Some Aspects of Seed Infection and Control of the Collar-rot Complex of Peas (*Pisum sativum* L.), caused by *Mycosphaerella pinodes* (Berk. and Blox.) Verstergr., *Phoma Medicaginis* var. *pinodella* (Jones) Boerema, and *Ascochyta pisi* Lib.

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SUMMARY

1. The collar-rot complex of peas caused by *Mycosphaerella pinodes*, *Phoma medicaginis* var. *pinodella* and *Ascochyta pisi* is present in the Manawatu, but only mycosphaerella blight (caused by *M. pinodes*) is prevalent.

2. The component diseases and causal fungi of the complex are readily identified on the basis of their symptoms and pycnidiospore morphology.

3. Symptoms induced by *A. pisi* are distinct from those of *M. pinodes* and *P. medicaginis* var. *pinodella*. Although symptoms induced by the latter two pathogens are indistinguishable, in the field situation *M. pinodes* generally induced more profuse lesioning.

4. The pycnidiospores of *M. pinodes* and *A. pisi* are large and uniseptate, those of *M. pinodes* being broader (11.5 x 4.3 u) than those of *A. pisi* (13.2 x 3.7 u). Pycnidiospores of *P. medicaginis* var. *pinodella* are non-septate (or occasionally uniseptate) and smaller (7.8 x 2.9 u). *Mycosphaerella pinodes* can also be identified by the production of ascostromata and ascospores on agar and on infected plants.

5. The disease complex was present in 60% of 86 New Zealand produced pea seedlines from the 1969/70 and 1970/71 harvests.

The level of infection in individual seedlines was low, only 18% being infected to more than 1%.

6. Mycosphaerella pinodes constituted 66.9% of the collar-rot fungi isolated to agar from commercial seedlines, the remainder being P. medicaginis var. pinodella. In no instance was A. pisi detected.

7. The infection level of individual seed lines can be increased by macroscopic selection on the basis of seed discolouration, fluorescence under ultraviolet light and smaller seed size.

8. All three pathogens are highly sensitive to benomyl and thiram, mycelial growth being suppressed by 50% (ED_{50}) at rates of less than 10 ug/ml a.i.

9. Benomyl is taken up and systemically translocated in pea plants when applied as a soil drench, foliar spray and seed dressing.

10. When applied as a soil drench benomyl is taken up and translocated at concentrations of 250 to 1,000 ug/ml a.i. In plants drenched with the higher concentrations translocation is more widespread and accumulation is higher.

11. By the third week after drenching at 250 to 1,000 ug/ml a.i. the distribution of benomyl in all treated plants is bimodal, being concentrated mainly in the leaves of the lower and upper

nodes (nodes 2 and 10 respectively). Within individual leaves, benomyl is mainly concentrated in the leaf tip and margins.

12. When drenched at 500 ug/ml a.i., benomyl persists up to nine weeks in treated plants and peak accumulation occurs at approximately the third week. The chemical is translocated to leaves, pedicels, sepals, podwall and maturing seeds, but is not detected in the petals.

13. When applied as a foliar spray at concentrations of 62.5 to 250 ug/ml a.i., benomyl is taken up by pea plants but translocation is not widespread.

14. In glasshouse trials benomyl applied as a soil drench and a foliar spray was systemically absorbed and provided significant but not complete protection.

15. Benomyl applied to pea seeds as a dust or slurry (1 to 6 oz Benlate/bushel) is taken up by germinating seeds and accumulates mainly in the cotyledons and the hypocotyl/ epicotyl regions of the seedlings. In glasshouse and field trials the absorbed chemical inactivated deep seated mycelium of M. pinodes and P. medicaginis var. pinodella in most but not all infected seeds.

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Chapter I

INTRODUCTION.

The green pea, Pisum sativum L., is one of the principal field crops in New Zealand (Anon, 1970a), and essentially two varieties, P. sativum var. sativum (garden peas) and P. sativum var. arvense (field peas) are grown. Peas of the garden variety are used for human consumption and may be consumed fresh, processed or marketed as split peas. The field variety is mainly used for animal consumption. However both varieties are widely grown for seed production and are important to New Zealand in international seed trade.

The cultivation of green peas in New Zealand has increased steadily over the last few years. In 1966-67 approximately 28,000 acres were sown, while in 1968-69 this had increased to approximately 50,000 acres (Anon, 1970a). The main pea producing areas are Canterbury, Wellington, Marlborough, Hawkes Bay and Otago, with Canterbury alone being responsible in 1967-68 for three-quarters of the total production in New Zealand (Anon, 1969). In 1968-69 export of seed peas and artificially dehydrated peas alone resulted in total earnings of more than two million dollars (Anon, 1970b).

The average yield of peas for the last few years has been approximately 35-40 bushels/acre (Anon, 1969). However this does not reflect the genetic potential of the crop owing to the influence of a variety of adverse environmental factors, of which diseases frequently play an important part. Some fifteen fungal diseases have been recorded

on peas in New Zealand (Dingley, 1969), and of these the collar-rot complex, and pea wilt (Fusarium orthoceras Appel. and Wollenw. var. pisi Linford; synonym Fusarium oxysporum Schlecht. f pisi) are considered the most important (Brien et al., 1955).

The so-called collar-rot complex is in fact three distinct diseases grouped for convenience on account of the similarity of the symptoms induced and the close mycological relationship of the three causal fungi. The causal organism and common name of each component of the complex is as follows:

(i) Mycosphaerella pinodes (Berk. and Blox.) Vestergr. (Synonym: Ascochyta pinodes Jones), causing mycosphaerella blight

(ii) Phoma medicaginis var. pinodella (Jones) Boerema, causing ascochyta footrot

(iii) Ascochyta pisi Lib., causing ascochyta leaf and pod spot.

Ascochyta pisi attacks pods, seeds, leaves and sometimes stems, while P. medicaginis var. pinodella and M. pinodes cause lesions on pods, seeds, leaves, stems and roots (Hare and Walker, 1944; Brien et al., 1955). In all cases, where infections are severe the result is a significant reduction in both the quantitative and qualitative yield of seeds (Jones, 1927; Wallen, 1965; Maude, 1966).

The collar-rot complex is of world wide distribution (Map 1) and has been the subject of a great deal of research. The essential features of the epidemiology of each pathogen has been determined and these are summarised in Table I.

From the above table it is apparent that for all three components of the complex seed is considered an important source of primary infection.

Map 1. World distribution of the collar-rot complex in peas.

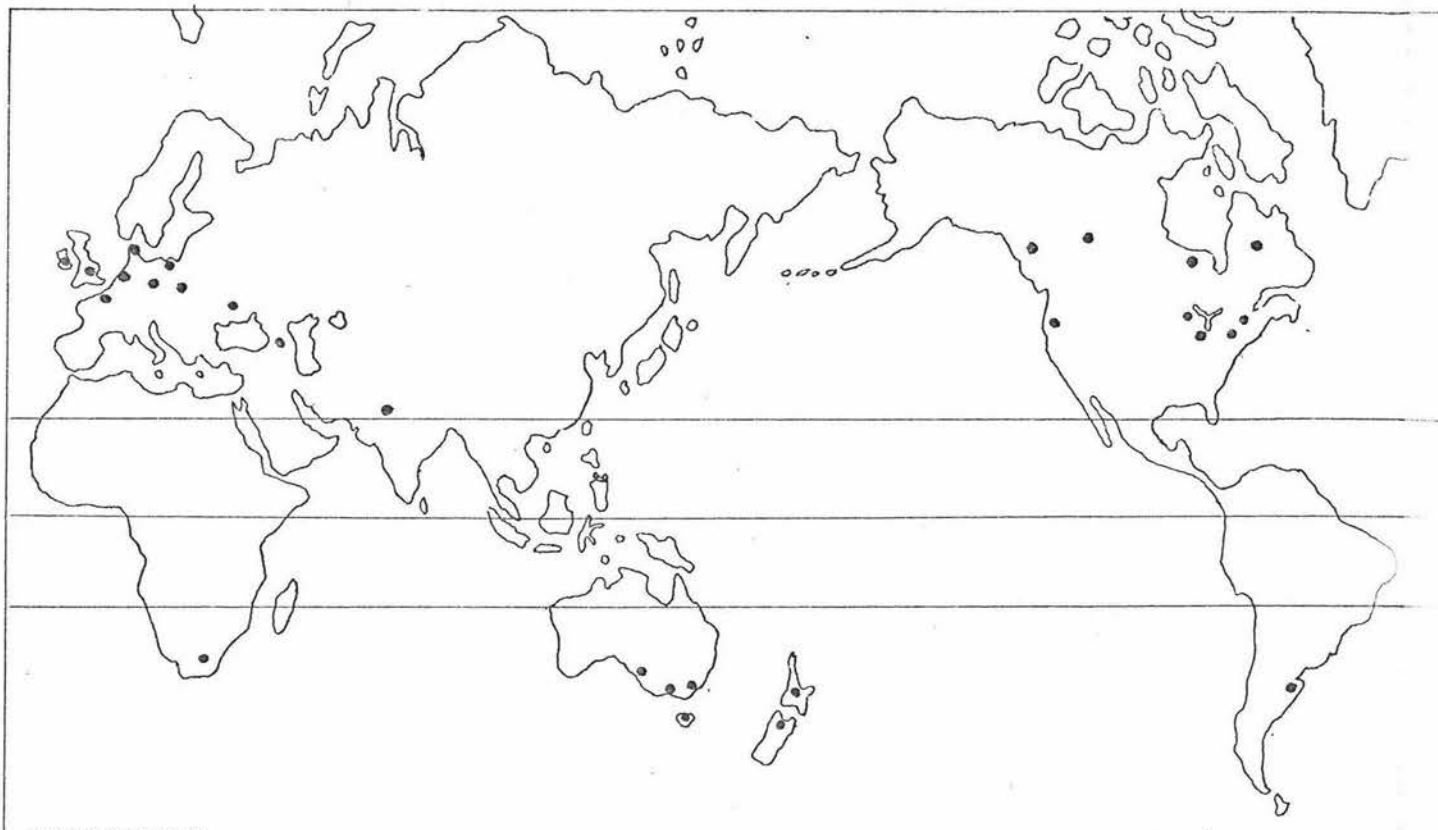

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Table I. Salient features of the epidemiology of the diseases caused by *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi* (Jones, 1927; Hare and Walker, 1944; Carter and Moller, 1961 and Wallen *et al.*, 1967b).

Phase of disease cycle	*Mycosphaerella pinodes*	*Phoma medicaginis* var. *pinodella*	*Ascochyta pisi*
Overwintering Phase	1. In seed. 2. In infected pea plant debris. 3. In soil.	1. In seed. 2. In infected pea plant debris. 3. In soil.	1. In seed. 2. In infected pea plant debris.
Primary Inoculum	1. From infected seeds. 2. Pycnidiospores from infected pea plant debris. 3. Ascospores from plant debris. 4. From soil-borne inoculum.	1. From infected seeds. 2. Pycnidiospores from infected pea plant debris. 3. From soil-borne inoculum.	1. From infected seeds. 2. Pycnidiospores from infected pea plant debris.
Secondary Inoculum and spread	1. Localised spread by rainsplashed pycnidiospores from primary lesions. 2. Long distance spread by airborne ascospores from primary lesions.	1. Localised spread by rainsplashed pycnidiospores from primary lesions.	1. Localised spread by rainsplashed pycnidiospores from primary lesions.

For this reason considerable emphasis has been placed on the establishment of crops using pea seed free of infection (Cruickshank, 1957; Wallen, 1965). Ideally seed should be produced in climatic areas not conducive to disease development, thereby ensuring the production of pathogen free seed. In all pea seed producing areas in New Zealand climate does have some restrictive influence on the collar-rot complex but insufficient to guarantee freedom from the component pathogens. For this reason there is need for a practical seed treatment method to inactivate inoculum associated with seed. Non-systemic fungicidal dusts applied either in dry form or as a slurry are relatively ineffective on account of the pathogens being established deep within the seed (Maude, 1966; de Tempe, 1968b), and hot-water seed treatments have failed to eradicate the pathogens without impairing seed viability (Ogilvie, 1933). The thiram soak method of seed treatment developed by Maude (1966) is effective against M. pinodes and A. pisi, but is impractical on account of the difficulty of drying large quantities of seed.

A recent development showing great promise for the control of seed-borne diseases has been the introduction of fungicides with systemic activity. When used as a seed dressing such fungicides are absorbed and inactivate deep-seated infection without adversely affecting seed germination (Catling, 1969; Maude and Shuring, 1969). In the United Kingdom, Maude and Kyle (1970) have demonstrated complete control of A. pisi using the systemic fungicide benomyl as a seed dressing, but no reference was made to its effectiveness in controlling P. medicaginis var. pinodella or M. pinodes. Since Cruickshank (1957) has shown these latter two species to be also commonly associated with New Zealand produced seed peas there is obvious need to explore the effectiveness of benomyl

against all three components of the collar-rot complex under New Zealand conditions. Such an investigation constituted the main theme of the present study.

However before this work could proceed it was first necessary to conduct symptomatological and mycological studies to enable ready identification of the component diseases and their causal fungi. Further, to ascertain the relative prevalence of the three fungi in New Zealand produced seed a survey was undertaken over two years of the state of health of commercially available seed lines.

In summary then, the main objectives of the present investigation were:

(i) to determine the distinctive features of the component diseases and their causal fungi

(ii) to survey the health status of pea seed lines harvested in New Zealand in 1969/70 and 1970/71

(iii) to investigate the effectiveness of some recently available fungicides for the control of the collar-rot complex when applied as a seed dressing.

Chapter II

METHODS OF IDENTIFYING THE THREE DISEASES AND FUNGAL PATHOGENS IN THE COMPLEX

Recognition of the component diseases of a complex is usually based on the distinctive symptomatology expressed by infected plants, supplemented by knowledge of the morphology of each pathogen. In the present investigation comparative symptomatological and mycological studies were made to enable a comparison with overseas descriptions, and to identify those features which would allow ready recognition of each disease.

A. SYMPTOMATOLOGY.

Symptoms of the three diseases have been widely studied overseas and essentially similar observations have been reported (Table II).

Symptoms induced by the three fungi were studied under both field and glasshouse conditions and compared with those summarised in the above table. Particular note was made of specific points of difference between the diseases as described overseas and expressed under local conditions.

1. Field Observations.

Disease development was followed in several commercial pea crops in the Manawatu during the 1970/71 growing season. In the early stages of growth there was very little evidence of infection but as the plants approached anthesis aerial symptoms became more evident and were

Table II. Summary of overseas descriptions of symptoms caused by M. pinodes, P. medicaginis var. pinodella and A. pisi (Jones, 1927; Linford & Sprague, 1927; Sattar, 1934; Hare & Walker, 1944; Skolko et. al., 1954; Zaumeyer, 1962).

	Mycosphaerella pinodes	P. medicaginis var. pinodella	Ascochyta pisi
1. Parts infected	Leaf, petiole, stem, collar, flower and pod.	Leaf, petiole, stem, collar and pod.	Leaf, petiole, stem and pod.
2. Leaf symptoms	Brown to purplish-black, irregular, dendritic spots without definite margin; under wet conditions, enlarging to circular, zonate lesions 5-6 mm in diameter; leaves may shrivel and turn brown. Pycnidia erratically produced, minute, black and scattered.	Indistinguishable from M. pinodes, but less prolific; symptoms may develop into more regular, somewhat circular zonate lesions.	Definite, sunken, tan to brown circular spots with prominent dark-brown margin. 2-10 mm in diameter. Pycnidia numerous, minute, tan to brown and grouped at centre.
3. Petiole and stem symptoms	Brown to black or purplish streaks up to 10 mm long, more pronounced at the nodes and advancing above or below them. Pycnidia erratically produced. Ascostromata abundant on dead tissues; minute, black, scattered.	As for M. pinodes, but less abundant and severe.	Essentially similar to leaves except elongated, tan to dark-brown.

Continued over ...

Table II. Summary of overseas descriptions of symptoms caused by M. pinodes, P. medicaginis var. pinodella and A. pisi - continued.

	Mycosphaerella pinodes	P. medicaginis var. pinodella	Ascochyta pisi
4. Collar symptom	Severe collar rot, may extend up the stem and down the tap-root to the main laterals; collar often girdled.	As for M. pinodes, but more severe and often girdling the collar.	No collar infection or only very slight lesioning.
5. Flower symptom	Small, dark lesions on petals; flowers wither and drop.	Not described.	Not described.
6. Pod symptom	Brown to blue-black or purplish specks; enlarging and coalescing to produce sunken pits; young pods distorted and often absciss.	Not described.	Pitted, tan to brown with definite dark-brown margin.

Figure 1. Early field symptoms on garden peas, caused by M. pinodes.

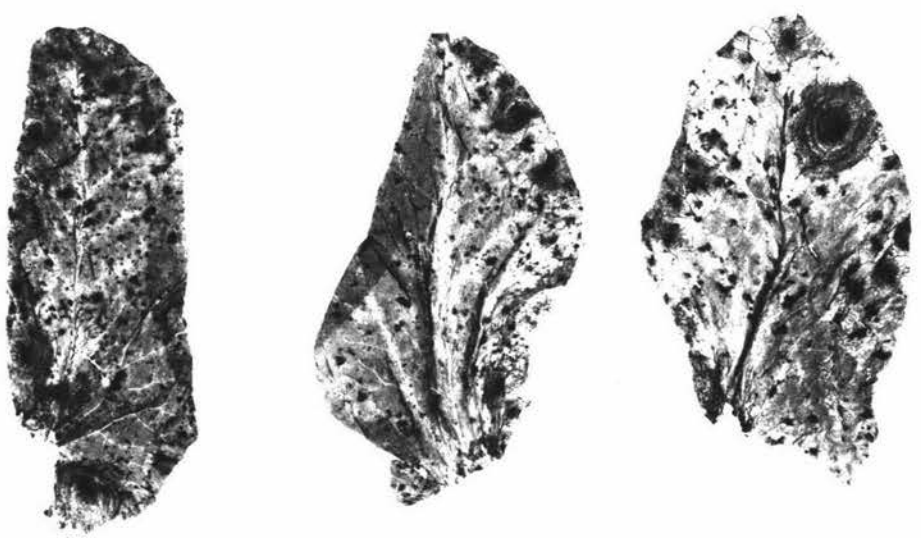


Figure 2. Advanced field symptoms on pea leaves, caused by M. pinodes.

Figure 3. Pea blossoms naturally infected with M. pinodes.

Figure 4. Early (left) and advanced (right) field symptoms on pea pods, caused by M. pinodes.

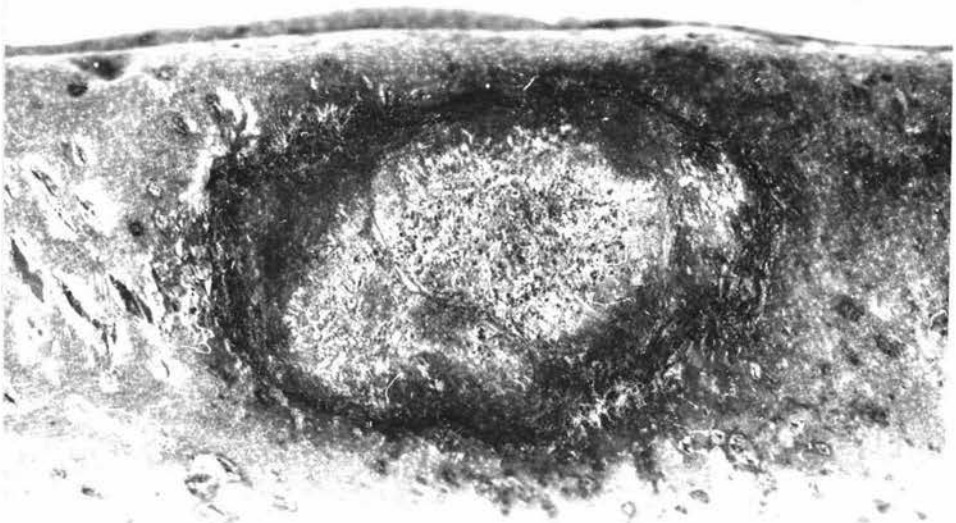


Figure 5. Pea pod naturally infected by M. pinodes showing well developed lesions with pycnidia.

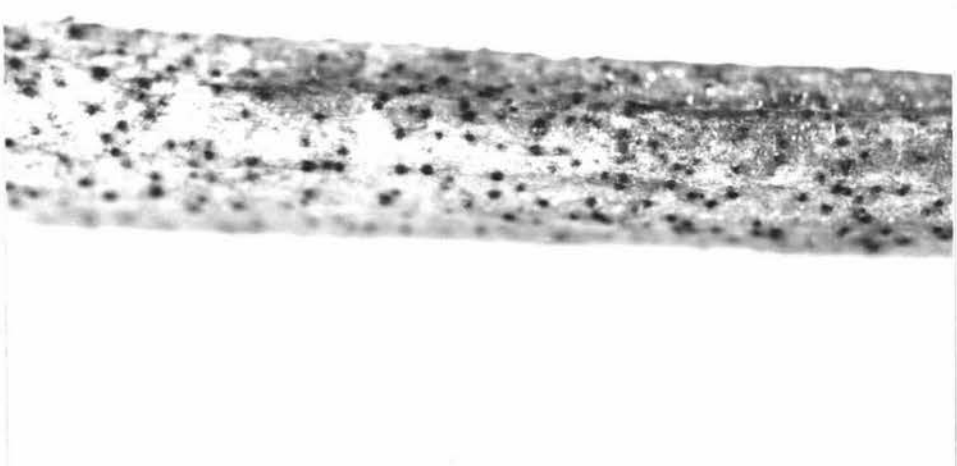


Figure 6. Ascostromata of M. pinodes produced on field infected pea stem.

widespread towards pod maturity. The symptoms expressed on infected plants in most properties corresponded to those recorded for M. pinodes in the literature (Table II), and isolation studies confirmed this species to be the dominant pathogen. Figures 1-6 depict symptoms typical of field infections.

On rare occasions P. medicaginis var. pinodella was isolated from leaf lesions which were indistinguishable from those induced by M. pinodes. Invariably such lesions were associated with necrotic areas infected by M. pinodes, with the latter pathogen predominating. It appeared that the presence of P. medicaginis var. pinodella was largely obscured by the more prolific M. pinodes infections.

The observation in the literature that P. medicaginis var. pinodella caused a more severe collar-rot than M. pinodes was not found to be generally true in local fields. M. pinodes was observed to produce a collar-rot as severe as that caused by P. medicaginis var. pinodella (Figures 57 and 58).

Symptoms attributed to A. pisi were only observed in one crop in the Manawatu. Typical pod, stem and leaf lesions (Table II) were observed on infected plants and these are illustrated in Figures 7-10.

2. Glasshouse Observations.

As field infections caused by P. medicaginis var. pinodella were rarely encountered the complete range of symptoms produced by the disease complex could not be studied. Further observations were therefore made in conjunction with artificial inoculations carried out in the

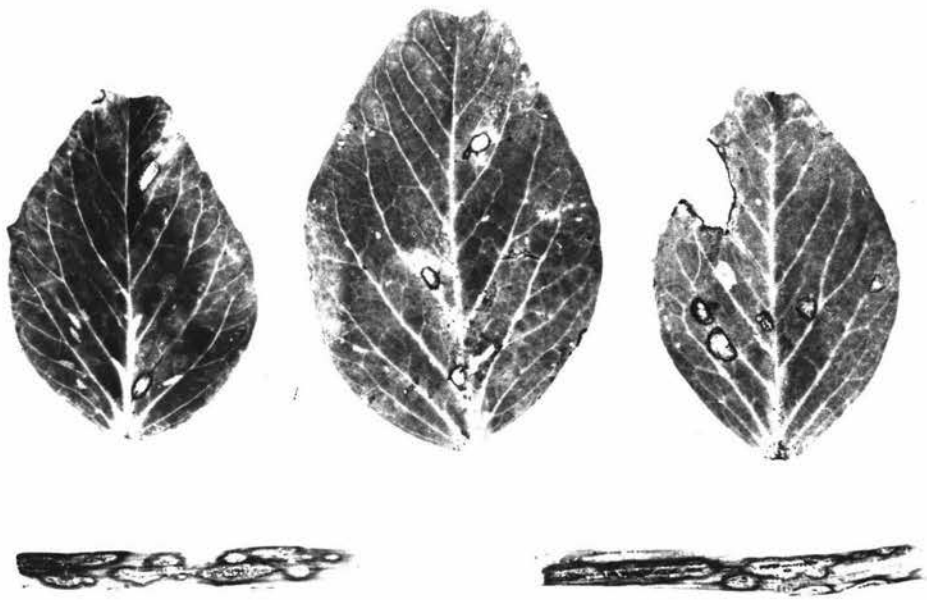


Figure 7. Pea leaves and stems naturally infected with A. pisi.

Figure 8. Pea stem naturally infected by A. pisi showing well-developed lesions with pycnidia.

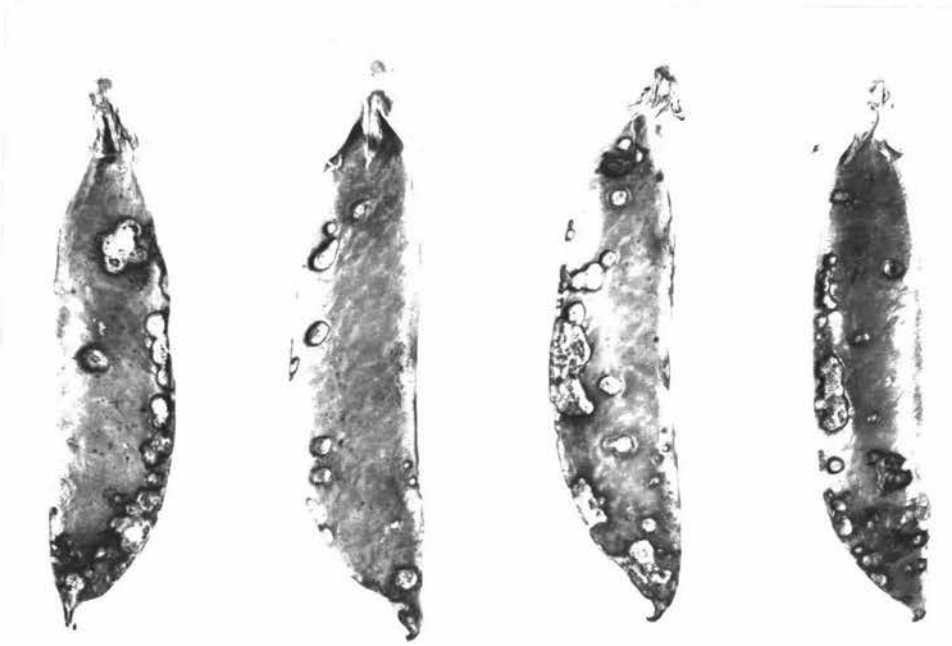


Figure 9. Pea pods naturally infected by A. pisi showing advanced lesioning.

Figure 10. Pea pods naturally infected with A. pisi showing well developed lesions and pycnidia.

glasshouse to meet the requirements of "the rules of proof of pathogenicity", or Koch's Postulate (Appendix III).

While the symptoms produced by each species were essentially similar to those observed in the field and reported overseas (Table II), minor differences were noted.

(a) Mycosphaerella pinodes.

(i) On leaves less profuse lesioning was produced in the glasshouse than in the field (Figure 11)

(ii) Severe lesions were produced at the nodes which caused most of the inoculated plants to wilt and die. This was assumed to be due to the inoculum flowing down the leaf and collecting in the nodal region.

(b) Phoma medicaginis var. pinodella.

(i) Leaf lesions (Figure 12) were not profuse and were similar to those produced by M. pinodes in the glasshouse

(ii) Lesions produced at the nodes were generally less severe than those caused by M. pinodes and inoculated plants only occasionally wilted and died.

(c) Ascochyta pisi.

(i) Moderate lesioning was observed on inoculated plants (Figure 13), and these were quite typical of overseas descriptions (Table II)

(ii) Nodal lesions were produced infrequently and were

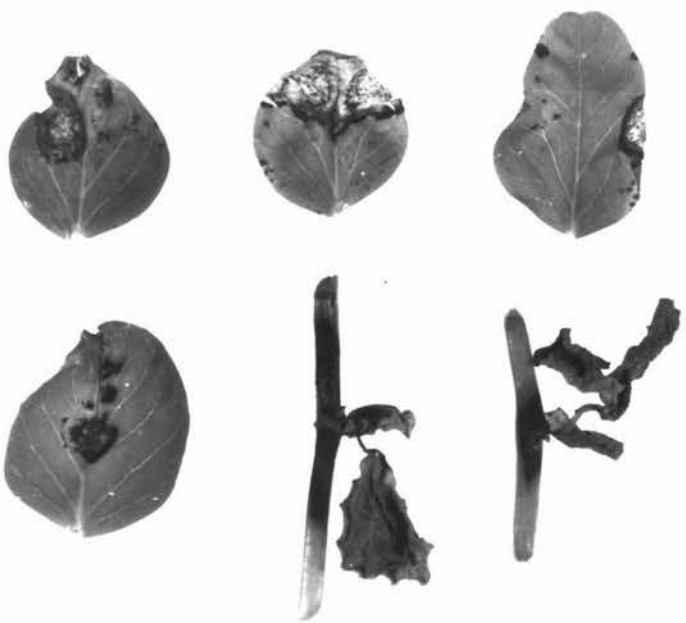


Figure 11. Symptoms on leaves and stems artificially inoculated with M. pinodes.

Figure 12. Symptoms on leaves and stems artificially inoculated with P. medicaginis var. pinodella.

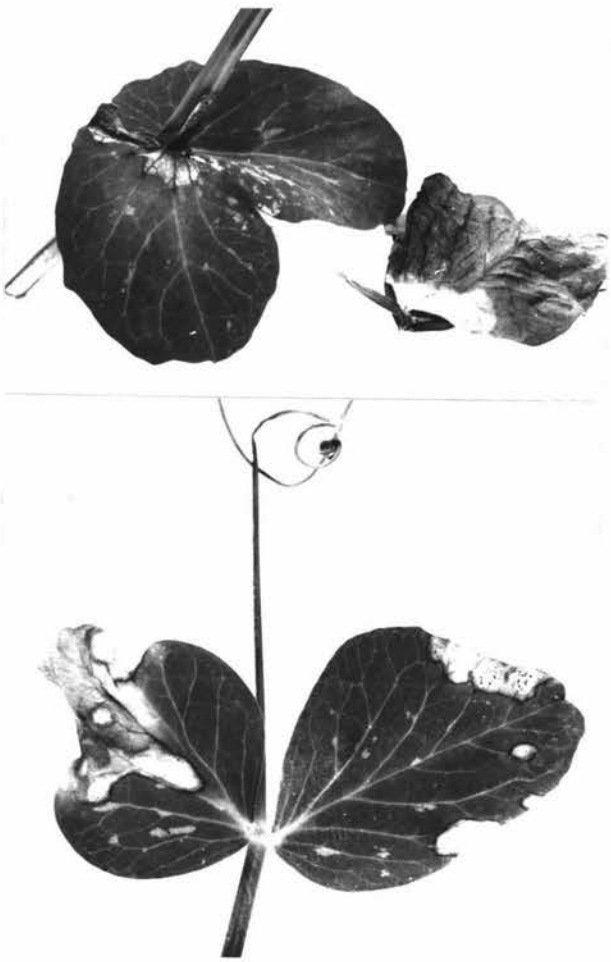


Figure 13. Symptoms on leaves and stems artificially inoculated with A. pisi.

less severe than those caused by the other two species.

3. Discussion.

The above studies revealed that symptoms induced by each of the three pathogens under local conditions were essentially similar to those reported overseas. Further, it was found that except for minor differences similar symptoms were expressed locally in the field and glasshouse.

Lesions produced by A. pisi were distinct from those of M. pinodes and P. medicaginis var. pinodella and can be distinguished from them by the following features:

(i) On leaves and pods A. pisi produced discreet, circular, tan lesions with dark-brown margins, while M. pinodes and P. medicaginis var. pinodella produced irregular, dendritic lesions of uniform dark-brown or purplish-black colour. These later enlarged to become circular and zonate under wet field conditions.

(ii) In the glasshouse A. pisi rarely produced collar or nodal lesions and these were generally less severe. However M. pinodes and P. medicaginis var. pinodella commonly produced severe collar lesions, often accompanied by stunting or wilting of the infected plants.

(iii) On artificially inoculated plants lesions caused by A. pisi were observed five to seven days after inoculation, while lesions caused by M. pinodes and P. medicaginis var. pinodella were evident one to three days after inoculation (Appendix III).

Both in the field and in the glasshouse extreme difficulty was

experienced in differentiating M. pinodes and P. medicaginis var. pinodella infections since lesions on leaves, stems and pods were essentially indistinguishable. However in the field, because of the intense production of ascostromata by M. pinodes on dead tissues (Figure 6), abundant ascosporic inoculum ensured that practically all plants in a crop were infected with this fungus, and further, that on individual plants lesioning was prolific. That is, in the field situation if one encountered widespread and intense lesioning one could be confident that M. pinodes was the pathogen involved.

Hence in practice, symptomatology was of limited value in distinguishing infections caused by each of the three fungi. A. pisi infections could be readily recognised, but differentiation between M. pinodes and P. medicaginis var. pinodella infections was extremely difficult.

B. MYCOLOGICAL FEATURES.

The mycological features of the three fungi on the host and on agar have been described overseas (Jones, 1927; Linford and Sprague, 1927; Sattar, 1934; Hare and Walker, 1944; and Warmelo, 1966), but considerable variations were observed between isolates within each species (Linford and Sprague, 1927; Madhosingh and Wallen, 1968). Such variations could also be expected between isolates from different regions and thus local isolates may not conform strictly to those described overseas. If such variations were great and consistent it follows that the mycological features employed overseas would have limited value here.

In New Zealand no formal studies have been conducted on the mycology of the three fungi. Accordingly studies were undertaken to compare their mycology both on the host and on agar. Mycological features on the host are least prone to environmentally induced variation and for this reason are generally accepted as being more typical of a species. Cultural studies may however provide supporting evidence for identification.

1. Mycological features on the host.

A feature of all three pathogens was the production of reproductive fruiting bodies on infected tissues. These structures, which included pycnidia, ascostromata (only in the case of *M. pinodes*) and their associated spores were examined to determine their value in differentiating the three species.

(a) Materials and Methods.

