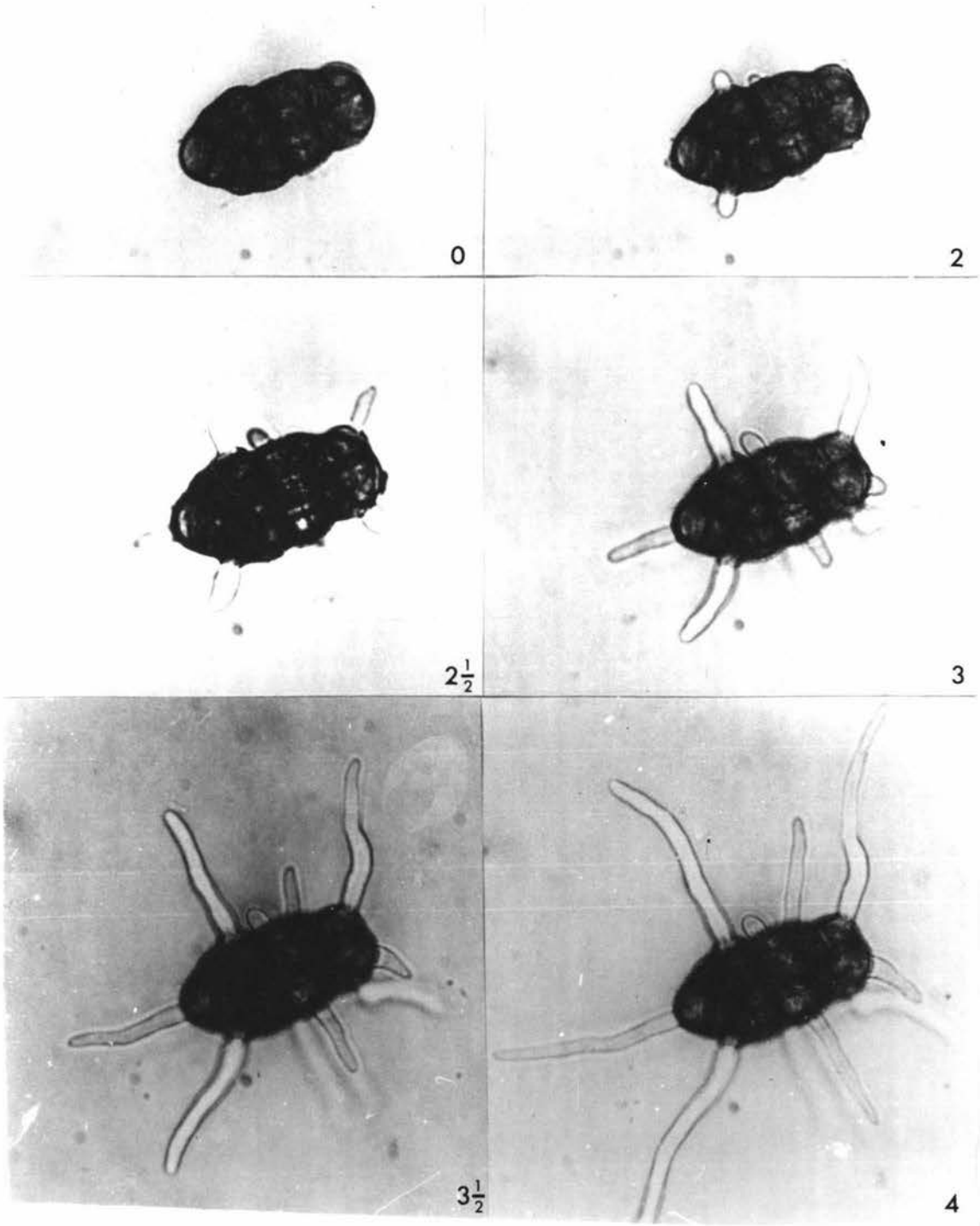

FIGURE 17.
Pattern of conidium germination of S. botryosum on PDA_L at 24°C (x400).
(Left to right, top to bottom;
0, 2, 2½, 3, 3½, 4 hours).

A further culture was forwarded to Dr E.G. Simmons, Natick, Mass., U.S.A., who in reply commented that it might be S. vesicarium and intimated that he would later advise me of his conclusion.* Thus there was the suggestion that S. vesicarium may be coexistent with S. botryosum as a pathogen of lucerne in the Manawatu. However, conidia produced on infected leaflets subsequently collected from several local crops were in all instances typical of S. botryosum.

Stemphylium vesicarium was erected by Simmons (1969) and differs from S. botryosum in the shape of juvenile conidia, and in the morphology of both mature conidia and immature and mature ascospores (Table 22). To determine whether isolate 3 was in fact S. vesicarium the morphology of juvenile and mature conidia were compared with those of isolate 2, the latter being considered typical of S. botryosum. Comparisons of the morphology of ascospores were not possible because isolate 3 only produced pseudothecia.

1. CONIDIUM ONTOGENY

According to Simmons (1969), juvenile conidia of S. vesicarium are oblong almost from the time of initiation, whereas those of S. botryosum are usually spherical (Figure 18). Comparison of the two isolates in this regard was made by following conidia ontogeny on agar using an apparatus described by Harvey and Wenham (1971).

An agar block from a PDA_L plate was transferred to a sterile coverslip and inoculated with conidia and mycelial fragments at points where the agar and coverslip met. The coverslip was inverted over the aperture of the growth chamber and sealed in place with paraffin wax.

---

*To date (September 1973) a further communication from Dr Simmons on this matter has not been received.

TABLE 22. Comparison of juvenile conidium shape, mature conidium, and immature and mature ascospore morphology of S. botryosum and S. vesicarium (Simmons, 1969).

	S. botryosum	S. vesicarium
Juvenile conidium shape	Spherical almost from time of initiation.	Oblong.
Mature conidium morphology	Once-constricted at median transverse septum; 23 x 33u; L/B ratio 1.0 - 1.5	Constricted at 3 major transverse septa; 18 x 45u; L/B ratio 2.5 - 3.0
Immature and mature ascospore morphology	Immature oblong, obtusely rounded ends, constricted at 1 or more of initial transverse septa by time longitudinal septa produced. Mature have broadly rounded apex, flat base, 1 complete series longitudinal septa plus incomplete series.	Immature ellipsoidal, upper half narrowly tapered, constricted at initial 3 transverse septa by time longitudinal septa produced. Mature have obtusely pointed apex, broadly rounded base, incomplete series longitudinal septa of different lengths.

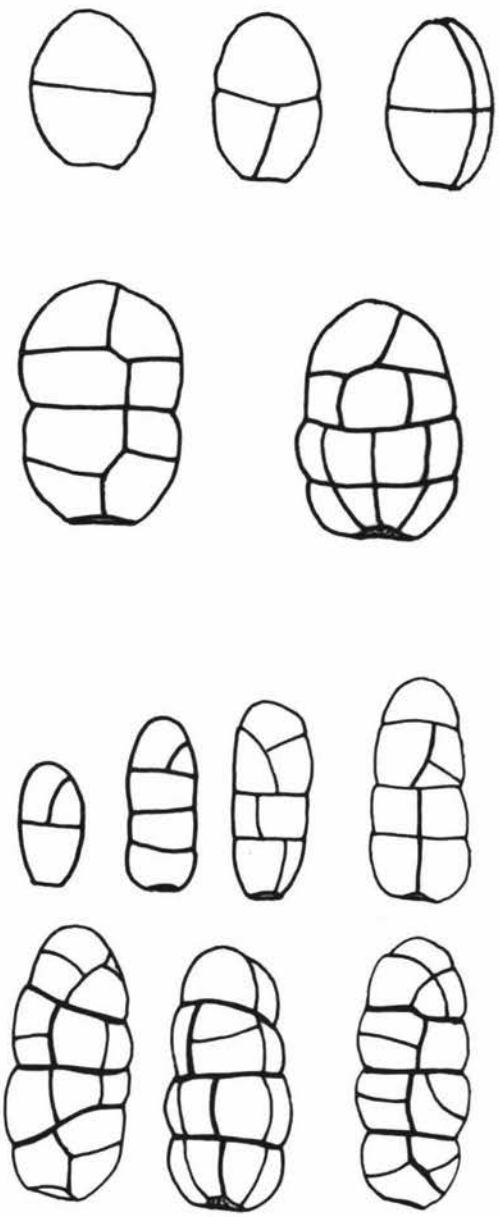

FIGURE 18.
Comparison of juvenile conidia from a culture of S. botryosum (upper) and S. vesicarium (lower), after Simmons (1969).

When sporulation was observed to occur close to the coverslip (after 2-3 days at 24°C), a photographic sequence of a suitable conidiophore and conidium was begun. However, difficulty was experienced in obtaining a complete sequence due to a combination of factors such as lack of sporulation, water condensation on the coverslip, and dense vegetative growth obscuring conidial development. Accordingly, a series of photomicrographs were taken of several developing conidia selected to depict stages in conidium development. As the maturation process was followed several times for both isolates the photographs represent a typical ontogenetic sequence.

The results (Figures 19 and 20) show that conidium ontogeny in the two isolates were essentially similar, and therefore failed to provide support for the contention that isolate 3 was S. vesicarium. In both series juvenile conidia were spherical almost from the time of initiation (Figures 19b and 20b), and hence were typical of S. botryosum. With time several transverse septa were produced by both isolates, with only the median septum constricted, a further feature considered to be characteristic of S. botryosum isolates (Simmons, 1969).

2. MORPHOLOGY OF MATURE CONIDIA

According to Simmons (1969) the conidia of S. vesicarium from agar cultures are constricted at one or more commonly three of the major transverse septa and have an L/B ratio ranging from 2.5 - 3.0, whereas conidia of S. botryosum are constricted at the median transverse septum and have an L/B ratio ranging from 1.0 - 1.5.

To determine whether the above stated differences in the morphology of mature conidia applied to isolates 2 and 3, each was grown on V-8 juice agar at 24°C in the dark for ten days, by which time both were sporulating profusely. Slide preparations were then made and mature conidia compared.

The results recorded photographically in Figure 21 and summarised in Table 23 further confirm that isolate 3 is more typical of S. botryosum than S. vesicarium.

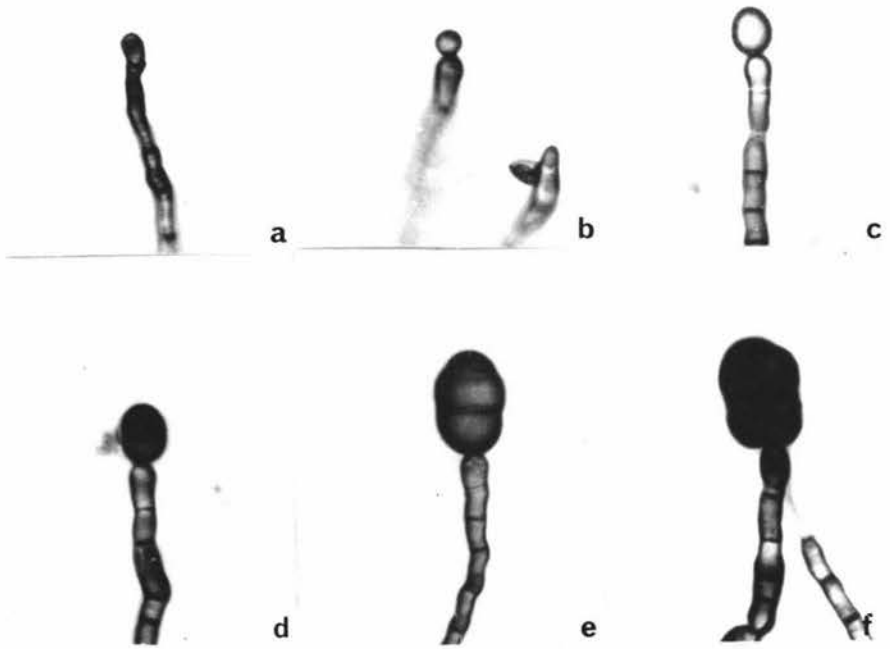

FIGURE 19.
Conidium ontogeny of isolate 2 on PDA_L at 24°C.

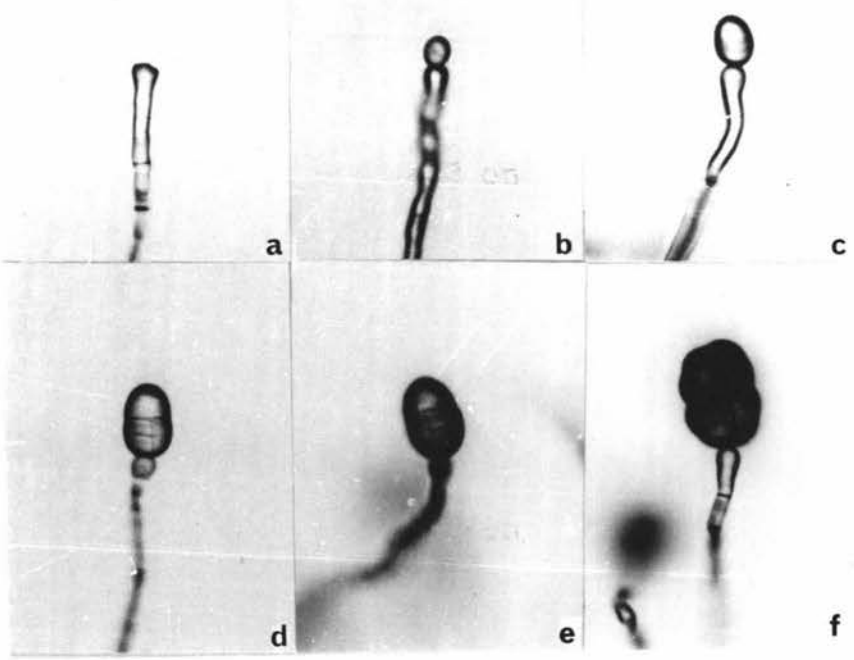

FIGURE 20.
Conidium ontogeny of isolate 3 on PDA_L at 24°C.

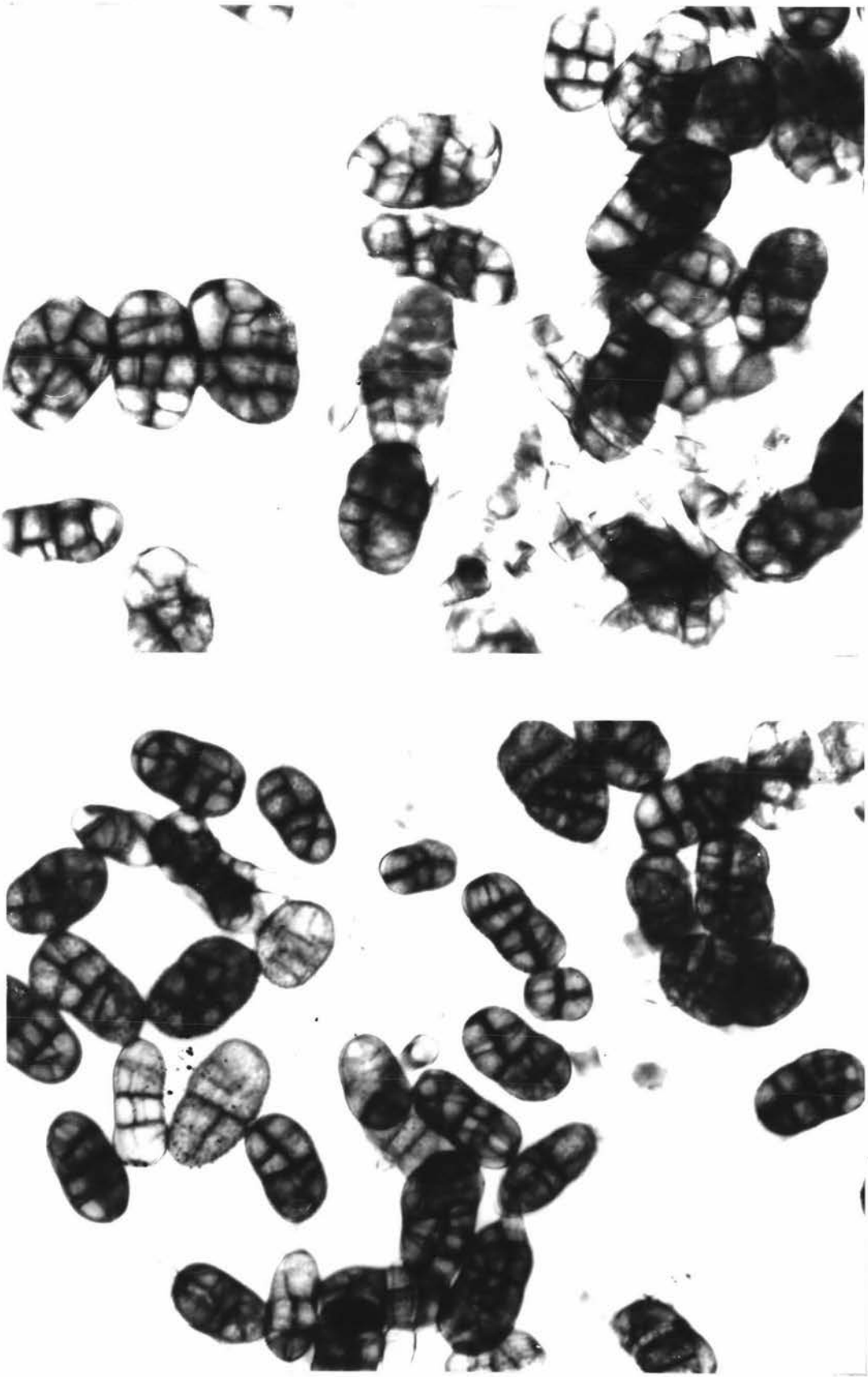

FIGURE 21.
Comparison of conidia of isolate 2 (upper) and isolate 3 (lower) after incubation on V-8 juice agar at 24°C for ten days in the dark. (x400)

TABLE 23. Comparison of mature conidia dimensions and L/B ratio of isolates 2 and 3, S. botryosum and S. vesicarium.

Isolate or species	Mean length (u)	Mean breadth (u)	L/B ratio	Authority
Isolate 2	33.7	24.1	1.40	Author
Isolate 3	25.9	15.0	1.73	Author
S. botryosum	33.0	23.0	1.50	Simmons (1969)
S. vesicarium	45.0	18.0	2.50	Simmons (1969)

From the photographs it is clear that the majority of conidia of each isolate had a single median constriction. As regards the L/B ratio the isolates were similar (1.40 isolate 2; 1.73 isolate 3), and most approximated that recorded by Simmons (1969) for S. botryosum (1.50).

These results, and the studies on the ontogeny of conidia of the two isolates fail to support the contention that isolate 3 was S. vesicarium.

3. EFFECT OF TEMPERATURE ON CONIDIUM MORPHOLOGY

Since the culture of isolate 3 examined by Laundon had been incubated at 8°C for four weeks it was possible that this low temperature had influenced the morphology of the conidia to the extent that they resembled those of S. vesicarium. That is, it was postulated that the temperature at which cultures were incubated may influence morphology of S. botryosum isolates from lucerne in a manner similarly reported for Stemphylium floridanum Hannon and Weber by Leach and Aragaki (1970). This hypothesis was investigated as follows.

Duplicate plates of V-8 juice agar were centrally inoculated with either isolate 2 or 3 and incubated at the following temperatures:

8, 12, 16, 20, 24, 28 and 32°C.

Four weeks later slides were prepared from all colonies and the morphology of the conidia compared. The dimensions of conidia were also recorded for each series, with the exception of the 12 and 32°C cultures.

TABLE 24. Comparison of conidia dimensions of isolates 2 and 3 grown on V-8 juice agar at five temperatures for four weeks.

Temperature (°C)	Isolate 2					Isolate 3				
	Length (u)		Breadth (u)		L/B ratio	Length (u)		Breadth (u)		L/B ratio
	Range	Mean	Range	Mean		Range	Mean	Range	Mean	
8	36.2-48.9	40.0	33.3	26.4	1.51	28.2-41.6	37.6	19.8-30.6	22.5	1.67
16	31.5-44.0	37.2	21.7-30.0	25.1	1.48	30.4-40.9	35.4	18.2-28.1	21.8	1.62
20	30.0-44.0	39.7	21.8-32.9	25.6	1.55	28.6-39.7	35.8	17.8-25.9	21.3	1.68
24	25.9-40.0	32.5	16.0-25.0	20.3	1.60	25.2-38.6	30.4	16.2-24.4	19.3	1.58
28	23.1-36.1	31.4	16.3-21.6	19.6	1.60	23.9-35.2	28.6	14.8-20.7	18.2	1.57

The dimensions of conidia are summarised in Table 24.

As regards isolate 2, at low temperatures (8 and 12°C) conidia were more septate, larger, less pigmented and less echinulate. As incubation temperatures increased conidia were increasingly less septate, smaller, strongly pigmented and more echinulate. Conidia of isolate 3 also showed a similar trend in morphology with increasing temperature, but were smaller at all temperatures than conidia of isolate 2.

Hence at 8°C the conidia of isolate 3 were longer and more septate than at 24°C, and similar in many respects to conidia of S. vesicarium. That is, conidia produced at 8°C could easily be misidentified as S. vesicarium, whereas at a higher temperature (24 or 28°C) they were more typical of S. botryosum.

