SOME EFFECTS OF FUNGI ON BRACKEN PROTHALLI

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Some Effects of Fungi on Bracken Prothalli.

Summary

The first part of the thesis reports the development of techniques for the study of interclonal variation in susceptibili of prothalli to attack by Botrytis cinerea. This involved the establishment of suitable conditions for growth in aseptic culture the establishment of clonal populations by proliferation of protha and the establishment of consistent inoculation and disease-record techniques.

The second part of the thesis reports the study of the inter-clonal variation in resistance. This was shown to be associated both with resistance to infection, and with resistance to spread of the disease from infected sites.

The third part of the thesis reports a detailed study of some aspects of the host-pathogen relationship of prothalli of two selected clones which differed in resistance to the fungus. A detailed study of the morphology of infection in both clones was made. This gave some indication that the differences were likely to be associated with the reaction of the walls of the cell The effects of aqueous extracts of uninfected and infected cells of both clones on the germination of the spores and the growth of the germ tubes of the pathogen were studied. No evidence of any differences in reaction to cell extracts was seen. This, of course does not discount the possibility of the existence of some difference in the intact, living cytoplasm. The reaction of the wall

to the enzyme, protopectinase, produced by the fungus *in vitro* was investigated but no differences were found between the clones which differed in susceptibility to the pathogen. A study of the nature of the cell wall by electron microscopy was carried out. This part of the work provided the first known record of the presence of ectodesmata in bracken prothalli and their possible implication in the infection process is discussed. New informati of the detailed reaction of the wall is given, but no differences which could be consistently related to the variation in resistanc were seen.

CONTENTS

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INTRODUCTION

INTRODUCTION

The gametophyte of bracken (*Pteridium aquilinum* Kühn) has been shown to be a useful tool in the study of the interaction of fungi and green plant cells (Hutchinson and Fahim, 1958). The prothalli are relatively simple, almost undifferentiated flat plates of green cells with cellulose walls and they can be readily grown under controlled, aseptic conditions in the laboratory. The single layer of cells makes it possible to study the intracellular reactions of the host and parasite by direct observation of the living material.

Clonal populations can be obtained from single-spore cultures and these clones may be maintained indefinitely. This thesis is concerned with the study of the susceptibility of different clonal populations of prothalli to attack by the fungus, *Botrytis cinerea* Pers., and the study of the nature of the variation in susceptibility of these populations.

The investigation falls into three convenient parts:-

1) The establishment of clonal populations and the development of certain methods used in the second part of thesis.
2) The demonstration of interclonal variation in susceptibility to attack by *B. cinerea*.

3) The analysis of the nature of the interclonal variation in resistance.

PART ONE

METHODS

INTRODUCTION

This part of the thesis describes the development of the methods used in Part Two and comprises

1) The study of cultural conditions and methods for obtaining clonal populations.

2) The development of a reproducible inoculation technique.

3) The design and testing of a method of recording the disease intensity.

CULTURE METHODS

THE GROWTH OF PROTHALLI IN CULTURE

Introduction and literature review

Prothalli of many different species of fern have been grown both in mixed culture and in pure culture. The methods of the previous workers have been studied in order to determine the methods most suitable for the investigations to be carried out in this thesis.

The early work on prothallial growth in culture was carried out using natural media, for example Campbell (1892) used unsterilized damp soil for the culture of prothalli of Osmunda spp. Mottier (1927) reviewed some of the earlier work on the growth of prothalli of various fern species on different media and concluded (1924) that contamination was a troublesome factor in long-term cultivation of prothalli on sterilized soil. His cultures contained algal, moss and fungal contaminants. Conway (1949) also experienced contamination when using sterilized soil in the form of upturned, rotted grass sods for the growth of prothalli of Pteridium aquilinum. Blue-green algae appeared as contaminants in cultures of Plagiogyria spp. on various media even after washing the fronds vigorously with a dilute solution of copper sulphate at the time of collection (Stokey

and Atkinson, 1956).

Growth on mineral media under aseptic conditions has been studied. Hurel-Py (1950) has reviewed some of this work and has concluded from her own investigations that germination was more rapid in liquid media than on solid media, the most favourable for prolonged cultivation being sterile Knop's mineral medium plus 1 to 2 per cent glucose, solidified by the addition of two per cent agar. Sterile Knop's agar medium has been used by other authors with favourable results (Conway, 1949; Wilkie, 1954, 1956; and Hutchinson and Fahim, 1958), and the same medium without agar was used by Hepden (1960). Other media which have been used have included Moore's agar medium (Hepden, loc. cit. and Bell, 1958), Benecke's solution and Shive's three-salt solution, with or without agar (Charlton, 1938), and Knudson's agar medium (Steeves, Sussex and Partanen, 1955). Knop's solution was modified by the addition of dextrose, yeast extract and 0·8 or 0·5 per cent agar by Steeves et al (loc. cit.). Analysis of these records has shown that most authors have chosen any arbitrary medium which supported adequate growth for their purposes. Hurel-Py (1950) and Soussountzov (1948) made more calculated investigations of growth requirements and showed that some media were more favourable than others. Since the differences in growth of prothalli on the above media did not appear significant in relation to our requirements of maintaining an adequate

stock of prothalli for experimental purposes, and as Knop's agar has been found satisfactory in previous work in this department (Hutchinson and Fahim, 1958), it was used as the standard medium.

Methods

Spores of bracken (*Pteridium aquilinum* Kühn) were collected from several localities in Scotland. The spores were surface-sterilized before sowing by the method of Fahim (1955). This consisted of presoaking the spores in distilled water at 24°C for about 18 hours and after removing the spores by centrifuging, agitating them for one minute in a 0·1 per cent solution of mercuric chloride. This method gave adequate control of surface contaminants without adversely affecting the germination of the bracken spores.

For maximum germination, spores of the current season were used for sowings. Spores were germinated and prothalli grown on a modified Knop's agar medium of the following formula.

KCl	0·60 g.
$MgSO_4$ $7H_2O$	0·90 g.
$Ca(NO_3)$ $4H_2O$ ...	1·00 g.
KH_2PO_4	0·60 g.
$NaNO_3$	0·36 g.
Ferric Tartrate ...	0·06 g.
agar-agar	20·00 g.
distilled water ...	1000 ml.

The medium was autoclaved at 15 pounds per square inch pressure for 20 minutes.

Spore germination was carried out on the above medium contained in Petri dishes. The decontaminated spore suspension was spread over the surface of the agar, the density of the suspension having been adjusted so that on germination the prothalli were spaced sufficiently far apart over the agar to facilitate the removal of single prothalli.

The culture apparatus consisted of a thermo-controlled room where the temperature was maintained at 20°C. The cultures were accommodated on shelves, each of which had a light source of two 40 watt, warm white, reflector-type fluorescent lamps (Plate 1). These were 4 feet long and were placed 30 cm apart and 20 cm above the glass shelf. The effect of heat from the lights was minimised by good ventilation and the fitting of a heat filter, in the form of a glass strip, in front of each tube. The cultures were illuminated for fifteen hours daily. A slight decrease in light intensity occurs towards the ends of the fluorescent lamps and so all prothallial cultures were placed on the shelves between two points about 20 cm from each end of the lamps. Under the conditions described, condensation did not usually collect on the inside of culture containers.

Germination took place in the manner described for other fern species (see Hurel-Py, 1950 for description, and Steeves, Sussex and Partanen, 1955 for bracken in particular), and a good proportion of the Petri dish cultures remained

PLATE 1

The apparatus used in prothallial culture.
(x 1/6)

sterile for about two months. For longer-term culture without airborne contamination, the prothalli were placed in one ounce, wide-necked McCartney bottles containing sloped Knop's agar medium. The cap of each bottle had been bored with a 20 mm diameter hole, and covered with a double layer of surgical lint (Plate 2). This cap permitted gaseous exchange to take place freely between the interior and exterior of the bottle without permitting contamination and was more convenient in use than a cotton wool plug.

