IDENTITY, TAXONOMY AND SEED-BORNE ASPECTS OF THE GRAY LEAF SPOT ORGANISM ON BLUE LUPIN

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C O N T E N T S

F I G U R E S

P L A T E S

TABLES

A C K N O W L E D G E M E N T S

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I N T R O D U C T I O N

While conducting studies in the Manawatu on the brown spot disease of blue lupins[1/] caused by *Pleiochaeta setosa* (Kirchn.) Hughes, Milne (1964) frequently encountered a *Stemphylium* disease characterised by necrotic lesions on leaves, stems and pods. A disease caused by a species of this genus had not previously been reported on blue lupins in New Zealand, but in the United States of America Wells, Forbes, Webb and Edwardson (1956) described two previously unrecognised diseases on this host, namely "little leaf spot" caused by *Stemphylium botryosum* Wallroth and "gray leaf spot" caused by *S. solani* Weber. Milne considered his isolates to be *S. botryosum* but was confused by the symptoms being typical of those recorded for *S. solani* (gray leaf spot). He did not pursue the matter further and at the completion of his studies on *P. setosa* there remained the unresolved question of the identity of the *Stemphylium* species present on blue lupin in the Manawatu.

The anomally revealed by Milne (1964) was explained in a later publication by Wells, Forbes and Edwardson (1961a) in which they reported the discovery of a virulent strain of the little leaf spot organism (*S. botryosum*) that caused gray leaf spot symptoms identical to those induced by *S. solani*. It seemed probable therefore that

1/ *Lupinus angustifolius* L.

Milne had in fact isolated virulent strains of S. botryosum from diseased blue lupins. In the present study the identity of the species causing gray leaf spot is established and consideration given to field identification of the disease.

The second part of this work concerns taxonomy of the genus Stemphylium. Until recently the accepted concept of Stemphylium was based on a compromise proposal put forward by Wiltshire in 1938. This was necessary, if not strictly correct, due to the original concept of Stemphylium being misinterpreted soon after the genus was erected in 1833 and a different group of fungi thereafter being commonly attributed to Stemphylium. However in a recent paper Simmons (1967) restored the original concept by proposing that the other group be transferred to another genus, namely Ulocladium Preuss. This makes it possible for the first time in over 100 years to consider the genus Stemphylium strictly within the limits of the original concept laid down by Wallroth.

The final part of the study is concerned with the seed-borne nature of the gray leaf spot disease of blue lupins in New Zealand. The presence of necrotic lesions on developing pods and seeds, together with a report that the pathogen is seed-borne (Milne 1964) suggested that both seed yield and seed performance may be affected. Further, if viable inoculum of the pathogen is associated with harvested seed from blue lupin crops, this could be of significance in the establishment of primary infection foci when such seed is used for further cropping.

These three areas of study can be summarised as follows:

I the pathogen - its identity, and field identification of the disease it causes;

II taxonomy of Stemphylium - the tenability of present species, and an evaluation of species delimitation in this genus;

III significance of the pathogen in blue lupin seed crops - its effect on seed yields and seed performance, its presence in seed lines and the significance of this in the establishment of primary infection foci.

I THE PATHOGEN - IDENTITY

PURE CULTURE STUDIES

FIELD IDENTIFICATION

A. IDENTITY OF THE SPECIES CAUSING GRAY LEAF SPOT

1. PRELIMINARY ISOLATIONS

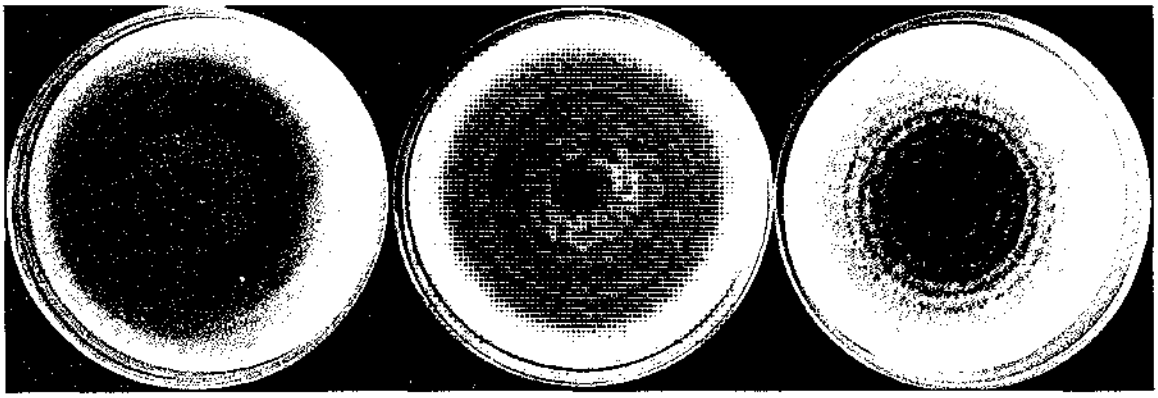
From a maturing seed crop of blue lupin (var. Borre) showing severe defoliation from this disease, random samples of lesioned leaves, stems and pods were regularly removed for the purpose of isolating the causal organism and completing the requirements for proof of pathogenicity (Koch's Postulates). These were completed in the following manner.

(a) Isolation to Pure Culture

(i) Sporulation was induced by subjecting lesions to high humidity in Petri dishes lined with moistened filter paper and held at 24°C for a minimum of 24 hours. Using a needle seeker, single conidia were transferred aseptically to culture media in either Petri plates or slopes.

(ii) Lesions were surface sterilised in a solution of mercuric chloride (0.001% w/v), rinsed in three changes of sterile water, dried between sterile filter papers, and aseptically transferred to culture media. Experience showed that 30 to 90 seconds immersion in the sterilant was sufficient to inactivate most of the associated saprophytes. Following four to nine days incubation at 24°C, colonies of one particular fungus developed from most tissue pieces (Plate 1). On the basis of conidial and conidiophore morphology the isolated

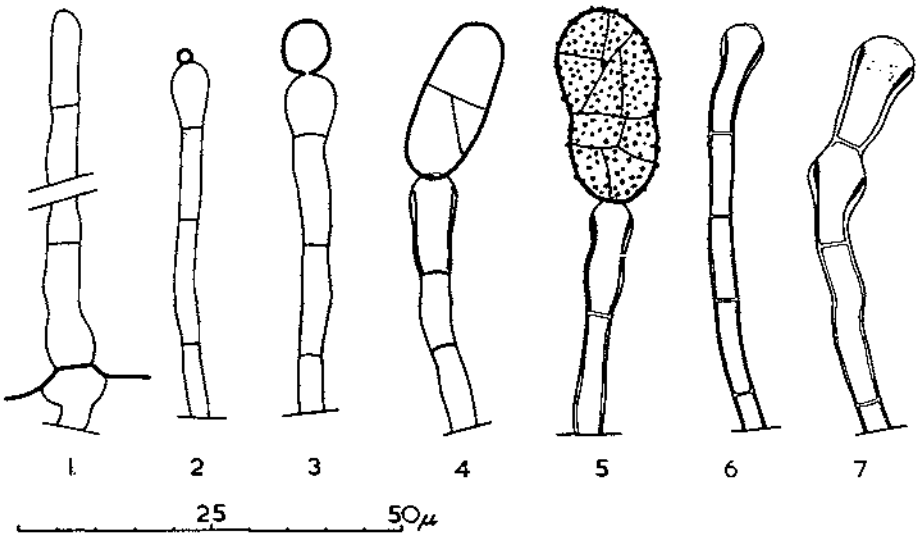
Plate 1:



Cultural Characters of the Causal Organism on

Potato Dextrose Agar Malt Agar Prune Agar

Figure 1:

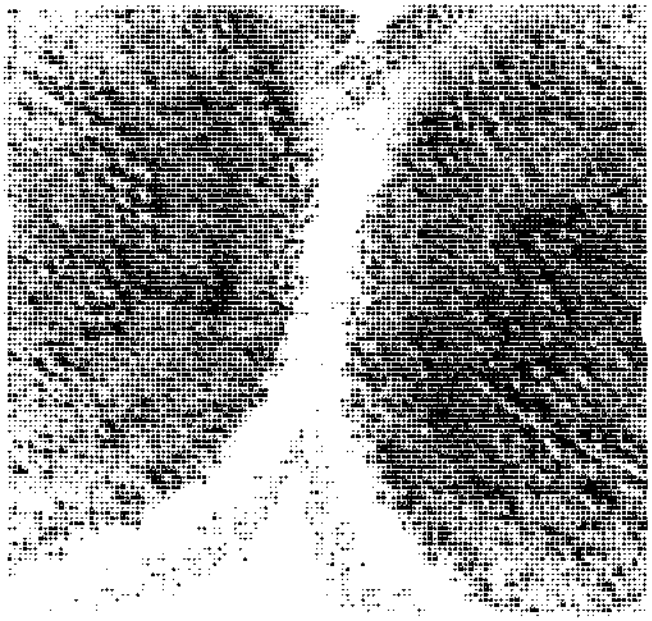


Developmental Morphology (asexual sporulation apparatus) of the Causal Organism on Artificially Inoculated Blue Lupins. (Camera Lucida reproduction)

fungus was identified as Stemphylium botryosum Wallr. (Figure 1). After some months on agar, monosporous isolates occasionally produced an ascigerous stage identified as Pleospora herbarum (Pers. ex Fr.) Rabh. (Plate 2). Cultures derived from single ascospores, following the method described by Slade (1961) produced in turn the asexual conidial stage in identical colonies.

Early in the study it was determined that non-pathogenic strains of S. botryosum, morphologically indistinguishable from pathogenic strains, were occasionally associated with lesions as superficial contaminants. In such instances, when isolation method (i) was followed these non-pathogenic strains were sometimes inadvertently isolated and when undetected, were responsible for confusion regarding the interpretation of unsuccessful artificial inoculations. By contrast, method (ii), judiciously employed, greatly decreased the chances of non-pathogenic strains appearing in the plating medium, and resulted in the pathogen being consistently isolated. Accordingly, in most subsequent experimentation this latter isolation method was followed, monosporous isolates of the pathogen then being prepared by needle-tip transfer of conidia from parent colonies to agar slopes.

Plate 2:



Ascostromata Production on PDA
(Surface Scraped Colonies) X6 Magnification

Mature Bitunicate Ascus
with Ascospores

Mature Ascospore with Seven
Transverse Septa

(b) Artificial Inoculation

The pathogenicity of S. botryosum isolates obtained from naturally infected blue lupin plants was demonstrated on several occasions using both Bitter Blue lupin and the sweet selection Borre. The inoculum was a conidial suspension (10,000 - 30,000 conidia/ml) prepared from a series of malt agar plates incubated at 24°C for a minimum of ten days. The plants were inoculated by "misting" to the point of run-off, using a de Vilbiss atomiser. To facilitate the establishment of infection, high humidity conditions were promoted for two days following inoculation by covering the pots with clear polythene bags securely sealed at the opening. Controls were provided by plants atomised with distilled water, but otherwise given identical treatment. The plants of all series were set out in a glasshouse where temperatures ranged from 60-80°F. 1/

First symptoms usually appeared two or three days following inoculation as a light spotting of leaflets, petioles and stems, and within two weeks these lesions had developed characteristics identical to those observed in the field and considered to be caused by S. botryosum. In all series of inoculations, the control plants remained disease free.

The pathogen was readily reisolated to PDA following the previously described tissue plating method. Macroscopically the colonies were identical with those from which the inoculum was

1/ For routine materials and methods employed in the glasshouse, see Appendix I.

initially prepared, and microscopic examination revealed conidia and conidiophores typical of S. botryosum. To establish beyond doubt that the same pathogenic strain was here involved several of these isolates were used to inoculate a further series of blue lupin plants, with positive results.

From these results it was concluded that the virulent strain of S. botryosum reported by Wells et al (1961a) is present in the Manawatu causing a foliage disease on blue lupin with symptoms identical to those reported overseas. In all probability this is the same strain isolated earlier by Milne (1964). No other species of Stemphylium were detected during these regular isolations which were continued over one growing season.

2. SURVEY OF BLUE LUPIN CROPS IN MANAWATU, TURAKINA AND WANGANUI AREAS

Having determined that S. botryosum is present as a causal organism of gray leaf spot a field survey was conducted to confirm its widespread occurrence and determine to what extent S. solani was contributing to the disease in lupin crops.

A third species S. loti has been recorded pathogenic to blue lupin (Graham and Zeiders 1960) but only to varieties exhibiting joint resistance to the other two species (Wells, Forbes and Edwardson 1961b). Since the only varieties now extensively grown in New Zealand are Bitter Blue and Borre, (both very susceptible to S. botryosum), S. loti was not expected to be a contributing factor to gray leaf spot in this area.

Method -

Ten widely scattered stands of blue lupin exhibiting varying intensities of gray leaf spot were visited over the 1966-67 growing season. From each of these crops random samples of spotted leaflets were removed and stored by pressing between newspaper. Two weeks after the final collection, 24 leaflets of each crop sample were laid out in high humidity Petri plates at room temperatures (20-24°C) to encourage sporulation of any fungi present. As conidial production commenced each leaflet was regularly scanned at 60 to 120 magnifications to detect the presence of Stemphylium conidia. Any conidial populations obviously atypical of S. botryosum were noted, and then isolated and mounted as follows:

(a) four of the most atypical conidia from each population were aseptically plated to PDA slopes;

(b) the portion of leaflet bearing this conidial population was removed and mounted in lactophenol on a slide. 1/

With these removed the remaining leaflets of each collection were shaken over a watch glass containing lactophenol, and mounts made of the resulting spore suspensions. In addition four monosporous isolates per collection were made. To establish the pathogenicity of isolated stemphylia conidial suspensions were prepared (as for S. botryosum) and potted plants in the glasshouse

1/ All mounts were cleared of air bubbles by warming over a bunsen with the coverslip in place.

immediately inoculated, with uninoculated controls used for comparison. This pathogenicity test was necessary since the survey method did not automatically select out non-pathogenic species.

RESULTS AND DISCUSSION

Results are summarised in Table 1. In all lupin stands sampled (these generally showing a high level of foliage lesioning and defoliation towards the seed setting period) S. botryosum proved to be the pathogen involved in the great majority of cases. Neither S. solani nor S. loti was found to be associated with gray leaf spot, although from several crops other stemphylia were detected. From one stand a pathogenic Stemphylium was found in one lesion, but this could not be identified because conidial and cultural characteristics did not correspond with any existing species diagnosis. Two leaflets from another stand also yielded a pathogenic Stemphylium and this likewise could not be identified as spore characteristics appeared to alter markedly from the initial detection to when cultured on PDA. None of the other atypical spore forms proved pathogenic to blue lupin. One of these, present on three leaflets from each of two crops corresponded well with the diagnosis for S. trifolii Graham. The other, present on one leaflet from one crop was similar to S. botryosum on PDA, but when first detected on the leaflet the spores exhibited atypically high Length:Breadth ratios of up to more than 3:1.

It was concluded that in the Manawatu, Turakina and Wanganui areas S. botryosum was primarily responsible for the gray leaf spot symptoms occurring in blue lupin crops over the 1966-67 season.

TABLE 1: Relative Incidence and Pathogenicity of *Stemphylium* species in Blue Lupin Crops.

Locality	Variety	Number of leaflets producing:			
		S. botryosum	*S. solani*	*S. loti*	Others
Massey University	Borre	24 *	-	-	1 *
	Bitter Blue	24 *	-	-	3 ø
Bulls	Borre	24 *	-	-	-
"	Bitter Blue	24 *	-	-	2 *
Turakina	" "	24 *	-	-	-
"	" "	24 *	-	-	1 ø
Waverley	Borre	24 *	-	-	-
"	"	24 *	-	-	3 ø
Palmerston North	"	24 *	-	-	-
Foxton	Bitter Blue	24 *	-	-	-

* Isolates pathogenic to Blue Lupin

ø Isolates not pathogenic to Blue Lupin

B. STUDIES CONCERNED WITH ARTIFICIAL INFECTIONS

1. SPORULATION IN PURE CULTURE

While completing the requirements of Koch's Postulates it became apparent that before further inoculation experiments could be undertaken there was a need for an investigation into the conditions which would ensure convenient and intense sporulation on agar media. A study of relevant literature revealed that restricted conidial production on agar is a feature common to many species of *Stemphylium* and that special techniques have often been employed in the preparation of inoculum. For certain purposes, such as resistance screening and host range studies, this problem has been overcome by the use of blended agar colonies (Nelson 1955, Wells et al 1956, Petzer 1958, Graham and Zeiders 1960). However in studies requiring the use of conidial suspensions for inoculations, stimulation of sporulation is necessary. For example, Diener (1952, 1955) working with *S. solani*, found that culturing on V8 juice agar at 20 - 30°C, with intermittent exposure to ultra violet radiation induced abundant sporulation.

In the present study the influence on sporulation of such environmental factors as substrate, temperature and light was investigated. In all experiments, Petri dishes of agar media were inoculated with one or more agar plugs (2mm diameter) taken from a ten day old malt agar culture of a monosporous isolate. The intensity of sporulation was determined by following the method used by Slade (1961), and Tammen (1963), which was as follows:

After a minimum of ten days incubation, plates were flooded with ten mls of distilled water, scraped clean of aerial mycelium and conidia, and the suspension funnelled into a 20 ml McCartney bottle containing a quantity of glass beads. After vigorous shaking the conidia were separated from agar and mycelium by straining the suspension with butter muslin (four layers), then flushing this strainer with a further five mls of distilled water. The conidial concentration of the resulting spore suspension expressed as conidia/ml, was estimated with a Neubauer haemocytometer.

(a) Effect of Substrate

The intensity of sporulation on the following media was compared.

Potato dextrose agar	PDA
Malt agar	MA
V8 juice agar	V8
Potato carrot agar	PCA
Lupin seed agar	LSA
Lupin decoction nutrient agar (var. Borre)	LDNA(Bo)
Lupin decoction nutrient agar (Bitter blue)	LDNA(Bi)

Four plates of each medium were inoculated at two points.

The results, following 12 days incubation at 24°C (Table 2) show that on average, the greatest sporulation occurred on LSA and PDA. However in subsequent experiments where a bulk supply of conidia was required, PDA was used because of the ready availability of its constituents, and the relative ease of preparation.

TABLE 2: Effect of Media on Conidial Production.

Culture Medium	Conidia/ml (x 1000)				
	Replicate 1	2	3	4	Treatment Mean
PDA	20	21	20	28	22
LSA	15	22	16	48	25
LDNA (Bo)	15	16	10	13	13
LDNA (Bi)	8	10	19	15	13
PCA	10	11	10	12	11
V8	10	6	11	8	9
MA	8	10	11	7	9

(b) Effect of Light

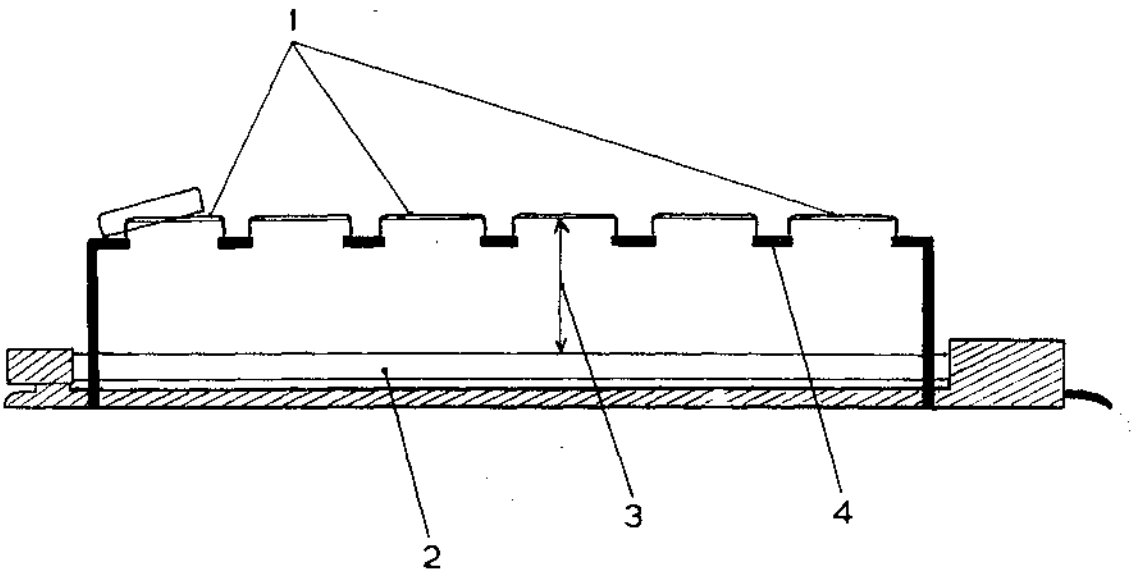
The following treatments were compared.

(i) Continuous total darkness, provided by enclosing Petri plates inside two layers of aluminium foil.

(ii) Exposure to normal diurnal periods of diffuse daylight.

(iii) Total darkness as before, for all but ten minutes daily exposure to an ultra violet (UV) light source held at a distance of 15 cm below the inverted plates with lids removed (Figure 2). This UV radiation was provided by a Westinghouse Sterilamp type S.B.

Figure 2:



Apparatus for Irradiating Colonies with UV Light

1. Inverted Petri Plates with Lids removed to Expose Colonies

2. UV light source

3. Distance from Light Source to Colonies of S. botryosum = 15cm

4. Plywood Box Restricting Irradiation to Plates only

There were four replicates for each treatment and each plate was inoculated at four equidistant points. Results were recorded at 14 days. Plates of all treatments were sited on a bench near a west window in a random fashion, direct sunlight being avoided by the manipulation of venetian blinds. This prevented local temperature rises in treatment (ii), shown by regularly reading thermometers both protruding from the foil bags, and placed beside the exposed petri plates of treatment (ii). In this way, all treatments received the same temperature conditions, which varied diurnally from 18° - 24°C.

The results (Table 3) show that sporulation in treatments (ii) and (iii) were consistently higher than in treatment (i). The beneficial effect of light on sporulation was clearly demonstrated, whether in the form of daylight for ten hours daily or as UV light for ten minutes daily. The stimulation effects of UV light measured here are in agreement with similar experience obtained by Diener (1952, 1955) working with S. solani, and Leach (1963) working with Pleospora herbarum.

TABLE 3: Effect of Light Conditions on Conidial Production.

Light Treatment	Conidia/ml (x 1000)				
	Replicate 1	2	3	4	Treatment Mean
Darkness	1	6	7	2	4
Daylight/ darkness	9	28	70	61	42
UV light/ darkness	14	39	14	33	25

(c) Effect of Temperature

The influence of temperature on sporulation was determined by incubating plates at the following temperatures:

(i) $15^{\circ}C \pm 0.5^{\circ}$

(ii) $20^{\circ}C \pm 1^{\circ}$

(iii) $25^{\circ}C \pm 0.5^{\circ}$

(iv) $30^{\circ}C \pm 0.5^{\circ}$

There were five replicates at each temperature, with each plate being inoculated at five equidistant points. The plates were kept in total darkness in foil bags except for ten minutes daily exposure to an UV light source positioned in front of the open doors of the incubators. The results after fourteen days incubation (Table 4), show that constant temperatures of 20° and $25^{\circ}C$ consistently induced the greatest sporulation. The higher average sporulation at $20^{\circ}C$ is partly attributable to the greater temperature variation that occurred in this treatment (assuming that diurnal fluctuation about an optimal mean temperature would be a greater stimulus to conidial production than constant temperatures). This was caused by the fluctuation of room temperatures above the operating temperature of an incubator not fitted with a refrigeration unit.

TABLE 4: Effect of Temperature on Conidial Production.

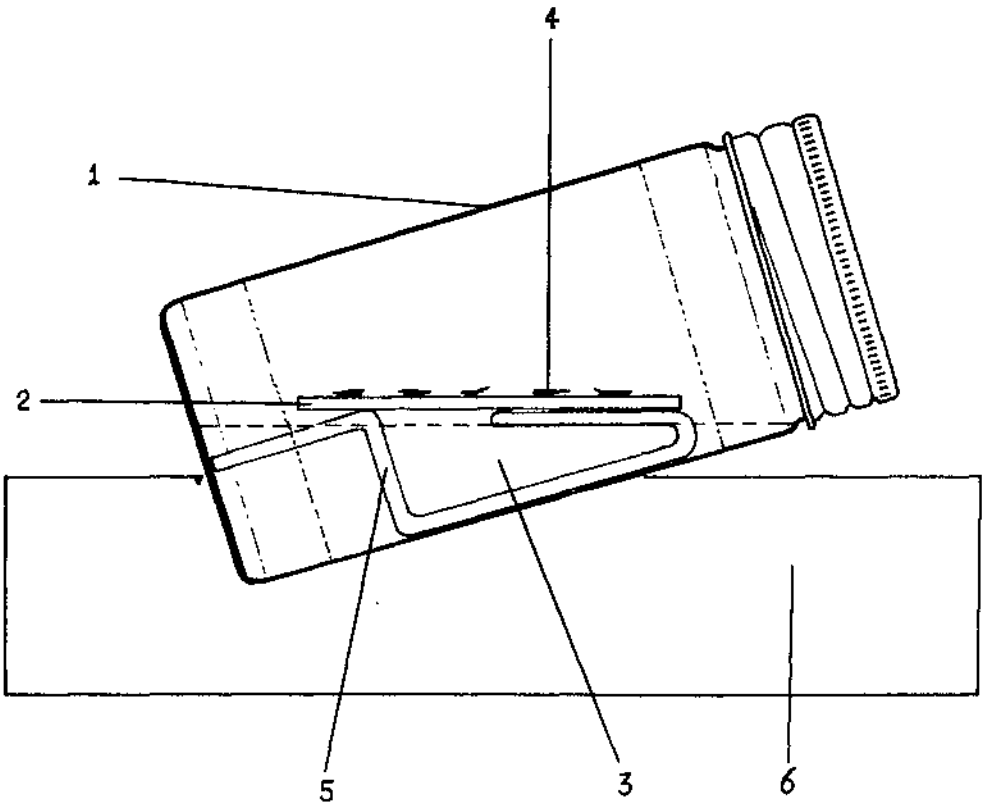
Temperature °C	Conidia/ml (x 1000)					
	Replicate 1	2	3	4	5	Treatment Mean
15	4	1	4	0	1	2
20	20	23	31	24	31	26
25	21	23	24	21	18	21
30	1	3	0	3	2	2

(d) Effect of Humidity

In general a high level of relative humidity (RH) favours growth and sporulation of fungi and in Petri plate studies this is provided by evaporation from the media surface during incubation. Accordingly, it was considered that little would be contributed by experimenting to determine the optimum level of RH for sporulation on agar in Petri plates. However, during taxonomic studies it was necessary to determine the minimum level of RH which would still allow the production of conidia, and the results of an experiment in this regard are here presented as confirmatory evidence that very high levels of RH are a pre-requisite to abundant sporulation.

In this experiment, PDA was replaced by artificially inoculated leaflets of blue lupin (var. Borre) held above constant-humidity solutions on dry glass slides at constant temperatures (Figure 3).

Figure 3:



Constant-Humidity Apparatus

1. Screw-top coffee jar
2. Glass slide
3. Constant-humidity solution
4. Inoculated leaflets
5. Glass-rod platform
6. Polystyrene base

The results (Table 5) show that at constant temperatures conidial production was inhibited by levels of RH lower than 95%, even though some mycelial growth occurred at 90% RH.

TABLE 5: Effect of Humidity on Conidial Production and Mycelial Growth.

% RH	Temperature °C	Mycelial Growth	Conidial Production
50-80	25	none	none
90	"	some	"
93	"	"	"
95	20	moderate	sparse
97	25	"	"
98	"	heavy	moderate
100	"	"	heavy

(e) Conclusions

As a consequence of the above experiments the following method was adopted for obtaining bulk supplies of conidia.