Observations were carried out on both naturally and artificially infected plants. Pycnidia and pycnidiospores of the three fungi were studied solely on glasshouse inoculated leaves while ascostromata and ascospores of *M. pinodes* were examined on both glasshouse and field infected stems.

When examining pycnidia, tissue clearing was carried out by gently warming small lesioned areas in a mixture of glacial acetic acid and absolute alcohol (1:1). They were then boiled for approximately three minutes in lactophenol, cooled and finally cleared in a saturated solution of chloral hydrate. This clearing enabled detailed observations

of the fruiting bodies in the infected tissues and facilitated accurate measurement of their diameter. The average of two measurements at right angle to each other were recorded under the low power of the compound microscope using an eyepiece micrometer.

Whereas with A. pisi many pycnidia were present on field diseased tissues, in the case of P. medicaginis var. pinodella and M. pinodes they were relatively rare. However with these latter two fungi pycnidia production was readily induced by exposing infected tissues to an environment of high humidity.

Slides of pycnidiospores were prepared in the following manner. Pieces of infected leaves, preferably with abundant pycnidia, were rinsed twice in sterile water, dried between clean filter papers and set out on a flamed glass slide. This was placed on a clean glass triangle sitting on moist filter papers in the petri dish (Figure 14) and incubated at 24C. Cirrhi or "spore blobs" produced after two to four days incubation were transferred to a drop of either lactophenol acid fuchsin or Shear's mounting fluid on a glass slide, using a flamed needle. The length and breadth of the pycnidiospores were measured under the high power of the compound microscope, using an eyepiece micrometer.

Ascospore production was similarly induced using infected stems with abundant ascostromata. These were taped onto the lower surface of the lid of a petri dish directly over a clean glass slide which had been lightly smeared with vaseline (Figure 15). Ascospores liberated after one to two days incubation at 24C settled on the slide, which was then removed, stained with lactophenol acid fuchsin or Shear's mounting fluid and examined. The length and breadth of the ascospores were similarly

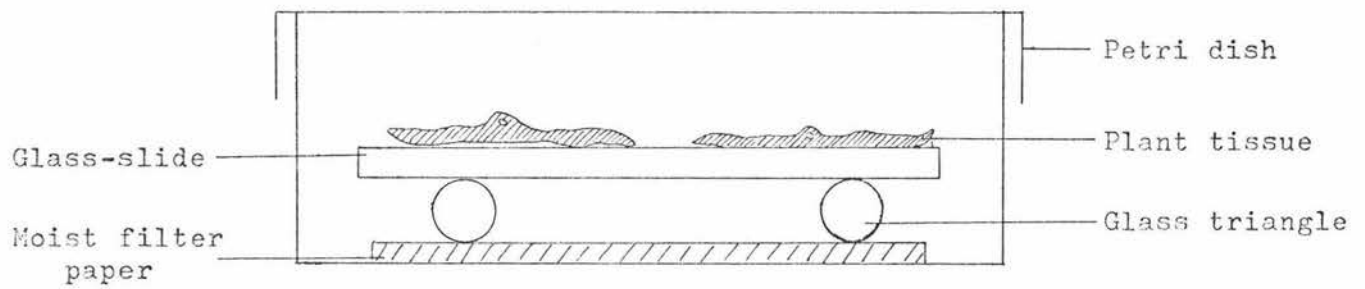


Figure 14. Apparatus to induce pycnidiospore production on infected leaves.

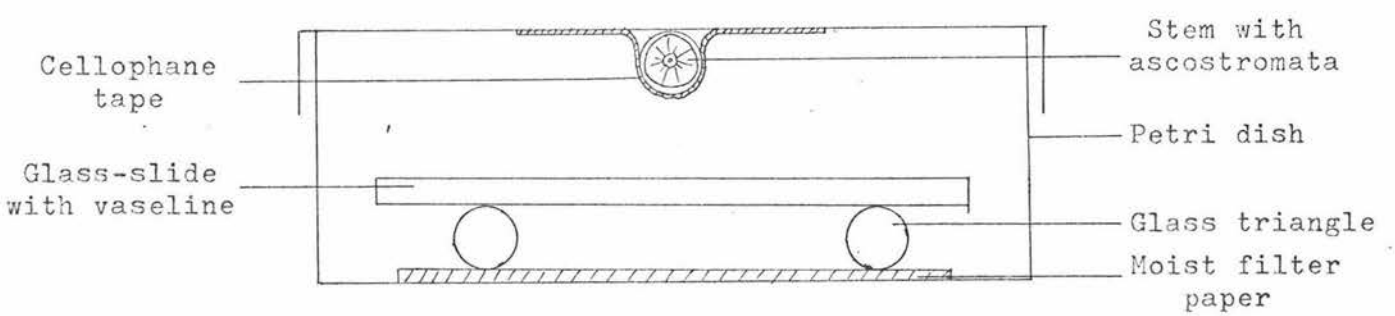


Figure 15. Apparatus to induce ascospore liberation from infected stems bearing ascostromata.

measured under the high power of the compound microscope, using an eyepiece micrometer.

(b) Results and Discussion.

To simplify comparison of the three fungi their essential mycological features on infected hosts are summarised in Table III.

The mycological features described in the table corresponds with those recorded overseas by Jones (1927), Linford and Sprague (1927), Hare and Walker (1944), and Warmelo (1966). Pycnidia were of little value for distinguishing the three fungi on the host as they were too variable in size and colour, the extreme of one species commonly overlapping that of another. Pycnidiospore features and the presence or absence of ascostromata were the only useful criteria observed, and these are summarised as follows:

(i) Mycosphaerella pinodes.

a. Pycnidiospores were slipper-shaped, mainly uniseptate, with a distinct constriction at the septum. The spores were larger than those of P. medicaginis var. pinodella, but approximated those of A. pisi, except that spores of the latter were narrower.

b. Ascostromata were produced on infected tissues in the field and glasshouse.

(ii) Phoma medicaginis var. pinodella.

a. Pycnidiospores were elliptical, uni or non-septate with no constriction at the septum. The spores were the smallest of the three fungi.

b. Ascostromata were not produced by this species.

Table III. Comparison of microscopic structures produced by M. pinodes, P. medicaginis var. pinodella and A. pisi on infected pea tissues in the field and glasshouse.

Fungal Structures	M. pinodes	P. medicaginis var. pinodella	A. pisi
1. PYCNIDIA			
a. Morphology	Solitary, erumpent, ostiolated, dark-brown to carbonaceous, with brown spore-blobs or cirrhi.	Solitary, erumpent, ostiolated, dark-brown to carbonaceous, with brown spore-blobs or cirrhi.	Solitary, erumpent, ostiolated, tan to dark-brown, with light-brown spore-blobs or cirrhi.
b. Diameter			
Range	76.6-165.7 u	66.8-178.5 u	89.7-247.0 u
Average	101.0 u	95.1 u	127.7 u
2. PYCNIDIOSPORES			
a. Morphology	Hyaline, mainly uniseptate, slipper-shaped. Commonly constricted at the septum. Commonly guttulate.	Hyaline, uniseptate or non-septate, elliptical. No distinct constriction at the septum. In some instances slightly guttulate, especially at the ends.	Hyaline, mainly uniseptate, cylindrical, with no marked constriction at the septum. No guttulation.
b. Dimensions			
Av.Length (L)	11.5 u	7.8 u	13.2 u
Av.Breadth (B)	4.3 u	2.9 u	3.7 u
L/B ratio:	2.7	2.0	3.6

Continued over ...

Table III. Comparison of microscopic structures produced by M. pinodes, P. medicaginis var. pinodella and A. pisi on infected pea tissues in the field and glasshouse - continued.

Fungal Structures	M. pinodes	P. medicaginis var. pinodella	A. pisi
3. ASCOSTROMATA a. Morphology	Similar on naturally and artificially infected plants. Solitary, erumpent, ostiolated, dark-brown. Asci clavate, hyaline, bitunicate and arising from a centrum of pseudo-parenchymatous cells, eight ascospores, biseriate.	Not produced.	Not produced.
4. ASCOSPORES a. Morphology	In field and glasshouse, ascospores hyaline, uni-septate, slipper-shape, with distinct constriction at the septum.	No ascospores produced.	No ascospores produced.
b. Dimensions			
Av.Length (L)	15.3 u		
Av.Breadth (B)	6.6 u		
L/B ratio:	2.2		

(iii) Ascochyta pisi.

a. Pycnidiospores were cylindrical, mainly uniseptate with no constriction at the septum. The spores were larger than those of P. medicaginis var. pinodella, but approximated those of M. pinodes except that the latter were broader.

b. Ascostromata were not produced by A. pisi.

2. Mycological features on agar.

Cultural studies involving observation of the characteristics of the three fungi on agar and their physiological behaviour with respect to temperature and media were carried out. The cultural characters studied were gross colony features such as shape, colour, and growth rate (macroscopic features) and the morphology of pycnidia, pycnidiospores, chlamydospores, ascostromata and ascospores (microscopic features). In the physiological studies, changes in gross colony characteristics and growth of the three species were compared.

(a) Materials and Methods.

Cultures of the three fungi were prepared using 2-3 mm agar cubes derived from monosporous isolates used in the Koch's Postulate studies (Appendix III). After three days incubation at 24C, agar blocks (1 mm diameter) were removed aseptically and placed individually in the centre of the agar plates on which observations were to be made. In inoculating the plates care was taken to ensure that at least one pycnidium was present on the agar blocks since such inoculi were found to

give more prolific pycnidia production on agar than an agar block free of pycnidia. Similar agar blocks were also used by Dorenbosch (1970).

(i) Cultural characteristics.

In view of references in the literature to considerable variations in cultural characteristics between isolates of the same species (Linford and Sprague, 1927; Madhosingh and Wallen, 1968), five isolates each of M. pinodes and P. medicaginis var. pinodella and three isolates of A. pisi were studied in the present investigation.

The isolates are listed below and their origin is indicated in Appendix III:

Mycosphaerella pinodes -	Isolates 44A, 30N, 55/65, 55/61P, and 55/61B
Phoma medicaginis var. pinodella -	Isolates 37, 35, 71/7A, 71/7B and 55/63A.
Aschochyta pisi -	Isolates LV, PN, EG.

The isolates were grown on PDA_o and incubated at 24C. Each isolate was replicated on four plates and the diameters of the colonies were compared. Measurements were taken on the third, seventh and tenth day by taking the average of two measurements at right angle to each other. The macroscopic features of the isolates were also observed periodically during the first two weeks of growth.

To induce the production of ascostromata, two weeks old cultures of M. pinodes were removed to a 16C incubator. Hare and Walker (1944) reported that ascostromata were readily produced at this temperature.

In the microscopic studies, small portions of the colonies were removed aseptically at regular intervals, mounted in lactophenol acid fuchsin or Shear's mounting fluid and observed under the compound microscope. The dimensions of pycnidia and pycnidiospores were measured on the seventh day, chlamydospores on the third week and ascostromata and ascospores on the sixth week following transfer to the 16C incubator. Pycnidiospores, ascospores and chlamydospores were measured with an eyepiece micrometer under the high power of the compound microscope. Similar measurements were recorded for pycnidia and ascostromata but under the low power of the compound microscope.

(ii) Physiological features.

The reaction of the three fungi to temperature and media was compared by periodic observation of their gross colony characteristics. Their radical growth was also compared following ten days incubation.

a. The effect of temperature.

The effect of temperature on growth on PDA_o was determined using two isolates of each of the three fungi. Each isolate was replicated on four plates at each temperature. The isolates used are listed below and their origin indicated in Appendix III.

Mycosphaerella pinodes -	Isolates 94M and 83J.
Phoma medicaginis var. *pinodella* -	Isolates 37 and 35.
Ascochyta pisi -	Isolates PN and LV.

There were ten temperatures in the series, namely,

2, 5, 8, 14, 18, 22, 24, 28, 32 and 36C.

b. The effect of media type.

The effect of media on the three fungi was studied at 24C. The media used were oxoid potato dextrose agar (PDA_o), laboratory potato dextrose agar (PDA_L), malt agar (MA), water agar (WA), prune agar (PrA), cornmeal agar (CMA), Czapek Dox (CD), oat agar (OA), and pea agar (PA). These were prepared as described in Appendix I. Two isolates each of the three fungi were studied. The isolates are listed below and their origin indicated in Appendix III.

Mycosphaerella pinodes -	Isolates 44A, 83J.
Phoma medicaginis var. *pinodella* -	Isolates 37, 35.
Ascochyta pisi -	Isolates PN and LV.

Each isolate of the three fungi was replicated on three plates of each of the nine media.

(b) Results and Discussion.

(i) Cultural characteristics.

a. Macroscopic features.

To facilitate comparison of the developmental morphology of the three fungi their essential macroscopic features on the third, seventh, tenth and fourteenth day are summarised in Table IV and their surface topography on the seventh day illustrated in Figures 16-19. The average growth rate of isolates of the three fungi is illustrated graphically in Figure 20.

Table IV. Comparison of the macroscopic colony characteristics of M. pinodes, P. medicaginis var. pinodella and A. pisi after three, seven, ten and fourteen days on PDA_o at 24C.

Macroscopic Features	M. pinodes	P. medicaginis var. pinodella	A. pisi
THIRD DAY			
1. Colony shape	Roughly circular, flat with smooth margin.	Circular, flat with smooth margin.	Circular, slightly raised with smooth filamentous margin.
2. Colony colour	Translucent.	Translucent, some with greenish-yellow sectors.	White.
3. Aerial mycelium	Lacking.	Lacking.	Moderate, fluffy, cottony-white.
4. Pycnidia production	Moderate, brownish to black, scattered towards centre or radiating from it in a line or arc.	Moderate, carbonaceous, scattered around colony centre. Occasionally a few aligned in radial lines.	Abundant, partly covered by aerial mycelium, scattered brownish-red in colour.
5. Sporulation	Few light-brown spore blobs or ooze.	Few pale-brown or milky spore blobs or ooze.	Scarce, pinkish spore blobs or ooze.

Continued over ...

Table IV. Comparison of the macroscopic colony characteristics of M. pinodes, P. medicaginis var. pinodella and A. pisi after three, seven, ten and fourteen days on PDA_o at 24C - continued.

Macroscopic Features	M. pinodes	P. medicaginis var. pinodella	A. pisi
SEVENTH DAY			
1. Colony shape	As above.	As above.	As above, some irregular.
2. Colony colour	Translucent except for pale to greenish-brown centre.	Translucent except greenish-olive at centre. Some with dark or greenish-black sectors.	White.
3. Aerial mycelium	As on third day.	As on third day.	As on third day.
4. Pycnidia production	As on third day.	As on third day.	As above, very dense, some greenish-grey.
5. Sporulation	As on third day but very dense.	As on third day but very dense.	As on third day but very dense.

Continued over ...

Table IV. Comparison of the macroscopic colony characteristics of M. pinodes, P. medicaginis var. pinodella and A. pisi after three, seven, ten and fourteen days on PDA_o at 24C - continued.

Macroscopic Features	M. pinodes	P. medicaginis var. pinodella	A. pisi
TENTH DAY			
1. Colony shape	As on seventh day.	As on seventh day.	As on seventh day.
2. Colony colour	Deepened to buff-green or cinnamon except at edge. Some with white crystal formation on underside, dendritic or feathery.	Deepened to buff-green or greenish-black except at the edge. Some with white crystal formation on underside, dendritic or feathery.	White, no crystal formation.
3. Aerial mycelium	As on seventh day.	As on seventh day.	As on seventh day.
4. Pycnidia production	As on seventh day.	As on seventh day.	As on seventh day.
5. Sporulation	Pycnidiospores as on seventh day. Chlamydospores production towards centre.	Pycnidiospores as on seventh day. Chlamydospores production towards centre.	Pycnidiospore production as on seventh day. No chlamydospores.

Continued over ...

Table IV. Comparison of the macroscopic colony characteristics of M. pinodes, P. medicaginis var. pinodella and A. pisi after three, seven, ten and fourteen days on PDA_o at 24C - continued.

Macroscopic Features	M. pinodes	P. medicaginis var. pinodella	A. pisi
FOURTEENTH DAY			
1. Colony shape	As on seventh day.	As on seventh day.	As on seventh day.
2. Colony colour	Darker hue of the same colour. Crystal pattern well formed.	Centre dark with greenish-black middle and translucent edge. Crystal pattern well formed.	White, no crystal formation.
3. Aerial mycelium	As on seventh day.	As on seventh day.	As on seventh day.
4. Pycnidia production	As on seventh day.	As on seventh day.	As on seventh day.
5. Sporulation	Pycnidiospore blobs or ooze congealed to form shiny black crystalline mat over the pycnidia. Abundant dark-brown aerial or submerged chlamydospores.	Pycnidiospore blobs or ooze congealed to form shiny black crystalline mat over pycnidia. Abundant dark-brown aerial or submerged chlamydospores.	Pycnidiospore blobs or ooze remaining pink, no chlamydospores.

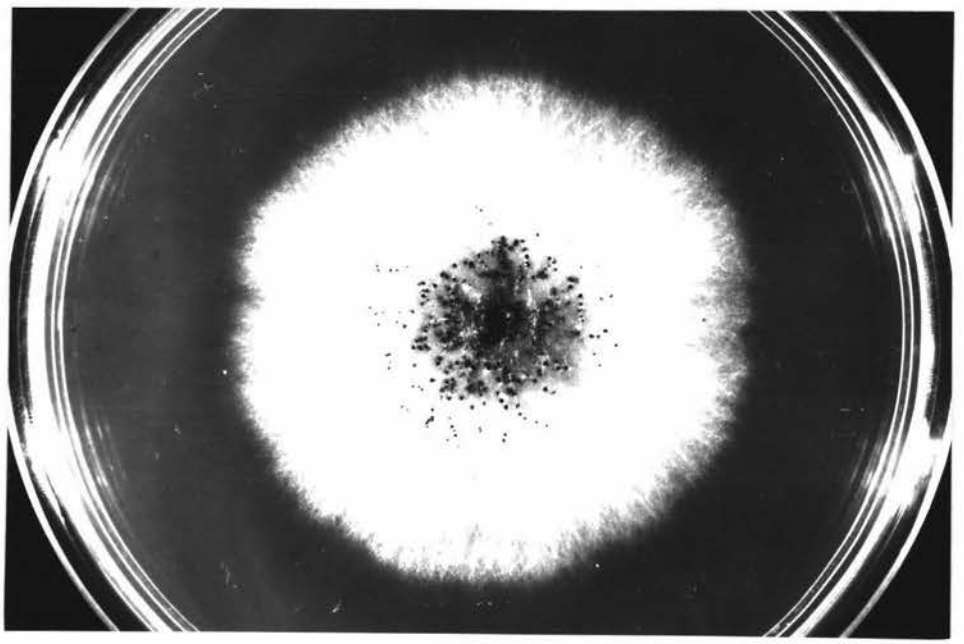


Figure 16. Surface topography of M. pinodes colony (isolate 44A) after seven days growth on PDA_o at 24C.

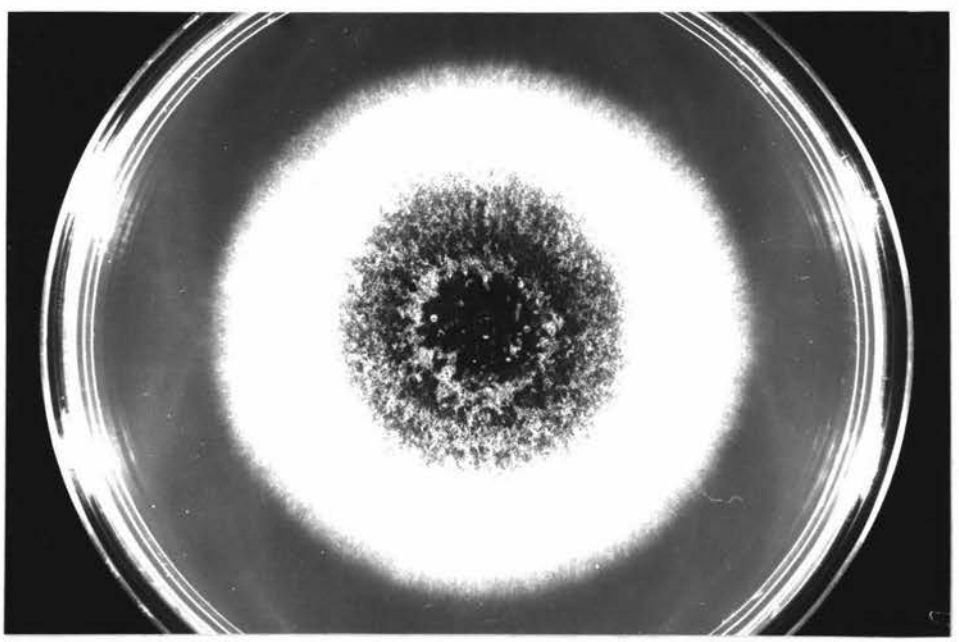


Figure 17. Surface topography of P. medicaginis var. pinodella colony (isolate 37) after seven days growth on PDA_o at 24C.

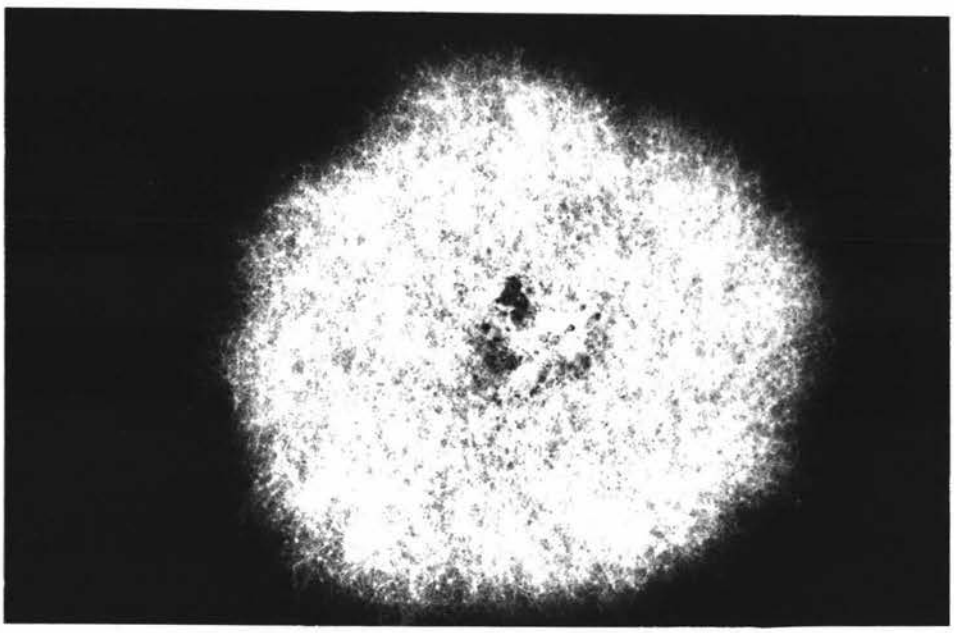


Figure 18. Surface topography of A. pisi colony (isolate PN) after seven days growth on PDA_o at 24C.

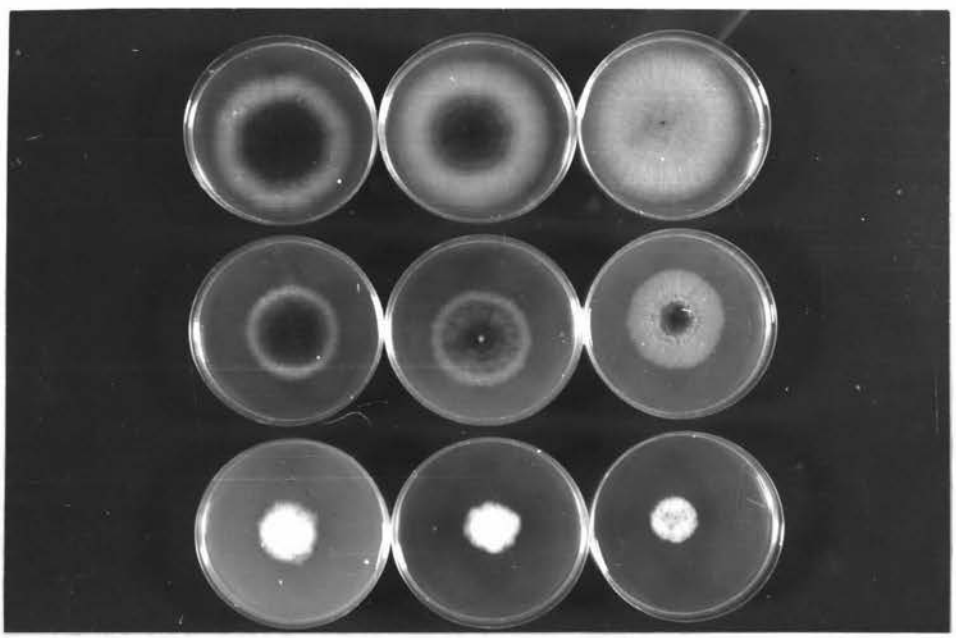


Figure 19. Cultural variations exhibited by three isolates each of P. medicaginis var. pinodella (top), M. pinodes (middle) and A. pisi (bottom) after seven days growth on PDA_o at 24C.

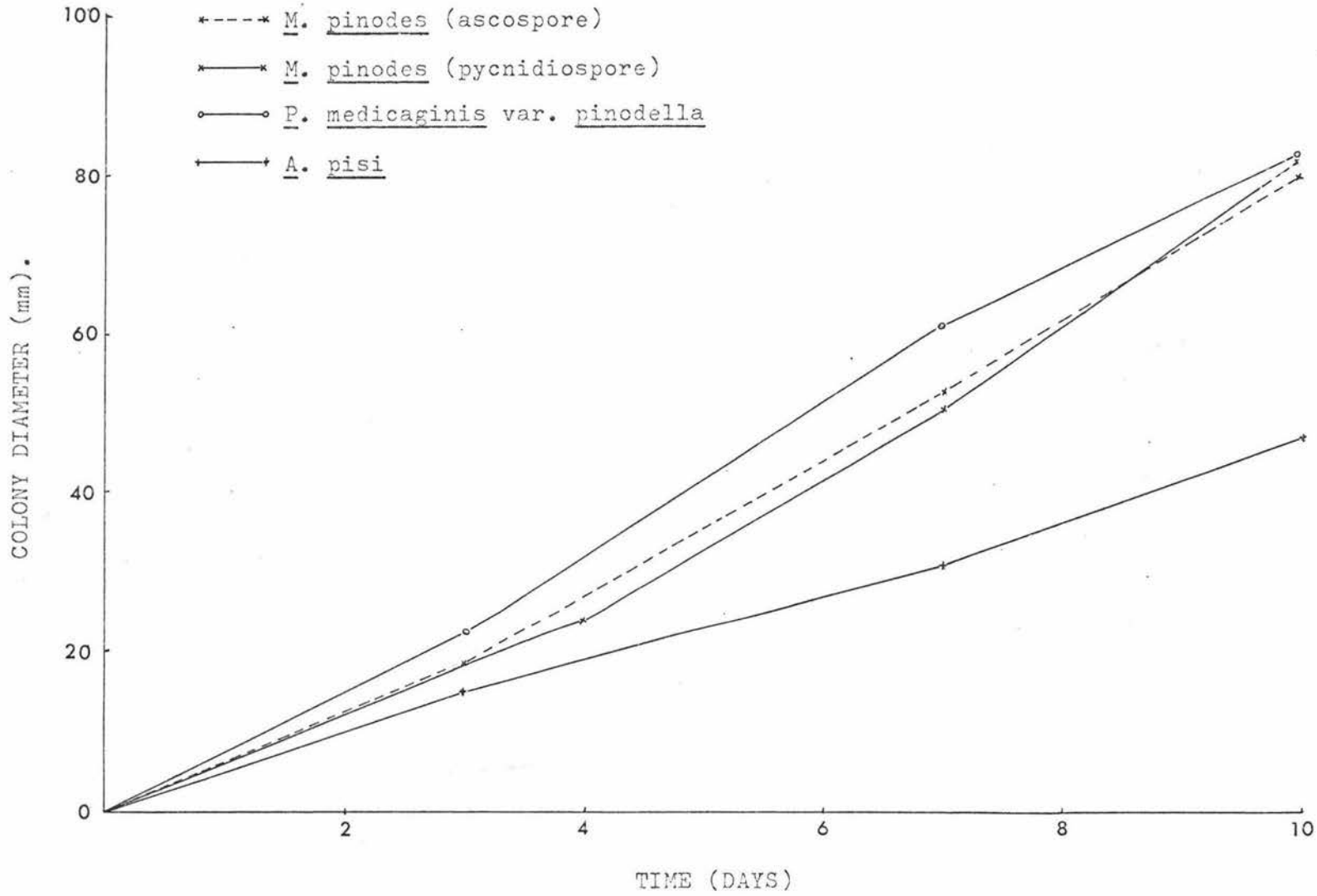


Figure 20. Growth rate of M. pinodes, P. medicaginis var. pinodella and A. pisi on PDA_o at 24C.

b. Microscopic features.

The microscopic structures of importance for identification are the pycnidia, pycnidiospores, chlamydospores, ascostromata and ascospores. The salient features of these structures are summarised in Table V for easier comparison, and illustrated in Figures 21-27.

The cultural characters observed in local isolates of the three species were essentially similar to those observed overseas by Jones (1927), Linford and Sprague (1927), Sattar (1934), and Hare and Walker (1944). The macroscopic and microscopic features of M. pinodes and P. medicaginis var. pinodella were more similar to each other, but were quite different to those of A. pisi.

Mycosphaerella pinodes and P. medicaginis var. pinodella were more difficult to differentiate because they possessed several common characteristics, and tended to be more variable. However M. pinodes was generally observed to have the following distinctive features:

(i) An intermediate growth rate, being slower than P. medicaginis var. pinodella, but more rapid than A. pisi (Figure 20)

(ii) Cultures were often in shades of brown or cinnamon and were lighter than P. medicaginis var. pinodella, but darker than A. pisi (Figures 16 and 19)

(iii) Unlike A. pisi, chlamydospores (Figure 26) and crystals (Figure 35) were produced by older cultures. These were produced slightly later than those of P. medicaginis var. pinodella, presumably because of its slower growth rate

Table V. Comparison of microscopic structures produced by M. pinodes, P. medicaginis var. pinodella and A. pisi on PDA_o at 24C.

Structure	M. pinodes	P.medicaginis var.pinodella	A. pisi
1. PYCNIDIA			
a. Disposition	Solitary, erumpent.	Solitary, erumpent.	Solitary, erumpent or immersed.
b. Morphology	Globose, brownish-black, ostiolated.	Globose, brownish or greenish-black, ostiolated.	Globose, greenish-buff or reddish-brown, ostiolated.
2. PYCNIDIOSPORES			
a. Colour	Hyaline, commonly with moderate guttulation.	Hyaline, commonly with an oil globule at each end.	Hyaline, no guttulation.
b. Shape	Slipper-shaped, some beaked at narrow end, constricted at septum.	Elliptical to bacilliform, no constriction.	Cylindrical, some beaked at narrow end, no constriction.
c. Septation	Uniseptate, at middle.	Uniseptate at middle or non-septate.	Uniseptate at middle.
d. Aging	In older cultures, pycnidiospores changed to clear cells with dark, thickened walls similar to chlamydospores.	As in M. pinodes.	No morphological changes with age.
e. Dimensions			
Av.Length (L)	13.2 u	7.9 u	13.8 u
Av.Breadth (B)	5.1 u	3.4 u	3.7 u
L/B ratio:	2.6	2.3	3.8

Continued over ...

Table V. Comparison of microscopic structures produced by M. pinodes, P. medicaginis var. pinodella and A. pisi on PDA_o at 24C - continued

Structure	M. pinodes	P.medicaginis var.pinodella	A. pisi
3. ASCOSTROMATA			
a. Production	Variable, generally after further 3-5 weeks at 16C.	No sexual fruiting bodies.	No sexual fruiting bodies.
b. Disposition	Erumpent when solitary; erumpent or immersed when grouped. When immersed in groups sometimes covered by tuft of cottony aerial mycelium.		
c. Morphology	Globose, carbonaceous, ostiolated with slight beak. Contain numerous bitunicate, transparent sac-like asci. Each ascus with 4-8 ascospores arranged in one or two rows.		
d. Diameter			
Range:	103.0-235.6 u		
Mean:	168.1 u		

Continued over ...

Table V. Comparison of microscopic structures produced by M. pinodes, P. medicaginis var. pinodella and A. pisi on PDA_o at 24C - continued

Structure	M. pinodes	P. medicaginis var. pinodella	A. pisi
4. ASCOSPORES			
a. Colour	Hyaline.	No ascospores.	No ascospores.
b. Shape	Barrel to slipper-shape, distinct constriction at septum.		
c. Septation	Uniseptate at middle.		
d. Dimensions			
Av.Length (L)	15.9 u		
Av.Breadth (B)	6.9 u		
L/B ratio	2.3		
5. CHLAMYDOSPORES			
a. Production	Variable, moderate to lacking.	Common, moderate to dense.	No chlamydospores.
b. Disposition	Common at centre or middle, immersed or aerial.	Common at centre or middle, immersed or aerial.	
c. Morphology	Intercalary, uni- to multicellular, in chains, or knots. Olive to light-brown.	Intercalary, uni- to multicellular, in chains or knots, olive-yellow to greenish-brown.	

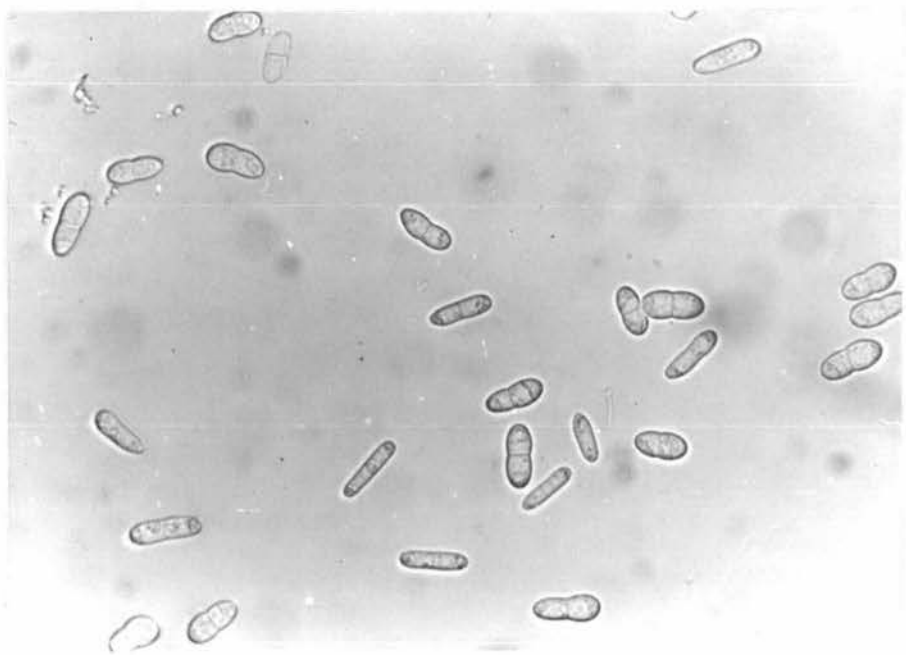


Figure 21. Pycnidiospores produced by M. pinodes after five days incubation on PDA_o at 24C.