PLATE 2

Standard lint-capped culture bottle.
(x 1)

PROLIFERATION OF PROTHALLI AND THE FORMATION OF CLONAL POPULATIONS

Introduction and literature review

Proliferation to form colonies was first observed by Hofmeister (1851) in Notochlaena and since then it has been reported in many genera. Mottier (1927) described the formation of lateral shoots or proliferations which developed both on the margins and in the growing point region at the apical sinus of young and old prothalli of Osmunda claytoniana and Matteuccia nodulosa. Campbell (1892) had found similar prothallial proliferations in his studies of the Osmundaceae. Both Mottier and Campbell reported the production of sex organs on these adventitious shoots. Albaum (1938b) has reviewed the earlier work of Heim, Goebel, Linsbauer and others who found that prothalli lacking an apical region developed adventitious sprouts. Prothalli which were cut in various ways formed proliferations polarized in their origin, tending to appear close to the cut surface and toward the apical notch (Albaum, 1938a). The same author (Albaum, 1938b) has stated that intact prothalli grown under controlled conditions of light, temperature and humidity, never gave rise to adventitious outgrowths. The conditions under which they arose were all abnormal. Other authors, however, (Steeves, Sussex and

Partanen, 1955) found that individual prothalli gave rise to colonies when transferred to fresh medium. Stokey and Atkinson (1956) found proliferations on prothalli growing on peat and just over one year old. After 15 months the regenerating prothalli had grown all over the ventral surface, the dorsal surface and the margins. The young prothalli arose from green healthy tissue and fell off easily on handling. Hurel-Py (1950) obtained prothallial colonies from single-spore cultures by removing single prothalli from a liquid germination medium, and placing them on the same medium solidified by the addition of agar, proliferation occurring on the solid medium.

The maintenance of clonal populations of prothalli by removing pieces of prothalli and placing them on fresh culture medium has been recorded (Hurel-Py, 1950; Steeves, Sussex and Partanen, 1955).

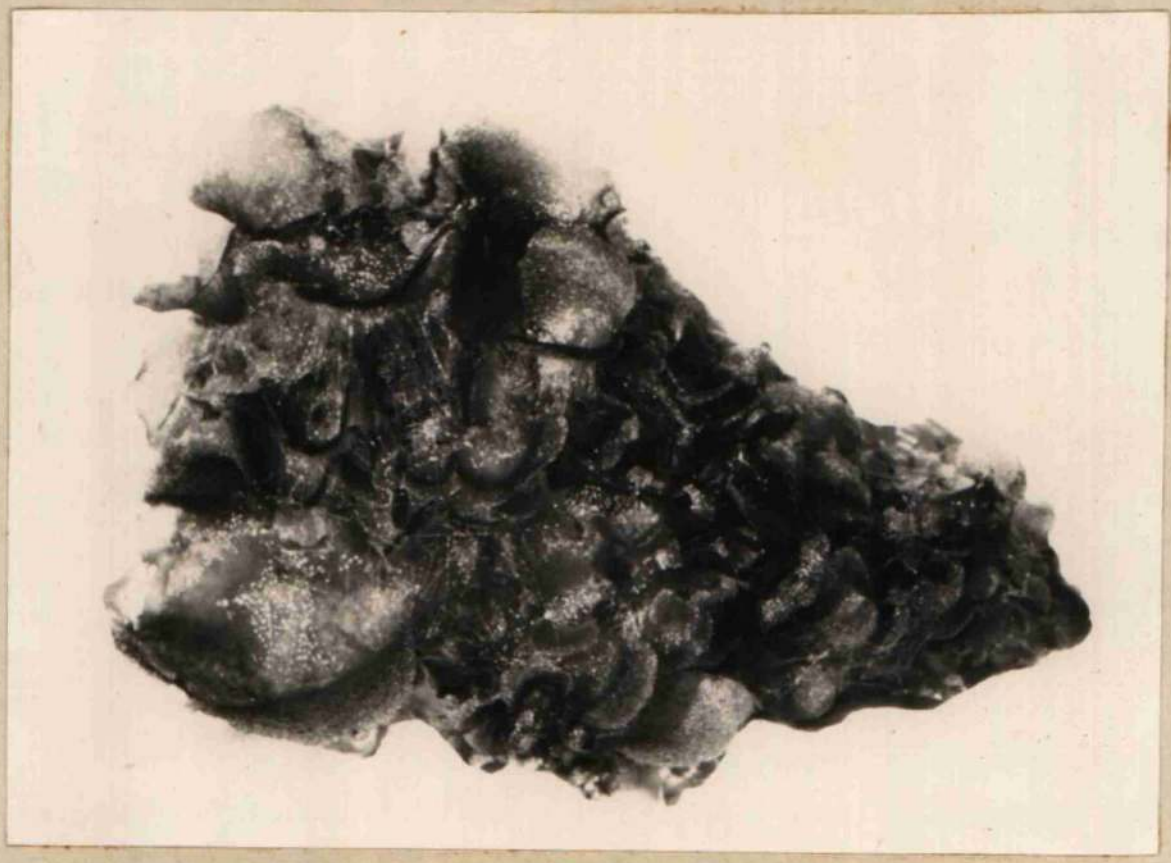
The proliferations described above have resulted in the formation of colonies of normal prothalli. Abnormal types of proliferation have also been reported in cultures of fern prothalli, however, (Morel and Wetmore, 1951; Steeves and Sussex, 1952; Sussex and Steeves, 1953; Steeves, Sussex and Partanen, 1955; and Partanen and Steeves, 1956).

Methods

To establish clonal populations, single prothalli were removed from the Petri dishes and each was placed on Knop's agar in the standard McCartney bottles with the special tops described above (cf. Hurel-Py, 1950). Such cultures were kept in the thermo-controlled room under the same conditions as the Petri-dish cultures.

A single prothallus left under the standard culture conditions for two to three weeks developed proliferations without requiring to be cut or damaged (cf. Albaum, 1938b) or placed on fresh medium (cf. Steeves et al, 1955 and Hurel-Py, 1950). At first the young prothalli were formed mainly in the apical indentation, and around the edges of the wings. As the young prothalli enlarged and developed rhizoids, they became free from the parent prothallus and lay on its surface, closely attached to each other by their rhizoids. The original prothallus gradually became very large and very irregular in shape and small proliferations arose over its upper surface. These also enlarged and the bottle eventually contained a colony of prothalli of various ages and sizes and in most cases irregularly-shaped by mutual pressure (Plate 3). As reported in other species (Campbell, 1892; and Mottier, 1927) the prothalli in such colonies have normal sex organs. From the original

PLATE 3



A colony of prothalli derived
from a single prothallus. (x 5)

selection of single prothalli removed from the Petri dishes, it was noted that some individuals were more prolific in the production of adventitious growths than were others. These freely-proliferating prothalli were selected to establish clonal populations. At intervals the colonies were divided by sterile dissection, the daughter prothalli being transferred to fresh medium where they gave rise to further daughter prothalli by the same process. In this way, therefore, clonal populations of prothalli were established.

The prothalli in stock cultures of clones maintained in this way are irregular in shape owing to the crowded conditions of growth. To obtain more regularly-shaped prothalli for experimental work individuals between 0·5 mm and 3 mm across the wings were removed aseptically from colonies and placed on Knop's agar. Careful handling was required to avoid damaging these delicate prothalli and so the rhizoids only were gripped very lightly when the prothallus was removed and this ensured that the prothallial surface was undamaged. By using 20 ounce flat medicine bottles (Plate 4) containing Knop's agar medium, approximately 50 prothalli of several different clones could be grown together under identical conditions.

PLATE 4

Standard Method of maintaining stock cultures of experimental material.

(x ½)

INOCULATION TECHNIQUE

Introduction and literature review

A variety of methods has been used for the inoculation of plants with pathogenic fungi, the method in each case being dependent on the type of infection process involved. Where the pathogen is incapable of active penetration, it is necessary to introduce it into the host and various methods have been used (for examples see Moore, 1936; Vanderweyen, 1960b; and Poehlman, 1945). In cases where the pathogen enters the host directly through the cuticle, it is only necessary to place mycelium or a suspension of spores of the fungus on the host surface (examples given by Murakishi, Honma and Knutson, 1960; Podhradszky, 1961; and Deverall, 1960).