Results of the above experiment serve to emphasize the danger associated with identifying species on the basis of study of one isolate. Since environmental factors can significantly influence such morphological factors as spore dimensions, colour and septation (Neergaard, 1945; Williams, 1959; Leach and Aragaki, 1970) it follows that any account of the morphology of a fungus must be accompanied by a precise statement of the environmental conditions under which the organism was grown. Further, in any taxonomic study it is imperative that the variability of specific morphological characters be tested by growth in controlled environments or by extensive sampling of field collections.

STUDIES ON LEPTOSPHAERULINA

PEPPER SPOT OF LUCERNE

CHAPTER V

INTRODUCTION

The genus Leptosphaerulina (= Pseudopleа) includes several species associated with leaf diseases of forage plants, the most common of which are pepper spot of lucerne caused by L. briosiana, and pepper spot of clovers caused by L. trifolii. The two diseases are so called on account of the numerous, small brown to black sunken lesions observed on leaves and petioles of host plants. The diseases are widely distributed throughout the world (Europe, North America, Australia, Brazil, Taiwan), especially in humid, temperate areas (Graham and Luttrell, 1961; Booth and Pirozynski, 1967a).

In New Zealand pepper spot of lucerne was first recorded from Putaruru in 1969, and was subsequently found at Murupara, Hastings and in Mid-Canterbury (Close, 1972). According to Close (1972) the causal organism in each instance was L. trifolii.

On clovers pepper spot was first recorded in New Zealand on white clover (Trifolium repens L.) by Brien and Dingley (1955), and later on suffocated clover (T. suffocatum L.) by Dingley (1969). The causal organism in each case was cited by Dingley (1969) as Sphaerulina trifolii (= L. trifolii). During the present study the causal organism was also isolated from red clover (T. pratense L.), this being a new host record for the pathogen in New Zealand.

At the present time controversy exists as to whether the pepper spot diseases of lucerne and clovers are caused by the one species of Leptosphaerulina. Graham and Luttrell (1961) when reviewing the taxonomy and morphology of the legume-infecting species of Leptosphaerulina considered that the pathogens causing pepper spot of lucerne and clovers were distinct, and should be named L. briosiana and L. trifolii, respectively.

They separated the two pathogens on the basis of pathogenicity, cultural characteristics, ascospore septation, and dimensions of pseudothecia, asci and ascospores. However, Booth and Pirozynski (1967a) consider that only one species is the causal agent of pepper spot on lucerne and clovers, namely, L. trifolii. They commented that although on host material L. trifolii exhibits wide variation in spore size, isolates from the Leguminosae grown on standardised potato-dextrose agar under controlled conditions produce ascospores which are uniform in both size and septation. Thus currently there exists two opposing schools of thought as to whether pepper spot of lucerne and clovers is caused by the one morphological species.

To clarify the situation the pathogenicity, cultural characteristics, ascospore septation, and dimensions of pseudothecia, asci and ascospores of isolates from lucerne and red clover crops in the Manawatu were compared. Preliminary results indicated that the two series of isolates were morphologically very similar. Accordingly, for reason of convenience, the tentative conclusion was drawn that only one species of Leptosphaerulina was involved, viz. L. trifolii. Throughout the present study this binomial has been applied to all isolates, irrespective of the host source.

In the course of screening New Zealand produced lucerne seed-lines, Percival (pers. comm.) noted the development of Leptosphaerulina colonies from seed plated on agar. Although pepper spot of Medicago spp. and Trifolium repens L. (var. Ladino) caused by L. trifolii is listed as a seed-borne disease (Noble and Richardson, 1968), evidence has not been provided which demonstrated unequivocably that seedling infection may arise from use of seeds carrying viable inoculum. Hence, besides the above stated taxonomic considerations, the question of pepper spot being a seed-borne disease of lucerne was also investigated.

CHAPTER VI

PROOF OF PATHOGENICITY

As with S. botryosum the Rules of Pathogenicity were completed by firstly isolating the organism to pure culture, then artificially inoculating healthy host plants to produce symptoms typical of the disease, and finally re-isolating the organism from the inoculated plants and comparing it with the original culture.

A. ISOLATION TO PURE CULTURE

As the pathogen is characterised only by an ascigerous state and failed to readily produce pseudothecia on infected leaflets, the only practical method of isolating the pathogen to agar was by the tissue plating method. Initial isolations were made by surface-sterilising infected tissue pieces contained in a muslin bag by immersion in a 1:7 dilution of commercial Janola for three minutes, and then aseptically transferring the tissue pieces to PDA, or V-8 juice agar plates. However, in these isolations contaminants were often present and the results obtained were inconsistent. This was considered to be in part consequent on the method of surface-sterilising the tissue pieces.

Previous workers have commonly utilised a 0.5% sodium hypochlorite solution to surface-sterilise tissue pieces infected with L. trifolii. Martinez and Hanson (1963) immersed tissue pieces for one minute, but neither Graham and Luttrell (1961) nor Kreitlow and Kilpatrick (1967) specified the treatment time. The latter workers also used both 0.1% mercury bichloride and washing in running tap water for several hours.

In the present study surface-sterilising infected tissue pieces by immersion in a sodium hypochlorite solution and by washing in running tap water were compared, with treatment time in each instance a variable. Associated with this experiment the effect of adding antibiotics to the plating medium was also examined.

Infected tissue pieces were loosely wrapped in muslin bags and subjected to the following treatments:

1. Immersion in a 1:7 dilution of commercial Janola (approximately 1.3% available chlorine) for 0.5, 1.0, 1.5, 2, 3 and 4 minutes. The bags were constantly agitated to remove trapped air bubbles and ensure separation of all tissue pieces, and on removal were rinsed in sterile distilled water.

2. Washing in running tap water for 1, 2, 4 and 6 hours. The water was allowed to flow at a rate so as to provide a constant agitation of the bags.

For each treatment there were 60 tissue pieces.

At the completion of each treatment the tissue pieces were aseptically transferred to six agar plates at the rate of ten tissue pieces per plate. All plates were of PDA_L, but to three of them the antibiotics penicillin and streptomycin sulphate had been incorporated, each at a concentration of 50 ppm. All plates were then incubated in the dark at 24°C for six days.

As a control, infected tissue pieces were plated directly to PDA_L plates, with and without antibiotics added.

The results are presented in Table 25, and discussed as follows:

1. Antibiotic medium versus non-antibiotic medium

In all treatments except two (treatments 8 and 22), where antibiotics were incorporated into PDA_L bacterial contamination was non-existent. The benefits were most apparent in the series where tissue pieces had been water-washed. Both control series showed a relatively high level of bacterial contamination with only a slight reduction in colony numbers in plates containing antibiotic medium. By contrast, and as expected, incorporation of antibiotics in agar did not prevent growth of saprophytic fungi.

2. Janola versus water-wash

In all instances the water-wash was superior to the Janola as a surface-sterilisation method.

TABLE 25. Effect of surface-sterilisation treatment and addition of antibiotics to PDA_L on isolation of *L. trifolii* from infected lucerne leaflets.

Treatment	Duration	Antibiotic	Number of colonies on the 3 replicates				Treatment code
			L. trifolii	Other fungal	Bacterial	Total	
1:7 dilution Janola solution	0.5 min.	-	16	5	2	23	1
		+	13	6	0	19	2
	1.0 min.	-	8	2	2	12	3
		+	8	2	0	10	4
	1.5 min.	-	2	1	0	3	5
		+	1	3	0	4	6
	2.0 min.	-	2	1	3	6	7
		+	5	3	1	9	8
	3.0 min.	-	2	3	0	5	9
		+	0	2	0	2	10
	4.0 min	-	1	2	3	6	11
		+	2	6	0	8	12

Continued over....

TABLE 25. (contd).

Treatment	Duration	Antibiotic	Number of colonies on the 3 replicas				Treatment code
			L. trifolii	Other fungal	Bacterial	Total	
Water-wash	1 hr	-	20	8	2	30	13
		+	23	7	0	30	14
	2 hr	-	21	7	0	28	15
		+	23	7	0	30	16
	4 hr	-	19	11	0	30	17
		+	23	7	0	30	18
	6 hr	-	22	8	0	30	19
		+	18	12	0	30	20
Control	-	-	3	16	8	27	21
		+	1	18	6	25	22

In the water-wash series considerable numbers of *L. trifolii* colonies developed, whereas in the Janola series colony numbers were greatly reduced, especially at the longer treatment times. Most satisfactory results were obtained where tissue pieces were water-washed for 2, 4 or 6 hours and plated to antibiotic agar.

3. Comparison of Janola for different soaking durations

(a) Plating to antibiotic medium

In all treatments fungal contamination remained at a relatively high level, whereas bacterial contamination was suppressed in all treatments except one (treatment 8). Also there was a suppression in the number of colonies of the pathogen which developed as the soaking duration increased beyond one minute, whereas fungal contaminant numbers remained static. This latter result would suggest that the pathogen was more superficially located than the fungal saprophytes.

(b) Plating to non-antibiotic medium

At all soaking durations tested, moderate fungal contamination was present. Further, some bacterial contamination was present in all treatments except two (treatments 5 and 9). This indicated the ineffectiveness of the Janola soak treatment in eliminating saprophytic fungi and bacteria, and also emphasised the need for inclusion of antibiotics in the medium.

4. Comparison of water-wash for different soaking durations

(a) Plating to antibiotic medium

At all washing durations tested bacterial contaminants were totally suppressed without any adverse effect on pathogen colony numbers. The number of fungal contaminants remained at a relatively high, consistent level, even after a 6 hour water-wash (treatment 20).

(b) Plating to non-antibiotic medium

A similar trend in the number of pathogen, and fungal and bacterial contaminant colonies to that just described above was recorded. Only in treatment 13 (involving a 1 hour water-wash) were bacteria present.

From the above results it was concluded that surface treating infected tissue pieces by washing in running tap water for 4-6 hours, plating to antibiotic PDA_L, and then incubating at 24°C was the most satisfactory method for isolating the pathogen from infected lucerne foliage. This method was followed in subsequent isolation work.

Using the above isolation method cultures were obtained that macroscopically and microscopically were typical of Leptosphaerulina trifolii (Rost.) Petr.

Isolates of the pathogen were also obtained from infected lucerne seed following the method of Percival (1972), which involved the plating of seed to antibiotic malt agar.

B. ARTIFICIAL INOCULATIONS

Pseudothecia were readily produced on PDA_L or V-8 juice agar colonies incubated for 10 days at 23-27°C under 12 hours per day of fluorescent light. Healthy potted plants (var. Wairau) were inoculated by inverting sporulating cultures over the plants for 24 hours in a high humidity cabinet (90-100% R.H.) at 20°C. The plants were held for a further 48 hours under high humidity, and then transferred to a glasshouse bench.

Controls were provided by plants not inoculated but otherwise given identical treatment.

Symptoms were first apparent within 48 hours of inoculation as numerous, black, minute necrotic spots which gradually increased to approximately ½ mm in diameter. They were circular to irregular in shape, became lighter in colour with a dark margin, and were slightly sunken and occasionally surrounded by a chlorotic area. This latter lesion type was characteristic of those observed locally in the field and was typical of overseas descriptions where the causal organism is considered to be either L. trifolii or L. briosiana (Graham and Luttrell, 1961; Martinez and Hanson, 1963; Booth and Pirozynski, 1967a).

C. RE-ISOLATION OF THE CAUSAL ORGANISM AND COMPARISON WITH THE ORIGINAL CULTURE

The causal organism was readily re-isolated to agar using the method previously described. The colonies were macroscopically similar to the original cultures, and on microscopic examination were found to be morphologically identical.

By fulfilling the requirements of Koch's Postulates it was established that a Leptosphaerulina sp. was the causal organism of a leaf spot of lucerne not previously reported from the Manawatu.

CHAPTER VII

THE DISEASE

A. SYMPTOMATOLOGY

1. INTRODUCTION

Diseases referred to as Leptosphaerulina leaf spot, or pepper spot, have been reported on various legumes throughout the world since the latter part of the nineteenth century (Graham and Luttrell, 1961). From a study of the literature there appear to be few differences in the symptoms of the disease as described on lucerne and clovers. In discussing clover diseases, Dickson (1956) described symptoms caused by Pseudoplea trifolii (= L. trifolii) on lucerne and clover that differed only in the final colour and size of the lesions. Similarly, Graham and Luttrell (1961) who considered the two pathogens on lucerne and clover to be distinct, gave descriptions of the symptoms that again only differed in lesion colour.

One of the criteria used by Graham and Luttrell (1961) to justify separation of the two pathogens was based on the results of cross-inoculation tests. They found that L. briosiana and L. trifolii differed in severity of infection and symptoms they produced on Medicago and Trifolium species, and on such other legumes as Melilotus alba Med. and Pisum sativum L.

In the present study the field and glasshouse symptoms of pepper spot on lucerne and red clover were examined, and cross-inoculation studies of lucerne and red clover isolates to M. sativa (lucerne), T. pratense (red clover) and P. sativum var. arvense (field pea) were conducted to determine whether symptom expression and pathogenicity to the respective hosts provided supporting evidence for recognition of the two species.

2. FIELD OBSERVATIONS

Symptoms caused by L. trifolii on lucerne and clover crops in the Manawatu are compared with those reported overseas in Table 26.

TABLE 26. Comparison of symptoms caused by L. trifolii on lucerne and clover crops in the Manawatu and overseas.

Local (Author)		Overseas (Graham & Luttrell, 1961)	
Lucerne	Red clover	Lucerne	Clover
On leaves. Lesions numerous, circular, sunken, ¼-½ mm dia., black to brown centre and occasionally with darker margin; surrounded by a chlorotic halo.	On leaves and petioles. Lesions numerous, circular, sunken ¼-1 mm dia., black to dark-brown, with a surrounding chlorotic halo.	On leaves and petioles. Leaf symptoms are irregular to round eyespots, 1-3 mm dia., with light-brown to bleached centres and dark-brown margins, often with surrounding chlorotic halo.	On leaves and petioles. Lesions numerous, circular, small, black and sunken; under favourable conditions spots develop up to 3 mm dia.

(a) Lucerne

The disease in the Manawatu still appears to be restricted in distribution. Of several crops examined only in one was the disease observed, being present as a trace infection. It was most prevalent during early spring, and to a lesser extent in the autumn.

Symptoms observed on leaves (Figure 22) were essentially similar to those reported overseas (Table 26). Lesions were numerous, circular, black, slightly sunken, ¼-½ mm in diameter, occasionally with brown centres and dark margins, and often with a surrounding chlorotic area. The lesions were randomly located predominantly on the upper leaflet surface. It was this lesion type that was difficult to distinguish from those caused by other foliage pathogens of lucerne present in the Manawatu.

Lesions on petioles were not found.

According to Close (1972), in severe infections there is a yellowish-brown burnt appearance of the leaf margins with some leaf shedding.

Although the production of pseudothecia on lesioned tissues is regarded overseas as a common feature of the disease (McDonald, 1958; Elliot and Wilcoxson, 1964), in no instance were they observed in local crops. However, by inoculating excised leaves with ascospores in the laboratory protopseudothecia were readily produced which matured to produce asci and ascospores typical of L. trifolii (Chapter VIII A).

(b) Red Clover

A surprising feature of the disease was the fact that, although widespread and severe in several red clover pastures in the Manawatu, it had not been reported on this species in New Zealand prior to the present study.

Symptoms were observed on leaves (Figure 23) and occasionally on petioles, and were essentially similar to those reported overseas (Table 26). On leaves the first evidence was numerous, black, pin-point spots, occasionally surrounded by a chlorotic area.

(a) Lucerne

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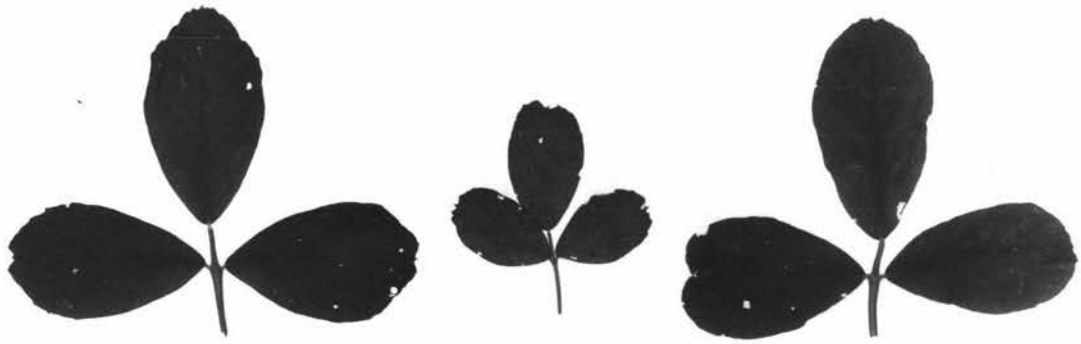


FIGURE 22.
Symptoms on lucerne leaves naturally infected with L. trifolii.

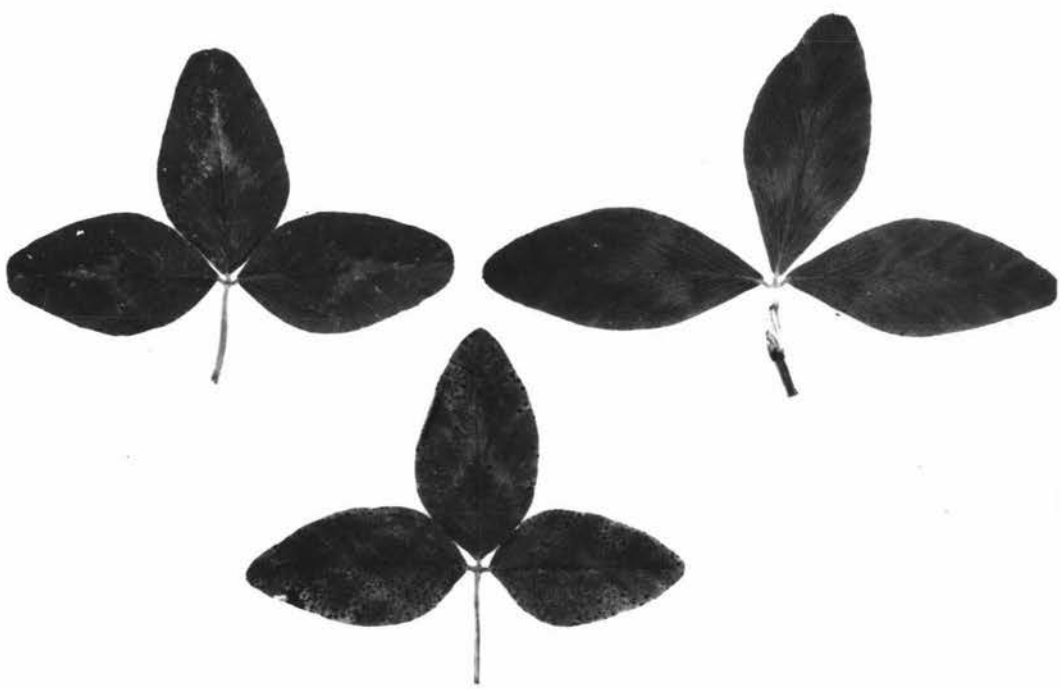


FIGURE 23.
Symptoms on red clover leaves naturally infected with L. trifolii.

These spots were randomly located and were predominantly on the upper leaflet surface. Pepper spot, the name commonly applied to this disease aptly describes the symptoms as expressed at this stage. With time the lesions became circular, sunken, black to dark-brown, ¼-1 mm in diameter, with a surrounding chlorotic halo. When infections were severe, lesions had enlarged and coalesced causing leaves to wither and turn yellowish-brown. Again, neither protopseudothecia nor pseudothecia were observed in mature lesions or on host debris.

Small, black, elongated lesions were also observed on petioles.

3. GLASSHOUSE OBSERVATIONS

In each instance symptom development was observed in conjunction with studies conducted to fulfil the requirements of Koch's Postulates.

(a) Lucerne

Symptoms first appeared on leaves within 48 hours of inoculation as numerous, black, minute, circular, necrotic spots which were randomly located, mainly on the upper leaflet surface. At this stage there was no evidence of petiole symptoms. On the tenth day following inoculation lesions ranged from pin-point up to ½ mm in diameter, were circular to irregular in shape, slightly sunken, black, occasionally with brown centres and dark margins, and often surrounded by a chlorotic area (Figure 24). On the lower leaflet surface faint, black, pin-point spots were now apparent corresponding to well-developed lesions present on the upper surface. Where infections were severe, lesions coalesced causing the leaflet tips and margins to wither and turn chlorotic.

Within 12 days of inoculation a few small, black, elongated lesions had developed on petioles.

Protopseudothecia were not found on any lesioned tissue, even 20 days after inoculation.

Although more severe infections were obtained on artificially inoculated plants, the glasshouse symptoms were, in general, characteristic of those observed locally in the field.

FIGURE 24.
Symptoms on lucerne leaves artificially inoculated with a L. trifolii isolate from lucerne.

