A STUDY OF SEED-BORNE ASPECTS OF THE SPRING BLACK STEM DISEASE OF LUCERNE

A Thesis presented in partial fulfilment of the requirements for the Degree

of

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by

Neil Seeley Percival

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SUMMARY

1. Morphological, cultural, and pathogenicity studies confirmed the causal organism of the spring black stem disease of lucerne (Medicago sativa L.) present in New Zealand to be Phoma medicaginis Malbr. et Roum..

2. Of eighty six New Zealand lucerne seed lines screened during 1970 and 1971, eighty three were infected at levels ranging from one half to forty nine percent; twenty three of the seed lines had an infection level greater than twenty percent.

3. In all seed lines (both infected and uninfected) there was a gradation from dark to pale coloured seed. In infected lines the infection level of the dark seed was greater ($\geq$ 3:1) than that of the pale seed.

4. Over eighteen months the level of seed infection decreased at a rate faster than the natural decrease in germination capacity; after this period the infection level was less than ten percent of the original value.

5. Improved moist blotter and agar plate tests were developed following a critical examination of established methods for detecting fungal pathogens associated with lucerne seed.

6. Seed-borne inoculum of P.medicaginis induced both pre-emergence and post-emergence damping-off. While the pathogen was most pathogenic to seedlings at 14 C, at this temperature not all detectable seed-borne inoculum caused damping-off.

7. In most instances pre-emergence damping-off resulted from destruction of tissues concerned with seedling elongation.

8. Of ten seed dressings evaluated benomyl and thiram provided most effective control of seed-borne inoculum of P.medicaginis. However before these therapeutants can be recommended for general use there is a need of further glasshouse and field trials.

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INTRODUCTION

The area of lucerne (Medicago sativa L.) in New Zealand has increased rapidly in recent years as farmers have recognized the role it can play in farming enterprises (Table I). There are several reasons to account for this increase:

(i) A gross margin analysis of lucerne as a cash crop shows a return that is equal to or greater than for comparable crops such as barley or peas (Tocker, 1970; Lamb, 1969; Anon., 1970).

(ii) Lucerne has a greater versatility than other crops as it can be utilized in several ways to produce many final products (Fig. 1).

(iii) Lucerne provides a more assured feed supply than conventional rye grass/clover pastures in those areas subject to droughts and with light soils (Oliver, 1971).

In parts of the South Island, the area of lucerne grown is small because of the difficulty experienced in stand establishment (Oliver, 1971). The importance of even and rapid establishment of lucerne cannot be underestimated, since a resulting vigorous stand with a minimum of weed infestation will give the greatest nett annual return. Several factors have been implicated as causing poor stand establishment in lucerne:

(i) Nutrient status of the seedbed; a deficiency in the essential mineral nutrients or a soil pH below 6.2 are likely to result in poor stand establishment (McFarlane, 1970).

(ii) Seedbed preparation; lucerne seedlings require a firm, fine, weed-free seedbed with adequate moisture for successful establishment (Bonner, 1970).

(iii) Efficiency of nodulation with Rhizobium meliloti Dangeard; non-nodulated seedlings rapidly show general yellowing and poor growth (Close, Whitelaw, and Taylor, 1971).

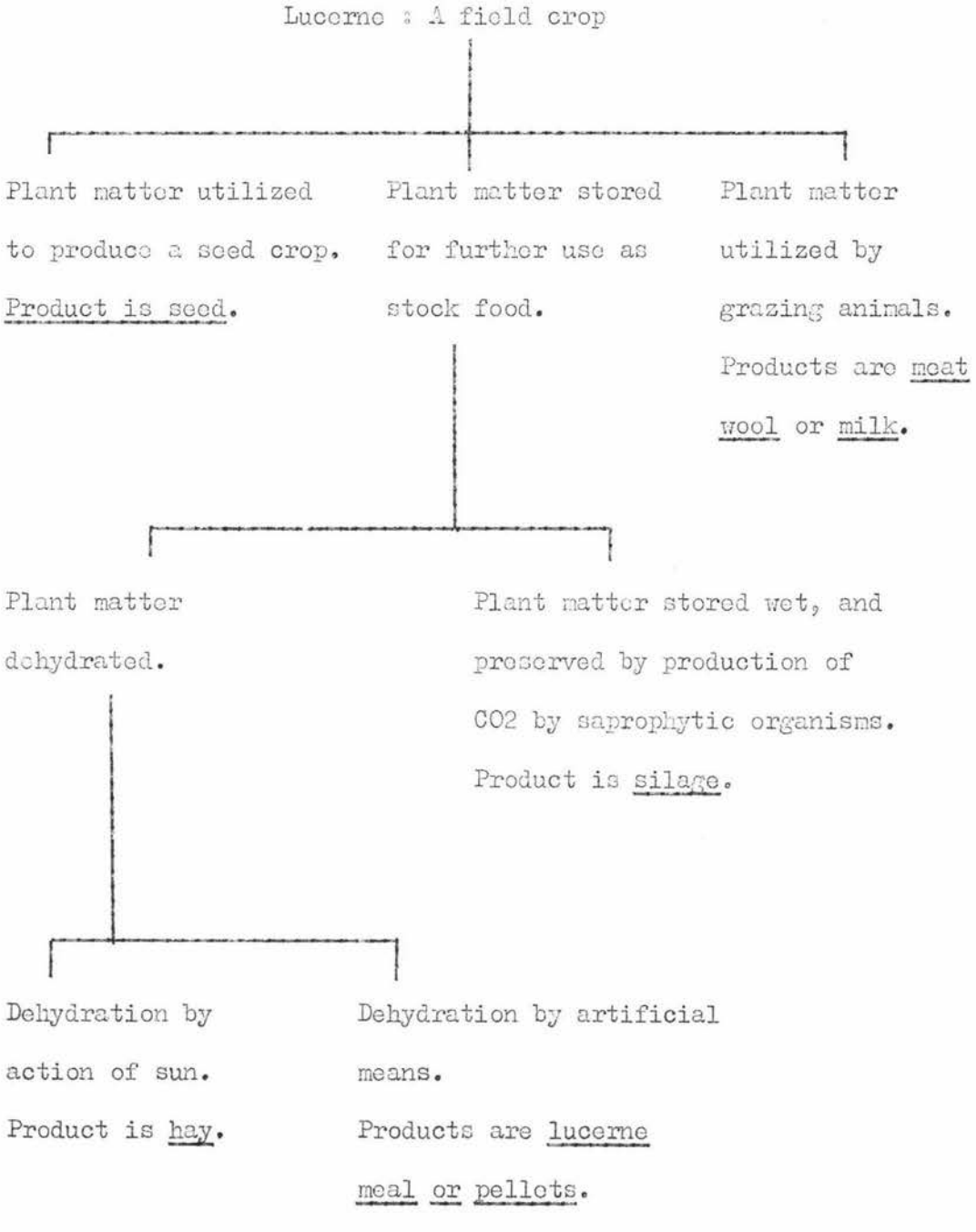
Table 1. The area of lucerne grown in New Zealand utilized as hay, silage or seed crops.

Year	Area (acres)	Authority
1958-59	144,516	N.Z. Yearbook, 1964.
1968-69	203,010	Ibid., 1970.
1970-71	300,000 x	Meeklah & Allen, 1971.

x estimate only.

(iv) Disease factor; several fungal pathogens cause pre-emergence and/or post-emergence damping-off in New Zealand, including species of Fusarium, Pellicularia, Pythium, and Phoma (Close, 1967). The pathogenic inoculum is derived from either soil or seed. As regards this latter aspect, twelve fungal pathogens of lucerne are known to be seed-borne (Cormack, 1945; Leach and Elliott, 1951; Noble and Richardson, 1966). Seven of these are present in New Zealand (Dingley, 1969), although only Phoma medicaginis Malbr. et Roum. (var. medicaginis Boerema) has been recorded as being seed-borne in this country (Matthews, 1970).

Figure 1. The uses and respective products of lucerne.



In broad outline, the present study was concerned with establishing the validity of the hypothesis that poor stand establishment as experienced in New Zealand may in part be consequent on fungal pathogens associated with seed at the time of sowing. That is, such seed-borne inoculum may induce pre-emergence and/or post-emergence damping-off.

This hypothesis is tenable in view of the following facts:

(i) Overseas work has shown that several fungal pathogens, in particular P.medicaginis, are seed-borne in lucerne and may cause damping-off (Table II).

(ii) P.medicaginis is prevalent in New Zealand lucerne crops being saved for seed x.

(iii) Preliminary studies in this laboratory earlier established that P.medicaginis was commonly associated with certified lines of New Zealand produced seed (Wenham, 1970).

More specifically the study involved

(i) establishing by morphological, cultural, and pathogenicity investigations that the tentative identification of P.medicaginis on lucerne seed was correct

x An account of the New Zealand history of blackstem of lucerne and a description of the disease is presented in Appendix I.

(ii) a critical examination of methods for the detection of *P. medicaginis* in lucerne seed

(iii) a survey of the health status of commercially available New Zealand lucerne seed

(iv) an investigation into the significance of seed-borne inoculum of *P. medicaginis* on seedling emergence

(v) a study of methods of reducing the fungal inoculum load associated with commercially available New Zealand lucerne seed.

Table 2. Fungal seed-borne pathogens of lucerne known to cause damping-off.

Causal Organism	Authority
Blackpatch (causal organism never identified)	Leach and Elliott, 1951.
Botrytis cinerea Pers. ex Fries.	Zakopal et al., 1966.
Colletotrichum trifolii Bain & Essary	Weber, 1952. Roberts et al., 1959.
Fusarium spp.	Leach, C.M., 1960. Hofer and Crosier, 1962.
Phoma medicaginis	Cormack, 1945. Kernkamp and Hemerick, 1953. Mead, 1953. Leach, C.M., 1960.
Stemphylium botryosum Wallr.	Nelson, 1955. Leach, C.M., 1960.
Sclerotinia trifoliorum Drayton and Groves.	Cormack, 1946.

CHAPTER I

MORPHOLOGICAL, CULTURAL, AND PATHOGENICITY STUDIES

A. INTRODUCTION

Morphological, cultural, and pathogenicity studies were undertaken to confirm that the causal fungus isolated from seed, seedlings, and mature plants in the current investigation was in fact P. medicaginis.

Isolation Techniques

The fungus was isolated to agar using two methods:

(1) High humidity method. Typical lesions with pycnidia (Fig.2) were subjected to high humidity for 36 hours, which had the effect of inducing pycnidiospores to escape through the ostiole. Using a flamed needle and with the aid of a binocular microscope, the resultant spore mass (spore blob, Fig.3) was transferred to a drop of sterile distilled water to produce a spore suspension. Using a flamed platinum loop the spores were then streaked over the surface of potato-dextrose agar (PDA) or malt extract agar (MA) and incubated for three days at 24 C in the dark. If required, monosporous isolates were obtained by removing whole colonies to plates of PDA or MA.

(2) Tissue Plating Method. This technique utilized the ability of mycelium established within infected tissues to grow saprophytically on artificial media.

Small pieces of tissue (approximately $1mm^2$) were cut from the margin of lesions and using a sterile needle tip were transferred to a specialized agar medium (P. 75) at the rate of five tissue pieces per 9cm Petri dish. After incubation for five days at 24 C in the dark, colonies emanating from the tissue pieces were readily recognizable as P.medicaginis. Pure cultures were obtained by transferring blocks of agar with mycelium onto plates of either PDA or MA.

Figure 2. Lucerne stem naturally infected with *P. medicaginis*.

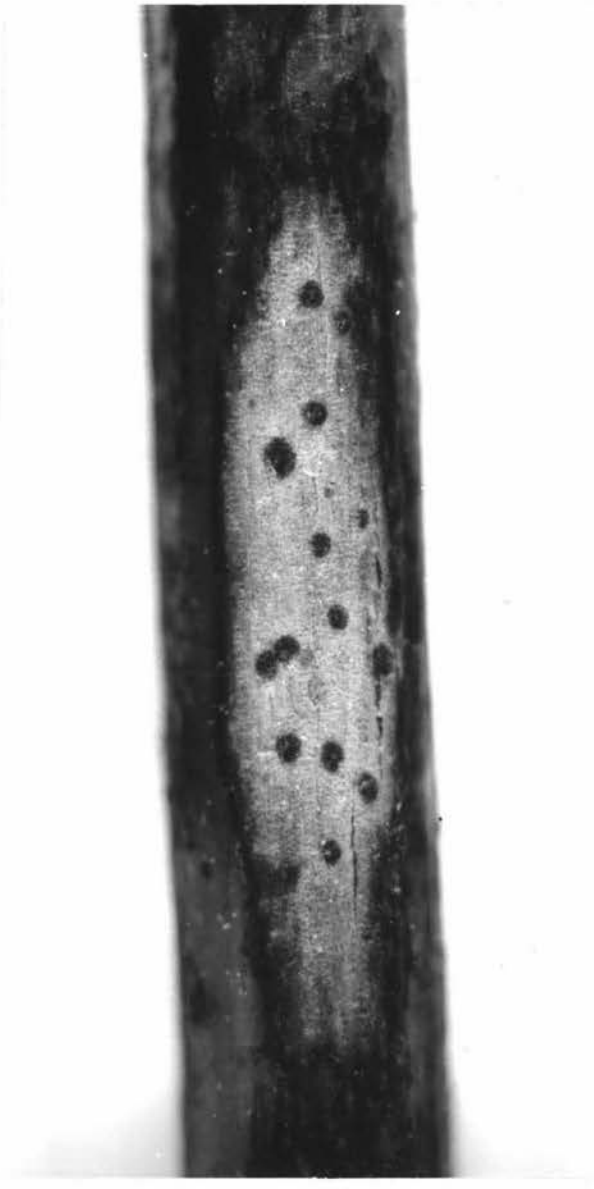
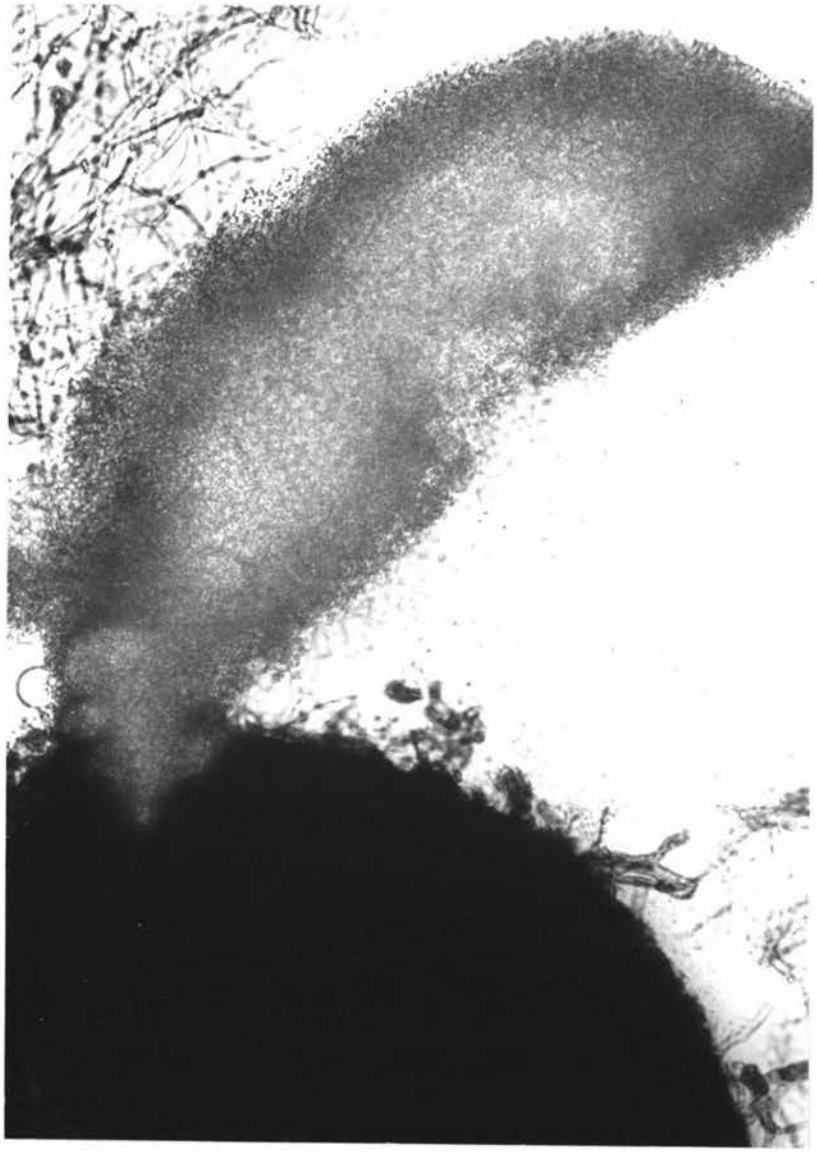


Figure 3. Pycnidiospore mass exuding from pycnidium of P.medicaginis (produced on MA following incubation for 12 days at 24 C in the dark).



B. MORPHOLOGICAL STUDIES

Pycnidiospore features are of major importance in identifying species belonging in the Sphaeropsidales. With regard to P.medicaginis, several workers have reported considerable variation in the size, shape, and septation of pycnidiospores, be they produced on host material or artificial media (Johnson and Valleau, 1933; Toovey et al., 1936; Schenck and Gerdemann, 1956; Ellingboe, 1959a; Rössner, 1968). This is not surprising since it is well established that field environmental conditions and the nutritional status of agar exert a considerable influence on spore morphology (Hughes, 1953; Luttrell, 1963; Simmons, 1966).

Accordingly, in the present study observations were made on the shape, size, and septation of pycnidiospores produced both on host tissues and culture media. In all cases cultures were derived from the same field collections used in determining spore morphology on host tissues.

Experimental Procedure. Naturally infected foliage and seed samples were subjected to conditions of high humidity as previously described (P. 8). Spore blobs which formed over the ostioles of pycnidia were aseptically transferred to drops of sterile distilled water to form spore suspensions. This inoculum was used for determining the morphological features of pycnidiospores on host tissue, and also to prepare monosporous PDA isolates for spore feature studies on agar. These latter isolates were incubated for 11 days at 24 C in the dark.

Results and Discussion. The results are summarized in Table III. On host material the mean pycnidiospore length varied from 4.2 to 5.8 microns(u) with a range over all four isolates of 2.1 to 9.4u. On PDA the mean pycnidiospore length varied from 4.4 to 6.0u, with a range of 3.4 to 11.6u. Thus it is apparent that both the mean and range of pycnidiospore lengths were generally similar, when derived from either host material or PDA. For individual isolates pycnidiospore length was approximately the same on both host material and PDA. That is, the isolate with the largest mean length on host material also had the largest mean length on PDA, and vice versa for the shortest pycnidiospores.

The pycnidiospore widths were not recorded because of the inaccuracies inherent in measuring spores of very small size. However it was observed that most were two to three microns wide on both host material and PDA.

The pycnidiospore dimensions recorded in the present study are in general agreement with those of other workers (Table IV). However Toovey et al. (1936) found a mean length of 9.5u on Dox's agar after incubation for 182 days at 5 C. This result does not invalidate those recorded on PDA in this investigation as Rössner (1968) found that pycnidiospore length increased as incubation temperature decreased. Further Schenck and Gerdemann (1956), and Ellingboe (1959a) recorded pycnidiospore lengths on PDA similar to those on the same medium in the present study.

Pycnidiospore shape varied considerably, ranging from oval to cylindrical with rounded ends, to straight or slightly curved (Fig. 4). Most pycnidiospores were guttulate. Although no specific studies on pycnidiospore shape were conducted, the types illustrated in drawings by Johnson and Valleau (1933), and Toovey et al. (1936) were all regularly observed.

As regards septation, most isolates had very few septate pycnidiospores, a finding in general agreement with previous reports (Table IV). However one isolate was located on host material with a large proportion of bicelled spores (Fig. 5). Such isolates appear to be uncommon.

Table III. Pycnidiospore dimensions of *P. podicaginis*.

Isolate code	Substrate	No. measured	Incubation temperature (C)	Incubation time (days)	Pycnidiospore length (microns)	
					Mean ± S.E.	Std.Dev.
OL230	Seed	120	-	-	4.16 ± 0.05	0.33
OL230	PDA	110	24	11	4.36 ± 0.05	0.30
OL263	Seed	117	-	-	4.78 ± 0.07	0.58
OL263	PDA	120	24	11	4.45 ± 0.05	0.29
DSIR (a)	Stem	110	-	-	5.81 ± 0.07	0.69
DSIR (a)	PDA	121	24	11	6.03 ± 0.11	1.47
OL279	Seed	120	-	-	5.10 ± 0.07	0.71
OL279	PDA	120	24	11	4.71 ± 0.06	0.40

Key:

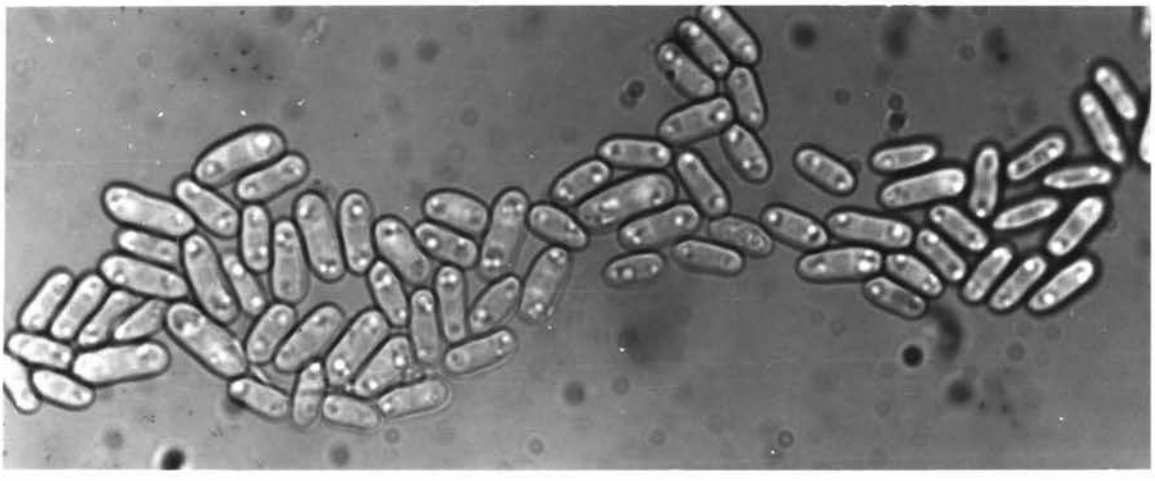
S.E. = Standard Error.

Std. Dev. = Standard Deviation.

Table IV. Previously recorded pycnidiospore dimensions of P.medicaginis.

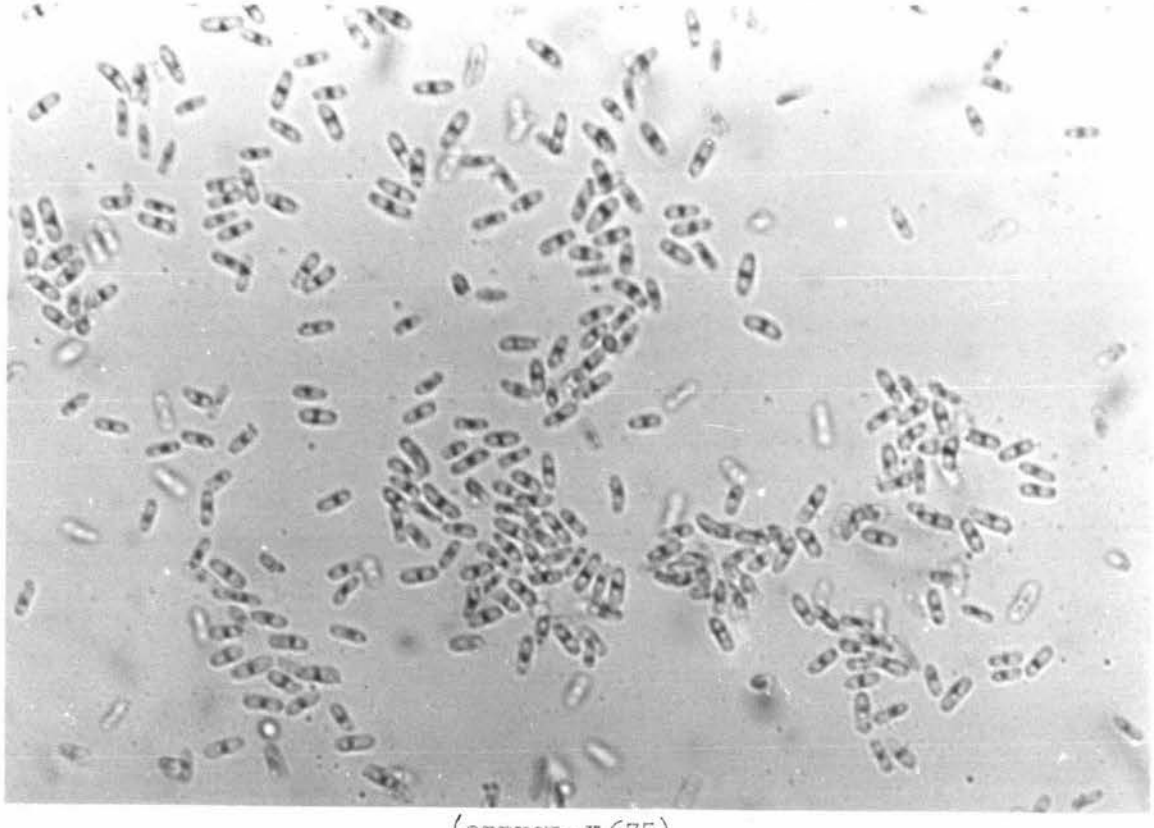
Authority	Subtrate	No. measured	Incubation temperature (C)	Incubation time (days)	Pycnidiospore length (microns) Mean	Range	Pycnidiospore width (microns) Mean	Range	% Septate spores
Malbr.et Roum., 1886	-	-	-	-	-	3-7	-	1-2½	-
Stewart et al., 1908	-	-	-	-	-	6-12	-	2.5-3.5	few
Peck, 1912	-	-	-	-	-	6-15	-	2.5-4	few
Johnson and Valleau, 1933	host	-	-	-	-	5-12	-	1.5-2.5	few
Toovey et al., 1936	host	-	-	-	6.4	4.0-10.0	2.6	2.1-3.5	-
	host	-	-	-	7.1	5.0- 9.4	2.7	2.3-3.7	-
	host	-	-	-	7.3	5.0- 9.0	2.8	1.7-3.1	-
	Dox's agar	-	5	182	9.5	5.0-17.8	3.0	1.8-4.0	-
	Dox's agar	-	room	31	5.0	3.5- 7.0	2.2	1.5-3.1	-
Peterson and Melchers 1942	-	-	-	-	-	6-15	-	2.5-4	-
Schenck and Gerdemann, 1956	PDA	100	room	14	5.12	4.5- 6.3	-	-	38
	host	100	-	-	6.40	5.5- 7.3	-	-	0-22
	host	100	-	-	6.76	5.4-10.0	-	-	0-22
Ellingboe, 1959a	PDA	-	-	-	5	4- 7	-	-	0-100

Figure 4. Non-septate pycnidiospores of P.medicaginis (produced on MA following incubation for 12 days at 24 C in the dark).



(approx. x 1500)

Figure 5. Septate pycnidiospores of *P.medicaginis* (produced on lesioned lucerne stem subjected to high humidity (approximately 100%) for 48 hours at room temperature).



(approx. X 675)

C. Cultural Studies

A cultural study of the fungus tentatively identified as P.medicaginis was conducted for two reasons. Firstly, the cultural characteristics of this fungus were used to aid its identification, and secondly, it was necessary to ascertain the most suitable agar medium and conditions of incubation to ensure a rapid growth rate in later experiments.

I. The Influence of Media Type on Gross Colony Characteristics

Sixteen agar media were prepared and poured into 90 mm gamma sterilized plastic Petri dishes [x]. These were subsequently inoculated with a circular plug of inoculum (4 mm diameter) taken from the margin of an actively growing culture and incubated at 24 C for 11 days in the dark. Each treatment was replicated six times. This method of inoculation was followed in all growth rate studies.

Observations were made on the following characters:

(i) Colony colour at the periphery, medial, and centre positions.

(ii) Colony shape; outline circular or irregular.

(iii) Colony delineation; edges entire or diverse.

[x] Details of the media used and their preparation are presented in Appendix II.

(iv) Mycelial density; sparse, medium, or dense.

(v) Whether the mycelium was raised noticeably above the agar surface; raised or flat.

(vi) Relative number, size, and distribution of pycnidia.

(vii) Mean pycnidiospore length (4 media only).

(viii) Colony diameter.

The results are summarized in Table V, and indicate the wide diversity in the observed characters. In no instance were the gross colony characteristics of the pathogen on two different media identical.

Of the sixteen media examined, potato-carrot, malt extract, lab. potato-dextrose, Oxoid potato-dextrose, and V-8 juice agars proved the most satisfactory for culturing P.medicaginis because each allowed

(i) rapid growth

(ii) abundant pycnidiospore production (Fig. 6a)

(iii) ready identification based on the distinctive colour of colonies (Fig. 6b).

Accordingly in all subsequent experiments three of these media, malt extract agar (MA), lab. potato-dextrose agar (PDA), and Oxoid potato-dextrose agar (Oxoid PDA) were used.

The principle features of the fungus observed in this study are in close accordance to the descriptions of P.medicaginis given by various workers (Johnson and Valleau, 1933; Toovey et al., 1936; Cornack, 1945; Edmunds and Hanson, 1960; Boerema, Doronbosch, and Leffring, 1965).

Note. A feature of P.medicaginis on PDA was the formation of crystals up to 2 cm long in the agar after incubation for 3 weeks at 24 C in the dark (Fig. 7).

Table V. Influence of media type on cultural characteristics of P. medicaginis.

Type of agar medium	Media pH	Colony colour			Colony shape	Colony delineation	Mycelium density	Relative no. pycnidia	Pycnidial size	Pycnidial distribution	Pycnidiospore length ± S.E. microns	Colony diameter mm
		Fringe	Medial	Centre								
Potato-carrot	6.0	white	olive green	dark olive/ green	R	E	medium/flat	1	B	W	-	55.8
Prune	5.0	white	olive green	olive/ green	R	E	medium/flat	2	S	W	-	50.9
Nutrient	7.5	white/ cream	white/ cream	white/ cream	R	E	dense/raised	0	-	-	-	34.0
Water	6.0	white	white	white	I	D	sparse/flat	0	-	-	-	37.7
Malt extract	5.5	white	olive/ green	dark olive green	R	E	dense/flat	1	B	W	5.95 ± .14	59.9
Lucerne seed	7.0	yellow/ cream	yellow/ cream	cream	R	E	medium/flat	½	S	C	5.76 ± .14	46.1
Czapek	6.8	white	olive/ grey	grey	R	E	dense/raised	2	S	C	5.06 ± .09	52.3
Lab potato-dextrose	5.5	white	dark olive/ green	brown	R	E	dense/flat	2	B	W	5.71 ± .17	59.4
Difco potato-dextrose	6.0	white	mouse grey	mouse grey	R	E	dense/flat	2	S	W		44.0
Oxoid potato-dextrose	6.0	white	olive/ green	brown	R	E	medium/flat	1	B	C	-	65.0
Nutrient gelatin	6.8	failed to grow			-	-	-	-	-	-	-	-
V-8 juice	4.5	white	light brown	grey	I	E	medium/flat	2	S	W	-	48.2

Table V. cont.

Type of agar medium	Media pH	Colony colour			Colony shape	Colony delineation	Mycelium density	Relative no. pycnidia	Pycnidial size	Pycnidial distribution	Pycnidiospore length ± S.E. microns	Colony diameter mm
		Fringe	Medial	Centre								
Tomato juice	6.0	grey	grey/ red	grey/ red	I	E	dense/raised	1	S	C	-	33.9
Milk	6.0	white/ cream	white/ cream	white/ cream	R	E	dense/raised	0	-	-	-	24.1
Tryptone soya	7.5	trans- lucent	mouse grey	mouse grey	R	E	dense/flat	0	-	-	-	35.0
Kligler iron	7.0	mouse grey	mouse grey	mouse grey	R	E	dense/raised	0	-	-	-	26.4

Key:

Colony shape : R = regular, I = irregular.
Colony delineation : E = entire, D = diverse.
Mycelial density: sparse/medium/dense.
Mycelial height : raised/flat.
Relative pycnidial numbers : 0 = absent, 2 = abundant.
Pycnidial size : B = large, S = small.
Pycnidial distribution: C = present in centre of colony only,
W = present over whole of colony.

Figure 6a. Colony characteristics of P.medicaginis (Incubated on lab. PDA for 11 days at 24 C in dark).

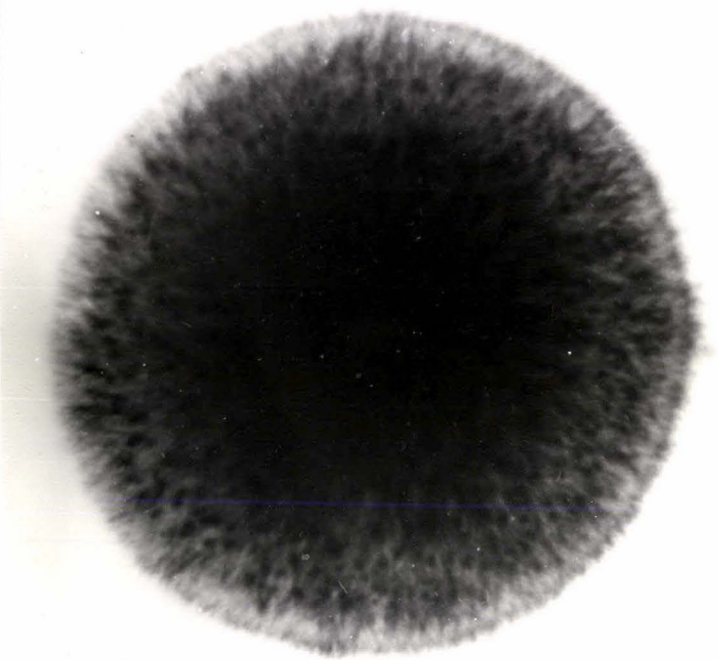


Figure 6b. Colony characteristics of P.medicaginis (Incubated on lab. PDA for 14 days at 24 C in dark).

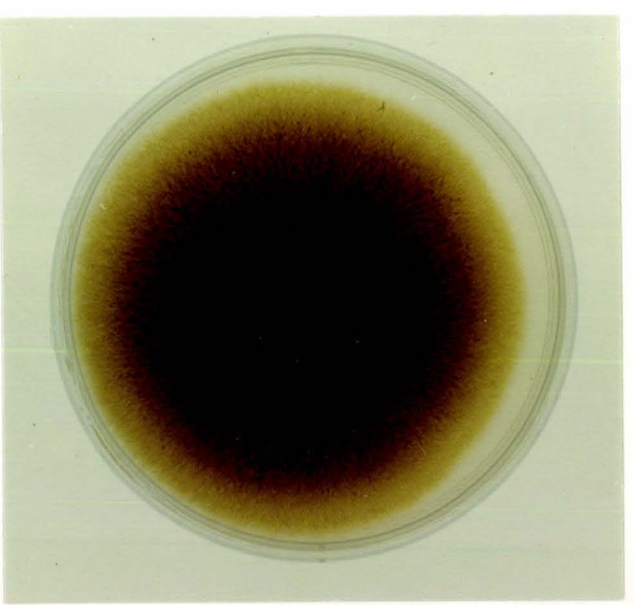
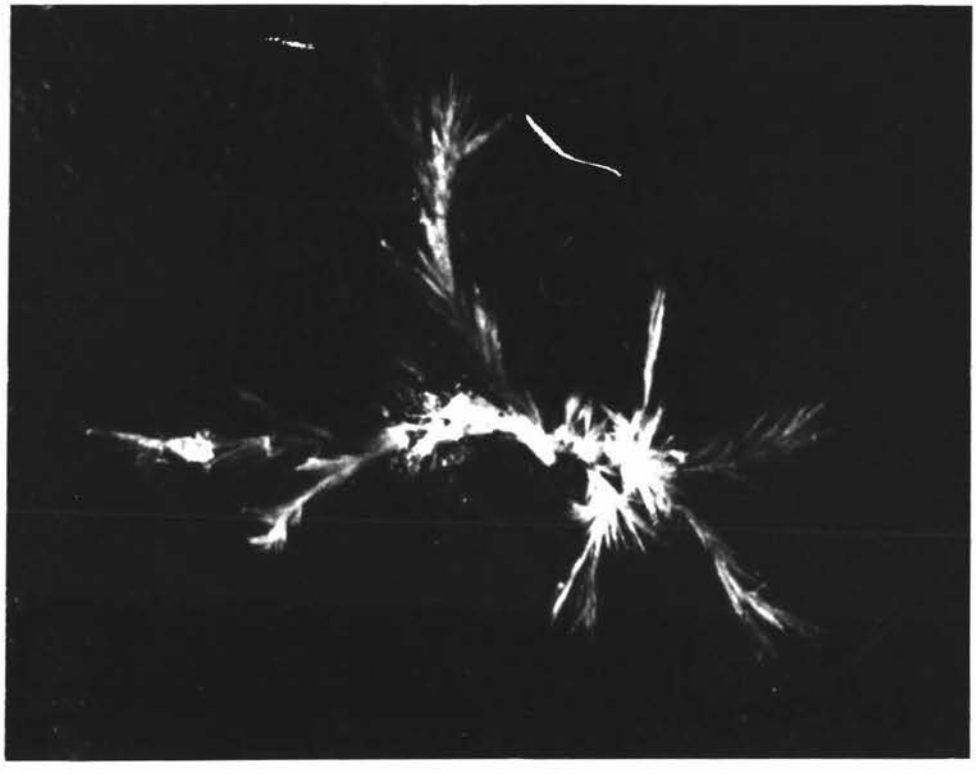


Figure 7. Crystals formed by *P.medicaginis* on lab. PDA.



II. Growth Studies

In studies of several diverse fungal species, Brancato and Golding (1953) established that colony diameter on artificial media was a reliable measure of the effect of environmental and nutritional factors on growth. However, Ellingboe (1959b) when referring specifically to *P.medicaginis* stated:

> "Colony diameter on PDA in Petri plates is not always the best measure of growth because the method fails to take into consideration the compactness and fluffiness of the colony".

Ellingboe contended that a superior measure of growth rate was the dry weight of mycelium produced, and for this reason conducted his growth rate trials in liquid shake cultures. It must be noted that Ellingboe was primarily concerned with comparisons of growth rate between isolates.

In the present study it appeared justifiable to use colony diameter as a measure of growth rate because the comparisons were mainly being made between treatments of one isolate, and not between treatments of several isolates. The exception to this was the effect of temperature on growth rate, where several isolates were compared.

Two measurements of colony diameter at right angles to each other were made on each Petri plate in all growth studies.

(a) Influence of media type on growth.

These experiments were conducted in conjunction with those on gross colony characteristics (P. 19).

The results are expressed in Table V and indicate considerable variation in the rate of growth, being greatest on Oxoid PDA and least on milk agar. Substantial growth also occurred on Czapek, lab. PDA, lucerne seed, malt extract, potato-carrot, prune, and V-8 juice agars.

The explanation for the variation in growth rate can possibly be attributed to factors such as the acidity, concentration, and availability of certain nutrients in the agar media. As regards the latter factor, studies by Mead (1961; 1962a; 1962b) indicate that sucrose, glucose, aspartic acid, glutamic acid, urea, and potassium nitrate are all necessary for P. medicaginis to sporulate and show growth on artificial media.

(b) Influence of temperature on growth.

The effect of temperature on colony growth was studied using monosporous isolates on Oxoid PDA in 90 mm Petri dishes. The plates were inoculated by the method described earlier (P. 19), and incubated for 11 days in the dark at temperatures ranging from 2 to 35 C.

Two series of studies with different objectives were conducted. Firstly, the effect of temperature on the growth rate and gross morphology of one isolate was examined, and the second study involved measurement of

differential effects of temperature on the growth rate between three isolates.

