A Study of Two Seed-Borne *Alternaria* Diseases on Choumoellier

A Thesis presented in partial fulfilment
of the requirements for the Degree of
Master of Agricultural Science
at Massey University

by

Franklin Henry Wood
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INTRODUCTION

The livestock industry in New Zealand is based on a pastoral farming economy, an important aspect of which is the utilisation of fodder crops as supplementary feed during seasons of poor pasture growth. Playing an important role in this regard are the two selections of choumoellier (Brassica oleracea var. acephala D.C.) usually referred to as giant choumoellier and medium stemmed choumoellier. The growing importance of choumoellier in feed crop rotations is indicated by the increased area sown, from 8,000 acres in 1933 to 130,000 acres in 1963 (New Zealand Farm Production Statistics, 1962-63). In the past, choumoellier seed has been imported from the United Kingdom, but more recently the trend has been to promote locally grown seed. At the present time New Zealand's requirements are met in most years by South Island growers producing seed under the authority of a Government seed certification scheme.

In discussing brassica crops in New Zealand, Palmer (1966) stated that - "apart from weather variations, the main uncontrollable causes of yield variations are fungus diseases, insect pests and associated virus diseases". Seven fungus diseases are recorded in New Zealand on choumoellier (Table 1), five of which are evidenced by foliage lesioning in field stands.

TABLE 1

Pathogenic fungi recorded on choumoellier in New Zealand

Pathogen	Authority
* Alternaria brassicicola (Schw.)Wilt.	Brien and Dingley, 1957
* Alternaria brassicae (Berk.)Sacc.	Morton, 1964
* Peronospora parasitica (Pers.)Tul.	Brien and Dingley, 1959
* Phoma lingum (Tode ex Fr.)Desm.	Neill and Brien, 1933
* Mycosphaerella brassicicola (Fr.)Lind.	Brien, 1939
Sclerotinia sclerotiorum (Lib.)Mass.	Cunningham, 1927
Plasmodiophora brassicae Woron.	Cunningham, 1922

Preliminary surveys in the Manawatu established that leaf infections caused by A.brassicae and A.brassicicola are prevalent and on the basis of the extent of the disease development these two fungi are considered to be of some significance in affecting yields of local choumoellier crops. Both pathogens are world-wide in distribution and have been studied on a number of brassica crops (Weimer, 1924, 1926; Neergaard, 1945; Rangel, 1945; Domsch, 1957; McDonald, 1959; Changsri and Weber, 1963). In general, published work reveals both species to be seed-borne and frequently of economic significance in :

* Species which cause foliage symptoms

(a) causing seed and seedling death and thereby considerably affecting stand establishment;

(b) directly reducing yields in seed crops by infecting siliquas and seed.

To the author's knowledge there has been no formal study conducted on the seed-borne nature of the pathogens in choumoellier, but seed health surveys reported in the 1963 and 1964 annual reports of the Government Seed Testing Station (Palmerston North), show that the Alternaria species were present in a high percentage of the seed lines screened, thus indicating the prevalence of the diseases in choumoellier crops in this country.

In view of the lack of detailed research relating to the seed-borne nature of both pathogens in choumoellier seed, and the fact of both fungi being recorded in a high percentage of New Zealand seed lines, studies were undertaken, the main objectives of which were :

(i) to determine the extent to which both pathogens are associated with New Zealand certified seed lines;

(ii) to investigate the significance of A.brassicae and A.brassicicola in causing seed bed losses;

(iii) to investigate the role of both pathogens in causing reductions in yields of choumoellier seed crops;

(iv) to critically examine methods of health screening for A.brassicae and A.brassicicola in brassica seed.

CHAPTER I

IDENTIFICATION OF THE *ALTERNARIA* PATHOGENS ON CHOUMOELLIER

A. INTRODUCTION

Two species of Alternaria distinguished primarily on the basis of spore size and beak characteristics have long been recognised as pathogens on crucifers. There has been considerable confusion as to the correct nomenclature of the two species, but the matter has been resolved by Wiltshire (1947) who established the correct binominals as A.brassicicola for the small spored fungus, and A.brassicae for the long beaked large spored species. Wiltshire (1947) also revised the descriptions of the two species, and both have been the subject of detailed studies by Groves and Skolko (1944), and Neergaard (1945).

In the present work initial studies were concerned with establishing the identity of Alternaria isolates from choumoellier seed and foliage as A.brassicicola and A.brassicae.

B. MATERIALS AND METHODS

1. Measurement of conidiophores

Discs approximately 3 mm in diameter were punched from foliage lesions and placed on glass slides in Petri dishes containing moistened filter paper to provide a high humidity chamber. After 48 hours incubation at 24°C the tissue pieces were transferred to mounting fluid on a slide and a cover slip gently lowered over the disc. Conidiophores arising from the perimeter of the lesioned tissue were measured using an eyepiece micrometer.

2. Measurement of conidia

Conidia produced on host material and malt agar were measured. Spores were harvested from host material after incubation of leaf lesions under high humidity conditions for 48 hours at 24°C. Conidia produced on malt agar were taken from a position approximately 1 cm from the perimeter of a 10 day colony grown at 24°C. A dilute spore suspension was prepared in each case to ensure ready measurement of all spores in any one field. By systematically altering the position of the mechanical stage measurement of spores more than once was avoided.

3. Cultural studies

Throughout the study both pathogens were grown on malt agar (MA) and potato dextrose agar (PDA). Details of the preparation of media are presented in Appendix I. Observations on cultural characters are based on the study of many isolates of each species.

4. Temperature and colony growth

The effect of temperature on colony growth was studied using monosporous isolates on Petri dishes of MA. A dilute spore suspension was prepared and streaked over the surface of agar slides and incubated at 24°C. Ten hours later the slides were microscopically examined and single germinating spores were transferred to the centre of Petri plates containing approximately 20 ml of MA. The isolates were then incubated for 14 days at a series of temperatures ranging from 5 to 37°C.

Brancato and Golding (1953) established that colony diameter

is a valid measure of the effects of environmental factors and this criterion has been used throughout the study. Colony growth was measured for six isolates per treatment by taking the average of two diametrical measurements made at right angles to each other.

5. Spore germination and penetration

Spore germination was studied on agar slide preparations.

Microscope slides were wiped with alcohol and flamed. A film of water agar was then run over the surface of the slides using a 10 ml pipette. Water agar proved the most favourable medium in minimising contaminant problems while providing a moist substrate for germination. The prepared slides were placed in Petri dish high humidity chambers containing a filter pad moistened with sterile distilled water. The plates were incubated for one hour prior to inoculation to ensure equilibration with the incubator temperature. Slides were removed and examined at 10 minute intervals.

Leaf penetration was investigated using excised leaves from an eight week old choumoellier plant.

The lid of a 9 cm Petri dish was placed in a 14 cm dish to form a raised platform and approximately 35 ml of water flooded around the base. A wad of moistened cotton wool was wrapped around the petiole of the excised leaf and placed in the "moat" to form a wick which assisted in the maintainance of leaf turgidity. The leaf blade was positioned on the platform so as to prevent contact with the water and the upper surface inoculated by streaking with a spore suspension of the respective pathogens. After an incubation period of 72 hours

at 24°C the leaves were removed and cleared in a 1:1 solution of glacial acetic acid and 95% alcohol (Latch and Hansen 1962). Following a rinse in a beaker of water the leaves were stained with lactophenol cotton blue for four minutes. Excess stain was removed using a wash bottle and areas of the prepared leaf mounted on water agar slides for microscopic examination. The presence of the agar was found to prevent rapid drying out of the tissue piece.

C. RESULTS

1. A.brassicae

(a) Conidiophores

The conidiophores were light olive in colour, generally unbranched, upright, septate, with 10 - 25 /u between septa. They were 2.5 - 10.0 /u in diameter, extremely variable in length (10 - 200 /u), cylindric but often tapering to a slightly swollen base and with well rounded apices and a prominent apical pore.

(b) Conidia

Conidia were obclavate, long beaked, usually straight but sometimes curved, constricted at the septa, generally smooth but in some cases slightly echinulate and of a light olive to gold-brown colour.

Conidia from host material were 60 - 220 /u long (Figure 1) and 7.5 - 30.0 /u wide (Figure 2). The majority of spores were transversely septate with 4 - 14 septa, but longitudinal and oblique septa (1 - 6) were also present in many conidia. Beak length varied

between 12.5 - 82.5 /u (Figure 3), with up to six transverse septa (Table 2).

Conidia produced on MA varied in length from 57.5 /u to 237.5 /u (Figure 1), and in width from 10 /u to 35 /u (Figure 2). Beak length ranged from 12.5 to 107.5 /u (Figure 3), with 0 - 7 transverse septa (Table 2). Spores exhibited longitudinal and oblique septa (Table 2).

(c) Cultural characters

Young colonies appear as a fluffy white growth with loose cottony aerial mycelium 1 - 2 mm from the surface of both malt agar and PDA. With age, colonies develop a tea-green to deep olive colour as sporulation occurs but often retaining the fluffy white centre, and usually with a perimeter of hyaline to greyish-olive submerged mycelium which gives a whitish halo effect (Plate 1).

Growth was generally faster and sporulation more prolific on PDA. Plates exposed to 12 hour alternating dark and light cycles exhibited conidial formation in a series of concentric rings which gave an overall "target board" appearance to the colony. On MA aerial mycelium was more abundant, and in many cases resulted in a greyish-olive to white colony.

Cultural degradation occured in stock cultures as evidenced by sub-isolates bearing little resemblance to the parent colonies, with a marked reduction in the capacity to sporulate. For this reason it was necessary to obtain fresh isolates from host material.

(d) Temperature and colony growth

Growth occurred between 5°C and 25°C with the maximum occurring at 22°C (Figure 7). However, at 28°C only three plates were recordable due to contamination and the steep decline of growth indicated at this point must be regarded with reservation.

(e) Spore germination and penetration

Spore germination was rapid on water agar slides. Germ tubes were first apparent after an incubation period of ½ hour and were well established after three hours (Figure 4). They were 1.0 - 5.0 /u in diameter, septate, and often branching. A longitudinal or oblique septum was frequently observed in the cell from which the germ tube emerged. Many cells of the one conidum produced germ tubes including the terminal body cell and beak cells.

Leaf penetration occurred only through stomata (Plate 3); in no instance was direct leaf penetration observed.

2. A.brassicicola

(a) Conidiophores

Conidiophores were olivaceous, 2.5 - 5.0 /u in diameter, cylindric but often tapering to a slightly swollen base, variable in length (5 - 300 /u) with a prominent apical pore at the rounded terminal end (Plate 4), septate, 3 - 50 /u between septa, often branched, fasciculate but rarely geniculate. Frequently a septum was delimited immediately above the point of bifurcation and another separated the new conidiophore soon after branching (Plate 4).

(b) Conidia

Conidia were olivaceous to dark brown in colour, formed in chains of 1 - 25 which were often branched, smooth, not beaked but with a short cone-like apical cell which was generally lighter than the rest of the spore (Plate 5). The spore body was obclavate to oblong with marked constrictions at the septa.

Spores harvested from host material were 15 - 70 /u long (Figure 5), 5 - 15 /u wide (Figure 6) with 1 - 9 transverse septa (Table 3). The majority of spores were without longitudinal septa but occasionally some were observed with 1 or 2 such septa.

The dimensions of spores on MA were 12.5 - 50.0 by 7.5 - 15.0 /u (Figure 5), with 1 - 7 transverse septa. Longitudinal septa were rarely observed. Conidia produced in culture showed greater uniformity in shape and size (Figure 5), were generally cylindric to oblong rather than obclavate, and with readily visible pores in the cross walls.

(c) Cultural characters

Young colonies on MA and PDA were deep olive in colour with a perimeter of hyaline to greyish-white submerged radiate mycelium (Plate 1). Conidial production was prolific, often forming concentric rings on the agar. Growth was slower on malt agar and aerial mycelium, although sparse and cottony, was in greater evidence on this medium. Colonies darkened with age to an olivaceous black colour, with the profuse conidial production giving an overall velvety appearance.

(d) Temperature and colony growth

Growth occurred between 5°C and 32°C with a maximum at 22°C (Plate 6).

(e) Spore germination and penetration

Spores germinated readily on water agar at 24°C (Plate 7), but the initial appearance of germ tubes was 2 - 3 hours slower than for A.brassicae. Germ tubes were 1.0 - 2.5 /u in diameter, usually unbranched, septate, with anastomosis of adjacent germ tubes frequently occurring. Such anastomosis was rarely observed for A.brassicae.

Penetration was both direct and by way of stomata, the former being more frequent (Plate 7). Associated with direct penetration was the formation of distinct appressoria and penetration pegs.

D. DISCUSSION

The range of spore measurements recorded in this study fall within the limits reported by previous workers for the two pathogens (Table 6), and agree with the detailed measurements tabulated by Neergaard (1945) (Tables 4 and 5). Further, cultural and morphological characters correspond to the descriptions and figures presented by Neergaard (loc.cit.), Groves and Skolko (1944) and Wiltshire (1947). Temperature growth relationships on culture media compare favourably with those recorded by Neergaard (loc.cit.) and reproduced in Figure 8. The observations on leaf penetration made in this study confirm the findings of Changsri and Weber (1963);

they also found stomatal penetration to be characteristic of A.brassicae while A.brassicicola exhibited both stomatal and direct leaf penetration.

In view of these results it is concluded that the two species of Alternaria under study were in fact A.brassicae and A.brassicicola.

TABLE 2

Spore dimensions of *A.brassicae*

Substrate	Total spore length (microns)		Spore body length (microns)		Spore beak length (microns)		Spore width (microns)		No. of transverse septa	Spores with longitudinal septa		Spores with septa in beak	
	Mean ± S.E.	Std. Dev.	Mean ± S.E.	Std. Dev.	Mean ± S.E.	Std. Dev.	Mean ± S.E.	Std. Dev.	Mean	No. of spores	Mean no. per spores	No. of spores	Mean no. per spore
MA	153.0 ± 2.96	29.66	102.07 ± 2.02	20.22	50.7 ± 1.95	19.5	17.82 ± 1.29	12.9	9.85	83	2.5	93	2.4
Choumoellier Leaf	131.02 ± 3.06	30.66	87.27 ± 1.92	19.22	43.4 ± 1.58	15.81	17.57 ± 1.64	16.4	9.06	83	2.22	78	1.756

TABLE 3

Spore dimensions of *A.brassicicola*

Substrate	Spore length (microns)		Spore width (microns)		Mean no. of transverse septa
	Mean	Std. Dev.	Mean	Std. Dev.	Mean
PA	26.57 ± 0.26	2.66	9.62 ± 0.15	1.56	3.35
Chaumoellier leaf	32.79 ± 0.35	3.56	10.18 ± 0.17	1.73	4.10

TABLE 4

Previously recorded spore dimensions of A.brassicae

Authority	Substrate	Total spore length (microns)	Spore body length (microns)	Spore beak length (microns)	Spore width (microns)	No. of transverse septa	No. of longitudinal septa
Ellis and Martin (1882)	Horse-radish	200 - 225	-	-	21 - 26	Many	Few
Saccardo (1886)	Cabbage	120 - 140	-	-	20 - 25	6 - 8	-
Eliasson (1897)	Cabbage	115 - 240	-	-	20 - 25	6 - 11	-
Massee (1901)	Br. comp. v. sarsonii	160 - 200	-	-	25 - 35	-	-
Bolle (1924)	Cherry agar	90 - 350	-	-	14 - 42	7 - 19	0 - 8
Bolle (1924)	-	92 - 204	-	-	13 - 30	-	-
Weiner (1926)	-	125 - 225	-	-	16 - 28	-	-
Goidanich (1937)	-	130 - 229	-	-	15 - 24	-	-
Groves and Skolko (1944)	-	100 - 238	-	-	16 - 35	5 - 12	Relatively few
Neergaard (1945)	-	39 - 350	33 - 147	9 - 148	9 - 33	3 - 18	0 - 15
Changsri and Weber (1963)	-	148 - 184	96 - 114	46 - 65	17 - 65	10 - 11	0 - 6

TABLE 5

Previously recorded spore dimensions of *A.brassicicola*

Authority	Substrate	Spore length (microns)	Spore width (microns)	No. of transverse septa	No. of longitudinal septa
Elliot (1917)	Cabbage leaves	13.0 - 59.0	5.0 - 9.0	-	-
Elliot (1917)	10 different agar media	8.0 - 51.0	5.0 - 17.0	-	-
Milbraith (1922)	Cabbage leaves	30.0 - 61.0	8.7 - 12.3	-	0 - few
Milbraith (1922)	Standard lima bean agar	13.0 - 70.0	6.5 - 14.0	1 - 9	0 - few
Weiner (1924)	Cabbage and agar media	11.0 - 75.0	6.5 - 16.5	1 - 10	0 - few
Bolle (1924)	Cabbage and agar media	29.0 - 108.0	8.0 - 25.0	3 - 11	0 - 5
Groves and Skolko (1944)	-	30.0 - 50.0	10.0 - 15.0	3 - 7	Relatively few
Neergaard (1945)	-	7.5 - 67.5	3.0 - 19.0	1 - 9	-
Wiltshire (1947)	-	18.0 - 130.0	8.0 - 30.0	1 - 11	-
Changsri and Weber (1963)	-	44.0 - 55.0	11.0 - 16.0	5 - 8	0 - 4

TABLE 6

Spore dimensions of *A.brassicicola* in microns
(after Neergaard, 1945)

Isolate	Medium	Length of spore		Width of spore		Range of variation Length of spore body x width of spore
		Mean value M	Std. Dev.	Mean value M	Std. Dev.	
Iberis	MA	22.8 ± 0.67	6.73	9.8 ± 0.16	1.62	12.0 - 45.0 x 6.0 - 15.0
	SA *	19.8 ± 0.64	6.41	8.1 ± 0.21	2.07	7.5 - 34.5 x 6.0 - 10.5
	Iberis (seedling)	27.5 ± 0.29	2.88	10.0 ± 0.25	2.57	10.5 - 63.0 x 6.0 - 18.0
	Cabbage (seedling)	28.0 ± 1.06	10.64	10.2 ± 0.25	2.46	12.0 - 67.5 x 6.0 - 18.8
Cabbage	MA	20.9 ± 0.58	5.75	9.5 ± 0.14	1.36	9.0 - 39.0 x 7.5 - 13.5
	SA	20.7 ± 0.20	2.02	8.3 ± 0.14	1.40	4.5 - 45.0 x 3.0 - 12.0
	Iberis (seedling)	24.7 ± 0.72	7.15	9.7 ± 0.17	1.68	10.5 - 45.0 x 6.8 - 13.5
	Cabbage (seedling)	26.6 ± 0.77	7.61	10.1 ± 0.18	1.80	15.8 - 54.8 x 7.5 - 18.0

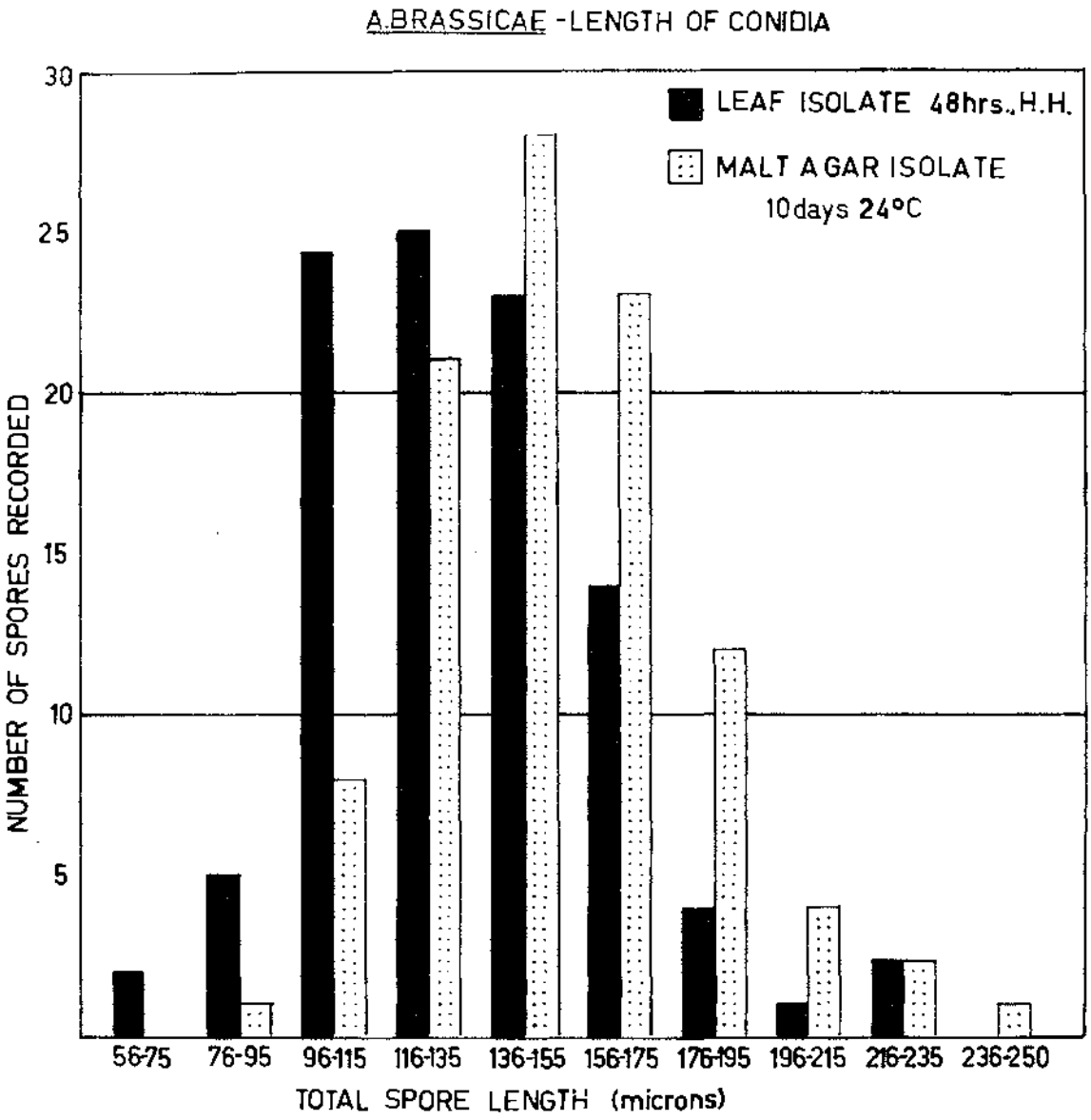
* Standard nutricat agar

TABLE 7

Spore dimensions of *A. brassicae* in microns
(after Neergaard, 1945)

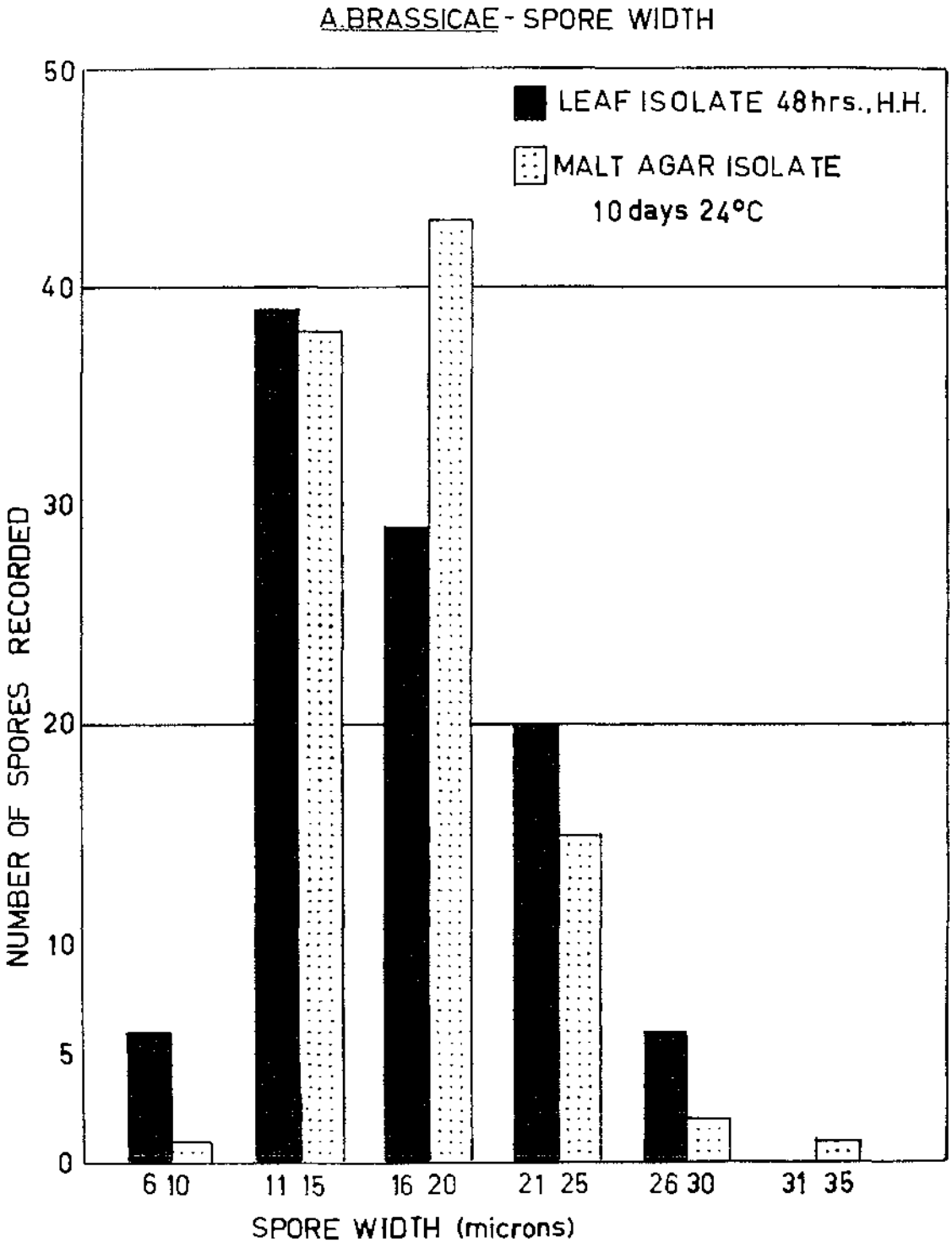
Isolate	Medium	Length of spore body		Length of beak		No. of spores with beak	Width of spore		Total length of spore. Range of variation
		Mean value M	Std. Dev.	Mean value M	Std. Dev.		Mean value M	Std. Dev.	
Radish	MA	98.5 ± 2.12	21.21	63.1 ± 3.25	32.50	100	16.4 ± 0.26	2.58	82.5 - 276.0
	SA	80.5 ± 1.53	15.34	31.1 ± 2.18	21.71	88	15.9 ± 0.33	3.26	52.5 - 219.0
	Cabbage	83.4 ± 1.87	18.68	32.7 ± 1.04	10.32	99	20.3 ± 0.41	4.11	60.0 - 172.5
	Radish	68.0 ± 1.43	14.31	28.9 ± 1.10	11.00	100	18.1 ± 0.45	4.49	39.0 - 156.0
Horse-radish	MA	76.0 ± 2.22	22.24	43.7 ± 2.10	21.04	100	12.8 ± 0.20	1.97	52.5 - 210.0
	SA	66.2 ± 1.54	15.35	34.6 ± 2.01	20.09	100	12.5 ± 0.14	1.42	30.0 - 199.5
	Cabbage	61.5 ± 1.32	13.18	41.7 ± 1.36	13.55	100	14.0 ± 0.27	2.70	57.0 - 186.0
	Radish	59.7 ± 1.13	11.26	28.5 ± 1.61	16.10	100	14.9 ± 0.27	2.67	52.5 - 186.5

Figure 1



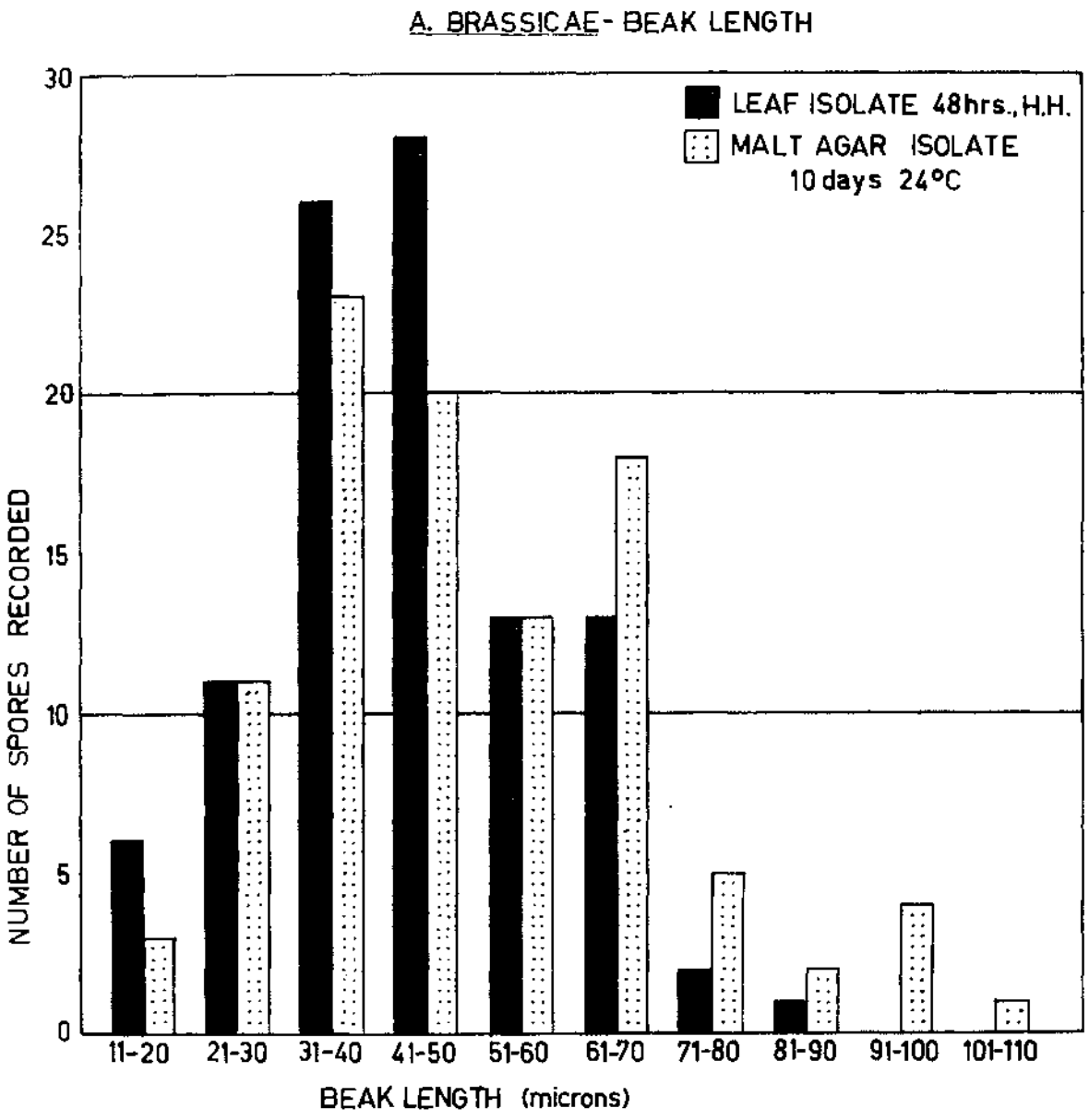
[illegible] - Distribution of spore length

Figure 2



A.brassicae - distribution of spore width

Figure 7



...brassicae - distribution of beak length

Figure 4

SPORE GERMINATION OF ALTERNARIA BRASSICAE

(on water agar slides)

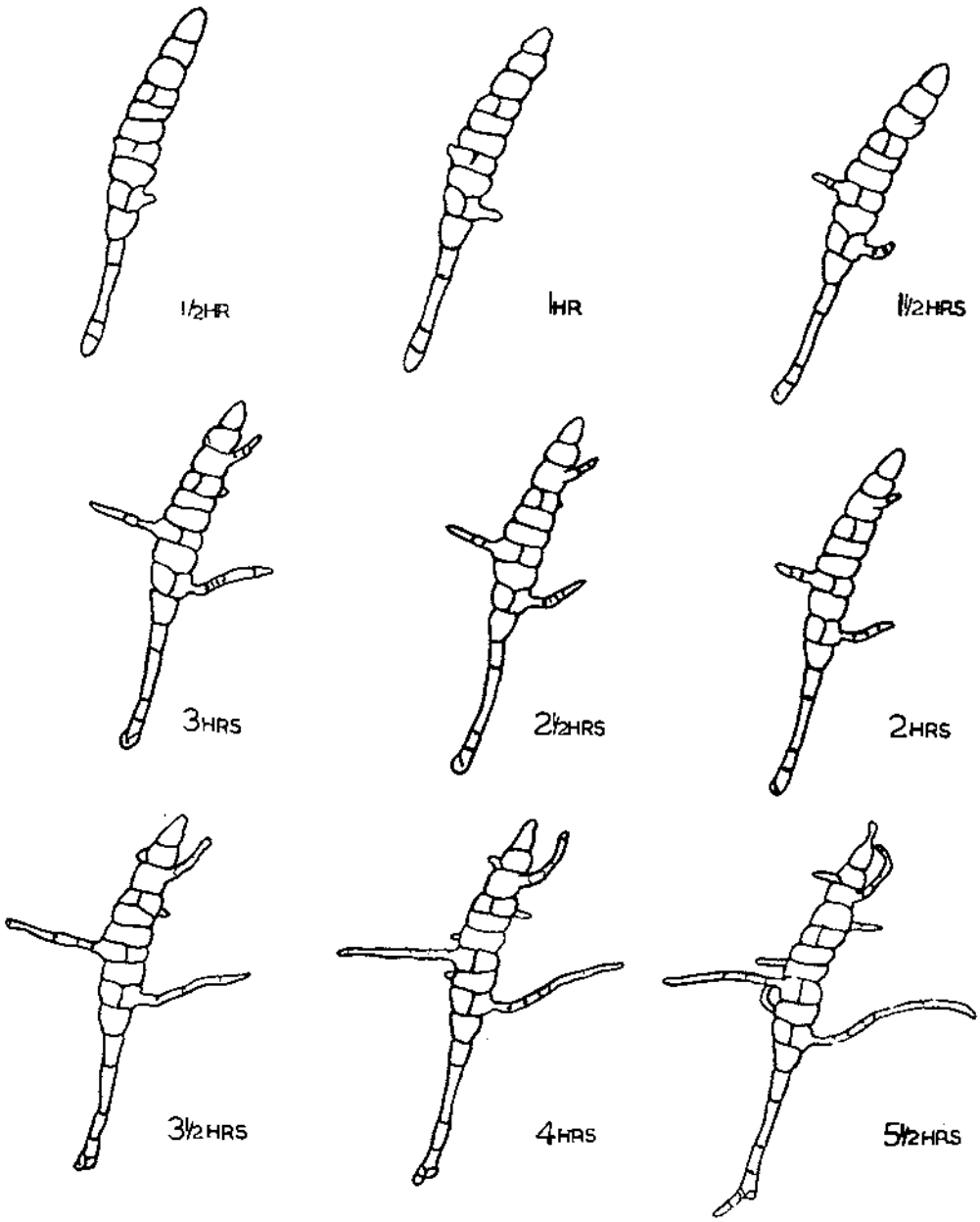


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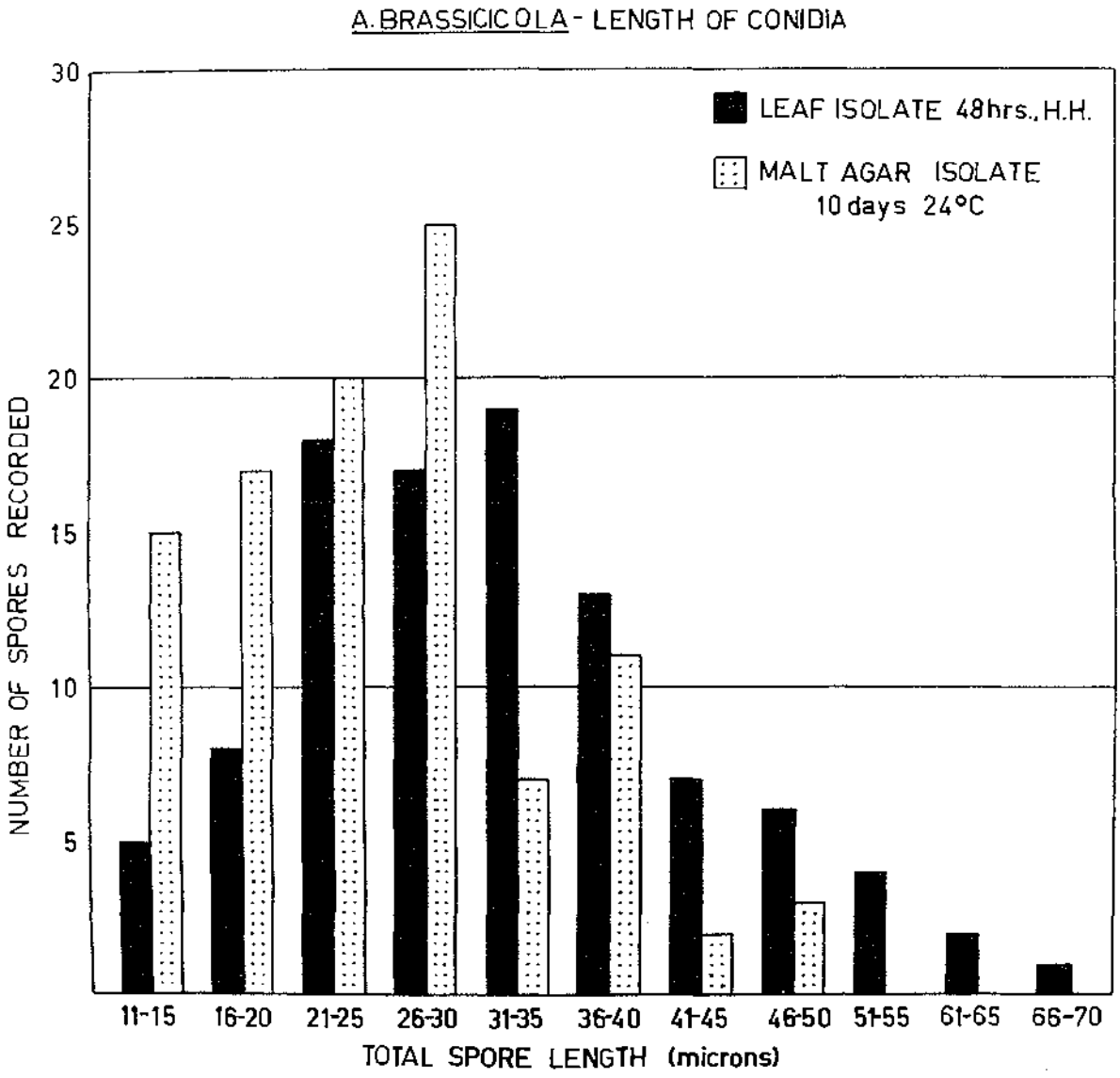
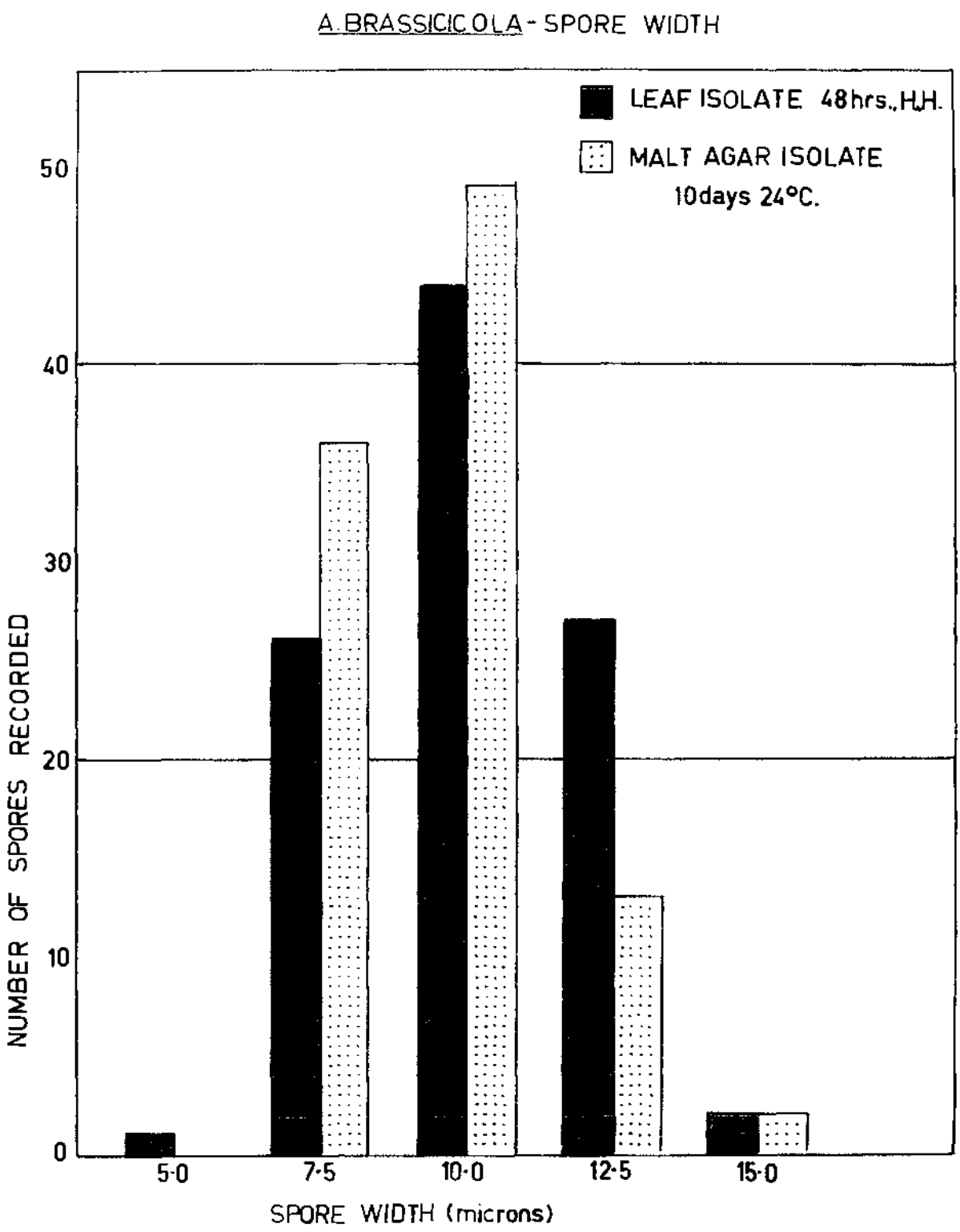
Figure 5