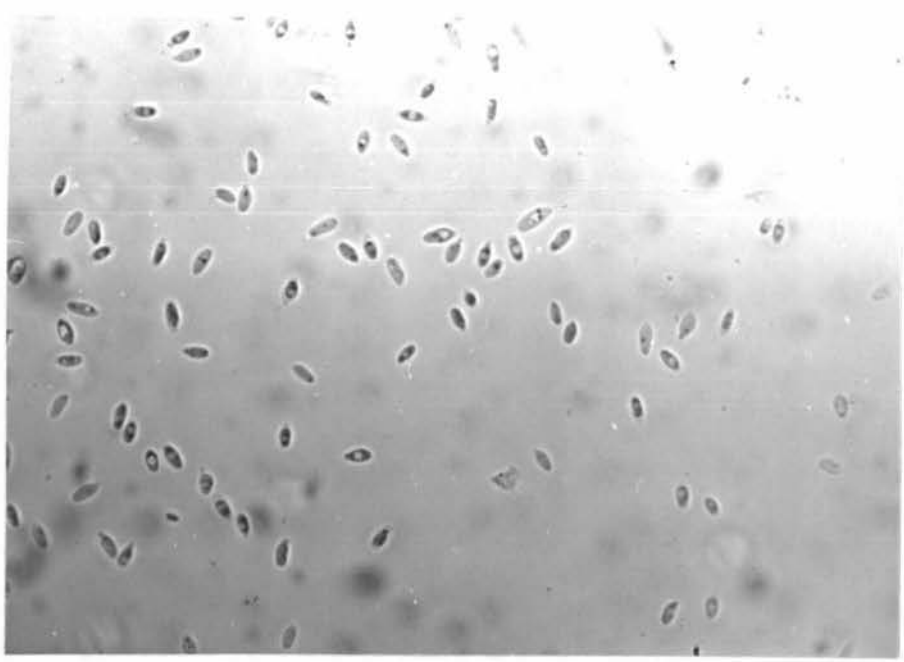


Figure 22. Pycnidiospores produced by P. medicaginis var. pinodella after five days incubation on PDA_o at 24C.

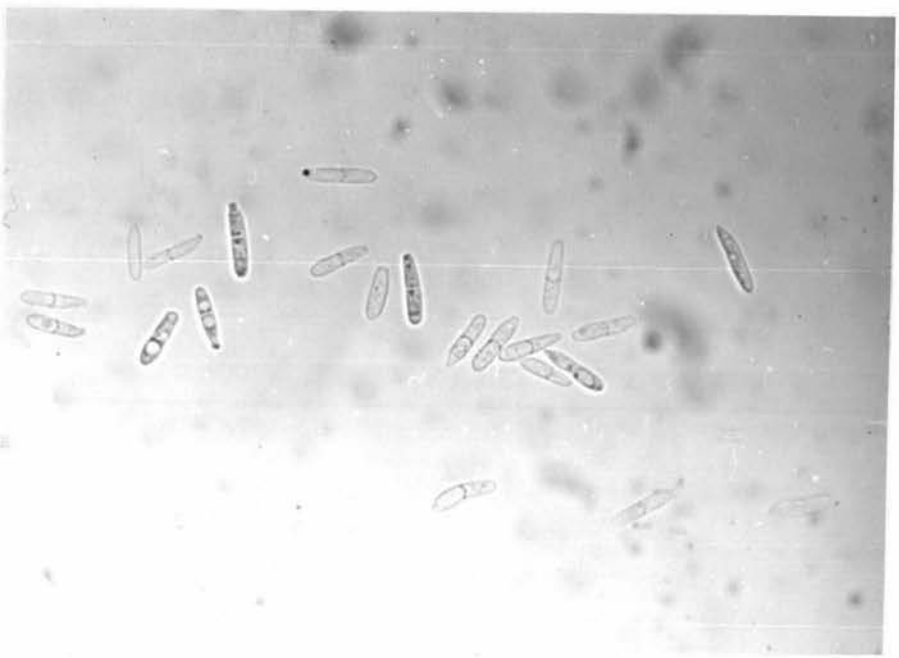


Figure 23. Pycnidiospores produced by A. pisi after five days incubation on PDA_o at 24C.

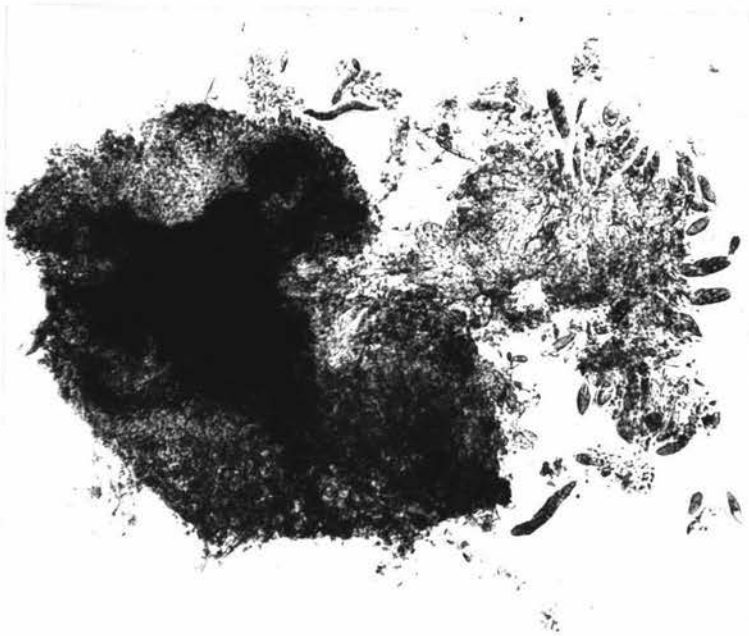


Figure 24. Ascostromata from a PDA$_o$ colony of M. pinodes incubated consecutively for two and five weeks at 24C and 16C.

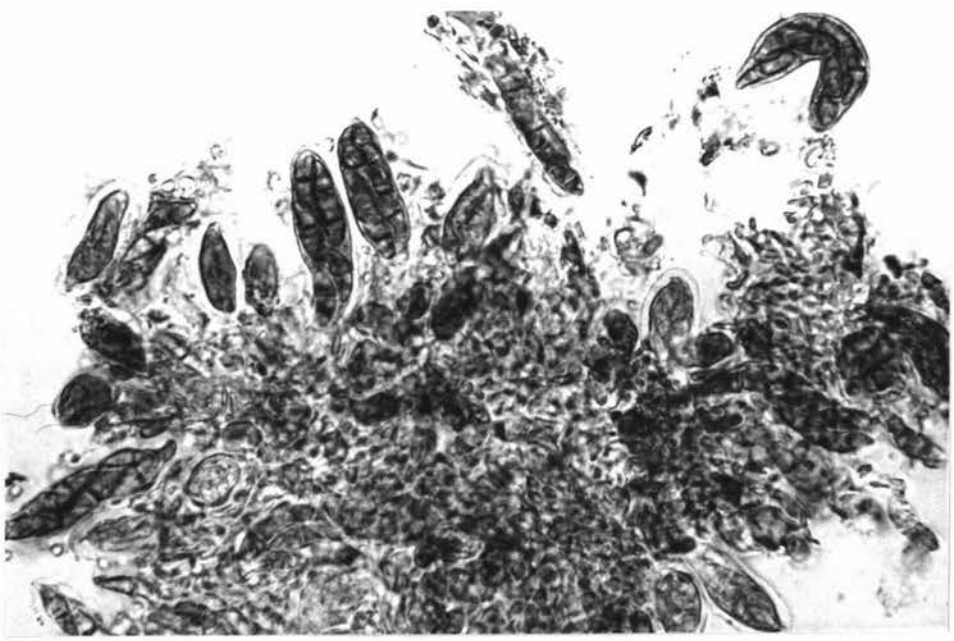


Figure 25. Asci containing ascospores from a PDA$_o$ colony of M. pinodes.

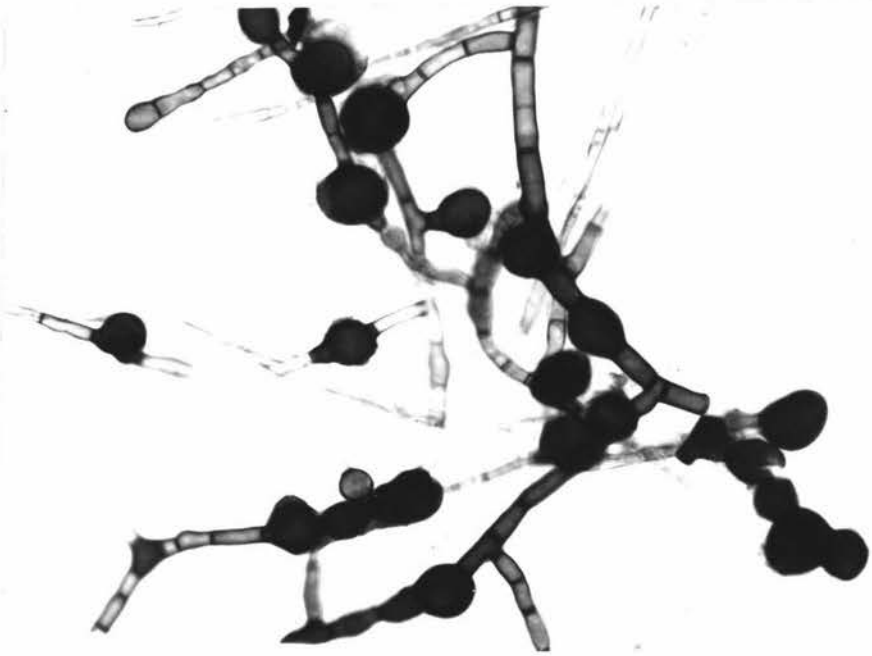


Figure 26. Chlamydospores from a PDA colony of M. pinodes after two weeks incubation at 24C.

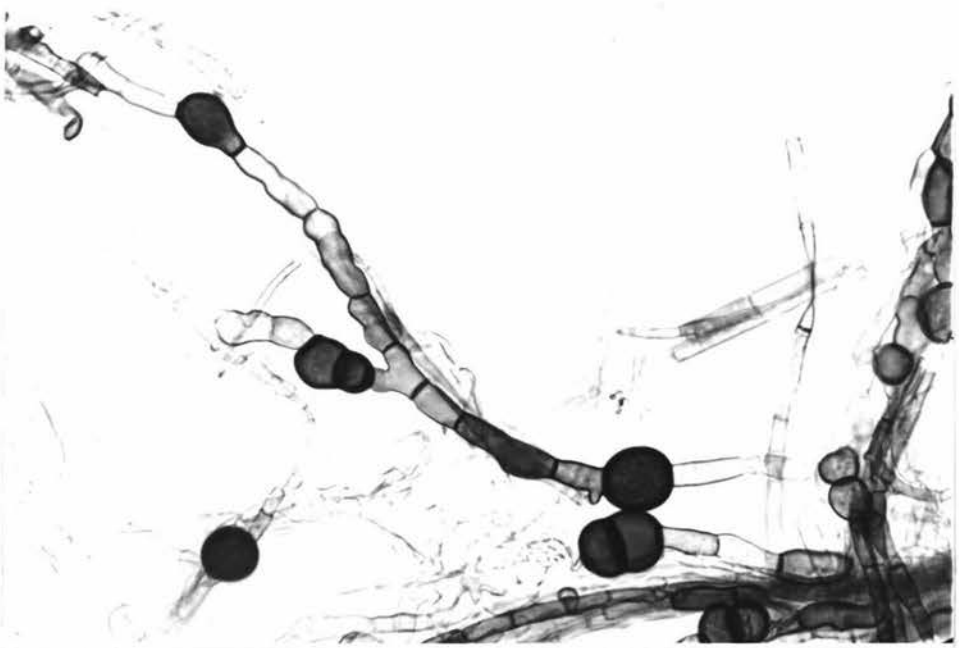


Figure 27. Chlamydospores from a PDA colony of P. medicaginis var. pinodella after two weeks incubation at 24C.

(iv) Pycnidia were often aligned radially in lines or arcs from the centre

(v) The pycnidiospores were large and uniseptate, generally with a slight constriction at the septum (Figure 21)

(vi) Ascostromata and ascospores were produced in older cultures incubated at 16C (Figures 24 and 25).

The distinctive features of P. medicaginis var. pinodella when compared with M. pinodes may be listed as:

(i) The growth rate of P. medicaginis var. pinodella was more rapid than that of M. pinodes (Figure 20)

(ii) Cultures of P. medicaginis var. pinodella were generally darker in colour, although this varied in individual cases (Figures 17 and 19)

(iii) Pycnidia of P. medicaginis var. pinodella were generally concentrated around the centre and were rarely aligned in radial arcs, as in M. pinodes

(iv) Pycnidiospores of P. medicaginis var. pinodella were smaller (Table V) and were often non-septate (Figure 22).

Ascochyta pisi can be distinguished from M. pinodes and P. medicaginis var. pinodella by the following mycological features:

(i) The growth rate of A. pisi was slowest (Figure 20)

(ii) Cultures of A. pisi were white and covered by a dense, fluffy, cottony-white mat of aerial mycelium (Figures 18 and 19). Cultures of the other two fungi were darker and generally lacking in aerial mycelium

(iii) Chlamydospores and crystal formation were not observed in A. pisi

(iv) Pycnidia of A. pisi were more abundant and evenly distributed over the colony. They were also brighter in colour

(v) Pycnidiospore blobs or ooze were distinctly pink in colour and did not congeal into dark shiny mats over the pycnidia

(vi) The pycnidiospores were larger than those of P. medicaginis var. pinodella, but approximately the same as those of M. pinodes (Table V). However on average they were slightly longer but narrower than those of M. pinodes, and did not show any constriction at the septum (Figure 23).

It should be noted that the above are generalisations of the features observed in most isolates of the three fungi and should not be regarded as constant features for all isolates of a particular species. This applies particularly to M. pinodes and P. medicaginis var. pinodella where variations between isolates were common and the extreme features of an isolate of one fungus overlapped those considered typical of the other. However, when taken as a whole, the above features can strengthen the identification of the particular fungus concerned.

(ii) Physiological features of the three fungi.

a. The effect of temperature.

The effect of temperature on radial growth of M. pinodes, P. medicaginis var. pinodella and A. pisi is illustrated in Figures 28, 29 and 30 respectively. Their radial growth on the tenth day is shown

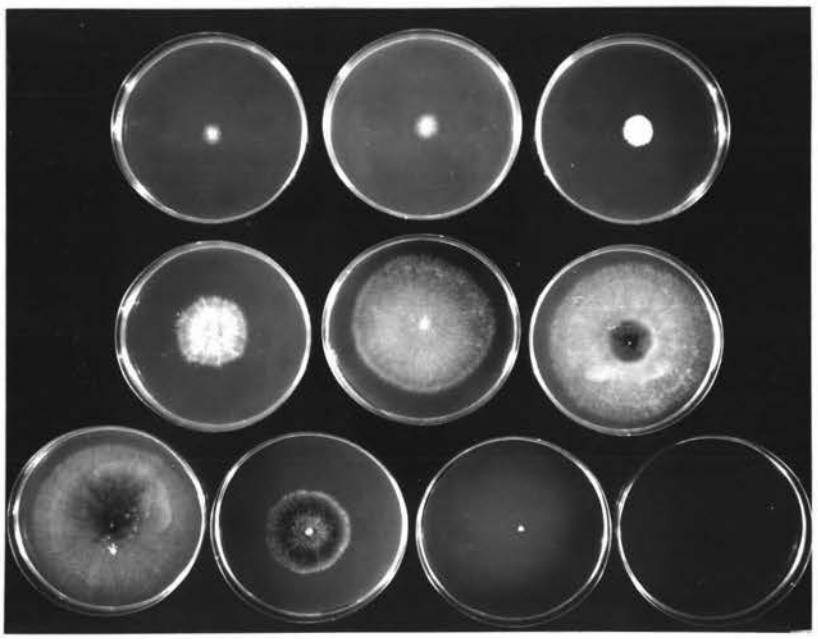


Figure 28. The effect of temperature on the growth of M. pinodes on PDA after ten days incubation. (Left to right, top to bottom: 2, 5, 8, 14, 18, 22, 24, 28, 32, 36C).

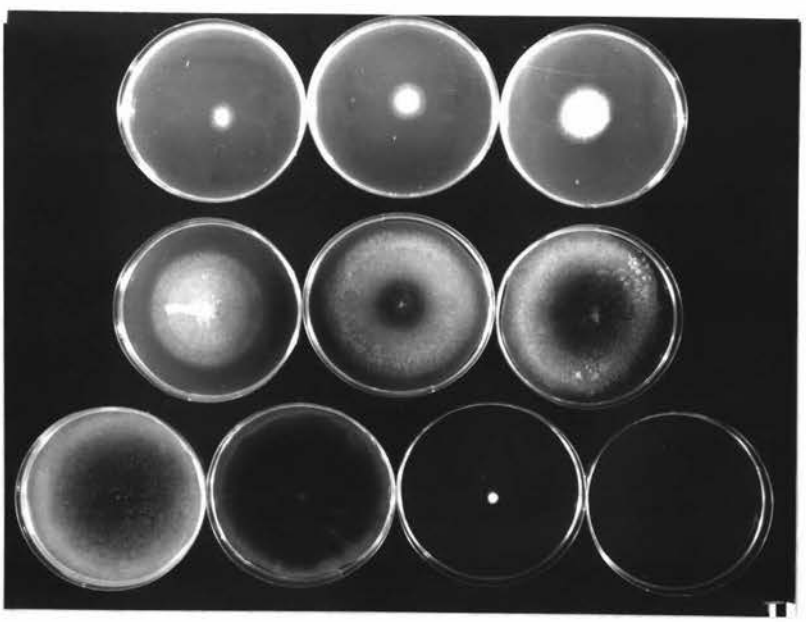


Figure 29. The effect of temperature on the growth of P. medicaginis var. pinodella on PDA after ten days incubation. (Plates as arranged in Figure 28)

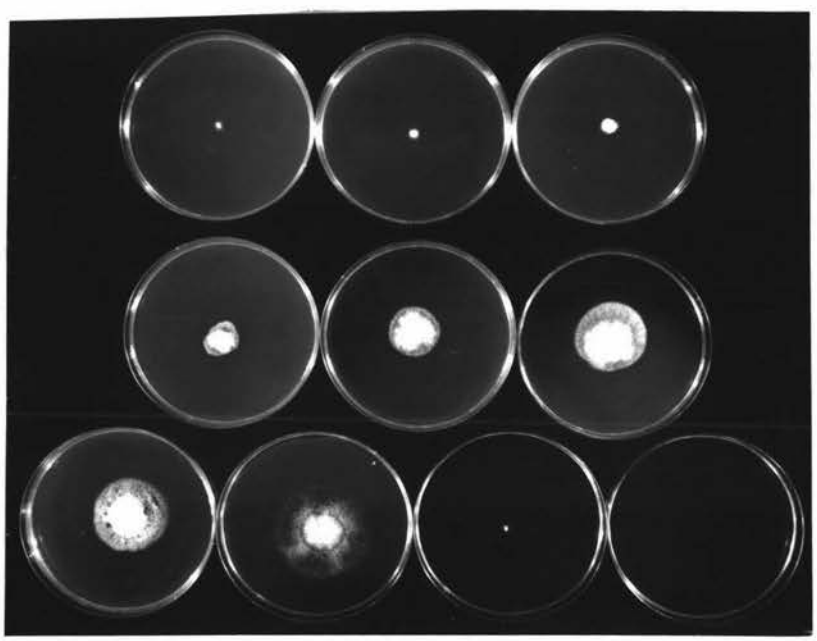


Figure 30. The effect of temperature on the growth of A. pisi on PDA_o after ten days incubation. (Plates as arranged in Figure 28)

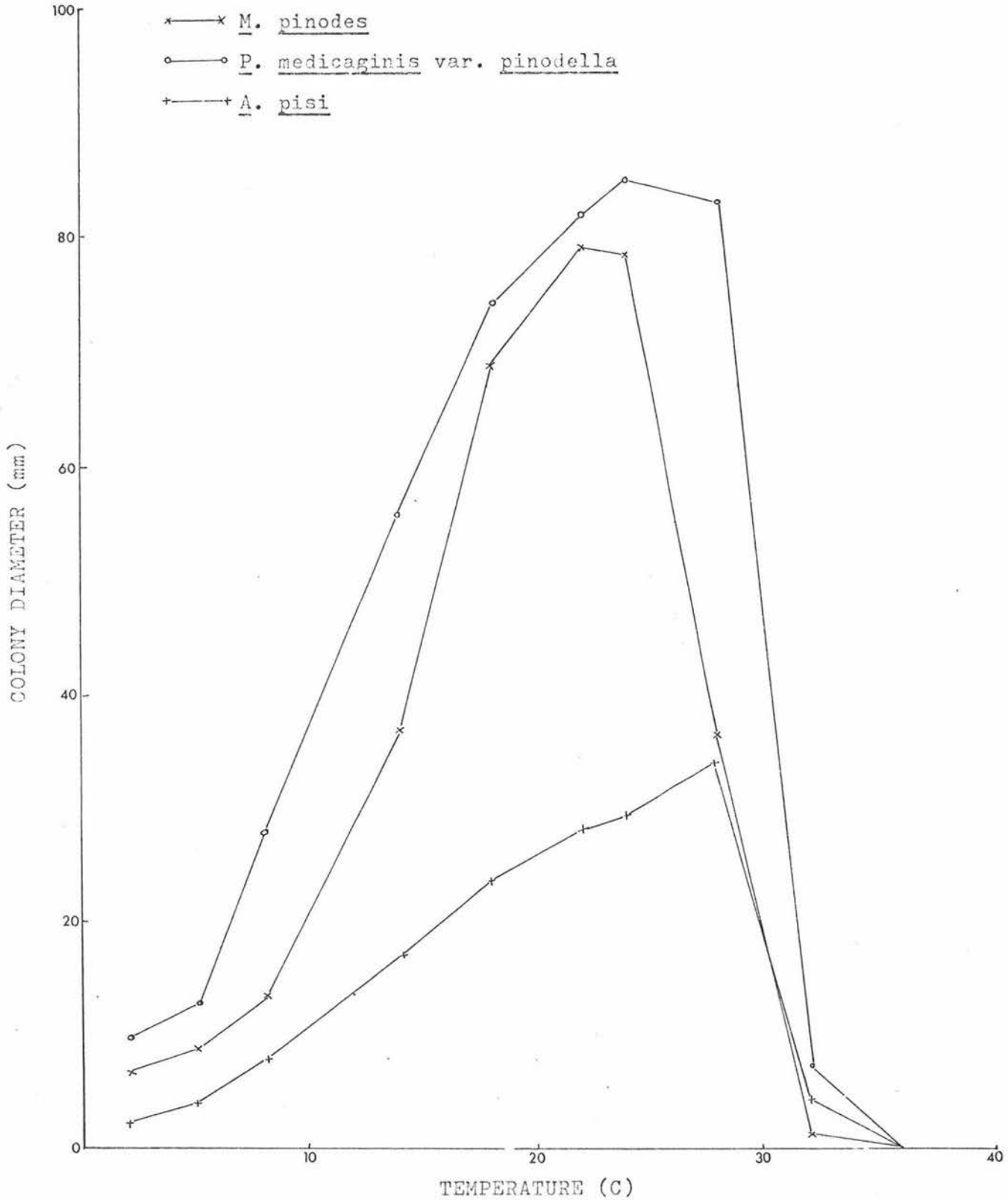


Figure 31. Effect of temperature on radial growth of M. pinodes, P. medicaginis var. pinodella and A. pisi on PDA_o after ten days.

graphically in Figure 31. Essentially similar responses were observed for all three fungi and their cardinal temperatures are compared in Table VI.

Table VI. Cardinal temperatures for growth of M. pinodes, P. medicaginis var. pinodella and A. pisi on PDA_o.

FUNGI	Minimum	Optimum	Maximum
Mycosphaerella pinodes	< 2C	22C	36C
P. medicaginis var. pinodella	< 2C	24C	36C
Ascochyta pisi	< 2C	28C	36C

At all the temperatures studied P. medicaginis var. pinodella maintained its relatively fast growth, followed respectively by M. pinodes and A. pisi, the exception being at 28C at which temperature the radial growth of A. pisi was slightly greater than that of M. pinodes. However it should be noted that the above only refers to the average growth of two isolates of each of the three fungi, and individual isolates can differ slightly from the above.

Morphological changes with temperature were essentially similar in all three fungi and were of little value in differentiating the three species. Aerial mycelium was sparser at the lower and higher temperatures and relatively dense between 8C and 18C, except for A. pisi where aerial mycelium remained dense at temperatures up to 28C. Cultures of the three fungi were also generally darker at temperatures above 24C.

Hence the above results indicate that temperature variations were of little importance in providing additional distinctive features for the differentiation of the three fungi. Distinctive features

revealed at 24C on PDA_o were essentially similar at all the other temperatures studied.

b. The effect of media type.

The effect of media on the cultural characteristics of M. pinodes, P. medicaginis var. pinodella and A. pisi is illustrated in Figures 32, 33 and 34 respectively, and summarised in Table VII. Cultural characteristics of the three fungi were similarly modified on all nine media. The modifications can be broadly grouped, as follows:

(i) Growth was generally good on PA, CD, PDA_o, PDA_L and MA, with cultures having moderate to dense aerial mycelium and pycnidia production

(ii) Cultures of the three fungi were less dense on CMA, OA, and PrA. Aerial mycelium was sparse and except for OA, pycnidia production was poor

(iii) On WA, the three fungi were limited to sparse growth lacking in aerial mycelium and pycnidia production.

The effect of media type on radial growth of the three fungi is illustrated graphically in Figure 36. In general, P. medicaginis var. pinodella had the greatest growth on all the media. Ascochyta pisi had the least growth except on MA where it was greater than M. pinodes. It should be noted that the histogram only records the average of two isolates of each of the three fungi, and that variations between isolates did occur.

The above results indicate that compared with PDA_o (Table IV), there were no additional distinctive cultural features in the test series of media which would allow further differentiation of the three fungi.

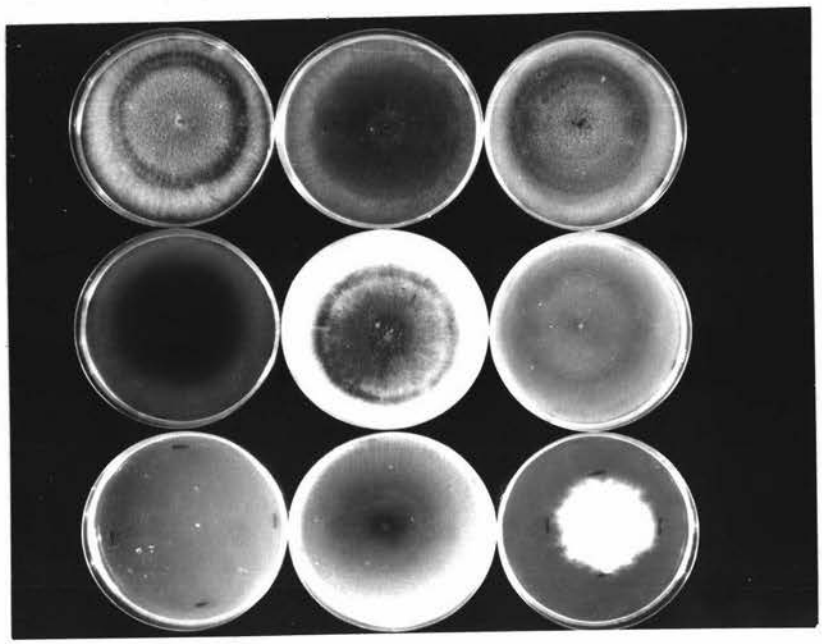


Figure 32. The effect of media type on the growth of M. pinodes after ten days at 24C. (Left to right, top to bottom: PDA_L, PDA_o, MA, PrA, PA, CMA, WA, OA and CD.)

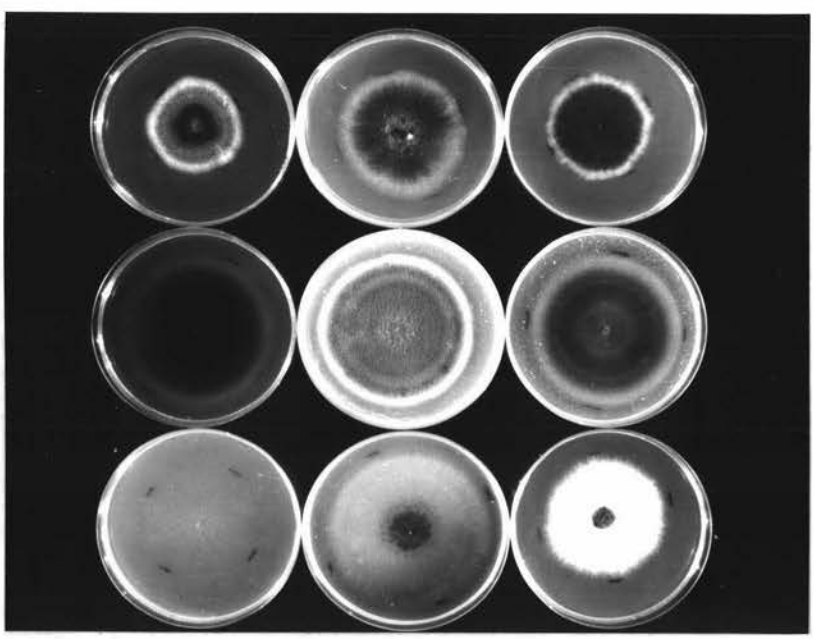


Figure 33. The effect of media type on the growth of P. medicaginis var. pinodella after ten days at 24C. (Plates as arranged in Figure 32).

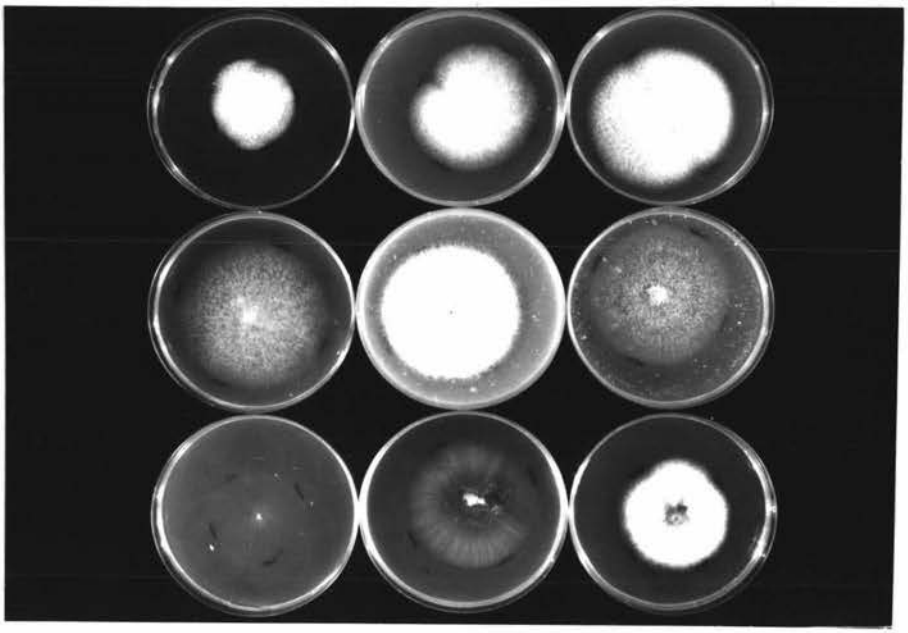


Figure 34. The effect of media type on the growth of A. pisi after ten days at 24C. (Plates as arranged in Figure 32).

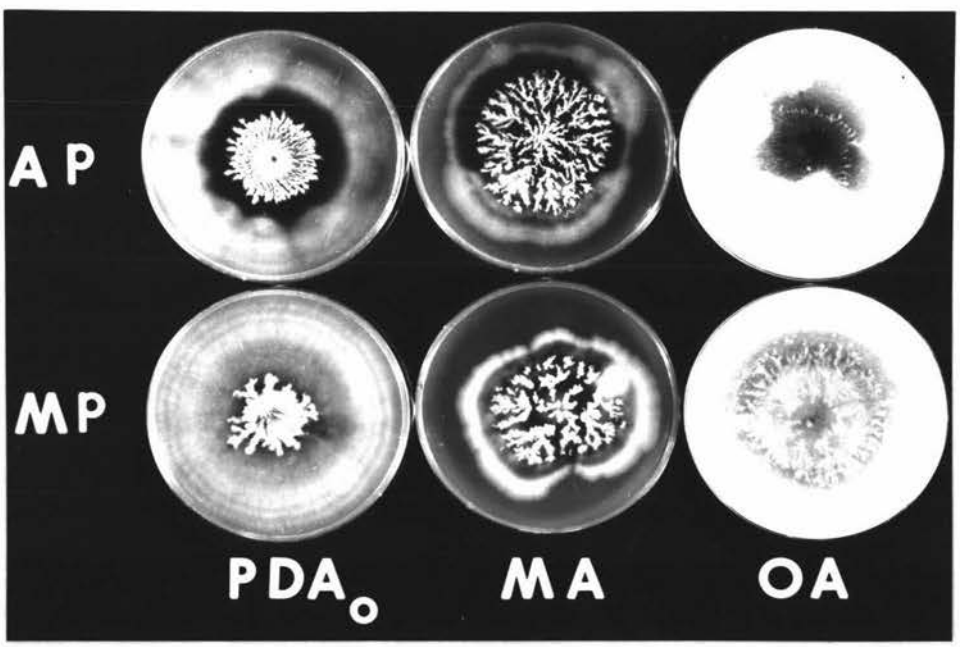


Figure 35. Crystal production on the undersurface of two weeks old cultures of M. pinodes (MP) and P. medicaginis var. pinodella (AP) on PDA$_o$, MA, and OA at 24C.