Plates of laboratory PDA were inoculated at five to seven equidistant points, or streaked with spores and mycelium prepared from a stock culture. The plates were placed at room temperatures of 20 - 24°C near a window during summer months, and in winter months incubated at 24°C in a glass fronted incubator exposed to a west window. In the latter case, the plates were also exposed

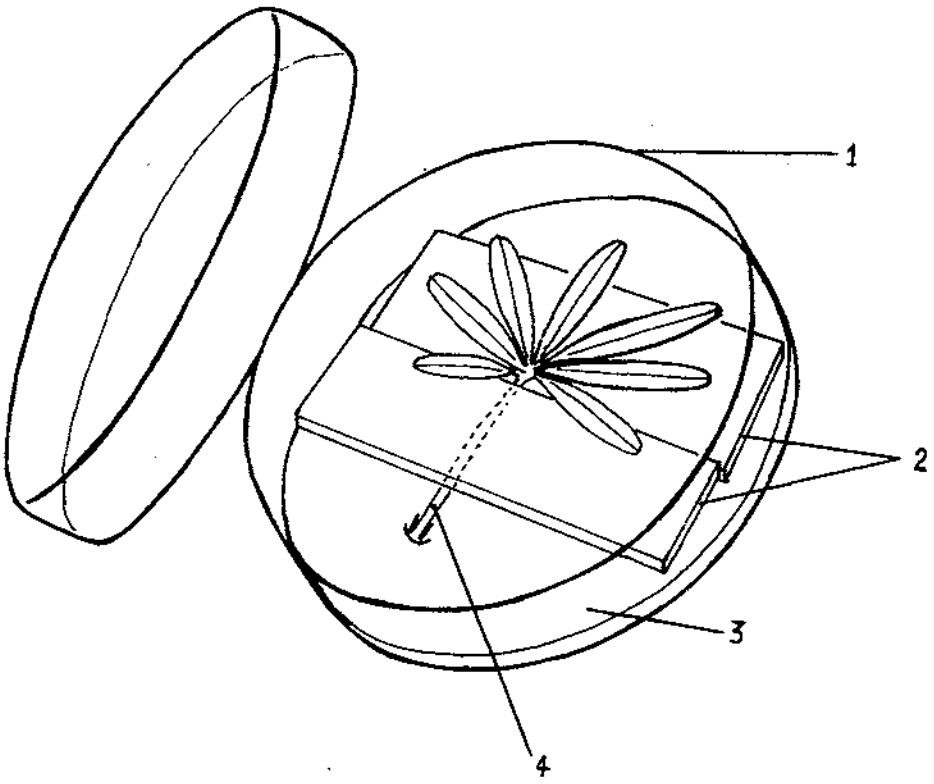
to UV light for ten minutes on at least three daily occasions towards the end of the incubation period. At the end of ten days, the conidia were harvested as previously described, the maximum yield being obtained by repeatedly rinsing to separate the conidia from the mycelium and agar sediment lodged in the butter muslin. Suitable conidial concentrations were obtained by centrifugation followed by haemocytometer-estimation of conidial concentration, and then simple adjustment of the dilution if found to be necessary.

2. ARTIFICIAL INOCULATION

During preliminary pathogenicity studies there was considerable variation in the extent to which plants became infected and this was considered to be in part consequent on the variation in the inoculum strength used, and the temperature and relative humidity conditions following inoculation. Experiments were therefore conducted aimed at determining the combination of these factors required to rapidly achieve intensities of foliage lesioning at least of the order commonly encountered in nature. These experiments were conducted using both excised leaves in the laboratory (a convenient method advocated by Yarwood 1933), and potted plants in the glasshouse.

In the former method, whole leaves (including the petiole) of the same age were removed from eight week old lupin plants (var. Borre) and "tumble" rinsed within a gauze covered beaker to remove contaminant micro-organisms. The leaves were then rinsed in distilled water and set out in high humidity Petri plates (Figure 4). Conidial suspensions prepared from 12 day old subcultures of a

Figure 4:



Apparatus for Excised Leaf Method in Inoculation Studies

Natural Size

1. Petri plate
2. Glass slides
3. Moist filter paper
4. Excised whole leaf with petiole inserted into filter paper

monosporous isolate were atomised over these wetted leaves to the point of surface saturation. The control series consisted of one replicate of leaves treated identically to the inoculated series (several replicates) except that the distilled water used for atomising contained no conidia. This guarded against the possibility of wrongly interpreting symptoms caused by conditions of the experiment not associated with the inoculation of leaflets (for example, extreme temperatures). In no instances was recording delayed beyond the point at which leaflet abscission occurred in the control series (usually five days at temperatures of 20 - 24°C), as this indicated that conditions were becoming too artificial.

In the pot-plant experiments, humidity cages consisted of open wire netting cylinders covered with clear polythene bags. The blue lupin variety used was the sweet selection Borre. (It had previously been found that no varietal differences in susceptibility or disease expression existed for this host). Pots were randomly set out in a moist sand bed on the bench tops, the glasshouse temperatures being maintained within the range 60 - 78°F by a thermostatically controlled electrical heating system.

(a) Humidity

The intensity of foliage lesioning depends on the number of separate infections that occur, and a pre-requisite for this is the maintenance of a saturated atmosphere from spore germination to the time when the fungus becomes firmly established in the host tissues. In order to determine the minimum period of high humidity required at which sufficient separate infections would occur

to produce a moderate intensity of foliage lesioning, the following experiments were conducted.

(i) Excised Leaf Method

Excised leaves in Petri plates were inoculated with a spore suspension (20,000 conidia/ml) and exposed to 100% RH for 12, 24, 36, 48 and 60 hours. After each exposure period the plate covers were removed, thereby reducing the RH at the upper leaf surface. Filter paper liners were kept saturated to prevent leaf dehydration and the effectiveness of the treatments were measured at three days by determining the mean number of infection points/leaflet (recognised by the separately located pinpoint lesions on the leaf surface).

The results (Table 6) show that the minimum duration of high humidity required to produce infection was 24 hours. However, for a moderate intensity of pinpoint lesions to result, at least 36 hours was required. The controls were healthy at the time of assessment.

TABLE 6: Effect of High Humidity Duration on Disease Intensity.

High Humidity Duration in Hours	Mean Number of Lesions / Leaflet			
	Replicate			Treatment Mean
	1	2	3	
12	-	-	-	-
24	1	1	-	0.6
36	5	7	5	5.6
48	5	6	8	6.3
60	10	8	7	8.3

(ii) Pot Plant Method

A similar experiment was conducted using potted plants. Each pot contained five plants at three weeks growth, two pots being used per treatment. Plants were atomised with a conidial suspension (50,000 conidia/ml) to the point of run-off and covered with humidity-cages for the respective durations. Two pots "inoculated" with distilled water and covered for the longest duration (72 hours) served as controls. The effectiveness of the treatments were assessed at five days with the use of a disease intensity rating.

The results (Table 7) show that a consistently moderate disease intensity resulted only after a minimum period of 36 hours of high humidity treatment. The controls remained healthy throughout the experiment.

TABLE 7: Effect of High Humidity Duration on Disease Intensity.

High Humidity Duration	Disease Intensity *	
	Replicate 1	2
24	+	0
36	++	++
48	++	++
72	++	+++

* 0 = no lesions present
\+ = light lesion intensity
++ = moderate lesion intensity
+++ = heavy lesion intensity

These results confirmed those of the previous experiment and further, showed that excised leaves may be used in experiments of this nature.

(b) Temperature

Temperature has a considerable influence on the rate at which most phases of the disease cycle are completed. The experiment conducted here was concerned with identifying the approximate temperature at which the disease cycle starting with spore germination and ending with disease expression would be most rapidly completed. This was achieved by subjecting inoculated excised leaves to a range of constant temperatures (15°, 20°, 25°, 30°C) and recording for each the extent of symptom expression at 24, 48 and 72 hours respectively. The inoculum strength used was 47,000 conidia/ml, and the lids of all plates, both inoculated and control, were removed at 36 hours to reduce RH. The recording at each time interval reflected the extent of lesion development for the three inoculated plates per temperature level.

The results (Table 8) show that the disease cycle was completed more rapidly at 20° and 25°C than at 15° and 30°C.

The control leaves remained unblemished at all temperatures except 30°C, at which temperature a darkening and mottling of the leaf surface caused confusion regarding symptom expression.

TABLE 8: Effect of Temperature on the Rate of Disease Development.

Temperature °C	Extent of Symptom Expression on Inoculated Leaves		
	24 hours	48 hours	72 hours
15	No symptoms	Very few pinpoint lesions	A few pinpoint lesions starting to enlarge
20	A few pinpoint lesions	Moderate lesioning, some coalescence	75% of leaf surface lesioned
25	Many pinpoint lesions	Lesions enlarging and coalescing	Whole leaf surface lesioned
30	Very few pinpoint lesions	A few pinpoint lesions	Mottling due to high temperature confusing symptom expression

The rate of lesion development in this experiment provided an explanation why S. botryosum caused the rapid defoliation observed in the field during this study. At 20°, 25° and 30°C the early stages of the infection cycle had been completed and symptoms were already showing within 24 hours of inoculation. At less favourable temperatures this stage had been reached by 48 hours. It was decided that for subsequent inoculations, temperatures maintained within the range 20 - 25°C would produce the most satisfactory results.

(c) Inoculum Concentration

The Inoculum Potential of the host environment is an important factor affecting the intensity of disease establishment. In this experiment the intensity of foliage lesioning resulting from the following inoculum concentrations was determined.

5,000	conidia/ml	
27,000	"	"
41,000	"	"
69,000	"	"
144,000	"	"

These inoculum concentrations were obtained by centrifugation of a conidial suspension harvested from ten plate cultures, followed by serial dilution. Five singly potted plants per treatment were atomised to the point of run-off with the respective conidial suspensions. In addition two pots were inoculated with distilled water as a control. Plants were eight weeks old at the time of inoculation. Humidity cages were removed from all pots at 36 hours, and temperatures were maintained throughout the experiment within the range 68° - 77°F. Treatments were compared at two days using an infection intensity index. This was calculated by sampling the centre leaflet (or the one most in line with the petiole where leaves comprised an even number of leaflets) for every expanded leaf (averaging 17 leaflets per plant at this stage of development), and totalling the number of separate infection points.

The infection index was computed as follows:

$$\text{Infection Index (or Lesions/Leaflet)} = \frac{\text{Total Infection Points}}{\text{Number of Expanded Leaves on Plant}}$$

The results (Table 9) show that moderate infection, represented by an Infection Index of 4, was consistently obtained using a conidial concentration of 69,000/ml. The heaviest concentration produced a degree of symptom coalescence and defoliation in some replicates higher than that normally encountered in the field, while the lower concentrations produced degrees of foliage lesioning commonly present under natural situations.

The control plants remained disease free throughout the experiment.

TABLE 9: Effect of Inoculum Concentration on Intensity of Infection.

Inoculum Concentration Conidia/ml	Infection Index					
	Replicates					Treatment Mean
	1	2	3	4	5	
5,000	1	-	1	2	3	1.4
27,000	2	3	3	1	1	2.0
44,000	2	3	4	5	1	3.0
69,000	5	5	6	3	2	4.2
144,000	13	9	2	4	3	6.2

(d) Discussion

As a result of the above experiments, where potted plants were spray-inoculated with conidial suspensions, the following conditions applied:

> Conidial concentrations of approximately 50,000 per ml were atomised onto foliage to the point of run-off and the plants covered by high humidity cages for 36 hours. A temperature range of 68° - 77°F (20° - 25°C) was maintained throughout. Under these conditions symptoms characteristic of the disease could be expected within one to two weeks.

It was considered that the high conidial concentrations required to produce moderate intensities of infections was a consequence of both the use of water as the carrying medium, and the inherent hydrophobic nature of the foliage surfaces. These factors (observed by microscopic examination) combined to prevent conidia from effectively contacting leaf surfaces (as opposed to the natural situation) and to cause the droplets holding them in suspension to round up, run together and fall to the ground. The majority of infection points resulting from this situation were confined to those parts where water droplets were held for long enough to allow the completion of the spore germination and penetration phases of the disease cycle (such as leaf edges and petiole-stem junctions). By contrast, natural distribution patterns of infection on the host resulting from movement of conidia by air currents, were completely random.

In subsequent pathogenicity studies therefore, the above environmental treatments were adhered to, while methods of inoculation were occasionally modified to suit the particular experiments concerned. For example, for rapid pathogenicity tests, single attached leaves of potted plants were "rub" inoculated lightly between the thumb and forefinger (dipped previously in a spore suspension of the above concentration) or "brush" inoculated by drawing sporulating leaf tissue pieces across healthy leaves. In later seed studies, another modification was developed aimed at minimizing the time and material involved, but in all cases situations involving an artificial host-pathogen relationship that could not be confidently related to the natural situation were avoided.

C. FIELD IDENTIFICATION

The literature on *S. botryosum* as a pathogen of the genus *Lupinus* is very limited. The most relevant papers available to the writer was that by Wells et al (1956) where they reported and briefly described little leaf spot (caused by *S. botryosum*) as a hitherto unrecognised disease of blue lupins in the United States, and one by Wells et al (1961a) reporting that virulent strains of *S. botryosum* caused symptoms identical to those of gray leaf spot, caused by *S. solani*. These two papers appeared to present a reasonable account of the disease symptoms but in practice the present author found diagnosis to be complicated by the presence of two other foliage

diseases frequently present in local lupin crops, namely brown spot caused by Pleiochaeta setosa (Kirchn.) Hughes, and stem canker caused by a species of Ascochyta.

Brown Spot has been the subject of special study in New Zealand (Milne 1964), but there is no account of the other two diseases under New Zealand conditions. Comparative symptomatology studies for the three diseases were therefore undertaken with the objective of determining those features characteristic of each to facilitate accurate diagnosis of field infections caused by S. botryosum. This particular area of study was approached in two ways:

1. specimens of the different symptom types observed in the field were collected and the pathogen determined by isolating to agar using the tissue-plating method described previously. Experience showed that S. botryosum in particular was often present as a secondary invader in lesions caused by Ascochyta. At the incubation temperature of 24°C normally used, the former fungus dominated the agar surface and obliterated the causal organism. This was overcome by incubating at 20°C at which temperature Ascochyta exhibited a radial growth rate sufficient to prevent domination by either S. botryosum or P. setosa;

2. potted blue lupins (vars. Borre and Bitter Blue) were inoculated with the three pathogens and the development of symptoms observed on leaves, stems, pods and cotyledons. In all cases the inoculum was a spore suspension prepared

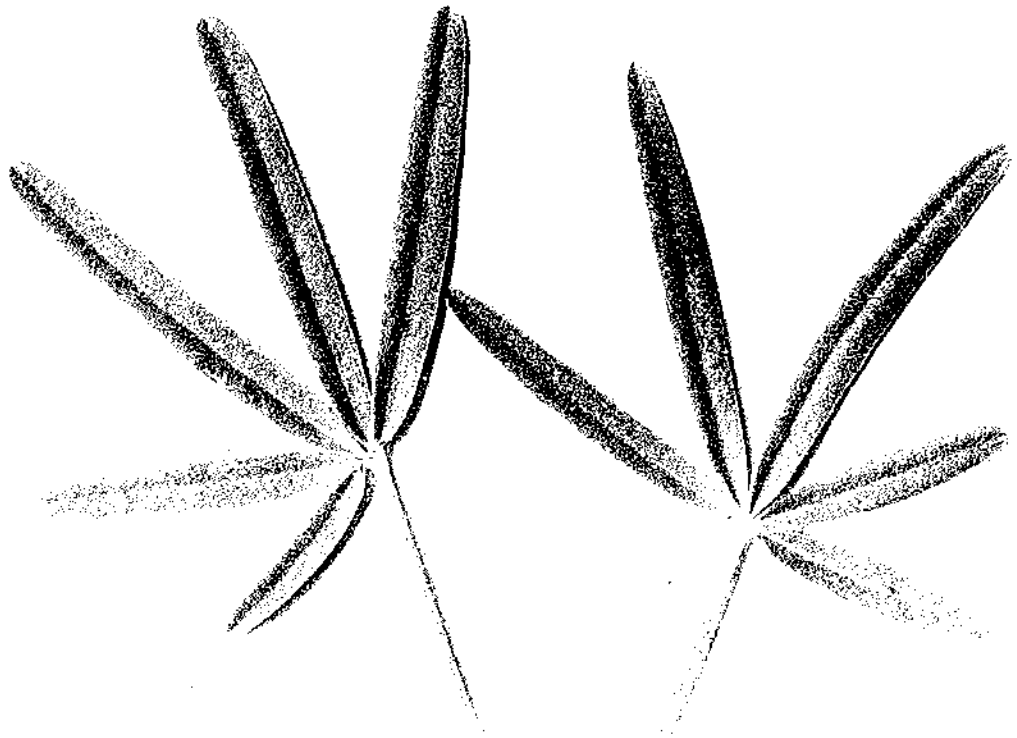
from pure cultures. Ascochyta was found to sporulate well on PDA, but to obtain sufficient inoculum of P. setosa, this latter fungus was grown on PCA for ten days at 24°C (Milne 1964), and the surface of colonies then scraped and washed to induce conidial production. Following inoculation plants were subjected to conditions of high humidity for two to three days and temperatures were maintained throughout within the range of 64° - 77°F (18° - 25°C).

It was realised early that disease expression was influenced by the prevailing environmental conditions. Both in isolating and artificially inoculating it was apparent that Ascochyta was basically a low temperature pathogen, while S. botryosum and P. setosa were most destructive in warm weather. This meant that one fungus could produce lesions with different characteristics at different times of the year. Due to the different methods of asexual spread, so too were there differences in the infection sites. Whereas Ascochyta was present mainly on pods and stems, S. botryosum infection was randomly distributed over the aerial parts of plants.

In the earliest stages of symptom development on any plant structure, accurate diagnosis without attempting to isolate the fungus was practically impossible. This is illustrated in Plate 3a for leaf symptoms. Here S. botryosum and P. setosa were artificially inoculated to blue lupin leaves and later reisolated. At the stage of development depicted, the problem of accurate diagnosis is very

Plate 3:

(a)



(b)

Symptom Differentiation at Early Stages of Expression

(a) Two days following inoculation
(b) Three days following inoculation

apparent. After a suitable period of incubation however, symptoms could be readily differentiated. Plate 3b shows these same leaves after a further 24 hours at 22 - 26°C. The differences between the two fungi are very marked at this stage.

Comparative symptomatology is now discussed:

1. LEAVES

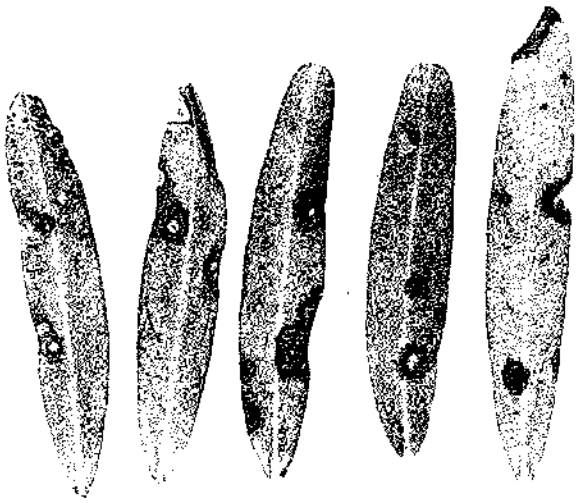
(a) Leaflets

The distinguishing features for the three fungi are the peripheral characteristics and the colour of the lesion centre. These are illustrated in Plates 4 and 5.

S. botryosum lesions were commonly circular or semi-circular (infection at the edge of a leaflet), and measuring one to five mm in diameter. Viewed from the upper leaf surface, they were bounded by a chocolate band fairly clearly demarcated from both the surrounding healthy green tissue and the bleached fawn-coloured centre (This bleached centre was absent from immature lesions). From below, they were uniformly grey from the centre to the periphery. Secondary development of lesions [1] was often initially accompanied by a purple hue of the surrounding healthy tissue. Those leaflet lesions exhibiting the bleached collapsed centres were the only lesions produced on the plant on which sporulation was observed. In no case were similar-sized lesions on stems, pods or cotyledons observed in the

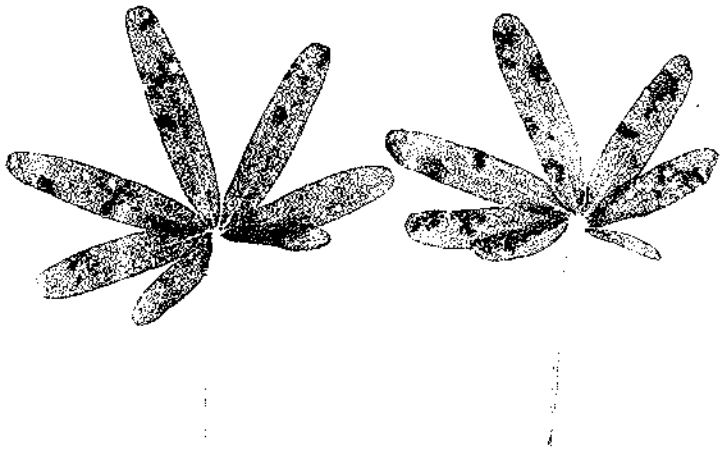
1/ Secondary development implies that the pathogen, previously inactive, has resumed growth and is again advancing into healthy leaf tissue from a clearly differentiated lesion.

Plate 4:



S. botryosum

Ascochyta sp.



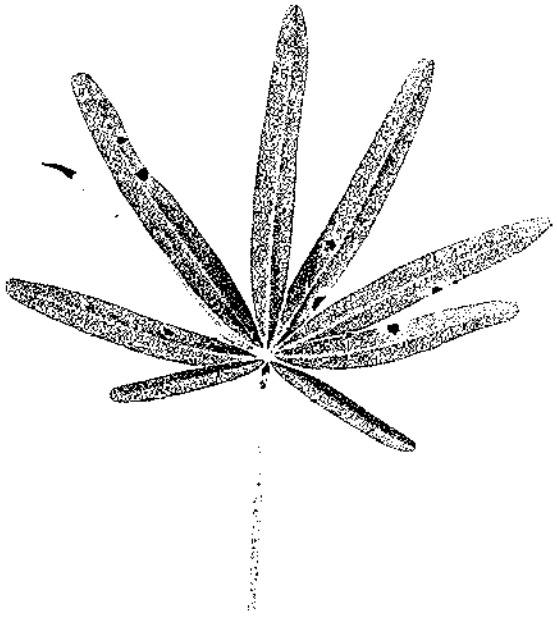
P. setosa

Leaflet Symptoms

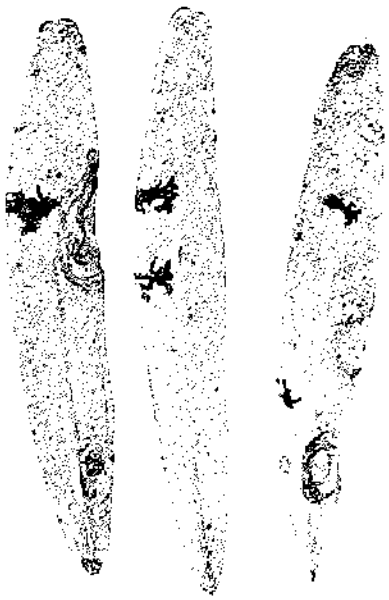
Plate 5:

(a)

(b)



(c)



Leaflet Symptoms

(a) S. botryosum (Natural Infection) Affected Leaflet Chlorotic and about to Abcise

(b) Ascochyta sp. (Artificial Infection)

(c) P. setosa (irregular spot) and S. botryosum (Natural Infection) on pressed leaflets

field or induced in the laboratory to sporulate. The presence of a single lesion on a leaflet was usually sufficient to result in a general yellowing followed by abcission of the affected leaf portion. (Plate 5).

Ascochyta produced two types of lesion, the most characteristic one (Plate 4) being circular to semi-circular and developing to a similar size as those for S. botryosum but with a thin, black, sharply-demarcated periphery. When viewed with transmitted light an indistinct clearing of the green surrounding tissue could be seen, a feature not associated with S. botryosum lesions. The lesion centre was dark brown and the light coloured region between this and the perimeter was concentrically zonate due to the presence of rows of pycnidia. The less characteristic lesions (Plate 5) probably represented the initial stage of the large lesions. They were small, round, dark-brown to black, and sharply demarcated from surrounding green tissue, with a pronounced "halo" of cleared photosynthetic tissue. These symptoms were usually produced by artificial inoculations in warm weather.

P. setosa lesions, which have been referred to as "cobweb" leaf spots (Milne 1964), were very irregular in outline with dendritic clearly demarcated black outgrowths ramifying into green photosynthetic tissue. Lesion centres were either bleached and deeply penetrating or remained black and superficial. Weimer (1952) described, and Milne (1964) reproduced artificially, a circular leaf spot phase on blue lupin where the pathogen advanced in a broad front (presumably during favourable conditions for pathogenesis). In the present study this symptom was not observed in the field. Further, attempts to

reproduce it in the glasshouse, although successful on yellow lupin (Lupinus arboreus), were not so on blue lupin under similar conditions.

(b) Petioles

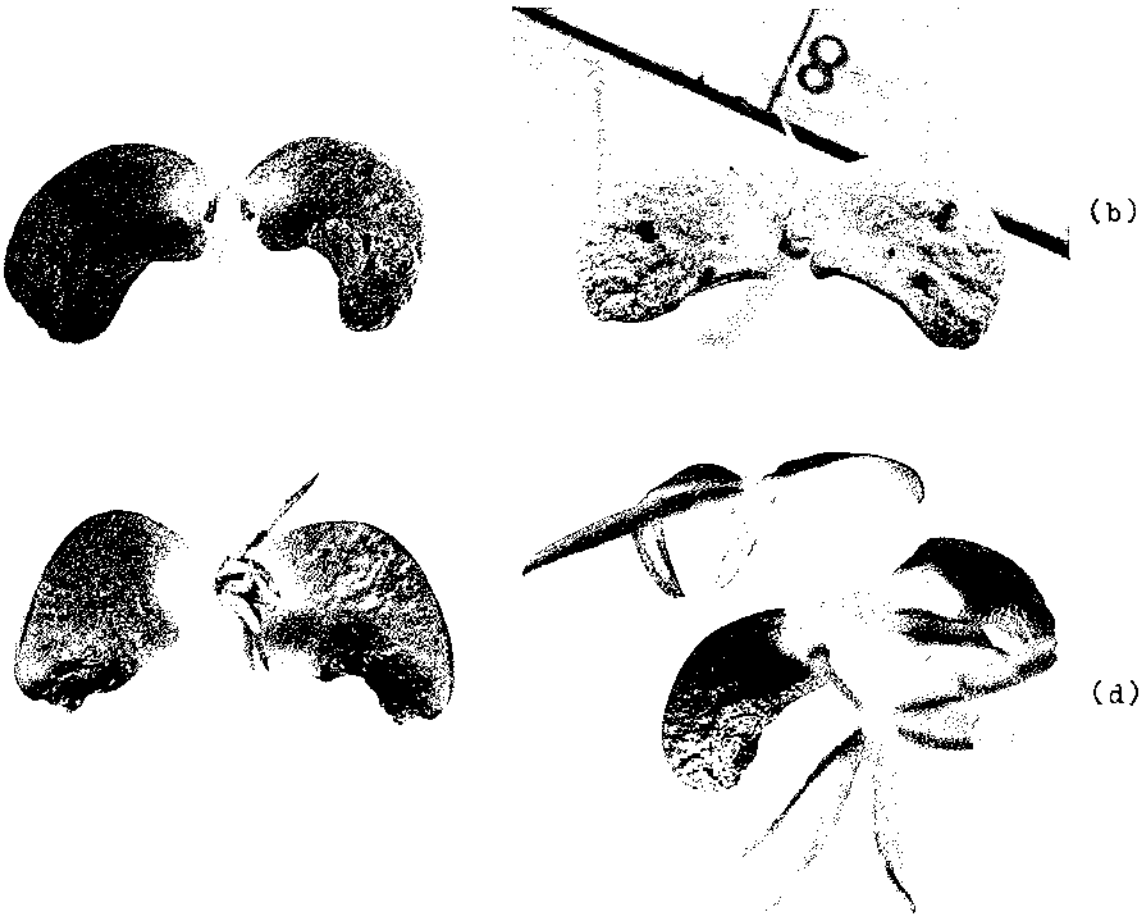
For the three fungi, a single lesion on a petiole usually resulted in the abcission of all leaflets on that leaf. It was not possible to distinguish the three causal agents without isolation techniques being employed. Where Ascochyta was the pathogen there was a tendency for the lesion to begin at the base of the petiole and grow onto the stem, causing leaflet abcission and large pycnidia-studded stem cankers. P. setosa spores also tended to lodge at the junction of petiole and stem whereas S. botryosum, being primarily air dispersed, initiated infection more randomly on the petiole.

(c) Cotyledons

Lesions observed in the field were invariably caused by S. botryosum and their random distribution suggested they had resulted from aerial dissemination from neighbouring infected plants. They were grey, circular lesions with a central depressed spot and surrounded by an indistinct grey halo (Plate 6). Rapid cotyledon collapse and abcission usually occurred following the development of even a single lesion.

Ascochyta lesions were observed only during emergence trials and they were assumed to have resulted from the sowing of seed with embryo infection, contracted during seed maturation in the parent crop. The lesions were small, black, hard and semi-circular, producing secondary growth in an even front during cool humid conditions (Plate 6) with concentric zones of pycnidia being a feature. Seedling collapse was

Plate 6:



Cotyledon Symptoms

Artificial Infection

(a) S. botryosum (b) P. setosa

Natural Infection

(c) Ascochyta sp. (d) Ascochyta sp.

rapid if fungal advance reached the hypocotyl.

Lesions caused by P. setosa were never observed. However, in artificial inoculations, small black circular lesions that did not enlarge (Plate 6) were produced under conditions favourable to S. botryosum infection.