The concentration of spores in the infection drop has been shown to affect the infection of plants and Fahim (1955) has reviewed the literature concerned with this feature of infection. He confirmed Brown (1922) who found that in the case of Botrytis cinerea there was an optimum spore concentration for maximum infection with a falling off in degree of infection on either side of the optimum. More recently Deverall (loc. cit.) inoculated leaves of field beans with drops of spore suspensions of Botrytis cinerea

and B. fabae of pre-arranged concentrations and found that increasing the number of spores increased the probability of lesion development and spread. Deverall does not mention the presence of an optimum spore concentration. Vanderweyen (1960a) has stated that, when using artificial methods of inoculation, it is essential that the plants be given equal inocula so that the results of tests will be comparable.

A standard method of inoculum production has been used and is described below. Early experiments showed variation in the amounts of infection within clones and this variation was attributed to a) variation in the size of the inocula and b) the inability of drops to remain on the surface of some prothalli so that most of the spores ran off in the drops or dried out.

Methods

Preparation of inoculum and standardisation of spore concentration.

Boiling tubes containing 20 ml sloped potato extract in 2% agar were inoculated with a spore suspension of *Botrytis cinerea* Pers. and placed in the dark for 12 days at 20°C. These conditions encouraged good sporulation (cf. Fahim, 1955). The spore suspension was prepared by flooding a culture with 10 ml of sterile distilled water and freeing the spores by agitation of the tube. The spores were then separated by forcing the suspension through a finely-drawn Pasteur pipette. By means of haemocytomater counts, the concentration of spores in the suspension was determined and adjusted by suitable dilution with sterile distilled water in order to maintain a standard concentration between experiments. It was found, however, that the concentrations obtained in five of the six experiments recorded in the second part of this thesis were relatively constant without requiring dilution (range - $0{\cdot}81 \times 10^6$ to $1{\cdot}02 \times 10^6$ spores per cc of suspension). The relatively constant value obtained can be attributed to the standard conditions observed with regard to the inoculum preparation.

The standardisation of the spore concentration ensured that the results of different experiments could be

reasonably compared since other experimental conditions were kept constant.

Inoculation and Standardisation of drop size.

The spore suspension was delivered by means of a modified hypodermic syringe of 1 ml capacity, fitted with a threaded plunger which, when rotated, delivered the inoculum through a number 19 gauge needle. A fine degree of control could be exercised over the size of the inoculum, and the standard inoculum used was that resulting from one quarter turn of the plunger.

From visual observation of the drops as they were delivered, it was thought that some variation in size between successive drops was occurring, and to test for this variation, the following procedure was carried out. A series of 48 consecutive drops of distilled water was dispensed and each was weighed singly on a clean, dry coverglass using an electric balance sensitive to 0·1 mg. To prevent variation arising from evaporation, the time elapsing between the delivery of each drop was kept to a minimum (less than 30 secs.). Table I shows the weights of the 48 drops arranged in 4 columns corresponding to the 4 quarter turns of the plunger from which they were derived.

Table I

The weights in milligrammes of 48 consecutive drops of distilled water, delivered by a hypodermic syringe fitted with a screw plunger

Quarter Turn			
1	2	3	4
4·0	10·9	7·7	5·3
4·0	8·6	9·1	6·6
4·1	8·4	10·6	5·9
3·3	9·6	10·1	6·4
2·9	6·8	11·3	5·6
4·3	6·1	13·0	5·3
4·5	4·8	13·0	5·6
4·3	4·9	12·2	6·7
4·2	6·5	7·2	9·1
5·3	3·1	6·9	6·7
3·2	5·6	10·5	6·6
4·6	9·4	10·4	5·4

From empirical observation of the results of this table it can be seen that there is variation in drop size between successive drops from the syringe. The range is relatively large (2·9 to 13·0) and since the suspension normally contained approximately 1 x 10^6 spores per cc the numbers of spores delivered in the drops will range between approximately 2,900 and 13,000. In order to minimise the effect of inoculum variation on the amounts of disease shown by prothalli, it was decided to use inocula produced from the first quarter turn of plunger only and to discard the remainder since the least variation in drop size appears in those from the first quarter turn.

The variation occurring between prothalli of a single clone is negligible compared to that occurring between clones (Part Two) and since the inoculum effect is only part of the total small variation found, it has not been investigated in greater detail.

The fixing of the inoculum to the prothallial surface.

It was noted that when some prothalli were inoculated with an aqueous spore suspension from the syringe, the inoculation drops did not remain on the surface but ran off on to the agar block. In some cases drops ran off prothallial surfaces which were quite flat although other prothalli with

almost vertical wings allowed the drops to remain. This inability of the drop to remain on the surface may be attributed to the physical or chemical nature of the outer wall of the prothallial cells. In cases where this happened, such few spores which were left on the surface without an infection drop, dried out and no infection was shown by the prothalli even five or six days after inoculation. This feature occurred in prothalli of different clones, but was not a standard characteristic of any one clonal population. An investigation into the distribution of this character in the clones was not carried out as the apparently random distribution suggested that it was not a major factor in clonal resistance to infection.

The immediate problem concerned the fixing of the infection drop to the surface of the prothallus sufficiently long to enable infection to take place without the spores drying out or running off in the drop. It was decided to use a non-fungitoxic substance which would cause the drop to stick to the prothallus and so gelatine was chosen. A series of concentrations of gelatine in water was prepared and it was found that the lowest concentration which was in the gel state at 20°C was four per cent.

The above inoculation technique was modified by mixing the inoculum with an equal volume of sterile 8 per cent gelatine in the liquid state and then using it in the syringe

as before. The drops were delivered when the inoculum was almost a gel, and the very viscous drops stuck to the surfaces of the prothalli. While the gelatine affected infection, this factor was uniform throughout the experiments and hence it can be disregarded.

Conclusion

A reproducible inoculation technique has been developed and this has minimised the variation found in the earlier experiments. Variability in spore concentration between experiments is slight and Table I has shown that the drops from the first quarter turn of the plunger are less variable than from the other turns and so this series of drops was used. The use of gelatine in the infection drop enabled the spores to remain on the surfaces of all prothalli sufficiently long to germinate and be capable of producing infection.

MEASUREMENT OF DISEASE INTENSITY

Introduction and literature review

The recording of the results of the experiments carried out in the second part of this thesis requires that severity of disease in prothalli be expressed quantitatively, so that the comparative degrees of susceptibility of different clonal populations may be determined. Various authors have suggested methods of evaluating "disease intensity" and the method used in each case has depended upon the particular features of the host, pathogen and environment involved. Chester (1959) has dealt quite thoroughly with the literature and has reviewed the problems and methods of disease measurement in plants. In cases involving the total destruction of the plant, it is sufficient to know the percentage of diseased individuals in the population in order to get a measure of the plant's disease intensity. In other cases (including bracken prothalli infected with Botrytis cinerea), the amount of disease present varies with each plant so that the amount of disease in each must be determined by some method, and there are several ways of doing this (see Chester loc. cit.). The number of plants in each of several classes can be recorded, for example the classes may be chosen as 0-10%, 11-20% ..... to 91-100% diseased. Horsfall (1945) has

described a grading system which made use of the fact that the human eye can distinguish differences in area as small as 3%. His grades in per cent were 0, 0 to 3, 3 to 6, 6 to 12, 12 to 25, 25 to 50, 50 to 75, 75 to 87, 87 to 94, 94 to 97, 97 to 100 and 100. Descriptive scales separating the plants into 'light', 'moderate' and 'severe' disease categories have been used and sometimes the classes ascribed numbers, for example, 0, 1, 2 .... to 5 (Chester, 1959). Barnes (1960) compared two methods of estimating the severity of stalk rot of Corn (causal fungus, Giberella zeae) and found that visual estimation was as accurate as the quantitative method used.

The method used in this thesis involved the visual estimation of the amount of disease present in the individual prothallus and is described below. A test of the accuracy of the method is also recorded.

Method

Using a binocular dissecting microscope, the area of a prothallus which was occupied by a lesion or lesions was estimated visually as a percentage of the total area of the prothallus. This value was determined to the nearest 10 per cent and was taken as a measure of the disease intensity of a prothallus.