A.brassicicola - distribution of spore length

Figure 6



A.brassicicola - distribution of spore width

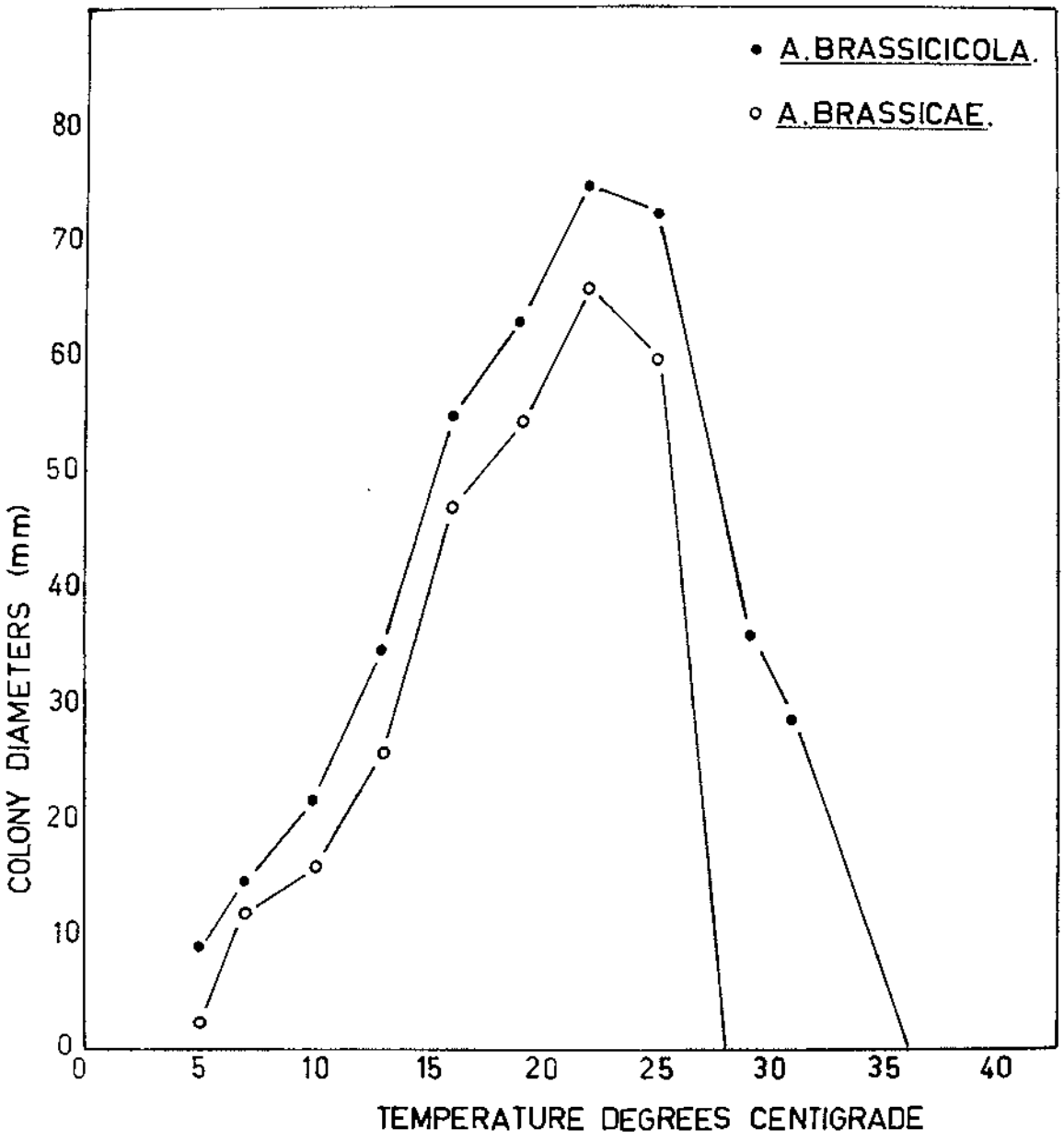
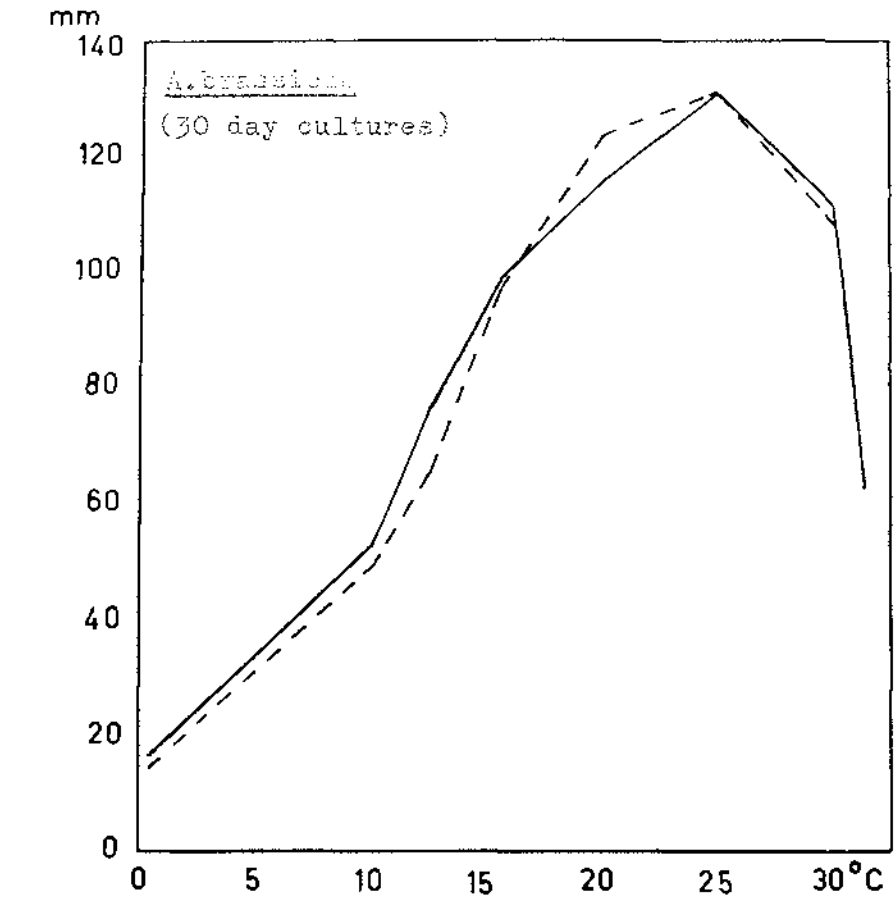
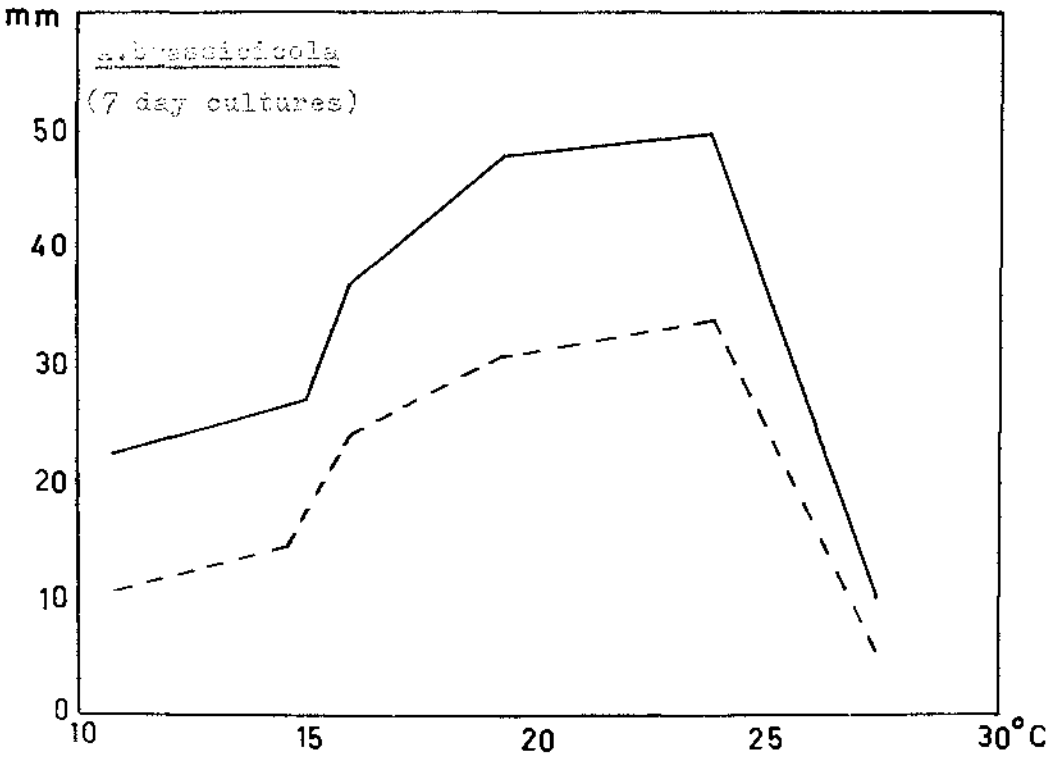

• A.BRASSICICOLA.
○ A.BRASSICAE.
COLONY DIAMETERS (mm)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40
TEMPERATURE DEGREES CENTIGRADE

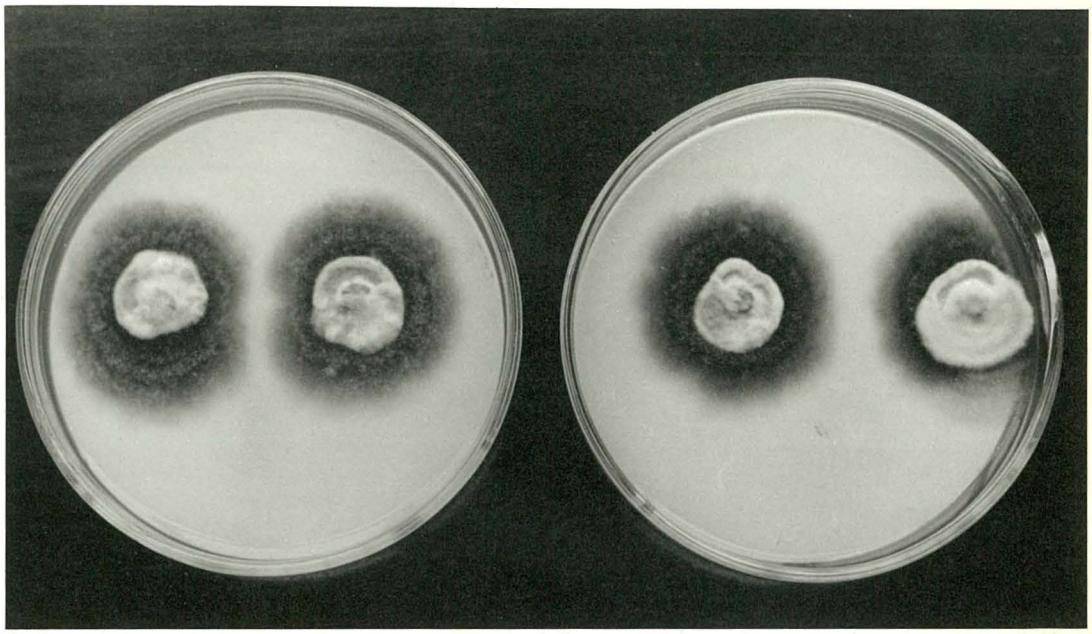
Figure 8



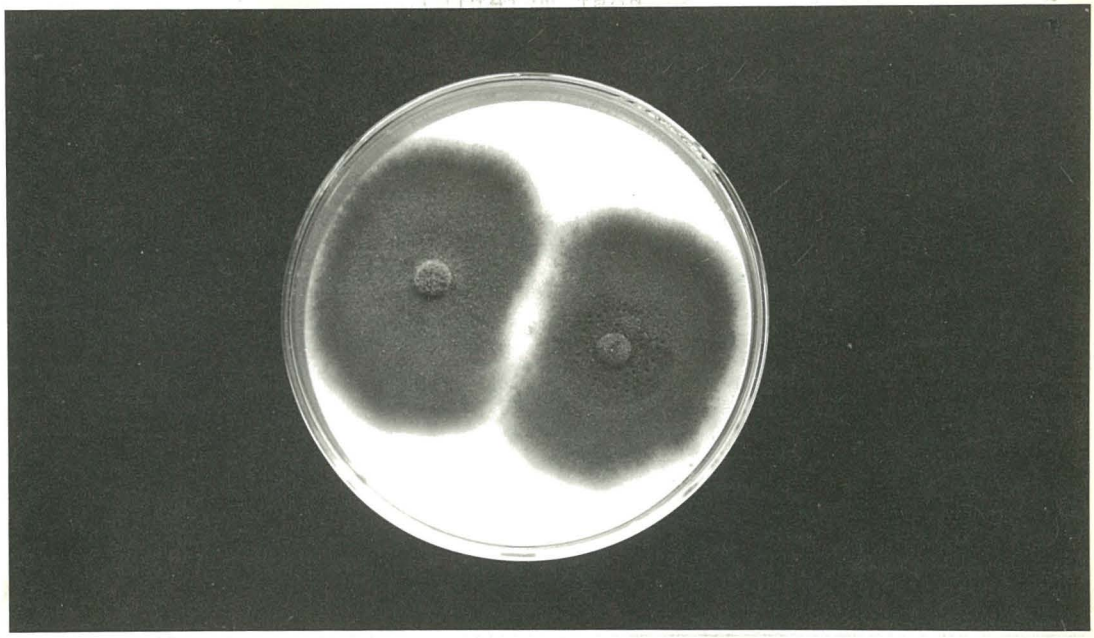


Temperature effects on growth
(Rossgaard, 1975)

Plate 1



A.brassicae

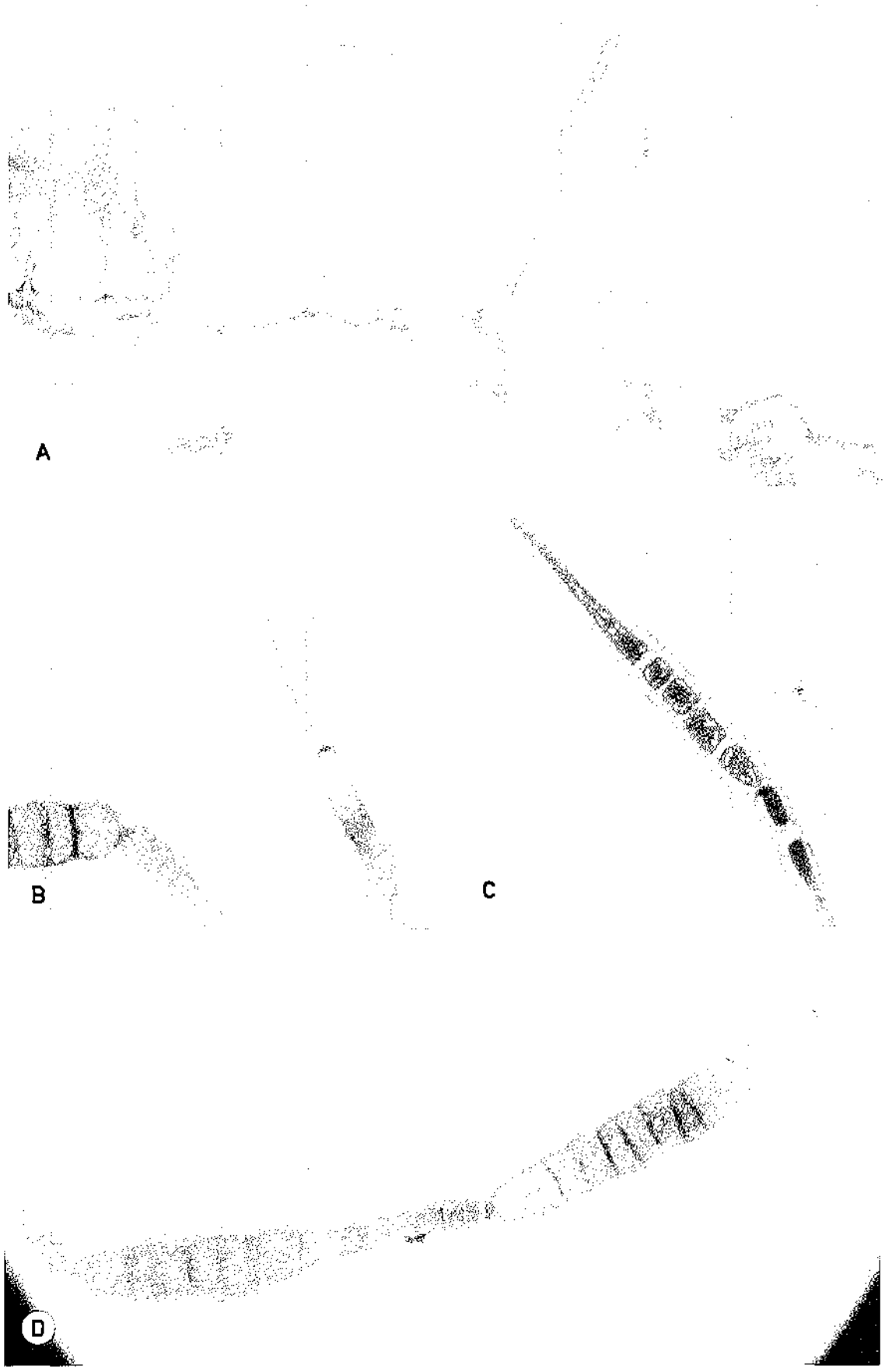


A.brassicicola

Colony characteristics of A.brassicae and A.brassicicola

A	On PDA
B - D	Stages of conidial formation on host material

Plate 2



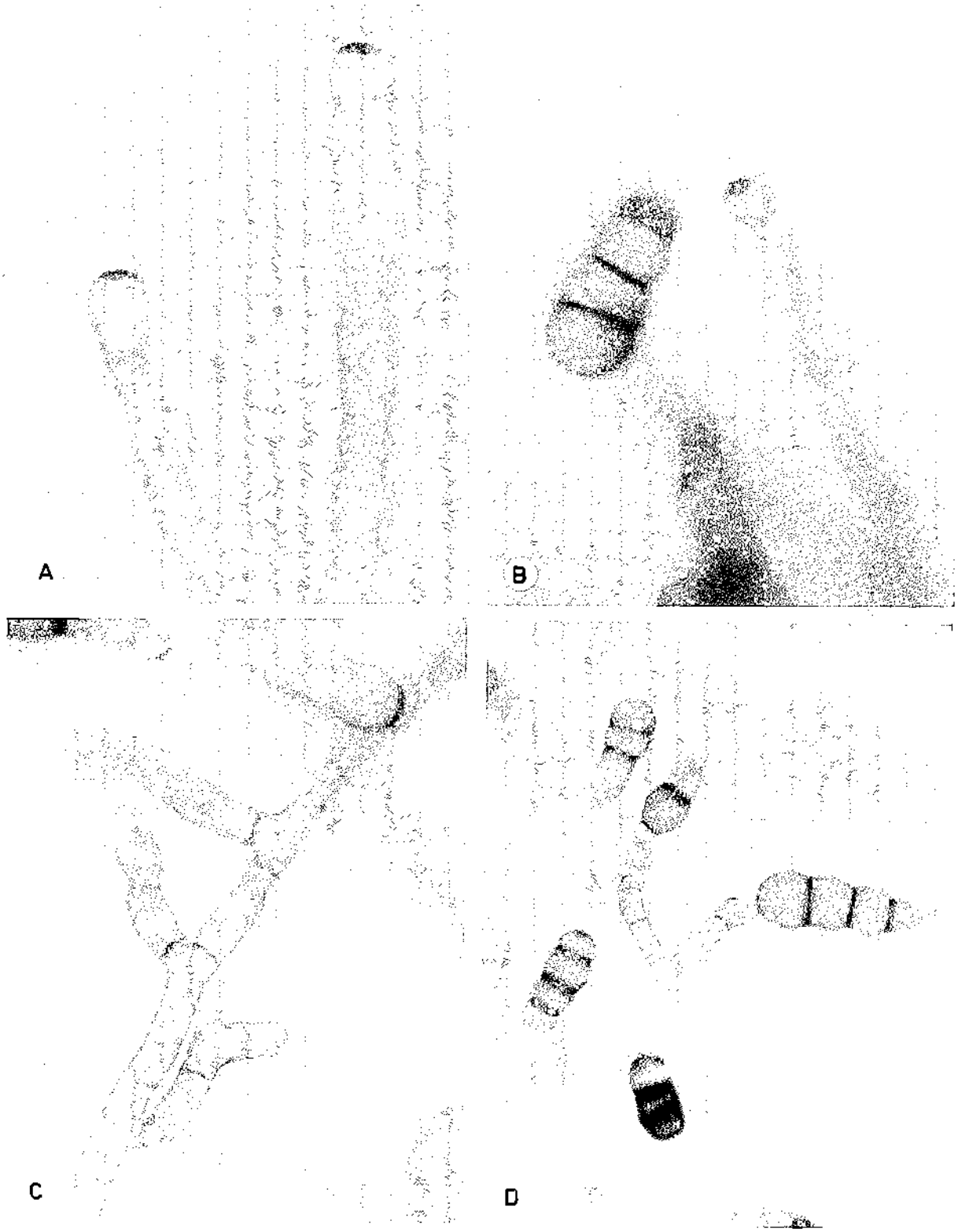
Conidial production by A.brassicae

Plate 3

Leaf penetration by A.brassicae

A	Conidiophores
B	Apical pore
C	Conidiophore branching and septation
D	Conidial production

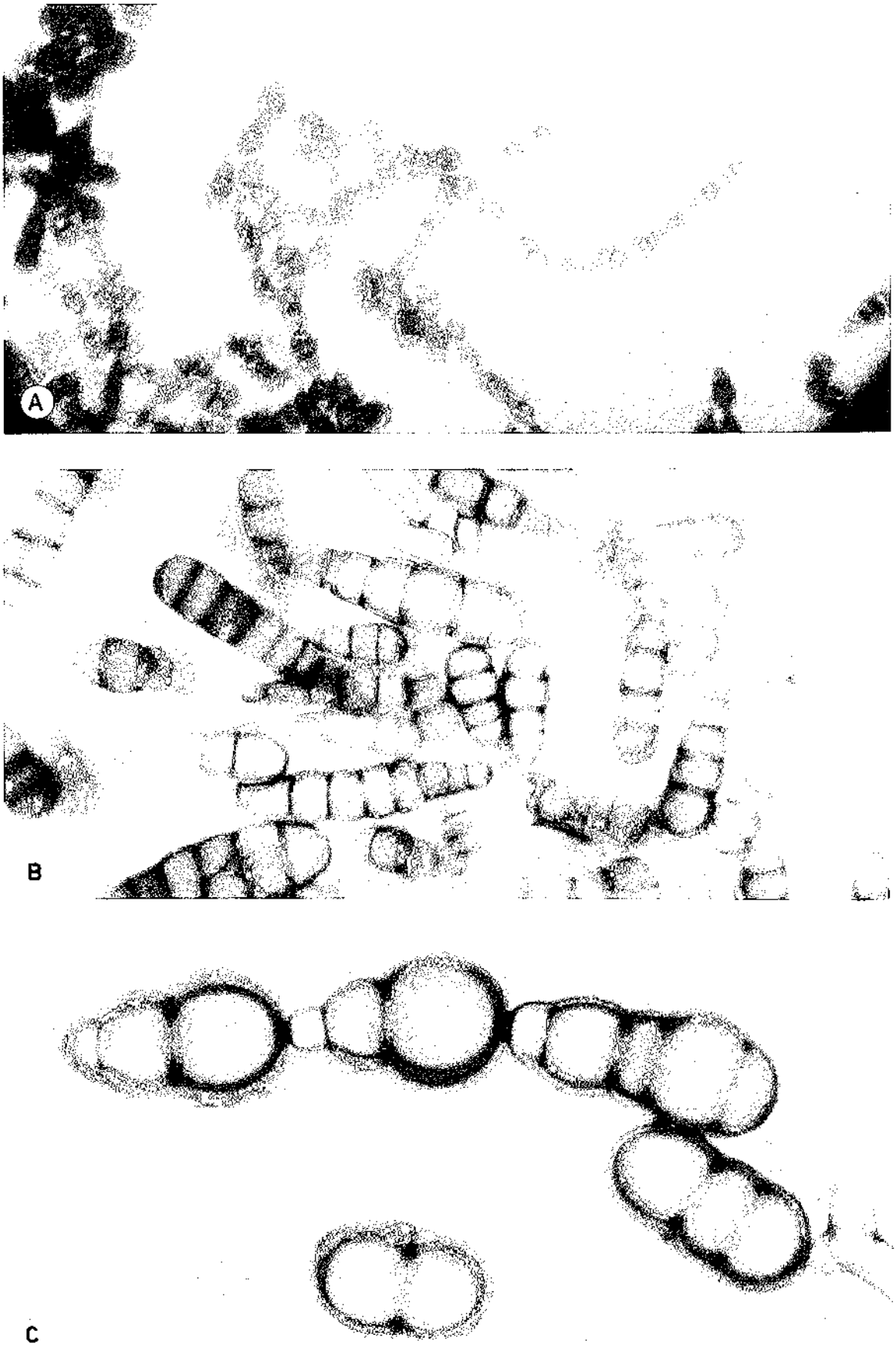
Plate 4



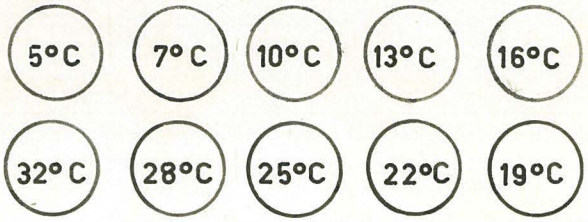
Conidiophore characteristics of A.brassicicola

A	Chain formation on PDA
B	Characteristics of spores from host material
C	Conidial apical cone

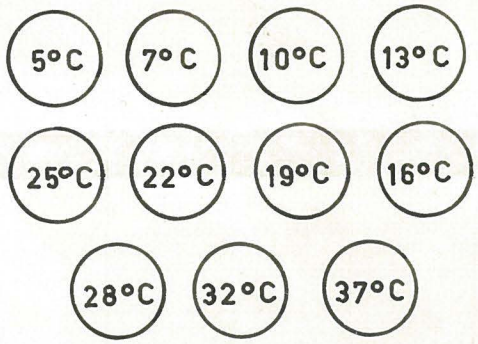
Plate 5



Conidial characteristics of A.brassicicola

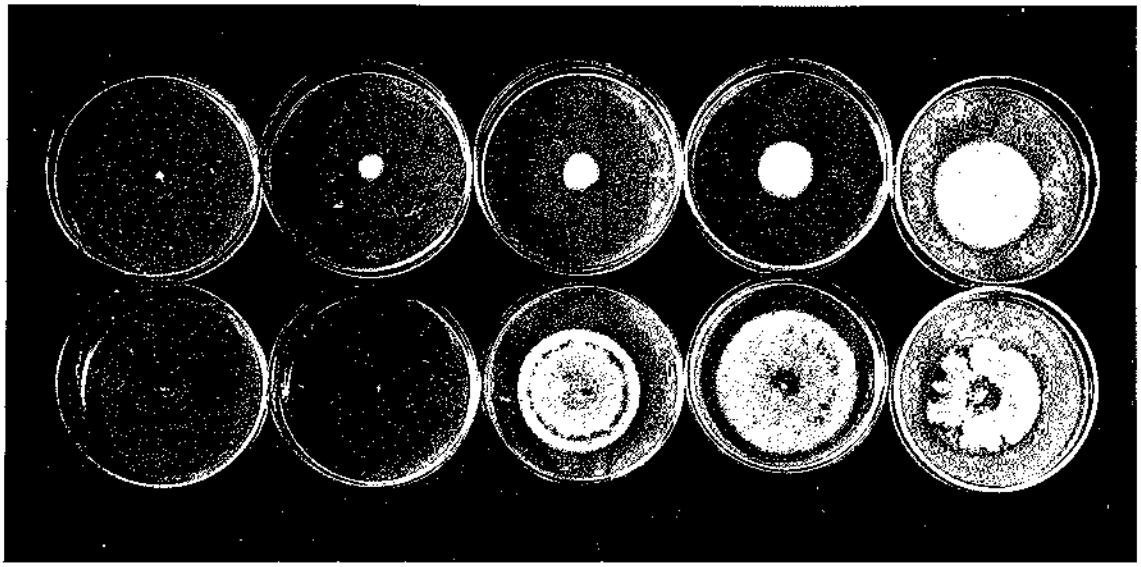


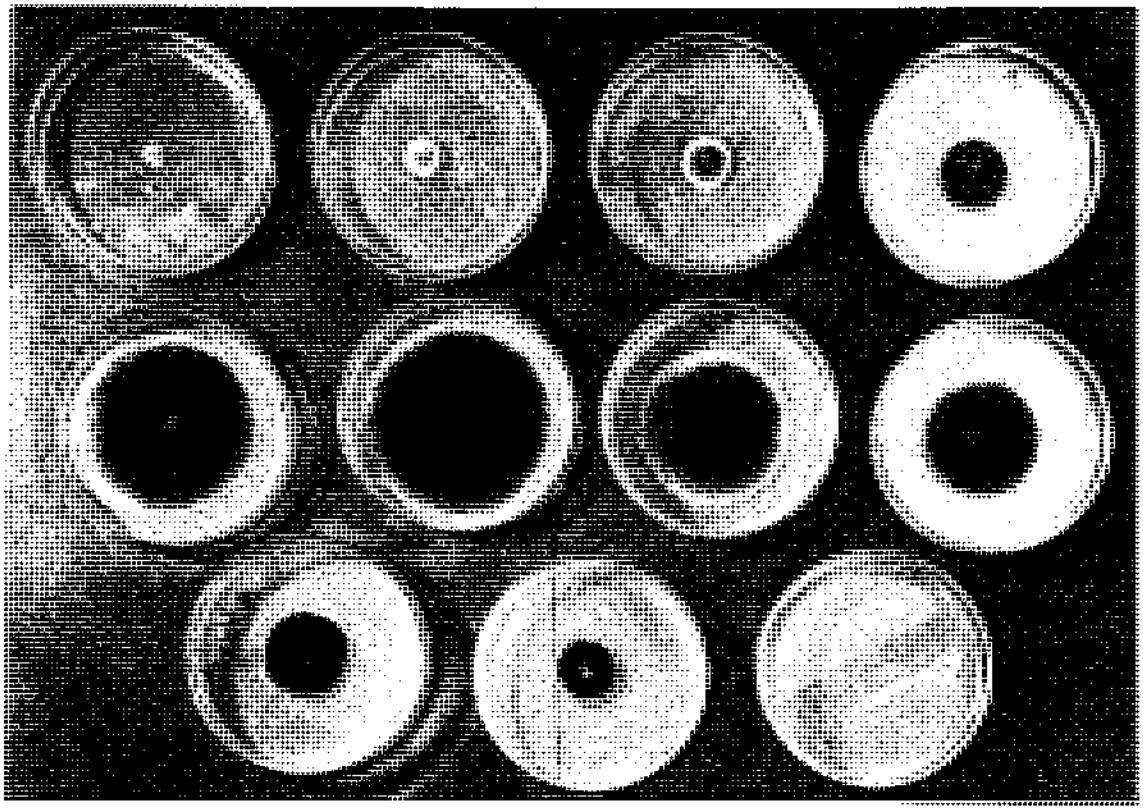
A A.BRASSICAE



B A.BRASSICICOLA

Plate 6



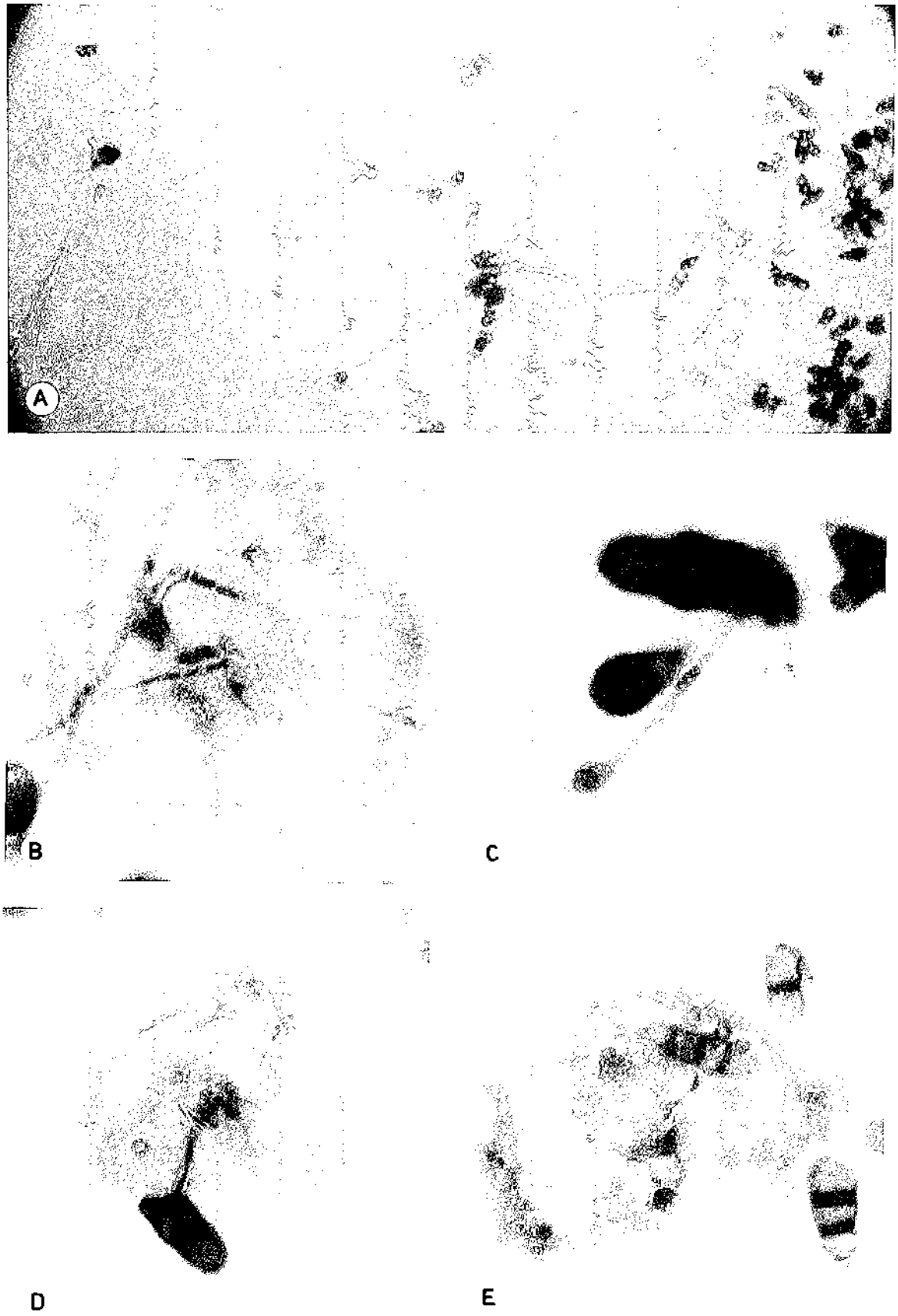


Temperature effects on colony growth

(Growth measured after incubation for 14 days on MA)

A	Spore germination on water agar
B	Stomatal penetration
C	Appressorium formation
D	Stomatal penetration
E	Developing lesion

Plate 7



Spore germination and penetration by A.brassicicola

CHAPTER II

A.BRASSICAE AND *A.BRASSICICOLA*

AS SEED-BORNE PATHOGENS

A. INTRODUCTION

Overseas workers have shown both A.brassicae and A.brassicicola to be present in brassica seed lines. For example, Neergaard (1945) found more than 90% of the 746 cabbage seed samples analysed between 1935 and 1941 to have A.circinans (Berk. and Curt.) Bolle (1924) (syn. A.brassicicola) present. In England, Schimmer (1953) found "infection was 96-100% in 1950; 31% in 1951; and 63-77% in 1952" in summer cauliflower seed harvested from crops raised under glass. In a series of referee tests conducted under the supervision of the International Seed Testing Association (I.S.T.A.) in 1958, A.brassicicola was shown to be present in samples of cabbage (B.oleracea var. capitata L.) brussels sprouts (B.oleracea var. gemmifera D.C.) and red cabbage (Table 8).

The work of McDonald (1959) has demonstrated the seed-borne nature of A.brassicae in cruciferous crops. On examination of 25 seed samples of rape (B.napus L.) he found infections of up to 4% in 14 lines.

In New Zealand, pathogenic species of Alternaria have been shown to be present in commercial lines of choumoellier seed. Screening trials conducted at the Government Seed Testing Station, Palmerston North (G.S.T.S.) in 1963 and 1964, revealed the presence of A.brassicicola in a large percentage of the 128 seed samples tested (Table 9). A.brassicae was associated with some lines, but was less prevalent than the small spored species (Table 10).

TABLE 8

I.S.T.A. health tests on distributed samples of brassica seed (I.S.T.A., 1958)

Sample	Test method	Mean infection % with *A. brassicicola*
a. Cabbage	2,4D - Blotter	3
	Agar plate	2
	Blotter	11
b. Brussels sprouts	2,4D - Blotter	22
	Agar plate	33
	Blotter	49
c. Red cabbage	2,4D - Blotter	4
	Agar plate	4
	Blotter	15

TABLE 9

Prevalence of A.brassicicola in choumoellier seed lines screened at G.S.T.S.

Year	No. of lines screened	No. of seed lines with an infection of . . .				
		0%	1-5%	6-10%	11-15%	16-20%
1963	30	0	19	8	2	1
1964	98	32	63	3	0	0

100
66

TABLE 10

Prevalence of A.brassicae in choumoellier seed lines screened at G.S.T.S.

Year	No. of lines screened	No. of seed lines with an infection of . . .					
		0%	< 1%	1%	2-3%	4-5%	14%
1963	30	23	5	0	2	0	0
1964	98	90	0	6	0	1	1

23
8 7

In instances where diseases are seed-borne, the nature of the association between pathogen and host varies, but two broad categories are generally recognised :

(i) seed infection - where mycelium is established within the seed coat, embryo, or other seed tissue;

(ii) seed contamination - where spores or mycelial fragments adhere to the seed coat, or are trapped within the surrounding bracts, as in cereals and grasses.

Rangel (1945) studied Alternaria species on crucifers and showed that seed-borne inoculum of both A.brassicae (Berk.) Sacc. (syn. A.brassicicola) and A.herculea (Ell. and Mart.) Elliot, (syn. A.brassicae) may occur both as latent mycelium in the seed and as spores on the seed coat. His studies included investigations on kale (B.oleracea var. viridis L.) seed and by a series of surface sterilisation experiments he demonstrated the presence of seed infection.

In view of the apparent lack of detailed research on the seed-borne nature of A.brassicae and A.brassicicola in choumoellier seed, the following areas were investigated :

(i) surveys were conducted to determine the prevalence of the two pathogens in New Zealand certified choumoellier seed, and the nature of their association with seed;

(ii) studies on the viability of seed-borne inoculum with time;

(iii) the effects on germination of the elimination of A.brassicicola from seed.

B. MATERIALS AND METHODS

The following refers to materials and methods common to this chapter; those relating to specific experiments are detailed in the appropriate sections.

1. Seed sampling method

All seed lines tested were obtained from the G.S.T.S. and were lines submitted to them for purity and germination tests as a certification requirement. Following vigorous agitation each sample was spread over a sheet of paper and sub-divided using the straight edge of a ruler. Sub-dividing in this manner was continued until a sub-sample of approximately 4 gm was obtained. This constituted the final sample for testing.

2. Surface sterilisation of seed

Test samples were sub-divided and approximately half of each placed on muslin squares which were closed with an identification band to form a loose bag. These were then briefly dipped in tap water and gently squeezed to remove air locks before immersion in the sterilant chemical.

(a) Mercuric chloride treatment

The seed bags were immersed in a solution of 0.001% mercuric chloride for 10 minutes (McLean 1947), rinsed in three washes of sterile distilled water, after which the seed was spread between filter pads to dry.

(b) Sodium hypochlorite treatment

The seed bags were first dipped in 70% alcohol prior to immersion in a sodium hypochlorite solution (1% available chlorine) for 10 minutes (Matthews 1965). Drying was again carried out between filter pads.

3. Germination tests

Three 100 seed replicates for each line were incubated on moist germination pads (25 seeds per pad at $\frac{1}{2}$ inch seed placements centres). To overcome seed dormancy the blotters were moistened with a 0.2% solution of potassium nitrate at the beginning of the test. They were then incubated under plastic high humidity covers at an alternating 30°C day, and 18°C night temperature cycle. Routine germination counts involving the removal of healthy seedlings were carried out by a G.S.T.S. seed analyst. Counts were made at 3 days and 7 days (interim) with a final count at 10 days. The germination percentage of a line is the average of the 10 day recording for 3 x 100 replicates. Percentage figures are also recorded for dead seed and abnormal seedlings. According to the rules of the I.S.T.A. (1966), a seedling is considered to be abnormal if it exhibits :

(i) damage to essential structures;

(ii) deformity or unbalanced development;

(iii) decay or disease arising from seed infection;

(iv) cotyledon development from the micropyle, or radicle development from a part of the seed other than the micropyle.

4. Hot water treatment of seed

Hot water treatment was carried out by immersing loose muslin bags containing the seed in a thermostatically controlled water bath (Plate 8) for a specific exposure period. A mechanical agitator kept the water constantly in motion, and the muslin bags were suspended in a wire mesh basket to prevent blockage of the circulatory system. Following treatment the seeds were cooled by plunging the bags into cold water, and air dried.

5. Moisture tests

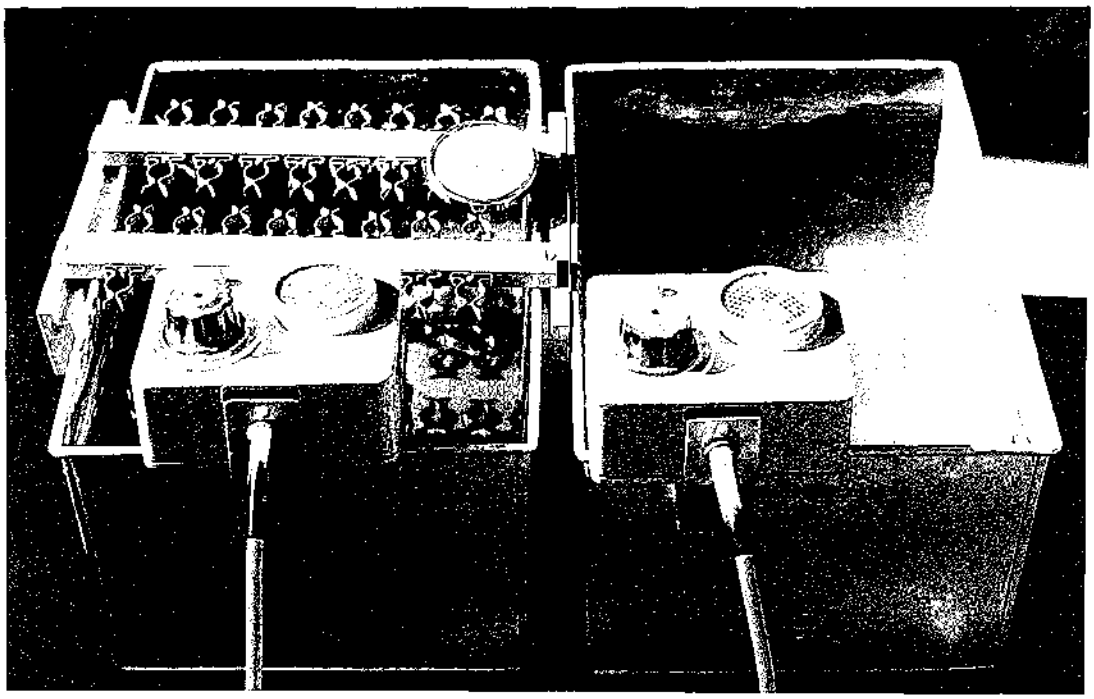
Samples were weighed before and after drying for two hours at 103°C in a Wilco oven. Longer drying periods have been found to cause the distillation of oils in certain seeds (Hill 1966).

C. INOCULUM ASSOCIATED WITH SEED LINES

1. Seed infection

The extent to which New Zealand certified choumoellier

Plate 8



Apparatus used in hot water treatment of seed
(Grant instruments type SV2)

seed is infected with the two pathogens was studied in a survey conducted during 1965 (40 seed lines) and 1966 (25 seed lines). This work in effect was a continuation of the G.S.T.S. surveys of 1963 and 1964 and thus the combined results furnish information covering a continuous four year period.

The survey method was essentially similar to that used at the G.S.T.S. and involved plating of surface sterilised seed to MA. In the Seed Testing Station experiments, Matthews (1965), used a sodium hypochlorite solution (1% available chlorine) as the sterilant material. However with the constant need for standardisation of this solution over the survey period an alternative mercuric chloride method was used as recommended by McLean (1947).