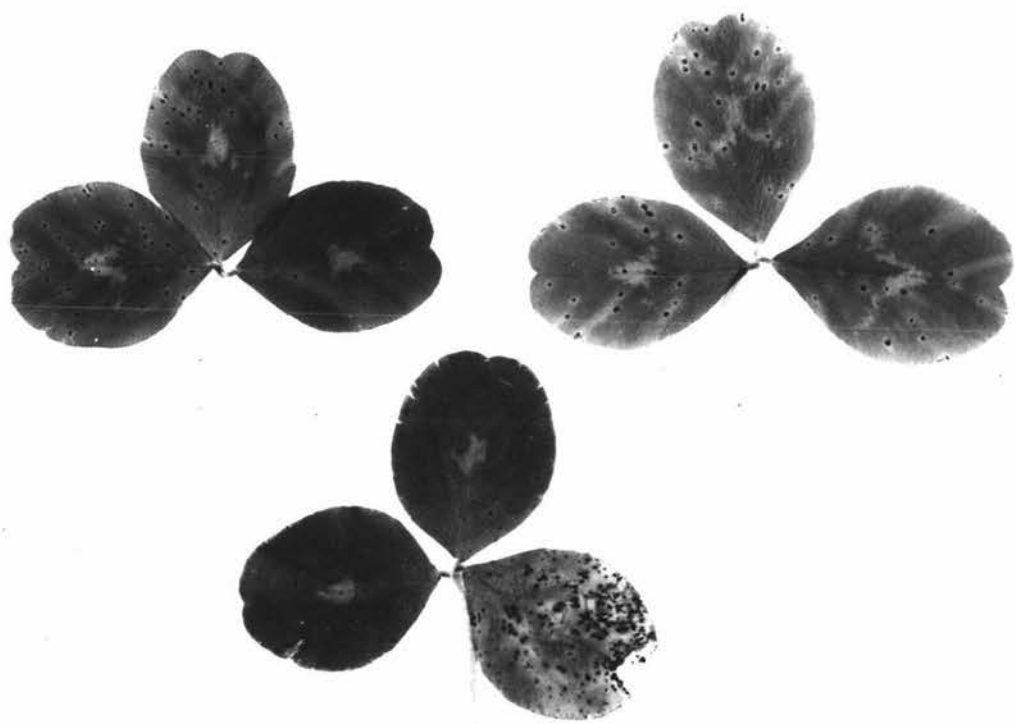


FIGURE 25.
Symptoms on red clover leaves artificially inoculated with a L. trifolii isolate from red clover.

(b) Red Clover

Symptoms first appeared on leaves within 24-48 hours of inoculation as numerous, black, pin-point spots which were randomly located, mainly on the upper leaflet surface. At this stage the lesions were very similar to those observed on lucerne at a similar time interval following inoculation. On the tenth day lesions ranged from pin-point up to 1 mm in diameter, were circular, sunken, with tan centres and dark-brown margins, and surrounded by a chlorotic halo (Figure 25). Lesions initially observed on the upper leaflet surface were now also visible on the lower surface. Where infection was severe lesions had coalesced causing a yellowish-brown burnt appearance of leaflets.

A relatively few lesions developed on petioles which in appearance were similar to those found in the field.

Protopseudothecia in or around lesions were not observed.

Symptoms expressed by artificially inoculated plants differed from those of naturally infected plants only in that the lesions were generally larger.

4. CROSS-INOCULATION STUDIES

One of the criteria used by Graham and Luttrell (1961) for the separation of L. briosiana and L. trifolii was differences in pathogenicity as evidenced by reciprocal cross-inoculation studies. They observed that L. briosiana usually occurred only on Medicago spp. in the field, but from artificial inoculations it was found to cause mild infections on other legumes. By contrast, L. trifolii has only been reported from the field on Trifolium spp., but their inoculation studies showed it had the greatest potential host range, causing severe infections on Melilotus alba and Pisum sativum, plants on which L. briosiana produced little or no disease.

Graham and Luttrell (1961) also found that cross-infection occurred with *L. briosiana* and *L. trifolii* on *M. sativa* and *T. repens*, but when isolates were returned to the original host the disease was more severe.

In the present study inoculations were carried out to determine whether, in fact, isolates from lucerne and red clover could be differentiated on the basis of symptomatology and the severity of infection on *M. sativa*, *T. pratense*, and *P. sativum*.

Six potted plants of each host species were inoculated, three with isolates from lucerne and the remainder with isolates from red clover, by inverting sporulating cultures over the plants for 24 hours in a high humidity cabinet (90-100% R.H.) at 20°C. The plants were held for a further 48 hours under high humidity, and then transferred to a glasshouse bench. The type of symptom and severity of infection produced were recorded 10 days after inoculation.

The results are photographically recorded in Figures 26-28.

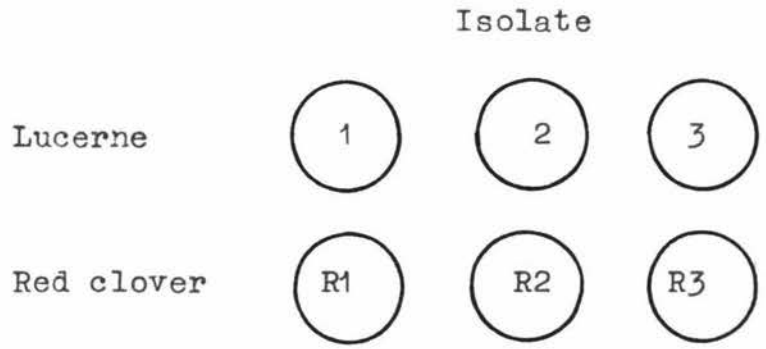
(a) *Lucerne* (*M. sativa*)

Lesions caused by the lucerne isolates were typical of those previously obtained in artificial inoculations; that is, circular to irregular in shape, large, black or with tan centres and dark margins, and in turn surrounded by a chlorotic area. Lesions produced by the red clover isolates were similar, being circular in shape, small, mostly black but occasionally brown, but invariably lacked the chlorotic area.

The severity of infection produced by all isolates was very similar. In general, it varied no more between isolates from different host species that it did between isolates from the same host species.

The observation by Graham and Luttrell (1961) that the disease was more severe on the original host when *L. briosiana* and *L. trifolii* were cross-inoculated to *M. sativa*, was not substantiated. However, the differences they described in symptom type and also observed in this study were essentially the same.

Key to Figures 26-28 :



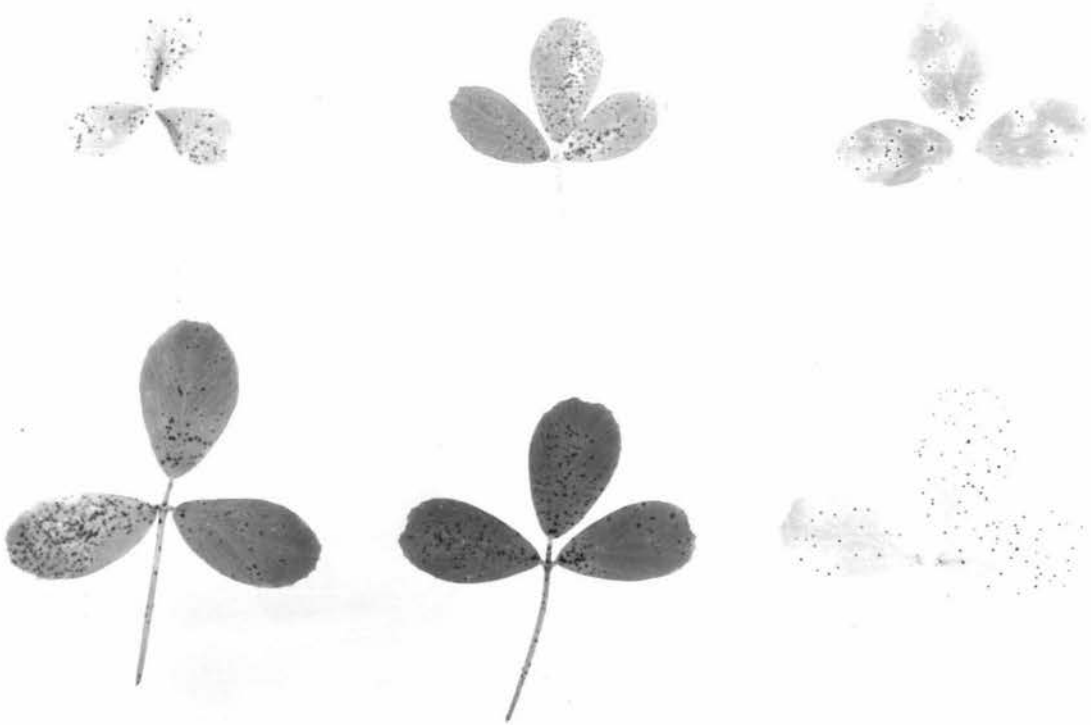


FIGURE 26.
Comparison of symptoms on lucerne leaves artificially inoculated with Leptosphaerulina isolates from lucerne and red clover.

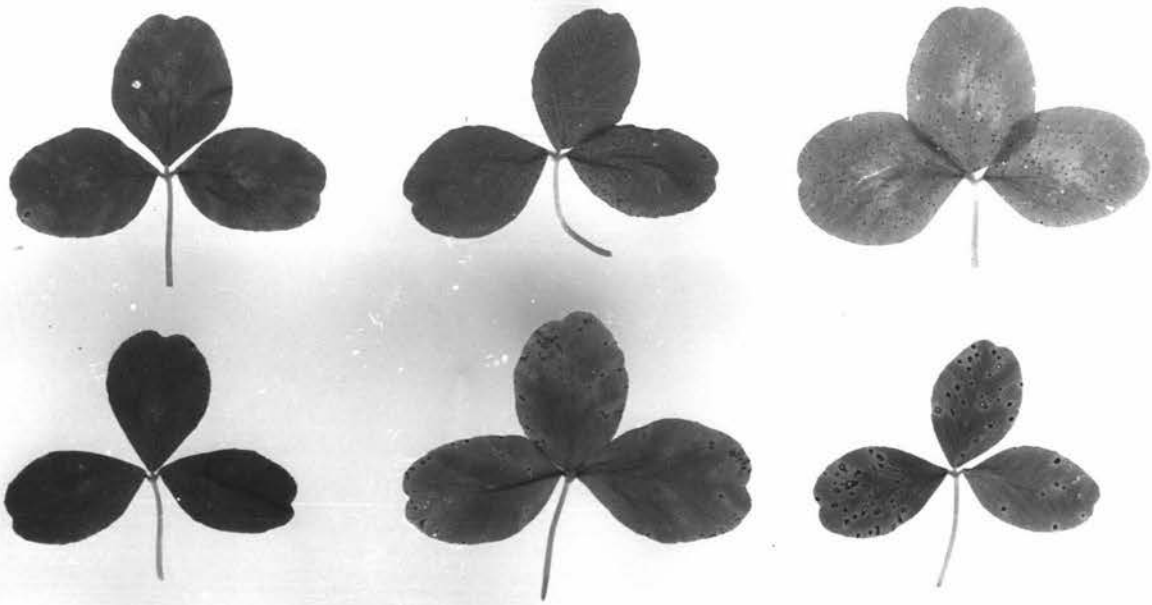


FIGURE 27.
Comparison of symptoms on red clover leaves artificially inoculated with Leptosphaerulina isolates from lucerne and red clover.

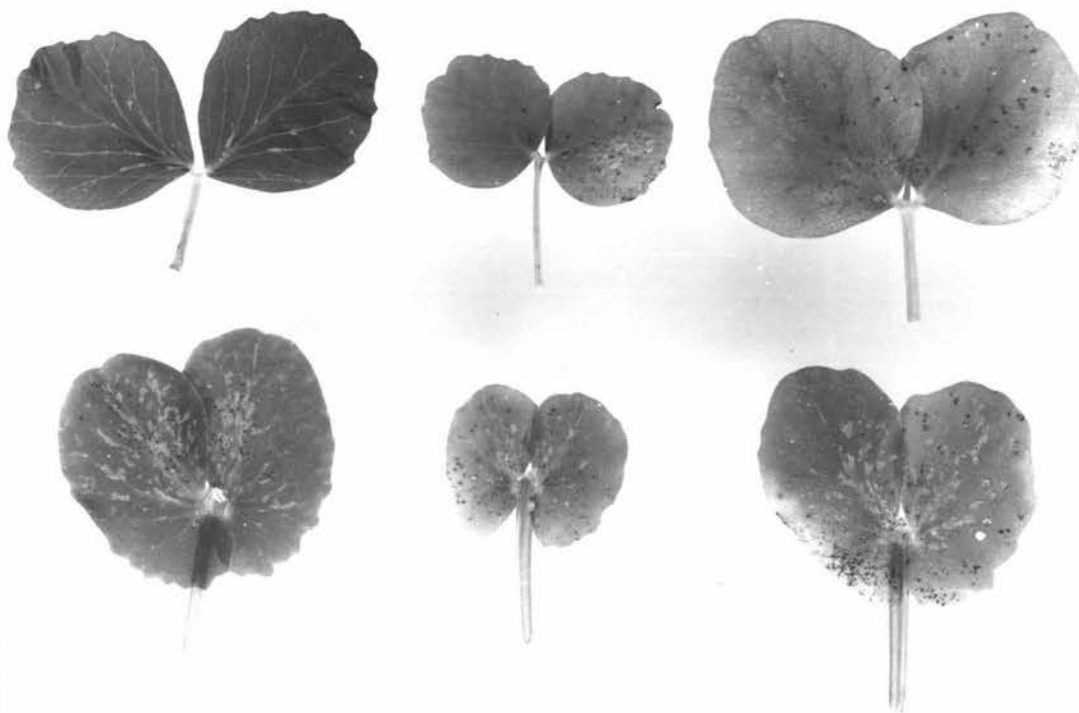


FIGURE 28.
Comparison of symptoms on pea leaves artificially inoculated with Leptosphaerulina isolates from lucerne and red clover.

(b) Red Clover (T. pratense)

On this host the lucerne isolates produced circular, pin-point, black spots with little or no chlorosis, whereas the red clover isolates produced circular to irregularly shaped, large spots, with tan centres and dark margins, and with a chlorotic area around each lesion.

The clover isolates caused a more severe infection than the lucerne isolates, confirming the findings of Graham and Luttrell (1961) that the disease is more severe on the original host. However, this contrasts with the results produced by the two groups of isolates on lucerne, where little difference in severity was noted.

(c) Field Pea (P. sativum)

Circular, pin-point, black lesions, that developed no surrounding chlorotic area were produced by the lucerne isolates, whereas circular, small, dark-brown lesions, that also developed no chlorotic halo were produced by the red clover isolates.

The clover isolates caused a more severe infection than the lucerne isolates, but the difference was not as great as that indicated by Graham and Luttrell (1961), who found that L. trifolii caused severe infection on P. sativum, a plant on which L. briosiana produced only slight disease.

In conclusion, small differences in severity of infection and symptom type, particularly lesion size and the presence of a chlorotic halo were observed, but these were not distinctive or substantial enough to allow separation of the two species, as claimed by Graham and Luttrell (1961). Such differences that did exist are the result of a host-parasite interaction and are not an attribute of the pathogen itself. If the same fungus is inoculated onto different host species then such differences can occur even though the pathogens are morphologically identical.

The traditional concept that fungi belonging to the same genus could be separated by their distinctive pathogenicity on different host genera or species is now of questionable use in modern taxonomy.

This practice leads to numerous 'species' being erected, separated only on a host basis and correlated with minor morphological differences. Pathogenicity should only be used to differentiate categories below the species level (formae speciales) and not as a criterion for the separation of species, as proposed by Graham and Luttrell (1961). Species separation should be based only on morphologic criteria, with due regard to genetically and environmentally induced variation.

B. SEED-BORNE INOCULUM

Claims have been made that pepper spot is seed-borne in both Medicago spp. (Miles, 1925; Graham and Luttrell, 1961; Booth and Pirozynski, 1967a; Noble and Richardson, 1968) and Trifolium spp. (Hopkins, 1923; Kilpatrick, 1958), but the evidence provided is not convincing.

By plating seeds of Medicago polymorpha L. (bur clover) with what he referred to as "sclerotia-like bodies" (= protopseudothecia?), Miles (1925) isolated colonies which in growth characteristics and pathogenicity were identical with isolates of Pseudoplea medicaginis (= L. trifolii) from field infected plants. On the basis of these results he commented:

> "It is probable that they (infected seeds) may serve as a capable and prolific agent in the spread of this disease in the field from one locality to another".

It should be noted that Miles provided no evidence to support his suggestion that infected plants could arise from use of such seed.

In claiming that L. trifolii is seed-borne in lucerne both Graham and Luttrell (1961) and Booth and Pirozynski (1967a) cite as their authority a short note by Carr (1957) reporting Pseudoplea trifolii (= L. trifolii) on lucerne as a new record for Great Britain.

The relevant section of Carr's note reads:

> "The apparent genetic difference between infected and uninfected families is further substantiated by the following observation. The families in trial on the field were the progeny of a diallel cross in which one parent was derived from a cross between a plant of the Canadian variety Rhizoma and one of the Aberystwyth bred S. 205. Only those families derived from this particular parent were affected. The Rhizoma material was imported as seed. However, these observations suggest that resistance and susceptibility are under fairly strict genetic control".

It is clear from the above quotation that Carr makes no direct claim of L. trifolii being seed-borne in lucerne. Further, one must conclude that the information provided by Carr does not justify the claim by Graham and Luttrell (1961) and Booth and Pirozynski (1967a) that the pathogen is seed-borne in this host species.

In their list of seed-borne diseases, Noble and Richardson (1968) stated that evidence concerning the seed-borne nature of the organism was incomplete. The only evidence rested on Miles observation of "sclerotia" on seed of bur clover, and as already noted above, he provided no evidence to show that seedling infection could arise from use of such seed.

In the course of screening 38 New Zealand produced lucerne seed-lines for fungal pathogens, Percival and Wenham (1972) found two to be infected with L. trifolii at levels of 0.4-5%. However, no attempt was made to demonstrate whether when so seed-borne L. trifolii could induce seedling infection.

In a study on Sphaerulina leaf spot of clover, Hopkins (1923) found lesions of the disease on the calyx and corolla. He suggested that this was significant as it indicated that seed infection may occur and thus account for the general distribution of the pathogen.

He further commented that:

> "Of a large number of commercial samples of white clover seed examined, mycelium was found in practically every instance in some of the seed coats. No successful isolation cultures were obtained from these seed to establish the identity of the fungus although very short periods of disinfection were used. It was finally concluded that the samples were old and the mycelium was dead".

Hence, as Hopkins failed to obtain viable cultures of the pathogen from seed he was unable to demonstrate that the disease was seed-borne.

In a survey of the kind and relative prevalence of fungi isolated from seeds of Trifolium repens, Kilpatrick (1958) isolated L. trifolii from seeds obtained from naturally infected plants. No evidence was provided of the establishment of infection in plants arising from use of this seed.

From the above review it is apparent that the question of whether pepper spot is a seed-borne disease of lucerne and clovers has not been resolved. For evidence to be conclusive that a disease is seed-borne in a specified host crop, the following two steps of study must be completed:

1. The presence of viable inoculum associated with the seed must be demonstrated.
2. Seedling infections must result from sowing such seed.

The possibility of pepper spot being a seed-borne disease of lucerne in New Zealand was investigated, as follows.

1. EVIDENCE OF VIABLE, PATHOGENIC INOCULUM BEING ASSOCIATED WITH LUCERNE SEED

Two hundred and fifty seeds of 10 of the 38 lines screened by Percival (1972) were re-screened for the presence of L. trifolii, using the agar plate method as described by Percival and Wenham (1972).

Only four colonies of L. trifolii developed from the total of 2,500 seeds plated, three from one seed-line, and a single colony from a second seed-line (Figure 29). Following the preparation of pure cultures of each, the production of ascospores was induced by subjecting the isolates to cyclic fluorescent light (Chapter VII C). To determine the pathogenicity of each isolate the foliage of healthy potted lucerne plants in the glasshouse was then inoculated, by inverting the sporulating cultures over the plants. In each case typical symptoms of the disease subsequently developed.

Thus viable inoculum of L. trifolii was found to be associated with New Zealand produced lucerne seed-lines, as shown by Percival and Wenham (1972), and further its pathogenicity was established.

2. SEEDLING INFECTION FROM SEED CARRYING VIABLE INOCULUM

Two experiments were conducted to provide evidence that seedling infection could result from use of lucerne seed carrying viable inoculum of L. trifolii.

(a) Copenhagen Test

In a Copenhagen germinator 250 seeds of the heavier infected line were placed on moist blotter pads (25 seeds/pad) and incubated at 15–19°C under natural light conditions. Eleven days later seed and seedlings were examined microscopically for the presence of protopseudothecia of the pathogen.

In this test seeds and seedlings infected with L. trifolii were observed (Table 27; Figures 30 and 31). The presence of the pathogen was seen as black protopseudothecia and white mycelium on the seed testa and hypocotyl. Such protopseudothecia when transferred to PDA_L plates produced colonies of L. trifolii. This demonstrated that viable inoculum associated with seed could give rise to seedling infection.

