Some Mycological Problems Associated with the Prothalli of Pteridium aquilinum Kuhn.

by Moustafa Mohammed Fahim.

This thesis describes a series of investigations into the mycological problems affecting the prothalli of Pteridium aquilinum Kuhn. This prothallus has been chosen because it consists of a plate of almost unspecialized cells, only one cell thick over a large part of its area. It can be grown easily unde precisely controlled conditions and it appears to be an excellent "tool" for the study of certain host-parasite relationships. The investigation has involved four phases:

(1) The establishment of satisfactory methods for bracken spore decontamination and the study of the factors affecting spore germination and the growth of prothalli in pure cultures.

Treatment with 0.1% mercuric chloride solution for one minute has been found to be a satisfactory method for decontamination. It has been found that part of the falling off of power of spore germination and prothallial growth in pure cultures is associated with ageing of the spores. The results show that a proportion of the prothalli are non autotrophic and that these require a factor or factors which can be provided by some contaminant fungi.

(2) A general survey of the effect of common pathogens on prothalli was carried out with two objects:

(a) The isolation of pathogens producing disease in nature. Only two actively pathogenic species of fungi have been found and a diagnosis and description are given of one of these as new species of Coniothyrium.

(b) The study of the effect of common pathogens of higher plants on prothalli. The prothalli have been found to be susceptible to the attack of a wide range of rot, mould and damping-off fungi but not by those causing wilt and certain other diseases.

(3) Detailed study of the effect of Botrytis cinerea Pers. on prothalli.

(a) A preliminary study of the effect of temperature and media on growth and sporulation of Botrytis cinerea has been carried out. Abundant sporulation occurs at 20°C. on media with a relatively low carbohydrate conten

(b) A detailed morphological, histological and physiological study of the attack of the fungus on the host has been made.

(i) Initial penetration of the cell wall is associated with the formation of an appressorium and infection peg which suggests that mechanical pressure is

site of original infection. It appears that the initi failures to infect are due to slight differences betwee the inocula or between cells in different areas of the prothallus and not due to general resistance of the host plant concerned. Where spread of infection is arrest this is due to changes in the host cell walls in the areas immediately surrounding the lesion. These chang are incited by the action of some fungal metabolites on the living cells of the plant.

4. The effect of variation in external conditions on disease incidence.

(a) It has been observed that both increases or decreases from a certain optimum in spore density cause a reduction in dise incidence and that increase in nutritive materials in the inocula is associated with increase in the amount of disease developed.

(b) It has been found that variation in the period of illumination during growth of the host has a significant effect on disease incidence. It also has an effect on the morphology a physiology of the fungus, but it has been shown that this is not the cause of the difference in incidence of the disease.

(c) It has been shown that there are significant differenc in disease development on prothalli grown on media containing different concentrations of potassium. Histological study of th

living hyphae growing within the host suggests that this effect is due to variation in rate of penetration of the cell walls. The results of experiments using different concentrations of nitrate nitrogen in the media did not show that such differences in the medium caused any significant difference in the amount of disease developed.

SOME MYCOLOGICAL PROBLEMS ASSOCIATED WITH FERN PROTHALLI *Pteridium aquilinum* Kühn.

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by

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Introduction.

The complex structure of the host plant has often caused difficulty in the interpretation of the results of studies of host-parasite relationship. It was thought that this problem might be overcome by the choice of a morphologically and histologically simpler type of host. The gametophyte of Pteridium aquilinum Kühn has been chosen as such a host because it consists of a plate of almost unspecialized cells, only one cell thick over a large part of its area and it can be grown easily under precisely controlled conditions.

The investigation has involved four phases :-

(1) The establishment of satisfactory conditions for growth of the plants in pure culture.

(2) The general survey of the occurrence of pathogens on prothalli under natural conditions, and of the effects of common pathogens of higher plants on them.

(3) A detailed study of the pathology of Botrytis cinerea on the prothalli.

(4) A study of the effect of variation in selected external conditions on disease development and resistance in prothalli.

The summary of the results is given at the end of the thesis.

Part I.

Study of methods for bracken spore sterilization and the factors affecting spore germination and the growth of prothalli in pure cultures.

(1) Study of methods for spore sterilization.

Previous work

It was not practicable to collect spores by sterile dissection of ripe unopened sporangia, and the spores collected in bulk from scrapings of mature sori were normally found to have a heavy surface infestation of bacteria and fungi. This has been a common problem in working with the spores and gametophytes of the Pteridophyta, and previous investigators have used many methods to overcome it. Stokey (1930) decontaminated prothalli of species of Cyatheaceae growing on a sterilized peat medium by washing them with 0.004% aqueous potassium permanganate as recommended by Twiss (1910). In cases of bad fungal infection the prothalli were washed with the solution, transferred to a fresh dish of peat, and were given daily application of the solution until either the prothalli or the contaminants died. He also found that an unmeasured stronger solution which he described as being "decidedly pink" in colour was equally satisfactory. Knudson (1940) found that ten to fifteen minutes immersion in a freshly made 8% aqueous solution of calcium hypochlorite successfully decontaminated spores of Polypodium aureum. Schwabe (1951) found that washing in 0.036% aqueous mercuric chloride

decontaminated spores of Pteridium aquilinum and that 1% calcium hypochlorite was lethal. He found however that the germination and growth of the spores were much retarded by using mercuric chloride, and that washing in ten to twelve changes of sterile water was sufficient to eliminate practically all the surface contaminants. Castle (1953) exposed strobili of Equisetum arvense L. to an aerosol hypochlorite treatment and dissected out spores from the sporangia into a small volume of nutrient solution under sterile conditions. Steeves, Sussex and Pastanen (1955) found that the spores of Pteridium aquilinum were successfully decontaminated by vigorous shaking ten times in a 5% solution by weight of "Pittcher" in water, followed by several washings in sterile water.

The wide variety of agents and methods used and the reports of reduction in germination in some cases suggested that a preliminary investigation should be made to determine a convenient and satisfactory method for treating the large number of spores which would be required for the project.

Methods.

Mercuric chloride, calcium hypochlorite and sulphur dioxide were chosen for study, and their effects were examined by the following methods:

1. - Pretreatment. Immediately before each test, spores were suspended in tap water, and held at 24°C. for 18-24 hours. They were then washed in two changes of sterile distilled water, centrifuging the suspension each time to recover them. This treatment was given to remove gross contaminants and to encourage the germination of any resistant spores of residual contaminants, so making them more sensitive to the action of the disinfectants.

2. - Treatment with mercuric chloride. Aqueous solutions of 1.0% and 0.1% mercuric chloride were tested in 1 minute and 5 minutes treatments. Each solution was added to a sample of pretreated spores, and at the end of the timed period each suspension was diluted 100 times with sterile distilled water.

3. - Treatment with calcium hypochlorite. The treatment used was a modification by Wilkie (1954) of Knudson's method (1940). One ml. of a freshly prepared and filtered 1.0% aqueous solution of commercial bleaching powder was added to a sample of pretreated spores. After 1 minute the

chlorine content was neutralized by the addition of approximately 0.1 ml. of 25% aqueous potassium iodide solution, which produced a brown precipitate in the suspension. This precipitate was taken up by the addition of approximately 0.1 ml. of a 25% aqueous solution of commercial sodium thiosulphate and the suspension then diluted 100 times with sterile distilled water.

4. - Treatment with sulphur dioxide. A sulphur dioxide solution was prepared immediately before use by mixing 1.25 ml. N hydrochloric acid and 1.25 ml. of 10% potassium thiosulphate in 97.5 ml. of distilled water. One ml. of this solution was added to the pretreated spores and the suspension shaken vigorously for 5 minutes. Sufficient 2.5 N aqueous potassium permanganate solution was then added so as just to oxidise the sulphur dioxide, which was shown by the retention of a faint pink colouration by the suspension. The suspension was then diluted 100 times with sterile distilled water. A second sample of the spores was shaken in the sulphur dioxide for 15 minutes, and then neutralized and diluted in the same way.

A control sample of the same pretreated spores was tested by dilution in distilled water in parallel with each of the above tests, and after treatment all spores were spread on

the surface of a modified Knop's medium agar in petri dishes. This medium had been found to be a convenient one supporting good prothallial growth in previous departmental work. The dishes were held under 15 hours daily illumination and the percentage of germination and growth were recorded after 14 days.

Modified Knop's medium,
employed for culturing prothalli.

KCl	12.0 gm.	per litre.
$MgSO_4 . 7H_2O$	13.0 gm.	" "
$Ca(NO_3)_2 . 4H_2O$	20.0 gm.	" "
KH_2PO_4	12.0 gm.	" "
Fe tartrate	1.2 gm.	" "
$NaNO_3$	14.4 gm.	" "

Results.

i. - Mercuric chloride.

There was no germination of any spores treated with 1.0% solution of aqueous mercuric chloride. Table I details the results of two replicate experiments using 0.1% solution.

Table I.

Decontamination of Bracken spores by Mercuric chloride

Treatment	No. of plate	Exp. I No. of spores counted	Exp. I % of germ.	Exp. I Remarks	Exp. II No. of spores counted	Exp. II % of germ.	Exp. II Remarks
0.1% for 1 min.	1	242	40	no contamination in all dishes. very poor growth.	142	44	no contamination in all dishes. very poor growth.
	2	200	41		166	37	
	3	217	38		156	47	
	4	278	41		141	50	
	5	180	43		125	41	
0.1% for 5 mins.	1	94	14	no contamination in all dishes. very poor growth.	125	4	no contamination in all dishes. very poor growth.
	2	147	19		169	13	
	3	87	15		157	7	
	4	156	16		130	0	
	5	72	19		182	11	
control	1	197	74	all cultures contaminated with fungi & bacteria. good growth	109	76	all cultures contaminated with fungi & bacteria. good growth
	2	227	74		106	74	
	3	158	89		122	75	
	4	154	82		129	73	
	5	132	83		132	77	

ii. - Bleaching powder.

Table II details the result of two replicate experiments carried out in which 1.0% Bleaching powder was used for 1 minute.

Table II.

Decontamination of bracken spores by bleaching powder.

		Exp. I			Exp. II		
Treatment	No. of plate	No. of spores counted	% of germ.	Remarks	No. of spores counted	% of germ.	Remarks
1.0% for 1 min.	1	114	33	no contamination in all dishes. very poor growth.	225	62	No contamination in all dishes. very poor growth.
	2	85	39		179	60	
	3	118	31		218	56	
	4	84	33		192	57	
	5	89	37		166	57	
control	1	74	78	all cultures contaminated with fungi & bacteria. good growth.	232	82	all cultures contaminated with fungi & bacteria. good growth
	2	61	76		154	81	
	3	110	78		186	77	
	4	89	80		153	92	
	5	105	76		141	82	

iii. - Sulphur dioxide.

Table III details the results of two replicate experiments carried out in which spores were treated with Sulphur dioxide for 5 minutes and 15 minutes.

Table III.

Decontamination of Bracken spores by Sulphur dioxide.

		Exp. I			Exp. II		
Treatment	No. of plate	No. of spores counted	% of germ.	Remarks	No. of spores counted	% of germ.	Remarks
5 mins.	1	152	36	good growth contaminated.	157	23	no contamination in all dishes. very poor growth.
	2	109	28	no contamination in all dishes, very poor growth.	173	22	
	3	133	23		149	13	
	4	152	16		108	20	
	5	102	49		149	14	
15 mins.	1	130	19	no contamination in all dishes. very poor growth.	140	0	no germination. no contamination in all dishes.
	2	145	9		155	0	
	3	182	13		129	0	
	4	270	23		144	0	
	5	210	23		180	0	
control	1	215	72	all cultures contaminated with fungi & bacteria. good growth.	186	74	all cultures contaminated with fungi & bacteria. good growth.
	2	155	76		167	75	
	3	175	78		156	77	
	4	162	80		161	70	
	5	135	81		168	72	

In all the experiments, prothalli which developed from decontaminated spores in uncontaminated cultures did not develop beyond the one or two celled stage (Fig.I), while prothalli in control cultures developed normally (Fig. II). When air born contaminants developed on cultures containing decontaminated spores the prothalli around the contaminant colony started growing again and developed into normal adults (Fig.III), this stimulation being less evident in proportion to the distances from the edge of the growing colony. The amount of spore germination was not changed.

Discussion.

The methods of decontamination tested had similar effects on both the percentage of germination and on the amount of growth of the prothalli which developed. The experiments were not designed to investigate the cause of these effects, but it is noted that they may be due either to a direct toxic or inhibitory action of the disinfectant or to the absence of some factor in the environment which is contributed by the contaminants in cultures of untreated spores, or to the presence of traces of the disinfectant material in the diluted suspensions which were plated onto the agar.

Fig. I. Microphotograph showing Prothalli developed from decontaminated spores in uncontaminated cultures. Approx. x 180.

Fig. II. Microphotograph showing prothalli developed from untreated spores in contaminated control cultures. Approx. x 180.

Fig. III. Microphotograph showing prothalli developed from decontaminated spores in contaminated cultures. Approx. x 180.

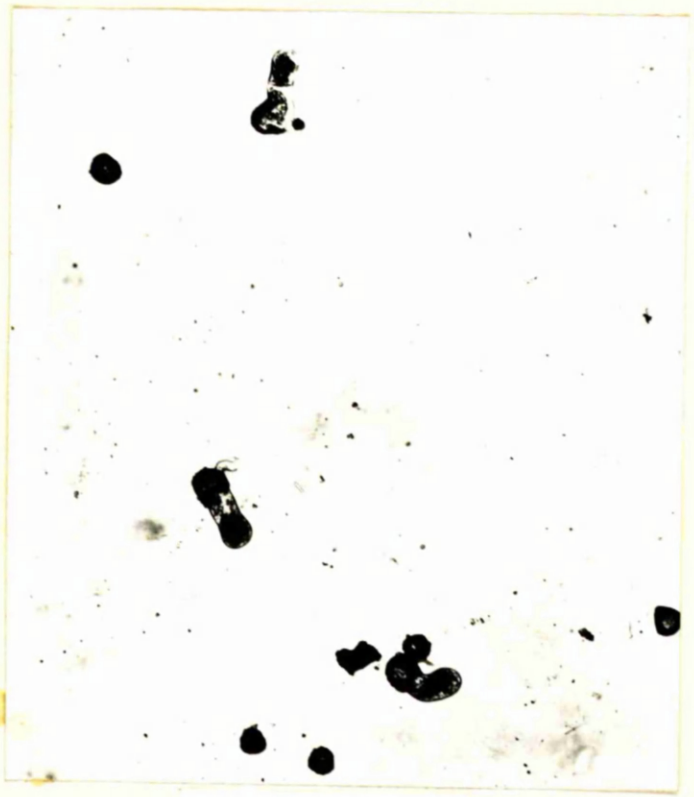


Fig. I

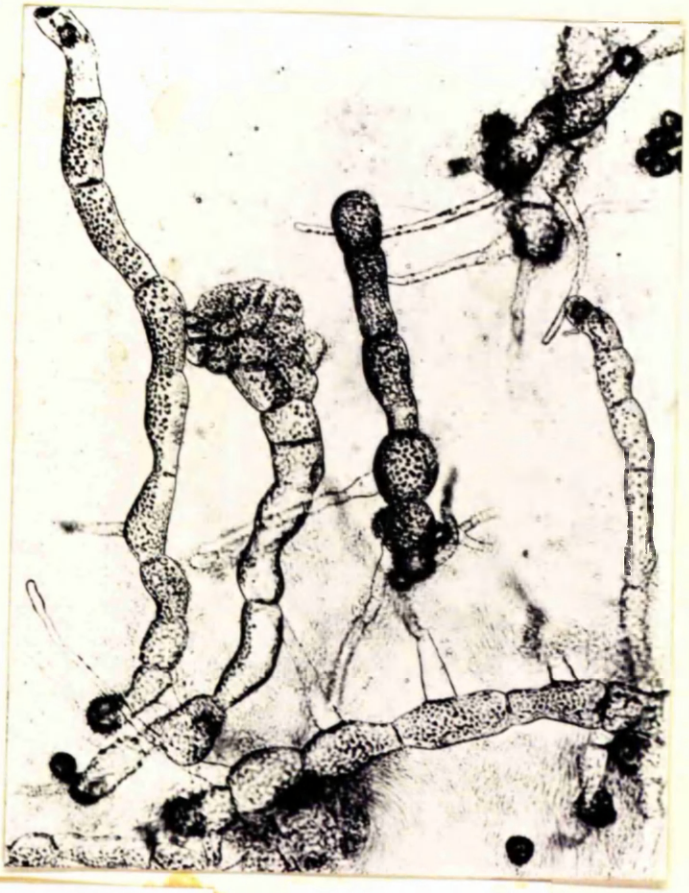


Fig. II

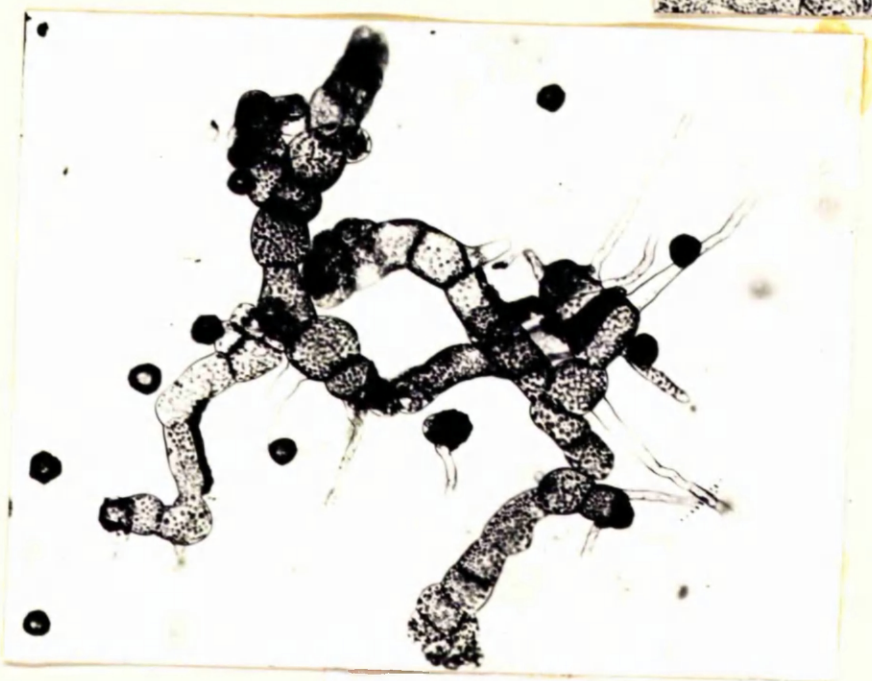


Fig. III

It is noted that the effect of contaminants on plates of disinfected spores was limited to the stimulation of prothallial growth and that it did not affect the germination of the "dormant" spores. This suggests that the factor influencing the prothallial growth is some metabolite produced from the contaminant, but that the reduction in germination is more likely to be due to a toxic effect of the disinfectant. Further experiments to investigate these phenomena are reported below.

The object of this investigation was, however, achieved by the determination that treatment with 0.1% mercuric chloride solution for 1 minute gives an adequate control of contamination. As the results of this simple method were as satisfactory as those got from the more elaborate ones it was adopted for all routine work.

(2) Study of the Factors affecting Germination and prothallial growth in pure Culture.

Previous work.

The work reported above showed that the amount of germination of spores and the growth of the prothalli was reduced when they were decontaminated and held in pure culture. The amount of germination varied with spores of varying ages and sources, and the check to growth of the prothalli was overcome if contaminant fungi appeared in the cultures.

The previous workers who have grown prothalli of Pteridophytes in pure culture have not recorded this problem, though Conway (1949) stated that the viability of spores of Pteridium aquilinum decreases with age. Hurel-Py (1942) grew the Gametophytes of Asplenium in pure culture on Knop's medium. She found that the growth was stimulated by the presence of glucose and in 1943 she reported further studies of the gametophytes of Nephrolepis cordifolia and showed that glucose was stimulating only up to 2% concentration. She concluded that in both cases the stimulation was due to the effect of the glucose on chlorophyll formation. Castle (1953) stated that prothalli of Equisetum arvense grew normally on Moor's solution ($NH_4 NO_3$, 0.5 gm; $KH_2 PO_4$, 0.2 gm; $Mg SO_4 . 7H_2O$, 0.2 gm; and $CaCl_2 . 2 H_2O$, 0.1 gm. in 1 litre of distilled water) and that growth was stimulated by the addition

of 50 to 100 mg. per litre of casein hydrolysate, or by the addition of 500 mg. per litre of sucrose, or by the addition of combinations of these two. Sussex and Steeves (1953) found that prothalli of Pteridium aquilinum made good growth on 1% agar medium containing Knudson's solution of inorganic salts (1925), Berthelot's trace element solution, 2% glucose, and 0.5 gm. per litre yeast extract. Morel (1950) and Morel and Whetmore (1951) reported the spontaneous appearance on prothalli of Osmunda cinnamomea growing in pure culture of undifferentiated parenchymatous "calluses" which were capable of unlimited growth when subcultured onto agar media containing organic salts, sugar and B. vitamins.

The variation in results obtained in early work in this department caused difficulty in the maintaining of a regular supply of pure culture of prothalli. The following experiments were therefore designed to examine the effect of certain factors on:

(a) the germination of the spores

(b) the growth of the gametophytes.

The phenomena were examined with a view to determine a satisfactory routine method for the maintenance of pure culture of prothalli required for the investigation, and they

are not necessarily related. The experiments give information about both, however, and it will therefore be convenient to consider them simultaneously in this account.

Experimental.

i. Study of the effect of the age of spores on germination AND On the growth of the gametophyte.

Spores were collected in the autumn of each of the years 1951, 1952, 1953 and 1954 from the same group of plants on Drumelog Moor, Milngavie, Dumbartonshire. In November 1954 representative samples of each collection were disinfected using 0.1% mercuric chloride for 1 minute, a similar untreated sample was suspended in sterile water and each was spread on the surface of plates of Knop's agar held under 15 hrs. daily illumination. Records of germination and amount of prothallial growth were made two weeks after sowing. Results are shown in Table IV.

Table IV

The effect of age of spores on germination and on the growth of the Gametophyte

		Treated spores			Control (Untreated spores)		
Sample	plate	No. of spores counted	% of germ.	Remarks	No. of spores counted	% of germ.	Remarks
1951	*1	281	0	No contamination.	*214	0	No contamination.
	*2	305	0	" "	*230	0	" "
	3	250	0	" "	290	0	" "
	4	266	0	" "	277	0	" "
1952	*1	180	0	No contamination.	285	20	Very poor growth contaminated.
	*2	195	0	" "	367	13	" "
	3	177	0	" "	276	0	No contamination.
	4	201	0	" "	331	0	" "
1953	1	246	12	No contamination in all dishes, poor growth.	275	29	All dishes were contaminated. Good growth.
	2	274	18		448	25	
	3	170	12		417	20	
	4	187	17		474	24	
1954	1	315	29	No contamination in all dishes, good growth.	216	82	All dishes were contaminated, very good growth.
	2	266	23		184	80	
	3	365	25		204	70	
	4	245	30		209	87	

These results show that there is a reduction in the amount of germination and of growth using older spores. Comments on the effect of fungal contamination on this property are made below (Table VII). The study was supported by a record of empirical observations of the amount of germination and of growth in all routine cultures set up between October 1953 and July 1955. These are detailed in Table V. These observations show that there was a considerable variation in germination in successive experiments using spores of the same age, but that there was an overall reduction in the amount of germination and growth as the spores became older.

Table V.

Observations of the amount of germination and growth in culture set up between October 1953 and July 1955

Spores used below were collected in September 1953.

Time of sowing	Place from which spores were collected	Remarks
15.10.1953	Mugdockbank	Normal germination, good growth, no contamination.
2.12.1953	"	5 petri dishes had normal germination and growth but were contaminated. The remaining 5 dishes had normal germination, poor growth and no contamination.
1. 1.1954	"	Normal germination, good growth, no contamination.
5. 3.1954	"	Normal germination, good growth, no contamination.

Table V (cont.).

4. 4.1954	Mugdockbank	Normal germination, two dishes had poor growth, others had good growth, no contamination.
4. 6.1954	"	Normal germination and good growth in all petri dishes. 6 dishes, only, contaminated.
1. 7.1954	"	No germination, no contamination.
8. 7.1954	"	Poor germination, many germinated spores failed to continue growth, poor growth in general, no contamination.
1.10.1954	"	Normal germination, many prothalli were growing but still very small in size. Few plants reached the normal size (1.0 cc. in diameter) within 80 days, no contamination.

Spores used below were collected in September 1954.

4. 9.1954	Mugdockbank	Normal germination, very good growth, no contamination.
20.10.1954	"	Normal germination, good growth, no contamination.
31. 3.1955	"	Normal germination, good growth, no contamination.
4. 4.1955	"	Poor germination and growth in seven petri dishes, no contamination, the other three had good growth but were contaminated.
21. 4.1955	"	No germination, no contamination.
21. 5.1955	"	Normal germination, good growth but very slow, no contamination.
9. 7.1955	"	Two petri dishes had poor germination and growth, no contamination. The other 8 dishes had good germination and growth but all were contaminated.

ii. Study of variations in amount of germination and prothallial growth in spores from different localities.

The question of genetic differences in the prothalli has not been studied in detail, but the results in Table VI show that there may be substantial differences between various population in this respect.

The samples of spores were collected from Drumclog Moor, Milngavie, and from a patch of bracken on the roadside at Mugdockbank, Milngavie, about ½ a mile from Drumclog Moor. To prevent confusion all work other than this was carried out using spores from Mugdockbank.

Table VI.

Table VI.

Observation of variations in amount of germination and prothallial growth between spores* from different sources.

Time of sowing	Place from which spores were collected	Observations
3.11.1953	Mugdockbank	Normal germination, good growth, no contamination.
17.11.1953	Milngavie	Normal germination, good growth, no contamination.
14.12.1953	"	Normal germination, poor growth, no contamination.
19. 1.1954	Mugdockbank	Normal germination, good growth, no contamination.
11. 5.1954	"	Normal germination, very good growth, no contamination.
20. 7.1954	Milngavie	No germination, no contamination.
9. 8.1954	Mugdockbank	Normal germination, poor growth, no contamination.
1.10.1954	Milngavie	Very poor germination, no growth, no contamination.

(*) Both kinds of spores were collected in September 1953.

iii. Study of the effect of Fungal metabolites on spore germination and prothallial growth.

(a) The effect of the presence of contaminating Fungi.

The differences between germination and prothallial growth in contaminated control cultures and in pure cultures containing decontaminated spores are recorded in Table IV. A further experiment to determine whether these differences

were due to the presence of the contaminants or to the action of the decontaminating process was set up using the plates in which no growth had occurred in the above experiment. After the examination referred to in Table IV, the lids of half of these plates, marked * in the table were removed for half an hour. The whole group was then maintained under 15 hours daily illumination for a further month. The results seen on re-examination are detailed in Table VII.

Table VII.

The effect of the presence of contaminant fungi.

		Treated spores			Control (Untreated spores)		
Sample	Plate	No. of spores counted	% of germ.	Remarks	No. of spores counted	% of germ.	Remarks
1951	* 1	≥250	0	Contaminated	*>250	0	Contaminated
	* 2	>250	0	"	*>250	0	"
	3	>250	0	No contamin-	>250	0	No contamin-
	4	>250	0	ination	>250	0	ination
1952	* 1	411	12	Contaminated, few prothalli were growing.			
	* 2	432	13				
	3	>250	0	No contamin-			
	4	>250	0	ants.			