2. PODS

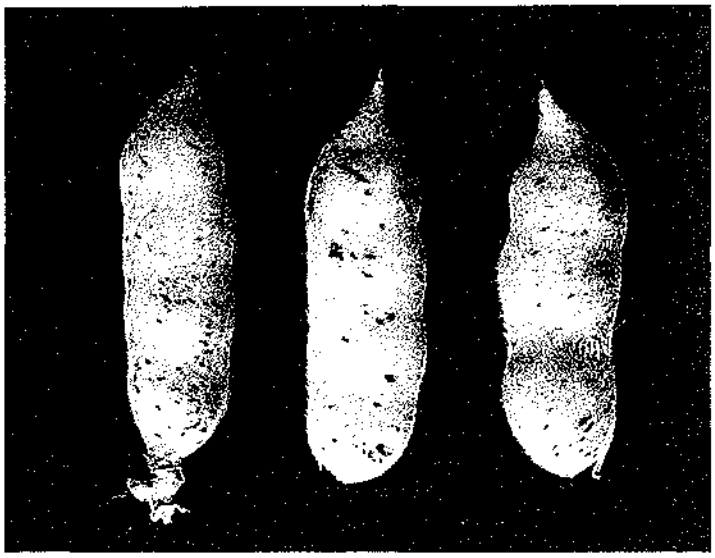
The differential characteristics exhibited by the three fungi are illustrated in Plates 7 and 8.

S. botryosum lesions were circular, superficial at first, one to five mm in diameter, with a "target spot" appearance caused by a distinct narrow "halo" encircling the central brown region of the lesion. Upon further development the halo became incorporated in the main sunken area which became to resemble a pock mark. About the stage of normal pod wall collapse, the fungus penetrated deeper into the pod wall, sometimes reaching the seed. (See Part III).

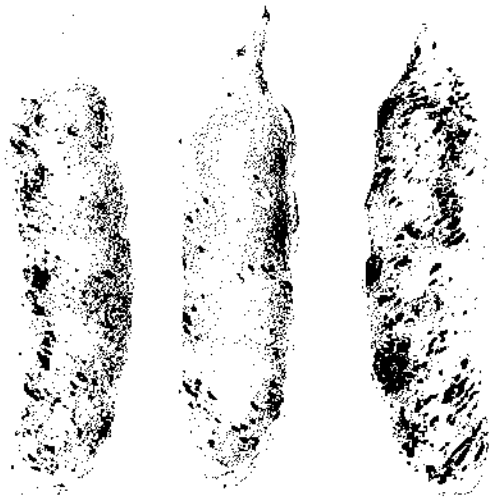
Ascochyta lesions were indistinguishable from those of P. setosa, being rectangular or sliver-shaped flecks, dark brown to black in colour, 1-2 mm in length, and orientated at a 45^{o} angle with the longitudinal axis of the pod. Under certain conditions (particularly towards pod maturity) lesions coalesced and occasionally developed into large sunken areas studded with pycnidia. At the stage of normal pod wall collapse the pathogen when present commonly ramified through the pod wall producing many pycnidia, and sometimes entered the seed.

Plate 7:

S. botryosum



P. setosa



Ascochyta sp.

Pod Symptoms. Natural Infection

Plate 8:

(a)

(b)

(c)

Pod Symptoms. Artificial Inoculation

(a) P. setosa
(b) Ascochyta sp.
(c) S. botryosum

P. setosa lesions remained identical to those of Ascochyta, were separate or coalescent, and superficial until normal pod wall collapse.[1/] Penetration of seed was never encountered in seed studies, although this has been reported overseas (Weimer 1952).

3. STEMS

The main characteristics produced by the three fungi are illustrated in Plates 9 and 10. Apart from slight modifications, lesions on stems were in general identical to those on pods.

S. botryosum lesions were more often longitudinally oval to elliptical than circular, but otherwise were identical to pod lesions caused by this fungus.

Ascochyta lesions, at first indistinguishable from those caused by P. setosa, (Plate 10a shows the early stage), invariably developed to produce very characteristic large, oval, sunken cankers up to five cms long, the colour ranging from dark-brown at the periphery to fawn at the centre, and studded with zonate bands of pycnidia (Plate 10b). These cankers could completely girdle the stem, causing a collapse at that point.

P. setosa lesions on stems during certain conditions merged into large dark-brown to black, irregularly-shaped superficial cankers (Plate 9). These symptoms corresponded with those described by Weimer (1952). In contrast to Ascochyta they did not penetrate into the woody layers of the stem to produce sunken lesions, and lodging

1/ "Normal pod wall collapse" occurs when seeds have fully developed, and is preceded by a general browning and dying-off of the senescent photosynthetic tissues involved.

Plate 9:

(a) (b)

Stem Symptoms. Natural Infection

(a) S. botryosum (b) P. setosa

Plate 10:

(a) (b)

Stem symptoms. Ascochyta sp. Natural Infection

(a) Early stage (b) Advanced stages

was therefore not an associated feature.

4. FLOWERS

Floral symptoms were never observed in the field. Milne (1964) on rare occasions encountered this phase of P. setosa attack in his study of brown spot, and Weimer (1952), had previously described these as sliver-shaped flecks similar to those produced on pods and stems. In the present study attempts to infect flowers with S. botryosum proved unsuccessful.

II TAXONOMY OF STEMPHYLIUM - REVIEW AND EVALUATION OF SPECIES DELIMITATION IN THIS GENUS

A. INTRODUCTION

The genus Stemphylium was erected by Wallroth (1833), with S. botryosum as type species. According to Wiltshire (1938), the salient diagnostic characters implied in Wallroth's definition of the genus are:

1. conidiophores swollen at the apex which bears a single terminal spore;
2. growth of the conidiophore may be continued through the terminal scar, the successive swellings recognisable in an old conidiophore marking the places where conidia have been borne;
3. spore shape oval or subangular, muriform, frequently constricted markedly at the median transverse septum.

In 1871, Harz assigned a fungus found on honeycomb to the genus, naming it S. lanuginosum. This identification was incorrect by reason of the conidiophore features being quite foreign to the original conception of the genus. Largely because of his excellent description and illustrations of S. lanuginosum this modified concept of Stemphylium came to be followed by most systematists. Abetting the error was the identification of an isolate by Oudemans as S. botryosum, and which Bolle (1924) regarded as agreeing with Wallroth's description, but later demonstrated by Wiltshire (1938) to be either closely allied to, or even synonymous with, S. lanuginosum. That is, the characters of this latter species came to be widely accepted as typical of the genus. These characters

may be stated as follows:

1. conidiophores without apical or nodular swellings;
2. growth of the conidiophore may be continued by a lateral outgrowth just below the apex, giving a cymose arrangement to an old conidiophore;
3. conidia ovate and borne singly. Marked constrictions not associated with the median transverse septum.

Wiltshire (1938) critically considered the whole question of Stemphylium taxonomy and nomenclature and concluded that Wallroth's characters for Stemphylium must be retained.

To avoid the necessity of transferring species of Stemphylium sensu Harz to another genus, he proposed that both concepts be retained in the one genus, the essential characters of which would be the dark-coloured, muriform, ovate or subangular spore. To differentiate the two types he proposed two subgenera:

Subgen. Eustemphylium - those species with the original characters of the genus as laid down by Wallroth (1833);

Subgen. Pseudostemphylium - those species allied to S. lanuginosum Harz.

While Wiltshire's sectional treatment of Stemphylium became widely accepted in principal (Groves and Skolko 1944, Neergaard 1945), it was not put into practice nomenclaturally as the subgeneric interpretation of subsequently described species in both groups was never indicated (for example, S. loti Graham 1953, a Eustemphylium

species, and S. dichroum Petrak 1950, a member of Pseudostemphylium.)

C.V. Subramanian (1961) in part rectified this inadequacy by elevating Pseudostemphylium to generic rank, with P. lanuginosum as the type species. Some acceptance of this proposal was evident when Hoes, Bruehl and Shaw (1965) described a new species P. chlamydosporum on winter wheat.

A proposal likely to receive wide acceptance has recently been presented by Simmons (1967) for overcoming the problem of differentiating pseudostemphylioid fungi from true stemphylia. From a comparative study of several hundred collections and cultures in addition to type species, Simmons concluded that fungi of the S. lanuginosum group had the greatest overall resemblence to the type species Ulocladium botrytis Preuss 1851. Accordingly, he proposed that S. lanuginosum Harz be transferred to Ulocladium, and this imposed the taxonomic synonymy of Stemphylium subgen. Pseudostemphylium Wiltshire and Pseudostemphylium (Wiltshire) C.V. Subr., since both are typified by S. lanuginosum. This in turn meant that Wallroth's original concept of Stemphylium was restored.

General acceptance of Simmon's proposal is highly desirable because it is based on a comparison of developmental morphology of the sporulation apparatus in Alternaria, Ulocladium and Stemphylium. This modern approach to classification of hyphomycetous fungi (on the basis of developmental morphology) has in recent years been advocated by Hughes (1953), and Tubaki (1958) and is considered to provide a much more natural foundation for classification than the Saccardian system. This latter scheme is based on mature-spore form rather than developmental morphology with the result that true relationships between asexual fungi are not always reflected in the classification.

Hughes system has been acclaimed as the best alternative yet to the Saccardian System (Alexopoulis 1962, Hawker 1966, Simmons 1967).

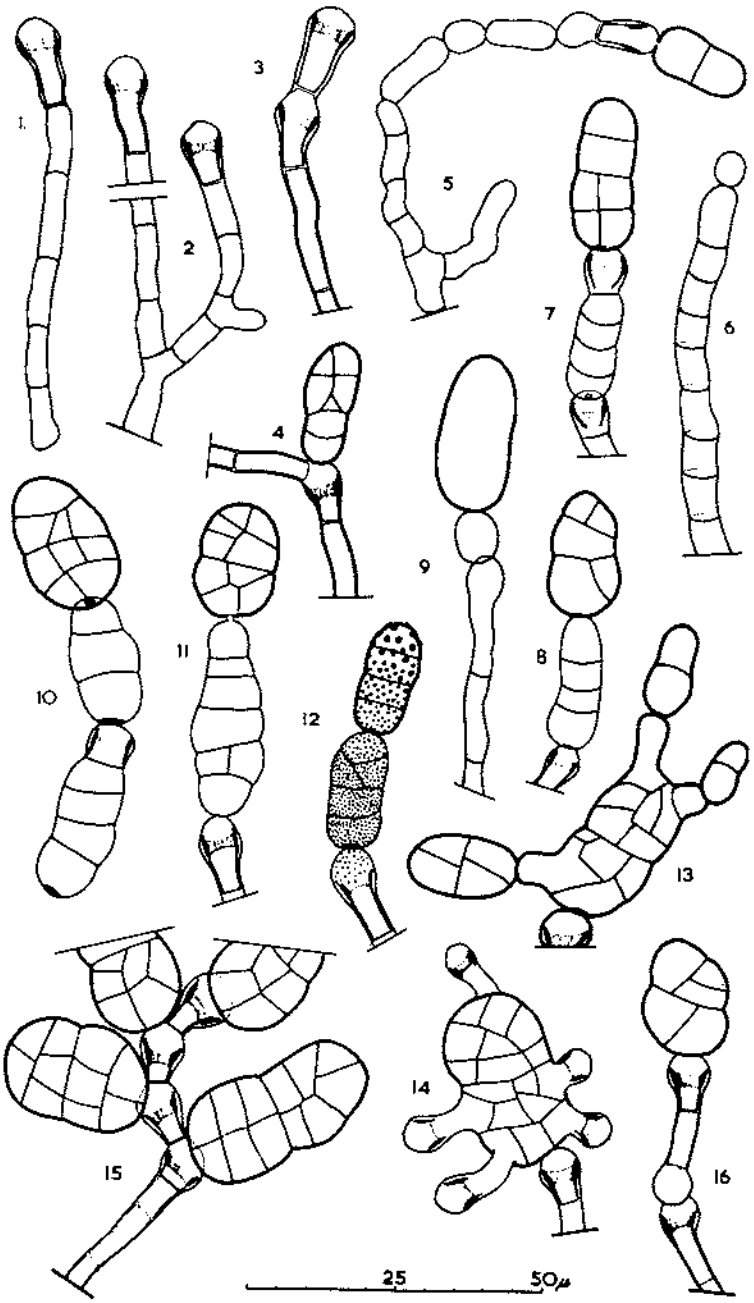
For the sake of clarity, a more expanded description of the genus is here presented, based on Wiltshire (1938) and Simmons (1967), and in no way modifying Wallroth's (1833) concept of the genus:

Genus Stemphylium Wallroth 1833.

1. Conidiophores* terminally and nodally bulbous,[3] each node having produced a single porospore (Hughes 1953) during the growth of the conidiophore. Where successive spores are not immediately shed, a variable botryose spore-cluster results,[15] the proliferated conidiophore being geniculate and curved.[15] This proliferation is always through the swollen apical region from which the previous porospore was extruded.[3,4,15]
2. Conidia variously ovcid,[4] obclavate,[24] obturbinate,[4] spherical,[26,21] broadly ellipsoid[18] to subdoliiform,[3,7] sarciniform,[7,12,31] darkly pigmented, muriform, with pronounced constrictions normally associated with the main transverse septa. In some forms, apex variously pointed.[16,18,24] Conidial proliferation may occur prior to the primary conidium being shed thus producing numerous secondary and tertiary conidiophores and conidia.[13,14]

* The numbers which follow refer to conidiophore characters which have been depicted by way of line drawings in Figure 5. In the case of conidial characteristics, the numbers attached refer to Figure 7.

Figure 5:



Conidiophore characters and Conidial Arrangements observed for Stemphylium during the Present Study. (Camera - Lucida Reproductions)

Acceptance of Simmon's proposals as the correct solution to a complicated taxonomic problem dating back to the mid 1800's means that the time is opportune for a major review of the genus Stemphylium. To this end studies were initiated with the following objectives.

1. To decide which recorded species are tenable in Stemphylium in its restored sense.
2. To examine and evaluate in relation to modern taxonomy the basis used for species delimitation in Stemphylium Wallr.

B. DETERMINATION OF TRUE STEMPHYLIA

1. INTRODUCTION

Other than Stemphylium, the genus Macrosporium provides a potential source of stemphylia for the following reasons:

(a) from a study of both the diagnoses and exsiccati representing 85 species of Macrosporium (listed by Saccardo 1882 - 1913), Elliot (1917) concluded that 20 of these should be transferred to Stemphylium. His concept of Stemphylium at that time embraced those of both Wallroth and Harz;

(b) Wiltshire (1933) in his examination of the foundation species of Alternaria and Macrosporium found that sarciniform spores typical of Stemphylium Wallr. had been placed in Macrosporium. His cited examples were

various synonyms of S. botryosum (M. alliorum, M. symplocarpi, M. parasiticum), S. sarcinaeforme (M. sarcinaeforme) as well as M. sophorae, M. nigricantium, M. Eriobotryae, M. camelliae, and a probable synonym of Ulocladium consortiale (Thüm.) Simmons (M. verruculosum). His widely accepted proposal was that Macrosporium be suppressed as a nomen ambiguum in favour of Alternaria, and although many former macrosporia have accordingly been transferred or reduced to synonymy, many still await consideration. That is, many species of Macrosporium erected before 1933 remain to be transferred to either Stemphylium, Alternaria or Ulocladium. In addition, because some workers prefer to recognise Macrosporium as a valid genus, a number of new species have been erected since Wiltshire's publication in 1933.

In order to determine which species presently included in Stemphylium and Macrosporium are tenable in the former genus (in line with the proposals of Wiltshire, 1938 and Simmons, 1967) a literature search was conducted aimed at assembling all the species so far described in these two genera and then deciding which represented Stemphylium Wallr.

2. METHOD

Generic affinity was determined by examining the original species diagnoses and illustrations, aided by any subsequent papers relating to taxonomy of the species concerned. (For "new species" described

prior to 1920, diagnoses listed in Saccardo's Sylloge Fungorum were used.) Examination of type material of the large number of species involved was not practicable in this study, but it was considered that valid conclusions could still be drawn regarding generic affinity because of the characteristic and distinct nature of true stemphylia amongst closely related genera (provided that diagnoses were adequately representative). The following simple key [1] was used as the basis for these decisions.

Key to *Stemphylium*, *Alternaria* and *Ulocladium*

A Conidiophores terminally bulbous, with proliferation through the swollen apical surface ..... *Stemphylium*

A_1 Conidiophores *not* terminally bulbous, with proliferation lateral to and slightly below the apical surface.

B Conidia fundamentally ovoid and distally beaked ..... *Alternaria*

B_1 Conidia fundamentally obovoid and *not* distally beaked ..... *Ulocladium*

Where conidiophores were described but not illustrated it was assumed that if no reference was made to "torulose", "nodulose" or "moniliform" features, then the species was not representative of *Stemphylium* Wallr.

To facilitate the most complete coverage of the literature, the following reference works were consulted:

Saccardo, P.A. Sylloge Fungorum (1882-1931)
Vols 4,10,11,14,16,18,22,25 (Hyphomycetes)
Vols 19,20 (Index to illustrations)

1/ This key was based on the typification of the three genera by Simmons (1967).

Petraks Lists, Index of Fungi reprints (CMI)(1921-1939)
Index of Fungi (CMI)(1940-1967)

In addition, extensive use was made of the publications by Wiltshire (1933 and 1938), Groves and Skolko (1944), Neergaard (1945) Joly (1964), and Simmons (1967).

3. RESULTS

(a) Examination of genus Stemphylium

A total of 74 erected species were assembled. 63 species could be classified into three groups, depending on the clarity and detail of diagnoses and illustrations available for examination. (The rest have at various times been reduced to synonymy and are therefore excluded from this study).

Group (i) those representing true stemphylia (14 spp.)
Group (ii) those no longer tenable in Stemphylium (41 spp.)
Group (iii) those with uncertain affinity 1/ (18 spp.)

Group (i) is presented in Table 10, Groups (ii) and (iii) being included in Appendices III and IV respectively. (Subspecific taxa have been excluded).

1/ In these cases attempts to acquire original diagnoses were unsuccessful.

TABLE 10: Species Representing Stemphylium Wallr.

Species	Author	Date	Source
S. botryosum	Wallroth	1833	Wiltshire (1938), Trans.Brit.Mycol.Soc.21:211-239
S. sarcinaeforme	(Cav.)(Tehon et Dan.)Wilts.	1938	Cavara 1890 Dif. dei parass. n.4
S. loti	Graham	1953	Phytopath. 43; 577-579
S. trifolii	Graham	1957	Phytopath. 47; 213-215
S. solani	Weber	1930	Phytopath. 20; 513-518
S. lycopersici	(Enjoji) Yamamoto	1960	Enjoji (1931), Trans.Mycol.Soc.Japan 12; 9-13
S. bolicki	Sobers et Seymour	1963	Phytopath. 53; 1443-1446
S. callistephi	Baker et Davis	1950	Mycologia 42; 447-486
S. astragali	(Yoshii) Yamamoto	1960	Journ. Plant Prot. 16; 536
S. chisha	(Nisikado et Hiura)Yamamoto	1960	Nôgaku Kenkyû 39; 40
S. triglochinicola	Sutton et Pirozynski	1963	Trans.Brit.Mycol.Soc. 46; 505-522
S. nabarii	Sarwar	1965	Mycopath.et Mycol. Appl. 29: 320-322
1/ S. melanopus	(Schw.) Hughes	1958	Schw.(1832), Trans.Amer.Phil.Soc. 11; 283
2/ S. pyriforme	(Corda) Bonorden	1851	Wiltshire (1938) Trans.Brit.Mycol.Soc. 21: 211-239

1/ S. melanopus (Schw.) Hughes 1958

This species was originally described under Dactylium, but Hughes upon examination of the type material transferred it to Stemphylium. The original diagnosis although meagre certainly indicates conidiophores of a nodulose nature.

2/ S. pyriforme (Corda) Bonorden 1851

According to Wiltshire (1938), this was the first fungus to be placed in the genus after it was erected by Wallroth in 1833. Wiltshire considered it to be a true Stemphylium from Corda's description, as it had conidiophores swollen at the tips and more or less ovate conidia, with a prominent central constriction. However, Wiltshire could not identify it more precisely than to the genus Stemphylium in the absence of a type specimen, so that until one can be raised, S. pyriforme must remain a nomen confusum.

(b) Examination of genus Macrosporium nomen ambiguum

A total of 247 erected species were assembled. It was concluded that 49 of these may in fact represent true stemphylia since the conidiophores were referred to as either nodulose, moniliform or torulose. One could not be certain of their affinity due to inadequate diagnoses and the lack of illustrations in many cases. Of the remaining 198 species, 95 have already been reduced to synonymy either in Alternaria or Stemphylium, 76 do not represent true stemphylia, while 27 have uncertain affinity.[1/]

A group of 49 species possibly representing true stemphylia is listed in Table 11.

4. DISCUSSION

As a result of the above survey the following points emerged.

(a) Only 14 of the 65 species of Stemphylium so far erected (excluding binomials shown to be synonymous) can definitely be regarded as truly representative of Wallroth's genus. Forty-three species are no longer tenable here and need to be transferred to other genera. The majority of these in all probability have their greatest affinities with Ulocladium Preuss. Many of the earlier-erected of these species (appearing in Saccardo's Sylloge Fungorum) may prove to be merely collections of the same species. Elliot (1917) demonstrated that at least 34 of the 85 species of

1/ Attempts to acquire the original diagnoses were unsuccessful. These species are listed in Appendix V.

TABLE 11: Macrosporia with Possible Affinity to Stemphylium Wallr.

	Species	Author	Date	Source
	M. heteronemum	(Desm.) Saccardo		Saccardo 1882 Sylloge Fungorum 4
	M. leptotrichum	C. et E.		"
2/	M. rosarium	Penz.		"
1/	M. Catalpae	Ell. et. Mart.		"
1/	M. antennaeforme	B. et C.		"
	M. Lagenariae	Thümen		"
1/	M. Floridanum	Cooke		"
1/	M. leguminum	Cooke		"
	M. diversisporum	Thüm.		"
1/	M. Maydis	C. et. E.		"
	M. Gynerii	Thüm.		"
	M. consors	(Thüm.) Sacc.		"
1/	M. bulbotrichum	Cooke		"
	M. Ravenelii	Thüm.		Sacc. 1886 Syll. Fung. 10
2/	M. Valerianellae	Roum.	1886	"
	M. nigricans	Atkins.	1891	"
2/	M. schemnitziense	Bäuml.		"
	M. Polytrichi	Peck		"
	M. hybrideum	Ell. et. Ev.	1894	Sacc. 1892 Syll. Fung. 11
	M. Violae	Pollacci	1897	Sacc. 1895 Syll. Fung. 14
	M. Mac-Alpineanum	Sacc. et. Syd.		"
	M. Malvae-vulgaris	Eliasson	1897	"
	M. Velutinum	McAlp.	1898	"
1/	M. Amaranthi	Peck	1895	"
2/	M. toruloides	Ell. et Ev.	1895	"
	M. Asphodeli	Pat.	1892	"
	M. striaeforme	Syd.	1900	Sacc. 1899 Syll. Fung. 16
	M. Lunariae	Oud.et van Hall	1904	Sacc. 1902 Syll. Fung. 18
	M. epicarpium	McAlp.	1902	"
	M. Sydowianum	Farneti	1905	Sacc. 1906 Syll. Fung. 22
	M. Dictamni	Lindau	1908	"
	M. verrucosum	Lutz.	1906-7	"
	M. Cirsii	Lindau	1908	"
	M. Sparganii	Lindau	1908	"
3/	M. verruculosum	Bubàk	1916	Sacc. 1913 Syll. Fung. 25
	M. mycophilum	Bubàk et Dearness	1916	"
	M. Jurisicii	Ranojevic	1914	"
	M. lineare	Sacc.	1913	"
	M. Phormii	Speg.	1912	"
	M. engonatum	Sacc.	1915	"
	M. vesiculiferum	V.Hohn	1915	"
	M. Hesperidearum	Pantanella	1913	"
	M. cleghornianum	Sacc.	1913-14	"
	M. laminarianum	Sutherl.	1916	"
4/	M. Sophorae	Turconi et Maffei	1912	Wiltshire 1933 T.B.M.S.18(Part 2)
4/	M. nigricantium			pp.135-(151)-160.
4/	M. Eriobotryae			"
4/	M. camelliae			"

Table 11: (Cont'd)

1/ Species listed by Elliot (1917) as probably belonging to Alternaria.

2/ Species listed by Elliot (1917) as probably belonging to Stemphylium. (These, in view of Simmon's paper are almost certainly true stemphylia).

3/ This is not the same as M. verruculosum Zimmerman 1878, according to Saccardo.

4/ According to Wiltshire (1933) these species had sarciniform-shaped conidia.

Macrosporium he studied were inseparable, as they were delimited on spore measurements, the differences between which could be attributed solely to personal error. The same argument could well apply to Stemphylium species erected during that period. Examination of type material, where it is available, is required.

(b) One species (S. bizarrum Viegas 1961) has no real affinities either with Stemphylium, Alternaria or Ulocladium for the reason that spore and conidiophore morphology are foreign to these genera. (This is discussed in Appendix VI).

(c) Anomalies concerning the nomenclature of two species exist. The first, (S. floridanum Hannon and Weber, 1955) according to Yamamoto (1960) has previously been described under a different specific epithet ("lycopersici") by Enjoji (1931) and this epithet must therefore be the valid one.

The second (S. tritici Deshpande and Deshpande, 1965) was formerly applied to a different fungus by Patterson, Charles and Veihmeyer (1910). Accordingly S. tritici Deshp. and Deshp. 1965 is invalidated.

(d) Some confusion concerning S. solani Weber (1931) exists. This species was transferred by Sawada (1931) to Thyrospora but according to Yamamoto (1960) was not the same as the one Sawada had studied on tomatoes in Formosa and named Thyrospora solani. This latter fungus is considered by Yamamoto (1960) to be in fact synonymous with S. lycopersici (Enjoji) Yamamoto 1960 and consequently S. solani Weber remains a valid species. Sawada incorrectly considered his fungus to be identical with that described by Weber. Both fungi can be found on tomatoes and Sawada's description and illustrations are certainly typical of S. lycopersici.

(e) From a total of 148 erected species of Macrosporium (excluding 95 species reduced to synonymy), 49 have possible affinity with Stemphylium Wallr., since in all cases Saccardo's diagnoses referred to conidiophores of a "nodulose" nature. The examination of type material is again required for confirmation.

C. THE BASIS FOR SPECIES DELIMITATION IN STEMPHYLIUM

1. THE SPECIES CONCEPT

It is now generally accepted that for a taxonomic species to be of functional value to the mycologist or phytopathologist concerned with non-specialised fungi,[1/] it must be:

1/ Taxonomic principals applying to such fungi of low specialisation do not necessarily hold for highly specialised parasites such as the smuts (Fischer and Shaw, 1953) or downy mildews (Yerkes and Shaw, 1959).

(a) delimited solely on stable morphologic criteria;

(b) clearly distinct amongst closely related species;

(c) fully representative of the fungus as it occurs in the field (the biologic species).

In order to meet these requirements, species delimitation must take into account that fungi are variable. This necessitates identification and use of those morphologic features (characterising the biological species) that are least prone to genetic variations and most stable under changing environmental conditions.

The truth of these generalisations is well illustrated by taxonomic studies conducted on the genus Fusarium since 1930. In 1935 Wollenweber and Reinking divided this genus into 16 sections, with a total of 65 species. This classification proved highly unworkable in practice because insignificant and unstable features of macroconidial form (for example, degree of curvature and dimensions) had been used in the delimitation of both sections and species. Fusaria were difficult to identify under this system and this led to misdeterminations.

In contrast, by determining the extent of inherent and induced morphologic variability in fusaria, Snyder and Hansen (1940, 1941, 1945, 1954) identified those characters most stable and therefore most suitable as taxonomic criteria. As a result they reduced the number of Fusarium species to nine. These species were clearly-defined morphologic entities, fully representative of the biological species and therefore readily distinguished by non-taxonomists. That is, their basis for speciation was the use of stable morphologic criteria. In using conidial morphology the stable criterion was

the "overall form" as opposed to conidial dimensions, or the form of any component part.

The concepts of fungal taxonomy propounded by Snyder and Hansen and applied to the genus Fusarium have been widely accepted and extended (Cain 1962, Nelson 1965, Ainsworth 1962).

It was considered that any appraisal of the genus Stemphylium, which like Fusarium represents a group of non-specialised fungi, should be based on these concepts.

2. MORPHOLOGIC COMPARISON OF STEMPHYLIUM SPP.

To determine whether the basis for species delimitation in Stemphylium conformed with the present day species concept discussed above a critical study was made of published descriptions and illustrations of twelve Stemphylium species definitely representing Wallroth's genus. 1/

The morphologic criteria utilised by the authors in erecting these species were identified, and then used to "chart" all species in a comparative table, so enabling their distinctness in form to be evaluated. These criteria were:

(a) Categories of Mature Conidial Form

- (i) number of prominent lateral constrictions
- (ii) base and apex morphology
- (iii) longitudinal symmetry
- (iv) epispore sculpture
- (v) length:breadth ratio;

1/ This was possible in the absence of type material because in each instance the descriptions and accompanying illustrations clearly revealed the essential features of the conidiophores and conidia.

(b) Function of Stromatic Bodies

(i) developing into ascostromata

(ii) remaining undifferentiated. 1/

Subdivision of the above categories of conidial form is presented in Table 12. The interpretation of descriptive terms appearing in this Table is illustrated with sketches in Figure 6. (redrawn in part from Ainsworth, 1966).2/ The application of the various spore form categories is illustrated by camera lucida reproductions of conidia sampled from field collections (Figure 7).3/ All possible literature sources dealing with the taxonomy of the 12 species were taken into account during the comparison (Appendix VII).