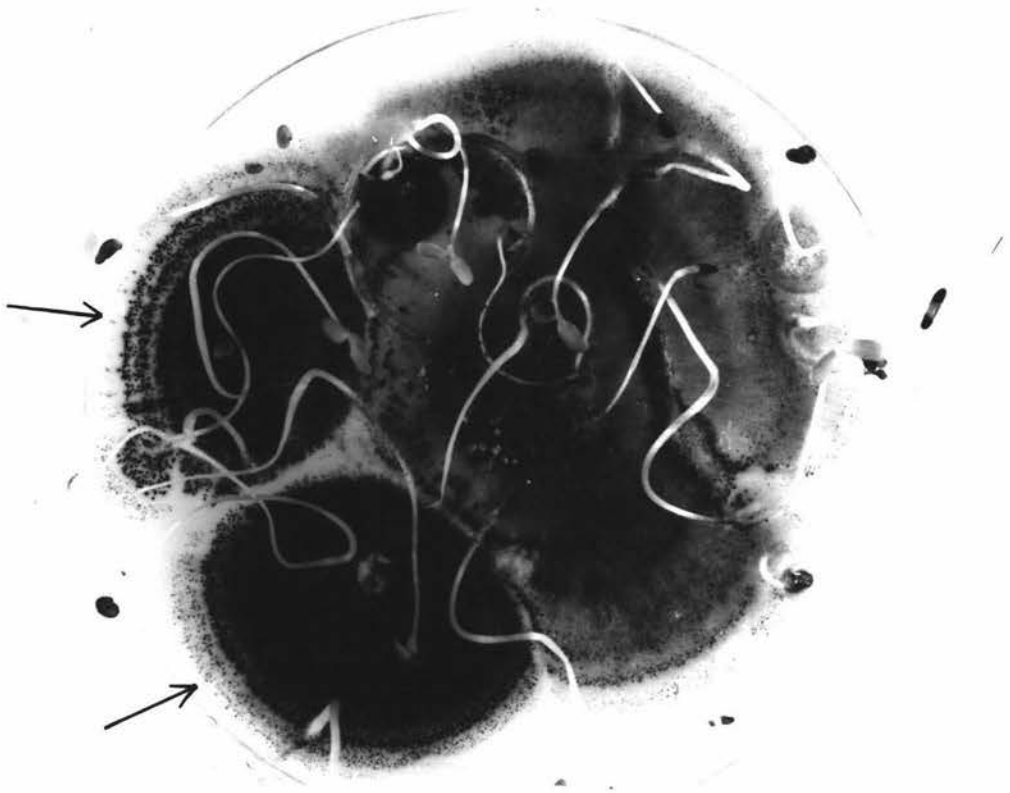


FIGURE 29.
Colonies of L. trifolii (arrowed) growing from seeds plated on antibiotic malt agar.

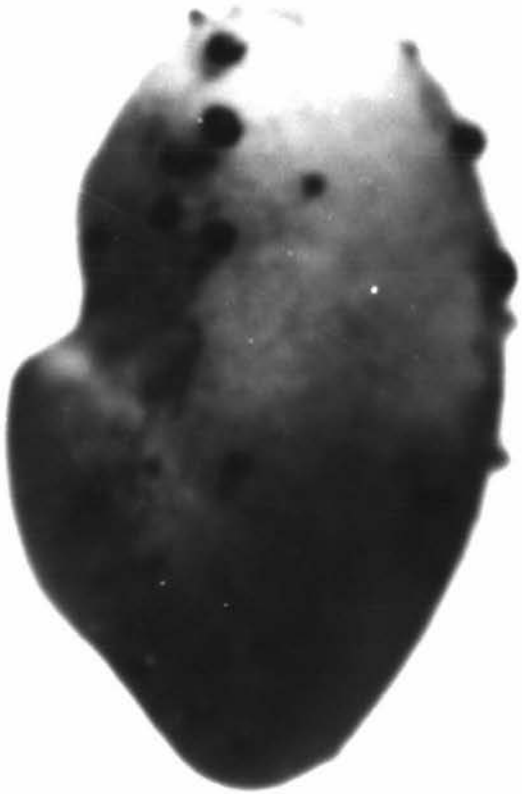


FIGURE 30.
Ungerminated seed from Copenhagen germinator with protopseudothecia of L. trifolii on the testa.

FIGURE 31.
Dead lucerne seedling from Copenhagen germinator with protopseudothecia and mycelium of L. trifolii on the testa and hypocotyl.

(b) Glasshouse Test

In the glasshouse 500 seeds of the same seed-line were sown evenly in vermiculite in plastic trays. Seedlings were examined after three weeks for disease symptoms caused by the pathogen.

This experiment was unsuccessful in that seedling infections were not observed (Table 27) due to the low infection level of the seed-line.

TABLE 27. Number of lucerne seeds and seedlings found infected with L. trifolii in the Copenhagen and glasshouse tests.

	Copenhagen Test	Glasshouse Test
No. seeds in test	250	500
No. seedlings	197	426
No. infected seeds	2	-
No. infected seedlings	4	-

In conclusion, seedling infection can result from seed infected with L. trifolii, but as the level of seed infection is low the effect on seedling establishment of sowing such seed would be minimal.

However, as the seed sowing rate is very high only an extremely low level of seed infection is required to provide numerous primary infection focii within a crop. Further, due to the close proximity of seedlings and the fact that the pathogen is disseminated by air-borne ascospores, rapid secondary spread and the establishment of numerous secondary infections could readily occur. Obviously then the presence of only a few infected seedlings within a crop, as in this instance, may be sufficient to initiate an epidemic outbreak under favourable conditions.

C. EFFECT OF LIGHT ON MATURATION OF PROTOPSEUDOTHECIA ON AGAR

Although intense production of protopseudothecia readily occurred on PDA_L or V-8 juice agar plates when incubated at 24°C, it was only on exposure to a light source that a relatively few matured to form asci and ascospores. Hence, before inoculation studies and morphological studies of the pathogen on agar could be conducted there was need for an investigation into those factors that would ensure ready and intense sporulation. Several environmental factors are known to stimulate maturation of protopseudothecia including substrate, incubation temperature and light (Graham and Luttrell, 1961; Martinez and Hanson, 1963; Leach, 1972). Of these, light was investigated in the present study.

Graham and Luttrell (1961) reported that the majority of their isolates of L. briosiana and L. trifolii produced ascospores only when exposed to a continuous fluorescent light source. Of their 11 isolates of L. briosiana, only two, both of which were prolific sporulators, produced ascospores in total darkness. A single isolate of L. trifolii produced a very few ascospores in the absence of light. These ascospores developed only in a sector of one colony of this isolate. Martinez and Hanson (1963) observed that an isolate of L. briosiana formed protopseudothecia in both darkness and light, but that cyclic fluorescent light was necessary for ascospore formation. The effect of light quality on ascospore formation was investigated by Thomas and Halpin (1964) for L. trifolii and L. australis McAlp. They observed that ascospore production was greatest under green and grey light and absent in darkness or red light. Leath (1971) reported that of 49 isolates of L. briosiana, all formed protopseudothecia in darkness or when wavelengths shorter than 340nm (i.e. NUV light) were excluded from fluorescent light, and all, except one, only matured when exposed to light of wavelengths less than 340nm.

Leach (1972) found that protopseudothecia of L. trifolii may develop in culture in darkness, but were usually more abundant in colonies exposed to light. Further, he found that pseudothecia formation in a cultural mutant was stimulated only by UV wavelengths less than 370nm and that longer wavelengths, including visible light, were ineffective.

Although the findings of Thomas and Halpin (1964) contradict the results of Leath (1971) and Leach (1972), the general trend is that protopseudothecia formation can occur in either darkness or light, but that fluorescent or more specifically UV light is necessary for their maturation.

In the present experiment inoculated V-8 juice agar plates were initially incubated in the dark at 22-24°C for 14 days to allow abundant production of protopseudothecia. The plates were then exposed for seven days to the following light treatments:

(i) Total darkness, provided by enclosing the plates in a metal cylinder.

(ii) Continuous near-ultraviolet light provided by a Phillips 40 watt black light blue tube held 40 cm above the plates.

(iii) Cyclic (12 hour cycle) fluorescent light provided by a Phillips 80 watt cool white fluorescent tube held 40 cm above the plates.

(iv) Natural diurnal periods of diffuse light provided by placing the plates on a laboratory bench out of direct sunlight.

There were four replicates per treatment, and the intensity of sporulation was recorded at the completion of the light treatments. This was carried out by inverting each plate over a glass slide for one hour and then counting, under the microscope, the number of ascospores ejected onto a square centimetre.

The results are summarised in Table 28. All Leptosphaerulina isolates used in this study required light for sporulation. This beneficial effect of light was clearly demonstrated by the fact that ascospore production in all treatments exposed to a light source was significantly higher than in total darkness.

Greatest sporulation occurred under fluorescent light and least under natural/diurnal light. It thus appeared that a wider range of wavelengths was required for sporulation than that provided by NUV light. Thus, this may be why the fluorescent light gave a higher intensity of sporulation. These findings are in agreement with those of Graham and Luttrell (1961), Martinez and Hanson (1963) and Sundheim and Wilcoxson (1965), but in contrast with those of Leath (1971) and Leach (1972) who both concluded that sporulation was stimulated by NUV light.

TABLE 28. Effect of light conditions on the intensity of ascospore production of L. trifolii.

Light treatment	Ascospores/cm^2				
	Replicate				Average
	1	2	3	4	
Darkness	0	0	0	0	.0
Continuous NUV	33	34	15	15	24.2
Cyclic fluorescent	37	20	55	34	36.5
Natural/diurnal	7	8	3	6	6.0

On the basis of the above experiment the following procedure was adopted to induce the development and maturation of protopseudothecia of L. trifolii on agar:

> Colonies on V-8 juice agar (when available) or PDA$_L$ were initially incubated at 24°C in the dark for ten days to allow protopseudothecia formation. They were then exposed to a cyclic fluorescent light source (12 hour cycle) for 5-7 days to induce their maturation.

CHAPTER VIII

MYCOLOGY OF THE PATHOGEN

Since the present investigation constitutes the first study of the pepper spot disease on lucerne in New Zealand it was deemed necessary to determine whether isolates of the causal organism in this country conformed with the several overseas descriptions of the pathogen on host tissue (Miller, 1925; Miles, 1925; Graham and Luttrell, 1961; Booth and Pirozynski, 1967a), and on culture media (Miles, 1925; Graham and Luttrell, 1961; Martinez and Hanson, 1963).

The origin of the isolates studied were as follows:

Isolate	Source	Locality
1	Lucerne leaves	D.S.I.R. Lincoln
2	Lucerne leaves	Kairanga
3	Lucerne seed (var. Wairau)	Christchurch
R1	Red clover leaves	Kairanga
R2	Red clover leaves	D.S.I.R. Palmerston North
R3	Red clover leaves	Massey University

A. MYCOLOGICAL FEATURES ON THE HOST

Production of the perfect state of isolates 2 and 3 on lucerne was induced on autoclaved, excised leaves in petri dishes, following the method of McDonald (1958). This involved inoculation of the leaves by inverting sporulating cultures over the petri dishes for 24 hours, and then transferring the dishes to an incubator (24°C). Four days later the petri dishes were placed outside the laboratory on a wall facing east. The leaves were examined after a further four, seven and seventeen days for the presence of pseudothecia. The experiment was repeated several times between April and August when air temperatures and daylength were low.

At four days numerous protopseudothecia of both isolates were in evidence, and by seven days the majority of protopseudothecia of isolate 3 had matured, ejecting ascospores onto the petri dish lids.

By seventeen days isolate 2 had also formed pseudothecia. Slides were prepared of pseudothecia, asci and ascospores and their morphology determined under the high power (x 400) of a compound microscope.

In Table 29 the morphological features of the perfect state are summarised and compared with those recorded overseas.

The black, globose pseudothecia were erumpent in the host tissue and contained several bitunicate, saccate asci. The asci (Figure 32) were thick-walled at the apex and had average dimensions of 40.1 x 78.7u. The typically hyaline ascospores were irregularly clustered in the ascus, were oblong, ellipsoid or clavate in shape, phragmosporous or muriform, and surrounded by a thin gelatinous sheath. Their average dimensions were 14.2 x 35.3u with a length/breadth ratio of 2.50.

Allowing for genetic and environmentally induced variability between isolates the above description of pseudothecia, asci and ascospores conforms closely to that of Miller (1925), Graham and Luttrell (1961) and Booth and Pirozynski (1967a) for Leptosphaerulina trifolii.

B. MYCOLOGICAL FEATURES ON AGAR

Graham and Luttrell (1961) claimed that L. briosiana and L. trifolii may be separated on the basis of such cultural criteria as gross colony characteristics (including growth rate), dimensions of pseudothecia, asci and ascospores, and ascospore septation. To confirm their conclusions, the mycological features of lucerne and red clover isolates were compared on culture media, as follows:

(a) Cultural characteristics.

Three isolates each from lucerne and red clover grown on 20% V-8 juice agar were compared, the specific features studied being the shape, colour and growth rate of colonies, and the morphology (including dimensions) of pseudothecia, asci and ascospores.

(b) Physiological studies

The effect of temperature and media type on the growth rate and gross colony characteristics of two lucerne isolates was determined. Three isolates each from lucerne and red clover were similarly compared on five media at 24°C.

TABLE 29. Comparison of the morphological features of *L. trifolii* from infected lucerne tissue.

Fungal Structure	Author	Miller (1925)	Graham and Luttrell (1961)	Booth and Pirozynski (1967a)
Pseudothecia				
(i) Morphology	Globose, black, with short necks and ostiolate; erumpent in tissue.	Globose to pyriform, black carbonaceous with short, thick necks and ostiolate; arise subepidermally, erumpent when mature.	Spherical, pale brown, membranous, with short necks and ostiolate; erumpent at apex.	Globose, pale brown, membranous, ostiolate; immersed in leaf tissue, erumpent at apex.
(ii) Diameter	105.0 - 186.0u	110 - 140u	83 - 152u	100 - 200u
Asci				
(i) Morphology	Bitunicate, saccate, apex thick-walled, contain 8 ascospores irregularly clustered.	Broadly clavate, truncate, smooth at apex, rounding and narrowing to pedicellate base, thick-walled.	Bitunicate, saccate, embedded in and separated by centrum parenchyma tissue.	Bitunicate, saccate, thick-walled, embedded in and separated by thin-walled, hyaline parenchyma.
(ii) Dimensions				
a) Length				
-range	68.4 - 92.6u	70 - 85u	53 - 98u	50 - 90u
-mean	78.7 ± 0.8u	-	-	-
b) Breadth				
-range	32.6 - 45.8	30 - 38u	31 - 48u	40 - 60u
-mean	40.1 ± 0.4u	-	-	-

Continued over....

TABLE 29. (contd)

Fungal Structure	Author	Miller (1925)	Graham and Luttrell (1961)	Booth and Pirozynski (1967a)
Ascospores				
(i) Morphology	Oblong, ellipsoid or clavate; typically hyaline; 3-5 x 0-3 septate; surrounded by a thin gelatinous sheath.	Elliptical to fusiform; becoming smoky-brown when mature; 3-4 x 1-3 septate.	Oblong, ellipsoid, or short cylindrical; hyaline but often becoming brown; 3-5 x 0-2 septate; surrounded by a thin gelatinous sheath.	Oval, clavate, or ellipsoid; hyaline, when mature often become slightly coloured; 3-4 x 0-2 septate.
(ii) Dimensions				
a) Length				
-range	27.2 - 41.8u	30 - 40u	26 - 46u	25 - 50u
-mean	35.3 ± 0.3u	-	32.2 - 39.7u	-
b) Breadth				
-range	12.6 - 17.5u	11 - 14u	11 - 18u	10 - 20u
-mean	14.2 ± 0.2u	-	14.3 - 16.2u	-
c) L/B ratio	2.50	-	2.27 - 2.45	-

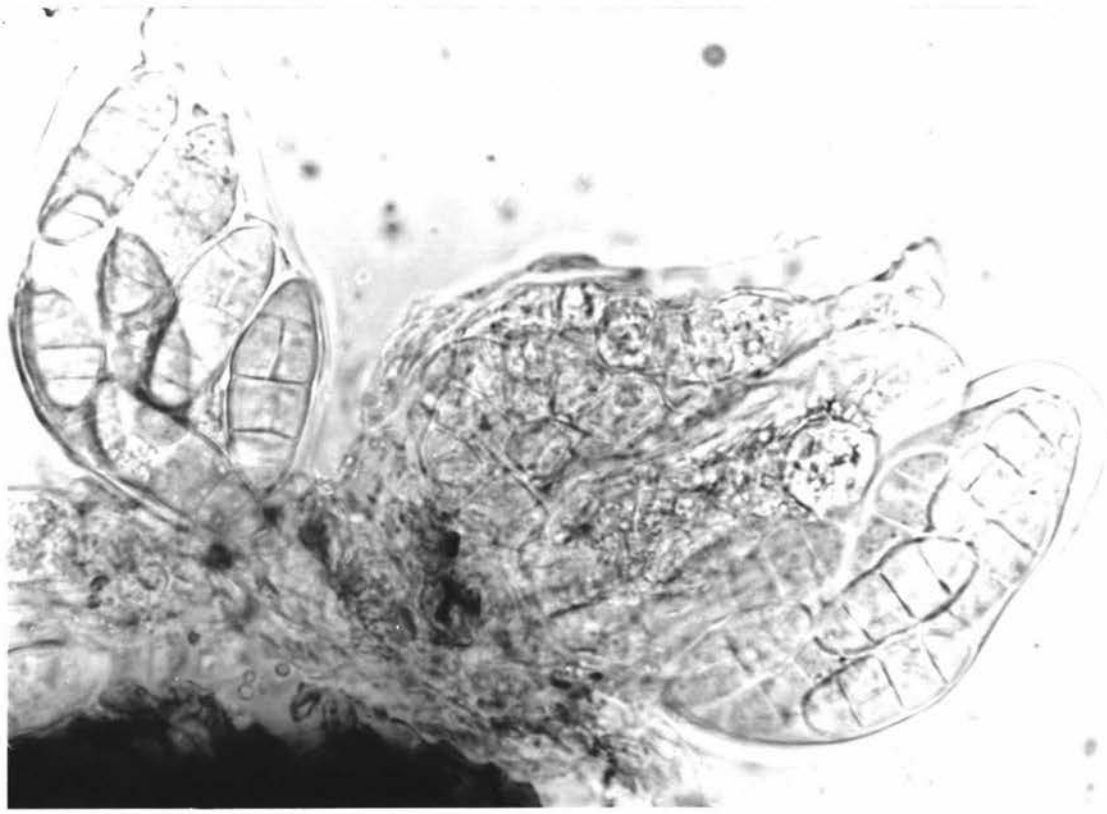


FIGURE 32.
Asci and ascospores of L. trifolii produced on artificially infected lucerne leaves (x 400).

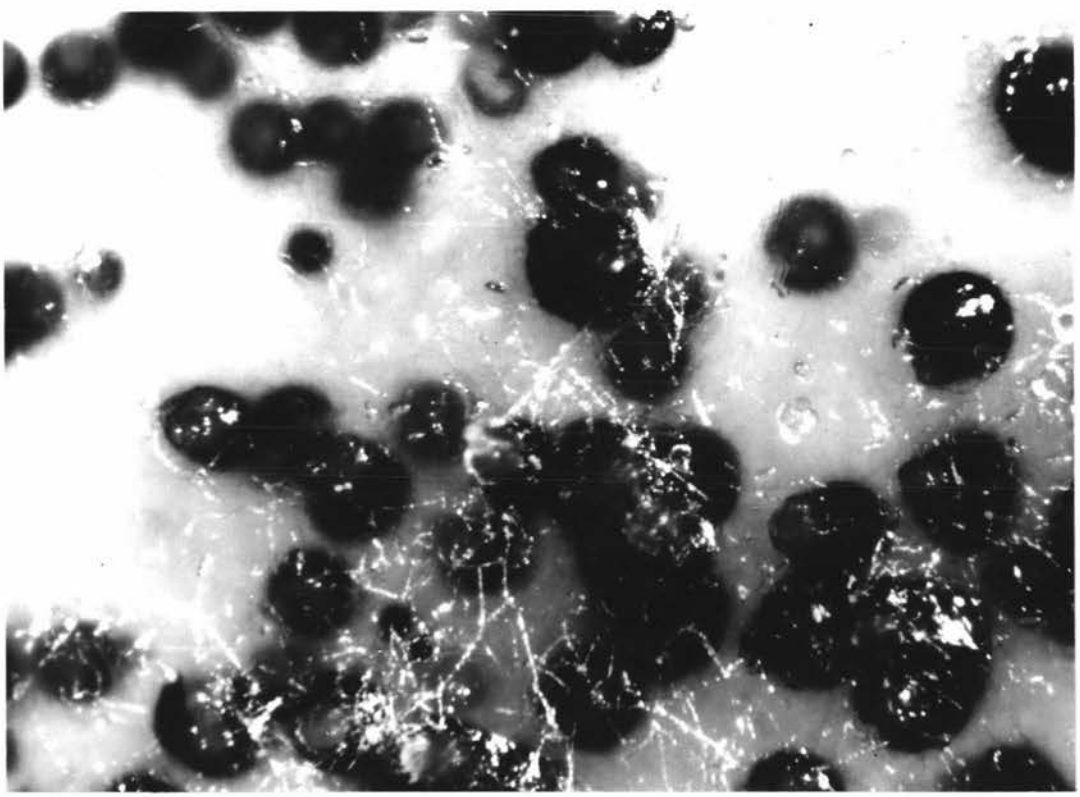


FIGURE 33.
Sporulation of a L. trifolii isolate from lucerne on V-8 juice agar after ten days incubation in the dark at 24°C and six days under cyclic fluorescent light.