RESULTS AND DISCUSSION

The results presented in Table 11 show that A.brassicicola was present in a high percentage of seed samples in both years, to the extent of 72.5% in 1965 and 72% in 1966. A.brassicae was present in a few lines (21% in 1965 and 32% in 1966), but in all cases at low infection levels (Table 12). These results are essentially similar to those of the G.S.T.S. surveys (Tables 9 and 10).

Surface sterilisation as a pre-treatment before plating seed was a necessary practice to remove contaminating saprophytic fungi which grew rapidly on artificial media, masking the growth of the Alternaria pathogens. Such a treatment also eliminated

TABLE 11

The prevalence of A.brassicicola in choumoellier seed lines screened in 1965 and 1966

Year	No. of lines screened	No. of seed lines with an infection of . . .				
		0%	1-5%	6-10%	11-15%	16-20%
1965	40	11	23	5	1	0
1966	25	7	15	3	0	0

TABLE 12

The prevalence of A.brassicae in choumoellier seed lines screened in 1965 and 1966

Year	No. of lines screened	No. of seed lines with an infection of . . .				
		0%	<1%	1%	2-3%	4-5%
1965	40	33	4	3	0	0
1966	25	17	4	4	0	0

contaminating spores of pathogenic species and hence the results expressed above pertain to seed infection only.

2. Seed contamination

In general, methods used for the assessment of seed contamination involve recovery of the contaminating elements by agitating a seed sample in water and concentrating the dissociated spores by centrifuging. The relative numbers of contaminating spores may then be estimated using a measuring apparatus such as a haemocytometer, or by application of the spore suspension to a growing plant and recording a relative infection index. There are however certain inherent limitations in such "centrifuge" techniques for estimating seed contaminants. In a spore count system no differentiation can be made between viable and dead spores. Further, the estimate per ml of the spore suspension has to be related to the number or weight of seeds treated, and if an average inoculum load per seed or per unit of seeds is assigned, it must be assumed that inoculum is evenly distributed throughout the sample. While a pathogenicity method involving inoculation of plants does select for viable spores, the results overstate the importance of such inoculum when associated with seed. The application of spores to a leaf surface under high humidity conditions gives all spores an equal chance to germinate and infect, whereas spores present on seed coats are less likely to make direct contact with growing tissue.

In the present work, screening for seed contamination

was carried out using the following method which overcame some of the above difficulties.

Method

Three replicates of 100 seeds from each of 16 low germinating lines were subjected to a standard germination test. At the conclusion of the test dead seed and abnormal seedlings were retained and investigated for the presence of the Alternaria pathogens by microscopic examination and isolation to PDA slopes. The results obtained are an expression of seedling attack resulting from both seed-borne infection and seed-borne contamination by the pathogens. It was reasoned that if a higher percentage of infection with the Alternaria species was recorded by this method than by using the agar method, then the difference could be attributed to seedling attack by contaminating pathogenic elements.

RESULTS AND DISCUSSION

It is evident from the difference between results obtained using both methods (Table 13), that considerable numbers of contaminating spores and mycelial fragments potentially capable of causing seedling infection are eliminated by surface sterilisation. In all of the 16 lines tested a considerably higher percentage infection was recorded for the pathogen A.brassicicola by examination of the dead seed and abnormal seedlings from a germination test.

It is possible that secondary infection as a result

TABLE 13

Percentage of infected seedlings resulting from contaminating inoculum

Seed Line No.	% pathogens recorded on examination of dead seed and abnormal seedlings		% pathogens recorded by the agar plate method		Difference between methods (%)	
	A.brassicicola	A.brassicae	A.brassicicola	A.brassicae	A.brassicicola	A.brassicae
1	26.6	2.0	11.3	0.6	15.3	1.4
2	11.6	0.3	9.3	1.0	2.3	- 0.7
3	9.3	0.3	3.3	0.3	3.3	0.0
4	20.0	0.0	4.6	0.0	15.4	0.0
5	11.6	0.3	4.0	0.0	7.6	0.3
6	7.0	0.0	4.0	0.0	3.0	0.0
7	16.0	0.0	5.6	0.0	10.4	0.0
8	16.0	0.0	6.3	0.0	9.7	0.0
9	11.6	0.0	2.0	0.0	9.6	0.0
10	8.3	0.0	3.6	0.0	4.7	0.0
11	11.9	0.0	8.0	0.0	3.9	0.0
12	11.6	0.3	2.6	0.0	99.0	0.3
13	10.6	0.0	1.3	0.0	9.3	0.0
14	16.0	0.3	5.0	0.0	9.0	0.3
15	8.3	0.0	4.3	0.0	4.0	0.0
16	13.3	0.0	3.6	0.0	9.7	0.0

of contact between healthy seedlings and neighbouring diseased seedlings may have occurred, in which case the results would be biased in favour of the germination test. As against this, it could also be argued that many seedlings which were removed during interim counts in the germination test may have developed symptoms by the time the test was terminated, had they remained on the blotters until the final count (a further three days). However, it seems reasonable to regard the results obtained as indicative of seedling infection caused by both contaminating and infecting inoculum.

D. VIABILITY OF SEED-BORNE INOCULUM

Having demonstrated the presence of Alternaria species, particularly A.brassicicola, in choumoellier seed lines it was desirable to investigate the longevity of this inoculum in stored seed. For the pathogens to be significant in seedling emergence the inoculum must retain its viability for a period at least equivalent to the interval between harvest and sowing.

Spores of Alternaria species have been shown by previous workers to be capable of surviving for long periods. Rangel (1945) found the spores of A.brassicicola to retain their viability and pathogenicity for more than six months. Hill (1965) has demonstrated the viability of contaminating spores of Alternaria zinniae Pape on seed on Zinnia elegans Jacq. for up to 2½ years,

and seed infections for over three years. In the light of this evidence it seemed likely that seed-borne inoculum of A.brassicae could also remain viable for considerable periods in stored seed.

1. Seed infection

Seed samples obtained from lines harvested in the 1963, 1964 and 1965 seasons were tested for the presence of both Alternaria species. Where possible, lines were surveyed which had been previously tested by Matthews at the G.S.T.S., thus enabling a comparison of results before and after storage. For this reason the surface sterilisation technique utilizing sodium hypochlorite (1% available chlorine) as used by Matthews (1965) was employed. Three hundred seeds per line were so treated and incubated for six days at 24°C on MA. The results for surveys made before and after storage are presented in Tables 14, 15 and 16.

RESULTS AND DISCUSSION

Although no comparative results are available for the 1963 lines tested, it is apparent that A.brassicicola can survive as viable infections in seed tissues for a period in excess of three years. A drop in the number of seeds infected in a line occurred following a two year storage period and in many samples this represented two thirds of the originally recorded infection percentage. A smaller but consistent drop occurred in infections recorded over a one year storage interval.

A.brassicae was not isolated from any of the lines

TABLE 14

Viability of seed infections after 3 years 4 months storage
(Seed harvested in 1963)

Seed line No.	Infection % recorded in 1966	
	A.brassicicola	A.brassicae
T 1	0.00	0.00
T 2	0.00	0.00
T 3	0.00	0.00
T 4	0.00	0.00
T 5	0.66	0.00
T 6	0.00	0.00
T 7	0.00	0.00
T 8	0.66	0.00
T 9	1.33	0.00
T 10	0.00	0.00

TABLE 15

Viability of seed infections after 2 years 5 months storage

Seed Line No.	Infection % recorded in the year of harvest (1964)		Infection % recorded in 1966	
	A.brassicicola	A.brassicae	A.brassicicola	A.brassicae
F 1	3.00	14.00	1.66	0.00
F 2	9.00	0.30	3.33	0.00
F 3	6.30	0.00	1.33	0.00
F 4	2.70	0.00	0.00	0.00
F 5	3.30	0.00	2.00	0.00
F 6	3.30	0.00	1.66	0.00
F 7	9.00	0.30	3.33	0.00
F 8	6.70	4.30	3.00	0.00
F 9	-	-	1.66	0.00
F 10	-	-	0.00	0.00
F 11	-	-	2.00	0.00
F 12	-	-	0.00	0.00

TABLE 16

Viability of seed infections after 1 year 6 months storage

Seed line No.	Infection % recorded in the year of harvest (1965)		Infection % recorded in 1966	
	A.brassicicola	A.brassicae	A.brassicicola	A.brassicae
Fv 1	0.00	0.00	0.00	0.00
Fv 2	1.00	0.00	0.00	0.00
Fv 3	4.00	0.00	1.33	0.00
Fv 4	8.00	0.00	5.00	0.00
Fv 5	2.66	0.00	2.00	0.00
Fv 6	9.33	1.00	8.00	0.00
Fv 7	4.33	0.00	5.00	0.00
Fv 8	3.00	0.33	2.66	0.00
Fv 9	6.33	0.00	2.00	0.00
Fv 10	0.00	0.00	0.00	0.00
Fv 11	6.66	1.66	2.66	0.00
Fv 12	6.66	0.33	4.33	0.00
Fv 13	4.0	0.00	2.33	0.00
Fv 14	0.00	0.66	0.00	0.00
Fv 15	2.00	0.00	2.33	0.00

subjected to analysis in 1966. Although of the 37 lines screened, only nine were known to be infected with A.brassicae, the evidence suggests that this species is short-lived in seed. That is, it would appear that A.brassicae is not likely to be active in causing seedling infection at the time of sowing.

2. Seed contamination

Trials were carried out using the same seed lines screened in the previous section, with the addition of 10 lines harvested in 1966. The samples were investigated approximately six months after the summer harvest period.

Initial trials to develop a method for demonstrating the presence of viable contaminating inoculum in seed lines involved the use of a membrane filter and graduated filter pads. After filtration of a spore suspension obtained from seed, the pads were sprayed with agar and incubated under high humidity conditions at 24°C for five days. It was hoped that the number of viable spores per square on the filters could be determined by colony counts. Unfortunately, the long incubation period required for mycelial development of the pathogen also favoured profuse growth of associated saprophytes which masked the presence of the pathogens. Further experimentation led to the adoption of an agar slide technique enabling identification of individual germinating spores before contaminants obscured the results.

Method

A 5 gm sample from each seed line was agitated for

two minutes in 10 ml of sterile distilled water and the suspension recovered by filtering through a wire gauze funnel. The suspension levels in the test tubes were adjusted to a common level and after centrifuging for 10 minutes at 1450 r.p.m. approximately 7 ml of the supernatant was decanted off. A 0.01 ml aliquot from the concentrated suspension was then applied to a water agar slide preparation and spread over an area delimited by two parallel lines drawn 1 cm apart on the under surface of the slide. Ten fields were examined after incubation of the slides under high humidity conditions for 24 hours. These were taken at random by focusing the low power objective on one end of the slide between the defined spore preparation limits and moving the mechanical stage 4 mm along the horizontal axis between readings. The number of germinating and non-germinating spores per field was recorded for each species of pathogen.

RESULTS AND DISCUSSION

All spores recovered from 1963 and 1964 lines of choumoellier seed were non-viable. Eight of the 15 lines of 1965 seed tested exhibited germination of A.brassicicola spores on agar at an average ratio of one viable to seven non-viable spores. All of the 1966 seed lines tested (10), showed the presence of viable contaminating inoculum with an average ratio of viable to non-viable spores of 1:4.5. From these results it appears that spores of A.brassicicola can survive on seed for 18 months, which is sufficient time to ensure their

successful carry-over on seed from harvest to sowing in the next season.

A.brassicae spores were observed to germinate in only one of the 10 lines tested in 1966. This would suggest that A.brassicae is not capable of surviving between seasons by means of spores adhering to the seed coat.

E. THE EFFECTS ON GERMINATION OF THE ELIMINATION OF A.BRASSICICOLA FROM SEED

In seed-borne disease two general situations exist with regard to the effect of seed infection on seed health :

(i) where the embryo is killed at, or shortly after the time of infection. That is, the seed embryo is killed before a sowing is made;

(ii) where the embryo is not killed before sowing, but may be attacked at, or soon after germination is initiated.

Elimination of the seed infections of the first type would not result in an increased germination percentage of a line as exemplified by A.raphani Groves and Skolko, the cause of pod and stem blight of radish (Raphanus sativus L.). McLean (1947) has shown that the factor responsible for low germination of radish seeds was operative before the seeds

were marketed, or even before they were harvested. He found that although hot water treatment of low germinating lines of seed for 25 minutes at 50°C eliminated the pathogen as evidenced by plating to agar, this treatment did not increase the germination percentage of the lines. That is, in this situation the embryo was killed by the pathogen.

Elimination of the infection from seed of the second category, not only ensures against perpetuation of the disease, but may result in an increased germination percentage of the treated line. For example, Crosier and Patrick (1940) found that the percentage germination of Copenhagen cabbage seed was "increased from 64% to 76% and 80%" by hot water treatment at 50°C for 25 minutes. The fungi involved were considered to be A.brassicae, A.brassicicola and Rhizopus nigricans. This indicated that the pathogens had not impaired the germinability of the seed embryo.

In view of the fact that A.brassicicola remains viable in seed over the storage period between harvest and sowing, experiments were conducted to investigate the effect on germination resulting from elimination of the pathogen. It was considered that by effectively hot water treating seed, then germination would be increased if the embryo was not damaged by A.brassicicola and results analagous to those recorded by Crosier and Patrick (1940) would be obtained. If the fungus killed the embryo then a situation similar to that

shown by McLean (1947) could be expected.

1. Identification of an effective hot water treatment

For this experiment a specific temperature-time relationship had first to be found which would eliminate the Alternaria pathogen without damaging seed vitality. Many hot water treatments have been recommended for cruciferous seeds (Table 17). Preliminary screening trials showed that a 20 minute treatment at 45°C (Huber and Gould, 1949) was not sufficient to completely eliminate A.brassicicola from seed, but that the 50°C treatments investigated appeared to be successful. A consideration of these led to the selection and subsequent testing of three treatments covering the range most frequently advocated in the literature.

Method

Seeds were subjected to hot water treatment as follows :

(i) 55°C for 10 minutes (Weimer, 1924);

(ii) 50°C for 18 minutes (Schimmer, 1953);

(iii) 50°C for 25 minutes (Crosier and Patrick, 1940).

To investigate the efficiency of the respective treatments in elimination of the pathogens and their effect on seed germination, the seeds were examined by the following tests :

TABLE 17

Recommended hot water treatments for cruciferous seeds

Authority		Host	Temperature	Time Minutes
Weimer	1924	Cabbage	55°C	10
Nielsen	1937	Cabbage	45°C	30
Nielsen	1937	Cabbage	50°C	20
Crosier and Patrick	1940	Cabbage	50°C	25
McLean	1947	Radish	50°C	25
Huber and Gould	1949	Cabbage	45°C	20
Walker	1952	Crucifers	50°C	30
Schimmer	1953	Cauliflower	122°F (50°C)	18
McKay	1956	Cabbage	122°F (50°C)	25
McKay	1956	Cauliflower	122°F (50°C)	18
Chupp and Sherf	1960	Cabbage	122°F (50°C)	25
Chupp and Sherf	1960	Cauliflower	122°F (50°C)	20

(i) plating 300 seeds per treatment to MA after surface sterilisation with mercuric chloride;

(ii) a germination test at the G.S.T.S.;

(iii) examination of the dead seed and abnormal seedlings for the presence of the Alternaria species at the conclusion of the germination test;

(iv) recording emergence figures of four 100 seed replicates per treatment sown to glasshouse flats.

For each trial there was a control series using untreated seed of the same line.

RESULTS AND DISCUSSION

The results of experiments (i), (ii) and (iv) are presented in Tables,18, 19 and 20. As regards experiment (iii), analysis of dead and abnormal seedlings resulted in two identifications of A.brassicicola infections on seed treated at 50°C for 10 minutes nor 50°C for 25 minutes.

The treatment at 50°C for 25 minutes was the most efficient; in none of the experiments was A.brassicicola isolated from treated seed, and no adverse effects on germination were recorded.

Plating to agar (Table 18) indicated successful elimination of the pathogen in all treated samples. However, the analysis of dead seed and abnormal seedlings showed that the 18 minute treatment at 50°C was not completely efficient.

TABLE 18

Examination of hot water treated seed by an agar plate method

Treatment	Infection %	
	A. brassicicola	A. brassicae
55°C for 10 minutes	0.0	0.0
50°C for 18 minutes	0.0	0.0
50°C for 25 minutes	0.0	0.0
Control - untreated	6.7	1.7

TABLE 19

Examination of the germination of hot water treated seed

Treatment	Germination % Mean ± S.E.
55°C for 10 minutes	90 ± 2.9
50°C for 18 minutes	95 ± 0.7
r0°C for 25 minutes	96 ± 1.8
Control - untreated	93 ± 2.3

TABLE 20

Examination of the emergence of hot water treated seed

Treatment	Emergence % Mean ± S.E.
55°C for 10 minutes	63 ± 2.6
50°C for 18 minutes	80 ± 2.7
50°C for 25 minutes	87 ± 2.2
Control - untreated	84 ± 2.8

Although no adverse effects on germinability were apparent in the G.S.T.S. germination test, a low three day count for seeds treated at 55°C indicated that the embryo had been weakened. This was supported by the germination reduction in glasshouse sowings of seed treated at 55°C. That is, under the favourable conditions of a blotter germination test, seed germinated, but under the more stringent environment of a soil medium, many seeds failed to produce a seedling.

As the results of the preliminary experiment applied to one line of seed only, a number of seed lines with various initial germination capacities were tested to determine if the above results were applicable to most lines.

2. Effect of hot water treatment on germination

It is generally recognised that aging increases the physiological weakness of seed. In this regard, the opportunity was taken to investigate the effect of hot water treatment on the germination percentage of two age groupings of seed, namely seed six months and 18 months old.

Gloyer (1938) found that the effect of hot water treatment on china aster seed (Callistephus chinensis Nees) was dependent on the initial moisture content of the seed. In the present experiment the moisture content of each seed line was determined before hot water treatment to invesigate a possible relationship between this factor and germination

after treatment.

Method

Samples of seed lines with a known moisture content and infection percentage of A.brassicicola were each subjected to a G.S.T.S. germination test both before and after hot water treatment at 50°C for 25 minutes.

In analysis of the effect of hot water treatment on germination a "t" test was made using the method of paired comparisons (Bailey, 1959).

RESULTS AND DISCUSSION

The results presented in Tables 21 and 22 show that in both age groupings of seed there was an overall significant germination reduction due to hot water treatment of seed, but the treatment did not cause a reduction to an extent that would prohibit the use of this recommendation as a control measure. It appears that aging up to 18 months has not weakened the seed as germination of the older seed has not been affected any more severely than the six months old seed. It is also apparent that there was no relationship between moisture content and the final germination percentage. It will be noted that in three cases there was a slight increase in germination following hot water treatment. This could possibly be attributed to inactivation of the pathogen present, but the inconsistent relationship between final germination percentages and the infection percentages at the time of treatment discount such a conclusion.

TABLE 21

Effect of hot water treatment on germinability of 6 months old seed

Seed Line No.	Germination %		Relative advantage of untreated over hot water treated seed	Infection % of A.brassicicola	Moisture %
	Untreated seed	Hot water treated seed			
	Mean ± S.E.	Mean ± S.E.			
1	91 ± 1.1	91 ± 1.5	0	2.0	8.5
2	83 ± 1.9	85 ± 2.2	- 2	4.3	7.9
3	78 ± 3.7	69 ± 2.6	+ 9	10.0	8.4
4	98 ± 1.2	90 ± 3.2	+ 8	2.3	8.6
5	83 ± 2.4	75 ± 1.3	+ 8	6.0	7.7
6	68 ± 3.6	60 ± 1.5	+ 8	0.7	8.4
Means	83.5	78.33	+ 5.166 * *		

* * Significant difference at the 1% level of probability

TABLE 22

Effect of hot water treatment on germinability of 18 months old seed

Seed Line No.	Germination %		Relative advantage of untreated over hot water treated seed	Infection % of A. brassicicola	Moisture %
	Untreated seed	Hot water treated seed			
	Mean ± S.E.	Mean ± S.E.			
1	84 ± 2.1	82 ± 1.5	+ 2	4.0	8.2
2	98 ± 0.4	95 ± 0.9	+ 3	1.0	7.8
3	77 ± 2.6	72 ± 2.3	+ 5	5.3	8.9
4	98 ± 1.1	88 ± 2.9	+ 10	1.0	8.1
5	81 ± 2.3	71 ± 4.0	+ 10	4.3	8.5
6	92 ± 1.5	93 ± 1.2	- 1	1.3	7.7
7	91 ± 1.5	93 ± 2.7	- 2	3.3	8.9
8	92 ± 2.1	88 ± 0.9	+ 4	1.00	8.0
Mean	89.125	84.0	+ 3.875 *		

* Significant difference at the 5% level of probability

F. DISCUSSION

A.brassicicola was present in a high percentage of the seed lines screened. As the combined G.S.T.S. surveys and those conducted in this study cover a four year period it seems reasonable to suppose that this pathogen is present in a major proportion of New Zealand seed lines in most years. A.brassicae was not as prevalent and did not appear to survive in stored seed for long periods. These results support the findings of Neergaard (1945) who states, "in my laboratory examinations of seeds of Cruciferae I have found A.brassicae only in 3-4% of the seed lots attached by siliqua moulds and practically always as weak infections". On the other hand, seed-borne inoculum of A.brassicicola has been shown to remain viable for long periods in storage and could therefore be capable of causing seedling infections on sowing.

In germination trials incorporating the analysis of dead seed and abnormal seedlings (Section C 2.) to establish the presence of pathogenic contaminating inoculum of the Alternaria pathogens, the percentage recorded was higher than that obtained by the agar plating method. This demonstrates that surface sterilisation in health tests eliminates or reduces the amount of inoculum capable of causing a seedling infection. The infection percentages obtained using such a technique in health tests must be regarded as representing something less than the true level of disease potential.

It has been shown that hot water treatment of seed at 50°C for 25 minutes eliminates *Alternaria* species from choumoellier seed, but this was attended by a reduction in germinability. The adverse effect on germination did not appear to be associated with either the presence of *Alternaria* infections, the seed age, or the moisture content of the seed at the time of treatment. However seed weakness probably results from the synergistic action of many factors and thus the results expressed after hot water treatment reflect an overall reaction which may have obscured the expression of any single "seed weakness" factor such as infection by *A.brassicicola*.

In preliminary trials on the viability of contaminating inoculum, seed from an "*Alternaria* free" line was surface sterilised and contaminated with spores of *A.brassicicola*. After storage under dry conditions for six months it was found that 14% of the sample was infected with *A.brassicicola*. This means that the fungus is capable of causing seed infection as a result of heavy contamination in storage. Hewett (1965) has shown that while hot water treatment of cereal seeds kills most of the seed-borne micro-flora, massive superficial recontamination by moulds may occur, and he suggests that handling of seed during treatment may increase seed coat damage, facilitating reinfection. By similar reasoning it is suggested that damage to choumoellier seed

caused by threshing and cleaning could be important in the same way. With the dispersal of spores from crop debris during harvest and the presence of seed at various moisture levels providing a favourable micro-climate within a bag, then the possibility of seed infection by contaminating spores during the harvesting, handling, and storage processes of choumoellier seed production cannot be discounted.

CHAPTER III

EFFECT OF THE PATHOGENS ON SEEDLING DEVELOPMENT

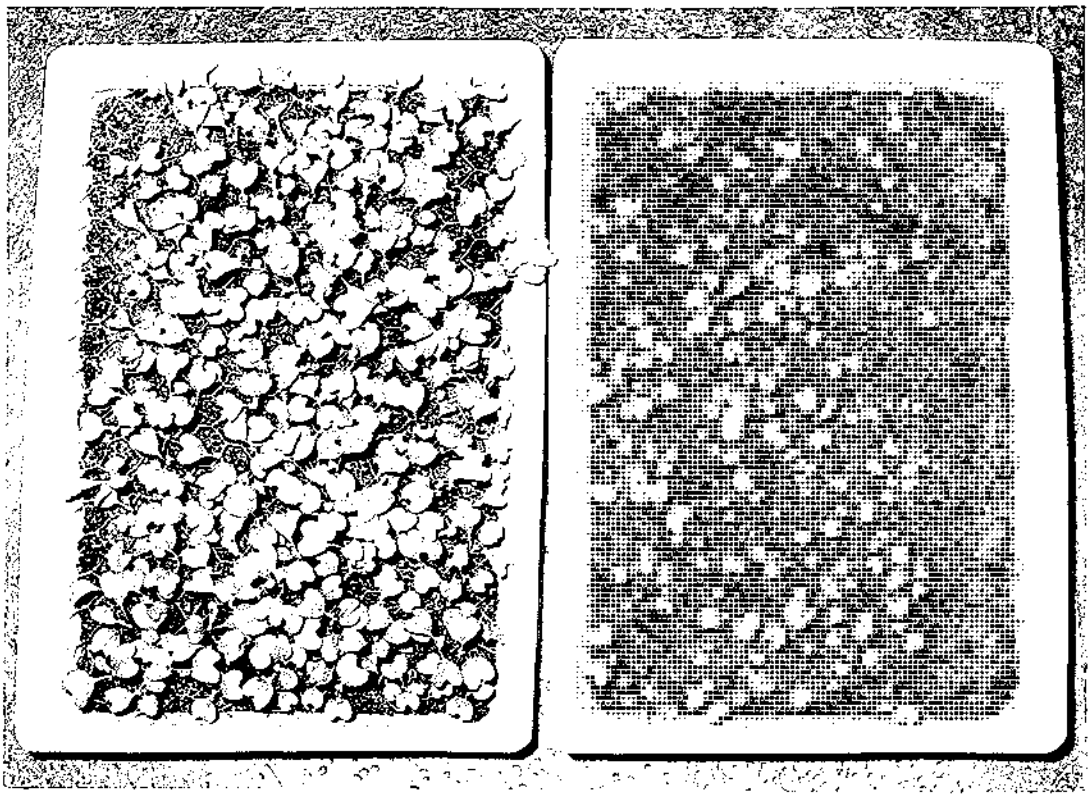
A. INTRODUCTION

The association of a pathogenic fungus with seed ensures a close host-pathogen relationship during seedling development. Many seed-borne fungi have been demonstrated to be active in causing seed-bed losses due to seedling attack, and such losses are usually discussed under the relative terms of pre-emergence damping-off and post-emergence damping-off. Pre-emergence losses are considered to occur when a germinating seed is killed before above ground evidence of the seedling is apparent, while in post-emergence damping-off the seedling is killed following emergence from the soil.

Overseas workers (Weimer, 1924, 1926; Rangel, 1945; Neergaard, 1945; Schimmer, 1953; McKay, 1956; McDonald, 1959; Changsri and Weber, 1963) have shown seed-borne inoculum of A.brassicae and A.brassicicola to be active in causing pre-emergence and post-emergence seedling damping-off of crucifers which may result in severe reductions in stand establishment.

Examination of lesioned seedlings, collapsed seedlings, and non-germinating seed in high humidity blotter germination tests, proved both pathogens to cause seed and seedling death. Further, preliminary glasshouse trials indicated that A.brassicicola could be instrumental in pre-emergence and post-emergence seedling losses when present on the seed as adhering spores (Plate 9) or as deep seated infection. A.brassicae also caused some seedling injury but did not appear to be as pathogenic

Plate 9



Seedlings grown from clean seed (control)

Seedlings grown from contaminated seed

Effect on emergence of seed artificially contaminated with A.brassicicola

as the small spored species. As a result of these findings and in view of the seedling destruction in brassica seed sowings demonstrated by overseas workers, trials were initiated to determine the importance of A.brassicicola and A.brassicae in causing seed-bed losses.

B. MATERIALS AND METHODS

In the following section materials and methods generally used throughout the chapter are discussed. Those specific to particular experiments are detailed in the appropriate sections.

1. Seed line used in the artificial contamination and infection of seed for emergence trials

A seed line (line 13819) with a 100% germination percentage was identified as free from infection of both Alternaria pathogens by a series of screening trials. A bulk supply of this line was purchased for use in subsequent emergence experiments.

2. Seed treatment in emergence studies

(a) Surface sterilisation of seed

Surface sterilisation was effected by immersing the seed in a 0.001% solution of mercuric chloride for 10 minutes (McLean, 1947), rinsing in sterile distilled water and drying the seed between filter paper pads.

(b) Hot water treatment of seed

Hot water treatment was carried out in a thermostatically controlled water bath at 50°C for 25 minutes (Crosier and Patrick, 1940). Following treatment, seed was cooled rapidly by immersion in cold water and air dried.

(c) Seed contamination

(i) A.brassicicola

A 20 gm sample of surface sterilised seed was shaken in a Petri plate containing a sporulating colony of A.brassicicola grown on MA at 24°C.

(ii) A.brassicae

Contamination of seed with A.brassicae was achieved by agitation of a 2 gm sample of surface sterilised seed in a sporulating colony of this pathogen grown on PDA at 24°C.

3. Seedling production

Plastic seed boxes (14" x 11" x 2¼") were sterilised with a dilute sodium hypochlorite solution and wiped down with alcohol before use. A standard UC type C soil mix (Baker, 1957) was employed for seedling emergence studies. Seeds were sown in a wire mesh grid (Plate 9), one seed per cell, using forceps and a mechanical counter. The grid enabled systematic counting of seedlings in observations made during the trials. Trays were labelled using a random lettering system, and unless

otherwise specified, watering was carried out by steeping the seed boxes in shallow water troughs.

Emergence trials were conducted during the winter months in a glasshouse warmed by thermostatically controlled heaters. A hydrothermograph run in conjunction with the experiments showed temperature extremes of 54° and 70°F but generally ranged between 60° and 65°F. Humidity varied between 45 and 70%.

4. Examination of seedlings

At the completion of the emergence trials, the germination percentage was recorded and individual seedlings were assigned to infection categories on the basis of the severity of the attack and location of the infection on the seedling. Categories used are illustrated in Figure 9 and are based on observations made in preliminary trial work.

5. Re-isolation from infected seedlings

Re-isolation from infected tissue pieces was carried out using one of two methods.

(a) Chemical method

Tissue pieces were enclosed in muslin bags, sealed with identification bands and immersed in a 1:1 solution of 0.001% mercuric chloride and 95% alcohol (Weimer, 1926) for 15 seconds, rinsed in three washes of sterile distilled water, dried between filter pads and plated to MA.

Figure 9

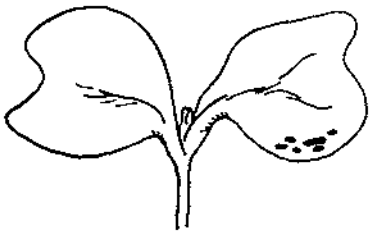
Definition of terminology used in describing symptoms expressed during germination by abnormal seedlings and infected seedlings parasitized by A.brassicicola

Code -

I	Cotyledon lesions	:	Category 1
II	Cotyldeon lesions	:	Category 2
III	Cotyledon lesions	:	Category 3
IV	Post-emergence damp-off symptoms		
V	Abnormal development		
VI	Hypocotyl lesions	:	Category 1
VII	Hypocotyl lesions	:	Category 2
VIII	Hypocotyl lesions	:	Category 3

(Overleaf)

CATEGORIES USED IN GERMINATION TRIALS



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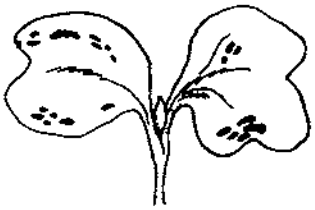

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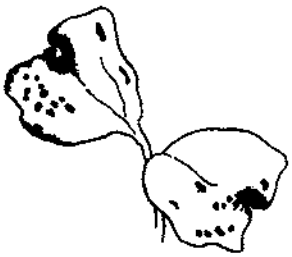


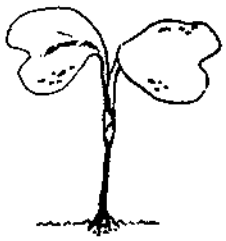
III

III

III

IV

IV



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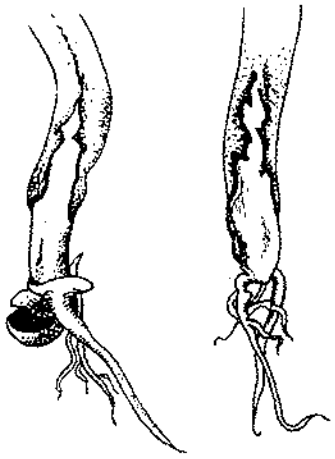

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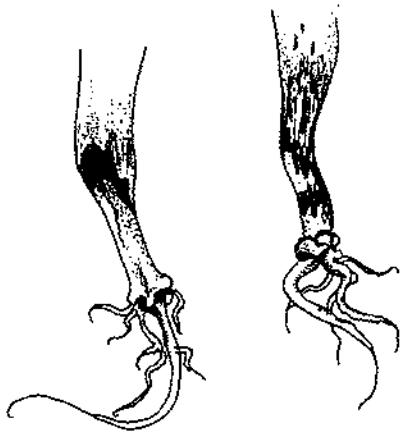
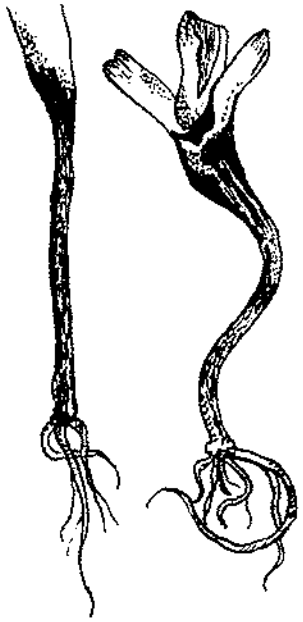


VII



VIII

(b) Washing method

Tissue pieces were enclosed in muslin bags and washed for two hours in running tap water (Milne, 1964). They were then rinsed in sterile distilled water before drying between filter paper and plating to MA.

Results were recorded for both methods after six days incubation at 24°C.

C. EFFECTS ON EMERGENCE

In the screening trials no lines were identified as carrying high percentage infections of one pathogen and not the other. Therefore, to assess the potential of each of the fungi in causing seed bed losses it was necessary to synthesise a 100% diseased line. Infection and contamination levels of 100% were considered desirable to minimise the sampling error and to obtain maximum disease expression under the experimental conditions.

1. The potential effect of contamination on emergence

Method

Seed was surface sterilised and samples contaminated with A.brassicae and A.brassicicola respectively. Four replications of 100 seeds were sown for each treatment with a further control series of clean seed. Watering was carried out by steeping the flats in a shallow water bath and in addition,

seedlings were misted with a de Vilbiss atomiser which gave a fine spray sufficient to wet the foliage without causing splashing between seedlings. Results were recorded at 10, 14, 18, and 22 days (Appendix II) to enable observation of disease development, with a final analysis at 30 days (Appendix III). In the interim observation, cotyledon attack and post-emergence damping-off symptoms were indexed to the categories illustrated in Figure 9. In the final examination at 30 days, individual seedlings were lifted, enabling a more comprehensive assessment of symptoms.

RESULTS AND DISCUSSION

The results presented in the following table and detailed in Appendix III show that seed contamination by both pathogens has caused a reduction in the number of seedlings established 30 days after sowing.

TABLE 23

The potential effect of seed contamination with A.brassicae and A.brassicicola on emergence

Treatment	Emergence (%) Mean ± S.E.
Seed contaminated with A.brassicicola	66.2 ± 2.0
Seed contaminated with A.brassicae	86.0 ± 2.4
Control - clean seed	94.7 ± 1.1

A 28.5% emergence reduction was caused by A.brassicicola

and in addition many of the surviving seedlings were infected with the small spored pathogen (Appendix III). In 57% of the seedlings attacked at ground level (35% of the total emergence) the infection was manifest as a discolouration and splitting of the cortex of the hypocotyl starting near the point of root initiation (Plate 10). The remaining 43% (26.5% of the total emergence) showed a definite wire stem collapse (Plate 10) severe enough to have a possible effect on subsequent development, and were indexed to categories 2 and 3 (Figure 9).

Until the 18 day scoring, most of the post-emergence damping-off symptoms recorded were due to cotyledonary necrosis. At the 30 day examination, 52.5% of the seedlings with cotyledonary lesions were indexed to categories 2 and 3, (31.5% of the total emergence), but it was considered that in the majority of instances necrosis of the cotyledons would not influence seedling development directly. After this period the seedlings were generally at the two true leaf stage, and despite severe cotyledonary lesions, the young leaves were free from evidence of infection. On the other hand, it is possible that a diseased cotyledon could collapse and adhere to the hypocotyl of the young plant providing a source of inoculum in close contact with the healthy tissue, or that a shrivelled infected cotyledon could lodge close to the foot of the plant after being shed. However, severe cotyledon lesioning in the early growth stages of the seedling did appear to affect

Plate 10

Hypocotyl lesions caused by A.brassicicola

subsequent development. For example, those seedlings indexed to "category 3" at the 10 and 14 day recordings were largely responsible for early post-emergence losses, and frequently, surviving seedlings were reduced in size.

A.brassicae caused an 8.7% emergence reduction and of the remaining seedlings 12.5% exhibited hypocotyl lesioning and 15.0% cotyledon lesions. However, in the majority of such attacks, infection was not severe and was unlikely to affect subsequent seedling development.

By assuming that 14 days was the interval after which maximum emergence occurred in each sowing it was possible to estimate the extent of pre-emergence and post-emergence losses. This assumption was based on the fact that after the 14 day reading, emergence figures for the A.brassicicola treatments decreased. The slight increase in the emergence percentage recorded in the control sowings after this interval was considered to compensate for post-emergence losses caused by the failure of abnormal seedlings to develop. Thus by subtracting the average percentage recorded at 18, 22 and 30 days from the average figure for the 14 day reading, an estimate of post-emergence damping-off was obtained. Similarly, pre-emergence attack by the pathogens was estimated by adding the average final emergence at 30 days to the estimated post-emergence damping-off losses for the respective trials and subtracting this total from the average final emergence figure

expressed by the corresponding control treatment. The data presented in Table 24 indicates that both pathogens caused approximately three times more seedling losses due to post-emergence damping-off compared with pre-emergence losses.

While the above results demonstrate a "potential" emergence loss under the sowing conditions due to contamination of seed lines, it seemed unlikely that inoculum loads would be as high in naturally contaminated samples. To assess the inoculum load of naturally infected lines relative to that of the artificially contaminated lines, samples were examined using a standardised centrifuge technique and comparative spore loads estimated on the basis of a haemocytometer count. From the 40 commercial lines investigated, the average spore load for A.brassicicola was 2,500 spores/ml and the heaviest load recorded for any one line was 14,000 spores/ml. Similarly, for A.brassicae the average was 800 spores/ml and the heaviest load 7,000 spores/ml. The spore loads recorded from the artificially contaminated samples were 200,000/ml for A.brassicicola and 86,000/ml for A.brassicae.

It is apparent that under natural conditions it is in fact unlikely that contamination of seed by the two Alternaria pathogens would reach the proportions of the samples used in the previous experiment. Contamination of individual seeds may reach high levels, but uniform distribution of spores throughout a sample does not seem likely.

TABLE 24

Pre- and post-emergence seedling death in emergence trials

Treatment	Post-emergence damping-off (%)			Pre-emergence damping-off (%)
	18 days	22 days	30 days	
	Total	Total	Total	
Seed contaminated with A. brassicae	0	2.75	6.00	2.25
Seed contaminated with A. brassicicola	4.75	13.50	20.75	7.25
Clean seed	0	0	0	0

2. The potential effect of seed infection by A.brassicicola on emergence

To assess the potential of seed infection in causing seed-bed losses it was first necessary to perfect a method for artificially infecting all seed of a line with the Alternaria pathogens. In preliminary trials seed was placed between moistened folds of newspaper and misted with a concentrated spore suspension. This method was unsuccessful as the seed coat split and germination occurred before infection was achieved. Attempts to prevent germination by alternating moist and dry conditions were also unsuccessful. Further research led to the following method which utilised the differential moisture requirements for spore germination and penetration, and seed germination.

Plastic cabinets were adapted to provide an infection apparatus (Plate 11). Each tray consisted of three cells, of which the centre one contained moistened blotter paper and was separated from the neighbouring cells by perforated plastic divisions. The two outside cells contained light aluminium foil trays in which artificially contaminated seed was placed. The cabinets were sealed in plastic bags to promote high humidity conditions during the experiment which caused a softening of the seed coat and favoured spore germination and seed infection. Although a seed line 100% infected with A.brassicicola was obtained using this method,

Plate 11

Infection cabinets used in artificial infection of seed

(as demonstrated by a blotter health test) successful infection with A.brassicae was not achieved. It is possible that the inability of A.brassicae to form an appressorium precluded penetration of the seed coat. However, in view of the relatively low occurrence of A.brassicae in seed lines screened in previous trials further attempts to infect seed with this pathogen were considered to be unwarranted. Hence the following emergence experiment pertains only to the effect of seed infection by A.brassicicola.

Method

Seed to be infected was surface sterilised in mercuric chloride and then contaminated with spores of A.brassicicola. After incubation in the infection cabinets for 25 days, seed was air dried for 24 hours and then surface sterilised to ensure that only seed infection was involved. Four replications of 100 seeds each were sown to soil in glasshouse flats. A control series of four 100 seed replicates of clean seed which had also been exposed to high humidity for 25 days was sown under the same conditions as the artificially infected line.

After 30 days individual seedlings were lifted and indexed to the categories previously illustrated in Figure 9.

RESULTS AND DISCUSSION

The results presented in Table 25 show that a marked reduction in emergence has resulted from seed infection with A.brassicicola.

TABLE 25

The effect of artificial infection of seed with A.brassicicola on emergence

Treatment	Mean ± S.E.
Artificially infected seed	38.2 ± 3.9
Clean seed exposed to high humidity	84.2 ± 2.6

Of the seedlings that emerged from infected seeds 46.5% exhibited cotyledon or hypocotyl lesions but in most cases development did not appear to be affected by the attack.

3. Effect of seed-borne inoculum on emergence in commercial seed lines

Seed-borne inoculum has been demonstrated to cause seedling death when present on seed under artificial conditions of high inoculum loads. An experiment was conducted to ascertain if such seedling losses were significant in sowings of commercial seed lines.

Method

Four seed lines with infection percentages of A.brassicicola ranging from 5 - 10% (determined by an agar plate test) were selected for the trial. Samples of each seed line were sown to glasshouse flats after treatment as follows :

(i) untreated seed;

(ii) hot water treated seed;

(iii) surface sterilised seed.

Results were recorded after 30 days by individual examination of seedlings and by indexing lesions to the categories illustrated in Figure 9.

RESULTS AND DISCUSSION

The presence of A.brassicicola in the seed lines sown did not cause a significant reduction in emergence under the conditions of this experiment (Table 26). That the pathogen was present and active in causing seedling infection is evidenced by the presence of diseased seedlings in the stands. However, most of the seedlings were not severely infected and in the majority of cases would probably have "grown through" the infection. Elimination of the surface inoculum by surface sterilisation of seed resulted in no increase in emergence and further, hot water treatment reduced emergence. In view of the germination reduction due to hot water treatment demonstrated in Chapter II the above reduction was also considered to be a treatment effect.

4. Effect of seedling infection by A.brassicicola on seedling development

Observations in the previous emergence trials indicated that seedlings which survived cotyledonary and hypocotyl attack

TABLE 26

Effect of seed infection on the emergence of commercial seed lines *

Seed Line	Treatment	Emergence (%)	Hypocotyl lesioning (%)			Cotyledon lesioning (%)		
		Mean ± S.E.	1 Mean	2 Mean	3 Mean	1 Mean	2 Mean	3 Mean
X_1	Untreated	88 ± 3.1	5.25	1.00	0.50	9.50	3.5	0.50
	Surface sterilised	85 ± 2.6	1.00	0.00	0.50	5.75	2.50	1.50
	Hot water treated	82 ± 2.8	0.25	0.25	0.00	3.75	0.25	0.00
X_2	Untreated	62 ± 3.1	6.0	1.25	0.50	8.00	2.25	1.00
	Surface sterilised	67 ± 4.6	2.0	2.00	0.50	7.00	1.00	0.25
	Hot water treated	51 ± 4.2	0.75	0.75	0.00	4.00	0.00	0.00
X_3	Untreated	65 ± 2.3	5.75	1.25	0.50	11.0	3.00	0.75
	Surface sterilised	68 ± 5.1	1.50	1.75	1.50	7.5	2.00	1.25
	Hot water treated	64 ± 1.9	2.25	0.00	0.00	1.0	0.75	0.00
X_4	Untreated	80 ± 3.7	1.50	2.00	1.25	5.75	2.50	3.00
	Surface sterilised	76 ± 3.6	0.00	0.25	0.25	5.50	2.00	1.75
	Hot water treated	63 ± 2.6	1.25	0.00	0.00	2.50	0.25	0.00

* Four replicates of 100 seeds were sown per treatment for each seed line

in the early stages of development were often reduced in size. These seedlings appeared to "grow through" the infection in many cases, but is suggested that this initial setback could be reflected in later development of the plant. An experiment was carried out to enable a more objective assessment of the effect of this reduction in seedling size.