* Lids of plates were removed for half an hour at the beginning of the experiment.

(b) Comparison of the effect of different fungal species in pure cultures.

The general observations recorded above were extended by a study of the effect of growing pure cultures of decontaminated spores in association with pure cultures of common soil fungi. Petri dishes of sterile Knop's agar were inoculated with pure cultures of the test fungi detailed below, and inoculated for 48 hours at 24°C. The surface of the fungal colony was then covered with a sheet of sterile cellophane, and a suspension of decontaminated spores spread over the surface of the cellophane. The plates were then held under 15 hours daily illumination for two weeks. The spores had been collected from Tarbet, Dumbartonshire, eight months before the experiment was carried out. The results are detailed in Table VIII.

Table VIII.

Table VIII.

Comparison of the effect of different fungal species in pure culture on spore germination and prothallial growth.

Treatment	No. of plate	No. of spores counted	% of germination	Average size of prothelli
Botrytis cinerea Pers. Imperial College culture	1	183	84	4-6 cells
	2	186	80	
	3	116	80	
	4	131	72	
Penicillium notatum departmental collection culture	1	143	80	3-5 cells
	2	184	65	
	3	168	77	
	4	154	73	
Pythium de-baryanum Hesse. C.M.I. culture 48558	1	111	66	2-3 cells
	2	117	74	
	3	204	61	
	4	184	81	
Aspergillus niger Van Tiegh C.M.I. culture 17454	1	168	88	7-12 cells
	2	188	86	
	3	192	85	
	4	120	80	
Pure culture	1	164	78	one cell
	2	192	45	
	3	165	60	
	4	155	53	
Cultures of untreated spores with miscellaneous contaminants	1	106	81	8-14 cells
	2	198	71	
	3	194	85	
	4	140	85	

It was noted that the prothalli formed in cultures containing Pythium de-baryanum Hesse. were much distorted. The effect of other fungi was generally similar, differing only in the degree of stimulation produced. The greatest stimulation was that produced by cultures containing Aspergillus niger Van Tiegh, in which the prothalli were considerably larger, and in which lateral division of the cells occurred when the prothalli had become two to three cells long.

(c) Detailed study of effect of Aspergillus niger.

Experiments to determine whether the stimulatory effect of Aspergillus niger was due to the presence of some fungal metabolite which diffused out into the medium were carried out by two methods:

i. Plates of sterile Knop's agar were covered with sterile cellophane, and spores of the fungus were inoculated onto the surface of the cellophane. The cultures were incubated at 24°C. for four to five days, by which time the fungus had grown almost to the edge of the cellophane, but had not grown over it onto the agar surface. The cellophane with the fungal colony was then removed with sterile forceps, the margin of the paper being folded into the centre initially

to prevent the dispersal of spores from the surface during handling. A suspension of decontaminated bracken spores was then spread on the agar surface from which the fungus had been removed, and the culture held under 15 hours daily illumination for two weeks: the results are shown in Table IX.

Table IX.

Germination and growth on Knop's agar on which Aspergillus niger had been grown previously.

Treatment		No. of spores plate counted	% of germination	average size of prothalli
Cultures treated with Aspergillus niger	1	124	10	one cell
	2	106	11	
	3	124	17	
	4	112	14	
Cultures not pretreated with A. niger	1	102	20	one cell
	2	121	14	
	3	115	17	
	4	128	15	
Cultures of untreated spores with miscellaneous contaminants	1	103	39	8-10 cells
	2	129	43	
	3	101	36	
	4	120	37	

ii. Flasks containing a sterile double strength Knop's solution plus 2.0% glucose were inoculated with Aspergillus niger and inoculated at 24°C. for 5 days. The medium was then filtered off through a bacteriological filter, diluted by the addition of an equal volume of warm sterile 4% agar in distilled water, and 20 ml. aliquots were dispensed into sterile petri dishes. A control medium was set up using precisely the same procedure without inoculation of the fungus. After cooling, a suspension of decontaminated bracken spores was spread on the surface of both media, control plates of the control medium were spread with untreated spores and the whole were held under 15 hours daily illumination for 14 days. The results are detailed in Table X.

Table X.

Table X.

Germination and growth in medium made from filtrate of a culture of Aspergillus niger Van Tiegh.

Treatment	No. of plate	No. of spores counted	% of germination	Average size of prothalli
Filtrate medium from liquid culture of A.niger	1	130	11	one cell
	2	108	10	
	3	121	14	
	4	105	11	
Culture not pretreated with A. niger	1*	106	31	3-4 cells
	2	114	15)	
	3	125	12)	one cell
	4	110	16)	
Cultures of untreated spores with miscellaneous contaminants	1	103	41	8-12 cells
	2	123	39	
	3	108	28	
	4	112	38	

(*) Plate No.1 of this group developed a secondary contaminant. The % of germination increased as shown; and prothalli were 3-4 cells long on examination.

iii. Control test. The spores used in both the above tests were 16 months old, and had been collected from Drumclog Moor, Dumbartonshire. A parallel experiment set up by the method described in paragraph (c) above shows that the fungus stimulated both growth and germination of the same spores in these conditions. The results are detailed in Table XI.

Table XI.

Germination and growth on Knop's agar in the presence of Aspergillus Niger Van Tiegh.

Treatment	No. of plate	No. of spores counted	% of germination	Average size of prothalli
Cultures contain Aspergillus niger	1	125	12	3-5 cells
	2	140	14	
	3	121	8	
	4	110	9	
Pure cultures	1	108	6	one cell
	2	137	8	
	3	142	8	
	4	171	11	
Cultures of untreated spores with miscellaneous contaminants	1	204	33	7-10 cells
	2	172	35	
	3	277	31	
	4	189	31	

iv. Study of the effect of addition of glucose, Yeast extract and Peptone to the medium.

The work by Hurel-Py, Castle and By Sussex and Steeves (loc. Cit.), suggests that the addition of additional carbohydrates, trace elements or growth factors may have a significant effect on germination and growth. The effect of the addition of some of the above substances to the medium was therefore tested by spreading a suspension of decontaminated spores onto plates of Knop's agar with the additions stated, and holding the plates under 15 hours daily illumination for 14 days. Two replicate experiments were carried out and the results are recorded in Table XII.

Table XII.

Table XII.

Effect of addition of various substances to the medium.

		Exp. I			Exp. II		
Treatment	Plate	No. of spores counted	% of germ.	Average size of prothalli	No. of spores counted	% of germ.	Average size of prothalli
1.5% Yeast extract	1	154	8	1-2 cells	89	10	1-2 cells
	2	75	17		89	15	
	3	119	13		92	13	
	4	140	10		87	9	
1.5% Peptone	1	146	17	2-4 cells	107	11	1-2 cells
	2	157	17		73	5	
	3	200	13		135	13	
	4	190	12		108	9	
2% Glucose	1	277	20	3-6 cells	301	18	3-5 cells
	2	185	19		193	18	
	3	194	14		201	16	
	4	167	15		270	19	
Knop's agar with no additions	1	272	21	3-7 cells	109	20	3-6 cells
	2	255	15		136	17	
	3	265	16		128	18	
	4	246	19		117	22	
Cultures of untreated spores with miscellaneous contaminants	1	132	34	3-12 cells	110	35	9-12 cells
	2	163	34		142	45	
	3	218	40		119	31	
	4	179	31		124	35	

Discussion.

The object of this investigation was attained by finding that pure cultures of the prothalli could be obtained in satisfactory numbers by the use of young spores, and that the falling off of powers of germination and ability for completely autotrophic prothallial growth was associated with ageing of the spores. The results do not however explain the cause or nature of these changes. It is not clear whether the reduced percentage of germination of the spores after decontamination in Table IVis due to the inability of the majority to germinate autotrophically, or to a toxic action of the decontaminating process, or to some interaction of the two. In a separate experiment (Table VIII) it is clear that the decontaminating process had little effect on the young spores used, and the "recontamination" of older spores (1952) recorded in Table VII resulted in a germination of similar order to that of the control contaminated spores. It is also noted in Table VII that there was no germination of spores in the two control plates which by chance developed no contaminations. It is therefore apparent that at least part of the failure to germinate in pure cultures is due to the absence of some essential factors in them.

The results confirm that a proportion of the prothalli

are non autotrophic, and that these require a factor or factors which can be provided by many contaminant fungi. Aspergillus niger was found to be the most prolific source of the factor of the fungal species tested.

The results in Table VIII show that the stimulating factor is a product which can diffuse through cellophane, and the failure to demonstrate its presence in media in which the fungus had been grown may be due to its instability, or to it being produced in too small concentration in these conditions. The fact that the stimulating effect is limited to an area closely in contact with the contaminant colony on plates, can be explained by either theory.

Work on this problem has not yet been developed further, as the primary object of the investigation was the study of parasitic relationships of the prothalli. It is, however, clearly an interesting field for study.

Part II.

Survey of the fungal pathogens of Prothalli.

Survey of the effect of common pathogens on Bracken prothalli.

A general survey of the effect of common pathogens on prothalli was carried out with two objects:

(a) the isolation, identification and study of pathogens found naturally in cultures of prothalli,

(b) the study of the effect of common pathogens of higher plants on prothalli.

Previous work

Although much work has been done on the growth of prothalli in culture, there are few records of study of parasites which have been found on them. Verdoorn (1938) reported that the prothalli of the Pteridophyta live in so much intimate connection with the soil and under moist conditions that they very readily fall victims to the damping-off fungi. He stated that Cephalothecium roseum CDA., which is usually a saprophyte, was found in a greenhouse in Indiana causing severe damping-off of prothalli of Pteris longifolia. He stated also that Pythium de-baryanum Hesse had been recorded by Sadebeck as a parasite on the prothallia of Equisetum arvense, E. palustre and E. limosum.

Methods.

(a) Isolation of disease producing pathogens from nature.

During the course of this work many thousands of prothalli have been grown from unsterilized spores planted on Knop's agar. Any diseased prothalli found in these conditions were removed, and isolations made of fungi on them. The isolates were tested for pathogenisity by inoculation onto sterile prothalli, those causing disease being reisolated for further examination. It was realized that this process limited the possibility of study to the Bracken spore born and air born parasites, and experiments to examine the possible occurrence of soil born parasites were set up by planting sterile spores into the surface of samples of soil from a wide variety of Bracken infested localities in the west of Scotland. The soil samples were held in plant pots in damp and warm conditions in the department. The growing plants were examined periodically to isolate any diseased prothallus.

(b) Examination of effect of common pathogens of higher plants on prothalli.

To supplement the study of parasites isolated from infected prothalli, a series of tests were carried out using common parasites of higher plants. The following

parasites were chosen from those readily available, so as to include as wide a range of disease types as possible, and representatives of the main classes of the Fungi. They are grouped in the following list according to the type of disease they most commonly incite.

The cultures used were subcultures from the Commonwealth Mycological Institute collection which were received on 29th October 1954, two weeks before the tests were carried out, unless otherwise stated. The details of their catalogue number and medium are given in the relevant paragraphs below, and the virulence of some of those which did not attack the prothalli was tested against cress seedlings as detailed below.

I. Fungi which cause rot diseases:

(1) Phytophthora parasitica Dast.
Culture C.M.I.(22176) - maintained on Oatmeal agar.

(2) Rhizopus nigricans Ehrenb.
Culture C.M.I.(42844) - maintained on Potato dextrose agar.

(3) Fusarium solani (Mart.) Sacc. var. Martii.
Culture C.M.I.(49360) - maintained on Potato-carrot agar.

(4) Fusarium caeruleum (Lib.) Sacc.

Culture C.M.I.(44742) - maintained on Potato-carrot agar.

(5) Helminthosporium sativum Pamm, King & Bakkle.

Culture C.M.I.(21195) - maintained on Potato-carrot agar.

II. Fungi which cause mould disease:

(6) Aspergillus niger Van Tiegh

Culture C.M.I.(17454) - maintained on Potato dextrose agar.

(7) Botrytis cinerea Pers.

Culture Imperial College received on 10th November 1953 - maintained on Potato agar.

(8) Botrytis allii Munn.

Culture C.M.I.(42078) - maintained on Oatmeal agar.

(9) Trichothecium roseum Link ex Fri.

Culture C.M.I.(32236) - maintained on Malt agar.

III. Fungi which cause damping-off:

(10) Corticium solani (Prill & Delacr) Bourd & Galz.

Culture C.M.I.(69933) - maintained on Potato-carrot agar.

(11) Pythium de-baryanum Hesse.

Culture C.M.I.(48558) received on 6th July 1953 - maintained on Oatmeal agar.

(12) Cephalothecium roseum Corda Anleit.

Culture C.M.I.(60661) - maintained on Potato dextrose agar.

IV. Fungi which cause wilt disease:

(13) Fusarium vasinfectum Atk.

Culture C.M.I.(43528) received on 6th July 1953 - maintained on Malt agar.

(14) Verticillium dahliae Kleb.

Culture C.M.I.(45492) - maintained on Oatmeal agar.

(15) Verticillium albo-atrum Reinke & Berth.

Culture C.M.I. received on 6th July 1953 - maintained on Malt agar.

V. Miscellaneous species:

(16) Sclerotinia sclerotiorum (Lib.) de-Bary.

Culture C.M.I.(17473) - maintained on Starch agar.

(17) Phoma betae Frank.

Culture C.M.I.(52777) - maintained on Potato-carrot agar.

(18) Rhizoctonia solani Kühn.

Culture C.M.I.(42844) - maintained on Potato dextrose agar.

(19) Tilachlidium nigrum Hutchinson.

Departmental collection - maintained on 2% Malt agar.

(20) Alternaria brassicae (Berk) Sacc.

Culture C.M.I.(51115) - maintained on Potato-carrot agar.

Artificial inoculations

Mature prothalli of similar age and size were transplanted on Knop's medium in sterile petri dishes, 5 plants in each petri dish. Each fungus was tested on 20 plants in each experiment by placing a small piece of agar containing mycelium onto the plant surface. Varying numbers of experiments were carried out with each fungus, and some contaminated plants were discarded in each experiment. Table XIV records the whole number of plants actually observed in each case.

Disease records were taken 10 days after inoculation. Each infected plant was placed in one of these three classes by empirical observation:

(a) Generally diseased. (b) Locally diseased.

(c) Healthy.

The amount of disease developed was also recorded by visual estimate of the amount of prothallus invaded on the following scale:

0 = no visible infection
1 = up to 1/5 of prothallus infected
2 = 1/5 to 2/5 " " "
3 = 2/5 to 3/5 " " "
4 = 3/5 to 4/5 " " "
5 = 4/5 to whole prothallus infected

This scale was used for this and all subsequent experiments and it is referred to as the "Standard Scale" below.

Results.

The isolation of disease producing pathogens found in nature.

The common contaminants of culture on Knop's agar and on soil, inoculated with both surface sterilized and non sterilized spores, were species of Penicillium, Aspergillus, Trichoderma and Bacteria. They rarely attacked the prothalli directly, though in favourable conditions for their growth they occasionally smothered slowly growing

young prothalli.

Only two species acting as active parasites were isolated from the many hundreds of cultures examined:

(1) Tilachlidium nigrum Hutchinson. This new species of Tilachlidium was isolated previously by Dr. Hutchinson in 1949, and a report of his study of its character is being published separately.

(2) Fungus X. This fungus was found in January 1954 on diseased prothalli growing from spores which had been collected at Mugdockbank, Dumbartonshire in the autumn 1953. These spores had been stored in a non sterile glass tube in the Department at room temperature, and inoculated onto Knop's agar plates in December 1953.

The diseased plants showed typical symptoms, with very extensive development of brown pigment in the cell walls. The spread of the disease was rapid, and plants were completely destroyed within 6 to 7 days of the first sign of the disease being visible on them. Fungal mycelium bearing pycnidia was abundant on the diseased area. (Fig.IV). The fungus was isolated on 2% Malt agar and subsequently maintained on Czapek Dox agar.

Its virulence was tested by the inoculation of each of 60 healthy prothalli growing in pure culture with a small

piece of agar containing the fungal mycelium and spores. Disease symptoms precisely similar to those of the original prothalli developed from the site of inoculum. The following table records the rate at which the disease spread through the plants.

Table XIII.

Days after inoculation	1	2	3	4	5	6	7	10
Total % of disease in population on Standard Scale	0	1.5	37	48	72	91	97	100%

Pure cultures of the test fungus only were reisolated from the diseased prothalli.

A description of this fungus in culture is as follows:- (References are to Plates in Ridgway (1912)).

"On Czapek Dox agar in the dark at 24°C. the fungus form a white colony the centre of which after 5-7 days growth changes through "Drab"(XLVI) with "Hair Brown" spots (XLVI) to "Dark Greyish Olive" (XLVI) by 14 days, the under surface becoming"Green Blue Slate" (XLVIII) and the edge of the colony remaining white throughout."

The mycelium is made up of brown walled septate branched hyphae 2μ to 4μ in diameter. The Pycnidia (Fig.Va) are spherical, separate, superficial, dark brown, hirsute

non stellate, ostiolate at maturity, 150 μ to 370 μ in diameter, conidia are brown in the interior of the pycnidium. They are unicellular (Fig.Vb), cylindrical, occasionally allantoid, 1.5 μ to 3 μ x 3 μ to 7 μ, and at maturity have dark brown cell walls which give a black appearance to spore masses. The pycnidial cavity contains hyphae radiating centripetally singly or in fascicles from the lower half of the wall, but no evidence has been seen of the conidia being budded off from these hyphae or from the other parts of the locule wall. In many cases the appearance of sections suggests that the whole ground tissue of the locule turns into spores, but this requires more detailed examination before a firm statement can be made.

The fungus resembles a species of the genus Coniothyrium except in the details of conidial formation, and it has provisionally been filed at the Commonwealth Mycological Institute under reference Coniothyrium sp. C.M.I.60947. The Institute state that the fungus is new to their collection, and agree that further work on it is required before it can be classified precisely.

Fig. IV. Microphotograph showing fungal mycelium bearing pycnidia on the diseased area of a prothallus. Approx. x 30.

Fig. V. Camera lucida drawing shows :

a. Pycnidia. b. Conidia.

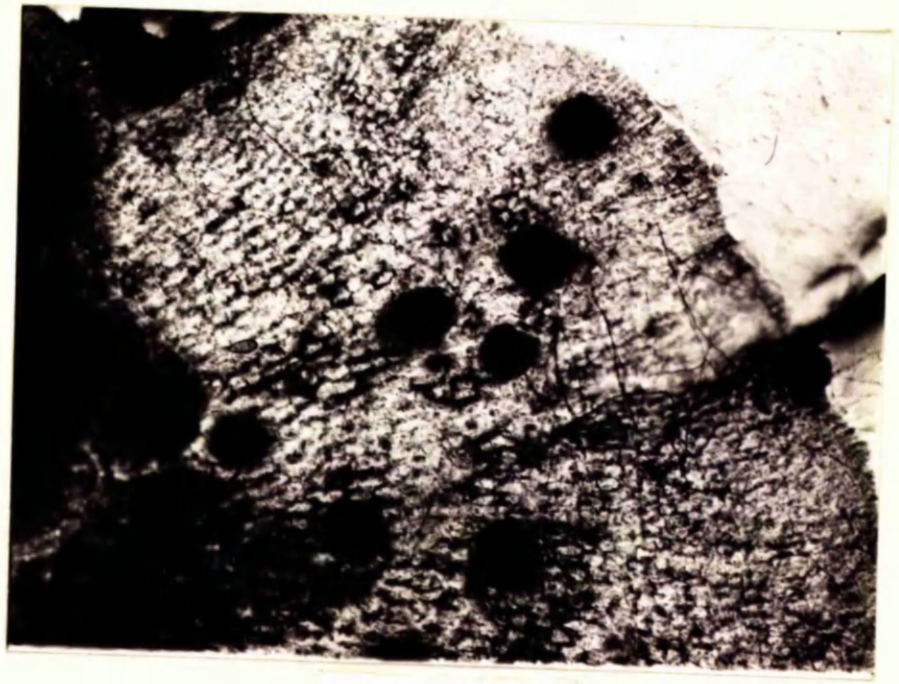


Fig. IV

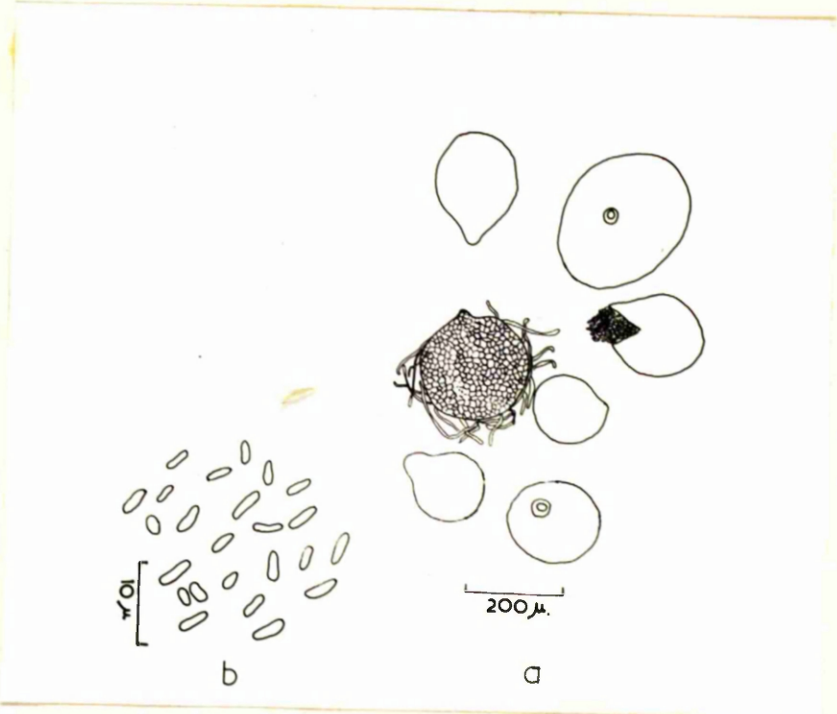


Fig. V

Study of the effect of common pathogens of higher plants on prothalli.

(1) General account of symptoms.

All parasites studied incited similar symptoms on successful invasion of the host, the difference between them being only in rate and degree of development of the disease in the several species.

The first visible host reaction was in most cases the formation of brown pigment in the walls immediately around the sites of initial penetration by the fungi. The contents of cells penetrated by the fungus later became disorganised, the chloroplasts usually disappearing early in the attack. In isolated cases with each pathogen the cells remained green until a late stage of the attack. The walls of attacked cells often subsequently became brown, but in some cases, particularly in those in which the disease developed very rapidly, they remained colourless. Later the cell contents disintegrated completely, the cell walls fell apart and broke down and the whole infected area became a disorganised mass. These symptoms were generally similar in the attack by most of the virulent parasites, and they are referred to as the "typical symptoms" in the account below.

In some individuals of each batch of plants the pathogens

successfully caused initial infections but did not spread generally through the host tissues. In these cases the attacked area was normally surrounded by a continuous zone of dark brown host cell walls, the significance of which is examined below. Table XIV details the amount of disease developed on prothalli by each of the fungi tested.

Table XIV.

The effect of common pathogens of higher plants on prothalli.

Fungi	Number of plants tested	Number of plants which were: (a) Generally diseased	(b) Locally diseased	(c) Healthy	Percentage of infection
Phytophthora parasitica	60	19	19	22	38
Rhizopus nigricans	60	12	12	36	23
Fusarium solani	40	0	0	40	0
Fusarium caeruleum	60	8	4	48	17
Helminthosporium sativum	59	49	6	4	75
Aspergillus niger	58	0	16	42	7
Botrytis cinerea	110	95	25	0	86
Botrytis allii	60	37	6	17	62
Trichothecium roseum	60	3	15	42	15
Corticium solani	40	3	13	24	10

Table XIV.(Cont.)

Fungi	Number of plants tested	Generally diseased	Locally diseased	Healthy	Percentage of infection
Pythium de-baryanum	100	100	0	0	100
Cephalothecium roseum	70	69	1	0	95
Fusarium vasinfectum	82	9	15	57	15
Verticillium Dahliae	60	4	4	42	7
Verticillium albo-atrum	90	0	13	77	3
Sclerotinia sclerotiorum	40	27	9	4	71
Phoma betae	40	0	0	40	0
Rhizoctonia solani	60	43	7	10	71
Tilachlidium nigrum	89	54	25	10	61
Alternaria brassicae	56	0	7	49	3

(2) Brief account of the effect of each pathogen on Bracken prothalli.

I. Fungi which cause rot diseases:

(1) Phytophthora parasitica Dast.

Symptoms : typical symptoms, the fungus was moderately virulent.

(2) Rhizopus nigricans Ehremb.

Symptoms : The fungus had little effect. The majority of the plants became covered with fungal mycelium which caused no visible symptoms of disease. The few diseased plants turned light brown in colour but there was little breakdown of the cell contents, even the generally diseased plants were still alive 10 days after inoculation.

(3) Fusarium solani (Mart.).

Symptoms : No effect.

Test of virulence of the parasite against cress plants.

A control test was carried out to study the virulence of the fungus against its normal host. Fourteen days old cress plants, grown asceptically on medium described by Chen and Thornton (1940), were inoculated with an aqueous spore suspension. Other uninoculated plants served as

control. Disease record taken periodically showed that inoculated plants were dead within 15-20 days. First symptoms were accompanied by a general yellow discolouration of the leaves, and the roots became blackened. Finally the plant collapsed and died. Control plants were still alive and healthy.

(4) Fusarium cheruleum (Lib.) Sacc.

Symptoms : The fungus had little effect. No visible symptoms despite the abundant mycelial growth which covered the plants. The few diseased plants had typical symptoms.

(5) Helminthosporium sativum Pamm, King & Bakkle.

Symptoms : Typical symptoms. The fungus was very virulent.

II. Fungi which cause mould disease:

(6) Aspergillus niger, Van Tiegh.

Symptoms : The fungus had a slight effect. It caused only local lesions at the place of infection. No general disease occurred and the plants remained healthy.

(7) Botrytis cinerea Pers.

Detailed study elsewhere.

(8) Botrytis allii Munn.

Symptoms : Symptoms were typical. The fungus was virulent.

(9) Trichothecium roseum Link ex Fri.

Symptoms : The fungus had little effect. The very few diseased plants were dead but no noticeable change in their green colour occurred. Some plants were locally diseased, and the other majority were covered with fungal mycelium but the plants remained healthy.

III. Fungi which cause damping-off:

(10) Corticium solani (Prill & Delacr) Bourd & Gals.

Symptoms : The fungus had little effect. Very few plants were generally attacked, some others were locally diseased. The majority of the plants remained healthy despite that they became covered with mycelium.

(11) Pythium de-baryanum Hesse.

Symptoms : The fungus was extremely virulent. No plant which was infected showed any sign of resistance nor was its infection even localised. Complete disorganisation occurred

to the plants and they became extremely mushy. No brown colour developed and the plants turned only pale green in colour.

(12) Cephalothecium roseum Corda Anleit.