Testing the Accuracy of the Method

In order to determine the amount of variation in personal observation which occurred using this method, the following procedure was carried out.

In one particular experiment, the amounts of disease shown by each prothallus, on the third day after inoculation, was estimated by the above method. The results were recorded and placed out of sight and the same set of prothalli were immediately re-examined and the results noted. This procedure was carried out four times in succession, the Petri dishes being taken for examination in a different sequence on each occasion, the total time elapsing being approximately 30 minutes. The values obtained are shown in Table II.

Table II

The values obtained in four successive and independent estimations of the percentages of the total area of prothalli occupied by lesions. The results were taken on the third day after inoculation of the prothalli with Botrytis cinerea.

Clone	D_1				M				N			
Estimation / Dish	1	2	3	4	1	2	3	4	1	2	3	4
1	70	80	80	80	50	50	60	60	70	70	70	70
2	30	30	40	30	60	60	60	70	60	60	70	70
3	70	70	70	70	60	60	60	70	40	50	40	40
4	50	50	50	40	90	90	90	90	70	70	70	70
5	60	60	70	70	20	20	20	20	40	40	40	40
6	40	30	40	30	70	80	80	80	70	70	70	70
7	60	60	60	70	80	80	80	80	40	50	50	50
8	60	60	60	60	50	60	60	50	100	100	100	100
9	100	100	100	100	10	20	20	20	40	40	40	40
10	80	90	90	90	40	40	30	40	100	100	100	100

The results have not been analysed statistically since it is obvious from empirical observation that the values, obtained for each prothallus, do not vary more than ten per cent between the four estimations. In thirteen cases no variation in results was found. The method appears to yield consistent results between estimations and the degree of accuracy is sufficient for the purpose of comparing the relative degrees of susceptibility of different clonal populations.

PART TWO

INTERCLONAL VARIATION IN RESISTANCE TO ATTACK BY BOTRYTIS CINEREA PERS.

Introduction and Literature Review

Plants differ in their reaction to fungal attack and this variation in resistance to attack occurs between species, varieties and individuals of a single variety. Ausemus (1943), discussing variation in host-pathogen relations, has said that it is necessary to deal with two biological organisms, the host and the pathogen, both of which are variable and contain heritable factors conditioning the reaction of the host to the disease-producing organism. Resistance of the host, therefore, depends on the genotype and is not something acquired by mere association of host and parasite (H.D. Barker in Coons, 1953). Inheritance of resistance is due to single or multigenic characters (Walker and Stahmann, 1955) and the genetical aspects of resistance have been reviewed by Wingard (1941), and Walker (1941; 1953).

The breeding of crop plants resistant to disease is of economic importance and is based on the widely observed fact that, within a variety or a species, there occur individuals which are naturally more resistant to a given disease than the remainder of the population (Walker and Stahmann, loc. cit.). The fact of varietal differences in resistance appears unquestionable (Vavilov, 1951).

The literature dealing with the variation in susceptibility to disease, within and between varieties, is voluminous. Walker (1950) has dealt with some of the earliest reports and has mentioned that the observation that varieties of economic plants differed in disease susceptibility was recorded about the third century B.C. by Theophrastus. Since these early reports (Walker loc. cit.), much routine testing of economic plants for resistance to various pathogens has been carried out and much of this work has already been reviewed (Walker, 1941; 1953).

It was decided to investigate the hypothesis that different clonal populations of bracken prothalli differed in their degree of resistance to attack by Botrytis cinerea.

Method

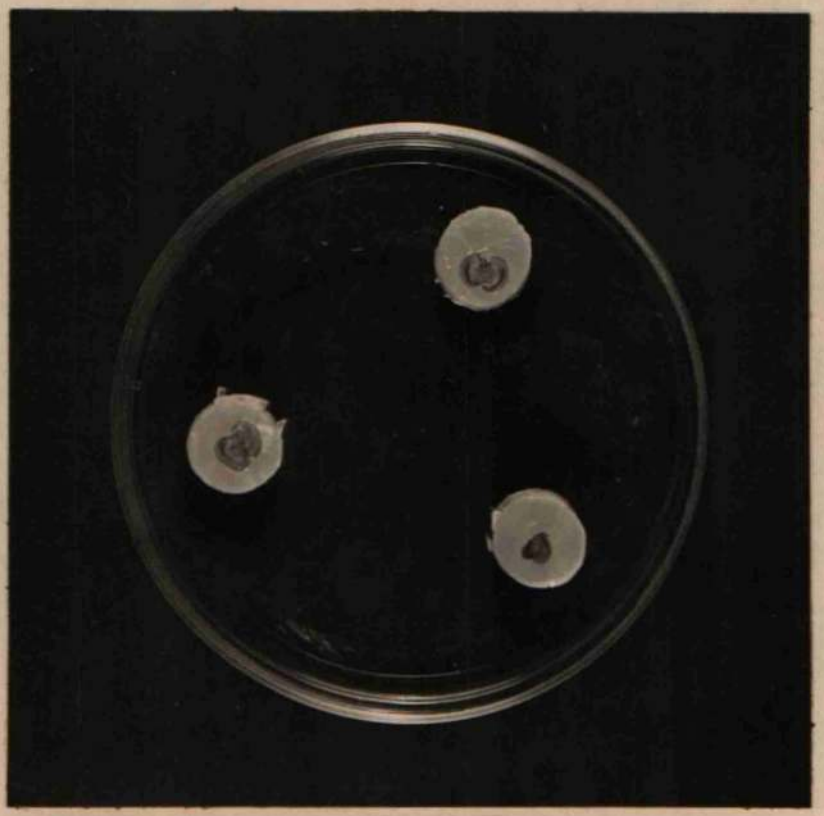
Three clones were normally tested simultaneously in each experiment. Prothalli of similar size and age were selected from the clonal populations and each was placed on a disc of Knop's agar medium, 15 mm in diameter. The discs were placed in sterile Petri dishes so that each dish contained one prothallus of each clone being tested (Plate 5). By appropriate placing within the replicates the clones were randomised throughout the experiment. Each experiment contained ten replicates.

Before inoculation, the Petri dishes containing the prothalli were placed in the constant temperature room under normal cultural conditions for 24 hours to enable the prothalli to recover from any metabolic changes they may have undergone as a result of being handled.

The prothalli were inoculated, in the manner described in Part One, with a spore suspension of Botrytis cinerea and the Petri dishes then replaced in the constant temperature room. The positions of the dishes on the shelf were changed daily to allow for any small local variation in the environment.

The amount of disease which developed in each prothallus on the second day after inoculation was estimated by the method described in Part One, and this

PLATE 5



Petri dish containing prothalli of three different clones on agar blocks.

(x 4/5)

was taken as a measure of the degree of infection. The increase in lesion size between the second and third days after inoculation was taken as a measure of the rate of spread of the infection.

The clones involved in each experiment are shown in Table III.

Table III

The combinations of the clonal populations of bracken prothalli used in six experiments

CLONE	EXPERIMENT NUMBER					
	1	2	3	4	5	6
B	-	-	-	+	+	-
J	-	+	+	-	+	-
M	+	+	-	-	-	+
N	+	+	+	-	+	-
O	-	-	-	+	-	-
U	-	-	+	+	-	-
T	-	-	-	-	+	-
*D_1	+	-	-	-	-	+

\+ ..... used in experiment.

\- ..... not used in experiment.

* ..... the prothalli of this clone were diploid.