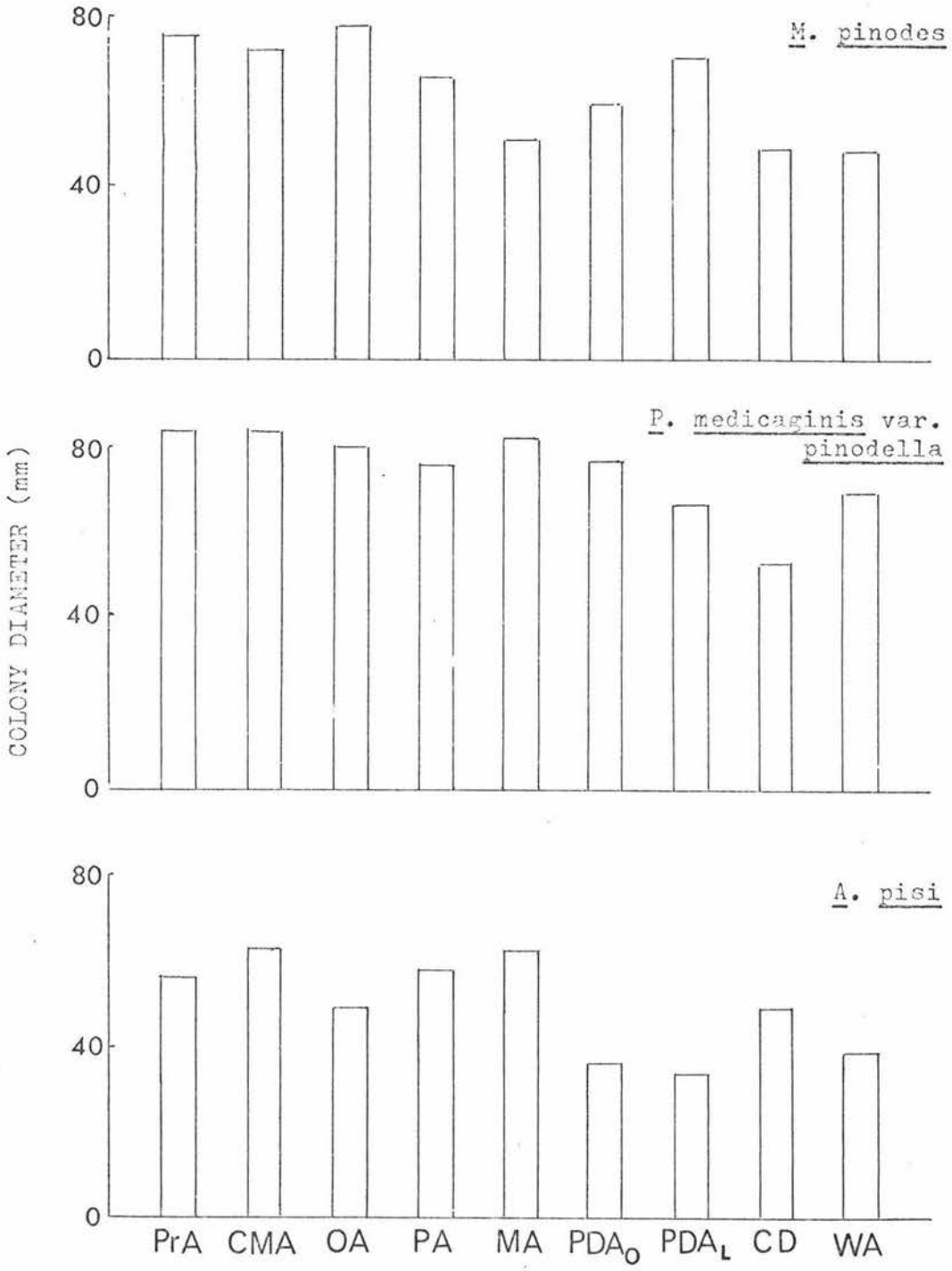


Figure 36. The effect of media type on radial growth of M. pinodes, P. medicaginis var. pinodella and A. pisi after ten days at 24C.

Table VII. Effect of media type on the morphology of M. pinodes, P. medicaginis var. Pinodella and A. pisi incubated at 24C for ten days.

	PDA_L	PDA_O	MA	PA	OA	CD	CMA	PrA	WA
M. pinodes									
SURFACE TOPOGRAPHY									
Shape	Cr	Cr	Ir/Cr	Ir/Cr	Cr	Cr	Cr	Cr	Ir/Cr
Elevation	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft
Zonation	NZ/Z	NZ	NZ/Z	NZ/Z	Z	NZ	NZ	NZ	NZ
COLONY COLOUR									
Fringe	T	T	T	T	T	T	T	T	Fm
Middle	Bn	Bn-Gn	Gn-Gy	Bn	T	Bn	L-Gn	L-Gn	Fm
Centre	Gy-Bl	Bn-Gn	Gn-Bl	D-Bn	L-Gn	Gn-Bn	Gy-Gn	Gy-Gn	Fm
AERIAL MYCELIUM									
Quantity	3-4	1-2	2-4	4	3-4	2-4	1-2	0	0
Location	G/M-C	M-C	G/M-C	G/M-C	G/M-C	G/M-C	M-C	-	-
Morphology	Ct/Fk	Ct/Fk	Pw/Fk	Ct	Ct/Pw	Ct	Fk	-	-
Colour	W	W	W	W	Gn-Gy	W	W	-	-
CHLAMYDOSPORES PRODUCT.									
Quantity	-	-	-	2-3	-	-	-	-	-
Location	-	-	-	M-C/I	-	-	-	-	-
Morphology	-	-	-	U-Mt/Q-B	-	-	-	-	-
Colour	-	-	-	Bn	-	-	-	-	-
PYCNIDIAL FEATURES									
Quantity	3-4	3-4	2-4	4	2-3	1	2	0-1	0-1
Location	C-M/E	C-M/I-E	C-M/E	C-M/E	C-M/I-E	C-M/E	C-M/E	C/I	C/E
Colour	D-Bn	Bl	Bl	D-Bn	D-Bn	Bl	Bl-Bn	Bl	Y-Bn
PYCNIDIOSPORE PRODUCTION									
Emission	Oz/Bb	Oz	Oz	Oz/3b	Oz	Oz/Bb	Oz	Oz	Oz
Colour	L-Bf	L-Bn	L-Bn	L-Bn	L-Bn	L-Bn	L-Bn	L-Bn	L-Bn
PROPORTION OF PLATES WITH CRYSTALS (14th day)	-	5/6	6/6	-	5/6	-	-	-	-
P. med. var. pinodella									
SURFACE TOPOGRAPHY									
Shape	Ir/Cr	Ir/Cr	Ir/Cr	Cr	Ir/Cr	Cr	Cr	Cr	Ir/Cr
Elevation	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft	Ft
Zonation	Z/Sc	NZ/Z	NZ/Z	Z/Sc	Z/Sc	NZ/Z	NZ/Z/Sc	NZ	NZ
COLONY COLOUR									
Fringe	T	T	T	T	T	T	T	T	Fm
Middle	Bn	Gn	Gn-Bn	D-Bn	L-Gn	Bn	L-Gn	L-Gn	Fm
Centre	Gn-Bn	Gn-Bl	Gn-Bl	L-Bn	Gn-Bl	Gn-Bn	L-Gn	Gn-Bl	Fm
AERIAL MYCELIUM									
Quantity	2-3	1-2	1-2	2-3	2-3	3	0-1	0-1	0-1
Location	G	M-C	M-C	G	G/M-C	G	G/M-C	G	G
Morphology	Pw/Fk	Fk	Pw/Fk	Pw/Fk	Fl	Fl/Fk	St/Fk	St	St
Colour	W	Gy	Gy	W	Gy-W	W	W	W	W
CHLAMYDOSPORES PRODUCT.									
Quantity	3	1-3	0-3	3-5	2-4	3-4	1-2	3-4	1-2
Location	M-C/I-S	M-C/I-S	M-C/I-S	M-C/I-S	M-C/I-S	M-C/I-S	M-C/I-S	M-C/I-S	C/I-S
Morphology	U-Mt/Q-B	U-Mt/Q-B	Mt/Q	U-Mt/Q-B	U-Mt/Q-B	U-Mt/Q-B	U-Mt/Q-B	U/B	U-Mt/Q-B
Colour	Y-Bn	Ol-Bn	Ol-Bn	Y-Bn	Ol-Bn	Gn-Bn	Ol-Bn	Ol-Bn	Ol-Y
PYCNIDIAL FEATURES									
Quantity	0-1	0-3	0-4	0-3	0-1	1-3	0	0	0-1
Location	C/E	C/E	C-M/E	C-M/I-E	C/E	C/E	-	-	C/E
Colour	Bl	Bl	Bl	D-Bn	D-Bn	D-Bn	-	-	Ol-Y
PYCNIDIOSPORE PRODUCTION									
Emission	Oz	Oz	Oz	Oz	Oz	Oz	-	-	Oz
Colour	L-Bf	L-Bn	L-Bf	L-Bn	L-Bn	L-Bf	-	-	L-Bf
PROPORTION OF PLATES WITH CRYSTALS (14th day)	1/6	3/6	6/6	-	4/6	-	-	-	-

A. pisi									
SURFACE TOPOGRAPHY									
Shape	Ir/Cr	Ir/Cr	Ir/Cr	Ir/Cr	Ir/Cr	Ir/Cr	Ir/Cr	Cr	Ir/Cr
Elevation	SR	SR	SR	SR	SR	SR	SR	SR	SR
Zonation	NZ/Z	NZ	NZ	NZ	NZ/Z	NZ	NZ/Z	NZ	NZ
COLONY COLOUR									
Fringe	T	T	T	T	T	T	Fm	Fm/T	Fm
Middle	W	W	W	W/P	L-Gn	W	T	T	Fm
Centre	W	W	W	W/P	L-Gn	R-Bn	T	T	Fm
AERIAL MYCELIUM									
Quantity	2-3	2-3	3-4	1-2	3-2	3-4	1-2	0-1	0-1
Location	Cr	G/F-M	G	G	G/F-M	G	G/F-M	G/M-C	-
Morphology	Ct	Ct	Ct	Ct	Ct	Ct	Pw	St	-
Colour	W	W	W	W	Gy-W	W	W	T	-
CHLAMYDOSPORES PRODUCT.									
Quantity	-	-	-	-	-	-	-	-	-
Location	-	-	-	-	-	-	-	-	-
Morphology	-	-	-	-	-	-	-	-	-
Colour	-	-	-	-	-	-	-	-	-
PYCNIDIAL FEATURES									
Quantity	3-4	3-4	3-4	4	3-4	3	3-4	2-3	
Location	G/I-E	M-C/I-E	M-C/I-E	M-C/I-E	M-C/I-E	M-C/I-E	M-C/I-E	G-C/I-E	
Colour	Y-R	Y-R	Y-R	Bn-R	Bn-R	Bn-R	Y-R	Y-R	
PYCNIDIOSPORE PRODUCTION									
Emission	Oz/Bb	Oz	Oz/Bb	Bb	Oz/Bb	Oz/Bb	Oz/Bb	Oz	
Colour	P	P	L-R	L-R	P	L-R	P	P	
PROPORTION OF PLATES WITH CRYSTALS (14th day)	-	-	-	-	-	-	-	-	

KEY:

Shape: Ir - irregular; Cr - circular

Elevation: Ft - flat; Sr - slightly raised

Zonation: Z - zonation; NZ - no zonation; Sc - sectoring

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

Location: G - general; F - fringe; M - middle; C - centre; I - immersed; S - superficial; E - erumpent

Morphology: Ct - cottony; Pw - powdery; St - straggling; Fk - flecky; Fl - fluffy; U - unicellular; V - multicellular; Q - muriform; B - beaded

Colour: L - light; D - dark; T - translucent; Fm - filamentous; Bf - buff; Ol - olive; Bn - brown; Y - yellow; Gn - green; W - white; P - pink; Gy - grey; Bl - black; R - red

Pycnidial emission: Oz - ooze; Bb - blob

Except for WA, the distinctive macroscopic features described previously on PDA_o were still generally applicable to the other media studied. As before, P. medicaginis var. pinodella had the greatest growth and was generally darker than the other two species. Ascochyta pisi had the least growth but developed a more luxuriant cover of cottony aerial mycelium.

In the studies on cultural characteristics reported earlier (pages 38 and 47), reference was made to crystal production by both P. medicaginis var. pinodella and M. pinodes on PDA_o. In the present experiment considering the growth on a range of media it was noted that crystal formation was a feature of both P. medicaginis var. pinodella and M. pinodes on OA, MA as well as on PDA_o (Figure 35). Additionally, with P. medicaginis var. pinodella crystal formation occurred on PDA_L.

C. SUGGESTED KEY FOR ROUTINE DIAGNOSIS OF M. PINODES, P. MEDICAGINIS VAR. PINODELLA AND A. PISI.

Studies conducted in this chapter indicated the value of both host symptoms and mycological features of the pathogens both on host and agar for identifying the specific diseases of the complex. This information is summarised as a diagnostic key (Table VIII). The key is divided into three sections to enable progressive diagnosis beginning with tentative field identification and progressing through to confirmation in the laboratory.

Table VIII. Key for the routine diagnosis of the component diseases of the collar-rot complex of peas as caused by M. pinodes, P. medicaginis var. pinodella and A. pisi.

Infected plants showing collar and/or aerial lesions; pycnidia produced, especially when

Chapter III

SURVEY OF NEW ZEALAND PRODUCED PEA SEED LINES FOR INFECTION BY THE THREE PATHOGENS.

A. INTRODUCTION.

The seed-borne nature of the three fungi in the collar-rot complex has long been known (Jones, 1927; Orton, 1931), in each instance the pathogen being established as dormant mycelium within the testa and cotyledons (Maude, 1966). Very high levels of seed infection (up to 48%) have been reported overseas (Jones, 1927; Hickman, 1940; Skolko *et al.*, 1954; Wallen *et al.*, 1967a), with *A. pisi* being the most prevalent species in the majority of cases (Jones, 1927; Hickman, 1940; Skolko *et al.*, 1954). Only in Australia (Carter and Moller, 1961), Canada (Wallen *et al.*, 1967a) and Poland (Bajan, 1968) has *M. pinodes* been reported as the dominant species associated with seeds. In no instance has *P. medicaginis* var. *pinodella* been cited as the most prevalent species.

In New Zealand very few studies have been conducted on the disease complex, especially with respect to its seed-borne nature. Brien *et al.* (1955) reported the complex to be seed-borne, and Cruickshank (1957) reported on a four year survey of the incidence of *Ascochyta* species in 175 lines of New Zealand produced pea seed. His findings indicated all three species were present (except for 1954/55 when *A. pisi* was absent) with *M. pinodes* consistently the dominant species and *P. medicaginis* var. *pinodella* the least prevalent. The infection level of most seedlines was low, being less than 5%.

In view of more than ten years having elapsed since Cruickshank's survey, an investigation was undertaken of the health status of New Zealand produced seed to determine the current situation as regards the relative prevalence of the three fungi, and to determine the infection levels of each within individual seed lines.

Many techniques have been employed for routine screening of pea seeds infected with the collar-rot fungi. These can be broadly grouped as follows:

(i) macroscopic examination of seed lots
(ii) blotter method
(iii) agar plate method.

In macroscopic examinations infected seeds are detected by two main features, namely, the discolouration of infected seeds, and their fluorescence under ultraviolet irradiation. The discolourations are in fact lesions produced as a result of seed infection and have been described by Crosier (1936) as being "slightly discoloured to light brown". As regards fluorescence, four main types (blue or yellow spots, with either a diffuse or clear border) were distinguished by Anselme and Champion (1962) on pea seeds irradiated with a 3600Å ultraviolet light source. Wharton and Ensor (1970) recommended this method as a rapid means of accepting or rejecting pea samples for sowing, the method being used to identify seed lines with infection levels above or below 5%.

The blotter method has frequently been employed for detecting the collar-rot fungi in pea seeds (Crosier, 1936; Hickman, 1940; Anselme and Champion, 1962; de Tempe, 1968a). In this method seeds are spaced out evenly on or between moist blotters which are then either enclosed directly in a moist environment, or rolled up into cylinders, and then

incubated. Depending on the particular worker, incubation is for three to seven days in the light or darkness at temperatures of approximately 20C. After this, the seeds or seedlings are examined under a low power stereoscopic microscope and the pathogen involved identified by its characteristic growth features on the seed and/or the nature of the lesions produced on the seedling host.

In the agar method, various modifications have been employed (Jones, 1927; Skolko et al., 1955; Cruickshank, 1957; Wallen et al., 1967a; and de Tempe, 1968a). Depending on the particular worker, the seeds are surface sterilised by soaking in chlorine solutions (1-2%) for 10-15 minutes, drained and plated onto an agar medium. Oat agar, prune agar, pea agar, malt agar and potato-dextrose agar have all been used. The plates are then incubated at 20-24C for a period ranging from four to ten days, at which time the fungi are identified on the basis of their characteristic growth features on the agar media, and morphology as observed in microscopic preparations.

In choosing a method for routine screening of seeds the two main features required are ease of operation, and sensitivity. A sensitive test may be described as one which not only yields high infection percentages, but is also capable of detecting slight infections in seeds (de Tempe, 1968b).

With respect to the sensitivity of the above methods, macroscopic examination is of limited use as seed discolouration is a feature of only deep seated infection, slightly infected seeds appearing perfectly normal (Maude, 1966). In the case of seed fluorescence, Anselme and Champion (1962) could observe no significant correlation between fluores-

cence and infection of pea seeds by the collar-rot fungi.

The sensitivity of the blotter and agar plate method have been compared by Anselme and Champion (1962), Matthews (1964) and de Tempe (1968a), and all agreed that the agar plate method is more sensitive for detecting pea seeds infected with the collar-rot fungi. Matthews (1964) further observed that the method has the added advantages of greater ease and speed of operation.

In view of the above findings the agar method as detailed by the International Seed Testing Association (Anon, 1966), was adopted in the present survey, with slight modifications.

In preliminary seed screening trials it became apparent that the success of the seed health survey was dependent on the author becoming proficient in differentiating the three pathogens as they developed from infected seeds. That is, it was essential that each pathogen be positively identified on the basis of its growth characteristics and morphological features as expressed on agar. To this end an identification key was prepared (Table IX) based in part on studies reported in Chapter II, and on preliminary plating trials using artificially inoculated seeds.

B. MATERIALS AND METHODS.

A total of 86 lines of pea seeds harvested in New Zealand in 1969/70 and 1970/71 (43 lines from each year) were screened. Three replicates of 100 seeds were used from each submitted line.

Table IX. Key for differentiating M. pinodes, P. medicaginis var. pinodella and A. pisi in routine seed screening tests on malt agar.

(1) Pea seed covered with a velvet mat of white to greyish-white aerial mycelium. Pycnidia produced on seed or agar; globose, dark, ostiolated; containing hyaline didymospores (occasionally amerospores). ... ASCOCHYTA SPP.

(a) Colony mderate to fast growing, translucent to various shades of brown or greenish-black, lacking in aerial mycelium. Dark-brown or black pycnidia with light-brown or milky exudate.

(i) Pycnidiospores slipper-shape, large (11.5 x 4.3 u), uniseptate, with distinct constriction at the septum. ... MYCOSPHAERELLA PINODES

(ii) Pycnidiospores elliptical to bacilliform, small (7.8 x 2.9 u), uniseptate, occasionally aseptate, commonly with an oil globule at each end. ... PHOMA MEDICAGINIS VAR. PINODELLA

(b) Colony slow growing, covered with dense, fluffy, cottony-white aerial mycelium. Pycnidia reddish-brown with pinkish exudate.

(i) Pycnidiospores large (13.2 x 3.7 u) cylindrical, uniseptate, with no constriction at the septum. ... ASCOCHYTA PISI

To obtain the working samples, submitted lines were subsampled by a modified halving method. Each packet of seed was mixed and spread evenly over the workbench. The seeds were then divided into eight approximately equal groups in two rows of four each. Four alternate groups were taken and the mixing and dividing repeated until there were approximately 100 seeds in each of the eight groups. One hundred seeds were then counted from any of three alternate groups.

Each group of 100 seeds was wrapped loosely in a muslin bag so as to ensure free movement of the seeds during surface sterilisation. Each bag was then dipped in ethyl alcohol (60%) to wet the seeds, and immersed in 1:7 dilution of commercial "Janola" (approximately 1.3% $^{w}/_{w}$ chlorine after dilution) for ten minutes. The seeds were constantly agitated with a glass rod to free any trapped air bubbles. On removal from the solution the bags were briefly shaken to remove excess "Janola" and placed in a large sterilised tray. Each bag was then unwrapped and the tray tilted to allow excess chlorine solution to drain from the seeds. The seeds were then plated aseptically onto 2% malt agar (Appendix I), ten seeds per plate, and incubated at 24C.

Although the International Seed Testing Association recommends that the seeds be examined after five days incubation (Anon, 1966), this time period was found to be insufficient for significant growth and sporulation on agar. In the present study recordings were made on the eighth day. The identification key (Table IX) was used to identify the three pathogens, and the percentage of seeds infected by each was recorded. It should be noted that the lowest infection level that could be detected was one in three hundred, or approximately 0.3%.

C. RESULTS AND DISCUSSION.

1. Prevalence and levels of the disease complex.

The prevalence of the disease complex and its level in individual seedlines are illustrated in Table X. The complex as a whole was prevalent in the 1969/70 harvest, being present in approximately 60% of the lines. In 1970/71 the disease was less prevalent, being detected in approximately 30% of the lines.

The level of infection in the majority of the lines in both years was low. If less than 1% infection is taken as the arbitrary value to denote low infection (the value used by Cruickshank in 1957), then 69% and 94% of the seedlines submitted in 1969/70 and 1970/71 respectively would be classified as having low infection levels.

The prevalence and level of infection of the complex was considered by Cruickshank (1957) to be largely controlled by weather conditions existing in the parent seed crop towards pod filling and maturity, a significantly higher incidence and higher infection level being associated with wetter summers (January to March). In the present survey, a similar relationship was observed (Table X), the higher incidence and higher infection level in seedlines harvested in Hawkes Bay in 1969/70 and 1970/71, and Canterbury in 1969/70 being a reflection of high rainfall figures for January and March (approximately 7 inches). Conversely, the low incidence and low infection level in Canterbury seed in the 1970/71 harvest may be attributed to the low rainfall in January to March of the same year (3.25 inches). However, seed from the Kairanga district does not conform to the above suggested relationship. In 1970/71,

Table X. Prevalence and level of infection of the collar-rot complex in New Zealand produced pea seed lines.

Year	District	Rainfall inches (Jan.-Mar.)	% of seedlots in each infection class					Total No. of lines
			0	0.1-1.0	1.1-5.0	5.1-10.0	>10.0	
1969/70	Hawkes Bay	7.63	35.3	29.4	35.3	-	-	17
	Canterbury	6.26	50.0	23.1	11.5	11.5	3.9	26
1970/71	Hawkes Bay	6.74	62.5	25.0	12.5	-	-	8
	Canterbury	3.25	78.6	21.4	-	-	-	14
	Kairanga	11.68	71.4	23.8	4.8	-	-	21

Table XI. Prevalence of M. pinodes, P. medicaginis var. pinodella and A. pisi in seed samples.

Year	Percentage of cultures identified			Total no. of cultures
	M. pinodes	P. med. var. pin.	A. pisi	
1969/70	81.3	18.7	-	166
1970/71	52.4	47.6	-	42

Table XII. Level of infection of seed lines by *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi*

Year	Pathogen	No. of seed lots in each infection class					Comments
		0	0.1-1.0	1.1-5.0	5.1-10.0	>10	
1969/70	Non-infected	19	-	-	-	-	
	M. pinodes	-	6	6	3	1	3 lines are mixed infection
	P. med. var. pin.	-	8	3	-	-	
	A. pisi	-	-	-	-	-	
1970/71	Non-infected	30	-	-	-	-	
	M. pinodes	-	6	1	-	-	2 lines are mixed infection
	P. med. var. pin.	-	7	1	-	-	
	A. pisi	-	-	-	-	-	

the rainfall between January and March was relatively high (11.68 inches), yet both prevalence and infection levels were low. No reasonable explanation can be offered for this apparent anomaly.

2. Prevalence and level of infection by each of the component fungi.

The prevalence and level of infection by the three fungi are illustrated in Table XI and Table XII. The former table shows that M. pinodes (Figure 37) was most prevalent in both years although the proportion of P. medicaginis var. pinodella (Figure 38) was also quite high in 1970/71. The latter situation was not due to an increase of P. medicaginis var. pinodella but rather to the sharp drop in the incidence of M. pinodes.

A. pisi was not detected in any of the seedlines screened during the two years.

Table XII reveals that the level of infection by P. medicaginis var. pinodella in individual seedlines was low for both seasons and rarely exceeded 1%. Conversely, the level of infection by M. pinodes was relatively high in many seedlines harvested in 1969/70, in one instance exceeding the 10% level. However its infection level was markedly lower in seed lines harvested in the 1970/71 season.

The relatively high incidence and high infection levels of M. pinodes, when compared with P. medicaginis var. pinodella can possibly be attributed to basic differences in the mycology of the two fungi. In both species localised secondary spread occurs by way of pycnidiospores, but in the case of M. pinodes there is also a well developed sexual phase. That is, ascospores are produced and the fact that they are air-borne

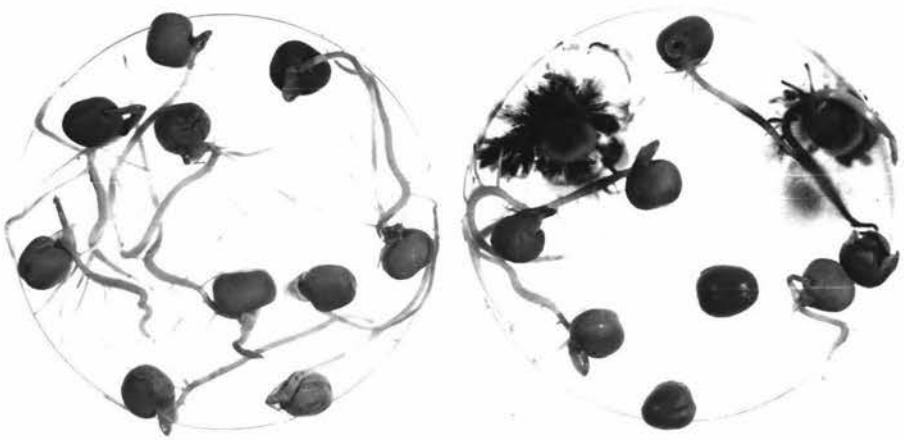


Figure 37. Healthy (left) and infected seedline of M. pinodes (right) exposed by the agar plate screening technique.

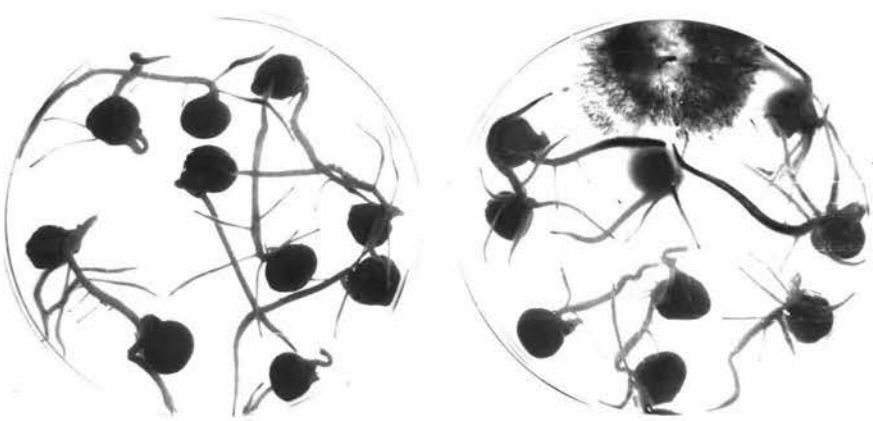


Figure 38. Healthy (left) and infected seedline of P. medicaginis var. pinodella (right) exposed by the agar plate screening technique.

increases the likelihood of pod and seed infection.

Regarding M. pinodes, the marked difference in seed infection level between 1969/70 and 1970/71 (Table XII) was largely brought about by the Canterbury figures, this being the only locality showing wide fluctuations in infection levels between the two seasons (Table X). In 1969/70 ten lines were infected at levels greater than 1%, and of these seven were produced in Canterbury. In no instance was Canterbury seed of the 1970/71 harvest infected with M. pinodes at levels higher than 1%. In the other centres the infection levels induced by M. pinodes was maintained at approximately the same level during the two seasons.

The reduction of M. pinodes in Canterbury in 1970/71 may be regarded as reflecting the relatively low rainfall recorded there during the growing season from January to March (3.25 inches compared with 6.26 inches in the 1969/70 season). It is known that under dry conditions ascospore maturation and liberation are severely restricted (Hare and Walker, 1944; Carter and Moller, 1961; Carter, 1963). In Canterbury, during the dry 1970/71 season one can assume therefore that inoculum available for secondary spread was limited to some extent, thereby restricting the level of seed infection.

A remarkable feature of the two year survey was the complete absence of A. pisi. This result was surprising particularly in view of the fact that some fourteen years earlier Cruickshank (1957) found this species to constitute 15.8% of the collar-rot fungi isolated to agar in the course of his seed health survey of New Zealand pea seed. One could postulate that the seed screening technique used in the present survey was unsatisfactory in that it failed to detect A. pisi, but this

explanation cannot be seriously considered for two reasons. The method followed was essentially similar to that recommended by the International Seed Testing Association (Anon, 1966), and shown to be effective for detecting this species (Anon, 1968). Secondly, the writer readily detected A. pisi from a seedline of United Kingdom origin.

In comparing the results of the present survey with that conducted earlier by Cruickshank (1957), three main points of difference emerge:

(i) the complex as a whole has become less prevalent, the percentage of infected lines decreasing from 55% to 41%

(ii) the level of infection by the complex as a whole has declined, the percentage of seedlines with an infection level greater than 1% decreasing from 48.6% to 16%

(iii) in no instance was A. pisi present in lines screened during the two year survey.

These points of difference collectively suggest that the state of health of New Zealand produced pea seed has generally improved in recent years. Whether in fact this is the situation, or whether the results of the current survey are merely a reflection of two dry seasons must remain a matter for speculation.

Chapter IV

MACROSCOPIC SELECTION OF INFECTED SEEDS

A. INTRODUCTION.

The availability of seed lines with high infection levels was a prerequisite for studies on the control of the disease complex by fungicidal seed treatment. Such lines were not detected during the two year survey (Chapter III). This necessitated macroscopic selection of infected seeds within a line and bulking to provide a sample with a high percentage infection level.

Features exhibited by infected seeds that could possibly be utilized in such macroscopic selection were:

(i) seed discolouration
(ii) fluorescence under ultra-violet lights
(iii) reduced seed size.

The use of seed discolouration and fluorescence as seed screening methods were reviewed earlier (Chapter III). As regards seed size, Jones (1927) reported that when seeds from an infected line were size graded the infection level of the smaller seeds was considerably higher than the rest. That is, infection of individual seed had the effect of suppressing seed development.

The relative effectiveness of the above three methods as means of obtaining seed samples with high infection levels was investigated.

B. MATERIALS AND METHODS.

Seed from three bypassed crops were used, one infected with *M. pinodes*, one with *P. medicaginis* var. *pinodella*, and one with both of these species. Features of the three seedlines are summarised in Table XIII. Seedlines infected with *A. pisi* were not available.

Table XIII. Germination and infection percentage of seedlines used to test the efficiency of macroscopic methods for selecting infected seeds.

Line No.	Germination % (blotter test)	Infection % (Agar test)	
		M. pinodes	*P. med.* var. *pin.*
5-1	95	4.7	-
7-2	70	0.3	5.4
7-7	82	-	4.3

Working samples of 500 gm were obtained by the modified halving method previously described (Chapter III).

1. Selection of discoloured seeds.

Seeds showing light-brown discolouration (Figure 39) were removed from the working samples until at least 300 of both discoloured and unblemished seeds were obtained. The seeds were then screened for the collar-rot fungi, using the agar plate method (Chapter III). Three replicates of 100 seeds each were screened.

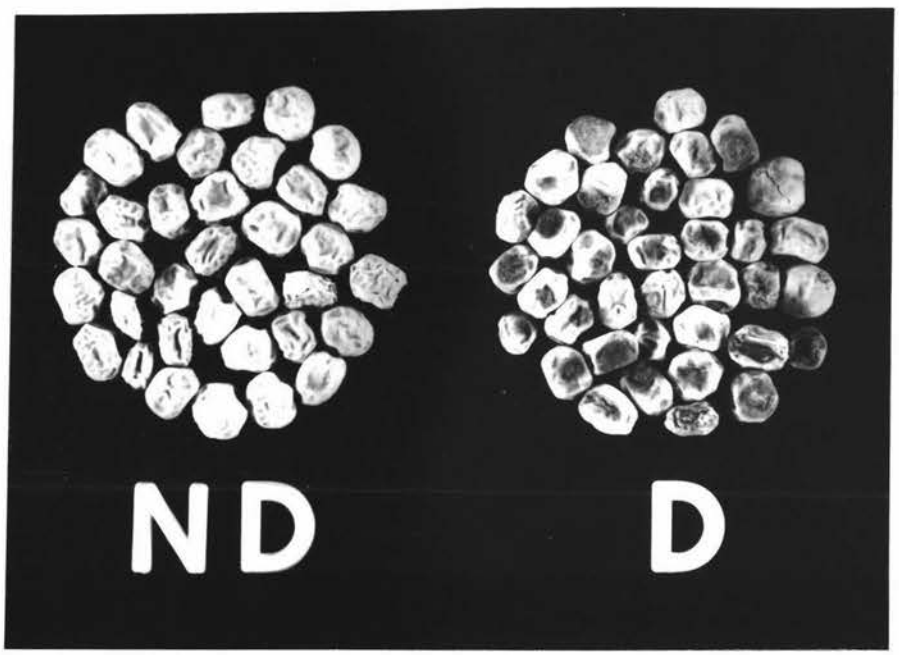


Figure 39. Discoloured (D) and non-discoloured (ND) peas separated macroscopically.