1. MATERIALS AND METHODS

(a) Cultural characteristics

All isolates from lucerne and red clover were grown on 20% V-8 juice agar at 24°C, with each isolate replicated on three plates. The colony diameters were measured on the fourth, seventh, tenth and fourteenth day by taking the average of two measurements at right angles to each other. The macroscopic features of the isolates were also recorded on the seventh and fourteenth day.

On completion of the above growth rate study the production of pseudothecia by each isolate was induced by placing the cultures (all of which had produced protopseudothecia) under a cyclic fluorescent light source (12 hours light/12 hours dark) at 20-24°C for six days (Figure 33). The essential role of light in inducing maturation of protopseudothecia had earlier been established in this study, and also reported by Graham and Luttrell (1961), Martinez and Hanson (1963) and Pandey and Wilcoxson (1970).

To observe pseudothecia and asci, slide mounts were prepared by transferring pseudothecia to a drop of Shear's mounting fluid on a glass slide and squashing under a coverslip. Slide mounts of mature ascospores were obtained by inverting a sporulating culture over a glass slide for 1-2 hours on the sixth day following transfer of cultures to the fluorescent light. Using an eye-piece micrometer the dimensions of pseudothecia, asci and ascospores were also determined.

(b) Physiological studies

(i) The effect of temperature

The gross colony characteristics and growth rate of lucerne isolates 1 and 3 on PDAL were determined. Each isolate was replicated on four plates at each of the following nine temperatures:

4, 8, 12, 16, 20, 24, 28, 32 and 36 ± 1°C.

(ii) The effect of media

The gross colony characteristics and growth rate of lucerne isolates 1 and 3 incubated at 24°C were determined.

Each isolate was replicated on three plates for each of the following nine media: Laboratory potato-dextrose agar (PDA_L), Maknur potato-dextrose agar (PDA_M), oatmeal agar (OMA), cornmeal agar (CMA), malt agar (MA), prune agar (PrA), V-8 juice agar (V-8), water agar (WA), and lucerne decoction agar (LDA).

(iii) Comparison of lucerne and red clover isolates on five media

The gross colony characteristics and growth rate of the three lucerne and three red clover isolates incubated at 24°C were compared on the following media: Laboratory potato-dextrose agar (PDA_L), V-8 juice agar (V-8), cornmeal agar (CMA), lucerne decoction agar (LDA) and water agar (WA). Each isolate was replicated on three plates for each medium.

In each of the above three experiments growth rates were recorded after four, seven, ten and fourteen days incubation; gross colony features were recorded only on the fourteenth day. All media were prepared as described in Appendix I.

2. RESULTS AND DISCUSSION

(a) Cultural characteristics

(i) Macroscopic features

The essential macroscopic features of the three lucerne and three red clover isolates on 20% V-8 juice agar after seven and fourteen days incubation at 24°C are summarised and compared in Table 30. The gross colony characteristics of isolate 2 after fourteen days incubation is illustrated in Figure 34, and the average growth rates of the three lucerne and three red clover isolates are illustrated graphically in Figure 35.

When the macroscopic colony characteristics of the lucerne and red clover isolates were compared after 14 days incubation only minor differences were in evidence, such as the intensity of protopseudothecia production.

TABLE 30. Comparison of the macroscopic colony characteristics of lucerne and red clover isolates on V-8 juice agar after seven and fourteen days at 24°C.

Macroscopic features	Lucerne isolates	Red clover isolates
Seventh day		
1. Colony shape	Circular, flat with entire to undulate margin.	Circular, flat with entire margin.
2. Colony colour	Centre dark-brown to black due to brown mycelium and developing black protopseudothecia; margin(4-5 mm wide) white.	Centre black due to developing protopseudothecia; margin (3-5 mm wide) white. Mycelium slightly brown.
3. Aerial mycelium	Sparse, white and arising from whole of colony.	Sparse, white and arising from whole of colony.
4. Protopseudothecia production	Intense production at centre, with gradation to within 4 mm of margin; are black and submerged.	Scattered over whole of colony, except to within 6-7 mm of margin; are black, submerged and sparser than in the lucerne isolates.
Fourteenth day		
1. Colony shape	As on seventh day.	As on seventh day.
2. Colony colour	As on seventh day.	Except at very margin, mycelium is olive-brown; otherwise same as on seventh day.
3. Aerial mycelium	As on seventh day.	As on seventh day.
4. Protopseudothecia production	As on seventh day.	As on seventh day.

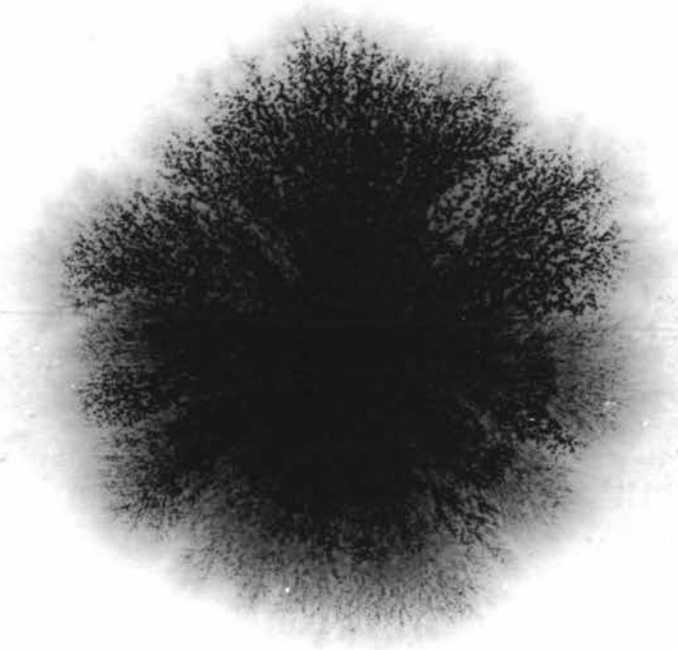


FIGURE 34.
Colony of L. trifolii (isolate 2) after fourteen days growth on V-8 juice agar at 24°C in the dark.

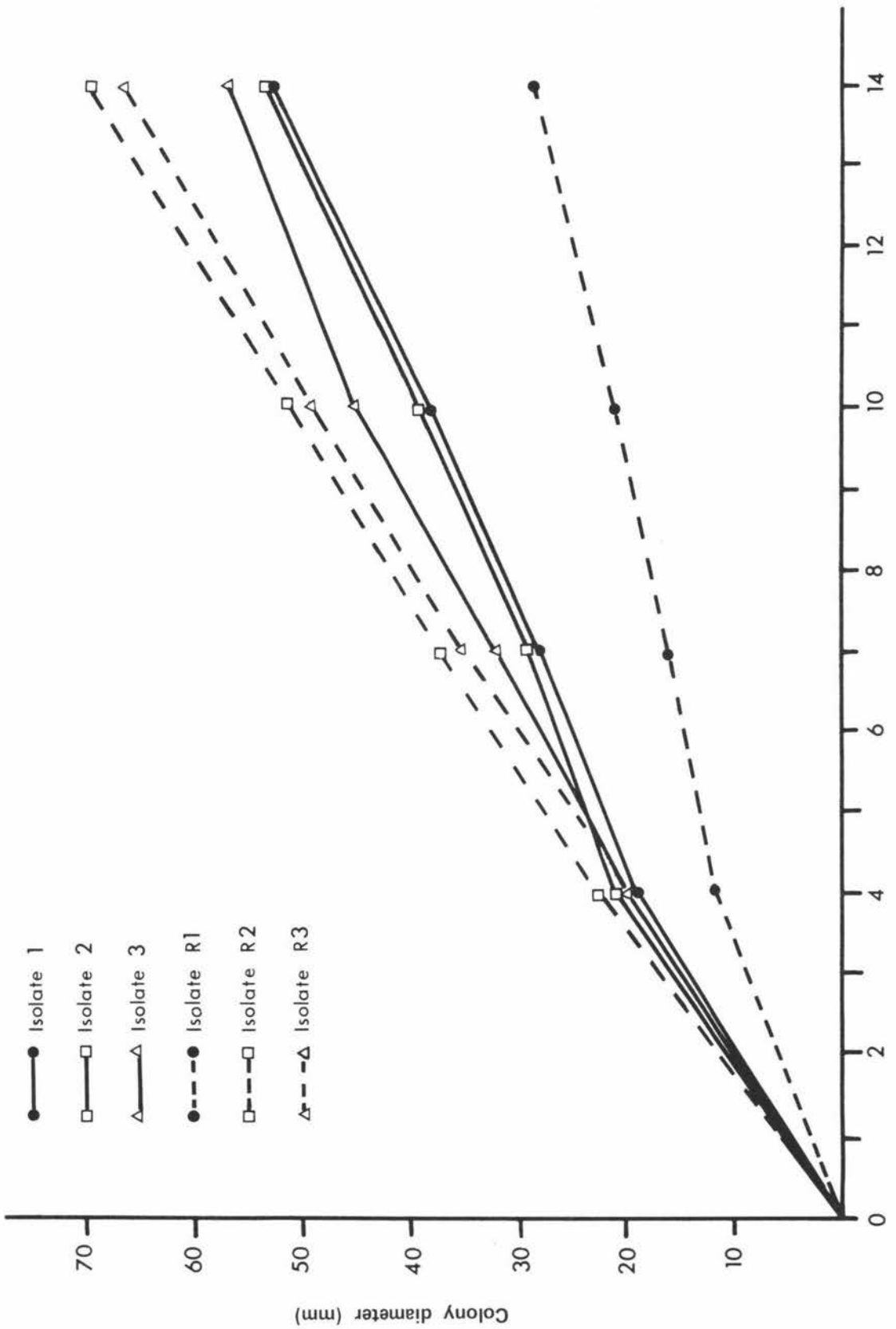


FIGURE 35. Growth rate of L. trifolii isolates from lucerne and red clover on V–8 juice agar at 24C.

Otherwise colonies of both groups of isolates were circular, flat, with an entire margin and with sparse, white aerial mycelium arising from the total colony surface. Viewed from above the colonies were dark-brown to black at the centre with a white margin. In the older central area of the colonies, intense production of black protopseudothecia was evident. This description of the macroscopic features of both series of isolates in culture essentially agrees with the description given overseas by Martinez and Hanson (1963) for L. briosiana.

The average growth rate of each group of isolates was very similar (lucerne 5'.4 mm; red clover 5'.8 mm). Further, the variation in growth rate between each group of isolates was just as great as within each group. Hence the finding of Graham and Luttrell (1961) that L. briosiana isolates (from lucerne) were faster growing than L. trifolii (from red clover) was not substantiated. That is, separation of the two species on the basis of this character was not possible.

(ii) Microscopic features

On agar the morphology of pseudothecia and asci of the lucerne and red clover isolates were essentially similar. Ascospores of the two series were likewise morphologically similar, except that they could be differentiated on the basis of the degree of septation.

Pseudothecia were black, globose, erumpent and had a well-defined apical beak. They contained several saccate, bitunicate asci which had thickened apical walls penetrated by a pore, and each with eight ascospores arranged in an irregular manner (Figures 36 and 37). Mature ascospores were oblong, ellipsoid or short cylindrical, with obtusely rounded ends, phragmosporous or muriform, rarely with 2 but usually with 3-5 transverse septa and 0-3 longitudinal septa, typically hyaline but often becoming brown, and surrounded by a thin gelatinous sheath (Figure 38).

According to Graham and Luttrell (1961), L. trifolii in culture produced larger pseudothecia, asci and ascospores than L. briosiana, with ascospores of the former being typically phragmosporous and predominantly with 3-transverse

FIGURE 36.
Asci containing eight ascospores of a L. trifolii isolate from lucerne on V-8 juice agar after five days incubation in the dark at 24°C and seven days under cyclic fluorescent light (x 400).

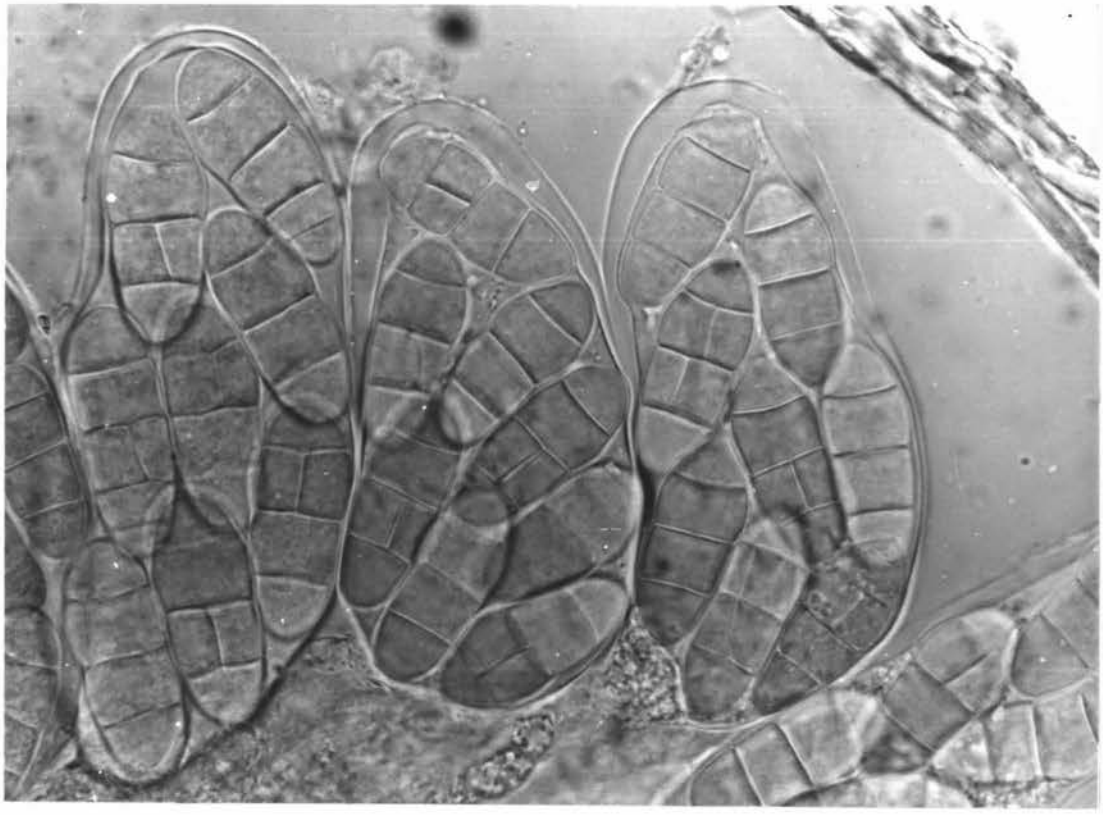


FIGURE 37.
Asci containing eight ascospores of a L. trifolii isolate from red clover on V-8 juice agar after five days incubation in the dark at 24°C and seven days under cyclic fluorescent light (x 500).

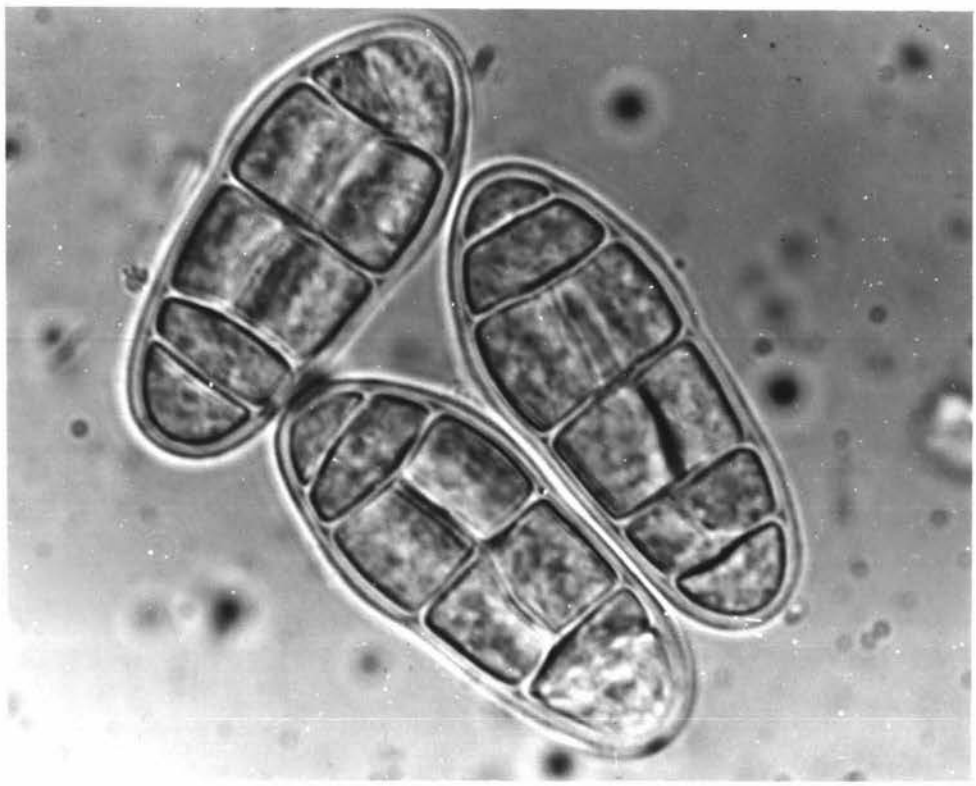


FIGURE 38.
Mature ascospores of a L. trifolii isolate from lucerne on V-8 juice agar after five days incubation in the dark at 24°C and seven days under cyclic fluorescent light (x 900).

septa, whereas ascospores of L. briosiana tended to be muriform and with 4-transverse septa. However, Booth and Pirozynski (1967a) stated that if isolates from infected hosts in the Leguminosae were grown under standardised controlled conditions, the ascospores produced would be uniform in both size and septation. These authors provided no evidence to support this prediction.

Collectively the lucerne and red clover isolates could likewise not be separated on the basis of pseudothecia, asci and ascospore dimensions since, as shown in Table 31, the degree of variation between each group of isolates was just as great as within each group. A possible exception was isolate R1 which produced larger pseudothecia and ascospores than any of the lucerne isolates, but similar sized asci, and hence would perhaps justify being identified as L. trifolii. Thus the claim by Graham and Luttrell (1961) that lucerne and red clover isolates could be differentiated by the size of pseudothecia, asci and ascospores was not confirmed in this study.

As stated above, the degree and type of ascospore septation was another major criterion by which the two species have been separated by Graham and Luttrell (1961). During this study separation of the two groups of isolates only on the degree of ascospore septation was found possible. Ascospores of the red clover isolates were typically 3-transversely septate with a low percentage being 4-septate, whereas the lucerne isolates had a high percentage of 4-septate with a correspondingly lower percentage of 3-septate ascospores. This is shown graphically in Figure 39. Although the range in septation shown by the red clover isolates was narrower it still overlapped considerably with the range found within the lucerne isolates (Figures 40 and 41). Further, the percentage of muriform ascospores varied greatly between all isolates and would prove to be an extremely unstable criterion on which to separate the two species.

Hence the report by Graham and Luttrell (1961) claiming that L. trifolii and L. briosiana have typically 3-septate and 4-septate ascospores, respectively, was substantiated, whereas their claim that the percentage of muriform ascospores could be used to separate the two species was not proven.

TABLE 31. Comparison of dimensions of pseudothecia, asci and ascospores of lucerne and red clover isolates grown on V-8 juice agar.