Symptoms : This fungus was extremely virulent. Total disorganisation occurred to all plants. The cells shrank and the plants became mushy. The plants were mostly covered with abundant growth of mycelium and they turned yellow green in colour. No local lesions.

IV. Fungi which cause wilt disease:

(13) Fusarium vasinfectum Atk.

Symptoms : The fungus had little effect. The plants remained healthy although they were completely covered with the fungal mycelium. Few plants only were generally or locally diseased.

(14) Verticillium Dahliae Kleb.

Symptoms : No effect.

Test of the virulence of the fungus against cress plants.

The virulence of the fungus against the cress plants was tested in the same manner as described above (page 50).

The fungus was found virulent. Diseased leaves curled up at the margins and their colour became yellow green. Plants soon collapsed and within three weeks the majority died.

(15) Verticillium albo-atrum Reinke & Berth

Symptoms : Very little effect. No general infection but few plants were locally diseased.

V. Miscellaneous species:

(16) Sclerotinia sclerotiorum (Lib.) De Bary

Symptoms : Typical symptoms. The fungus was extremely virulent.

(17) Phoma betae Frank

Symptoms : No effect.

(18) Tilachlidium nigrum Hutch.

Symptoms : Typical symptoms. The fungus was virulent. Wide patches of brown cells developed round the place of infection.

(19) Rhizoctonia solani Kühn.

Symptoms : Typical symptoms. The fungus was very virulent.

(20) Alternaria brassicae (Berk.) Sacc.

Symptoms : No effect.

Discussion.

The prothalli are found to be potential suscepts to a wide range of rot, mould and damping-off fungi. The majority of these pathogens incite similar extensive symptoms; the difference between them being in rate and degree of development of the disease. Invasion is associated in most cases with disorganisation and the development of brown colouration of the cells. Actually most of these diseases are soil inhabitant and are likely to cause disease in nature to the prothalli, although none of them were found attacking a prothallus in nature. The only two fungi which have been isolated from apparently diseased prothalli are (a) Coniothyrium sp., and (b) Tilachlidium nigrum. Hutch.

The most effective pathogens among the tested parasites are Pythium debaryanum Hesse, Cephalothecium roseum Corda Anliet, Botrytis cinerea Pers., Helminthosporium sativum Pamm, King & Bakke, Botrytis allii Munn and Sclerotinia sclerotiorum (Lib.) De Bary. Rhizoctonia solani Kühn, Tilachlidium nigrum, Hutch., and Phytophthora parasitica Dast. are also active on prothalli but less virulent than the above mentioned pathogens.

The remaining list of fungi in general are considered to be unable to attack the prothalli. It is shown by the virulence tests that some of these fungi are active and capable of causing disease to their normal hosts. Therefore lack of aggressiveness in the case of the prothalli cannot be due to the fungus itself. Factors responsible for that were not examined but in the interim it may be suggested that the failure to cause disease can be due either to the secretion of toxins not active on prothalli or to a state of resistance incited in the plants or to a combination of both factors.

Part III.

Study of the pathology of the disease produced by Botrytis cinerea on prothalli.

(1) Study on the effect of variation in temperature and medium on the growth and sporulation of Botrytis cinerea Pers.

Department cultures are normally grown at 24°C., which is a convenient temperature for abundant growth for most fungi. The cultures of Botrytis cinerea Pers. with which this work was carried out grew erratically and did not sporulate at this temperature. It was apparent from preliminary experiments that the cultures on media kept at room temperature produced abundant spores. It was not clear whether this was due entirely to the more abundant growth which developed in these conditions or to a specific effect of temperature on sporulation. A preliminary investigation on the effect of temperature and media on sporulation and growth was therefore carried out to determine the most convenient conditions which would support the regular production of the large number of spores required for inoculation.

Previous work

While there has been much work on the effect of variation of cultural conditions on both growth and sporulation in other fungi, previous work on Botrytis spp. has mostly recorded effects on growth only. Brooks and

Cooley (1917) noted that Botrytis cinerea Pers., which was grown on Dox's solution plus 2% dextrose, had an optimum temperature of growth of about 25°C. The amount of growth fell rapidly as the temperature was raised above the optimum and only slight growth occurred at 30°C. Walker (1926) found that Botrytis neck rots of onions, Botrytis allii Munn., Botrytis byssoidea Walker and Botrytis squamosa Walker grew on potato-dextrose agar over a range of 3°C. to 33°C. with most profuse development from 27°C. but most rapidly from about 19°C. to 27°C. He stated that Botrytis allii Munn. and Botrytis byssoidea Walker sporulated at temperature from 4°C. to 25°C. or higher, but Botrytis squamosa Walker seldom produced spores at temperatures above 20°C. Paul (1929) stated that the optimum temperature for growth appeared to be very similar for three strains of Botrytis cinerea Pers. grown on potato extract agar. It fluctuated between 21°C. and 25°C. and was probably nearer the latter temperature. He concluded that the growth rate fell off very rapidly even a few degrees above the optimum temperature. Newton and Hastings (1931) in their experiments on the production of conidia by Botrytis tulipea (Lib.) Lind. found that good crops of mycelia were produced but no conidia on barley meal, corn meal and on nitrate synthetic agar, while on potato disc

abundant crops of both mycelia and conidia were produced. They stated that, in general, media unfavourable for the growth of mycelia induced conidia production. They also found that low temperature favoured the production of conidia and above 25°C. spores were rarely produced. Yu (1938) stated that the max., opt. and min. temperatures for the growth of Botrytis cinerea Pers. on potato-dextrose agar were about 30-32°C., 20-25°C., and 4°C. respectively. He concluded that the opt. temperature for spore production was 21-23°C. and added that Czapek's was the best medium both for conidium and sclerotium production. Wade (1945-1947) observed that when a Botrytis sp. of Corn rot was grown on Gladiolus dextrose agar, in the absence of light in an incubator at 25°C., no conidia were produced. When grown on this medium in the presence of light on the leboratory bench some conidia were produced but not more than on potato-dextrose agar under the same conditions. He also stated that factors which were not favourable to continued vegetative growth, were favourable to sporulation.

Experimental

A series of experiments were carried out in which four kinds of media, (Czapek agar, Malt agar, potato-dextrose agar and potato agar), were used. Groups of sterile petri dishes,

each containing 20 ml. of medium, were set up on each of the media, and inoculated with an aliquots of a spore suspension in distilled water. The inoculum was spread evenly over the surface by use of sterile glass rods and the plates transferred to the required conditions. All dishes were kept in the dark except for the short period required for experimental observations.

Experiment I

The effect of variations in temperature on the growth and sporulation was first examined by holding four cultures of each medium at R.T. (Room Temperature) (16°C. - 21°C.) and four at 24°C.

Growth and sporulation of all dishes were recorded after 5 and 10 days from the time of inoculation. Growth was recorded empirically by observations against the following scale:

+	poor growth	+ +	moderate growth
+ + +	good growth	+ + + +	abundant growth

To examine the amount of sporulation, three discs of equal diameter were cut from each culture. The discs were then shaken in 5 ml. distilled water and were examined to see that the spores had been dislodged and thoroughly distributed through the solution. The number of spores was

then counted by using a hemmocytometer. Results are recorded in Table XV.

Table XV.

The effect of variations in temperature on growth and sporulation of B. cinerea growing on 4 different media.

Kind of medium	Days of incubation	24 °C Growth	24 °C No. of spores in 1.0 c.c.	R.T. Growth	R.T. No. of spores in 1.0 c.c.
Czapek agar	5	+	0	++++	6 x 10^3
	10	++	0	++++	76 x 10^3
Malt agar	5	+	0	++++	0
	10	++	0	++++	54 x 10^3
Potato-dextrose agar	5	+	0	++++	54 x 10^3
	10	+	0	++++	82 x 10^3
Potato agar	5	+	0	++	63 x 10^3
	10	+	0	+++	204 x 10^3

Experiment II

It was realised that the difference in number of spores produced in Experiment I might have been associated with either the difference in total growth or to a specific effect of variation in temperature on sporulation. A second experiment was therefore carried out to study the effect of the different temperatures on sporulation of cultures which

had already grown for 5 days at 24°C. Twelve petri dishes of each of the four media were placed in the incubator at 24°C. for 5 days. In these conditions the cultures had all grown to a similar extent but no sporulation occurred. Four of these petri dishes of each medium were then transferred to 5°C. and R.T. respectively. The other 4 petri dishes were left at 24°C. Results are shown in Table XVI.

Table XVI.

The effect of transferring cultures of B. cinerea, which were grown at 24°C. for 5 days, to 5°C., R.T. and 24°C.

Kind of medium	Days of incubation	5 days at 24°C. + 5 days at 5°C.		5 days at 24°C. + 5 days at R.T.		10 days at 24°C.	
		Growth	No. of spores in 1.0cc.	Growth	No. of spores in 1.0cc.	Growth	No. of spores in 1.0cc.
Czapek agar	5	++	0	++	0	++	0
	10	++++	63 x 10^3	++++	65 x 10^3	++	0
Malt agar	5	++	0	++	0	++	0
	10	++	6 x 10^3	++++	42 x 10^3	++	0
Potato-dextrose agar	5	+	0	++	0	+	0
	10	+	7 x 10^3	++++	6 x 10^3	+	0
Potato agar	5	+	0	++	0	+	0
	10	+	19 x 10^3	+++	115 x 10^3	+	0

Experiment III

A parallel control experiment was also set up by incubating 12 plates for 5 days at R.T., then transferring 4 dishes to 5°C. and 4 to 24°C. and the other four were left in R.T. This was carried out on Czapek and Malt agar only. Results are shown in Table XVII.

Table XVII.

The effect of transferring cultures of B. cinerea, which were grown at R.T. for 5 days, to 5°C., R.T. and 24°C.

Kind of medium	Days of incubation	5 days at R.T. + 5 days at 5°C.		5 days at R.T. + 5 days at 24°C.		10 days at R.T.	
		Growth	No. of spores in 1.0cc.	Growth	No. of spores in 1.0cc.	Growth	No. of Spores in 1.0cc.
Czapek agar	5	++++	34×10^3	++++	26×10^3	++++	33×10^3
	10	++++	64×10^3	++++	44×10^3	++++	196×10^3
Malt agar	5	++++	17×10^3	++++	13×10^3	++++	18×10^3
	10	++++	26×10^3	++++	16×10^3	++++	38×10^3

The results were not examined statistically as empirical observations gave adequate information for the limited objects of the investigation. A summary of the results in Tables XV, XVI and XVII is given below.

I. On Czapek medium

(a) at 24°C. - Moderate growth, no sporulation.

(b) at R.T. - Abundant growth, moderate sporulation.

(c) at 5°C. - Abundant growth, poor sporulation.

II. On Malt agar

(a) at 24°C. - Moderate growth, no sporulation.

(b) at R.T. - Abundant growth, poor sporulation.

(c) at 5°C. - Moderate growth, very poor sporulation.

III. On Potato-dextrose agar

(a) at 24°C. - Poor growth, no sporulation.

(b) at R.T. - Abundant growth, poor to moderate sporulation.

(c) at 5°C. - Poor growth, very poor sporulation.

IV. On Potato agar

(a) at 24°C. - Poor growth, no sporulation.

(b) at R.T. - Moderate to good growth, abundant sporulation.

(c) at 5°C. - Poor growth, poor sporulation.

Discussion.

The effect of variations in temperature was similar in all media, sporulation being entirely suppressed and growth reduced at 24°C. This effect was shown both in cultures which had been grown continuously at 24°C. and in cultures which had been grown at R.T. then transferred to 24°C., though in the latter case the inhibition was not complete or immediate on transfer. The control experiment also showed that the effect was specifically an inhibition of sporulation as mycelium which had grown at 24°C. sporulated readily on transfer to R.T. The results also showed that sporulation is inhibited at low temperature, though to a less extent than at high temperature, and that this occurs irrespective of the amount of previous growth of the mycelium. It was noted throughout the experiments that sporulation occurred on the older mycelium, and that the increase in number of spores produced after transfer from high to low temperature was not due to spores being formed on the new mycelium which had developed after the transfer.

Variations in the media affected both types of growth and sporulation. The four media were chosen to include one mineral and three complex organic media, the potato agar and the potato-dextrose media having a high carbohydrate level,

the malt having a high nitrogen and vitamin level. Mycelial growth was similar in mineral, malt and potato-dextrose agar media and noticeably less in potato agar and the greatest number of spores was obtained in potato agar cultures at R.T.

The reason for these results has not been examined in detail as the object of the experiments was successfully attained by the establishment of the fact that a regular and abundant supply of spores could be obtained by growing the fungus on potato agar at Room Temperature. This medium was also convenient as it supported a culture with numerous spores and relatively few vegetative hyphae from which it is relatively easy to obtain a clear suspension of spores.

The results suggest, however, that a high level of sugar tends to increase vegetative growth and reduce sporulation. This can readily be examined by similar experiments using a standard formula with varying carbohydrate level etc.

(2) Detailed Study of the Pathology of the disease.

Previous work

The pathology of Botrytis cinerea Pers. has been extensively investigated to higher plants, though it has not been studied in the fern gametophyte. It was chosen, therefore, to be studied in detail.

The works of De Bary (1886), Kissling (1889) and Ward (1889) were the first to call attention to the mode of parasitism in fungi which had not been previously recognised. They found that in certain fungi penetration and colonization of the host was brought about by the secretion of a soluble substance by the mycelium which killed and disintegrated the host tissue at a considerable distance from the filaments, thus affording them practically saprophytic nourishment. This substance was thought by each of these investigators to be of the nature of "soluble ferment or enzyme" possessing the power of dissolving cellulose.

De Bary reported that boiling the extract of Sclerotinia libertiana stopped its activity. Behrens (1889) found that boiling the extract of Botrytis mycelium did not destroy its activity and he stated that the toxic principle of certain fruit-rotting fungi was a thermostable substance.

Busgen (1893) while discussing the importance of appressoria in bringing the fungus and host into close contact, assumed that the formation of these organs is to serve as an agent for the accumulation and penetration of toxic materials into the host plants. Smith (1902) in his study of the parasitism of Botrytis cinerea Pers. on lettuce plants found that the cells were killed by a thermostable toxic substance before penetration of the fungal hyphae. He stated that the attack of the fungus took place in two successive phases. The first phase was caused by a poison substance (oxalic acid) which killed the cells. The second was caused by a variety of enzymes which caused the breakdown of the cell walls.

The classical work of Brown (1915) on Botrytis cinerea Pers. demonstrated that initial penetration was entirely due to mechanical means. Brown stated that chemical action being excluded as a tool of penetration, penetration of the cuticle must take place in a purely mechanical way. He rejected the idea that maceration and killing were caused by two different substances. He criticised the methods employed by the previous workers to obtain a fungal extract, particularly in their methods of preparing fungal extract from old mycelium and from old organs which have been overrun by the fungus.

He said that extracts from old mycelium contained stale products while these from the invaded tissues contained toxic substances originating from both fungus and host. He stated that the active substance in the extract of Botrytis cinerea Pers. appeared to be the enzyme pectinase which produced a macerating action on the cell wall and was also responsible for the lethal action of the extract. He thought that the death of the cells was brought about either by direct action of the enzyme on the protoplasmic membrane, or indirectly as a result of the action upon the cell walls. He found that the macerating and lethal properties of the extract were completely deactivated if it was heated to 65°C. Blackman and Welsford (1916) concluded that penetration could occur without the development of appressorium and the piercing of the cuticle of the leaf of the Broad bean (Vicia faba) is due solely to the mechanical pressure exerted by the germ tube of Botrytis cinerea Pers. as a whole or by an apical outgrowth from it. They observed that the death of the epidermal cells in advance of the penetration of the cuticle was never found to occur. Dey (1919), in his work dealing with the infection of bean by Colletotrichum lindemuthianum, stated that the passage of the hypha through the cells after it entered through the cuticle was associated with a swelling and

dissolution of the cellulose layers. He added that the infection hypha, after growing a short distance into the host, produced at its end a small vesicle from which one or more branches emerged and spread through the host tissue. Hawkins and Harvey (1920) made observations on the growth of the fungus Pythium de-baryanum Hesse. within the cells of potato. They said that in passing through the cell wall between two cells, the hypha approached the cell wall nearly at a right angle, formed a swelling of the end, then bent slightly and penetrated the wall into the next cell, where the hypha expanded to its normal diameter. They stated that there was quite clear evidence of the mechanical pressure. They stated that the fungus secreted a toxin which killed the cells of the potato and it also secreted an enzyme which broke down the middle lamellae of the cell walls. Boyle (1921) described the early stages of infection of Bean leaves by Sclerotinia libertiana. He stated that a very narrow infection hypha arose from the tip of each hypha which was in contact with the surface of the plant. He stated that the cuticle may be markedly indented at the point of contact due to the pressure exerted by the infection hyphae. He added that after penetration disorganisation occurred to the tissue and extended in advance of the infection hypha.

Leach (1923) found that when the germ tube of Colletotrichum lindimuthianum became attached to the cuticle, a small infection thread was sent into the epidermal cell, apparently by mechanical pressure. He added that the penetration of the inner cell walls took place through a very small hole without any staining or swelling of the cell wall. He stated that the fungus penetrated through numerous cells without killing the host protoplast, and concluded that the bending and swelling of the hyphae during cell-wall penetration indicated that mechanical pressure is the main factor in the process. Young (1926) described the penetration of the germ tubes of Alternaria diplodia in wheat coleoptiles. He stated that a slender outgrowth (penetration hypha) appeared at the lower side of each appressorium which entered the epidermal cells. Young observed a characteristic appearance of stained rings or discs around points of incipient infection. He described these red rings and said that perhaps some chemicals diffused from the penetration hypha in the callosity into the cell wall and caused differential chemical alteration of the wall in different places. He concluded that there was insufficient microchemical evidence to identify the chemical nature of callosities or the nature of the alteration of the cell wall around infection points.

Higgins (1927) concluded that the killing of the host cells by Sclerotium rolfsii Sacc. was due to the toxic action of oxalic acid which was secreted by the fungal hyphae. Brown and Harvey (1927) pointed out that the only satisfactory theory of membrane penetration by fungi was a purely mechanical one. Pearson (1931) stated that the fungus Gibberiella saubinetii (Mont.) which caused a seedling blight of corn penetrated the internal cell walls by means of a fine filament. He considered that it could not be conclusively demonstrated that the process of penetration was either a mechanical or a chemical one; there was only slight evidence that a chemical action might take place at the point of penetration. He reported, however, that invasion was accompanied by an accumulation of dark staining material in the walls and intercellular spaces of the host tissues. The area in which this accumulation occurred extended considerably beyond the limits of the fungus penetration. Brown (1936) stated that while the initial penetration of host cuticle by Botrytis cinerea was by mechanical action, the fungus subsequently spread through the host by the action of some principle which killed the cells and macerated the cell walls in advance of the position of the hyphae. He concluded that some fungi could

grow through host tissues entirely by mechanical action, and that where microscopic study showed that "infection pegs" were present one is justified in assuming that mechanical penetration had occurred. In these cases the host cytoplasm might be killed by the mere presence of the parasite hypha within the cell. On the other hand where pronounced rotting was present chemical action by the fungus was probably predominant. Ainsworth, Oyler and Read (1938) described a characteristic ring like spots caused by Botrytis cinerea Pers. on tomato fruits. They stated that evidence was presented to show that, under conditions of high humidity, Botrytis spores lying on the surface of immature fruits germinated, penetrated the epidermis, and the spots resulted from the pectinase enzyme secreted by the germ tubes. They observed that when drops of the enzyme were placed on the surface of the fruit, without injury to the underlying epidermis, they caused no damage even when they persisted for more than 24 hours, but when a puncture was made through a drop of active enzyme lying on the surface of the fruit, the surrounding tissue turned brown within 12 hours. Nelson (1951) studied the effect of Botrytis cinerea Pers. on the tissue of infected Tokey and Emperor grapes. He observed that infection through the cuticle took place by means of a

small infection peg produced by an appressorium in contact with the surface of the grape. The host cells were separated to a depth of 6-10 layers by the enzymatic action of subcuticular mycelium. Infected materials stained with ruthenium red showed that the middle lamella had disappeared in the area of cell maceration. Priston and Gallegly (1954) made observations on leaf penetration of susceptible Cobbler potato by potato race A of Phytophthora infestans (Mont.) de Bary. They reported the formation of appressoria and that penetration was accompanied by mechanical pressure exerted by infection pegs which indented the cell wall at the beginning of penetration. They stated that it is possible that mechanical pressure was responsible for penetration of the cuticle and that enzymic action was responsible for penetration of the remainder of the epidermal cell wall, either alone or in combination with mechanical pressure. Wood (1955) defined "protopectinase" as the term applied to the enzyme or group of enzymes which by their action bring into solution the protopectin of the cell wall and produce maceration or the separation of cells from each other. He stated that so far the enzyme has been demonstrated only by its action on plant tissues and the types of chemical reactions involved must remain hypothetical until more is known of the nature of protopectin itself.

The problem was studied in this case in two ways:

(a) a detailed morphological and histological study was carried out, taking full advantage of the suitability of the plant for the study of disease developed in vivo.

(b) a physiological study to determine the mechanism of attack was carried out, in particular to see whether Brown's findings regarding the mechanism of attack of higher plants also applied to this one.

(2a) Morphological and histological study.

Experimental.

Gametophytes were grown on Knop's agar medium in petri dishes under 15 hours daily illumination. After 8-10 weeks growth, the plants were transplanted on the same media, six plants in each petri dish, and then inoculated with an aqueous spore suspension of the fungus prepared by flooding a test tube culture (10-15 days old) with about 10 ml. of sterile distilled water. The general development of the disease was studied by direct observations of living material for which the structure of the gametophyte was particularly suitable.

Detailed study of the method of penetration and development of the fungus within the cells was made by killing

and fixing suitable material in weak chrom-acetic (Johanson 1940), staining with Harris's hematoxylin and counterstaining with erythrosin. Whole mount materials were used and sections were also made by embedding in paraffin wax and cutting at 12μ.

The growth of the mycelium within the cells was observed in living plants. A plant was transplanted on a disc (2 c.m. in diameter) of Knop's agar medium, in a petri dish and inoculated with a drop of an aqueous spore suspension. Three days later, the plant was covered with a sterile cover slip, and a hypha was traced, using the microscope, as it grew within the cells. Records were taken of the time required to penetrate the cell walls and rate of growth of the hypha inside the cells.

I. Prepenetration phase

The majority of spores germinated within 24 hours. From some the germ tubes grew on the surface of the host until they reached the length of about 5 to 10 times the spore diameter (Fig.VI). Other spores grew for a short distance only before penetrating the plant surface (Fig.VII). The width of the germ tubes were equivalent to or slightly less than the spore diameter. Before penetration occurred, the tip of the germ tube formed a typical swollen appressorium (Figs.VI and VII).

Fig. VI. Camera lucida drawing showing germ tubes which reached the length of 5-10 times the spore diameter; the formation of appressorium and the entrance of the hyphae which enlarged to its normal diameter and resumed its growth.

Fig. VII. Camera lucida drawing of a germ tube growing for a short distance before penetration.

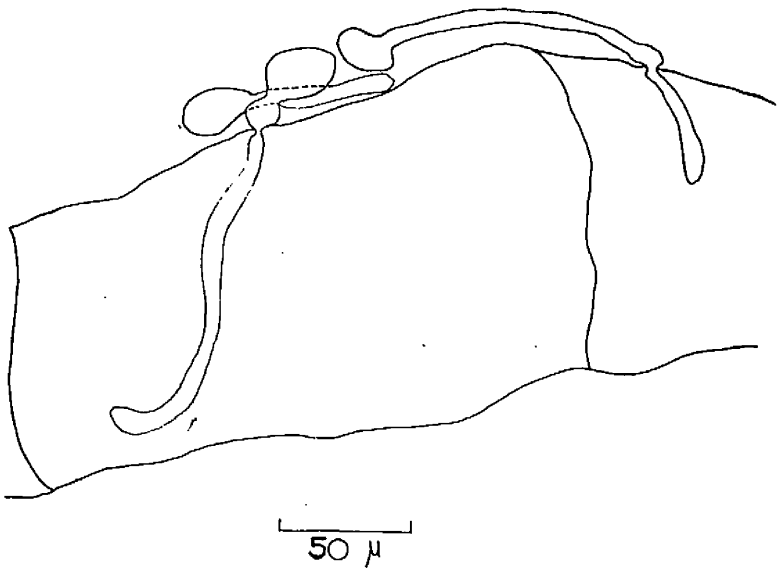


Fig. VI

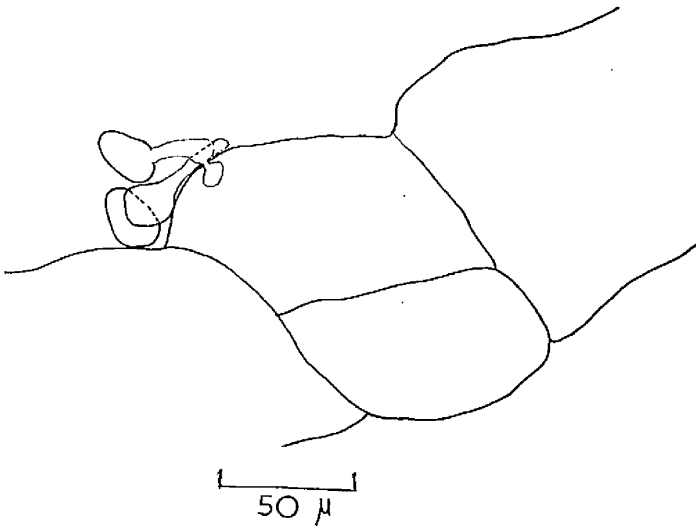


Fig. VII.

Within the limits of the infection drop, where most of the spores germinated, the underlying cells were disorganised (Figs.VIII and IX). This occurred frequently in the first two days after inoculation, before the germ tubes penetrated the cells.

II. Penetration phase and the accompanied symptoms.

The cell wall of the host became brown in the area around any appressorium (Fig.X). The area extended to about twice the width of the appressorium. A typical infection peg was formed which penetrated through the brown wall into the interior of the cell. This appeared as a bright spot in the centre of each area in surface view (Fig.XI). Subsequently varying amounts of brown colouration developed in the host. In some cases, this was limited to an area immediately adjacent to some or all the superficial hyphae of the parasite, particularly around the hyphae running in the depressions in the host surface at the lines of cell junction. In other cases, the colour developed generally in all cells in contact with the fungus, and in some adjacent cells.

In some cases, plants were not attacked by the fungus despite the presence of germinated spores in the infection drop on the plant surface. In these plants many cells in

Figs. VIII & X. Microphotograph showing an infection drop where most of the spores germinated (Fig.III); the underlying cells were apparently disorganised (Fig. IV). Approx. x 360.