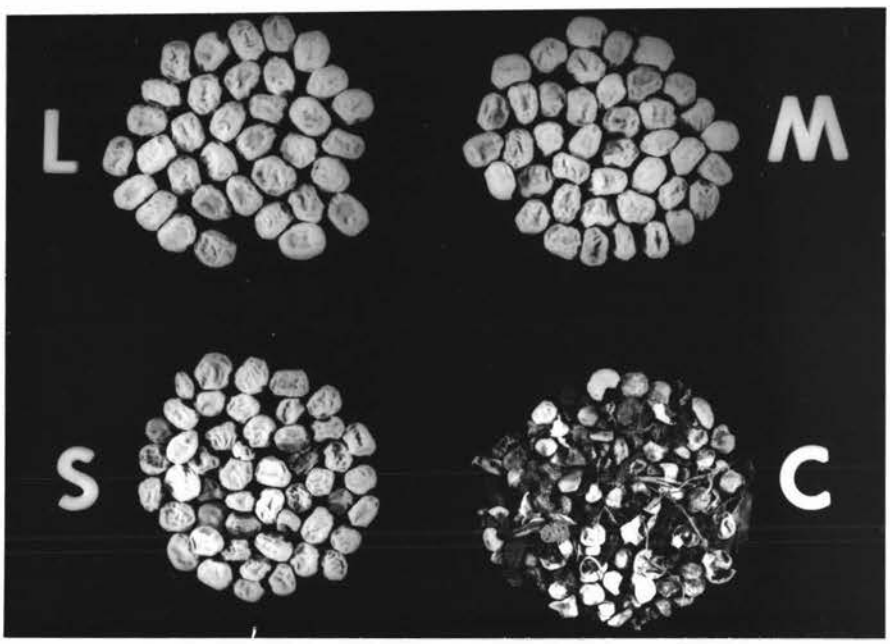


Figure 41. Large (L), medium (M), small (S) and chaffy (C) seeds separated with sieves.

2. Selection of fluorescing seeds.

The working samples were sorted macroscopically under an ultra-violet light ("Mazda" mercury vapour lamp of 200 W and 220 V) in a darkened room. All fluorescing seeds, regardless of colour, were removed (Figure 40) until slightly more than 300 of both fluorescent and non-fluorescent seeds were obtained. The seeds were then screened for the collar-rot fungi, using the agar plate method (Chapter III). Three replicates of 100 seeds each were screened.

3. Size separation of seed.

Using each of the three lines in turn, seeds were passed successively through three sieves of decreasing pore size so that four size categories were obtained, namely, large, medium, small and chaffy (Figure 41). The pores in the sieves differed in shape and size, as follows:

(i) Sieve I - circular pores, $^{5}/_{16}$ in. in diameter
(ii) Sieve II - elliptical pores, $^{3}/_{4}$ in. x $^{7}/_{32}$ in.
(iii) Sieve III - circular pores, $^{7}/_{32}$ in. in diameter.

The large, medium and small seeds were retained by sieve I, sieve II and sieve III respectively, while the chaffy seeds passed through sieve III. Three replicates of 100 seeds each from the large, medium and small seeds were then screened for the collar-rot fungi, using the agar plate method (Chapter III). No attempt was made to determine the pathogens associated with the chaffy seeds as this consisted mainly of soil debris and split seeds.

C. RESULTS AND DISCUSSION.

To enable ready comparison of the relative efficiency of the three seed selection methods, results are summarised in Table XIV. They indicate that in line 5-1, and line 7-7, selection on the basis of seed discolouration was the most efficient means of obtaining high infection levels, whereas in line 7-2 the fluorescent seed method was superior.

The fact that for different seed lines a different selection method was superior was not unexpected since the host-parasite relationship in individual lines may differ. In the case of the seed discolouration method, for example, the accuracy of the method is dependent on:

(i) the depth of infection, and therefore the degree of discolouration

(ii) whether seeds are present with lesions caused by factors other than the collar-rot fungi.

Similarly, the accuracy of the seed fluorescence method is dependent on:

(i) the ability of the particular physiological strain of pathogen to fluoresce

(ii) the capacity of other micro-organisms associated with both infected and non-infected seeds to fluoresce.

In studies on control of seed-borne inoculum by seed treatment with fungicides (Chapter V), infected seeds were identified and bulked using both the seed discolouration method (lines 5-1, 7-7) and the fluorescence method (line 7-2).

Table XIV. Total infection by the collar-rot fungi in different categories of pea seeds separated on the basis of discolouration, fluorescence and size.

Line/replicate	Percentage infection by agar plate test							
	Unsorted seeds	Seed discolouration		Seed fluorescence		Seed size		
		+	-	+	-	Small	Medium	Large
Line 5-1								
Replicate I	5.0	52.0	5.0	29.0	12.0	9.0	4.0	2.0
II	5.0	45.0	7.0	21.0	8.0	13.0	9.0	3.0
III	4.0	44.0	3.0	29.0	5.0	21.0	8.0	4.0
Average	4.7	47.0	5.0	26.3	8.3	14.3	7.0	3.0
Line 7-2								
Replicate I	7.0	20.0	2.0	34.0	6.0	6.0	8.0	4.0
II	5.0	18.0	-	34.0	2.0	7.0	3.0	2.0
III	5.0	22.0	-	32.0	4.0	8.0	4.0	5.0
Average	5.7	20.0	0.7	33.3	4.0	7.0	5.0	3.7
Line 7-7								
Replicate I	3.0	30.0	2.0	2.0	-	12.0	6.0	4.0
II	4.0	28.0	6.0	4.0	2.0	11.0	7.0	-
III	6.0	38.0	4.0	12.0	2.0	8.0	6.0	1.0
Average	4.3	32.0	4.0	6.0	1.3	10.3	6.3	1.7

Chapter V

ASSESSMENT OF FUNGICIDAL SEED TREATMENT AS A POTENTIAL CONTROL MEASURE

A. INTRODUCTION.

Research has established that infected seeds, rather than crop debris, constitute the main source of primary inoculum (Jones, 1927; Maude, 1966; Wallen et al., 1967a). Thus control of the collar-rot complex has largely centred on means of inactivating viable inoculum associated with seed lines. Past investigations towards this end may be grouped into two broad categories, namely, thermotherapy and chemotherapy.

In thermotherapy, infected seeds are subjected to high temperatures with the objective of killing deeply established infections, but without impairment of germination. Ogilvie (1933) reported that exposure of infected seeds to hot water at 50C for 25 minutes failed to eradicate the pathogens. Maude (1966) found that wet heat was more efficient than dry heat but concluded that thermotherapy was unlikely to be useful since he could not find a temperature-time combination that was totally effective.

Chemotherapy, "the control of plant diseases by compounds that, through their effect on the host or the pathogen, reduce or nullify the effect of the pathogen after it has entered the host" (Dimond, 1959) appears to have greater potential since seed viability is often less adversely affected. Various fungicides applied in dry form as a seed dressing have been evaluated in the past and although many have given a

high degree of control, total inactivation of the seed-borne inoculum has not been achieved (Jones, 1927; Maude and Kyle, 1970). This has been due in part to the deep seated nature of the pathogen in seed, and to poor deposition and/or poor penetration properties of the fungicides. Thus various alternative methods of seed treatment have been tested, namely:

(i) slurry treatment of seed
(ii) seed fumigation
(iii) chemical soak of seed.

Slurry treatment of seed was evolved primarily to achieve greater and more even deposition of the chemical over the seed. Wallen et al. (1967a) tested the effectiveness of the protective fungicides captan, thiram and chloranil applied as a slurry to infected pea seed and found that although the proportion of healthy plants was increased from approximately 50% to more than 90%, total eradication was again not achieved.

Similarly, seed fumigation with chloropicrin was reported to be unsuccessful for total eradication of the seed-borne inoculum, although a high degree of control was obtained (Kennedy, 1961).

The use of chemical soaks to achieve deeper penetration of the fungicide into seed has been investigated by a number of workers. As far back as 1927, Jones attempted (unsuccessfully) to eradicate the deep seated infection by soaking peas in 0.25% "Uspulun" at 90F for 30 minutes. Antibiotic soaks using rimocidin and pimaricin at 75 ug/ml for 24 hours also did not provide total eradication (Dekker, 1957). However, Maude (1966) obtained 100% control of A. pisi and M. pinodes by soaking pea seeds in a 0.2% suspension of thiram for 24 hours at 30C. Laboratory

germination and field emergence were not adversely affected. Unfortunately this method is impractical on a field scale owing to the large quantities of peas involved, but as stated by Maude (1966), it is of value "for treatment of nuclear seed stock" where smaller quantities of seeds are handled.

The methods of seed treatment reviewed above have not proved entirely satisfactory, either because of the poor penetration properties of the chemicals, or the impracticability of the method of application. If non-phytotoxic fungicides were available that could be absorbed when applied as a seed dressing, then complete eradication would not only be possible but also practical. Of great significance in this regard is the recent introduction of systemic fungicides; that is "compounds that are directly toxic against the pathogen and act to kill or inactivate it inside the plant" (Dimond, 1959).

The effectiveness of systemic fungicides for controlling the collar-rot complex in peas was demonstrated by Maude and Kyle (1970). They reported total eradication of A. pisi in peas by dusting infected seeds with the systemic fungicide benomyl, at the rate of $1\frac{1}{2}$ to 2 oz/28 lb of seed. This method has great practical value since seeds are at present routinely dusted with non-systemic fungicides, such as captan. It would appear that the potentialities of such systemic fungicides in controlling the collar-rot complex in peas are great.

In view of the relative effectiveness of chemotherapy in general as a means of control, and of systemic fungicides in particular, some newly introduced systemic and non-systemic fungicides were screened for their effectiveness against the collar-rot complex in peas. Since

benomyl has been widely reported to be systemic in plants (Delp and Klopping, 1968; Catling, 1969; Hine *et al.*, 1969) and shown to give total control of *A. pisi* (Maude and Kyle, 1970), particular attention was paid this fungicide. The areas investigated were its uptake and translocation by pea plants following various methods of application, and a consideration of its effectiveness against the other two pathogens in the complex (*M. pinodes* and *P. medicaginis* var. *pinodella*), when applied as a seed dressing.

B. PRELIMINARY SCREENING OF FUNGICIDES AGAINST THE COLLAR-ROT FUNGI BY THE POISON FOOD METHOD.

1. Introduction.

Fungicides under test are evaluated initially against specific pathogens in pure culture. The various methods available for such *in vitro* evaluations have been reviewed by McCallan *et al.* (1959) and Torgeson (1967). Of the methods, the so-called poison food or agar plate method measures chemical inhibition of mycelium (rather than of spores), and for this reason was adopted in the present study since the objective was to inactivate vegetative mycelium associated with seed. The method was initially developed for work with wood rotting fungi (Horsfall, 1956) and has since been used by many workers to evaluate fungicides *in vitro* (Bollen and Fuchs, 1970; Edgington *et al.*, 1971; Fuchs *et al.*, 1971).

2. Preliminary broad screening of fungicides.

(a) Materials and Methods.

Preliminary tests were conducted on five fungicides using two isolates of each of the three causal fungi. The fungicides used were:

	Trade name	Common name	Chemical name	Properties
1.	Benlate	benomyl	50% $^{w}/_{w}$; methyl 1-(butylcarbamoyl) -2-benzimidazole carbamate	systemic
2.	Vitavax	carboxin	75% $^{w}/_{w}$; 5,6-dihydro-2-methyl-1, 4-oxathiin-3-carboxaniline	systemic
3.	Methyl topsin	methyl thiophanate	50% $^{w}/_{w}$; 1,2-bis (3-methoxycarbonyl -1,2-thioureido) benzene	systemic
4.	Thiram 80	thiram	80% $^{w}/_{w}$; bis (dimethylthiocarbamoyl) disulphide	non-systemic
5.	Sclex	dichlozoline	30% $^{w}/_{w}$; 3-(3,5-dichlorophenyl)-5, 5-dimethyl oxazolidinedione-2,4.	non-systemic.

The isolates of the three fungi were:

Mycosphaerella pinodes - Isolates 44A, 30N
Phoma medicaginis var. *pinodella* - Isolates 35, 37
Ascochyta pisi - Isolates LV, PN.

Each fungicide was tested at concentrations of 0, 10, 20, 40, 60, 80 and 100 ug/ml active ingredient (a.i.) in PDA_o.

To prepare the poison food plates, thirty-five flasks containing 250 ml of double strength PDA_o were sterilised and maintained at 51C. A stock solution of each of the five fungicides at 500 ug/ml a.i. was prepared by adding the appropriate weight of the fungicide to one litre of

sterile water. With the aid of a pipette the required volume of each fungicidal stock solution was added to the sterile water to obtain 250 ml aliquotes, each double the concentration to be tested. Each was then added to one of the flasks of double strength PDA_o maintained at 51C. The flasks were vigorously shaken to mix the agar and the fungicide, and the contents poured into twelve petri dishes at the rate of 20 ml/ plate. The plates were allowed to cool after which they were each centrally inoculated with a mycelial block (one millimeter diameter) removed from three days old test cultures. Four replicates were used for each treatment.

The inoculated plates were incubated in the dark at 24C and the radial growth of each fungus on the seventh day was recorded by averaging two measurements of its diameter at right angle to each other.

(b) Results and discussion.

The results (Figures 42-44) clearly indicate that benomyl and thiram were highly effective against the three causal fungi, radial growth being completely suppressed even at very low concentrations of less than 20 ug/ml a.i. Conversely, dichlozoline, methyl thiophanate and carboxin were relatively ineffective, moderate growth occurring even at high concentrations of 100 ug/ml a.i. On the basis of the dosage required to cause 50% reduction in radial growth (ED_{50}), the effectiveness of the five fungicides can be ranked in the order - benomyl, thiram, dichlozoline, methyl thiophanate and carboxin (Table XV).

No one species of test fungus stood out as being more sensitive than the others to all five fungicides. That is, depending on the

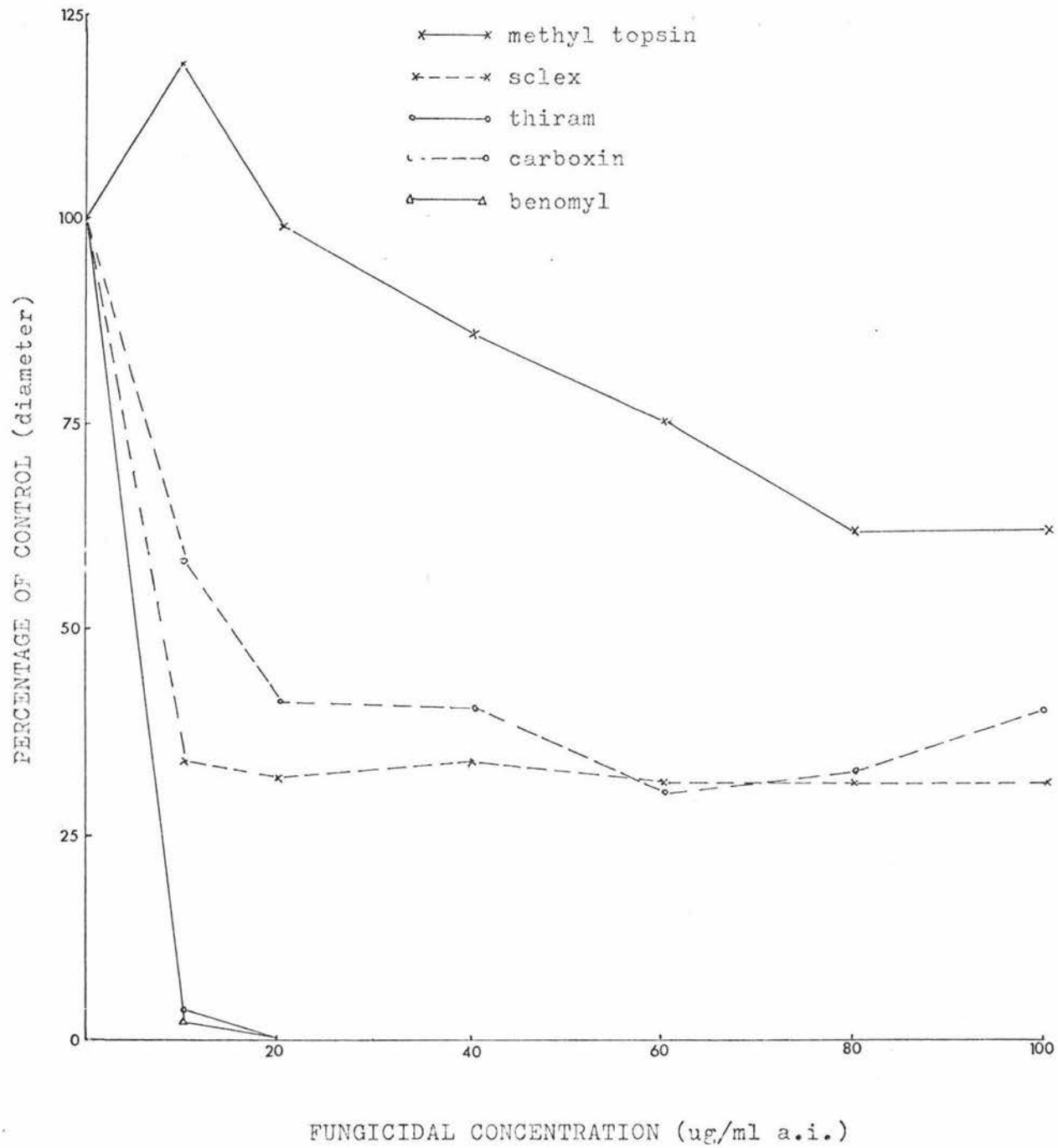


Figure 42. Effect of benomyl, thiram, sclex, methyl topsin and carboxin on radial growth of M. pinodes.

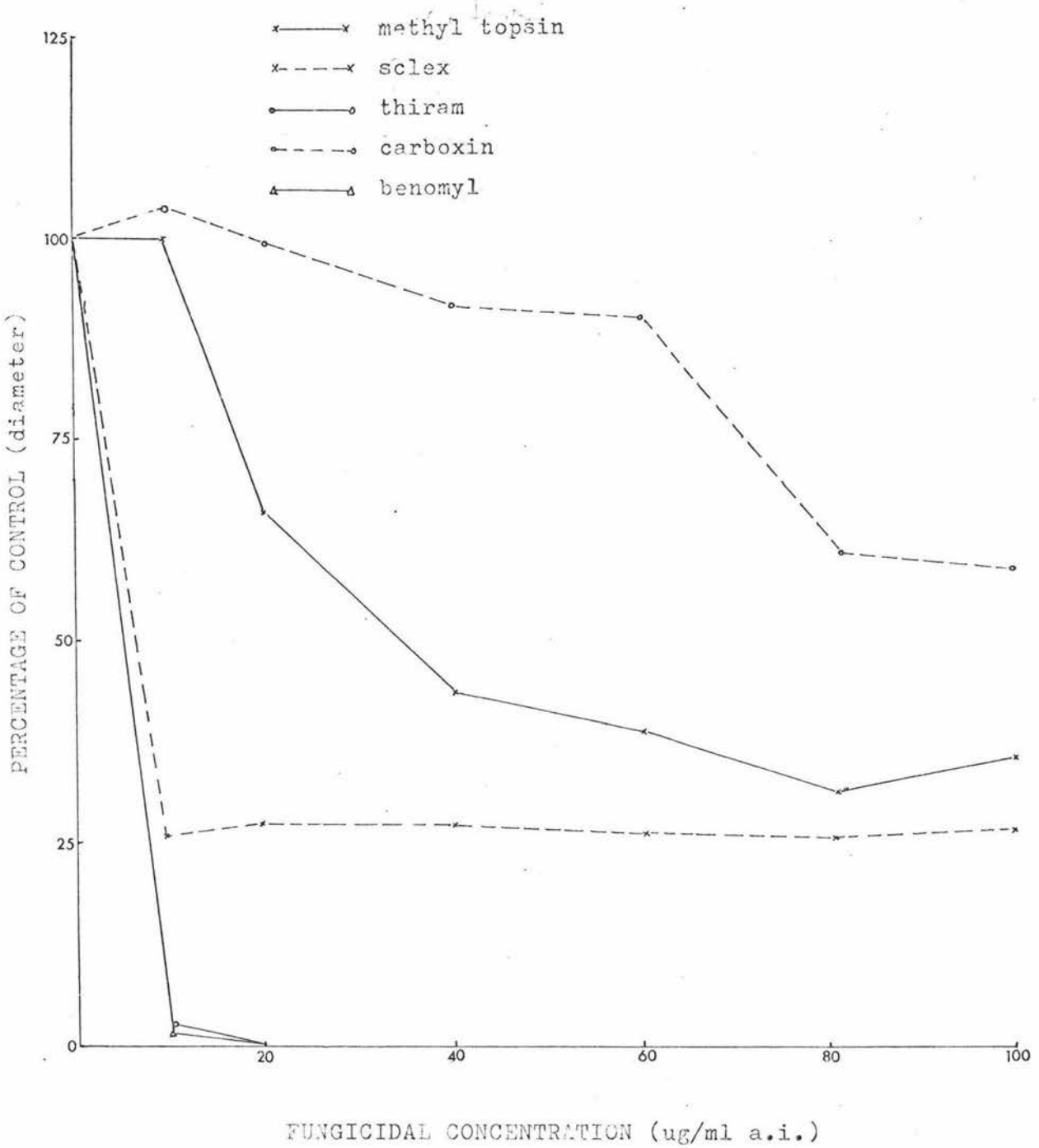


Figure 43. Effect of benomyl, thiram, sclex, methyl topsin and carboxin on radial growth of P. medicaginis var. pinodella.

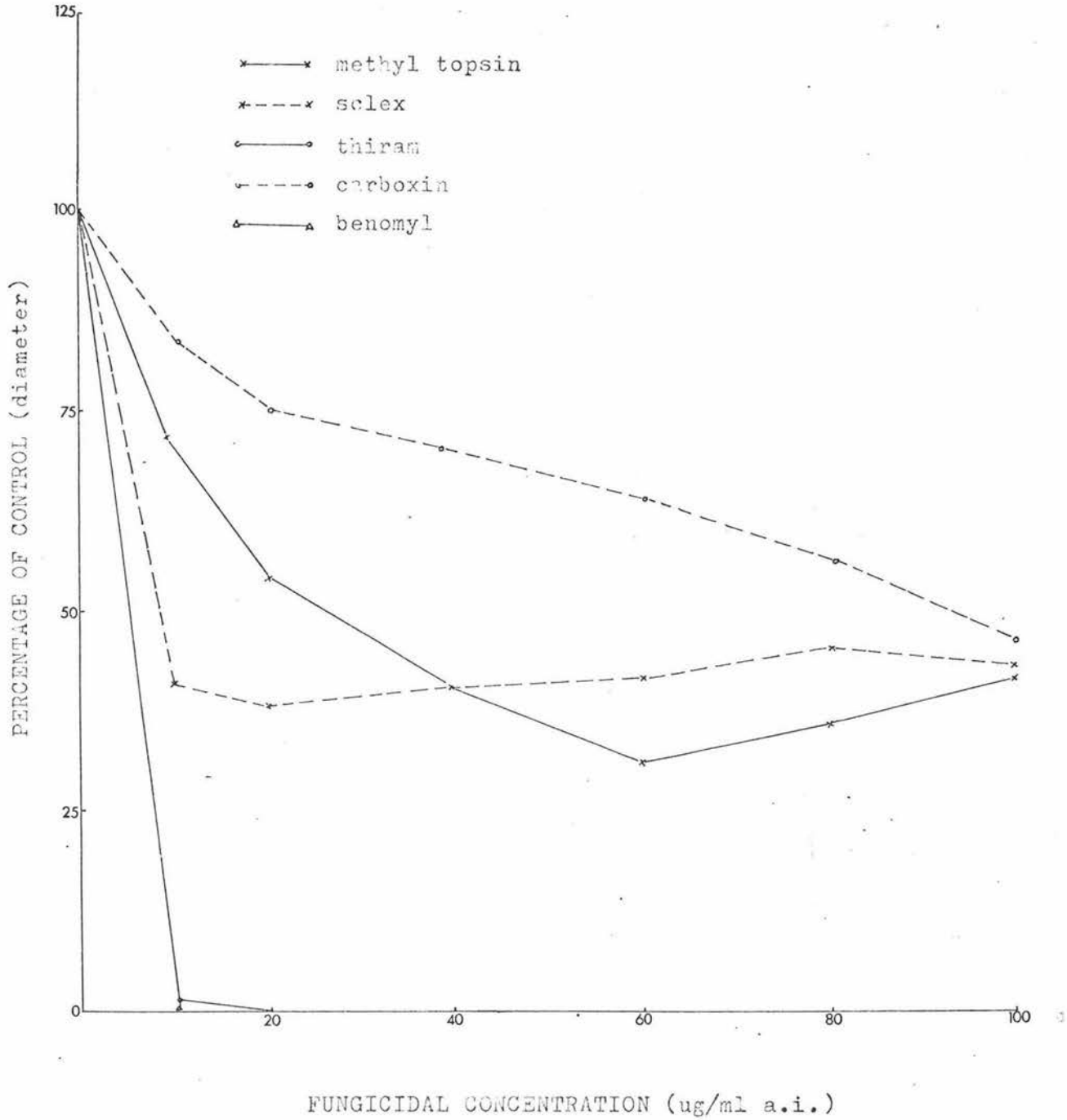


Figure 44. Effect of benomyl, thiram, sclex, methyl topsin and carboxin on radial growth of A. pisi.

fungicide concerned so the relative sensitivity of the three fungi varied. Thus with carboxin, M. pinodes was most sensitive and P. medicaginis var. pinodella least, while with methyl thiophanate, A. pisi was most sensitive and M. pinodes was least (Table XV).

Table XV. The ED_{50} values of benomyl, thiram, sclex, methyl topsin and carboxin against the three collar-rot fungi.

FUNGI	APPROXIMATE ED_{50} VALUES (ug/ml)				
	Benomyl	thiram	sclex	methyl top.	carboxin
M. pinodes	5	5	8	100	15
P.medicaginis var.pinodella	5	5	6	33	100
A. pisi	5	5	8	25	93

An interesting observation was the apparent stimulatory effect of methyl thiophanate on M. pinodes, and of carboxin on P. medicaginis var. pinodella at concentration of approximately 10 ug/ml (Figures 42 and 43). A similar stimulatory effect by other fungicides at low concentrations was observed by Ogawa et al. (1968) and Findlay (1970).

The fact that benomyl and thiram were lethal to all three fungi, even at low concentrations, indicated the need for further screening to define more precisely the extent of growth inhibition by these two fungicides at concentrations of less than 15 ug/ml.

3. Screening of benomyl and thiram at low concentrations.

(a) Materials and Methods.

The materials and methods were identical to those used in the previous studies, except that lower concentrations of both benomyl and thiram were tested, namely, at 0, 2, 4, 6, 8, 10, 15 and 20 ug/ml a.i. The poison food plates were inoculated with the same series of isolates, and the results were recorded on the seventh day.

Tests were also conducted to determine whether the action of the two fungicides was fungistatic or fungicidal. At the end of the above described experiment mycelial blocks which showed no evidence of growth were transferred to PDA_o plates free of fungicides. The growth of the fungi in these plates was observed after a further seven days incubation at 24C. If growth was resumed it would indicate the fungicide was only inhibiting growth; that is, fungistatic action. If however, growth was not evident, the fungicide must have killed the fungi, in which case the action was fungicidal (McCallan and Wellman, 1942).

(b) Results and discussion.

The effect of benomyl and thiram on the three fungi is illustrated in Figures 45 and 46 and Table XVI. The results indicate that although both fungicides were equally lethal to the three fungi, benomyl was slightly more effective at equivalent concentrations. For benomyl, growth of the three fungi was totally suppressed at less than 10 ug/ml a.i.

Comparing the sensitivity of the three fungi to the two fungicides, *A. pisi* was most sensitive to benomyl and *M. pinodes* least

sensitive. In the case of thiram, A. pisi and M. pinodes were approximately of equal sensitivity, whereas P. medicaginis var. pinodella was relatively less sensitive (Table XVI).

Table XVI. The ED_{50} values of benomyl and thiram against the three collar-rot fungi, by screening at low fungicidal concentrations.

FUNGI	ED_{50} Values (ug/ml)	
	Benomyl	Thiram
M. pinodes	3.2	3.7
P. medicaginis var. pinodella	2.5	7.5
A. pisi	1.7	4.4

The effect of benomyl was shown to be fungistatic since mycelial growth was resumed by all three fungi in nearly all cases when re-transferred to fresh PDA_o plates. On the other hand, the effect of thiram was fungicidal since no growth was observed. However it should be noted that both fungistatic and fungicidal activities can commonly be produced by the same chemical depending on its concentration and its time of exposure to the fungi (Lily and Barnett, 1951). That is, if the inoculum had been exposed to higher concentrations of the chemicals or for a longer duration, the effect may have been fungicidal, rather than fungistatic.

Although benomyl and thiram were both equally lethal to the three collar-rot fungi in vitro, benomyl is obviously more useful than thiram in inactivating the deep seated mycelium within seeds on account of its systemic properties. Its superiority in this regard has been

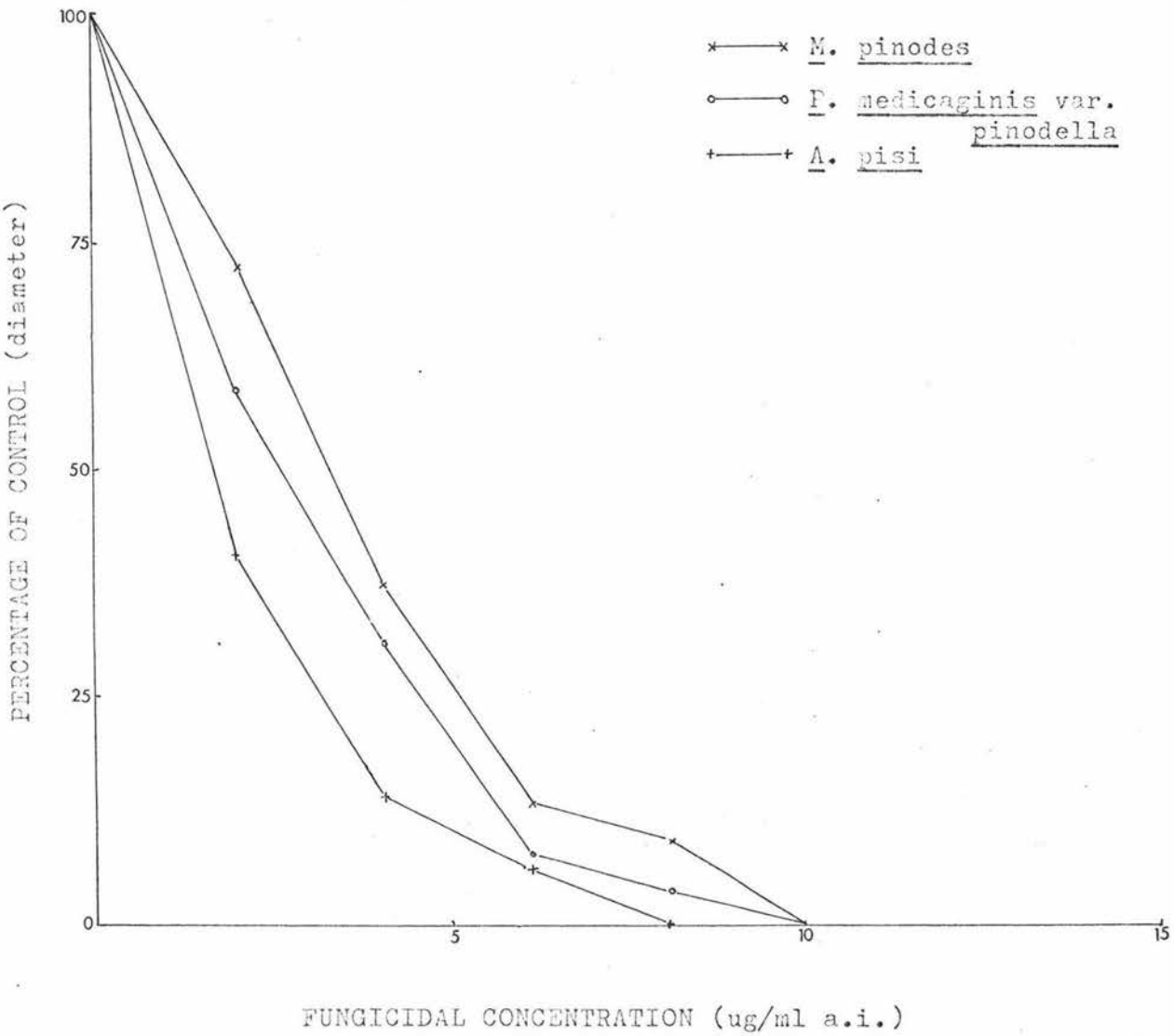


Figure 45. Effect of low concentrations of benomyl on radial growth of M. pinodes, P. medicaginis var. pinodella and A. pisi.