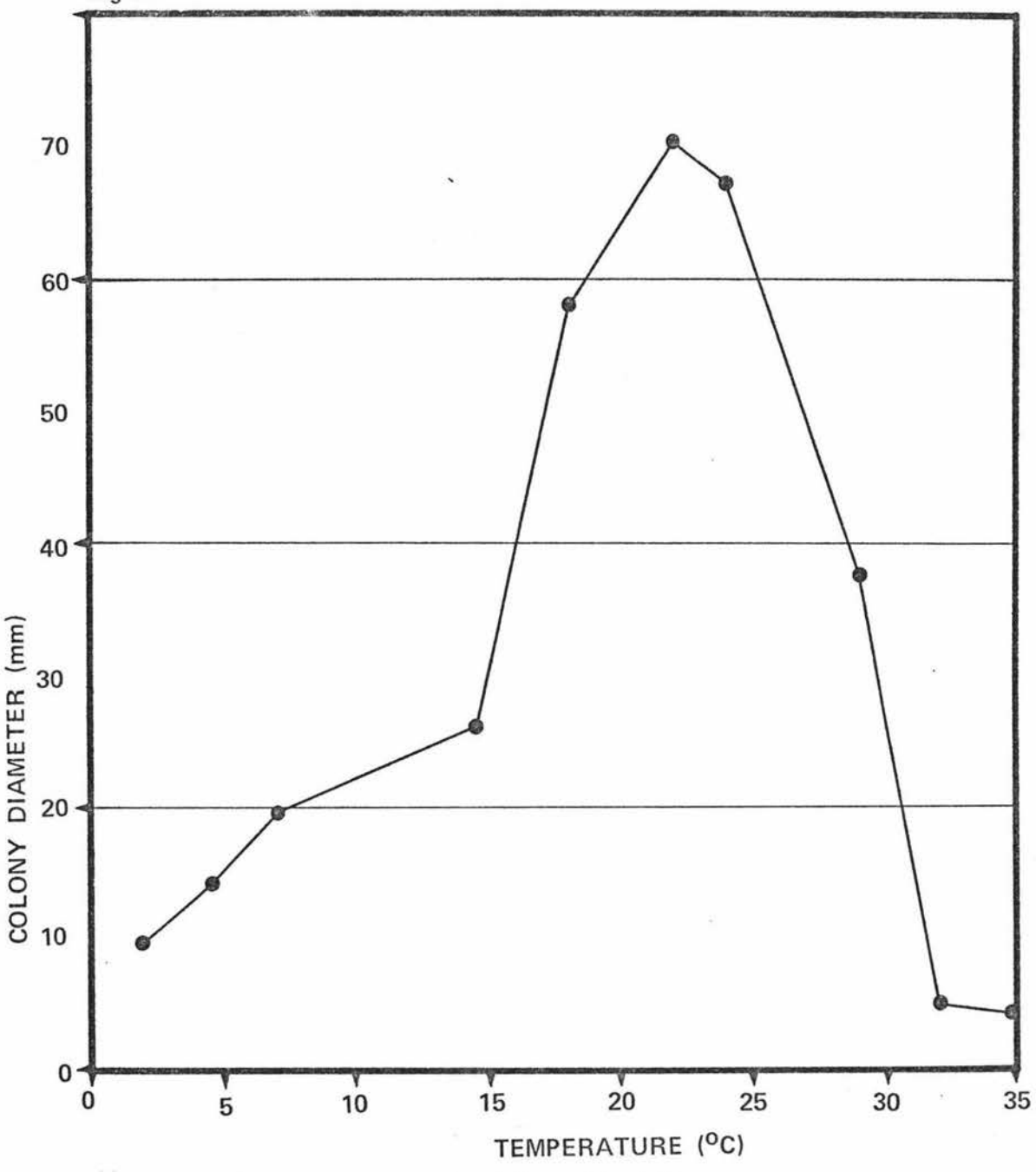
(1) From the results presented in Fig. 8 it is evident that growth occurred between 2 and 32 C, with the maximum at 22 C (also see Fig. 10). This is in accordance with previous reports (Peterson and Melchers, 1942; Cormack, 1945; Mead, 1963).

At temperatures up to 7 and above 32 C, the margin of the colonies was diverse, while from 14.5 to 29 C the margins were entire. All colonies at all temperatures had regular shape. Pycnidia were produced in the temperature range of from 14.5 to 29 C, but a previous study by Cormack (1945) suggests that colonies at all temperatures will eventually sporulate with time. The pycnidia were largest in cultures incubated from 18 to 24 C and were most numerous at 29 C.

Cultures incubated at between 7 to 24 C were olive-green with white margins of varying extent. Those incubated at 2 C were white, while those at 4 C were cream-white with a white fringe. Cultures incubated at 29 C were dark grey with a small white fringe while those at 32 C were white overall. These colony colour observations closely match those made by Peterson and Melchers (1942).

(2) The results of the study on the variability between isolates are presented in Fig. 9. The considerable variation in amount of growth between the three isolates at any one temperature was similar to that observed by Mead (1963) in a comparable study.

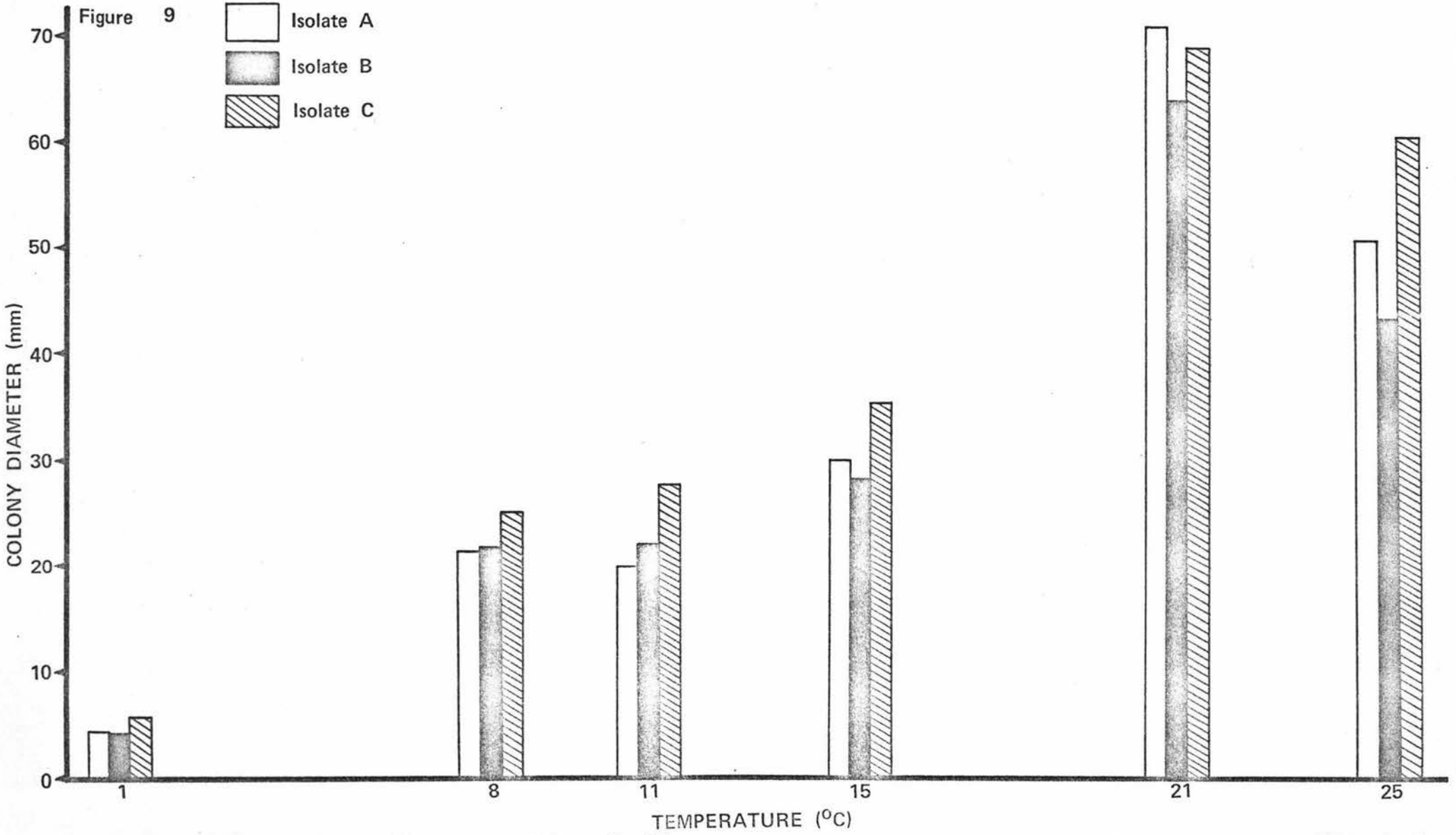
Figure 8



Effect of temperature on growth of P. medicaginis

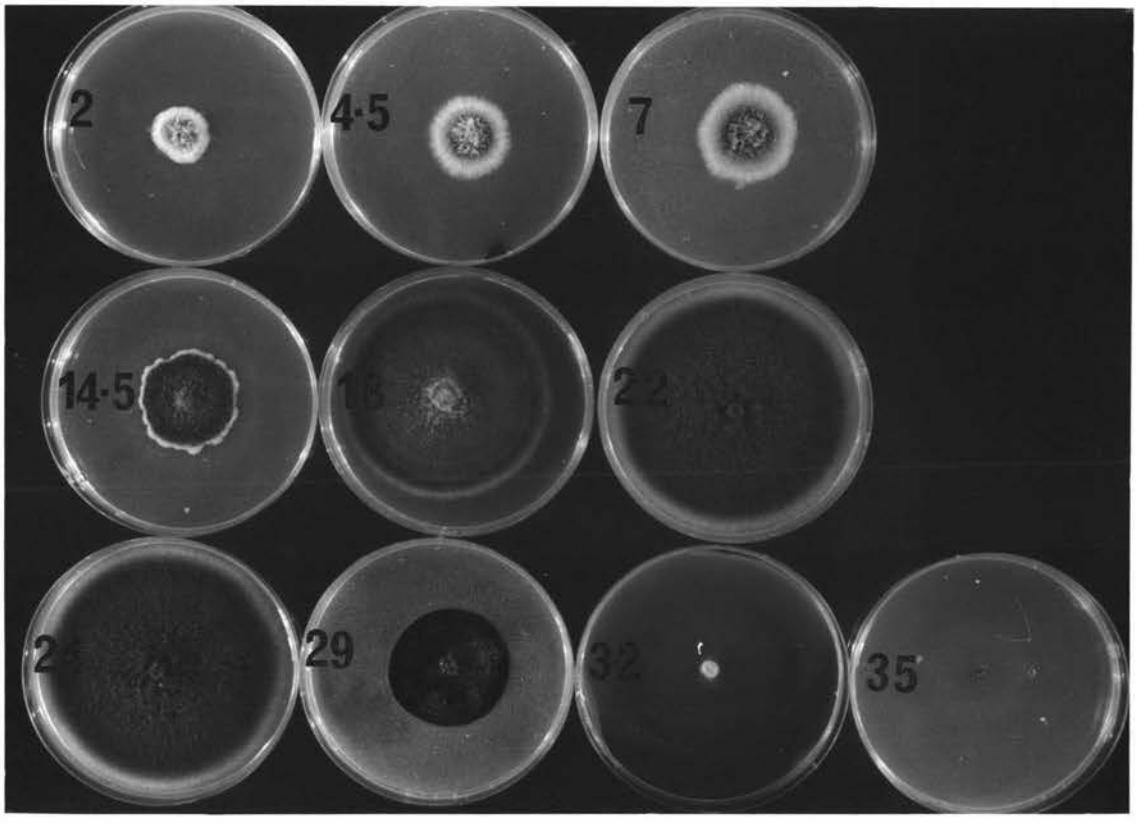
(Growth measured after incubation on Oxoid PDA for 11 days in dark)

Figure 9



Temperature/growth histograms of three isolates of P. medicaginis (Growth measured after incubation on Oxoid PDA for 11 days in dark)

Figure 10. Effect of temperature on colony growth (growth measured after incubation for 11 days on Oxoid PDA in dark).



(c) Influence of pH on growth.

Fungi vary in their response to the pH value of the growth medium. Some species are sensitive to a deviation of one pH unit away from the optimum, while others can tolerate and exhibit growth over a broad pH spectrum. The value of knowing the optimum pH and range over which a fungus exhibits growth is that all growth rate studies can be conducted at the optimum pH. In addition some agar media can be prejudged as unsuitable in view of their pH values significantly deviating from the optimum.

Experimental Procedure. Buffered Oxoid PDA was modified from its normal pH of 5.6 to values between 2 and 8 by addition of either 2N.HCl or 1N.NaOH, following the method used by Milne (1964). Details of the media and buffer are presented in Appendix IV. The plates were inoculated and replicated as described earlier (P. 19). Incubation was for 11 days at 24 C in the dark.

The influence of media pH on growth was determined by making two measurements of colony diameter at right angles to each other on each Petri plate.

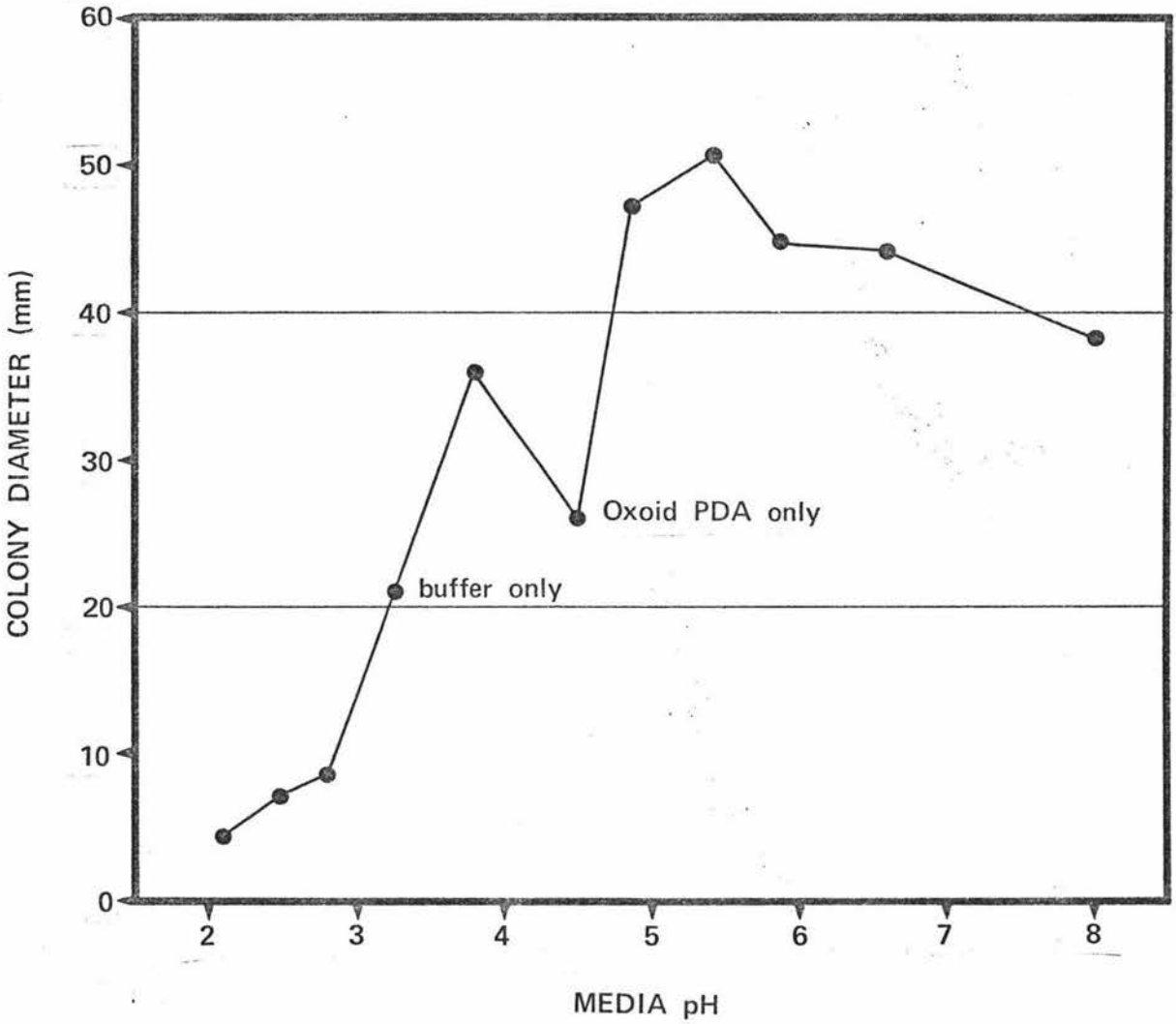
Results and Discussion. The results are summarized graphically in Fig. 11.

P.medicaginis showed tolerance to a wide range of pH values with strong growth between pH 4 to at least pH8. Maximum growth occurred at pH 5.4.

A questionable result was obtained with unmodified Oxoid PDA, in that growth on this medium was less than that observed in a previous experiment (P. 22). The pH of the medium in question was only 4.5, whereas the manufacturer's value is 5.6. The slow growth was attributed to the low pH of the media. Subsequent studies with Oxoid PDA confirmed the manufacturer's figure. No explanation can be offered on why this anomaly occurred.

There are no previous reports on the effect of media pH on growth of P.medicaginis. However, partial confirmation of these results is provided by Mead (1962a), who observed that pycnidial production on agar media varied little between pH3 and 8.

Figure 11



Growth of P. medicaginis on Oxoid PDA adjusted to different pH values (incubation for 11 days at 24°C in the dark)

D. PATHOGENICITY STUDIES

The final step in verifying that the tentative identification of the fungus under study as P.medicaginis involved demonstrating its pathogenicity to lucerne. In effect, this meant completing the requirements of "Kochs' postulates". Applied to fungi they are as follows (Agrios, 1969):

(i) The pathogen must be found associated with the disease in all the diseased plants examined.

(ii) The pathogen must be isolated and grown in pure culture on nutrient media, and its characteristics described (non-obligate parasites), or on a susceptible host plant (obligate parasites) and its appearance and effects recorded.

(iii) The pathogen from pure culture must be inoculated on healthy plants of the same species or variety on which the disease appears, and it must produce the same disease on the inoculated plants.

(iv) The pathogen must be isolated in pure culture again and its characteristics must be exactly like those observed in step two.

Pathogenicity tests were conducted using both seedlings and mature plants.

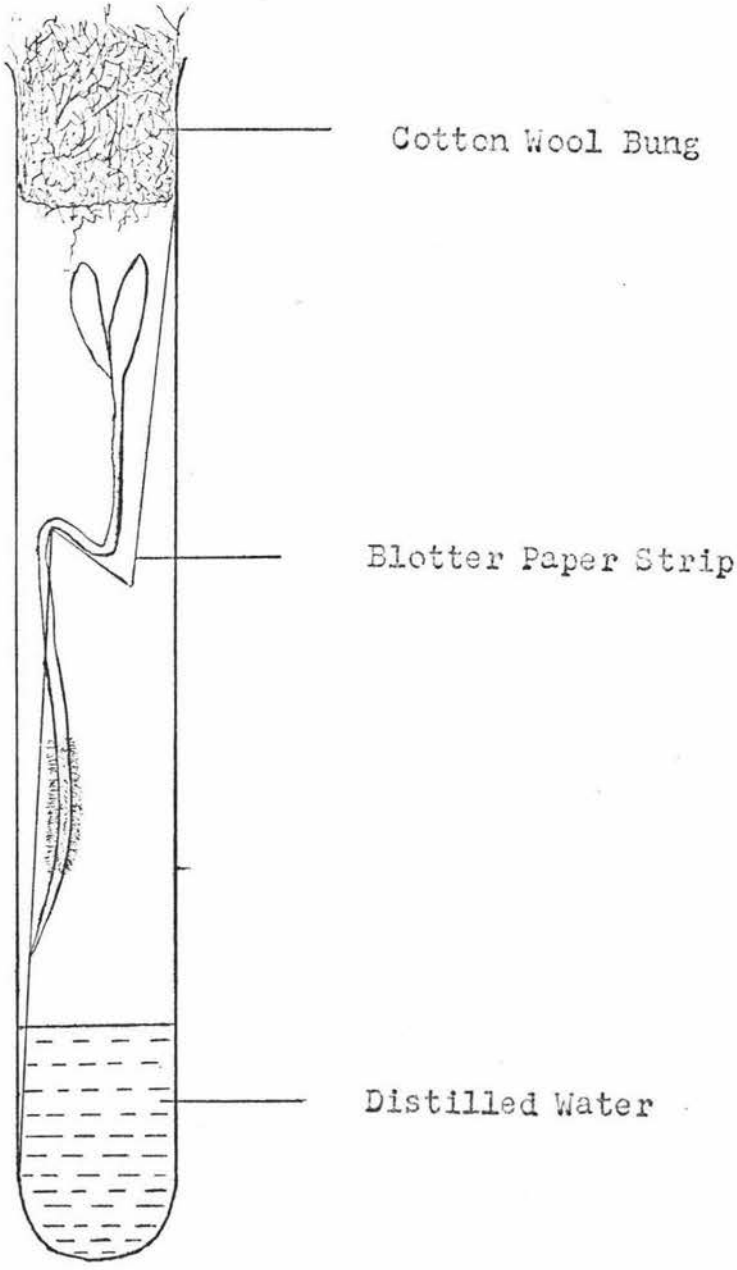
Experimental Procedure. The inoculum used was from monosporous isolates originally derived from lucerne seed and cultured on either MA or PDA.

(1) Seedlings. Since a major part of this study was an investigation into the role of seed-borne inoculum in causing damping-off, it was imperative to determine if this fungus was in fact pathogenic on lucerne seedlings, as a negative result would obviate the need for further studies in this direction.

The apparatus used in these experiments consisted of a test tube (1.7 x 15 cm) into which was placed a filter paper strip (1.5 x 14.5 cm), folded such that a groove was formed on which a seed could germinate (Fig. 12). Five ml of distilled water were added to provide moisture for both germination and subsequent seedling growth, and to maintain humidity within the chamber near to saturation. The tube was sealed with cotton wool and the whole unit sterilized prior to placement of one seed on the groove.

Ten seeds free of fungal pathogens were sown on the "filter paper platforms". When the resultant seedlings had shed the testa from the cotyledons (approximately five days) each was inoculated by placing a mycelium/agar block (approximately $1mm^3$) in direct contact with the hypocotyl. A similar number of seedlings were inoculated with sterile agar to act as controls.

Figure 12 . Apparatus for artificial inoculation of lucerne seedlings with P.medicaginis.



The seedlings were examined daily for evidence of infection.

(2) Mature Plants. Earlier reports indicated considerable variation in the nature of the inoculum applied to foliage of mature plants:

(a) Pycnidiospores suspended in water (Sprague, 1929; Toovey et al., 1936; Cormack, 1945; Ellingboe, 1959a).

(b) Pycnidiospores suspended in a nutrient solution (Bantarri and Renfro, 1962).

(c) Agar cultures homogenized in water (Mead, 1964b).

A comparative study conducted by Bantarri and Renfro (1962) showed the method using pycnidiospores suspended in a nutrient solution gave more extensive and more rapid appearance of foliar lesioning than pycnidiospores suspended in water. Accordingly, in the present study spore suspensions were prepared by flooding either MA or PDA cultures of P.medicaginis with a 1% dextrose in sterile distilled water solution. A sterile dextrose solution (1%) provided controls.

The method of inoculation involved spraying a pycnidiospore suspension onto foliage of mature plants which had been and subsequently were kept in a high humidity chamber (Fig. 13) for a period, to allow infection to take place. It was apparent from preliminary experimentation that standardization of the

following factors was necessary in order to ensure infection:

(a) Inoculum concentration. Studies showed that a pycnidiospore concentration of at least 50,000/ml was necessary to cause infection. A concentration of at least 100,000/ml was used in subsequent investigations.

(b) Pretreatment applied to foliage. Plants were maintained in conditions of humidity approaching saturation for 24 hours prior to inoculation. This is in line with a recommendation by Renfro and Wilcoxson (1963) who found increased infection on plants that were so pretreated.

(c) Humidity. Trials to determine the minimum period of high humidity necessary following inoculation to allow substantial infection were conducted. Plants maintained in conditions of humidity near saturation for 48 hours developed few lesions compared with those so treated for 72 hours. A period of at least 72 hours was used in subsequent experiments.

(d) Post-inoculation environment. Inoculated plants were placed under a bench to avoid direct sunlight. A similar practice was adopted by Cormack (1945).

In summary, the method used in foliage inoculations of mature plants was as follows:

Plants which had been held in conditions of humidity near saturation for 24 hours were atomized to the point of runoff with a pycnidiospore suspension (100,000/ml) in a 1% dextrose solution. The plants were then returned to the high humidity chamber for 72 hours. All plants were kept out of contact with direct sunlight for the duration of the trial. Plants spray inoculated with a 1% dextrose solution served as controls.

Inoculated plants were examined daily for evidence of infection.

Results and Discussion. (1) Seedlings. General browning of the hypocotyl in the region adjacent to the mycelium/agar block was observed three days after inoculation, and within ten days seedlings had collapsed at this point (Fig. 14). P.medicaginis was readily reisolated from infected tissues by the method previously described (P. 8).

The pathogenicity of P.medicaginis on lucerne seedlings confirmed similar observations by Cormack (1945) and Mead (1953).

(2) Mature Plants. Five days after inoculation numerous minute dark lesions could be seen at the edge or middle of the leaflets. These enlarged with time, eventually coalescing to form brown to black lesions reaching their maximum size of 3 x 2 mm after 14 days (Fig. 15a and 15b). Some lesions were zoned. Severely infected leaflets turned yellow (Fig. 16) and abscised. Again, P.medicaginis was readily reisolated from these lesions by the method described previously (P. 8), so fulfilling the requirements of "Kochs' postulates".

Some lesioning occurred on the stipules and petioles of inoculated plants but these were not as numerous as on the leaflets. Stem lesions were not apparent until such time that some stems had started to die, and were then only observed following subjection of the plant to an additional period of high humidity for 72 hours.

The symptoms observed on mature plants closely matched those observed in the field (Fig. 17), and reported in previous studies (Johnson and Valleau, 1933; Toovey et al., 1936; Peterson and Melchers, 1942; Cormack, 1945; Kernkamp and Homerick, 1953).

Figure 13. Method used to provide conditions of high humidity to lucerne plants.

Figure 14. Symptoms expressed by lucerne seedlings following artificial inoculation with agar/ mycelium block.

A

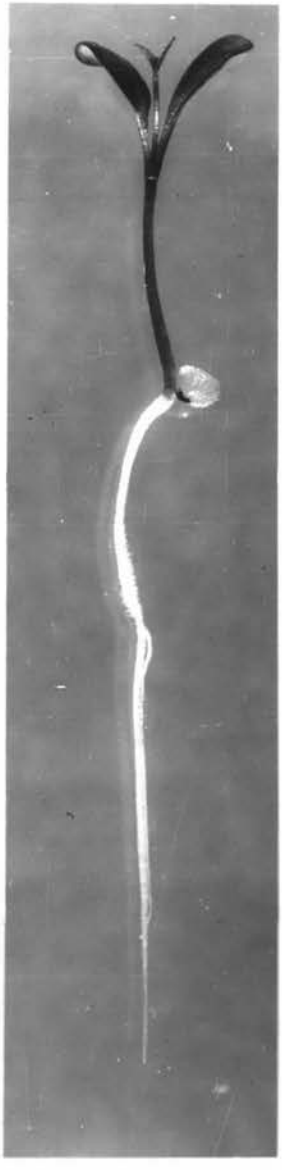
Control (inoculated with sterile agar).

B

Hypocotyl inoculation.

C

Radicle inoculation.



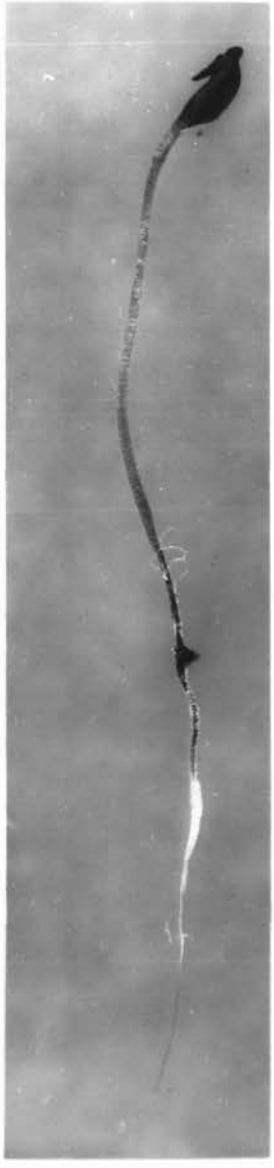
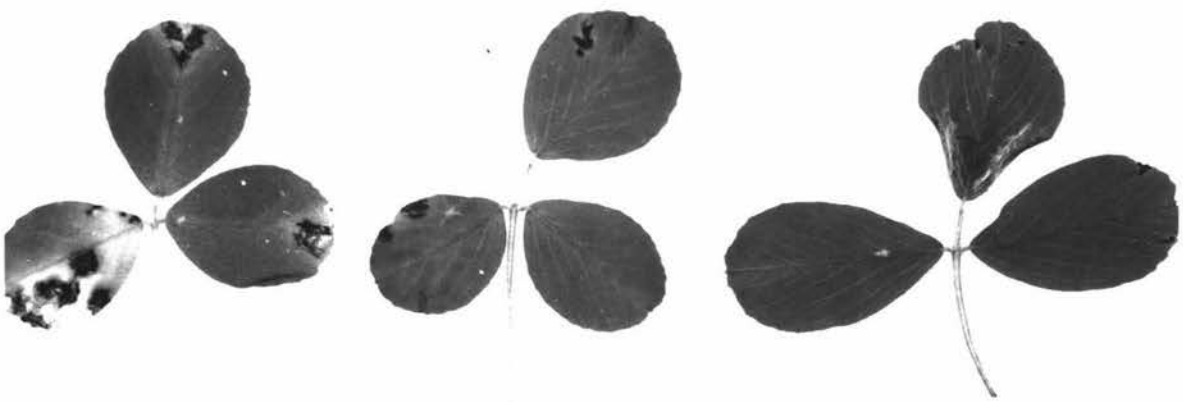


Figure 15. Symptoms expressed on lucerne leaves following artificial inoculation with *P.medicaginis*.

A.



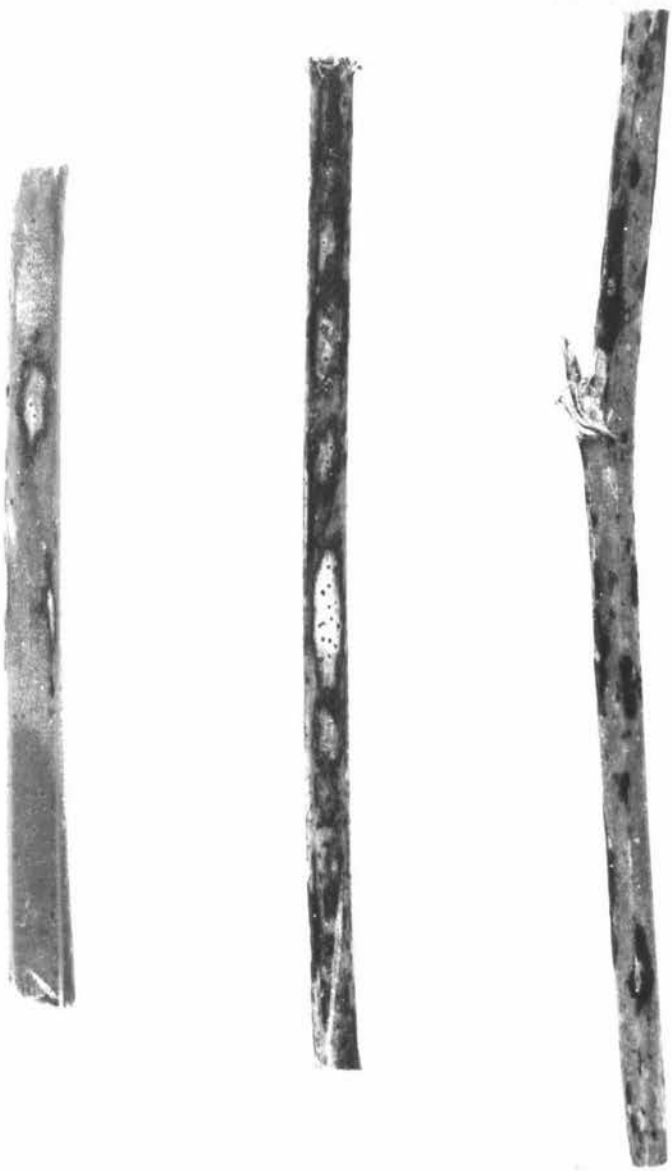
B.

Figure 16. Symptoms on lucerne leaves artificially inoculated with P.medicaginis.

Figure 17. Symptoms on lucerne leaves (A) and stems (B) naturally infected with P.medicaginis.

A.

B.



E. CONCLUSIONS

The evidence accumulated from the morphological, cultural, and pathogenicity studies established that the fungus isolated from lucerne was in fact *P. medicaginis*.

The correct generic and specific epithets of this fungus have been the subject of considerable misunderstanding. The following binomials or trinomials have been ascribed (Edmunds and Hanson, 1960; Jones, 1918):

Sporonema phacidioides Desm., (1847).

Phoma herbarum f. *medicaginum* West. ex Rab., (1862).

Phoma medicaginis Fuckel, (1869).

Ascochyta medicaginis Fuckel, (1869/1870).

Phyllosticta medicaginis (Fuckel) Sacc., (1884).

Phoma medicaginis Malbr. et Roum., (1886).

Gloeosporium norianum Sacc., (1892).

Diplodina medicaginis Oud., (1903).

Ascochyta imperfecta Peck, (1912).

Ascochyta pisi var. *medicaginis* Sacc., (1920).

Phoma medicaginis Malbr. et Roum. (var. *medicaginis* Boerema (1965)).

The explanation for such a large number of binomials having been applied may be attributed to the following:

(i) Unawareness of previous reports of the same fungus.

(ii) Nature of the taxonomic criteria used; in modern taxonomical theory, a physiological character when used by itself may not be used in species identification, but is of some value when used as a complement to a suitable morphological character. In addition, all criteria used must have proven stability. Characters such as pycnidiospore length and the percentage of septate pycnidiospores as used by Toovey et al. (1936), and Edmunds and Hanson (1960) as evidence that P.medicaginis was a member of the genus Ascochyta were shown to be unstable by Ellingboe (1959a) and Rossner (1968).

A comparative taxonomic study of the fungi causing blackstem of lucerne and red clover, and the footrot pathogen of pea was conducted by Boerema, Dorenbosch, and Loffring (1965). Only characters of known stability were used. Evidence was provided that the fungi infecting lucerne, red clover, and pea were in fact the same species, which could be divided on the basis of physiological criteria into two distinct varieties. One of these mainly attacked lucerne, and to a lesser extent pea and red clover, with the opposite occurring for the other variety.

In determining the correct generic position of the fungus they employed the following characters:

(1) Structure of the pycnidia.

(2) Mode of origin of the pycnidiospores.

(3) Nature of the pycnidiospore septum (if present).

Following comparison with the type species of the form-genera Ascochyta, Diplodina, Phoma, and Phyllosticta it was evident that the fungus was a member of the form-genus Phoma.

The specific epithet was determined from the oldest valid binomial in the form-genus Phoma. Prior to the study by Boerema, Dorenbosch, and Leffring (1965) the fungus had been placed in the form-genus Phoma on three occasions. The oldest of these, P.herbarum f.medicaginum West. ex Rab., (1862) was considered unsuitable because the fungus was too dissimilar to the type species, P.herbarum West., (1852). The next oldest name, P.medicaginis Fuckel, (1869) was invalid since it was only mentioned in connection with an unproven perfect stage, Pleospora medicaginis, Fuckel (1869). The most recent name, P.medicaginis Malbr. and Roum. (1886) was accepted as correct because it accorded full species rank while referring unequivocally to the fungus involved.

Varietal epithets were added to recognize the two physiologic types.

The full trinomials given to the two varieties are:

Phoma medicaginis Malbr. and Roum. (1886) (var. medicaginis Boerema (1965)), (primarily a pathogen of lucerne but also infects red clover and pea although to a lesser extent);

Phoma medicaginis Malbr. and Roum. (1886) (var. pinodella (Jones) Boerema (1965)), (primarily a pathogen of red clover and pea but also infects lucerne although to a lesser extent).

In common use the varietal epithet of the fungus primarily pathogenic to lucerne is omitted (Chung and Wilcoxson, 1971; Focke, 1967; Dickason, Leach, and Gross, 1968).

The name proposed by Boerema, Dorenbosch, and Loffring for the fungus on lucerne was accepted in the present study because

(i) the criteria used to delimit it had been shown to be stable

(ii) the fungus from lucerne described by Boerema, Dorenbosch, and Loffring appears to be identical with that isolated from lucerne in the present investigation.

CHAPTER II.

P.MEDICAGINIS AS A SEED-BORNE PATHOGEN.

INTRODUCTION.

A CONSIDERATION OF HEALTH-SCREENING METHODS.

STATE OF HEALTH OF COMMERCIALLY AVAILABLE LUCERNE SEED.

EFFECT OF *P.MEDICAGINIS* ON SEEDLING DEVELOPMENT.

METHODS OF CONTROLLING SEED-BORNE INOCULUM OF *P.MEDICAGINIS*.

CHAPTER II. SECTION A.

INTRODUCTION

The causal agent of spring black stem of lucerne was first reported to be seed-borne by Cormack (1945) and this has been confirmed in many subsequent reports (Kernkamp and Homerick, 1953; Mead, 1953; Weltzien, 1957; Leach, C.M., 1960; Csaplinka, 1967; Maude, Vigor, and Shuring, 1969; Matthews, 1970). Where the level of infection was recorded by the above workers, considerable variation was evident (Table VI). While a proportion of this variation undoubtedly reflects a real difference in the infection level, part may also be consequent on the nature of the seed samples examined ("dressed or field collected seed"), and the screening method applied.

Table VI. Previous reports of the level of *P.medicaginis* associated with lucerne seed.

Authority	Lines infected (percent)	Level *P.medicaginis* Average (percent)	Level *P.medicaginis* Highest recorded (percent)
Cormack, 1945.	50.5	-	40
Mead, 1953.	74	11.5	33
Leach, C.M., 1960.	35	1	3
Matthews, 1970.	88	-	25

Studies by Cormack (1945) and Mead (1953, 1964a) indicate that the fungus persists mainly as resting mycelium in the testa, but may also be present as a contaminant associated with infected stem, leaf, or pod debris remaining from threshing. In no instances were pycnidiospores detected on the testa.

The effect of P.medicaginis on a lucerne crop grown for the purpose of seed production was investigated by Kernkamp and Hemerick (1953). The results indicated that the seed crop could be virtually destroyed if heavy infection occurred during flowering. Considered overall, the seed crop was affected in several ways:

(1) The quantity of seed, as expressed by total bushel yield, was substantially reduced. Further, a large proportion of the seed harvested was light and as such was likely to be screened out during the seed cleaning process, thus also reducing the yield of "dressed seed".

(2) The quality of the seed was reduced;

  (a) those light seeds not dressed out were of low germination capacity and/or vigour;

  (b) a large proportion of seeds in all size classes were infected. Overall the infection level of the light weight seed classes was greater than that of seed in the heavier weight groups.

Most seed infection was the result of hyphal penetration of the pods and subsequent establishment in the developing seeds.

It has been established by several workers that seed-borne inoculum of P.medicaginis can cause seedling damping-off (Table II), but the field significance of this has not been determined.

In the present study investigations were conducted on the level, significance, and possible control of inoculum of P.medicaginis associated with New Zealand lucerne seed.

CHAPTER II. SECTION B.

A CONSIDERATION OF HEALTH-SCREENING METHODS.

INTRODUCTION.

THE MOIST BLOTTER TEST.

THE AGAR PLATE TEST.

SCREENING OF LUCERNE SEED FOR FUNGAL PATHOGENS OTHER THAN *P. MEDICAGINIS*.

I. INTRODUCTION.

The development of seed health testing did not commence until 1917 (Neergaard, 1965), although it is probable that the necessity of being able to both identify infected lines and to determine the levels of infection was recognized well before this. The first routine seed health testing was started in the 1920's. The testing procedures used have mainly evolved within each country to suit its particular requirements, facilities, and personnel (Noble, 1951). An ever increasing world trade in seeds, coupled with a requirement by many governments for seed health certificates on seed being imported gave rise to a need to standardize health testing methods. Since 1957 the committee on plant diseases of the International Seed Testing Association (I.S.T.A.) has been responsible for ensuring that seed health testing methods are standardized throughout the world.

Most seed health testing methods are based on an examination of seeds or seedlings after a period of incubation under controlled conditions of temperature and humidity (Neergaard, 1965). To be acceptable, a routine health test must satisfy several stringent requirements. These have been defined as follows (de Tempe, 1970):

(i) The pathogen must be recognizable with ease and certainty.

(ii) The method must provide results that are reproducible for any one sample and comparable for different samples.

(iii) Except in cases of quarantine inspection, the results should be informative for the possible field performance of the seed, which means that the relationship between laboratory test results and field development should be close.

(iv) The method should be simple, cheap, and quick.

(v) It should be fit for exact standardization with respect to international use.

From a study of the literature it was apparent that little routine seed health screening of lucerne has been conducted in the English speaking world. However, I.S.T.A. does recommend three months for detecting P.medicaginis (Anon., 1966):

(i) Direct Inspection; severely infected seed is brown, flat, and permeated by hyphae; slightly infected seed may only have black lines and dots, these being resting hyphae and pycnidia.