Isolate	Pseudothecia		Asci				Ascospores			
	Diameter (u)		Length (u)		Breadth (u)		Length (u)		Breadth (u)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
1	176.0 -356.0	266.66 ±5.96	72.5 -135.2	104.53 ±2.13	29.5 -60.2	44.47 ±1.02	20.0 -43.8	32.60 ±0.42	10.2 -14.0	12.32 ±0.14
2	167.0 -227.0	191.84 ±1.91	83.3 -127.7	109.52 ±1.47	35.6 -59.2	46.53 ±0.71	33.0 -44.7	37.73 ±0.84	13.7 -18.5	16.14 ±0.18
3	184.0 -291.0	228.54 ±3.57	88.6 -115.9	100.58 ±0.81	35.3 -47.7	41.68 ±0.46	34.0 -43.8	38.65 ±0.86	13.2 -19.3	15.86 ±0.38
R1	214.0 -405.0	304.36 ±6.76	84.6 -122.5	103.98 ±1.42	42.4 -67.9	52.80 ±0.76	39.7 -50.9	45.94 ±0.81	16.1 -25.8	21.52 ±0.17
R2	153.0 -230.0	184.66 ±2.50	87.5 -112.8	98.83 ±0.97	37.6 -56.5	45.61 ±0.59	32.7 -39.0	35.41 ±0.16	14.0 -17.4	15.45 ±0.13
R3	140.0 -286.0	189.84 ±4.41	84.2 -122.1	101.90 ±1.30	35.3 -54.4	43.48 ±0.58	33.6 -45.9	38.81 ±0.34	15.4 -19.3	16.78 ±0.12

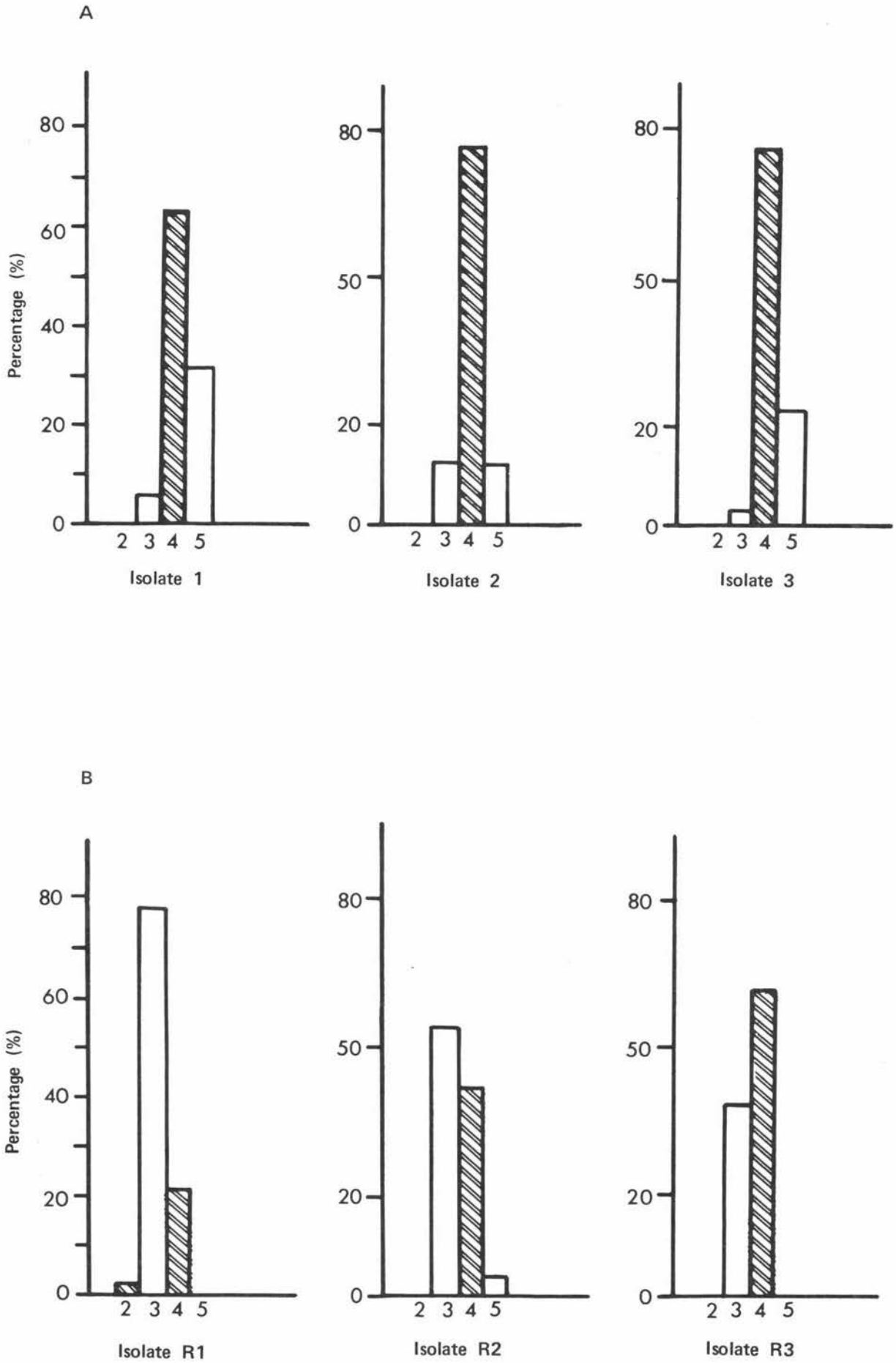


FIGURE 39. Percentage of 2, 3, 4 and 5 transversely septate ascospores of Leptosphaerulina isolated from lucerne (A) and red clover (B).

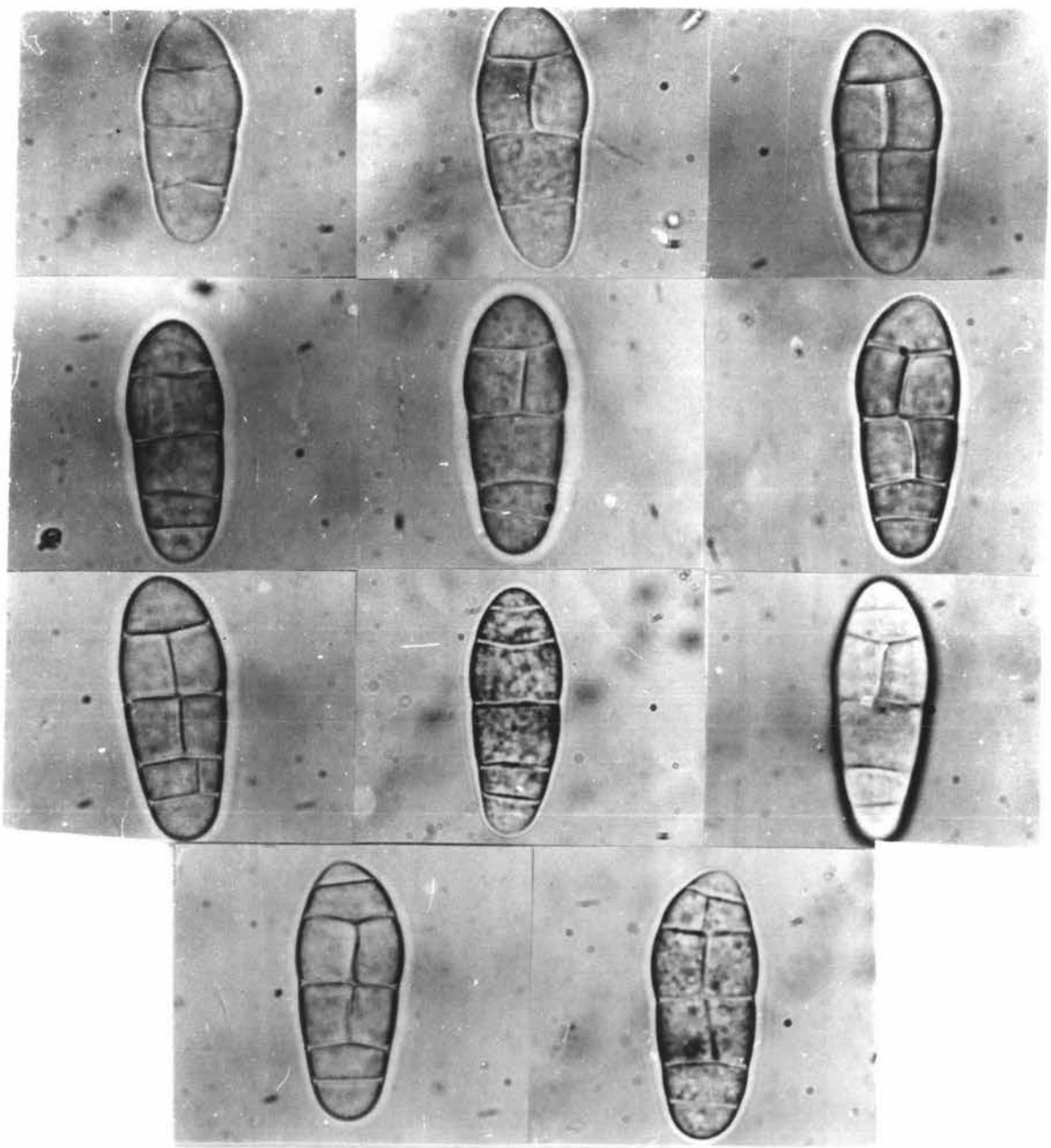


FIGURE 40.
Range in ascopsore septation of a L. trifolii isolate from lucerne on V-8 juice agar after five days incubation in the dark at 24°C and seven days under cyclic fluorescent light (x 900).

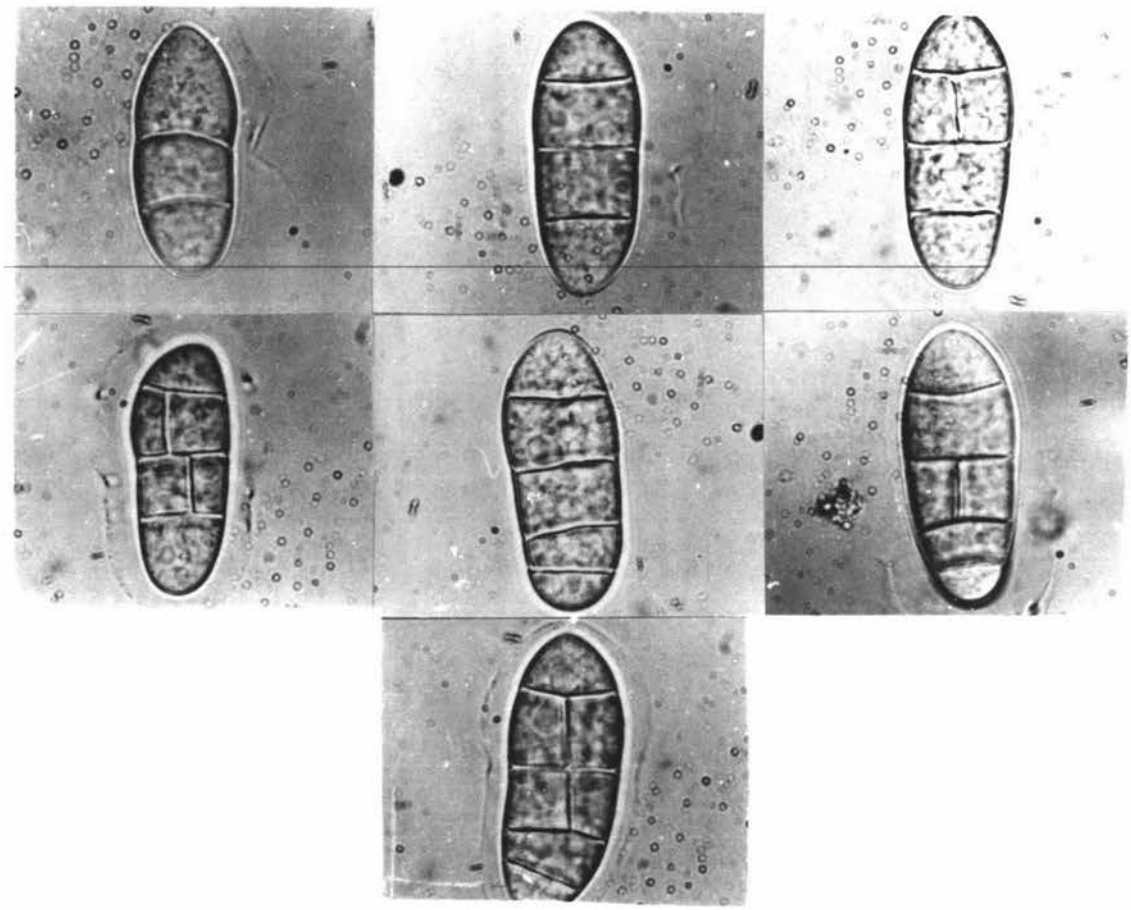


FIGURE 41.
Range in ascospore septation of a L. trifolii isolate from red clover on V-8 juice agar after five days incubation in the dark at 24°C and seven days under cyclic fluorescent light (x 900).

In general, the results did not directly support the contention of Booth and Pirozynski (1967a); although the ascospores produced by all isolates under standardised, controlled conditions were similar in size and septation, they still expressed considerable variation. However, as only three isolates from each host were examined general trends as provided by sampling large populations would not be detected.

In conclusion, results of the present study mainly did not support the claims of Graham and Luttrell (1961) in that isolates from lucerne and red clover could not be separated on the basis of the size of their pseudothecia, asci and ascospores, or type of ascospore septation. That is, the present findings did not provide evidence to support the contention that the two diseases under study are caused by distinct morphological species of the genus Leptosphaerulina.

(b) Physiological studies

(i) The effect of temperature

The gross colony characteristics of lucerne isolates 1 and 3 changed with temperature, with colonies having a black centre and white margin between 16 and 32°C, and being completely white at 8 and 12°C. White, fluffy aerial mycelium was sparsely produced centrally on all colonies incubated between 8 and 28°C, but was absent at all other temperatures. Black protopseudothecia were produced in cultures between 8 and 32°C, with the most intense production between 16 and 28°C. At these latter temperatures protopseudothecia were produced at the colony centre, and were initially submerged becoming erumpent with time.

The effect of temperature on radial growth of L. trifolii from lucerne is illustrated in Figure 42, and shown graphically in Figure 43.

The cardinal temperatures for vegetative growth of both isolates on PDA_L after fourteen days were 4, 24 and 36°C, these closely agreeing with the temperatures reported by previous workers (Miles, 1925; Graham and Luttrell, 1961; Martinez and Hanson, 1963). Growth at all times was greatest at 24°C, but nearly as great at 28°C, especially by the tenth day.

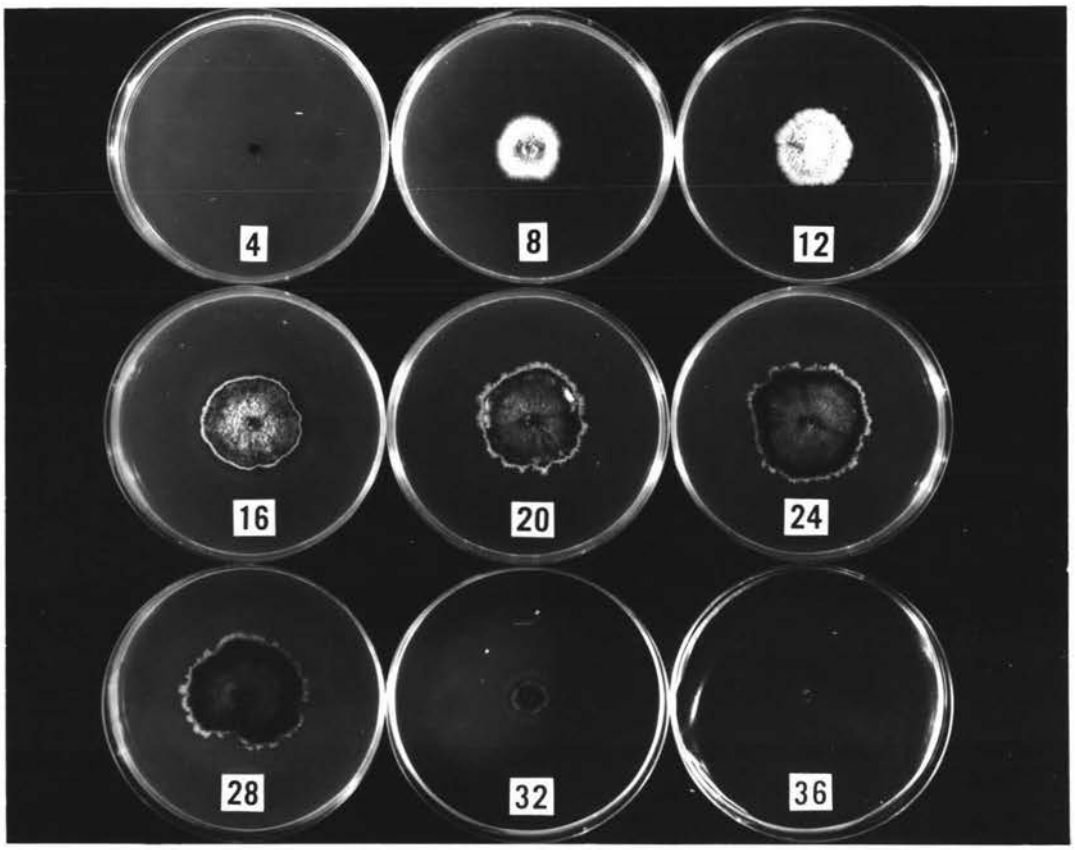


FIGURE 42.
Effect of temperature on the growth of L. trifolii from lucerne (isolate 1) on PDA_L after fourteen days incubation in the dark.

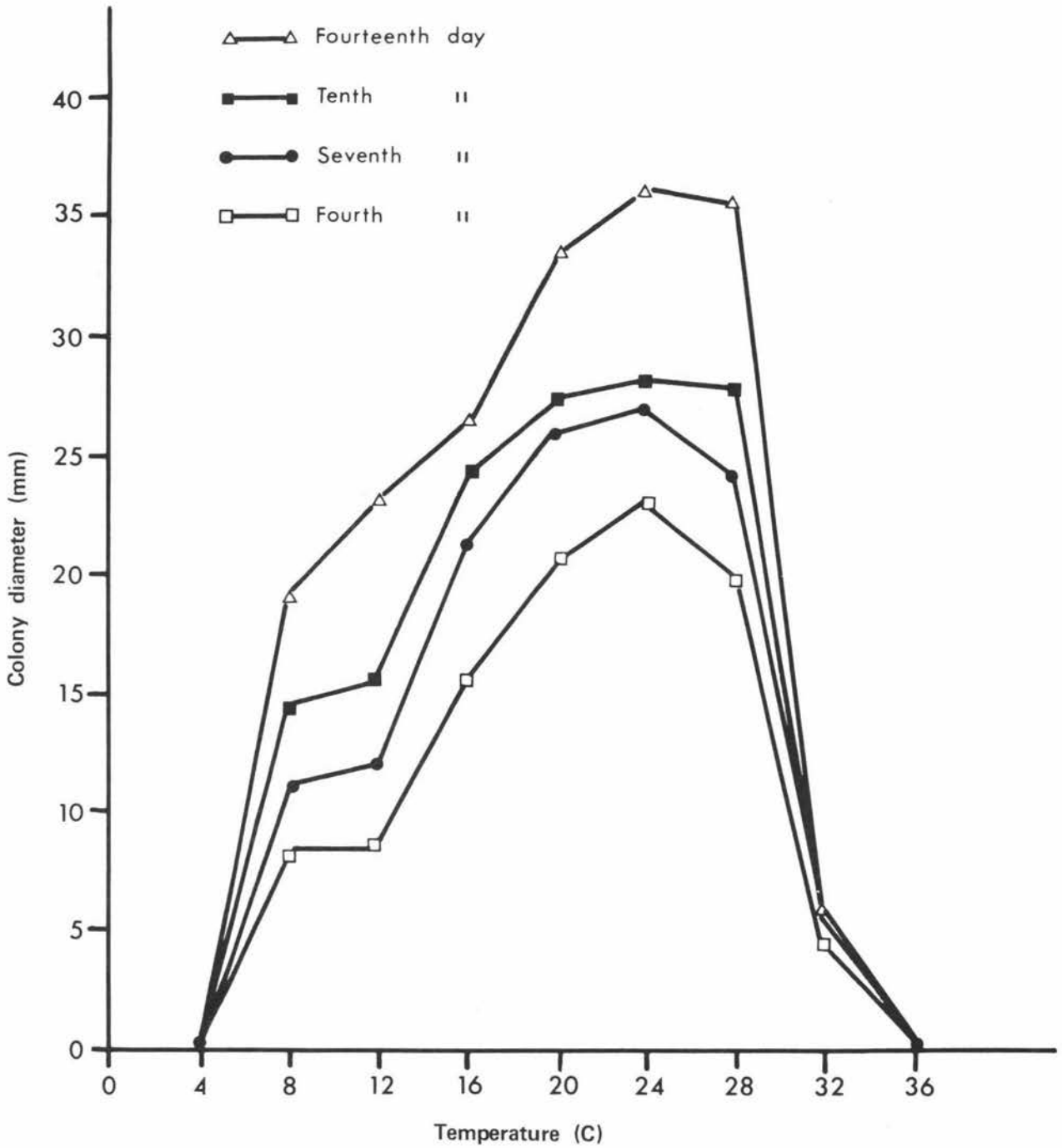


FIGURE 43. Effect of temperature on radial growth of L. trifolii from lucerne on PDA_L after four, seven, ten and fourteen days incubation.

It should be noted that these results refer to the average growth of two isolates, and that other individual isolates would differ slightly from the above.

(ii) The effect of media

The effect of media type on gross colony characteristics is illustrated in Figure 44 and summarised in Table 32.

Growth of both isolates was dense on LDA, V-8, PrA, MA and PDA_L, but very sparse on WA. Surface topography and colour of colonies were essentially the same on all media. In general, the colonies were circular to slightly irregular, flat, and when viewed from above light olive-green to black in colour at the centre, with a white margin. On V-8 and PDA_L the colony centres were black, while on WA they were completely white. Aerial mycelium was lacking on all media except LDA, V-8, MA and PDA_L, where it was sparsely produced at the colony centre.

On LDA, V-8, MA and PDA_L production of black protopseudothecia was very intense, whereas on all other media only sparse to moderate production occurred. Protopseudothecia were produced at the colony centre, and were either submerged or erumpent. Protopseudothecia production on CMA is illustrated in Figure 45.

The effect of media type on radial growth is illustrated graphically in Figure 46. After fourteen days incubation at 24°C growth was greatest on OMA, but nearly as great on LDA, V-8 and PrA; it was least on WA. It should be noted that the histograms record the average growth of two isolates, and that some variation between isolates would exist.