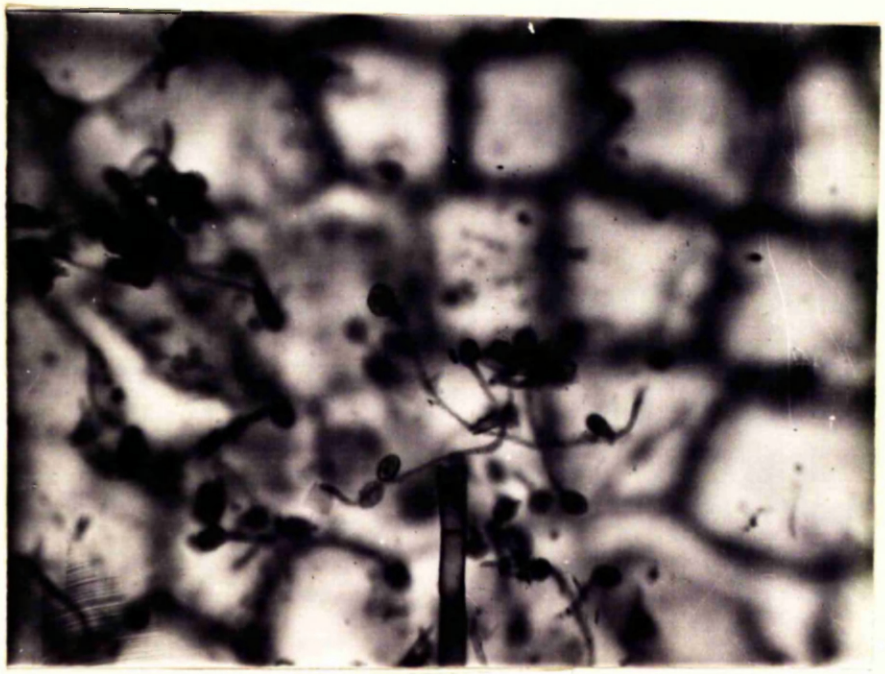


Fig. VIII

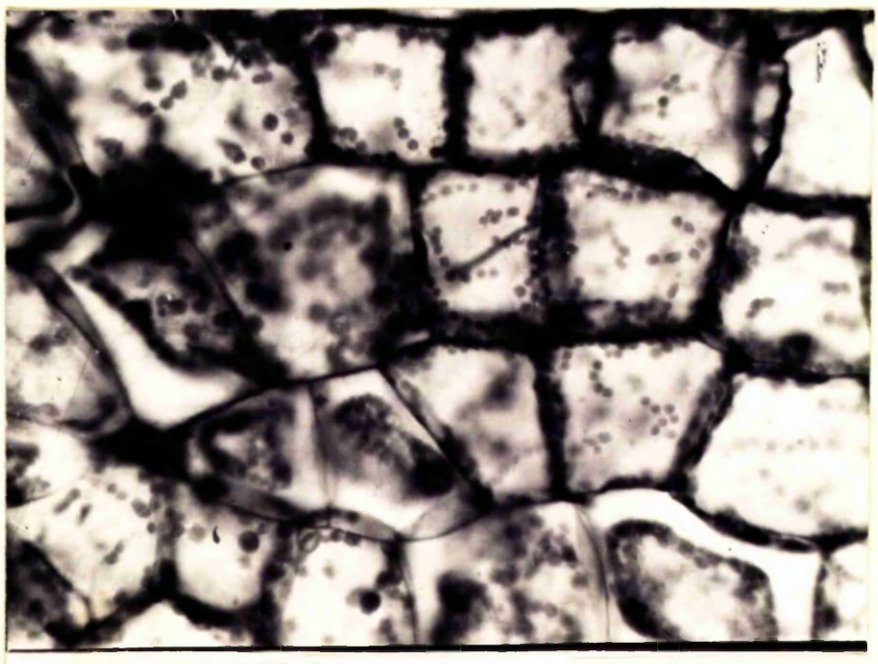


Fig. IX

Fig. X. Camera lucida drawing shows a cell wall of the host which became brown in colour in the area around the appressorium; a typical infection peg was formed which was just passing through the brown wall. (The germ tube was detached from the cell surface during staining and embedding process.)

Fig. XI. Microphotograph showing many brown areas which developed around the appressoria with a bright spot in the centre of each area in surface view. Approx. x 620.

Fig. XII. Microphotograph showing a plant which was not attacked by the fungus and many cell walls in the region of the infection drop turned brown but the fungus did not penetrate the host. Approx. x 30.

Fig. XIII. Microphotograph showing the edge of a region of cells occupied by the fungus. Disorganisation of these cells was shown by disturbance in the regular arrangement of the chloroplasts and by the reduction in their number. Neighbouring cells were partly disorganised. Approx. x 160.

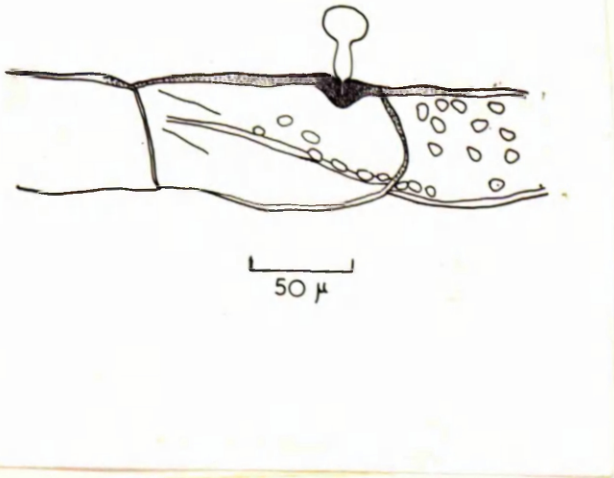


Fig. X

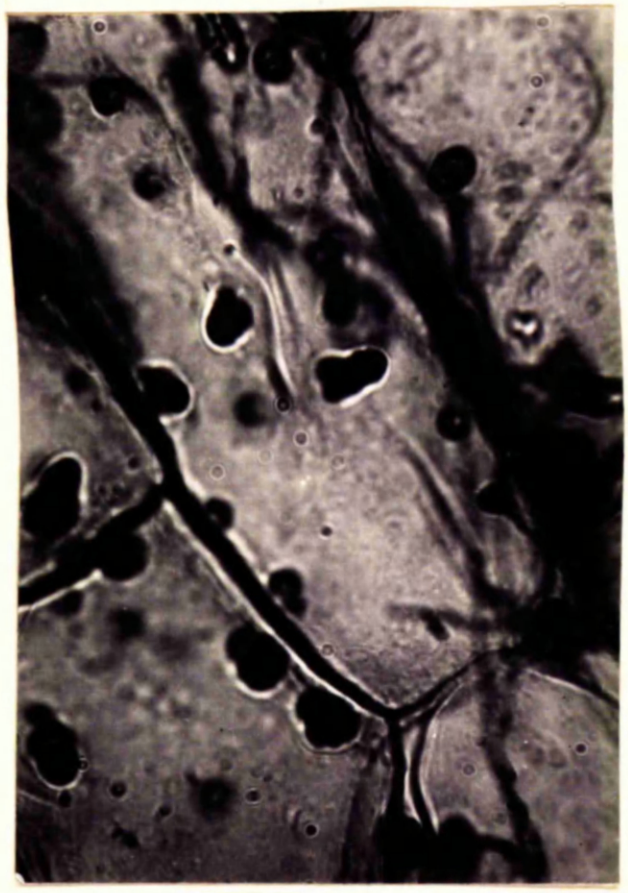


Fig. XI

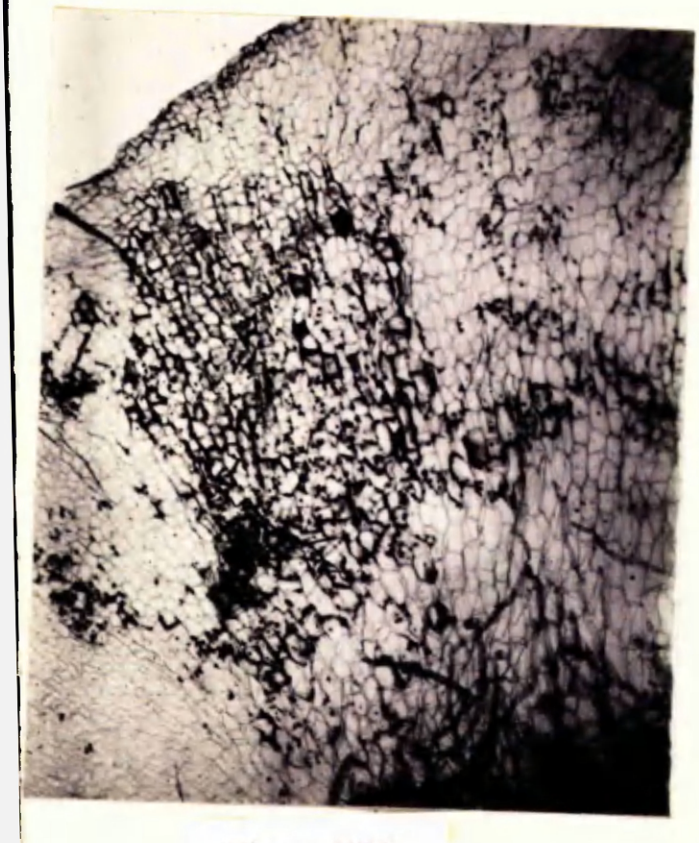


Fig. XII

Fig. XIII

the region of the infection drop turned brown, but the fungus did not penetrate the host. (Fig.XII).

III. Postpenetration phase, (the colonization and the reaction induced in the host)

After penetration and the entrance of the parasite into the cell, the hypha resumed its normal diameter (Fig.VI and VII), and the site of penetration seemed to extend slightly by the further growth in diameter of the infection peg.

When the fungus established itself within the cells, it started growing in different directions causing disorganisation of the cells and the disintegration of the cell walls. Disorganisation was first shown by a disturbance in the regular arrangement of the chloroplasts, and by reduction in their number in a cell (Fig. XIII). At a late stage in the disease, the plant cells died and the walls lost their coherence, so that the plant collapsed and fell apart when lifted on the point of a needle. It was observed, in general, that after the fungus entered the plant cells and grew successfully beyond the primary infected area, no brown discolouration developed in the newly attacked cells. The chloroplasts in these cells turned pale green, and the cell eventually became colourless and died.

In other cases, infection took place, but after the

fungus developed through a few cells it came to a standstill and seemed to be unable to continue to penetrate living cells. In these cases the infected area was mostly surrounded by dark brown cell walls which had formed ahead of the growing fungus (Fig.XIV). The rate of disorganisation of adjacent cells often varied considerably, and in some cases groups of cells remained green and apparently unaffected for several days although entirely surrounded by dead brown cells.

The hyphae normally grew within the cells in several directions and often branched (Fig.XV) and at a late stage the old hyphae (4-5 days old) usually expanded to about twice the diameter of the primary infection hyphae illustrated in Fig.VI.

Detailed observations were made of the growth of the hyphae in the living plant cells. A typical record of a hypha in the cavity of a cell, three days after infection, is as follows:

The hypha reached the cell wall at "a" (Fig.XVI) and before penetration occurred the tip of the hypha became rounded and somewhat swollen within 4-5 minutes. The place where the hyphal tip was resting indented slightly into the neighbour cell B. Penetration at "a" was complete in 14

Fig. XIV. Microphotograph shows a local lesion which was surrounded by dark brown cell walls formed ahead of the growing fungus. Approx. x 180.

Fig. XV. Camera lucida drawing shows hyphae which normally grew within the cells in several directions and often branched. These old hyphae expand to about twice the diameter of the primary infection hyphae illustrated in Fig.VI.

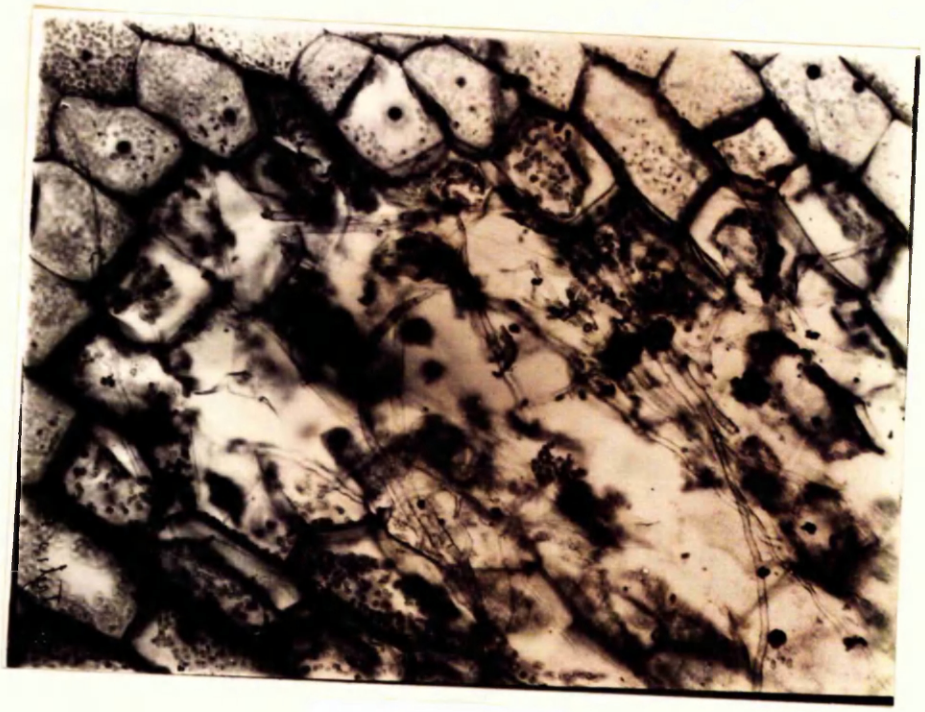


Fig. XIV.

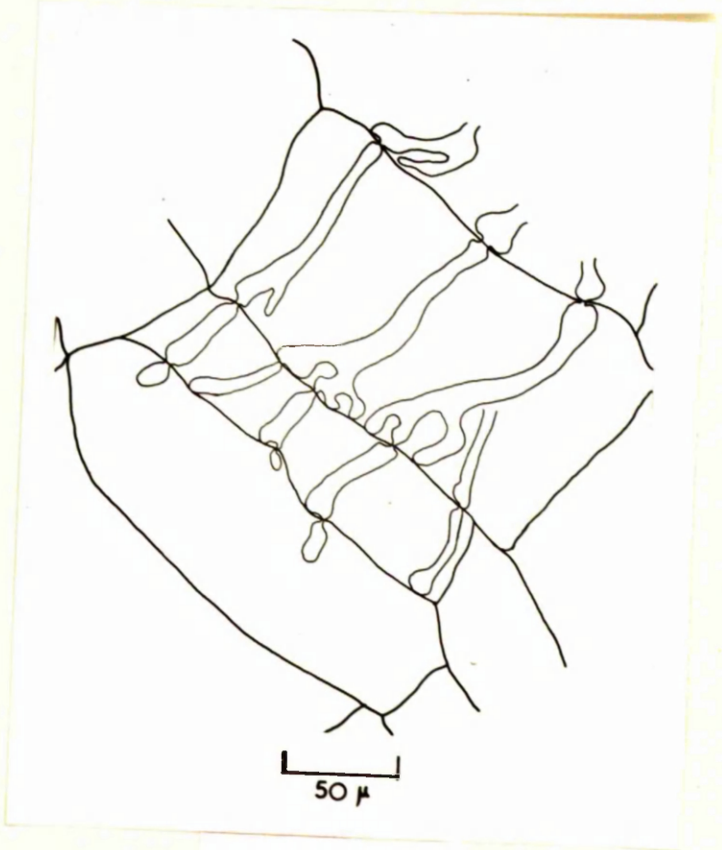


Fig. XV

minutes and a very fine globular growth could be seen emerging from the other side of the cell wall in cell B. As soon as penetration occurred, most of the swelling disappeared and the growing hypha reached "b" after 50 minutes. Penetration at "b" was complete in 13 minutes, with the same sequence of events as occurred at point "a". Forty-three minutes later "c" was reached. In the meantime the lateral end of the hypha bent slowly towards the cell wall and another branch started to grow. Penetration at "c" was complete in 14 minutes, and 47 minutes later "d" was reached. The hypha then grew along the side of the cell wall to reach "e" after 13 minutes. The tip of the hypha, at that point became rounded, but later most of the swelling disappeared soon after penetration, which occurred in 12 minutes. In the meantime the lateral end of the hypha between "d" and "e" formed a curve downwards in cell D.

The growth of the hypha within the cell was accompanied by the disappearance of most of the plastids around the advanced hyphal tip.

In another case the hypha reached a brown cell wall (Fig.XVII), and after 5 minutes at a standstill, it turned to grow along the side of the cell wall. Although the hypha did not penetrate the intercellular walls, the contents of the neighbouring cell showed typical early disease symptoms

Fig. XVI. Camera lucida drawing shows a hypha within the cavity of cells three days after inoculation.

Fig. XVII. Microphotograph shows a hypha which reached a brown cell wall then turned after 5 minutes at standstill to grow along the side of the cell wall. The neighbouring cell was disorganised. The hypha continued its growth, after resting at the corner for more than ten minutes, along the neighbouring cell wall. Approx. x 470.

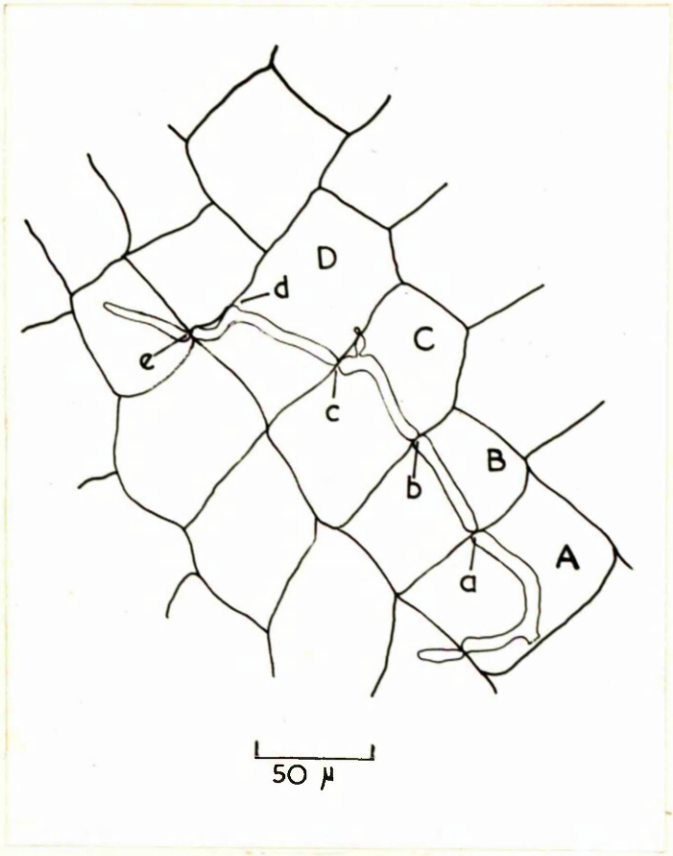


Fig. XVI

Fig. XVII

of disorganisation of the chloroplasts. The hypha, after resting at the corner for more than ten minutes, continued its growth along the neighbouring cell wall. Many similar observations were made of other hyphae, and it was noted that the hyphal tips were very frequently checked by brown cell walls although they could readily penetrate normal walls.

Discussion.

These observations show that initial penetration is associated with the formation of an appressorium and an infection peg such as has commonly been reported in the mechanical penetration of other plants. The swelling of the hyphal tip, on coming into contact with the intercellular walls and the invagination of these walls before penetration occurs also suggest that mechanical pressure is exerted in this stage. This has been supported by observations on living materials, that when penetration occurs of an intercellular wall, a very small growth of hypha can be seen emerging from the side of the cell wall. The small growth is apparently the apical part of the very fine tube which has already passed through the wall.

The mechanical damage and irritation associated with penetration might account for some of the disorganisation of the protoplast in the invaded cell, but it is noted that in many cases disorganisation is apparent before the fungus has penetrated the cell. Experiments to determine whether this is due to a secondary effect of injury to neighbouring cells or to the action of extra cellular metabolites of the fungus are reported below.

The enlargement in diameter of the hyphae which develops 3-4 days after penetration is similar to that reported by Leach (1923). The cause of the enlargement has not been examined, but it may be due partly to retardation of the apical growth of each hypha caused by the resistance of the intercellular cell walls to penetration process or partly to the presence of abundant food supply with the cells.

It is apparent that, while there is no hyperplastic resistance reaction by the host, it does possess or acquire some resistant properties during the development of the disease. The brown colouration seems to be associated with this reaction and presence of groups of apparently unattacked cells surrounded by dead ones may be due to an acquired resistance or to exeny. More detailed studies of this problem are also reported below.

(2b) Physiological study of the mechanisms of attack.

Experimental.

Preparation of fungal filtrate

An active preparation of protopectinase was prepared by Brown's method (1915) as modified by Wood (personal communication 1953). A spore suspension was prepared by scraping the spores from the surface of 10 days old potato agar petri-dish cultures into 10 ml. of sterile Wood's solution.(1) The suspension thus obtained was filtered through sterile muslin and 5.0 ml. of this suspension was used to inoculate each of a series of 35 ml. aliquots of the same solution contained in 250 ml. flasks. After 5 days incubation at 20°C. in the dark the liquid media were removed by filtration through muslin and centrifuged out for 5 minutes to eliminate the remaining hyphae and spores.

Estimation of pro-pectinase activity

Filtrate prepared as mentioned above was used at once and its activity was tested in each experiment by Brown's method (1915). This involved the preparation of transverence

(1) Wood's solution : Glucose 20 g., Asperagine 2.5 g., Potassium phosphate 1.0 g., Magnesium sulphate 0.3 g., and one litre of distilled water.

sections of 500 μ thickness, taken from a cylinder 1.5 cm. in diameter of potato tuber. These sections were injected under the vacuum with water to render them more turgid and to remove intercellular air. After thorough washing in distilled water, some were placed in the filtrate and others in unfermented Wood's solution to serve as control. Activity was estimated as the inverse of the time taken for the tissue to lose coherence. This was judged to take place when the discs as tested by hand offered no perceptible resistance to a pulling stress. Each record is the average reading from a test of 4 discs.

Study of the Effect of fungal filtrate on prothalli

Mature prothalli of standard age and size were injected under the vacuum with water to remove intercellular air and to ensure that all cells were equally turgid. It was found that intercellular air caused plants to float at the surface of the test solutions unless this was done. They were then drained over a clean filter paper, placed in the filtrate and periodically examined under the microscope. The degree of maceration of the cell walls was determined by lifting the plants on the point of a needle. Macerated plants were observed to collapse over the needle while those unaffected

remained stiff. Maceration was also recorded by pulling apart the plant when macerated plants cleaved along the lines of the middle lamellae (Fig.XXII), while non macerated plants broke irregularly across the cell walls (Fig.XXIV).

The amount of disorganisation of the cells was estimated by empirical observation. The observation of the death of the cells was confirmed by transferring plants to 0.75 M. Sucrose which was hypertonic to normal living cells and in which dead cells could then be identified by their failure to plasmolyse.

Number of dead cells was recorded as an approximate percentage of the total cells of the prothallus as estimated by eye. In all cases each record is the average of the readings from four prothalli.

Effect of active fungal filtrate

Potato discs in 5 replicate experiments were macerated in between 20 and 40 minutes. Control discs in unfermented Wood's solution were apparently unaffected.

Prothalli in 5 replicate experiments were affected within 10-30 minutes of immersion in the active filtrate. The first sign of activity was in groups of cells in each plant which became disorganised, the chloroplast becoming scattered in each cell. The peripheral regions of many cells became

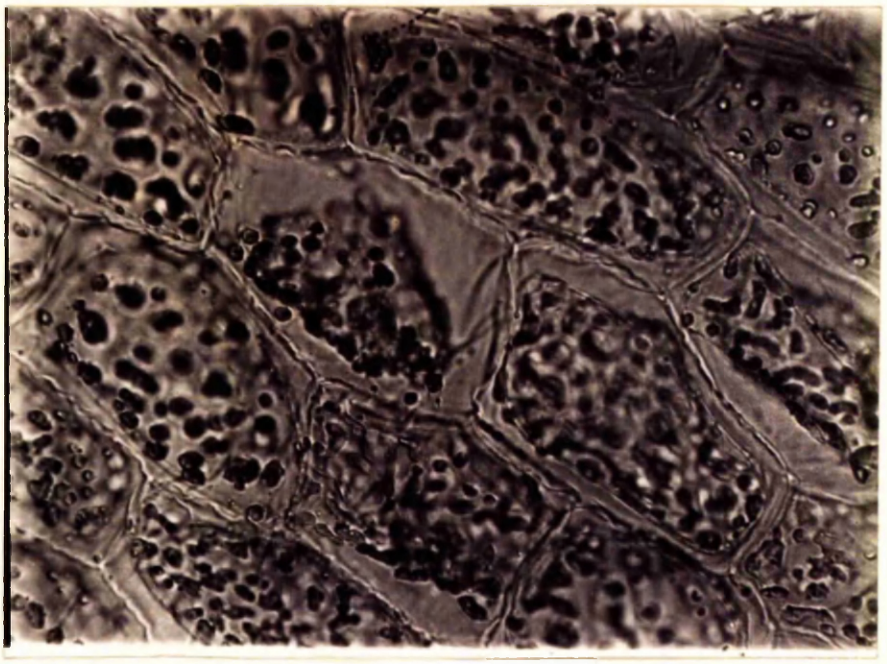


Fig. XVIII

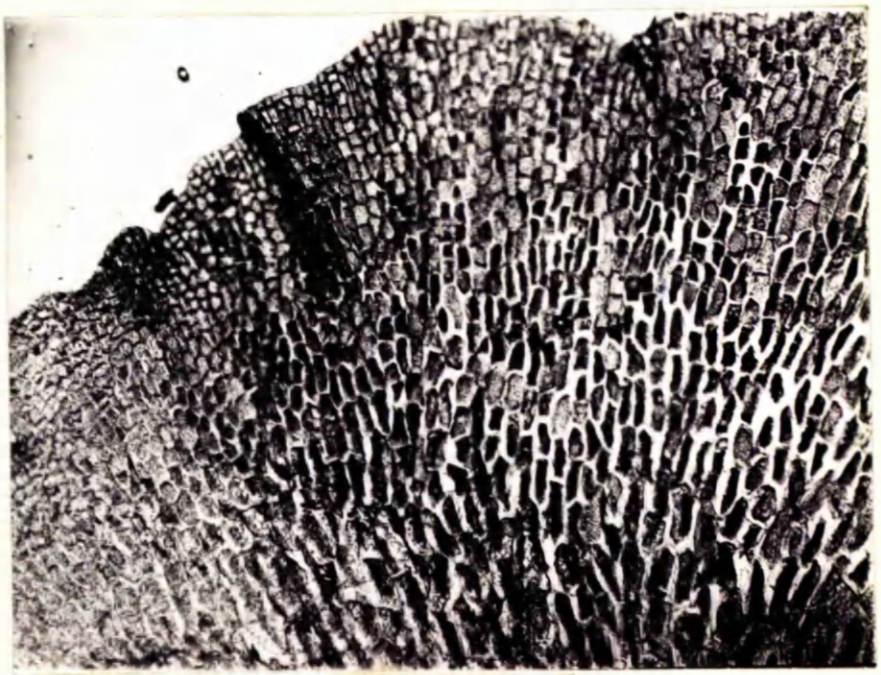


Fig. XIX

colourless and the cell walls became less visible eventually appearing as a contorted thin line (Fig.XVIII). After 3 hours the number of disorganised cells increased and the cell walls became more distorted. Complete loss of coherence of the plant cells eventually occurred, and within four to six hours from the beginning of each experiment, the plant was completely affected (Fig.XIX). In this stage they could hardly be handled without collapsing.