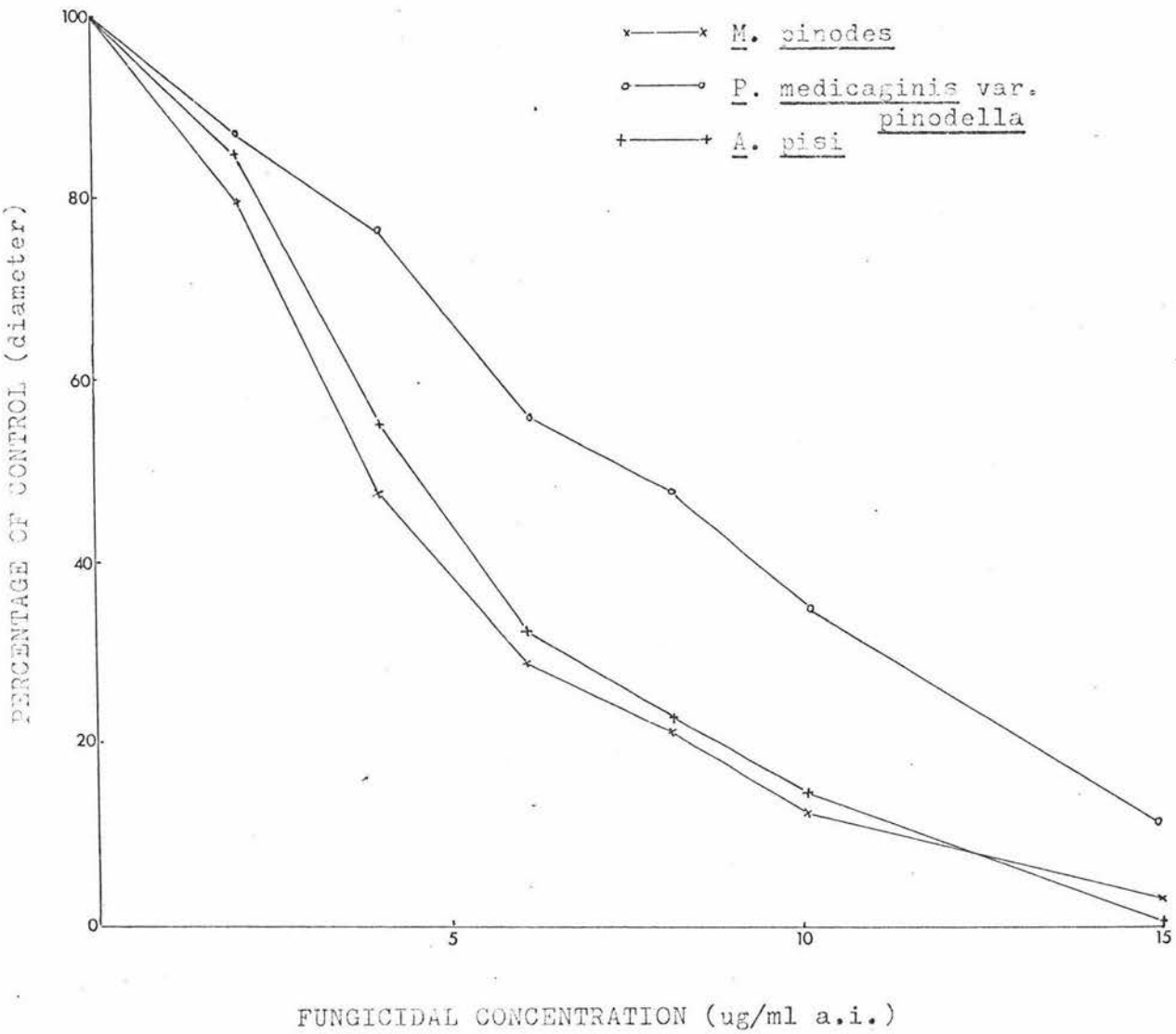


Figure 46. Effect of low concentrations of thiram on radial growth of M. pinodes, P. medicaginis var. pinodella and A. pisi.

confirmed by Maude and Kyle (1970) who reported that seed-borne infection of A. pisi in peas was totally controlled by dusting seeds with benomyl, but not with thiram. Evidently the latter fungicide could not penetrate effectively into the seeds, except following prolonged soaking (Maude, 1966).

In view of the distinct superiority of benomyl when used as a dry seed dressing, further investigations were concentrated on this fungicide. Since this superiority was a direct consequence of its systemic properties, studies in the first instance were concerned with its uptake and translocation in peas.

C. SYSTEMIC ACTIVITIES OF BENOMYL IN PEA PLANTS.

1. Introduction.

Since its introduction by Delp and Klopping (1968) benomyl has attracted much attention owing to its unique combination of protective, eradicative and systemic fungicidal properties (Al-Beldawi and Pinckard, 1970; Cole et al., 1970; Jacobsen and Williams, 1970; McCain, 1971). Its importance as a fungicide is further emphasised by its wide fungitoxic spectrum (Edgington and Khew, 1970; Bollen and Fuchs, 1970; Edgington et al., 1971) and its low mammalian and plant toxicity (Catling, 1969; Gould and Miller, 1970). The latter factor is especially important since many systemic fungicides introduced in the past were unacceptable owing to their relatively high phytotoxicity (Erwin, 1969).

Benomyl is a highly unstable compound in solution and has been demonstrated by paper chromatographic bioassay to break down rapidly into

a relatively stable and fungitoxic compound called methyl 2-benzimidazole carbamate (Clemons and Sisler, 1969). Methyl 2-benzimidazole carbamate (BMC) has been suggested as the main chemical that is translocated within plant tissues and responsible for the fungitoxic activities of benomyl at sites remote from its point of application (Clemons and Sisler, 1969; Peterson and Edgington, 1970).

The systemic activities of benomyl or its derivatives have been demonstrated in a number of plants including many cucurbits, legumes, solanaceous plants, ornamentals, grasses and fruit trees (Schroeder and Provvidenti, 1968; Hammett, 1968; Harper, 1968; Hardison, 1968; Porter, 1970; Biehn and Dimond, 1969; Netzer and Dishon, 1970; Arif Anwar and Goode, 1970; Biehn, 1970; Al-Beldawi and Pinckard, 1970; Thanassoulopoulos *et al.*, 1970; Gould and Miller, 1970; Forsberg, 1970; Cole *et al.*, 1970; Jackson, 1970; Cimanowski, 1970; McDonnell, 1970; Hearn and Fenton, 1970; McCain, 1971; Engelhard, 1971; Vargas and Laughlin, 1971). The fungicide has been reported to be systemic following application as a seed treatment, foliar spray or as various soil amendments such as soil incorporation, soil drenching, side placement or subirrigation (Catling, 1969; Peterson and Edgington, 1969; Thapliyal and Sinclair, 1970; Netzer and Dishon, 1970; Jacobsen and Williams, 1970; Biehn and Dimond, 1970a and b). In the case of seed treatment, the fungicide is relatively localised in distribution and transient (Thapliyal and Sinclair, 1970), but with foliar sprays, it is more widely translocated and persistent (Netzer and Dishon, 1970). With soil amendments the fungicide is translocated to all parts of the plant and can be detected for a longer period (Peterson and Edgington, 1970; Biehn and Dimond, 1970b).

Benomyl is only translocated unilaterally up the plant and no downward movement has been reported (Catling, 1969; Jhooty and Behar, 1970). This is because the main axial flow of the chemical is confined to the xylem and although some fungitoxic chemical may be detected in the phloem of treated plants, this is due to lateral transfer from the xylem rather than its direct involvement in chemical translocation (Peterson and Edgington, 1970).

Benomyl is taken up by a number of plant organs including the cotyledons of germinating seeds, hypocotyl, stems, leaves, petals, bracts and fruits (Biehn and Dimond, 1969; Thapliyal and Sinclair, 1970; Gray and Sinclair, 1970; Netzer and Dishon, 1970; Peterson and Edgington, 1970 and 1971). The ability of an aerial organ to accumulate benomyl was reported to be dependent on the presence of functional stomata. Hence, geranium petals and poinsetta bracts do not accumulate benomyl owing to the absence of stomata in the former and the lack of functional stomata in the latter (Peterson and Edgington, 1971).

Of all the above organs, the accumulation of benomyl in leaves has been most widely studied. Peterson and Edgington (1969) reported that there was no difference in the concentration of benomyl in the three leaflets of bean plants when applied as a soil drench. However within individual leaves benomyl was initially found at the mid-vein or base, but thereafter became more concentrated at the leaf margins and apices (Peterson and Edgington, 1970). Some benomyl was always present at the central leaf regions since the chemical was being continuously supplied from the roots. Its concentration at the leaf margins and tip was suggested to be due to physical force rather than the active participation

of the plant, since this could be mimicked artificially with dye and paper models (Peterson and Edgington, 1971).

In peas, the systemic action of benomyl has been demonstrated by a number of workers (Harper, 1968; Jhooty and Behar, 1970; Fuchs et al., 1970), but its translocation pattern and persistence within the plant have not been fully determined. As such information is required before control measures giving maximum systemic fungitoxicity in the plant can be formulated, studies were initiated to trace the translocation and persistence of benomyl when applied as a seed dressing, foliar spray or soil drench. However, before such studies could be initiated an efficient bioassay method for detecting and measuring benomyl in host tissues had to be selected.

Bioassay methods for detecting and measuring the movement of chemicals in plants have been reviewed by Wain and Carter (1967). In the case of benomyl, two assay methods have been widely used:

(i) Paper chromatographic bioassay or bioautograph techniques (Peterson and Edgington, 1969; Fuchs et al., 1970)

(ii) Assay of intact or mascerated plant tissues on agar plates seeded with a sensitive organism (Erwin et al., 1968; Biehn and Dimond, 1969; Davies and Pinckard, 1969; Netzer and Dishon, 1970).

In paper chromatographic bioassay the extracted sap to be assayed is separated into different fractions in a paper chromatogram which is then sprayed with an agar-spore suspension of a sensitive test organism. After incubation the fungitoxicant is located by the clear spots on the chromatogram. These are then quantitatively analysed.

In the agar plate bioassay the fungitoxicant diffuses from the intact or mascerated tissues to the surrounding seeded agar and inhibits the growth of the test fungus. The amount of fungitoxicant is measured by the diameter of the resultant clear zone of inhibition.

In the present study the agar plate bioassay method was preferred since it does not require specialised equipment, is simple to operate and is sensitive (Erwin et al., 1968; Erwin, 1969; Davies and Pinckard, 1969). Various modifications of the method have been used by different workers, especially with respect to the medium and the test fungus. For this study, PDA_o and Penicillium spp. were employed since the former is readily available in the laboratory and the latter is a common aerial contaminant which is highly sensitive to benomyl (Bollen and Fuchs, 1970). Species of Penicillium have commonly been used as the test organism in bioassays of benomyl (Erwin et al., 1968; Hine et al., 1969; Biehn and Dimond, 1971). The method adopted in the present study for detecting benomyl in pea plants was as follows:

2. Materials and Methods.

(a) The agar plate bioassay method.

(i) Preparation of the double strength spore suspension.

The species of Penicillium used as the test fungus for bioassay was an aerial laboratory contaminant. Cultures of the fungus were prepared by streaking a loopful of conidia from the stock culture onto PDA_o plates and incubating for seven days at 24C. The plates were then flooded with sterile water and the resulting conidial suspension poured into a flask of sterile water and shaken thoroughly for five minutes to

disperse the conidia. The suspension was then adjusted to give a final concentration of 45 to 55 x 10^4 conidia per ml (double strength). A haemocytometer was used to determine the spore concentration and in this process, conidial aggregates were each regarded as "single conidium".

(ii) Preparation of seeded agar plates.

A flask of molten, double strength PDA_o (commonly of 250 ml to 500 ml depending on the number of tissues to be bioassayed) was maintained at 51-52C. This was then added to an equal volume of the above standardised conidial suspension, the latter being continually agitated as the agar was added. After thorough mixing, the seeded agar was poured into petri dishes (20 ml/plate) and allowed to solidify. These plates were invariably used within six hours after pouring.

(iii) Bioassay of tissue pieces.

Tissue pieces to be bioassayed were in all cases randomly selected from treated plants. Depending on the particular structure assayed, the nature of the samples removed from the plant varied, as shown in Table XVII.

The samples removed from the plant were surface sterilised by drawing them rapidly across a burner. They were then spaced out on the seeded agar (two to five/plate) and gently smoothed down with a sterilised rod to ensure good contact between the tissues and the agar surface. The plates were then incubated at 24C for three days.

(iv) Recording of results.

The results were recorded on the third day of incubation by

Table XVII. Nature of the samples removed from various plant structures for bioassay, and calculation of their effective inhibitory zone.

Plant structure	Nature of sample	Actual or estimated* diameter	Calculation of effective diam. of inhib. zone (mm)
1. Leaves	7 mm discs	7 mm	x - 7
2. Calyx	Whole calyx	9 mm	x - 9
3. Petal (Standard)	7 mm discs	7 mm	x - 7
4. Developing cotyledons (in soil drench expt.)	7 mm core/half cotyledon	7 mm	x - 7
5. Seed cotyledons (in seed dressing expt.)	2 x 7 mm core/half cotyledon	7 mm	x - 7
6. Pedicel	10 mm, split at centre	5 mm*	(a + b) - 10
7. Epicotyl	10 mm, above cotyledon, split	5 mm*	(a + b) - 10
8. Hypocotyl	10 mm below cotyledon, split	5 mm*	(a + b) - 10
9. Pod wall	7 mm discs from half wall	7 mm	x - 7

Note: * = estimated diameter of tissue pieces as these are not in the form of discs.

x = actual diameter of the zone of inhibition on agar plates.

(a + b) = sum of the greatest length and width of the zone of inhibition on agar plates. Note that the zones are not circular but elliptical in these cases.

taking two measurements of the inhibition zone at right angle to each other. Only the size of the clear zone was recorded and the intervening narrow annulus of partial fungal growth was ignored. To obtain the effective diameter of the inhibition zone the actual or estimated size of the plated tissues (Table XVII) was deducted from the size of the inhibition zone. The average effective diameter of the inhibition zone of all replicates was used for comparison between treatments.

The quantity of benomyl in plated tissues can be estimated by referring to the standardisation curve in Appendix IV. The curve shows the relationship between the quantity of benomyl and the average effective diameter of the zone of inhibition.

(b) Seed treatment.

The systemic action of benomyl following seed treatment was investigated using 100 gm samples of seed (Victory Freezer) obtained randomly by the modified halving method described in Chapter III. The seeds were either dusted or slurry treated in 250 ml Ermeyer flasks with Benlate (50% benomyl) at the rate of 0, 1, 2, 4 and 6 oz/bushel. The procedure followed is described in Appendix V.

When testing the uptake and translocation of benomyl following seed treatment seeds from each treatment were sown in three pots of perlite-peat mixture (Appendix II) in the glasshouse, at the rate of four seeds per pot. The pots were continuously subirrigated by standing them in trays of water. After emergence (eleven days from sowing), four seedlings from each treatment were randomly selected and the soil removed by washing. Discs of cotyledons, first nodal leaves and sections of

hypocotyl and epicotyl were removed from each seedling (Figure 47 and Table XVII) and assayed.

(c) Foliar spray.

The uptake of benomyl following foliar spraying was investigated using four weeks old pea plants (Victory Freezer) grown in a perlite-peat mixture (Appendix II) in 4 inch pots. Two plants were grown in each pot and eight pots were used for each of four treatments.

Half litres of benomyl at the concentrations 0, 62.5, 125 and 250 ug/ml a.i. were prepared by dilution of a stock solution (500 ug/ml a.i.) obtained by adding one gram of Benlate (50% benomyl) to a litre of sterile water. Using a pipette, 0.1 ml of a surfactant (90% dodecyl ether of polyethylene glycol) was added to each 500 ml aliquot, and thoroughly shaken.

When spraying the plants the top of each pot was covered with wads of tissue paper to prevent any chemical from being deposited on the soil and being absorbed by the roots. Using an atomiser, the plants were thoroughly sprayed to the point of run off (approximately 30 ml/plant). The paper wads were then removed and the pots subirrigated by standing them in trays of water.

Of the eight pots in each treatment, five were used for bioassay. The distribution of benomyl within individual leaves was investigated on the third day after spraying by randomly removing eight leaves from the fourth node of plants in each treatment. Four of these were rinsed in distilled water while the remaining four were left unwashed. Seven millimeter discs were then removed from the base, centre, tip and

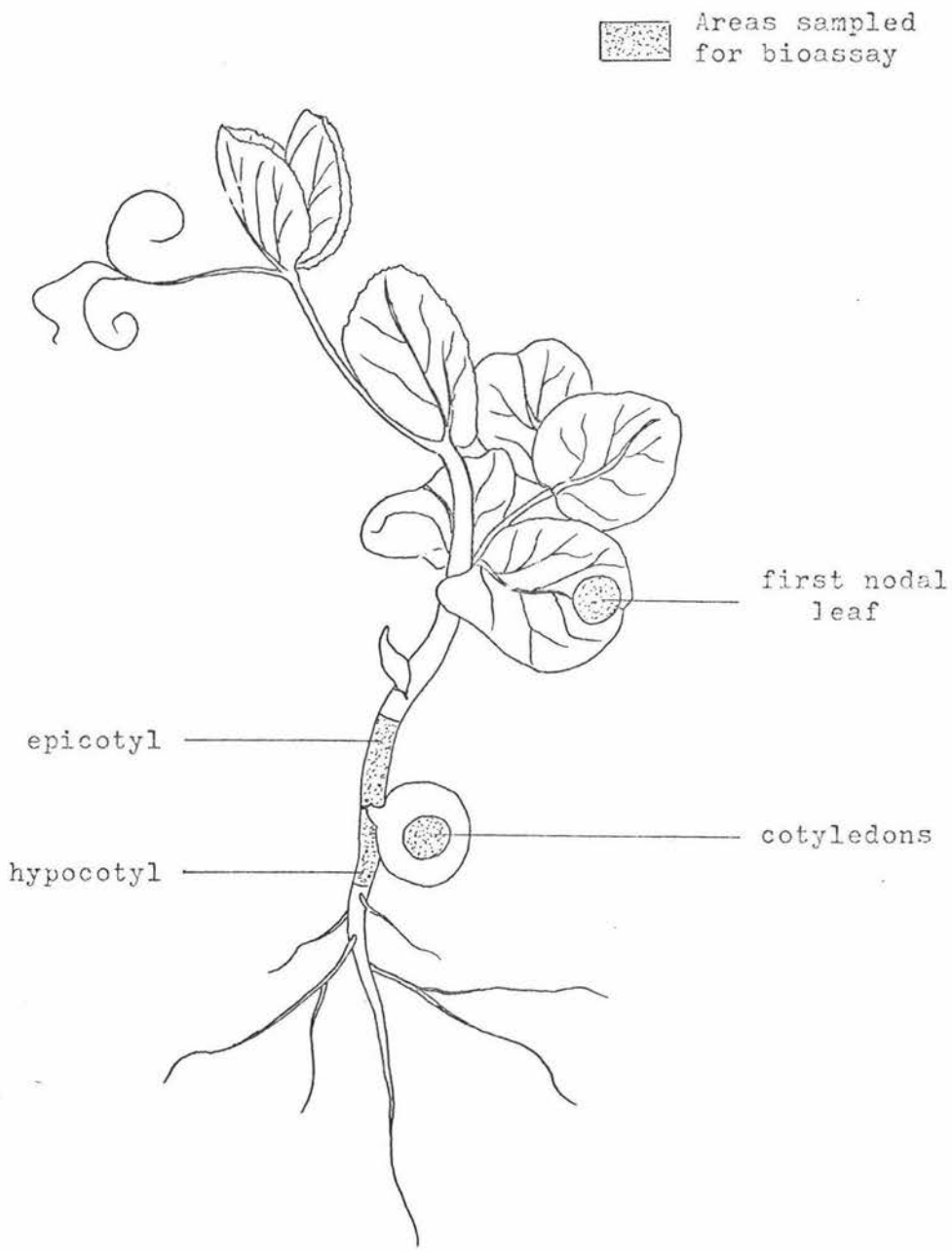


Figure 47. Location of areas from which samples of various seedling structures of peas were removed for bioassay.

the two lateral edges of each leaf for bioassay.

The distribution of benomyl within treated plants was further investigated by removing four leaves from nodes 1, 4 and 8 on the third day and four leaves from nodes 1, 8 and 10 on the seventh day. In each case, 7 mm discs from the tip of each leaf were assayed.

Treated plants in the remaining three pots of each treatment were inoculated on the third day with a pycnidiospore suspension of M. pinodes (Isolates 44A and 30N; 5×10^4 pycnidiospores/ml) to test whether they were protected against infection. Preparation of the pycnidiospore suspension and the inoculation procedure were as described in Appendix III. Infection was assessed two weeks after inoculation and the pair of leaves at each node was scored 0, 1, 2 or 3, depending on whether they were non-infected or slightly, moderately or severely infected.

(d) Soil drenching.

The systemic action of benomyl following soil drenching was studied in the glasshouse using pea plants (Victory Freezer) grown in perlite-peat mixture (Appendix II) in 4 inch pots. Two plants were grown in each pot and twelve pots were used for each of five treatments. All plants were subirrigated by standing them in trays of water.

One litre of benomyl at each of the concentrations 0, 250, 500, 750, 1000 ug/ml a.i. were prepared by dilution from a stock solution (1000 ug/ml a.i.) obtained by adding six grams of Benlate (50% benomyl) to three litres of distilled water. When the plants were four weeks old, twelve pots were drenched with each of the above concentrations of benomyl, 60 ml being applied to each pot.

Of the twelve pots, nine were used for the following bioassay.

(i) The effect of time on the quantity of benomyl in treated plants.

This study was carried out using plants treated with 500 ug/ml a.i. of benomyl. Four leaves from nodes 1, 4 and 10 were randomly selected each week after drenching, and 7 mm discs removed from the centre of the leaves for bioassay.

(ii) The distribution of benomyl within treated plants at the third week after drenching.

The distribution pattern was traced by assaying four randomly selected leaves from alternate nodes of plants in each treatment on the third week after drenching. Seven millimeter discs from the centre of each leaf were again bioassayed.

(iii) The distribution of benomyl within individual leaves.

This study was conducted on the third week after drenching by randomly removing four leaves from nodes 4 and 10 from the plants in each treatment. Seven millimeter discs were removed from the base, centre, tip and the two lateral edges of each leaf and bioassayed.

(iv) Comparison of the quantity of benomyl in nodal and branch leaves.

The comparison was carried out using plants treated with benomyl at 500 ug/ml a.i. Four nodal leaves and four branch leaves (Figure 48) were removed from each of the nodes 1, 4 and 6 at one, two and three weeks after drenching. Seven millimeter discs removed from the centre of each leaf were bioassayed.

(v) The distribution of benomyl in the reproductive structures.

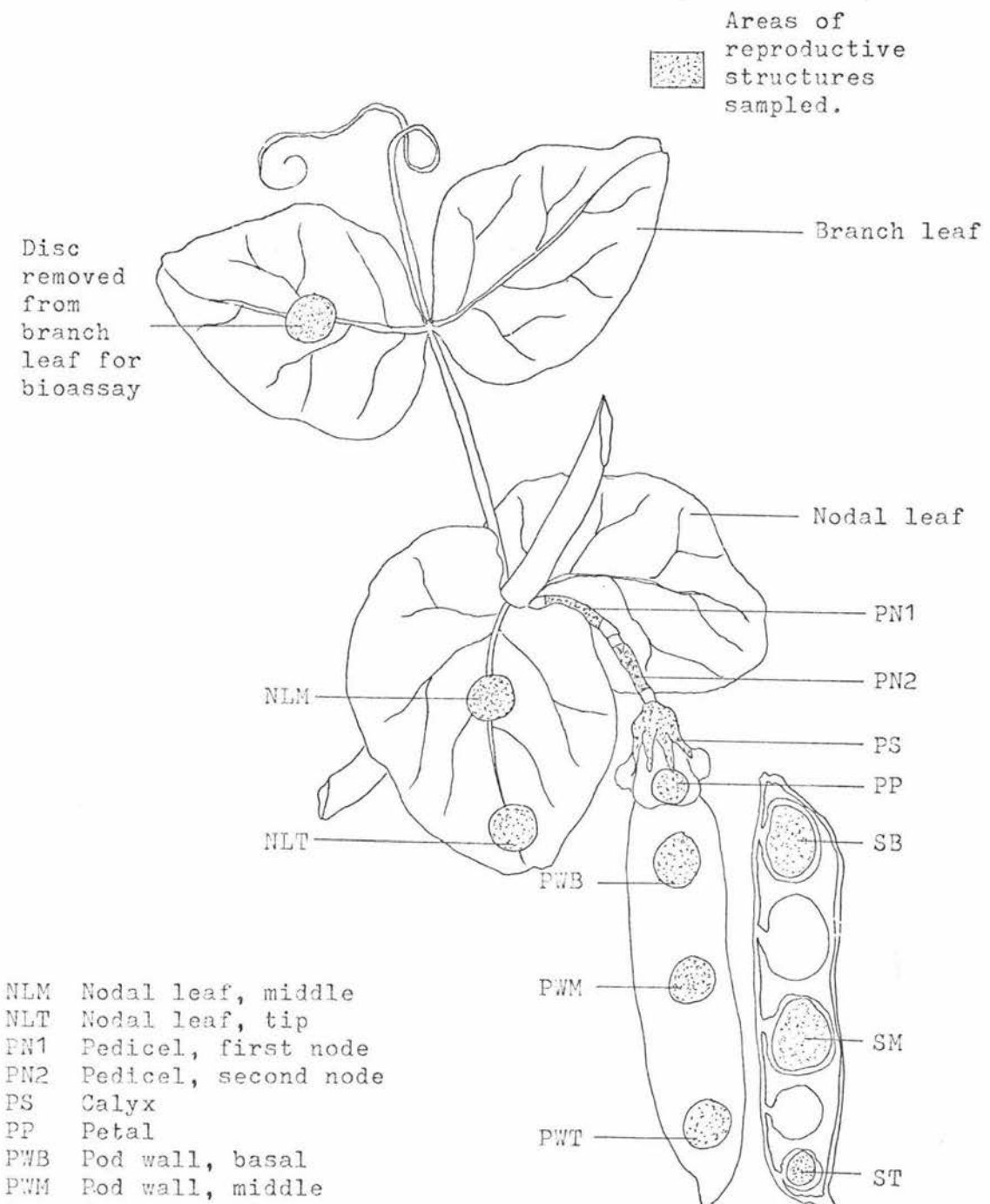
Bioassays were carried out with plants which had been drenched with benomyl at 500 ug/ml a.i. Two pods were randomly removed five, seven and nine weeks after drenching, at which time their stage of development was approximately that of pod elongation, early pod filling and pod maturity, respectively. The structures assayed are illustrated in Figure 48.

The remaining three pots of plants from each treatment were inoculated on the third week after drenching, using a spore suspension of M. pinodes (Isolates 44A and 30N; 7×10^4 pycnidiospores/ml) to test whether they were protected against infection by the systemically absorbed benomyl. The preparation of the spore suspension and the inoculation procedures were as described in Appendix III. Infection was assessed two weeks after inoculation by scoring the pair of leaves at each node as either 0, 1, 2 or 3 depending on whether they were non-infected, or slightly, moderately or severely infected, respectively.

3. Results and discussion.

(a) Seed dressing.

Benomyl applied to pea seeds as a dust or slurry at 1 to 6 oz Benlate (50% $^{w}/_{w}$)/bushel was taken up and translocated to the hypocotyl, cotyledons, epicotyl and first nodal leaves of seedlings by the eleventh



NLM Nodal leaf, middle
NLT Nodal leaf, tip
PN1 Pedicel, first node
PN2 Pedicel, second node
PS Calyx
PP Petal
PWB Pod wall, basal
PWM Pod wall, middle
PWT Pod wall, tip
SB Seed, basal
SM Seed, middle
ST Seed, tip.

Figure 48. Location of areas from which samples of reproductive structures were taken for bioassay.

day after sowing (Table XVIII and Figure 49).* In all treatments, highest concentration of benomyl was detected in the cotyledons, followed by the hypocotyl (except dusting at 1 oz/bushel where nodal leaves were higher).

Table XVIII. Distribution of benomyl in seedlings of treated seeds on the eleventh day after sowing.

Treatment	Average diameter of effective zone of inhibition (mm)			
	First nodal leaf	Epicotyl	Cotyledon	Hypocotyl
Slurry treatment				
1 oz/bushel	1.1	0.3	12.2	1.2
2 oz/bushel	-	1.5	7.1	2.3
4 oz/bushel	5.4	7.4	15.1	12.3
6 oz/bushel	2.6	4.8	19.8	8.4
Dusting				
1 oz/bushel	1.7	0.8	3.1	1.4
2 oz/bushel	1.4	0.8	10.4	2.2
4 oz/bushel	1.8	4.7	11.0	5.4
6 oz/bushel	4.8	6.1	18.3	10.1

Lowest concentration of the chemical was usually detected in the first nodal leaves, especially when higher levels of benomyl were applied.

In dusted seeds the amount of benomyl in the epicotyl, cotyledons and hypocotyl increased with increasing concentration of the chemical

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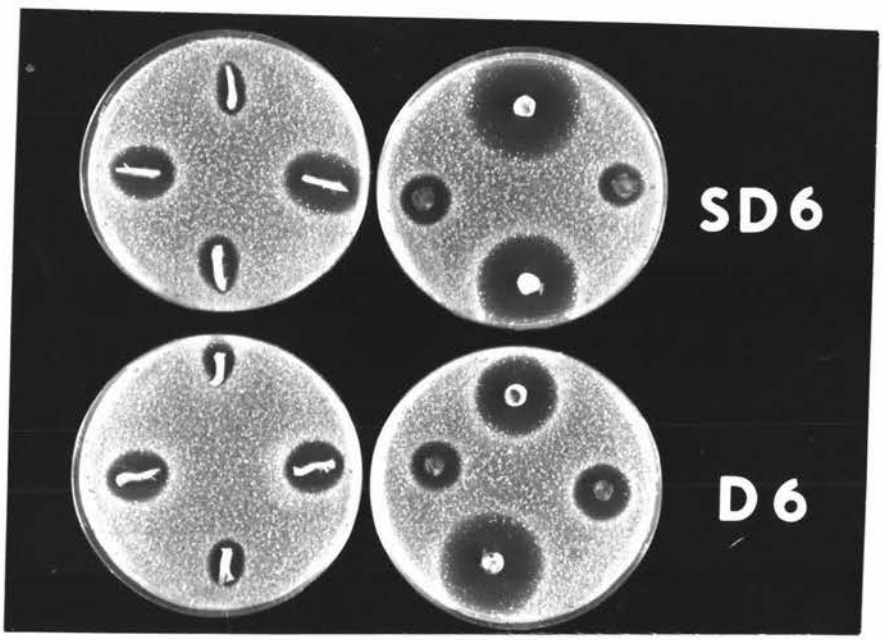
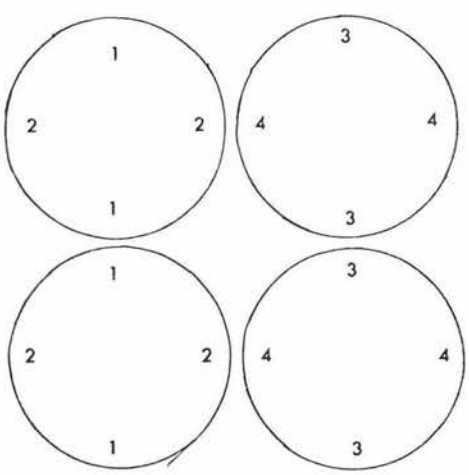
* Note: This experiment was conducted in the summer (Oct./Nov.) but initial experiments conducted in the winter (May to July) failed to detect benomyl in the first nodal leaves of seedlings up to six weeks after sowing. The complete absence of benomyl or its presence at only undetectable concentrations in the seedling leaves in winter may be a reflection of differences in weather conditions, especially those affecting transpiration. Differences in translocation of benomyl under different weather conditions have been observed by Biehn and Dimond (1971).





1 - epicotyl

2 - hypocotyl

3 - cotyledons

4 - first nodal leaf

Figure 49. Inhibition zones produced by different parts of pea seedlings developed from seeds slurry treated or dusted with benomyl at the rate of 6 oz/bushel.

applied to the seeds. However such a trend was not observed with tissues removed from seedlings of slurry treated seeds.

The decreasing concentration of benomyl in the axial structures (epicotyl and hypocotyl) of seedlings with increasing distance from the soil indicates that the chemical had been absorbed by plant structures located in the soil. Both roots and cotyledons may be involved because:

(i) high concentrations of benomyl were detected in the hypocotyl, and since the fungicide can only move upwards and the roots are the only structures located below the hypocotyl, it follows that the chemical was absorbed by the roots

(ii) high concentrations of benomyl were detected in the cotyledons, indicating the chemical was either absorbed via the testa and/or micropyle, or translocated from the roots. Absorption from the seed surface probably accounted for most of the benomyl present in the cotyledons for the following reasons:

  (i) direct absorption of fungicides by pea seeds has been demonstrated by Maude (1966) and Maude and Kyle (1970)

  (ii) there are no xylem tissues in the cotyledons through which high concentrations of the chemical may be translocated from the roots

  (iii) it is highly unlikely that benomyl absorbed by the roots would accumulate in the cotyledons since stomata, which determine the ability of plant structures to accumulate the chemical (Peterson and Edgington, 1971) are not found in the cotyledons.

The relatively low concentration of benomyl detected in seedling leaves indicates that these structures are poorly protected following seed treatments. However, the high concentration of benomyl in the cotyledons of treated seeds indicates the fungicide can be absorbed deep into the seed and may therefore inactivate deep seated infection.

(b) Foliar spray

The distribution of benomyl in individual leaves on the third day following foliar spraying is shown in Table XIX. It can be seen that in all treatments benomyl was located randomly within leaves, rather than consistently accumulated in any specific location. As similar observations were also noted in washed leaves which measured only systemically absorbed benomyl, it would seem that although the chemical was taken up within three days of spraying, once absorbed it remained largely in that

Table XIX. Distribution of benomyl within individual leaves on the third day after foliar spraying.

Location on leaf	Average effective diameter of inhibition zone (mm)							
	Control		62.5 ug/ml		125 ug/ml		250 ug/ml	
	UW	WH	UW	WH	UW	WH	UW	WH
Base	-	-	6.1	1.2	8.2	5.6	8.9	10.6
Centre	-	-	2.1	0.1	8.8	2.9	14.6	11.0
Tip	-	-	1.5	1.1	9.6	5.6	12.4	8.3
Left edge	-	-	4.6	-	11.4	11.4	13.0	13.5
Right edge	-	-	3.0	-	9.9	5.1	8.9	7.0
Av. for whole leaf	-	-	3.5	0.5	9.7	6.1	11.6	10.1

Note: UW = unwashed leaves; WH = washed leaves.

immediate area. That is, no extensive translocation within the leaf tissues had occurred.

Comparing the quantity of benomyl within the leaves following each treatment (average effective inhibition zone/whole leaf), the results (Table XIX) indicate that higher quantities of benomyl were detected in leaves originally sprayed with higher concentrations of the chemical. However the amount of benomyl in unwashed leaves was consistently higher than that of the washed leaves, suggesting that only a proportion of the sprayed chemical had been absorbed by the time of bioassay. As the proportionate difference between washed and unwashed leaves was greater with decreasing concentrations of benomyl (Table XIX), it appears that the chemical was less efficiently taken up by the leaves when sprayed at lower concentrations.