Method

Two hundred seeds were contaminated with A.brassicicola and sown to glasshouse flats. A further 200 clean seeds were sown under the same conditions. Records were taken after seven weeks by individual examination of seedlings and by recording the weight of the total aerial tissue. Root systems were not included due to difficulties involved in dislodging adhering sand and peat fragments, without also removing plant tissue. Both treatments were overhead watered using a sprinkler system.

RESULTS AND DISCUSSION

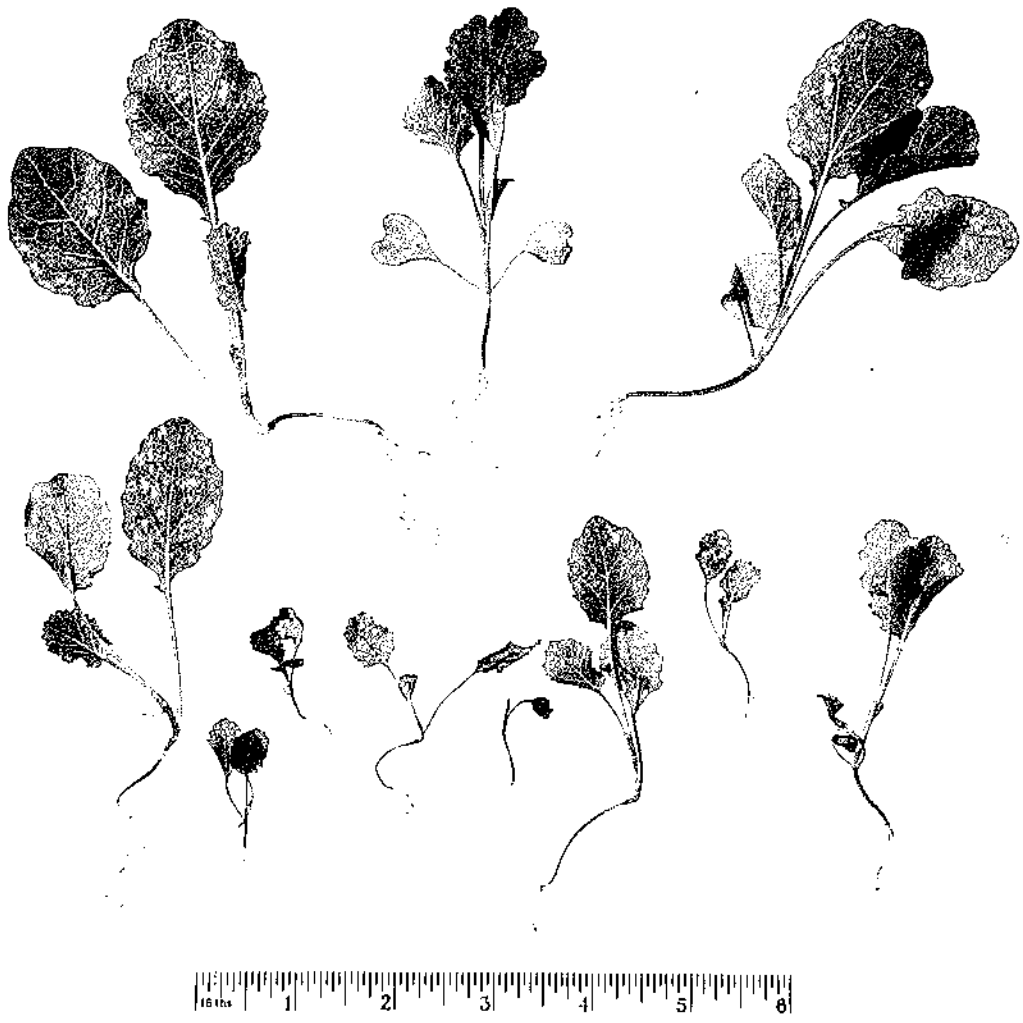
A marked reduction in the weight of aerial tissue was recorded for seedlings grown from contaminated seed (Table 27). The average weight of tissue from individual seedlings was 0.246 gm (approximately 40%) less than the average weight from seedlings grown from clean seed in the control series. Examination of individual seedlings indicated that this was mainly the result of stem constrictions due to ground level infection. Such seedlings exhibited a "woody" wire stem symptom, and were frequently reduced in size (Plate 12).

TABLE 27

The Effect of Seed Contamination on Seedling Development

Treatment	Emergence (%)	Total green weight of the above ground material (gm)	Average weight per plant (gm)	Hypocotyl lesions (%)			Leaf Lesioning (%)
				1	2	3	
Contaminated seed	52.0	40.04	0.385	9.5	7.5	10.5	18.0
Control - clean seed	94.0	118.70	0.631	2.5	1.5	0	1.5

Plate 12



Effect of infection on seedling development

Top - healthy seedlings

Bottom - diseased seedlings

The reduction in plant size was not uniform in the sowing. Many seedlings exhibited normal development from contaminated seed, while others were extremely stunted and probably would not have survived under field conditions.

D. MECHANISM INVOLVED IN SEEDLING ATTACK

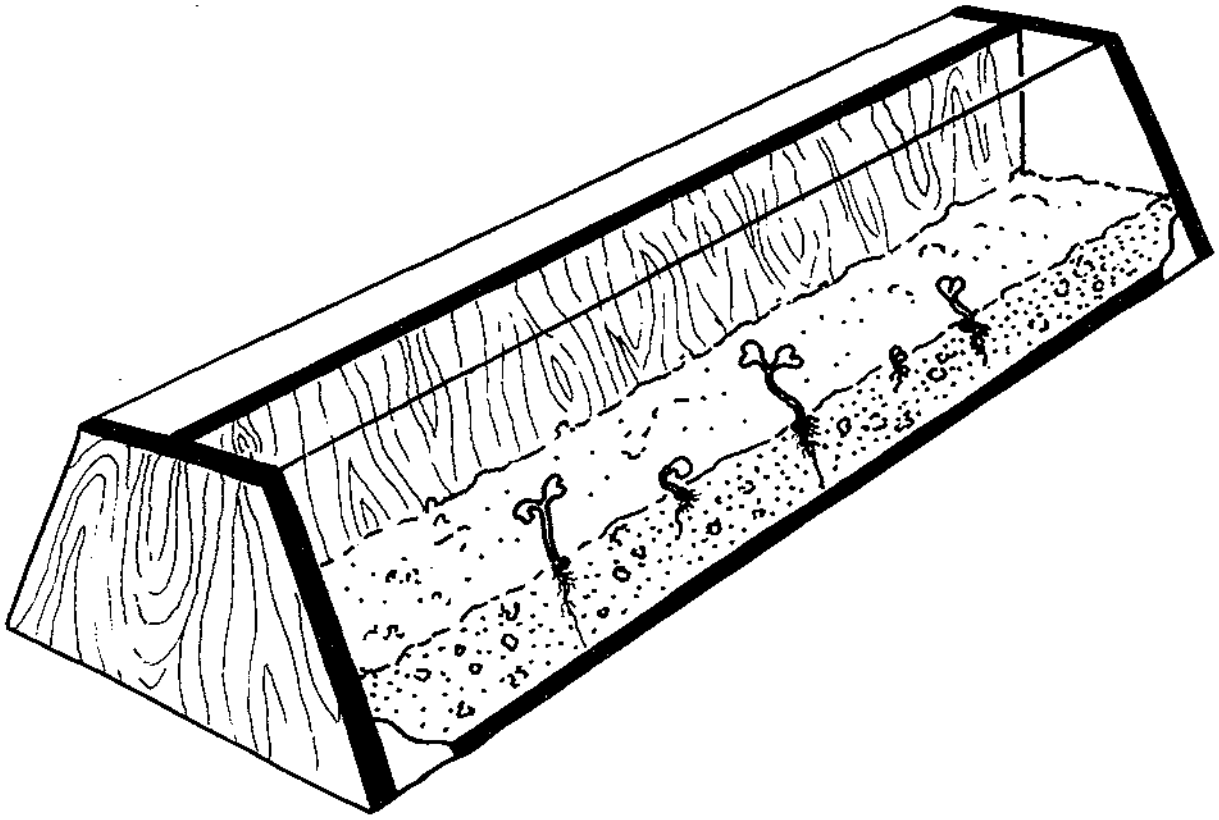
Observations on infected seedlings in emergence trials indicated that seedling collapse resulted from cotyledonary infection or decay of the hypocotyl at ground level. To examine the mechanism of seedling attack more closely an experiment was devised in which infected growing seedlings could be observed from day to day and disease progress noted.

Method

Seed-boxes were constructed with glass "slide in" walls (Figure 10) inclined at a slight angle from the vertical to encourage growth close to the glass face enabling observation of individual seedlings.

Two samples of surface sterilised seeds from a disease-free line were contaminated with spores of A.brassicicola and A.brassicae respectively. From each treated sample, 35 seeds were planted in a vermicullite medium in close proximity to the glass face. A further 35 seeds from a line of seed artificially infected with A.brassicicola were sown, and finally a control series of clean seed was set out. The seed-boxes

Figure 10



Development of glass-faced seed boxes used to enable close observation of symptoms on diseased seedlings

were held at room temperature and kept moist by a daily misting with a De Vilbiss atomiser.

Isolations from lesioned tissue were made to MA following surface sterilisation in a 1:1 solution of 0.001% mercuric chloride and 95% alcohol.

RESULTS AND DISCUSSION

Several forms of injury were observed to cause seedling collapse :-

(i) The young radicle was attacked soon after germination was initiated, arresting development at that stage. Root hairs did not develop and the radicle tip discoloured and died (Plate 13). This type of injury occurred frequently where seed was infected with A.brassicicola and in emergence studies would probably be assessed as pre-emergence damping-off. A similar attack was observed at an early post-emergence stage of development (Plate 13), in which cotyledons had developed but were severely lesioned.

(ii) Commonly, damping-off occurred as the result of the collapse of severely lesioned cotyledons and the decay extending to the upper hypocotyl region. In most cases the infected or contaminated seed coat remained attached to the cotyldeon and was carried above ground during seedling development (Plate 14). Lesioning initiated as necrosis and decay in the immediate tissue

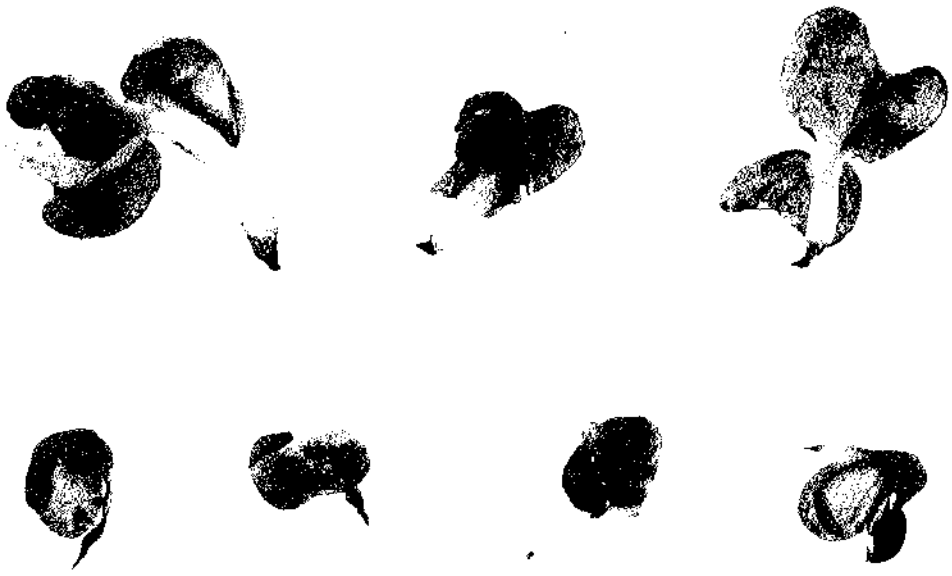
surrounding the seed coat. In some instances the infected cotyledons wilted and following collapse became attached to the hypocotyl providing a focus for stem infection.

Cotyledonary attack by A.brassicicola was frequently manifest as sharply defined dark-brown to black spots, often with a shrunken appearance and giving an overall "fleck symptom". A.brassicae caused purple-black to brown lesions similar to the small spored species, but generally more localised.

(iii) Ground level attack was characteristic of both species. Hypocotyl lesions were similar for both, and appeared as narrow dark-brown to black "streak-like" lesions about 1 mm long. Severe infection at early stages of development resulted in a soft waxy hydrotic area at the base of the stem and the seedlings collapsed and died.

More frequently, infection by A.brassicicola caused a collapse of the cortical tissue of the hypocotyl forming a layer of dead cells and resulting in a constriction and wire-stem symptom (Plate 15). In many cases seedlings survived such hypocotyl attack but the stems were distorted, the root systems reduced, and the growth of the plant impaired. Less severe infection was apparent as splitting of the cortex of the hypocotyl along a line of lesioning extending up the stem from

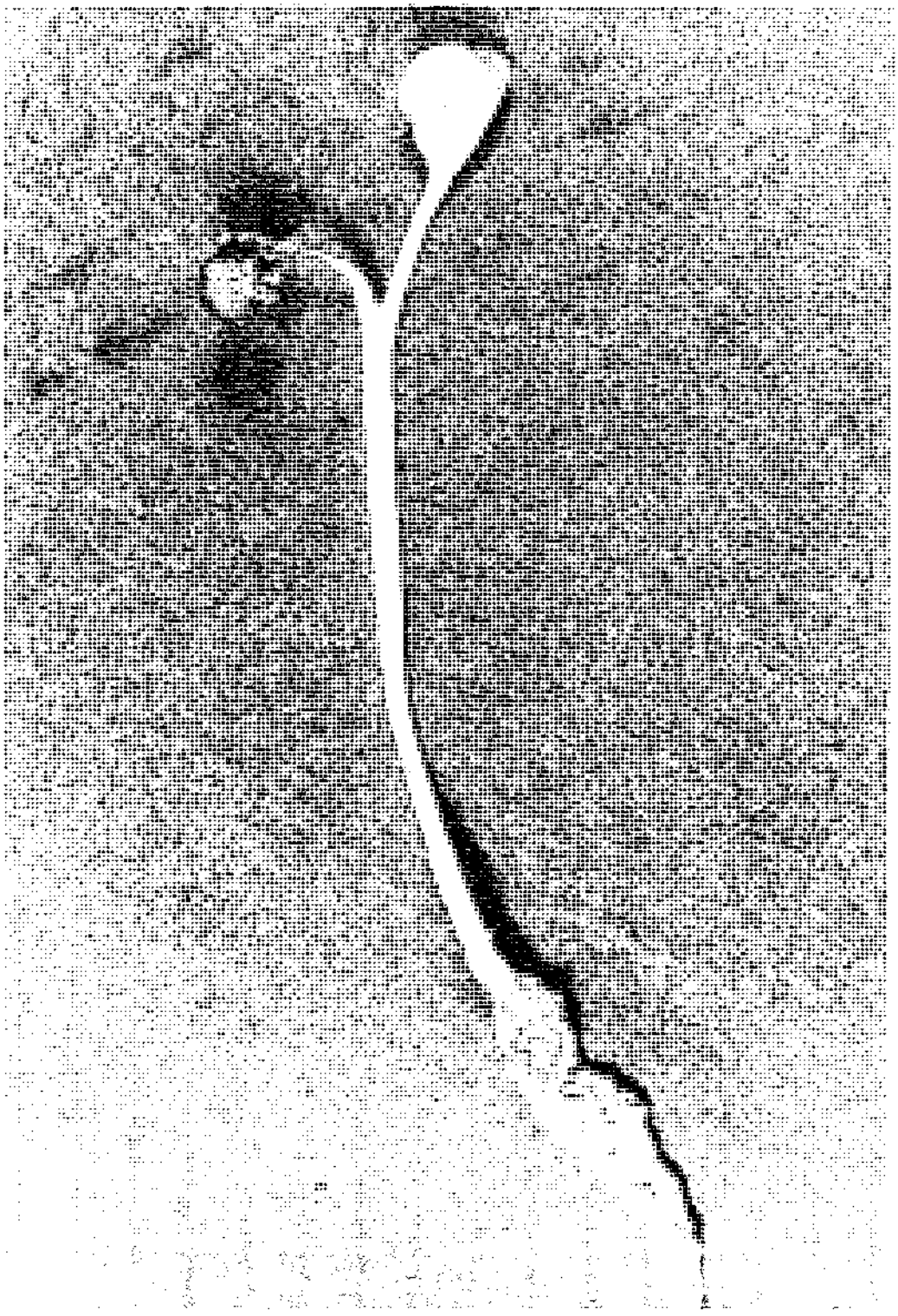
Plate 13



Early stage symptoms of damping-off
caused by A.brassicicola

Top - early post-emergence
Bottom - pre-emergence

Plate 14



Cotyledon infection of A.brassicae
originating from the attached testa

the point of root initiation (Plate 15). The growth and development of these seedlings did not appear to be markedly affected. In most cases of severe hypocotyl attack the seed coat had previously been shed from the cotyledons and remained clasping the base of the hypocotyl or lying in a position close to it.

Microtome sections of decayed stems showed mycelium of the pathogen ramifying through the infected tissue (Plate 16). The mycelium appeared at first to be intercellular, but later the diseased tissue was permeated with hyphae.

E. DISEASED SEEDLINGS AS A SOURCE OF INOCULUM IN THE SEED BED

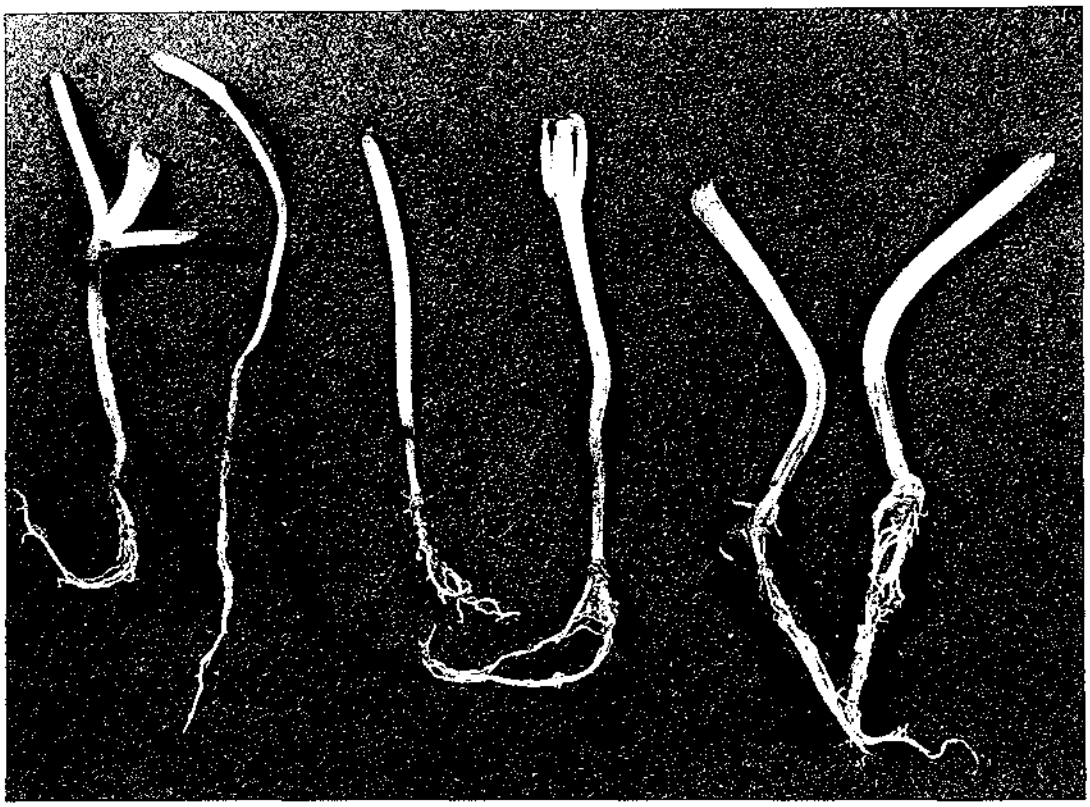
Seed-borne diseases may influence the development of a crop in two ways :

(i) through a direct effect on seed and seedling health causing pre-emergence and post-emergence losses which are reflected in a poor stand establishment;

(ii) by sporulation on infected and decaying seedlings providing a source of inoculum for spread to healthy plants with ultimate spread of the disease throughout the crop.

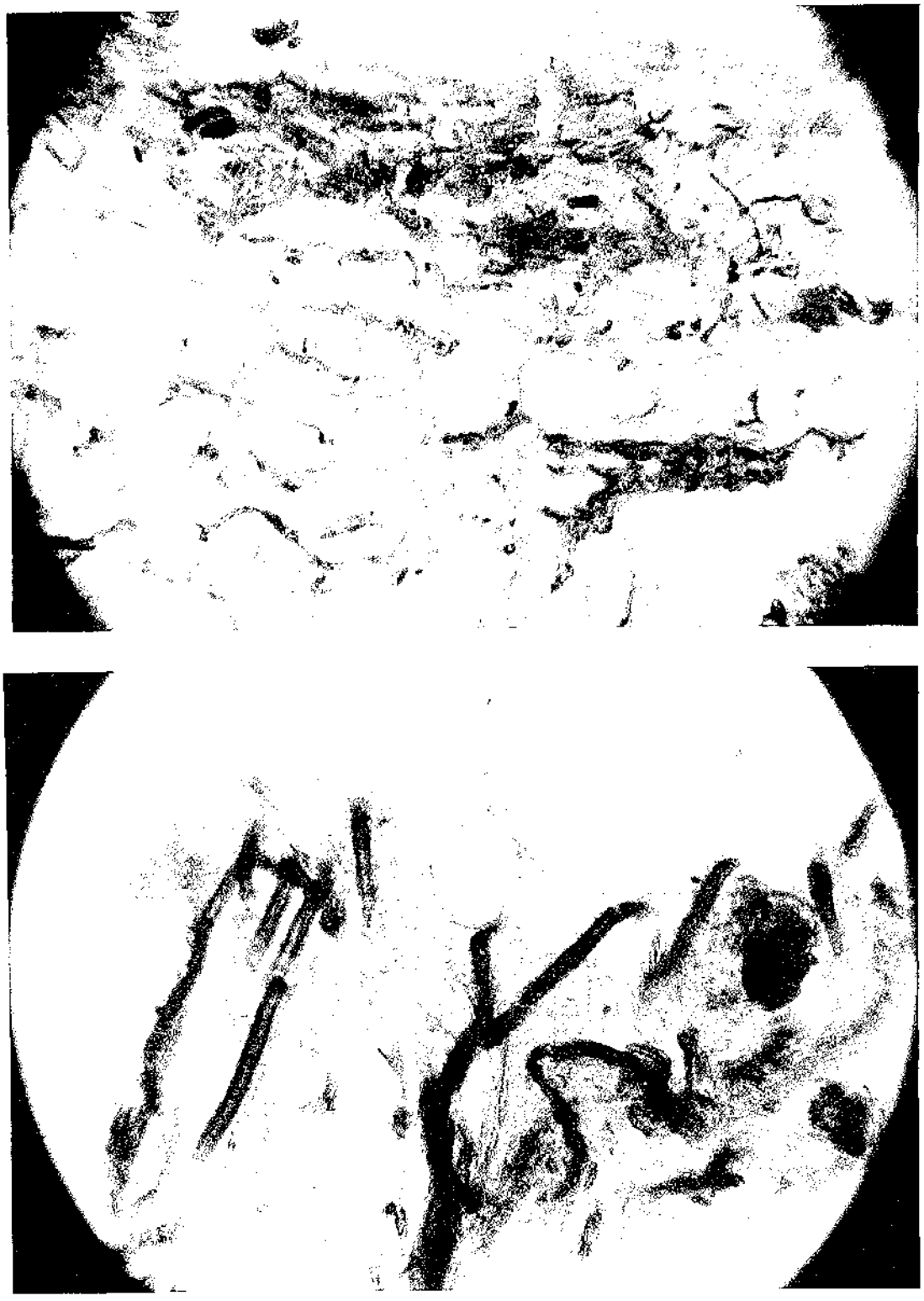
A.brassicicola has been shown to cause a significant

Plate 15



Wire-stem symptoms and cortical splitting of the hypocotyl caused by A.brassicicola

Plate 16



Microtome sections of hypocotyl tissue infected with A.brassicicola

reduction in stand establishment when associated with seed at high levels of contamination and infection. But in this study, the presence of the pathogen in commercial seed lines did not cause significant seedling losses. However, no consideration was allowed for the potential spread of inoculum from diseased seedlings in the seed bed.

Pound et al (1951) considered that the spread of A.brassicicola was practically nil in cabbage seed beds in Wisconsin. Conversely, Rangel (1945) notes the importance of infected cabbage seedlings in a box providing a source of inoculum for the initiation of secondary spread, and in addition, McDonald (1959) considered that spores of A.brassicae produced on diseased seedlings of rape were possible inoculum sources for the spread of the pathogen to healthy plants. In support of this, spore dispersal by wind and rainsplash in mature field crops has been demonstrated in preliminary investigations in this study. Spores of both Alternaria species were trapped on prepared agar slides placed at different levels in choumoellier stands. It seemed likely that dispersal of inoculum from sporulation on decaying seedlings could also be instrumental in disease spread in the seed bed. Experiments were conducted to test this supposition.

1. Glasshouse trials

Method

Four replicates of 100 seeds were contaminated with

A.brassicicola prior to sowing. The flats were watered at three day intervals by an overhead sprinkler system which caused a 'water-splash' effect between seedlings. Seedlings were lifted and individually examined for evidence of infection after 30 days. By comparing the results recorded with those obtained in the previous emergence experiment on contaminated seed (Appendix III), an estimate of disease spread due to spore dispersal was made. A control sowing of clean seed was also planted.

RESULTS AND DISCUSSION

The results presented in Appendix IV show that seed contamination with A.brassicicola caused a marked emergence reduction. A comparison of the emergence percentage recorded above with that obtained in the immersion watered series (Appendix III) shows that under these conditions spore dispersal by splashing did not increase seed mortality (Table 28). An important feature of the comparison is the effective dispersal to, and infection of the first true seedling leaves in the overhead watered sowings. Such infections may carry the pathogen through to the mature crop phase where high humidity conditions within the stand would promote sporulation on lesioned tissue, thereby providing inoculum for further disease development.

However, the experiment was conducted under an artificial situation in that seedlings were crowded and grown

TABLE 28

Effects of watering on seedling infection

Treatment	Total Emergence %	Hypocotyl lesioning (%)			Cotyledon lesioning (%)			Leaf lesioning %
		1	2	3	1	2	3	
	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
Overhead watering	71.5 ± 3.12	24.75 ± 2.65	12.25 ± 1.38	12.75 ± 1.38	14.0 ± 1.87	21.5 ± 1.68	6.5 ± 1.18	8.5 ± 1.85
Immersion watering	66.25 ± 2.04	23.25 ± 1.50	8.5 ± 0.91	9.0 ± 1.78	19.0 ± 1.47	13.0 ± 1.23	7.75 ± 1.12	0.25 ± 0.00

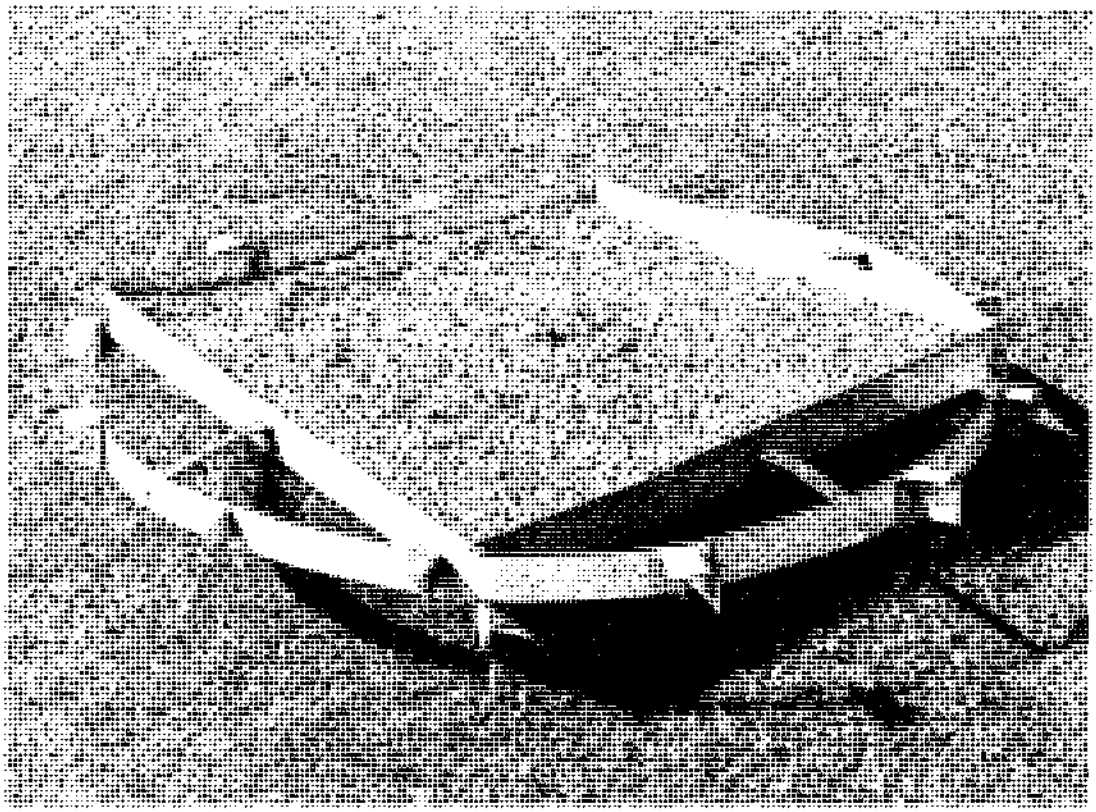
under relatively high humidity conditions. Hence, while the results may indicate a potential spread in the seed bed it was considered desirable to repeat the experiment under conditions more appropriate to a field sowing.

2. Field trials

Method

A shallow seed box measuring 2 ft. 6 in. square was constructed from galvanised iron. This was mounted on a shaft and bearing to allow it to rotate, thereby ensuring equal environmental conditions over the tray. A circular hoop with a series of "vanes" attached to the outside was fixed around this to form the driving mechanism (Plate 17). By directing the flow of water from a hose into the vanes with a 90^{o} angle of attack from the centre of the apparatus, a turning motion was achieved. Splashing was overcome by constructing each driving vane with a prominent upper lip which diverted the splashing away from the box. By adjusting the water flow the rotation of the apparatus was regulated to approximately four to five revolutions per minute. The bottom of the box was drilled out at intervals to allow drainage, and a wire mesh disc placed over each hole to retain the vermicullite medium. At the centre of the box six seedlings (two weeks old) were placed out in a circle and spores of A.brassicicola brushed over the cotyledons. At distances of 3, 6, 9, 12 and 15 inches from these seedlings eight healthy plants per spacing interval

Plate 17



Apparatus for measuring the
effective dispersal of spores

were placed out to form a "broken spiral". Over a three week period the apparatus was exposed to wind and rain for intervals of four hours and then incubated under a plastic "tent" for 72 hours to promote high humidity conditions. The seedlings were then examined for the presence of lesion symptoms. The humidity within the tent varied between 50 and 70% and temperatures ranged from 40°F to 80°F.

RESULTS AND DISCUSSION

In no instance was positive spore dispersal demonstrated. After one period of heavy rain isolated lesions were observed on a seedling at the three inch spacing interval but A.brassicicola was not successfully isolated on plating to MA. On the basis of these results spore dispersal in the seed bed does not appear to be important. Even so, the possibility of isolated secondary infections occurring cannot be discounted, but it does seem that spore dispersal is unlikely to cause further emergence losses.

F. DISCUSSION

Both pathogens were demonstrated as causing pre-emergence and post-emergence damping-off under artificial conditions of extreme inoculum loads. However, at the levels of infection and contamination recorded in certified seed lines severe emergence losses did not occur. It does not necessarily follow that the pathogens are not important in causing seed bed losses. Conditions at sowing vary from year to year, and

although in most seasons seedling death may be negligible, under certain conditions severe losses may occur. The presence of symptoms on seedlings raised from commercial seed indicated that seed-borne inoculum can cause seedling infection. However, the fact that from these initial foci there was little subsequent disease development would suggest that environmental factors were unsatisfactory. Possibly the conditions favoured host growth more than the pathogen, thus enabling the seedling to grow through an infection which may otherwise have caused death.

Seed artificially contaminated with spores of A.brassicicola caused a reduction in emergence, and although many infected seedlings survived the attack, there was an adverse effect on subsequent development as shown by a reduction in weight of the aerial tissues. It is debateable whether in field crops this growth reduction is of importance since these weaker seedlings would probably be crowded out and the losses compensated by stronger growth of neighbouring healthy plants. A more important role of such diseased seedlings could be in providing inoculum for secondary infection cycles in the developing crop with subsequent foliage losses. Although in glasshouse trials spore dispersal by water splash resulted in leaf infection, spread did not occur in experiments carried out under more natural conditions. With the capacity for prolific spore production exhibited by A.brassicicola it would seem that such infections

could possibly provide foci for spread of the disease throughout the stand, particularly when localised microclimatic conditions became more favourable with foliage development.

Examinations of diseased seedlings illustrated the significance of epigeal germination in establishment of cotyledonary infection. The retention of an infected or contaminated seed coat on the developing cotyledons provided a source of inoculum in contact with the host and frequently resulted in seedling damping-off. In those cases where the seed coat was not retained, it was characteristically lodged in close proximity to the hypocotyl and often resulted in wire-stem symptoms.

CHAPTER IV

THE EFFECT OF THE PATHOGENS IN SEED CROPS

A. INTRODUCTION

Overseas work has shown that A.brassicae and A.brassicicola may attack cruciferous seed crops and cause severe direct reductions in the quality and quantity of the harvested seed (Weimer, 1924; Nielson, 1933; Wraa-Jensen, 1938; Neergaard, 1945; Green, 1947; Schimmer, 1953; Van Schreven, 1954; McKay, 1956; Domsch, 1957; McDonald, 1959). Such losses are reported to result from pod infections causing siliquas to dry early with subsequent seed loss due to premature shedding of seed, or infections causing shrinkage and death of seed which may be removed from a line during machine dressing. Further, seed in the dressed line may be weakened as a result of infection by the pathogens. The importance of A.brassicicola and A.brassicae in causing seed losses in New Zealand choumoellier crops has not been studied. However in view of the large proportion of seed lines infected with A.brassicicola, and to a lesser extent A.brassicae, it would seem that either or both are generally present in our seed crops. With this evidence in mind, and the reported seed losses caused by the two fungi in overseas brassica crops, research was conducted to investigate the significance of the pathogens in reducing the yield and quality of seed harvested from choumoellier. Further studies were made to determine the mechanisms involved in such seed loss.

An associated difficulty in this study was the

identification of the pathogens involved in attacks on seed crops as both Alternaria species cause very similar symptoms on choumoellier. The disease caused by A.brassicicola has been called black leaf spot (Weimer, 1924) and small siliqua mould (Neergaard, 1945) to distinguish it from that caused by A.brassicae which has been described as grey leaf spot (Weimer, 1926) and large siliqua mould (Neergaard, 1945). The field expression is further confused by the presence of other diseases expressed as lesions on leaves and seed heads. Hence a pre-requisite to seed yield experiments was a close study of the comparative symptomology in mature seed crops.

B. MATERIALS AND METHODS

(a) Isolation from field material

Small sections of tissue were dissected from the margin of lesions on pod, stem and leaf material. Identification of the causal organism was facilitated through one of the following methods :-

(i) For rapid identification, sporulation was induced by placing lesioned areas in Petri plate high humidity chambers for 48 hours. The pathogens were identified by microscopic examination of spores and isolation to PDA slopes.

(ii) Tissue pieces were sealed in muslin bags, washed for two hours in running tap water, dried between filter pads and plated to a selective streptomycin sulphate/PDA medium (see Section D.1, Chapter V). Pathogens were identified on the basis of colony characteristics after incubation for 10 days at 24°C.

(iii) Tissue pieces were surface sterilised by immersion in a 1:1 solution of 0.001% mercuric chloride and 95% alcohol (Weimer, 1926) for 15 seconds, rinsed in sterile distilled water and plated to MA. Identification was again made on the basis of colony characteristics after incubation for 10 days at 24°C.

(b) Inoculation of seed heads

Plastic bags were placed over individual seed heads and misted to the point of run-off with spore suspensions of the respective pathogens (approximately 100,000 spores/ml) using a de Vilbiss atomiser (Figure 11). Inoculations were carried out during calm evenings with little air movement. Individual treatments were identified with coloured labels, and a distance of at least 6 feet maintained between heads inoculated with each species. Plastic bags were used to cover inoculated heads, and were sealed at the base to promote high humidity conditions for 48 hours following inoculation.

Spore suspensions were prepared from sporulating colonies of the respective pathogens and spore concentrations measured using a haemocytometer. Seed heads in concurrent control series were "inoculated" with sterile distilled water.

(c) Harvesting and storage

Labelled seed heads were cut from the plants. Siliquas were then removed from the racemes and laid out on newspaper to air dry for three weeks. At the end of this time half of the pods were graded into "severe", "fairly severe" and "slight" index categories on the basis of external lesioning. To eliminate bias as far as possible an observer was invited to group the pods, selecting only against those that had suffered bird damage. The indexed pods were then harvested by hand over large trays to ensure that no seed was lost in the harvesting process. Harvested seed and the remaining half of the siliquas were stored under cool dry conditions in paper bags until further trials could be carried out.

(d) Sampling

Seed samples were taken using the sub-division method described in Chapter II, Section B.1. Pod samples were made by randomly spreading out the total harvest in a row and counting off the first 100 pods.

Figure 11

INOCULATION OF CHOUMOELLIER SEED HEADS

Method of artificial inoculation of choumoellier seed heads with atomised spore suspensions

(e) Re-isolation from seed

Seed samples were enclosed in muslin bags and surface sterilised by immersion in 0.001% mercuric chloride for 10 minutes (McLean, 1947), rinsed in sterile distilled water and plated to MA or PDA.

C. SYMPTOMOLOGY

1. Foliage symptoms

Repeated isolations and inoculation trials were carried out in which descriptive records were taken and lesion development studied.

(a) A.brassicicola

Lesions began as small black infection points, slightly pitted and indistinguishable from those of A.brassicae. Leaf tissue died in the region of infection and resulted in an overall appearance of a lesion of a brown-green to brown-grey colour. On advanced lesions the infected area was "peppered" with slightly raised blackish spots of the initial infection symptom (Plate 18). Severe attacks were expressed as a rapid withering and browning in the region of the infection compared with the more even yellowing and wilting caused by A.brassicae (Plates 19 and 20). Consequently, A.brassicicola symptoms may, in some cases, be confused with the symptoms of the black rot bacterium Xanthomonas campestris (Pam.) Dows.

(b) A.brassicae

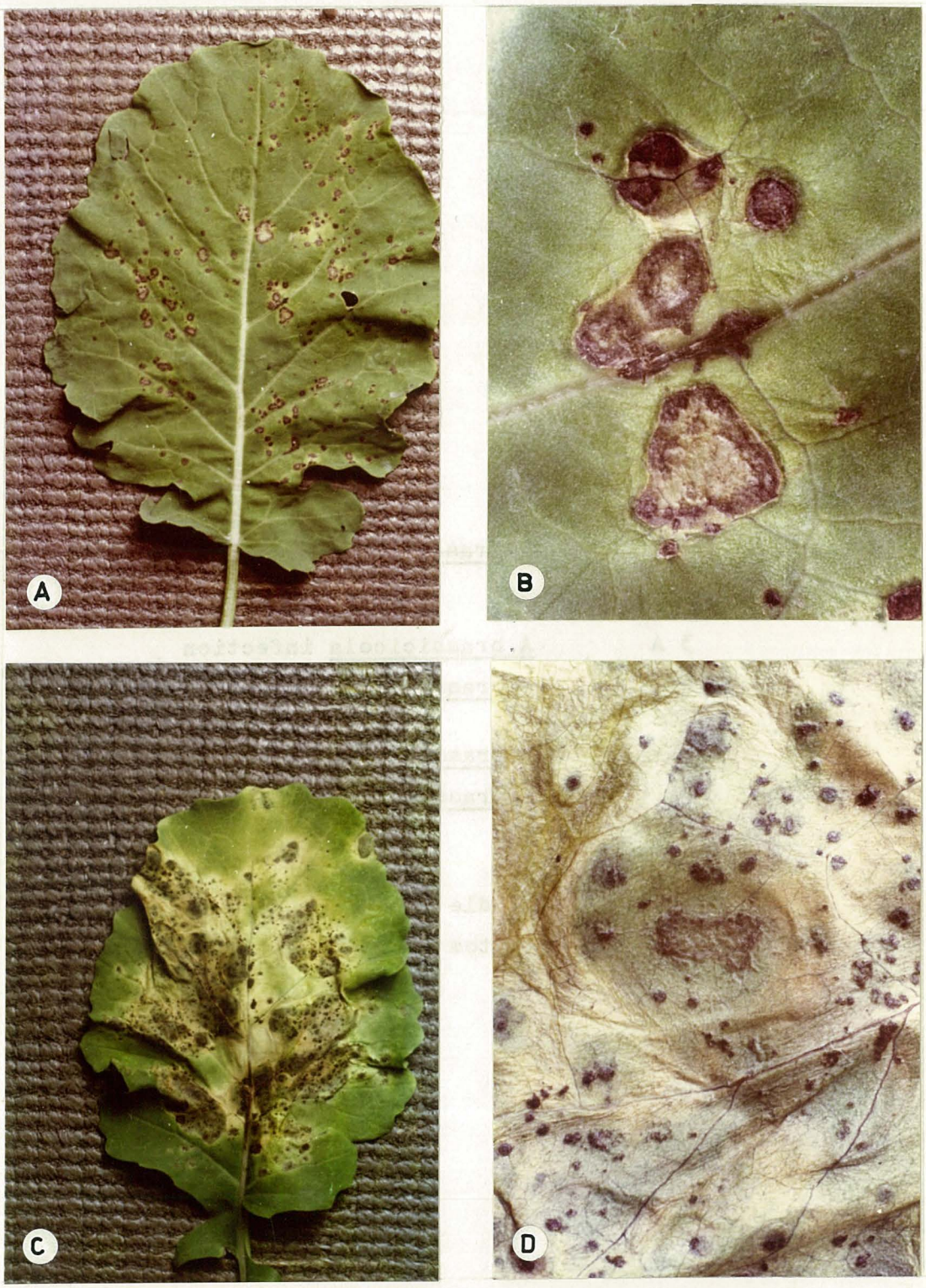
This pathogen was the most frequently isolated Alternaria species from choumoellier crops in the Manawatu. Spotting was often prevalent throughout the stands on both upper and lower surfaces of the leaves. Lesions began as minute purplish-black spots less than 1 mm in diameter which enlarged on older leaves to about 2-6 mm in diameter (Plates 18 and 19). Infected leaves frequently yellowed, wilted, and died off. Chlorosis began about the lesioned area but resulted in a uniform discolouration of the entire leaf (Plate 20). On older leaves degeneration of the lesion centre to give a "shot hole" symptom was common. On these leaves the lesions were brown to black and often exhibited the characteristic ridging effect. On young foliage of mature plants, death of the leaf was not rapid and lesions were slow to develop. These lesions appeared as whitish-grey spots varying from approximately 1-8 mm in diameter, with a well-defined brown to purplish-black perimeter (Plate 18).