A number of other criteria dealt with at length by Neergaard (1945) were not used either because they are generic characteristics (conidial colour, septation, and arrangement; conidiophore characteristics), or because they constitute criteria now widely recognised as being unstable under varying environmental conditions (conidial size, hyphal and cultural characteristics).

1/ Where black stromatic bodies were not referred to in species diagnoses it was assumed either that they were not produced by the species concerned, or that the authors had neglected to record their presence. This was the case for S. astragali, S. chisha, S. nabarii, S. triglochinicola.

2/ The descriptive term "punctate" does not appear in any diagnosis but was introduced here to categorise those conidia that are neither completely smooth nor moderately verrucose or echinulate, but variously described as "almost smooth", "slightly roughened", "very lightly verrucose" or "sparsely echinulate".

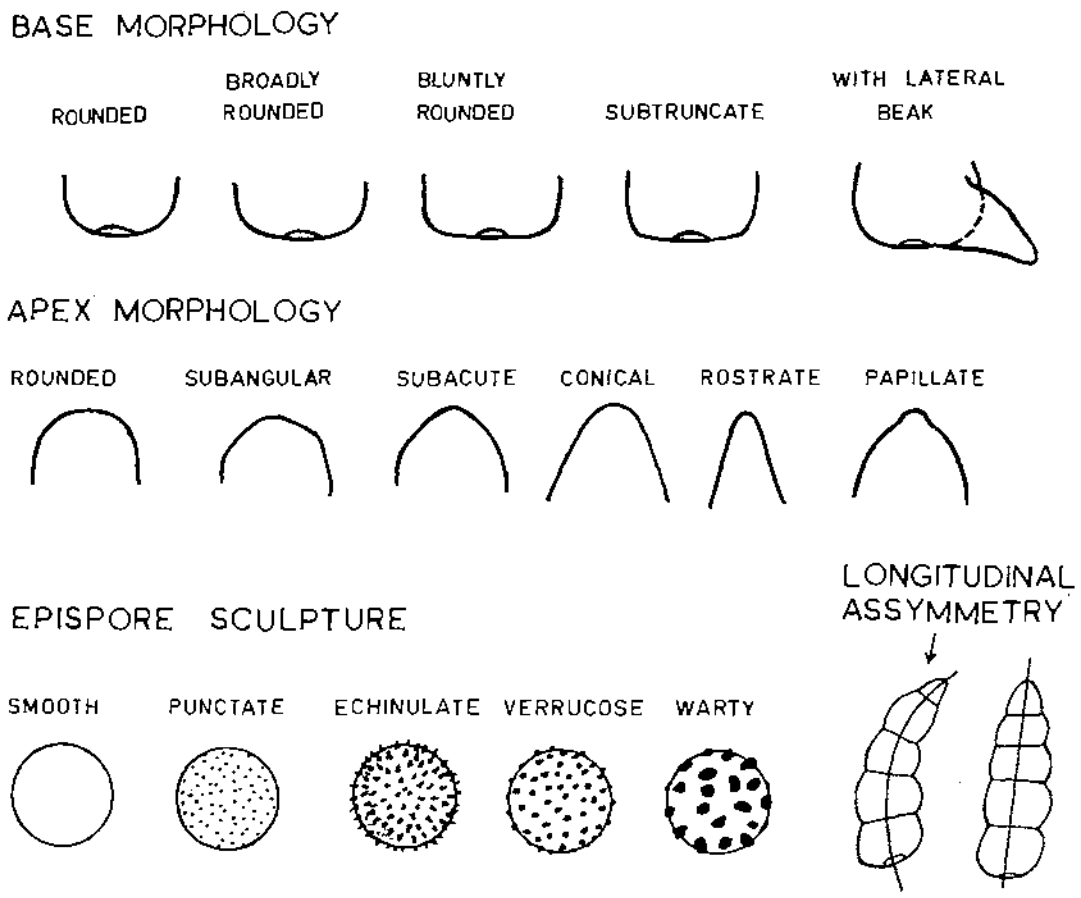
3/ All camera lucida reproductions represent plan sections focused midway through the spore. The apparatus used is illustrated in Plate 11.

TABLE 12: Subdivision of Conidial Form Categories.

Category				Subcategory	
1	Number of prominent lateral constrictions			a b c d	none one two or three more than three
2	Base and Apex Morphology	a	Base bluntly rounded to subtruncate;	i ii iii	Apex bluntly-rounded; rounded to subangular; subacute, conical, rostrate.
		b	broadly rounded to rounded;	i ii	Apex rounded to subangular; subacute to rostrate.
		c	with one lateral beak		Apex subacute to rostrate.
3	Longitudinal symmetry			a b	Symmetric Assymmetric
4	Epispore sculpture			a b c d	Smooth Punctate Echinulate Verrucose or warty
7	Length:Breadth ratio			a b c d e	1:1 3:2 2:1 3:1 more than 3:1

A comparison of the 12 species on the basis of the above stated conidial features and stromatic body association is presented in Figure 8. For each species conidial features are indicated by shaded squares located directly below the respective sub-categories of conidial form. The key to these categories and subcategories is presented in Table 12. The bars, crosses or circles on shaded squares

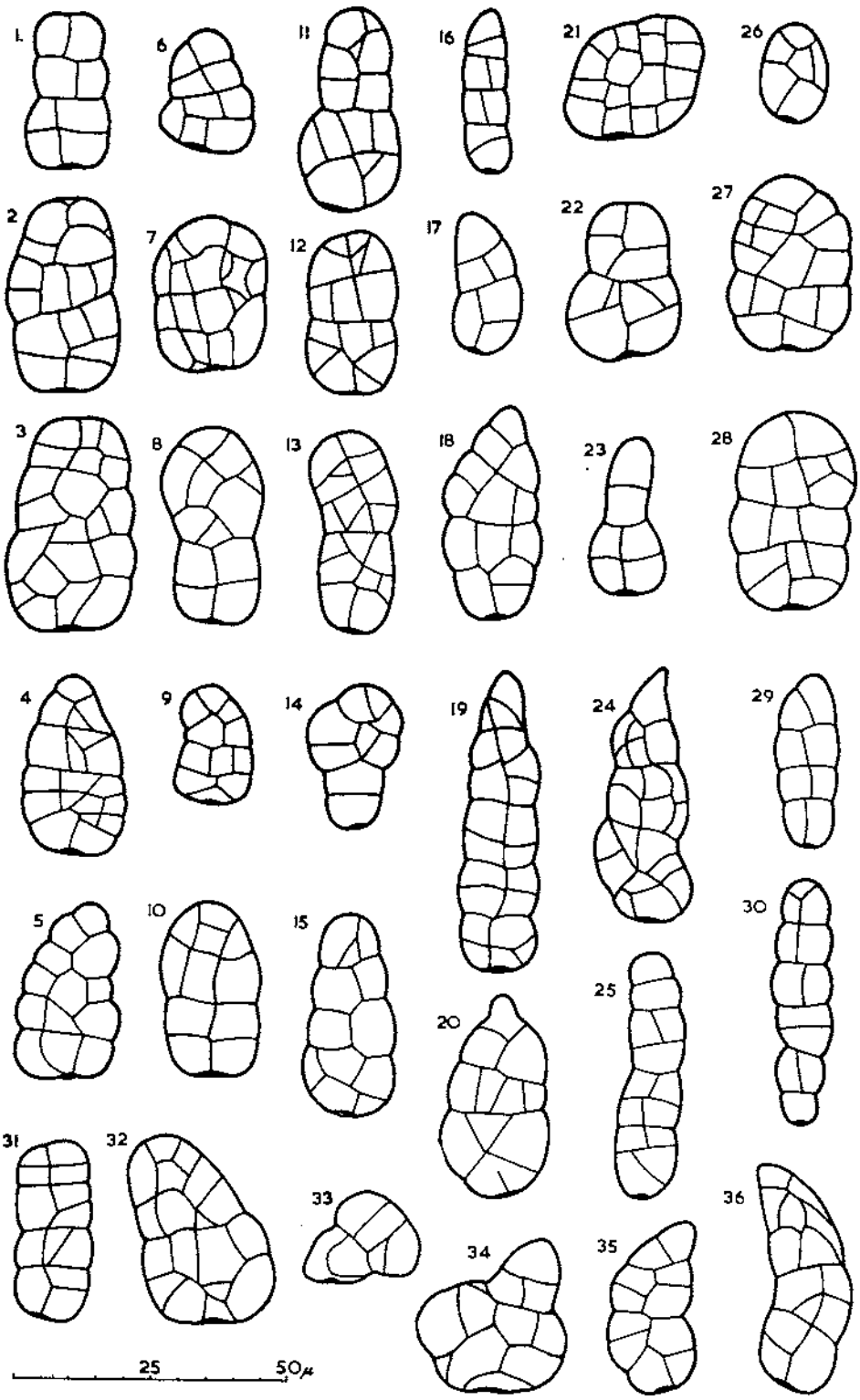
Figure 6:



Interpretation of Descriptive Terms used to Categorise Conidia

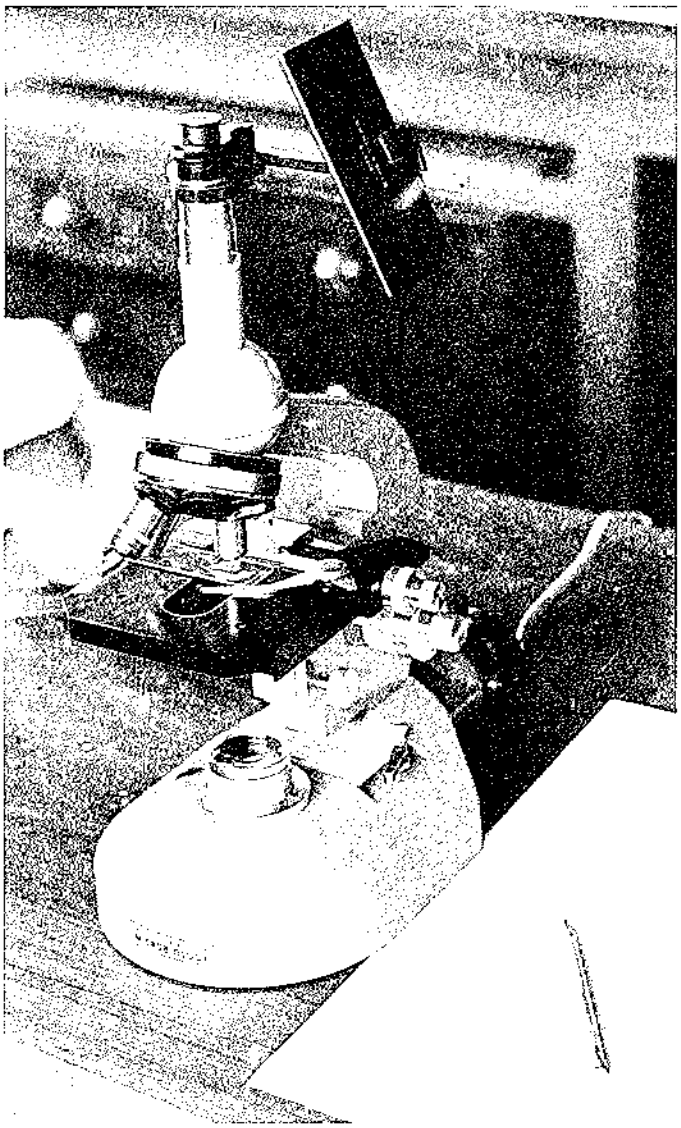
Subcategory of Table 12	Corresponding Spore Number Opposite
1a, b, (c), d.	26, 27, (28, 29), 30.
2ai.	1, 2, 3.
2aii.	6, 7, 8, 9, 10.
2bi.	11, 12, 13, 14, 15.
2bii.	16, 17, 18, 19, 20.
3a, (b).	31, (32, 33, 34, 35, 36).
7a, b, c, d, e.	21, 22, 23, 24, 25.

Figure 7:



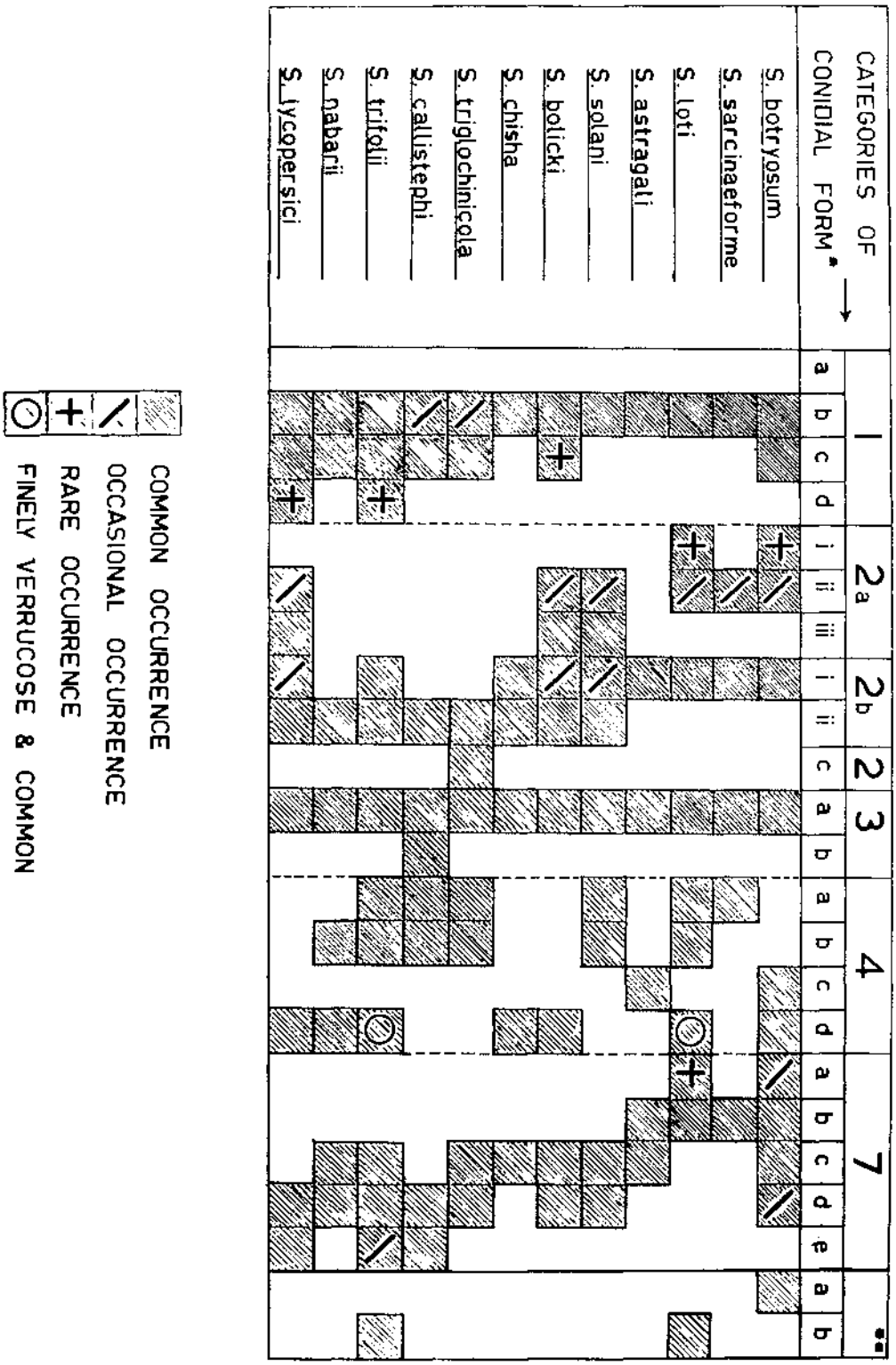
Naturally Occurring Examples of Stemphylium Conidia
Illustrating the Spore Form Categories of
Table 12

Plate 11:



Olympus Microscope fitted with Camera Lucida

Figure 8:



Morphological Comparison of 12 Stemphylium spp.

emphasize for each species the modal (most common) spore characteristics while the total series of squares from left to right depicts the range of form. The final vertical column designated** refers to the details of stromatic body association, namely:

(a) developing into mature ascostromata; or

(b) remaining undifferentiated.

A consideration of S. callistephi illustrates the interpretation of this chart:

> conidia have 1-3 prominent lateral constrictions (1b, 1c); are broadly rounded to rounded at the base and subacute to rostrate at the apex (2bii); are longitudinally symmetric (3a) or assymmetric (3b); are smooth (4a) or punctate (4b); with a length:breadth ratio of 3:1 (7d) or higher (7e). Black stromatic bodies are not produced.

3. DISCUSSION

The following points arise from the comparison:

(a) while all 12 species overlap to some extent for most details of conidial form, they differ in the extent to which these features occur. For example, while the range in number of prominent lateral constrictions for both S. bolicki and S. callistephi is one to three (subcategories 1b, 1c), most conidia produced by S. bolicki

have only one constriction, and for S. callistephi the majority have 2-3 constrictions. Only in a few species do the conidial features by themselves appear sufficiently distinct for clear demarcation (for example S. triglochinicola). In other species conidial form is very similar but the presence or absence of black stromatic bodies appears sufficient to distinguish them;

(b) the basis for delimitation of the 12 species has been mainly the emphasis of differences in the component features of the conidium, the "strength" of justification for recognition of a new species depending on the number of differences that could be assembled. In this way the genus has slowly accumulated new species. This approach is basically the classical approach used by early systematists (that of searching for differences however small), and discredited by Snyder and Hansen;

(c) statements made by the authors when describing their species suggest that several of the criteria used may be unstable under varying environmental conditions. For example, the following are reported to vary:

(i) length and breadth (Neergaard 1945, p.69) and therefore L:B ratio (Baker and Davis 1950, p.481);

(ii) number of prominent lateral constrictions (Graham 1957);

(iii) epispore sculpture (Neergaard 1945, p.71).

Further, longitudinal assymmetry is probably unstable. Graham and Zeiders (1960) emphasised the assymmetric nature of S. callistephi

conidia in pure culture when making comparisons with other stemphylia, whereas the original diagnosis and illustrations of this species described on host material (Baker and Davis, 1950) completely omitted reference to this feature. This suggests that assymmetry was not prominent on the host.

The above charting of the 12 species by examining published descriptions and illustrations was one means of establishing (in the absence of type material) that these species are not morphologically distinct. This conclusion is supported by the literature reports above which suggest that certain spore features are unstable. In order to provide further justification for this conclusion, another approach was followed. It was reasoned that if these 12 species were in fact morphologically distinct then it should be possible to incorporate them into a simple and reliable key. Conversely, if these requirements of simplicity and reliability could not be met it could be inferred that the present species delimitation was unrealistic, in the light of modern taxonomic concepts. During construction of the key therefore, every effort was made to exclude criteria reported to be unstable, as their inclusion would make the key unreliable in use.

The results (p. 53) show that it was not possible to key the 12 species without resorting to the use of unstable criteria, namely epispore sculpture and the number of prominent lateral constrictions. Further, since the key is in part based on stromatic body association it would be necessary in practice to culture the fungus on agar media.

From the above results it was concluded that, providing these reports of instability could be verified, then species delimitation in Stemphylium is not in accordance with modern taxonomic concepts. That is,

some species are not delimited solely on stable morphologic criteria, nor are they clearly distinct amongst closely related species.

KEY TO STEMPHYLIUM

A Conidia variously sarciniform, spherical, oval or short cylindrical with apex broadly rounded to subangular.

B Epispore sculpture smooth, punctate or finely verrucose.

C Stromatic bodies produced on host or pure culture ..... S. loti
C_1 Stromatic bodies not produced ..... S. sarcinaeforme

B_1 Epispore sculpture verrucose or echinulate

C_2 Stromatic bodies/ascostromata produced on host/agar ..... S. botryosum
C_3 Stromatic bodies/ascostromata not produced ..... S. astragali

A_1 Conidia as before, as well as ovate, obclavate or short cylindrical with apex subacute, conical, or rostrate.

B_2 Stromatic bodies produced on host or pure culture ..... S. trifolii
B_3 Stromatic bodies not produced

C_4 Epispore sculpture smooth, punctate or finely reticulate .. S. solani
C_5 Epispore sculpture verrucose.

D One main constriction laterally ..... S. chisha
D_1 One to three main constrictions laterally ..... S. bolicki
D_2 One to three or more main constrictions laterally ..... S. lycopersici

A_2 Conidia variously ovate, obclavate or short cylindrical with apex subacute to conical or rostrate.

B_4 Epispore sculpture smooth to punctate ..... S. callistephi
B_5 Epispore sculpture punctate to verrucose ..... S. nabarii

A_3 Conidia as for A_2, with a single lateral multicellular beak formed at the base when mature ..... S. triglochinicola

D. STABILITY OF TAXONOMIC CRITERIA IN STEMPHYLIUM:

1. INTRODUCTION

All published reports referred to in the previous section regarding instability of spore features were opinions based on casual observations rather than firm conclusions resulting from critical measurements. It was considered necessary to substantiate these reports by experimentation before final conclusions could be drawn. Experiments were therefore conducted to determine which conidial features exhibited by stemphylia and listed in Table 12 are least stable under changing environmental conditions. This would enable identification of those conidial features least prone to environmentally induced variability and therefore of most value in species delimitation.

2. METHOD

Mass-transferred subcultures of the one monosporous isolate of S. botryosum were subjected to different treatments of the following environmental factors: substrate, temperature, light, humidity. Following each treatment the conidial frequency distributions for the main features of mature spore form (Table 12) were recorded for a standard 100-spore random sample. An obvious change in conidial frequency for any one morphologic subcategory would indicate that this feature of spore shape was not stable. For example, if the percentage conidia with one prominent lateral constriction was 40% at 15°C and 60% at 30°C it would be taken that the "number of

prominent lateral constrictions" was unstable under varying environmental conditions and therefore unreliable as a criterion on which to base speciation.

Standardisation of experimental method

Each treatment was applied to ten subcultures [1] during growth and sporulation in humidity chambers.[2] Following the production of mature conidia all subcultures were mounted in lactophenol on glass slides and warmed over a bunsen to remove air bubbles and clear leaflet tissue. Staining was unnecessary due to the natural pigmentation in Stemphylium. From each sub-isolate thus mounted ten mature conidia were randomly sampled [3] and allocated to the appropriate categories. This resulted in a composite sample of 100 conidia per treatment.

It was important that only mature conidia be considered since the reading of either immature or proliferating conidia [4] would effect the results.

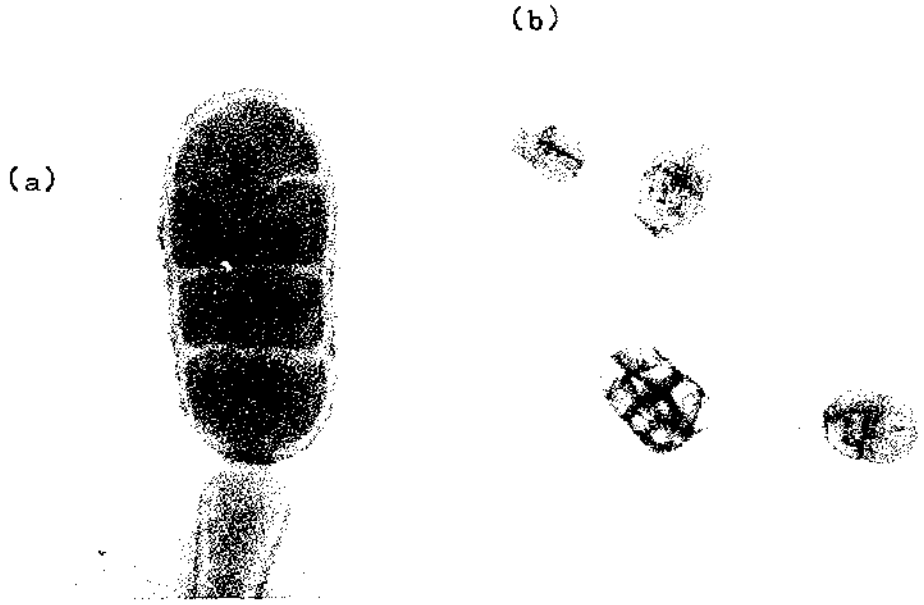
1/ Except for one treatment in the first experiment the substrate for each subculture was one excised Borre leaflet. The use of natural host substrate has been advocated by Alexopoulis (1960) for studies of this nature.

2/ In all experiments except where humidity was tested, these were Petri dishes lined with moist filter paper.

3/ This was done by taking a random sweep of the microscopic field (1500 magnifications) over a sporulating subculture and recording the first 10 mature and fully exposed conidia encountered.

4/ Examples of immature and proliferating conidia are illustrated in Plate 12. Immature conidia are characterised by a lack of differentiation and pigmentation. Proliferation of a conidium is the result of a mature conidium failing to dislodge and therefore becoming the supporting structure for further spore production.

Plate 12:



Immaturity (a) and Proliferation (b)
in Conidia of S. botryosum

Experience showed that:

(a) during mounting only mature or proliferating conidia were dislodged, while immature conidia invariably remained attached to conidiophores;

(b) proliferation occurred only after prolonged high humidity.

Therefore only dislodged, fully differentiated and non-proliferating conidia were recorded, the sampling being conducted as soon as sufficient mature conidia had developed. At that stage very little proliferation had occurred.

The following standards were adhered to when deciding to which subcategories of individual spore features the sampled conidia should be allocated:

(a) number of main constrictions - Figure 7 (26-30);

(b) apex and base morphology - Figure 7 (1-20) and Figure 6;

(c) longitudinal symmetry - Figure 7 (31-36) and Figure 6;

(d) dimensions - breadth was measured at the widest part at right angles to the longitudinal axis;

(e) L:B ratio - Figure 7 (21-25).

The subcategory applied was the one most nearly fitting the dimensions; where these fell midway between two subcategories both were recorded;

(f) epispore sculpture - Figure 6 1500 x magnification (oil immersion) and strong light intensity was used.

3. EXPERIMENTATION

(a) Substrate

The features of mature conidia produced on lab PDA and blue lupin leaflets were compared. Temperature, light and humidity were uniform for both treatments (24°C, diurnal light, and 100% RH respectively). All subcultures were initiated by mass mycelial transfers from the one monosporous isolate.

The results (Table 13) show that the unstable features of spore form were length, breadth, L:B ratio and the number of prominent lateral constrictions. The frequency distributions for length and breadth varied markedly from one substrate to the other, the host inducing the production of larger conidia. This in turn modified the L:B ratio due to one dimension being affected more than the other; that is, spores on the host were long and thin while those on PDA were short and thick. Similarly, the trend was for fewer main constrictions per conidium to be produced on PDA than on the host.

The stable features in this experiment proved to be the base and apex morphology (overall form), longitudinal symmetry and epispore sculpture.

(b) Temperature

Spore features produced under constant incubation temperatures of 15°, 20°, 25° and 30°C were compared. Substrate, humidity and light conditions were uniform for all treatments (Borre leaflets 1/, 100% and total darkness respectively). Subcultures were started by "mist

1/ Excised Borre leaflets were used as substrate in all subsequent experiments.

TABLE 13: Effect of Substrate on Conidial Morphology.

(Number or % of conidia sampled)

CONIDIAL MORPHOLOGY				Substrate	
CATEGORY		SUBCATEGORY		Borre	PDA
Number of prominent lateral constrictions		none;	1a	1	20
		one;	b	94	79
		two or three;	c	5	1
		more than three.	d		
Base and Apex morphology	Base bluntly rounded to subtruncate;	apex bluntly rounded;	2ai	2	2
		" rounded to subangular;	ii	29	27
		" subacute, conical, rostrate.	iii		
	Base broadly rounded to rounded;	apex rounded, subangular;	2bi	69	71
		" subacute, conical, rostrate.	ii		
	Base with one lateral beak.	apex subacute to rostrate.	2c		
Longitudinal symmetry		symmetric;	3a	99	98
		assymmetric.	b	1	2
Epispore sculpture		smooth;	4a		
		punctate;	b		
		echinulate;	c		
		verrucose or warty.	d	100	100
Length in μ.		≤20 - 20	5a	1	24
		20.5 - 25	b	11	45
		25.5 - 30	c	41	23
		30.5 - 35	d	37	8
		35.5 - 40	e	8	
		40.5 - 45	f	1	
		45.5 - 50	g	1	
		50.5 - 55	h		
Breadth in μ.		≤10 - 10	6a	2	5
		10.5 - 15	b	32	55
		15.5 - 20	c	63	37
		20.5 - 25	d	3	3
L:B ratio		1:1	7a		3
		3:2	b	29	63
		2:1	c	68	33
		3:1	d	3	1
		≥ 3:1	e		

inoculating" the 10 leaflets of each treatment with the one conidial suspension prepared from a monosporous isolate of S. botryosum.

The results (Table 14) show that the unstable characters were length and breadth, these being gradually reduced as the temperature was raised from 15° to 30°C. All other conidial characters (with the possible exception of epispore sculpture, at 30°C) were relatively unaffected by temperature.

(c) Light

Spore features produced in continual darkness 1/ and in diurnal diffuse daylight were compared. Substrate, temperature and humidity were uniform for both treatments. The experiment was conducted at room temperatures (19-24°C). No temperature variation was detected between treatments (shown by regularly reading thermometers, placed both beside the uncovered plates and protruding from the foil bag covering the "dark" treatment).