The plants remained green for a considerable time but later they faded to a yellowish green. No brown discolouration developed. Control prothalli in unfermented Wood's solution were apparently unaffected.

Methods employed to study the nature of the active materials in fungal filtrate.

The object of this study was to determine the nature of the materials which were responsible for cell wall maceration and death of the protoplast and in particular to determine whether the activity of protopectinase was the only inciting factor in the development of the symptoms seen.

Brown (1915) found that the macerating and lethal properties of the filtrate were completely deactivated when the filtrate was heated at 65°C. Behrens (1889) found that boiling the extract of Botrytis mycelium did not destroy its injurious effect upon plants. Fungal filtrates were

Fig. XVIII. Microphotograph showing the dissolution of the cell walls which became faintly visible and appeared as contorted thin line. Approx. x 320.

Fig. XIX. Microphotograph showing a plant completely affected by the active fungal filtrate within 6 hours. The cells were disorganised and the cell walls were macerated. Approx. x 30.

therefore prepared by Brown's method, and samples of equal quantities treated as follows :

I	Heated at	...	65°C. for 1 minute
II	" "	...	65°C. " 5 minutes
III	" "	...	98°C. " 1 minute
IV	" "	...	98°C. " 5 minutes
V	Autoclaved at 15 lbs. pressure for 10 minutes.		

In each experiment 5 test tubes, each containing 5.0 ml. of active fungal filtrate, were heated in a water bath as above. To avoid loss of volume by heating each test tube was tightly plugged with cotton wool through which a thermometer was placed to serve the double purpose of stirring the contents of the tube and recording the rise of temperature. The effect of each treated filtrate on potato discs and on prothalli was examined in the manner described above.

Table XVIII details the results of four replicate experiments. In each experiment the active fungal filtrate macerated potato discs in 20-40 minutes and induced total maceration and death of the prothalli within six hours. Heated fungal filtrate did not cause maceration of the potato discs or of the cell walls of the prothalli in any experiment.

Table XVIII.

The effect of the heated filtrates on prothalli.

Number of Experiment	The percentage of disorganised cells of prothalli						
	Active Filtrate	65°C. 1 min.	65°C. 5 min.	98°C. 1 min.	98°C. 5 min.	Auto-claved filtrate	Unfermented Wood's solution
I	90%	15%	5%	10%	2%	0	0
II	100%	20%	10%	10%	0	0	0
III	100%	25%	10%	10%	2%	0	0
IV	100%	25%	15%	15%	5%	0	0

The results showed that heated fungal filtrate still possessed some lethal activity though much less than that of the active filtrate, while the plants in this solution remained rigid and microscopic examination indicated that no dissolution of the cell walls occurred (Figs.XX and XXI). Figs. XXII, XXIII and XXIV represent three plants treated with active filtrate, heated filtrate at 65°C. for 1 min. and with unfermented Wood's solution respectively. Each plant was gently torn. Separation occurred in the first plant along the line of the cell walls. In the second and third plants separation caused a ragged tear across the cells showing that maceration did not occur to the cell walls.

Study of the effect of dilution of active filtrate

It was thought that disorganisation recorded above might be due to the presence of small residual amounts of unaffected

Fig. XX. Microphotograph showing a plant treated with fungal filtrate heated to 65°C. for one minute. The heated filtrate caused disorganisation to some cells. Approx. x 30.

Fig. XXI. Microphotograph showing a group of cells of the same plant (Fig. XX) which were disorganised by heated filtrate while cell walls are still rigid. Approx. x 30.

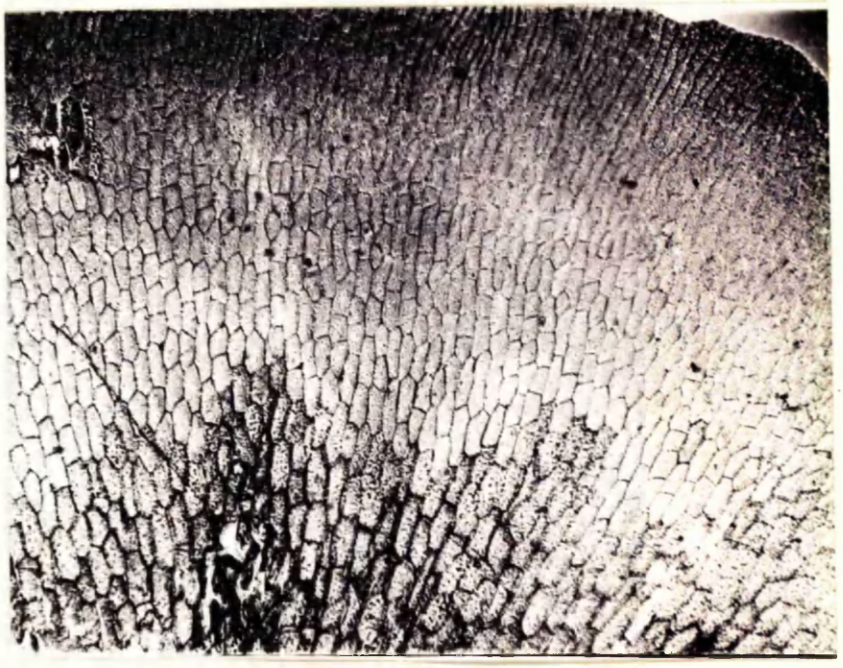


Fig. XX

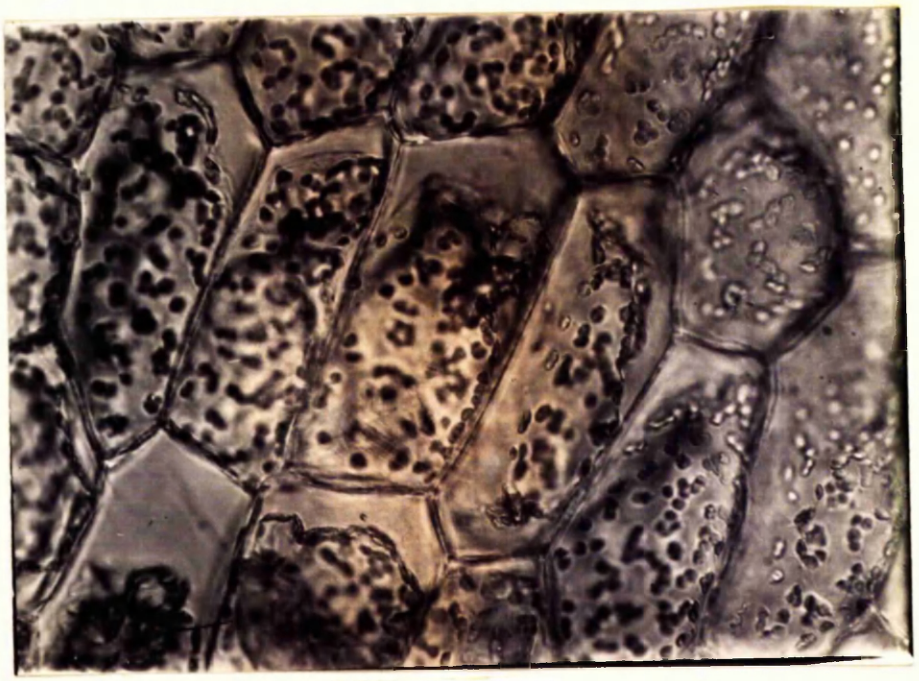


Fig. XXI

Fig. XXII. Microphotograph showing that separation occurred in plant treated with active filtrate along the line of the cell walls. Approx. x 60.

Fig. XXIII. Microphotograph showing that separation caused a ragged tear across the cells of plant treated with heated filtrate at 65°C. for one minute. Approx. x 60.

Fig. XXIV. Microphotograph showing that separation caused a ragged tear across the cells of plant treated with unfermented Wood's solution. Approx. x 60.

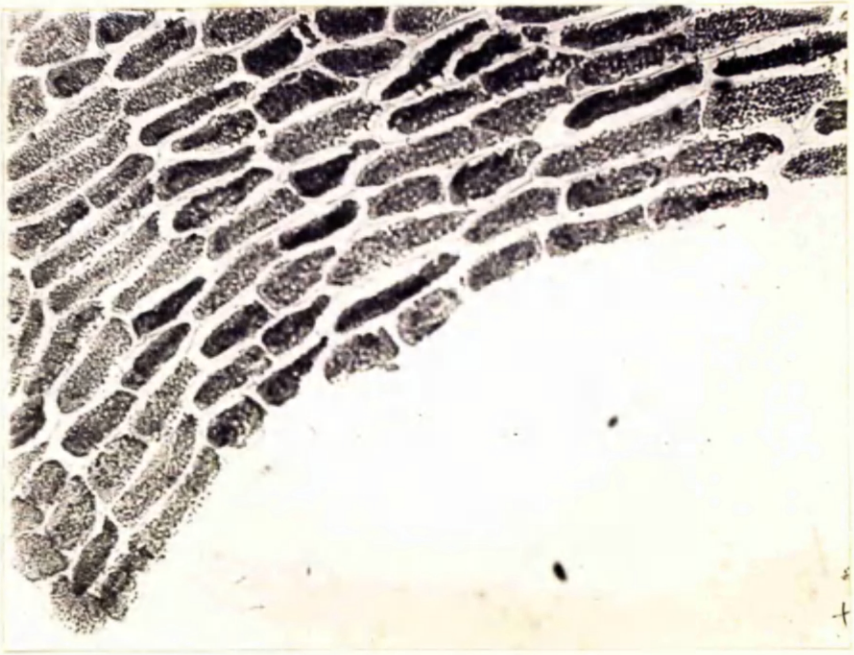


Fig. XXII

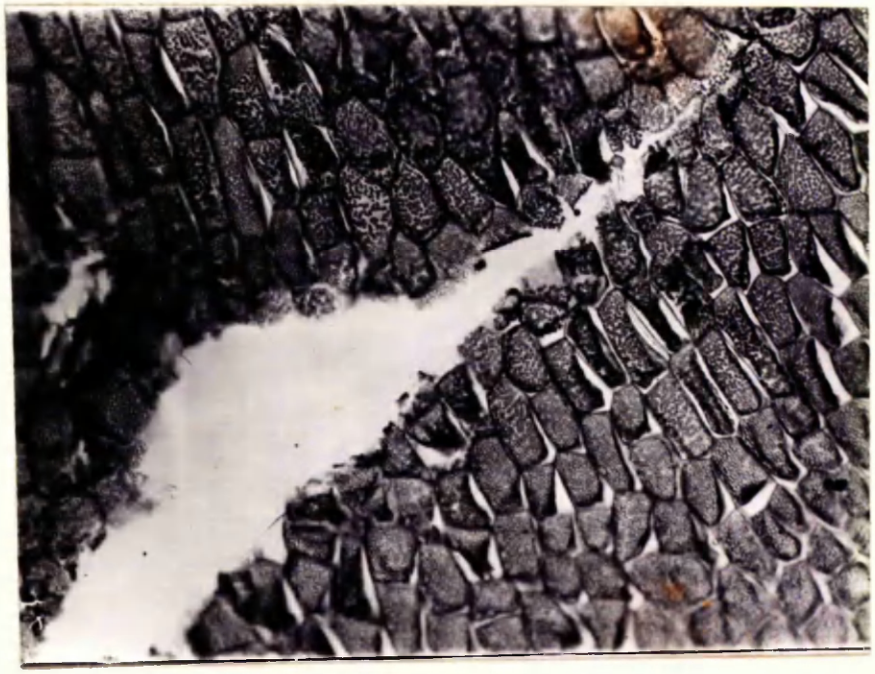


Fig. XXIII

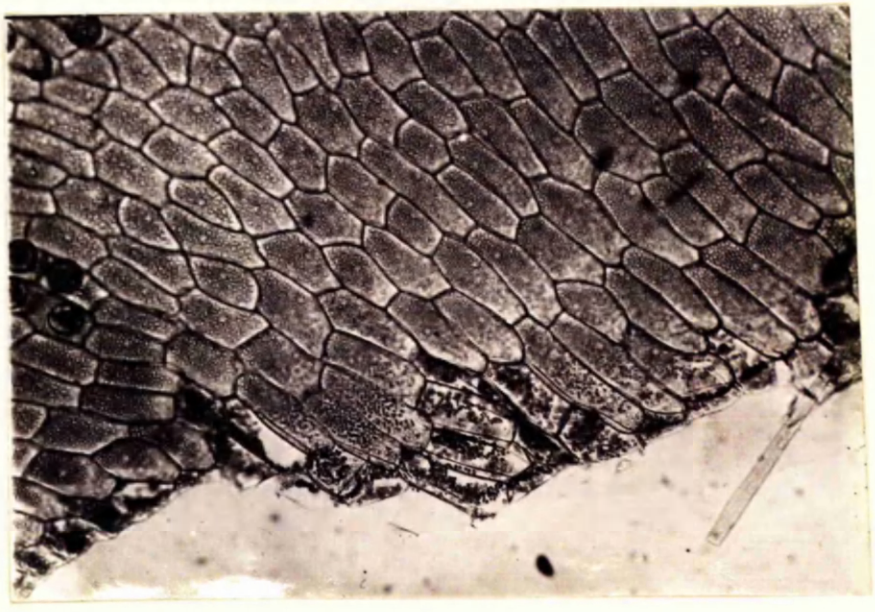


Fig. XXIV

enzymes in the heated filtrate. The effect of different dilutions of active filtrate was therefore examined. In a preliminary experiment the following wide range of dilutions were used:- 1 : 1, 1 : 3, 1 : 7, 1 : 20, 1 : 50 and 1 : 100. The test solutions were examined on potato discs and prothalli in the same manner as described above and the results are recorded in Table XIX.

Table XIX.

The effect of different dilutions on the activity of fungal filtrate.

Note : + maceration occurred to cell walls of the prothalli
- maceration did not occur

Dilutions	Active filtrate	1 : 1	1 : 3	1 : 7	1 : 20	1 : 50	1 : 100
Time required to macerate potato discs	0.3 hr.	1.5hr.	2.15hr.	2.5hr.	5.5hr.	8.5hr.	>12 hr.
Percentage of disorganised cells in prothalli	100	100	100	100	60	20	-
Maceration of cell walls of prothalli	+	+	+	+	+	-	-

Two further replicate experiments were set out using a narrower range as follows :- 1 : 10, 1 : 15, 1 : 20, 1 : 25, 1 : 30, 1 : 40, 1 : 50, 1 : 75 and 1 : 100. The results are recorded in Table XX.

Table XX.

The effect of different dilutions on the activity of the fungal filtrate.

Note : + maceration occurred to cell walls of the prothalli

\- maceration did not occur

No. of Experiment	Dilutions	Active filtrate	1 : 10	1 : 15	1 : 20	1 : 25	1 : 30	1 : 40	1 : 50	1 : 75	1 : 100
I	(a) Time required to macerate potato discs	25 min.	1.5hr.	2 hr.	2 hr.	2.5hr.	3 hr.	3.5hr.	5 hr.	7 hr.	>12 hr.
	(b) Percentage of disorganised cells in prothalli	100	100	100	80	60	60	40	20	0	0
	(c) Maceration of cell walls of prothalli	+	+	+	+	+	+	-	-	-	-
II	(a) Time required to macerate potato discs	40 min.	2.5hr.	2.5hr.	3 hr.	3.5hr.	4.5hr.	4.5hr.	7 hr.	9 hr.	>12 hr.
	(b) Percentage of disorganised cells	100	100	80	80	60	50	20	5	0	0
	(c) Maceration of cell walls of prothalli	+	+	+	+	+	+	-	-	-	-

Discussion.

The results show that the mechanism of attack of Fern prothalli by Botrytis cinerea is essentially similar to that of its attack of higher plants as described by Brown. It is shown, however, that the presence of thermolabile protopectinase enzyme is not the only factor inciting disease symptoms. The study of the effect of heat on the filtrate shows that a solution in which the protopectinase activity is not perceptible by the tests used still possesses some lethal properties. The dilution experiments show that this property is not due to the presence of small amounts of undestroyed enzymes as dilutions which still showed perceptible macerating power did not cause disorganisation of the cell content. The nature of this second factor has not been examined. It is noted however that it is not completely thermostable, and that therefore it is not possible to say from these experiments how much of the lethal activity of unheated filtrate is due to this factor and how much is due to the protopectinase.

(3) Study of the internal factors affecting the initial infection and disease development of prothalli.

It was observed in the foregoing experiments that a certain number of each batch of inoculated plants did not develop general disease. In some cases the fungal mycelium was apparently unable to invade the host cells, the cell walls of these prothalli turning brown in the area of the mycelium. In other prothalli infection occurred, but the area of infection was limited to a few cells in the area around the inoculum. This area was usually surrounded by a densely brown stained series of cell walls.

Previous work

There are many reports of similar reactions to parasites by other hosts. Hawkins and Harvey (1920) stated that resistance of the White McCormike potatoes to Pythium de-baryanum Hesse. was due to the cell walls being more resistant to mechanical puncture than the cell walls of extremely susceptible varieties. Leach (1923) observed that Colletotrichum lindemuthianum seldom attacked more than one or two cells in a highly resistant variety of beans. He stated that the fungal hyphae soon disintegrated and during this process brought about the death and disintegration of the host protoplast, at the same time staining the centre

cell contents, as well as the walls, reddish brown. In a less resistant variety some cells might be attacked, but the mycelium always disintegrated and the host protoplast became stained. He concluded that this process could be interpreted as a nutrition phenomenon, the mycelium being destroyed by autolysis induced by starvation, the resulting products killing and staining the host cells. Blackman (1924) reported that in many cases, resistance to disease was achieved by keeping the enemy out by some physical barrier, or possibly by some special chemical environment in the absence of such a barrier. In other cases the parasite achieved entry and in a susceptible host made its way through the tissues comparatively unimpeded, while in a resistant variety the entry cells developed a wound reaction leading to the production of cork which hindered or completely blocked the progress of the invader. Blackman recorded that no general bodily reaction of the plant was apparent, each infection was highly localized, and each group of host cells fought a solitary battle independent of its neighbour. Cunningham (1928), (leaf spotting fungi), Fahmy (1931), (Rhizoctonia solani on cotton plants), Shaw (1934), (Bacillus amylovorus on different varieties of pear), and Simmons (1949), (Botrytis corn rot), reported that layers of cork which were

formed some little distance ahead of invading hyphae were the most familiar examples of the mechanical type of resistance. Cunningham found that the margins of lesions caused by certain leaf-spotting fungi might or might not show corky barriers though the advance of the parasite was equally arrested in both cases. Dufrenoy (1936) stated that whenever the progress of parasites in the hosts were checked phenolic compounds, mostly tannins of the gallic group, developed in abundance within the vacoules of the cell before the cell was actually infected. Pearson (1931) described the action of Gibberella saubinettii on corn seedlings and stated that the fungus invasion produced various modifications in the character of the cells of the host tissues. These modifications might be alterations in the chemical nature of the walls and of the protoplasm or they might be some other response by the part of the host plant adopted to check or prevent further penetration by the parasite. He said that the most conspicuous change in the host tissue was a markedly increased tendency for the cell walls to take up stain. He interpreted the deeply stained materials of corn seedling to a product resulting from the action of enzymes secreted by the fungus either upon substances (pectin-like materials and pentosans) normally present in the cell walls of the host tissues or upon similar substances secreted into these regions by the

protoplasm. He found that in extensively invaded areas this densely stainable material decreased somewhat in amount, perhaps having been used as food by the fungus. Nobecourt (1946) stated that the defensive mechanisms of natural immunity in plants might be classified as those existing before an attack and those appearing just at the beginning of the attack. He described the later group as "phagocytosis" acting only against microorganisms that had already entered the cell and each cell defending itself separately. He concluded that the infected cell might be incited to divide, forming a hyperplasia or on the other hand a barrier tissue against microorganism and finally forming antibodies.

In the discussion of Brown, Brooks and Bawden (1948) on the physiology of disease resistance in plants, Brown stated that the reaction of the host to the presence of the parasite might be to lay down mechanical barriers like gum or cork, which sealed off the lesion. He said that chemical responses, for example the localized formation of phenolic compounds, may occur but it was not clear how far these were effective. He reported that cells with lignified or otherwise altered walls were, in general, resistant to invasion and, in some cases, the presence of such cells

limited the area of spread of the parasite. Brooks stated that the formation of cork barriers was greatly dependent upon the state of metabolism of the host. He said that leaf spots caused by fungi were often delimited by a suberized zone of cells leading sometimes to the falling away of the affected tissue and the well-known shot-hole effect. He concluded that whatever interpretation was placed on the formation of such corky or suberized layers they certainly seemed to prevent wider spread ramification of the fungus. Some leaf spots, although circumscribed in size, were not delimited by cork barriers and there seemed to be no obvious impediment to extensive spread of the parasite. He finally stated that in such leaf spots, apart from some obscure reaction by the host, perhaps further growth of the fungus was stopped by accumulation of staling product arising from its own metabolism. Sompio (1950) reviewed previous work on resistance mechanism exerted by plants against their parasites and reported that among such resistance mechanisms were some of a strictly metabolic type. He considered that fundamentally every resistance mechanism was linked more or less directly to a specific type of metabolism like the creation of barriers of suberous cells which tended to restrain mechanically the spread of the parasite. Gaumann (1950) stated that resistance to spread might derive from

histological characteristics or from the resistance which the cell walls oppose to the progress of the pathogen through their mechanical properties or chemical structure. This resistance was distributed throughout the tissues or localized in certain tissues. He reported that whereas the structure and condition of the cell walls created a general resistance to the spread of pathogenic fungi in the tissues concerned, the barricade tissues merely brought about a local resistance to spread within the host organism.

The following investigations were carried out to study which if any of these reasons was associated with the occasional failure of disease development in prothalli.

Experimental.

Material : To obtain material for the study a large number of prothalli were inoculated with a suspension of spores of Botrytis cinerea in water. Plants were examined seven days later and classified into the following categories:

I. No infection: The spores germinated but the germ tubes showed a limited growth and host symptoms were limited to a brown spotting of cell walls immediately underlying the mycelium.

II. Localized infection: The fungus caused a local infection but did not spread to more than a few cells immediately surrounding the site of first infection.

III. General infection: The fungus spread through the plant.

Plants of categories I and II were transferred to petri dishes of Knop's agar medium, 5 plants being placed in each dish. It was found that after the transfer the distinction between the various groups was not absolute and that in each series of cultures a small number of the plants first classified into group I and II became generally infected shortly after transfer. The total number of these plants was however less than 20% of the number indicated, except in one case where it reached 28%, and it was thought that the classification could usefully be retained for experimental purposes.

1. The study of the causes of failure of infection in plants.

It was thought that the failure of infection might be due to either:-

(a) some condition of the infection drops, possibly associated with starvation or dessication of the germinated spores or to inhibition by some plant metabolite or "staling product" of the fungus

or

(b) some property of the host cell walls or cell contents which made it resistant to infection.

(a) Examination of the effect of conditions in the infection drop

The possibility of the failure of infection being associated with some conditions of the infection drops was examined by irrigating the original inocula on prothalli of group I with fresh material as described in Table XXI and recording the amount of disease 10 days from the time of treatment. The results are detailed in Table XXI.

Table XXI.

The effect of irrigating inocula with fresh material on the development of infection.

Treatment	No. of prothalli examined	% of infection developed	Remarks
Irrigated with sterile distilled water	24	37	E few germ tubes resumed growth but only 5 plants became seriously diseased.
Irrigated with Wood's solution	26	85	The majority of germ tubes resumed growth, and penetrated the host to incite extensive disease symptoms.
Control (Untreated plant)	26	38	A few germ tubes resumed growth after transfer of the prothalli and in 6 plants incited extensive disease symptoms.

(b) Examination of the effect of the host cell walls or cells

The possibility of the failure of infection being due to some property of the plant cell walls or cells was examined in two ways.

i. The cells underlying the original inocula on prothalli of group I were killed by puncturing them with a fine sterile needle while similar untreated plants served as controls. Observations were made 7 days later and are detailed in Table XXII.

Table XXII.

The effect of killing the cells by puncturing on the development of infection.

Treatment	No. of prothalli examined	% of infection developed	Remarks
Underlying cells killed by puncturing	26	71	The majority of the infection hyphae attacked the underlying cells and incited extensive disease symptoms in many plants.
Control (Untreated plants)	26	20	A few germ tubes resumed growth but few plants became seriously diseased.

A control test was carried out to show whether the increased disease developed on puncturing cells is due to the death of the cells or a stimulating effect on growth of the fungus by presence of cell contents from damaged cells. In this case, 40 plants were used of which 20 were inoculated with aqueous

spore suspension, the others were inoculated in the same manner after adding one part of prothallus juice to 3 parts of the aqueous spore suspension. The prothallud juice was prepared by grounding up about 20 plants, and the emulsion centrifuged to remove cell wall material. Readings of the amount of infection were taken every two days on the "Standard Scale" and the results are detailed in Table XXIII.

Table XXIII.

The effect of adding prothallus juice to the spore suspension on the development of infection.

Days after inoculation	Percentage of infection developed	
	spore suspension in water	spore suspension in water + prothallus juice
2	2	2
4	10	11
6	13	22
8	19	30
10	32	43

Analysis of these results taken on the sixth and eighth days showed that the differences between the amount of disease development incited by inoculae containing prothallus juice and those without were not significant. (variance ratio = <1.0) required value at 0.05 significance level (6.0). It was noted that plate to plate variance and error variance were both high.

ii. A series of other experiments were carried out to study the nature of plant reaction to more than one inoculum. The purpose of these experiments was to determine whether failure of infection was due to certain inherent resistances possessed by individual plants or to variation in conditions of the original infection drops :

(1) At first twenty plants which had been previously inoculated without subsequent infection were reinoculated at points far from the previously inoculated part. Twenty similar were not treated and served as control.

(2) A number of healthy plants were given two or three simultaneous inoculations on different parts of each plant.

The amount of disease was recorded after 7 days from the time of inoculation and the results are detailed in Table XXIV.

Table XXIV.

The nature of plant reaction to more than one inoculum.

Treatment	Reinoculated plants		Plants with double inocula		Plants with three inocula	
No. of plants examined	20		52		32	
	Kind of infection	No. of plants	Kind of infection	No. of plants	Kind of infection	No. of plants
No change occurred in control plants.	No infection	4	No infection	14	No infection	5
	General infection	16	No infection and one local infection	7	Two no infection and one local infection	4
			One Local infection and one general infection	6	Two no infection and one general infection	3
			No infection and one general infection	12	Two general infection and one no infection	8
			General infection by both inocula	13	Two local infection and one general infection	2
					Two general infection & one local infection	10

2. The study of factors affecting local lesions formation.

It was thought that one or more of the following factors might be responsible for the formation of the local lesions :-

(a) Death of the fungus within the lesions.

(b) Presence of inhibitory substances or properties in the surrounding living cells.

(c) The properties of the brown cell walls.

(a) Death of the fungus within lesions. Experiments to study this factor were carried out as follows:-

(1) Healthy parts of diseased plants were inoculated with diseased cells taken from local lesions of the same plants.

(2) Healthy plants were inoculated with cells from local lesions of other diseased plants.

The results of both treatments, taken 10 days later are recorded in Table XXV.

Table XXV.

Death of the fungus within lesions as a factor affecting their formation.