(ii) Blotter Test; seeds are germinated on moist blotters and examined following nine days incubation at 18-22 C under near ultra-violet (UV) light arranged in a 12 hour light/12 hour dark cycle. Evidence of infection is pycnidia on seeds and seedlings and dark lesions on seedlings.

(iii) Agar plate test; seeds are subjected to a pre-treatment of 1% chlorine for ten minutes, and then set out on PDA or MA prior to incubation for seven days at 18-22 C preferably under near UV light in a 12 hour light/12 hour dark cycle. Evidence of infection is indicated by growth of characteristic dark olive-green colonies, and by pycnidia on the testas.

In addition to P.medicaginis, eleven other fungal pathogens of lucerne are reported as being seed-borne (Leach and Elliott, 1951; Cormack, 1945; Noble and Richardson, 1966). Techniques described in the literature for detection of these are basically modifications of the three above methods.

In preliminary screening trials using the blotter and agar methods, it soon became apparent that P.medicaginis was the most common pathogen present in New Zealand produced seed. In view of its prevalence and alleged importance when seed-borne, the present study was orientated more specifically towards screening for this pathogen.

Repeated comparative trials using the blotter and agar methods initially gave very variable results. Thus as a pre-requisite to large scale screening of seed it was first necessary to evaluate and standardize both the blotter and agar methods. The direct inspection method was abandoned as being unreliable, since flat brown seeds could be found in uninfected lines just as frequently as in infected lines.

II. THE MOIST BLOTTER TEST

This method is based on the capacity of pathogenic fungi associated with seed to induce signs and/or symptoms on the seed or seedling host. Four environmental variables may potentially influence the reliability of this test:

(a) The temperature at which the germinator operates.

(b) The light regime to which the seeds are subjected.

(c) The moisture level of the blotter pads.

(d) The relative humidity level within the germinator.

Studies were conducted to determine the optimum environmental conditions revealing maximum percentage infection of lucerne seed with P.medicaginis.

(a) Temperature. The effect of temperature on disease expression is complicated by the fact that we are dealing with two biological systems; the host and the pathogen. Thus the objective is to determine the optimum temperature within the temperature growth range of the host at which maximum symptom development occurs. Agar studies revealed the optimum temperature for growth on culture media, but this figure may not necessarily be optimum for a blotter pathogenicity test on account of host variation in susceptibility as influenced by temperature. In a field study on the effect of temperature on the development and incidence of spring blackstem of mature lucerne plants, Mead (1963) found it was most prevalent and developed most rapidly at 12.5 to 14.5 C, with little disease development at 20 C, which is near the optimum temperature for growth of P.medicaginis on artificial media.

In the present study, lack of suitable equipment prevented a precise definition of this host influence. Hence it was necessary to accept the 18-22 C temperature recommendation of I.S.T.A. The previous study on the level of P.medicaginis in New Zealand lucerne seed was conducted at 20-21 C (Matthews, 1970).

(b) Light Regime. The light regime under which seeds are germinated (in particular the near UV wave length) is known to influence the ability of some fungal species to sporulate through slowing down mycelial development and stimulating sporulation (Limonard, 1966). Since the production of pycnidia on seeds and seedlings is in part used in identifying P.medicaginis, a trial was conducted to determine whether the presence of a strong light source supplementing natural light increased the number of seeds and seedlings exhibiting infection. Two hundred seeds of a line previously shown to be infected were evenly spaced on moist blotters and placed under two 40 watt fluorescent tubes. For comparison 200 seeds of the same line were similarly incubated but under natural light.

Whilst there were no significant differences in the percentage infection, seedlings produced under artificial light were less etiolated and the testas were more readily shed from the cotyledons. This facilitated the recording of infection levels since cotyledonary lesions present were more easily observed. Subsequent experiments confirmed this indirect benefit of supplementary light.

Accordingly, during routine blotter tests two fluorescent tubes (40 watts) were suspended above the Copenhagen germinator and set to provide supplementary light for thirteen hours between 7 a.m. and 8 p.m.

(c) Blotter Pad Moisture Level. Studies conducted in Holland by de Tempe and Limonard (1966) showed that for certain seed-borne fungal pathogens a high moisture level in the blotter could markedly lower the infection percentage. This phenomenon was named the "wet blotter effect" (WBE). Subsequent experiments (Limonard, 1967) demonstrated that the WBE was caused by antagonism to the pathogen from the associated seed-borne saprophytic bacteria, and was more often found in those seeds that imbibed water rapidly at the commencement of germination, such as small seeded species.

The WBE was observed for B.cinerea, Ascochyta linicola Naoum. & Vass., and Alternaria linicola Gr. & Sk. of flax, B.cinerea of lettuce, Alternaria dauci (Kühn) Gr. & Sk. and Stemphylium radicinum (M., & E.) Neerg. of carrot, Alternaria brassicicola(Schw.) Wilts. of cabbage, but not for Fusarium spp. of wheat, Helminthosporium spp. of barley, and Phoma lingam (Tode ex Fr.) Desm. of cabbage (de Tempe, 1968). Limonard (1967) found that the bacterial antagonism that resulted in the WBE was eliminated through addition of the antibacterial antibiotic terramycin at the rate of 50 parts per million (ppm) to the water serving as blotter moisture.

Because lucerne is a "small seeded legume", trials were conducted to determine whether the WBE was significant in the screening of lucerne seed.

Experimental Procedure. Three lines of seed were subjected to a blotter test in dry, medium, and wet conditions. A "dry blotter" was one that was holding its own dry weight of water, a "medium blotter" was holding twice its own dry weight of water, and a "wet blotter" was holding three times its own dry weight of water. The moisture of the blotters was determined daily and adjusted as necessary (Appendix IV).

Two hundred seeds were examined per treatment. Incubation was for eight days at 18-22 C in a Copenhagen germinator (Fig. 18) with natural light supplemented by artificial light for 13 hours daily from two fluorescent tubes.

Results and Discussion. The results presented in Table VII clearly indicate the existence of a WBE in lucerne seed. Accordingly, all subsequent moist blotter tests were conducted on "dry blotters".

Table VII. The influence of blotter moisture on the expression of seed infection.

Seed line	Level of *P. medicaginis* (percent)		
	"Dry"	"Medium"	"Wet"
OL38	18.5	18	11
OL259	31	26.5	20.5
OL263	17	13	10.5

Figure 18. Copenhagen germinator used for routine moist blotter tests.

(d) Percentage Humidity Within the Germinator. The relative humidity within the Copenhagen germinators was continuously monitored with a thermohydrograph and found to be always near saturation. High humidity levels are known to be conducive to maximum establishment of infection induced by plant pathogens (Yarwood, 1956).

Following completion of the studies outlined, the wet blotter test as used in this investigation was standardized as follows:

(i) Two hundred seeds of the line under test are set out on blotters (Fig. 37).

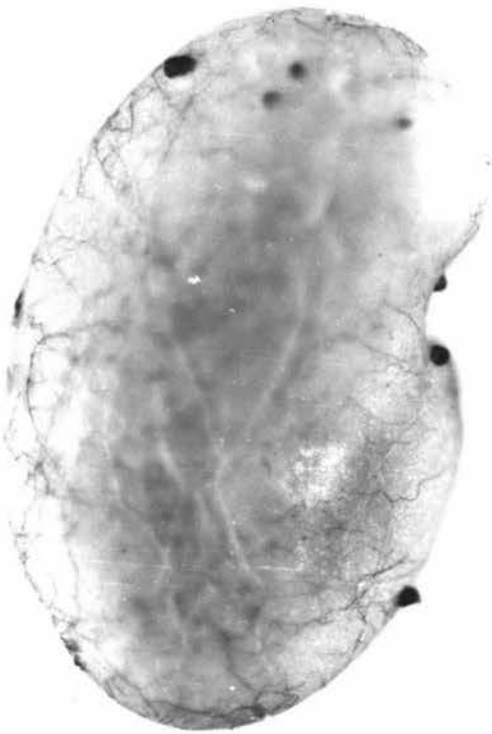
(ii) Sufficient water is added to double the original dry weight of the blotter. The moisture level is checked daily thereafter and adjusted accordingly.

(iii) The tray of seeds is placed in a refrigerator for four days at 5 C. (This is standard practice in lucerne seed germination studies, the objective being to maximize germination percentage).

(iv) The seed tray is transferred to a Copenhagen germinator (Fig. 18) held at 20 C ($\pm$ 2). Humidity within the germinator is maintained at close to saturation. The seeds have natural light supplemented by fluorescent lighting for 13 hours a day.

(v) Following eight days incubation within the germinator, seeds and seedlings are examined for the presence of P.medicaginis (Fig. 19, 25, 26, 27, 28) and other fungal pathogens.

Figure 19. Pycnidia and hyphae of P.medicaginis on testa of lucerne seed.



III. THE AGAR PLATE TEST.

This test utilizes the saprophytic growth ability of seed-borne fungal and bacterial pathogens, and as such is only applicable to those that can be cultured on artificial media. Seed is placed on the surface of an agar growth medium in Petri dishes, and after several days incubation the presence of pathogens is evidenced by macroscopically recognizable colonies which have developed from inoculum associated with the seeds.

According to de Tempe (1963), no one seed health test satisfies every requirement in the detection of a particular pathogen. A frequently criticized aspect of the agar plate test is that results do not distinguish between pathogenic and saprophytic strains within a species. However, in the present study seeds on agar were observed to germinate rapidly, with pathogenic species present attacking either the radicle, hypocotyl, cotyledons, or the whole seedling.

In considering the objective of a seed health test, one must decide whether the result will reflect the actual pathogenic value of the seed-borne inoculum, or the proportion of seed with potentially pathogenic inoculum. An individual seed must be considered as an ecosystem, comprising the host seed and its microbiota, of which part is the saprophytic and pathogenic microflora.

While in theory a fungal colony can develop from a single mycelial strand, this is improbable unless the

antagonistic seed-borne saprophytic flora is suppressed, or the amount of pathogenic inoculum is sufficiently great to counteract the antagonists. As a consequence factors such as soil temperature, moisture, and nutrient status must affect the relative importance of the pathogenic and saprophytic inocula, thus making it difficult to forecast the effect of pathogenic seed-borne inoculum on the field performance of seed. For this reason, all agar plate screening of lucerne seed for P.medicaginis was directed to finding the proportion of seeds with some inoculum present, while recognizing this would not necessarily give an indication of the severity of infection in individual seeds.

Considered overall, the reliability of the agar plate test may be influenced by several factors:

(a) Incubation temperature.

(b) Incubation time.

(c) Seed germination within the agar plate.

(d) Activity of the saprophytic microbial seed-borne flora.

In this regard, several factors of potential importance were considered;

(1) media type;

(2) role of antibiotics as a media additive;

(3) chemical pretreatment of seed;

(4) position of the seed in or on the agar.

Studies were conducted to determine the significance of each of these factors.

(a) Incubation Temperature. Since the health testing of lucerne seed was directed primarily to the detection of P.medicaginis it was important that the incubation temperature be such that it favoured rapid development of the pathogen on agar. The cultural studies indicated that growth rate of P.medicaginis was greatest at 21 C, with a small decrease at 18 and 24 C. While it was theoretically preferable to hold the plates at 21 C, lack of a suitable incubator resulted in all being held at 24 C.

(b) Incubation Time. Even though many recognizable colonies of P.medicaginis had developed from seed after five days incubation, full expression of the inoculum present was not achieved until 11 days. However in order to safeguard against biased results through the plates being over-run with species of Mucor and Rhizopus (which if present could mask P.medicaginis through envelopment of the whole plate), an interim count was made at eight days.

(c) Seed Germination within the Agar Plate. When lucerne seeds are set out on agar most germinate within several days. Two factors associated with this were noted that in some instances could affect the reliability of the agar plate test. Firstly, adjacent seedlings sometimes made contact, thereby allowing the possibility of cross infection from diseased to healthy seedlings. Secondly, lucerne seeds germinate in an epigeal manner, resulting in the testa being lifted clear of the agar by rapid elongation of the

hypocotyl, thereby giving rise to the possibility that inoculum on the inner surface of the testa may not have sufficient time to grow onto the agar.

Several workers have overcome these problems through suppressing seed germination by the addition of sodium 2,4 dichlorophenoxyacetic acid (2,4-D) to the culture medium (Hagborg et al., 1950; Kilpatrick and Johnson, 1956; Lloyd, 1959; Wood, 1966). Its success as a method depends on the chemical suppressing germination while at the same time having no adverse affect on the growth of the pathogen. In the present study the possible application of this principle to the agar plate test for P.medicaginis was examined. However it was first necessary to determine to what extent the addition of 2,4-D suppressed growth and/or modified cultural characters of the pathogen, and its effect on seed germination.

(1) Preliminary Studies.

Experimental Procedure. A one percent stock solution of the sodium salt of 2,4-D was prepared and added to PDA in quantities calculated to give final 2,4-D/agar concentrations of 0.05%, 0.1%, 0.2%, and 0.4%.

The 2,4-D/agar mixture was then sterilized by autoclaving at 15 p.s.i. for 20 minutes, in accordance with the method used by Lloyd (1959).

To observe the effect of 2,4-D on germination, 25 seeds per treatment were placed onto agar and incubated

at 24 C for six days in the dark (only 25 seeds were used per treatment as the result desired here was either a positive or negative effect; that is all or not one would germinate).

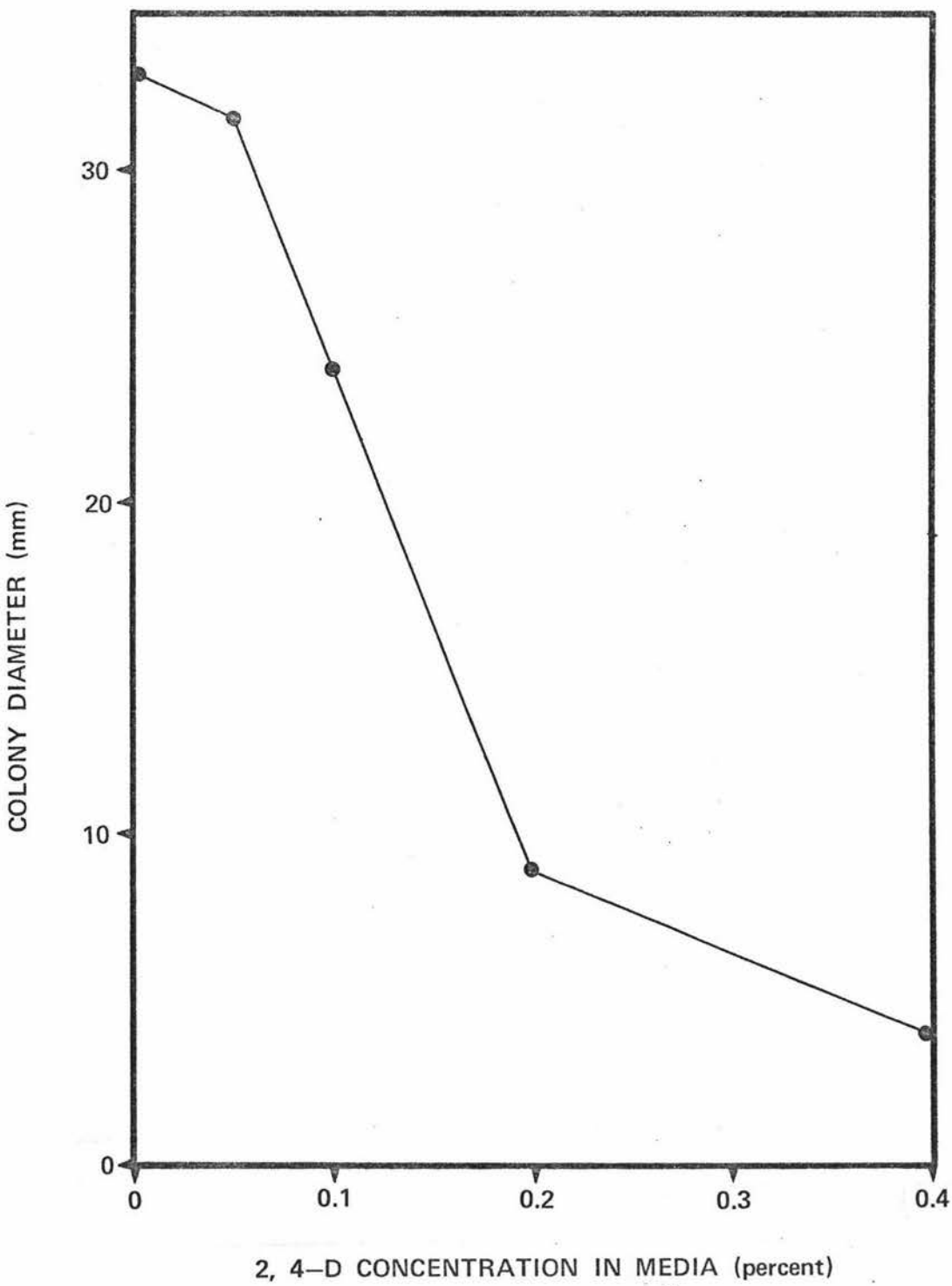
The effect of 2,4-D on the growth rate of P.medicaginis was determined by inoculating plates using the 4mm disc method described previously (P. 19). Incubation was for six or fourteen days at 24 C in the dark.

Observations were made on gross colony characteristics, and growth rate was determined by taking the average of two diametric colony measurements at right angles to each other.

Results. Seed germination was not observed on any of the plates to which 2,4-D had been added. The seeds did imbibe water but the radicle failed to emerge from the testa.

The results presented in Fig. 20 indicate that the addition of sufficient 2,4-D to PDA to give a final concentration of 0.05% had little effect on the growth rate of P.medicaginis, but at levels above this it caused severe retardation. In a second experiment in which 2,4-D was added to both MA and PDA at the 0.05% level, the results confirmed that at this low concentration it had little or no effect on growth rate (Table VIII). Further, the gross colony characteristics of P.medicaginis were unaffected by the presence of 2,4-D at the 0.05% level.

Figure 20



The effect of various concentrations of sodium 2, 4–D in PDA on colony growth of P. medicaginis (results recorded following incubation for six days at 24°C in the dark)

Table VIII. The effect of 0.05% sodium 2,4-D in PDA and MA on colony diameter of P.medicaginis (incubation for 14 days at 24 C in the dark).

Medium	2,4-D conc. (percent)	Colony diameter (mm)
P.D.A.	none	76
"	0.05	75
M.A.	none	74
"	0.05	78

(2) The role of 2,4-D in the agar plate test.

Having shown that incorporating 2,4-D at low concentrations in agar media suppressed seed germination and that growth of the pathogen was not adversely affected, it remained to demonstrate the application of the method to routine agar plate screening.

Experimental Procedure. 2,4-D was incorporated in MA to give a final concentration of 0.05%, before autoclaving at 15 p.s.i. for 20 minutes. Malt agar without additives provided controls. Two hundred and fifty seeds per treatment were drawn from four infected lines and placed on the agar surface prior to incubation for 11 days at 24 C in the dark.

Observations were made on the percentage infection, gross colony characteristics of P.medicaginis, and the number of seeds germinated.

Results and Discussion. The results presented in Table IX, indicate that the addition of 2,4-D to MA did not give a consistent increase or decrease in the infection percentage. That is, in three lines there was a decrease in the infection level over the controls whereas in the fourth line an increase occurred. However in all lines the presence of the chemical uniformly suppressed germination, thereby preventing both the possibility of cross contamination and elevation of the seed before agar colonization could occur.

This experiment failed to provide evidence to support the contention that the addition of 2,4-D would improve the agar plate test. The reason for this was that in some instances the addition of the chemical had an adverse effect in that the gross colony characteristics were atypical. Such an effect was not observed in those isolates studied in the preliminary experiments. It was considered that the possible benefit arising from the addition of 2,4-D (suppression of seed germination) would be less than the disadvantage of the gross colony characteristics being such that macroscopic identification of colonies would be unreliable.

Accordingly, the addition of 2,4-D to agar was not adopted in subsequent agar plate screening tests.

Table IX. The effect of incorporating 0.05% 2,4-D in MA on the expression of seed infection (incubation for 11 days at 24 C in the dark).

Seed line	Level P.medicaginis (percent)	
	Malt Agar	0.05% 2,4-D in MA
OL259	36	28
d259	20	12
IL129	32.4	40.2
IL356	12	10

(d) Activity of the Saprophytic Microbial Seed-Borne Flora.

(1) Type of media.

The type of agar media on which the seeds are incubated can markedly influence the expression of P.medicaginis through factors such as the nutrient status and pH affecting the relative growth of the various components of the seed-borne flora.

The I.S.T.A. recommends that lucerne seeds be placed on either lab. PDA or MA, and incubated for 8 days at 24 C (Anon., 1966). However preliminary tests using lab. PDA showed this medium was unsuitable for screening work because bacterial colonies developed rapidly from the seed and masked the presence of P.medicaginis. On MA bacterial colonies also developed, but not to the extent that P.medicaginis could

not be distinguished. Malt agar has a lower pH than does PDA and the reduced bacterial growth on the former medium is generally attributed to this factor.

(2) Role of antibiotics in the agar plate test.

Antibiotics have been extensively used to suppress bacterial growth in both the moist blotter and agar plate seed health tests. Limonard (1968) recommended the addition of terramycin to overcome the bacterial antagonism that gives rise to the wet blotter effect, and de Tempe (1970) considered it preferable to add either terramycin (25 ppm) or streptomycin (50 ppm) to agar media for the same purpose. In the present study an investigation was conducted into the possibility of adding penicillin and streptomycin sulphate antibiotics to agar to suppress the bacterial flora associated with lucerne seed.

(a) Preliminary studies.

Before the two antibiotics were evaluated in agar plate tests on seed lines known to be infected with P.medicaginis, it was necessary to establish whether these were effective in suppressing the seed-borne bacterial flora, and also if their presence in agar media affected the colony characteristics and growth rate of the pathogen.

Experimental Procedure. Sterile stock solutions of streptomycin sulphate and penicillin were prepared and added in combination to both Oxoid PDA and MA at rates calculated to give final

concentrations of 50 and 100 ppm. In addition both were added separately to MA in quantities sufficient to give a final concentration of 50 ppm.

To observe the extent to which the incorporation of antibiotics in agar suppressed the saprophytic seed-borne flora (both fungi and bacteria), a sample of 250 seeds per treatment was placed on the surface of the above media and incubated for 11 days at 24 C in the dark.

The effect on the pathogen's growth rate of the two antibiotics (when incorporated in MA both separately and in combination at 50 ppm) was determined through inoculating plates by the 4 mm disc method (P. 19). All plates were incubated for 12 days at 24 C in the dark. The growth rate was determined by taking the average of two diametric colony measurements at right angles to each other on each plate. In addition, observations were made on the gross colony characteristics.

Results and Discussion. Both antibiotics evaluated were partially effective in suppressing the growth of the seed-borne bacterial flora, with streptomycin causing greater suppression. When incorporated in combination at 50 and 100 ppm to both Oxoid PDA and MA the effect was greater than either antibiotic alone. Since the extent of bacterial suppression was not increased by incorporating them at 100 ppm, in subsequent experiments the antibiotics were added to give a concentration of 50 ppm. While bacterial suppression was not noticeably different between MA and

Oxoid PDA, on the latter medium the saprophytic seed-borne fungal flora grew more rapidly, so making the detection of the pathogen more difficult.

Neither antibiotic had an effect on the growth rate (Table X) or the gross colony characteristics of the pathogen.

Table X. The effect of penicillin and streptomycin sulphate when incorporated in MA on colony growth of P.medicaginis (incubation for 12 days at 24 C in the dark).

Antibiotic concentration (ppm)		Colony diameter
Streptomycin	Penicillin	m m
-	-	65
50	-	66
-	50	66
50	50	66

(b) The use of penicillin and streptomycin antibiotics in routine agar plate screening tests.

From the results of the preliminary studies it was possible to theorize that the accuracy of the agar plate test would be improved by incorporating both penicillin and streptomycin into the MA medium. This hypothesis was tested using four infected seed lines.

Experimental Procedure. Plates of MA with streptomycin and penicillin both incorporated at 50 ppm were prepared. Malt agar without additives provided controls. Two hundred and fifty seeds per treatment were drawn from each of four infected lines and placed on the agar surface. Incubation was for 11 days at 24 C in the dark.

Observations were made on the following characters:

(1) Percentage infection with P. medicaginis .

(2) Gross colony characteristics of P. medicaginis.

(3) Extent of the seed-borne bacterial flora.

Results and Discussion. The addition of penicillin and streptomycin to MA had the effect of increasing the percentage infection in three of the four lines studied (Table XI). Further, in all seed lines the seed-borne bacterial flora was effectively suppressed, but not eliminated.

Table XI. The effect of incorporating streptomycin sulphate and penicillin (each at 50 ppm) in MA on the expression of seed infection (incubation for 11 days at 24 C in the dark).

Seed line	Level P. medicaginis (percent)	
	Control (MA only)	M.A. Plus Antibiotics
OL38	8.4	5.2
OL181	3.5	6.0
OL259	7.6	14.4
OL272	4.4	10.2

The extent to which the addition of antibiotics to agar allows a more accurate revelation of the percentage seed infection with P.medicaginis is dependent on the nature and susceptibility of the associated bacterial species. Although considerable variation was noted in the bacterial load present in individual seed lines, in all cases the incorporation of penicillin and streptomycin (each at 50 ppm) greatly facilitated identification of the pathogen.

Accordingly, the addition to MA of penicillin and streptomycin sulphate (each at 50 ppm) was adopted for routine agar plate screening of lucerne seed.

(3) Chemical pretreatment of seed.

In agar plating tests it is customary to surface sterilize seed to inactivate or minimize the associated saprophytic fungal flora, that in some circumstances can prevent identification of a pathogen (Neergaard, 1970; de Tempe, 1970).

The most common method involves soaking seed in a sodium hypochlorite solution (1% available chlorine) for ten minutes, but for individual pathogens the type and strength of chemical or soaking time are varied. A second but not widely used method involves dry heat treatment of seed at 100 C for one hour prior to placement on the agar surface (Malone, 1962). These techniques only have application where the pathogen is not present as either a seed contaminant or in the surface tissues of the testa.

As regards P.medicaginis, studies by Mead (1953, 1964a) and Cormack (1945) indicate it is found mainly in the inner and outer tissues of the testa. Thus it is reasonable to postulate that the pretreatment recommended by I.S.T.A. for lucerne seed (Anon., 1966) would reduce the recorded infection percentage. Comparative tests were conducted to establish if surface sterilization of lucerne seed would improve the agar plate test for P.medicaginis.

Experimental Procedure. The investigation was conducted in two phases. In a preliminary study, 100 seeds from a line known to be infected were immersed in a 1% chlorine solution at room temperature for ten minutes prior to incubation on MA for 13 days at 24 C in the dark. The control series were not treated in any way. Later, 250 seeds per treatment were similarly soaked for either one, three, five, or ten minutes (two lines), or ten minutes (a further two lines) before incubation on MA for 11 days at 24 C in the dark. The controls were as above.

Observations were made on the level of infection and the extent of the associated saprophytic flora.

Results and Discussion. In four of the five seed lines examined the infection percentage was reduced following surface sterilization treatment for ten minutes (Table XII). Variation in the extent of the reduction most probably reflected differences in the depth to which pathogenic inoculum had penetrated the tissues of the testa.

The infection percentage of the fifth line showed a slight increase which was probably the result of inconsistency in the sampling technique, rather than any real difference resulting from the treatment. Surface sterilization for less than ten minutes gave inconclusive results.

The extent to which bacteria developed was unaffected by the treatment. However there was some reduction in the saprophytic fungal flora but this was of little significance as such fungi did not appear to mask the presence of the pathogen.

Considering the above results, there was no apparent advantage from surface sterilizing seed for ten minutes or a lesser period. Accordingly, the technique was not adopted for routine agar plate screening tests.

Table XII. The effect of surface sterilizing lucerne seed on the seed infection level (results recorded following incubation on MA at 24 C in the dark).

Seed line	Number seeds per treatment	Length of incubation (days)	Level *P.medicaginis* (percent) Immersion time (minutes)				
			0	1	3	5	10
OL48	100	13	18	-	-	-	0
d259	250	11	29.6	22.4	24.4	26.2	24.4
d263	250	11	24.5	25.8	25.2	26.2	26.4
IL97	250	11	40.0	-	-	-	11.2
IL129	250	11	49.6	-	-	-	30.8

(4) Positioning of the seed within the agar plate.

It was theorized that a more accurate assessment of the infection level would be obtained if growth of the aerobic bacterial seed-borne flora was suppressed by completely burying the seeds under test in agar. An additional benefit of this treatment would be to eliminate cross infection as the lack of oxygen would also hinder seed germination.

To test the above hypothesis an experiment was designed in which the infection level of seed placed both in or on an agar medium was compared.

Experimental Procedure. Two hundred and fifty seeds per treatment from a seed line infected with P.medicaginis were placed on or under the surface of the following media:

(i) Unmodified MA.

(ii) Malt agar with streptomycin sulphate and penicillin antibiotics added (each at 50 ppm).

All plates were incubated for 11 days at 24 C in the dark.

Records were taken of the number of colonies of P.medicaginis that developed, the expression of other seed-borne flora (bacteria and fungi), and the extent of seed germination.

Results and Discussion. The results (Table XIII) indicate that burying seed in agar reduced the expression of the pathogen. The reduction was greater on unmodified MA, than MA with streptomycin and penicillin added, thus suggesting that covering seed completely with unmodified MA failed to suppress the bacterial flora.

Table XIII. Effect of seed position in the agar plate on expression of P.medicaginis (incubation for 11 days at 24 C in the dark).

Type Medium	Position of seed with agar plate	Level P.medicaginis (percent)
MA	On surface	12.8
MA	Buried	5.3
MA + Antibiotics	On surface	10
MA + Antibiotics	Buried	8.8

Covering seed with unmodified MA reduced both number and size of the bacterial colonies emanating from seed. Further, the incorporation of the antibiotics almost eliminated the expression of bacteria from both buried and surface plated seeds. The saprophytic fungal flora was not completely suppressed through covering seed with MA, as mycelium of Alternaria spp. and Penicillium spp. grew to the agar surface from some buried seeds. However this was of little significance as these do not normally mask the presence of P.medicaginis in routine agar plate screening tests.

While most buried seeds still exhibited some signs of germination very few made contact, thus eliminating the possibility of cross infection.

The practice of completely burying seed in agar was not adopted for routine seed health screening work for the following reasons:

(i) The experiment conducted did not demonstrate any advantage in using this method for the detection of P.medicaginis.

(ii) The method was time consuming, costly in terms of the amount of MA used, and difficulties were experienced in ensuring each seed was completely covered with agar while still maintaining even seed distribution within the plate.

The agar plating method as finally adopted for use in routine seed health tests was as follows:

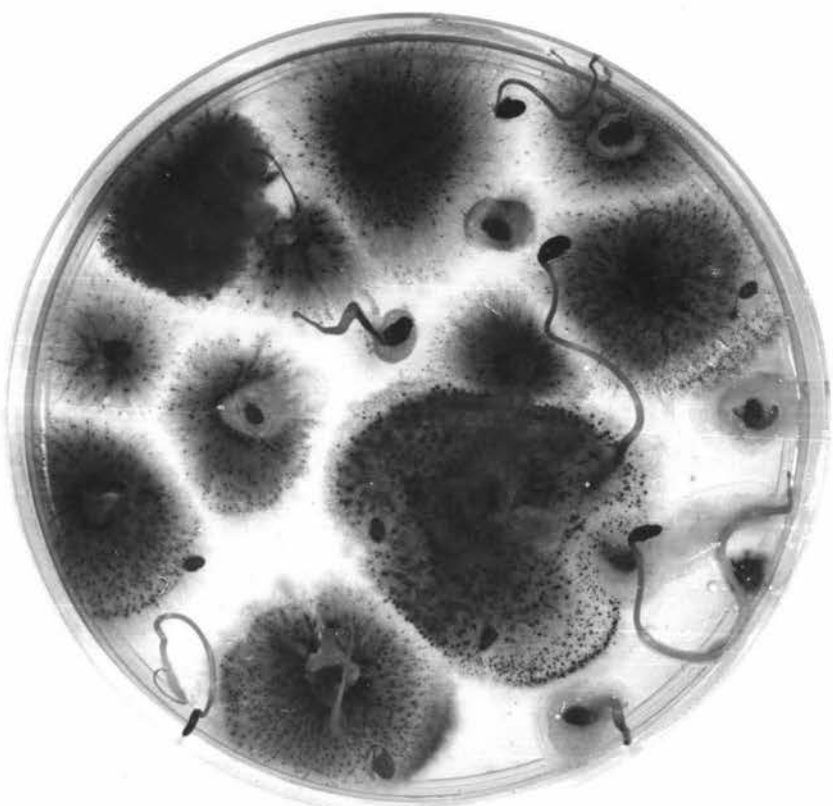
(i) A random sample of 250 seeds from the line under test are set out evenly spaced at the rate of 25 seeds per plate on MA with penicillin and streptomycin sulphate each incorporated at 50 ppm.

(ii) The plates are incubated at 24 C in darkness.

(iii) Results are recorded twice; an interim count is made at eight days and the final at eleven days. Evidence of seed transmission of viable

inoculum was indicated by development of dark green/olive colonies with numerous pycnidia (Fig. 21). The presence of other fungal pathogens was also recorded.

Figure 21. Development of fungal colonies from lucerne seed placed on the surface of MA with penicillin and streptomycin sulphate added (each at 50 ppm). All plates were incubated for 8 days at 24 C in the dark.



IV. SCREENING OF LUCERNE SEED FOR FUNGAL PATHOGENS OTHER THAN P.MEDICAGINIS.

While the primary objective of screening lucerne seed was to determine the level of P.medicaginis it was recognized that pathogenic inoculum of other seed-borne fungi could also be present. However the two routine health tests used did not necessarily allow the expression of these.

The moist blotter test relies on the ability of pathogenic inoculum associated with seed to induce seedling disease. Since only seven of the twelve recorded fungal seed-borne pathogens of lucerne are reported to induce seedling collapse (Table II) it is probable that the other five would not be readily detected in a blotter test. In addition, factors such as the duration, temperature, light regime, and blotter moisture level during incubation would have considerable influence on the expression of those pathogens known to induce seedling disease.

Similarly, in the agar plate test the expression of pathogens other than P.medicaginis would be limited by the media type, seed pretreatment, and the length, temperature, and light regime used during incubation.

The incidence of fungal seed-borne pathogens other than P.medicaginis was recorded. However, it was considered necessary to conduct separate tests in order to detect Verticillium albo-atrum Reinke & Berthold, since an incubation period of longer than 48 hours is reported to

be conducive to the antagonistic fungal flora to mask its presence (Noble, 1970). A screening method recommended by Noble was adopted:

> Incubate seeds on moist dark coloured blotters for 24 to 48 hours and examine for the characteristic verticillate conidiophore arrangement. Confirm identification by streaking out conidia directly onto agar.

Twenty-two of those seed lines screened for P.medicaginis were also screened for V. albo-atrum using the above method.

CHAPTER II.

SECTION C.

STATE OF HEALTH OF COMMERCIALLY AVAILABLE LUCERNE SEED.

During 1970 and 1971 samples were obtained of 86 seed lines submitted by commercial firms to the Seed Testing Station, Department of Agriculture, Palmerston North, for purity and germination tests. Using tests previously described, the samples were screened for fungal pathogens.

Experimental Procedure. The decision whether to use the moist blotter or agar plate test was governed by the chronological sequence of their development. The moist blotter test was standardized first and as a consequence most seed screening conducted during 1970 was by this method, although a modified agar plate test was used on some 25 lines [X]. Following standardization of the agar plate test early in 1971, it was apparent that this technique was simpler and less time consuming than the moist blotter test. As a consequence only the agar plate test was used for routine screening during 1971.

Twenty-two seed lines were also screened for Verticillium albo-atrum using the method outlined by Noble (1970).

Results and Discussion. The results are summarized in Table XIV, while details of individual lines are presented in Appendix V.

[X] Details of the agar plate test used for individual lines are recorded in Appendix V.

The survey clearly established P.medicaginis as the fungal pathogen most prevalent in seed of New Zealand origin, being present in all but two of the 86 lines tested. In addition, Stemphylium botryosum and Fusarium spp. were observed in over 50% of the seed lines examined. The higher incidence of S.botryosum in individual lines screened during 1971 was attributed to the type of test used, rather than to an absolute increase in the percentage infection. That is, S.botryosum was more readily expressed in the agar plate test than in the moist blotter test. However, pathogenicity tests (method P. 36) showed that only a small percentage of the S.botryosum isolates from seed were pathogenic to seedlings. All isolates of Fusarium spp. tested were shown to be pathogenic to seedlings.

In addition to the above pathogens, Leptosphaerulina briosiana (Poll.) Graham and Luttrell was also detected in two seed lines screened during 1971. The incidence of this pathogen was less than one percent in both lines.

No correlation was observed between the level of infection with P.medicaginis and the district from which the seed samples originated.

V.albo-atrum was not observed in any of the 22 lines tested.

The extent and level of lucerne seed infection by P.medicaginis detected in the current investigation was considerably higher than previous New Zealand and overseas reports (Cormack, 1945; Meads, 1953; Leach, C.M., 1960;

Matthews, 1970). This result may in part be consequent on higher susceptibility (genetic or environmentally induced) of the main New Zealand lucerne cultivar ('Wairau') to P.medicaginis, or to improved methods of screening lucerne seed.

Since there was considerable variation between lines in the level of transmission of P.medicaginis it may be hypothesized that this would have great bearing on the extent to which seed-borne inoculum could be of significance in affecting stand establishment.

Table XIV. Range in level of transmission of P.medicaginis in seed lines examined during 1970 and 1971.

Range infection (percent)	No. of lines	
	1970 (46 lines)	1971 (40 lines)
0	2	1
0.5-5	24	5
5.1-10	6	10
10.1-20	5	13
> 20.1	9	11

CHAPTER II.

SECTION D.

THE EFFECT OF P.MEDICAGINIS ON SEEDLING DEVELOPMENT

INTRODUCTION

GENERAL EXPERIMENTAL PROCEDURES

EFFECT ON EMERGENCE

MECHANISM INVOLVED IN SEEDLING ATTACK

I. INTRODUCTION

Having shown that pathogenic inoculum of *P.medicaginis* was associated with the majority of New Zealand lucerne seed lines screened for infection, studies were undertaken to determine the significance of this inoculum in inducing damping-off. Such studies were justified in view of work conducted both in New Zealand (Latch and Greenwood, 1964) and overseas (Cormack, 1945; Kernkamp and Hemerick, 1953; Mead, 1953) implicating seed-borne inoculum of *P.medicaginis* as a cause of both pre-emergence and post-emergence damping-off. Since Canadian studies had shown that the pathogen exists as strains which vary considerably in their pathogenic potentiality (Mead and Cormack, 1961), it was also necessary to establish whether inoculum associated with New Zealand seed was sufficiently pathogenic to effect stand establishment.

II. GENERAL EXPERIMENTAL PROCEDURES

Only those methods having general application to this chapter are discussed. Those specific to particular experiments are described in the relevant section.

The influence of P.medicaginis on seedling development was determined by comparing the rate of and total seedling emergence from infected and healthy seed. A standard procedure was developed to ensure that

(1) all seeds were sown at the same depth
(2) seed spacing was even
(3) all seeds commenced germination simultaneously
(4) seedling emergence was a reflection of only seed vigour.

Preliminary emergence trials were conducted in a glasshouse environment using plastic seed boxes (6" x 4" x 2"). Vermiculite (grade two aggregate size) was used as the potting medium. This proved particularly suitable for the following reasons:

(1) Ease of handling.
(2) Relatively inexpensive.
(3) Aggregates were not formed, thus simplifying root examination at the conclusion of a trial.
(4) It provided a sterile environment.
(5) Being inert, growth before emergence reflected only seed vigour, rather than the nutritional status of this potting medium.

(6) Many trays could be moistened simultaneously, thus allowing those seeds capable of germination the opportunity of doing so.

(7) Since moisture is held within the particles and not between them, the Vermiculite did not easily become water saturated.

Twenty five seeds were sown per seed box in five rows each of five seeds, at a depth of one inch. Care was taken to ensure that the seed sample used was representative of the line under study. The seed boxes were watered by steeping in trays until the surface became uniformly moist.

At specific intervals counts were made of seedling emergence and the number of seedlings which subsequently collapsed.