The results (Table 15) show that the unstable features were dimensions and L:B ratio. Length and breadth were reduced by diurnal exposure of the developing colonies to normal daylight. L:B ratio was slightly increased due to the uneven change in dimensions; that is, spores became longer and thinner.

1/ The petri plates were enclosed in aluminium foil bags.

TABLE 14: Effect of Temperature on Conidial Morphology.

Number or % of conidia sampled.

Category *	Temperature °C			
	15	20	25	30
1a		3	3	5
b	100	95	87	93
c		2	10	2
d				
2ai		3	2	2
ii	7	12	8	7
iii				
2bi	93	85	90	90
ii				1
2c				
3a	100	98	100	98
b		2		2
4a				7
b				
c				
d	100	100	100	93
5a	4	5	8	57
b	6	20	25	30
c	39	48	34	10
d	51	23	25	3
e		4	5	
f			3	
g				
h				
6a	1		5	8
b	8	20	47	72
c	74	77	47	18
d	17	3	1	2
7a	4		2	17
b	87	83	43	75
c	26	38	72	25
d		1	8	
e				

* see Table 13.

TABLE 15: Effect of Light Conditions on Conidial Morphology.

Number or % of conidia sampled.

Category *	Diurnal Light	Continuous Darkness
1a	4	
b	95	97
c	1	3
d		
2ai	1	2
ii	17	30
iii		
2bi	82	68
ii		
2c		
3a	98	100
b	2	
4a		
b		
c		
d	100	100
5a	3	3
b	25	15
c	60	37
d	12	40
e		5
f		
g		
h		
6a		
b	49	16
c	51	82
d		2
7a		
b	54	71
c	53	41
d	1	
e		

* see Table 13.

TABLE 16: Effect of Humidity on Conidial Morphology.

Number or % of conidia sampled.

Category *	% Relative Humidity	
	95	100
1a		
b	75	94
c	25	6
d		
2ai	1	
ii		1
iii		
2bi	99	98
ii		1
2c		
3a	98	98
b	2	2
4a		
b		
c		
d	100	100
5a		
b	20	13
c	53	62
d	26	23
e	1	2
f		
g		
h		
6a		
b	63	28
c	37	71
d		1
7a		
b	24	59
c	82	63
d	11	1
e		

* see Table 13.

(d) Humidity

The features of spores produced at 95% and 100% RH were compared.[1/] Substrate, temperature and light conditions were uniform for both treatments (Borre leaflets, 20°C and total darkness respectively).

The results (Table 16) show that the unstable features were breadth, and therefore L:B ratio and the number of prominent lateral constrictions.

4. DISCUSSION

(a) The results confirmed several of the reports quoted earlier that referred to the instability of criteria with changing environmental conditions. Dimensions and associated criteria (L:B ratio, number of prominent lateral constrictions) are extremely variable features of spore shape in Stemphylium, and by themselves would be unreliable for species delimitation. Similar results for spore dimensions have also been obtained in other genera (Harter 1939, Williams 1959, Rangaswami and Sambandam 1960). Williams (1959) concluded that dimensions could only be used to characterise a species if the environmental conditions are completely standardised. In Stemphylium this would also apply to the associated criteria stated above. Referring to the key of Stemphylium prepared earlier (p.53)

1/ The apparatus used, involving constant humidity solutions (O'Brien 1948, Spencer-Gregory and Rourke 1957), is illustrated in Figure 3.

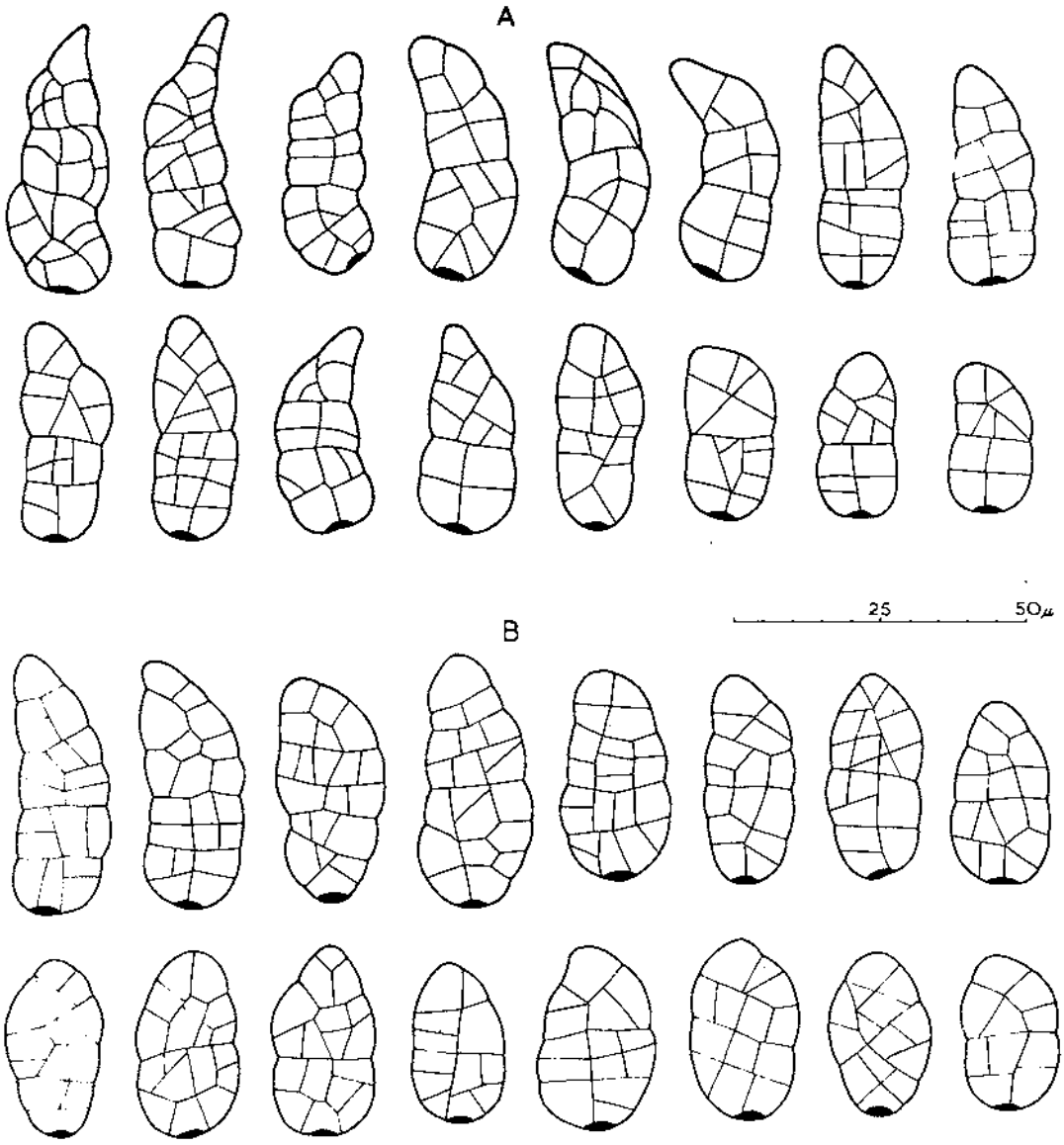
it is clear that reliance on the number of prominent lateral constrictions could present difficulties in diagnosis if for the species concerned [1] these criteria are as variable as they appear for S. botryosum and S. trifolii.

(b) Two of the reports regarding instability of criteria were not confirmed in the present study. Epispore sculpture did not appreciably change from one environmental situation to the next, and the stability of longitudinal assymmetry could not be determined here since it is not a feature of S. botryosum. However, some evidence was revealed during the survey conducted at the beginning of this study [2] demonstrating that these features of conidial form are not stable in some species. During the course of this survey several Stemphylium forms atypical of S. botryosum were encountered on blue lupin leaflets. A prominent feature in one of these forms was the longitudinal assymmetry of conidia. However, when isolated to PDA and again when inoculated to blue lupin, this feature was almost totally absent, the spores assuming a more symmetric shape. This is illustrated both in Figure 9, and Table 17. The latter accurately represents the frequency of spores in the various morphologic categories. These 50 spores were a random sample taken both from the initial lesion (A) and after reinoculation to blue lupin (B). This

1/ S. lycopersici, S. bolicki, S. chisha.

2/ Survey of stemphylia associated with gray leaf spot.

Figure 9:



Frequency of Conidial Form First Observed on Blue Lupin (A)
and after Reinoculation to Blue Lupin (B)

comparison also confirms the above experimentation that dimensions, L:B ratio and the number of prominent lateral constrictions can vary from one asexual generation and set of environmental conditions to the next. Further, epispore sculpture initially was predominantly smooth to punctate, but upon reinoculation to blue lupin was predominantly verrucose (Table 17). The fact that epispore sculpture can vary so greatly in one species is very significant since this feature of spore shape is generally regarded as an important criterion of species.

In another form identified as S. trifolii, although initially epispore sculpture was verrucose, when plated to PDA 32% of conidia produced were smooth or punctate (Table 18). In addition, dimensions, L:B ratio and the number of prominent lateral constrictions varied markedly at the three stages (Table 18 and Figure 10).

(c) The nett result of both these experimental and observed effects is that only one of the seven features of conidial form used in species delimitation (Table 12) can be regarded as consistently stable. This is the basic spore shape, that is, whether the apex is variously rounded or variously pointed, and therefore conferring on the whole conidium a variously sarciniform or variously obclavate shape respectively. The second of these groups can in turn be subdivided depending on whether or not a lateral beak is formed at the base when the conidium matures. In this way the genus can be divided into three distinct conidial forms, in contrast to the many indistinct forms currently recognised as species.

TABLE 17: Frequency of Conidial Form First Observed on Blue Lupin (A) and After Reinoculation (B).

Category *	Number of Conidia Sampled 1/	
	A	B
1a		1
b	18	41
c	32	8
d		
2ai		
ii		
iii	6	4
2bi	6	12
ii	38	34
2c		
3a	28	40
b	22	10
4a	21	
b	23	19
c		
d	6	31
5a		
b	2	2
c	6	9
d	7	17
e	16	12
f	13	7
g	5	3
h	1	
6a		
b	25	1
c	25	38
d		11
7a		
b		19
c	29	30
d	28	3
e	2	

* see Table 13

1/ From a total of 50 conidia.

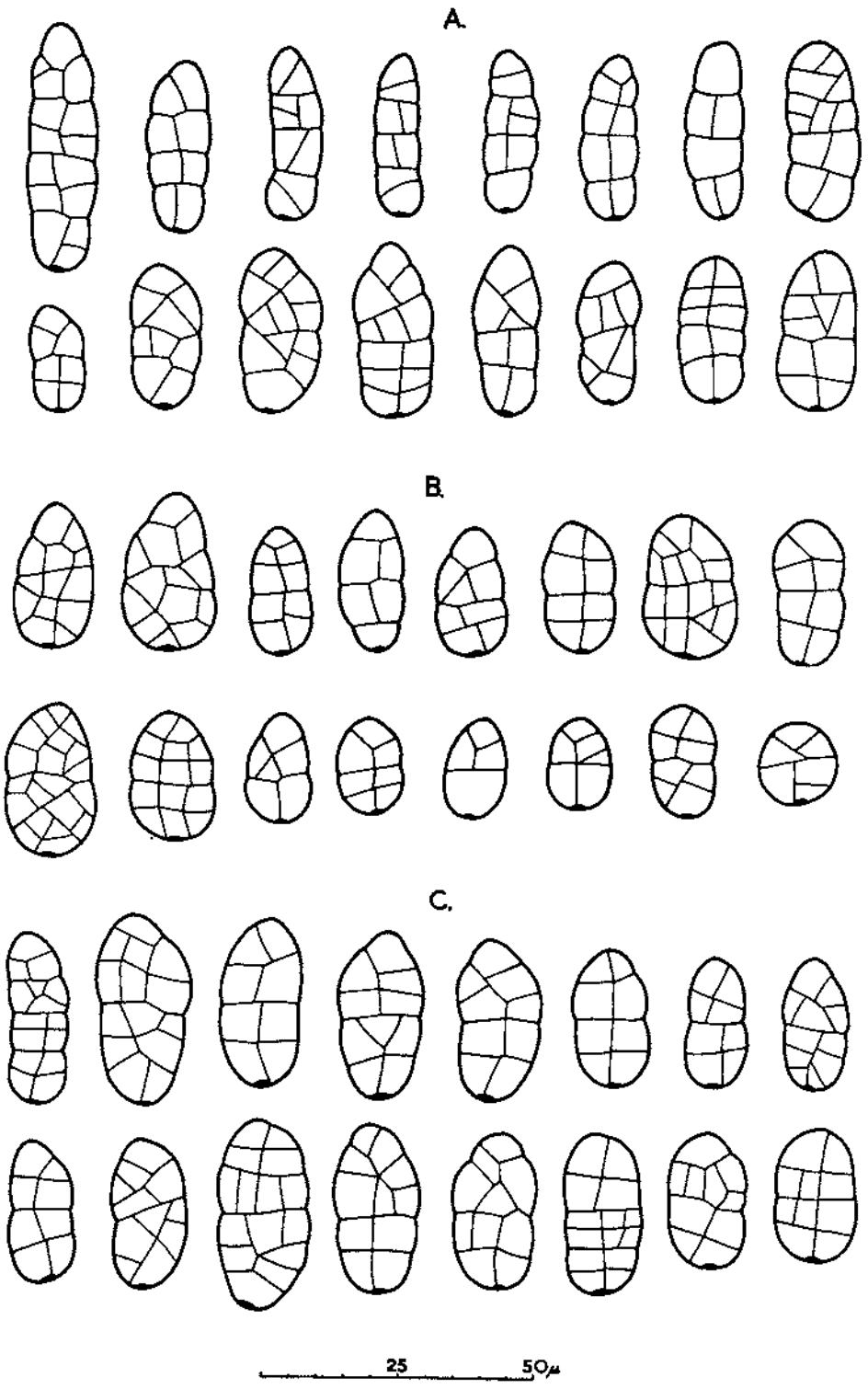
TABLE 18: Frequency of Conidial Form First Observed on Blue Lupins (A), during Subculture on PDA (B), and again on Blue Lupin Leaflets (C).

Category *	Number of Conidia Sampled 1/		
	A	B	C
1a	< 1	7	
b	21.5	40	46
c	28	3	4
d			
2ai			
ii			
iii	< 1	1	
2bi	26	20	20
ii	24	29	30
2c			
3a	39.5	49	43
b	10.5	1	7
4a		10	2
b	1	6	
c			
d	49	34	48
5a	3	16	
b	10	19	12
c	15	14	26
d	13	1	10
e	6		2
f	1.5		
g	< 1		
h			
6a	13	1	1
b	35	35	38
c	2	14	11
d			
7a		5	
b	1	24	8
c	29	23	44
d	25	1	4
e	3		

* see Table 13.

1/ From a total of 50 conidia.

Figure 10:



Frequency of Conidial Form First Observed on Blue Lupins (A), During Subculture on PDA (B) and Again on Blue Lupin Leaflets (C)

E. GENERAL DISCUSSION

The only previous comparison of true stemphylia was that by Graham and Zeiders (1960) in which seven species and three unknowns were grown on V8 juice agar and compared on the basis of conidial dimensions and morphology, stromatic body association and cultural characters. Although these authors clearly distinguished the seven species and three unknowns the validity of their conclusions is questionable since a total of only 20 isolates were used in the comparison. This number could not represent more than a very narrow cross section of each species. As stated by Snyder and Hansen (1954):

> "When only a few individuals are the subject of a taxonomic study it is easy to distinguish between them and these are therefore often named as different species. But when many isolates are assembled it becomes much more difficult to distinguish between the first named species. This difficulty usually means that they are all individuals of the same species and that the differences first seized upon were not valid as species criteria."

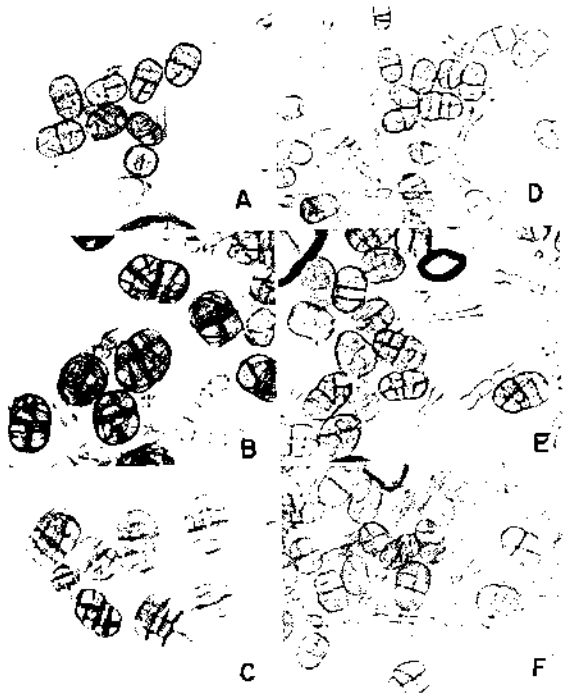
The criteria Graham and Zeiders (1960) used to distinguish the seven species included the number of prominent lateral constrictions, conidial size, smoothness of outer spore walls, longitudinal symmetry, production of perithecial or sclerotial bodies and cultural characters, most of which have been demonstrated in the present study to be unstable features in Stemphylium. It can therefore be suggested that if Graham and Zeiders had assembled a great many individual isolates of each species instead of just one or two, then speciation in Stemphylium would not have appeared as clear cut as indicated by their comparisons. It appears

that these authors did not have clear in their minds what exactly should be taken as valid species criteria. This is borne out by the fact that although the three "unknowns" were critically compared with the seven known species, the authors were not prepared to either assign them to existing species or describe them as new species. This tendency to obscure fundamental differences by emphasising minor differences appears to have been encouraged by the exhaustive treatment given by Neergaard (1945) to the criteria suitable for species delimitation in Stemphylium. Snyder and Hansen demonstrated convincingly for Fusarium that the only stable morphologic feature of the conidium was its overall form, and not the component features such as the degree of curvature or dimensions. This is a parallel situation to that in Stemphylium for which as the present study shows, basically only three distinct spore shapes occur. Differences in detail of conidial shape are not stable and therefore unreliable as species criteria, the only reliable criterion being the overall form.

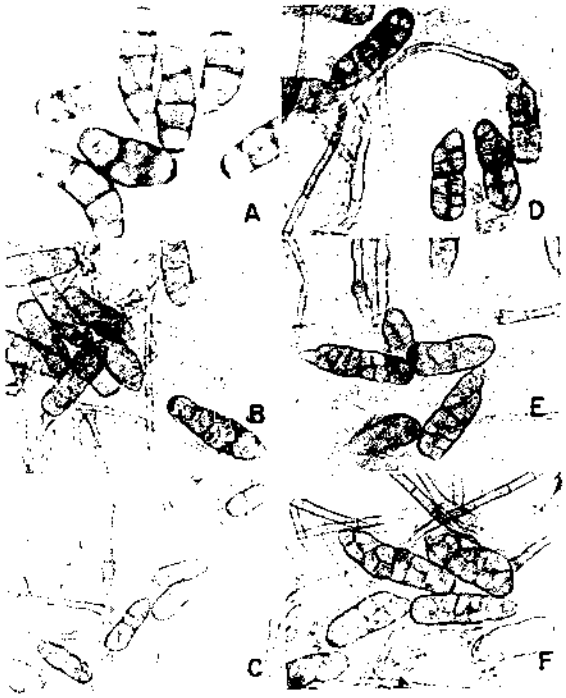
Graham and Zeiders (1960) in fact seized upon two of the three spore types suggested above to broadly group their seven species and three unknowns. These two groups are reproduced here (Plate 13) together with the third group which is represented solely by S. triglochinicola. It is clear that if one disregards individual differences and looks for overall similarity, then only three distinct spore forms exist. Further, if the following quotation by Snyder and Tousson (1965 p.836) is seriously considered, one is tempted to suggest that these three forms should represent only three species.

Plate 13:

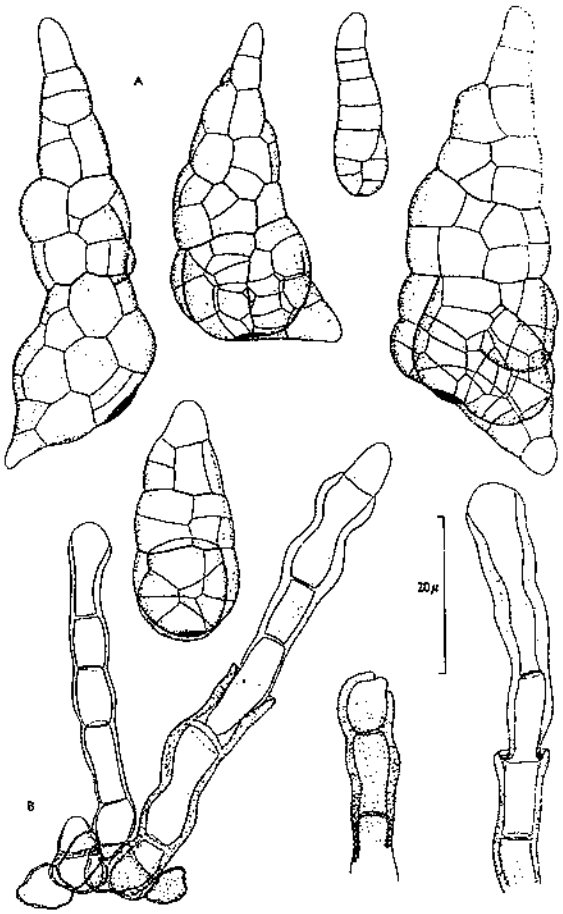
(a)



(b)



(c)



Suggested spore groups for Stemphylium

(a) basically sarciniform

(b) basically obclavate

(c) obclavate with lateral beak at base

> "Species are based on similarities among the different individuals that make up the species. If species are based on the differences, then every individual becomes a species."

In this discussion until now, the criterion of stromatic body association, which of necessity features strongly in the key presented earlier has not been considered. In the absence of type material the reliability of this cultural characteristic as a species criterion was not determined. If stromatic bodies are a constant feature of the species which are said to produce them, then a three-species scheme for Stemphylium would have to be increased to at least five species. This would involve increasing both the sarciniform-spore and the obclavate-spore groups to two species. The fact that S. botryosum produces a perfect stage should not affect this scheme since it is common experience that many isolates considered to be S. botryosum (which is homothallic) produces stromatic bodies that cannot be induced to mature into ascostromata even after long intervals of time. This was the case in the present study, and similar experience is reported by Linn (1942) Benedict (1954) and McDonald (1958).

Beyond exposing the present situation regarding species delimitation in Stemphylium as being taxonomically unsatisfactory, and suggesting an alternative basis for speciation, the limitations of the present study do not permit the formal proposal of an alternative solution. This would necessarily follow an intensive study of type material along the lines of that outlined by Snyder and Hansen (1954); that is, the world

wide accumulation of isolates, an experimental analysis of variability using single spore cultures, and finally the synthesis of species based on this experimental approach.

III SIGNIFICANCE OF *S. BOTRYOSUM* IN BLUE LUPIN SEED CROPS -

EFFECT ON SEED YIELDS AND SEED PERFORMANCE - PRESENCE IN SEED LINES AND EFFECT OF THIS ON SEEDLING ESTABLISHMENT AND DISEASE TRANSMISSION

A. INTRODUCTION

In a leguminous crop grown for the purpose of seed production, infection by a foliage pathogen may result in an adverse effect expressed both in the current crop, and in the crop resulting from use of that seed in the following season.

In the current season, seed development can be prematurely arrested by defoliation, stem and pod lesioning, and seed infection. As a result of this, and the existence of minimum-grade standards for commercial seed lines, the proportion of saleable seed harvested is reduced and the commercial grade will in all probability contain fewer plump seed. In addition to this reduction in the number (and therefore bulk) of saleable seed, the quality of seed would be reduced by reason of the pathogen becoming seed-borne. That is, viable elements of the causal fungus may be present as free propagules contaminating the seed surface, or as vegetative mycelium established within the seed.

In the following season an adverse effect in the resultant crop is possible in two ways.

1. Evidence is available indicating that inferior seed performance (expressed as poor quality stands) may result from use of smaller seed (Thiele 1965).
2. Contaminated and/or infected seed may germinate to produce seedlings that either succumb to pre-emergence or post-emergence damping-off, or emerge in a weakened state through being infected. In turn these latter diseased

seedlings may be of major importance in providing primary infection foci from which secondary cycles of infection are likely to develop.

In reviewing the literature relating to S. botryosum as a parasite of a great range of seed crops the following facts emerged:

1. premature defoliation and pod infection causes a reduction in seed size, and therefore reduced seed yields (Nelson 1955, Edwardson, Wells and Forbes 1961);
2. S. botryosum has been recorded both contaminating and infecting seed of a great range of vegetable, field crop and pasture species (Gentner 1918, Groves and Skolko 1944, Neergaard 1945, Nelson 1955, Malone and Muskett 1964);
3. in some crops the strain of S. botryosum associated with seed is pathogenic to the host species (Neergaard 1945).

The review also revealed that, irrespective of host species, very little has been published on the seed-borne phase of this cosmopolitan fungus. In view of this plus the current prevalence of S. botryosum in local blue lupin seed crops, and the fact that a high proportion of seed is dressed out through being of small size, studies were undertaken with the following objectives:

1. to determine whether pod lesioning in lupin crops causes reduced seed size (and therefore yield), and if so, the effect of reduced seed size on seed performance;

2. to determine whether both contamination and infection with pathogenic strains of S. botryosum occurs in commercial blue lupin seed lines;

3. to determine the significance of seed infection on seed performance and in providing primary infection foci.

B. EFFECT OF CROP INFECTION ON SEED YIELD

1. INTRODUCTION

It was postulated that the overall seed yield reduction caused by S. botryosum infection in blue lupin crops could come about in the following ways:

(a) reduction in photosynthetic tissue through defoliation, and stem and pod lesioning;

(b) arrestment of pod-wall development through pod-wall lesioning;

(c) restriction of nutrient translocation to developing seeds by pod-stalk lesioning;

(d) seed infection (invasion) following pod infection.

Statements (a), (b) and (c) can be considered indirect causes of reduction in seed yield, while statement (d) is a direct cause.

Edwardson et al (1961) compared the yields of blue lupin varieties resistant and susceptible to the Stemphylium leaf spot complex. They found that resistant selections (during three years

of Stemphylium attack) produced total seed weights per acre ranging from three to 27 times as high as that from susceptible varieties. The differences these authors attributed primarily to defoliation before and during seed maturation. Considering the above list of possible effects resultant on infection, this assumption is indisputable as far as the effects of photosynthetic tissue reduction on seed yield is concerned. (The comparative contribution of stem and pod lesioning to this effect would be considerably less). To be a correct assessment of causation however, their yield reduction due to defoliation must also have been greater than that due to factors (b), (c) and (d) above, (which theoretically may also reduce seed size and therefore weight). In order to assess the importance of these factors in nature their effects on seed yield were examined.

2. INDIRECT EFFECTS OF POD INFECTION

One would expect the presence of lesions on both the stalk and walls of developing pods to significantly affect seed size both directly and indirectly as indicated above. However, in the present study the occurrence of lesions on pods prior to attaining practically the full normal dimensions was rare. Further, these lesions appeared to remain superficial until late stages in pod development. This suggested that:

1. causal factors (b), (c) and (d) would have little influence on final seed size, due to the seed being largely developed before the pods became infected;

2. shrivelled seed which was quite common in maturing pods with only slight spotting was not caused by *S. botryosum* invasion of either pods or seeds.

In order to determine whether the combined causal factors (b) and (c) above would normally reduce seed yield of blue lupin two experiments were conducted in which the seed harvested from artificially infected pods was compared with that from the same number of "control" pods.

Method -

Randomly selected whole seed-heads in the field were inoculated by painting the individual pods with conidial suspensions (50,000 conidia/ml) of *S. botryosum* and then covered with high humidity cages for 2 - 3 days. A similar number of seed heads were "inoculated" with distilled water as controls. In each seed head when inoculated, the majority of pods were at semi-advanced to mature stages, the remaining few being at early stages of development. All pods were harvested just prior to the stage when seed shedding normally begins, (several harvests being necessary). From each series replicates of 200 pods were randomly sampled, all seed from all pods being carefully shelled into trays to prevent loss, then stored for two months before recording results to permit normal reduction in moisture content (since this would affect seed size). The total seed weight and

percentage of seed in three size grades [1] was finally compared for both series.