Mycosphaerella brassicicola was the pathogen most frequently observed in choumoellier crops but was distinguished by the characteristic large definite "ringspot" symptom and the presence of small black spermagonia and perithecia in the lesioned area. Developing lesions of the fungus were very similar to those caused by the Alternaria species but generally appeared lighter in colour, with a less well-defined

A - B *A.brassicae* inoculations

C - D *A.brassicicola* inoculations

Plate 18



Symptoms expressed by choumoellier leaves artificially inoculated with A.brassicae and A.brassicicola

1

A.brassicae infection

2

3 A *A.brassicicola* infection

B *M.brassicicola* infection

4 A *A.brassicae* infection

B *M.brassicae* infection

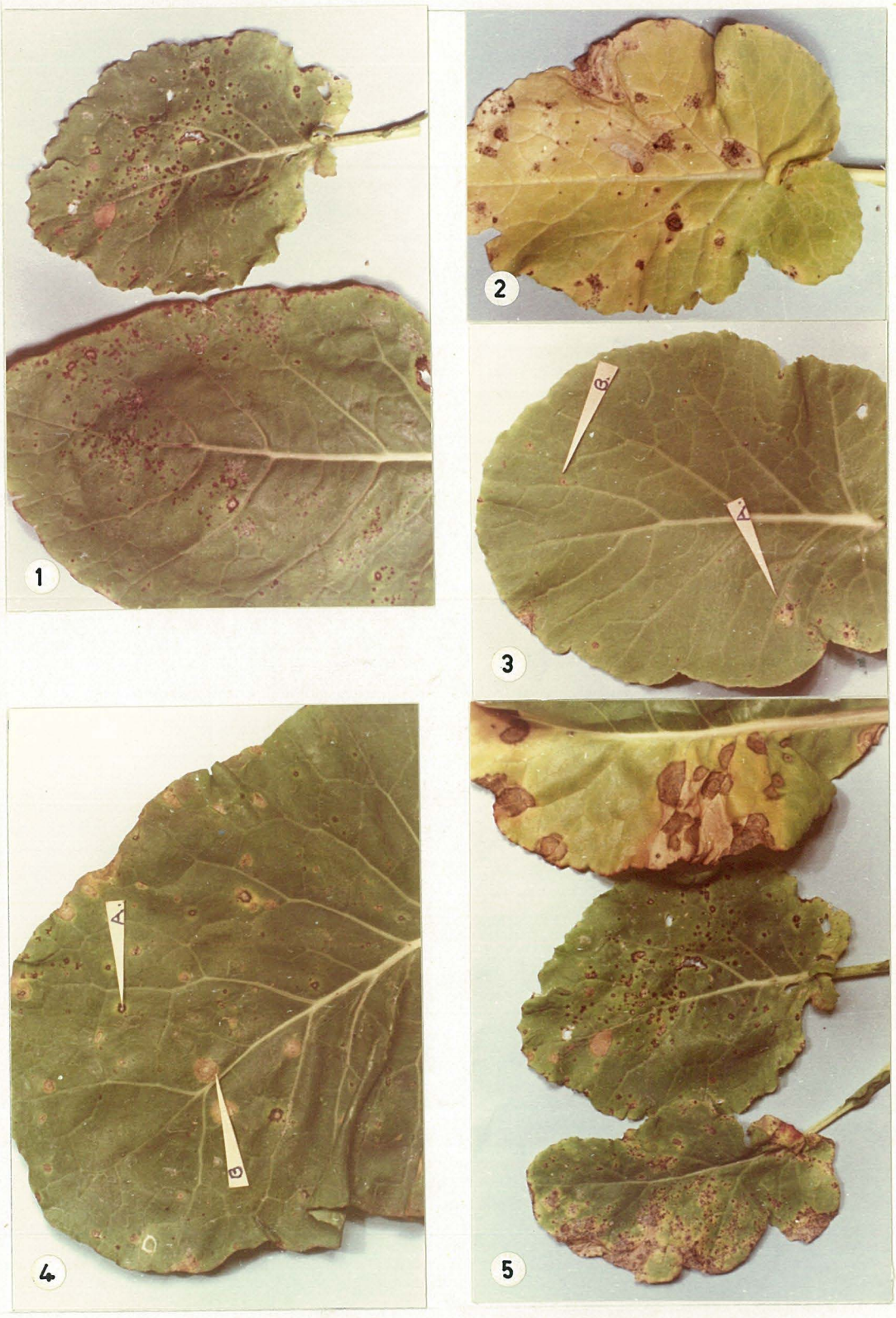
5

Top - *M.brassicicola* infection

Middle - *A.brassicae* infection

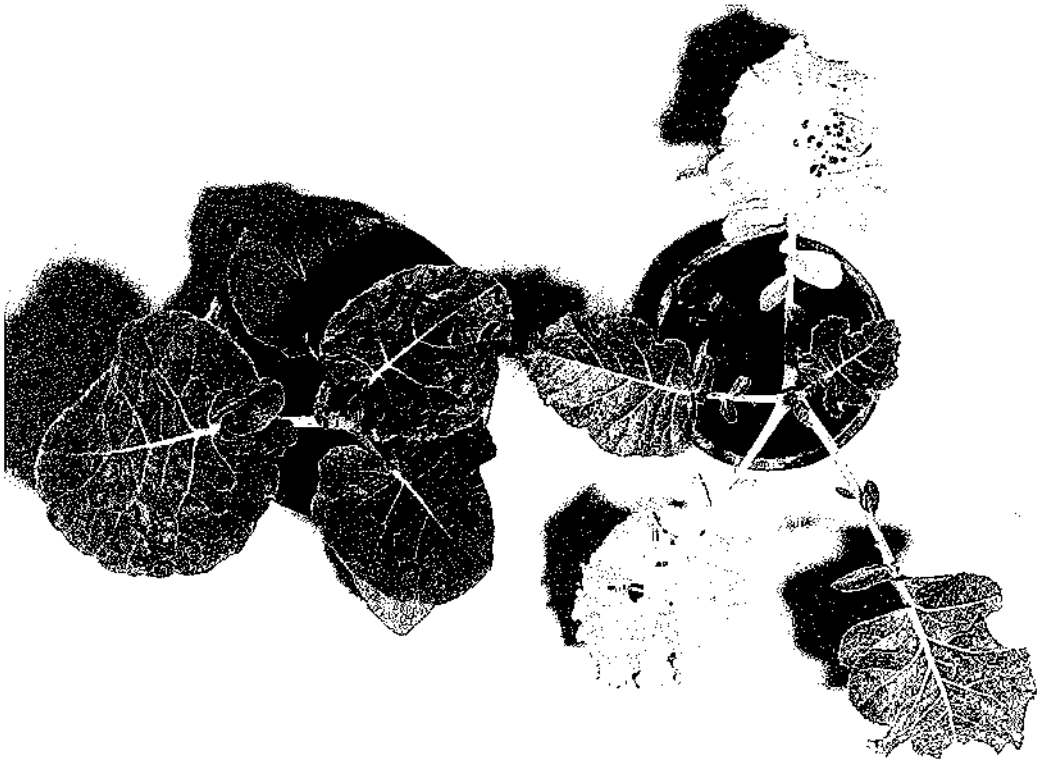
Bottom - *A.brassicicola* infection

Plate 19



Field symptoms on choumoellier leaves
caused by A.brassicae, A.brassicicola
and M.brassicicola

Plate 20



Plant artificially inoculated with A.brassicae
showing advanced stages of infection

Plate 21

Plant artificially inoculated with A.brassicicola showing advanced stages of infection

perimeter (Plate 19).

2. Seed head symptoms

As the Manawatu is not a choumoellier seed cropping area it was not possible to conduct surveys on field expression of the diseases. To overcome this problem seed heads were artificially inoculated with spore suspensions of the respective pathogens and symptom expression studied. In each case a spore suspension of approximately 20,000 spores per ml was misted over seed heads bearing a few flowers and young developing pods through to near mature siliquas.

(a) A.brassicicola

On removal of the high humidity bags, numerous small black "infection points" were manifest on pods and stems. The styler ends of the siliquas were discoloured on most pods. At four days lesions were more prominent and the inoculated heads lower down in the crop appeared to be most severely infected. Lesions had not enlarged six days after inoculation but were slightly raised giving a characteristic "small spot" or "pepper spot" symptom. At 10 days lesions appeared to be superficial and on pod dissection no seed discolouration was observed. Examinations made one week later showed that generally lesions had not advanced but in some cases had penetrated to the developing seeds. In a final observation after 30 days a discolouration of severely infected pods was noted which was associated with a shrinkage of tissue about the

lesioned area and infection of seeds (Plate 24 and 25).

(b) A.brassicae

At two days little evidence of lesioning was observed, but isolated infection points were apparent as minute purplish-black lesions, which were more uniformly distributed two days later. Lesions were larger and more sunken six days after inoculation, giving a crater like appearance. Dissection of infected pods 10 days after inoculation showed infected tissue deep into the pod wall and discolouration of some seeds in close proximity to the lesion was observed. By 30 days infection had resulted in shrinkage and decay of the pod wall giving rise to lesions 1-7 mm long. In some cases these encircled the whole pod but the effect was localised to the lesioned area and general pod discolouration did not occur (Plates 23 and 25).

Pod lesions caused by natural infection of M.brassicicola were very similar to the symptoms caused by A.brassicae. Differentiation between the two proved difficult, but close observations indicated that M.brassicicola lesions were lighter in colour with a more violet-brown appearance compared with the purple to black spots caused by A.brassicae. The raised lesion centres characteristic of the Alternarias were not so obvious for M.brassicicola and lesion perimeters appeared to be less well defined.

Plate 22

Artificially inoculated racemes of choumoellier

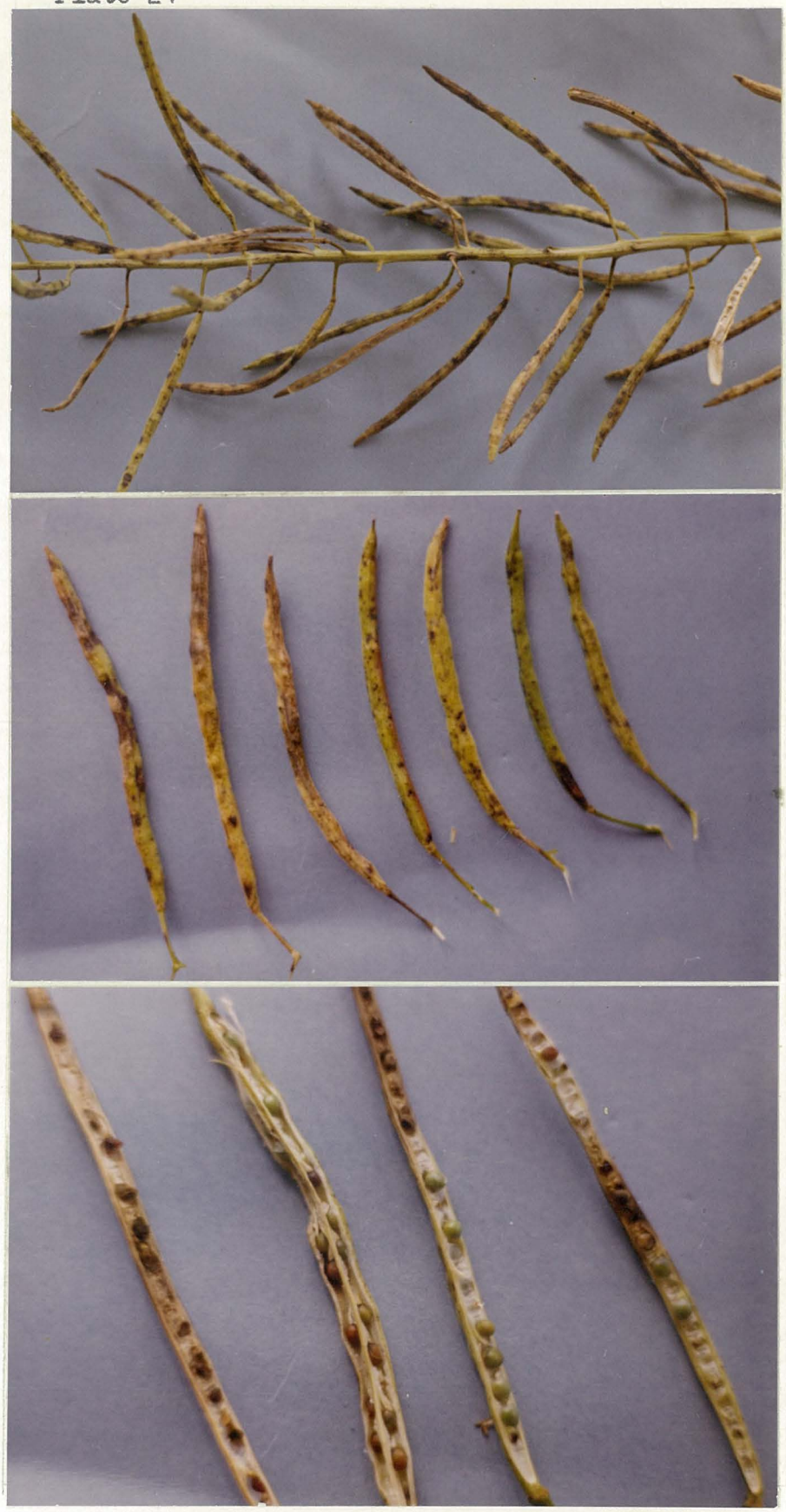
In the bottom print severely diseased siliquas are dissected to show infected seed

Plate 23

Symptoms on siliquas artificially inoculated with A.brassicae

In the bottom print severely diseased siliquas are dissected to show infected seed

Plate 24

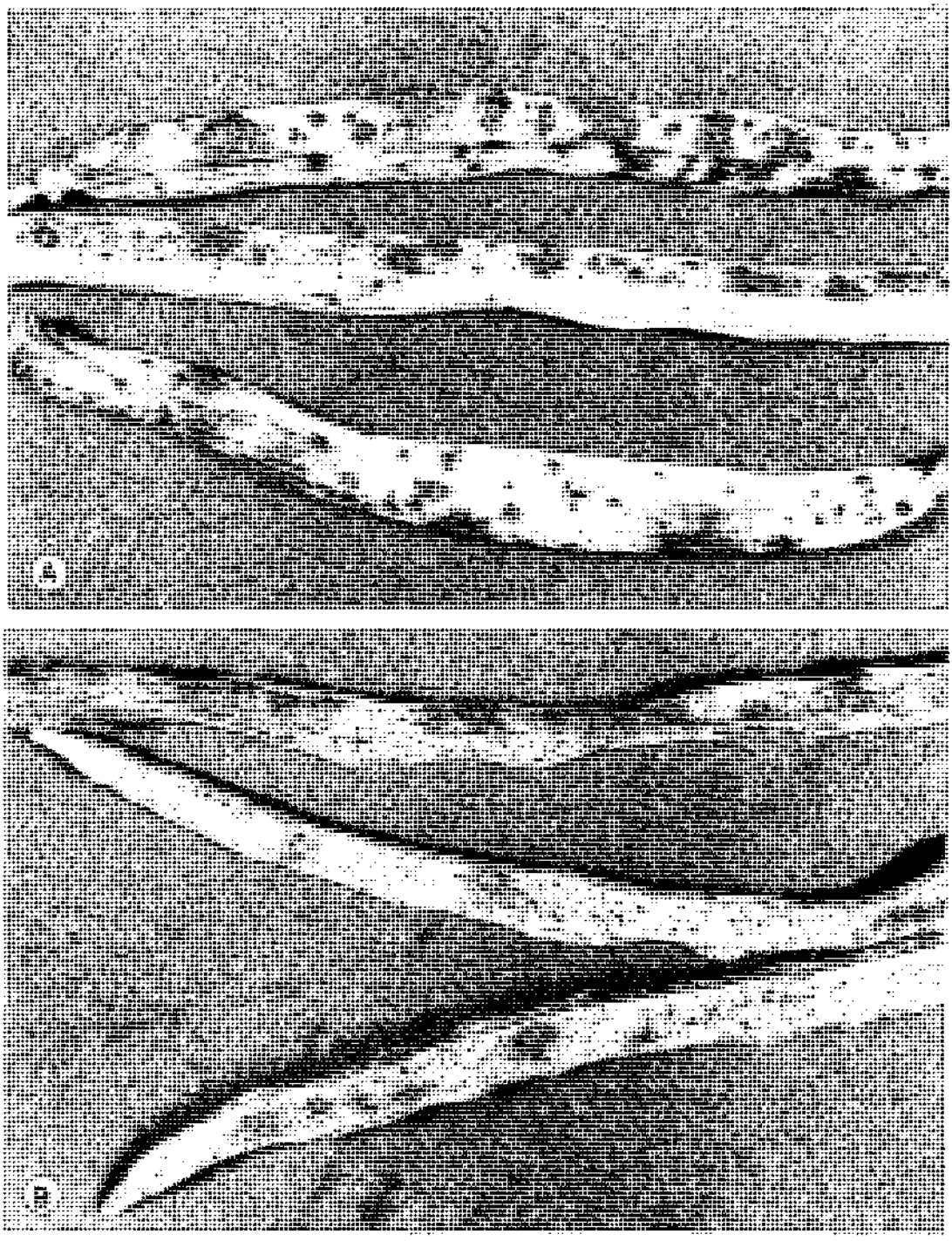


Symptoms on siliquas artificially inoculated with A.brassicicola

A A.brassicae

B A.brassicicola

Plate 25



The extent of lesion development on siliquas artificially inoculated with A.brassicae and A.brassicicola

D. THE YIELD AND QUALITY OF SEED HARVESTED FROM ARTIFICIALLY INOCULATED SILIQUAS

Having studied the symptomology of the two pathogens in seed crops and observed that discolouration of seed in infected siliquas does occur, trials were conducted to investigate the effects of A.brassicae and A.brassicicola on seed yields.

1. Effect of siliqua infection on seed yield

In this experiment siliquas were harvested from artificially inoculated seed heads seven weeks after inoculation. After drying, the pods were stored before examinations were made to investigate the quantitative and qualitative effects of the inoculated pathogens on seed yield.

(a) Quantitative effects

Method

For each treatment, that is, the A.brassicae and A.brassicicola inoculations and the control series, three lots of 100 siliquas were sampled and the weight of each lot recorded. The pods were then carefully harvested by hand and the seed yield for each group was weighed.

The data presented in Appendix VI was subjected to an analysis of variance (analysis of variance tables are presented as Appendices) and the significance of treatment differences determined by a "t" test.

RESULTS AND DISCUSSION

(i) Effect of siliqua infection on siliqua weight

TABLE 29

Comparison of the mean weight of 100 siliquas harvested from inoculated seed heads (see Appendix VII)

Treatment	Mean weight (gm)	Probability of a significant difference from the control
A.brassicicola inoculations	18.171	* *
A.brassicae inoculations	20.501	* *
Uninoculated control	25.545	
S.E. ± 0.01		
$d._{05}$ = 2.442		
$d._{01}$ = 3.707		

The results of this analysis show that the weight of 100 siliquas from plants inoculated with both A.brassicicola and A.brassicae were significantly less ($P < 0.01$) than the control. In addition, A.brassicicola was very nearly significantly lower than A.brassicae (difference required at the 5% probability level was 2.442; actual difference was 2.330). It is apparent then, that siliqua infection by both pathogens caused a restriction in development, and it is possible that this was also reflected in reduced seed yields.

(ii) Effect of siliqua infection on seed weight harvested

TABLE 30

Comparison of the mean weight of seed harvested from 100 siliquas (see Appendix VIII)

Treatment	Mean weight (gm)	Probability of a significant difference from the control
A.brassicicola inoculations	5.588	* *
A.brassicae inoculations	5.700	* *
Uninoculated control	8.599	
S.E.	± 0.26	
$d._{05}$ =	0.919	
$d._{01}$ =	1.392	

The analysis shows that there was no significant difference in weight of seed obtained from 100 siliquas harvested from seed heads inoculated with A.brassicicola and A.brassicae. However there was a highly significant ($P < 0.01$) reduction in the weight of seed harvested for both of the inoculated series when compared with the control. On a percentage basis, seed harvested from pods inoculated with A.brassicicola yielded 35.0% less seed than the control and a similar reduction of 33.7% was caused by the A.brassicae inoculations.

(b) Qualitative effects

Method

In this experiment, the seed from each 100 siliqua replicate was divided; one half being subjected to a G.S.T.S. germination test, and part of the other half plated to MA after surface sterilisation in mercuric chloride. In each case three replicates of 100 seeds were tested.

TABLE 31

Comparison of the mean germination percentages of seed harvested from infected siliquas (see Appendix IX)

Treatment	Mean ~~weight~~ (~~gm~~)	Probability of a significant difference from the control
A.brassicicola inoculations	71	* *
A.brassicae inoculations	79	NS
Uninoculated control	85	
S.E. ± 2.5		
$d._{05}$ = 7.3		
$d._{01}$ = 9.9		

The analysis shows that infection of siliquas with A.brassicicola resulted in a seed line of lowered germination capacity ($P < 0.01$) compared with the control, and compared with the seed harvested from the A.brassicae inoculations ($P < 0.05$).

NS No significant difference

The difference between A.brassicae and the control was nearly significant at the 5% level ($d._{05}$ = 7.3; actual difference = 6). Inoculation of seed heads with A.brassicicola has therefore caused a reduction in the germinability of the line, that is, its quality has been reduced. A.brassicae was shown in the previous experiment to reduce the quantity of seed harvested, but on the basis of the above results, does not appear to cause a qualitative yield reduction.

(iii) Effect of siliqua infection on seed infection

TABLE 32

Percentage infection of seed harvested from inoculated siliquas

Treatment	Replicate	Percentage infection			Mean
		1	2	3	
Siliquas inoculated with A.brassicicola	1	7	5	9	7.00
	2	14	8	8	10.00
	3	8	7	12	9.00
Siliquas inoculated with A.brassicae	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
Siliquas inoculated - control	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0

The results in Table 32 suggest that seed infection by A.brassicae had not occurred. However positive isolations made in the "symptomology" investigations indicated that some infection

had taken place. In view of the results obtained in Chapter II Section D.1, on the viability of seed infection, it would appear that A.brassicae has not survived the five months storage period between the harvest of the seed and the time at which the health screening trial was carried out. This in turn may be associated with the relatively small reduction in germination percentage caused by A.brassicae as compared with A.brassicicola.

In the above trials it appeared that the amount of "pinched" and small seed was proportionately greater from inoculated siliquas than from the controls. This was shown to be the case by grading a 100 pod harvest for each of the treatments into size categories (Plate 26) using sieves of different mesh diameters. Further experiments were conducted to examine this effect more closely. That is, to investigate the effects of siliqua infection on seed size, and in turn, the effect of the small seed on the quality of the harvested line. In addition, the effect of the severity of pod lesioning on yield was investigated.

2. Effect of siliqua infection on the size of seed harvested

(a) Quantitative effects

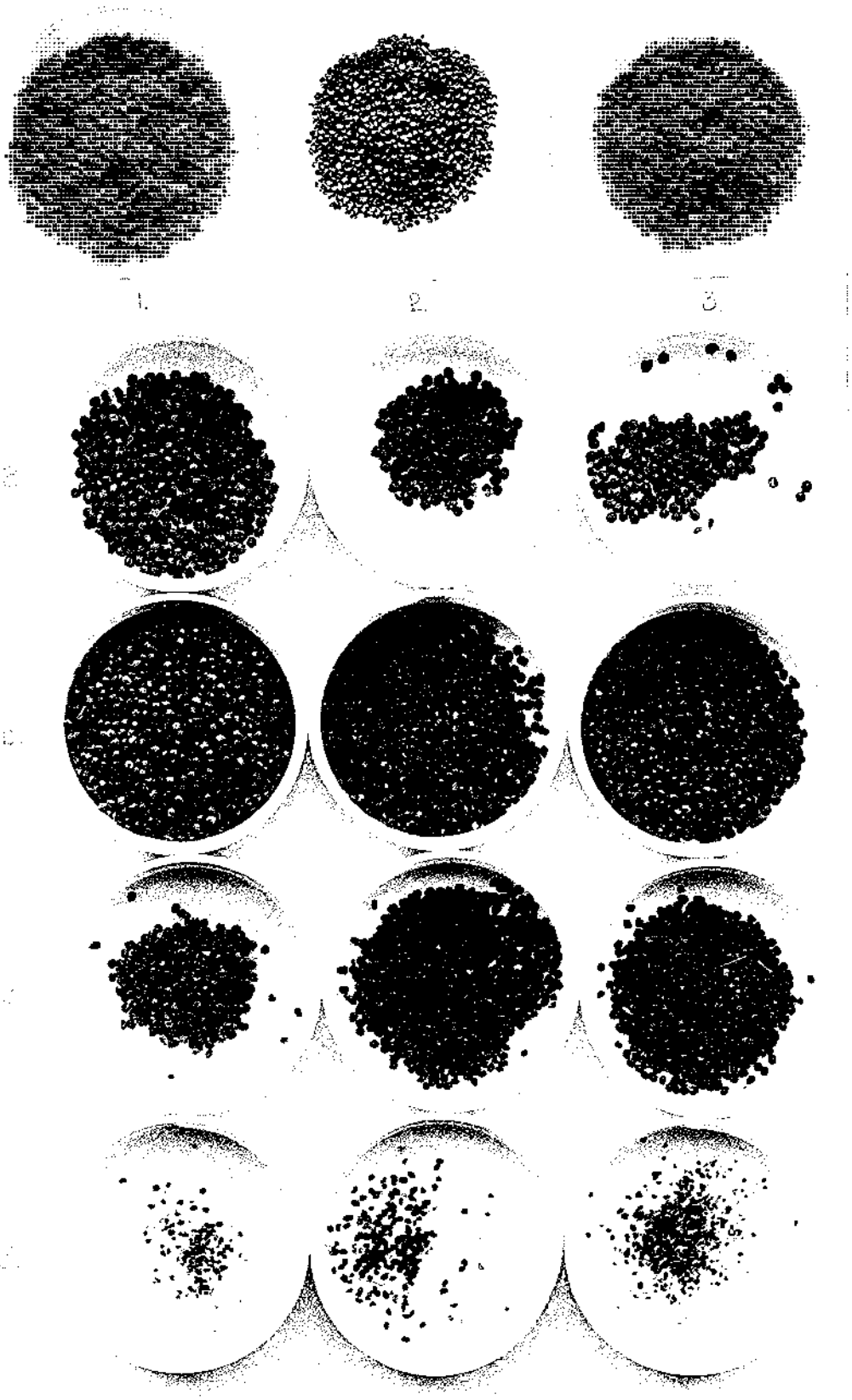
Method

Three 5 gm samples were taken from the bulked supplies of seed harvested from siliquas indexed to the categories of "mild", "fairly severe", and "severe", on the basis of external siliqua lesioning. Each sample was sieved through a series riddles of

1	Control
2	*A.brassicicola* inoculations
3	*A.brassicae* inoculations

a	> 2.007 mm	
b	> 1.651 mm	< 2.007 mm
c	> 1.270 mm	< 1.651 mm
d	> 0.838 mm	< 1.270 mm

Plate 26



Effect of siliqua infection on
the quantity of seed harvested
from 100 pods

the following diameters : 2.007 mm; 1.650 mm; 1.270 mm, and 0.838 mm. The weight of seed retained between each riddle size was recorded.

The "raw" data presented in Appendices X, XI, XII and XIII was subjected to analyses of variances and the significance to treatment differences determined by a "t" test.

RESULTS AND DISCUSSION

(i) Effect of siliqua infection on the percentage weight of seed retained by the 2.007 mm sieve

TABLE 33

Comparison of the mean percentage weight of seed from inoculated siliquas retained by the 2.007 mm sieve (see Appendix XIV)

Treatment		Mean percentage weight	Probability of a significant difference from the control
A.brassicicola	Slight	18.1	NS
	Fairly severe	18.9	NS
	Severe	16.6	NS
A.brassicae	Slight	13.3	* *
	Fairly severe	15.7	NS
	Severe	8.3	* *
Control		18.9	

S.E. $\pm$ 1.3

$d._{05}$ = 3.9

$d._{01}$ = 5.5

The analysis shows that compared with the control, pod infection with A.brassicicola did not affect the weight of seed retained at a sieve size of 2.007 mm. "Slight" and "severe" but not "fairly severe" siliqua lesioning with A.brassicae reduced the percentage weight ($P < 0.01$) significantly, compared with the control.

(ii) Effect of siliqua infection on the percentage weight of seed retained by the sieve size grouping
>1.651 mm < 2.007 mm

TABLE 34

Comparison of the mean percentage of seed from inoculated siliquas retained at the sieve size groupings >1.651 mm < 2.007 mm (see Appendix XV)

Treatment		Mean percentage weight	Probability of a significant difference from the control
A.brassicicola	Slight	59.8	* *
	Fairly severe	58.4	* *
	Severe	58.0	* *
A.brassicae	Slight	61.0	* *
	Fairly severe	57.7	* *
	Severe	61.0	* *
Control		73.3	

S.E. ± 1.5

$d._{05}$ = 4.6

$d._{01}$ = 6.4

The analysis shows that irrespective of the degree of pod lesioning or the inoculated species involved, when compared with the control, all infected siliquas produced less ($P < 0.01$) percentage weight of seed retained at sieve size category > 1.651 mm < 2.007 mm.

(iii) Effect of siliqua infection on the percentage weight of seed retained by the seed size category > 1.270 mm < 1.651 mm

TABLE 35

Comparison of the mean percentage weight of seed from inoculated siliquas retained at the sieve size groupings > 1.270 mm < 1.651 mm (see Appendix XVI)

Treatment		Mean percentage weight	Probability of a significant difference from the control
A.brassicicola	Slight	23.0	* *
	Fairly severe	23.2	* *
	Severe	24.4	* *
A.brassicae	Slight	24.7	* *
	Fairly severe	24.8	* *
	Severe	29.8	* *
Control		7.7	

S.E. ± 1.3

$d._{05}$ = 4.07

$d._{01}$ = 5.65

The analysis shows that a greater percentage weight ($P < 0.01$) of seed was retained from the infected siliquas (irrespective of the inoculated pathogen or the degree of siliqua lesioning) than the controls, by a sieve size grouping >1.270 <1.651. It appears then that the main effect of both pathogens has been to increase the amount of small seed (>1.270 <1.651) relative to the next largest size grouping (>1.651 <2.007).

(iv) Effect of siliqua infection on the percentage weight of seed retained by the sieve size grouping >0.838 mm <1.270 mm

TABLE 36

Comparison of the mean percentage weight of seed from inoculated siliquas retained at the sieve size grouping >0.838 <1.270 (see Appendix XVII)

Treatment		Mean percentage weight	Probability of a significant difference from the control
A.brassicicola	Slight	1.1	*
	Fairly severe	0.8	NS
	Severe	1.4	*
A.brassicae	Slight	2.2	* *
	Fairly severe	0.8	NS
	Severe	0.8	NS
Control		0.1	

S.E. $\pm$ 0.3

$d._{05}$ = 0.98

$d._{01}$ = 1.35

The analysis shows that there has been an increase in percentage weight ($P < 0.05$) of seed retained by the > 0.838 < 1.270 size grouping for seed harvested from "slight" and "severe" siliquas inoculated with A.brassicicola, and a highly significant increase ($P < 0.01$) for seed from "slight" siliquas inoculated with A.brassicae. However the small amounts of seed involved mean that even a few seeds missed at harvest could severely bias the results. The conclusion is made then, only that there are indications that the amount of seed of the size grouping > 0.838 < 1.270 mm, has been increased due to siliqua infection by A.brassicae and A.brassicicola.

(b) Qualitative effects

Method

In the previous trial three 5 gm replicates of seed were harvested from siliquas indexed to infection categories on the basis of external lesioning. The seed was then graded into size groupings, and in this experiment a seed sample from each of these groupings was subjected to a G.S.T.S. germination test, and a further sample plated to MA after surface sterilisation in mercuric chloride. Unless otherwise stated, three 100 seed replicates were investigated.

RESULTS AND DISCUSSION

(i) Effect of siliqua infection on the germination percentage of seed retained by the 2.007 mm sieve

TABLE 37

Comparison of the mean germination percentage of seed from inoculated siliquas retained by the 2.007 mm sieve (see Appendix XVIII)

Treatment		Mean germination (%)	Probability of a significant difference from the control
A.brassicicola	Slight	74.3	* *
	Fairly severe	78.6	NS
	Severe	68.0	* *
A.brassicae	Slight	86.0	NS
	Fairly severe	75.6	*
	Severe	86.0	NS
Control		85.6	

S.E. $\pm$ 2.48

$d._{05}$ = 7.53

$d._{01}$ = 10.45

The analysis shows that relative to the control, A.brassicicola siliquas indexed to the "slight" category, exhibited a reduced ($P < 0.01$) germination percentage. Seed from the "severe" category also had a reduced ($P < 0.01$) germination percentage and seed from the "fairly severe" category was almost significantly reduced ($d._{05}$ = 7.53; actual difference 7.00). In the A.brassicae series only the "fairly severe" group was significantly ($P < 0.05$) reduced in germination.

(ii) Effect of siliqua infection on the germination percentage of seed retained by the sieve size grouping >1.651 mm <2.007 mm

TABLE 38

Comparison of the mean germination percentage of seed from inoculated siliquas retained by the >1.651 mm <2.007 mm sieve size grouping (see Appendix XIX)

Treatment		Mean germination (%)	Probability of a significant difference from the control
A.brassicicola	Slight	70.6	* *
	Fairly severe	77.6	NS
	Severe	74.3	*
A.brassicae	Slight	86.0	NS
	Fairly severe	81.0	NS
	Severe	91.6	NS
Control		85.0	

S.E. $\pm$ 2.66

$d._{05}$ = 8.07

$d._{01}$ = 11.19

The analysis shows that compared with the control A.brassicicola "slight" has decreased germination ($P < 0.01$) percentage, as also has A.brassicicola "severe" ($P < 0.05$); seed from pods indexed to "fairly severe" was almost significantly reduced ($d._{05}$ = 8.07; actual difference shown 7.33). Again the infection of siliquas by the pathogen A.brassicae has not significantly reduced germination percentage of the seed.

(iii) Effect of siliqua infection on the germination percentage of seed retained by the sieve size grouping >1.270 mm <1.651 mm

TABLE 39

Comparison of the mean germination percentage of seed from inoculated siliquas retained by the >1.270 mm <1.651 mm sieve size grouping (see Appendix XX)

Treatment		Mean germination (%)	Probability of a significant difference from the control
A.brassicicola	Slight	75.6	* *
	Fairly severe	59.0	NS
	Severe	40.6	*
A.brassicae	Slight	49.6	NS
	Fairly severe	61.0	NS
	Severe	74.6	* *
Control		55.0	
	S.E.	± 4.25	
	$d._{05}$ =	12.87	
	$d._{01}$ =	17.862	

There appears to be no relationship with the severity of pod spotting and the percentage germination of seed. Only seed harvested from A.brassicicola "severe" pods is significantly lower ($P < 0.05$) in germination capacity than the control. In fact seed harvested from "slightly" infected siliquas from both the A.brassicicola and A.brassicae inoculations has a germination capacity significantly greater ($P < 0.01$) than that of the control.

(iv) Effect of siliqua infection on the percentage weight of seed retained by the sieve size grouping >0.838 mm <1.270 mm

The results presented in Appendix XII demonstrate that even on a moist agar medium this seed is nearly always incapable of germination, and is unlikely to produce a normal seedling.

(v) Effect of siliqua infection on seed infection for various seed size groupings

The results presented in Table 40 are further evidence for the lack of persistence of A.brassicae as a viable seed infection. In total, a considerably larger percentage of the seed harvested from siliquas "severely" infected with A.brassicicola, were also infected by this pathogen. It does appear that generally there is an increase in seed infection with an increase in the severity of siliqua lesioning, and that small seed obtained from infected siliquas is more often infected than large seed.

3. Discussion

It has been shown that siliqua infection by both pathogens resulted in reduced pod weight. Similarly, the yield of seed harvested from the infected siliquas was significantly less than that harvested from the controls. Specific investigations on seed of various sizes showed that in both cases this effect was manifest as a decrease in seed of

TABLE 40

Effect of siliqua infection on seed infection for various seed size groupings

Treatment	Degree of pod lesioning	Sieve sizes		Infection %			
				1	2	3	Mean
A. brassicicola inoculations	Slight	>2.007		2	2	2	2.00
		>1.651	<2.007	1	4	4	3.00
		>1.270	<1.651	5	9	6	6.66
		>0.838	<1.270	14	12	-	13.00
	Fairly severe	>2.007		2	1	3	2.00
		>1.651	<2.007	9	9	8	8.66
		>1.270	<1.651	5	7	8	6.66
		>0.838	<1.270	6	5	-	5.50
	Severe	>2.007		5	4	3	4.00
		>1.651	<2.007	9	5	4	6.00
		>1.270	<1.651	6	9	7	7.33
		>0.838	<1.270	12	16	-	14.00
A. brassicae inoculations	Slight	>2.007		0	0	0	0
		>1.651	<2.007	0	0	0	0
		>1.270	<1.651	0	0	0	0
		>0.838	<1.270	0	0	0	0
	Fairly severe	>2.007		0	0	0	0
		>1.651	<2.007	0	0	0	0
		>1.270	<1.651	0	0	0	0
		>0.838	<1.270	0	2*	-	1.0
	Severe	>2.007		0	0	0	0
		>1.651	<2.007	0	0	0	0
		>1.270	<1.651	0	0	0	0
		>0.838	<1.270	0	0	-	0.0
Control		>2.007		0	0	0	0
		>1.651	<2.007	0	0	0	0
		>1.270	<1.651	0	0	0	0
		>0.838	<1.270	2*	0	-	1.0

* A. brassicicola

the >1.651 <2.007 mm size grouping and a corresponding increase in the seed of the >1.270 <1.651 size grouping.

Seed harvested from racemes inoculated with A.brassicicola was considerably reduced in germination capacity relative to the control, and studies on seed of various sizes showed a germination reduction at each size grouping down to the >1.270 <1.651 mm size. Seed in this latter category was generally reduced in germination irrespective of the treatment. On the other hand, seed harvested from racemes inoculated with A.brassicae did not cause a marked germination reduction and this was also evident in germination trials on the various seed size groupings. It is tempting to conclude that the effect of A.brassicae on seed yield is solely a quantitative one. However Thiele (1965) showed that there was a substantial difference in performance (germination, rate of germination, vigour, soil growth and weight of crop) between small seeds and large seeds of a number of different kinds of vegetables. In his trials the germination percentage of cabbage seed was 92% (large seed) and 56% (small seed) and further, the average fresh weight of seedlings grown from small seed was only 57% of the weight of those grown from large seed, six weeks after plating. It follows then, that any factors which affects yield through reducing seed size is likely to have an indirect effect on the quality of the harvested line.

Although in terms of total yield, a crop may be poor, the quality of the line finally marketed can be "improved" through the cleaning and dressing processes but the losses incurred due to the elimination of small seed are a direct loss to the grower and constitute a reduction in the quantity of his saleable product. In the above experiments reductions in the weight of seed harvested in the order of 35% were associated with the A.brassicicola inoculations, and yet the harvested line exhibited only a 7-10% infection on plating to agar. However it must be conceded that the above results were obtained under atypical conditions in that firstly, the seed heads were artifically inoculated with the pathogens which in turn were given every opportunity to successfully infect; and secondly, the crop was grown in an area far removed from the normal choumoellier seed cropping areas of the South Island. An attempt to relate the above results to South Island conditions was made through the examination of seed and crop samples received from South Island growers.

E. EXAMINATION OF NATURALLY INFECTED FIELD CROP SAMPLES

Contact was made with growers of choumoellier seed crops through the co-operation of Field Officers in the Department of Agriculture. As a result samples of foliage from six crops were received for inspection over the period between August and November, 1965. A.brassicicola was isolated

from four of these crops and both A.brassicae and A.brassicicola from one. On request, samples of seed "ex the mill" were received from five crops after harvest and the seed was investigated for infections of A.brassicicola.

Method

Three replicates of 100 seeds each from the "firsts" and "seconds" (where possible) of each line were surface sterilised in mercuric chloride and incubated on MA for six days at 24°C.

RESULTS AND DISCUSSION

The results presented in Table 41 show that although there was extreme variability between replicates within each sample, the general pattern is for the small seed grouped as "seconds" to exhibit a higher percentage infection of A.brassicicola, than the "firsts". This seed represents the first loss to the grower, and as much of the top seed in all samples was also "pinched" and shrivelled", further losses would be incurred with machine dressing. However no control over the field sampling procedure was possible either for the seed samples or the foliage material received. But from comments made by Field Officers from the Department of Agriculture, from the growers themselves, and from laboratory examinations of leaf material and crop debris it was apparent that more than one pathogen was present in each crop. The "ring spot fungus" M.brassicicola was most frequently referred to and was in evidence on all the samples

TABLE 41

Isolations from field samples of choumoellier seed

Seed Line	Percentage of A. brassicicola isolated							
	"Firsts"				"Seconds"			
	1	2	3	Mean	1	2	3	Mean
N_1	4	4	2	3.33	2	3	10	5.00
N_2	8	5	2	5.00	20	15	13	16.00
N_3	0	2	5	2.33	-	-	-	-
N_4	2	0	3	1.66	-	-	-	-
N_5	11	11	6	9.33	7	18	10	11.66

received. Symptoms typical of the "black rot" organism X.campestris were also observed on samples from three crops. It can only be concluded then, that A.brassicicola was present as one pathogen in a complex of diseases which may have affected the ultimate crop yield. The percentage infections of A.brassicicola in seed of two crops (N_2 and N_5), were comparable to those obtained in seed from the artificially inoculated trials. It is possible that in these crops, losses due to A.brassicicola infections may have been comparable with those recorded in the artificial inoculation series.

F. MECHANISM OF SEED INFECTION AND SEED LOSS

Two facts were established in the previous experiments of this Chapter, namely :

(i) that pod infection by either species of Alternaria can cause a reduction in seed quantity and quality;

(ii) that pod lesions may extend deep into the siliqua wall, causing discolouration of seed and often arresting development.