Treatment	No. of plants	% of infection developed	Observations
Locally diseased plants inoculated with diseased cells taken from their local lesions.	10	80	The majority of plants which were inoculated with fragments of diseased cells developed extensive symptoms of disease.
Healthy plants inoculated with diseased cells taken from other locally diseased plants.	10	100	This indicated that the fungus was alive within the local lesions but unable to spread.

(b) Presence of inhibitory substances or properties in the surrounding living cells.

Two different treatments were carried out.

(1) Cells surrounding local lesions of ten plants were killed by puncturing with a fine sterile needle.

(2) Twenty locally diseased plants were reinoculated near the original local lesions.

Other 10 locally diseased plants not treated served as control. The results are recorded in Table XXVI.

Table XXVI.

Presence of inhibitory substances or properties in the surrounding living cells.

Treatment	No. of plants	% of infection developed after ten days	Observations
Cells surrounding local lesions punctured	10	0	No further increase of the disease occurred.
Inoculation of locally diseased plants	20	71	16 plants were generally diseased and fungal hyphae were seen growing within cells surrounding the original local lesions.
Control (untreated plants)	10	0	No change.

The failure of the initial inocula to spread out from the lesion when the surrounding cells were killed indicates that the formation of the lesion was due either to some change in the cell walls around the attacked area, or to the presence of some permanent inhibitory metabolite within the surrounding cells. The fact that the hyphae from the second inoculum in (2) above were seen to grow within the cells immediately around the original lesion indicates however that the formation of the lesion is not due to the presence of an inhibitory product within these cells.

(c) Examination of the properties of the brown cell walls.

(1) Effect of brown cell walls on hyphal penetration

Microscopic examination of the edges of the local lesions showed that in all cases the fungal hyphae had been unable to penetrate the brown cell walls. Detailed observations of movements of living hyphae during growth are recorded on pp. 80 which confirm this observation. A further experiment to test whether increased nutrition could assist the fungus to overcome this "resistance" was carried out by irrigating local lesions on prothalli with Wood's solution. In each case the fungus in the lesion resumed growth and caused general disease to the remainder of the plant, but microscopic examination showed that this spread was due to hyphae growing out from the surface of the lesions over the host surface, and reinfected cells beyond the brown area. In no case did a hypha penetrate the brown walls.

(2) Examination of the factors affecting the formation of and the nature of the brown cell wall.

(i) Study of factors affecting the formation of brown cell walls

The factors affecting the formation of the brown cell wall was examined as follows.

(a) Twenty plants were punctured at several places with a fine sterile needle.

(b) A drop of active fungal filtrate prepared as on p.83 was placed at several places on the surface of a similar group of 20 plants. The filtrate macerated potato discs in 30 minutes.

(c) A drop of the same fungal filtrate which had been autoclaved for 10 minutes at 15 lbs. pressure was placed at several places on the surface of a similar group of 20 plants.

(d) Test (a) and (b) were repeated using prothalli which had been killed by exposure to formalin for 8-12 hours immediately before the experiment.

Records of each experiment were taken 48 hours after the beginning of each treatment, and the results are detailed in Table XXVII.

Table XXVII.

Factors responsible for the formation of the brown cell wall.

Treatment	Observations
Living plants punctured.	No brown colouration developed in 16 plants while brown spots appeared in 4 plants* where the cells were injured.
Living plants with drops of fungal filtrate.	Brown colouration appeared below drops on all plants.
Living plants with drops of autoclaved fungal filtrate.	No brown colouration developed.
Dead plants punctured.	No brown colouration developed.
Dead plants with fungal filtrate.	No brown colouration developed.

* There was no evidence of contaminants in any culture under the microscopic magnification used, but a test for the presence of contaminants which might be present in such small number as not to be visible microscopically was carried out by transferring the plants to Bouillon agar slopes. Bacterial colonies developed around all the four prothalli marked * in which brown pigmentation had developed, but none were seen around the others. These uncontaminated plants were retransferred to Knop's agar medium in a petri dish and again punctured with a sterile needle. No brown colonies had developed when they were examined two days later.

(ii) Study of the nature of the brown cell walls

The following microchemical tests of the walls were made (Johansen 1940).

(a) Cutin and Suberin

Plants with local lesions were placed in cold 50% chromic acid for 20 minutes. At the end of this period it was seen that the whole plant including the brown cell walls was totally dissolved.

(b) Lignin

Plants with local lesions were bleached by immersing in commercial "Milton" for 30 minutes, washed in water for 30 minutes, and placed in a drop of alcoholic phloroglucin (1% phloroglucin in 95% Ethyl alcohol). A cover slip was placed on the specimen and after 2 minutes the mount was irrigated with 25% hydrochloric acid. A control section of a woody stem was treated in parallel on each slide. The typical red violet colour reaction developed in this control section in each case, but no colour appeared in the test material.

Discussion.

(1) Causes of the failure of initial infection.

The results show that the failures of infection were not due to an absolute resistance of the host plants concerned, as all those of group I which were examined were subsequently infected by various reinoculation treatments. It therefore appears that the initial failures were due to slight differences between the inocula, or between cells in different areas of the prothallus. More work is required to determine the relative significance of the two factors.

It is noted that in cases where infection had failed, reflooding the infection drop with water caused some increase in disease, while reflooding the drops with nutrient solution caused great increases. Previous workers have shown that in many diseases increased nutrition in the infection drop enables the parasite to overcome certain types of host resistance and the work reported in Part IV "I" confirms that disease incidence is higher when inocula contain nutrient material. In this case however it is not yet clear whether any such resistance is in fact possessed by the group of cells below the inoculum, or whether the original failure was due to some minor difference in the infection drop which was adjusted by reflooding.

The results of tests with multiple inocula show that there is much variation between the effect of several inocula on the same prothallus. This may similarly be due to minor variations in the individual inocula, or to very localized differences in the susceptibility of host cells.

The results of the puncturing experiments suggest that any resistance factor present must be destroyed by death of the host cell. The addition of a much larger proportion of plant cell extract in the control experiment did not cause a significant stimulation to fungal growth, and it would seem unlikely that the successful infection of killed cells was due to the stimulus of the fungus by the small amount of plant juice seeping from the dead cells.

(2) <u>The factors affecting local lesion formation.</u>

It has been shown that the fungus within the local lesions is alive and capable of reinfecting the same host once it has overcome the local resistance to spread which is present immediately around the lesion. It has also been shown that resistance of the surrounding cells is not a property of the cell cytoplasm, as hyphae from other infection of the same fungus were seen to penetrate them and grow freely without apparent check. On the other hand it was seen that the "brown" walls served as an almost complete barrier to the

pathogen, no hyphae being able to penetrate them once they are completely developed.

The examination of the factors affecting the formal of the brown walls shows that they are the product of an interaction between some fungal metabolite and the living plant cell. They are not formed by mechanical damage to uncontaminated host cells, nor are they formed by the action of the fungal metabolite on dead host cells. In the case of Botrytis cinerea this metabolite is deactivated by heat and it may be part of the protopectinase enzyme complex examined in Part III "2". It is shown that a similar effect may be produced by the presence of other contaminated organisms, but the factors involved in these cases have not been examined.

It was not possible to identify the actual constitution of the brown colouration by microchemical tests used. Results indicated that neither cutin, suberin nor lignin were present. It is known that many previous examinations of apparently similar brown colourations in cell walls which have developed as a result of fungal attack have been unsuccessful in identifying the chemical action involved.

Part IV.

Study of the effect of variation in external conditions on the development of disease incited by Botrytis cinerea.

(1) Study of the Effect of Variation in the conditions in the infection drop.

Previous work

Among the external factors which may determine the occurrence of infection of plants is the variation in the conditions in the infection drop. Blackman and Welsford (1916) stated that the germination of spores of Botrytis cinerea Pers. in water was very slow and they very often failed to infect a leaf of Broad bean (Vicia faba), but when sown in highly nutrient medium the spores grew rapidly and caused severe infection. Brown (1922b) reported that spores of Botrytis cinerea Pers. which germinated in pure water could be inhibited from doing so by sowing them sufficiently densely. He showed that this inhibiting effect was removed by the addition of a sufficiency of nutrient, and interpreted his results as due to the effect of increase of spore concentration on competition for the limited food supply available. Brown (1922a) found that the addition of extraneous nutrient to the infection drop accelerated the incidence of attack of B. cinerea Pers. on petals of Rosa sp. He also found that in extreme cases B. cinerea attacked leaves of Broad Beans when nutrients were present in the infection drop, but not in their absence. Wilson (1937) found that

when dilute spore suspension (0.1 ml. in 200 ml. water) of Botrytis cinerea Pers. was used "non aggressive" infection occurred, but when a dense suspension (0.1 ml. in 50 ml. water) was used, aggressive infection developed. He found that while hyphae were abundant in the lesions of "aggressive infection" only a sparse mycelium was present in those of "non aggressive infection". Gaumann (1950) stated that by increasing the quantity of inoculum up to an optimum, not only the incidence but also the severity of the disease was increased. He said that in rare cases increase of inoculum density above the optimum has no effect, but that in general the results of infection are diminished by such increases. He attributed this reaction to a deficiency in oxygen in the infection drop or to mutual inhibition by metabolic products, or in many cases simply to competition for nutrients. Gaumann also stated that in order that a germ tube might acquire and retain the necessary vitality it must have at its disposal sources of a saprophytic nourishment in addition to the maternal store.

From reviewing previous work it was decided to study two factors:

(a) The influence of inoculum density on the infection of prothalli

and :

(b) The effect of previous nutrition in the infection drop on disease development.

Experimental.

(a) The influence of inoculum density on the infection of prothalli with spores of Botrytis cinerea Pers.

The concentration of inocula were chosen to give extremes of conditions so as to bring out the factor under examination most clearly, and it is noted that the density of suspensions used were very much greater than any that would be likely to be met with in natural infections.

Spores were collected from 10 days old potato agar petri dish cultures into sterile distilled water. Suspensions thus obtained were filtered once through sterile muslin. The spores were centrifuged about four times in changes of sterile water to remove fragments of mycelium. Dilutions of the wet volume of centrifuged spores were made in sterile distilled water to give suspensions of S, S : 100, S : 1,000 and S : 10,000 where S denotes a suspension containing 1.0 ml. of wet spores in 10 ml. liquid.

Mature prothalli of standard size and age were transplanted to Knop's agar medium in petri dishes, 5 plants

in each plate. Sets of 20 such prothalli were inoculated by a loopful of each spore suspension respectively. Cultures were then kept under 15 hours illumination daily and records were taken each two days of the amount of disease developed on the "Standard Scale."

Table XXVIII shows the percentage of infection caused by each suspension in the first experiment.

Table XXVIII.

The influence of inoculum density on the infection of the prothalli with spores of Botrytis cinerea Pers.

Days after inoculation	Relative density of spores in the inoculum (1)			
	S	S : 100	S : 1000	S : 10,000
	Percentage of infection			
2	2	5	15	7
4	6	15	26	14
6	11	26	41	20
8	13	35	51	28
10	17	48	63	32

Table XXIX shows the percentage of infection in two further replicate experiments in which the actual number of spores were not counted, and the average of the three experiments is illustrated in Graph I.

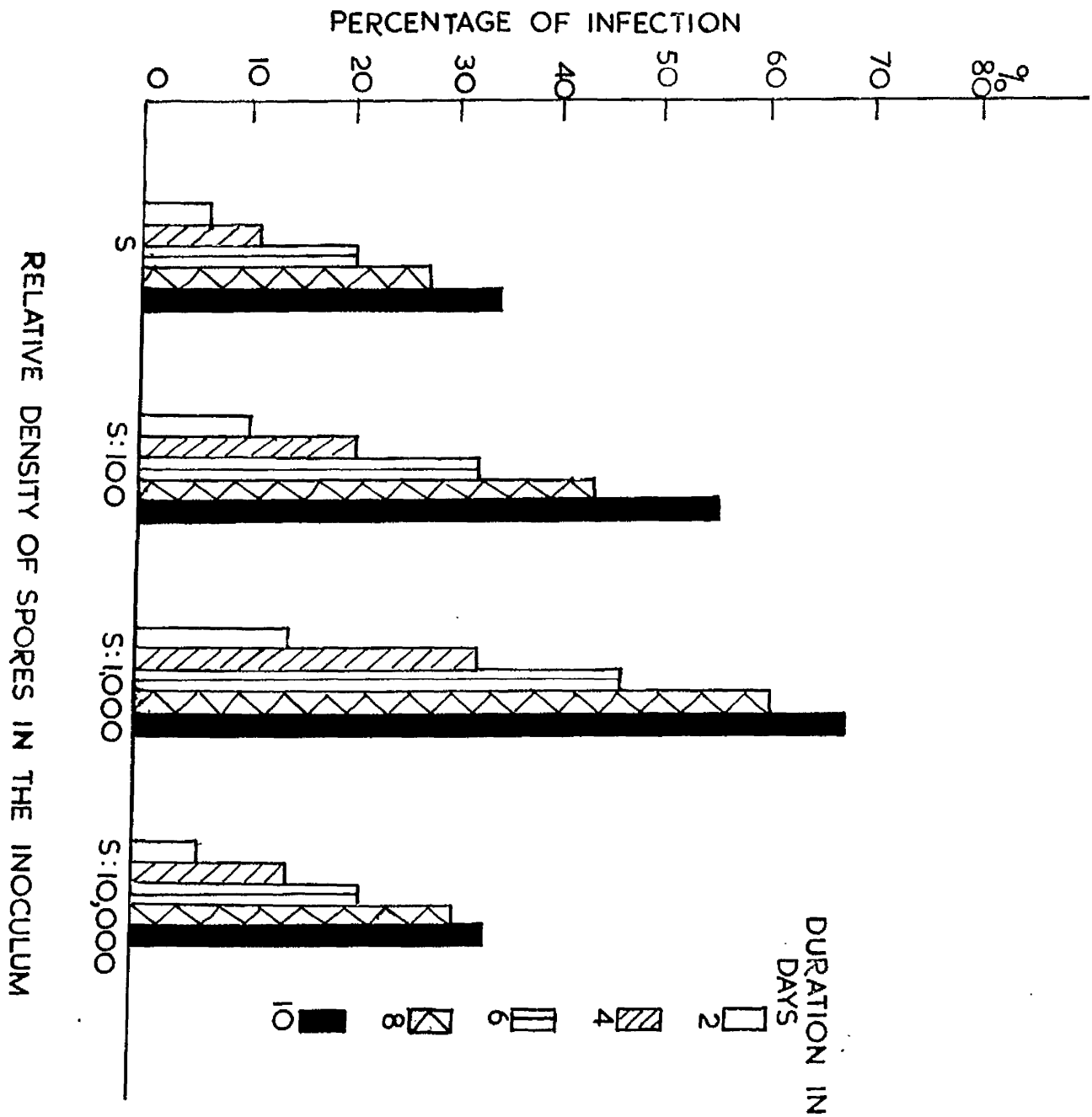
(1) The average number of spores in this suspension was 13×10^4 per 1.0 c.c.)

Table XXIX.

The influence of inoculum density on the infection of the prothalli with spores of B. cinerea.

	Days after inoculation	Relative density of spores in the inoculum S	S : 100	S : 1000	S : 10,000
		Percentage of infection			
Exp.I	2	8	13	12	2
	4	15	18	29	9
	6	24	35	50	14
	8	35	42	63	22
	10	42	53	67	24
Exp.II	2	9	13	18	9
	4	12	27	40	18
	6	25	35	46	29
	8	33	52	65	39
	10	42	65	75	43

Analysis of the results of the 6th day after inoculation show that the differences in amount of disease increase are significant in experiments I and II (var. ratio = 28 and 4.8) but that they are not significant in experiment 3 (var. ratio = 1.7, required value 5.9). In this experiment the experimental variation was high, and it is noted that the order of difference



THE INFLUENCE OF INOCULUM DENSITY ON THE INFECTION OF THE PROTHALLI WITH SPORES OF B. CINEREA PERS.

Graph I.

is similar to that of the other experiments.

Microscopic examination of the infection drops showed that the majority of spores in the most concentrated infection drops had not germinated. The spores in the drops of S : 1000 and S : 10,000 suspensions had germinated normally, and the germ tubes were well developed.

A control test of the effect of variations in spore density on germination was carried out by placing drops of various suspensions on a clean glass slide within a glass ring covered with a cover slip. The glass slides were placed on a glass rod triangularly formed in petri dishes containing a little sterile water to maintain humid concentrations. The petri dishes were placed in the incubator at 20°C. for 20 hours. Measurements were taken from 8 glass slides in each case. The results are recorded in Table XXX.

Table XXX.

The effect of the spore density on the germination and growth in vitro. (20 hr. incubation at 20°C.)

	Relative density of spores in the suspension			
	S	S : 100	S : 1,000	S : 10,000
Percentage of germination (based on at least 1500 counts)	0	5.2	52.0	44.0
Average length of germ tubes (based on 100 counts)	0	42.8 μ	107.0 μ	128.0 μ

The results have not been analysed statistically, as it is thought to be clear from empirical observation that decrease in spore density caused an increase in germination percentage and an increase in the rate of elongation of germ tubes.

(b) The effect of previous nutrition in the infection drop on disease development.

Spore suspensions of 10 days old cultures of Botrytis cinerea were made in three different media :

(1) sterile distilled water

(2) half strength Wood's solution

(3) full strength Wood's solution.

Each suspension was used to inoculate 24 plants, 4 plants being placed in each of six petri dishes on Knop's agar medium. Readings of the amount of disease development were taken at three intervals and recorded on the "Standard Scale". The results of two experiments are recorded in Table XXXI.

Table XXXI.

The effect of previous nutrition in the infection drop on disease development.

	Days after inoculation	Percentage of infection: Water	½ strength Wood's Solution	full strength Wood's solution
Exp.I	2	6	13	28
	4	22	81	87
	6	25	83	94
Exp.II	2	12	26	36
	4	30	64	83
	6	34	73	93

Analysis of the results on the fourth day of both experiments shows that increase in nutrition in the inocula was associated with a highly significant difference in the amount of disease developed (variance ratio= 24 and 16).

Spores in half and full strength of Wood's solution were able to germinate quickly and to incite extensive symptoms. Spores in water germinated in most cases but few incited extensive disease symptoms, others failed to cause infection or formed local lesions.

Discussion.

The results obtained confirm earlier observations of Brown (1922b) who found that with decrease in the number of spores in the inoculum, there was an increase in the amount of infection until a certain density was reached following which there occurred a proportionate decrease in infection.

The low infection incited by using very concentrated suspensions was associated with poor germination and growth of the spores, presumably due to lack of oxygen or competition in the infection drop. They also confirm his observation that the amount of infection is reduced when the spore concentration of the inoculum falls below an optimum, which may be due to the dilution of the extracellular metabolites of the fungus which are involved in the attack.

It is also shown that the addition of nutrient materials to the inocula caused a substantial increase in the amount of

disease developed. This agrees with many previous workers' reports, and it may be due to the provision of extra energy for the pathogen in the penetration stage, or to the presence or increase of some essential metabolite to support attack.

(2) Effect of variation in period of illumination of the host on disease development.

Previous Work

There have been many investigations of the effect of variation in period of host illumination on disease development. The results have shown that the effect varies with different hosts, different pathogens and in different conditions, and in many cases the precise effect of light has not been clearly analysed. It has been shown that variation in amount of disease development under varying light treatments may be due to the effect on the host or to the effect in the pathogen, or both, and the previous work may conveniently be recorded under two headings.

(1) Effect of variation of host illumination on disease development

Wilson (1937) found no difference in rate or intensity of infection of Botrytis cinerea Pers. between groups of broad beans and two varieties of field beans which were experimentally infected and then confined in dark and diffused daylight respectively.

Grainger(1949) found that the broad bean had similar amount of chocolate spot when grown in both short-day and long-day conditions.

Pohjakallio, Laila, and Paasi (1951) stated that lack of light increased destruction by Botrytis cinerea Pers. in red

clover and Pythium spp. in radish and that increase of light increased resistance of red clover and radish to these diseases, apparently by accelerating photosynthesis. They concluded that the resistance of red clover to Botrytis cinerea Pers. based on photosynthesis was probably "spontaneous" and hence not due to infection by the parasite.

Sempio (1950) found that wheat or lettuce plants, confined in the dark for the first 3 or 4 days after inoculation with Oidium moniloides, developed more severe disease than those exposed to normal daylight. He said that the main cause of the more severe attack encountered in plants placed in the dark during the early days of infection was the total block of photosynthesis. For this reason, he continued, the initial stimulus of photosynthesis should be interpreted as a defensive reaction. In addition, he found that keeping healthy wheat and lettuce plants in the dark for a few days produced a marked disturbance in the Glucolysis-respiration balance and both these functions were depressed. He also found that continuous artificial light increased the resistance of wheat to Oidium moniloides. He stated that the absence of the normal nocturnal halt in photosynthesis appeared to be the chief cause of the very considerable depression in Oidium attack on the plants exposed to continuous light for 12 days. He found that exposure to continuous light during the first 4 days after inoculation only was less effective in reducing disease severity.

Gaumann (1950) stated that light as a rule affected the host almost exclusively, an exception being the Erysiphaceae. Light particularly influenced the carbohydrates and protein metabolism and thus it modified the disposition of the host both prior to infection and during the post-infectional course of the parasitic relationship. He noted that under artificial conditions an increase in duration of light unsually reduced the susceptibility of the host to eusymbionts and increased it to parabionts, but the various manifestations of the pathogen host relationship are affected differently. Vavilov (1949-1950) in his discussion of the role of light stated that the influence of light was definitely related to the display of susceptibility to parasitic disease. He said that, although in relation to rusts the question of influence to light was comparatively clear, with respect to other parasites, the effect of this factor had not been explained and the data were contradictory.

(2) Effect of variation of period of illumination on fungi in cultural medium.

No records of study of the effect of light on Botrytis cinerea Pers., grown in culture, has been found. Coons (1916) reviewed previous work on otherspecies and stated that light appeared to be a factor of widely varying importance on organisms although the effect on vegetative growth had commonly been shown to be prejudicial.

For some it was a morphogenic factor of great influence; for others it was of no moment. He summarised his results on the effect of light on the growth and the pycnidium formation of Plenodomus fuscomaculans and pointed out that light was a decisive factor which determined in certain cultures whether reproduction takes place or not, and that the action of this factor was irrespective of the richness or the poverty of the substratum in nutrients. He stated that, as a morphogenic factor, its action was to inaugurate fruit-body formation but it was not essential to the process, once inaugurated. He added that, associated with its effect in initiating reproduction, it had its repressing effect on growth. He finally stated that the action of light, when pushed to a last analysis and when considered in view of the experiments in which hydrogen peroxide and other oxidizing agents replaced it, was seen to be of either an oxidizing or a catalytic type. Houston and Oswald (1946) found that abundant normal sporulation was obtained within 48 hours on agar culture of the fungus Helminthosporium gramineum Rab., the causal agent of the barley disease, when held outdoors to expose them to diurnal changes of environment. Light, preferably outdoor daylight, was necessary for the production of sporulation. In the absence of light no sporulation was obtained on agar slant culture or on mycellium growing from diseased leaves either outdoors or indoors.

Barnett and Lilly (1950) observed that conidia of the Choanephora cucurbitarum (B. and Rav.) failed to form in continuous darkness and in continuous light, and were produced in cultures exposed to alternate light and dark periods of approximately 12 hours each. They found that light had little or no effect upon mycelial growth. Timnick, Lilly and Barnett (1951) reported that continuous light permitted the development of stromata and perithecia of Diaporthe phaseolorum var. batatis, but delayed the development of ascospores. Continuous darkness allowed the formation of only a few perithecia, but these produced abundant mature ascopores. Alternate light and darkness was much more favourable for the production of perithecia, asci, and ascocpores. The object of this investigation is to study in some detail the relation between light duration and the susceptibility of prothalli to Botrytis cinerea Pers.

Experimental

1. The study of the effect of light on disease development.

Ten prothalli aged between 8-10 weeks, which had grown under 15 hours daily illumination, were placed in each of a series of petri dishes of Knop's agar, and groups of these were then placed in cardboard boxes covered by loosely fitting glass sheets.

The boxes were then covered with black paper covers which excluded all light, and placed under a series of fluorescent tubes. The boxes were moved daily to equalize the effect of any possible variation in light intensity under different parts of the tubes. The lights were left on continuously throughout the experiment and the paper covers removed to illuminate the plants for the periods stated.

(a) Examination of the effect on disease incidence of continued exposure to varying periods of illumination

Prothalli growing as above were exposed to the periods of daily illumination specified below. After two weeks treatment the prothalli were inoculated by placing a loopful of a suspension of spores of B. cinerea Pers. in ½ strength Wood's solution (qv.) onto their surface. Daily readings of the amount of disease developed were taken and recorded on the "Standard Scale". Four replicate experiments were carried out, each experiment consisting of the exposure of two plates (i.e. twenty prothalli) to each illumination. The results are recorded in Table XXXII and the average of the four results is plotted in Graph (11)

Table XXXII.