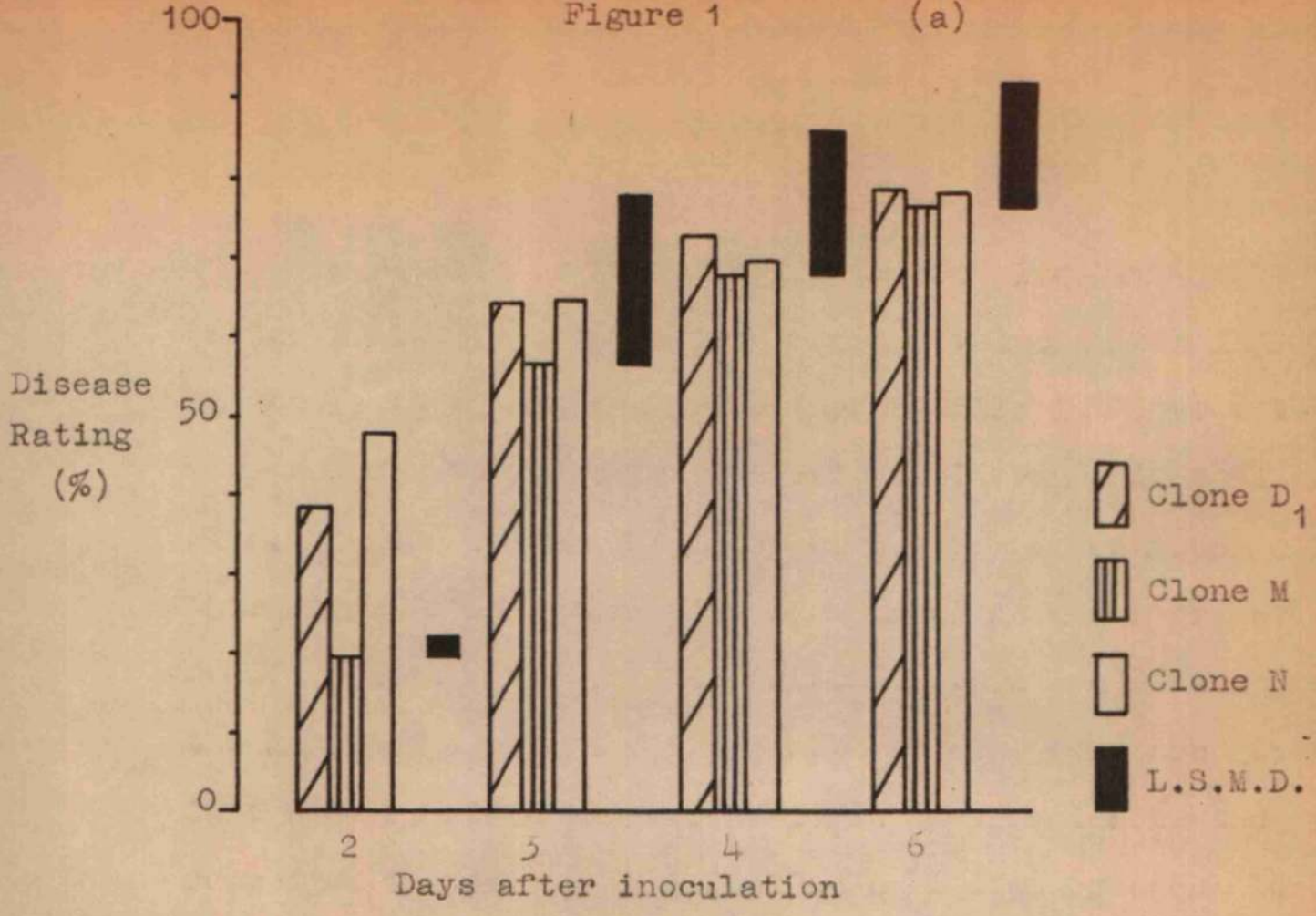
Results

The results of six experiments are given in Tables IV to XV in Appendix A and the values shown in Tables IV to IX are summarised graphically in Figures 1-3. The Tables X to XV record the rate of increase in lesion size, and the values obtained between the second and third days after inoculation were taken as a measure of resistance to spread. Comparison of variance ratios and the least significant differences between means are given in the appropriate places. Figure 4 consolidates the record of the