III. EFFECT ON EMERGENCE

(a) The Significance of Seed-Borne Inoculum of P.Medicaginis in Inducing Damping-off

Variation exists in the germination capacity of lucerne seed lines free of P.medicaginis. Accordingly, it was not possible to assess the significance of seed-borne inoculum in any one line simply by comparing the resultant stand establishment with that of uninfected seed lines. Thus with each infected line it was first necessary to determine its emergence capacity when free of inoculum, for comparison with the emergence of non-treated seed of the same line. This required some method of seed treatment which would eliminate the pathogen without inducing seedling abnormality. In this regard, several workers have shown thiram to be highly effective when applied as a dust or soak (Cormack, 1945; Mead, 1953; Latch and Greenwood, 1964; Maude et al., 1969), and this was later confirmed in the present study (P.149). Further, Cormack (1945) and Jacks (1956) found no evidence of phytotoxicity when thiram was applied as a dust.

Experimental Procedure. Samples of six infected and three uninfected seed lines were treated with Thiram 80[x] by thoroughly shaking the seed with a slight excess of the dust which was later removed by screening through a wire mesh seive. The extent to which the fungicide reduced the inoculum load was determined by placing 200 seeds from

[x] IWD Thiram 80 (80% W/W)

each sample onto MA prior to incubation for 11 days at 24 C in the dark.

The significance of seed-borne inoculum in inducing damping-off was determined by comparing the emergence of dusted and non-dusted seeds of lines sown in Vermiculite. Emergence counts were made regularly until 19 days after sowing (except line OL175 which was recorded until 21 days after sowing).

Results and Discussion. The results summarized in Table XV indicate that in five of the six infected lines, thiram seed treatment gave improved emergence over that of the controls. However in all those lines not infected with P.medicaginis and also in one infected line, thiram seed treatment gave decreased emergence. Since the seed was sown in a sterile and inert medium, the improved emergence in the majority of the thiram dusted infected lines is interpreted as being consequent on inactivation of pathogenic inoculum associated with seed at the time of sowing. The decreased emergence in the thiram dusted uninfected lines was assumed to be expression of slight phytotoxicity by the fungicide. Thus it can be theorized that if the phytotoxicity factor was eliminated, the increase in emergence following reduction in the level of P.medicaginis would have been even greater.

Table XV. The effect of fungicidal seed treatment on emergence of lucerne seedlings.

Seed line code	Treatment	Level seed infection (percent)	No. seeds sown	Total no. seedlings emerged after 19 days	No. live plants after 19 days	Pre-emergence damping-off (percent)	Post-emergence damping-off (percent)	Total damping-off losses (percent)
259	Non-treated	37.0	200	147		+9.5		+9.5 [x]
	Thiram dusted	6.0	"	166				
d259	Non-treated	37.6	350	183	183	+8.6	0	+8.6
	Thiram dusted	8.0	"	213	213		0	
263	Non-treated	22.0	300	174	158	+4.7	5.3	+10.0
	Thiram dusted	3.5	"	188	183		1.7	
38	Non-treated	20.0	200	175		+2.0		+2.0 [x]
	Thiram dusted	0	"	179				
181	Non-treated	11.0	300	205		+2.7		+2.7 [x]
	Thiram dusted	3.0	"	213				
272	Non-treated	12.5	300	236		-3.0		-3.0 [x]
	Thiram dusted	1.0	"	227				
173	Non-treated	0	300	206		-0.3		-0.3 [x]
	Thiram dusted	0	"	205				
266	Non-treated	0	300	268		-0.7		-0.7 [x]
	Thiram dusted	0	"	266				
175	Non-treated	0	200	146		-8.0		-8.0 [x]
	Thiram dusted	0	"	130				

[x] Damping-off losses calculated from pre-emergence figures only.

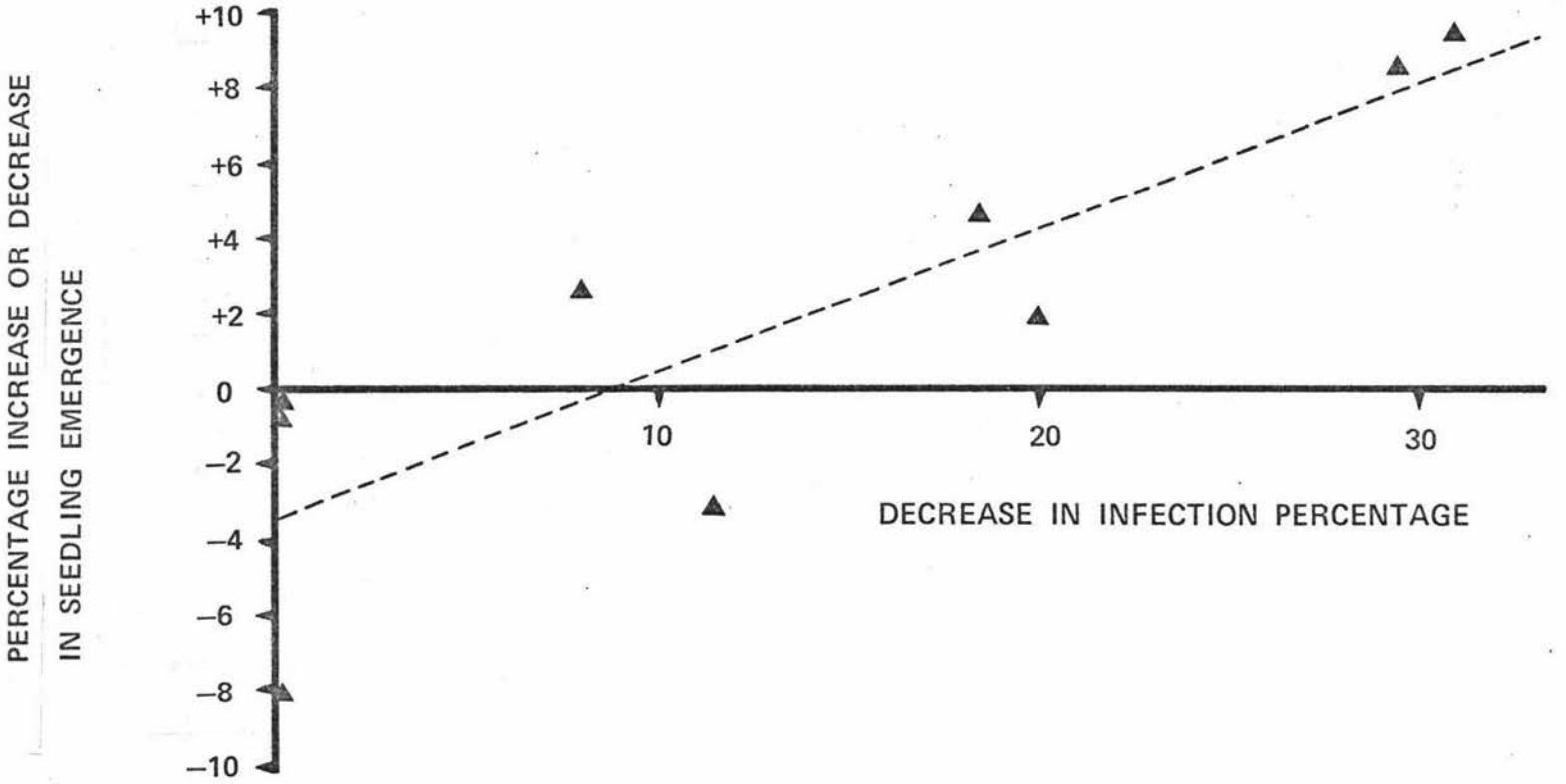
Under the circumstances of this experiment pre-emergence, rather than post-emergence damping-off was the most important phase of the disease. In no one seed line did a significant number of the emerged seedlings subsequently collapse.

In all seed lines analysis of variance applied to the difference in emergence between treated and non-treated seed did not indicate statistical significance. Thus interpretation of the results must be confined to a discussion of trends observed.

In considering the results (Table XV), a trend is apparent in that the magnitude of the increase or decrease in seedling emergence in any one line was dependent on the extent to which the infection percentage was decreased following thiram seed treatment. This relationship is illustrated graphically in Fig. 22. A regression line has been fitted to the available data (details are presented in Appendix VI), but no statistical tests of significance of the regression coefficient were possible because it is unclear whether or not the relationship between the two parameters is linear. Further, regression data based on percentage changes is considered unsuitable for analysis in that form (Searle, 1971). Nevertheless the regression line does illustrate a trend, thus allowing the dual effects of thiram on seedling emergence to be expressed on one graph. That is, increases in emergence are attributed to a reduction in the level of

P. medicaginis associated with the seed, while decreases in emergence are a function of fungicidal phytotoxicity on the unemerged seedlings.

Figure 22



Graph illustrating effect of thiram seed treatment on seedling emergence

Seed samples of both treatments from five of the nine lines under study were submitted to the Government Seed Testing Station, Palmerston North, New Zealand, for germination tests in order to ascertain if the changed emergence pattern could be detected. The results were inconclusive because there appeared to be no relationship between the emergence and final germination figures. Further, the germination percentage failed to reveal phytotoxicity of thiram towards lucerne seed.

In addition to the studies on absolute emergence, records were kept on the rate of seedling emergence until 19 days from sowing. These are presented graphically in Fig. 23 a-i, and indicate that trends observed with total (absolute) emergence were generally present from when the first counts were made. That is, if thiram seed treatment gave increased emergence 19 days after sowing then a similar result was usually observed from when the first seedlings appeared, and vice versa.

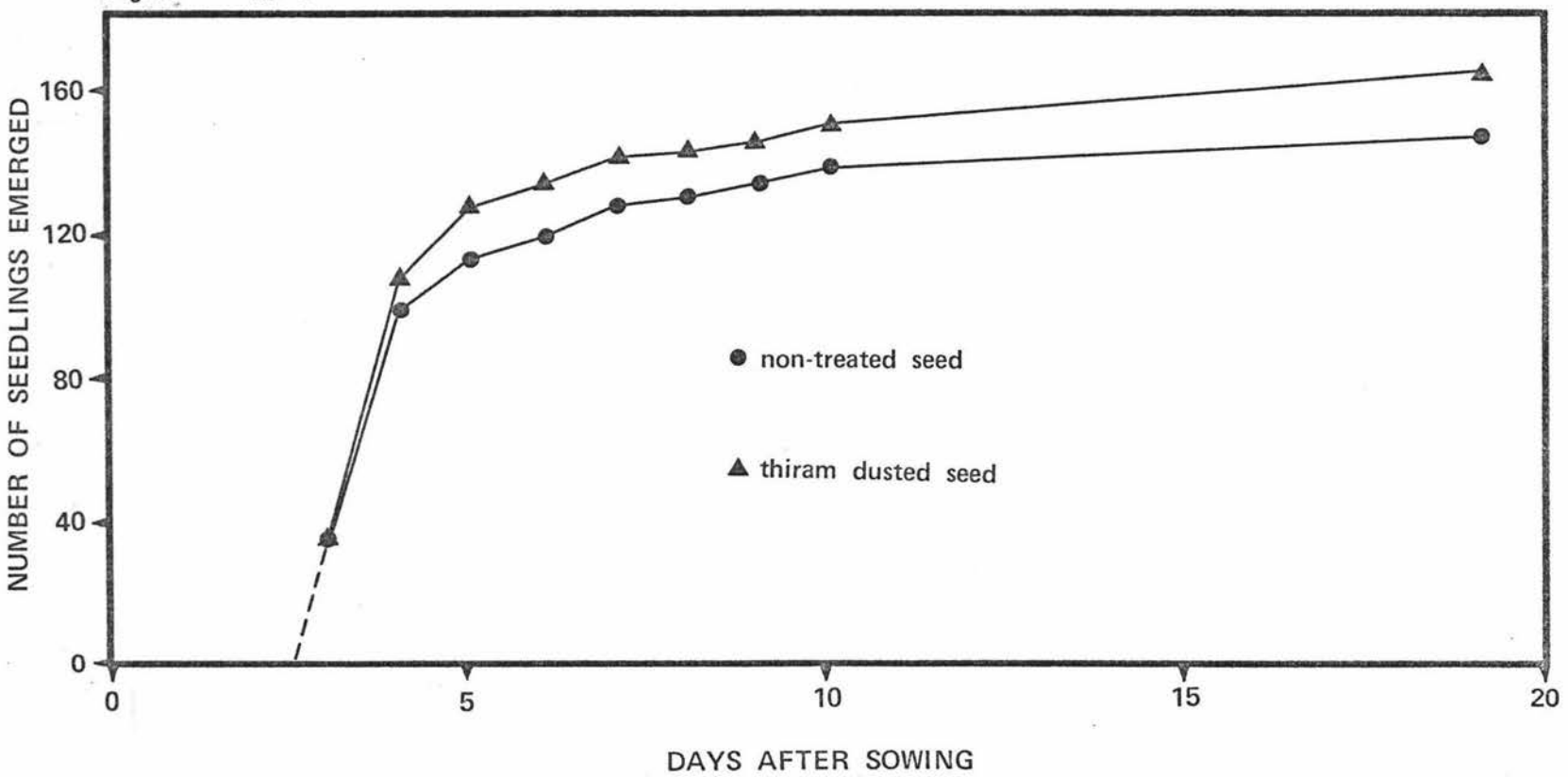
While the obvious benefit accruing from control of the pathogen is expressed as increased emergence, further benefit is probably associated with the increase in the emergence rate. One may speculate that competition with weed species for light, nutrients, and water by those seedlings exhibiting more rapid growth would give the latter a competitive advantage over slower growing seedlings, and as a consequence a greater chance of developing into mature plants.

The results obtained in the present study support the conclusion of earlier workers that seed-borne inoculum of P.medicaginis is capable of causing both pre-emergence and post-emergence damping-off (Cormack, 1945, Kernkamp and Homerick, 1953; Mead, 1953; Latch and Greenwood, 1964), while bearing in mind that the results from two of these studies must be considered with caution.

Firstly, the conclusion reached by Mead (1953) was the result of studies using surface sterilized seed artificially inoculated with a pycnidiospore suspension. His emergence results are difficult to evaluate since in the field situation the pathogen is located as dormant mycelium in the tissues of the testa, rather than as surface-borne pycnidiospores. A further point is that in Mead's studies the inoculum load was likely to be greater than that of naturally infected seed, thereby enhancing the degree of damping-off. It was for these two reasons that in the present study, naturally infected rather than artificially inoculated seed was used in all emergence experiments.

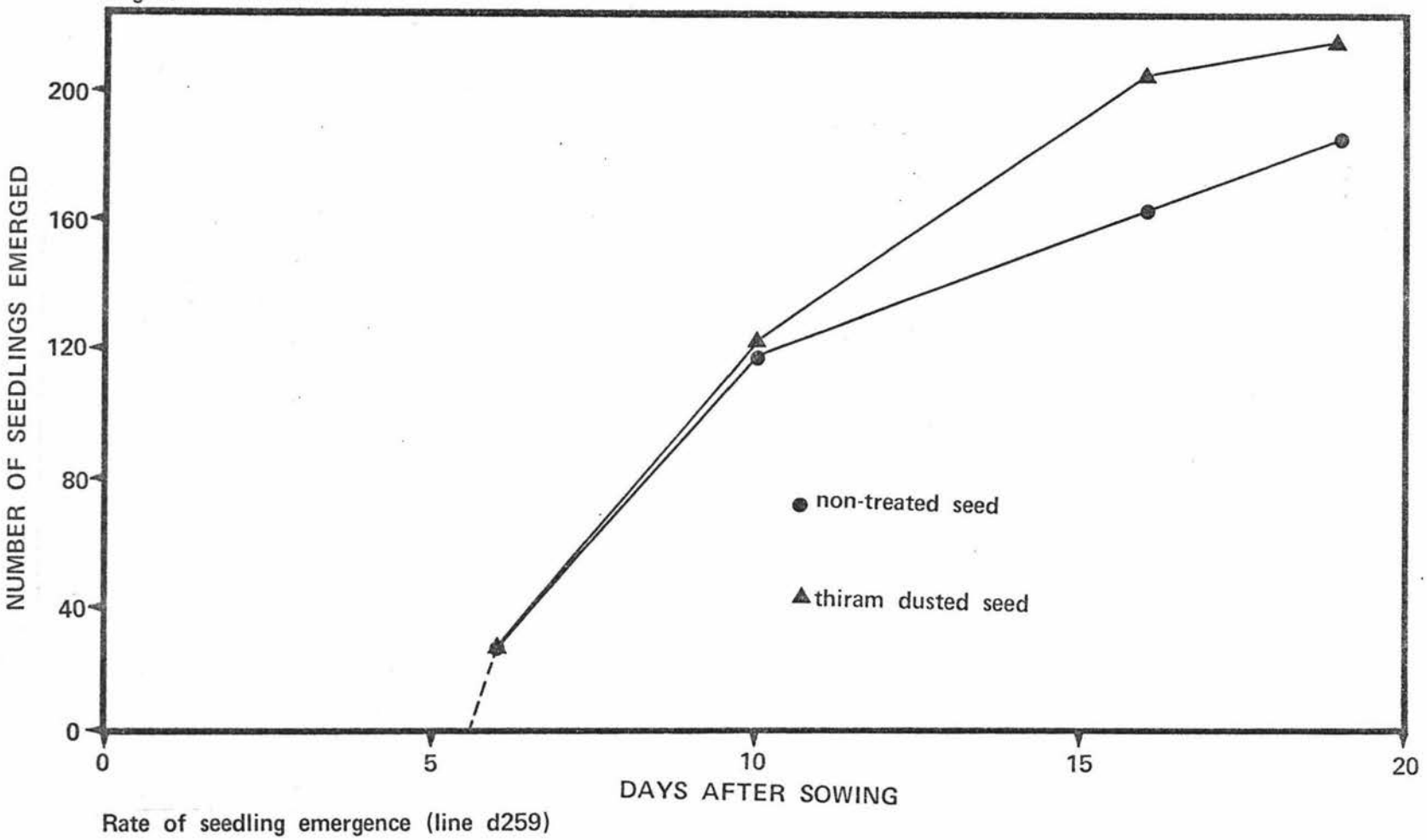
The emergence experiments conducted by Kernkamp and Homerick (1953) were also unsoundly based, since the emergence of five infected seed lines was compared without taking into account genetic differences in the germination capacity of each line when free of the pathogen. In the present study this factor has previously been acknowledged to be of considerable importance (P. 96).

Figure 23 a



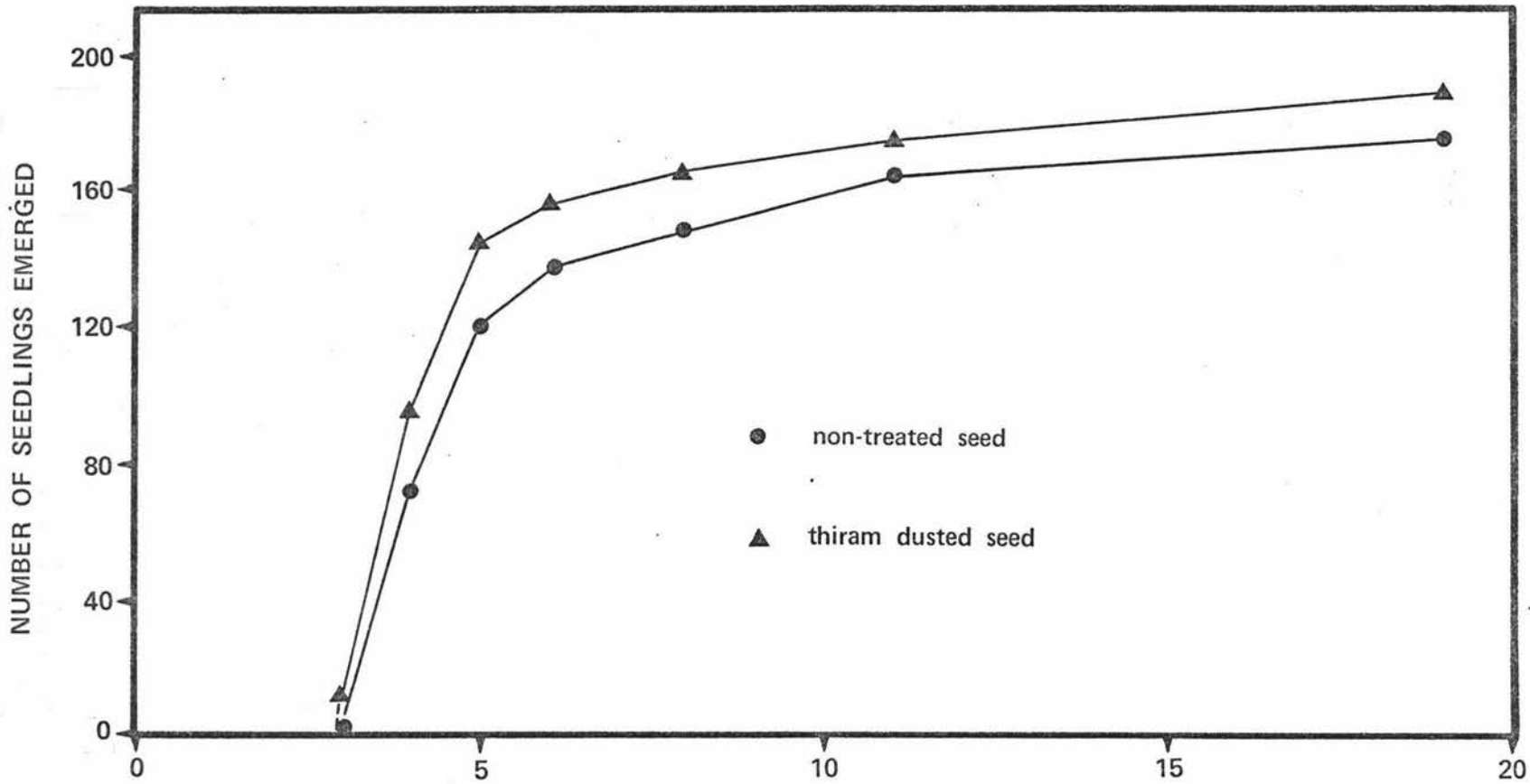
Rate of seedling emergence (line OL259)

Figure 23 b



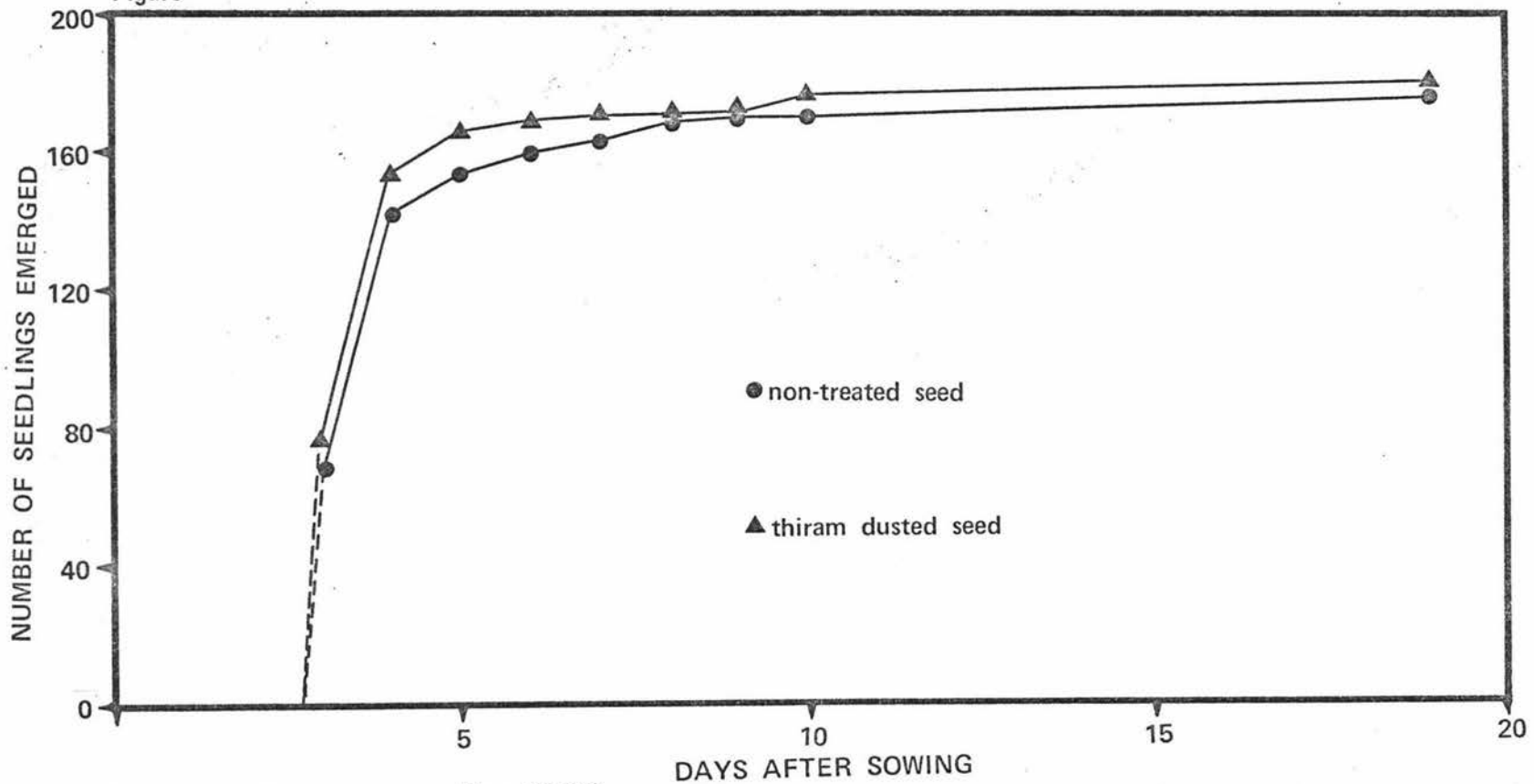
Rate of seedling emergence (line d259)

Figure 23 c



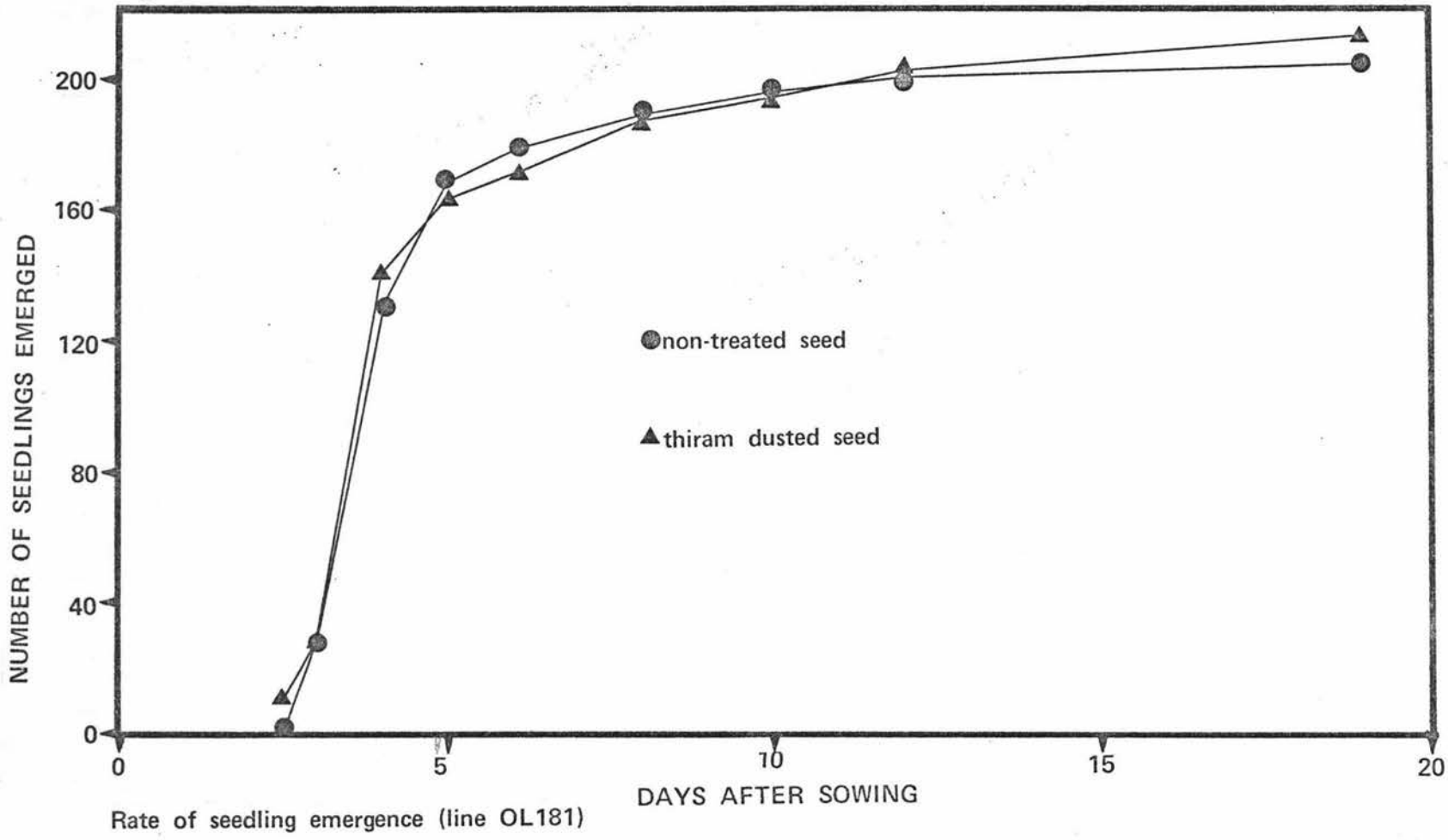
Rate of seedling emergence (line OL263)

Figure 23 d



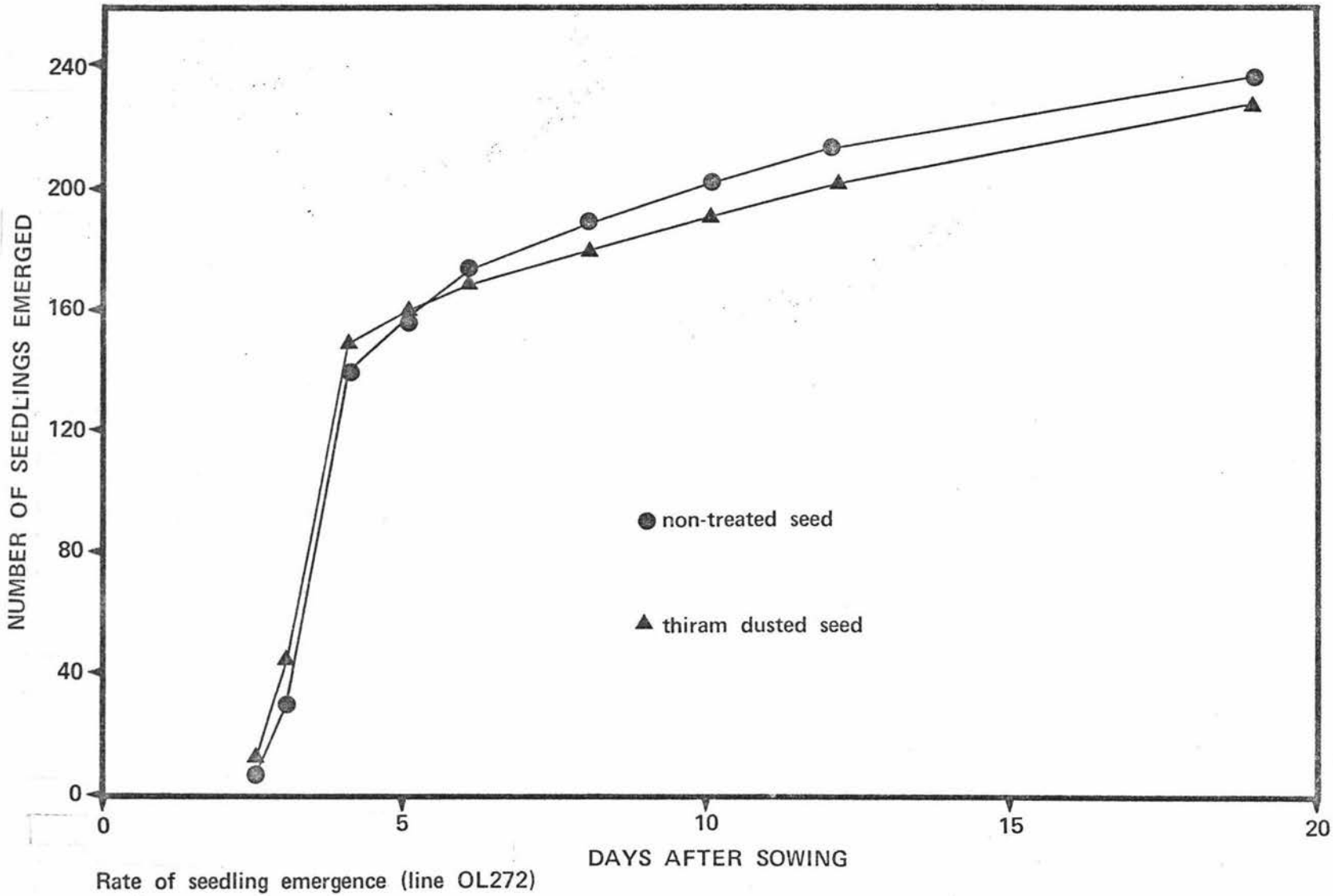
Rate of seedling emergence (line OL38)

Figure 23 e



Rate of seedling emergence (line OL181)

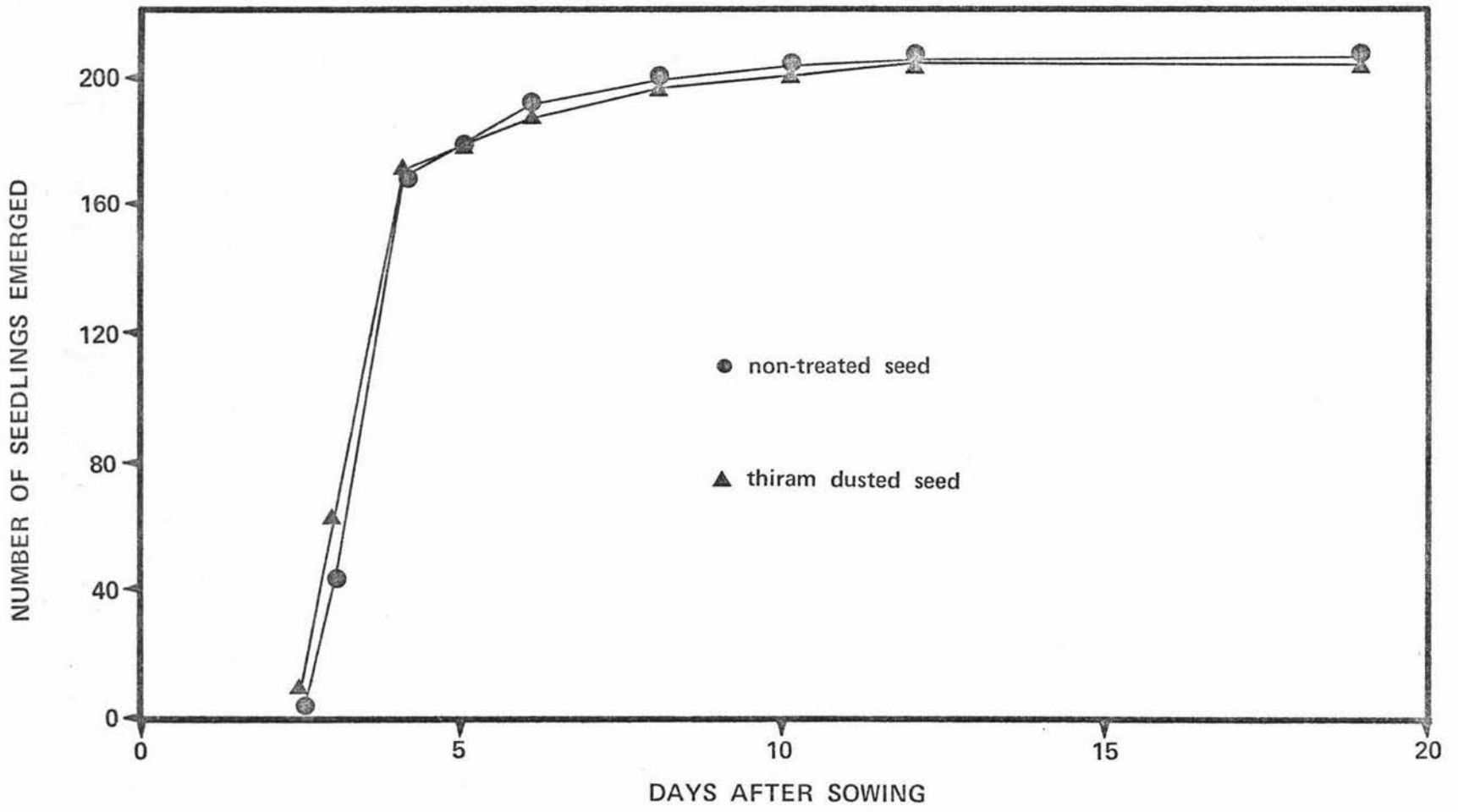
Figure 23 f



Rate of seedling emergence (line OL272)

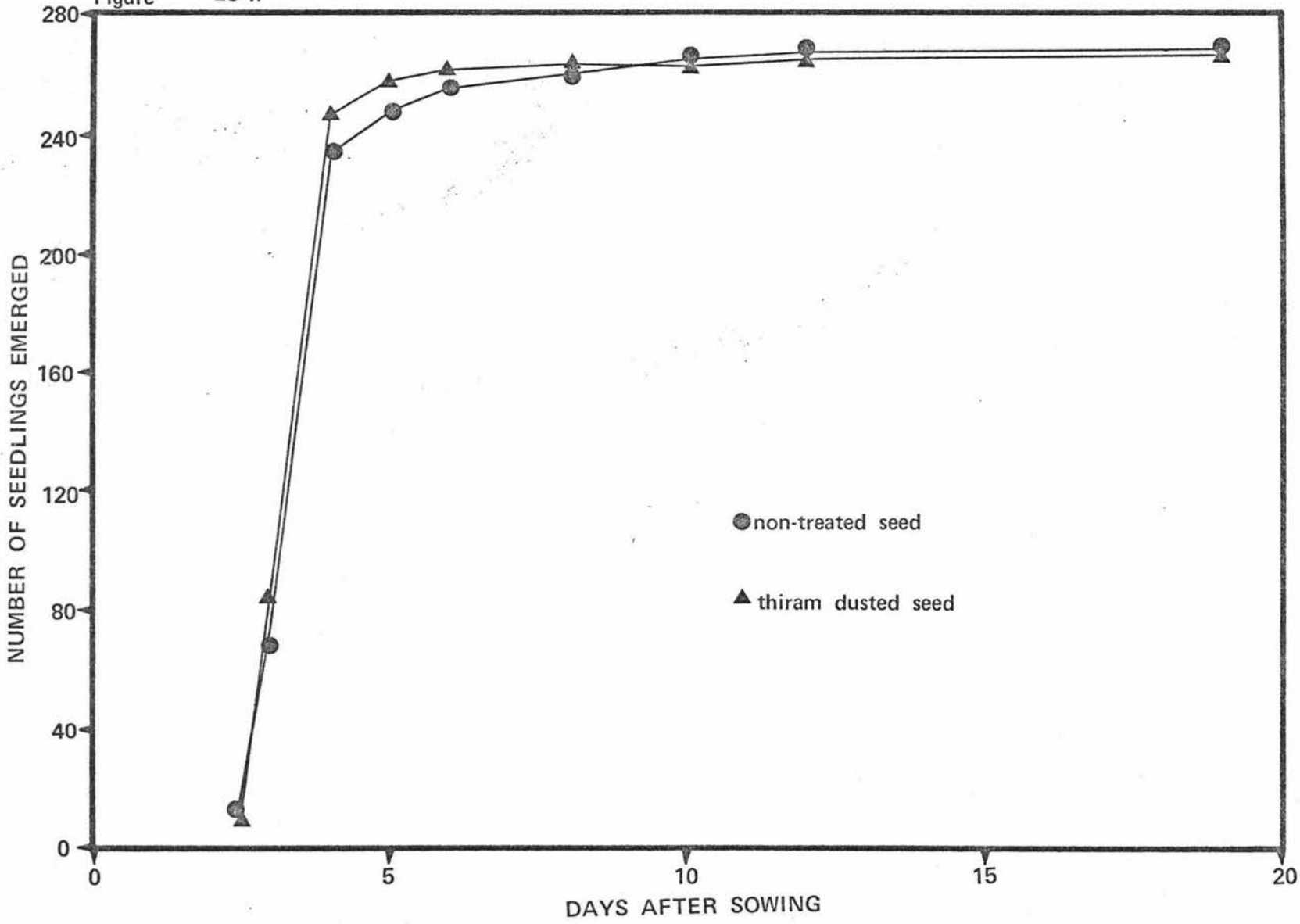
104f

Figure 23 g



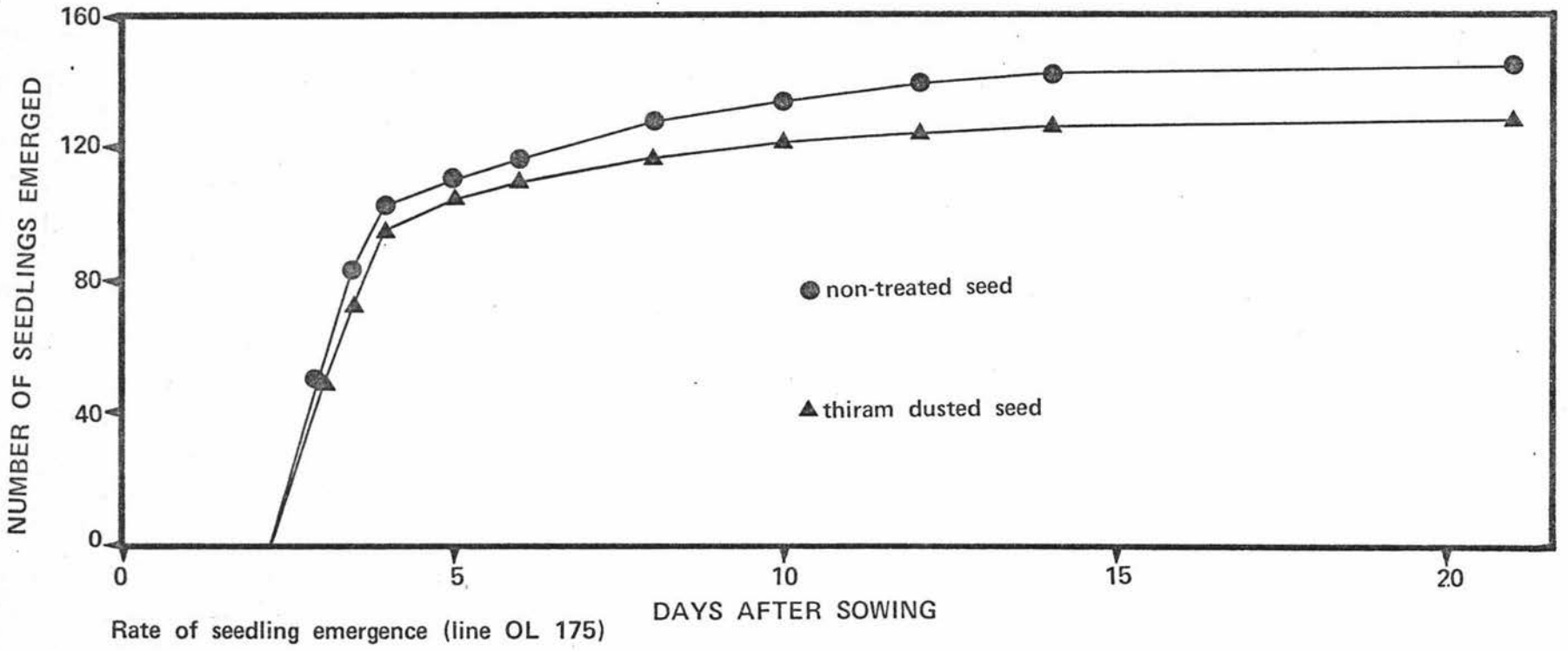
Rate of seedling emergence (line OL173)

Figure 23 h



Rate of seedling emergence (line OL266)

Figure 23 i



Rate of seedling emergence (line OL 175)

(b) Effect of Temperature on Damping-off Induced by Seed-Borne Inoculum of P.medicaginis.