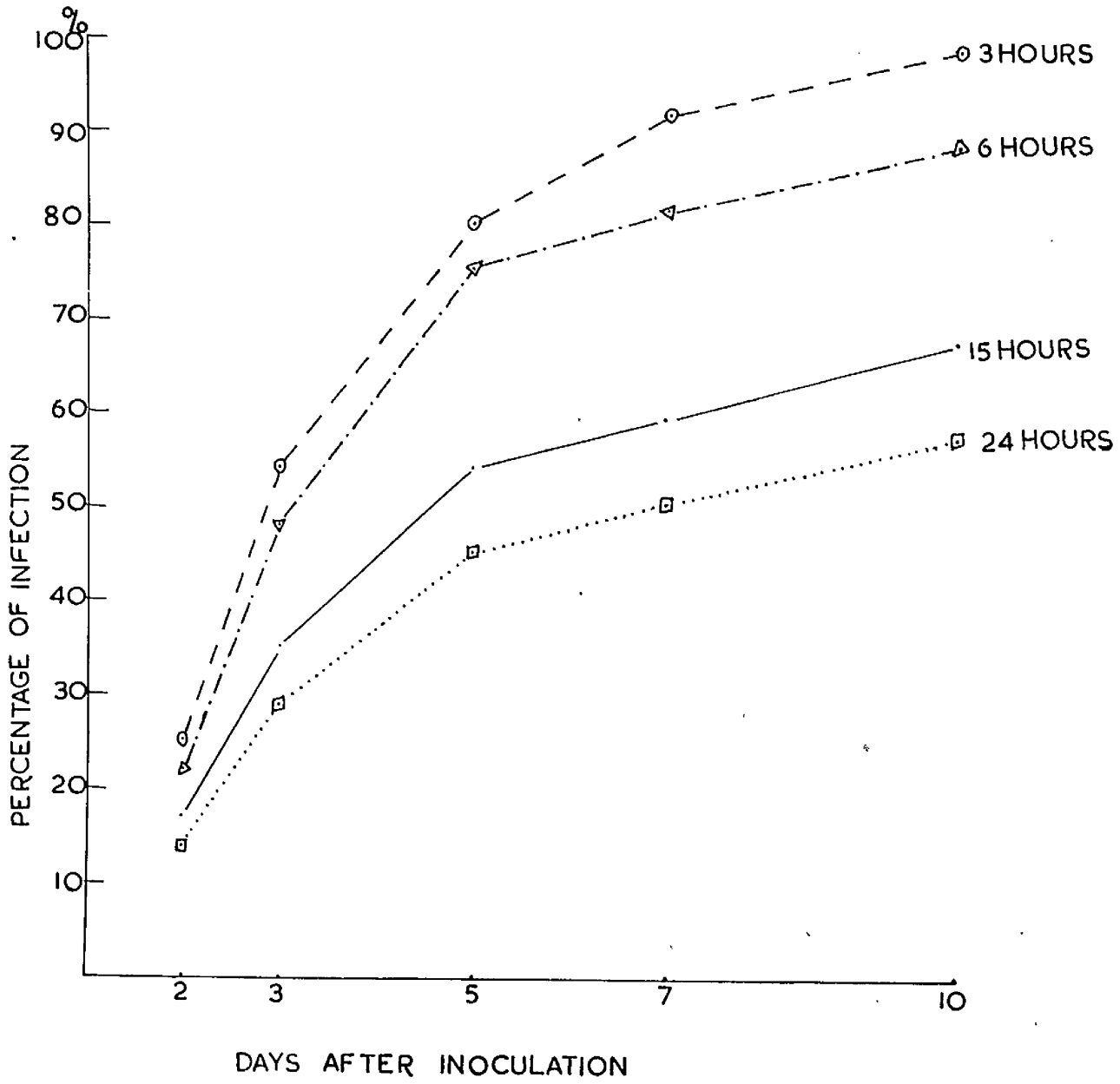
Disease Development under Varying Periods of Illumination

Table XXXII

Disease Development under Varying Periods of Illumination

Days after inoculation		Period of daily illumination: 3 hours					6 hours					15 hours					24 hours				
		2	3	5	7	10	2	3	5	7	10	2	3	5	7	10	2	3	5	7	10
Exp. I	Plate 1	6	16	34	45	50	6	16	26	33	39	11	17	27	30	30	2	7	15	16	16
	Plate 2	8	26	44	49	50	7	17	38	41	42	7	18	30	31	34	3	10	14	16	16
Exp. II	Plate 1	13	27	36	41	44	18	30	45	45	50	10	19	24	25	37	12	19	31	33	37
	Plate 2	22	45	45	50	50	13	27	39	44	46	11	26	38	40	46	8	18	28	35	38
Exp. III	Plate 1	16	31	43	48	50	11	26	33	36	39	7	17	30	32	32	10	20	26	26	32
	Plate 2	14	21	36	41	41	16	27	38	40	41	8	11	16	17	21	10	19	30	33	37
Exp. IV	Plate 1	13	34	44	45	50	7	20	36	39	42	7	20	35	39	41	4	10	19	21	25
	Plate 2	9	29	39	45	49	9	27	41	44	46	6	12	17	22	26	4	11	11	21	25
Total		101	215	320	364	384	87	190	296	322	345	67	140	217	236	267	53	114	180	201	226
Percentage of infection		25	54	80	91	98	22	48	74	81	88	17	35	54	59	67	14	29	45	50	57

BOTRYTIS DISEASE DEVELOPEMENT ON PROTHALLI
UNDER VARYING PERIODS OF ILLUMINATION.



Graph II.

Analysis of the readings taken on the seventh day showed that the variation in illumination had a highly significant effect on disease development (variance ratio = 22.9). At the 5% level of significance the least significant difference between any two means is 5.87, hence the mean difference between 3 hours and 6 hours illumination is just significant (5 . 9), that between 6 hours and 15 hours is highly significant, (10 . 8), and that between 15 hours and 24 hours is not significant (4 . 4).

Microscopic study of the diseased prothalli showed that those exposed to short periods of illumination were rapidly penetrated by the parasite, with little development of brown pigment at the site of penetration or elsewhere. The amount of brown colour which developed increased with longer illumination, and in many plants under 15 hours or 24 hours illumination, the disease remained localised in an area limited by a line of strongly pigmented dark brown cell walls. During this microscopic study a number of actively growing hyphae were examined as they were growing in the cells of living prothalli which had received 3 hours and 15 hours illumination for two weeks. The results are recorded in Tables XXXIII and XXXIV.

Table XXXIII

Rate of movement of hyphae through host tissues growing under varying illumination

	Average distance travelled per minute by hyphae within cell cavity												
Hyphae No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Illumination													
3 hours	46.5	18.5	29.3	37.0	33.3	35.3	62.8	53.8	61.5	64.8	55.8	56.8	56.9
15 hours	50.0	52.7	32.6	17.5	57.1	45.8	22.9	20.4	21.3	22.2	26.7	23.2	

The figures are arbitrary divisions of an eyepiece micrometer in which one division = 4.18

1st.

Table XXXIV

Average time in minutes taken to penetrate a cell wall

Hypha No.	1	2	3	4	5	6	7	8
Illumination								
3 hours	8	8	8	9	9	7	9	7
15 hours	12	12	14	11	16	12	13	

Analysis of these results shows that the differences between the rates of movement across the cell cavities are nearly significant (variance ratio = 2.12, required ratio at 0.05% level 2.6), while those for penetration of the cell walls are just significant (variance ratio = 3.7).

(b) Examination of the effect on disease incidence of transferring plants which had grown in short periods of illumination to long periods of illumination, and of transferring plants which had grown in long periods of illumination to short periods.

A series of experiments were carried out to examine whether the high rate of disease development in prothalli exposed to short periods of illumination was due to change in the host or to change in the parasite, and to examine the rate at which this "susceptible condition" developed or was overcome during growth.

In the first series, plants which had grown for 8 weeks under 15 hours illumination were transferred to boxes with 3 hours and 6 hours illumination periods immediately before inoculation. In the second series, similar plants aged 6 weeks were transferred to the low illumination boxes for 2 weeks, then retransferred to 15 hours illumination immediately before inoculation. All plants were inoculated by placing a loopful of a suspension of spores in ½ strength Wood's solution onto their surface. Daily readings of the amount of disease developed were taken and recorded on the "Standard Scale". Two replicate experiments were carried out in each case, each experiment consisting of the exposure of two plates (i.e. twenty prothalli) to each treatment. The results are recorded in Tables XXXV and XXXVI.

Table XXXV.

The effect of transferring plants held in short period illumination to long period.

		Illumination period for 14 days before inoculation														
		3 hours					6 hours					15 hours				
Days after inoculation		2	3	5	7	10	2	3	5	7	10	2	3	5	7	10
Exp. I	Plate I	13	37	47	48	48	12	32	36	38	45	10	24	40	41	41
	Plate 2	13	31	42	43	44	10	27	46	46	47	8	17	28	31	33
Exp. II	Plate 1	11	26	31	31	32	4	12	17	19	19	7	14	21	23	27
	Plate 2	7	10	12	13	16	12	23	36	39	39	7	14	23	23	24
Total		44	104	132	135	140	38	94	135	142	150	32	69	112	118	125

The plants were all transferred and maintained in 15 hours illumination daily at the time of inoculation.

Table XXXVI.

The effect of transferring plants grown in long period illumination (15 hours) to short period illumination after inoculation.

		Illumination period after inoculation														
Days after inoculation		3 hours					6 hours					15 hours				
		2	3	5	7	10	2	3	5	7	10	2	3	5	7	10
Exp. I	Plate 1	11	21	27	30	42	6	18	36	46	47	16	32	39	44	44
	Plate 2	11	19	35	42	44	9	27	34	35	40	13	24	35	37	37
Exp. II	Plate 1	8	19	34	38	38	8	21	33	40	44	10	16	38	35	41
	Plate 2	7	18	31	35	36	5	12	16	22	30	11	12	31	33	33
Total		37	77	127	145	160	28	78	119	143	161	50	94	143	149	155

It is clear from empirical observation that there was little difference between the amount of disease developed under the various treatments, and analysis of the readings at the second and seventh day showed that there was no significant difference between them.

11. The effect of light in vitro on the rate of growth and sporulation of B. cinerea in culture.

A study of the direct effect of variation in light on the development of B. cinerea in vitro was carried out as a control. In each experiment sets of petri dishes of potato dextrose agar were inolulated in the centre with agar bearing mycelium and spores of B. cinerea and placed in the covered boxes similar to those used in the above experiments. One set of 4 petri dishes was exposed to each light for 3 hours, 6 hours, 15 hours and 24 hours respectively and one set was kept in the dark. Boxes were treated in the same manner as described above (p.127) and removed daily to equalize any difference in intensity of illumination for different parts of the light. Daily records were taken of the diameter of the colonies and of the sporulation produced.

Sporulation was recorded empirically by observations against the following scale: - no sporulation; + poor sporulation; + + moderate sporulation; + + + good sporulation; + + + + abundant sporulation. The results are summarised in Tables XXXVII and XXXVIII.

Table XXXVII

Average diameter of colony in c.m. under varying period of illumination

Days after inoculation	Period of daily illumination				
	Dark	3 hours	6 hours	15 hours	24 hours
2	2.1	2.4	2.4	2.4	2.5
3	4.8	4.5	4.5	4.5	4.7
4	7.9	7.1	6.9	6.8	7.9
5	8.9	8.7	8.6	8.1	8.5

Table XXXVIII

Amount of Sporulation in varying periods of illumination

Days after inoculation	Period of daily illumination				
	Dark	5 hours	6 hours	15 hours	24 hours
2	-	-	-	-	-
3	+	+	+	-	-
4	+ + +	+ +	+	-	-
5	+ + + +	+ + +	+ +	-	-
6		+ + + +	+ +	-	-
7			+ + +	-	-
8			++ + +	very few	-
9				+	-
10				+	-

Empirical study of the results shows clearly that no difference can be seen in the rate of growth of the fungus when exposed to the several illuminations and no statistical analysis is thought to be required. The density of the mycelium was the same in all conditions, but it was dark in colour in cultures kept in the dark or exposed to short period illumination, while it was pale white in cultures exposed to 15 hours or 24 hours daily illumination.

The spores were first produced in a short time in all cultures kept in dark or exposed to short period illumination. The sporulation increased rapidly in these cultures and within 6 days there was a plentiful spore production particularly in the peripheral regions of the colonies. Cultures exposed to 15 hours daily illumination produced very few spores after 7 days while no spores whatever were produced in cultures exposed to continuous light for 10 days.

In a further experiment two plates of the later cultures, which produced no spores, were kept in dark while the other two plates were kept under the same condition. Spores were produced in abundance within 24 hours in the plates kept in the dark while the others got no spores.

The whole experiment was carried out twice and the same results were obtained.

Discussion.

The results show that the amount of disease developed when prothalli are grown under short periods of illumination is significantly greater than that developed under long periods of illumination. It appears that the amount of disease developed decreases as illumination is increased up to a certain limit, but that no further decrease occurs if illumination is increased above this limit. Conway's (personal communication) finding that 16 hours daily illumination is the optimum for vegetative growth may have some relation with this result.

It is also shown that light has a direct effect on the metabolism of the fungus, both pigmentation and sporulation being substantially different when it is grown in different periods of illumination. The result of transplanting plants which had grown in short period illumination to long period illumination at the time of inoculation and vice versa (Tables XXXV and XXXVI) shows, however, that differences in illumination during the growth of the fungus within or upon the host tissue do not result in changes in the disease developed. The factor concerned therefore appears to be some change in the host metabolism. The transferring experiments also show that the factor is one that is counteracted quickly when plants which have been exposed to

short period illumination are returned to long period illumination, and that it does not develop immediately a plant is placed in low period illumination. More work is necessary to identify the factor or factors concerned, but it is noted that the results are not incompatible with the theory that it is some form of energy relationship within the host cell.

"Reserves" of the factor might be accumulated during periods of long illumination in sufficient quantity to "survive" short periods of short illumination. Similarly, it might be formed rapidly when a plant is placed in long period illumination, enabling a transferred cell to attain a similar level of "resistance" to that of one grown continuously in long period illumination before the inoculum had developed sufficiently to attack it.

Study of the effect of variation in Potassium and Nitrogen concentration in the medium on disease development

Previous Work

Previous work had shown that the susceptibility or resistance of many host cells to the attack of a parasite could be modified by change in their nutrition, and that in particular variation in nitrogen and potassium concentration had an effect in many cases. Marshal (1901 - 1902), (*Bremia lactucae* on lettuce), Pryor (1940), (Club-root on crucifers), Alten (1945), (*Phytophthera infestans* on potato), Walker and Hooker (1945), (Fusarium disease on young cabbage plants), and Walker and Forster (1946), (Fusarium wilt of tomato), reported that high level of nitrogen ion-concentration and low level of potassium ion-concentration favoured disease development and the reverse occured when low nitrogen and high potassium ion-concentration were used. Delacroix (1905), (*Botrytis cinerea* Pers. on grapes), Gassner and Franke (1934) (Leaf spot diseases of tobacco plants), Thomas and Ark (1938-1939) *Bacillus amylovorus* on plants of *Pyracantha angustofolia*), and Keyworth and Hewitt (1948) (*Verticillium albo-atrum* on the Hop plant), found that an increase in nitrogen in the medium was followed by an increase in the amount of disease development.

Beaumont (1933) and Glynne (1936) found that chocolate spot occured in an excess on bean grown in soils which were deficient in potash.

Scott Watson (1936) stated that a fungus of the genus Botrytis which caused disease of beans was found to take epidemic character when there was a shortage of potash. Wilson (1937) stated that any factor tending to weaken the crop of bean (Vicia faba L.) such as potash deficiency rendered it more liable to aggressive infection by Botrytis cinerea Pers. Petri (1930) reported that the conditions of nutrition of a plant determined in a large measure the degrees of its resistance or susceptibility to pathogen; its histological or morphological structure or properties and the function of the organs and tissue might be modified in such a way as to retard or hasten the attack of the pathogen. He stated that excess in nitrogen, in general, tended to increase crop susceptibility to adverse factors, potassium to increase resistance against the same factors. He added that no general "law" could be established since in one case the conditions may make a plant susceptible and in another resistant. Naghski, Harris, Haley and Reid (1939) made a study of the reaction between variations in the nitrogen uptake of tobacco plants and variations in resistance to leaf spot disease. They stated that other things being equal, the higher the nitrogen level of the plant the greater the susceptibility of the plant to disease but they concluded that this was not the true relation between nitrogen uptake and disease susceptibility. They considered that the results

indicated that it was not a question of how much nitrogen a plant absorbed within reasonable limits that determined susceptibility, but the stage in life of the plant when significant quantities of nitrogen were absorbed. Wingard (1941) in his discussion on the effect of nutrition on the parasitic diseases of plants stated that there were workers who believed that excess nitrogen predisposed plants to disease whereas potassium tended to make them more resistant. He reported that the literature on the subject, however, showed that one should not generalise on the effect of nutrition, and what is true of fungi will not necessarily hold true for disease caused by other pathogens. Walker (1946) stated that increases in susceptibility to disease resulting from high nitrogen manuring were more numerous than those to the contrary, whereas with potassium the reverse was the case. He found however that tendencies in the opposite direction for each element were not uncommon, but that in general decrease in potassium-ion increased disease progress. He also stated that since the type of host parasite inter-relation varied widely and many grades and types of parasitism existed it might be expected that the type of pathoginicity predetermined to some measure the effect of host nutrition on disease development. Butler and Jones (1949) stated that the factors which determined

the resistance or susceptibility of the host plant were often intimately associated with intercellular processes and that the substances concerned had seldom been identified. They said that variations in nitrogen level seemed to act mostly on the cell contents, solutes, amino acids or proteins while potash is known to intensify cell wall development and this had been correlated in several cases with resistance to parasite attack. Anderson (1951) found that infection of Phytophthora cinnamomi Rand. on pineapple growing in liquid culture solution was markedly decreased by increasing the amount of potassium. Gaumann (1950) stated that the action of potassium was generally opposite to that of nitrogen, reduced supplies of potassium having the same effect as a relative excess of nitrogen and excess of potassium having a comparable effect to relative lack of nitrogen.

Experimental.

(a) Examination of the effect of variation in potassium concentration on disease development.

Loomis and Shull (1937) modification of Knop's formula was used as a basic balanced medium, and the amounts of potassium in gram per litre etc. varied as shown below.

Table XXXIX.

Media	A	B	C	D	E	F	G
K Level	Standard 100	Standard 10	Standard	2.5 x Standard	5 x Standard	10 x Standard	
N2, Cl & Po_4 level							2.5 x Standard
K Cl	0.06	0.6	6.0	15.0	30.0	60.0	6.0
Na Cl	4.72	4.29	-	-	-	-	11.7
$KH_2 PO_4$	0.06	0.6	6.0	15.0	15.0	60.0	6.0
$N_2H_2PO_4 . H_2O$	5.77	5.5	-	-	-	-	12.8
$Mg SO_4 7H_2O$	9.0	9.0	9.0	9.0	9.0	9.0	9.0
$Ca (NO_3)_2 4H_2O$	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Ferric tartrate	0.6	0.6	0.6	0.6	0.6	0.6	0.6

These media were chosen to give a wide range of potassium concentration so as to demonstrate any effect clearly, and they have no necessary relation to the concentrations which may occur in nature. The level of chlorine and phosphate ions was maintained in media A & B by the addition of suitable amountx of sodium salts. Medium G was used as a control to check whether any effects noted in Medium D were due to the addition of potassium or of chlorine and phosphate ions, and to check whether any effects found in media A and B were due to the lowering of the potassium ion concentration or to increase in the sodium ion concentration.

Bracken spores were initially inoculated onto petri dishes of the various media and after one month's growth individual prothalli were transferred from the dishes onto fresh media in 1 oz. screw bottles and grown for a further month. This was found to be a convenient procedure to reduce the amount of secondary contamination by air borne fungal spores and to encourage the growth of weak individual prothalli. At the end of the second month prothalli were again transferred to fresh media in petri dishes, 6 prothalli being placed in each dish. In each experiment 5 dishes (i.e. 30 prothalli) of each medium were inoculated with aliquots of a spore suspension of Botrytis cinerea prepared as on page 75. Throughout growth all cultures were held under 15 hours daily illumination at room temperature (18°C. to 23°C.) in the illuminated cabinet, and moved daily to equalize the effect of any slight variation in light intensity at different places. The amount of disease developed was recorded daily on the "Standard Scale", the total figure for each dish being given in the tables below.

In the first two replicate experiments the effects of change of potassium level were tested using media A.B.C. and F. There was no visible difference in spore germination or in the size and general appearance of prothalli grown on media

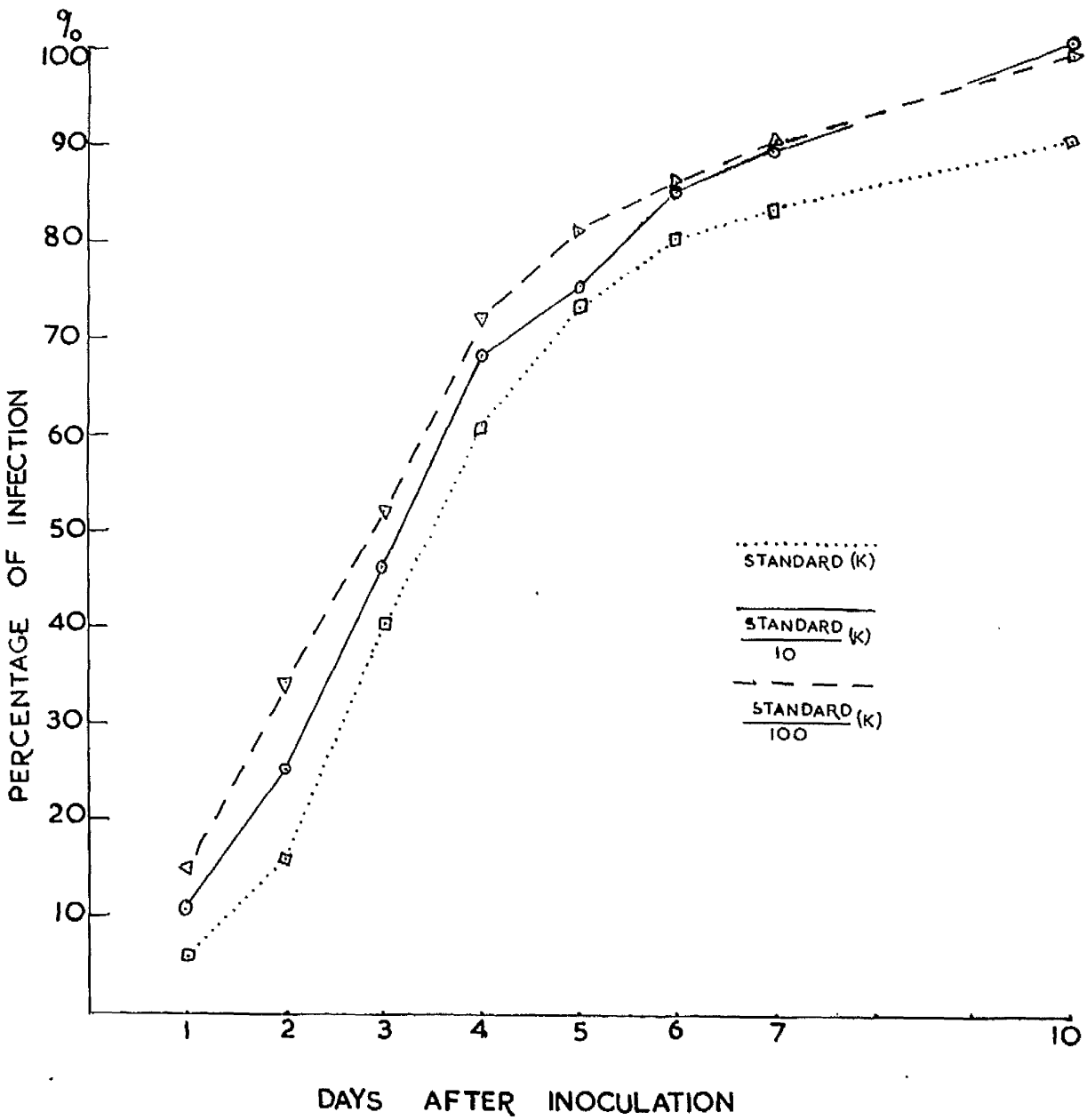
A.B.C., but there was no growth on medium F (x10 K). Two further replicate experiments were therefore set up to study the effect of smaller increases in potassium, using media C. D. and E., and medium G. was also used in these experiments as a control. In these experiments there was no growth in medium E (x5 K), but normal germination and growth in the other media occurred. The results are recorded in Tables XL and XLI below and the average of all results is plotted in Graph III and IV.

Table XL.

The effect of reduced levels of potassium on disease developed.

Days after inoculation		Levels of potassium concentration S : 100 K								S : 10 K								Standard							
		1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10
Exp. 1	Plate 1	6	13	19	30	30	30	30	30	4	7	12	22	24	29	30	30	1	3	14	24	30	30	30	30
	" 2	3	5	8	14	16	18	21	30	5	11	23	30	30	30	30	30	0	2	6	15	21	23	25	25
	" 3	4	9	14	22	26	27	29	30	4	4	6	10	13	18	23	30	4	6	16	19	20	20	20	20
	" 4	5	11	18	24	28	29	29	29	5	8	17	22	22	24	24	30	5	12	23	26	30	30	30	30
	" 5	5	12	24	29	30	30	30	30	4	6	13	25	25	30	30	30	1	4	9	22	26	27	30	30
Total		23	50	83	119	130	134	139	149	22	35	71	109	114	131	137	150	11	27	68	106	127	130	135	135
Exp. 2	Plate 1	5	13	16	21	24	27	30	30	3	9	12	17	20	24	26	30	0	2	13	23	30	30	30	30
	" 2	4	11	14	18	21	25	27	30	4	8	11	15	18	23	25	30	2	8	16	19	20	30	30	30
	" 3	4	12	18	22	26	28	30	30	3	8	18	25	28	30	30	30	3	6	12	20	24	25	25	25
	" 4	3	5	9	15	20	21	21	30	0	4	9	15	16	19	20	28	1	2	4	5	5	7	10	23
	" 5	6	12	16	20	21	22	24	29	2	10	17	22	29	29	30	30	1	3	7	11	12	18	20	27
Total		22	53	73	96	112	123	132	149	12	39	67	94	111	125	131	148	7	21	52	78	93	110	115	135
Total of Exps. I & II		45	103	156	215	242	257	271	298	34	74	138	203	225	256	268	298	18	48	120	184	220	240	250	270
Percentage of infection		15	34	52	72	81	86	90	99	11	25	46	68	75	85	89	100	6	16	40	61	73	80	83	90

THE EFFECT OF REDUCED LEVELS OF POTASSIUM ON DISEASE DEVELOPMENT.



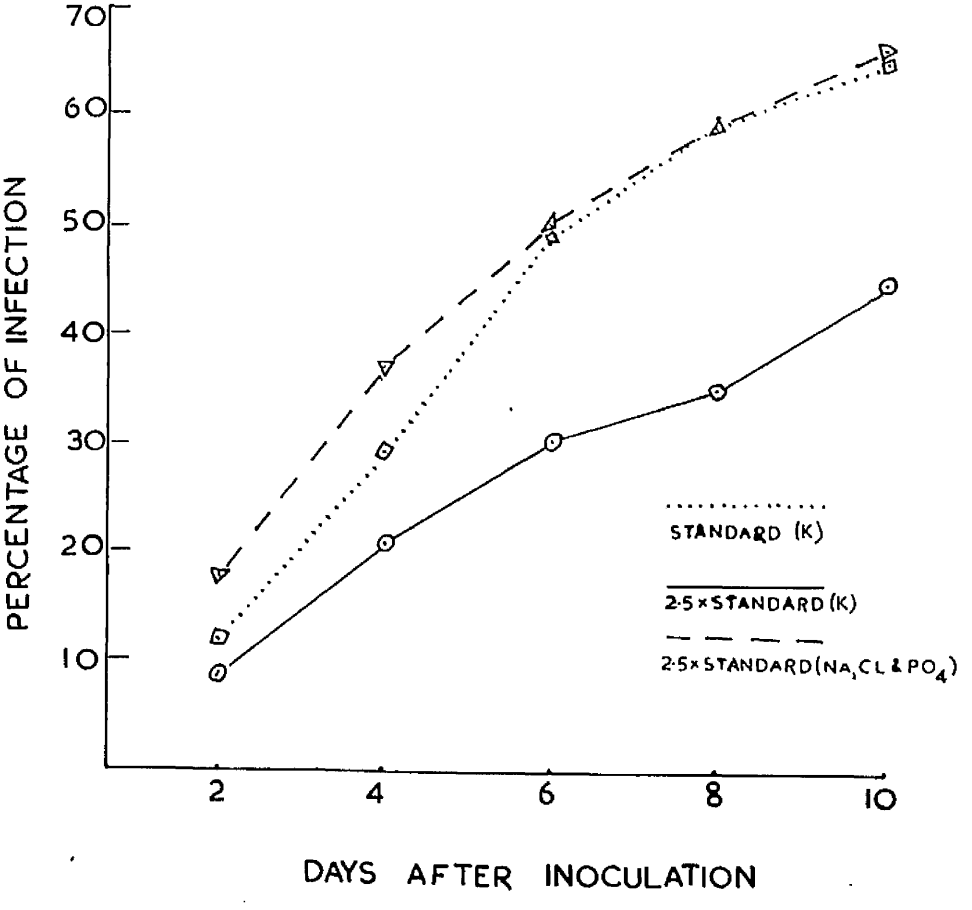
Graph III.