degree of infection exhibited by each clone in each experiment in which it was tested.

Note.

1) In Figures 1-4, the vertical scale represents the percentage of the total prothallial area which is occupied by lesion. Each value records the mean of ten prothalli.
2) In Figures 1-3, the term L.S.M.D. signifies the least significant difference between means.

Figure 1 (a)


100
50
0
Disease Rating (%)
2
3
4
6
Days after inoculation
Clone D1
Clone M
Clone N
L.S.M.D.

(b)

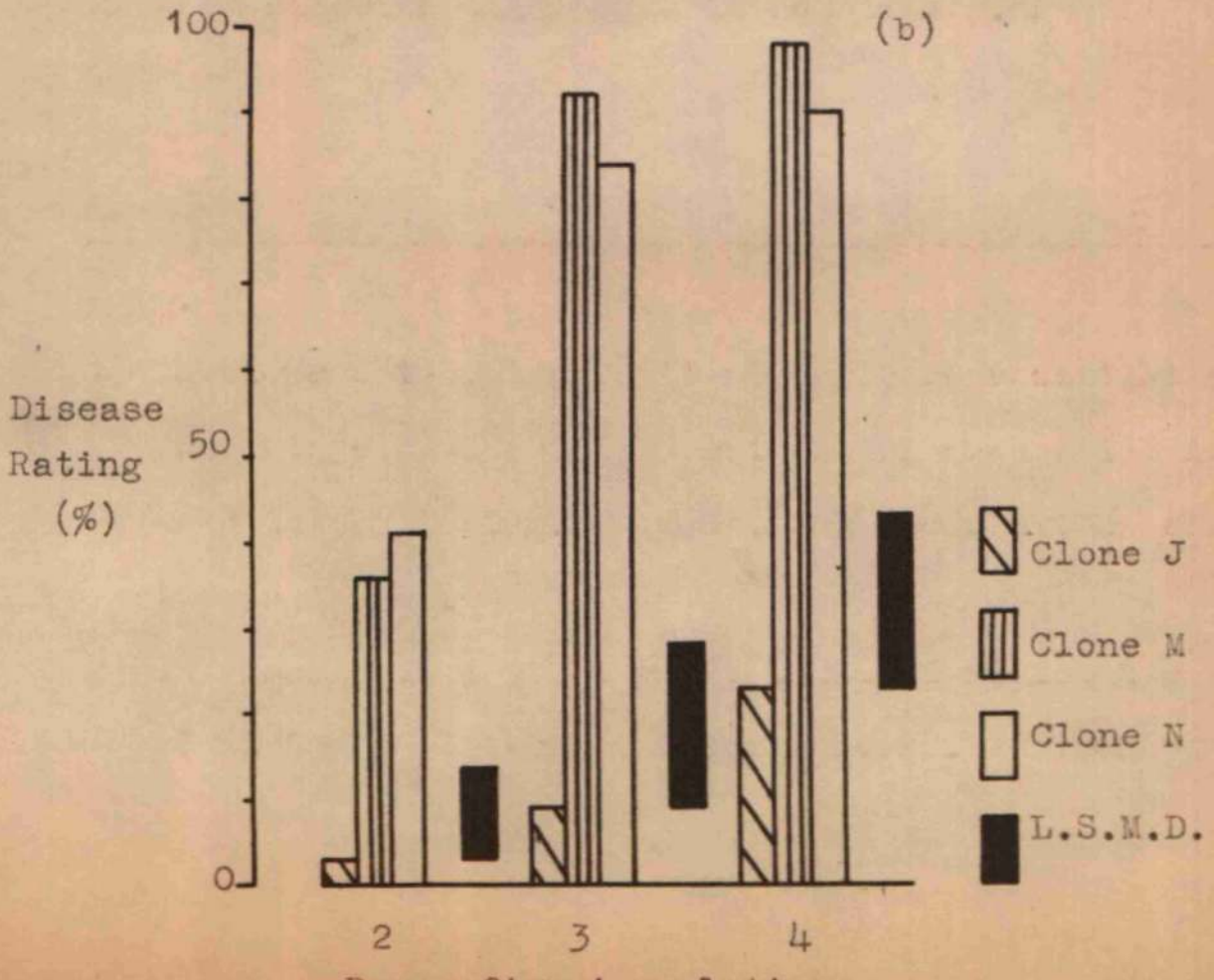
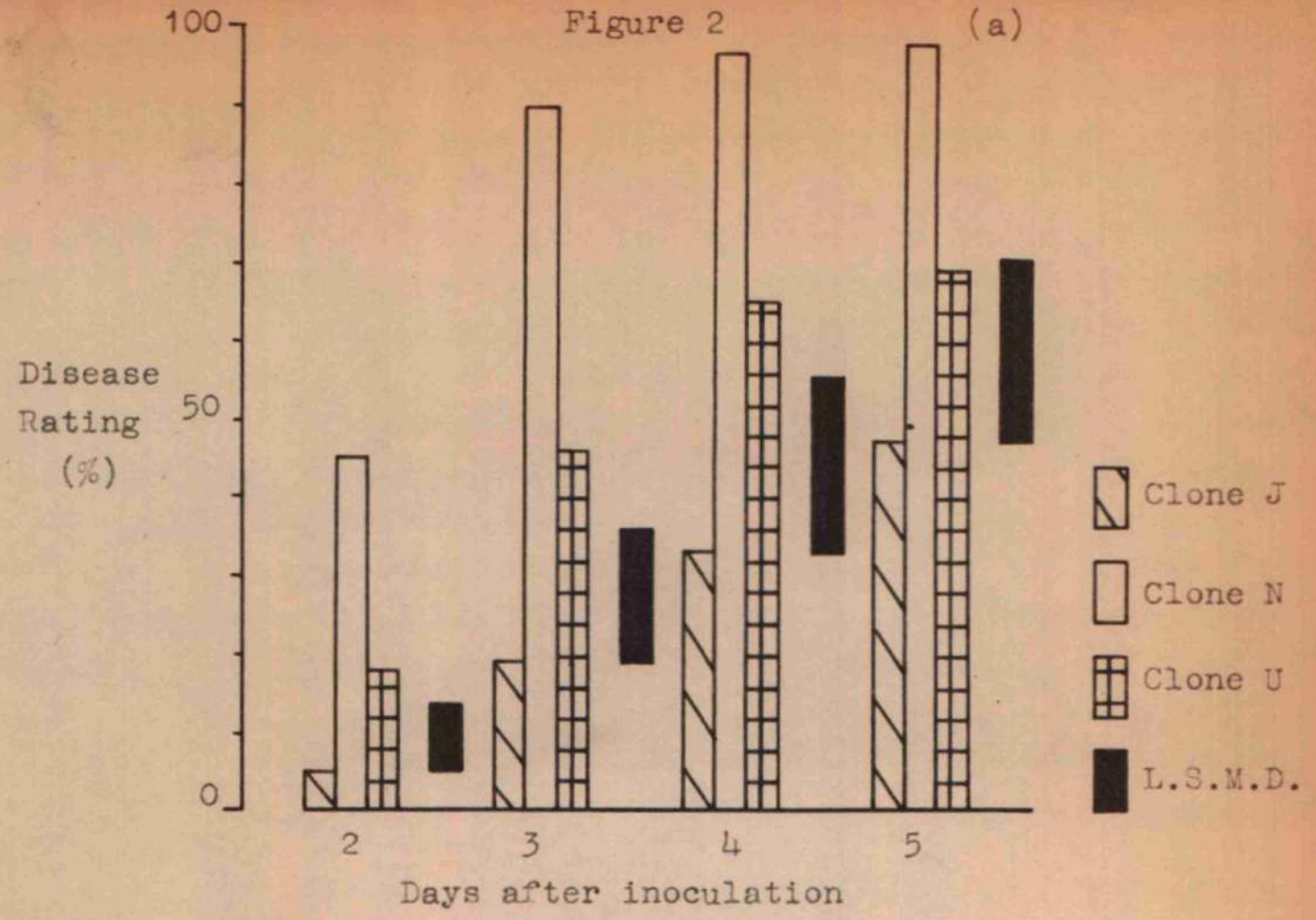