The latter observation supports the work of Weimer (1924) who showed by isolation of the fungus from cauliflower seeds taken from beneath the lesions on partly mature pods, that A.brassicicola can grow thrcugh the seed pods and infect seeds. Further, spores of the fungus were

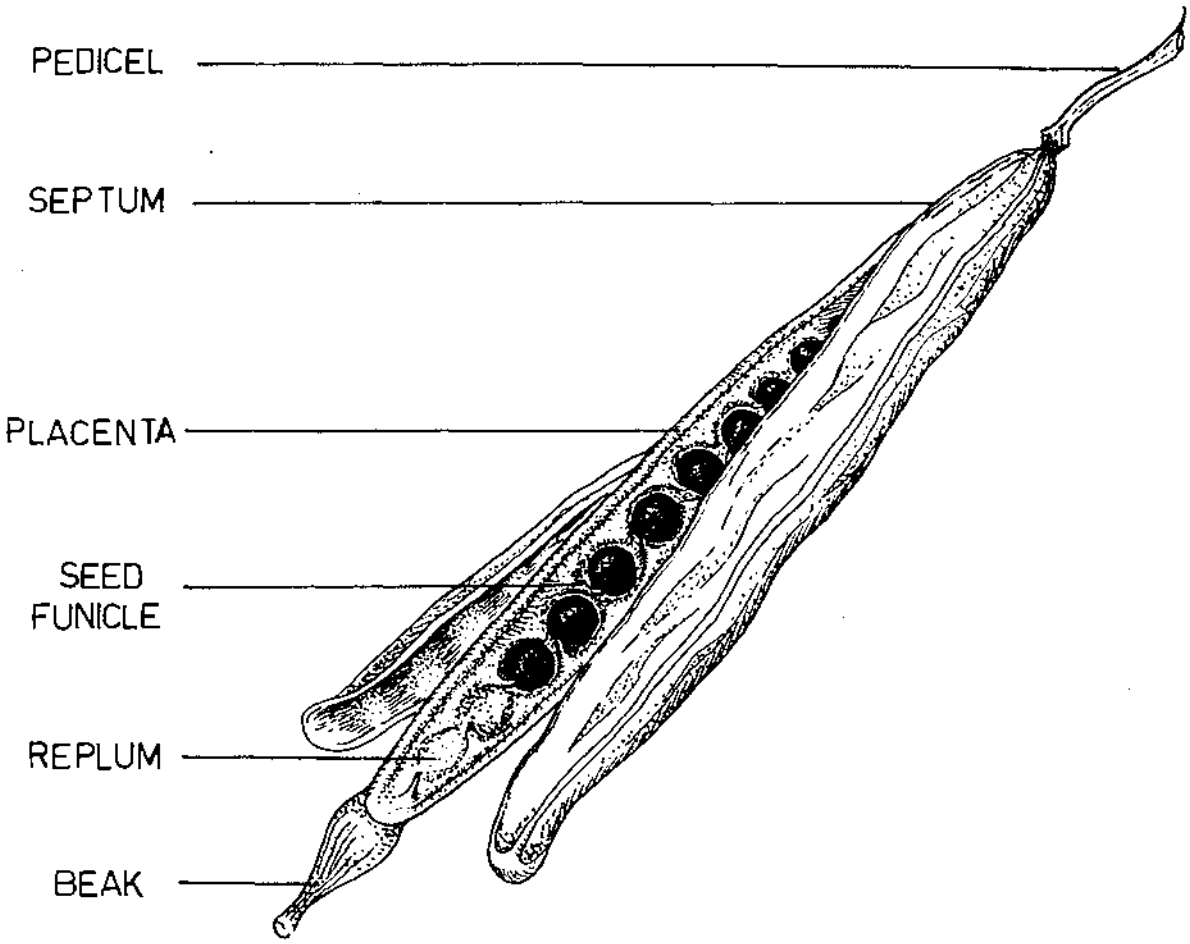
identified on seeds taken from pods which had not yet begun to dehisce. In similar work on radish, McLean (1947) mentions seed infection resulting from lesioning and mycelial penetration of pod tissue by A.raphani. McKay (1956) reported a similar action for A.brassicae and A.brassicicola, and McDonald (1959) found mycelium of A.brassicae to completely envelop the seed in some inoculations of rape plants.

While a direct effect of the pathogens on developing seeds of choumoellier causes some losses, it appears that other effects may be involved. For example, Neergaard (1945) reported severe losses due to premature drying out and splitting of cabbage seed pods caused by A.brassicicola infections. Examinations and isolations from infected siliquas were conducted in this study to investigate the manner in which seed infection and seed loss occurs in choumoellier.

1. A.brassicicola

Severely infected siliquas appeared shrunken and discoloured with a general darkening of the straw colour. Dissection of these pods showed that seeds immediately below the badly lesioned areas were shrivelled and in many cases had not developed at all. In the most severely attacked siliquas seed was shrunken throughout, but in others the effect was localised and confined to the immediate area below the lesion or extending to a few adjacent seeds. Often seed was observed

Figure 12



A choumoellier silique illustrating the structures referred to in the text

to have germinated in situ.

In some instances a splitting of the pod was noted and in many of these siliquas the placenta (Figure 12) was discoloured by a blackening which encompassed the replum and seed funicle. Shrivelled seed was also obtained from pods with a minimum of visible spotting and in many of these cases discolouration of the pedicel was apparent. Other siliquas exhibited a darkening in the stigma and beak region, often associated with separation of the carpel walls along the line of the placentas at the terminal end of the pod.

As a result of these examinations and the results obtained in yield trials it is postulated that the quantity and quality of seed harvested may be influenced in four ways by the action of the pathogen A.brassicicola on racemes of choumoellier :-

(i) The penetration of the siliqua wall and direct infection of seed in close proximity to the lesion. If the seed is infected at an early stage, development may be arrested, resulting in shrivelled seed. It appeared that spread from such an infected centre may occur within the pod, along the line of the placentas and through the soft papery tissue of the replum. This was evidenced by discolouration extending beyond the initially lesioned areas of the pod wall (Plate 28). The moist conditions within

the siliqua and vulnerability of the replum probably facilitate spread. In many siliquas the fungus had ramified throughout the interior of the pod causing the general discolouration often observed in severely diseased examples (Plate 28).

(ii) Diseased pods often exhibited splitting along the septum. Examination of these pods showed the placentas in the region of the dissociation to be darkened and constricted. It appeared that infection and lesioning resulted in shrivelling of this tissue giving rise to pressures from contraction with ultimate splitting of the siliqua (Plate 27) and subsequent seed loss.

Infection of the beak may also cause constriction at the terminal end of the siliqua, differential pressures again causing a splitting along the line of the septum (Plate 27).

(iii) Pedicel and pod attack early in the development of a pcd may restrict the flow of reserves to the seed and be expressed in an individual loss through seed being restricted in bulk but pathogen free.

(iv) Through splitting of the seed coat and premature germination in situ (Plate 27).

Siliquas exhibiting constriction and splitting were

A	Germination of seed in situ
B	Siliqua dehiscence
C	Siliqua dehiscence along the septum

Plate 27

A

B

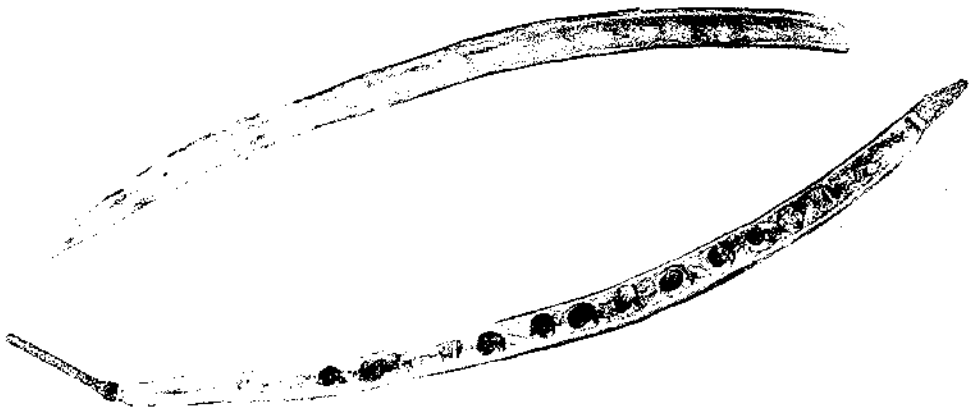
C

Mechanism of seed loss from siliquas
due to A.brassicicola infection

A Infection via the siliqua wall and spread through the papery replum

B Infected placental and replum dissected from an infected pod

Plate 28



A

B

Spread of A.brassicicola within infected siliquas

observed on numerous occasions but a series of isolations were carried out to substantiate suppositions (i), (iii) and (iv).

Method

Sections were removed from the internal tissue of seed pods using sterilised instruments and plated directly to MA. External structures such as pedicel and pod wall tissue were surface sterilised in a 1:1 mercuric chloride and alcohol solution.

RESULTS AND DISCUSSION

Plating shrivelled seeds which were in close proximity to the pod lesions gave a high percentage infection, indicating that penetration and infection of the pod wall may result in seed infection, (Table 42).

A high percentage of positive isolations from repla, placental tissue and seed funicles substantiates the theory that the fungus can spread from an infected seed centre to other seeds within the pods by infection of these parts. Further evidence is provided by the large proportion of infected seeds which were situated close to the discoloured placental tissue. In many cases this seed was taken from a chamber on the opposite side of the placenta from the lesioned pod wall. Plating plumped seeds from pods showing external lesioning resulted in a 12% infection indicating that only limited penetration to the seed had occurred. Lesioned pedicel tissue gave a high level of infection but seed from the pedicel end of such pods were not

TABLE 42

Isolation from siliquas infected with A. brassicicola

Origin and nature of the tissue plated	Treatment prior to plating	No. of isolations made	Percentage pathogen recorded
Severely lesioned pod wall tissue	Chemical surface sterilisation	40	77.5
Shrivelled seeds associated with severely lesioned siliqua wall tissue	None	100	80
Discoloured tissue of the replum	None	25	100
Discoloured and lesioned placental tissue	Chemical surface sterilisation	25	60
Seed funicles associated with infected replum and placental tissue	None	20	60
Seed adjacent to infected replum and placenta	None	50	58
Plumped seeds from pods showing severe lesioning without discolouration of the replum or placenta	None	100	12
Pinched and small seeds from pods with slight lesioning	None	50	6
Lesioned pedicel tissue	Chemical surface sterilisation	20	80
Seed from the lesioned pedicel end of pods with minimal spotting	None	50	4
Seed exhibiting germination in situ	None	20	75

generally infected. It is not likely then, that seed infection occurs via mycelial growth through the pedicel into the pod, but pedicel attack may be instrumental in causing a reduction of seed size due to constriction causing impedence of food material at the time of seed bulking. Further, on the basis of the above results the germination of seed in the pods is attributed to the effect of A.brassicicola.

2. A.brassicae

Pods were inspected after holding as in the previous investigation. In contract to siliquas infected with A.brassicicola, general discolouration of the pod was rare, the normal straw colour being retained although severely blemished with purplish-black localised lesions. Lesioning penetrated through the siliqua wall and seeds immediately below the discoloured tissue were frequently pinched and shrivelled. The replum and placental tissue was discoloured in some cases but not as frequently as in the A.brassicicola inoculated series. Splitting of pods along the line of the septum was observed, but again less frequently than for A.brassicicola.

Loss of seed yield caused by A.brassicae appeared to follow the pattern illustrated by A.brassicicola attacks.:-

(i) Through the direct affect of the fungus on the seed after penetration of the seed pod, with some spread occurring inside the pod.

(ii) The indirect loss due to failure of seed to bulk as a consequence of severe pedicel attack.

(iii) Premature splitting of pods and dehiscence of seed.

Tissue isolations were made to MA as in the previous trial.

RESULTS AND DISCUSSION

The results presented in Table 43, provide further evidence for the lack of persistence of A.brassicae infections in dried host material. However the isolation of some colonies from "pinched seed", even though only a small percentage, was in contradiction to the plating trials in the seed yield studies. In these experiments many hundreds of seeds were plated without successful isolation of A.brassicae. These results could be interpreted in two ways :

(i) that seed stored within the siliquas was not subject to as stringent drying conditions as the harvested seed and therefore some infection persisted;

(ii) that the infection was only superficially established in the seed coat and was eliminated by the surface sterilisation with mercuric chloride employed in the yield screening method.

Even if the latter was the case it was still apparent from the above results that very few seeds were in fact infected by A.brassicae.

TABLE 43

Isolation from siliquas infected with A. brassicae

Origin and nature of the tissue plated	Treatment	No. of isolations made	Percentage pathogen
Severely lesioned pod wall tissue	Chemical surface sterilisation	40	5
Lesioned pedical tissue	Chemical surface sterilisation	15	7
"Pinched" seed from severely infected pods	None	50	8
Discoloured tissues of the replum	None	15	0
Discoloured and lesioned placental tissue	None	15	0
"Plump" seeds from severely infected pods	None	50	0

G. DISCUSSION

In artificial inoculation trials, A.brassicae and A.brassicicola were shown to cause considerable reductions in the total weight of seed harvested per 100 siliquas, which was expressed mainly as a reduction in seed size. The increase in the amount of light seed harvested from inoculated pods is evidence for a direct effect of siliqua infection on the seed, but the yield reductions recorded must be regarded as a relative only, as further unaccounted for losses certainly occurred from premature dehiscence of pods in the field. In addition, factors such as inoculum load, environmental conditions and stage of growth at the time of infection may have had an influence on the final recorded effect. For example, Stevenson (1945) working on the pathogen A.ricini (Yoshi) Hansford, infecting racemes of castor-bean (Ricinus communis L.) noted that inflorescences at any stage of development were attacked. In the case of heavy infections young racemes and even flower primordia were killed. Schimmer (1953) observed in the course of her work that dying parts of the flower head were readily colonised by A.brassicicola, thus providing a source of inoculum close to the developing fruit. In the inoculation trials in this study most pods were well developed at the time of inoculation and hence it is possible that infections at an earlier stage could cause considerably greater losses. Finally, there is the possibility of foliage infection and premature defoliation causing a

reduction in the photosynthetic tissue area of the plant which may indirectly cause yield reductions.

Of the two Alternaria species, A.brassicicola was the most frequently isolated from crop material received from seed growers and was also isolated from field samples of Government nuclear seed crops of giant and medium stemmed choumoellier. However the complex of foliage and siliqua disease on choumoellier suggest that in the field, many pathogens could be involved in seed yield effects. To clarify the position as regards New Zealand produced choumoellier seed crops, more extensive work is required in field surveys of South Island stands.

CHAPTER V

A CONSIDERATION OF HEALTH SCREENING METHODS

A. INTRODUCTION

Routine seed health testing procedures are chiefly based on examination of seeds and seedlings after a period of incubation under controlled conditions of temperature and humidity. Testing methods have been developed more or less independently in various countries and differences in details between stations have resulted. For example, the containers used, the types of blotters, kinds of agar media, the incubation temperature selected and the length of the period of incubation often vary between stations (Neergaard 1965). The reason for this lack of uniformity in methods of disease diagnosis may be attributed primarily to the development of techniques by individual laboratories which were especially suited to their own requirements, facilities and personnel (Noble 1951).

In the report on the First International Conference on seed pathology (I.S.T.A., 1958) attention was drawn to the need for world-wide co-operation between seed pathologists for substantial progress to be made in this field. At the Conference the results of health screening tests on seed samples which had been distributed to many stations throughout the world were discussed. On the basis of the results obtained, the meeting proposed standardised testing methods for certain seed-borne diseases, which included a blotter method utilising 2,4-D* as a germination inhibitor for brassica species. In a later publication, Noble (1965) recommended a blotter test and

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* 2,4-Dichlorophenoxyacetic acid

an agar plate method specifically for the detection of the pathogens A.brassicae and A.brassicicola when associated with brassica seed. In this study in the course of preliminary seed screening tests using both these methods, disparities in the results between methods were observed, and further, certain inherent limitations in each technique were apparent. In view of the presence of both pathogens in New Zealand produced choumoellier seed (G.S.T.S., 1963 and 1964) and the ready availability of diseased lines, the whole question of screening choumoellier seed for the two pathogens was investigated. In this work full consideration was given to the following principles considered by de Tempe (1961) to be of major importance in any recommended seed health testing method :

(i) the pathogen should be easily identified;

(ii) the results obtained should be duplicable for one sample and comparable for different samples;

(iii) there should exist as close a relationship as possible between laboratory results and field performance;

(iv) the method should be cheap, simple and quick;

(v) the level of results should distinguish between disease-free, slightly, and severely infected samples and enable distinction between a seed crop of good and bad years.

B. THE BLOTTER METHOD FOR SEED HEALTH TESTING

A blotter method involves seed placement on moist blotters and incubation for a predetermined interval under controlled temperature and high humidity conditions. It is essentially the humid chamber method of plant pathological diagnosis adapted to seeds, and hence the pathogenic activity of the fungus on the growing plant is observed. This in turn enables an evaluation or indexing of the severity of the observed symptoms. Saprophytic species so often present on the seed do not cause infection and are therefore of little consequence in a blotter test, (Plate 29).

The method recommended by Noble (1965), for the identification of the two Alternaria species involved an incubation period of six days at 18-20°C in Near Ultra Violet Light in a 12 hour light/12 hour dark cycle. Identification of the pathogens was based on comparative symptomology and microscopic examination of sporulating material. Preliminary trials proved this method not to be altogether satisfactory. Considerable time was involved in the microscopic examinations and there was some doubt as to the reliability of visual appraisal of symptoms on seedlings. Further, from previous observations in germination trials (Chapter II) it seemed that seedling contact during growth on blotters could bias results in favour of a higher infection percentage. Hagborg et al (1950) used 2,4-D as an inhibitor of seed germination and this technique was

adapted by the I.S.T.A. (1958) for blotter health tests of brassica seed. Such a method could possibly overcome the problem of contact between diseased and healthy seedlings during the test.

In view of these criticisms experiments were conducted to examine closely :

(i) the reliability of eye appraisal in blotter tests;

(ii) the extent to which contact infection between seedlings may influence the results;

(iii) the value of germination inhibition by the use of 2,4-D.

1. Species identification

Two seed lines known to carry inoculum of both pathogens were subjected to a blotter test as described by Noble (1965), and examined independently by two analysts. Identification of the pathogens was made by microscopic examination of dead seeds and decaying seedlings and by following the symptom descriptions characteristic for each species. Two samples of 300 seeds from each seed line were investigated. Individual seedlings were examined and tentative identifications on the basis of symptoms either confirmed or discounted by plating the lesions to a selective "PDA/streptomycin" agar as developed for the "combination technique" (section D 1).

Plate 29

Seedling symptoms caused by
A.brassicicola during a blotter test

RESULTS AND DISCUSSION

It was apparent from the results presented in Table 46 that identification of pathogens to species level was not reliable. The main difficulty encountered was in the identification of A.brassicae lesions as there was a great variation in the symptoms expressed by this pathogen. It should also be noted, that in many instances cotyledonary lesions qualifying the description for A.brassicae were sterile on plating to agar. As the above results include the positive microscopic examinations it is apparent that the method as a whole may lead to extremely variable results the level of which would in part be dependent on the relative experience of the analyst.

2. Contact infection in blotter tests

de Tempe (1963a) concluded that a spacing of about 2 cm between seeds is necessary to prevent contact infection in a blotter test. In germination tests and blotter health tests conducted in this study 2 cm seed placement centres were used. However, although seeds were placed at this distance, on germination the position of the resultant seedling was not constant and contact between seedlings frequently occurred. This was especially so where severely attacked seedlings collapsed after emergence (Plate 30).

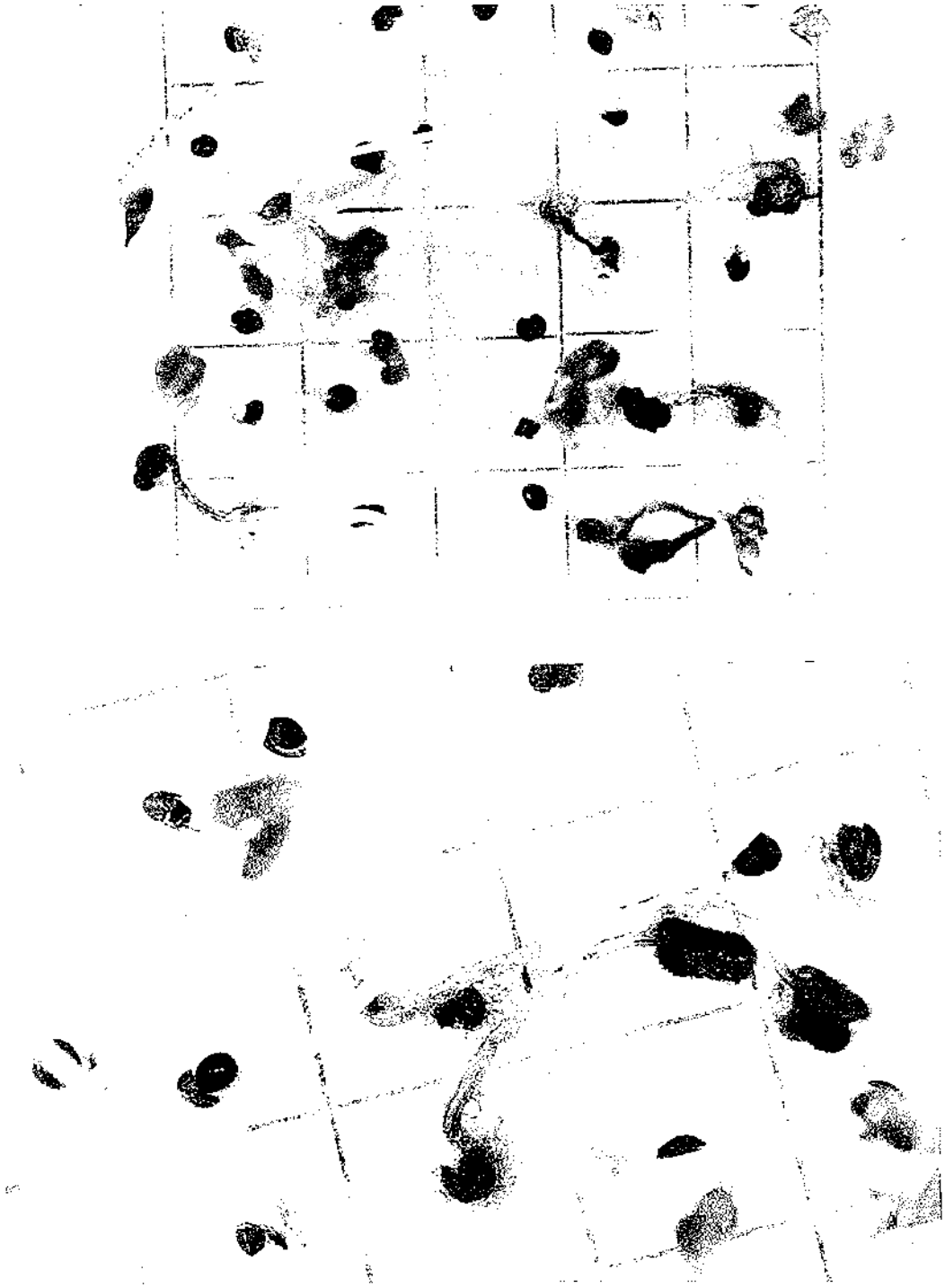
An experiment was conducted to investigate the possibility of contact infection occurring in a blotter test

TABLE 46

Identification of *Alternaria* infections of seedlings to species level

Seed Line	Analyst	*A. brassicicola* infections			*A. brassicae* infections		
		No. of infections identified	No. correctly identified	% correctly identified	No. of infections identified	No. correctly identified	% correctly identified
Q_1	A	46	30	65	15	5	33
	B	30	18	60	24	6	25
Q_2	A	28	25	89	12	3	25
	B	25	19	76	22	4	18

Plate 30



"Contact" infection between
seedlings during a blotter test

for choumoellier seed contaminated with A.brassicicola, thereby biasing the results against the seed line.

Method

Seed from the bulked supply of a disease-free line was surface sterilised, rinsed, and dried. A sample of this seed was then 100% contaminated with A.brassicicola.

Four 100 seed replicates were set out on blotters, only 5% of which were contaminated with the fungus. Two further series of four 100 seed replicates were similarly set out, but in these the level of contaminated seeds per series was 15% and 30% respectively. As controls, for each of the above three levels of contamination a parallel series of four 100 seed replicates were set out in individual cells formed by plastic "honeycomb" sheeting on germination trays. Each cell was 1.6 cm deep with seed placement centres of 2.5 cm. It was intended that the cells would provide a physical barrier and in effect prevent contact infection between diseased and healthy seedlings. It was reasoned that if contact infection occurred using the standard blotter test, then a higher infection percentage would be recorded for this method than for seedlings grown in individual cells.

RESULTS AND DISCUSSION

The results presented in Table 47 show that while some contact infection occurred, more particularly at high levels of seed contamination, this did not appear to be serious. At

TABLE 47

Contact infection in a blotter health test

Level of seed contamination %	% infected seedlings recorded			
	6 days		10 days	
	Standard blotters	Honeycomb cells	Standard blotters	Honeycomb cells
	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
5%	5.7 ± 0.2	5.5 ± 0.5	7.0 ± 0.9	6.5 ± 0.9
15%	17.5 ± 0.5	15.2 ± 0.2	21.0 ± 1.1	17.0 ± 0.7
30%	32.5 ± 0.8	30.5 ± 0.5	37.5 ± 1.7	32.7 ± 1.1

six days some severely diseased seedlings had collapsed and contacted neighbouring healthy plants, but it was only after a further four days that symptoms became obvious, indicating successful transference of infection. However, it was felt that an experienced analyst would recognise these as such. Spread of infection frequently occurred through root systems spreading to reach diseased seedlings, and this resulted in some browning of the radicle. But in most cases the discolouration was so slight that there was no danger of misinterpretation of such symptoms at six days. That is, it appeared that only minor secondary spread would occur over the normal duration of a blotter test.

It will be noted that in all of the control series some contact infection did occur and this was believed to have resulted from disturbance of the apparatus when recording results at the six day interval, thereby permitting root contact.

3. Germination inhibition in blotter tests

Sodium 2,4-D has been recommended in blotter health tests (I.S.T.A., 1958) for brassica seed. In this method a solution of the sodium salt of 2,4-D is used instead of water for wetting the blotters in the germination test. Lloyd (1959) used this method in screening seed of Brassica species for infections of Phoma lingam (Tode) Desm. In his experiments a 0.2% solution either prevented seed germination or caused

abnormal seedling development.

The possible use of 2,4-D for health screening of choumoellier seed was investigated by first determining an effective strength for suppression of germination and then ascertaining whether the use of this method in any way facilitated identification of the two pathogens when associated with seed.

(a) The effect of various concentrations of 2,4-D on seed germination

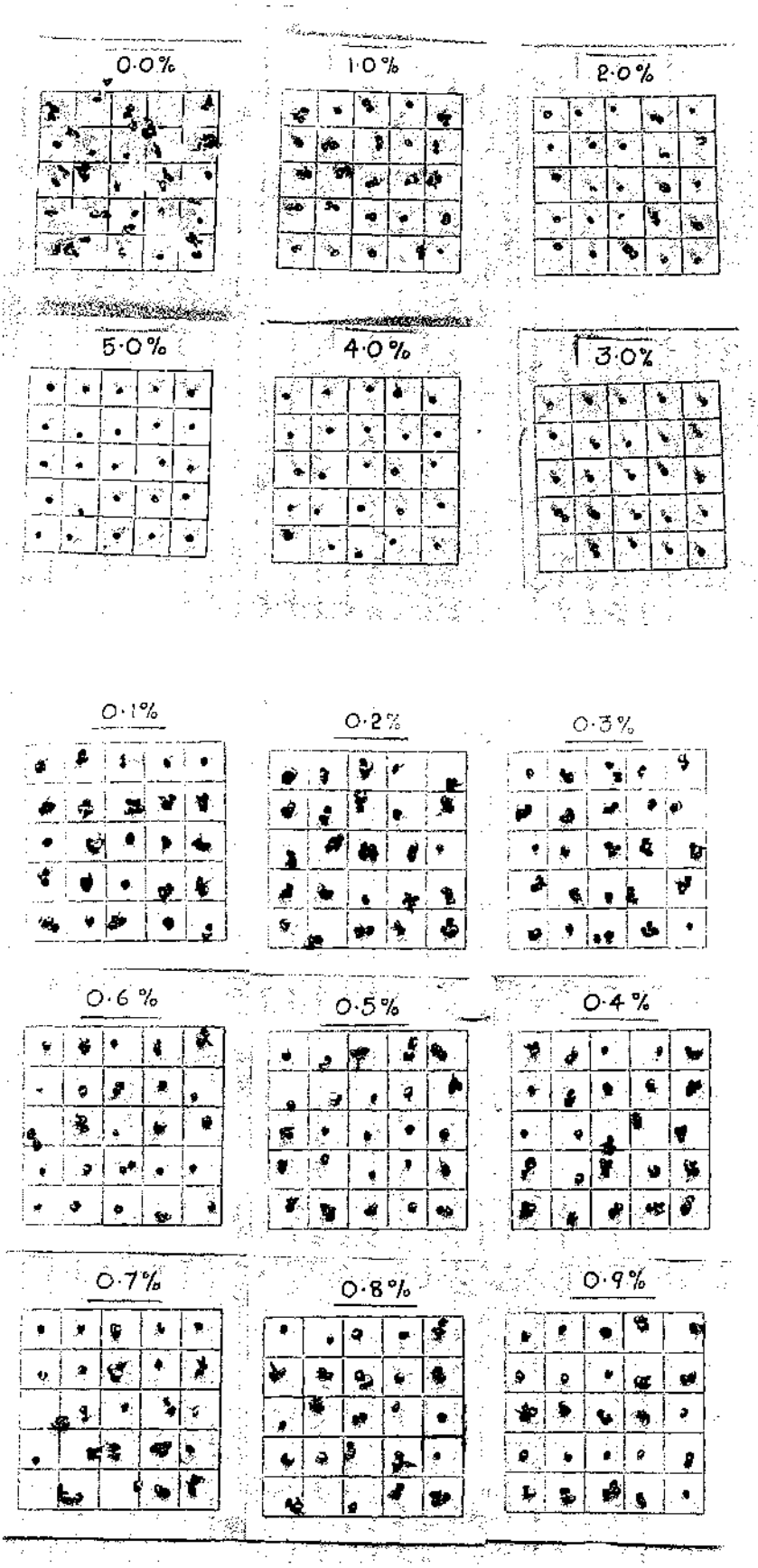
In preliminary experiments using a range of concentrations, germination was almost completely inhibited at the 1% level (Plate 31). A further experiment was then conducted with strengths ranging from 0.1% to 0.9% in 0.1% increments. The seeds were placed out on the blotters and incubated in a Copenhagen type seed germinator (Plate 32) for six days at 24°C.

At all strengths there was evidence of germination but seedling development was effectively suppressed (Plate 31). In subsequent experiments 0.1% concentration was therefore used.

(b) Use of 2,4-D in blotter tests

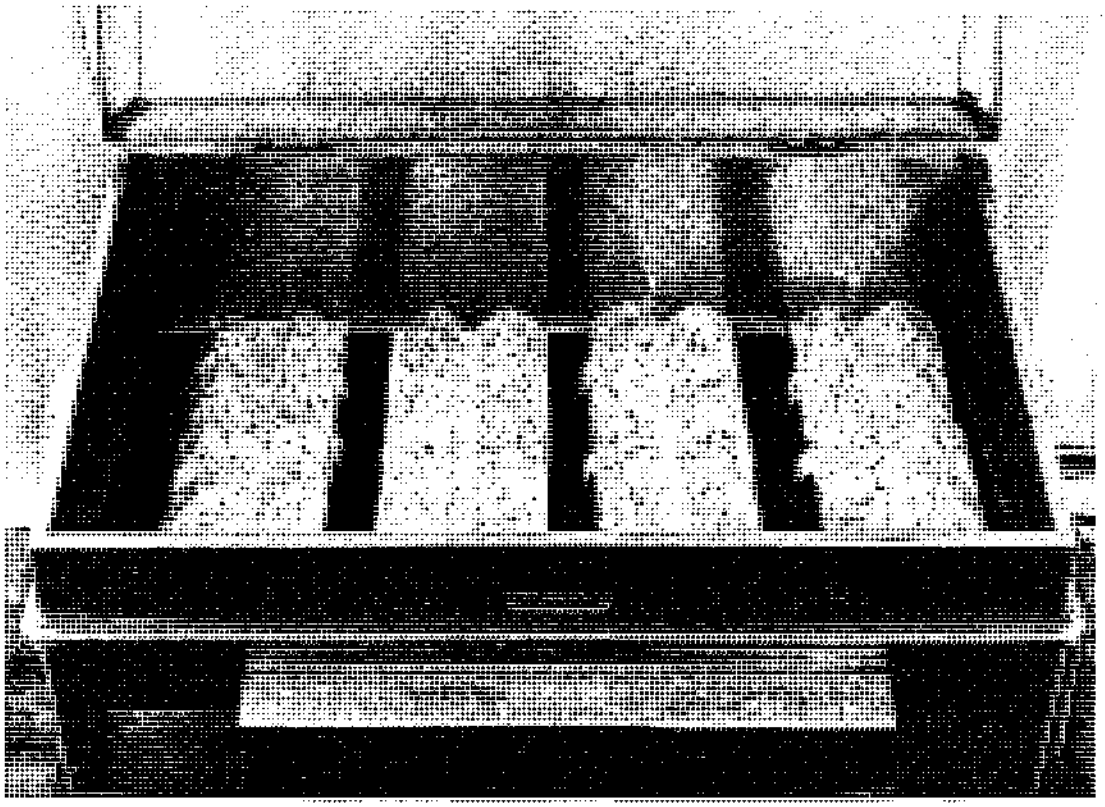
A number of seed lines infected with either or both A.brassicae and A.brassicicola were examined after incubation on blotters presoaked in a 0.1% 2,4-D solution. The objective was to determine whether the pathogen(s) present could be readily identified on the basis of either the symptoms expressed or sporulation on lesioned tissue.

Plate 31



Effect of varying concentrations of sodium 2,4-D on germination in a blotter test

Plate 32



Seed germinator used in seed
health trials

Several limitations of the method were immediately apparent. Symptoms were extremely difficult to identify and the microscopic examination of seedlings was both laborious and time consuming. Further, one of the main advantages of a blotter test is that it enables disease progress to be followed, and the severity of attack of individual seedlings to be assessed. By inhibiting germination these advantages are immediately lost, and the method thus became little more than a viability test. For these reasons germination inhibition by the use of 2,4-D was considered unsatisfactory in blotter health tests of choumoellier seed for A.brassicae and A.brassicicola.

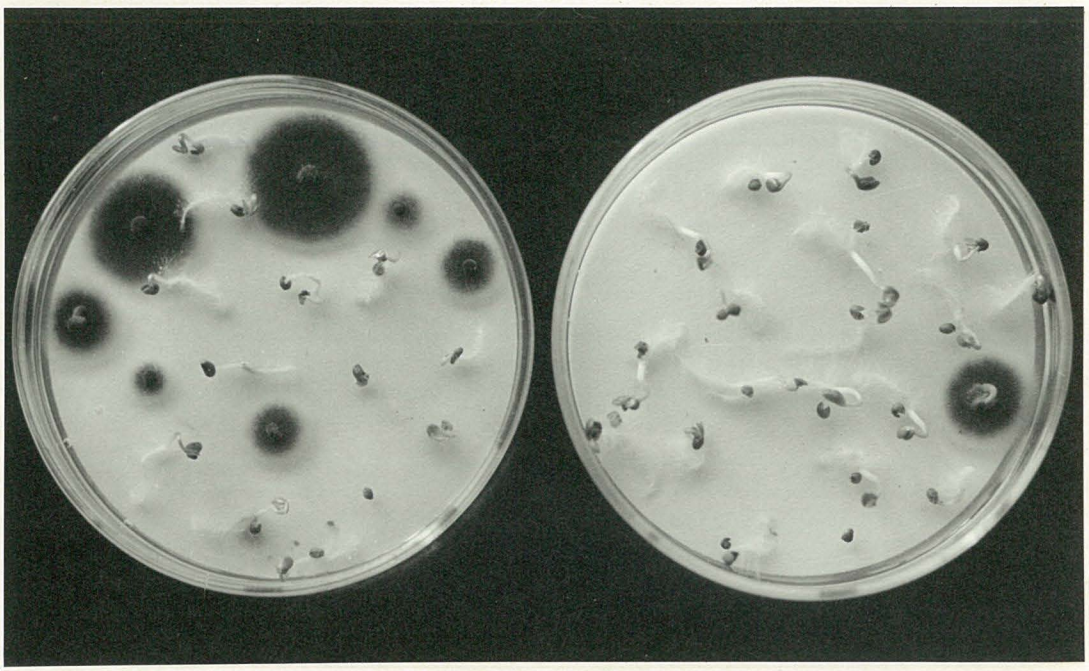
C. AGAR PLATING METHODS

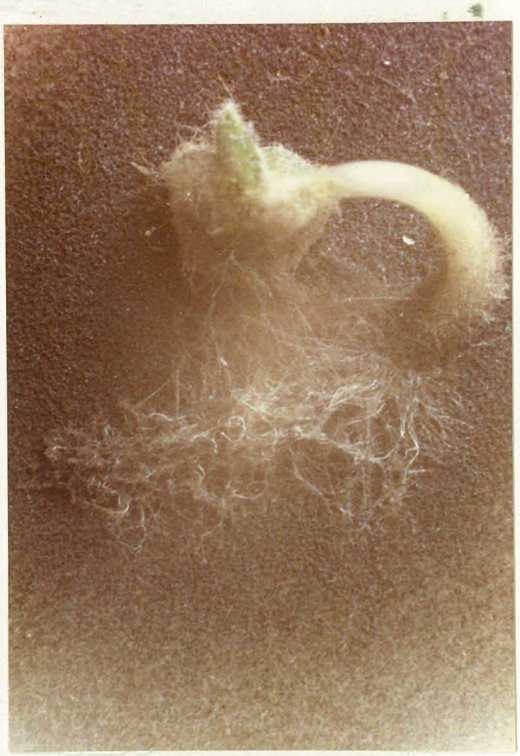
Agar plating methods are suitable for pathogens exhibiting quick and characteristic development on an agar medium; that is, the capability of saprophytic growth of the pathogens is utilised (Plate 33). Such a method does not discriminate between pathogenic and saprophytic fungi and hence the resultant colonies merely demonstrate the presence of viable inoculum. Where obligate saprophytes are associated with seed their growth may be so strong as to mask the growth of slower growing pathogenic species. This problem has been minimised by the development of surface sterilisation techniques designed to eliminate such associated contaminants. However these methods also remove contaminating pathogenic elements and the results obtained therefore only reflect seed infection.

A trial was carried out to investigate the possibility of dispensing with surface sterilisation and plating seed directly to agar, as in the Ulster method (Muskett and Malone 1941). Although favourable results were obtained for some seed lines, in the majority, the level of contamination necessitated pretreatment before plating to agar. In general, the older the seed, the greater the problem became.

Cyclohexamide has been successfully used in selective media for the suppression of contaminant mould growth (Phillips and Hanel, 1950). According to Whiffen,(1948) the growth of various genera of fungi is suppressed at different concentrations of this antibiotic. It was reasoned that there may be a particular concentration at which cyclohexamide would selectively eliminate some of the seed contaminants associated with choumoellier seed without adversely affecting growth of the pathogens. Trials were carried out using this chemical but the results were unsatisfactory. Unfortunately growth of A.brassicae was severely retarded at a concentration as low as 1.0 /ug/ml, and completely inhibited at 3.0 /ug/ml, while A.brassicicola was retarded at 5 /ug/ml and was inhibited at 15 /ug/ml. As against this, common saprophytes such as Penicillium species were not obviously affected at a strength as high as 20 /ug/ml. Accordingly, no further consideration was given to the use of cyclohexamide in agar screening methods for choumoellier seed.

Plate 33





Development of A.brassicicola colonies from infected seeds following incubation for 6 days at 22°C on P.D.A.

1. Comparison of seed surface sterilisation treatments

Several chemical methods have been used for surface sterilising seeds of cruciferous crops prior to plating to agar (Pound et al, 1951; McLean, 1947; McDonald, 1959; Noble, 1965; Matthews, 1965). With regard to choumoellier seed, to the author's knowledge the only established methods are those recommended by Matthews (1965) and Noble (1965), both of which utilise sodium hypochlorite (1% available chlorine) as the sterilant material. In the screening trials conducted in Chapter II it was found that the restandardisation of this material prior to each experiment was laborious and time consuming and for this reason an alternative method was sought. McLean (1947) had success with a 0.001% mercuric chloride solution for surface sterilising radish seed (Raphanus sativus L.) and as this material was readily prepared, a preliminary trial was conducted comparing these two methods. Since Milne (1964) had considerable success in surface sterilising seeds of lupin (Lupinus angustifolius L.) simply by washing in tap water this method was also included for comparison.

Seed from a naturally infected line was enclosed in muslin bags, and surface sterilised by one of the following methods :

(i) immersion in 0.001% mercuric chloride for 10 minutes (McLean 1947);

(ii) immersion in a sodium hypochlorite solution (1% available chlorine) for 10 minutes following a pretreatment dip in 70% alcohol (Matthews 1965);

(iii) washing for eight hours in running tap water using the tumbler technique (Milne 1964) (Figure 13).

After treatment the seed was in each case rinsed in sterile distilled water and dried between filter paper before plating to MA and incubating at 24°C for six days. The results are presented in the following table :

TABLE 48

Percentage infection from plating seed after surface sterilisation by three different methods

Treatment	% infection of* A.brassicicola
0.001% mercuric chloride - 10 minutes	5.7
1% chlorine solution - 10 minutes	5.0
Washing - 8 hours	14.0

A surprising feature of these results was the disparity between the chemical methods and washing as regards the percentage of seed which showed evidence of A.brassicicola. The chemical methods gave comparable results but the level of infection obtained after surface sterilisation by washing was more than twice as great. This difference may be interpreted

* Mean of three 100 seed replicates per treatment

in one of two ways :

(i) the washing technique was inefficient and did not remove all of the surface inoculum of the pathogen, thereby resulting in a relatively higher percentage figure;

(ii) washing was efficient and removed the surface contaminants, but the chemical treatments did more than this. That is, the chemical had penetrated the seed and eliminated some of the infection established in the seed.

If the latter supposition was in fact the case, then the results obtained after chemical surface sterilisation did not reflect the true infection percentage of seed. Since the primary objective of surface sterilisation techniques is to remove obligate saprophytes without inactivating establishing seed infection, any effect in this way is highly undesirable. To determine the precise cause of the discrepancy in the above experiment a further series of experiments were conducted, based on the following reasoning :

(i) by artificially infecting a seed line with A.brassicicola and then surface sterilising using the above three methods any differences in the resulting percentage infection must be attributed to a treatment effect on the fungus within the seed;

(ii) in any line of infected seed the depth to which the pathogen is established, no doubt varies. Hence it was considered desirable to test each method on seed with varying depths of infection. Such seed could be obtained simply by varying the incubation period in the course of artificially infecting that seed;

(iii) in an experiment where the objective is to determine if inactivation of established infection occurs, it is essential that surface inoculum inevitably present as a consequence of the method used in obtaining seed infection be eliminated;

(iv) thus a pre-requisite to the above was preliminary experimentation to establish the precise treatment time for each method which would eliminate all surface contamination. Henceforth this time interval is termed "the point of effectiveness".

(a) Determination of the "point of effectiveness" in surface sterilisation of seed

Method

Seed of a line previously shown to be pathogen-free were contaminated by vigorous agitation in Petri dishes containing sporulating colonies of A.brassicicola.

Sub-samples were then subjected to the three surface sterilisation methods for a range of treatment periods as follows:

(i) immersion in 0.001% mercuric chloride (5 - 11 minutes in one minute increments);

(ii) immersion in sodium hypochlorite (1% available chlorine) after a pretreatment dip in alcohol (5 - 10 minutes in one minute increments);

(iii) washing, using the tumbler technique (2 - 12 hours in two hour increments).

After all treatments seed was rinsed in sterile distilled water prior to plating to agar. In each case 300 seeds were tested per treatment and the results recorded after six days incubation at 24°C on PDA.

RESULTS AND DISCUSSION

The results presented in Appendix XXI show that the point of effectiveness for the mercuric chloride treatment was eight minutes and for sodium hypochlorite nine minutes was required. Although in the water washing there was a slight decrease in the amount of contamination, even at 12 hours, over 80% of the seeds gave rise to a colony of A.brassicicola on plating. It thus seemed highly probable that the difference in effectiveness of the chemical treatments and the washing method in the earlier experiment (Table 48), was largely due to inefficient

removal of surface inoculum from the seed. However there was still the possibility of the chemical treatments inactivating seed coat infection to some extent. To determine if this was the case an efficient non-chemical surface sterilisation method was needed to be used as comparison. Further experimentation was therefore carried out to develop an improved washing method which would totally eliminate surface borne inoculum on choumoellier seed.

(b) Determination of an effective washing method for surface sterilisation of seed

Artificially contaminated seed was surface sterilised by washing for varying periods using three different methods. In each case seed was then plated to MA to determine the relative efficiency of each method in removing this inoculum. Details of the three methods are as follows :

(i) "The gauze flask method" (Figure 13). Contaminated seed was placed in a gauze flask which was partially submerged in a 1000 ml beaker. The water jet thrusting at an angle promoted a circulatory current, and a fine air inlet to the hose caused further turbulence and agitation of the seed against the wire mesh of the container.