Table XLI.

The effect of increased potassium, sodium, chlorine and phosphate levels on disease developed.

		Level of potassium concentration 2.5 x K					Standard					Level of Na, Cl and Po_4 concentration x 2.5				
Days after inoculation		2	4	6	8	10	2	4	6	8	10	2	4	6	8	10
Exp. 3	Plate 1	7	11	17	18	22	3	7	11	12	12	6	11	13	15	16
	" 2	2	5	5	7	12	4	8	13	17	17	9	14	17	20	22
	" 3	3	7	9	12	13	7	15	25	28	30	9	17	23	26	26
	" 4	5	7	12	15	19	7	11	18	19	24	7	16	20	25	25
	" 5	5	7	9	9	12		9	1	16	18	7	11	15	20	21
Total		22	37	52	61	78	25	50	81	92	101	38	69	88	106	110
Exp. 4	Plate 1	4	11	13	16	18	1	5	11	16	17	4	10	14	16	18
	" 2	0	3	5	5	9	3	4	8	9	12	6	14	14	17	20
	" 3	0	3	4	4	6	3	11	18	24	27	0	1	4	4	8
	" 4	1	6	11	12	15	3	10	18	22	24	3	7	11	14	17
	" 5	1	3	5	6	9	2	6	13	13	15	3	10	17	19	24
Total		6	26	38	3	57	12	36	68	84	95	16	42	60	70	87
Total of Exps. III & IV.		28	63	90	104	135	37	86	149	176	196	54	111	148	176	197
Percentage of infection		9	21	30	35	45	12	29	50	59	65	18	37	49	59	66

THE EFFECT OF INCREASED POTASSIUM, SODIUM, CHLORINE AND PHOSPHATE LEVELS ON DISEASE DEVELOPMENT.



Graph IV.

Analysis of the results on the second day in Table XL shows that variation in potassium level was associated with a significant difference in the amount of disease developed in experiment 2 (variance ratio = 7.8) but that the differences in experiment 1 were not significant (variance ratio = 2.66, required value at 0.05 significance level 4.5).

The total differences between treatments for the two experiments are significant however, (variance ratio = 2.52) and it is noted that the residual variance in experiment 1 was almost double that in experiment 2.

Analysis of the results on the second day in Table XLI shows that neither treatment caused a significant difference in disease development from that on the standard medium. Empirical observations of the results for subsequent days show that in Table XLI the differences found in the early days after inoculation rapidly decreased, and that there was little apparent difference after 5 days development.

Study of the nature of the effect of potassium on disease resistance.

(b) Study of the rate of penetration of the cell wall and of the cell cavity by hyphae.

During the above experiments a microscopic study was made of the rate of penetration by hyphae into and across the cells of prothalli growing on standard ; 100 potassium and 2.5 x standard potassium media. The results are given in Tables XLII and XLIII.

Table XLII.

Rate of movement of hyphae through host tissues growing in media differ in the amount of potassium.

Average distance travelled per minute by hyphae within cell cavity

Hypha No.	1	2	3	4	5	6	7	8	9	10
Standard/100 K	42.6	60.8	55.0	65.7	38.7	48.4	47.8	61.9	66.6	69.2
2.5 x K	43.3	54.5	50.0	72.5	48.8	46.8	47.3	64.7		

The figures are arbitrary divisions of an eyepiece micrometer scale in which 1 division = 4.18 .
Analysis of these figures shows that the differences found are not significant (variance ratio= 1.06, required value at 0.05 significance level = 3.4).

Table XLIII.

Rate of Movement of hyphae through host tissues growing in media differ in the amount of potassium.

Average time in minutes taken to penetrate a cell wall

Hypha No.	1	2	3	4	5	6	7	8	9	10	11	12
$\frac{\text{Standard}}{100}$ K	9	8	10	7	10	12	9	11	7	7		
2.5 x K	15	16	15	18	15	13	20	18	16	12	22	16

Analysis of these figures shows that the differences found are nearly significant (variance ratio = 2.93, required value at 0.05 significance level = 3.1).

Study of the effect of cell sap from plants grown on media containing different amounts of potassium ion on mycelial growth of B. cinerea.

This test was carried out to see whether the increased resistance of plants grown with high level of potassium in the medium was due to the accumulation of potassium or to some stable metabolite in the cell sap. Thirty plants grown on each medium were ground up and the emulsion twice centrifuged to remove all cell wall material and the supernatant juice transferred to a clean sterile tube. Drops of each kind of juice were then placed on clean

sterile glass slides, spores of a 10 days old culture of Botrytis cinerea added to the drops, and the spore suspension so made was spread evenly over the surface of the slide. The slides were placed on stands in petri dishes containing water and incubated at 20°C. Four slides of each medium were set up and the amount of growth measured after 12 hr., 18 hr. and 24 hours incubation. The results are given in Table XLIV.

Spores germination was rapid and almost 100% on all slides and it was thought by empirical observation that any differences were so slight as to be negligible and not justifying specific measurements.

Table XLIV.

The effect of cell sap from plants grown on media containing different amounts of potassium ion on growth of germ tubes.

Average length of germ tubes based on at least 50 counts

Potassium level		Standard 100	Standard 10	2.5 x Standard	Standard	Water
Period of Incubation	12 hr.	28 μ	34 μ	34 μ	28 μ	27 μ
	18 hr.	52 μ	62 μ	92 μ	54 μ	67 μ
	24 hr.	80 μ	104 μ	138 μ	103 μ	100 μ

Analysis of the results at 24 hours showed that increase in the potassium ion concentration was associated with a significant increase in mycelial growth (variance ratio = 5.04).

Examination of the effect of variation of concentration of nitrogen as nitrate on disease development.

Experimental.

Loomis and Shull modification of Knop's formula was used as a basic medium and the amount of nitrate ion etc. modified as shown below (Table XLV).

Table XLV.

Formulae of mineral media used to study the effect of variation in the level of nitrogen concentration.

Media	A	B	C	D
NO_3 level	Standard 100	Standard 10	Standard	10 x Standard
	Concentration in gram per litre			
K Cl	6.0	6.0	6.0	6.0
$K H_2 PO_4$	6.0	6.0	6.0	6.0
$Mg SO_4 7H_2O$	9.0	9.0	9.0	9.0
$Ca(NO_3)_2 4H_2O$	0.1	1.0	10.0	10.0
$CaSO_4 2H_2O$	7.2	6.6	-	-
Ferric tartrate	0.6	0.6	0.6	0.6

The amounts were chosen for the same reason as in the potassium investigation, and Ca So_4 was used in medium A and B to maintain the Ca level.

Prothalli were grown and inoculated on the various media and disease records were taken as described in the potassium investigation. Two replicate experiments were set up and the results are recorded in Table XLVI.

Table XLVI.

The effect of variation of concentration of nitrogen as nitrate on disease development.

	Days after inoculation	Standard : 100								Standard ; 10								Standard								10 x Standard							
		1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10	1	2	3	4	5	6	7	10
Exp. 5	Plate 1	5	7	21	30	30	30	30	30	0	6	23	30	30	30	30	30	3	16	23	29	30	30	30	30	1	4	9	10	12	14	18	30
	" 2	1	4	8	11	17	29	30	30	2	4	15	25	29	30	30	30	5	13	25	30	30	30	30	30	6	13	21	26	28	30	30	30
	" 3	7	12	24	28	30	30	30	30	2	6	15	20	20	20	20	20	5	10	19	24	25	28	30	30	4	12	24	28	30	30	30	30
	" 4	6	15	22	28	30	30	30	30	2	7	22	30	30	30	30	30	1	6	15	19	23	25	25	25	5	11	22	25	25	25	26	30
	" 5	1	6	21	27	29	30	30	30	2	13	26	28	30	30	30	30	1	5	14	29	30	30	30	30	6	11	25	30	30	30	30	30
Total		20	44	96	124	136	149	150	150	8	36	101	133	139	140	140	140	15	50	96	131	138	143	145	145	22	51	101	119	125	129	134	150
Exp. 6	Plate 1	1	8	25	30	30	30	30	30	2	4	23	28	30	30	30	30	2	5	14	17	21	22	26	29	4	9	13	21	26	26	28	30
	" 2	2	9	19	23	26	30	30	30	3	7	15	26	27	27	28	30	0	7	18	24	25	27	28	30	2	13	23	25	25	25	27	30
	" 3	4	7	20	24	25	26	28	30	1	2	11	16	19	20	23	28	2	4	11	18	20	20	20	24	0	2	18	20	24	25	26	29
	" 4	2	3	18	23	27	30	30	30	2	3	7	14	15	15	17	19	0	2	4	7	9	13	17	18	2	13	16	24	26	28	30	30
	" 5	1	3	12	22	30	30	30	30	3	6	13	20	25	25	28	30	3	7	11	21	26	26	30	30	4	10	17	21	22	28	30	30
Total		10	30	94	122	138	146	148	150	11	22	69	104	116	117	126	137	7	25	58	87	101	108	121	131	10	46	87	111	123	133	141	149

Growth was poor on media A and B, the plants being pale or yellow in colour. Growth appeared normal and similar in media C and D.

Analysis of the results shows that the differences seen in amount of disease development in plants grown in various nitrate levels were not significant.

Discussion.

It is noted that plate to plate variance and experimental error was high in several of the experiments and it is possible that examination of larger samples under more rigorous experimental conditions might demonstrate differences which were not shown in these. There has not been time to carry out such experiments yet and it is therefore thought that firm conclusions should not be drawn from the records that are available. The results of the examination of the variations in potassium levels show that in one experiment decrease of potassium was associated with a significant increase in disease development and the differences in the other experiments were similar although not significant.

Empirical observations of the results of histological study suggest the probability of this effect being associated with a difference in the rate of penetration of the wall, which might attain a significant level with study of large samples. The results of the study of spore germination and growth in extracts of prothalli grown on various potassium levels show that reduction in disease in plants grown on a higher level of potassium is not due to a simple inhibition of fungal growth by potassium concentration in the host cells, as the cells extracts for the higher levels stimulate fungal growth in vitro.

Similarly although the results of examination of the effect of variation in nitrate levels appear to be consistent and to show that it has no significant effect on the amount of disease development in the conditions examined, further work in more rigorous conditions needs to be done before firm conclusions can be drawn.

Summary

This thesis describes a series of investigations into the mycological problems affecting the prothalli of Pteridium aquilinum Kühn.

Part I

Study of problems concerning the growth of prothalli in pure cultures

(1) A comparative study of several methods of spore disinfection has shown that immersion in 0.1% aqueous mercuric chloride for one minute gives satisfactory results, which are as good as those given by the more elaborate treatments used by previous workers.

(2) Previous investigations of the rapid fall of spore viability with age have been confirmed. It has also been shown that freshly gathered Bracken spores can germinate and give normal prothalli in a pure culture on a mineral medium. After a period of storage, however, these spores will not germinate in pure culture, and in many cases those which do germinate give rise only to very small prothalli.

(3) It has been shown that in the presence of contaminants in the cultures older spores frequently germinate

normally, and the prothalli which they form develop to normal size. The greatest stimulation to germination and normal growth had been found in cultures containing a mixture of natural contaminants, but the introduction of pure cultures of certain fungi into pure cultures of bracken prothalli produced a similar stimulation. Aspergillus niger produced the greatest stimulation of the species tested.

(4) A preliminary study of the stimulating effect on the growth of prothalli of Aspergillus niger has shown that it is due to a fungal metabolite which can diffuse through cellophane but that its activity can not be demonstrated by the use of a sterile filtrate extract from the fungal culture.

Part II

General survey of the pathology of prothalli

(1) Pathogens found in nature : Only two actively pathogenic species of fungi have been found in the examination of many hundred cultures of contaminated bracken spores on sterile and on natural media. A diagnosis and description is given of one of these as a new species.

(2) The effect on prothalli of a wide variety of common pathogens of higher plants has been examined by inoculation experiments in pure culture. The prothalli were attacked by the great majority of rot, mould and damping-off fungi, but not by those causing wilt and certain other diseases. The strains of these latter fungi were found to be virulent when inoculated onto cress seedlings.

Part III

Detailed Study of the Pathology of B. cinerea Pers.

The pathology of this fungus was chosen for detailed study because of the extensive knowledge which we have of its effect on other hosts.

(1) A preliminary study of the cultural conditions has been made to determine those most suitable for the production of the large number of Botrytis spores required for inocula[illegible]. This has shown that the optimum temperature for sporulation of the strain is approximately 20°C., that sporulation is much reduced by growth in temperatures slightly above the optimum, and that it is most abundant on media with a relatively low carbohydrate content.

(2) A detailed morphological and histological study of the attack of the fungus on the host has been made.

(3) A physiological study of the mechanism of attack by the fungus has been made. Repetition of certain experiments of Brown (1915) on the activity of protopectinase has shown that while this causes the major part of the disorganisation of the host, some disorganisation is also due to the action of a second fungal metabolite which has not been described previously.

(4) It has been shown that the prothalli are generally readily susceptible to infection, but that in a few cases inocula do not cause infection, and in other cases the spread of the fungus remains localised in a limited area around the site of original infection.

(5) It has been shown that the failure of infection by inocula is frequently overcome by reflooding the inoculum with fresh nutrient solution, and results suggest that the initial failures are due to a slight variation in the inoculum rather than to variation in host resistance.

(6) It has been shown that the localization of infection is due to changes in the host cell walls in the areas immediately surrounding the lesion. These changes are caused by the action of some fungal metabolite on the living cells of the host, and are not incited by mechanical damage in sterile conditions, not by

the action of the metabolite on dead host cells.

Part IV

The effect of variation in external conditions on disease incidence

(1) Previous work on the effect of variation in spore density and of concentration of nutrients in the inoculation drop has been confirmed, it being shown that increases or decreases from a certain optimum spore density both cause a reduction in disease incidence, and it is also shown that increase in nutrition in the inocula was associated with increase in the amount of disease developed.

(2) It has been shown that variation in the period of illumination during growth has a significant effect on disease incidence. A control experiment has shown that the variation in period of illumination has an effect on the morphology and physiology of the fungus, but that this is not the cause of the difference in disease resistance.

(3) Experiments to examine the effect of variation in potassium content of the media have been carried out. Significant differences in disease development with different potassium levels were found and histalogical study of the living hyphae growing within the host

suggested that this effect was due to variation in rate of penetration of the cell walls. No significant differences in disease development were found on plants grown on differing concentrations of nitrate. The experiments on the effect of these factors have been on a limited scale and the experimental error was high. It is thought that larger scale experiments under more rigorous conditions should be carried out before firm conclusions can be drawn.

References.

Ainsworth, G.C., Oyler, E. and Read, W.H. (1938). "Observations on the spotting of tomato fruits by Botrytis cinerea Pers." Ann. Appl. Biol. 25 : 308-321.

Alten, F. (1940). "Der Einfluss der Ernährung auf den Stickstoffhaushalt der Pflanzen". Qualitatsforschung. 45-59 Illus.

Anderson, E.J. (1951). "Effect of nutrient salt concentrations on infection of pineapple roots by Phytophthora cinnamomi Rands." Phytopath. 41:2.

Barnett, H.L. and Lilly, V.G. (1950). "Nutritional and environmental factors influencing asexual sporulation of Choanephora cucurbitarum in culture". Phytopath. 40 : 80-89.

Beaumont, A. (1933). "Chocolate spot of bean". Ann. Rep., Dept. Plant Path. Scale Hayne Agric. Coll. 1931-2, p.21.

Behrens, J. (1898). "Beitrage zur Kenntnis der Obstfäulnis". Centralbl. f. Bakt., Abt. 2, Bd. IV.

Blackman, V.H.P. (1924). "Physiological aspects of parasitism". Proc. Brit. Assoc. adv. Science. Section K. Toronto 235-246.

Blackman, V.H.P. and Welsford, E.J. (1916). "Studies in the Physiology of Parasitism" ii. Infection by Botrytis cinerea. Ann. Bot. 30 : 389-398.

Boyle, C. (1921). Studies in the Physiology of Parasitism VI. "Infection by Sclerotinia libartiana". Ann. Bot. London, 35 : 337-347, illus.

Brooks, C., and Cooley, J.S. (1917). "Temperature relations of apple rot fungi". Jour. Agr. Res. 8 : 139-164.

Brown, W. (1915). "Studies in the Physiology of Parasitism". I. The action of Botrytis cinerea Pers. Ann. Bot. 29 : 313-348.

Brown, W. (1922). a."Studies in the Physiology of Parasitism". VIII. On the exomosis of nutrient substances from the host tissue into the infection drop. Ann. Bot. 36 : 101-119.

Brown, W. (1922). b."Studies in the Physiology of Parasitism". IX. On the germination and growth of fungi at various temperatures and in various concentrations of oxygen and of carbon dioxide. Ann. Bot. 36 : 256-283.

Brown, W. (1936). "The physiology of host-parasite relations". Bot. Rev. 2 : 236-281.

Brown, W., Brooks, F.T. and Bawden, F.C. (1948). "Physiology of disease resistance in plants." Nature, London. 161 : 422-424.

Brown, W., and Harvey, C.C. (1927). "Studies in the Physiology of Parasitism". X. On the entrance of Parasitic fungi into the host plant. Ann. Bot. 41 : 648-662.

Busgen, M. (1893). "Über einige Eigenscheften der Keimlinge parasitischer". Pilze. Bot. Zeit. 51 : 53-72.

Butler, E.J., and Jones, S.G. "Plant Pathology". London, i-xii p. 979.

Castle, H. (1953). "Notes on the development of the Gametophyte of Equisetem arvense in sterile media". Bot. Gaz. 114 : 323-328.

Chen, H.K. and Thornton, H.G. (1940). "The structure of ineffective nodules and its influence on nitrogen fixation". Rec. Roy. Soc. B. 129 : p.208-229.

Conway, E. (1949). "The Autoecology of the Bracken (Pteridium aquilinum Kühn)". The germination of the spore, and the development of the prothallus and the young sporophyte. Pro c. Roy. Soc. Edin. B. 13 : 325-343.

Coons, G.H. (1916). "Factors involved in the growth and the pycnidium formation of Plenodmus fuscomaculous". Jour. Agr. Res. 5 : 713-769.

Cunningham, H.S. (1928). "A study of the histologic changes in leaves induced by certain leaf spotting fungi". Phytopath. 18 : 717-751.

De Bary, A. (1886). "Über einige Sclerotinien und Sclerotienkrankheiten". Bot. Zeit. 44 : 377-387, 393-404, 409-426, 433-441, 449-461, 465-474.

Delecroix, G. (1905). "Observations et cherches au sujet de la "pouriture grise", etc." Bull. Mensuel de Renseign. Agricoles. April 1905.

Dey, P.K. (1919). "Studies in the physiology of parasitism". V. Infection by Colletotrichum lindemuthianum. Ann. Bot. 33 : 305-312.

Dufrenoy, H.S. (1936). "Cellular immunity". Amer. Jour. Bot. 23 : 70-79.

Fahmy, T. (1931). "The sore-skin disease and its control". Egypt. Min. Agr. Plant. Prot. Sect. Bull. 108.

Gassner, G. and Franke, W. (1934). "Über den Einfluss der temperaturen auf Stickstoffgehalt und Rostresistenz junger Getreidepflanzen. Phytopath. Zeit. 7 : 315-326.

Gaumann, E. (1950). Principles of plant infection. London. I - xvi, pp. 543.

Glynne, M.D. (1934-1936). "Fungus and other diseases at Rothamsted and Woburn". Roth. Exp. St. Rep. 1935, 82-86.

Grainger, J. (1949). "The Tithe of plant disease". Trans. Royal High. and Agric. Soc. of Scotland. 61 : 56-76.

Hawkins, L.A. and Harvey, R.B. (1919). "Physiological study of the Parasitism of Pythium debaryanum Hesse. on the Potato tuber" Jour. Agr. Res. 18 : 275-297.

Higgins, B.B. (1927). "Physiology and parasitism of Sclerotium rolfsii. Sacc." Phytopath. 17 : 417-448.

Houston, B.R. and Oswald, L.W. (1946). "The effect of light and temperature on conidiumproduction by Helminthosporium gramineum Rab. in culture". Phytopath. 36 : 1049-1055.

Hurel-Py (1942). "Etude de quelques milieux permettant la culture illimitee d'un Prothalli d'Asplenium". Comp. Ren. de L'AC. des Sci., t, 214 : 571-573.

Johansson, D.A. (1940). "Plant Microtechnique". New York and London. i - xi, pp. 523.

Keyworth, W.G. and Hewitt, E.J. (1948). "Verticillium wilt of the Hop (Humulus lupulus)". V. The influence of nutrition on the reaction of the hop plant to infection with Verticillium albo-atrum. Jour. Hort. Sci. 34 : 219-227.

Kiesling, E. (1889). "Zur Biologie der Botrytis cinerea". Hedwigia. 28 : 227.

Knudson, L. (1925). "Physiological study of the Symbiotic Germination of Orchid seeds". Bot. Gaz 74 : 345-79.

Kundson, L. (1940). "Permanent changes of chloroplasts induced by X-rays in the Gametophyte of Polypodium aureum". Bot. Gaz 101 : 721-758.

Leach, J.G.(1923). "The parasitism of Colletotrichum lindemuthianum". Minn. Agr. Exp. Sta. Tech. Bul. 14, 41, illus.

Loomis, W.E. and Shull, C.A. (1937). "Method in plant Physiology". New York. I - xviii, pp. 742.

Marshal, E. (1901-3). "Recherches de biologie experimentale appliquc a l'agriculture". I.

Morel, G. (1950). "Sur la culture des tissue d'Osmundo cinnamomea. Comp. Ren. de l'Ac. des Sci. Paris, 230 : 2318-2320.

Morel, G. and Wetmore, R.H. (1951). "Fern callus tissue cultures. Amer. J. Bot., 38 : 1-31, 37-67.

Naghski, J., Harris, R.G., Haley, D.E. and Reid, J. (1939). "Plant nutrition and disease resistance". Jour. Bact. 38 : 234-235.

Nelson, K.E. (1951). "Some histological aspects of Botrytis rot of table grapes". Phytopath. 41 : 941.

Newton, W., and Hastin, R.J. (1931). "Botrytis tulipae (Lib) Lind. 'The production of conidia as influenced by various factors". Scient. Agric. 12 : 820-824.

Nobecourt, P. (1946). "Les mechanisms de l'immunite naturelle chez les vegetaux". Rev. Internat. Bot. Appl. et Agric. Trop. 26 : 529-542.

Paul, W.R.C. (1929). "A Comparative Morphological and Physiological Study of a number of Strains of Botrytis cinerea with special reference to their virulence". Trans. Brit. Myc. Soc. 14 : 118-135.

Pearson, N.L. (1931). "Parasitism of Gibberella saubinettii on Corn seedlings". Jour. Agric. Res. 43 : 569-596.

Petri, L. (1930). "Relation of mineral nutrients of plants to predisposition or resistance to pathogenic agencies". Boll. R. Staz. Patol. Veg. 10 : 121-152.

Pohjakallio, O., Laila, O. and Paasi, K. (1951). "Investigation into the Significance of Photosynthesis in Resistance to Plant Diseases". Maataloustieteellinen Arkakanskirja. 23 : 156-163.

Priston, R. and Gallegly, M.E. (1954). "Leaf penetration by Phytophythora infestans". Phytopath. 42 : 81-86.

Pryor, D.E. (1940). "The effect of some mineral nutrients on the development of clubroot of crucifors". Jour. Agric. Res. 61 : 149-160.

Ridgway, R. (1912). "Color Standards and color Nomenclature". Washington, i-iii pp. 43.

Schwabe, W.W. (1951). "Physiological studies of plant Nutrition". XVI. The mineral nutrition of Bracken. Ann. Bot. N. S. 15 : 417-446.

Scott Watson, J.A. (1936). "Notes on manuring". J. Minist. Agric. 43 : 178-181.

Sempio, C. (1950). "Metabolic Resistance to plant diseases". Phytopath. 40 : 799-819.

Shaw, L. (1934). "Studies on resistance of apple and other rosaceous plants to blight". Jour. Agr. Res. 49 : 283-313.

Simmons, S.A. (1949). "Research on Botrytis corn rot." Bull. N. Amer. Gladiolus Counc. 17 : 93-4.

Smith, R.E. (1902). "Parasitism of Botrytis cinerea". Bot. Gaz. 33 : 421-436.

Steeves, T.A., Sussex, I.M. and Pastanen, C.R. (1955). "In vitro studies on abnormal growth of Prothalli of the Bracken Fern". Amer. Journ. Bot. 42 : 232-245.

Stokey, A.G. (1930). "The prothallia of the Cyartheaceae". Bot. Gaz. 90 : 1-45.

Sussex, I.M. and Steeves, T.A. (1953). "Experimental and analytical studies of Pteridophytes". XXI. Sterile cultures of an abnormal growth from the Gametophytes of Bracken. Ann. Bot. N.S. 17 : 395-401.

Timnick, M.D., Lilly, V.G. and Barnett, H.L. (1951). "The influence of light and other factors upon Sporulation of Diaporthe phaseolorum var. batatatis". Phytopath. 41 : 327-336.

Tomas, H.E., and Ark, P.A. (1938-1939). "Some factors affecting the susceptibility of plant to fire blight". Hilgaidia 12 : 299-322.

Twiss, E.M. (1910). "The Prothallia of Aneimia and Lypodium". Bot. Gaz. 49 : 168-181.

Vavilov, N.I. (1949-1950). "Study of immunity of plants from Infectious Diseases". Chronica Botanica. 13 : 95-168.

Verdoorn, Fr. (1938). "Manual of Pteridology". Netherlands. I - xiv. pp. 640.

Wade, G.C. (1945-1947). "Botrytis Corn rot of the Gladiolus". Proc. Roy. Soc. Victoria N.A. 57-58; 81-124.

Walker, J.C. (1926). "Botrytis neck rots of onions". Jour. Agric. Res. 33 : 893-928.

Walker, J.C. (1946). "Soil management and plant nutrition in relation to disease development". Soil Sci. 61 : 47-54.

Walker, J.C. and Hooker, W.J. (1945). "Plant nutrition in relation to disease development". I. Cabbage Yellows. Amer. Jour. Bot. 32 : 314-320.

Ward, H.M.(1888). "A lily disease". Ann. Bot. 2 : 319-382.

Wilkie, D. (1954). "Ph. D. Thesis". Glasgow University.

Wilson, A.R. (1937). "The Chocolate Spot of Beans caused by Botrytis cinerea Pers". Ann. Appl. Biol. 24 : 253-289.

Wingard, S.A. (1941). "The nature of disease resistance in plants". I. Bot. Rev. 7 : 59-109.

Wood, R.K.S. (1953). "Personal communication". Imperial College.

Wood, R.K.S. (1955). "Studies of the Physiology of Parasitism". XVIII Pectic Enzymes secreted by Bacterium aroideae. Ann. Bot. N.S. 73 : 1-27.

Young, P.A. (1926). "Penetration phenomena and facultative parasitism in Alternaria diblodia and other fungi". Bot. Gaz. 81 : 258-279.

Yu, T.F. (1938). "A blossom blight of broad bean (Vicia faba) caused by Botrytis cinerea". Lingnan Scie. Jour. 17 : 551-566.

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