100
50
0
Disease Rating (%)
2
3
4
Days after inoculation
Clone J
Clone M
Clone N
L.S.M.D.

Figure 2


(a)
100
50
0
Disease Rating (%)
Clone J
Clone N
Clone U
L.S.M.D.
2
3
4
5
Days after inoculation

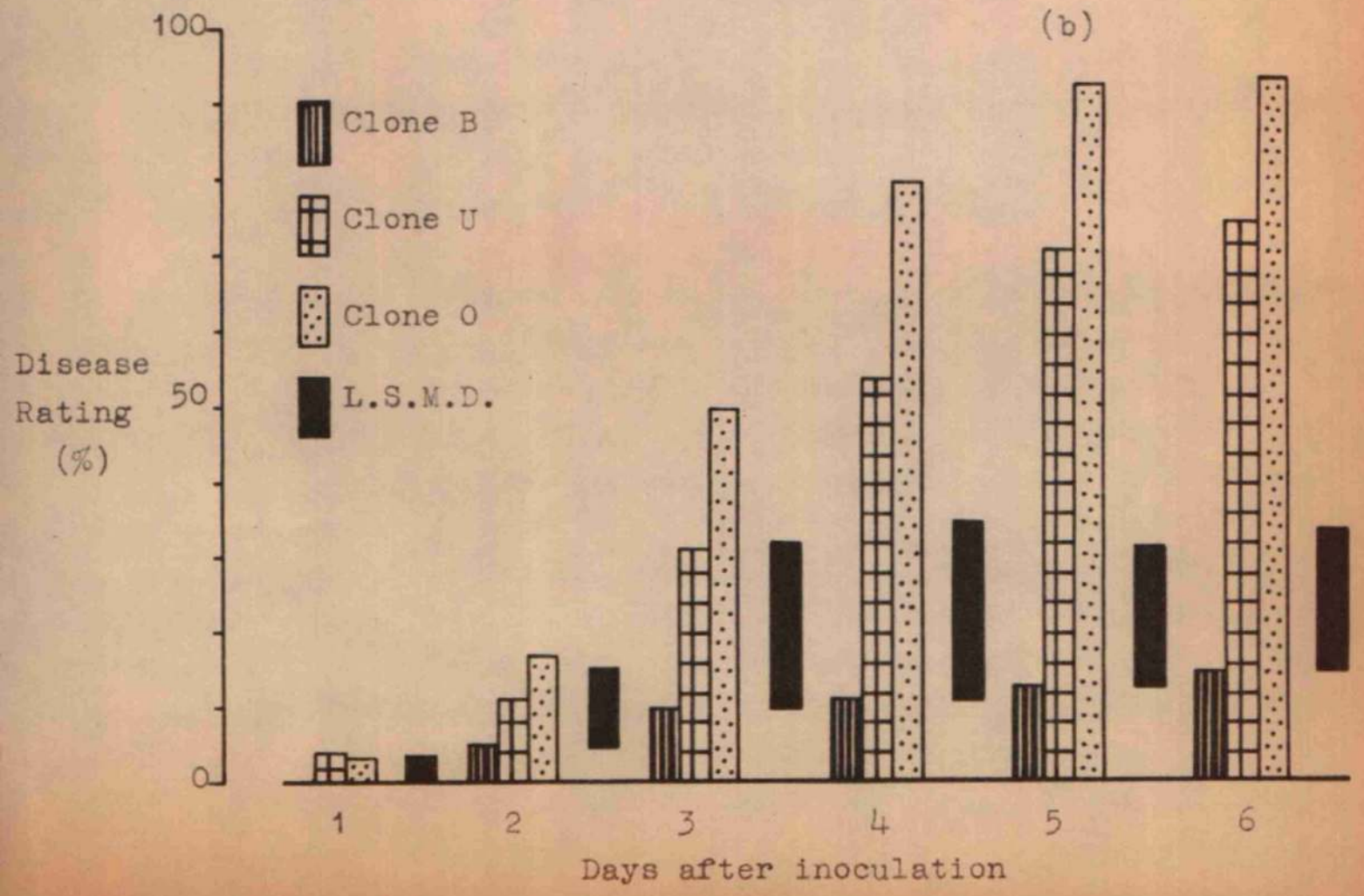
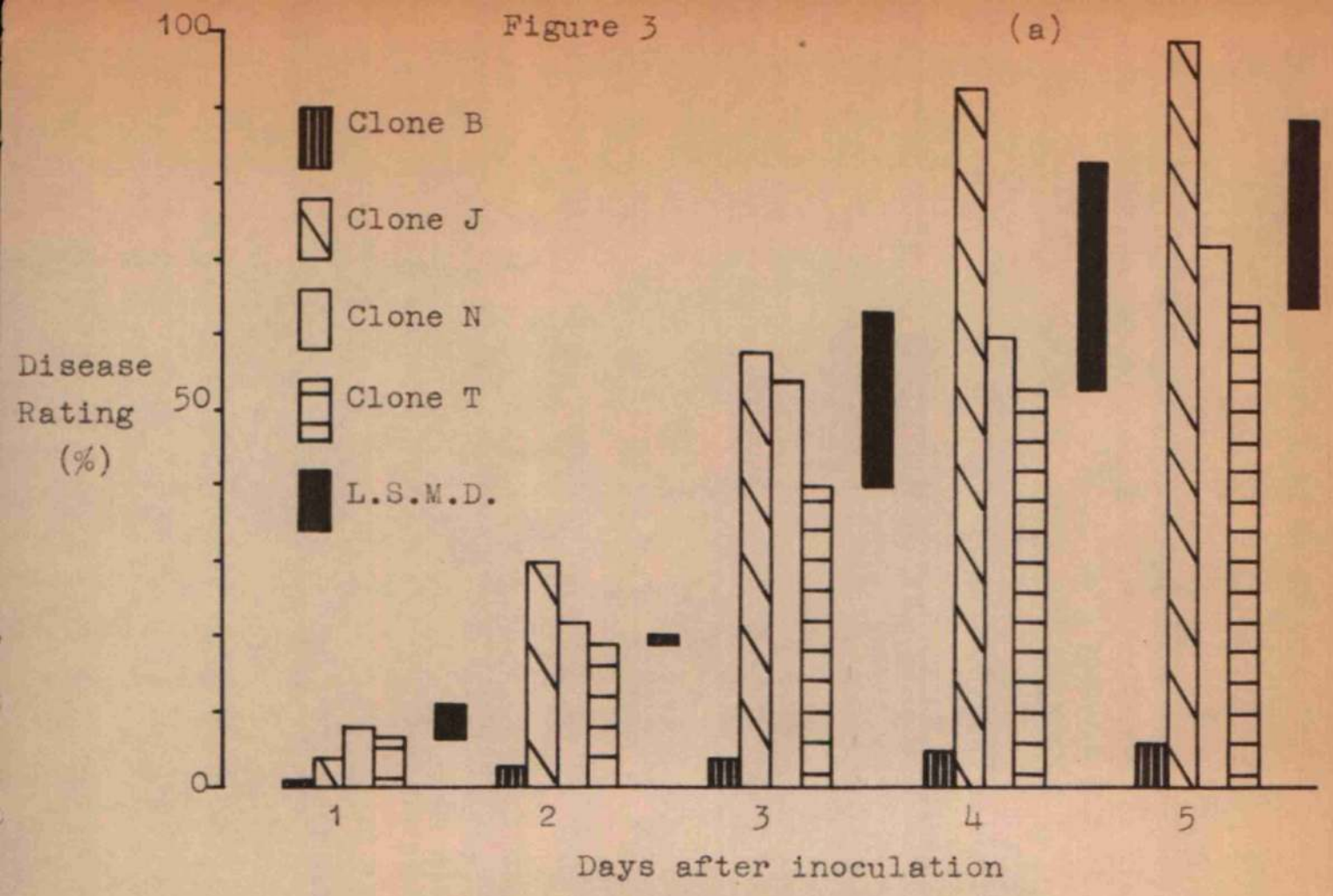
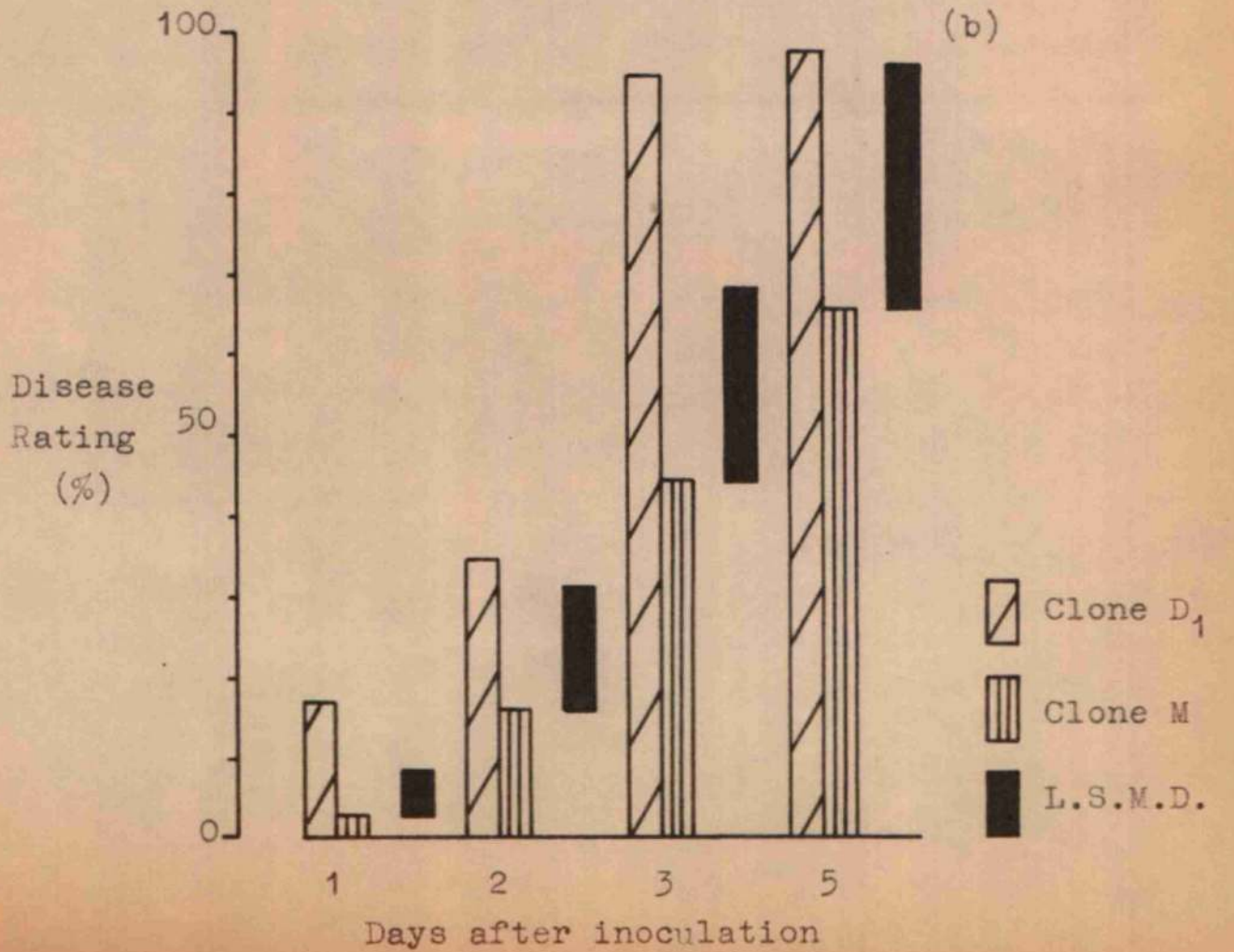

(b)
100
50
0
Disease Rating (%)
Clone B
Clone U
Clone O
L.S.M.D.
1
2
3
4
5
6
Days after inoculation

Figure 3


(a)
100
50
0
Disease Rating (%)
Clone B
Clone J
Clone N
Clone T
L.S.M.D.
1
2
3
4
5
Days after inoculation


(b)
100
50
0
Disease Rating (%)
Clone D_1
Clone M
L.S.M.D.
1
2
3
5
Days after inoculation