Results from the previous section clearly showed that a reduction in the level of P.medicaginis associated with seed led to a decrease in the degree of damping-off. However since in all instances the percentage increase in emergence (following seed treatment) was less than the decrease in percentage infection of the seed lines, it was clear that all infected seeds did not necessarily express damping-off. This apparent failure of all the inoculum detectable in routine seed health tests to induce damping-off can be attributed to either or both of the following factors:

(1) A reasonable assumption is that there is variation in the inoculum load associated with individual seeds. Thus one can visualize the situation where in some seeds the inoculum load (inoculum potential) would be insufficient to induce seedling collapse.

(2) The environmental conditions prevailing during the trials were such that the pathogen was limited in its ability to cause seedling disease. Studies conducted on environmental relationships using seedlings (Cormack, 1945) and mature plants (Rosemblit, 1950; Roberts, 1957; Mead, 1963) have demonstrated the fungus was most pathogenic under conditions

of nearly continuous moisture and at temperatures ranging from 12 C to 15 C.

In the present study experiments were conducted to determine the effect of temperature on the degree of damping-off consequent on the use of seed infected with P.medicaginis.

Experimental Procedure. Samples of thiram dusted and non-treated seed (400 seeds/treatment) from one line were sown in Vermiculite (grade two) by the method previously described (P.94). Each series was then placed in rooms held at the following temperatures for the duration of the experiment: 6 C; 10 C; 14 C; 17 C; 20 C; 25 C. These temperatures were selected following a study of the relevant New Zealand literature which revealed the average soil temperature at two inches over the year to range from 0.5 C to 21 C (Gabites, 1967, 1968, 1970). Additional figures of importance were the average two inch soil temperatures for September, October, and November (the three months in which most lucerne is sown) of 6.2 C, 10.5 C, and 13.3 C respectively.

The temperature of the six rooms was monitored using thermographs and maximum/minimum thermometers. These showed the temperature of all rooms was relatively steady with a maximum deviation of 0.5 C to 1 C from the mean.

All treatments were regularly steeped in trays of water to ensure that the moisture level of the potting medium was always close to its moisture holding capacity.

The effectiveness of the thiram seed treatment was again determined by the agar plate test. The dusted seed had an infection level of 3.5% whereas in the non-treated control the pathogen was detected in 22% of the seeds.

Regular counts were taken of the number of emerged and live seedlings. Each experiment was terminated when only a few seedlings were appearing between counts.

In this series of experiments it was important that interpretation of results be on the basis of an interaction between the two living systems involved, namely the host seed and associated pathogenic inoculum of P.medicaginis. Thus it was essential that the response of the fungus when grown on agar media first be known. Accordingly, as a preliminary experiment the effect of temperature on growth of three monosporous isolates from the seed line under study was determined. These results are presented in the form of temperature/growth histograms in Figure 9.

Results and Discussion. (1) Degree of pre-emergence damping-off

The effect of soil temperature on the degree of pre-emergence damping-off using thiram treated seed is illustrated graphically in Fig. 24 a-f. Since the response to temperature was not linear, interpretation was made with regard to both living entities.

Tests of significance on the data at individual points in time did not reveal statistically significant differences. Hence discussion of the results is again restricted to an evaluation of the trends represented by the two curves at each temperature.

At 6 C the emergence from both dusted and non-treated seed was relatively slow with the first seedlings appearing approximately 17 days after sowing. The decreased emergence consequent on thiram seed treatment can only be interpreted as reflecting thiram phytotoxicity, since little growth of the pathogen on the host would be expected at this temperature in view of its slow growth on agar media at 6 C. This assumes that growth on agar media at a particular temperature provides some indication of growth on the host tissues at that temperature.

The greatest increase in emergence consequent on thiram seed treatment occurred with those seedlings grown at 14 C. Thus at this temperature either the causal fungus was most pathogenic or the seedlings were most susceptible to infection. The pathogen was previously reported to be most pathogenic to both seedlings (Cormack, 1945) and mature plants (Mead, 1963) at a similar temperature. It is reasonable to assume that P.medicaginis would be pathogenic at 14 C, since although its growth on agar media at this temperature was approximately half of that exhibited at 21 C it is probable that its growth on host tissues (at 14 C) would still be

sufficient to cause infection. Mead (1962b, 1963) has attempted to explain the high degree of infection at 14 C in terms of increased susceptibility through the different proportions of carbohydrates and amino acids favouring the pathogen at the expense of the plant.

At 10 C and 17 C the difference in emergence between the thiram dusted and non-treated seed was small. This is interpreted as being manifestation of a balance between phytotoxicity of thiram and reduction in the level of P.medicaginis giving rise to a decrease and increase respectively in seedling emergence. Thus if the phytotoxicity factor was eliminated, a larger increase in emergence in the thiram dusted treatment over the non-treated controls would be expected.

At 20 C and 25 C thiram seed treatment eventually gave increased emergence but this advantage was not evident until ten days after the seed was sown. In interpreting these results it must be noted that the velocity of emergence was relatively rapid, with greater than 50% emergence occurring within five days of sowing. Thus even if P.medicaginis was highly pathogenic at these temperatures, it is probable that it would not have sufficient time to cause pre-emergence damping-off of those seedlings that rapidly emerged. The concept of velocity of emergence has previously been used to explain why high temperature crops are more subject to pre-emergence damping-off at low rather than at high temperatures, whereas low temperature crops often suffer less infection than

at intermediate or high temperatures (Leach, L.D., 1947).

The figures illustrating differences in emergence patterns following thiram seed treatment at various temperatures can also be considered in terms of the time to achieve 50% seedling emergence (T.A.E. 50). These results are presented in Table XVI.

Table XVI. The relationship between soil temperature and the time to achieve 50% seedling emergence from thiram dusted and non-treated seed.

Soil temperature (C)	Time to achieve 50% emergence (days)	
	Thiram-dusted seed	Non-treated seed
6	39.6	37.0
10	24.5	28.0
14	14.6	17.4
17	9.6	10.8
20	5.3	5.5
25	4.9	4.9

Seedling phytotoxicity at 6 C was clearly expressed in that the T.A.E. 50 for thiram dusted seed was 2.6 days longer than that of the non-treated seed. At 14 C the T.A.E. 50 was 2.8 days longer with the non-treated seed. That is, the reduction in the level of P.medicaginis consequent on seed treatment with thiram led to an increased rate of emergence. At 17 C the thiram seed treatment slightly decreased the T.A.E. 50. Thus for the 6 C, 14 C, and 17 C series this method of considering the results was in general agreement with the emergence curves. However the differences discernable at 10 C, 20 C, and 25 C did not accurately reflect the results presented in the rate of emergence curves for these temperatures. The differences in the

T.A.E. 50 at 20 C and 25 C were small or non-existent. This was attributed to the more rapid velocity of emergence at these temperatures not allowing sufficient time for any differences to be manifest. At 10 C the thiram dusted seed emerged at a faster rate than the non-treated seed. Some increase in emergence was expected at this temperature but the extent of the difference was greater than in the rate of emergence curves. No explanation can be offered to account for this result.

In the authors opinion the above method of presenting data is inferior to rate of emergence curves because the latter method allows one to consider results from the duration of the experiment, whereas the T.A.E. 50 only expresses the situation at one point in time. As a consequence, in later emergence experiments interpretation was based on rate of emergence curves.

(2) Degree of post-emergence damping-off.

The effect of environmental temperature on the degree of post-emergence damping-off is summarized in Table XVII. The losses for both the thiram seed treatment and the non-treated control were again calculated from the difference between the number of emerged (living and dead) and the number of living seedlings at any one point in time. The degree of post-emergence damping-off attributable to seed-borne inoculum of the pathogen was the difference between the losses calculated for the non-treated control and the thiram seed treatment.

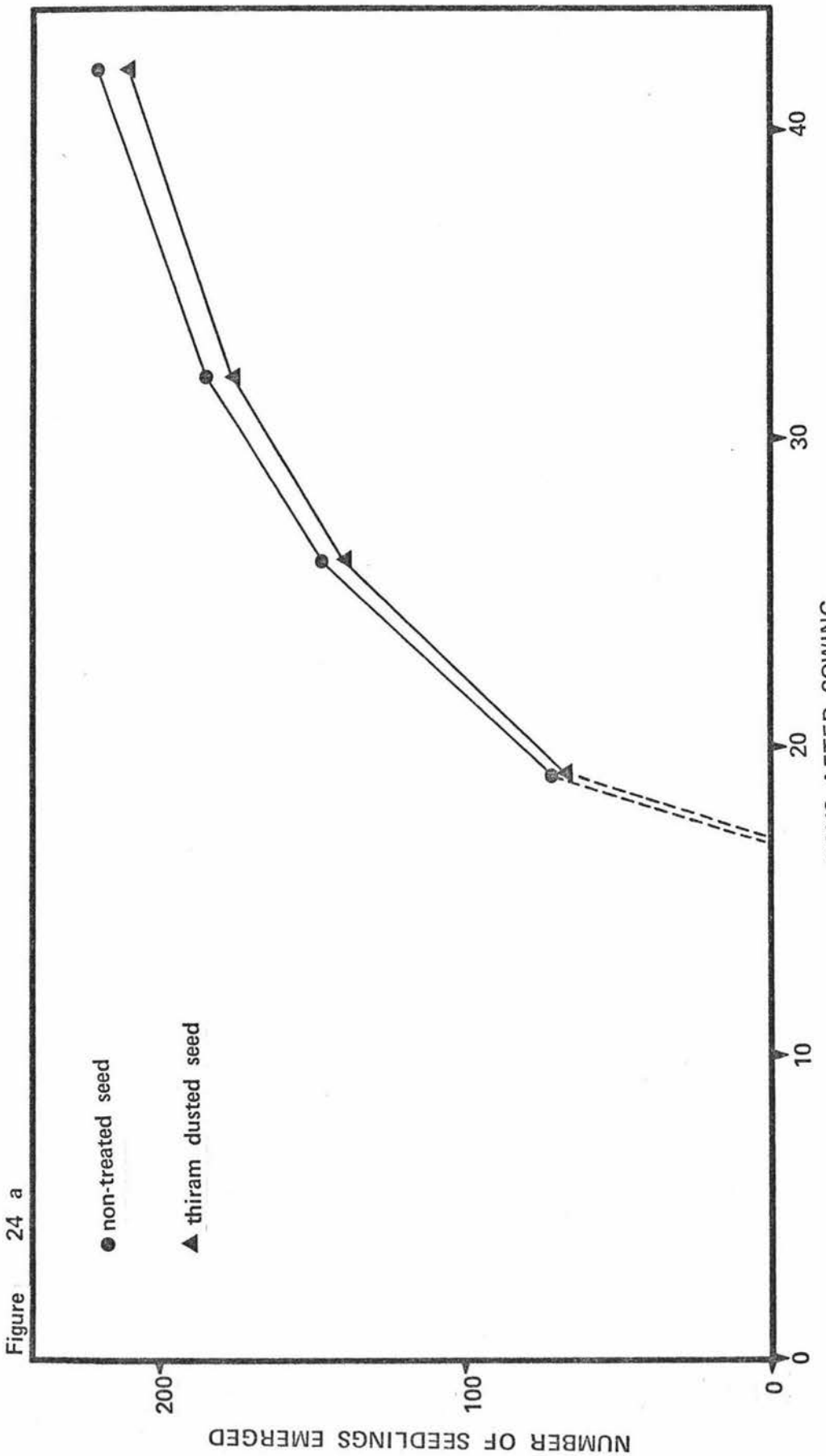
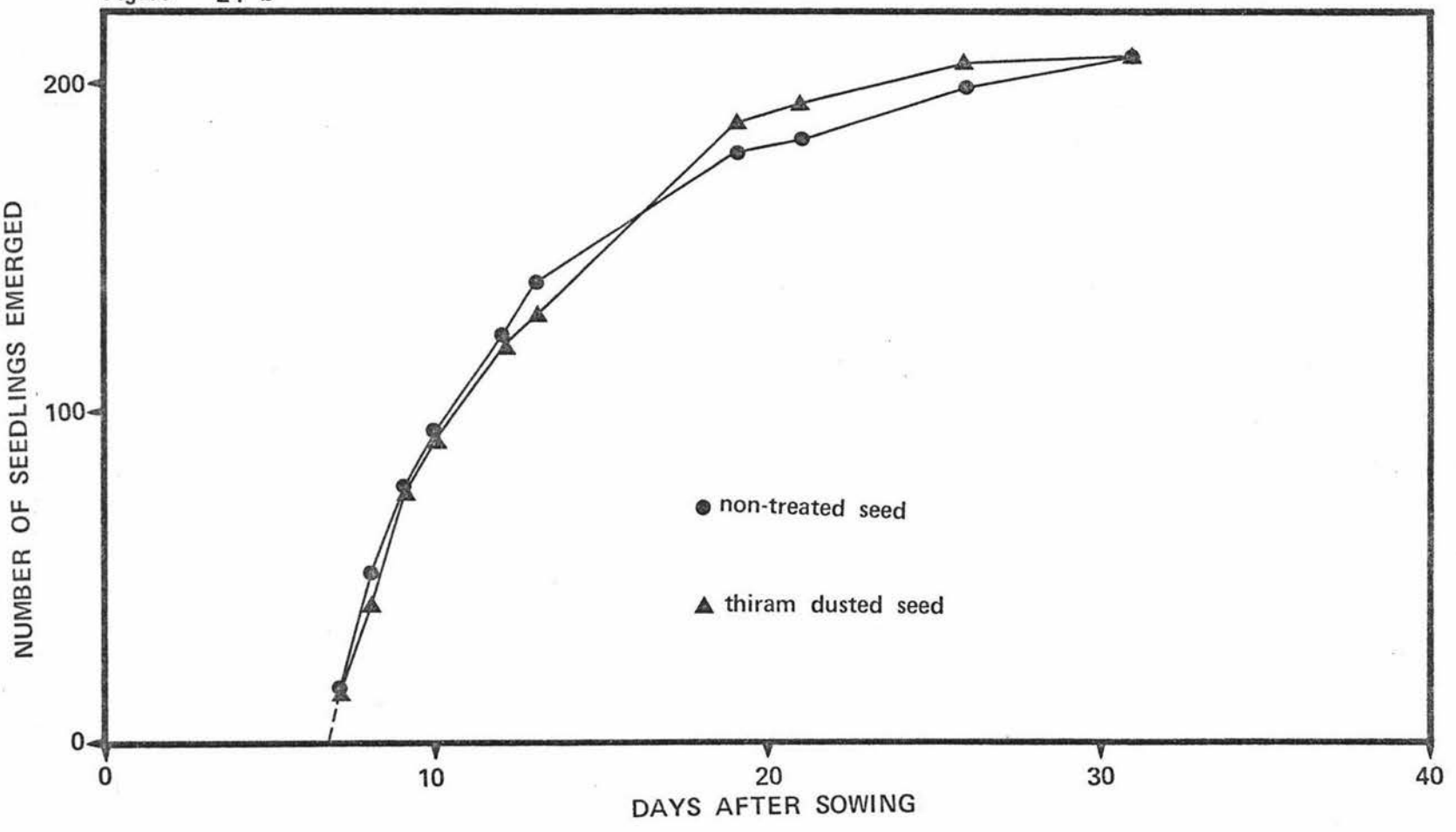


Figure 24 a

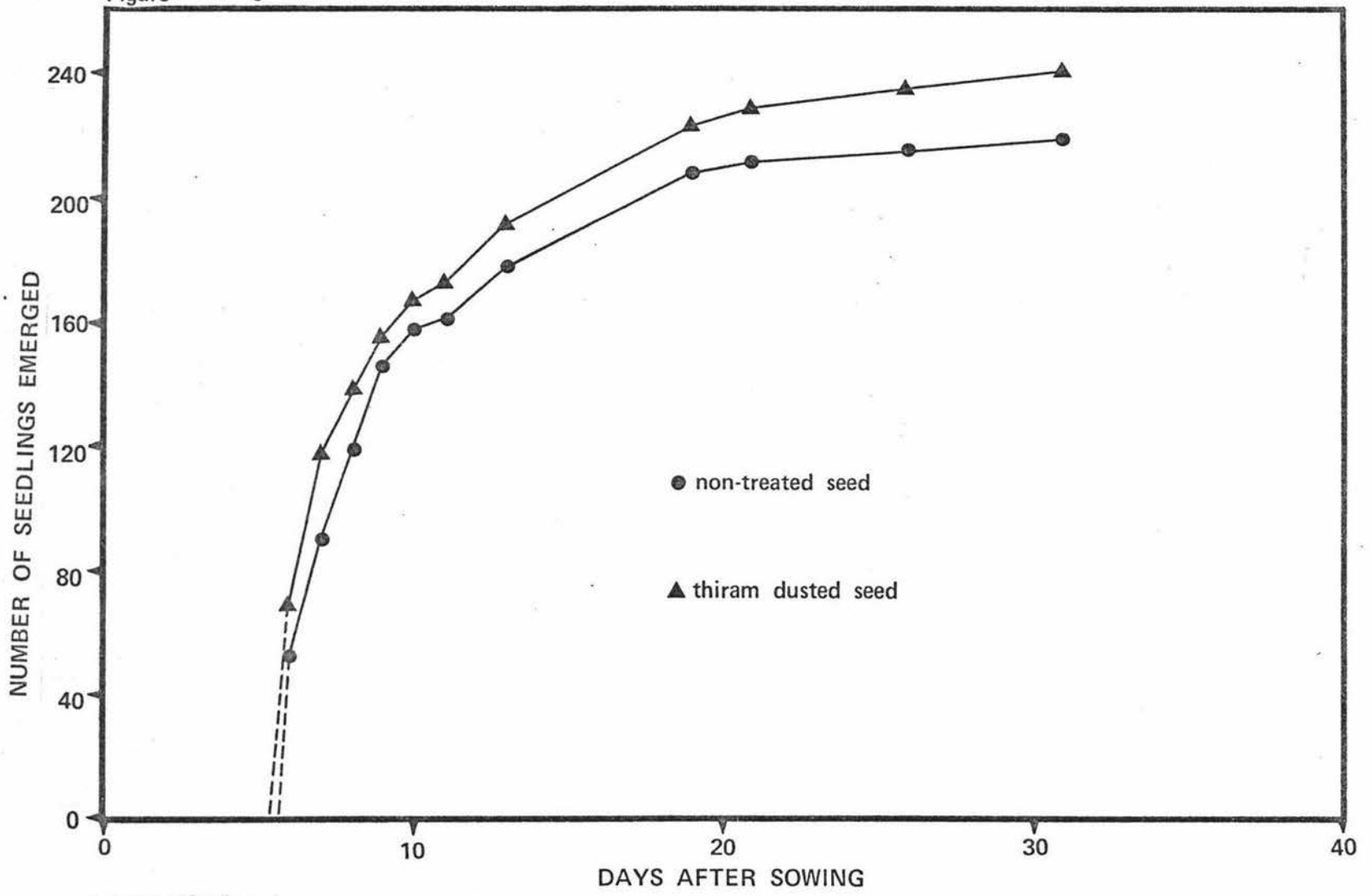
Rate of seedling emergence at 6°C

Figure 24 b

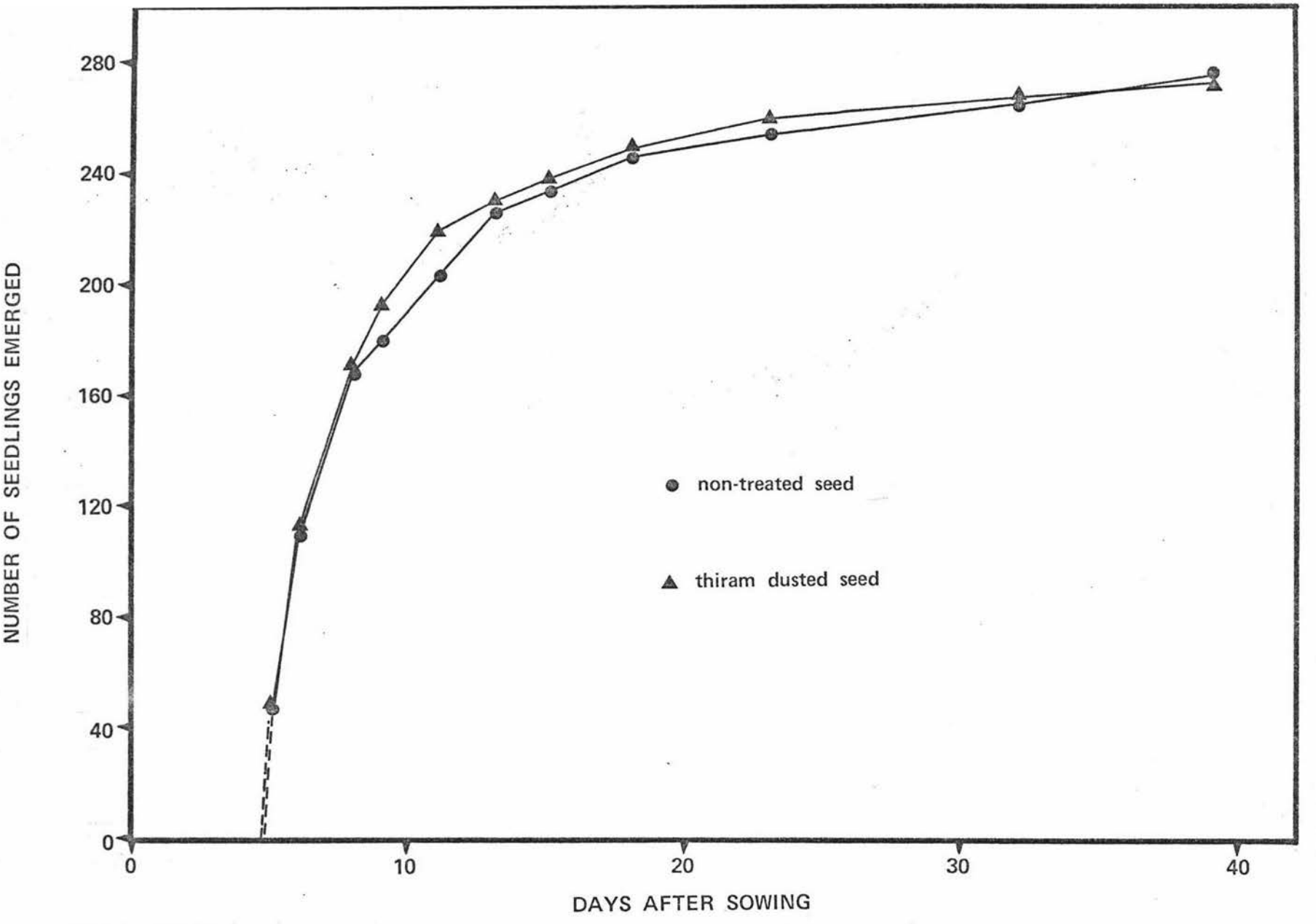


Rate of seedling emergence at 10°C

Figure 24 c

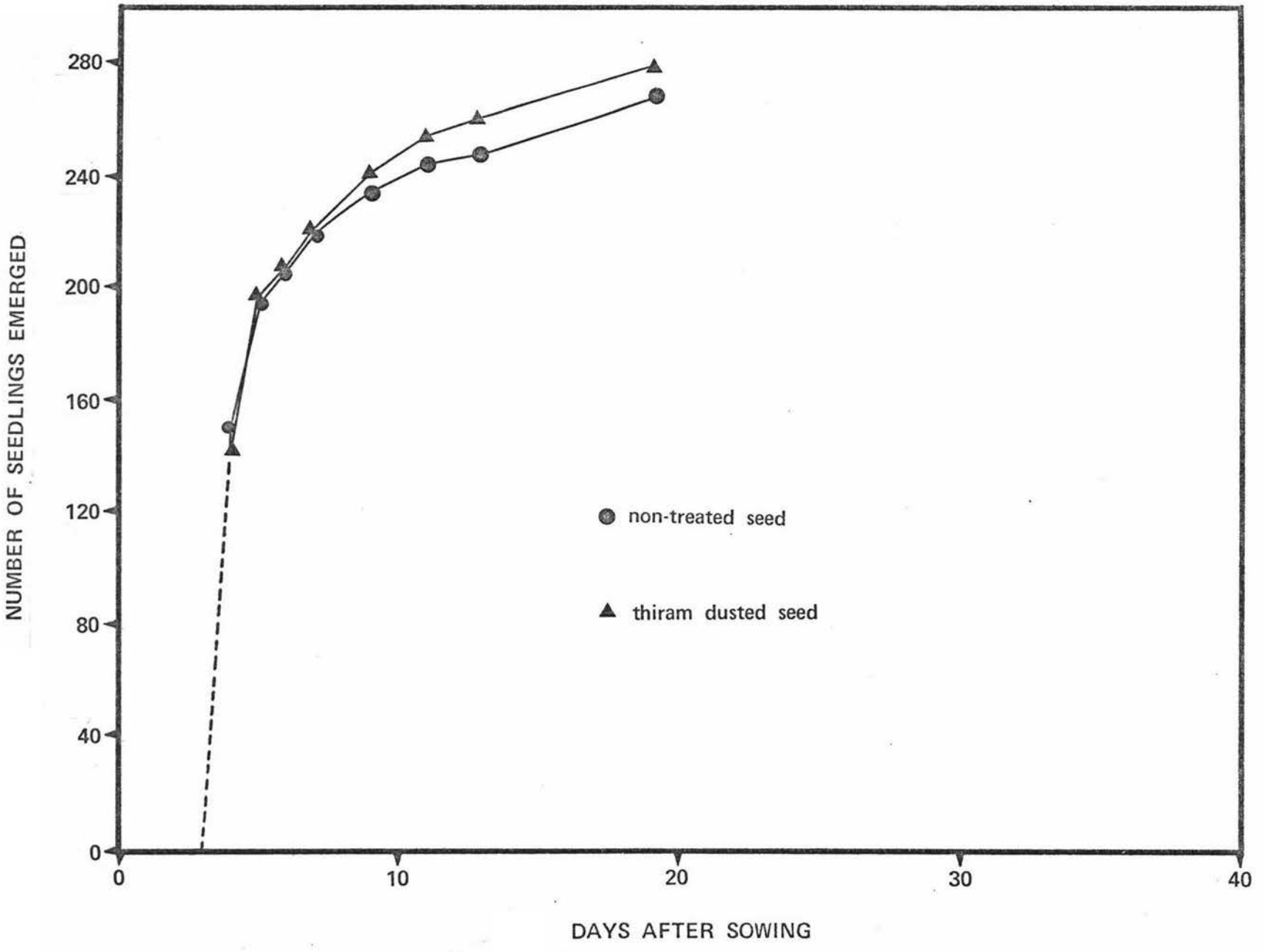


Rate of seedling emergence at 14°C



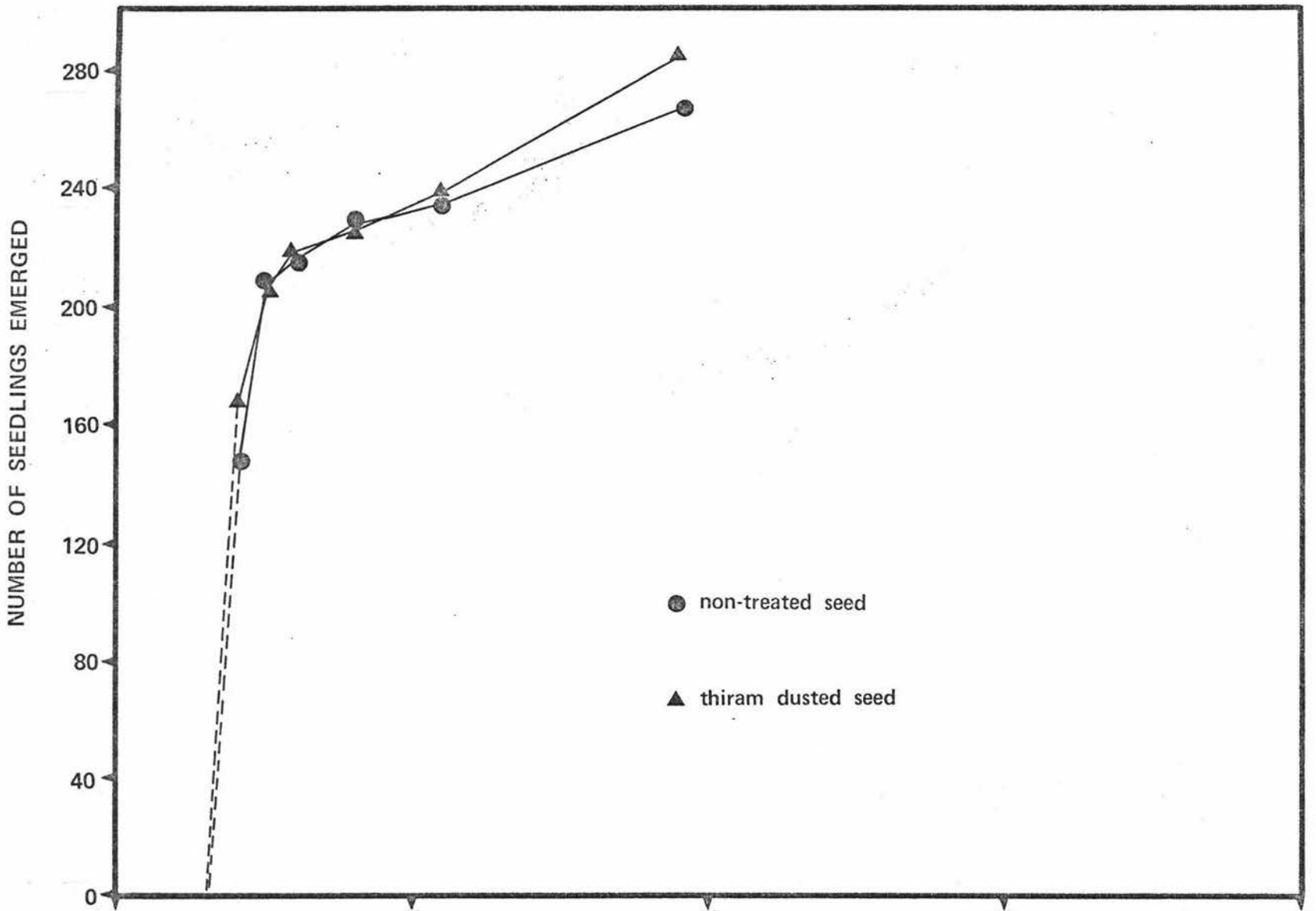
Rate of seedling emergence at 17°C

Figure 24 e



Rate of seedling emergence at 20°C

Figure 24 f



Rate of seedling emergence at 25°C

The extent of post-emergence damping-off at 25 C was greater than at any other temperature, with most seedling collapse occurring in the last eight days of the experiment. This tends to support the earlier contention that the lack of early expression of pre-emergence damping-off at 25 C (P.109) was consequent on the pathogen not having had sufficient time to attack the unemerged seedlings. That is, the degree of post-emergence damping-off at 25 C reflects the ability of lucerne seedlings to outgrow P.medicaginis in the ten days immediately following germination, the pathogen only by then having had sufficient time to induce seedling collapse.

Some post-emergence damping-off also occurred at 10 C, which was expected since some growth was exhibited by P.medicaginis on agar media at this temperature.

At 6 C no post-emergence damping-off occurred. This result was predictable because of the inability of the pathogen to grow at this temperature.

Post-emergence damping-off did not occur at 17 C and 20 C. No explanation can be offered for the apparent lack of pathogenicity at these temperatures.

Table XVII. Post-emergence damping-off attributable to P.medicaginis in relation to environmental temperature.

Environmental temperature (C)	Seed treatment	No. seedlings emerged	No. live seedlings	No.days after sowing	Post-emergence damping-off attributable to P.medicaginis
6	Thiram dusted	208	208	42	
	Non-treated	218	218		0
10	Thiram dusted	206	195	31	
	Non-treated	206	184		11
14	Thiram dusted	242	x	31	
	Non-treated	220	x		x
17	Thiram dusted	273	268	39	
	Non-treated	276	274		- 3
20	Thiram dusted	280	280	19	
	Non-treated	269	269		0
25	Thiram dusted	283	276	19	
	Non-treated	265	242		16

x Post-emergence damping-off could not be calculated for either the thiram dusted or non-treated seed at 14 C because an insect attack on large numbers of emerged seedlings made accurate recording impossible.

(c) Overall Discussion.

In a similar study in Canada using infected lucerne seed (dusted and non-treated), Cormack (1945) observed that the degree of pre-emergence damping-off was greater at 11 C and 21 C than at 17 C, a result in general agreement to those of the present study. However Cormack also reported that post-emergence damping-off did not occur above 17 C, which conflicts with the results of the present study. The data presented by Cormack must be viewed with caution as no mention was made of the duration of his experiments. That is, Cormack's experiments may have been terminated before the pathogen had sufficient time to induce seedling collapse.

In considering the total data obtained from these experiments it is apparent that the degree of pre-emergence and post-emergence damping-off was markedly influenced by environmental temperature. Thus it is reasonable to assume that the differences in emergence observed with commercial seed lines following seed treatment (P. 98) would have been greater had the experiments been conducted at a soil temperature of 14 C.

Although it was apparent that temperature could markedly effect the degree of both pre-emergence and post-emergence damping-off, in no case did more than half of those seeds infected with P. medicaginis express the disease. Because of the limited nature of these experiments it was not possible to draw the conclusion that only some of those infected seeds detected in agar plate tests had an inoculum load

sufficiently great to cause damping-off. That is, the possibility remains that under certain environmental conditions all the inoculum detectable could cause either pre-emergence or post-emergence damping-off.

It must be explained that the above results were obtained from glasshouse studies using an inert, sterile potting medium and do not necessarily apply to the field situation. To confirm that seed-borne inoculum of P.medicaginis does significantly affect stand establishment in the field, requires a series of outdoor trials.

A further point to be stressed is that the present studies have been solely concerned with the degree of seedling loss consequent on the use of infected seed. One may assume that seed-borne inoculum of P.medicaginis is also of significance in providing primary infection foci from which secondary infection cycles may develop. It follows that inactivating seed-borne inoculum may benefit a crop by improving stand establishment, and by delaying the establishment of primary infections.

IV. MECHANISM INVOLVED IN SEEDLING ATTACK.

In moist blotter seed health tests using infected seed it was apparent that the radicle, hypocotyl, and cotyledons could become infected. Further, it was observed that such infections occurred in the following manner:

(a) Radicle. First positive evidence of germination is the emergence of the radicle tip. At this early stage the radicle and testa (site of seed infection) are in close contact, allowing the possibility of cross infection to the radicle. Such infection is manifest as a general browning of the radicle tip (Fig. 25), with pycnidia sometimes developing on the lesioned area. The radicle may also be infected by a second method; as germination proceeds the testa usually becomes free of the cotyledons and is cast aside, but on some occasions may remain attached to the seedling, clasping the radicle. Cross infection may again occur, inducing general browning of that region in direct contact with the testa (Fig. 26). Again, pycnidia may develop on the lesioned tissues. Neither of these radicle symptoms are very common.

(b) Hypocotyl. In some instances the testa may clasp the hypocotyl after becoming free of the cotyledons, allowing cross infection to occur. The resultant lesions are usually dark brown or black (Fig. 27 & 31) and pycnidia may sometimes develop on these infected areas. Hypocotyl infection was more common than radicle infection.

(c) Cotyledons. Lucerne seed exhibits epigeal germination, the testa being carried aloft on the cotyledons. This allows cross infection from the testa to either or both cotyledons, followed by the development of several black lesions which frequently coalesce (Fig. 28). This type of seedling infection was equally as common as the hypocotyl infection.

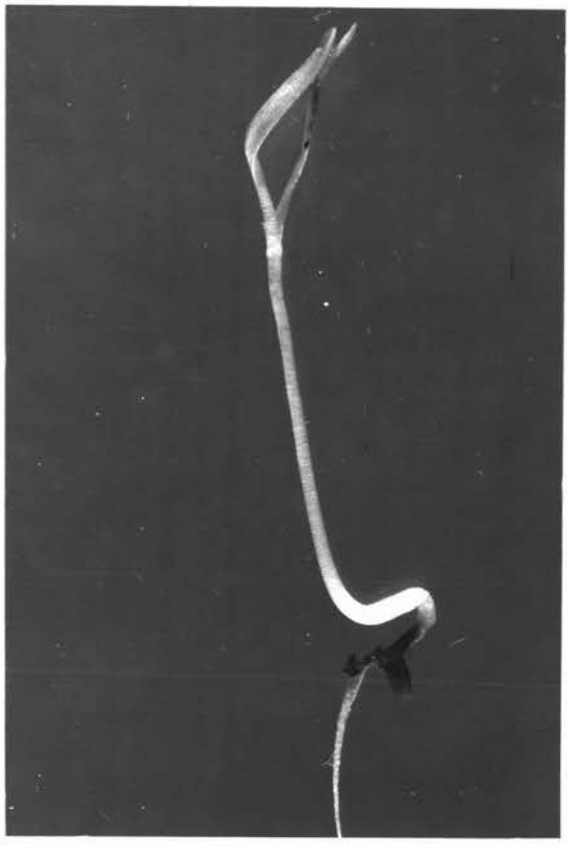
These observations suggested that in field sowings, radicle and cotyledon infections would be expressed as pre-emergence damping-off, whereas hypocotyl infections would be likely to induce post-emergence damping-off. The following experiment was conducted to determine whether this was the case.

Experimental Procedure. An apparatus which would allow microscopic examination of developing seedlings was constructed by modification of the plastic seed-boxes (6" x 4" x 2") used in previous emergence experiments. One of the long axis (6") walls was replaced by inserting a sheet of glass at 45° from the vertical (Fig. 29). A second glass plate was blackened and placed over the first to ensure that the seedlings grew up towards the light, and was removed only during seedling examination.