The results presented in Table XX show that in sprayed plants benomyl was detected in the lower leaves (nodes 1 and 4), but seldom in newly expanded leaves (nodes 8 and 10). This may be explained by the fact that at the time of spraying the newly expanded leaves were enclosed by older leaves (except some leaves from node 8), thus preventing any chemical from being deposited on their surface. As no chemical was detected in these leaves, even at one week after spraying, it suggests that benomyl was not extensively translocated from the basal to the apical leaves during the first week following foliar spraying.

Some infection occurred in all plants spray inoculated with pycnidiospores of M. pinodes three days after foliar spraying with benomyl (Table XXI), but such infections were mainly localised in the leaves of nodes 7 to 10. This was because at the time of spraying

Table XX. The distribution of benomyl in pea plants on the third and seventh day after foliar spraying.

Leaves bioassayed	Average effective diameter of inhibition zone (mm)							
	Third day				Seventh day			
	C	62.5 ug/ml	125 ug/ml	250 ug/ml	C	62.5 ug/ml	125 ug/ml	250 ug/ml
Node 1	-	4.0	5.8	5.8	-	5.5	3.4	6.1
Node 4	-	1.5	9.6	12.4	+	+	+	+
Node 8	-	-	-	3.4	-	-	-	-
Node 10	+	+	+	+	-	-	-	-

+ = samples not taken.

Table XXI. Infection of pea plants following inoculation with M. pinodes on the third day after foliar spraying with benomyl.

Foliar treatment	Nodes with infected leaves	Average score of infected leaves
Control	4 to 10	5.25
62.5 ug/ml a.i.	7 to 9	3.75
125 ug/ml a.i.	7 to 10	4.50
250 ug/ml a.i.	7 to 10	3.25

(with benomyl) the above leaves were enclosed by older leaves and were therefore not coated with the fungicide. However, three days later, at the time of inoculation with M. pinodes, these unprotected leaves were sufficiently expanded to allow infection by the germinating pycnidiospores. Conversely, the lower leaves of treated plants remained uninfected because they were adequately protected by the film of benomyl. In the case of the untreated control plants, both upper and lower leaves were infected.

(c) Soil drenching.

(i) The quantity of benomyl in treated plants with time.

Benomyl was detected in the leaves of drenched plants (500 ug/ml) up to six weeks after treatment (Figure 50). Although the leaves were not bioassayed after six weeks, bioassay of the reproductive structures (Table XXII) revealed persistence in treated plants even up to the ninth week following drenching.

The quantity of benomyl in the leaves of nodes 1, 4 and 10 increased gradually to reach a peak on the third week. Thereafter the quantity of fungitoxicant continued to decrease up to the sixth week (Figure 50).

In the first week the chemical was detected only in the leaves of the lower four nodes, but in subsequent weeks was detected in the leaves of node 10. Hence within the first week the chemical was already taken up from the soil by the treated plants and translocated to at least the fourth node.

In general, throughout the six weeks benomyl was detected at higher concentrations in the leaves of node 1, with decreasing quantities detected in nodes 4 and 10 respectively (Figure 50). This appears to indicate that the chemical was concentrated in the lower leaves of the treated plants and decreased with increasing distance from the base. The distribution of benomyl within the plant is considered in more detail in the following section.

(ii) <u>The distribution of benomyl within treated plants on the third week after drenching.</u>

The results (Figure 51) indicate that except for soils drenched at 250 ug/ml a.i. the distribution of the fungitoxicant on the third week after drenching was generally bimodal, with highest concentration detected in the leaves of nodes 2 and 10, and low concentration in the leaves of node 6. In plants drenched at 250 ug/ml a.i. benomyl was not detected in leaves beyond the fourth node.

Except for node 1 the concentration of benomyl in leaves up to node 6 decreased with increasing distance from the soil. Such decrease is probably a reflection of increasing distance from the source of the chemical (the soil). In leaves of node 1 the relatively low concentration of fungitoxicant in relation to its proximity to the chemical source may be due to its small surface area and/or early senescence. That is, transpiration was reduced and less chemical was translocated to the leaf tissue.

The increasing concentration of benomyl from nodes 6 to 10 may be due to the existence of some factors or activities in these leaves (probably increased transpiration) which enabled them to accumulate

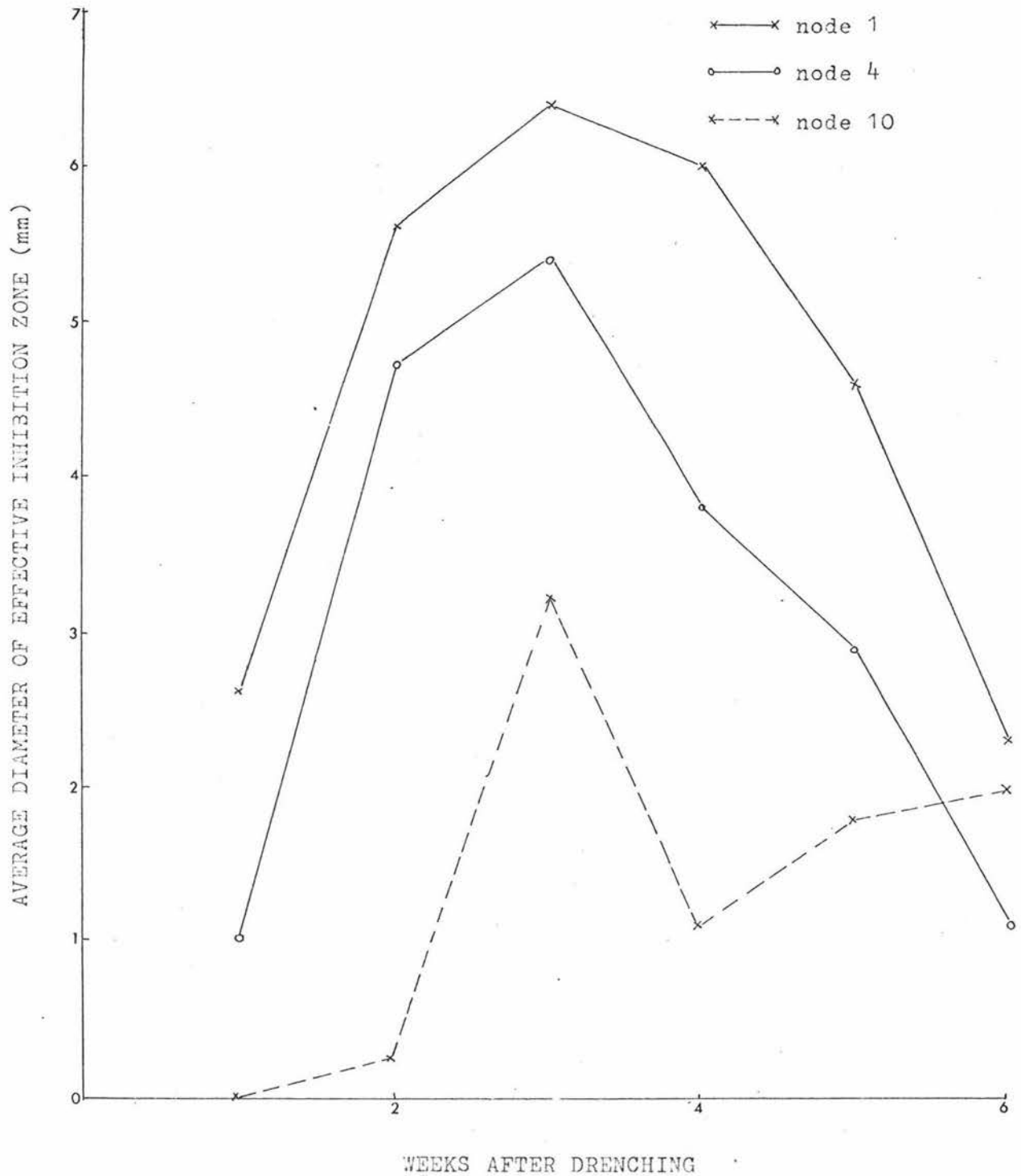
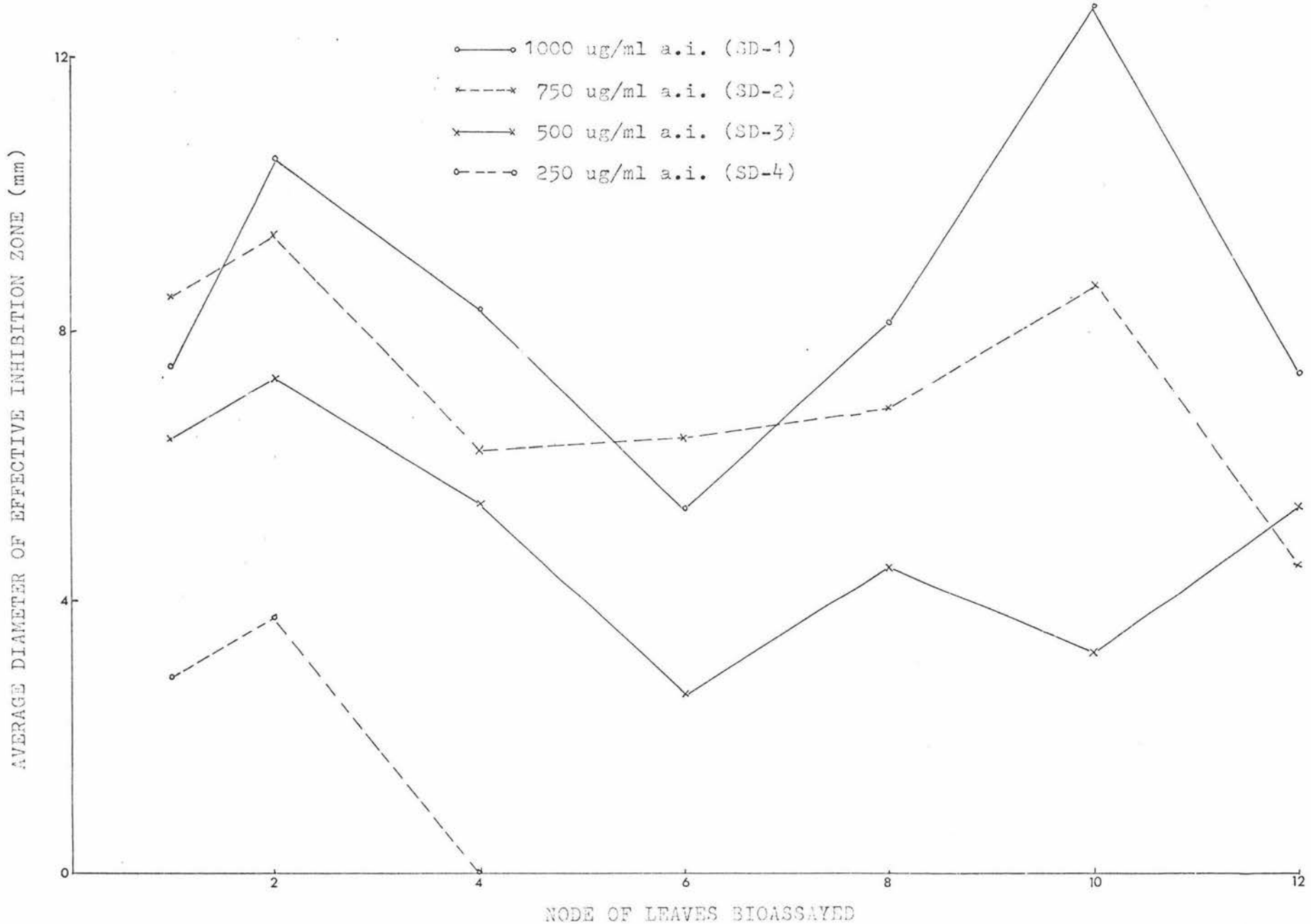


Figure 50. The effect of time on the quantity of benomyl in leaves of node 4 of treated plants (drenched at 500 ug/ml a.i.)

Figure 51. The distribution of benomyl within treated plants on the third week after drenching.



increasing amounts of the chemical. Such activities or factors were probably lacking in the young apical leaves (node 12) where lower levels of benomyl were detected (Figure 51).

In general, the amount of chemical in corresponding leaves of treated plants was higher in plants drenched with higher concentrations of benomyl. That is, larger quantities of benomyl were taken up by plants drenched with higher concentrations of the chemical.

(iii) The distribution of benomyl within individual leaves.

Benomyl translocated to the leaves (node 4) of all treated plants was mainly concentrated at the tips and the margins (Figure 52 and 54). Lowest concentration of the chemical was detected at the base of the leaves.

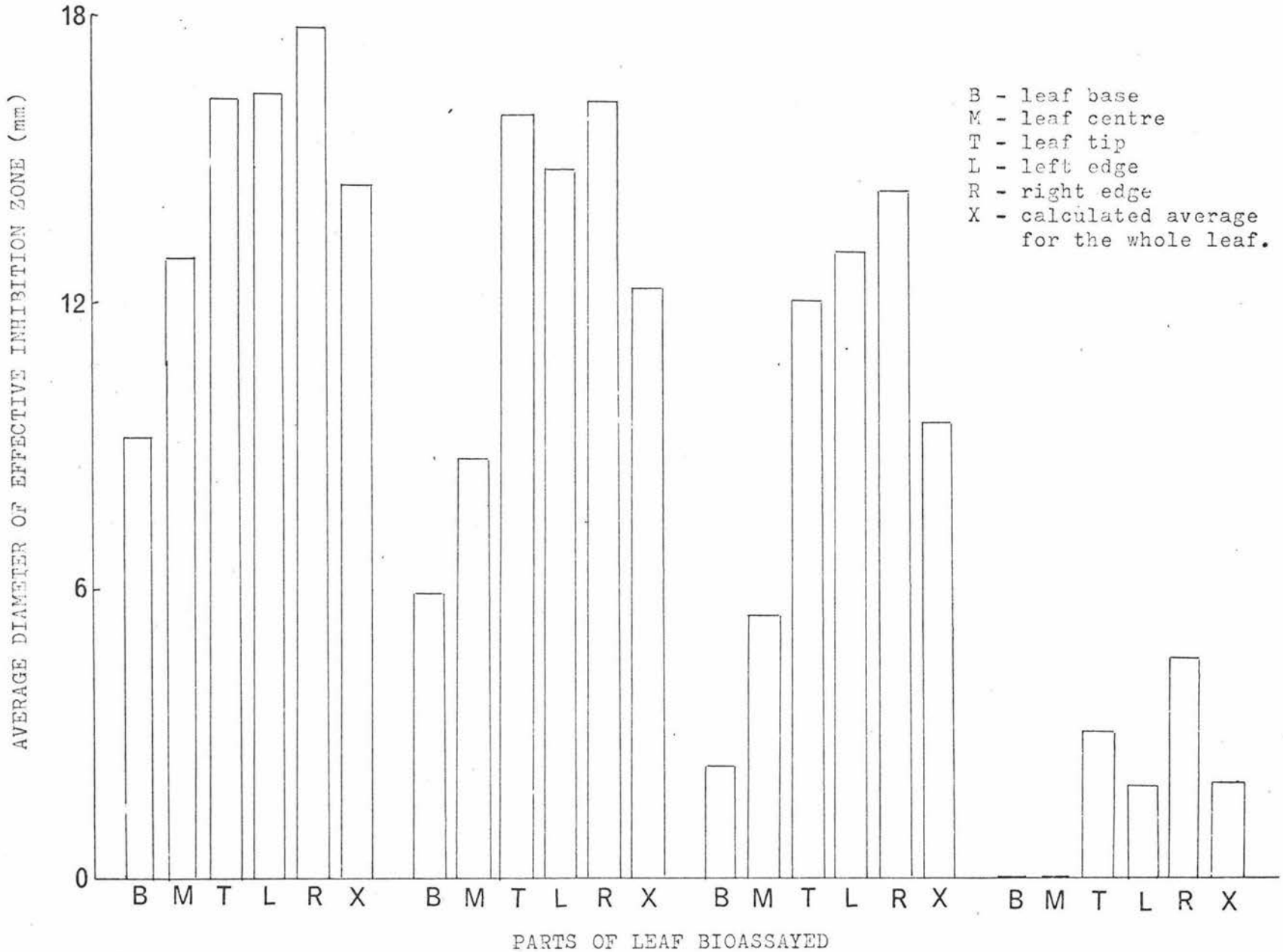
The average quantity of benomyl detected in the leaves of each treatment (x in Figure 52) increased with increasing concentration of the chemical. In plants drenched with lower concentrations (250 ug/ml), the fungitoxicant was not detected at the centre or base of the leaves.

(iv) Comparison of the quantity of benomyl in nodal and branch leaves.

Slightly lower quantities of benomyl were consistently detected in the branch leaves of all treated plants (Figure 53). As with the nodal leaves, highest concentration of benomyl was detected in the branch leaves of lower nodes.

The branch leaves of a particular node often contain higher concentrations of fungitoxicant than the nodal leaves on higher nodes.

Figure 52. The distribution of benomyl within leaves from node 4 of plants at three weeks after drenching (500 ug/ml a.i.)



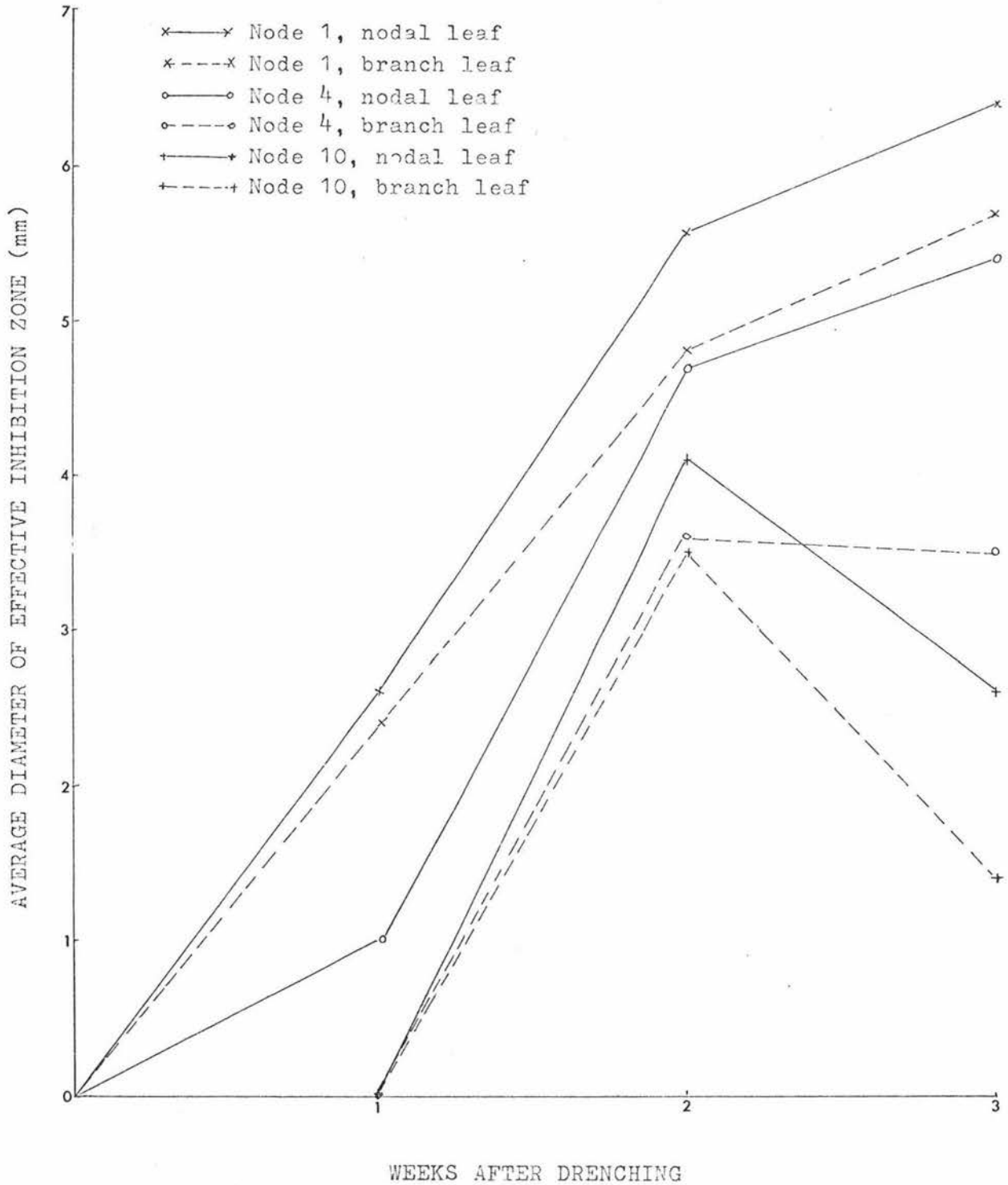
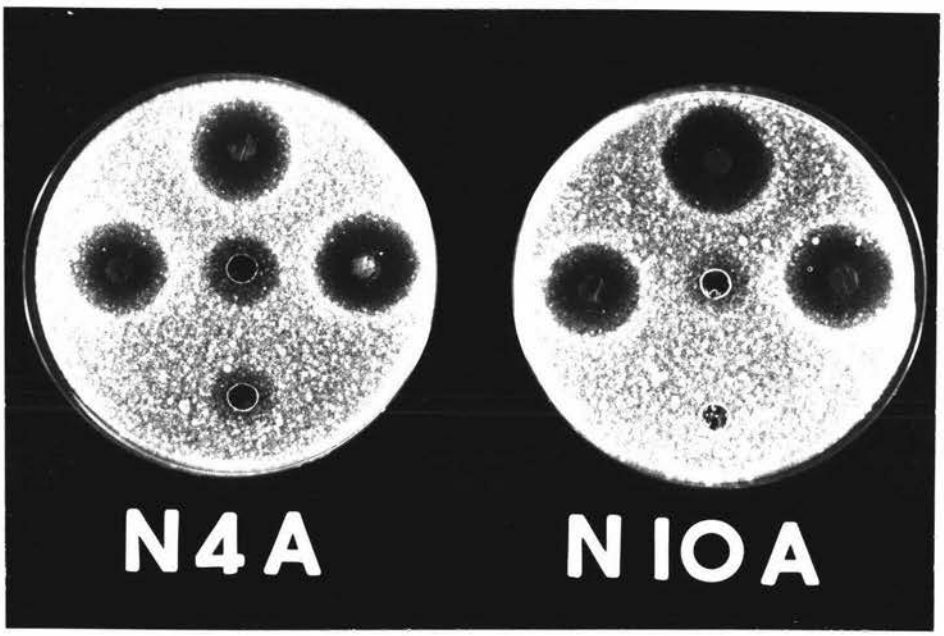
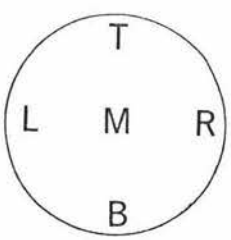


Figure 53. The quantity of benomyl in nodal and branch leaves of nodes 1, 4 and 6 at three weeks after drenching (500 ug/ml a.i.).





T - leaf tip

M - leaf centre

B - leaf base

L - left edge

R - right edge

Figure 54. Inhibition zones produced by different parts of leaves from node 4 (left) and node 10 (right) of soil drenched plants (500 ug/ml a.i.)

Hence the distribution of the chemical in leaves was affected to a greater extent by its point of origin on the stem (that is, lower or higher nodes) rather than the fact that it was a nodal or branch leaf.

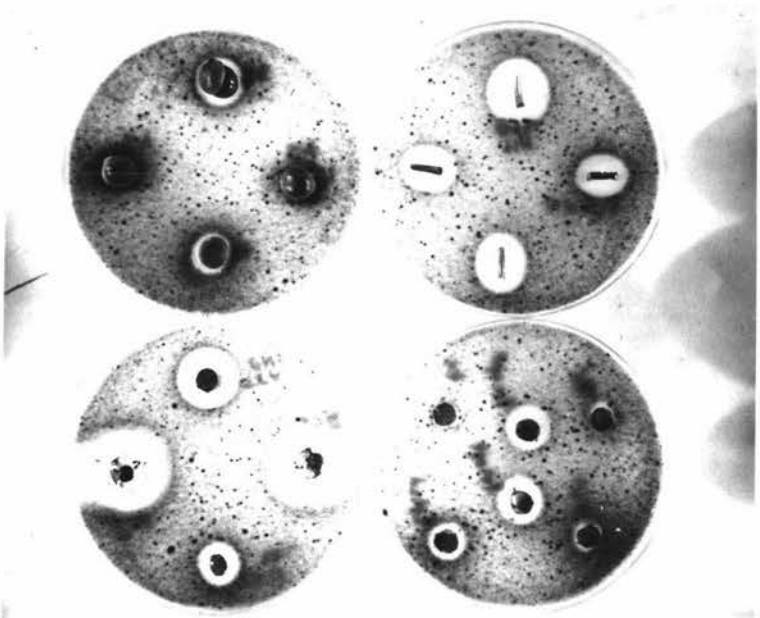
(v) The distribution of benomyl in the reproductive structures.

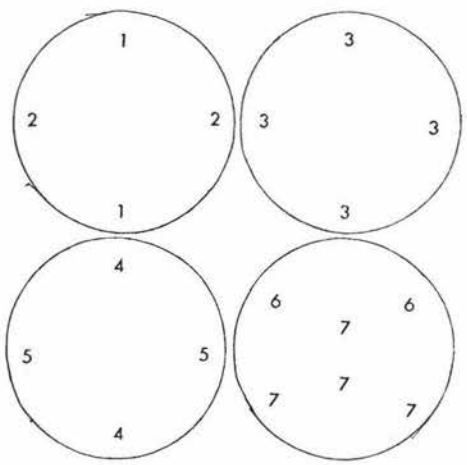
The results (Table XXII and Figure 55) indicate that within nine weeks of being applied as a soil drench (500 ug/ml a.i.), benomyl had accumulated in the reproductive structures of the plant (pedicel, sepals, pod wall and seeds). The chemical was not detected in the petals, however, either because it had not been translocated there, or because the amount present was insufficient for detection.

Table XXII. Distribution of benomyl within reproductive structures of pea plants at five, seven and nine weeks after soil drenching (500 ug/ml a.i.)

Code (refer Fig. 48)	Average effective diameter of inhibition zone (mm)		
	5th week	7th week	9th week
NLT	7.4	6.7	7.5
NLM	1.4	1.0	0.7
PN1	-	2.0	10.1
PN2	-	2.0	6.8
PS	-	10.5	11.5
PP	-	+	+
PWB	-	2.0	+
PWC	-	+	1.1
PWT	-	-	0.8
SB	-	-	4.8
SM	-	+	+
ST	-	-	0.5

Note: + indicates no samples taken.





1 Seed at pod base (PB)
2 Seeds at pod tip (ST)
3 Pedicel node 1 (PN1)
4 Nodal leaf, tip (NLT)
5 Calyx (PS)
6 Pod wall, tip (PWT)
7 Pod wall, centre (PWC)

Figure 55. Inhibition zones produced by the reproductive structures of pea plants drenched with benomyl (500 ug/ml a.i.)

The accumulation of benomyl in the reproductive structures followed a definite pattern. At first (fifth week after drenching), it was not detected in any parts of the young pods although the leaves of the same node contained appreciable amounts of the chemical. By the seventh week after drenching, benomyl was detected in the pedicel, sepals and wall of the basal parts of the pod, but not in the wall and seeds of the distal part. By the ninth week, when the pods were mature, the amount of benomyl in the basal pod area had increased, and the chemical had advanced and accumulated in all the reproductive structures (pedicel, sepal, pod wall and seeds). The basal seeds contained higher concentrations of benomyl than those in the distal parts of the pod, suggesting that accumulation of the chemical in individual seeds proceeded in the same direction as in pods.

Although benomyl was systemically absorbed by pea plants following soil drenching, complete protection was not provided by the absorbed fungitoxicant since most plants of all treatments showed some infection following inoculation with M. pinodes on the third week after drenching (Table XXIII). However significant protection was provided since infection in drenched plants was reduced from six to seventeen times when compared with undrenched plants. Except for plants drenched with benomyl at 500 ug/ml a.i., infection was lower in plants drenched with higher concentrations of benomyl.

Table XXIII. Infection of pea plants following inoculation with *M. pinodes* on the third week after soil drenching.

Origin of leaves.	Average score of infected leaves.				
	Control	250 ug/ml	500 ug/ml	750 ug/ml	1000 ug/ml
Node 1 (basal)	1.75	-	-	-	-
Node 2	1.25	0.20	-	-	-
Node 3	1.75	-	-	-	-
Node 4	1.25	-	0.17	0.17	-
Node 5	2.00	-	0.17	-	-
Node 6	1.75	-	-	-	-
Node 7	2.25	0.40	-	0.33	-
Node 8	2.50	0.25	0.33	0.67	-
Node 9	2.75	0.80	-	0.17	-
Node 10	2.00	0.20	-	0.33	0.17
Node 11	2.50	0.60	0.17	0.50	0.17
Node 12	2.25	0.80	0.60	0.17	0.33
Node 13	2.00	0.80	0.20	0.20	0.40
Node 14	2.67	0.80	0.20	0.25	0.67
Node 15	1.50	-	-	-	-
Total average score for treatment	30.17	4.85	1.84	2.79	1.74

From the above studies it is clear that the widespread translocation of benomyl in pea plants, when applied as a soil drench, provides considerable protection against foliar infections induced by the collar-rot pathogens. Further, the fact of the chemical accumulating in developing seeds means that some protection is also provided against the establishment of seed infection. Whether or not total protection is afforded is dependent on the concentration of benomyl reaching seeds, and its persistence in these tissues. Theoretically, if sufficient levels of benomyl were maintained through to seed maturity a benefit could be provided to the following seasons crop. That is, when such seed was sown the benomyl still present could contribute towards the prevention of pre- and post-emergence damping-off, as caused by non-pythiaceous fungi.

D. CONTROL OF SEED INFECTION BY TREATMENT OF SEED WITH BENOMYL.

In the field, benomyl controls a wide spectrum of fungal diseases of many fruit trees, field crops, vegetables and ornamentals (Delp and Klopping, 1968; Catling, 1969; Bollen and Fuchs, 1970). The chemical has been successfully used as a seed dressing, foliar spray, soil application, chemical soak or post harvest dip, and possesses both protective and eradicative properties (Gould and Miller, 1970; Hearn and Fenton, 1970; Johnston, 1970; Long, 1970; Maas and MacSwan, 1970; Netzer and Dishon, 1970).

With regard to the collar-rot fungi in peas, *in vitro* tests conducted in the present study (Chapter VB) demonstrated the chemical to

be highly lethal to all three fungi at low concentrations (less than 10 ug/ml a.i.). Bioassay studies also revealed that when applied as a seed dressing the chemical was translocated to all parts of the seedling, being concentrated mainly in the cotyledons and the hypocotyl/epicotyl region. Since infections in the seed and seedling are also generally localized in these tissues, it follows that treating seed with benomyl may provide a practical and highly effective means of controlling seed-borne inoculum.

Prior to this study the effectiveness of benomyl as a seed dressing against the collar-rot complex had only been demonstrated with pea seeds infected by A. pisi (Maude and Kyle, 1970). Since in New Zealand M. pinodes and P. medicaginis var. pinodella were the only species of the complex detected in pea seeds over the last two seasons (Chapter III), and since the two species were found in laboratory tests to be equally sensitive as A. pisi to benomyl there was obvious need to investigate the effectiveness of the chemical when applied as a seed dressing for the control of M. pinodes and P. medicaginis var. pinodella. Such studies were conducted both in the glasshouse and in the field.

1. Glasshouse screening.

(a) Materials and Methods.

Owing to the lack of highly infected pea seed lines, glasshouse screening was conducted with a single line of pea seed (line 5-1, Victory Freezer). The infection percentage was increased to 47% (all M. pinodes) by macroscopic selection of discoloured seeds (Chapter IV). The use of such discoloured seeds had an added advantage since, being deeply infected,

they would give a clearer indication of the effectiveness of benomyl when applied as a seed dressing.

Random samples (100 gm each) were obtained from the bulk of discoloured seeds by the modified halving method (Chapter III) and were dusted or slurry treated with benomyl and/or captan at the following rates:

Fungicide	Treatment	Code	Amount of fungicide/100 gm seeds	
			Benlate (gm)	Orthocide (gm)
Control	-		-	-
Benomyl	Slurry - 1 oz/bushel	BS-1	0.104	-
Benomyl	Slurry - 2 oz/bushel	BS-2	0.208	-
Benomyl	Slurry - 3 oz/bushel	BS-3	0.312	-
Benomyl	Slurry - 4 oz/bushel	BS-4	0.416	-
Benomyl	Slurry - 5 oz/bushel	BS-5	0.520	-
Benomyl	Dusting - 2 oz/bushel	BD-2	0.208	-
Benomyl	Dusting - 4 oz/bushel	BD-4	0.416	-
Captan	Slurry - 2 oz/bushel	CS-2	-	0.208
Captan & benomyl	Slurry-(2+2)oz/bushel	CBS-22	0.208	0.208

The fungicides were applied to the seeds as described in Appendix V.

Four replicates of 50 seeds each were randomly removed from each treatment and sown in a sterilised perlite-peat medium (Appendix II) in plastic trays (43 x 30.5 x 5 cm). Two lots of 50 seeds each were sown per tray, which was then continually subirrigated by standing in water.

The emergence was recorded on the second and fifth week after sowing. At the fifth week all plants were also carefully removed, washed and graded as either non-infected (N), moderately infected (MI) or severely infected (SI).

(b) Results and discussion.

Table XXIV. Efficiency of Benlate and/or Orthocide in controlling seed-borne infection by M. pinodes (47% seed infection) in the glasshouse.

Treatment Code	Average emergence (%)		Quality of seedlings at fifth week		
	2 weeks	5 weeks	% N	% MI	% SI
Control	60.0	45.0	7.0	13.0	25.0
BS-1	81.0	81.5	67.0	10.0	4.5
BS-2	78.0	78.5	65.0	10.0	3.5
BS-3	83.5	83.5	71.0	11.0	1.5
BS-4	82.0	83.0	71.5	10.5	1.0
BS-5	83.0	82.5	75.5	7.0	-
BD-2	83.5	82.0	73.0	7.5	3.5
BD-4	82.0	84.0	69.5	12.0	0.5
CS-2	70.0	59.5	26.5	11.0	22.0
CBS-22	63.5	62.5	60.5	2.0	-

The results (Table XXIV) indicate that Benlate (50% $^{w}/_{w}$ benomyl) was highly effective in controlling seed infection by M. pinodes. Although untreated seeds gave rise to severe infections (low emergence, severe post-emergence damping-off and high infection in surviving plants), seed treatments with Benlate (even at the rate of 1 oz/bushel) had the following distinct advantages:

(i) seedling emergence was greatly increased
(ii) post-emergence damping-off was totally overcome
(iii) the proportion of uninfected plants was greatly increased.