(iii) Comparison of lucerne and red clover isolates on five media

The effect of each medium on the gross colony characteristics of each isolate is illustrated in Figure 47, and their growth rates graphically illustrated in Figures 48 and 49.

On PDA_L the lucerne isolates had a fast growth rate, producing circular colonies with an undulate margin, whereas the red clover isolates had a slow growth rate, especially isolate R1, and also formed circular colonies, but with an entire margin.

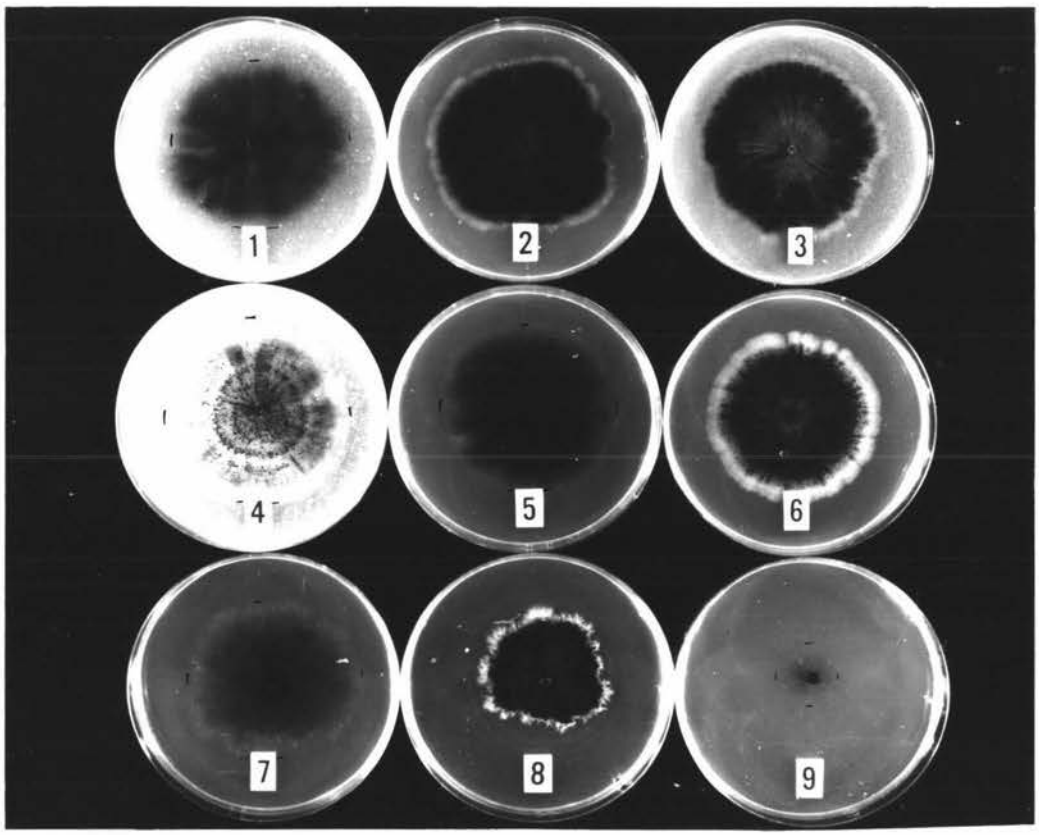


FIGURE 44.
Effect of media type on the growth and gross colony characteristics of L. trifolii from lucerne (isolate 1) after fourteen days incubation at 24°C in the dark.
(1-OMA, 2-LDA, 3-V-8, 4-CMA, 5-PrA, 6-MA, 7-PDA_M, 8-PDA_L, 9-WA).

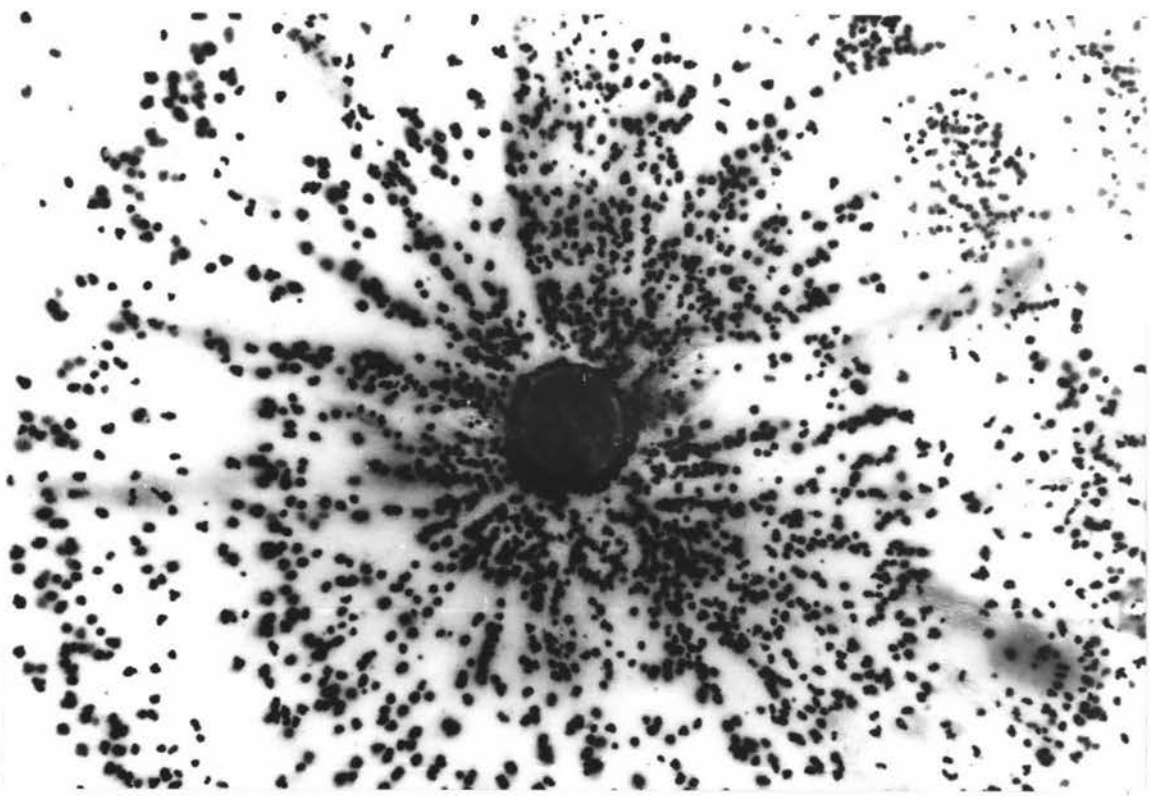


FIGURE 45.
Protopseudothecia production of a L. trifolii isolate from lucerne on CMA after fourteen days incubation at 24°C in the dark.

TABLE 32. Effect of media type on the gross colony characteristics of L. trifolii from lucerne incubated at 24°C for fourteen days.

	OMA	LDA	V-8	CMA	PrA	MA	PDA_M	PDA_L	WA
Medium pH	6.30	5.44	6.22	5.50	5.75	5.70	6.41	5.80	6.90
Surface Topography									
Shape	C	Sl Ir	Sl Ir	C	C	C	Ir	Ir	Ir
Elevation	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft
Zonation	NZ	Z	Z	Z	NZ	Z	NZ	NZ	NZ
Colony Colour									
Fringe	W	W	W	W	L Ol-Gn	W	W	W	W
Middle	L Ol-Gn	D Ol-Gn	Bl	L Ol-Gn	L Ol-Gn	D Ol-Gn	Ol-Gn	Bl	W
Centre	L Ol-Gn	D Ol-Gn	Bl	L Ol-Gn	L Ol-Gn	D Ol-Gn	Ol-Gn	Bl	W
Aerial Mycelium									
Quantity	0	1	1-2	0	0	1	0	1-2	0
Location	-	C	C-M	-	-	C	-	C-M	-
Morphology	-	Ct	Ct	-	-	Ct	-	Ct	-
Colour	-	W	W	-	-	W	-	W	-
Protopseudothecia Features									
Quantity	1	4	4	2	2	4	1	3-4	0-1
Location	C-M/E-I	C-M/I	C-M/E-I	C-M/E-I	C-M/I	C-M/E	C/I	C-M/E	C-M/I
Colour	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl	Bl

Key: See Table 19.

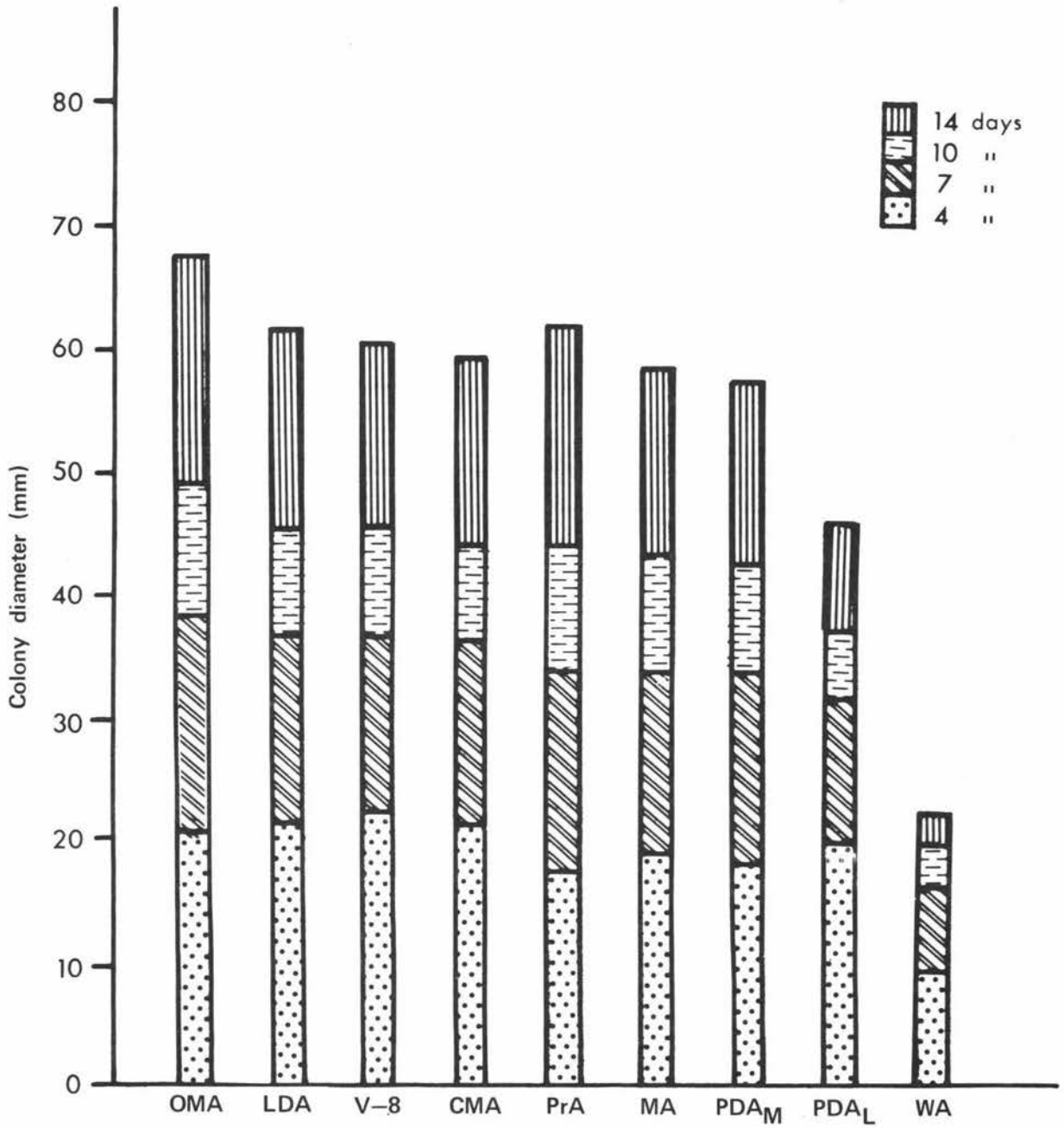
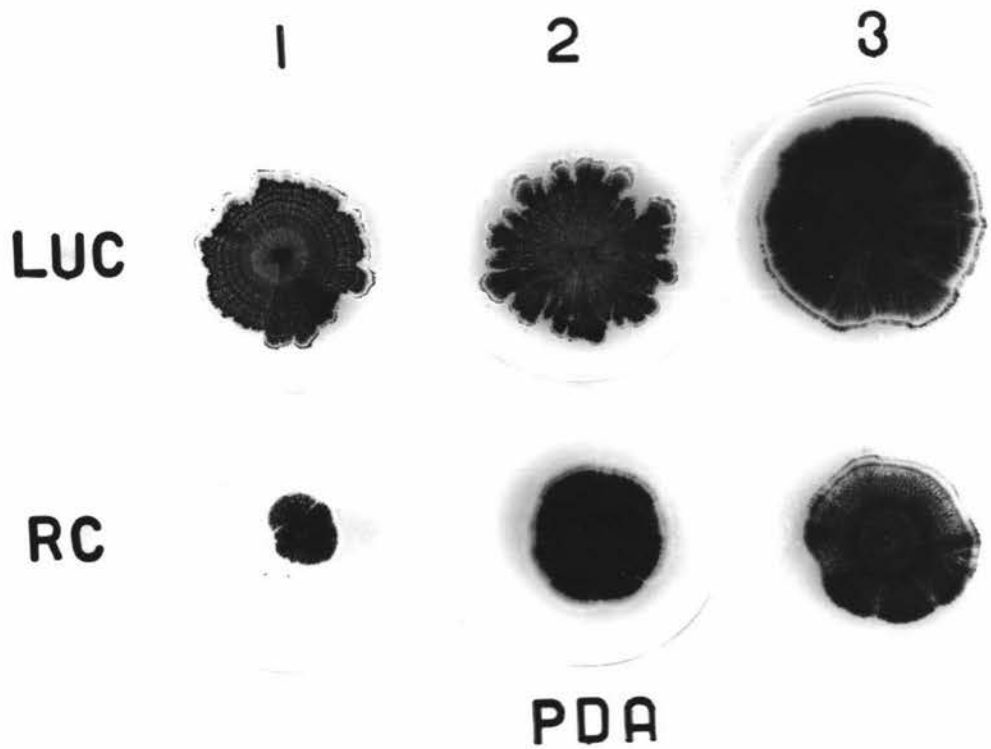


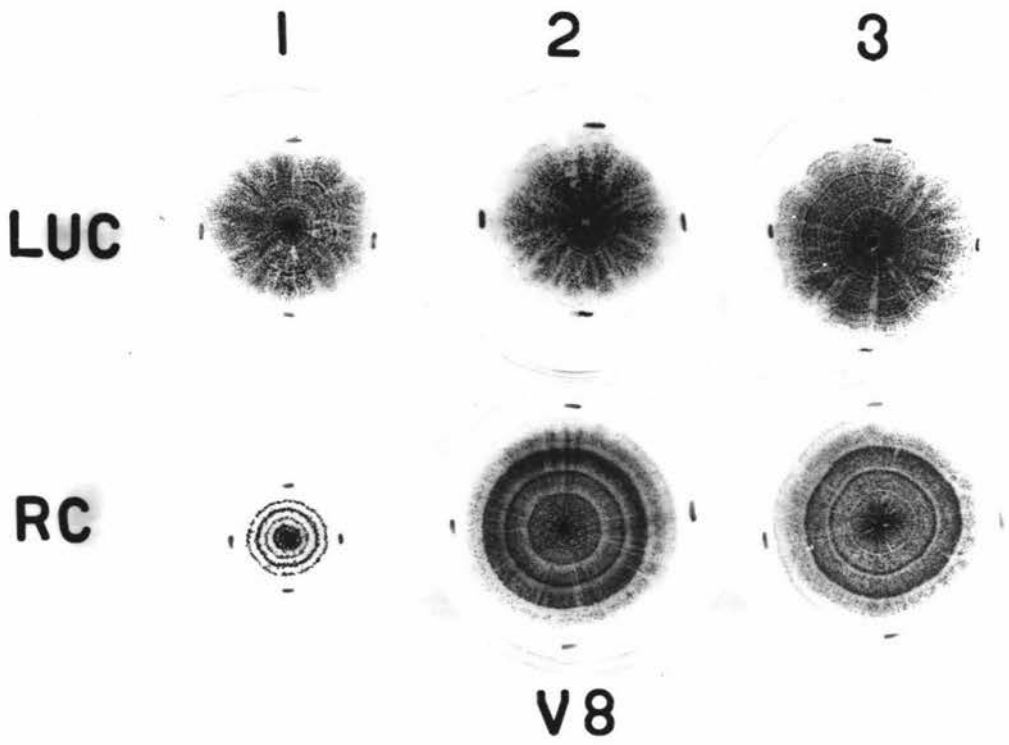
FIGURE 46. Effect of media type on radial growth of L. trifolii from lucerne after four, seven, ten and fourteen days incubation at 24 C.

FIGURE 47.
Comparison of growth and gross colony characteristics of Leptosphaerulina isolates from lucerne and red clover on five media after fourteen days incubation at 24°C in the dark.