Figure 25. Radicle browning consequent on contact between an infected testa and the radicle in the early stages of germination.

Figure 26: Radicle lesioning consequent on retention of the testa (site of seed infection) by the radicle.

(a) Whole seedling



(b) Close-up view of lesioned radicle with clasping testa.

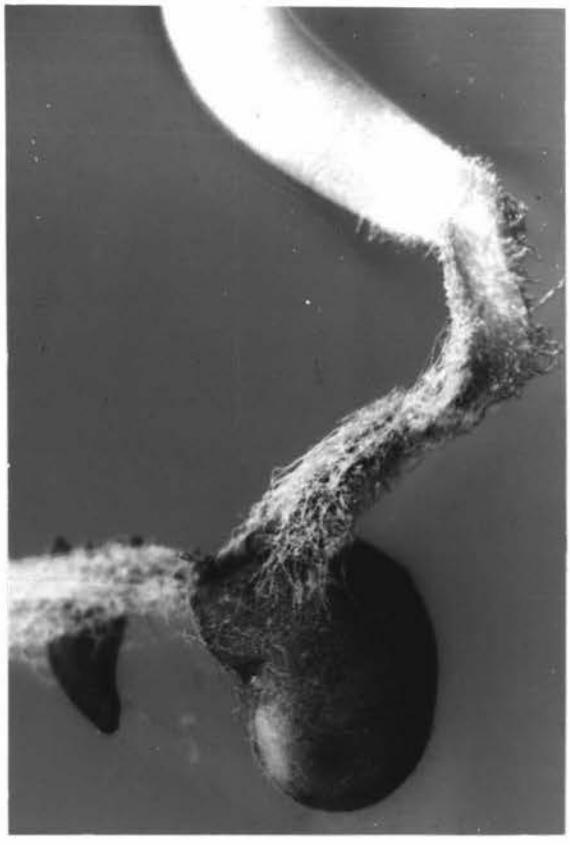
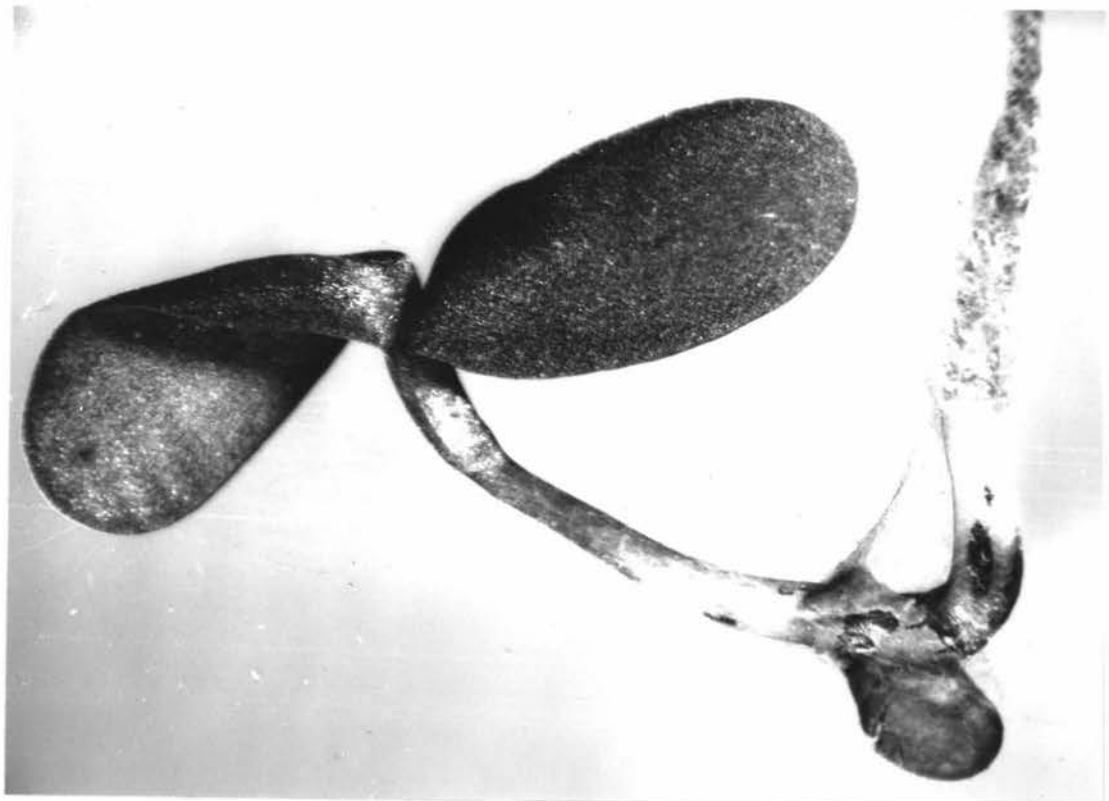


Figure 27. Hypocotyl lesioning with inoculum provided by an unshed testa.

(a) whole seedling

(b) close-up view of lesioned hypocotyl with clasping testa.

(a)



(b)

Figure 31. Close-up view of hypocotyl lesion commonly observed in moist blotter tests.
Note elongated nature of the lesions.

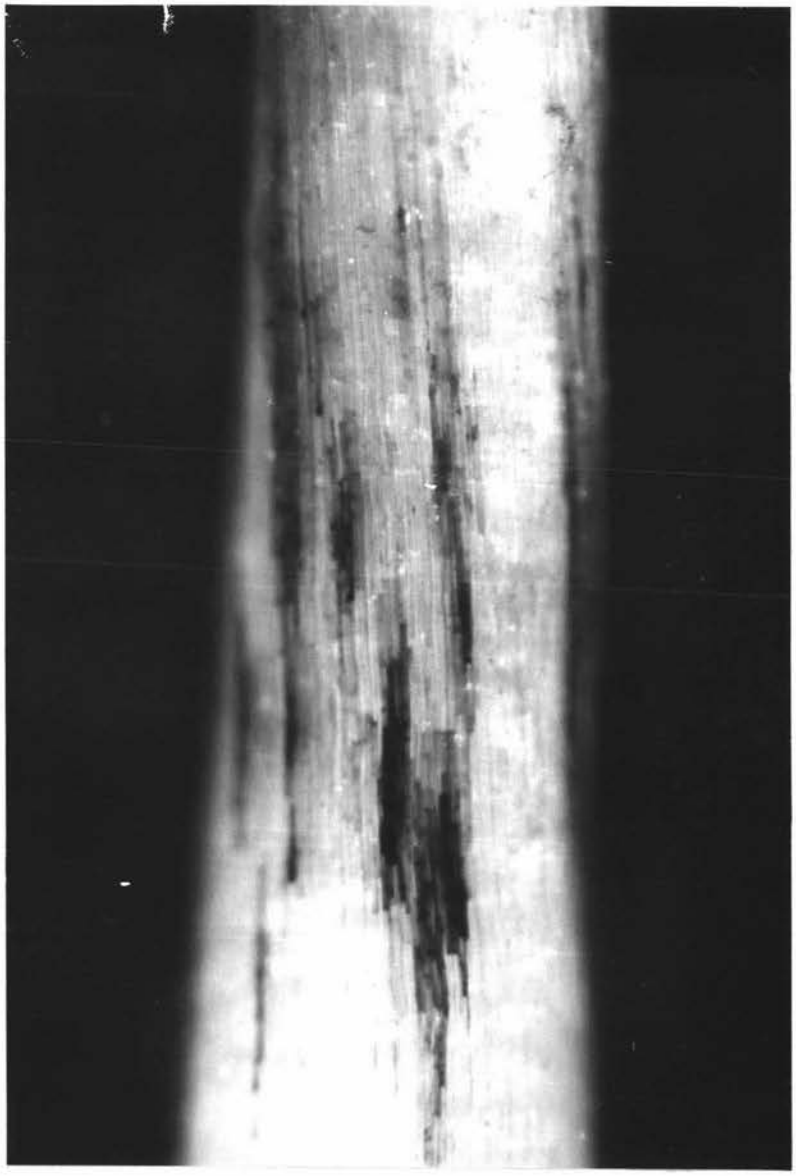


Figure 28. Cotyledon lesions consequent on infection by mycelium growing from an unshed testa.

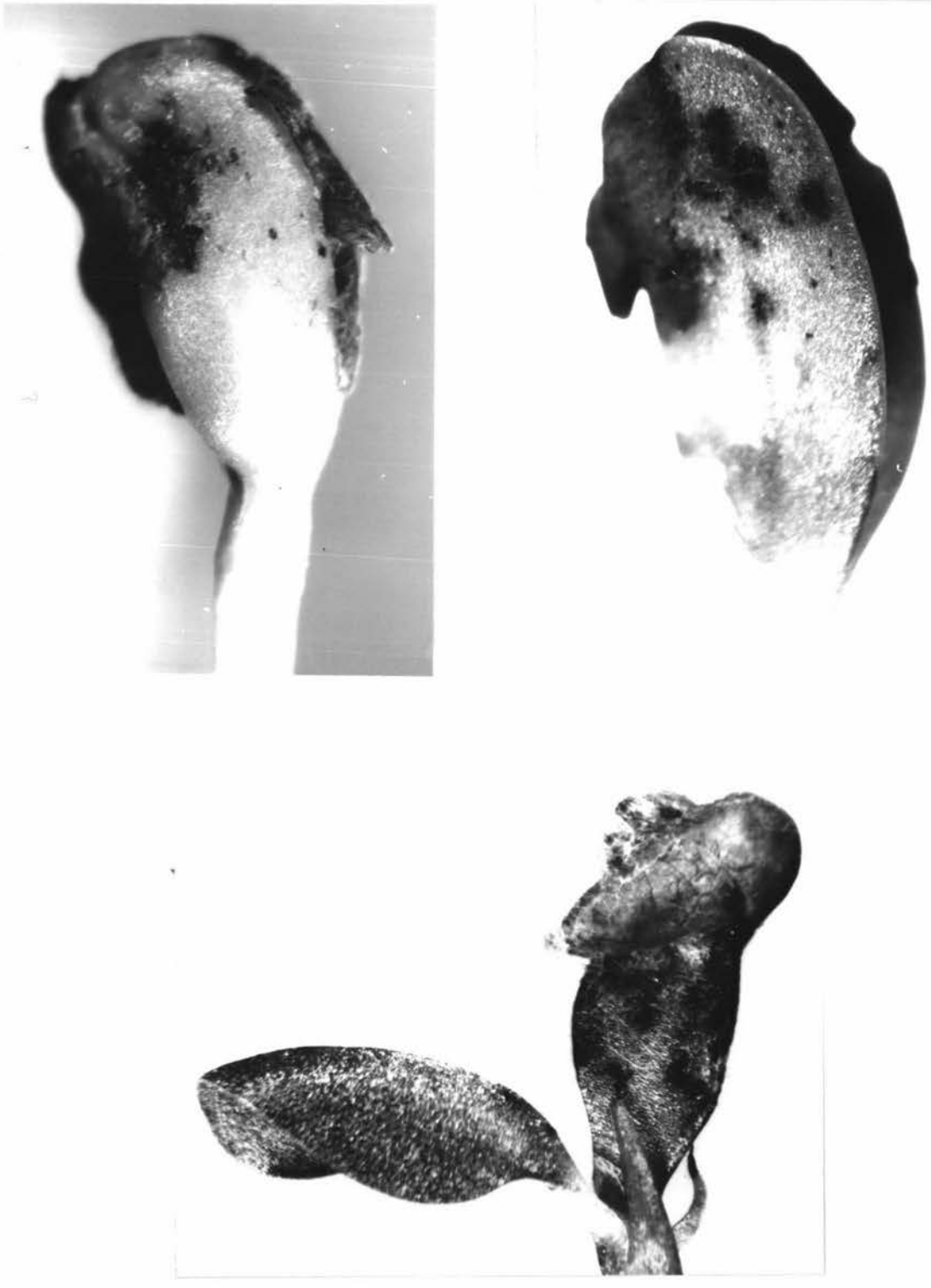
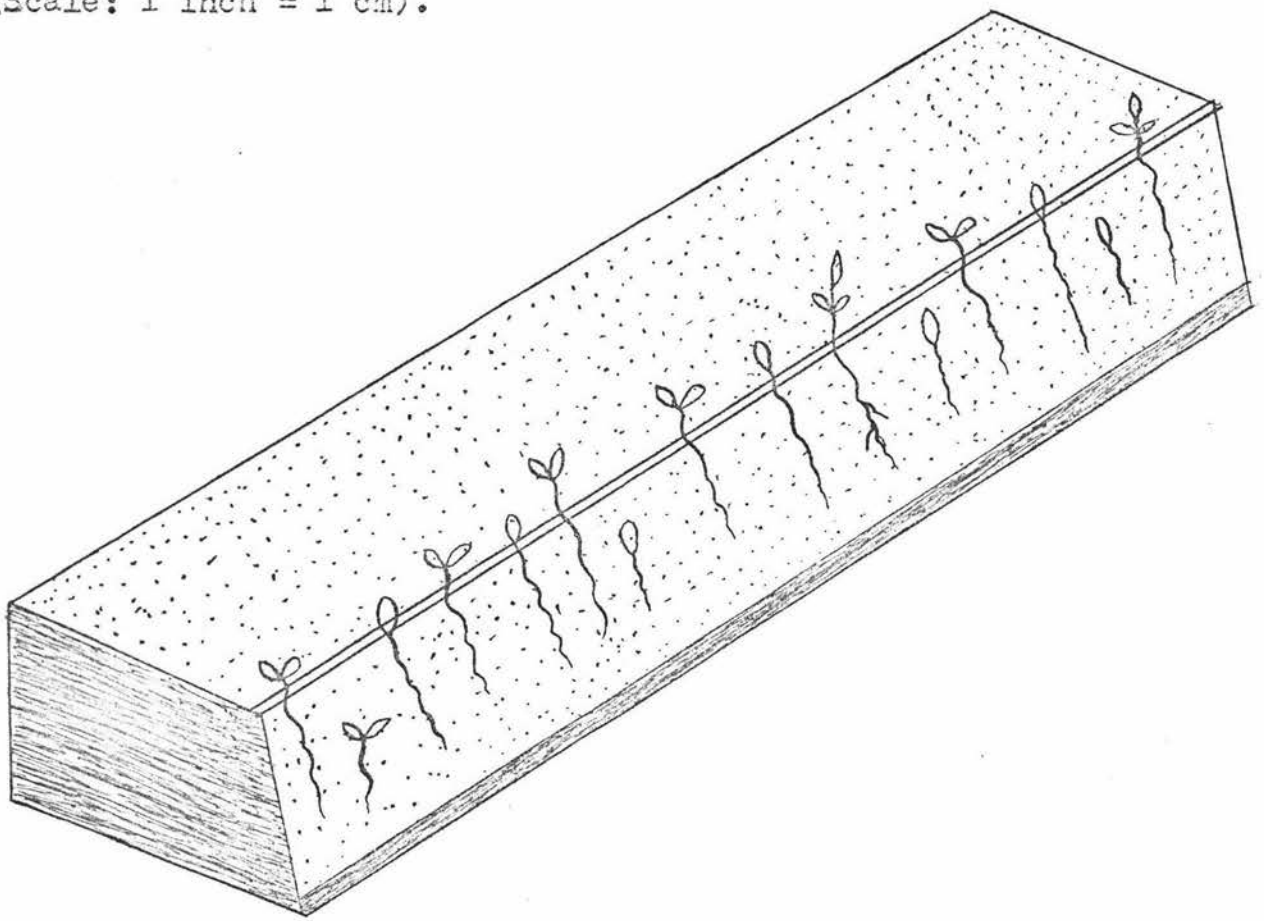


Figure 29 . Design of glass-faced plastic seed-boxes used to enable close observation of symptoms on diseased seedlings. (Scale: 1 inch = 1 cm).



Forty-five seeds from a highly infected line were sown approximately 0.5 inch apart in Vermiculite (grade two) at a depth of one inch and in physical contact with the glass wall. The apparatus was regularly steeped in trays of water to ensure that the Vermiculite was always close to its moisture holding capacity. Seeds and seedlings were examined daily using a stereoscopic binocular microscope.

Isolations from diseased tissues were by the methods previously described (P. 8).

Results and Discussion. Twenty-five of the 40 seedlings exhibited symptoms similar to those described in the moist blotter test. Three types of attack were again observed:

(a) Cotyledons. Pre-emergence damping-off most frequently resulted from infection of the cotyledons, with inoculum provided by mycelium growing from an unshed testa (Fig. 32). However, in several instances seedling emergence occurred following such infection.

(b) Hypocotyl. The second most frequently encountered method of infection occurred when the testa became free of the cotyledons but remained clasping the hypocotyl. Symptom expression then followed in that region in immediate contact with the seed coat (Fig. 30 and 31). Hypocotyl infection was not observed to result in pre-emergence damping-off. However it is theorized that this could occur if the infection was sufficiently severe so as to prevent nutrient flow within the seedling.

(c) Radicle. Both methods of radicle infection observed in the moist blotter test also occurred in the present study (Fig. 32). Although pre-emergence damping-off usually resulted the overall incidence of this type of infection was low.

In all cases P.medicaginis was readily isolated to pure culture from the infected tissues.

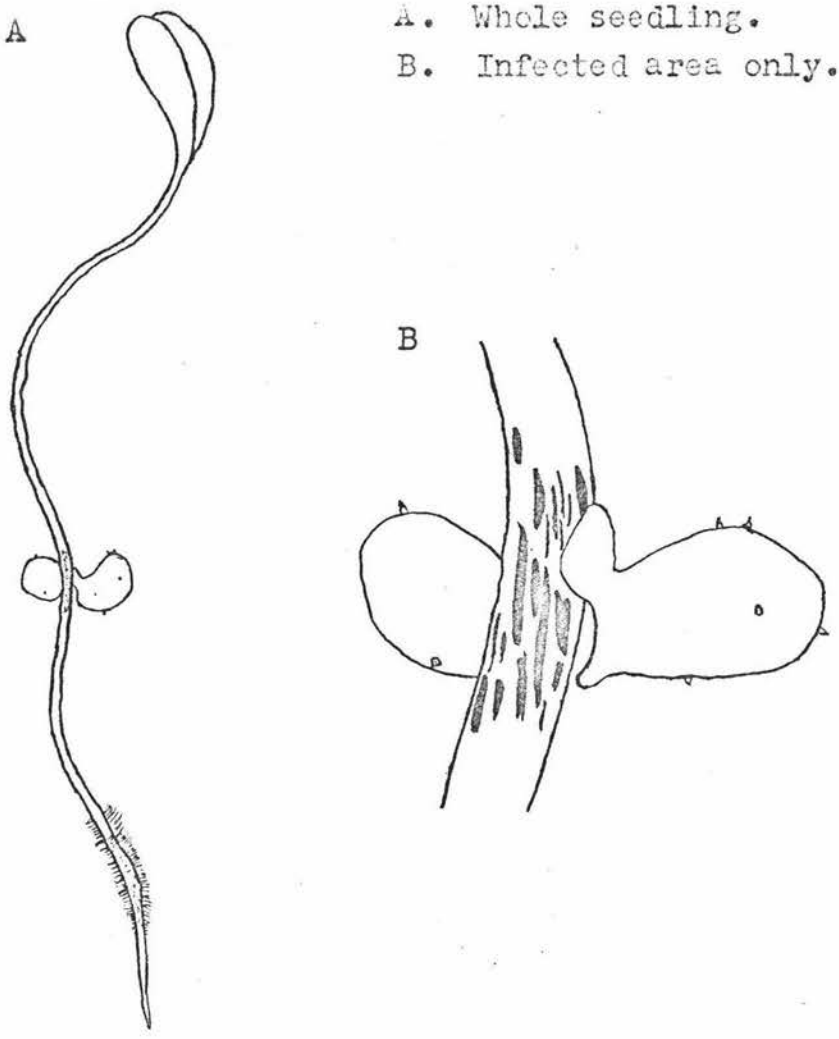
The observation that pre-emergence damping-off frequently resulted from cotyledonary or radicle infections is interpreted as being a function of the presence in that region of meristematic tissue concerned with the promotion of seedling elongation. With radicle or cotyledonary infections the destruction of meristematic tissue in or adjacent to these regions will invariably cause seedling collapse, as elongation depends on the continued activity of these cells. Most hypocotyl infections were observed to be near the base and thus physically isolated from those tissues concerned with root and stem elongation. As a consequence seedlings so infected continued to elongate as long as the pathogen did not cause complete breakdown of the tissues in that region. However, infection near the hypocotyl tip would be expected to cause seedling death through destruction of the apical meristematic tissue.

It is apparent that pre-emergence damping-off was caused by the destruction of those meristematic tissues concerned with seedling elongation. Further, it is postulated

that any instance of post-emergence damping-off would result from infection of the hypocotyl preventing nutrient flow or from weakening of those tissues concerned with providing rigidity.

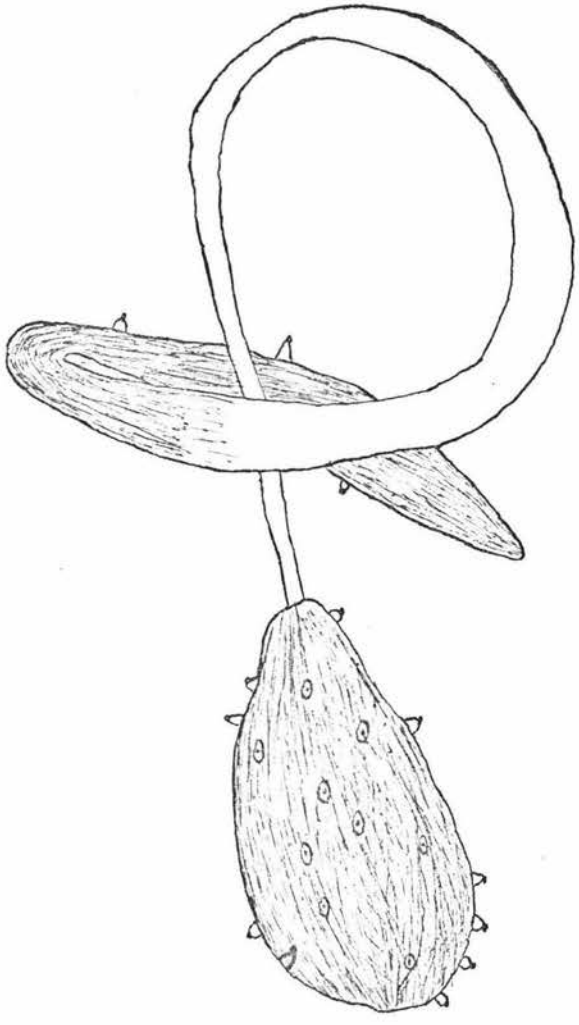
The results and conclusions from this study are similar to those recorded in a previous overseas investigation (Mead, 1953).

Figure 30 . Infection of hypocotyl by P.medicaginis.



A. Whole seedling.
B. Infected area only.

Figure 32 . Infection of radicle by P.medicaginis.



CHAPTER II.

SECTION E.

METHODS OF CONTROLLING SEED-BORNE INOCULUM OF P.MEDICAGINIS

INTRODUCTION

USE OF SEED SIZE AND COLOUR TO SEPARATE INFECTED FROM HEALTHY SEEDS

AGEING THE SEED

USE OF THERAPEUTANTS

DISCUSSION

I. INTRODUCTION.

Theoretically there are a number of ways in which control of the seed-borne phase of the spring black stem disease may be effected:

(a) Seedlings would be disease free if seed was sown when the soil temperature and moisture were such that growth of the pathogen was inhibited. This assumes that such conditions would allow seed germination to occur. However if the pathogen was only inactivated rather than lysed then conceivably its presence could later constitute a source of primary inoculum.

(b) If it is assumed that the only effect of seed-borne inoculum of *P.medicaginis* is to cause seedling damping-off then a simple control measure would involve increasing the seed sowing rate. However this method cannot be recommended until the validity of the above assumption is demonstrated. Palmer (1971) has shown that the sowing rate used by most New Zealand farmers is in excess of the amount required for successful stand establishment. Thus it is probable that a limited amount of damping-off would have a minimal effect on the resultant crop.

(c) If those seeds more likely to be infected with *P.medicaginis* could be visually identified on the basis of size and/or colour, then removal of such seeds would provide a practical means of significantly reducing the percentage infection level.

(d) If the level of seed infection in any one line decreased with time then a measure of control would be effected, provided seed viability was not significantly impaired by prolonged storage.

(e) Disease free seedlings would develop if the seed sown was previously treated with a fungicide to inactivate seed-borne inoculum. This assumes that such a treatment would not adversely affect the germination potential of seed or prevent nodulation with Rhizobium meliloti.

It was considered that the latter three methods provided the most practical possibilities for control. Accordingly, extensive studies were conducted on each.

2. USE OF SEED SIZE AND COLOUR TO SEPARATE INFECTED FROM HEALTHY SEEDS.

It was reasoned that if it was possible to identify those seeds more likely to be infected on the basis of size and/or colour, then removal of such seeds would provide a practical means of significantly reducing the percentage infection level.

As regards seed size, Cormack (1945) reported that no relationship existed between size and the level of infection. That is, highly infected samples were found in all size classes of seed examined. However, Kernkamp and Homerick (1953) stated that while some infected seeds could be found in all size classes, without exception the level of infection was considerably greater in the lightest class. Further, they established that infected seeds in the intermediate and heavy classes could only be identified if subjected to a moist blotter test.

This conflict of opinion can be attributed to the nature of the seed samples considered in each study. Kernkamp and Homerick used "undressed" seed samples obtained from artificially inoculated plants. That is, each sample comprised seed from all classes, including those light weight and shrivelled seeds that are removed in the "dressing" process. Conversely, it is probable (but not stated) that

Cormack examined "dressed" seed lines from which both light weight and shrivelled seeds had been removed, leaving a class of heavier seeds. The apparent conflict between Cormack, and Kernkamp and Hemerick is resolved when it is realized that both considered that infected seeds in the heavy class could not be identified without conducting further tests.

It is clear from the above studies that infected seeds in commercial seed lines cannot be visually identified on the basis of seed size. Accordingly, no further investigations on this method were conducted.

In the literature there is also disagreement as to whether an association exists between seed colour and infection. According to the I.S.T.A. handbook on seed-borne diseases (Anon., 1966), severely infected seeds are brown, flat, and permeated by hyphae, while those only slightly infected are recognizable by the presence of black lines and dots (resting hyphae). Conversely, Cormack (1945) found that the pathogen could be isolated as often from sound mature seeds as from discoloured or green seeds. The findings of Kernkamp and Hemerick (1953) were in agreement with those of Cormack, provided it is assumed that Cormack used commercial seed lines from which light weight and shrivelled seeds had been removed.

In the present study it was observed that all seed lines possessed a proportion of pale and dark seeds (Fig. 34). As a consequence of this and the apparent disagreement in the above literature, a study was conducted to determine if a relationship exists between seed colour and infection percentage.

Experimental Procedure. From each of three infected and one uninfected seed lines approximately 1000 of the darkest and a similar number of the lightest coloured seeds were separated (Fig. 34). To detect possible differences in average seed weight each whole sample was weighed. Each group of seeds was subsequently divided into samples of approximately 275, 350, and 375 seeds to enable the following tests to be conducted:

(a) The infection percentage was determined by plating 250 seeds to MA and recording the number of colonies present after incubation for 11 days at 24 C in the dark.

(b) Samples of pale and dark seed from two infected and one uninfected lines were submitted to the Government Seed Testing Station, Palmerston North, to determine vigour, percentage germination, and the proportion of hard, abnormal, and dead seeds.

(c) The rate of and total emergence were determined by sowing 300 seeds of both classes from the three lines to Vermiculite (grade two) by the method described previously (P.94).

Results and Discussion. The results presented in Table XVIII indicate that within infected seed lines, dark coloured seeds are more likely to be infected than pale seeds. That is, in each of the three infected lines the infection level of the pale seed was considerably less than that of the dark seed (also see Fig. 35).

No relationship was observed between seed colour and the average seed weight (Table XIX). That is, for two of the lines examined the weight of 1000 dark seeds was slightly greater than the weight of 1000 pale seeds, with an opposite trend for the other two lines. Thus while it is unlikely that a relationship exists between average seed weight and infection percentage, the possibility remains that infection of individual seeds may have an effect on the weight of those seeds.

In all lines the percentage germination of the dark seed was lower than that of the pale seed (Table XX). This cannot be interpreted as being the result of the pale seed having a lower infection level because the same trend was observed in the infected and uninfected lines. The proportion of dead seed and abnormal seedlings was considerably greater in the dark seed class. Again this finding cannot be correlated to percentage infection since the same trend occurred with all seed lines. In both infected lines the dark seed had greater vigour than the pale seed, while the opposite trend occurred in the uninfected line. This may be interpreted as being consequent on the presence of the pathogen in dark seed increasing the rate of germination.

The results of the emergence studies are summarized graphically in Fig. 33 a-c. Little can be drawn from these graphs as the differences observed between dark and pale seeds were not consequent on the presence of the pathogen, but rather on some property inherent in each seed class.

In an overall consideration of these results it is apparent that a relationship exists between seed infection and seed colour, but since dark seeds can also be found in all uninfected seed lines it is clear that seed infection by P.medicaginis does not necessarily cause the dark colouration. That is, dark coloured seeds are more likely to be infected because the same factor(s) that lead to some seeds being a brown colour are also conducive to infection of the pod (or part thereof) and its associated seeds.

The above results support the contention of the I.S.T.A. (Anon., 1966) that severely infected seeds are brown, but because a proportion of seeds in uninfected lines are similarly coloured the author is opposed to their recommendation that seed colour be used as a means of identifying infected seed lines. The observation by Cormack (1945) that the presence of the pathogen in the testa is not related to seed colour would appear to be incorrect, although there is a possibility that in some cultivars of lucerne this effect may not be manifest. Finally, the statement by Kernkamp and Hemerick (1953) that infected seeds of the intermediate and heavy weight classes can only be recognized when germinated on moist blotters (thus implying that seed infection was not related to seed colour), also appears to be erroneous.

Because a small proportion of pale seeds are infected, the practice of separating pale from dark seed was rejected as a means of controlling the seedling phase of the spring black stem disease.

Table XVIII. The relationship between seed colour and percentage infection with P.medicaginis.

Seed line	Level P.medicaginis (percent)	
	Pale seed	Dark seed
OL175	0	0
OL181	1.6	5.6
OL259	12.8	38.4
OL263	1.6	32

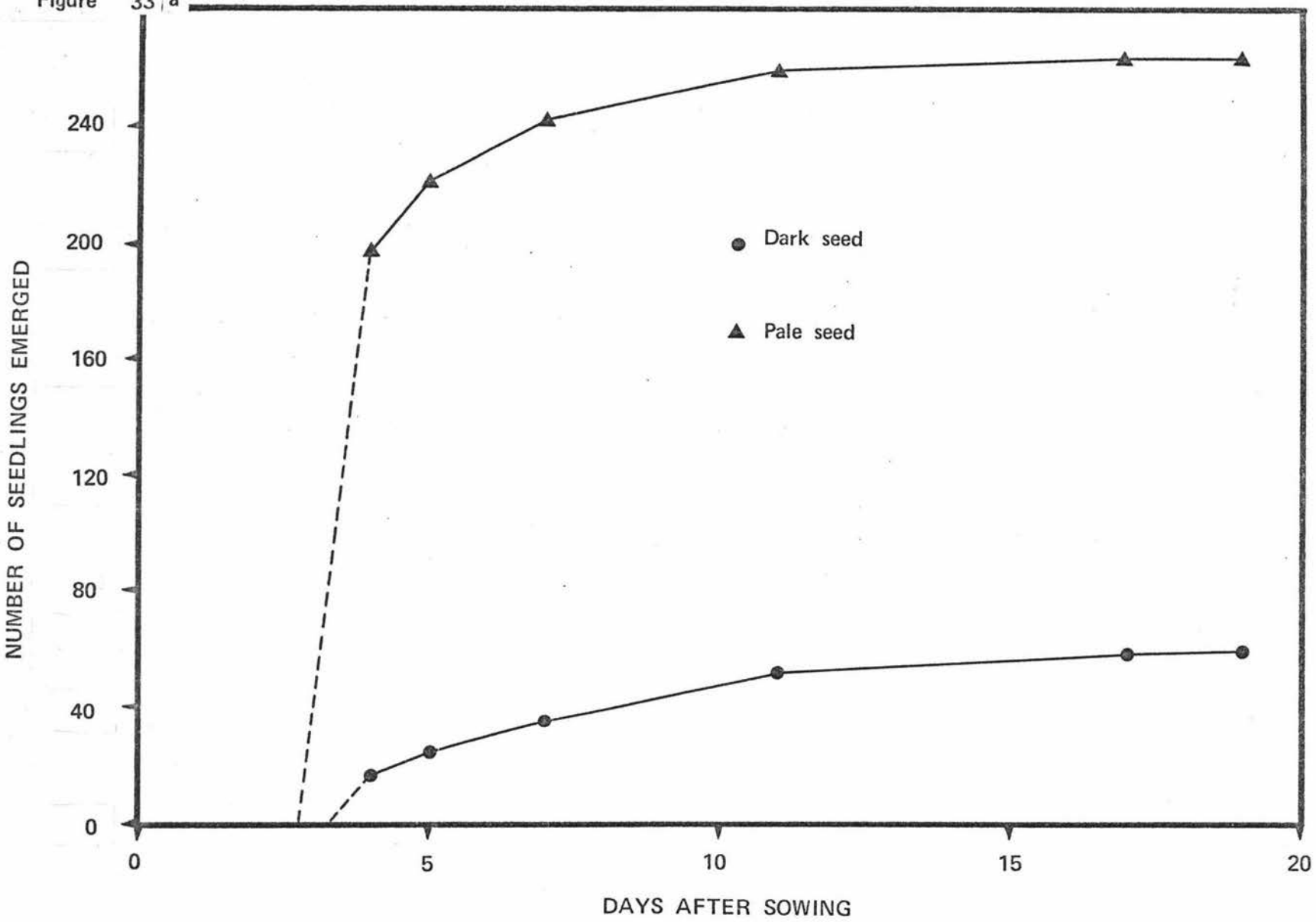
Table XIX. The relationship between seed colour and average seed weight.

Seed line	Weight per 1000 seeds (g)	
	Pale seed	Dark seed
OL175	2.537	2.324
OL181	2.263	2.194
OL259	2.346	2.441
OL263	2.660	2.784

Table XX. The relationship between seed colour and percentage germination.

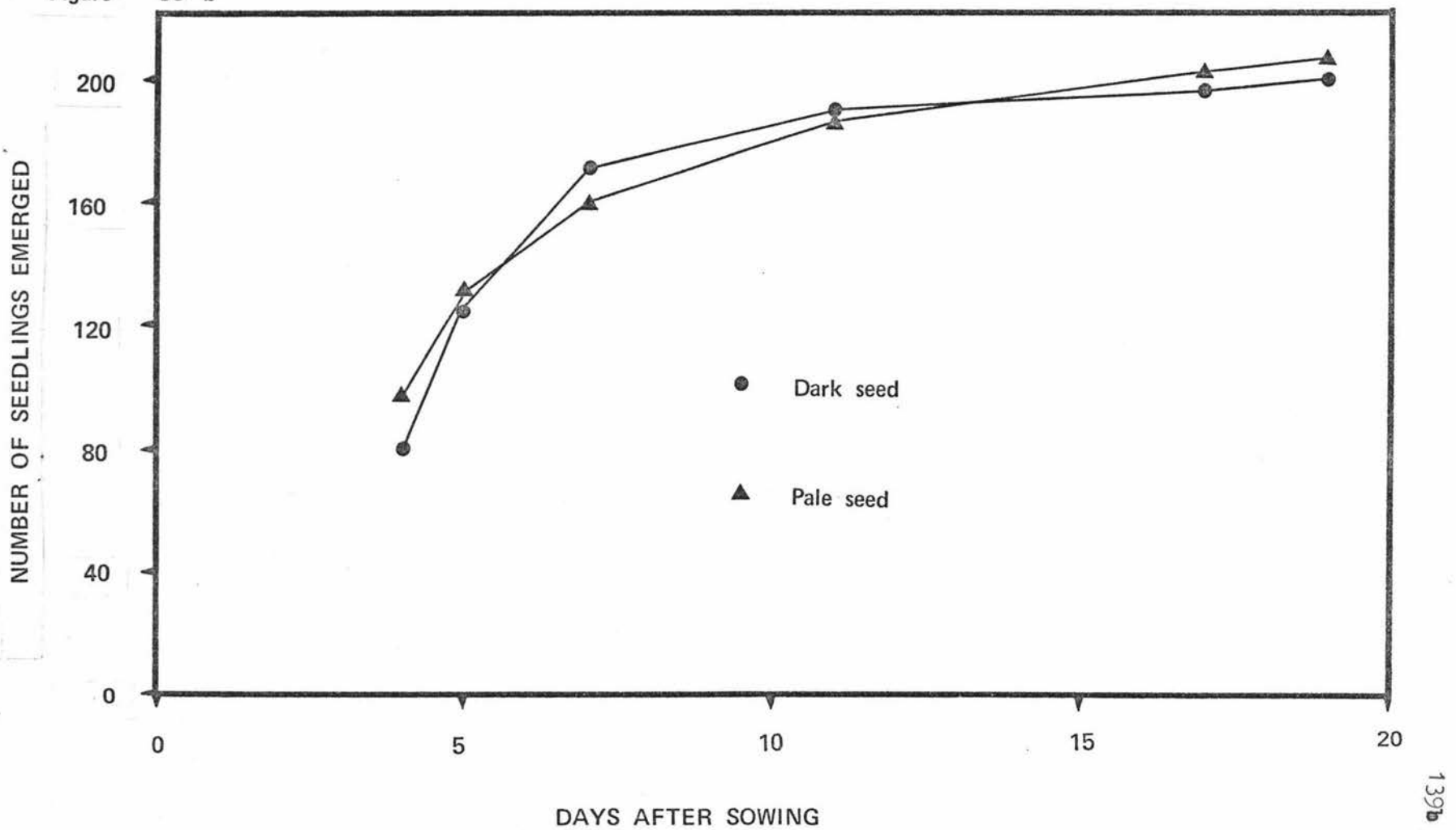
Seed line	Seed class	Interim germination (percentage in 4 days)	Final germination (percentage in 7 days)	Hard seed (per-cent)	Abnormal growth (per-cent)	Dead seed (per-cent)
OL175	Pale	75	86	10	2	2
	Dark	34	42	11	11	36
OL259	Pale	63	76	22	2	0
	Dark	64	71	6	18	5
OL263	Pale	49	70	29	1	0
	Dark	57	67	13	15	5

Figure 33 a



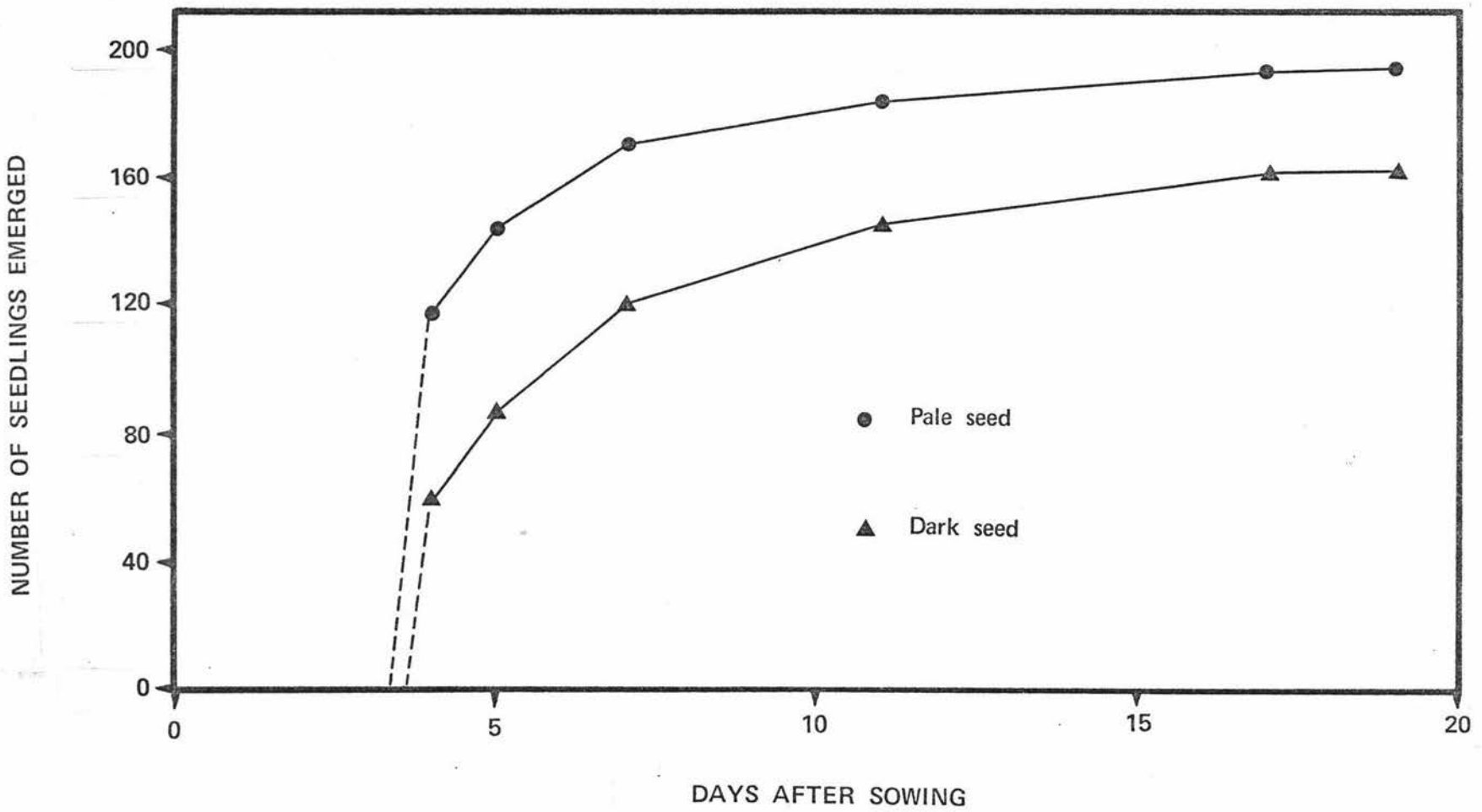
Rate of seedling emergence (line OL175)

Figure 33 b



Rate of seedling emergence (line OL259)

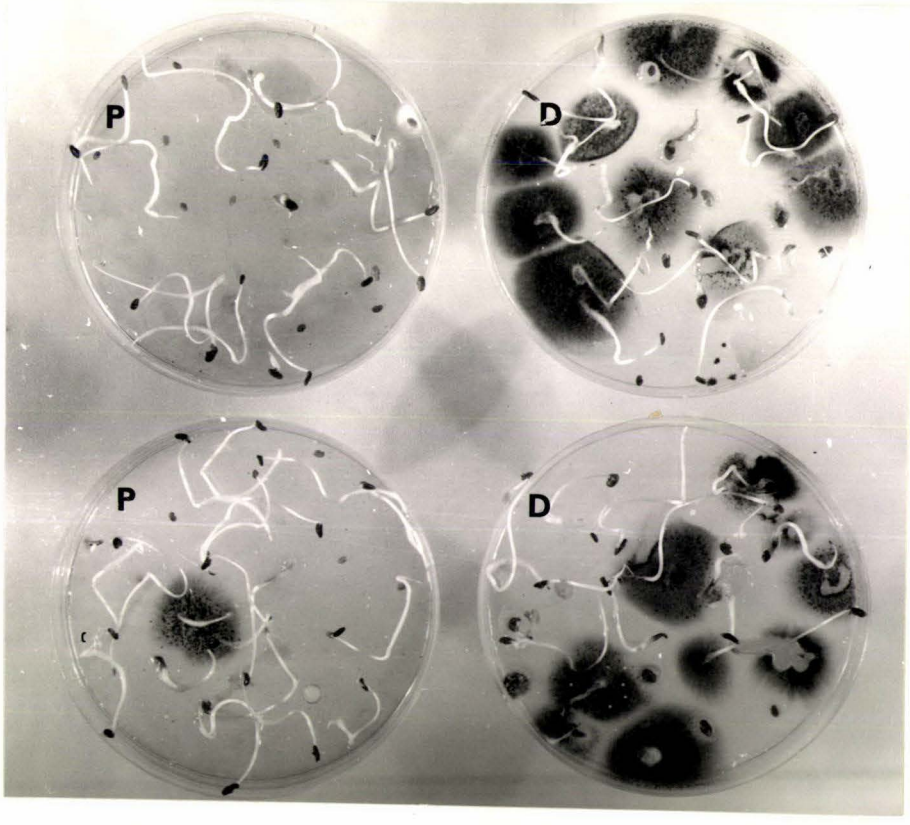
Figure 33 c



Rate of seedling emergence (line OL263)

Figure 34. Dark and pale coloured lucerne seed.