It should be noted that Benlate was equally effective both as a dust and as a slurry, and further, that there was no apparent advantage in

increasing the rate of Benlate from 1 to 5 oz/bushel.

Although Orthocide provided some control of seed infection by M. pinodes, it was inferior to Benlate at equivalent rates since:

(i) emergence was lower

(ii) post-emergence damping-off was not totally controlled

(iii) a high proportion of plants surviving at the fifth week were severely infected.

Use of the Benlate/Orthocide blend (each 2 oz/bushel) had the following effects:

(i) emergence at the second week was low and was maintained at the same level up to the fifth week, at which time it was approximately the same as that for the Orthocide treated seeds but lower than those treated with Benlate

(ii) as with Benlate treated seeds, post-emergence damping-off was overcome and a high proportion of the surviving seedlings on the fifth week were uninfected.

An important point to be noted is that in no instance was total eradication of seed infection achieved.

The above results clearly indicate the effectiveness of Benlate in controlling seed infection caused by M. pinodes. Although infection was very severe, Benlate inactivated the deep seated inoculum in most infected seeds. Its effectiveness in this respect is assumed to have been due to its ability to penetrate deep into the cotyledons and concentrate at the hypocotyl/epicotyl region (Chapter VC), where lesioning is most frequent and usually lethal. Conversely, Orthocide was less

effective, and this can be attributed to its lack of systemic activity. However it appears to be fairly effective against shallow infections since emergence was higher and more of the surviving plants at the fifth week were uninfected, when compared with the untreated seeds.

Blending Orthocide with Benlate lowered the efficiency of Benlate, as evidenced by the decrease of both seedling emergence and the proportion of uninfected plants. This may possibly be due to:

(i) phytotoxicity being induced by the addition of Orthocide

(ii) Orthocide interacting with Benlate and affecting its systemic properties, thereby preventing its penetration into the seed and the inactivation of deep seated inoculum.

It should be noted that the above findings were the result of glasshouse experiments and need not necessarily apply to the field situation. The following studies were undertaken to determine to what extent they have application under field conditions.

2. Field trials.

(a) Materials and Methods.

The field trial was conducted using three seed lines (Victory Freezer), the infection levels of which had been increased by the macroscopic selection method (Chapter IV). The salient features of these lines are presented in Table XXV.

Table XXV. Salient features of the seedlines used for testing the effectiveness of fungicidal seed treatments against the collar-rot fungi in the field.

Seed line	Method of selecting for infected seeds.	% infection with collar-rot fungi	Collar-rot pathogens present
5-1	seed discolouration	47.0	M. pinodes
7-7	seed discolouration	32.0	P. med. var. pinodella
7-2	seed fluorescence	33.3	M. pinodes and P. med. var. pinodella

With line 5-1, the following slurry treatments were tested using 100 gm sample of seed in each instance:

Fungicide	Treatment	Code	Amount of fungicide/100 gm seed.	
			Benlate (gm)	Orthocide (gm)
Control	-	C	-	-
Captan	2 oz/bushel	O-2	-	0.208
Benomyl	2 oz/bushel	B-2	0.208	-
Benomyl & Captan	(1+2)oz/bushel	B1-02	0.104	0.208
Benomyl & Captan	(2+2)oz/bushel	B2-02	0.208	0.208
Benomyl & Captan	(3+2)oz/bushel	B3-02	0.312	0.208
Benomyl & Captan	(4+2)oz/bushel	B4-02	0.416	0.208
Benomyl & Captan	(5+2)oz/bushel	B5-02	0.520	0.208
Benomyl & Captan	(6+2)oz/bushel	B6-02	0.624	0.208
Benomyl & Captan	(9+2)oz/bushel	B9-02	0.936	0.208
Benomyl & Captan	(12+2)oz/bushel	B12-02	1.248	0.208

With the remaining two lines (7-7 and 7-2) only four slurry treatments were tested, these being control, B1-02, B3-02 and B9-02.

Orthocide was included in most treatments to prevent damping-off by pythiaceous fungi which are not affected by Benlate. In the

absence of Orthocide the activities of the pythiaceous fungi may be accentuated owing to Benlate inactivating the majority of the immediate soil microflora, thereby decreasing natural microbial antagonism (Bollen and Fuchs, 1970; Close, 1971). Slurry treatments of seed with Orthocide and Benlate alone were included to act as controls.

Each sample (100 gm) of seed was slurry treated using the method described in Appendix V. For each treatment four replicates of 100 seeds each were sown in randomised plots in a silt loam soil which had not previously been sown with peas.

Seeds were sown at a depth of approximately two inches at distances of three inches within the row and six inches between rows. During the growing period (15th Sept. to 31st Oct.) the soil temperature (at approximately 2 inches) ranged from 10C to 31C, and the total rainfall for the period was 6.4 inches.

The percentage emergence of plants in all treatments was recorded on the second and fifth week after sowing. At the fifth week all plants were removed, washed, and graded as non-infected (N), moderately infected (MI) and severely infected (SI). Atypical lesioning (mainly water-soaked, straw colour lesions) were collectively grouped as "other lesions" (OT).

(b) Results and discussion.

The results (Table XXVI) reveal that in all three lines, seed treatments with combinations of Benlate and Orthocide significantly increased emergence and the proportion of uninfected plants (Figures 56-58). However no improvement resulted from increasing the rate of Benlate

Figure 56. Healthy seedlings produced from infected seeds (with M. pinodes); seed treated with a blend of Benlate and Orthocide at 2 oz/bushel each.

Figure 57. Collar lesions induced by seed-borne M. pinodes on pea seedlings in the field; seeds untreated.

Figure 58. Collar lesions induced by seed-borne P. medicaginis var. pinodella on pea seedlings in the field; seeds untreated.

Figure 59. Seedling lesions induced by seed-borne M. pinodes in the field; seed treated with Orthocide at 2 oz/bushel.

Table XXVI. Efficiency of Benlate and/or Orthocide in controlling seed-borne infection by M. pinodes and/or P. medicaginis var. pinodella in the field.

Line/ Treatment	Average emergence (%)		Seedlings in each grade at fifth week (%)			
	Second week	Fifth week	N	MI	SI	OT
5-1/Control	42.80	49.50	14.25	14.75	20.50	-
O-2	71.25	70.25	42.75	11.75	15.75	-
B-2	44.00	52.25	25.25	19.50	5.50	2.00
B1-02	76.50	80.50	71.50	2.75	1.00	5.25
B2-02	74.00	77.50	73.75	0.75	-	3.00
B3-02	65.50	68.50	66.50	-	-	2.00
B4-02	67.75	74.00	68.25	1.50	-	4.25
B5-02	68.25	77.25	73.75	-	-	3.50
B6-02	70.25	75.75	70.50	0.25	-	5.00
B9-02	67.00	73.75	71.00	0.25	-	2.50
B12-02	63.75	69.25	64.25	-	-	5.00
7-7/Control	49.00	50.75	26.00	9.00	13.25	2.50
B1-02	80.50	84.50	80.00	0.75	-	3.75
B3-02	76.75	81.50	76.75	0.25	-	4.50
B9-02	72.25	75.25	66.25	0.75	0.25	8.00
7-2/Control	34.75	33.25	13.75	7.50	12.00	-
B1-02	56.50	58.75	50.00	3.25	2.00	3.50
B3-02	55.00	61.50	56.25	2.00	-	3.25
B9-02	52.50	61.50	57.75	-	-	3.75

from 1 to 12 oz/bushel (Orthocide remaining at 2 oz/bushel). One would have predicted that increased rate of the Benlate/Orthocide combination would have been accompanied by a gradual increase in percentage control, culminating in total eradication, but such was not the case.

Some control was provided by Benlate and Orthocide applied alone to infected seeds, but such treatments were inferior to those where the two fungicides were combined. Emergence was lower and a higher proportion of the surviving plants were also infected (Figure 59). Of the two fungicides, Benlate was inferior to Orthocide in effectiveness.

E. DISCUSSION.

The studies reported in this chapter indicate that treating pea seed with Benlate (50% $^{w}/_{w}$) provides an effective, highly practical means of controlling seed infections caused by *M. pinodes* and *P. medicaginis* var. *pinodella*, which is expressed as increased emergence and improved plant quality. Further, the effectiveness of benomyl can be attributed to its high toxicity against the pathogens (Chapter VB), and its uptake and translocation when applied as a seed dressing (Chapter VC).

Although in the present study total eradication of *M. pinodes* and *P. medicaginis* var. *pinodella* was not obtained in any one treatment it should be noted that deeply infected seeds were used in the treatments, selected on the basis of severe lesioning. In practice, seed lines with such a high incidence of deeply infected seeds would be rarely encountered. Hence, it is highly probable that Benlate treatment of commercial seed

lines would in the majority of cases effect a very high degree of control.

As regards A. pisi, studies were not conducted on the control of this pathogen owing to the unavailability of infected seed lines. However Maude and Kyle (1970) found that seed dressings of benomyl at 1 oz/bushel achieved significant control of this species. It would thus seem that routinely dressing pea seeds with Benlate at 1 oz/bushel would achieve a very high degree of control of all three components of the collar-rot complex.

There is no suggestion that Benlate should replace captan as the fungicide for treating pea seeds since it is ineffective against pythiaceous damping-off fungi. Rather the suggestion is that Benlate at 1 oz/bushel be applied in addition to captan at 2 oz/bushel. The addition of Benlate not only controls the collar-rot pathogen when associated with seed, but achieves some control of soil-borne, non-pythiaceous pathogens (such as Fusarium and Rhizoctonia spp.,) thereby improving stand establishment.

The point is made that treating pea seed with Benlate is a highly practical recommendation since seed firms are already equipped to treat pea seeds. The only additional cost would be the fungicide which would amount to approximately $1.62 per acre, assuming a sowing rate of 3 bushels per acre, and Benlate retailing at $8.65 per pound.

APPENDIX I

COMPOSITION AND PREPARATION OF CULTURE MEDIA.

The media were prepared as described below and in all cases were sterilized by autoclaving in 250 ml flasks at 15 p.s.i. for 20 minutes.

(i) Laboratory potato-dextrose agar (PDA_L).

Agar (Davis)	15 gm
Potatoes (peeled and sliced)	200 gm
Dextrose	20 gm
Distilled water	1,000 ml

The sliced potatoes were cooked gently for approximately one hour in 500 ml of distilled water, after which they were filtered through clean cheesecloth. The agar and dextrose were melted in 500 ml of distilled water and the potato filtrate then added.

(ii) Oxoid potato-dextrose agar (PDA_o).

Potato-dextrose agar (Oxoid)	39 gm
Distilled water	1,000 ml

The potato-dextrose agar was soaked in the distilled water for approximately 15 minutes and autoclaved.

(iii) Malt agar (MA).

Agar (Davis)	15 gm
Malt extract (Oxoid)	20 gm
Distilled water	1,000 ml

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

(iv) Water agar (WA).

Agar (Davis)	12 gm
Distilled water	1,000 ml

The agar was melted in 500 ml of distilled water and the solution made up to 1,000 ml and autoclaved.

(v) Czapek-Dox (CD).

Czapek-Dox (Oxoid)	45.4 gm
Distilled water	1,000 ml

The Czapek-Dox was soaked in the distilled water for approximately 15 minutes and autoclaved.

(vi) Prune agar (PrA).

Prune agar (Difco)	24 gm
Distilled water	1,000 ml

The prune agar was gently soaked in the distilled water for approximately 15 minutes and autoclaved.

(vii) Cornmeal agar (CMA).

Agar (Davis)	15 gm
Cornmeal (crushed)	60 gm
Distilled water	1,000 ml

The cornmeal was cooked in 500 ml of distilled water for one hour and then filtered through clean cheesecloth. The filtrate was then added to the agar melted in 500 ml of distilled water.

(viii) Pea agar (PA).

Agar (Davis)	20 gm
Pea (whole seeds)	400 gm
Distilled water	1,000 ml

The peas were cooked in 500 ml of distilled water for one hour and then filtered through clean cheesecloth. The filtrate was then added to the agar melted in 500 ml of distilled water.

(ix) Oat agar (OA).

Agar (Davis)	15 gm
Oat (crushed)	100 gm
Distilled water	1,000 ml

The oat was cooked in 500 ml of distilled water for one hour and then filtered through clean cheesecloth. The filtrate was then added to the agar melted in 500 ml of distilled water.

APPENDIX II

THE PERLITE-PEAT MEDIUM.

The perlite-peat medium used in glasshouse studies had the following composition:

(i)	Peat (sterilised)	1.00 bushel
(ii)	Perlite	2.00 bushels
(iii)	Osmocote	494.70 gm
(iv)	Superphosphate	123.72 gm
(v)	Agricultural lime	123.72 gm
(vi)	Dolomite	247.50 gm
(vii)	Dried blood	92.70 gm
(viii)	Potassium sulphate	30.90 gm
(ix)	Iron chelates	1.35 gm
(x)	Borax	1.35 gm
(xi)	Urea	19.20 gm

APPENDIX III

PROOF OF PATHOGENICITY OF ISOLATES OF THE THREE COLLAR-ROT FUNGI.

Before a particular microorganism can be regarded as the causal pathogen of a disease the Rules of Proof of Pathogenicity (Koch's Postulates) must be adhered to. Briefly stated these are:

(i) constant association of the microorganism with the disease

(ii) isolation of the microorganism from the diseased tissues and a careful study of isolates in pure culture

(iii) production of the characteristic signs and symptoms of the disease by inoculation from pure culture onto healthy tissues

(iv) re-isolation of the microorganism from the inoculated diseased plants and growth on media in comparison with the original culture until such time as they are proved to be the same.

In the course of the present study isolates of the three collar-rot fungi were tested for their pathogenicity by adhering to the above rules.

A. Isolation of the causal organisms.

The causal fungi were isolated from infected leaves, pods and seeds of pea plants. The source of the various isolates used in this

study were as follows:

Source	Isolates of the collar-rot fungi.		
	M. pinodes	*P. med.* var. *pin.*	*A. pisi*
Infected seeds	55/61B, 55/61P 44A, 55/65.	37, 71/7A, 71/7B 44E, 55/63A	EG
Infected pods	94M	-	LV, PN
Infected leaves	30N, 83J	35	-

1. Isolation from infected seeds.

Isolations were carried out in conjunction with the seed health screening test described in Chapter III. Pycnidiospore blobs produced by the causal fungi on the surface of infected seeds or on the agar medium were aseptically transferred with a flamed needle to a drop of sterile water and agitated to disperse the pycnidiospores. The resultant suspension was then serially diluted by transferring a loopful to further drops of sterile water until only four to six pycnidiospores were observed in each loopful sample. Plates of malt agar were then streaked with a loopful of the diluted suspension and incubated for three days at 24C. Well separated colonies were individually transferred aseptically to PDA_o slopes.

2. Isolation from infected leaves and pods.

Diseased leaves and pods from the field were washed and rinsed with distilled water to remove associated debris, and dried between clean filter papers. Small pieces of fresh lesions were removed and placed in a high humidity chamber (Figure 14) to induce production of

pycnidiospore blobs.

Isolation of the fungi to agar was accomplished by aseptically transferring spore blobs to a drop of sterile water, using a flamed needle. This was agitated to encourage pycnidiospore dispersal and then serially diluted as before. The diluted pycnidiospore suspension was similarly streaked onto malt agar, incubated for three days at 24C and the resulting colonies transferred aseptically to PDA_o slopes.

B. Inoculation of pea plants.

All inoculation studies were carried out with four to six weeks old pea plants using pycnidiospore suspensions aseptically prepared from five days old colonies. These colonies were derived from the monosporous isolates by removing 1 mm mycelial cubes from the pure culture and incubating them on PDA_o at 24C.

The spore suspension was prepared by flooding the agar plates with sterile water and decanting the suspension through clean cheesecloth into a clean flask. The spore concentration was then adjusted to approximately 10-20 x 10^4 spores/ml, a haemocytometer being used to determine the concentration. A small drop of surfactant (Tween20) was then added to the suspension to ensure effective spore deposition on the leaves.

Pea plants used for inoculation studies were grown in sterilised perlite-peat medium (Appendix II) in the glasshouse, three to five plants

being inoculated with each isolate. Inoculations were carried out using a sterilised atomiser, each plant being sprayed with the spore suspension to the point of run-off. Inoculated plants were individually covered with a clean polythene bag which had been wetted to ensure high humidity around the plants. The plants were then left in a cool area for three days after which the polythene bags were removed and the pots spaced well apart on the glasshouse bench.

Control plants were similarly inoculated with sterile water using a clean atomiser, care being taken to eliminate the possibility of cross contamination.

The inoculation method was very successful since typical symptoms of each component disease were observed within a week. In all cases control plants remained disease free.

C. Re-isolation of the causal fungi.

Re-isolations were carried out using the same method as described above (Section A). The colonies which developed were macroscopically and microscopically identical to the original cultures. Hence Koch's Postulates were satisfied.

APPENDIX IV

STANDARDISATION OF THE DIAMETER OF THE INHIBITORY ZONE PRODUCED BY KNOWN QUANTITIES OF BENOMYL.

This standardisation test was conducted to enable estimation of the quantity of benomyl absorbed by different tissues of treated plants. This was achieved by correlating the diameter of the inhibition zone produced on seeded agar by such tissue pieces with that produced by known quantities of benomyl. The test was carried out as follows.

A. Materials and Methods.

Penicillium seeded agar was prepared as previously described (Chapter VC) by mixing 350 ml of double strength PDA_o (held at 51C) with an equal volume of a penicillium conidial suspension of 47×10^4 conidia/ml (double strength). Thirty two plates were poured using this seeded agar, at the rate of 20 ml per plate.

Benomyl solutions at 0, 1, 5, 10, 25, 50, 100, 500 ug/ml a.i. were used for standardisation. These were prepared from a stock solution (500 ug/ml a.i.) obtained by adding 0.1 gm of Benlate (50% $^w/_w$ benomyl) to 100 ml of sterile water. After thorough mixing the stock solution was serially diluted to give 10 ml of benomyl at each of the above concentrations.

Seven millimeter discs of filter paper (Whatman, No. 1) were removed with a cork borer and sterilised in an autoclave. For each concentration of benomyl eight of the discs were set out on the surface

of the seeded agar plates. The number of discs per plate varied between one and four (Figure 60). With the aid of a pipette, each series of eight discs was individually inoculated with 0.01 ml benomyl of the appropriate concentration, and the plates were then incubated at 24C for three days.

The diameter of the zone of inhibition was measured by taking the average of two measurements at right angle to each other. Only the middle clear zone was recorded, the intervening narrow annulus of partial fungal growth being neglected. To obtain the effective zone of inhibition, the diameter of the paper disc (7 mm) was deducted from the diameter of the zone measured above.

B. Results and discussion.

The results are presented in Table XXVII and illustrated in Figure 61. They indicate that the method can be effectively used to detect different quantities of benomyl or its derivatives since a measurable zone of inhibition was produced over a wide range of concentrations. The method was sensitive even at 0.1 ug of benomyl, the zone of inhibition increasing with an increase in the amount of benomyl (Table XVIII).

The sharp increase in the diameter of the inhibitory zone between 0.1 to 1.0 ug of benomyl (Figure 61) indicates that the method is especially sensitive over this range. Since tissues from benomyl treated plants commonly contained this amount of benomyl, the method was well suited for use as a bioassay. However it should be noted that

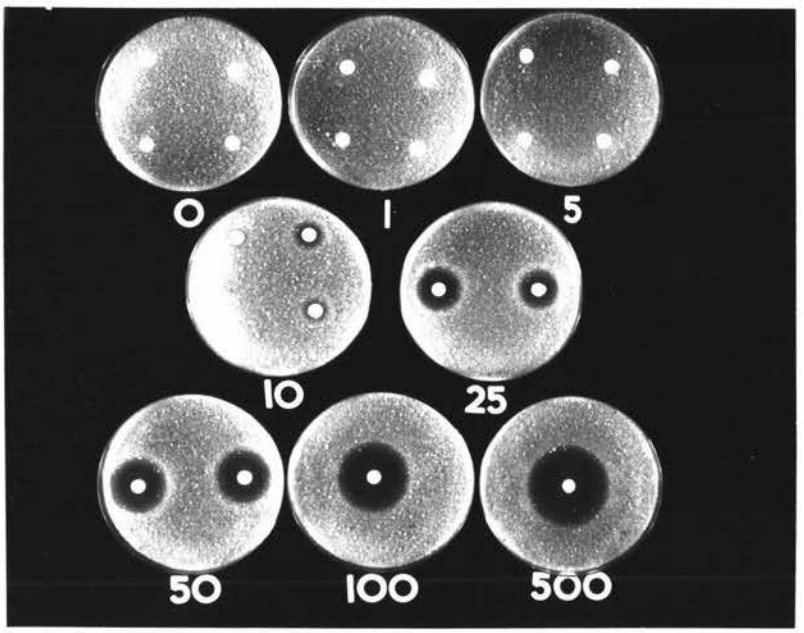


Figure 60. Inhibition zones produced by different quantities of benomyl (0.01 ml at concentrations of 0 to 500 ug/ml a.i. per 7 mm disc) on plates of PDA_0 seeded with conidia of a Penicillium sp.

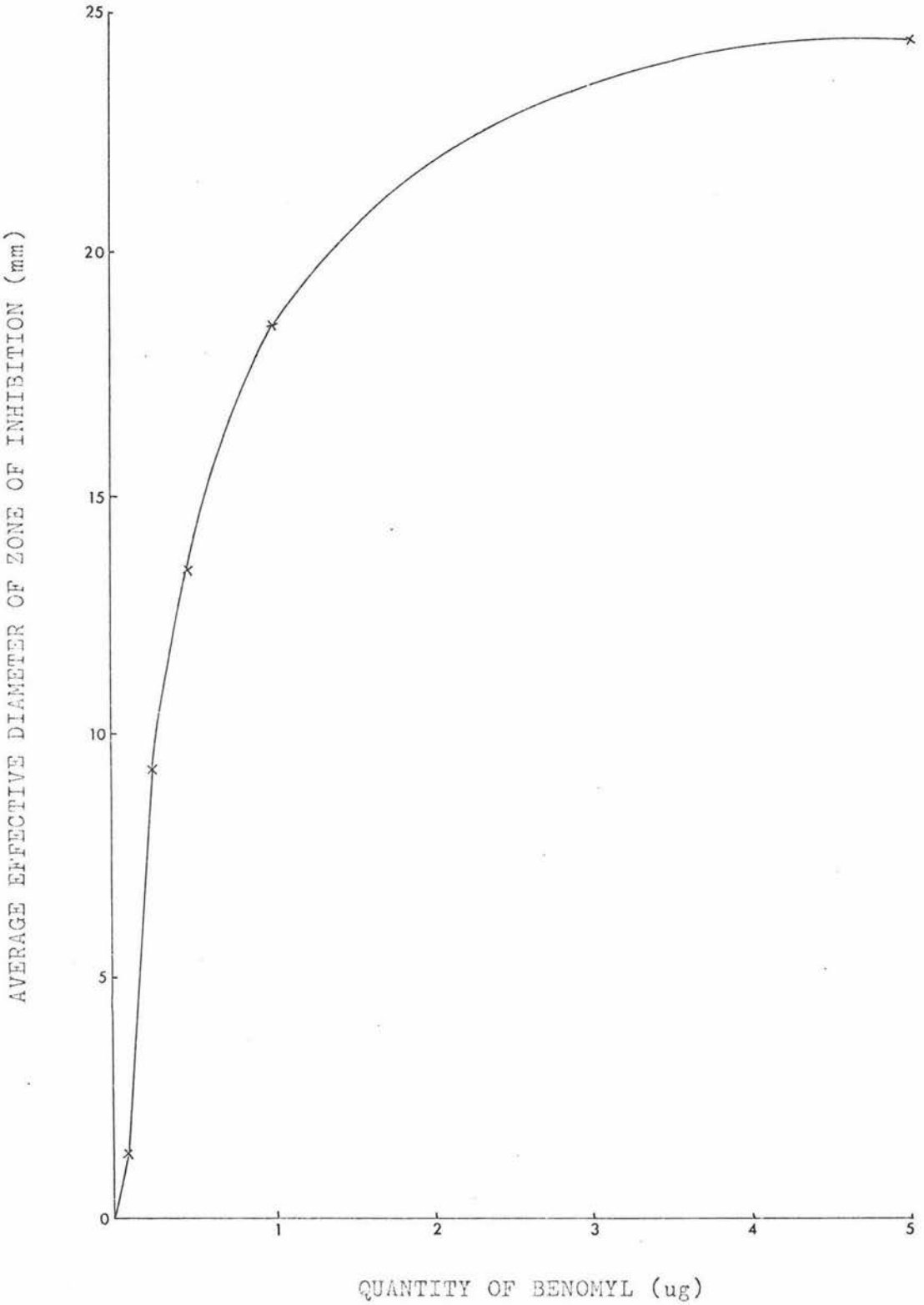


Figure 61. Correlation between the quantity of benomyl and the average effective diameter of the inhibition zone produced on Penicillium seeded PDA_o plates incubated for three days at 24C.

filter paper discs impregnated with benomyl were used in this experiment whereas in actual bioassay tests pieces of leaf tissue were used. As these may differ slightly in their ability to release benomyl to the surrounding agar small differences in the size of the inhibition zone may result even when they contain equivalent quantities of the chemical.

Table XXVII. Effective zone of inhibition produced by different quantities of benomyl on PDA_o plates seeded with a *Penicillium* sp. and incubated for three days at 24C.

Concentration of Benomyl used. (ug/ml a.i.)	Equivalent amount of benomyl (ug/7mm)	Average effective inhibition (mm).
0	-	-
1	0.01	-
5	0.05	-
10	0.10	1.25
25	0.25	9.25
50	0.50	13.31
100	1.00	18.50
500	5.00	24.31

APPENDIX V

THE QUANTITY OF BENLATE ADHERING TO PEA SEEDS FOLLOWING SEED DUSTING AND SLURRY TREATMENT.

It was necessary to ascertain the amount of benomyl actually adhering to seed following application as a dust or a slurry at different rates, for the following reasons:

(i) to determine which of the two treatments (seed dusting or slurry treatment) was more efficient in terms of the quantity of chemical adhering to seeds

(ii) to determine whether increasing rates of application would provide corresponding increase in the amount of chemical adhering to seeds

(iii) to determine whether the degree of control was correlated with the amount of fungicide adhering to seed.

This was carried out as follows:

Materials and Methods.

Random samples (100 gm) of two varieties of pea seed (Victory Freezer and Small Sieve Freezer) were dusted and slurry treated with Benlate (50% $^{w}/_{w}$ benomyl) at 1, 2, 4 and 6 oz/bushel. Three replicates of 100 gm seed samples were used in each treatment. The amount of Benlate added for each rate was as follows:

Rate of application	Weight of Benlate/100 gm seed
1 oz/bushel	0.104 gm
2 oz/bushel	0.208 gm
4 oz/bushel	0.416 gm
6 oz/bushel	0.624 gm

When dusting the seeds each 100 gm sample was placed in a weighed 250 ml flask. The appropriate amount of Benlate was added and the mouth of the flask secured with a piece of oil-paper. The flask and its contents were then shaken for 30 minutes in a rotary soil shaker.

Similar procedures were followed when slurry treating seeds, except that 0.5 ml of distilled water (equivalent to one gallon of water per 1950 lb of seed) was added to the fungicide and seed in each flask.

After shaking, the contents of the flask were poured through a clean sieve (approximately 2 mm mesh) onto a piece of clean oil-paper which had previously been weighed. The sieve was gently tapped a few times to remove loose aggregates of Benlate which was then carefully wrapped in the piece of oil-paper and placed in the 250 ml flask. The flask containing the oil-paper and remaining fungicide were weighed.

The amount of Benlate adhering to each 100 gm sample of pea seed was calculated by the following formula:

Weight of Benlate adhering to 100 gm of seed = (F + P + B) - T gm

where F = weight of empty flask
P = weight of clean oil-paper
B = weight of Benlate added per 100 gm of seed
T = final weight of flask with the oil-paper and remaining Benlate.

Results and Discussion.

The results presented in Table XXVIII and Figure 62 reveal that with one exception (dusting at 6 oz/bushel, variety Small Sieve Freezer), higher amounts of chemical adhered to seed following slurry treatments, compared with dust application. This was especially so with the variety Victory Freezer (Figure 62). With the variety Small Sieve Freezer there was little difference between the two methods of application, although the slurry method consistently provided greater adhesion.

When the rate of application (dust and slurry) was increased from 1 to 6 oz/bushel, increasing quantities of Benlate were deposited on seeds in all treatments. However the proportion of applied chemical adhering to seeds was lower at higher rates of application.

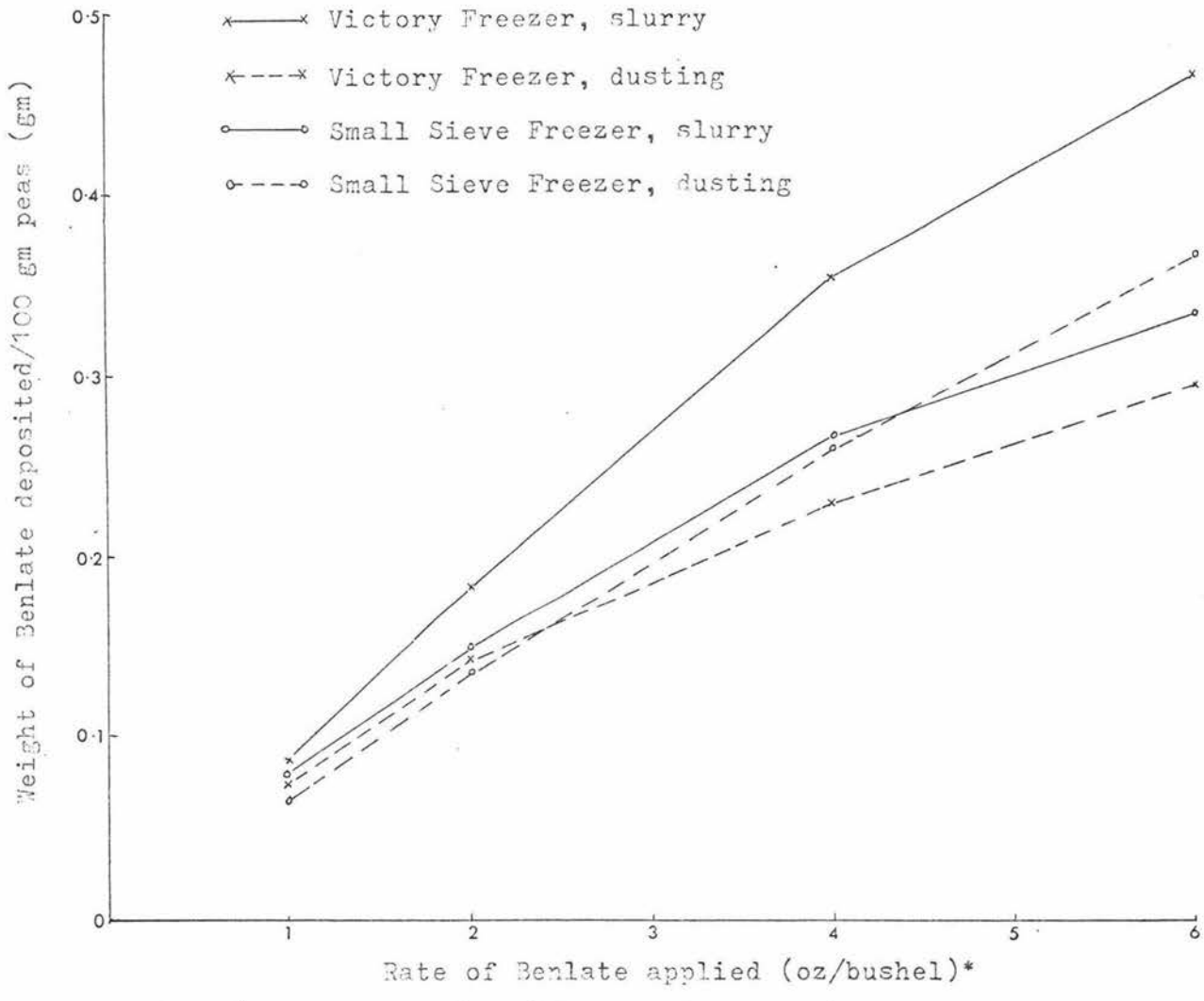
By relating the above results to the studies conducted in Chapter V, it can be concluded that 0.078 gm of Benlate adhering to 100 gm of pea seed (the lowest amount detected on Victory Freezer) is sufficient to allow absorption by the germinating seeds and accumulation in seedling tissues at detectable levels (0.1 ug benomyl a.i.). Further, this relatively low quantity of fungicide (equivalent to an application rate of 1 oz/bushel) is sufficient to eradicate deep seated infection in most seeds and to cause significant increase in stand establishment and plant quality in both the glasshouse and the field.

Table XXVIII. The amount of Benlate adhering to 100 gm of pea seed following dusting and slurry treatments at 1 to 6 oz/bushel.

Variety - application	Average amounts of Benlate adhering to 100 gm of peas							
	1 oz/bushel		2 oz/bushel		4 oz/bushel		6 oz/bushel	
	gm	% Dep.	gm	% Dep.	gm	% Dep.	gm	% Dep.
S.S. Freezer - slurry	0.080	76.93	0.150	72.11	0.266	63.94	0.333	53.35
- dust	0.068	65.39	0.138	66.34	0.264	63.46	0.369	59.13
V. Freezer - slurry	0.085	81.74	0.185	88.94	0.355	85.33	0.466	74.67
- dust	0.078	75.01	0.146	70.20	0.230	55.29	0.294	47.11

Note: % Dep. = percentage deposition $\left(\frac{\text{wt. of Benlate adhering to 100 gm of seeds}}{\text{wt. of Benlate applied to 100 gm of seeds}} \times 100\right)$

Figure 62. Weight of Benlate adhering to pea seeds dressed at rates of from 1 to 6 oz/bushel.



* 1 oz/bushel = 0.104 gm/100 gm of pea seeds.

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