(ii) "The gauze flask method, modified" (Figure 13). A paddle wheel was introduced as a refinement of the previous method for the purpose of increasing agitation

and turbulence.

(iii) The "free flow method" (Figure 13). Seed was placed in a cylinder, open at one end and with a gauze filter in the bottom. The apparatus was enclosed in a muslin bag which was fastened around the neck of a tap. The direct flow of water through the cylinder promoted a pounding and agitation of the contaminated seed. The muslin bag was pierced at the bottom to prevent air locks from forming and causing a "flush back" to disturb the washing action.

It can be seen from the results presented in Appendix XXII that of all the techniques, washing by the free flow method for five hours was the only one which was totally efficient in removing surface inoculum. With an effective non-chemical method identified it was now possible to use this as a control in determining the effect of chemical methods on the level of infection in artificially infected seed.

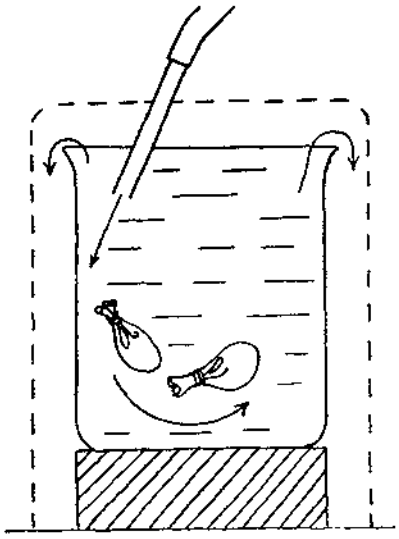
(c) Effect of surface sterilisation on the infection percentage of artificially infected seed

Method

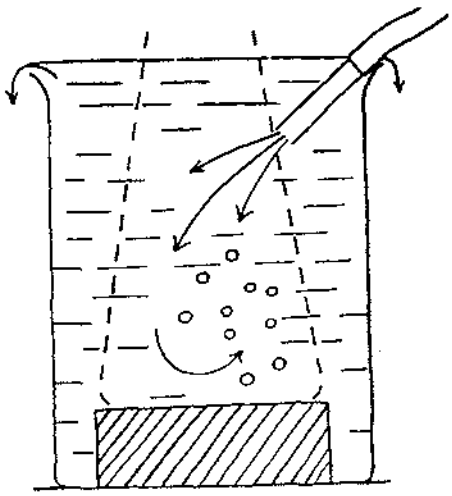
Seed infection was induced by placing artificially infected seed in the infection cabinets (Plate 11). To obtain varying depths of seed infection, samples were removed at $2\frac{1}{2}$, $7\frac{1}{2}$, 11, 14, 19 and 25 days. After drying for 24 hours a part of each sample was surface sterilised by immersion in

Figure 13

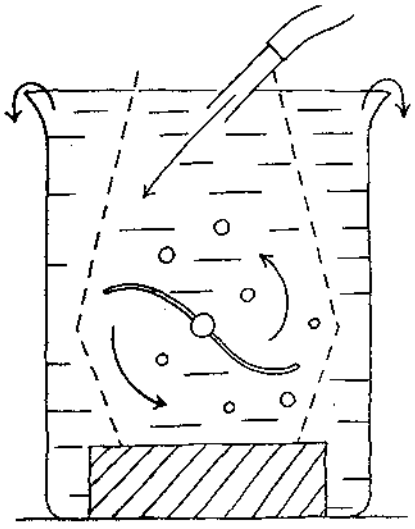
SURFACE STERILIZATION OF SEED BY WASHING



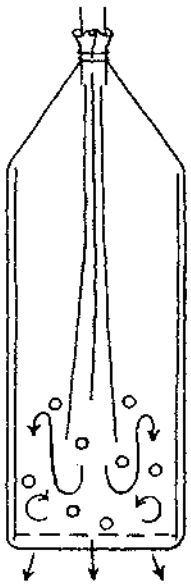
(i) TUMBLER TECHNIQUE.



(ii) GAUZE FLASK METHOD.



(iii) GAUZE FLASK METHOD (MODIFIED)



(iv) FREE FLOW METHOD

Apparatus used in seed washing trials

mercuric chloride (eight minutes), sodium hypochlorite (nine minutes) after a pretreatment dip in alcohol, and by washing using the free flow method (five hours). For each the infection percentage of three replicates of 100 seeds were assessed after incubation on blotters for eight days at 24°C.

RESULTS AND DISCUSSION

The variation between replicates of individual treatments and the lack of consistent differences between treatments at each test interval for the mercuric chloride and sodium hypochlorite methods (Table 49) preclude any conclusions regarding respective treatment effects on the infection level recorded. On the other hand, surface sterilisation of seed by washing resulted in a higher infection percentage at most readings. It seems then that under the conditions of this experiment the mercuric chloride and sodium hypochlorite treatments were in fact eliminating some of the mycelium in the seed coat. These results suggest that the difference in the percentage of A.brassicicola recorded in the preliminary trial (Table 48) was probably due to a composite effect. That is, the washing was ineffective as shown previously, but as well, the chemical did inactivate some seed infection. To substantiate this conclusion a further trial was carried out on naturally infected seed lines. In the experiment the free flow washing method was used as a control. As both the sodium hypochlorite treatment and the mercuric chloride treatment gave comparable

TABLE 49

Comparative surface sterilisation of artificially infected seed

Treatment	Infection percentage recorded after various incubation periods (days)					
	2½	7½	11	14	19	25
	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.	Mean ± S.E.
0.001% Mercuric Chloride	4.0 ± 1.1	17.0 ± 2.9	29.7 ± 1.2	37.3 ± 1.8	55.0 ± 3.2	90.7 ± 0.9
Sodium Hypochlorite (1% available chlorine)	4.0 ± 0.6	12.7 ± 1.2	30.7 ± 4.0	56.3 ± 3.3	59.3 ± 0.6	98.7 ± 1.3
Free Flow Washing Method	3.3 ± 1.3	36.0 ± 2.1	52.3 ± 3.4	65.7 ± 2.3	76.0 ± 2.3	95.0 ± 1.7

results in the previous experiments, only the latter was compared with washing.

(d) Effect of surface sterilisation with mercuric chloride on the infection percentage of naturally infected seed

Method

Five naturally infected seed lines were each surface sterilised by washing using the free flow method (5 hours), and by immersion in 0.001% mercuric chloride for eight minutes. In the former series seed was plated to "antibiotic agar" (PDA containing 80 iu/ml of streptomycin : see section D.1) which was generally selective against bacteria and eliminated the contamination problems experienced in previous washing trials. The number of seeds infected with either A.brassicae or A.brassicicola were recorded after six days incubation at 24°C.

RESULTS AND DISCUSSION

The results presented in Table 50 show that after chemical surface sterilisation the infection percentage recorded was generally less than that obtained after washing. Hence, this evidence lends weight to the supposition that chemical surface sterilisation does remove more than just surface spores from seed.

A surprising feature of these results was the high percentage of A.brassicae recorded for line S_4 after washing by the free flow method. These results suggest that the chemical sterilisation had all but eliminated seed infection by A.brassicae.

TABLE 50

Effect of chemical and non-chemical surface sterilisation on the infection percentage recorded for naturally infected seed

Seed Line	Infection percentage of *A. brassicae* and *A. brassicicola* *															
	A. brassicicola								*A. brassicae*							
	Mercuric chloride method				Free flow method				Mercuric chloride method				Free flow method			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
S_1	2	4	6	4.00	6	5	6	5.66	0	0	0	0.00	0	0	0	0.00
S_2	8	5	6	6.33	9	9	15	11.00	0	0	0	0.00	0	0	0	0.00
S_3	5	0	5	3.33	6	10	7	7.66	0	0	0	0.00	0	1	0	0.33
S_4	2	2	3	2.33	1	0	6	2.33	2	0	0	0.66	7	11	3	7.00
S_5	0	0	0	0.00	0	2	1	1.00	0	0	0	0.00	0	0	0	0.00

* Three replicates of 100 seeds were plated for each treatment of the five lines

If this is the case then it would appear that seed infection by A.brassicae was of a superficial nature, this pathogen penetrating only the outer layers of the seed coat. As mercuric chloride was the sterilant material in the earlier screening trials using seed harvested from artificially inoculated siliquas and certified seed lines, then the above reasoning could explain the inability to isolate A.brassicae in those experiments.

2. Modifications of the basic agar screening method

In previous screening trials (Chapter II) cotyledon lesions were observed on seedlings which had not developed a colony on the agar medium. While it is possible that such lesions were sterile, it is also possible that they may have been caused by weak infections or deep seated embryo infections which had not had sufficient time to establish growth in the agar before being lifted off the surface with epigeal germination. Further, as crowding often leads to confusion in identifying the seed from which a colony originated, only a limited number of seeds can be placed out per plate. Various workers have encountered these problems and as a result have modified the basic plating technique.

Newhook (1947), developed the "New Zealand Method" for screening flax seed. This involved covering the seed with a layer of agar after surface sterilisation which prevented seed germination and ensured that all of the seed surface was in contact with the medium. de Tempe (1963b) recommended inverting

the dishes after plating flax seed to agar using the Ulster method (Muskett and Malone 1941). In effect he was utilising the phototrophic response of seedlings to ensure cotyledonary development was in contact with the agar surface. Kilpatrick and Johnson (1956), following the work of Hagborg et al (1950) used the 2,4-D to inhibit germination in legume seeds. This technique was successfully adapted to brassica seed by Lloyd (1959) for the identification of seed infected with Phoma lingam (Tode) Desm. He found that seed coats were generally broken but germination did not often proceed beyond the stage at which the cotyledons and radicle started to emerge.

Experiments were conducted to ascertain whether such modifications improved the efficiency of agar plate tests in screening choumoellier seed for infections of A.brassicae and A.brassicicola. A pre-requisite to those experiment involved an investigation into the effects of 2,4-D on colony growth of the two pathogens, and on germination of choumoellier seed.

(a) The effect of 2,4-D on colony characters and on seed germination

At concentrations as low as 0.05% there was some adverse effect on germination. At a concentration of 0.15% or higher, neither root systems nor hypocotyls developed, germination being restricted to emergence of the radicle tip and cotyledons. Colony growth of A.brassicicola did not appear to be affected, but some reduction in growth of A.brassicae was apparent at the

0.25% level.

However, in these preliminary trials 2,4-D was added after tempering the agar to 45°C and this double handling of the media was not only time consuming, but also increased the liklihood of contamination.

Lloyd (1959) added 2,4-D to his media before autoclaving, thereby overcoming these difficulties. In view of his success an experiment was conducted in which 2,4-D was added to the media after autoclaving. MA was used since in previous screening trials, it had proved suitable from the viewpoint of colony identification.

Method

A 1% solution of the sodium salt of 2,4-D was prepared and added to molten MA in quantities calculated to give final 2,4-D agar concentration of 0.1%, 0.15%, 0.20%, 0.25%, 0.30% and 0.40%. The treated agar was then autoclaved at 15 lb/in^2 for 20 minutes and tempered to 45°C before plates were poured.

Three millimetre agar discs obtained from three week old sporulating colonies of each organism were placed face downward on the 2,4-D agar (six discs per 2,4-D concentration). The effect on colony growth was measured by taking the average of two diametric colony measurements made at right angles to each other after six days incubation at 24°C.

To observe the effect of 2,4-D on germination, 100

surface sterilised seeds were plated to the agar per treatment and the seeds examined after six days incubation. In assessing the extent of germination recordings were based on the following categories :

(i) no germination;

(ii) retarded germination,

A - emergence of the hypocotyl and radicle but no further development

B - splitting of the seed coat only;

(iii) normal germination.

RESULTS AND DISCUSSION

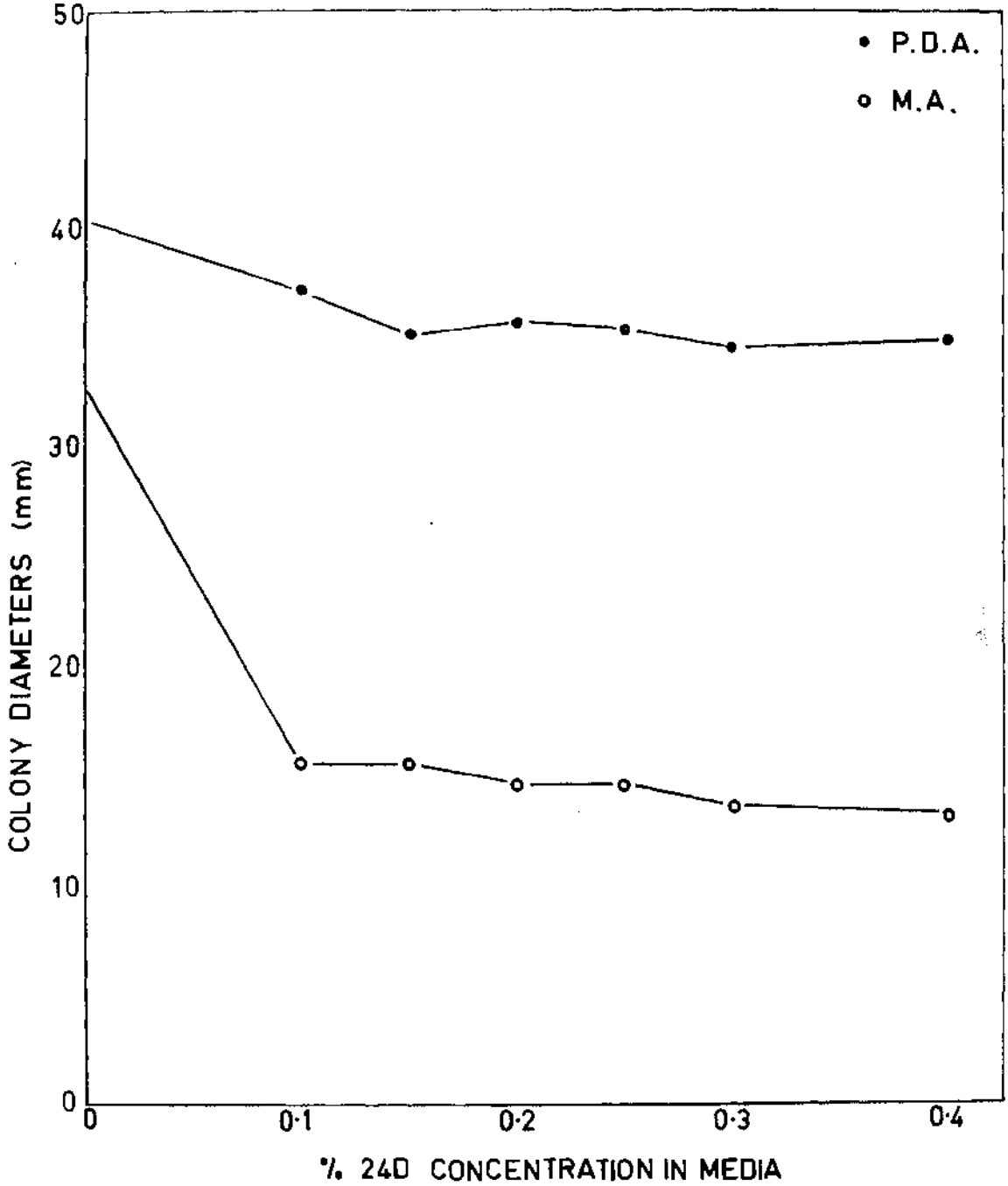
The results presented in Table 51 show that all concentrations of 2,4-D in MA had the desired effect of retarding germination. It seemed then, that the most suitable 2,4-D concentrations would be governed simply by the effect of the respective treatments on colony growth. However at all levels there was a severe reduction on colony development (Figures 14 and 15, Plate 34) which was not expected in view of the favourable results obtained in preliminary trials using PDA. Not only was growth reduced, but the colonies were devoid of the identifying characteristics associated with each fungus. This could be interpreted as either a media effect, or an effect of autoclaving on the properties of 2,4-D. To determine the actual cause, a further trial was carried out using 2,4-D in both MA and PDA.

TABLE 51

Effect of varying 2,4-D/MA concentrations on seed germination

2,4-D concentration in media	No germination (%)	Retarded germination (%)		Normal germination (%)
		A	B	
0.1%	16	56	28	0
0.15%	38	28	34	0
0.20%	60	12	28	0
0.25%	68	4	28	0
0.30%	68	8	24	0
0.40%	77	9	14	0
Control	7	0	4	89

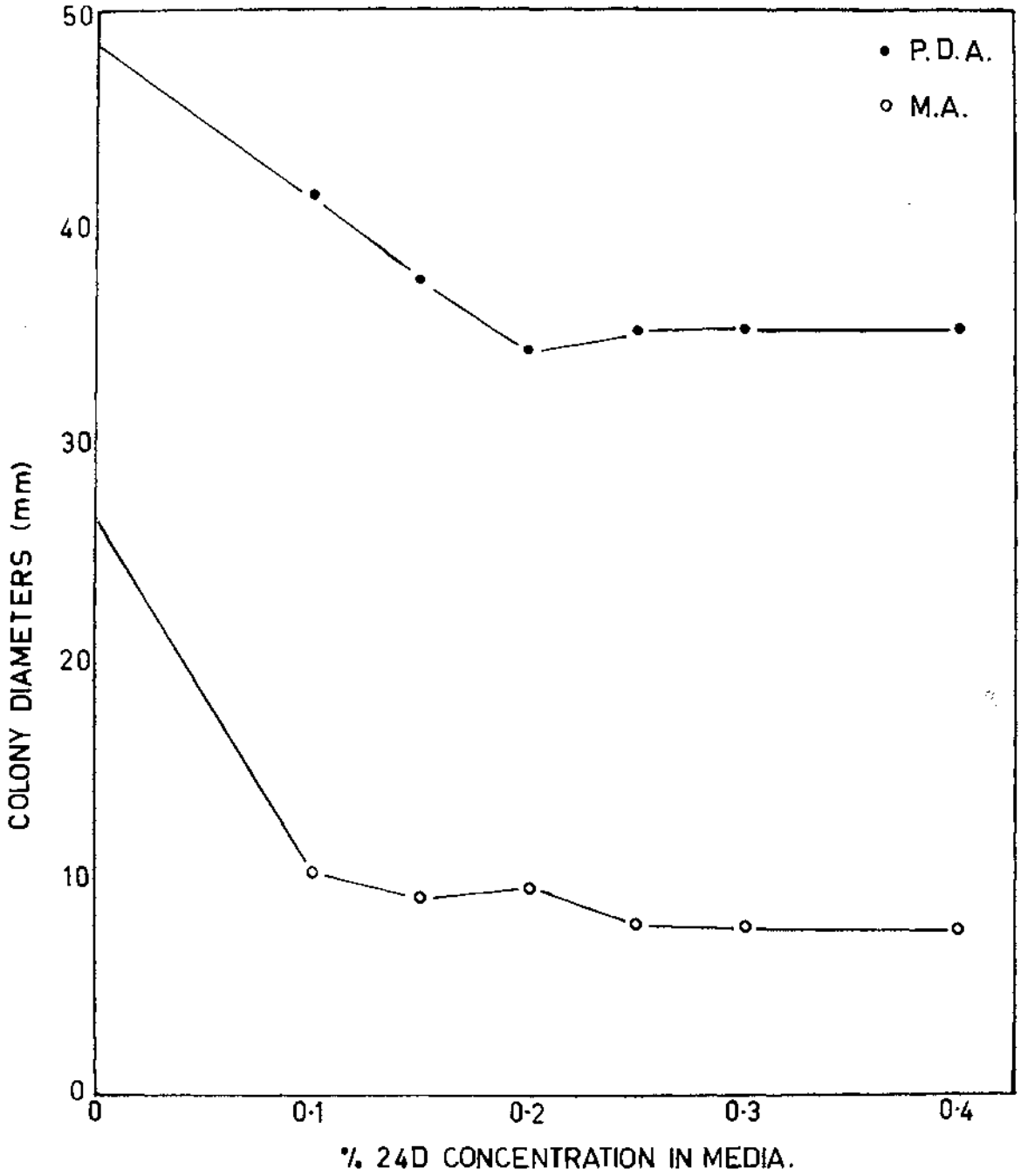
Figure 14



The effect of various concentrations of sodium 2,4-D in MA and PDA on colony growth of A.brassicicola

(results recorded following incubation for six days at 24°C)

Figure 15



The effect of various concentrations of sodium 2,4-D in MA and PDA on colony growth of *A.brassicae*

(results recorded following incubation for six days at 24°C)

(b) The effect of autoclaving 2,4-D/PDA and 2,4-D/MA on colony growth

Method

Using both media, 2,4-D/agar concentrations of 0.1% were prepared before and after autoclaving. To measure the effects on colony features and the extent of growth, six 3 mm diameter discs cut from sporulating colonies of A.brassicae and A.brassicicola were plated per treatment and incubated for six days at 24°C.

RESULTS AND DISCUSSION

The results presented in Table 53 show that colony growth was not markedly affected by the addition of 2,4-D to PDA. Colony characteristics were not altered (Plate 35) and the effect of autoclaving the media caused no adverse effect other than a slight reduction in growth. On the other hand, in the 2,4-D/MA treatments there was a severe growth reduction (Table 52) and colony characteristics were modified in that the capacity of both species to sporulate was impaired (Plate 34).

In view of these results PDA was considered to be the most satisfactory medium in 2,4-D agar preparations. To identify the most favourable 2,4-D/PDA concentration for screening work an experiment was carried out using autoclaved PDA with 2,4-D concentrations of 0.1%, 0.15%, 0.20%, 0.25%, 0.30% and 0.40%. Colony diameters and seed germination effects were recorded as above. The results expressed in Figures 14 and 15 and illustrated in plates 35 and 36 (see Appendix XXV) indicated

TABLE 52

Effect of autoclaving 2,4-D/MA on colony growth

Pathogen	Media autoclaved before or after adding 2,4-D	2,4-D concentration	Colony diameters (mm)						
			1	2	3	4	5	6	Mean
	Before	0.1%	13.5	13.0	12.5	14.0	13.5	13.0	13.25
A. brassicae	After	0.1%	13.5	14.0	13.0	13.0	12.5	13.0	13.16
	None	None	30.0	29.0	30.0	31.0	31.0	30.0	30.16
	Before	0.1%	20.0	20.0	20.5	22.5	19.0	19.5	20.25
A. brassicicola	After	0.1%	17.5	17.0	19.0	19.5	21.0	19.0	18.83
	None	None	35.0	38.0	36.0	37.0	34.0	32.0	35.33

TABLE 53

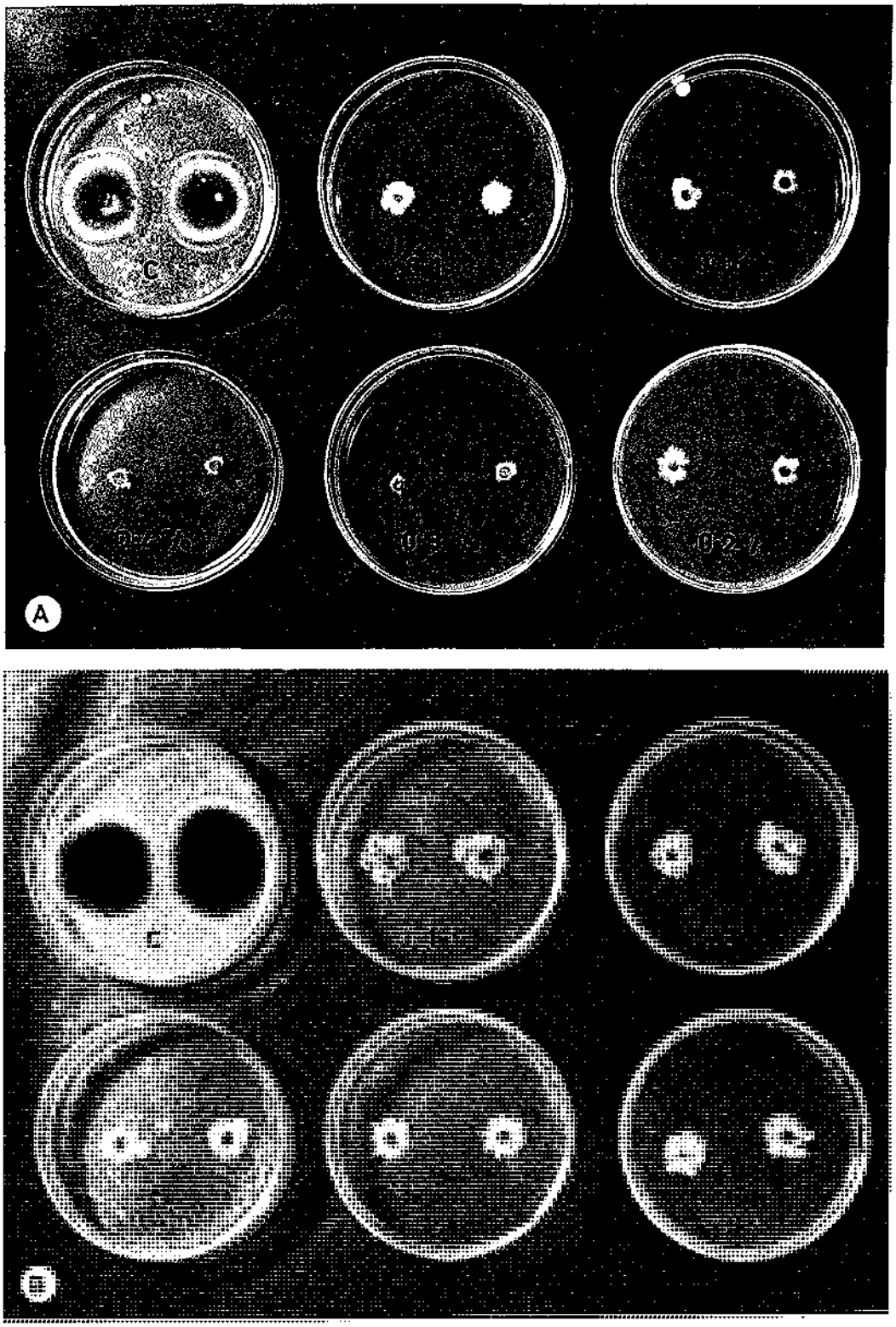
Effect of autoclaving 2,4-D/PDA on colony growth

Pathogen	Media autoclaved before or after adding 2,4-D	2,4-D concentration	Colony diameters (mm)						
			1	2	3	4	5	6	Mean
	Before	0.1%	28.5	32.5	30.0	29.0	27.0	28.5	29.25
A. brassicae	After	0.1%	26.5	25.0	24.0	25.5	27.5	28.0	26.0
	None	None	37.5	34.5	29.0	36.5	37.0	34.6	34.66
	Before	0.1%	42.0	38.5	40.0	40.5	42.5	41.5	40.83
A. brassicicola	After	0.1%	37.5	37.5	36.0	35.0	36.0	38.5	36.75
	None	None	45.5	45.0	43.5	43.0	43.0	44.5	44.8

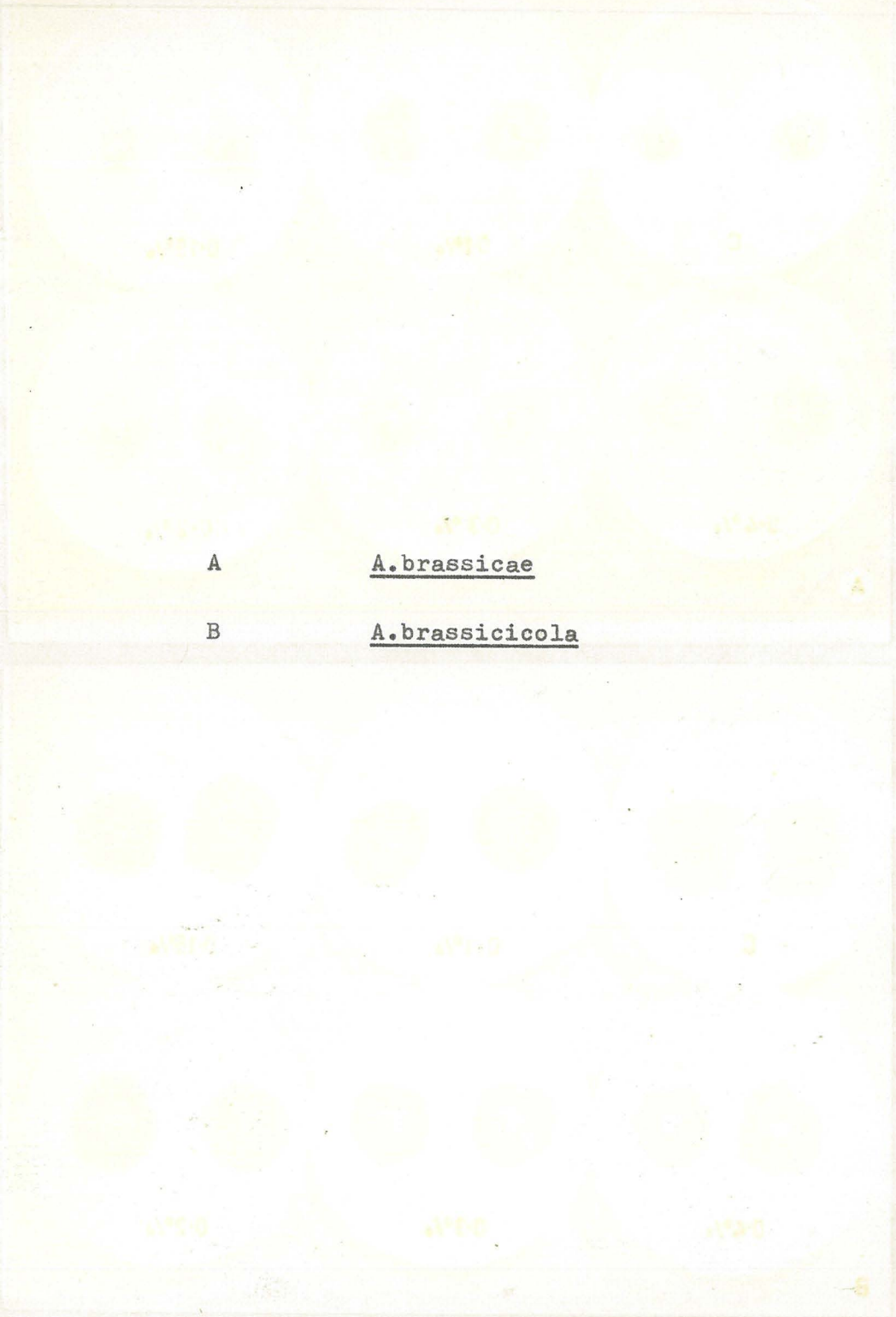
A A.brassicae

B A.brassicicola

Plate 34



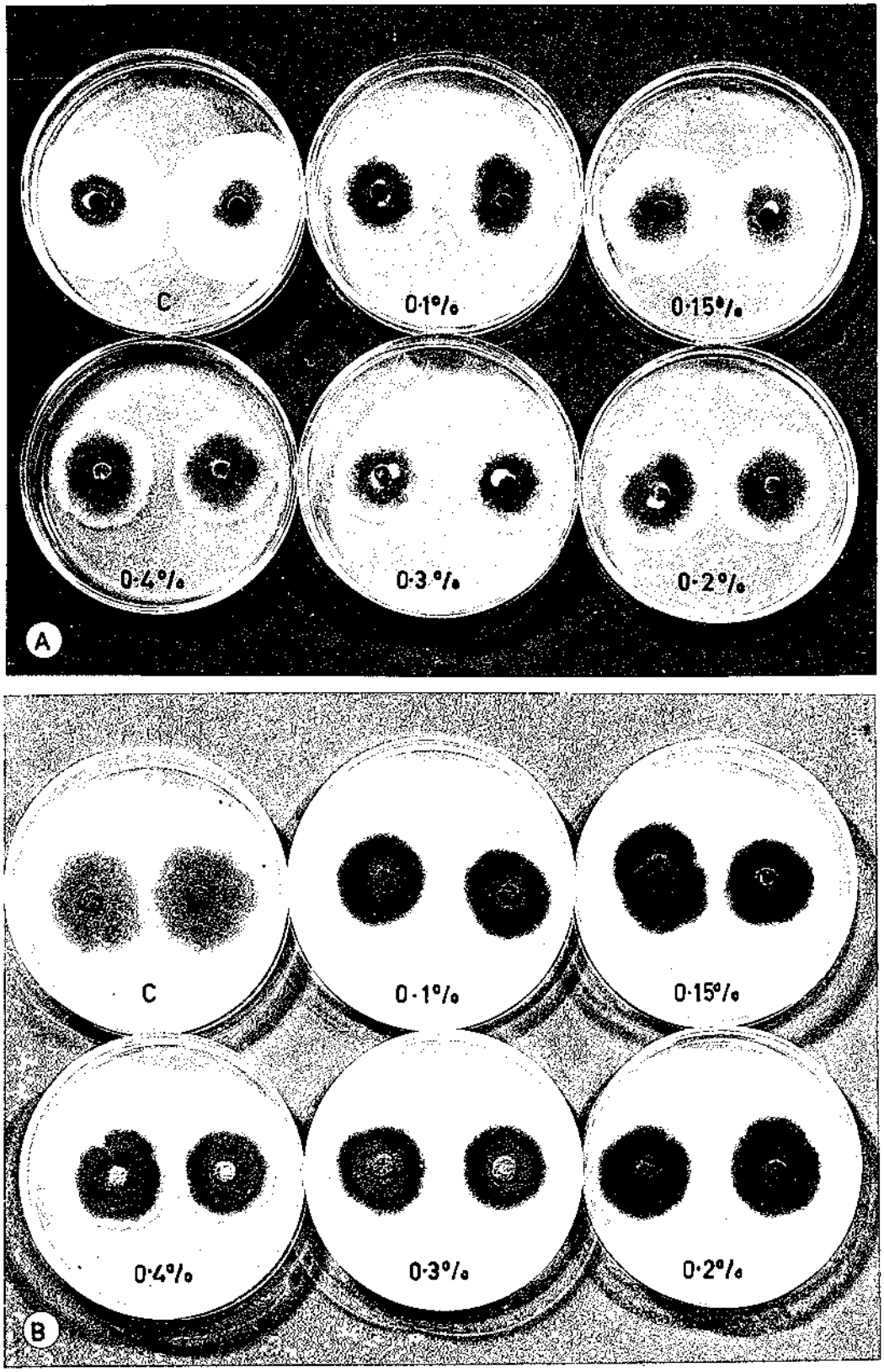
Effect of varying the concentration of sodium 2,4-D in MA on colony growth of A.brassicae and A.brassicicola



A A.brassicae

B A.brassicicola

Plate 35



Effect of varying the concentration of sodium 2,4-D in PDA on colony growth of A.brassicae and A.brassicicola

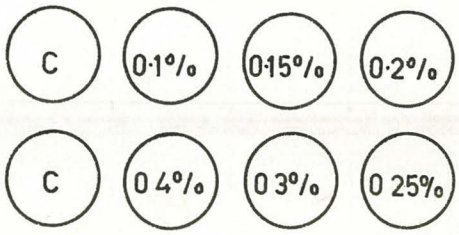
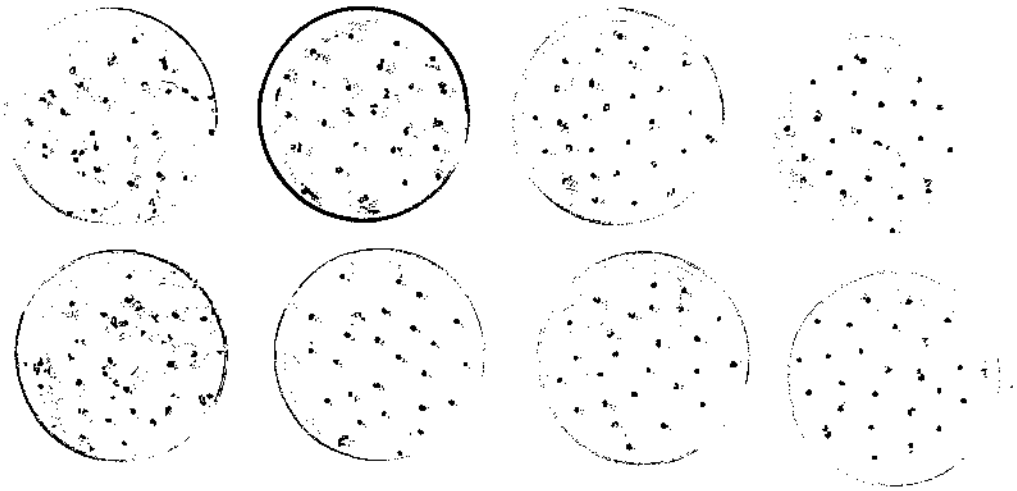

C
0·1%
0·15%
0·2%
C
0 4%
0 3%
0 25%

Plate 36



Effect of varying the concentration of
sodium 2,4-D in PDA on
germination of seed

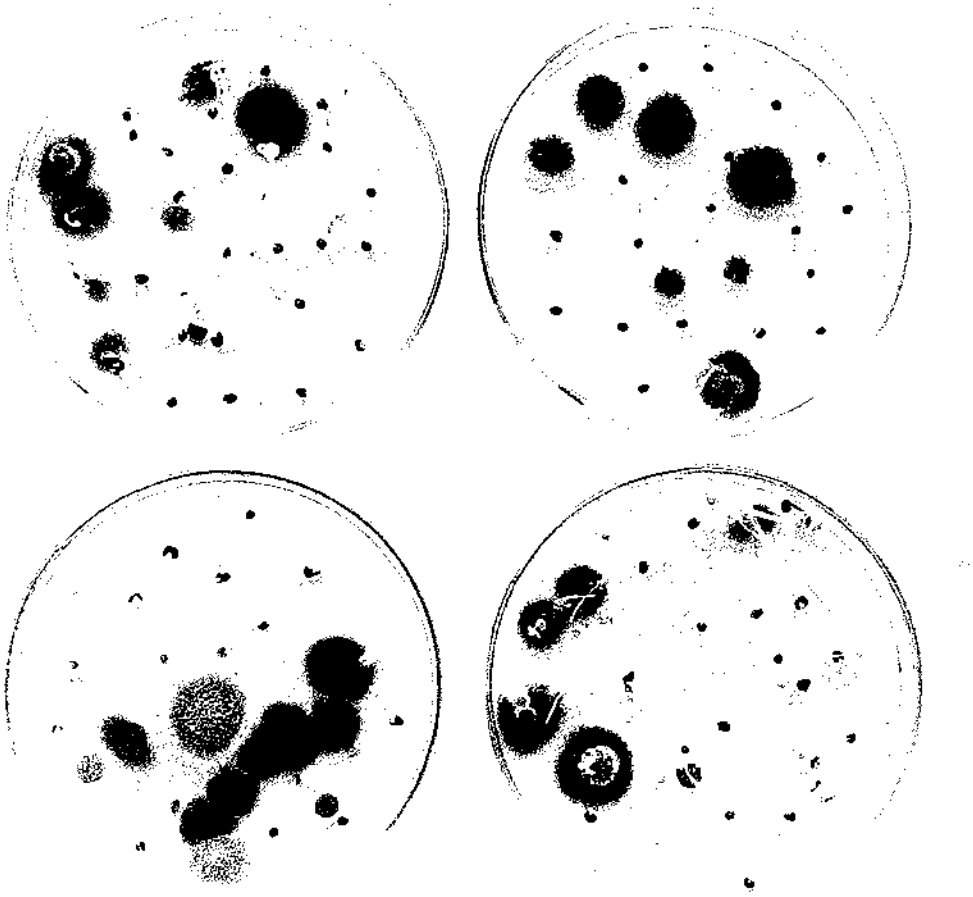
Top left - standard procedure

Top right - 2,4-D agar method

Bottom left - deep agar method

Bottom right- inverted plates

Plate 37



Comparison of agar methods used in screening choumoellier seed for *A.brassicae* and *A.brassicicola*

that colony growth was retarded to the least extent by a 0.1% 2,4-D/PDA concentration. Further, seed germination was inhibited at all levels as shown in Plate 36.

(c) Comparison of agar screening methods

Three methods have been mentioned which were devised to increase the efficiency of an agar plate screening test, namely, Newhook's "deep agar" method, de Tempe's "inverted plate" method, and Lloyd's "2,4-D/agar" method. Having identified a suitable modification of Lloyd's 2,4-D method the efficiency of this method was compared with those employed by the above workers in a trial using choumoellier seed, (Plate 37).

Method

Seed from four naturally infected lines was surface sterilised in mercuric chloride for 10 minutes, washed in sterile distilled water and dried between filter pads. The extent to which each line was infected with A.brassicicola was determined using the following methods :

(i) plating to PDA, as in standard procedure;

(ii) Newhook's deep agar method; a film of media was poured into each plate and allowed to solidify. The seed was placed in this layer and pressed firmly into the agar to enable complete immersion of the seed by a further pouring without disturbance of the placement positions;

(iii) de Tempe's inverted plate method; seed was plated to PDA as in standard procedure, but the plates were inverted before incubation;

(iv) seed was plated to a 0.1% 2,4-D/PDA medium.

Plates were incubated at 24°C and the results recorded after six days.

RESULTS AND DISCUSSION

The results presented in Table 54 show that no one method was more efficient in providing a means of determining the infection percentage of a seed line. However there were practical limitations involved with some.

In both the standard procedure method and de Tempe's inverted plate method seedling germination occurred which often made it difficult to identify the seed of colony origin where 25 seeds per Petri dish were plated. Further, in the former method there was the tendency for cotyledons to be lifted off the agar with germination. In some cases cotyledonary lesioning was observed but only diffuse colony growth was associated with the lesioned seedling. By inverting the plates as in de Tempe's method, cotyledons had remained in contact with the agar in most cases, but radicle growth was away from the surface and seedlings tended to come in contact through their sprawling growth pattern. The crowding problem could be minimised by plating less seeds per plate but this involves additional work and materials.

TABLE 54

Health screening seed using four agar plate methods

Seed Line		% A. brassicicola recorded +			
		Standard Plating	Inverted Plates	Deep Agar	2,4-D/PDA
A_1		5.00	12.00	10.00	10.00
		8.00	7.00	10.00	9.00
		7.00	7.00	10.00	8.00
	Mean	6.66	8.66	10.00	9.00
A_2		3.00	0.00	1.00	3.00
		0.00	2.00	1.00 *	2.00
		0.00	3.00	0.00	2.00
	Mean	1.00	1.33	0.33	3.33
A_3		5.00	1.00	1.00	4.00
		2.00	2.00	0.00	0.00
		6.00	4.00	1.00	2.00
	Mean	4.33	2.33	0.66	2.00
A_4		8.00	5.00	2.00	3.00
		3.00	4.00	5.00	1.00
		8.00	8.00	6.00	8.00
	Mean	6.33	6.00	4.33	4.00

+ Three replicates of 100 seeds were plated per treatment

* A. brassicae

Newhook's deep agar method was also considered unsuitable. The immersion of seed had the desired effect in suppressing germination, and with this there was no problem in identifying the seed from which the pathogen developed. However in some lines obligate saprophytes were established in the superficial tissue of the seed and grew out and masked the presence of the pathogen (e.g. line A_3). Further, the necessary double pouring of agar made this method rather time consuming.

The 2,4-D PDA method was equally as efficient as the above three methods but did not have the associated practical limitations. Germination was successfully inhibited and colony growth was localised. Crowding was not a problem even where 25 seeds were plated per dish, which in turn meant that less time, labour, and materials were required. No spread occurred due to seedling growth.

Since any agar method of seed screening is strictly a viability test, nothing is lost by suppression of seed germination; such as by the inclusion of 2,4-D into the media. Accordingly, the use of 2,4-D is recommended in agar plate health tests.

D. COMBINATION TECHNIQUE

The two generally accepted laboratory health screening methods involve the germination of seeds on blotters or the plating of seeds to an agar substrate. The relative merits of

each have been briefly discussed and it is apparent that both methods have limitations as well as advantages.

Agar methods have the advantage of enabling pathogen identification on the basis of colony growth from infected seeds. However surface sterilisation of seed is a pre-requisite to such tests and hence only the percentage of seeds infected is assessed. Further criticism may be levelled at the time and labour involved in preparation and standardisation of media and the space required for incubation of some 300 to 400 seeds in each test.