Figure 35. Development of P. medicaginis from dark and pale seed after incubation on MA for 11 days at 24 C in the dark.



Key:

P = Pale coloured seed.

D = Dark coloured seed.

3. AGEING THE SEED.

A phenomenon observed with many fungal seed-borne diseases is that the percentage of infected seeds decreases with time (Krout, 1921; Crosier, 1939; Cormack, 1945; Wallen and Seaman, 1963). Conceivably this could be used to reduce the level of P.medicaginis associated with lucerne seed, provided seed viability was not significantly affected. In a study on Canadian lucerne seed Cormack (1945) reported that the level of P.medicaginis was considerably reduced following storage at room temperature in envelopes for three years. Further, he noted that no infection could be detected after a similar period in those samples that initially had less than five percent infected seeds.

In the present study an investigation was conducted over two years to determine the extent of changes in the viability of inoculum of P.medicaginis associated with commercially available New Zealand lucerne seed. In addition, records were kept of the seed germination capacity and vigour over the same period.

Experimental Procedure. No specific experiments were conducted to measure changes in either infection percentage or germination with time. Instead, information was obtained by collating data from studies conducted over 18 months on two seed lines used extensively in previously described experiments.

In all cases infection percentage was determined by placing 250 seeds onto the surface of MA and counting the number of colonies present after incubation for 11 days at 24 C in the dark. The germination features were determined by submitting seed to the Government Seed Testing Station, Palmerston North. Seed of both lines was stored in sealed polythene bags at room temperature in the dark.

Results and Discussion. The results summarized in Table XXI confirm the earlier findings of Cormack (1945) that seed infection is decreased with time. The percentage germination also decreased somewhat over the 18 months but was still above 65% when observations ceased. Seed vigour as measured by the rate of germination increased over the duration of the experiments. This is interpreted as being consequent on the death of many weak and less viable seeds.

The practical significance of the above results is difficult to assess without conducting further studies. However the practice would appear to be of limited value for two reasons:

(1) The cost would be excessive on account of the storage space required and the amount of capital lying idle.

(2) Total control would not be effected.

Table XXI. Effect of duration of storage on percentage infection with P.medicaginis and seed germination.

Seed Line	Type Test	June '70	August '70	September '70	December '70	January '71	February '71	March '71	April '71	May '71	January '72
OL259	Germination (percent)	81				71				77	67
	Infection (percent)			45	31	35		22	29.6	14.4	3.2
OL263	Germination (percent)	95								68	65
	Infection (percent)		20				22	20.5	16.8		1.2

4. USE OF THERAPEUTANTS

The method offering the best prospects for total eradication of seed-borne inoculum of P.medicaginis was considered to be by fungicidal seed treatment, since both Cormack (1945) and Mead (1953) reported that the pathogen was present in the inner and outer tissues of the testa.

A series of experiments were conducted to determine the effect of various fungicides on the level of P.medicaginis in lucerne seed. Following this, more extensive studies were initiated to further investigate the possible commercial application of the two most promising chemicals.

(a) The Effect of Ten Fungicides on the Level of P.medicaginis in Seed.

Most of those workers concerned with studies on the seed-borne nature of P.medicaginis have advocated the use of fungicidal seed treatment in the form of dusts and/or soaks as a means of eradicating the pathogenic inoculum. The chemicals evaluated by previous workers and comments on their effectiveness are summarized in Table XXII. It is apparent that one fungicide (thiram) when applied as a dust provided satisfactory control in all but one study (Salunska, 1962). As regards seed soaking treatments, Maude et al. (1969) achieved complete eradication of P.medicaginis by soaking seed in a 0.2% thiram solution at 30 C for 24 hours. Preliminary experiments confirmed Maude's findings but the technique was considered impracticable as the soaking treatment impaired seed germination.

A modern approach to control of seed-borne diseases is by inactivating inoculum with systemic fungicides. As regards seed-borne inoculum of P.medicaginis, this was considered a distinct possibility in view of findings by Maude and Kyle (1970) that benomyl completely eradicated the closely related fungus Ascochyta pisi Lib. from pea seed. Further, Her (1972) found benomyl to be effective against established infections of Phoma medicaginis var. pinodella (Jones) Boerema and Mycosphaerella pinodes (Berk. and Blox.) Vestergr. in pea seeds.

In the present study representative samples drawn from five infected seed lines were dusted with ten fungicides. The rate and method of application of each fungicide is described in Table XXIII. Non-treated seed from each line provided controls. The relative effectiveness of each treatment was determined by placing seed onto agar media (details in Table XXIII) prior to incubation for 11 days at 24 C in the dark.

The results are presented in Table XXIII and can be summarized as follows:

(i) All treatments except chloroneb induced a considerable reduction in the level of P.medicaginis.

(ii) Benomyl, thiram, dichlofluanid, and NF44 were consistently the most effective fungicides.

It was considered that thiram and benomyl were the most suitable chemicals for further study. Thiram was

selected not only because it was highly effective against P.medicaginis (both in the present and previous studies), but also because this fungicide is known to provide protection against a wide range of soil-borne pathogens, including pythiaceous fungi (Kennedy and Brinkerhoff, 1959; Kreutzer, 1963). Besides proving to be the most effective fungicide against P.medicaginis (and presumably having the capacity to protect against non-pythiaceous soil-borne fungal pathogens), benomyl has the additional property of being systemic in a wide range of plants (Catling, 1969). Conceivably this latter factor applies to lucerne, in which case prolonged protection would be afforded established seedlings.

(b) Further Experiments Relating to the Possible Commercial Application of Benomyl and Thiram.

(i) Laboratory assessment of the concentration of benomyl required for total control.

There are no reports in the literature of laboratory studies on the effectiveness of benomyl against isolates of P.medicaginis from lucerne. Accordingly, this was investigated using the poison food bioassay method (Horsfall, 1956; Torgeson, 1967).

A stock solution of benomyl was prepared and incorporated into MA (held at 50 C) in quantities sufficient to give final benomyl/MA concentrations of 0.25, 2.5, 5, 25, 50, and 125 ppm (active ingredient), and poured into 9 cm Petri dishes [x].

[x] A preliminary study was conducted to investigate the feasibility of adding benomyl to MA before autoclaving in order to simplify media preparation. Results are summarized in Appendix VII.

Table XXII. Previous reports of fungicide application to lucerne seed for control of P.medicaginis

Source	Trade name	Common name	Chemical name	Rate application	Method application	Comment on effectiveness of treatment
Cormack, 1945	Arasan	Thiram	Bis(dimethylthiocarbamoyl) disulphide	Saturation	Shake seed with dust Remove excess	Very effective
Cormack, 1945	New improved Ceresan 1%	Ceresan	N-(Ethylmercury)-p-toluenesulphonanilide	Saturation		Very effective
Cormack, 1945	New Improved Ceresan 5%	Ceresan	N-(Ethylmercury)-p-toluenesulphonanilide	Saturation		Very effective; reduced germination
Cormack, 1945	Semesan	Semesan	Hydroxy mercuri chloropherol	Saturation		Reasonably effective; slight phytotoxicity
Cormack, 1945	Spergon	Chloranil	Tetrachloro-p-benzoquinone	Saturation		Reasonably effective; slight phytotoxicity
Mead, 1953	Arasan	Thiram	A.S.A.	0.25% of seed weight	Shake seed with dust	Reasonably effective; slight phytotoxicity
Mead, 1953	Ceresan	Ceresan	A.S.A.	0.09% of seed weight		Reasonably effective; reduced germination slightly
Mead, 1953	Semesan	Semesan	A.S.A.	0.25% of seed weight		Reasonably effective
Mead, 1953	Spergon	Spergon	A.S.A.	0.25% of seed weight		Reasonably effective
Weltzien, 1958	N.A.	N.A.	Mercurous chloride	0.1% solution	Soak 5 min	Prevented nearly all fungal and bacterial growth
Saluns'ka, 1962	Granosan	N.A.	N.A.	N.A.	Wet seed treat-ment Dry seed treat-ment	Degree of control unsatisfactory.
Saluns'ka, 1962	N.I.U.I. F.-I	N.A.	N.A.	N.A.		Degree of control unsatisfactory

Table XXII. cont.

Source	Material tested			Rate application	Method application	Comment on effectiveness of treatment
	Trade name	Common name	Chemical name			
Saluns'ka, 1962	Thiram	Thiram	A.S.A.	N.A.	Dry seed treatment	Degree of control unsatisfactory.
Latch and Greenwood, 1964	Captan	Captan	N-Trichloromethylmercapto-4-cyclohexene-1:2 dicarboximide	N.A.		Partial control but not sufficiently so to justify further consideration.
Latch and Greenwood, 1964	Dichlone	Phygon	2,3-Dichloro-1,4-naphoquinone	N.A.		Partial control but not sufficiently so to justify further consideration,
Latch and Greenwood, 1964	Ferbam	Fermate	Ferric dimethyldithiocarbamate	N.A.		Partial control but not sufficiently so to justify further consideration.
Latch and Greenwood, 1964	Phaltan	Folpet	N- (Trichloromethyl) thio phthalimide	N.A.		Partial control but not sufficiently so to justify further consideration.
Latch and Greenwood, 1964	Rhizoctol	Rhizoctol	Methylarsenic sulphide + Quinone-oxime-benzoyl-hydrozone	N.A.	Shake seed with dust	Partial control; seedling infection reduced by greater than 50%; slight interaction with nodule inducing Rhizobia.
Latch and Greenwood, 1964	Thiram	Thiram	A.S.A.	N.A.		Partial control; seedling infection reduced by greater than 50%; no interaction with nodule inducing Rhizobia.
Maude et al al., 1969	-	Water	-	-	Soak 24 hr. at 30 C	Level infection reduced; slight detrimental effect on germination.
Maude et al., 1969	Thiram	Thiram	A.S.A.	0.2% Solution		Infection eliminated; germination unaffected but testa becomes mucilaginous.

Key:

N.A. = Information not available

A.S.A. = As stated above

Table XXIII. Effect of ten fungicides on the level of P. medicaginis associated with lucerne seed.

Name of fungicide under test			Rate application	Method application	Seed screening method	Level P.medicaginis (percent)									
						Seed line OL38		Seed line OL259		Seed line d259		Seed line OL263		Seed line d263	
Trade name	Common name	Chemical name				Non-treated seed	Treated seed	Non-treated seed	Treated seed	Non-treated seed	Treated seed	Non-treated seed	Treated seed	Non-treated seed	Treated seed
Antracol	Propineb	N,N-propilen-1,2 bis (zinc-dithiocarbamate)	Saturation	A	X	20	+	35	+						
Benlate	Benomyl	Methyl-N-benzimidazol-2-yl-N(butylcarbamoyl) carbamate	1% of seed weight	B	Y					44.4	3.2				
Benlate	Benomyl	"	0.5% of seed weight	B	Y					44.4	3.6				
Benlate	Benomyl	"	1% of seed weight	C	Y					44.4	1.2				
Benlate	Benomyl	"	Saturation	A	X	20	0	35	4						
Ceresan	Ceresan	N-(Ethyl mercury)-p-toluenesulphonanilide	Saturation	A	X	20	2	35	10						
Demosan 65-W	Chloroneb	1,4 Dichloro-2,5-dimethoxybenzene	1% of seed weight	B	Y					44.4	40.4				
Demosan 65-W	Chloroneb	"	0.5% of seed weight	B	Y					44.4	44.8				
Euparin	Dichlofluanid	N,Dichlorofluoromethyl-thio NN-dimethyl-N1-phenylsulphamide	Saturation	A	X	20	0	35	7						
Methyl Topsin	N.F.44	Methyl thiophanate	Saturation	A	Z			22	4			20.5	1.5		
Rhizoctol-combi	Rhizoctol	Methylarsenic sulphide+ Quinone-oxime-benzoyl-hydrazone	Saturation	A	X	20	2	35	x						
Sclex	Dichlozoline	3-(3,5-Dichlorophenyl)-5,5- dimethyl-2,4-oxazolidinedione	Saturation	A	Z			22	15			20.5	2.5		
Thiram 80	Thiram	Bis(dimethylthiocarbamoyl) disulphide	1% of seed weight	B	Y					37.6	8.0			29.2	8.4
Thiram 80	Thiram	"	Saturation	A	X	20	0	35	6			22	3.5		
Topsin	N.F.35	Ethyl thiophanate	Saturation	A	Z			22	11.5			20.5	10		

Key:

+ Little effect on level of P. medicaginis. Treatment enhanced growth of saprophytic fungal contaminants.
x Treatment favoured growth of saprophytic contaminants (mainly Penicillium spp.), masking presence of P. medicaginis.

A Shake seed with fungicidal dust in glass container for 20 minutes; remove excess chemical through sieve.
B Shake seed with fungicidal dust in glass container for 30 minutes.
C Shake seed with fungicidal slurry (75 ml water/0. 1lb fungicide) in glass container for 30 minutes.

X Place 100 seeds on surface of MA. Incubate for 11 days at 24 C in dark.
Y Place 250 seeds on surface of MA with penicillin and streptomycin antibiotics incorporated (each at 50 ppm). Incubate as described above.
Z Place 100 seeds on surface of MA. Incubate as described above.

Malt agar without additives provided controls. All plates were inoculated with circular agar plugs obtained from the margin of an actively growing monosporous isolate using a 4 mm diameter cork borer. The control and each treatment were replicated six times. Incubation was for 11 days at 24 C in the dark.

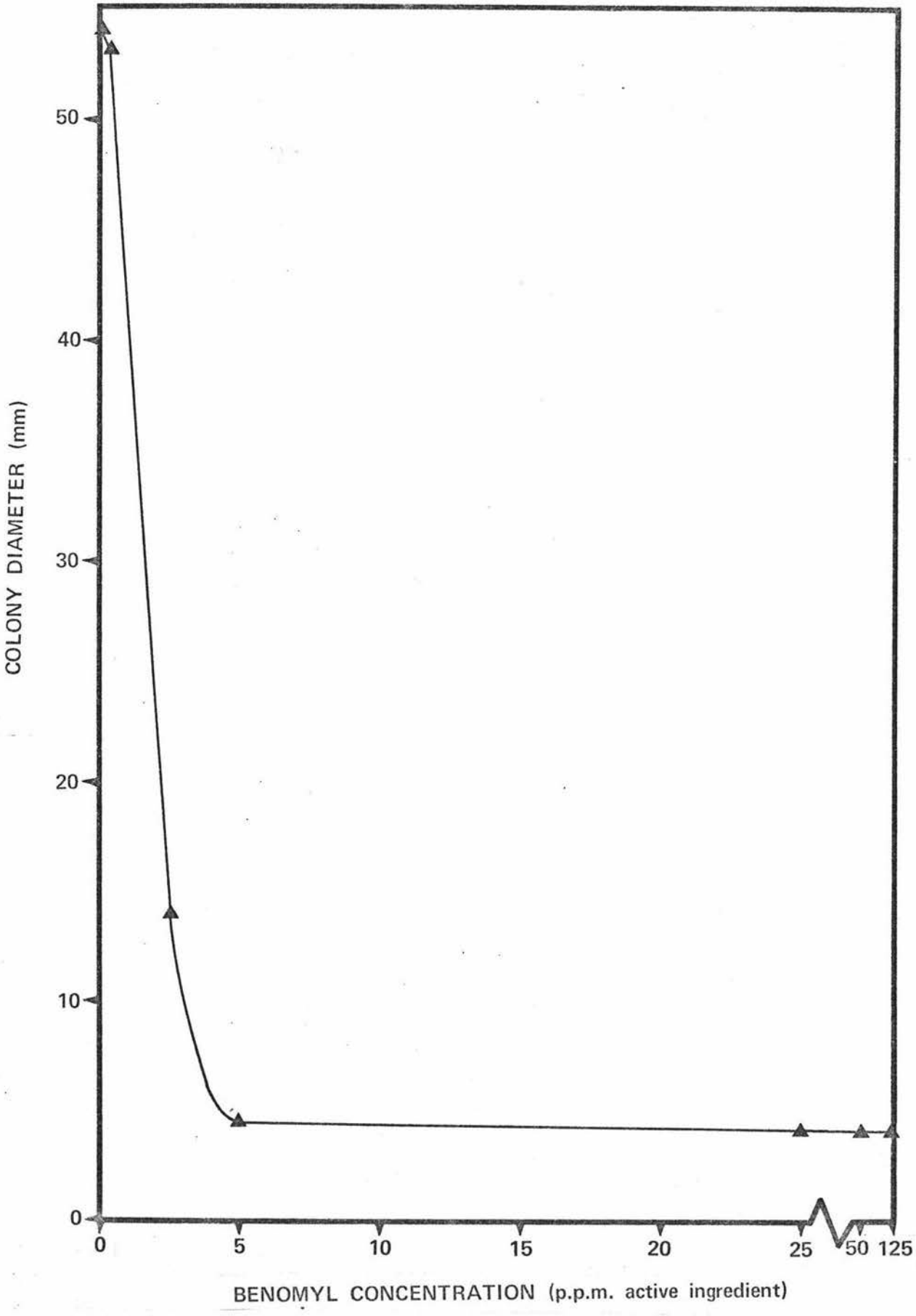
To determine the extent to which incorporation of benomyl affected growth of the pathogen, two diametric colony measurements at right angles were made on each plate.

The results summarized graphically in Figure 36 indicate that benomyl was highly effective against P.medicaginis to the extent that growth was almost completely suppressed at 5 ppm. This confirmed the earlier observations in seed dusting trials which demonstrated almost complete control of seed-borne inoculum.

(ii) Phytotoxicity of benomyl and thiram to lucerne seed and seedlings.

A therapeutant shown to be effective against a particular pathogen has potential practical application only if it does not induce phytotoxicity in the host species. Phytotoxicity is usually manifest through such parameters as lowered germination, reduced vigour and emergence, or a larger proportion of abnormal seedlings.

Figure 36



Effect of concentration of benomyl on radial growth of P. medicaginis (Growth measured after incubation for 11 days at 24°C in dark)

It was observed in previously described experiments (P. 98) that when thiram was applied at saturation levels to the testa of uninfected seed lines the emergence from Vermiculite was slightly less than that of non-treated seed. In similar preliminary experiments using benomyl conflicting results were obtained on the possible phytotoxicity of this fungicide. That is, when applied at the rate of 0.5 gram commercial product per 100 grams of seed total emergence decreased, but when applied at one gram of commercial product per 100 grams of seed an increase occurred (Appendix VIII).

The above results indicate that under certain circumstances thiram and benomyl may induce slight seedling phytotoxicity. Before conducting laboratory studies to assess the effectiveness of thiram and benomyl in controlling seed-borne inoculum it was essential that adverse effects consequent on the use of the chemicals be recognized. Accordingly, the following experiments were conducted to clearly define symptom expression of phytotoxicity as induced by benomyl and thiram.

Experimental Procedure. During agar plate screening tests it was observed that seed germinated rapidly on the agar medium, producing seedlings similar to those found in the field. It was considered that seed germinating on MA with benomyl and thiram incorporated at a range of levels (as in poison food bioassay) would express evidence of phytotoxicity.

Benomyl was added to MA (held at 50 C) in quantities sufficient to give final concentrations of 0.25, 0.5, 2.5, 5.0, 25.0, 50.0, and 125.0 ppm (active ingredient). Further, in order to suppress bacterial growth streptomycin sulphate and penicillin antibiotics were also added, each at the rate of 50 ppm [x]. Malt agar with only the two antibiotics added provided controls. Two hundred seeds each from three uninfected lines were placed on the surface of the antibiotic agar. Line OL175 was tested at eight different concentrations of benomyl while lines IL88 and Aust III were tested at four levels. Results were recorded following incubation for eight days at 24 C in the dark.

Similar experiments were conducted using thiram incorporated in antibiotic MA at 10, 50, and 100 ppm (active ingredient). Malt agar with only the two antibiotics added again provided controls. Two seed lines were examined using 125 seeds per treatment.

Observations were made on the following:

(i) Percentage germination.

(ii) Percentage of seedlings with etiolated hypocotyls.

(iii) Percentage of seedlings possessing root hairs.

(iv) Average seedling length (50 measured).

[x] A preliminary experiment indicated that the antibiotics did not effect any of the characters under study.

Table XXIV. The effect of benomyl on lucerne seedling characteristics.

Concentration benomyl in MA (ppm a.i.)	Germination (percentage)			Seedlings with etiolated hypocotyl (percent)			Seedlings with visual root hairs (percent)			Average seedling length (mm)		
	Seed line OL175	Seed line Aust III	Seed line IL88	Seed line OL175	Seed line Aust III	Seed line IL88	Seed line OL175	Seed line Aust III	Seed line IL88	Seed line OL175	Seed line Aust III	Seed line IL88
0	76.5	97.5	85.5	0.5	0	0	70.5	97	81	42.5	46.7	42.1
0.25	79			0			75					
0.5	74.5			0			69.5					
2.5	81			0			80					
5.0	83.5	97.5	82	0	0	0	79	96	75.5	37.8	46.5	44.8
25.0	85.5			3.5			77			54.3		
50.0	81	98.5	83	17	87.5	56	64.5	8	38	49.4	46.3	42.6
125.0	82.5	96.5	81	60	93.5	66	9	0	5	50.8	43.9	42.4

Results and Discussion. From the results presented in Table XIV it is apparent that at the greatest concentration (125 ppm) benomyl had no effect on either germination percentage or average seedling length. However evidence of phytotoxicity was clearly provided by the other two characters studied. At concentrations above 50 ppm benomyl inhibited the formation of root hairs, and also caused a distinct etiolation of the hypocotyl. It is probable that both these indications of phytotoxicity would precede seedling collapse.

The incorporation of thiram in MA had no effect on germination percentage, the proportion of seedlings with etiolated hypocotyls, or the development of root hairs. However at the greatest concentration (100 ppm) thiram caused a decrease in the average seedling length (Table XV). It is probable that this indication of phytotoxicity would affect the rate of and total emergence of thiram dusted seed.

Table XXV. The effect of thiram on average seedling length.

Concentration thiram (p p m a.i.)	Average seedling length (mm)	
	Seed line A	Seed line B
0	71.7	68.1
100	60.4	62.8

(iii) Effect of benomyl and thiram when incorporated in "Coated Seed" on the level of P.medicaginis.

Recent technological advances have led to the development of new methods of applying nodule inducing Rhizobium meliloti inoculum onto seed. One such technique involves coating the seed with peat based Rhizobia inoculum, followed by a further coating with a calcite slurry. The use of "coated seed" presents an excellent opportunity for adding fungicides either to the outer layer of the coat or in immediate contact with the testa under the calcite coating.

In the present study an investigation was conducted to determine the degree of control consequent on applying benomyl and thiram to seed both separately and in combination.

Experimental Procedure. Seed pelleting and fungicidal incorporation of two highly infected lines were carried out by the Fruitgrower's Chemical Company, Mapua, Nelson. Benomyl and thiram both separately and in combination were applied to seed as follows:

(i) Directly to the testa before coating.

(ii) Incorporated in the calcite coat.

Coated seed without fungicides provided controls. The fungicides were each applied at the rate of one gram of commercial product per 100 grams of seed.

The infection percentage and evidence of phytotoxicity were determined by subjecting 200 seeds from

each treatment to a moist blotter test (P.64). The identity of pathogens causing seedling infection was determined by the tissue plating technique (P. 8).

Results. From the results presented in Table XXVI it is apparent that all fungicidal treatments were either totally or almost totally effective against seed-borne inoculum of P.medicaginis. Further, all treatments either eradicated or reduced to very low levels other fungal pathogens present in these two lines. That is, in no instance was inoculum of Fusarium spp. detected; and the level of S.botryosum was less than 2.5% in all fungicidal seed treatments (for controls see Appendix V).

No evidence of phytotoxicity was observed in any fungicidal treatment, and the germination percentage in both lines was unaffected.

Table XXVI. The effect of incorporating benomyl and thiram into coated seed on the level of infection with P.medicaginis.

Code	Seed line	Treatment	Level of infection (percent P.medicaginis)
1	IL129	Control; seed coated only; no fungicidal treatment.	43
2	IL129	Benlate applied as slurry to the testa, and seed then coated.	0
3	IL129	Thiram-as per treatment (2).	0.5
4	IL129	Benlate and thiram applied as slurry to the testa, and seed then coated.	0
5	IL129	Apply normal seed coating but with Benlate incorporated into coat.	0
6	IL129	As for (5) but using thiram.	0
7	IL129	Benlate and thiram incorporated in the normal coating.	0
8	IL315	As for (1).	25.5
9	IL315	As for (2).	0
10	IL315	As for (3).	0.5
11	IL315	As for (4).	0
12	IL315	As for (5).	0
13	IL315	As for (6).	0.5
14	IL315	As for (7).	0

5. DISCUSSION.

It was presumed that any measure adopted for the control of P.medicaginis would have to eradicate (rather than reduce) the infection level, since it is unclear to what extent seed infection can provide primary infection focii. For this reason seed treatment with therapeutants was considered a more practical proposition than control based on seed colour or the relative longevity of the seed and pathogen.

While in the present study seed dressings of benomyl and thiram were shown to effectively control seed-borne inoculum of P.medicaginis, S.botryosum, and Fusarium spp., before field application can be recommended consideration must be given to the following:

(a) Effectiveness of benomyl and thiram in controlling soil-borne (as opposed to seed-borne) fungal pathogens of lucerne seedlings.

(b) Effect of benomyl and thiram on nodulation.

(c) Economics of applying benomyl and thiram to seed.

(a) Effectiveness of Benomyl and Thiram in Controlling Soil-Borne Fungal Pathogens of Lucerne Seedlings.

When considering control of the seed-borne and soil-borne fungal complex of lucerne it was obviously desirable that the fungicide(s) applied to seed provide effective control of all those pathogens capable of inducing damping-off. Close (1967, 1971) has implicated soil-borne inoculum

of Fusarium spp. and members of the Pythiaceae as causing damping-off in the Canterbury region, and shown these pathogens can be controlled by applying benomyl and chloroneb in combination to "coated seed" (each at the rate of one gram of commercial product per 100 grams of seed). In the present study benomyl was shown to reduce seed-borne inoculum of P.medicaginis to very low levels, whereas chloroneb had little or no effect on the infection level. Thus it was considered that thiram could be substituted for chloroneb since it is effective against the same group of soil fungi that chloroneb is purported to control, namely the Pythiaceae (Richardson, 1954; Kennedy and Brinkerhoff, 1959; Kreutzer, 1963). The benefit accruing from using thiram instead of chloroneb is that total eradication of P.medicaginis would be more probable.

A further possible benefit resulting from the use of benomyl is that in view of its reported systemic activity in a wide range of plants (Catling, 1969), protection may be afforded established seedlings against the post-emergence phase of damping-off. Thus on the basis of the above reasoning it was postulated that incorporating benomyl and thiram into "coated seed" would more effectively control both the seed-borne and soil-borne fungal pathogens.

(b) Effect of Benomyl and Thiram on Nodulation .

An important consideration when using fungicidal seed dressings on clover or lucerne seed is that the treatment must not suppress nodulation by Rhizobium meliloti. Jacks (1956), and Latch and Greenwood (1964) established that thiram

applied as a dust directly to the testa did not reduce nodulation. As regards benomyl, Close, Whitelaw, and Taylor (1971) observed that when incorporated in combination with chloronob into the outer layer of "coated seed", nodulation was not adversely affected. From these studies it was reasoned that incorporating benomyl and thiram into "coated seed" would likewise not prevent nodulation.

(c) Economics of Applying Benomyl and Thiram to Seed.

The measures applied for control of any plant disease are justified only if the increase in monetary return consequent on control of that disease is greater than the costs involved. While one can readily assess the additional costs so incurred, the benefits accruing from control of the disease are much more difficult to assess.

As regards the economics of incorporating benomyl and thiram into "coated seed", the only additional cost incurred is the seed treatment. On the credit side are three possible advantages. Firstly, the seed sowing rate could be lowered as each seed would have a greater chance of developing into a mature plant. Secondly, the plant spacing would be more uniform, allowing the possibility of maximum production from the whole crop instead of a part thereof. Finally, the cost associated with hand inoculating seed with Rhizobium meliloti immediately prior to sowing would be eliminated.

It must be emphasised that in the present study it is only possible to propose that applying benomyl and thiram to lucerne seed may be a practical proposition. Before these measures could be adopted for field use a series of follow up studies are required to determine

(i) whether such treatment would adversely affect nodulation

(ii) whether benomyl is adsorpted by the seedlings

(iii) by a series of field trials whether the benefit accruing from control of the seed-borne and soil-borne pathogenic fungal flora justifies the associated cost of seed treatment.

APPENDIX I

THE NEW ZEALAND HISTORY, AND SYMPTOMATOLOGY OF SPRING BLACK STEM OF LUCERNE.

Extensive studies have not been conducted in New Zealand on spring black stem of lucerne. The disease was first recorded in this country by Cunningham (1956). However an earlier report also by Cunningham (1922) on yellow leaf blotch of lucerne caused by Pyronopeziza medicaginis Fcl., syn. Ascochyta medicaginis Fcl., syn. Phyllosticta medicaginis (Fcl.) Sacc., could well refer to the same disease, as the latter pathogen was recorded by Jones (1918) as being synonymous with P.medicaginis.

In a survey of approximately one half of the lucerne seed lines certified in New Zealand during 1963 Matthews (1970) established that P.medicaginis was present in 88% of the lines examined, at levels up to 2%. Seed treatment and nodulation studies conducted during 1963 by Latch and Greenwood (1964) found that while several fungicides significantly reduced the amount of inoculum associated with lucerne seed, all except thiram prevented nodulation. However, the fungicidal action of thiram against P.medicaginis was not considered sufficiently satisfactory to warrant its recommendation as a routine seed dust.

In an article entitled "Diseases of Lucerne in New Zealand", Close (1967) commented on spring black stem as follows:

> "There is a need for further work to elucidate its importance, and to determine the usefulness of seed treatments for the control of the seed-borne phase of this disease and other seed-borne pathogens."

In the course of the present study spring black stem was observed in all lucerne crops inspected by the author. Blair (1971) in a survey of 160 South Island lucerne crops concluded that this disease was "ubiquitous, trace in all areas, and present in all crops." While there are no New Zealand reports of the spring black stem disease contributing to substantial losses in herbage yields, overseas reports (Toovey et al., 1936; Johnson and Valleau, 1933) indicate that severe infestation can result in total crop loss.

Several authors have adequately described the symptomatology of spring black stem of lucerne. Two such descriptions are recorded below:

(1) Toovey et al., (1936).

> "On the Stems. Sunken lesions of irregular shape, often at the base of the stipules, $\frac{1}{4}$-$2\frac{1}{2}$ in. long, varying in colour from dark brown to black with a light brown centre. Pycnidia were usually present in the old lesions. The lesions partly or wholly girdled the stem, sometimes forming "cankers" thereon, and frequently caused wilting and death of the shoots.
>
> On the petioles. Elongated blackish lesions similar to those on the stems; necrotic zones at the base caused the leaves to become prematurely yellow and to wither.

On the leaf laminae. Lesions of variable size, round or irregular in shape, either on the edge or in the middle of the leaflets, dark brown to black in colour, occasionally zoned, sometimes becoming paler in the centre with age and sometimes surrounded by a yellowish halo. The pycnidia were obscure and were not always present in the leaf lesions. Leaves bearing many spots rapidly turned yellow and withered."

(2) Kernkamp and Homerick, (1953).

"In the early stages of infection small black lesions appeared on the leaves and stems. As they progressed, those on the leaves caused profuse defoliation. Stem lesions enlarged and coalesced during the season until the entire stem became surrounded by a black sheath of fungus mycelium, and diseased host tissue. At this stage, all the leaves had dropped, peduncles and floral parts were infected, and flowers frequently had fallen."

APPENDIX II

COMPOSITION AND PREPARATION OF CULTURE MEDIA.

All media were sterilized by autoclaving at 15 p.s.i. for 20 minutes immediately after preparation.

(a) Potato-carrot Agar

agar	12g
carrot	20g
potato	20g
water (distilled)	1000ml

The potato and carrot were cooked gently for one hour in 500ml water, and the filtrate retained after straining the mixture through cheesecloth. The agar was added slowly to this and stirred until all was dissolved. Any additional water required was then added.

(b) Prune Agar

prune infusion	36g
bacto-agar	15g

24g of this mixture were suspended in 1000ml distilled water and boiled to dissolve the medium.

(c) Nutrient Agar

Oxoid nutrient agar	28g
water (distilled)	1000ml

Boil the solution to dissolve all the nutrient agar.

(d) Water Agar

agar	12g
water (distilled)	1000ml

The agar was dissolved in hot water before autoclaving.

(e) Malt Extract Agar

malt extract (Oxoid)	20g
agar	25g
water (distilled)	1000ml

The water was added slowly to the agar/malt extract mixture and shaken before autoclaving.

(f) Lucerne Seed Agar

lucerne seed	100g
agar	12g
water (distilled)	1000ml

The seed was reduced to powder in a Waering blender and boiled with 800ml water for 20 minutes. The preparation then as for (a).

(g) Czapek Dox Agar

Czapek Dox agar (Oxoid)	45.4g
water (distilled)	100ml

Soak agar in water for 15 minutes, shake well, and autoclave.

(h) Lab Potato-Dextrose Agar

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

Preparation as for (a), except add dextrose as well as agar.

(i) Difco Potato-Dextrose Agar

potato-dextrose agar (Difco)	42g
water (distilled)	1000ml

Preparation as for (c).

(j) Oxoid Potato-Dextrose Agar

potato-dextrose agar	39g
water (distilled)	1000ml

Preparation as for (g).

(k) Nutrient Gelatin

nutrient gelatin (Difco.)	12.8g
water (distilled)	100ml

Warm solution to 50 C, then autoclave.

(l) V-8 Juice Agar

V-8 juice (commercial)	100ml
agar	8.6g
water (distilled)	428ml

Preparation as for (c).

(m) Tomato Juice Agar

tomato juice agar (Oxoid)	52g
water (distilled)	1000ml

Preparation as for (c).

(n) Milk Agar

yeast extract	1.5g
peptone	2.5g
milk	5ml
agar	7.5g
water (distilled)	1000ml

Preparation as for (c).

(o) Tryptone-Soya Agar

tryptone-soya	40g
water (distilled)	1000ml

Soak agar in water for 10 minutes, shake well, and autoclave.

(p) Kliglor-Iron Agar

kliglor-iron (Oxoid)	27.5g
water (distilled)	500ml

Preparation as for (c).

APPENDIX III.

COMPOSITION OF AGAR MEDIA USED TO DETERMINE THE EFFECT OF pH ON GROWTH RATE.

The three components of the media were autoclaved separately at 15 p.s.i. for 20 minutes and then mixed together when the temperature had dropped to 45 C. It was established that if the three components were mixed prior to sterilization those media with a pH below 3.2 failed to solidify. All pH values were determined using a Beckman glass electrode pH meter.

(a) Composition of buffer. (Milne, 1964).

2.6g citric acid

3.8g glycine

3.8g sodium dihydrogen phosphate

100ml distilled water

(b) Composition of agar media.

100ml of Oxoid PDA were used in each treatment.

Treatment	Buffer ml.	2N HCl drops	1N NaOH drops	pH
1	5	30	-	2.1
2	5	20	-	2.5
3	5	10	-	2.8
4	5	-	-	3.25
5	5	-	20	3.8
6	5	-	60	6.6
7	-	-	-	4.5
8	5	-	28	4.85
9	5	-	40	5.85
10	5	-	34	5.4
11	5	-	71	8.0

APPENDIX IV.

METHOD USED TO CALCULATE BLOTTER MOISTURE LEVEL.

The moisture of the blotters was determined by placement of three small strips of blotting paper onto the blotter sheets (Fig. 37). The strips were removed daily and weighed collectively. Preliminary experiments had shown that the moisture level of the strips was the same as that of the blotters, after an equilibration period of several hours. From knowledge of the dry and wet weight of the strips, and the dry weight of the blotters, the moisture level of the blotters was calculated.