Experiment (i)

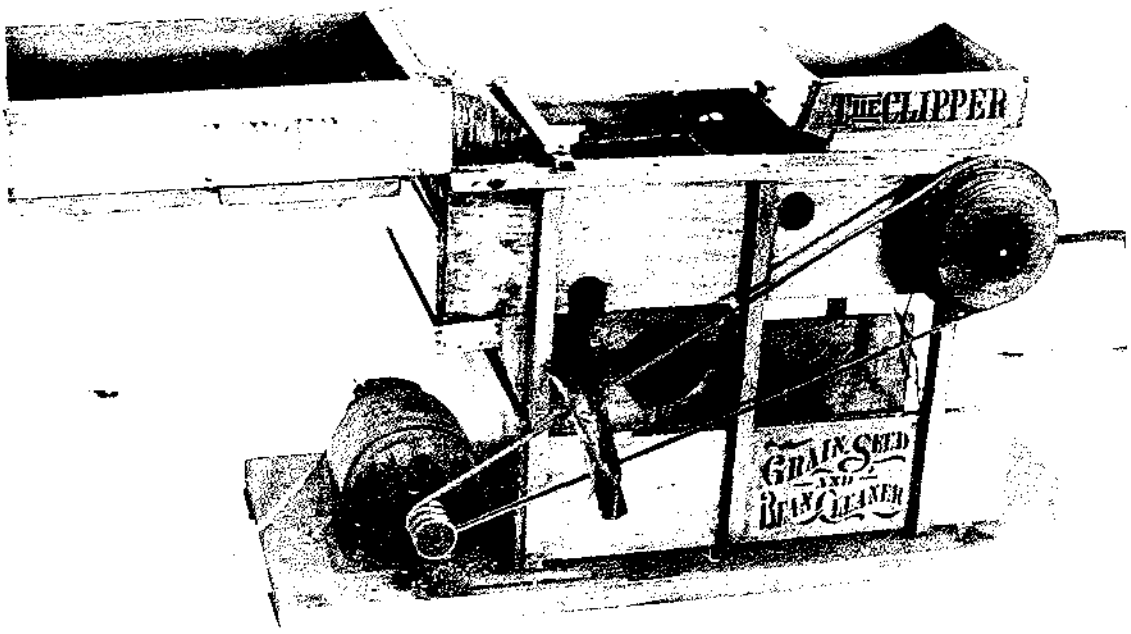
This was conducted on a small field plot of autumn-sown, seven month old blue lupins (var. Borre) free of S. botryosum infection. The pods of 16 seed heads were inoculated, a similar number being used for comparison. Periodic checks on disease development were made, and at the end of five weeks pods were harvested.

Results

In the inoculated series pods nearing maturity developed a degree of superficial lesioning that was uniformly heavy (Plate 15). In the youngest pods infection was not heavy or uniform. At three weeks it was evident that inoculated pods as a whole were one week closer to the normal pod-ripening phase of wall-collapse and drying out than the control pods. After harvest, from each series 200 pods (comprising almost the total number inoculated) were sampled. These yielded 844 seeds each. The total weight of seed and the respective proportions falling into the three size categories (following two months storage) are presented in Table 19. The figures show that the total weight of seed was reduced by pod infection, this being due to

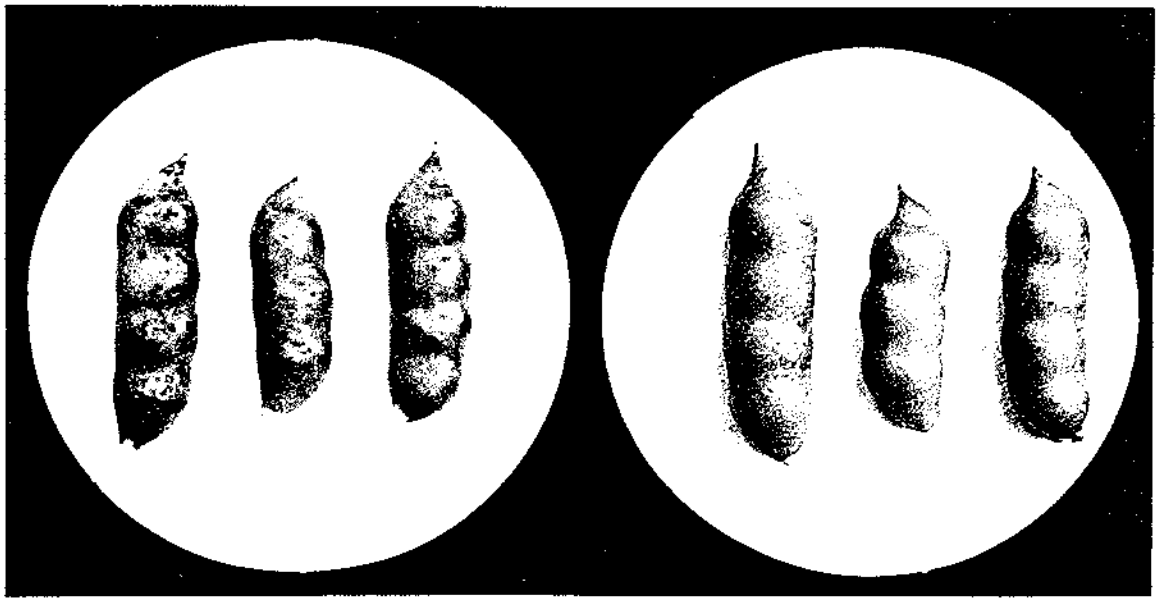
1/ The seed was graded into three sizes using a motorised seed grader and cleaner (Plate 14) fitted with two riddles. These in series separated the plump seed and moderate-sized seed in turn, while the undersized, pinched and shrivelled seed passed through both riddles. In practical terms, grades one and two combined corresponded to the commercial grade handled by seed merchants while the lowest grade (grade three) was equivalent to the reject grade resulting from seed dressing operations.

Plate 14:



Motorised Seed Cleaner used for Grading Blue Lupin Seed into Three Sizes

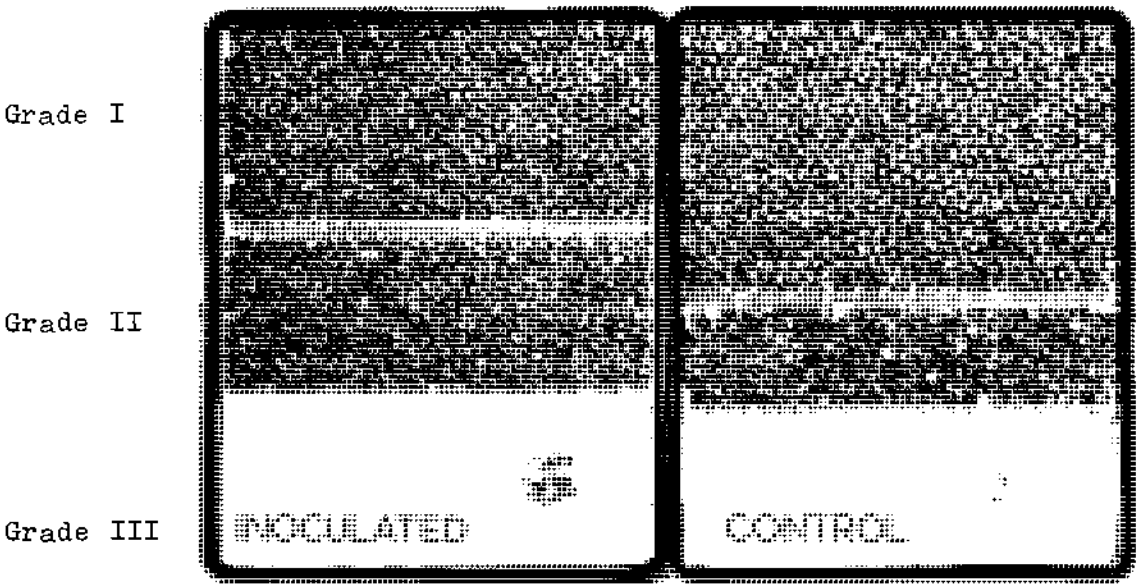
Plate 15:



Intensity of Pod Infection at 12 days following Inoculation.

Inoculated Series Control Series

Plate 16:



Effect of Pod Infection on Seed Size. (Both trays contain 844 seeds)

the reduction in numbers of plump seed produced (Plate 16).

TABLE 19: Effect of Pod Infection on Seed Size and Weight.

Pod Treatment	Total weight of seed harvested (gms)	% number of seed harvested		
		Seed size categories 1	2	3
Inoculated	171	50	47	3
Control	190	69	30	1

Experiment (ii)

This was a parallel experiment in which the pods of 25 seedheads of blue lupin plants (var. Bitter Blue) situated on a sand dune (and also free of S. botryosum infection) were inoculated and compared with a similar uninoculated control series. After six weeks all pods were harvested, and from each series three replicates of 200 pods were drawn.

Results

In this experiment, pods in the inoculated series did not become as uniformly lesioned as in the former experiment, possibly due to the cool conditions prevailing over the trial period. The total weight of seed harvested, and the proportions falling into the three grades are presented in Tables 20 and 21 respectively.

TABLE 20: Effect of Pod Infection on Total Weight of Seed Harvested.

Pod Treatment	Total weight (gms)			Treatment Mean
	Replicates			
	1	2	3	
Inoculated	163	167	167	166
Control	172	175	171	173

TABLE 21: Effect of Pod Infection on the Proportions of Seed in Three Size Categories.

Seed Size Category	Pod Treatment	% number of seed harvested			
		Replicate			Treatment Mean
		1	2	3	
1	Inoculated	37	40	41	39
	Control	49	50	46	48
2	Inoculated	56	57	55	56
	Control	47	46	49	47
3	Inoculated	7	3	4	5
	Control	4	4	5	4

The results of both experiments show that in the absence of defoliation effects the proportion of plump seed was reduced by pod infection, this being reflected in the reduced total weight of the three grades combined. In practice this would reduce the bulk of those seed meeting the commercial grade requirements (as more seed of grade two size are required to fill a bushel measure than are seed of grade one).

It was concluded that under the usual conditions of disease development where pods are well advanced in maturity before severe spotting develops, seed yield can be reduced.

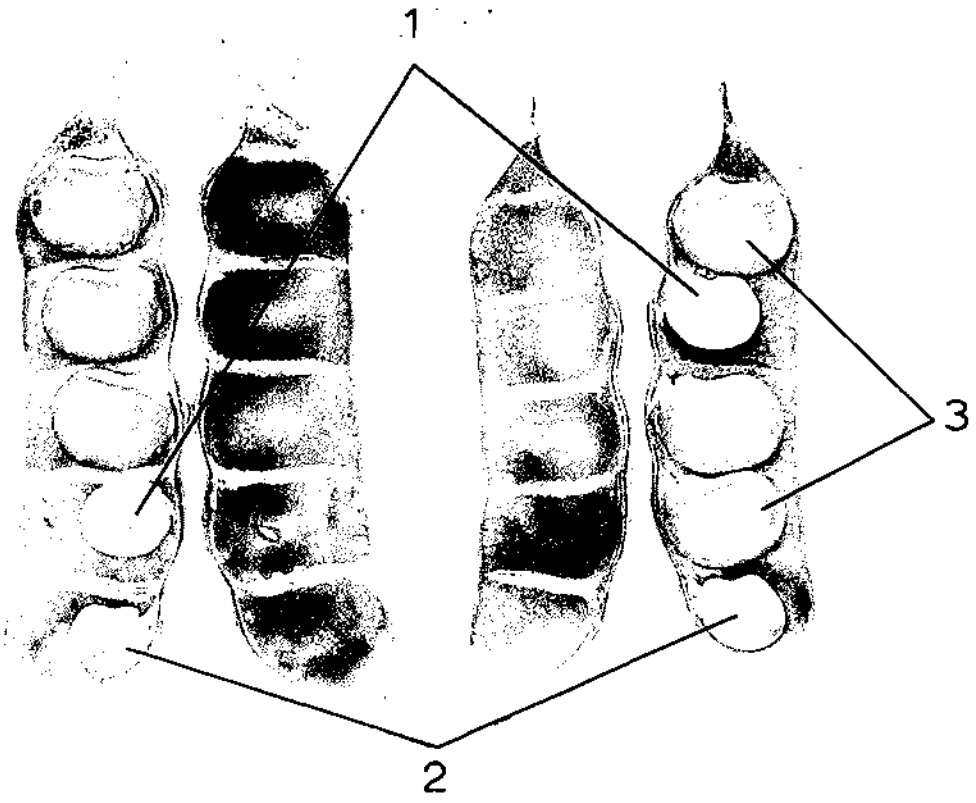
This reduction is the result of a diminished number of seed reaching the desirable "plump" size, this being caused by accelerated dying and drying-off of pod walls and translocating tissues.

3. DIRECT EFFECTS OF POD INFECTION

In the previous experiments the percentage of seed dressed out due to small size (grade three) was of little significance due to the generally advanced stage of pod development at the time of inoculation. However, the fact remained that small white or stained (lesioned) seed [1/] (Plate 17) was very common in undressed seed lines. It was postulated that where infection of immature pods occurs, the pathogen could penetrate these structures, invade the seed and arrest further development. That is, it was considered that stage of pod

1/ In seed lines these were shrivelled as a result of drying out.

Plate 17:



Small White (1) Small Lesioned (2) and Normally Developing (3) Seed of Blue Lupin

maturity at the time of infection may be of significance in determining the extent to which shrivelled seed are produced.

In an attempt to establish whether S. botryosum infection resulted in the occurrence of these shrivelled seed the following experiments were conducted.

Experiment (i)

To determine whether shrivelled seed was a result of pathogenic invasion of the seed itself, 50 shrivelled-white, and 50 shrivelled-lesioned seed, (a composite two month old sample representing the harvest of three diseased crops situated in widely scattered areas) were surface sterilized and plated to PDA to detect the presence of any pathogen.1/

Results

S. botryosum was isolated from 34% of the shrivelled-lesioned seed, and from 8% of the shrivelled-white seed, but none of these isolates proved pathogenic to healthy blue lupin plants. One lesioned seed yielded an isolate of Ascochyta which proved to be pathogenic to blue lupin.

These results showed that shrivelled seed is not necessarily the result of the pathogenic strain of S. botryosum invading the seed itself (assuming that at two months storage the invading fungus would still be viable). There remained the possibility however that other

1/ The development of the technique aimed at detecting seed infection and proving pathogenicity of the isolates obtained, is described in Section D.

fungi may be involved, namely Ascochyta, since one shrivelled-lesioned seed yielded this pathogen.

Experiment (ii)

To determine whether the presence of shrivelled seed in pods was consistently associated with pod lesioning the following experiment was conducted.

From a diseased blue lupin crop, 150 unblemished pods, and 150 lightly spotted pods were sampled. All were at an advanced stage of development similar to that illustrated for the control series of Plate 15. From both series the numbers of seed that could be classed as "normal", "small white", or "small lesioned" were counted, special notice being taken of the location of shrivelled seed in relation to superficial pod wall lesions. All "small" seed was aseptically plated to PDA after surface sterilization.

Results

Seed Class	Number of seed harvested from pods:		Number of seed yielding S. botryosum when "plated".
	infection free	infected	
Normal	583	567	ND
Small White	6	10	0
Small lesioned	0	3	0

ND = Not determined

In addition, the location of pod-wall lesions, (in the infected pods) which were superficial at this stage, bore no relation to the position

of small seed.

These results show that S. botryosum infection of pods was not the cause of either the white or lesioned shrivelled seed encountered in the sample. Further, since the pods were advanced in maturity when sampled, pod collapse during early pod development was not responsible.

Experiment (iii)

In an attempt to reproduce the shrivelled-seed symptoms, a total of 50 very young to semi-developed pods on glasshouse-grown Borre plants were "painted" with spore suspensions of either S. botryosum or Ascochyta, or both fungi combined. These, together with a few pods at similar developmental stages but "inoculated" with distilled water, were subjected to the same intermittent 12 hour periods of high humidity by separately covering with small polythene bags. Temperatures during the experiment were maintained between 18° and 24°C. Development of disease was kept under regular surveillance and after four weeks the size and condition of all seed was examined in relation to the extent of pod collapse. Such seed, together with its corresponding pod wall section, was then plated to PDA after surface sterilisation in order to ascertain whether pod or seed attack was the cause of any shrivelled seed present.

Results

These were quite variable. In all series some very young pods were arrested in development as a result of either wall or stalk infection (demonstrated by tissue plating) while others remained relatively unblemished. In the former situation seed development was also

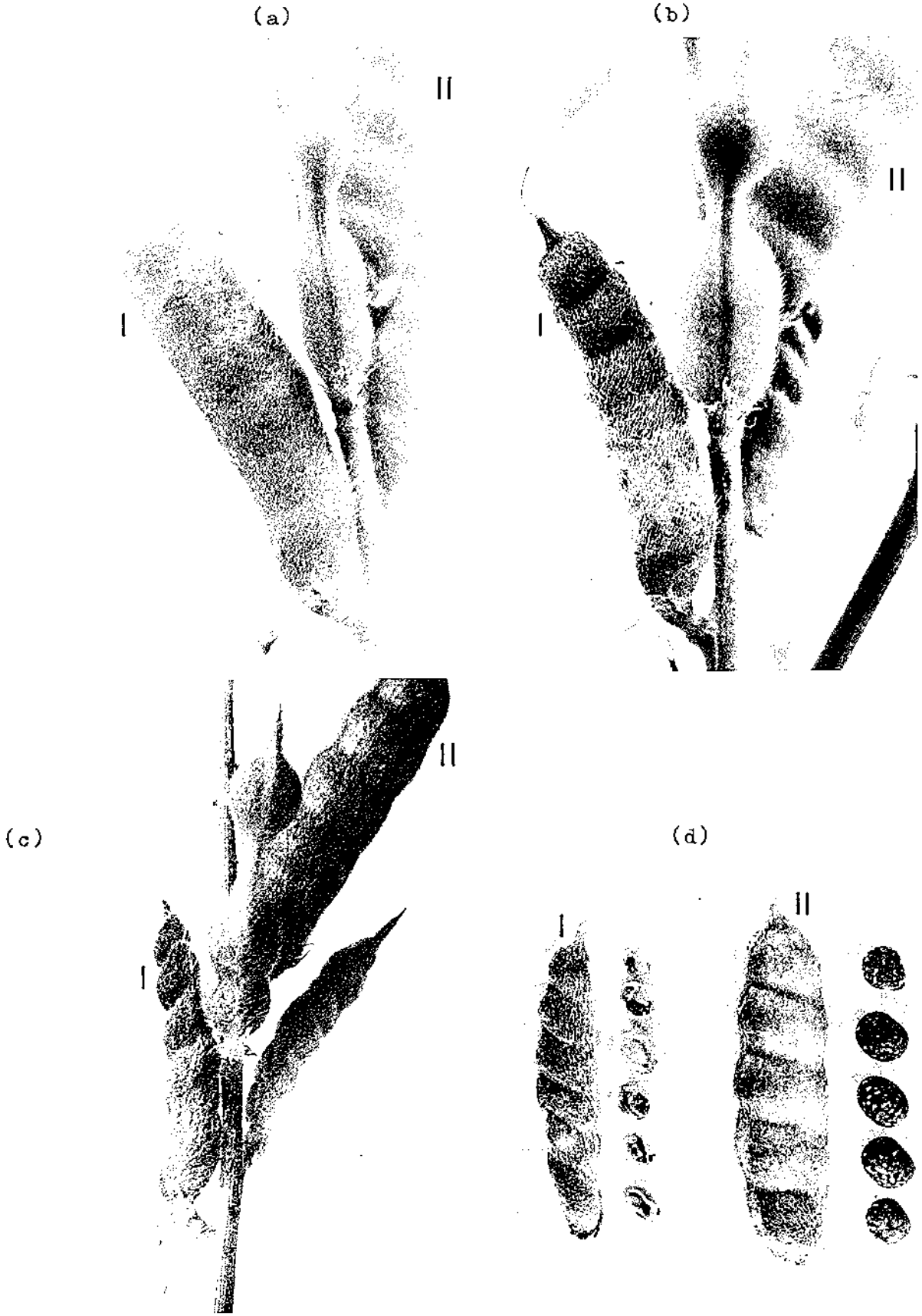
arrested as indicated by shrivelled seed. (This mostly occurred without seed infection).

In the series where a "blend" of inoculum of the two pathogens was used, adverse effects on pod development more severe than those caused by either fungus alone was the result. This is illustrated in Plate 18, which follows the development of pods subjected to the combined invasion of both fungi, compared with uninoculated pods of the same raceme. The final illustration in this series shows the effect on the contained seed. This shrivelled seed when plated to PDA yielded only Ascochyta, while the pod walls yielded both pathogens. (The control pod and its seed remained sterile when plated).

Overall, less than 20% of the seed was invaded, there being no great difference between any one series in numbers (14%, 21%, 17%) although the "blended inoculum" resulted in the most seed of a collapsed nature (Plate 18) being produced due to Ascochyta invasion. This suggested a synergistic action by both fungi.

In a few pods from both the inoculated and control series, seed lesions of the type illustrated in Plate 19 were present. This condition had been previously observed quite commonly in the field, but upon plating to PDA had consistently remained sterile. Since this condition was present in uninoculated pods here, and again remained sterile when plated, it was concluded that the condition was not necessarily connected with S. botryosum attack.

Plate 18:



Combined Effect of S. botryosum and Ascochyta sp.

on Pod and Seed Development

Plate 19:

Lesions of Unknown Cause on

Blue Lupin seeds

The above three experiments suggested that although under exceptional conditions S. botryosum could contribute to the occurrence of shrivelled lesioned seed, under normal circumstances this would not be so.

From the examination and plating of field material (experiments (i) and (ii)) it was shown that shrivelled seed had never been invaded by S. botryosum, and in fact were produced whether or not the pathogen was present in the pods.

Attempts to synthesise the situation (experiment (iii)) were successful, but only under highly artificial conditions. That is, pods were "blanket" inoculated with abnormally heavy conidial concentrations of both fungi (combined and separate) at an unusually early stage of development. Further, the maintained conditions of temperature and humidity were such as to encourage maximum disease development.

4. DISCUSSION

The reduction in seed yield due to pod attack was shown to be confined to indirect effects which prevented the maturing seed from reaching the final plumpness that would occur in healthy crops. Direct effects (seed invasion and collapse) were shown to be extremely unlikely in nature. Since defoliation is likely to reduce the number of harvested seed that meet the minimum-size standard, as well as their bulk, then the statement of Edwardson et al (1961) was well-founded. (The order of difference in yield they obtained means

that much of their seed harvested from susceptible varieties must have been of grade three size).

S. botryosum is generally considered a weak pathogen and this is probably the reason why it does not appear to invade seeds, causing total collapse. The reason for the lateness of pod infection observed in the field could be due to a combination of reasons, such as the high temperature optimum for disease development, possible changes in pod susceptibility as they mature, and the prevention of effective natural inoculation during early development because of the existence of a dense covering of hairs at this stage.

Except in occasional instances (through pathogenic invasion by Ascochyta) the occurrence of shrivelled seed is probably not connected with fungal attack. There is the possibility, however, that viral or physiological causes are responsible.

C. EFFECT OF SEED SIZE ON SEED PERFORMANCE

1. INTRODUCTION

Thiele (1965) found that seed size in a number of varieties of small-seeded vegetable species affected both the germination percentage and early performance of the subsequent seedlings. He quoted other workers who had similar results (Grose and Zimmer 1958, Cameron, Cole and van Maren 1962). Partly because of the high cost of blue lupin seed, it is the practice of some farmers concerned with

sand-stabilisation problems to economise by saving their own seed, omitting seed cleaning operations, and resowing the total harvest. In this situation, the detrimental effects of small seed size if they occur, would be realised.

To determine whether size is a factor likely to be of significance in germination, emergence and later performance of blue lupin seed, experiments were conducted to compare the performance at these stages of grades one and three of a seed line of blue lupin (var. Bitter Blue). Since the experiments were concerned solely with the effect of seed size on seed performance, all dead shrivelled seed occurring in the grade three samples were dressed out.

2. EFFECT ON GERMINATION

Four 100-seed replicates from both grades were subjected to a standard germination test. These were conducted by the Seed Testing Station of the New Zealand Department of Agriculture and involved the following procedure: each 100-seed replicate was spread out between moistened paper towels, which were then rolled into firm cylinders, and incubated at temperatures of 20°C day/18°C night. At four and ten days, the normally-germinating seed were counted and removed, the "abnormals" and "remainder" being both counted in the final tally and made available for inspection. A pretreatment of 5°C for four days to break seed dormancy preceded these tests.

The results (Table 22) show that the mean percentage total germination was the same for both large and small seed. In this experiment, if only normal germination had been compared, a false

impression of the effect of size would have been gained. This is because abnormal germs (in this case germinated seeds attacked by fungi) developed more commonly from the small seed.

TABLE 22: Effect of Seed Size on Germination.

Seed Grade	Mean % Total 1/ germination		Mean % Abnormal 2/ germs at 10 days	Mean % Normal germs at 10 days
	4 days	10 days		
1	87	99-100	1	99
3	88	99.	8	91

1/ "Normals" plus "Abnormals"

2/ These were all classed as such because of fungal attack

The results suggest that those of Thiele (1965) may not be as significant as his figures indicate if he did not take into account the possibility of more infection being associated with smaller than larger seed. It was concluded that reduced seed size (caused by infection in the parent crop) was unlikely to affect germination of the sown seed.

3. EFFECT ON EMERGENCE AND EARLY SEEDLING VIGOUR

Three replicates of 100 seeds from both grades (of the same samples used for the previous experiment) were pregerminated to the point of the first sign of radicle-emergence and sown at two inches depth, 1¼ inch spacing, in flats of sterilized potting soil.

Watering was standardised by initially steeping each flat for a set period in a tray of water and later by evenly sprinkling all flats with water. Flats were laid out randomly on the sand covered bench tops of a glasshouse in which temperatures were maintained within the range of 18° - 22°C. At six, ten and 14 days, percentage emergence was recorded, and at three weeks, average height and fresh top weight per replicate was determined. A correction was made in the results to account for the percentage of abnormal germs that would possibly be prevented from emerging due to fungal attack.

(a) Emergence

The results (Table 23) show that final emergence (14 days) was unaffected by seed size, but that rate of emergence, or seedling vigour, was clearly greater for plump seed than for pinched seed planted at this depth.

TABLE 23: Effect of Seed Size on Emergence.

Seed Grade	Mean % emergence			Correction for non-emerging abnormals	Corrected % emergence		
	6 days	10 days	14 days		6 days	10 days	14 days
1	42	97	98	1%	43	97	99
3	21	85	93	8%	29	93	100

(b) Early Seedling Vigour

The results (Table 24) show that both the height and fresh weight of seedlings grown from large seed were considerably greater than that for small seeds, to an extent roughly equivalent to the difference in fluid volume displaced by equal numbers of seed in both grades. In addition, the ground cover at three weeks afforded by seedlings of grade 3 seed was much inferior to that afforded by grade 1 seedlings which presented a dense front of vegetation at this stage.

TABLE 24: Effect of Seed Size on Early Seedling Vigour.

Seed Grade	Fluid vol. displaced by 400 seeds (mls)	Mean fresh weight per seedling at 3 weeks (gms)			Average seedling height at 3 weeks (cms)
		Replicate 1	2	3	
1	110	128	112	118	6.1
3	60	69	69	64	4.2

4. DISCUSSION

The reduced emergence rate and early seedling vigour demonstrated in the above experiments could seriously affect the success of sand-dune stabilisation, where rapidity and density of ground coverage is an important factor. The earlier a dense ground cover can be established, the less likelihood there is of surface sand drying out and being moved by wind.

An equally important factor revealed in these experiments was the likelihood of smaller seed being infected and capable of providing primary infection foci following germination and emergence. In the germination comparison 8% of the small seed germinated abnormally because of fungal attack, while this was practically absent for the large seed. In the emergence comparison 4% of the seedlings from small seed emerged with varying degrees of cotyledon lesioning while the grade 1 seedlings without exception emerged unblemished. Certain of these lesions underwent secondary development, producing mature pycnidia, the pathogen being identified as Ascochyta.

These incidental observations invite the following conclusions:

(a) pathogenic strains of Ascochyta in viable form may be associated with blue lupin seed;
(b) inoculum may be present both in grades I and III;
(c) levels in grade III can be rather high (up to 8%);
(d) such inoculum is potentially important in providing primary infection foci.

The seed line used for these experiments was harvested from a crop affected considerably by Ascochyta stem canker. This evidence suggests that Ascochyta may be of considerable significance as a seed borne pathogen in New Zealand-produced seed lines of blue lupin.

These obvious liabilities inherent in the use of undersized pinched seed would be eliminated in practice by sowing only the commercial grade of seed. To this extent, the practice of saving

and resowing the total seed harvest by the farmer probably amounts to false economy.

D. THE RELATIONSHIP BETWEEN SEED AND PATHOGEN

1. INTRODUCTION

As earlier stated, *S. botryosum* has been reported as both contaminating and infecting seed of a great range of plant species.

Contamination with viable fungal elements arises as a consequence of lesioned leaves being included with the seed heads during harvesting of the seed crop. As threshing proceeds conidia and mycelial fragments may be freed and so contaminate the seed surface.

In contrast, seed infection is usually a direct result of the reproductive structures of the host becoming infected during development, and the fungus then growing through into the seed coat (Testa Infection) or beyond this to the embryo (Embryo Infection).

A series of experiments were conducted to determine whether both the above situations exist in commercial blue lupin seed lines. Further, the manner in which seed becomes infected was investigated.

2. CONTAMINATION

To be of practical significance, methods demonstrating the presence of fungal propagules on seed should also reveal whether they are viable. Further, where the species concerned is comprised of

morphologically indistinguishable strains of differing pathogenicity, it is not enough to merely demonstrate viability. In these cases an assessment of pathogenicity is also required.