The blotter method has the advantage of being a pathogenicity method. Seed is allowed to germinate on blotters without any pre-test surface sterilisation to inactivate the associated contamination. This in turn means that seed contamination and seed infection is reflected in the results. Furthermore, the effect of pathogenic fungi on seedling health under a controlled set of circumstances can be assessed and if required, accorded an index of infection severity. The method is also cheap once the initial cost of a germinator has been met, requires a minimum of space, and the labour input is small compared with that required in an agar method. Against this, it has been shown that it is difficult to assess accurately the presence of pathogens to species level and in some cases it may be difficult to identify different genera on the basis of symptomology. Hence experience becomes a variable in the

assessment of results.

Experiments were conducted to investigate the possibility of combining the two established methods in a "combination" technique incorporating the favourable attributed of each, while minimising their respective limitations. This combination technique involved the placing out of seed on moist blotters and inspection and indexing of germinating seedlings for severity of disease, as in a standard blotter technique, but then plating lesioned tissues to agar. Identification would then be based on the colony features characteristic of each fungus. However the high humidity conditions of the germinator invariably encouraged an intense population of saprophytic bacterial contaminants which masked pathogen growth on plating to agar. For the combination technique to be successful the bacterial contamination had to be controlled. In this regard, the possibility of using a selective antibiotic plating medium was considered.

1. Antibiotics as bacterial suppressants

The use of antibiotics in selective media is an established technique. For example, Martin (1950), Hine and Butler (1957) and Johnson (1957) used antibiotics in media for suppressing bacterial contamination in isolating fungi from the soil. Further, Sheridan (1963) mentions the use of antibiotics in media for studying the viability of contaminating inoculum of seeds by examination of seed washings, and Wilhelm (1964)

used neomycin sulphate successfully.

Preliminary experiments involved the use of streptomycin sulphate, neomycin sulphate and chloramphenicol all of which were reported by White (1962) to have a broad spectrum of antibiotic activity. All of the materials showed promise, but as streptomycin was the most readily available it was chosen for further study. Experiments were conducted to determine the level at which bacterial contaminants were inhibited with a minimal suppression of pathogen growth.

Method

Flasks containing 100 ml quantities of PDA were held in a tempering bath at 45°C. The media was then cooled almost to the point of solidification, and while constantly agitating the flask, a streptomycin solution was added to give final concentrations of 20 iu*/ml, 40 iu/ml, 60 iu/ml, 80 iu/ml, 100 iu/ml, 200 iu/ml. Six plates at each concentration were inoculated with A.brassicicola using 3 mm agar discs, and a similar series were inoculated with A.brassicae. After incubation for six days at 24°C two diametric measurements of the resultant colonies were taken at right angles to each other as a measure of the streptomycin effect on pathogen growth. In addition, cotyledons from seedlings grown under the conditions of a blotter test were plated for each treatment to investigate the efficiency of the streptomycin inhibition of bacterial contaminants.

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* international units

The results presented in Figure 16 show that growth of both pathogens was not adversely affected by the addition of streptomycin sulphate to the media at levels up to 80 iu/ml (for "raw" data see Appendix XXVII & XXVIII). At 100 iu/ml A.brassicae was reduced in colony diameter and at 200 iu/ml A.brassicicola showed a comparable reduction, but even at 400 iu/ml some growth occurred. Inhibition of the growth of bacterial contaminants from cotyledons was achieved at 80 iu/ml. At a concentration of 60 iu/ml most bacterial were controlled but in some cases a weak growth did occur. In view of these results a streptomycin concentration of 80 iu/ml of PDA was used in the experiments which follow.

2. Use in seed screening

In preliminary trials to investigate the practicability of the combination technique as a general procedure, lesions were plated to Petri dishes containing streptomycin agar (PDA). In some instances however, fast growing saprophytic fungi grew from the plated tissue, often overgrowing the entire surface making readings impossible. To overcome this problem pyrex trays of antibiotic agar containing a series of individual cells were used (Plate 38). Lesioned tissue was plated to each cell and this restricted any saprophytic growth which occurred, thus enabling successful reading of adjacent cells. The apparatus provided a further advantage in that seedlings indexed to various categories could be plated to one tray, so long as the

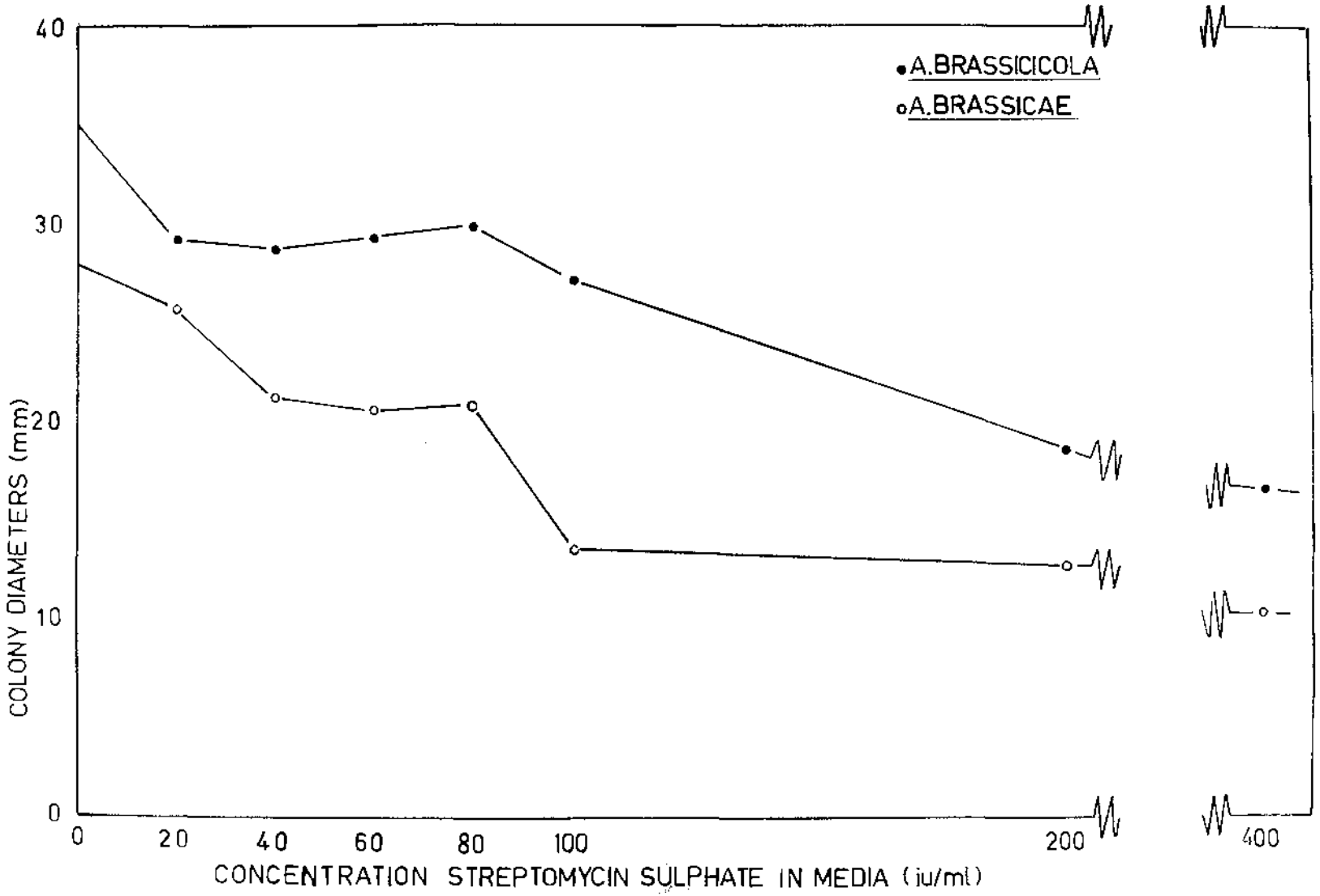
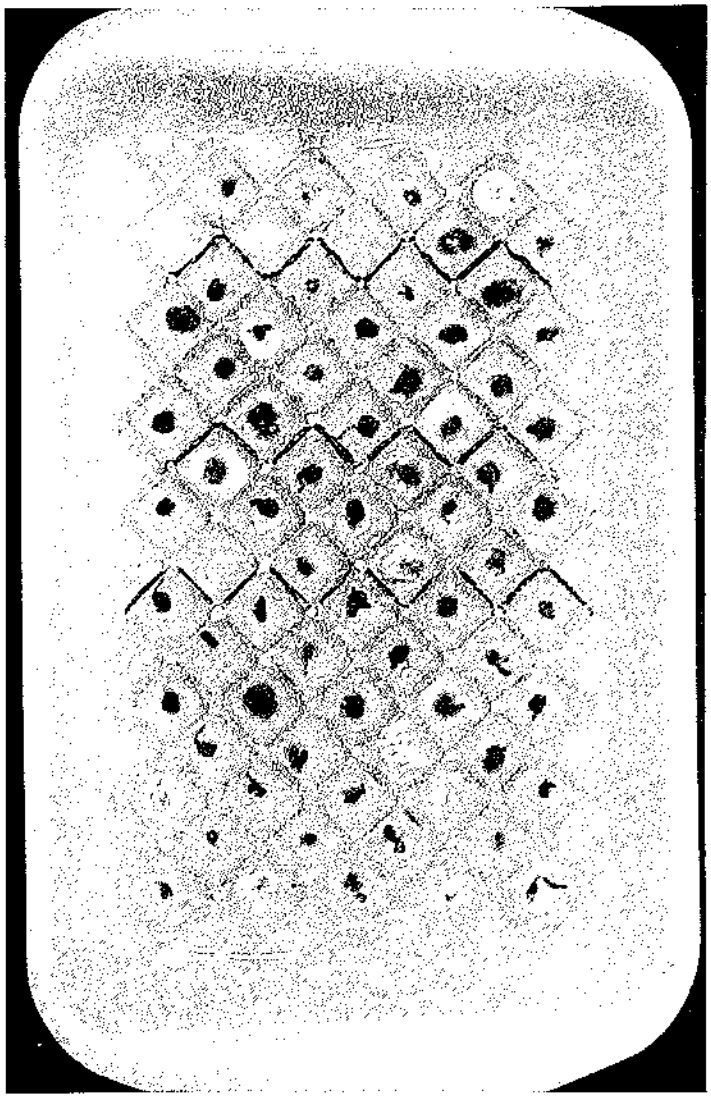


Figure 16

The effect of various concentrations of streptomycin in PDA on colony growth of A.brassicae and A.brassicicola

Plate 38



Apparatus used in the blotter-agar
combination method for screening
choumoellier seed

cells were identified with marker divisions. This overcame the difficulty of working a number of individual Petri plates (one for each index category) at the same time. Ideally the trays would be constructed to contain 100 cells to accommodate all seedlings in the extreme case of a 100% infected line.

Naturally infected seed lines were screened for the presence of A.brassicicola and A.brassicae using this modified technique.

Method

Three replicates of 100 seeds each from five field samples of seed lines were placed out on moistened filter papers in sterilised Petri dishes (20 seeds per dish). Petri dish high humidity chambers were used to ensure an uncontaminated environment. The plates were incubated at room temperature for eight days under normal day/night light variation. Prior to reading, the dishes were exposed to four hours continuous Ultra Violet light as a further precaution against bacterial contamination. Seedling infection was identified by the presence of sporulation, or lesions on the cotyledons and hypocotyl. Four indices for infection severity were recorded, as follows :

(i) dead seed exhibiting sporulation;

(ii) severe seedling necrosis with sporulation on the decayed areas;

(iii) severe lesioning on the hypocotyl and/or the cotyledons;

(iv) isolated lesions on the hypocotyl and/or the cotyledons.

A thin layer of streptomycin/PDA was poured into the tray and allowed to solidify. A further layer was poured and the honeycomb cell sheet placed in this before it hardened. Infected seedling material was then plated in each cell using forceps (flamed between seedlings) and the trays incubated in sealed plastic bags for six days at room temperature.

RESULTS AND DISCUSSION

The results presented in Table 55 give a measure of the success of the method. Pathogens could usually be identified on the basis of colony characteristics, but in some cases where saprophytes were also present, microscopic confirmation was necessary. As exemplified by the results recorded for line S_4 the pathogenicity method has been successfully combined with the viability method allowing positive identification to species level, as well as enabling indices for infection severity to be assigned. The high total infection percentages compared with those recorded for the same lines after surface sterilisation and plating to agar (see Table 50) are positive evidence for the argument that surface sterilisation removes a considerable amount of inoculum which is capable of causing seedling infection. Not all lesions plated gave rise to Alternaria pathogens, and

TABLE 55

Health screening seed lines by the "combination method"

Seed Line	Replicate	Infection percentage of A. brassicae and A. brassicicola									
		A. brassicicola					A. brassicae				
		Dead seed exhibiting sporulation	Severe seedling necrosis plus sporulation	Severe lesioning on hypocotyl and/ or cotyledons	Isolated lesioning on hypocotyl and/ or cotyledons	TOTAL	Dead seed exhibiting sporulation	Severe seedling necrosis plus sporulation	Severe lesioning on hypocotyl and/ or cotyledons	Isolated lesioning on hypocotyl and/ or cotyledons	TOTAL
S_1	1	5.00	3.00	7.00	12.00	27.00	-	-	-	-	-
	2	0.00	3.00	6.00	8.00	17.00	-	-	-	-	-
	3	2.00	4.00	8.00	5.00	19.00	-	-	-	-	-
	Mean	2.33	3.33	7.00	8.33	21.00	-	-	-	-	-
S_2	1	8.00	4.00	27.00	35.00	76.00	-	-	-	-	-
	2	5.00	17.00	12.00	27.00	61.00	-	-	-	-	-
	3	9.00	18.00	26.00	25.00	78.00	-	-	-	-	-
	Mean	7.33	13.00	21.66	29.00	71.66	-	-	-	-	-
S_3	1	0.00	2.00	5.00	7.00	13.00	-	-	-	-	-
	2	3.00	1.00	4.00	6.00	15.00	-	-	-	-	-
	3	0.00	4.00	6.00	5.00	15.00	-	-	-	-	-
	Mean	1.00	2.33	5.00	6.00	14.33	-	-	-	-	-
S_4	1	2.00	2.00	3.00	2.00	9.00	7.00	2.00	1	2	12
	2	1.00	2.00	0.00	2.00	5.00	3.00	0.00	3	1	7
	3	4.00	1.00	2.00	2.00	9.00	5.00	3.00	3	2	13
	Mean	2.33	1.66	1.66	2.00	7.66	5.00	1.66	2.33	1.66	10.66
S_5	1	3.00	0.00	0.00	4.00	7.00	-	-	-	-	-
	2	0.00	0.00	0.00	3.00	5.00	-	-	-	-	-
	3	1.00	2.00	2.00	2.00	5.00	-	-	-	-	-
	Mean	1.33	0.66	0.66	3.00	5.66	-	-	-	-	-

contamination was still a problem in some cells, but was not severe enough to preclude the use of the method. On average 75% of the dead seed exhibiting sporulation gave rise to colonies of the Alternaria pathogens. Colonies of A.tenuis Auct. accounted for some of the others and colonies tentatively identified as A.consortiale (Thum) Groves and Hughes were sometimes isolated, particularly from the line S_4. These colonies were very similar to growth of A.brassicicola, but were generally darker, without the olivaceous appearance and with less aerial growth. Overall, pathogenic species of Alternaria were recorded from 82% of the "severe seedling necrosis plus sporulation category", 95% of the "severe lesioning" group and 60% of the "isolated lesioning" group. It is apparent then, that an infection percentage recorded by the blotter method alone, would have been much higher than that recorded by the combination technique.

E. DISCUSSION

The nature of information required governs the type of method used in seed health screening. For example, soil methods are conducted to obtain information which reflects the average field performance of the seed. The criticism of these methods is the difficulty involved in controlling the seed bed environment. In laboratory tests using the blotter and agar plate techniques, the aim is primarily to determine

the extent to which a pathogen is present in a seed line. These methods are carried out under controlled conditions, thereby minimising environmental variation. An agar plate method is a viability test only, in that pathogens are identified on the basis of saprophytic growth on laboratory media. With this test the presence of obligate saprophytes on the surface of the seed necessitates surface sterilisation before plating. This means that surface inoculum of pathogenic species is also removed and the results expressed are only informative for the level of seed infection in a line. Blotter methods are pathogenicity tests in that the pathogens are identified on the basis of symptoms expressed on developing seedlings. Hence these methods reflect both seed infection and seed contamination and in addition, enable indices for the severity of seedling attack to be recorded, but pathogen identification on the basis of sporulation and symptoms on seedlings is often tedious and may be unreliable.

In the present study the objective was to identify a method which would accurately reveal the pathogenic species associated with choumoellier seed lines, and the extent to which these were present. The recommended blotter methods were studied and the standard test (Noble, 1965) shown to be the best. The use of 2,4-D as a germination inhibitor was not favoured as pathogen identification was tedious and further, the method precluded the use of seedling infection indices.

However, even Noble's method was not entirely suitable due to the difficulty and unreliability of species delimitation of the Alternaria pathogens.

Surface sterilisation techniques were considered in relation to agar plate methods and it was shown that water washing was the most efficient. Chemical treatments appeared to remove some of the seed infection and gave rise to a consistently lower infection percentage. On the other hand, washing seeds in running tap water incurred problems of bacterial contamination on plating. This was overcome by incorporating streptomycin into the plating medium. Further, the use of 2,4-D in media to inhibit seed germination was found to increase the efficiency of the agar plate method. Therefore to use both recommendations to advantage, that is, surface sterilisation by washing and suppression of seed germination, it was necessary to demonstrate the compatibility of streptomycin and 2,4-D in the media without affecting characteristic colony development of the pathogens. Preliminary trials indicated that the chemicals could be successfully combined for screening work. However there was still the inherent limitation involved in removal of surface inoculum by surface sterilisation and hence further experimentation led to the development of the combination technique which was considered to be superior to the established methods. This method was designed to overcome the limitations of the blotter and agar tests while incorporating the favourable

attributes of both. Seeds were first germinated on blotters thereby dispensing with the need to surface sterilise, and by plating lesioned tissues to agar, pathogen identification was based on colony growth. Bacterial contamination proved a problem, but this was overcome by using a selective antibiotic medium. Some contamination due to obligate saprophytes also occurred, but was minimised by the use of cells in the plating tray to prevent spread. In most of these instances pathogens could still be identified by a microscopic examination. Hence the method enabled the assessment of both contaminating and infecting inoculum of A.brassicae and A.brassicicola and further, provided the opportunity for indexing seedlings for the severity of infection.

SUMMARY

1. Two species of Alternaria causing leaf spot symptoms on choumoellier in the Manawatu were studied and the identity of the causal fungi established as A.brassicicola (Schw.) Wilt., and A.brassicae (Berk.) Sacc.

2. Surveys conducted on New Zealand produced certified choumoellier seed showed A.brassicicola present in more than 70% of the lines screened. A.brassicae was isolated from fewer lines (21% in 1965 and 32% in 1966) and was usually present at low levels only.

3. Seed infections of A.brassicicola remained viable for at least three years, thereby ensuring the presence of seed-borne inoculum on sowing. A.brassicae did not persist as a viable infection following one year's storage.

4. Hot water treatment at 50°C for 25 minutes eliminated A.brassicicola from seed but caused some germination reduction. This was independent of the seed age, the moisture content and the percentage infection.

5. A.brassicicola caused considerable emergence losses under artificial conditions of contamination and infection, but did not cause losses of any consequence in the commercial seed lines tested. In all experiments A.brassicae was of minor importance.

6. The retention of the testa on the cotyledons or its location close to the hypocotyl at ground level was an important factor in facilitating seedling infection.

7. Leaf and pcd symptoms of A.brassicicola and A.brassicae were studied and the distinguishing features described.

8. Lesioning and penetration of the siliqua wall resulted in seed infection. Severe infection caused premature drying and splitting of seed pods. A.brassicicola spread within the siliqua and caused premature germination in situ.

9. Siliqua infection by A.brassicicola caused a reduction of 35.0% in the weight of seed harvested whereas with A.brassicae the reduction was 33.7%. In both instances loss of seed weight was associated with an increase in the amount of small seed.

10. The germination percentage of seed harvested from siliquas infected with A.brassicicola was significantly reduced.

11. Established methods for health screening brassica seed were critically examined. An improved test incorporating features of the blotter and agar methods was developed for choumoellier seed.

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APPENDIX I

COMPOSITION AND PREPARATION OF MEDIA

(a) Potato - Dextrose Agar (lab PDA)

agar	12 gm
potatoes (sliced and peeled)	200 gm
dextrose	10 gm
water (distilled)	1,000 gm

The sliced potatoes were cooked gently for one hour in 500 ml of water and the filtrate retained by straining through cheese-cloth. Agar was dissolved in 500 ml of water, dextrose added and the solutions combined. The total volume was adjusted to 1,000 ml before autoclaving at 15 p.s.i. for 20 minutes. Approximate pH 5.8.

(b) Malt Agar

malt extract (Difco)	20 gm
agar	25 gm
water (distilled)	1,000 gm

Malt extract and agar were dissolved in 500 ml of water respectively, mixed together, and autoclaved as above. Approximate pH 5.5.

APPENDIX II

INTERIM EXAMINATIONS OF EMERGENCE TRIALS ON ARTIFICIALLY CONTAMINATED SEED

Treatment	Days after sowing	Replicate	Total Emergence	Abnormals	Post-emergence damping-off symptoms	Lesions on Cotyledons			No germination
						1	2	3	
Contaminated seed with A. brassicicola	10	1	75	2	1	8	6	9	25
		2	73	3	1	9	3	10	27
		3	76	3	2	3	7	8	24
		4	81	2	3	7	10	20	19
		Mean	76.25	2.5	1.75	6.75	6.5	11.75	23.75
	14	1	87	3	11	10	5	21	13
		2	83	3	8	8	10	13	17
		3	88	3	10	4	5	14	12
		4	90	2	11	7	9	24	10
		Mean	87	2.75	10.0	7.25	7.25	18.0	13
	18	1	84	-	12	8	9	16	-
		2	80	-	11	7	7	14	-
		3	84	-	8	6	9	11	-
		4	81	-	12	7	8	21	-
		Mean	82.25	-	10.75	7.0	8.25	15.5	
	22	1	74	-	21	13	4	17	-
		2	74	-	13	10	5	11	-
		3	75	-	14	9	4	7	-
		4	71	-	16	10	6	12	-
		Mean	73.5	-	16.0	10.5	4.75	11.75	-
Contaminated seed with A. brassicae	10	1	86	1	0	4	4	2	14
		2	75	1	0	2	3	0	25
		3	78	3	2	3	0	2	22
		4	65	2	0	2	5	0	35
		Mean	76.0	1.75	0.5	2.75	3.0	1.0	24
	14	1	96	1	1	6	3	3	4
		2	92	2	0	7	11	0	8
		3	93	3	2	5	1	1	7
		4	87	2	1	4	5	1	15
		Mean	92.0	2.0	1.0	5.5	2.5	1.25	8.5
	18	1	96	-	2	7	3	2	-
		2	93	-	1	6	0	0	-
		3	92	-	2	4	2	0	-
		4	89	-	1	4	4	1	-
		Mean	92.5	-	1.5	5.25	2.25	0.75	-
	22	1	94	-	1	6	3	2	-
		2	89	-	0	6	0	0	-
		3	90	-	1	4	1	1	-
		4	86	-	0	4	4	1	-
		Mean	89.75	-	0.5	5.0	2.0	1.0	-
Clean seed sown	10	1	80	1	-	1	-	-	20
		2	85	2	-	0	-	-	15
		3	68	3	-	0	-	-	32
		4	72	1	-	0	-	-	28
		Mean	76.25	1.75	-	0.25	-	-	23.75
	14	1	95	2	-	2	-	-	5
		2	95	4	-	0	-	-	5
		3	87	3	-	1	-	-	13
		4	90	2	-	0	-	-	10
		Mean	91.75	2.75	-	0.75	-	-	8.25
	18	1	96	-	-	2	0	-	4
		2	97	-	-	0	0	-	3
		3	92	-	-	0	1	-	8
		4	92	-	-	0	0	-	8
		Mean	94.25	-	-	0.5	0.25	-	5.75
	22	1	95	-	-	1	0	-	5
		2	97	-	-	0	0	-	3
		3	94	-	-	0	1	-	6
		4	93	-	-	0	0	-	7
		Mean	94.75	-	-	0.25	0.25	-	5.25

APPENDIX III

EFFECT OF ARTIFICIAL CONTAMINATION OF SEED ON EMERGENCE
(examination made 30 days after sowing)

Treatment	Replicate	Total Emergence	Hypocotyl Lesioning			Cotyledon Lesioning			Lesioning on Leaves
			1	2	3	1	2	3	
Contaminated seed	1	62	27	10	14	16	14	10	0
with *A. brassicicola*	2	71	22	8	9	22	11	5	0
	3	68	20	10	6	17	16	7	0
	4	64	24	6	7	21	11	9	1
	Mean	66.25	23.25	8.5	9.0	19.0	13.0	7.75	0.25
Contaminated seed	1	91	12	1	0	12	5	9	1
with *A. brassicae*	2	83	6	1	0	9	2	0	0
	3	89	8	0	1	8	1	0	0
	4	83	10	3	1	3	1	2	0
	Mean	86.5	9.0	1.25	0.5	8.0	2.25	2.75	0.25
Control - clean	1	96	3	0	0	1	0	0	0
seed	2	97	3	0	0	0	0	0	0
	3	92	1	0	0	1	1	0	0
	4	94	2	0	0	2	0	0	0
	Mean	94.75	2.25	0	0	1.0	0.25	0	0

APPENDIX IV

EFFECT OF ARTIFICIAL INFECTION OF SEED WITH A.BRASSICICOLA ON EMERGENCE
(examination made 30 days after sowing)

Treatment	Replicate	Total Emergence	Hypocotyl Lesioning			Cotyledon Lesioning			Lesioning on Leaves
			1	2	3	1	2	3	
Artificially infected	1	37	4	1	2	4	2	0	2
seed with	2	28	2	0	1	9	3	2	0
A.brassicicola	3	46	5	4	1	8	1	2	0
	4	42	6	1	2	8	3	0	0
	Mean	38.35	4.25	1.50	1.50	7.25	2.25	1.00	0.66
Clean seed	1	87	1	1	0	0	0	0	0
exposed to high	2	90	1	0	0	2	0	0	0
humidity	3	82	1	0	0	1	0	0	0
	4	78	2	0	0	3	2	0	0
	Mean	84.25	1.25	0.25	0.00	1.50	0.50	0.00	0.00

APPENDIX V

EFFECT OF SEED CONTAMINATION WITH A.BRASSICICOLA ON EMERGENCE AT 30 DAYS (overhead watered)

Treatment	Replicate	Total Emergence	Hypocotyl lesioning			Cotyledonary lesioning			Lesioning on leaves
			1	2	3	1	2	3	
Contaminated seed with A.brassicicola - overhead watered	1	75	22	14	11	9	22	5	4
	2	64	30	11	16	15	17	8	8
	3	78	26	15	14	18	20	9	13
	4	69	21	9	10	14	25	4	9
	Mean	71.5	24.75	12.25	14.0	21.5	6.5		8.5
Clean seed - overhead watered	1	90	0	1	0	3	1	0	0
	2	86	4	0	0	0	0	0	0
	3	89	1	0	0	2	2	0	1
	4	94	2	1	0	3	0	0	0
	Mean	98.75	1.75	0.5	0	2.0	0.75	0	0.25

APPENDIX VI

EFFECT OF SILIQUA INOCULATION WITH A.BRASSICAE AND A.BRASSICICOLA ON THE WEIGHT OF SILIQUAS, THE WEIGHT OF SEED AND GERMINATION PERCENTAGE OF THE HARVEST

Treatment	Replicate	Weight of 100 siliquas (gm)	Weight of seed from 100 siliquas (gm)	Germination (%)			
				1	2	3	Mean
Siliquas inoculated with A.brassicicola	1	17.567	5.543	62	75	68	68
	2	19.859	6.131	64	94	72	77
	3	17.087	5.091	80	59	67	69
Siliquas inoculated with A.brassicae	1	20.248	5.321	81	75	81	79
	2	21.725	5.988	79	74	76	76
	3	19.531	5.791	89	78	82	83
Control	1	26.623	9.043	79	83	94	86
	2	24.595	8.053	85	80	78	81
	3	25.417	8.581	85	91	90	88

APPENDIX VII

ANALYSIS OF VARIANCE OF THE WEIGHT OF 100 SILIQUAS HARVESTED FROM INOCULATED SEED HEADS

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	2	85.245	42.622	28.49	5.14	10.92	* *
Error	6	8.873	1.496				
Total	8	94.218					

APPENDIX VIII

ANALYSIS OF VARIANCE OF THE WEIGHT OF SEED PER 100 SILIQUAS HARVESTED FROM INOCULATED SEED HEADS

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	2	17.011	8.506	40.2	5.14	10.92	* *
Error	6	1.269	0.212				
Total	8	18.281					

APPENDIX IX

ANALYSIS OF VARIANCE OF THE GERMINATION PERCENTAGES OF SEED HARVESTED FROM INFECTED SILIQUAS

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	2	864.9	423.5	7.70	3.40	5.61	* *
Error	24	1347.8	56.2				
Total	26	2212.7					

APPENDIX XIV

ANALYSIS OF VARIANCE OF PERCENTAGE WEIGHT OF SEED RETAINED BY THE 2.007 mm SIEVE

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	264.50	44.09	8.75	2.85	4.46	* *
Error	14	70.53	5.04				
Total	20	335.03					

APPENDIX X

THE EFFECT OF VARIOUS DEGREES OF SILIQUA INFECTION BY A.BRASSICICOLA ON THE PERCENTAGE WEIGHT OF SEED OF GRADED SIZES

Treatment	Degree of pod lesioning	Mesh size of sieves (mm)	Percentage weight of seed recorded for each sieve size (5 gm sample)			
			1	2	3	Mean
A.brassicicola inoculations	Slight	>2.007	20.08	17.40	16.90	18.13
		>1.651 <2.007	58.94	59.25	61.27	59.82
		>1.270 <1.651	21.47	26.09	21.42	22.99
		>0.838 <1.270	1.49	1.25	0.42	1.05
	Fairly Severe	>2.007	17.05	19.43	20.28	18.92
		>1.651 <2.007	60.99	56.44	57.66	58.36
		>1.270 <1.651	25.09	23.19	21.17	23.15
		>0.838 <1.270	0.86	0.93	0.64	0.81
	Severe	>2.007	15.08	18.19	16.59	16.62
		>1.651 <2.007	59.05	54.04	60.90	57.99
		>1.270 <1.651	25.78	26.31	21.23	24.44
		>0.838 <1.270	1.40	1.47	1.28	1.38

APPENDIX XI

THE EFFECT OF VARIOUS DEGREES OF SILIQUA INFECTION BY A.BRASSICAE ON THE PERCENTAGE WEIGHT OF SEED OF GRADED SIZES

Treatment	Degree of pod lesioning	Mesh size of sieves (mm)		Percentage weight of seed recorded for each sieve size (5 gm sample)			
				1	2	3	Mean
A.brassicae inoculations	Slight	>2.007		9.75	15.96	11.03	11.83
		>1.651	<2.007	60.32	58.21	64.65	61.60
		>1.270	<1.651	27.52	25.66	21.02	24.73
		>0.838	<1.270	0.97	2.39	3.32	2.23
	Fairly Severe	>2.007		16.33	15.17	15.56	15.68
		>1.651	<2.007	58.88	55.84	58.41	57.31
		>1.270	<1.651	23.90	25.57	24.88	25.45
		>0.838	<1.270	0.87	0.30	1.15	0.72
	Severe	>2.007		7.09	8.08	9.68	8.28
		>1.651	<2.007	63.28	62.77	57.26	61.10
		>1.270	<1.651	28.83	28.88	31.81	29.84
		>0.838	<1.270	0.82	0.34	1.24	0.80
Control	-	>2.007		19.33	18.63	18.79	18.92
		>1.651	<2.007	72.51	71.48	75.39	73.13
		>1.270	<1.651	7.73	9.68	5.76	7.77
		>0.838	<1.270	0.41	0.02	0.00	0.14

APPENDIX XII

THE EFFECT OF VARIOUS DEGREES OF SILIQUA INFECTION BY A.BRASSICICOLA ON THE GERMINATION PERCENTAGE OF SEED GRADED ON SIZE

Treatment	Degree of pod lesioning	Mesh size of sieves (mm)	Germination (%)			
			1	2	3	Mean
A.brassicicola inoculations	Slight	>2.007	75	76	72	74
		>1.651 <2.007	71	69	72	71
		>1.270 <1.651	76	84	67	76
		>0.838 <1.270	0	0	-	0*
	Fairly Severe	>2.007	80	81	75	79
		>1.651 <2.007	81	82	70	78
		>1.270 <1.651	55	64	58	59
		>0.838 <1.270	2	3	-	2.5*
	Severe	>2.007	73	61	70	68
		>1.651 <2.007	77	77	69	74
		>1.270 <1.651	44	40	38	41
		>0.838 <1.270	2	2	-	2.0*

* Germination assessed in the agar plate heat test

APPENDIX XIII

THE EFFECT OF VARIOUS DEGREES OF SILIQUA INFECTION BY *A.BRASSICAE* ON THE GERMINATION PERCENTAGE OF SEED GRADED ON SIZE

Treatment	Degree of pod lesioning	Mesh size of sieves (mm)		Germination (%)			
				1	2	3	Mean
A.brassicae	Slight	>2.007		91	82	85	86
		>1.651	<2.007	92	78	88	86
		>1.270	<1.651	59	53	37	50
		>0.838	<1.270	3	0	-	1.5*
	Fairly Severe	>2.007		78	75	74	76
		>1.651	<2.007	85	80	78	81
		>1.270	<1.651	67	63	53	61
		>0.838	<1.270	3	2	-	2.5*
	Severe	>2.007		89	78	91	86
		>1.651	<2.007	93	92	90	92
		>1.270	<1.651	76	71	77	75
		>0.838	<1.270	1	0	-	0.5*
Control	-	>2.007		85	85	87	86
		>1.651	<2.007	84	82	89	85
		>1.270	<1.651	57	45	63	53
		>0.838	<1.270	0	0	-	0.0*

* Germination assessed in the agar plate heat test

APPENDIX XV

ANALYSIS OF VARIANCE ON THE PERCENTAGE WEIGHT OF SEED RETAINED BY THE SIEVE SIZE GROUPING >1.651 mm <2.007 mm

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	524.45	87.41	12.76	2.85	4.46	* *
Error	14	95.87	6.85				
Total	20	620.32					

APPENDIX XVI

ANALYSIS OF VARIANCE OF THE PERCENTAGE WEIGHT OF SEED RETAINED AT SEED SIZE CATEGORY 1.270 mm 1.651 mm

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	862.57	143.76	26.6	2.85	4.46	* *
Error	14	75.63	5.40				
Total	20	938.20					

APPENDIX XVII

ANALYSIS OF VARIANCE ON THE PERCENTAGE WEIGHT OF SEED RETAINED AT THE SIEVE SIZE >0.838 <1.270

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	7.69	1.28	4.13	2.85	4.46	* *
Error	14	4.39	0.31				
Total	20	12.08					

APPENDIX XVIII

ANALYSIS OF VARIANCE OF THE GERMINATION PERCENTAGE OF SEED RETAINED BY THE 2.007 mm SIEVE

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	951	158.5	7.47	2.85	4.46	* *
Error	14	297	21.21				
Total	20	1248					

APPENDIX XIX

ANALYSIS OF VARIANCE OF THE GERMINATION PERCENTAGE OF SEED RETAINED BY THE SEED SIZE CATEGORY >1.651 mm <2.007 mm

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	951					
Error	14	297	158.5	7.47	2.85	4.46	* *
Total	20	1248	21.2				

APPENDIX XX

ANALYSIS OF VARIANCE OF THE GERMINATION PERCENTAGE OF SEED RETAINED BY THE SEED SIZE CATEGORY >1.270 mm <1.651 mm

Source of Variation	df	ss	ms	F	F required		Result
					0.05	0.01	
Treatment	6	3654	609	11.26	2.85	4.46	* *
Error	14	757	54.1				
Total	20	2897					

APPENDIX XXI

DETERMINATION OF THE "POINT OF EFFECTIVENESS" FOR
SURFACE STERILISATION TECHNIQUES

Treatment	Treatment Time	*A. brassicicola* isolated (%)	Clean Seed (%)	Contaminants	
				Fungi (%)	Bacteria (%)
	Minutes				
0.001%	5	2.00	97.66	0	0.33
Mercuric Chloride	6	2.66	96.33	0.33	0.66
	7	0.66	98.33	0	1.00
	8	0.00	98.66	1.0	0.33
	9	0.33	99.00	0.66	0
	10	0.00	100.00	0.00	0.00
	11	0.00	99.96	0.00	0.33
Sodium Hypochlorite	5	5.33	93.33	0.00	1.33
(1% available Cl)	6	6.33	93.33	0.00	0.33
	7	5.00	94.66	0.33	0.00
	8	2.00	97.66	0.33	0.00
	9	0.00	99.33	0.33	0.33
	10	0.00	99.33	0.33	0.33
	Hours				
Tumbler	2	99.33	0.00	0.00	0.66
Technique	4	98.66	0.33	0.33	0.66
	6	93.33	5.66	0.00	1.66
	8	87.00	9.66	0.00	3.33
	10	83.00	9.00	0.00	7.66
	12	84.00	6.33	0.33	9.00

APPENDIX XXII

DETERMINATION OF THE "POINT OF EFFECTIVENESS" OF WASHING METHODS FOR SURFACE STERILISATION

Method	Time (hours)	A.brassicicola isolated (%)	Clean seed (%)	Contaminants	
				Fungi (%)	Bacteria (%)
Gauze Flask	2	100.00	0.00	0.00	0.00
Method	4	95.66	2.66	0.00	3.00
	6	70.33	2.33	0.00	27.33
	8	65.00	10.00	2.00	19.66
	10	49.66	23.00	1.33	26.00
	12	4.66	63.00	4.33	28.00
Gauze Flask	2	100.00	0.00	0.00	0.00
Method	4	98.33	0.00	0.00	1.66
Modified	6	90.33	1.33	0.00	8.33
	8	85.00	8.00	0.66	6.33
	10	72.66	12.33	0.00	15.00
	12	61.33	14.66	1.00	24.00
Free Flow	1	10.66	60.33	5.66	23.33
Method	2	7.00	60.00	7.00	26.00
	2½	7.66	60.66	5.66	19.00
	3	4.33	76.66	4.00	15.00
	3½	3.66	77.66	2.00	16.66
	4	1.00	77.66	3.33	17.33
	4½	3.33	80.33	1.00	15.33
	5	6.00	77.00	4.00	19.00

APPENDIX XXIII

EFFECT OF VARYING 2,4-D/MA CONCENTRATIONS ON COLONY GROWTH OF A.BRASSICAE
(measured after 6 days incubation at 24°C)

2,4-D Concn. in media	Colony diameters (mm)						
	1	2	3	4	5	6	Mean
0.1%	9.5	12.0	9.5	10.0	11.0	9.0	10.2
0.15%	9.0	9.0	8.5	9.5	10.0	9.5	9.2
0.20%	9.0	12.0	7.5	9.5	11.5	8.0	9.6
0.25%	8.5	7.0	7.0	7.5	9.5	8.0	7.9
0.30%	9.0	8.0	6.0	6.5	7.5	7.0	7.7
0.40%	7.5	7.0	7.0	6.5	7.0	7.5	7.1
Control	25.5	26.0	26.5	28.5	26.0	26.5	26.5

APPENDIX XXIV

EFFECT OF VARYING 2,4-D/MA CONCENTRATIONS ON COLONY GROWTH OF A.BRASSICICOLA
(measured after 6 days incubation at 24°C)

2,4-D Concn. in media	Colony diameters (mm)						
	1	2	3	4	5	6	Mean
0.1%	16.5	15.5	15.0	14.5	15.5	16.0	15.5
0.15%	14.5	15.5	15.0	14.5	16.0	17.5	15.5
0.20%	15.0	15.0	14.5	13.0	14.5	15.5	14.6
0.25%	14.5	14.5	15.0	14.5	14.0	15.0	14.6
0.30%	13.0	13.5	13.5	13.0	14.0	14.5	13.6
0.40%	14.0	15.0	12.0	13.5	12.5	13.0	13.3
Control	31.0	34.5	36.0	31.5	34.5	30.0	32.9

APPENDIX XXV

EFFECT OF VARYING 2,4-D/PDA CONCENTRATIONS ON COLONY GROWTH OF A.BRASSICAE
(measured after 6 days incubation at 24°C)

2,4-D concentrations in media	Colony diameters (mm)						
	1	2	3	4	5	6	Mean
0.10%	39.5	43.0	42.5	43.5	41.5	39.0	41.50
0.15%	35.0	36.5	40.0	38.0	36.0	39.0	37.43
0.20%	34.0	35.5	33.5	36.0	33.5	34.5	34.25
0.25%	35.5	37.5	31.0	38.08	36.0	37.5	35.25
0.30%	36.0	35.0	33.0	32.5	39.0	37.5	35.50
0.40%	36.5	35.5	34.5	35.5	33.0	36.5	35.25
Control	51.0	48.5	49.0	46.5	49.5	46.5	48.5

APPENDIX XXVI

EFFECT OF VARYING 2,4-D/PDA CONCENTRATIONS ON COLONY GROWTH OF A.BRASSICICOLA
(measured after 6 days incubation at 24°C)

2,4-D concentration in media	Colony diameters (mm)						
	1	2	3	4	5	6	Mean
0.10%	38.5	37.5	40.0	34.0	38.0	35.5	37.25
0.15%	36.0	37.0	35.5	34.5	32.0	35.5	35.0
0.20%	33.5	38.5	36.0	36.0	35.5	33.5	35.5
0.25%	35.5	35.5	34.0	35.5	34.5	36.5	35.25
0.30%	34.5	35.5	33.5	34.5	34.0	35.0	34.5
0.40%	35.0	35.0	34.5	36.0	34.5	34.0	34.8
Control	38.5	41.5	41.0	42.0	38.0	42.0	40.5

APPENDIX XXVII

EFFECT OF STREPTOMYCIN ON COLONY GROWTH OF A.BRASSICAE
(measured after 6 days incubation at 24°C)

Streptomycin concn. (iu/ml)	Colony diameters (mm)						
	1	2	3	4	5	6	Mean
None	28.5	29.0	25.5	27.5	26.5	28.5	27.7
20	27.0	26.0	26.0	24.5	25.5	25.0	25.7
40	20.5	21.5	20.5	20.0	23.5	21.5	21.2
60	22.0	21.5	21.0	20.0	19.0	19.0	20.4
80	20.0	21.0	21.5	20.5	22.0	20.0	20.8
100	15.0	12.5	13.0	15.0	14.0	11.5	13.5
200	12.5	13.0	13.0	13.5	11.5	12.0	12.6
400	11.0	9.5	9.5	12.0	10.0	8.5	10.1

APPENDIX XXVIII

EFFECT OF STREPTOMYCIN SULPHATE ON COLONY GROWTH OF A.BRASSICICOLA
(measured after 6 days incubation at 24°C)

Streptomycin concn. (iu/ml)	Colony diameters (mm)						
	1	2	3	4	5	6	Mean
None	34.0	34.5	36.0	36.0	34.5	35.0	35.0
20	29.5	29.0	28.0	28.0	31.0	29.0	29.1
40	27.0	30.0	29.0	29.5	27.5	28.5	28.6
60	30.0	29.0	28.0	30.0	29.5	29.0	29.25
80	30.0	31.0	29.0	29.5	29.5	29.0	29.7
100	29.0	28.0	25.0	28.0	25.0	27.0	27.0
200	19.0	16.0	18.0	19.019.0	19.0	19.0	18.5
400	16.0	18.0	17.0	16.5	15.0	16.0	16.4