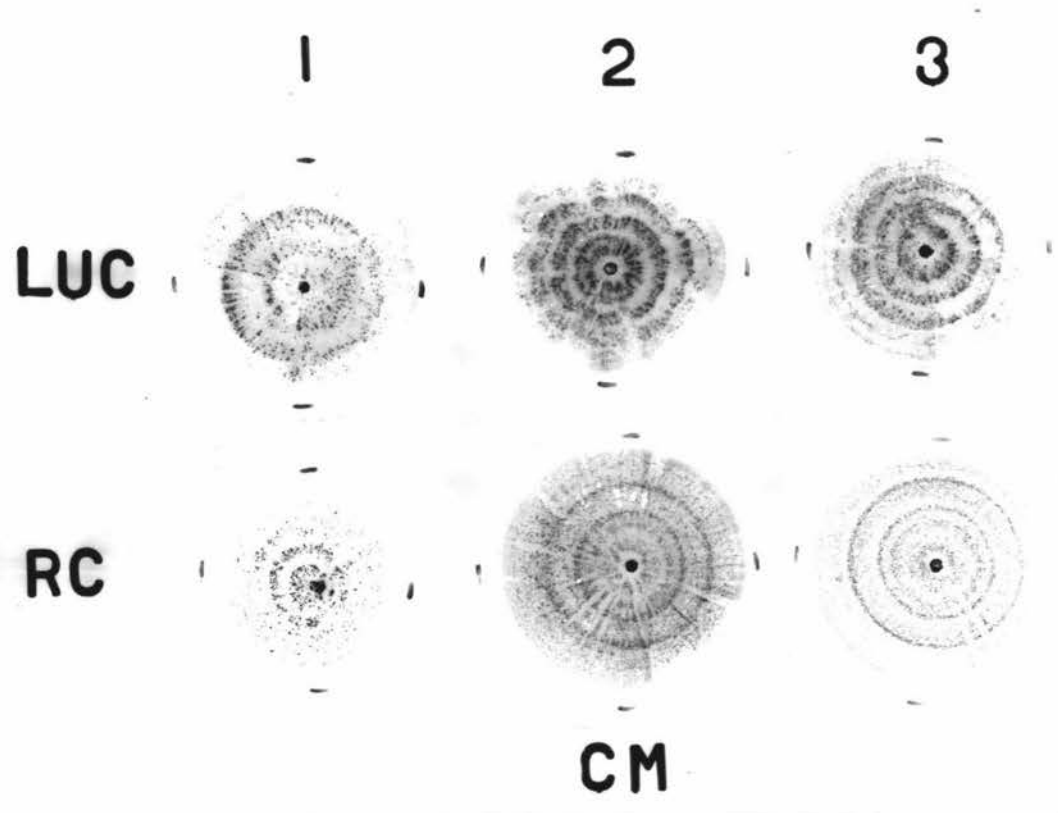
A. PDA_L
B. V-8
C. CMA
D. LDA
E. WA



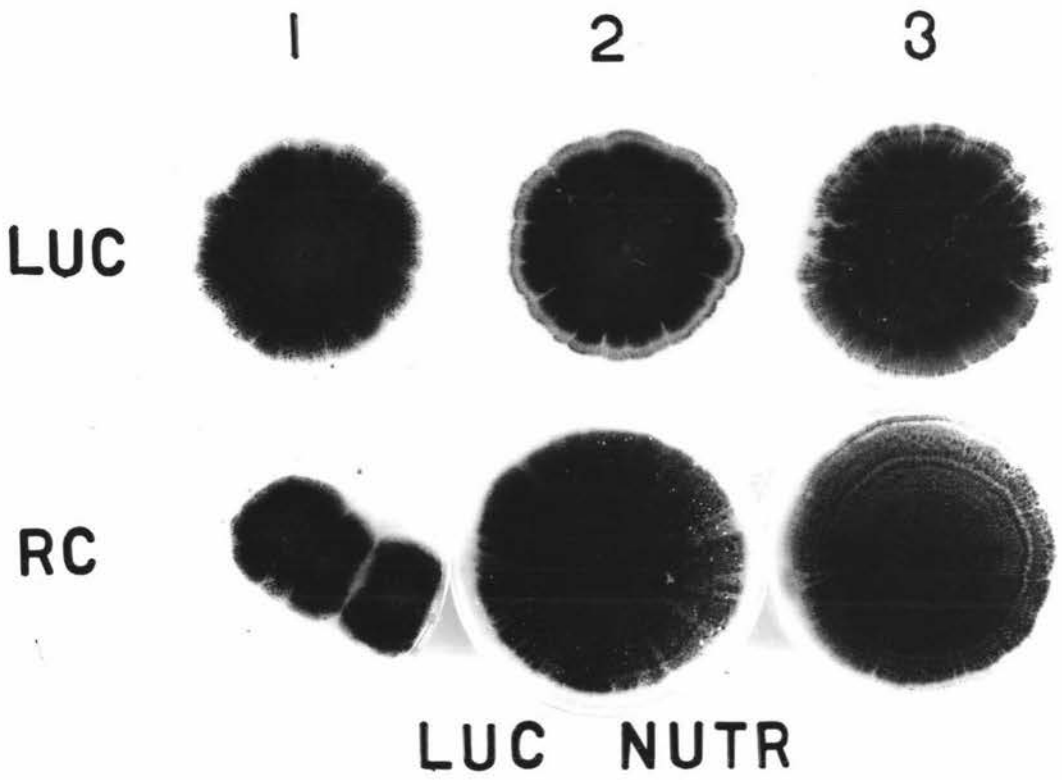
A



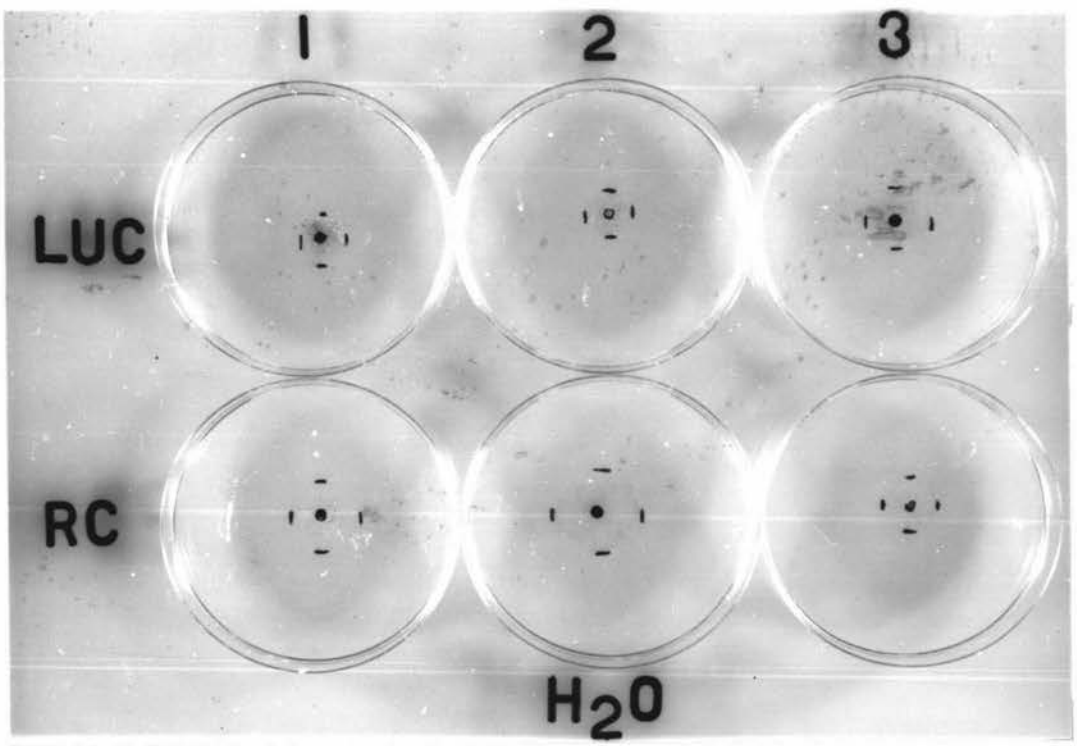
B



C



D



E

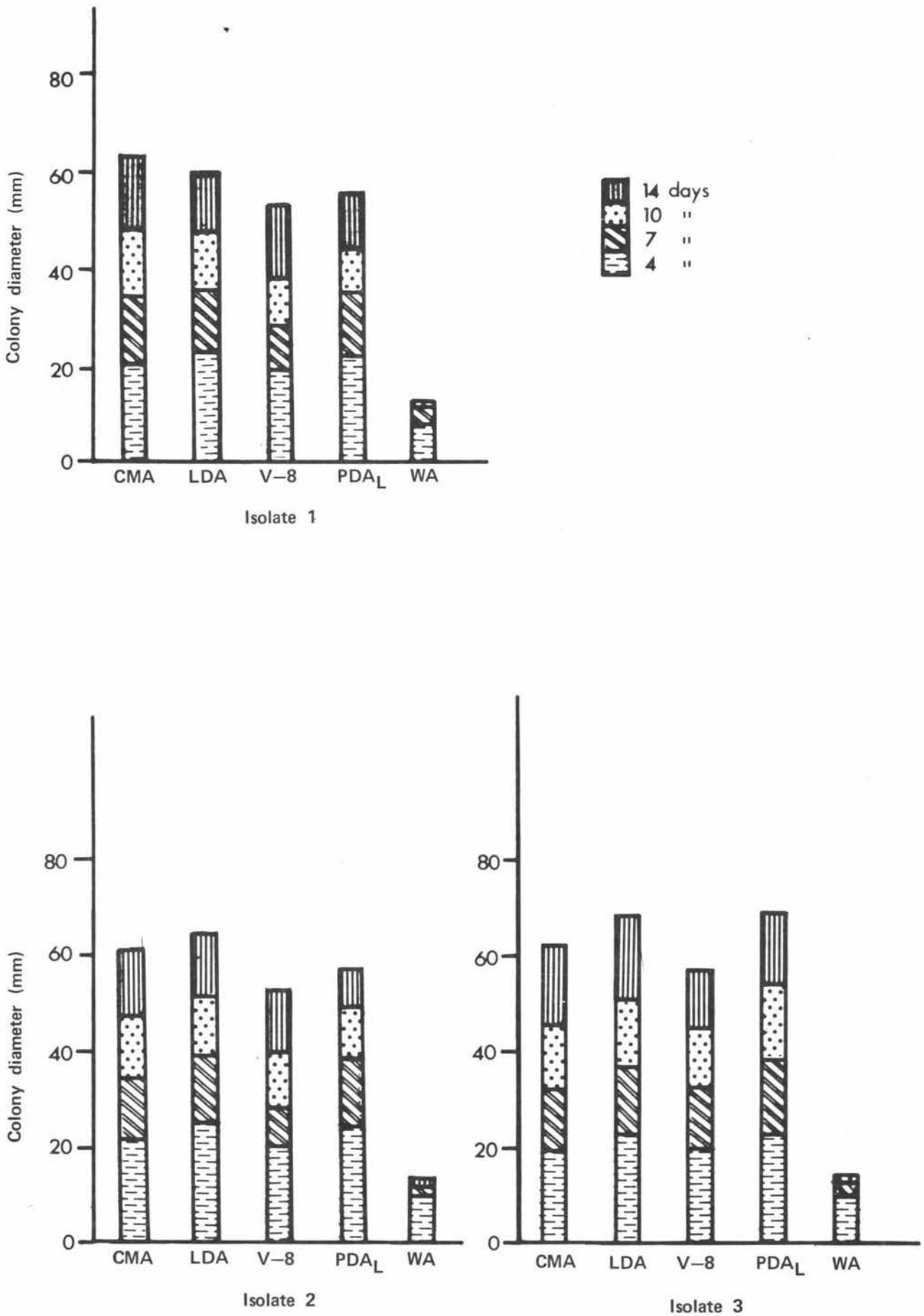


FIGURE 48. Effect of media type on radial growth of Leptosphaerulina isolates from lucerne after four, seven, ten and fourteen days incubation at 24 C.

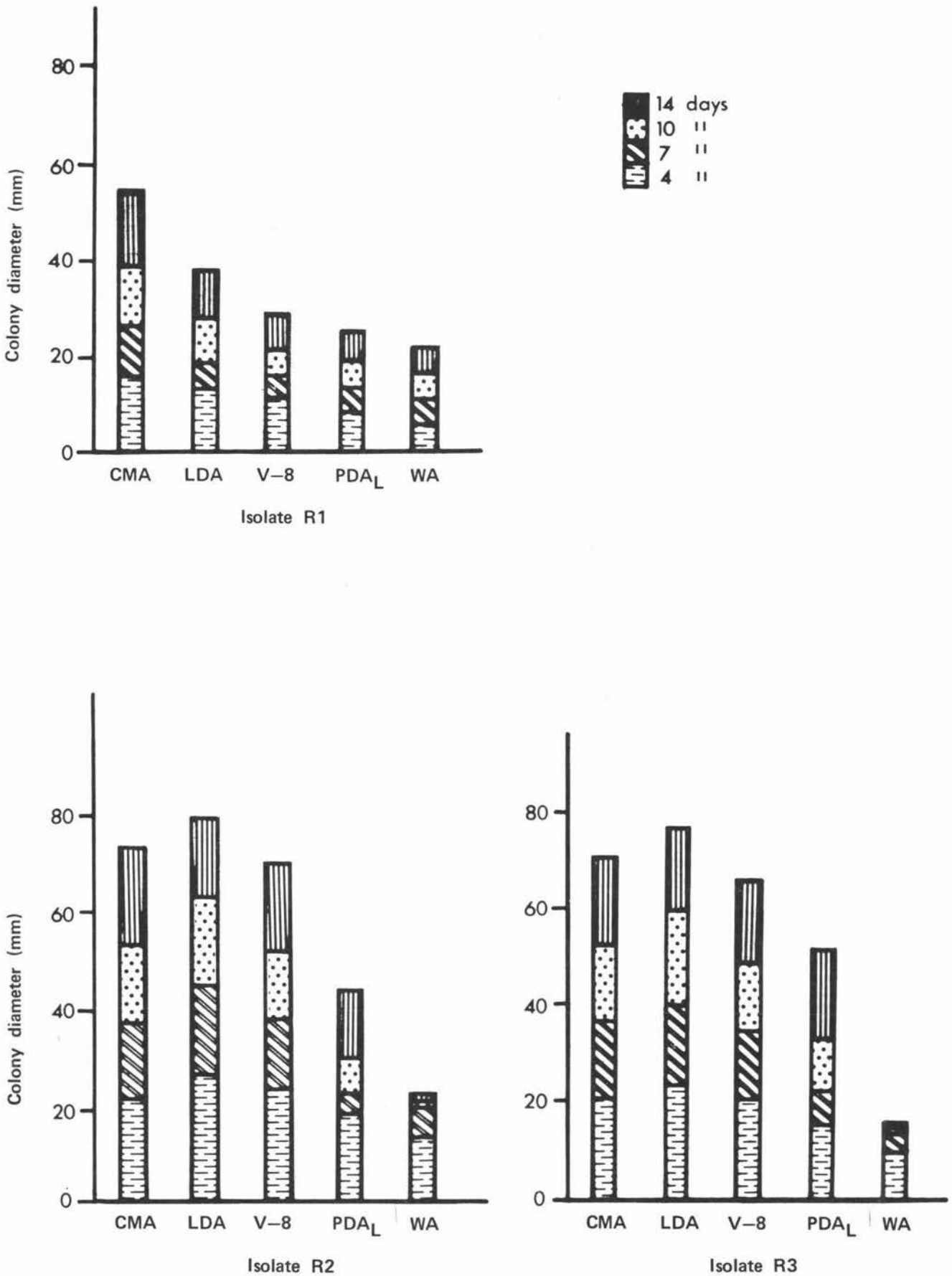


FIGURE 49. Effect of media type on radial growth of Leptosphaerulina isolates from red clover after four, seven, ten and fourteen days incubation at 24 C.

Otherwise colony colour, the density, location and morphology of aerial mycelium, and intensity and location of proto-pseudothecia were very similar for all isolates (Figure 47A).

On 20% V-8 juice agar the average radial growth for both groups of isolates was very similar, with again few differences being observed in colony colour, aerial mycelium and protopseudothecial features. The major differences, however, were in colony morphology (as described above for PDA_L), and that the red clover isolates developed proto-pseudothecia in concentric circles, whereas in colonies of the lucerne isolates they were randomly located (Figure 47B).

Both groups of isolates on CMA had a similar radial growth; the colonies produced were light olive-green in colour and with a white margin, they lacked aerial mycelium, and developed moderate numbers of black protopseudothecia in concentric rings. Again similar differences as above were found in colony morphology (Figure 47C).

The average radial growth, colony colour, aerial mycelium and protopseudothecial features on LDA were essentially the same for all isolates; the colony morphology differed, as before, in the shape of the colony margin (Figure 47D).

Finally, on WA colonies of each group of isolates remained small in size (lucerne 13.4 mm; red clover 19.6 mm), and had similar colony characteristics. Colonies were circular, flat, and with an undulate margin, completely white, lacked aerial mycelium, and developed few or no protopseudothecia (Figure 47E).

In conclusion, the above physiological studies did not provide results which warranted deviation from the earlier conclusion, that in the Manawatu pepper spot of lucerne and red clover is caused by the one species, namely, *L. trifolii*.

C. SPORE GERMINATION

In the present study the rapidity and pattern of ascospore germination was examined on agar at 24°C, and photographically recorded using a growth chamber similar to that described by Harvey and Wenham (1971). After inverting a sporulating culture of the fungus over a PDA_L plate for 1-2 hours and allowing natural ejection of ascospores onto the agar surface, a square of agar was transferred to a sterile coverslip.

The coverslip was then inverted over the growth chamber, and the edges sealed with paraffin wax. A moist air flow was maintained through the chamber to prevent dessication of the agar and ascospores. Germination patterns of one or more ascospores were periodically observed and photomicrographically recorded under the high power (x 400) of a compound microscope. Ascospores were considered to have germinated when the length of a germ-tube was equal to the breadth of the ascospore.

Generally, 5-20% of the ascospores had germinated after 1½ hours, and by 2½ hours approximately 80% of the spores had well developed germ-tubes. By the third hour 100% germination was attained at this temperature (24°C). A similar result was reported by Martinez and Hanson (1963) who found the optimum temperature for germination was 24°C, with 94% of ascospores germinating in three hours at this temperature.

The pattern of germination is shown in Figure 50. In all cases there was an initial expansion of the ascospore (up to 4u in length and 2u in breadth), presumably due to absorption of water from the substrate. Germ-tubes were in evidence within one hour originating from either the terminal or lateral cells of the ascospore. Within 3 hours of first evidence of germination individual ascospores had produced between 3 and 7 germ-tubes. Once emerged, germ-tubes elongated very rapidly (within 4 hours the germ-tube length was seven times greater than its breadth), becoming septate within 6 hours and branched within 10-12 hours.

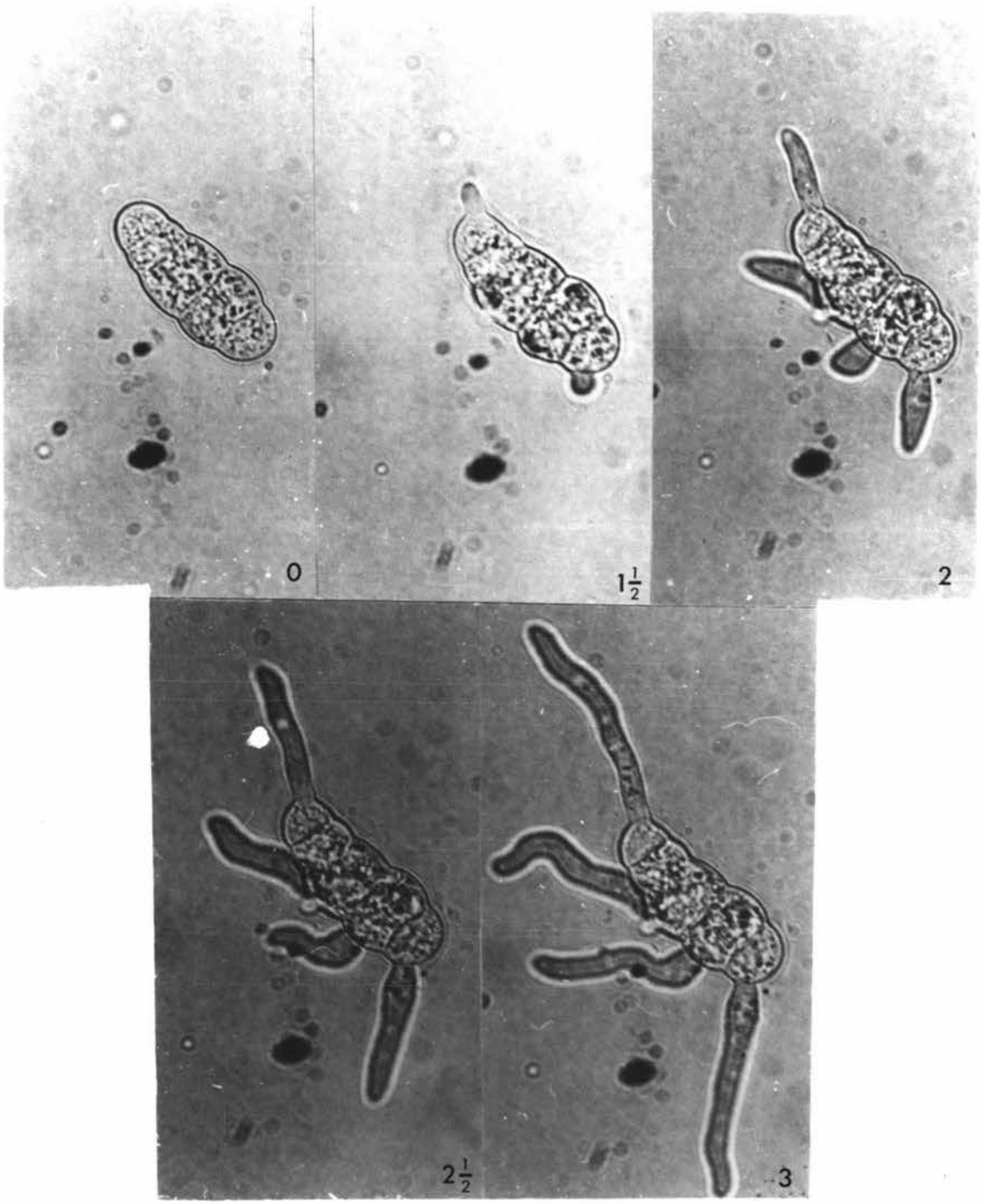


FIGURE 50.
Pattern of ascospore germination of L. trifolii from lucerne on PDA_L at 24°C (x 400).
(Left to right, top to bottom; 0, 1½, 2, 2½, 3 hours).

APPENDIX I

COMPOSITION AND PREPARATION OF CULTURE MEDIA

The media were prepared as described below. They were stored in 250 ml Erlenmeyer flasks, after being autoclaved at 15 p.s.i. for 20 minutes.

A. Potato-Dextrose Agar (lab PDA)

agar (Davis)	12 g
potatoes (sliced and peeled)	200 g
dextrose	10 g
distilled water	1000 ml

The sliced potatoes were cooked for approximately one hour in 500 ml of distilled water and the filtrate retained after straining through a clean cheesecloth. The agar and dextrose were melted in 500 ml of distilled water, the potato filtrate added, and the whole then autoclaved.

B. Oxoid PDA

potato-dextrose agar (Oxoid)	40 g
distilled water	1000 ml

The prepared dry medium is added to the distilled water, and then autoclaved.

C. Maknur PDA

potato-dextrose agar (Maknur)	36 g
distilled water	1000 ml

The prepared dry medium is added to the distilled water, and then autoclaved.

D. V-8 Juice Agar (20%)

agar (Davis)	15 g
$CaCO_3$	1.5 g
V-8 juice	200 ml
distilled water	800 ml

The agar is melted in the 800 ml of distilled water, the $CaCO_3$ and V-8 juice added, and the whole then autoclaved.

E. Cornmeal Agar

agar (Davis)	15 g
cornmeal (crushed)	60 g
distilled water	1000 ml

The cornmeal was cooked in 500 ml of distilled water for one hour at 60°C and then filtered through clean cheese-cloth. The filtrate was then added to the agar melted in 500 ml of distilled water, and the whole autoclaved.

F. Oatmeal Agar

agar (Davis)	15 g
oatmeal	20 g
distilled water	1000 ml

The method of preparation is the same as for cornmeal agar above.

G. Malt Agar

agar (Davis)	15 g
malt extract (Oxoid)	20 g
distilled water	1000 ml

The malt extract was warmed for a few minutes in 500 ml of distilled water, then added to the agar melted in the remaining 500 ml of water, and the whole autoclaved.

H. Prune Agar

prune agar	24 g
distilled water	1000 ml

The prune agar was gently heated in the distilled water to completely dissolve the medium, and then autoclaved.

I. Water Agar

agar (Davis)	12 g
distilled water	1000 ml

The agar was added to the distilled water, and then autoclaved.

J. Lucerne Decoction Agar

lucerne leaves	30 g
agar (Davis)	20 g
dextrose	20 g
distilled water	1000 ml

The leaves were comminuted in a Waring Blender with a small volume of water, and cooked in a 50°C water bath for two hours. After filtering, the filtrate was added to the dextrose and agar dissolved in water, the total being made up to 1000 ml. The whole is then autoclaved.

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