Methods demonstrating the presence of seed contamination are currently based on that developed by Bolley (1902), in which seed washings are centrifuged and the sediment microscopically examined for the presence of spores. A ready means of determining whether these spores are capable of infecting the host species is to spray healthy plants with this spore suspension (Hill 1965). Any subsequent development of infection indicates that viable spores of the pathogen were previously contaminating the seed coats.

In the present study the method used for determining both presence and viability of contaminant spores was based on that developed by Wood (1966). This involved spreading the sediment obtained by centrifugation over water agar and later microscopically examining (following incubation) for the presence of germinating conidia. To determine the pathogenicity of these germinating conidia, a method was developed in which they were recovered and cultured to produce sporulating colonies which were later used to inoculate healthy plants.

Experimentation -

(i) Presence and Viability of Inoculum

Three random samples each of 50 seeds, from a mechanically harvested and dressed seed line 1/ (var. Bitter Blue) were tested in the following way:

1/ This seed line had been harvested from a crop heavily infected with S. botryosum.

each sample was shaken vigorously in 10 mls distilled water for 20 seconds, the suspension centrifuged for five minutes, 9 mls of the clear supernatant decanted off and the remainder shaken. From this, aliquots of 0.1 ml were spread evenly over a 36 square cm area of water agar in marked Petri plates. After 4 - 12 hours incubation at 22°C, half the area was scanned (x 60 magnification) with transmitted light, aided by previous division of the area into field-width lanes and scanning each alternate lane. Both germinating and non-germinating conidia of S. botryosum were counted and the viable conidial load per seed estimated.

The results (Table 25) show that in the seed lines tested, viable conidia of S. botryosum were present contaminating the seed surfaces at an estimated load of three to four conidia per seed.

TABLE 25: Presence and Viability of Contaminating Conidia on Blue Lupin Seed.

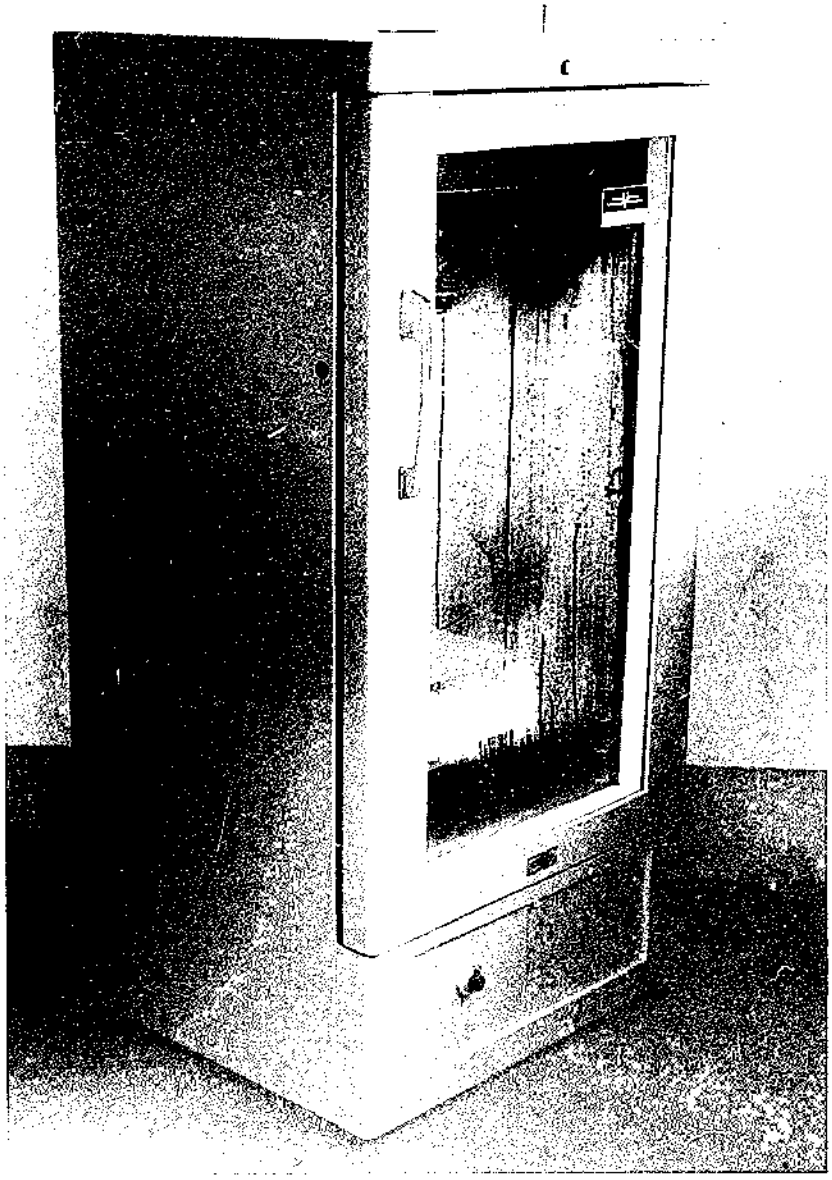
Details	Replicate 1	2	3
Total conidia detected	31	14	20
Germinating conidia detected	10	8	11
Estimated viable conidial load per seed	4	3	4

(ii) Pathogenicity

Germinating conidia detected in the above test were transferred to agar for the provision of inoculum. A differential medium was necessary to restrict growth of the associated bacterial contaminants and for this purpose PDA with potassium thiocyanate (KSCN) at 2.2% w/v added (Du Toit 1957) was found to be most satisfactory. This medium did not inhibit S. botryosum and in fact appeared to stimulate early sporulation. From the sporulating region of each fungus colony so produced, one agar plug of 2 mm diameter was cut with a cork borer and used to inoculate one newly emerged Borre seedling grown in sterilised soil. In each case the inoculum plug was placed with the sporulating surface down on the upper surface of one cotyledon, while the opposite cotyledon was used for comparison. Flats of these seedlings, so inoculated, were either placed in a high humidity seed germinating cabinet (Plate 20) and incubated at 22°C for two days, or left near a west window at room temperature under similar humidity conditions (Plate 21), care being taken in both instances to prevent cross-infections.

Following the high humidity treatment, each inoculated cotyledon was compared with its uninoculated opposite for evidence of infection. To check whether conditions were suitable for infection, a known pathogenic strain of S. botryosum was similarly inoculated to several seedlings in each flat. It was necessary for these to become infected for results to be valid. After two

Plate 20:



Marford Seed Germinator used for Maintaining High Humidity Conditions at Constant Temperatures

Plate 21:

Maintenance of High Humidity Conditions at Room Temperatures following Inoculation of Newly Emerged Seedlings

further days (at reduced humidity) progressive infections [1] were recorded. It was shown that non-pathogenic strains would not establish progressive infections under these favourable conditions even if the cotyledons were wounded.

From the total of 29 colonies of S. botryosum cultured on KSCN/PDA, 12 proved to be capable of establishing progressive infection resulting in rapid cotyledon collapse. That is, it was shown that viable inoculum of the pathogenic strain of S. botryosum may be present contaminating seed of commercial lines of blue lupin.

3. INFECTION

(a) Introduction

Methods for detecting the presence of seed infection usually involve two steps: firstly, the removal of contaminant micro-organisms from the seed surface, and secondly, the "plating"of such treated seed to agar media. Any fungi present in the seed are in this way able to resume activity, and as a result, grow out into the agar. Since the testa when mature is a non-living component of the seed, the results of plating techniques reflect not only parasitic or pathogenic infection, but also saprophytic testa colonisation. In previous sections it was shown that non-pathogenic strains of S. botryosum frequently occur in association with pathogenic strains.

1/ A progressive infection was considered to be one where the lesion appearing after two days incubation continued to enlarge, finally involving the whole cotyledon.

For these reasons, isolates obtained by plating seed after surface treatment were subjected to pathogenicity tests of the type described earlier (Section D.2). In determining a suitable surface treatment to remove all contaminant inoculum from seed, the use of both water washing and chemical steeps was investigated.

(b) Removal of Contaminant Propagules of S. botryosum from Seed

One would expect surface washing methods as used by Milne (1964) and Wood (1966) to give truer results than chemical steeps as the latter would conceivably cause some superficial testa infection to be eliminated through testa absorption of the chemical. Since Wood (1966) while working with choumoellier seed had developed a "free flow" method demonstrably superior to Milne's (1964) "tumbler" method of water washing, the former apparatus in modified form was tested (Figure 11).

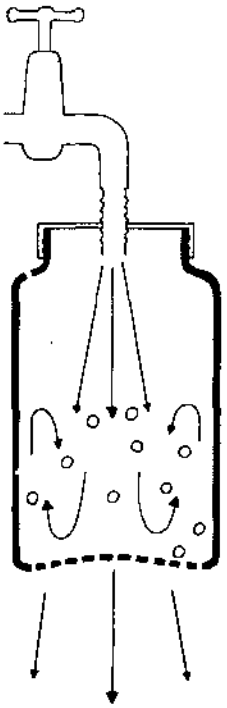
Experiments were conducted aimed at determining the duration of continual surface washing required to remove the last traces of contamination by viable propagules of S. botryosum. This was achieved by subjecting infection-free seed lots contaminated with conidia of S. botryosum to increasing durations of surface washing, and then assessing the effectiveness of each by plating the seed to agar.

Materials and Methods -

(i) Production of Infection-free Seed Lots

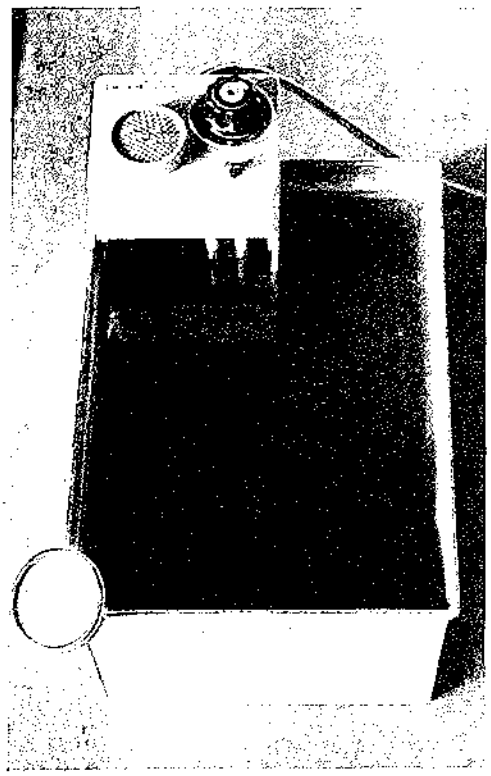
It was necessary to artificially produce infection-free seed since in preliminary tests all lines screened were shown to be

Figure 11:



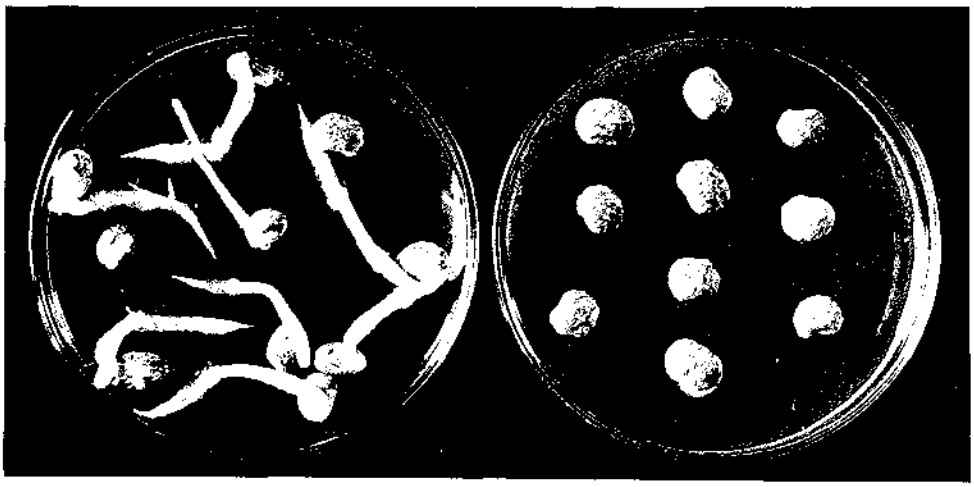
"Free Flow" Seed Washing Apparatus

Plate 22:



Constant Temperature Water Bath used for HWT of seed

Plate 23:



Effect of 2,4-D on Germination at seven days

PDA 2,4-D/PDA

infected to some extent. [1] This was attempted by hot water treatment (HWT - Plate 22). The tested lines were subjected to increasing durations of a range of time-temperature combinations. The combination finally adopted (immersion in water at 55°C for 60 minutes) was found to be the most severe blue lupin seed could withstand without adverse effects on germination, but this was still insufficient to remove all traces of infection by S. botryosum. Therefore, hot water treated seed at low known levels of infection (determined by plating immediately to PDA after a rinse in sterile water) was used, and these levels taken into account when assessing the water washing treatments.

(ii) Artificial Contamination of Seed

Hot water treated seed was rolled in sporulating 10 day old colonies of S. botryosum in Petri plates producing contamination levels of up to 1500 conidia/seed. The extent of contamination was determined by agitating 10 seed in 10 mls of water, estimating conidial concentration with a haemocytometer, and then calculating the mean number per seed.

(iii) Plating Medium

PDA was used as it supported good sporulation and familiar colony characteristics. Bacterial contamination was not a problem if careful hygiene was practised, and in the few cases of

1/ Seed lines were screened for percentage infection by treating random samples of 100 seed with mercuric chloride (0.001%) for 10 minutes, then plating to PDA after rinsing in sterile water.

chance contamination, the incubation temperature used (20 - 24°C) enabled radial growth quite sufficient for S. botryosum to rapidly grow clear. To economise on Petri plates, 17 seeds were sown on each, with six plates being required per 100-seed replicate. Seed germination was inhibited by the addition to the agar of 2,4-D [1] (Hagborg, Warner and Philips 1950) at a concentration of 0.1% (Plate 23). This strength was shown to have no effect on colony growth rate or colony characteristics on PDA and enabled ready identification of up to 17 colonies per Petri plate.

(iv) Assessment of Results

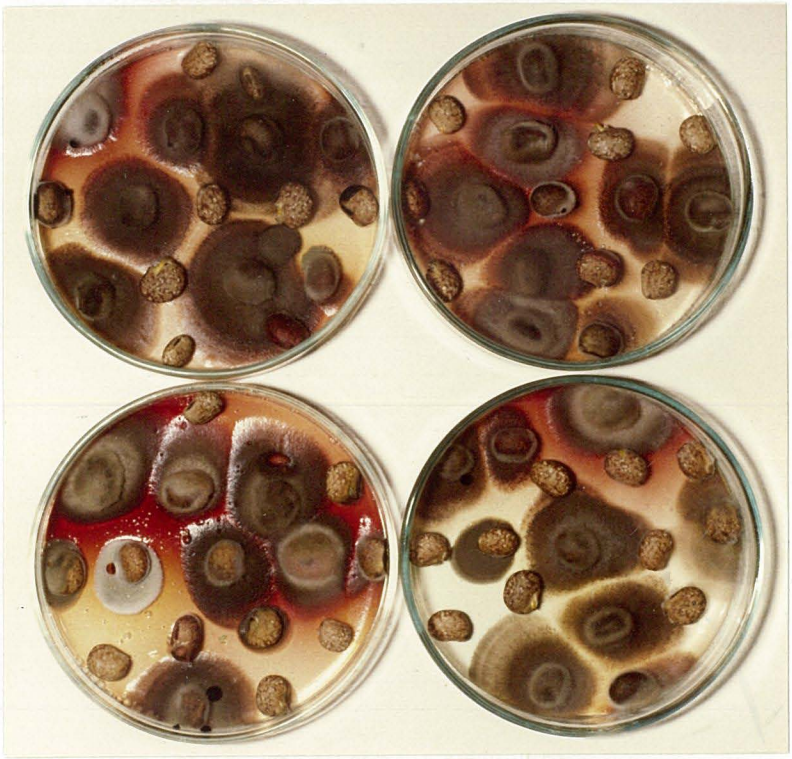
Residual viable contamination after treatment was assessed following eight days incubation at 24°C by counting the number of seeds producing colonies of S. botryosum and subtracting from this the percent infection known to be still present following HWT. Features that aided rapid identification of S. botryosum colonies growing out from plated seed were the production of a red diffusable pigment in the seed coats and surrounding agar (Plate 24), the characteristic ascostromata production and the readily identified conidia.

Results

Three "time series" experiments in a process of elimination showed that seven hours continual operation of the "free-flow" method removed the great majority of contaminant spores, but that invariably, even after longer treatment durations, a small percentage of seed still

1/ 2,4-Dichlorophenoxyacetic acid. In these experiments a wettable powder containing 80% of the sodium salt of this acid was used.

Plate 24:



Colony Characteristics and Pigmentation produced by S. botryosum in the presence of Blue Lupin Seed

produced colonies shown to have developed from contamination and not infection. In addition, this lengthy period of extreme treatment tended to fragment some seeds, and resulted in considerable bacterial contamination in subsequently plated lines. The reasons for this appeared to be in the fact that lupin seed (apart from hard seed) exhibit a sizeable cavity at the hilum in which conidia and bacteria readily lodge. Frequently it was noted that the only mycelial growth that developed emanated from the hilum during the plating tests utilised to assess the treatment. For these reasons, water-washing for the elimination of contaminant propagules was abandoned in favour of a chemical treatment incorporating mercuric chloride (0.001%), as used successfully by Milne (1964) and Wood (1966).

Parallel experiments to the above were conducted, aimed at determining the critical period of immersion in mercuric chloride (0.001%) that removed all contamination with the least effect on infection.

In these experiments the period of surface treatment was controlled by dunking the seed immediately following the immersion interval in three changes of sterile water before plating to 2,4-D/PDA.

Experiment (i)

Four 100-seed lots of a contaminated seed line containing 11% infected seed after HWT were subjected to immersion periods of one, two, four, and eight minutes respectively, and then plated.

The results (Table 26) indicate that one to four minutes effectively eliminated contamination by spores of S. botryosum. However since this experiment did not pinpoint the exact immersion time required, the following experiments that were conducted involved closer immersion intervals ranging from one to four minutes.

TABLE 26: Effect of Treatment Duration on Viability of Contamination.

Immersion time in minutes	% seed producing S. botryosum	
	From contamination and infection	From contamination alone
1	11	0
2	8	0
4	11	0
8	3	0

Experiment (ii)

Replicated 100-seed lots of a contaminated line containing 6% infection after HWT were subjected to 0.5, 1.0, 1.5, 2.0 and 4.0 minutes immersion interval and then plated.

The results (Table 27) show that the point of effectiveness was somewhere between two and four minutes.

TABLE 27: Effect of Treatment Duration on Viability of Contamination.

Immersion time in minutes	% seed producing S. botryosum			
	Replicate			Treatment Mean
	1	2	3	
0.5	10	8	6	8
1	9	5	10	8
1.5	7	4	6	6
2	3	0	1	1.3
4	0	0	0	0

Experiment (iii)

Replicated 100-seed lots of a contaminated line containing no infected seed after HWT were subjected to 2.5, 3.0, 3.5 and 4.0 minutes treatment respectively.

It was concluded from the results (Table 28) that four minutes immersion in mercuric chloride (0.001%) would be effective in eliminating all contaminant spores of S. botryosum with a minimum effect on superficial testa infection.

TABLE 28: Effect of Treatment Duration on Viability of Contamination.

Immersion time in minutes	% seed producing S. botryosum			
	Replicate 1	2	3	Treatment Mean
2.5	5	5	2	4
3	1	2	5	2.6
3.5	0	0	2	0.6
4	0	0	0	0

(c) Determination of Infection in Seed Lines of Blue Lupin

From representative samples of five commercial seed lines, the percentage seed carrying both pathogenic and non-pathogenic strains of S. botryosum was determined using the following method developed as a result of the above experimentation.

Subsamples of approximately 450 seeds were enclosed in a nylon gauze bag, washed briskly under a tap to remove the larger contaminating particles, then immersed for four minutes in a solution of mercuric chloride (0.001% w/v). Immediately following this treatment the seed was rinsed in three changes of sterile water, then roughly divided between four Petri plates containing dry filter paper (heat sterilised). From each Petri plate, the first 100 seed encountered were aseptically plated to

2,4-D/PDA. After eight days incubation at 24°C, combined with daily exposures for the last four days to UV radiation, the percentage of seed producing colonies of S. botryosum was recorded.

The results (Table 29) show that a high average percentage of seed colonised with S. botryosum occurred in these lines.

TABLE 29: The Presence of S. botryosum in Five Seed Lines.

Seed Line	% seed producing S. botryosum				
	Replicate 1	2	3	4	Treatment Mean
1	40	23	24	40	32
2	72	70	66	69	69
3	11	8	6	16	10
4	35	30	33	36	34
5	43	34	49	39	41

In order to determine the percentage of seed containing potentially pathogenic strains of S. botryosum, the isolates obtained from four of these seed lines were tested for pathogenicity by inoculating seedling cotyledons as described previously.

The results (Table 30) show that the proportion of seed infected with the pathogenic strain of S. botryosum was consistently very low, while that colonised by saprophytic strains ranged from 28 - 65%.

One would assume that since these latter strains are non-pathogenic they must be confined to the seed testa.

TABLE 30: Pathogenicity of Seed Isolates of S. botryosum.

Seed Line	Mean % 1/ Total Infection	Number of separate inoculations	% "pathogenic" seed infection	% Saprophytic testa colonisation
1	32	24	4	28
2	69	100	4	65
4	34	84	5	29
5	41	100	10	31

To demonstrate that this was actually the case, the seed line containing the highest level of Total Seed Infection (Line 2) was tested for percentage embryo infection alone, all resulting isolates being tested for pathogenicity.

The method used, involving the removal of testas and the plating of the resulting naked embryos was as follows:

Subsamples of 100 seed were soaked in cold water for one hour to loosen and soften the seed coats, these being removed with a pair of forceps by grasping folds of the expanded testa

1/ Both pathogenic infection and non-pathogenic colonisation combined.

and bodily tearing them off, care being taken to leave the radicle intact. The naked, whole embryos were then surface sterilised as for whole seeds and plated to 2,4-D/PDA. Recording was carried out at eight days.

From three 100-embryo replicates, one isolate of S. botryosum was obtained that proved pathogenic when inoculated to blue lupin seedlings. The infected embryo was slightly discoloured at the point on the cotyledon where the mycelium grew out onto the agar, while the other 299 embryos were unblemished. This showed that non-pathogenic strains of S. botryosum were confined to the testa in this seed line, and it was concluded that this would be so for all seed lines since the embryo is composed of living (if temporarily dormant) tissue. In addition, the results suggested that pathogenic infection would be normally confined to the testa, since only one embryo in 300 proved to be infected. The presence of 69% testa colonisation indicated that conditions at some stage of seed development or storage were very conducive to colonisation or invasion by S. botryosum. The fact that only a very low percentage of embryo infection was the result of such conditions is substantial evidence for this generalisation.

(d) Mechanism of Seed Infection by the Pathogenic Strain of S. botryosum

In Section B it was found that pods do not normally become infected until well advanced in maturity and further, that lesioning is superficial until late stages. This suggested that seed infection occurred at very late stages of pod maturity. To determine

both the path of seed infection and the stage in pod maturity at which this occurs in nature, the following experiment was conducted.

Three classes of pod maturity (Plate 25) all with uniformly heavy intensities of surface lesioning were sampled from a severely infected crop of blue lupin (var. Bitter Blue).

Class (i) green, almost mature, fully sized pods;

(ii) browning-off, mature, with pod walls collapsing;

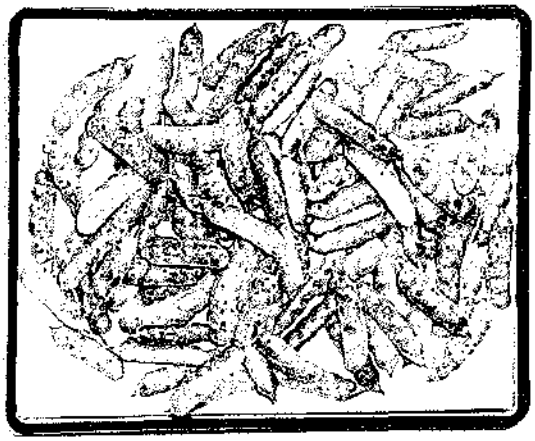
(iii) mature, almost ripe, drying-off.

The first 200 seeds encountered together with their funicles were surface sterilised for four minutes and 0.5 minutes respectively, then rinsed, dried and plated to 2,4-D/PDA. The shorter interval for the funicles had previously been shown suitable for these relatively small structures which, it was considered could be completely sterilised if treated for too long.

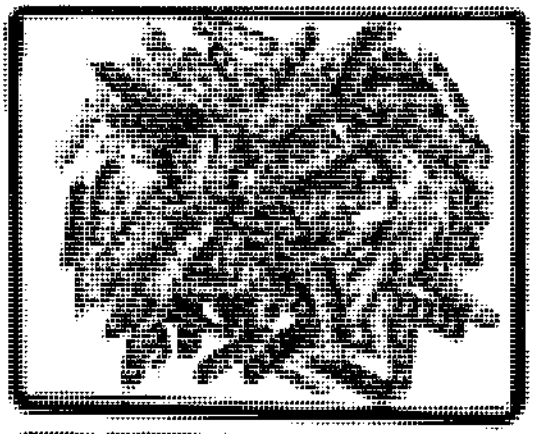
The results (Table 31) show that seed infection did not occur until the whole surface area of the pod walls had visibly collapsed. Since all the funicles remained sterile after plating to 2,4-D/PDA the path of seed infection must have been directly through the pod walls into the contacting seed coats.

Plate 25:

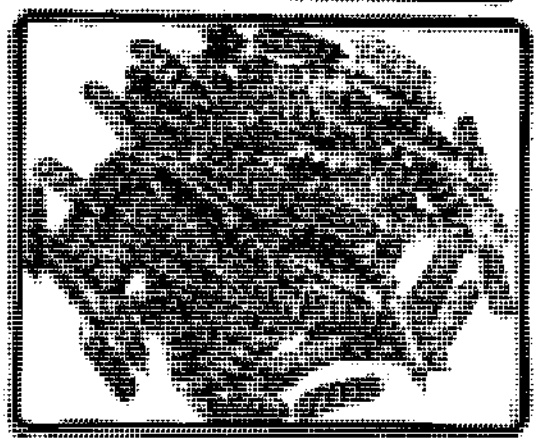
Class (i)



Class (ii)



Class (iii)



Pod Maturity Classes used in determining
Mechanism of Seed Infection

TABLE 31: Mechanism of Seed Infection.

Pod Maturity Class	% seed infected	% funicles infected
(i)	0	0
(ii)	6	0
(iii)	19	0

E. SIGNIFICANCE OF SEED INFECTION

Having shown that low percentages of commercial seed lines are infected with the pathogenic strain of *S. botryosum* it was necessary to determine whether in the following season such infected seed was of significance either in causing poor seedling establishment or in providing primary infection foci.

1. SEEDLING ESTABLISHMENT

Conceivably, infection of seed could induce poor seedling establishment in the following season in two ways:

(a) infection of seed could cause seed death thereby contributing towards lowered germination percentage of that seed line;

(b) infected but viable seed could begin germination, but death of the developing seedling could occur before emergence. That is, the effect would be expressed as a "poor strike".

However the results of previous experimentation suggested that seed infection would in fact be of little consequence when sown in the following season:

(a) seed infected with pathogenic strains did not exceed 10% in the lines tested and averaged only 6% (Section D3);

(b) where seedlings were raised for pathogenicity testing (Section D2) the emergence was 100% even though the seed line was 4% infected with the pathogenic strain, and the conditions provided were considered optimum for disease development.

To further clarify the part played by seed infection in stand establishment the following experiments were conducted.

Experiment (i)

A standard germination test using rolled paper towels was conducted on three commercial seed lines (previously surface sterilised) infected with the pathogenic strain to the extent of 4%, 10% and 5% respectively. At the completion of the test all abnormally germinating and dead seed were examined for the presence of S. botryosum.

The results (Table 32) show that in the one instance where dead seed were present (seed line 1) the absence of S. botryosum indicated that death was not due to this pathogen. In all seed lines there was some abnormal seed germination, but only in one line (seed line 1) was S. botryosum associated with the seedlings, and then only in two of the four seedlings involved. From these results it seems clear that natural infection in a commercial seed line could affect germination only in causing some slight increase in the number of abnormal seedlings. It should be stated that the conditions of the germination test under which these abnormals were produced in the experiment were continuously favourable to fungal development for 10 days. Under field conditions of germination therefore, one would expect fewer "abnormals" to develop with greater chances of normal emergence.

TABLE 32: Germination Performance of Seed Lines Naturally Infected with S. botryosum.

Seed Line	Mean % Infection		Mean % Normal Germination		Mean % Abnormal Germination		% seed not Germinating	
	Total	"Pathogenic"	4 days	10 days	Total	Due to S. botryosum	Total	Due to S. botryosum
1	69	4	87	94	4	2	2	-
2	41	10	90	99	1	-	-	-
3	34	5	90	99	1	-	-	-

Experiment (ii)

To determine whether the death of developing seedlings in soil could be brought about before emergence, seed line 1 (4% infection) was subjected to the following test:

> four 100-seed replicates were surface sterilised, pregerminated in water and sown at uniform depth (1½ inches) and spacing (1½ inches) in flats of sterilised soil kept moist at 20 - 22°C in a high humidity germinator.