Sample calculation

Dry weight strip (determined before start of experiment) = 2g

Wet weight strip (determined daily) = 3.5g

Dry weight blotter (determined before start of experiment) = 40g

$$\text{Wet weight blotter} = \frac{\text{dry weight blotter} \times \text{wet weight strip}}{\text{Dry weight strip}}$$

$$= \frac{40 \times 3.5}{2}$$

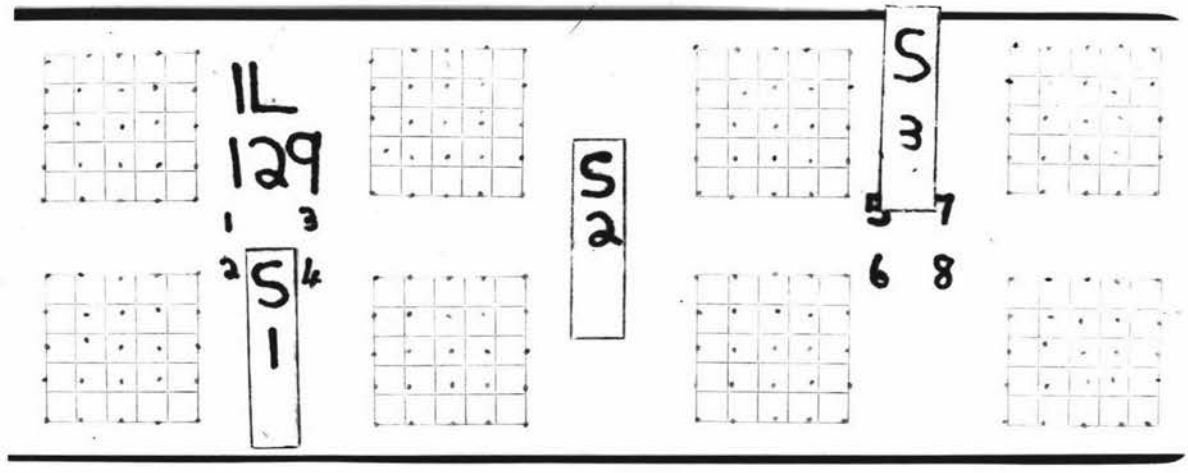
$$= 70g$$

Weight "dry" blotter = Dry weight blotter + same weight in water

$$= 80g$$

i.e. Add 10g (10ml) water to blotter

Figure 37. Seed layout on moist blotter. Note three blotting paper marker strips (S1, S2, S3) used to determine the moisture level.



SEED TRANSMISSION OF FUNGAL PATHOGENS IN COMMERCIAL LUCERNE SEED

Seed line	Date tested	Cultivar	Origin	Level *P. medicaginis* Moist Blotter (percent)	Agar Plate (percent)	Other pathogens (fungal) (percent)
9L347	24.3.70	Wairau	Blenheim	3.5		*S. botryosum* (1.5)
9L348	24.3.70	"	"	0.5		*S. botryosum* (1.5)
9L514	14.8.70	"	Waimate	3		*S. botryosum* (0.5)
9L561	20.4.70	Hunter River	Austr.	3		*Fusarium* spp. (1)
9L589	17.7.70	Wairau	Blenheim	6		-
9L602	12.8.70	"	Oamaru	2		*S. botryosum* (0.5)
OL 37	12.7.70	"	"	4.5		-
OL 38	5.7.70	"	"	21	23X	-
OL 40	9.7.70	"	"	2		-
OL 41	2.7.70	"	"	0	0X	-
OL 48	20.5.70	"	Blenheim	18.5	18X	-
OL 49	29.6.70	"	Chch	4.5	9X	-
OL 50	26.6.70	"	Leeston	2.5	6X	-
OL 51	20.4.70	"	"	11.5	9X	-
OL 52	15.7.70	"	Oamaru	7		-
OL 53	20.3.70	"	Blenheim	0.5		-
OL 54	19.4.70	"	"	22.5		-
OL 55	19.4.70	"	"	23		-
OL 56	20.4.70	"	"	29.5		*Fusarium* spp.(1.5)
OL 59	13.7.70	"	Chch	2		*S. botryosum* (1)
OL167	10.8.70	"	Timaru	2		-
OL168	20.7.70	"	Palm Nth	4	1X	*S. botryosum* (1)
OL171	22.7.70	"	Blenheim	5		-
OL172	24.7.70	"	Amberly	8.5	9X	-
OL173	26.7.70	"	A'burton	0.5		-
OL175	29.7.70	"	"	2	0X	-
OL177	3.8.70	"	Chch	1.5		-
OL178	3.8.70	"	In'gill	3		-
OL180	5.8.70	"	Chch	5		-
OL181	7.8.70	"	Oamaru	47.5		-
OL230	25.9.70	"	Timaru		15X	-
OL232	25.9.70	"	A'burton		23X	-
OL253	25.9.70	"	Blenheim		9X	-
OL258	25.9.70	"	"		14X	-
OL259	21.8.70	Chanti-cleer	Chch	49	45X	*S. botryosum* (0.5)
OL260	25.9.70	Wairau	"		7X	-
OL263	25.9.70	Chanti-cleer	"		20X	-
OL266	25.9.70	Wairau	Blenheim		1X	-
OL269	25.9.70	"	"		2X	-
OL272	21.8.70	"	"	22	24X	-
OL275	22.8.70	"	"	46.5	35X	-
OL279	25.9.70	"	"		28X	-
OLHTW	1.2.71	"	-	1.5	1X	-
Aust I	22.12.70	Hunter River	Austr.	0	0X	-
Aust II	22.12.70	"	"	1.5	0X	-
Aust III	22.12.70	"	"	1.5	0X	-

Seed line	Date tested	Cultivar	Origin	Level P.medicaginis Moist Blotter (percent)	Agar Plate (percent)	Other pathogens (fungal) (percent)
IL 52	18.6.71	Wairau	-	21.5	15.5+	S.botryosum (>60); L.briosiana (0.25)
IL 65	"	"	Blenheim		3.2+	S.botryosum (40)
IL 66	"	"	"		14 +	S.botryosum (31)
IL 67	"	Chanticleer	-		8 +	S.botryosum (81)
IL 86	12.7.71	Wairau	Chch		5.2+	S.botryosum (64.4)
IL 88	"	"	Blenheim		3.1+	S.botryosum (2); Fusarium spp.(0.4)
IL 92	"	"	Chch		10 +	S.botryosum (60.4)
IL 93	"	"	"		21.6+	S.botryosum (40.8) Fusarium spp.(1.6)
IL 94	"	"	"		18.0+	S.botryosum (44.0) Fusarium spp.(2.0)
IL 97	"	"	Oamaru		40.4+	S.botryosum (47.2) Fusarium spp.(2.0)
IL 99	"	"	Chch		2.4+	S.botryosum (73.6) Fusarium spp.(0.4)
IL103	"	"	"		11.2+	S.botryosum (32.0)
				L.briosiana (0.5)		Fusarium spp.(1.2)
IL114	13.7.71	"	Blenheim		14.0+	S.botryosum (34.8)
IL118	"	"	"		10.0+	S.botryosum (33.2)
IL123	"	"	Oamaru		26.4+	S.botryosum (36.0) Fusarium spp.(2.0)
IL126	"	"	Blenheim		3.2+	S.botryosum (54.4) Fusarium spp.(0.4)
IL127	"	"	"		23.6+	S.botryosum (41.6) Fusarium spp.(0.4)
IL129	"	"	Chch	43.0	48.0+	S.botryosum (28.4) Fusarium spp.(2.0)
IL138	"	"	Blenheim		15.2+	S.botryosum (54.0)
IL145	"	"	Chch		8.9+	S.botryosum (82)
IL260	22.8.71	"	Blenheim		8.0+	S.botryosum (61.6)
IL263	"	"	"		7.6+	S.botryosum (63.6) Fusarium spp. (0.8)
IL268	"	"	"		7.6+	S.botryosum (56.8) Fusarium spp.(0.8)
IL282	23.8.71	"	"		16.8+	S.botryosum (46)
IL305	"	"	"		16.8+	S.botryosum (30)
IL307	"	"	"		11.2+	S.botryosum (71.2)
IL308	"	"	"		0 +	S.botryosum (51.6)
IL310	"	"	"		2.8+	S.botryosum (74.4)
IL312	21.8.71	"	"		5.2+	S.botryosum (27.6)
IL313	"	"	"		32.4+	S.botryosum (40)
IL314	"	"	"		18.3+	S.botryosum (48)
IL315	"	"	Chch	26.0	32.8+	S.botryosum (45.2)
IL316	20.8.71	"	"		16.8+	S.botryosum (43.2)
IL317	"	"	-		34.4+	S.botryosum (45.6)
IL324	"	"	Chch		12.0+	S.botryosum (45.6)
IL342	"	"	Blenheim		7.6+	S.botryosum (31.6)
IL344	"	"	"		24.0+	S.botryosum (64.8); Fusarium spp.(.4)
IL347	21.8.71	"	"		11.2+	S.botryosum (53.6)
IL354	22.8.71	"	"		20.8+	S.botryosum (42)
IL356	"	"	"		37.2+	S.botryosum (29.6)

Key:

Chch = Christchurch

x = Seeds incubated on MA for 11 days at 24C in the dark.

\+ = Seeds incubated on MA with streptomycin sulphate and penicillin antibiotics incorporated (each at 50 ppm) for 11 days at 24 C in the dark.

APPENDIX VI:

CALCULATION OF LINEAR REGRESSION LINE FROM DATA REPRESENTING DECREASE IN INFECTION PERCENTAGE OF P.MEDICAGINIS AND THE RESULTING INCREASE OR DECREASE IN SEEDLING EMERGENCE.

	x	x^2	y	xy	y-y	$(y-y)^2$	y^2
	31.0	961.0	19.5	604.5	+ 1.0	1.00	380.25
	29.8	888.04	18.6	554.28	+ 0.5	0.25	345.96
	18.5	342.25	14.7	271.95	+ 0.9	0.81	216.09
	20.0	400.0	12.0	240.0	- 2.2	4.84	144.0
	8.0	64.0	12.7	101.6	+ 2.9	8.41	161.29
	11.5	132.25	7.0	80.5	- 4.0	16.00	49.0
	0	0	9.7	0	+ 3.0	9.00	94.09
	0	0	9.3	0	+ 2.6	6.76	86.49
	0	0	2.0	0	- 4.7	22.19	4.0
Sums	118.8	2787.54	105.5	1852.83	0.0	69.26	1481.17

Raw

s.s. 2787.54 1481.17

C.T. 1568.16 1236.69

1219.38 244.48

Sum Cross Products 1852.83

C.T. 1396.6

460.23

$$\text{Coeff. of regression} = \frac{\text{covariance xy}}{\text{var. x}}$$

$$= \frac{460.23}{1219.38}$$

$$= 0.3774$$

i.e. equation of regression line

$$y = 0.377x + 6.74$$

Code. x = Decrease in infection percentage

y = Increase or decrease in percentage emergence + 10

APPENDIX VII.

THE HEAT STABILITY OF BENLATE

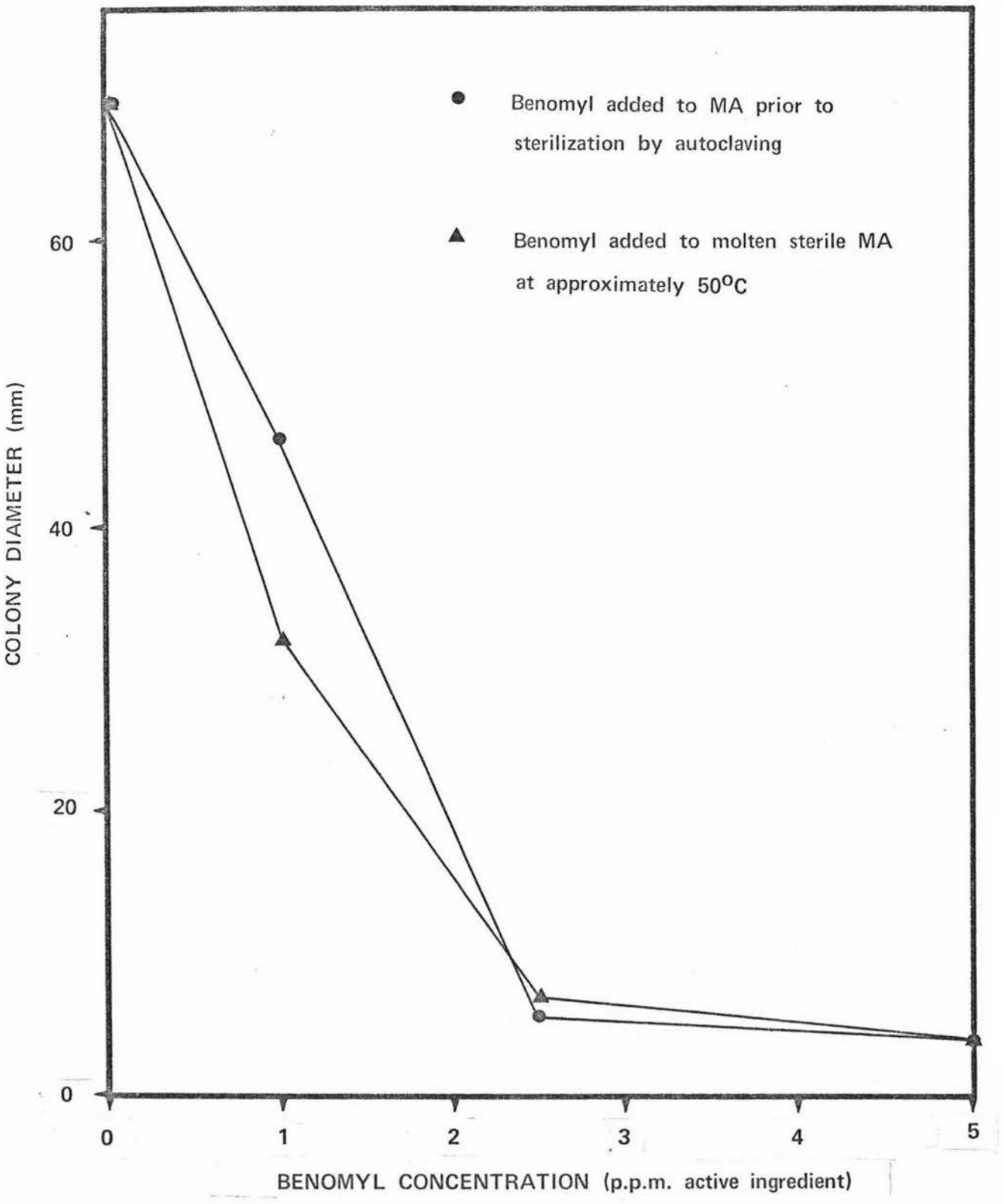
While conducting poison food bioassays with benomyl it was reasoned that the media preparation would be simplified if the fungicide was incorporated into the agar before sterilization (autoclaving at 121.5 C for 20 minutes). Recent work on the heat stability of benomyl indicated that its fungicidal action was not impaired by adding it to a sterile liquid medium prior to autoclaving at 120 C for 10 minutes (Rochecouste, 1971).

A comparative experiment was conducted to determine if the incorporation of benomyl to MA before autoclaving reduced its efficacy against P.medicaginis.

Two treatments were used; in the first the fungicide was added to molten MA in quantities sufficient to give concentrations of 1, 2.5, and 5 ppm (active ingredient) only when it had cooled to approximately 50 C. For the second the benomyl concentrations were the same but it was added to the medium prior to being sterilized by autoclaving at 121.5 C for 20 minutes. The controls for both treatments was MA without additives. The plates were inoculated and incubated as described previously (P.19).

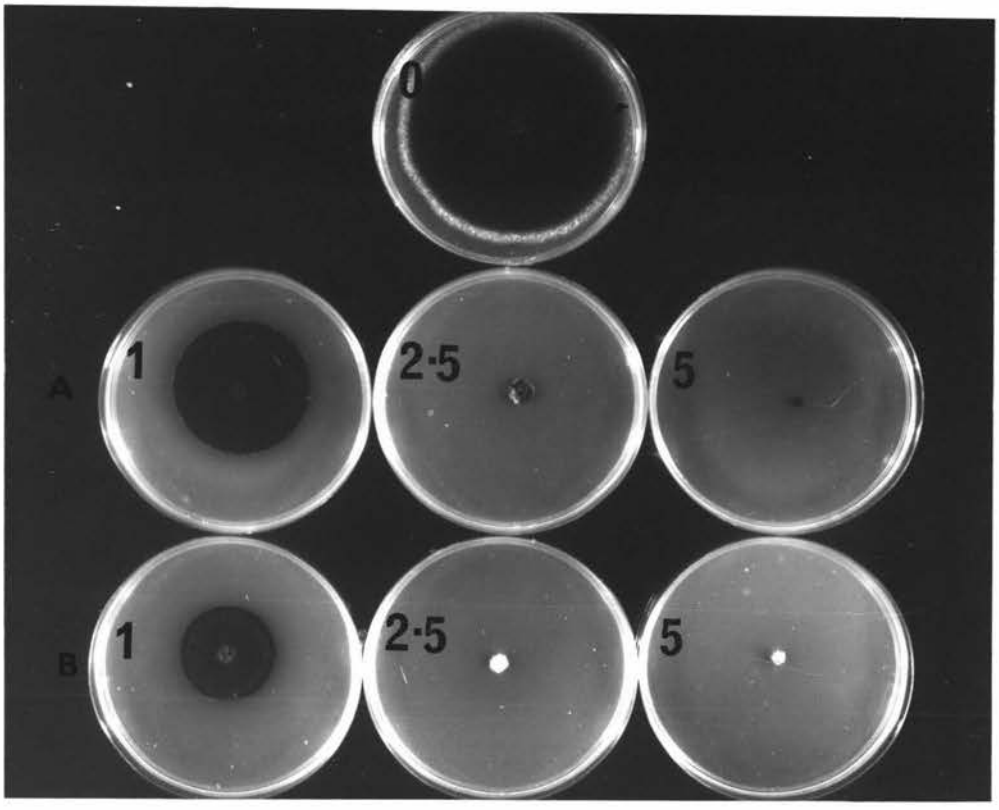
From the results presented in Fig. 38 and 39, it is apparent that at 1 ppm some inactivation of benomyl occurred during autoclaving. As a consequence, the practice of adding benomyl to MA before sterilization was not adopted.

Figure 38



Effect of incorporating benomyl into MA prior to autoclaving for 20 minutes at 121.5°C on growth of P. medicaginis (incubation for 11 days at 24°C in the dark)

Figure 39. The relative heat stability of benomyl when incorporated in MA (colony growth measured following incubation for 11 days at 24 C in the dark).



Key:

A. Benomyl incorporated prior to sterilization by autoclaving (121.5 C at 15 p.s.i. for 20 minutes)

B. Benomyl incorporated at approximately 50 C following autoclaving.

APPENDIX VIII.

INVESTIGATIONS INTO PHYTOTOXICITY OF BENOMYL WHEN APPLIED AS A DUST TO THE TESTA OF LUCERNE SEED.

Benomyl was applied as a dust to the testa of an uninfected seed line (OL175) at the rates of 0.5 and 1 gram of commercial product per 100 grams of seed, by shaking both the seed and the fungicide in a glass container for 30 minutes. Non-dusted but shaken seed provided controls.

Three hundred and fifty seeds from all three treatments were sown to Vermiculite (grade 2) as described previously (P. 95). Records were made on the number of seedlings emerged until 19 days after sowing.

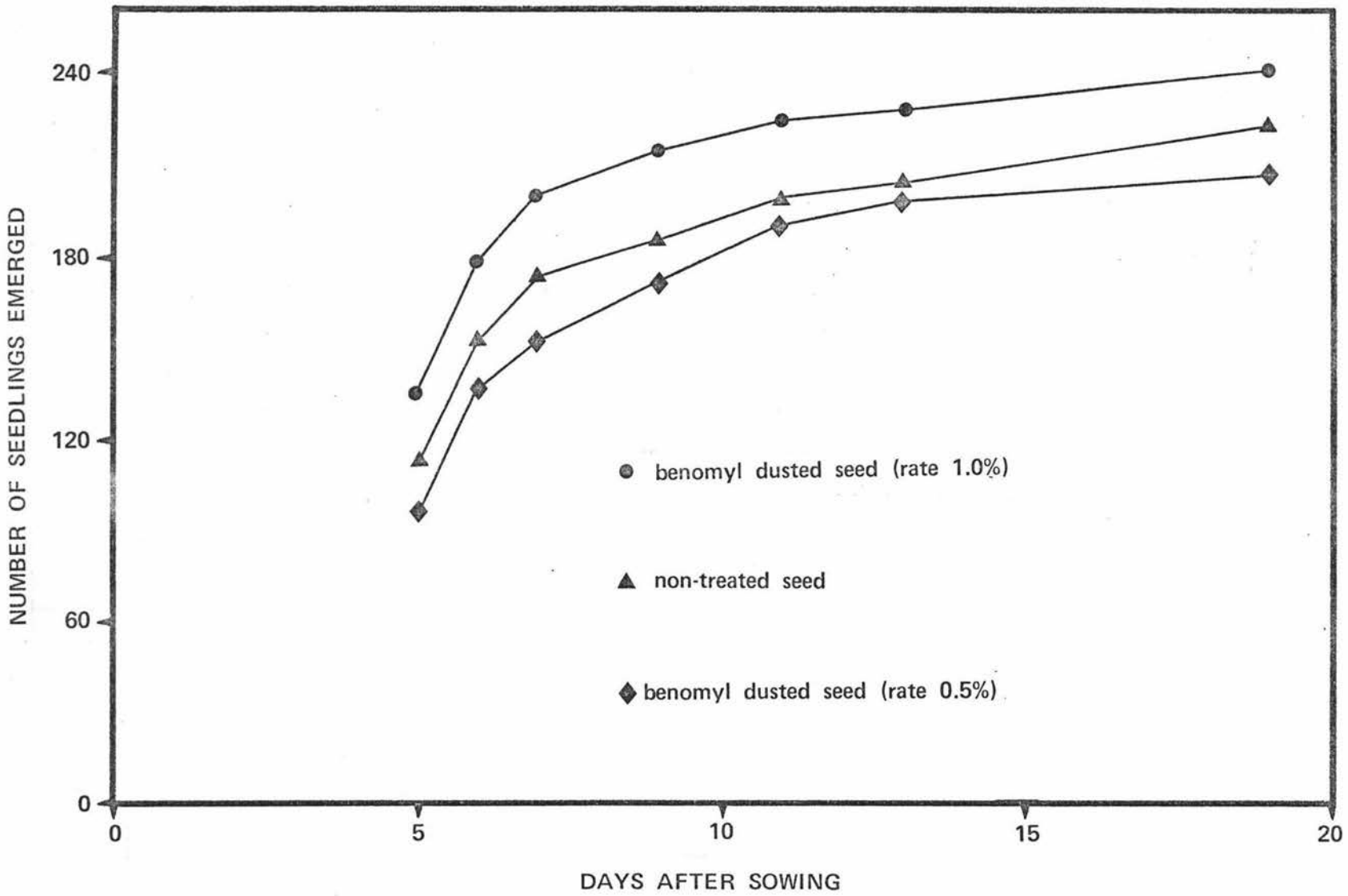
The results are presented in Fig. 40 and can be summarized as follows:

(i) At the lower rate of fungicidal application seedling emergence was decreased over that of the control.

(ii) At the higher rate of fungicide emergence was increased over that of the control.

Interpretation of these results is difficult. However, the possibility remains that the lowered emergence observed in one seed treatment could be consequent upon phytotoxicity of benomyl towards the seedlings.

Figure 40



Rate of seedling emergence (line OL175)

BIBLIOGRAPHY

AGRIOS, George N., 1969. Plant Pathology. Academic Press Inc., 629 pp.

ANON., 1966. Int. Seed Test. Ass. handbook on seed-borne diseases. Series 3, No. 41.

ANON., 1970. Dried lucerne - new cash crop in Mid-Canterbury. N.Z. Farmer 91, 3 : 28-31.

BAKER, K.F. and SMITH, S.H., 1966. Dynamics of seed transmission of plant pathogens. Ann. Rev. Phytopath. 4 : 311-334.

BANTARRI, E.E. and RENFRO, B.L., 1962. Development of spring blackstem in alfalfa as affected by nutrients added to spore suspensions at the time of inoculation. Phytopath. 52 : 724.

BLAIR, I.D., 1970. Agronomic survey of lucerne. Pers. Comm.

BOERMA, G.H., DORENBOSCH, MARIA M.J. and LEFTRING, LIDDY., 1965. A comparative study of the blackstem fungi on lucerne and red clover and the footrot fungus on pea. Neth. J. Plant Path. 71 : 79-89.

BONNER, R.G., 1970. Lucerne establishment with a barley cover crop. N.Z. Fertiliser Jour. 36 : 24.

BRANCATO, F.F. and GOLDING, N.S., 1953. The diameter of the mold colony as a reliable measure of growth. Mycologia 45 : 848 - 864.

CATLING, W.S., 1969. Benomyl - a broad spectrum fungicide. Proc. 5th Br. Insectic. Fungic. Conf. 2 : 298-309.

CHUNG, Hoo Sup and WILCOXSON, Roy D., 1971. Effects of temperature, light, carbon, and nitrogen nutrition on reproduction in Phoma medicaginis. Mycopath. et Myc. appl. 44 : 297-308.

CLOSE, R.C., 1967. The Lucerne Crop. 311 pp. R.H.M. Langer ed., A.H. & A.W. Reed, Wellington, New Zealand.

_____, 1971. Pers. comm.

CLOSE, R.C., WHITELAW, J.S. and TAYLOR, G.G., 1971. Pers. comm. (re Studies on the nodulation of lucerne in the field. I. After inoculation with Rhizobium meliloti granules. II. After using certain fungicides as seed treatments. Paper No. 12. Fourth Australian Legume Nodulation Conf. Canberra, Australia. 1971).

CORMACK, M.W., 1945. Studies on Ascochyta imperfecta, a seed - and soil-borne parasite of alfalfa. Phytopath. 35 : 838-855.

CORMACK, M.W., 1946. Sclerotinia sativa, and related species, as root parasites of alfalfa and sweet clover in Alberta. Can. J. Ag. Sc. 26 : 448-459.

CROSIER, W., 1939. Occurrence and longevity of Ascochyta pisi in seeds of hairy vetch. J.Ag.Res. 59 : 683-697.

CSAPLINSKA, S., 1967. Mycoflora of seeds of some Polish lucerne vars. derived from plantings in Yugoslavia and Poland. Biul. Inst. Hodowl. Aklim. Rosl. 76-77 : 129-132. (Abstr. in Rev. Appl. Myc. 47 : 1172. 1968).

CUNNINGHAM, G.H., 1922. Some recent changes in names of plant diseases, IV. N.Z. J. Agric. 24 : 96-102.

_____, 1956. Plant Diseases Division. Rep. Dep. scient. ind. Res. N.Z. 30 : 55-59.

DICKASON, E.A., LEACH, C.M. and GROSS, A.E., 1968. Clover root Curculio injury and vascular decay of alfalfa roots. J. Econ. Entom. 61 : 1163-1168.

DINGLEY, J.M., 1969. Records of Plant Diseases In New Zealand. Bull. New Zealand Dep. scient. ind. Res. 192. 298 pp.

EDMUNDS, L.K. and HANSON, E.W., 1960. Host range, pathogenicity, and taxonomy of Ascochyta imperfecta. Phytopath. 50 : 105-108.

ELLINGBOE, A.H., 1959a. A comparative study of the fungi causing the spring blackstem disease of alfalfa and red clover. Phytopath. 49 : 764-770.

ELLINGBOE, A.H., 1959b. Studies on the growth of Phoma herbarum var. medicaginis in culture. Phytopath. 49 : 773-776.

FOCKE, I., 1967. Taxonomic confusions in two leaf-spot pathogens of lucerne as examples for the elucidation of the taxonomic situation in the Deuteromycetes. Feddes Repert Z. Bot. Taxon. Geobot. 75 : 61-65. (Abstr. in Biol. Abstr. 49 : 14,735. 1968).

GABITES, J.F., 1967. Meteorological observations for 1965. N.Z. Met. Service Misc. Pub. 109 : 12-14.

_____, 1968. Meteorological observations for 1966. N.Z. Met. Service Misc. Pub. 109 : 14-16.

_____, 1970. Meteorological observations for 1967. N.Z. Min. Transport Misc. Pub. 109 : 14-16.

HAGBORG, W.A.F., G.M. WARNER and PHILLIPS, N.A., 1950. The use of 2,4-D as an inhibitor of germination in routine examinations of beans for seed-borne infection. Science 111 : 91.

HOFER, A.W. and CROSIER, W.F., 1962. Preinoculated alfalfa seed. Agron. J. 54 : 97-100.

HOR, Y.L., 1972. Some aspects of seed infection and control of the collar-rot complex of peas (Pisum sativum L.) caused by Mycosphaerella pinodes (Berk. and Blox.) Vestergr., Phoma medicaginis var. pinodella (Jones) Boerema and Ascochyta pisi Lib. M.Agr.Sc. Thesis. Massey University.

HORSFALL, J.G., 1956. Principles of Fungicidal Action. Chronica Botanica Co., 280 p.p.

HUGHES, S.J., 1953. Conidiophores, conidia and classification. Can. J. Bot. 31 : 577-659.

JACKS, H., 1956. Seed disinfection. XIV. Effect of seed dressings on damping-off and nodulation of lucerne. N.Z.J.Sci. Tech. 38A : 312-315.

JOHNSON, E.M., and VALLEAU, W.D., 1933. Blackstem of alfalfa, red clover and sweet clover. Ky. Agr. Expt. Sta. Bull. 339.

JONES, Fred R., 1918. Yellow-leafblotch of alfalfa caused by the fungus Pyrenopeziza medicaginis. J. Agr. Res. 13 : 307-330.

KENNEDY, B.W. and BRINKERHOFF, L.A., 1959. Comparison of four soil fungicides in the greenhouse for the control of seedling diseases of cotton. Pl. Dis. Reptr. 43 : 90-97.

KERNKAMP, M.F. and HEMERICK, G.A., 1953. The relation of Ascochyta imperfecta to alfalfa seed production in Minnesota. Phytopath. 43 : 378-383.

KILPATRICK, R.A. and JOHNSON, H.W., 1956. Purple stain of legume seeds caused by Cercospora species. Phytopath. 46 : 201-204.

KREUTZER, W.A., 1963. Selective toxicity of chemicals to soil microorganisms. Ann. Rev. Phytopath. 1 : 101-126.

KROUT, W.S., 1921. Treatment of celery seed for the control of Septoria blight. J. Agric. Res. 21 : 369-372.

LAMB, D.H., 1969. Growing lucerne for cash or feed. N.Z. J. Agr. 119 : 88-91.

LATCH, G.C.M. and GREENWOOD, R.M., 1964. Pers. comm. (re The effect of fungicide dusts on Ascochyta imperfecta and Rhizobium meliloti. Rhiz. Newsletter 2 : 146-147).

LEACH, C.M., 1960. Phytopathogenic and saprophytic fungi associated with forage legume seed. Plant Disease Reptr. 44 : 364-369.

LEACH, J.G. and ELLIOTT, E.S., 1951. The blackpatch disease of red clover and other legumes in West Virginia. Phytopath. 41 : 1041-1049.

LEACH, L.D., 1947. Growth rates of host and pathogen as factors determining the severity of preemergence damping-off. J. Agr. Res. 75 : 161-179.

LIMONARD, T., 1966. A modified blotter test for seed health. Neth. J. Pl. Path. 72 : 319-321.

_____, 1967. Bacterial antagonism in seed health tests. Neth. J. Pl. Path. 73 : 1-14.

_____, 1968. Ecological aspects of seed health testing. Proc. Int. Seed Test. Ass. 33 : 343-513.

LLOYD, A.B., 1959. The transmission of Phoma lingam (Tobo) Desm. in the seeds of swede, turnip, choumoellier, rape and kale. N.Z. J. Agr. Res. 2 : 649-658.

LUTTRELL, E.S., 1963. Taxonomic criteria in Helminthosporium. Mycologia 55 : 643-674.

MALBRANCHE, A. and ROUMEGUERE, C., 1886. Fungi Gallici exsiccati. Rev. mycol. Toulouse 8 : 91.

MALONE, J.P., 1962. The application of heat to seed oats as an aid in the detection of Pyrenophora avenae by the Ulster method. Proc. Int. Seed Test. Ass. 27 : 856-861.

MATTHEWS, D., 1970. Pers. Comm.

MAUDE, R.B., VIZOR, Ann S. and SHURING, Catriona G., 1969. The control of fungal seed-borne diseases by means of a thiram seed soak. Ann. appl. Biol. 64 : 245-257.

MAUDE, R.B. and KYLE, Ann M., 1970. Seed treatments with benomyl and other fungicides for the control of Ascochyta pisi on peas. Ann. appl. Biol. 66 : 37-41.

McFARLANE, I.F., 1970. More dry matter from lucerne. N.Z. Fertiliser Jour. 36 : 24.

MEAD, H.W., 1953. Studies on black stem of alfalfa caused by Ascochyta imperfecta Peck. I. Seed and seedling phases of the disease. Can. J. Ag. Sc. 33 : 500-505.

_____, 1961. Studies on Ascochyta imperfecta Peck. Differential utilization of nutrients by isolates from Canadian alfalfa seed. Can. J. Bot. 39 : 1591-1594.

_____, 1962a. Studies on Ascochyta imperfecta Peck. Factors affecting sporulation of Canadian isolates on natural and artificial media. Can. J. Bot. 40 : 263-271.

_____, 1962b. Studies on Ascochyta imperfecta Peck. Changes in physical characteristics of Canadian isolates on agar amended with various amino acids. Can. J. Bot. 40 : 1365-1370.

_____, 1963. Comparison of temperatures favouring growth of Ascochyta imperfecta Peck. and development of spring black stem on alfalfa in Saskatchewan. Can. J. Bot. 41 : 312-314.

_____, 1964a. Infection of alfalfa seed by Ascochyta imperfecta Peck. through the pod. Can. J. Bot. 42 : 1101-1102.

_____, 1964b. Resume of data on blackstem of alfalfa caused by Ascochyta imperfecta Peck. Can. Plant Dis. Survey 44 : 134-141.

MEAD, H.W. and CORMACK, M.W., 1961. Studies on Ascochyta imperfecta Peck. Parasitic strains among fifty isolates from Canadian alfalfa seed. Can. J. Bot. 39 : 793-797.

MEEKLAH, F.A. and ALLEN, F.C., 1971. Weed control in lucerne. N.Z. J. Ag. 122(1) : 26-31.

MILNE, K.S., 1964. A study of brown spot disease of Lupinus spp. caused by Pleiochaeta setosa (Kirchner) Hughes. M.Agr.Sc. Thesis, Massey University.

NEERGAARD, Paul, 1965. Historical development and current practices in seed health testing. Proc. Int. Seed Test. Ass. 30(1) : 99-118.

_____, 1970. Seed pathology, international co-operation and organization. Proc. Int. Seed Test. Ass. 35 : 19-42.

NELSON, R.R., 1955. Studies on Stemphylium leafspot of alfalfa. Phytopath. 45 : 352-356.

New Zealand Official Yearbook, 1964.

New Zealand Official Yearbook, 1970.

NOBLE, M., 1965. Introduction to Series 3 of the handbook on seed health testing. Proc. Int. Seed Test. Ass. 30 : 1045-1047.

_____, 1970. Pers. Comm.

NOBLE, M. and RICHARDSON, M.J., 1966. An annotated list of seed-borne diseases. Second edition. Proc. Int. Seed Test. Ass. 33 : 87-89.

OLIVER, J., 1971. Room for more lucerne. Christchurch Press. 16th July, 1971.

PALMER, T.P., 1971. Plant density and production in lucerne. (Medicago sativa L.). Proc. Agron. Soc., N.Z. Inst. Agr. Sc. Conf. 1 : 26-35.

PECK, C.H., 1912. Report of the state botanist, 1911. Species not before reported. N.Y. State Museum Bull. 157 : 21.

PETERSON, M.L. and MELCHERS, L.E., 1942. Studies on black stem of alfalfa caused by Ascochyta imperfecta. Phytopath. 32 : 590-597.

REITZ, L.P., GRANDFIELD, C.O., PETERSON, M.L., GOODING, G.D., ARNESON, M.A. and HANSING, E.D., 1948. Reaction of alfalfa varieties, selections and hybrids to Ascochyta imperfecta. J. Agr. Res. 76 : 307-323.

RENFRO, B.L. and KERNKAMP, M.F., 1963. Fungi isolated from black stem of alfalfa and the influence of temperature on lesion formation and disease severity. Phytopath. 53 : 774-777.

RENFRO, B.L. and SPRAGUE, E.W., 1959. Reaction of Medicago spp. to eight alfalfa pathogens. Agron. J. 51 : 481-483.

RENFRO, B.L. and WILCOXSON, Roy D., 1963. Spring black stem of alfalfa in relation to temperature, moisture, wounding, and nutrients and some observations on pathogen dissemination. Phytopath. 53 : 1340-1345.

RICHARDSON, L.T., 1954. The persistence of thiram in soil and its relationship to the microbiological balance and damping-off control. Can. J. Bot. 32 : 335-346.

ROBERTS, D.A., 1957. Observations on the influence of weather conditions upon severity of some diseases of alfalfa and red clover. Phytopath. 47 : 626-628.

ROBERTS, D.A., FORD, R.E., WARD, C.H. and SMITH, D.T., 1959. Colletotrichum anthracnose of alfalfa in New York. Plant. Dis. Rptr. 43 : 352-359.

ROCHECOUSTE, E., 1971. Pers. Comm.

ROSEMBLIT, A., 1950. The Ascochyta of lucerne in Argentina. Rev. Argent. Agron. 17 : 89-97. (Abstr. in Rev. Appl. Myc. 29 : 567.1950).

RÖSSNER, H., 1968. Untersuchungen über den Erreger der Phomakrankheit der Luzerne (Phoma medicaginis Malbr. et Roum). Phytopath. Z. 63 : 101-123.

SALUNSKA, Mme. N.I., 1959. Ascochytosis of lucerne in the Ukr. S.S.R. Nauk. Prats. Ukr. nauk.-dosl. Inst. Zakh. Rosl. 9 : 83-97. (Abstr. in Rev. Appl. Myc. 41 : 527.1962).

SCHENCK, N.C. and GERDEMANN, J.W., 1956. Taxonomy, pathogenicity, and host-parasite relations of Phoma trifolii and Phoma herbarum var. medicaginis. Phytopath. 46 : 194-200.

SEARLE, S.R., 1971. Linear Models. John Wiley and Sons, Inc. (Distributional properties : 99) 532 pp.

SIMMONS, E.G., 1966. The theoretical bases for classification of the Fungi Imperfecti. Quarterly Rev. Biol. 41 : 113-123.

SPRAGUE, R., 1929. Host range and life history studies of some leguminous Ascochytae. Phytopath. 19 : 917-932.

STEWART, F.C., FRENCH, G.T. and WILSON, J.K., 1908. Troubles of alfalfa in New York. N.Y. Agr. Expt. Sta. Bull. 305.

TAMIMI, S.A. and RUMBAUGH, M.D., 1963. Resistance of diploid alfalfa to Phoma herbarum var. medicaginis and Cercospora zebrina. Crop. Sci. 3 : 227-230.

DE TEMPE, J., 1963. On methods of seed health testing, principles and practice. Proc. Int. Seed Test. Ass. 28 : 97-105.

_____, 1968. The health condition of seeds in commercial channels: Development of methods for routine testing of seeds for seed-borne organisms. Govt. Seed Test. Stn. Wageningen. The Netherlands. 136 p.p.

_____, 1970. Routine methods for determining the health condition of seeds in the seed testing station. Proc. Int. Seed. Test. Ass. 35 : 257-296.

DE TEMPE, J. and LIMONARD, T., 1966. The influence of substrate moisture on the results of seed health testing in blotter medium. Proc. Int. Seed Test. Ass. 31 : 169-178.

TOCKER, H.H., 1970. Lucerne as a cash crop in Canterbury. N.Z. J. Agr. 120 : 5.65.

TOOVEY, F.W., WATERSTON, J.M. and BROOKS, F.T., 1936. Observations on the black-stem disease of lucerne in Britain. Ann. Appl. Biol. 23 : 705-717.

TORGESON, D.C., 1967. Determination and measurement of fungitoxicity. In Fungicides, An Advanced Treatise. Ed. Torgeson D.C. Academic Press Inc. Vol. I. 697 p.p.

WALLEN, V.R. and SEAMAN, W.L., 1963. Seed infection of soybean by Diaporthe phaseolorum and its influence on host development. Can. J. Bot. 41 : 13-21.

WEBER, Paul V.V., 1952. Effectiveness of seed treatment in controlling damping-off in alfalfa. Phytopath. 42 : 22.

WELTZIEN, H.C., 1957. Studies on the fungal infection of lucerne seed and its eradication by treatment. Z. Pflkrankh. 64 : 705-718. (Abstr. in Rev. Appl. Myc. 37 : 496 1958).

WENHAM, H.T., 1970. Pers. Comm.

WOOD, F.H., 1966. A study of two seed-borne Alternaria diseases on choumoellier. M.Agr.Sc. Thesis, Massey University.

YARWOOD, C.E., 1956. Humidity requirements of foliage pathogens. Plant Dis. Reptr. 40 : 318-321.

ZAKOPAL, J. and SYCHROVA, E., 1966. Unusual occurrence of Botrytis cinerea on seed lucerne. Ochr. Rost. 2 : 243-244. (Abstr. in Rev. Appl. Myc. 46 : 2767 1967).