The emergence, recorded at seven days, averaged 99% with no replicate below 98%. An average of three emerged seedlings per replicate were found to be infected with S. botryosum, in each case later shown to be pathogenic. Since a total of 16 seeds (4%) were known to be infected, and that a total of 12 seedlings emerged with S. botryosum infection, only four of the 16 infected and germinating seeds sown (1% of Total) could have been killed before emergence. Thus under the conditions of this experiment (considered to be optimum for pathogenesis) only one quarter of the seedlings developing from infected seed could have failed to emerge. In the field one would not normally expect these extended favourable conditions for pathogenesis and in such cases seedling emergence figures should be higher.

From these experiments and the previously assembled evidence it was concluded that as a result of the normally low levels of pathogenic seed infection occurring, plus the rather weak pathogenic capacity of this fungus, S. botryosum would not normally be a significant factor preventing seedling establishment.

2. ESTABLISHMENT OF PRIMARY INFECTION FOCI

The presence of a few infected seed in a seed line may be of great significance for disease transmission from the parent crop to the seed bed of the following season's crop. Provided that the pathogen can invade at least one emerging seedling and has a ready means of dispersal, then under favourable conditions secondary cycles of infection will probably occur. Since S. botryosum is not likely to cause much pre-emergence seedling loss, is present in up to 10% of the seed normally used for resowing, and has a very efficient air-borne method of local dissemination, the probability of successful disease establishment should be high.

(a) Embryo Infection

Above it was shown to be unlikely that S. botryosum would normally prevent seed germination and emergence. In Section D3 it was shown that seed with infected embryos can occur at very low frequencies (one in 300 seed). However, if three bushels of a commercial seed line are sown per acre, more than 500 seed per acre with infected embryos could result. 1/ If only 50% of these seeds successfully germinate, emerge and establish as infected seedlings, more than 250 primary infection-foci per acre could result. One of these would be more than sufficient under favourable conditions, to initiate disease spread.

1/ This was based on the following: A cylindric 0.5 litre capacity holds approximately 1850 seeds of grade one size, and one bushel capacity is equivalent to 36.4 litres.

(b) Testa Infection

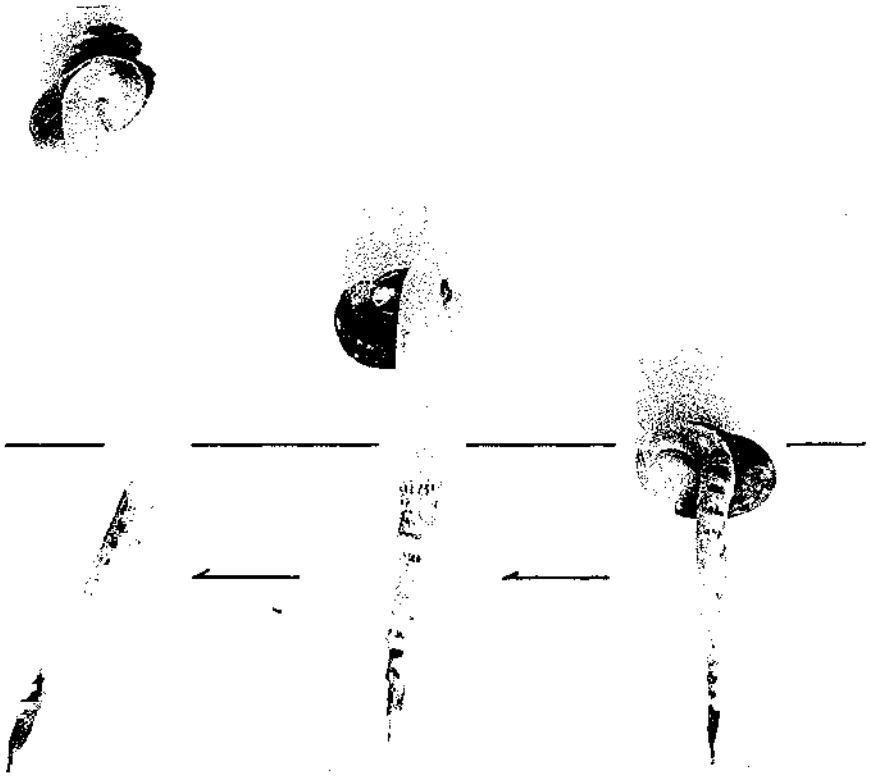
Many seed-borne diseases are known to be transmitted by testa infection. For hosts exhibiting epigeal seed germination, this is normally achieved by resumption of hyphal advance resulting in pathogenic invasion of the cotyledons prior to the testa being shed above ground.

Evidence for testa transmission of S. botryosum was revealed both in section E1 above and in Section D2 when the health of 400 seedlings raised for pathogenicity testing was examined. In the former case 3% of emerged seedlings were lesioned, in the latter, 4%. However, in both cases the lesions were confined to the hypocotyl (see later), the cotyledons and growing tips remaining unblemished. From these lesions only S. botryosum was isolated and in all cases subsequently shown to be pathogenic. For the following reasons it was considered that the pathogen was probably confined to the testa prior to germination:

(a) the hypocotyl could not have been infected before seed germination occurred because it does not exist as a structure of the seed embryo, but only arises as a result of cell division of the region between embryo radicle and cotyledons. (This is illustrated in Plate 26). Further, even after emergence the plumule and cotyledons remained free of infection;

(b) the pods and contained seeds for both seed lines had been harvested by hand, so reducing to a minimum the

Plate 26:



Development of Blue Lupin Hypocotyl and Manner of Emergence from Soil (X 1.5)

risk of seed contamination (c.f. machine harvesting and threshing).

It was postulated that the extent and site of lesioning on the affected seedlings could be explained only in the following way:

as the expanding cotyledons (enclosed in the partly split testa) are "dragged" upwards and out of the soil in the typical "crook" position (Plate 26) exhibited by many dicotyledonous plants, the testa makes contact with the developing hypocotyl region thus facilitating fungal invasion (from testa infection).

The net result of these observations was that the establishment of primary infection foci by S. botryosum had been demonstrated after four to five months storage of the seed (the interval after which the seed was used for experimentation). It still remained to be demonstrated that the pathogen could remain viable in the seed over the normal 8 - 12 months interval between the harvest of one seed crop and the sowing of the next.

This was accomplished by plating to PDA after surface sterilisation, a 100-seed sample of a 12 month old commercial line of seed (var. Borre). At eight days incubation, 13 seed had produced colonies of S. botryosum, and three of these proved pathogenic to blue lupin seedlings.

To provide further experimental evidence that the transmission of S. botryosum to the seedlings raised for pathogenicity testing (Section D2) was by testa infection, the following experiment was conducted:

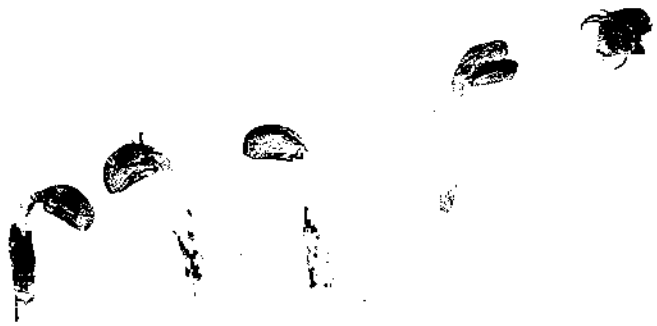
A random sample of 120 seed from the same seed line in question was surface sterilised prior to pre-germinating in water and sowing in flats of fresh potting mixture. They were then incubated at 20 - 22°C in a high humidity germinator for six days before removing to low humidity conditions near a west window.

From a total of 120 evenly-germinating seed sown, five seedlings severely lesioned on the hypocotyl were produced, the remaining 115 seedlings being unblemished. The degree of lesioning appeared to be related to seedling height (Plate 27) suggesting that severe infection can cause stunting of seedling development. The cotyledons and plumules on the worst-affected seedlings were also partially/completely lesioned (the pathogen here advancing in a broad front) while on the least affected seedling these structures were unblemished. (The former situation was not observed in the cases described previously).

In the least affected seedlings, hypocotyl lesions could be attributed to testa infection, while in the severely lesioned seedlings this could not be said as it could not be proved that the cotyledon and plumule infection observed at emergence was not there before germination. However, this could have still been the case if, due to the excessively favourable conditions for pathogenesis being provided, the cotyledons and plumules were invaded following germination.

The early-stage hypocotyl lesions illustrated in Plate 28 typify the stem lesions produced when aerially-borne conidia are the cause (see Plate 9 following p.31).

Plate 27:



Degrees of Stunting in Blue Lupin Seedlings as a result of Sowing Seed Naturally Infected with *S. botryosum*

Plate 28:

Typical Symptoms of *S. botryosum* on seedling hypocotyl of Blue Lupin as a result of sowing Naturally Infected Seed

As a result of these experiments and observations it was concluded that seed infection could be significant in providing primary infection foci. The experiments reflected the potential effect of the pathogen when established in the seed. If in the field only a small proportion of these infected seed actually germinated in conditions that would enable the pathogen to infect the emerging seedlings, the resulting number per acre would still be more than sufficient to initiate secondary disease cycles.

Having gained an initial foothold in seedlings, the pathogen could be expected eventually to sporulate and spread the disease. In the absence therefore of alternative methods of carry over between successive annual blue lupin crops (such as continually reseeding lupins in sandy regions, or alternate hosts) the probability of disease transmission by way of infected seed is high. In addition the possibility of introducing the pathogen to new regions is very real, and this could well have been the means by which the pathogen first became established in New Zealand.

F. GENERAL DISCUSSION

From the preceding study several points emerge.

1. *S. botryosum* is important in blue lupin crops grown for seed due to the severe defoliation that can occur at early stages of seed development. This is reflected in reduced seed yields. Since blue lupin seed realises a relatively high market price per

bushel, the application of fungicidal sprays to the crop from the first signs of flowering onwards could be an economic proposition worth consideration. One problem inherent in such an approach would be the risk of phytotoxicity, since blue lupin leaves are very sensitive and readily induced to abcise. Another problem would be that of effective and economic application since aerial spraying would most likely be necessary.

2. The value of crops utilised for livestock forage would not be appreciably affected by gray leaf spot since this is a warm weather disease that does nct normally become widespread in a crop until well after flowering. The usual practice of grazing a crop at the first signs of flowering therefore would in most cases enable one to escape the full effects of the disease.

3. In areas of sand country where the crop is left to reseed naturally (a common practice on raw sand) there could be some reduction of sand stabilisation if ground cover is reduced by defoliation during summer months. Again, if the average size of seed produced by the current crop is markedly reduced then this could be reflected in a poor initial volunteer stand arising in the autumn.

4. The disease caused by S. botryosum is seed-borne and can be transmitted via seed to new crops of blue lupin. This is very important if blue lupin seed is to be shipped any distance, since the pathogen would be shipped also. In these cases, prevention of the disease becoming established in a new area would involve seed

treatment, in such a way as to eliminate S. botryosum. The most common methods are hot water treatment and fungicidal steeps, the latter of which is not effective where the inoculum is present as deep seated infection. Due to the large size of blue lupin seed it is doubtful also whether hot water treatment would be effective, this being indicated in the present study. An alternative to this would be holding the seed in the hope that the pathogen would die out before germination capacity was reduced excessively. Prevention of disease introduction into new areas by eliminating the pathogenic strain of S. botryosum from imported blue lupin seed would depend on the pathogen not being already present on a wide range of hosts, but rather being host specific to blue lupins. In preliminary studies such pathogenic specialisation was strongly indicated. Repeated glasshouse inoculations to the following recorded hosts of S. botryosum proved unsuccessful:

Lettuce	Mangold	
Onion	Broad Bean	
Tomato	Tree Lupin	
Shallot	Russel Hybrids	Ornamental Lupins.
Red Clover	Pink Pearl	
White Clover	Hartwegii Annual Mixed	
Lotus sp.	Blue Gem	

If as indicated, the strain of S. botryosum causing gray leaf spot in blue lupins is in fact specific to this host, then it is probable that the pathogen was imported into New Zealand with infected seed when this forage species was first introduced.

5. The use of infected seed in sand stabilisation is not significant once the disease has become established, since volunteer plants arising in the autumn from reseeding crops (and in adjacent waste areas) are commonly observed to carry the disease through the winter months (in the lower North Island area at least). With its efficient means of local airborne spread, S. botryosum would readily move to adjacent developing crops. Disease transmission by infected seed would thus be of minor importance.

6. S. botryosum does not appear capable of causing seed death, except under highly artificial conditions. In nature pods are not attacked until late in development, and the pathogen remains superficially located almost until the stage of normal pod wall collapse. It is then that the pathogen grows through to the seed testa via the collapsed pod wall, only rarely penetrating as far as the embryo cotyledons.

The significance of seed infection does not lie with seed performance since neither germination capacity nor seedling emergence is appreciably affected. The fact that S. botryosum, while only a weak pathogen, can cause rapid defoliation is probably more a consequence of the sensitive nature of blue lupin foliage than to excessive virulence on the part of S. botryosum. Leaf fall in blue lupin can be induced quite easily by subjecting plants to excessive shading, water deficit or even water logging.

7. The frequent association of non-pathogenic strains of *S. botryosum* with the pathogenic strain, both in seed and on foliage lesions, emphasises the importance of frequent pathogenicity testing in any study concerned with this disease. It cannot be assumed from merely plating blue lupin seed to agar that all the isolates of *S. botryosum* arising represent the pathogen. If this is assumed, then a vastly inflated figure of "potentially pathogenic seed infection" could result. In the routine screening of commercial seedlines for "pathogenic *S. botryosum* infection" therefore, it would be essential to incorporate a suitable pathogenicity test for results of the agar plating test to be meaningful. This probably also applies to other fungi for other host species.

S U M M A R Y

1. A virulent strain of Stemphylium botryosum Wallr. was established as the cause of gray leaf spot, a foliage disease of blue lupins grown in the Manawatu, Turakina and Wanganui areas.

2. Field symptoms on blue lupins were compared and contrasted with those caused by two other common foliage pathogens, namely Pleiochaeta setosa (Kirchn.) Hughes, and an unidentified species of Ascochyta.

3. Taxonomy of the genus Stemphylium was studied. On the basis of a recent proposal in which the original concept of Stemphylium is restored for the first time in almost 100 years, not more than 14 of the 65 species so far described in Stemphylium Wallr. are tenable in this genus.

4. In the genus Macrosporium (which was invalidated in 1933 by Wiltshire), 49 of the remaining 148 species still to be disposed of could well represent Stemphylium since taxonomic characters described in the original diagnoses of these macrosporia are characteristics of Wallroth's genus.

5. A morphologic comparison of 12 true stemphylia showed that species delimitation was often based on unstable features of spore shape,

and that species were not morphologically distinct. Species delimitation based on overall conidial form was suggested as an alternative, and the need for an intensive study of all representative isolates of Stemphylium along the lines of that outlined by Snyder and Hansen (1954), was indicated.

6. S. botryosum causes seed yield reductions primarily through premature defoliation of seed crops, this resulting in less plump seed being harvested. The extent to which pod attack affects seed size is considerably less since this does not normally occur before pods (and seed) are approaching maturity, and because the pathogen does not kill seed outright.

7. Seed size has no effect on germination capacity. However the rate of emergence and resulting density of foliage may be inferior to that produced by plump seed.

8. S. botryosum becomes seed-borne at about the stage of natural pod collapse, (this stage being hastened by severe pod wall infection). The pathogen, together with non-pathogenic strains of S. botryosum, grows directly through the pod wall and enters the seed testa.

9. Commercial grades of blue lupin seed harvested during 1967 carried up to 10% pathogenic infection, and 65% non-pathogenic testa colonisation. Contamination with viable inoculum was also detected.

10. *S. botryosum* is incapable of killing seed outright under average climatic conditions and seldom establishes in the embryo. The occurrence of shrivelled dead seed in undressed seed lines is not a consequence of gray leaf spot.

11. Seed infection does not appreciably affect germination capacity or seedling establishment, but may be significant in providing primary infection foci in blue lupin crops (as a result of infected seed being sown). Since this pathogen can remain viable in seed for at least 12 months, the risk of long distance spread of the disease is high.

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APPENDIX I

Routine Materials and Methods in the Glasshouse

The experimental glasshouse was thermostatically heated during winter months, and manually cooled during summer by occasional "damping down" of walls and ceiling with a water spray. Pot plants were bedded into a wet sand layer covering the bench tops. This was supplemented by overhead watering every four to five days.

A low fertility potting mixture (Baker 1957) was used throughout in accordance with the low nutritional requirements of blue lupins. Potting mixtures were heat sterilised in an electric soil steriliser before fertiliser was added. Pots and flats were scrubbed, and soaked in 2% formalin for one night, then put aside for a few days before use.

Lupin seeds were pregerminated in water before sowing in flats or pots, and "Maxicrop", a foliar nutrient preparation, was used sparingly when plants were grown through to maturity.

Powdery mildew was controlled by Karathane sprays, and caterpillars, aphids, leafhoppers and thrips by several insecticides including DDT, malathion and carbaryl.

APPENDIX II

Composition and Preparation of Culture Media

Recipes are given below for all media. Distilled water was used in their preparation and 200 ml quantities were autoclaved in 250 ml Erhlenmeyer flasks and then stored for use. All media was sterilised at 15 p.s.i. for 20 minutes, immediately following preparation.

A. Potato-Dextrose Agar (lab PDA)

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

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

B. Oxoid PDA

Reference : Oxoid Manual (1962) reprint second edition.

The prepared dry medium was added to water as indicated on the label.

C. Lupin Seed Agar (LSA)

agar	12 gm
blue lupin seed	100 gm
water	1000 ml

The seed was comminuted in a Waring Blender with a small volume of water. The resulting suspension was then cooked for half an hour and filtered

through cheese cloth. The filtrate was made up to the required volume with water containing the dissolved agar.

D. Malt Agar (MA)

agar	25 gm
malt extract (Difco)	20 gm
water	1000 ml

The two constituents were dissolved in the water by heating and stirring.

E. V-8 juice agar (V8)

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

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

F. Potato Carrot Agar (PCA)

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

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

G. Lupin decoction Nutrient Agar (LDNA)

agar	20 gm
Blue lupin leaves	30 gm
Dextrose	20 gm
Water	1000 ml

The leaves were comminuted as for LSA, and cooked in a 50°C water bath for 2 hours. After filtering, the filtrate was added to the dextrose and agar dissolved in water, the total being made up to 1000 mls.

APPENDIX III

Species Untenable in Stemphylium Wallr.

Species	Author	Source
S. inflatum	Saccardo	Saccardo 1882 Sylloge Fungorum 4
S. soredosporum	Sacc.	"
S. ericoctonum	A. Br. et de Bary	"
S. glaucum	(Pr.) Sacc.	"
S. sphaerospermum	(Pr.) Sacc.	"
S. Magnusianum	Sacc.	"
S. atrum	(Pr.) Sacc.	"
S. pulchrum	(Berk.) Sacc.	"
S. botryoideum	(Cooke) Sacc.	"
S. phyllogenum	(K. et C.) Sacc.	"
S. sphaeropodium	Bon.	"
S. Alternariae	(Cooke) Sacc.	"
S. amoenum		"
S. viticolum	Passer	Sacc. 1886 Syll. Fung. 10
S. insidens	Cooke et Mass.	"
S. paraguayense	Speg.	"
S. opacum	Sacc.	"
S. puntiforme	Sacc.	"
S. albo-atrum	Karst.	Sacc. 1892 Syll. Fung. 11
S. juniperinum	Karst.	"
S. laxum	Ell. et Ev.	Sacc. 1897 Syll. Fung. 14
S. subradians	Ell. et Ev.	"
S. Elasticae	Patters.	Sacc. 1899 Syll. Fung. 16
S. butyri	Patters.	"
S. copallinum	Ell. et Ev.	Sacc. 1902 Syll. Fung. 18
S. Allii	Oud.	"
S. Tritici	F.Patters. Charles et Veihmeyer	Sacc. 1906 Syll. Fung. 22
S. maculans	Osterwalder	"
S. celosiae	Bremer et al	"
S. elegans	(Corda) Bonorden	1851 Wiltshire 1938 (see Biblio.)
S. dubium	(Corda) Bon.	1851 "
S. bulbotrichum	(Corda) Bon.	1851 "
S. graminis	(Corda) Bon.	1851
S. bizarrum	Viegas	1961 Bragantia 20(15) 529-31
S. Eugenia	Verw.et du Plessis	1933 Sth.Afr.Journ.Sci XXX; 222-33
S. subsphaericum	Fairman	1922 Proc.Roch.Acad.Sci.6(4),117-139
S. ramulosum	Sacc.	1878 Michelia 1(4) p.360
S. codii	Zeller	1918 Puget Sound Biol.Sta.Publ.2.
S. uredinis	(Ellis et Barth.)Thirum.	Proc.33rd Ind.Sc.Cong.Part 1946 III p.113
S. dichroum	Petrak	1950 Sydowia Annales Mycologici 4: p.585
S. iranicum	Esf.	1950 Ibid 4:34
S. maritimum	Johnson	1956 Mycologia 48: 841-51
S. tritici	Deshp. et Deshp.	1965 Mycopath.Mycol.appl.27:203-4

1/ 1/ 1/ 1/

1/ According to Wiltshire 1938.

APPENDIX IV

Species of Uncertain Affinity

Species	Author	Date	Source
S. heterosporum	Dom. Sacc.	1896	Sylloge Fungorum 14 (1899)
S. heterosporum	(Desm.) Buchw.	1852	Ann. Sci. Nat. Ser. III 18;358
S. Tabaci	Oud.		Sylloge Fungorum 18 (1906)
S. Berlesii	Oud.		"
S. macropodium	(Cda) Bonorden	1851	Trans. Brit. Mycol. Soc. 21;220 (Wiltshire 1938)
S. polymorphum	(Cda) Bonorden	1851	"
S. flavicans	Jasevoli	1924	*Bull. dell'Orto Bot. Univers. Napoli 7:232
S. crataegi	(Ell. et Ev.) Höhn	1929	*Mitteil.Bot.Inst. Techn. Hochsch. Wein VI, 1. Heft.p.13

* These papers were not obtained

APPENDIX V

Species of Macrosporium with Undetermined Affinity [1]

Species	Author	Date	Source
M. melophthorum	(Pr.et Dela.) Rostr.		[2]
M. eriobotryum			[2]
M. Cavarae	Parisi	1921	Riv. di Patolog. veget. 11: 1-16.
M. dioscoreae	Gonz. Frag.	1923	Petraks Lists. C.M.I. Reprints 1920-1939 inclusive
M. opuntiicola	Cif. et. Frag.	1925	"
M. bromelliphilum	Speg.	1926	"
M. gallarum	Bres.	1926	"
M. hypoxides	Cif. et. Frag.	1926	"
M. cannabinum	Bakhtin et Gutner	1933	"
M. lini-cathartici	Unanumo	1933	"
M. vaccariae	Savul. et Sandu	1933	"
M. pruni-mahaleb	Savul. et Sandu	1935	"
M. digitalis	Milovtzova	1937	"
M. centaureae	Roldan	1938	"
M. trisectum	Raabe	1939	"
M. astragalinum	Petrak	1939	Ann. naturh. Mus. Wein. 50: 513 (1940)
M. anatolicum	Săvulescu	1944	Bull. Sect. Sci. Acad. roum. 26: 709
M. convolvulus	Losa Espăna	1945	An. Jard. bot. Madr. vi, page 460.
M. poissoni	Bouriquet	1947	"Les maladies des plantes cultivées à Madagascar" p. 489
M. melandrii	Vasjagina	1957	Izv.Acad.Sci.Kazakh.S.S.R.,ser.Biol, 1(13) p.102.
M. cleomes	Sawada	1958	Bull.Govt.Formosa Expt.Sta.Meguro, Tokyo 105 p.100
M. sonchi	Sawada	1958	" p.101
M. calendulae	Nelen apud Nelen et Vasiljeva	1959	Bull. centr. bot. Gdn. Moscow 35: 90
M. rhapontici	"	1959	Ibid 35: 90
M. savulescui	Tóth	1959	Omagiu lui Traian Sávulescu, page 780.

1/ Papers not obtained.
2/ Source unknown.

APPENDIX VI

Discussion of the Taxonomic Position of S. bizarrum Viegas 1961

It is certain that this fungus has been wrongly included within Stemphylium. An English translation of the Latin diagnosis is as follows -

> Fungus "Hyphae septate subhyaline, ramifying, 4-5 μ diameter within the lesion and emerging towards maturity giving rise to superficial black extraordinary looking bulbils. Cells of bulbils globose, smooth walled, on rare occasions drawn out into a narrow elongated neck in the manner of a conidiophore. Spores not seen fixed to conidiophores."

This description does not indicate generic affinity, since conidiophore and conidial morphology are not described.

The accompanying Portugese description (in translation) indicates that although conidiophore-like structures were seen by the author, none were found with conidia attached, in which case their function as conidiophores is not beyond doubt. Illustrations show them to be simple, unbranched, neither nodulated nor proliferating by lateral outgrowth, but with a single pore at the apex. (This strongly suggests a conidiophore-like function).

The conidia are described as being quadrangular, opaque, smooth, 12-18X 10-12u at maturity, and were found only in very low abundance. The illustration shows them to be variable in shape, with a few spores

sarciniform, a few with a definite pore situated at one end, and most with very globose cells.

It appears that the author was not aware of either Wiltshire's 1938 or Neergaard's 1945 publications, both of which would have indicated the need for a greater understanding of the structure and mechanisms of the sporulation apparatus.

On the basis of the author's illustrations and descriptions, these appear to be as follows.

Mycelium ramifying in the region of the host epidermis in the central zone of the lesion appears to proliferate at isolated points, developing into a type of stromatic body of extremly variable shape. Certain peripheral cells of these bulbils become drawn out into conidiophores (short and conical) which produce porospores acrogeneously. Some cells which do not elongate into conidiophores may also produce porospores. (These observations were based on the fact that pores are illustrated both on conidiophore apices and conidial bases, as well as on non-attenuated peripheral cells of the bulbils).

Accepting this description as being correct, then on the basis of conidiophore characters, the genus Stemphylium Wallroth is excluded. On the basis of conidial form and colour, the genus Ulocladium Preuss (Syn. Pseudostemphylium (Wilts.) C.V. Subramanian) is excluded, as also is Alternaria Nees.

However, method of conidial production appears to resemble all three genera, and it is probable that this fungus is a close relation. 1/

1/ This is based upon the importance attached by Hughes (1953) to the mechanism of the sporulation apparatus as a criterion in classifying related groups of fungi.

APPENDIX VII

Literature Studied When Comparing *Stemphylium* Species

Species	Author	Date	Source
S. botryosum	Wiltshire	1938	Trans. Brit. Mycol. Soc. 21: 211-239
	Du Toit	1951	Sc. Bul. Dept. Agr. S. Afr. 301: 47 pp
	Petzer	1958	Sth. Afr. J. Agric. Sci. 1(1) : 3-24.
	Groves & Skolko	1944	Canad. Journ. Res. C.22 : 190-199
	Neergaard	1945	Danish species of *Alternaria* and *Stemphylium*. Einar Munksgaard, Copenhagen, 560 p.
	Ramsay	1935	J. Agric. Res. 51 : 35-43
	Graham & Zeiders	1960	Phytopath. 50 : 757-760
	Joly	1964	Encycl. Mycol. 33 : 1-250
S. sarcinaeforme	Cavara	1890	Dif. dei. parass. n.4
	Wiltshire	1938	
	Groves & Skolko	1944	
	Graham & Zeiders	1960	
S. loti	Graham	1953	Phytopath. 43 : 577-579
	Graham & Zeiders	1960	
	Sobers & Seymour	1963	Phytopath. 53 : 1443-1446
S. trifolii	Graham	1957	Phytopath. 47 : 213 - 215
	Graham & Zeiders	1960	
S. solani	Weber	1930	Phytopath. 20 : 513 - 518
	Hannon & Weber	1955	Phytopath. 45 : 11 - 16
	Graham & Zeiders	1960	
	Sobers & Seymour	1963	
S. lycopersici	Enjoji	1931	Journ. Plant Prot. 18 : 52
	Hannon & Weber	1955	
	Yamamoto	1960	Trans. Mycol. Soc. Japan 12(15) : 9-13
	Sobers & Seymour	1963	
	Sobers	1965	Phytopath. 55 : 1313-1316
	Graham & Zeiders	1960	
S. bolicki	Sobers & Seymour	1963	
	Sobers	1965	

APPENDIX VII (Cont'd)

Species	Author	Date	Source
S. callistephi	Baker & Davis	1950	Mycologia 42 : 477-486
	Hannon & Weber	1955	
	Graham & Zeiders	1960	
	Sobers & Seymour	1963	
S. astragali	Yoshii	1929	Jour. Plant. Prot. 16 : 536
	Yamamoto	1960	
S. chisha	(Nisikado & Hiura) Yamam.	1950	Nôgaku Kenkyû 39 : 40
S. triglochinicola	Sutton & Pirozynski	1963	Trans. Brit. Mycol. Soc. 46 : 505-522
S. nabarii	Sarwar	1965	Mycopath. et Mycol. appl. 29 : 320-322