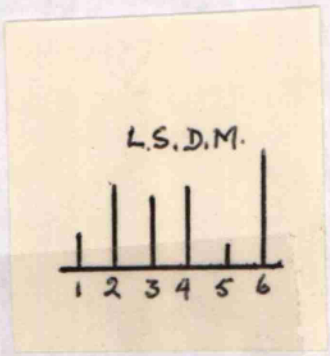
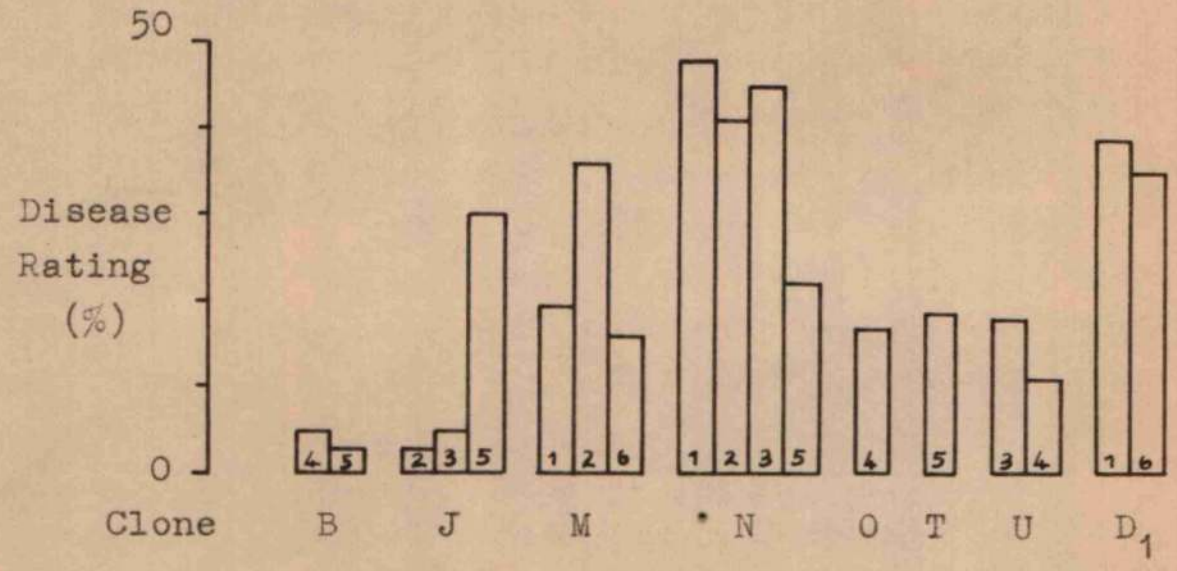

L.S.D.M.
1 2 3 4 5 6

Figure 4



The amounts of disease developed in prothalli of eight different clonal populations of Pteridium aquilinum, two days after inoculation with Botrytis cinerea.
Each value represents the mean of ten prothalli.
The enclosed numbers refer to the experiments.

Discussion

The results presented here show that interclonal variation in susceptibility occurs in the gametophyte of bracken when inoculated with Botrytis cinerea. The total variation in resistance can be attributed to both variation in resistance to infection and to variation in resistance to the spread of the pathogen.

The differences in infection found between clones are apparent from the histogram summarising the results of the second day after inoculation (Figure 4). These show that Clones B and J were much more resistant to infection than the others, except in experiment 5. The reason for this result with Clone J in experiment 5 is not known. The results were consistent in this experiment (Table VIII) and the other clones which were tested in it behaved normally. No variation in conditions could be identified to explain the difference in reaction of Clone J. Clones N and D_1 appear quite susceptible to infection. There was some variation between the total results of the various experiments but the proportional difference between the clones remained similar in all experiments except number 5. This is shown by the pattern

of results in figure 4.

The differences in degree of resistance to spread are shown in the Tables X to XV. These differences are parallel to the differences in resistance to infection. For example, Clones B and J were resistant to spread, again with the exception of Clone J in experiment 5 (Table XIV), for which no explanation can be offered.

PART THREE

ANALYSIS OF THE NATURE OF THE INTERCLONAL VARIATION IN RESISTANCE

INTRODUCTION AND LITERATURE REVIEW

Investigation of the nature of disease resistance characters in plants has produced a vast amount of literature on the subject and reviews have appeared from time to time (Blackman, 1924; Brown, 1936; Walker, 1941; Wingard, 1941, 1953; Ausemus, 1943; Gaumann, 1950; Allen, 1954; Walker and Stahmann, 1955; and Horsfall and Dimond, 1959-60).

In considering the nature of disease resistance it is necessary to distinguish initially between extreme resistance or immunity, and disease escape (Freeman, 1911; Butler, 1918 and Brick, 1919). Freeman (1911) has stated that true resistance lies in protoplasmic activity and that it is independent of inoculation accidents. True resistance is dependent on structural or physiological characters of the host which prevent successful invasion by the parasite (Butler, 1918 and Brick, 1919) and true resistance can be readily detected under proper experimental conditions (Wingard, 1953). From consideration of the experimental methods of Part Two it is evident that a detailed discussion of disease escape can be omitted from this introduction since most cases of disease escape are relevant to field experiments where factors such as earliness or lateness of ripening or late opening of stomata

may affect the expression of disease.

In cases of true disease resistance, the factors involved can be separated into two broad categories, (a) mechanical factors and (b) physiological factors. Each of these groups may affect penetration by the fungus or spread of the fungus. Accordingly the literature will be considered under this classification. Conflicting opinions have been given concerning the relative importance of the two main groups in disease resistance. Wingard (1953) has said, "when we consider the causes for true immunity and resistance, we find ourselves involved in a maze of anatomical, physiological, biochemical evidence and theories which have been offered, some to explain specific cases of resistance and the others to explain the problem in general". He himself considered that research into the cause of disease resistance should be concentrated in the field of biochemistry since natural immunity does not depend on the anatomical peculiarities of plants but on properties of their cytoplasmic cell contents and an active resistance of host plant cells. This is usually accompanied by a complicated physiological reaction in response to penetration by the parasite although other workers just as strongly contend that resistance in plants is due to morphological characters. Newton, Lehmann and Clarke (1929) considered that there appeared to be two main

groups of factors controlling or modifying infection, namely mechanical or structural factors and biochemical or physiological factors. The former was thought to be of more importance in the entrance of the parasite into the host and the latter in the establishment of nutritive relations. Several authors have reported the possibility of a morphological relationship between infection and resistance (Ainsworth, Ayler and Read, 1938; Akai, Yasumori and Terasawa, 1959; Nutman and Roberts, 1960 and Hoffmann, 1960) while Akai (1959) and Flentje (1959) have reviewed some of the literature. Akai (loc. cit.) has considered the mechanical barriers to penetration which exist before infection and also those defence structures which appear postinfectionally in response to pathogenic invasion. Flentje (loc. cit.) has reviewed the work in connection with failure of penetration. The penetration of epidermal cell walls is usually by means of a fine infection peg and is regarded as a purely mechanical action (Flentje, 1959 and Wood, 1960a). The work on the ability of fungi to penetrate the host barriers has been reviewed by Dickinson (1960) and Wood (1960a). It has been suggested (Flentje, 1959) that failure to effect penetration could be due to the mechanical toughness of the cell wall, and Brown & Harvey (1927) have shown that above a certain degree of hardness, gelatine membrane penetration by germ

tubes of Botrytis cinerea was prevented. Similar investigations were carried out by Rosenbaum and Sando (1920) who correlated resistance of tomato fruits to Macrosporium tomato Cooke with resistance of the skin to mechanical puncture. Recently, Mower (1962) found that the main factor involved in resistance of a variety of Poa pratensis to Helminthosporium spp. was the resistance of the epidermal walls to penetration. Species of Berberis, resistant to Puccinia graminis, were found to have thicker epidermal walls than had species which were susceptible (Melander & Craigie, 1927) and there have been other reports of resistance being correlated with hardness or thickness of the cuticle and epidermis, e.g. in brown rot of plums (Valleau, 1915 and Willaman, Pervier and Triebold, 1925) in potato scab (Lutman, 1919) and rice blast (Ōtani, 1959). That the relationship between cuticle and wall penetration and the fungus may be specialized is shown by the failure of Erysiphe graminis hordei, from barley, to penetrate the walls of other cereals (White & Baker, 1954) and also by the failure of a root attacking strain of Pellicularia filamentosa (Pat.) Rogers to penetrate wheat coleoptile (Flentje, 1957). At the point of penetration in this latter case, the cell wall rapidly became thickened. There have been other reports of cellulose thickenings, or cellulose with lignin and suberin, occurring after

inoculation (Stevens, 1921; Fellows, 1928 and Thatcher, 1943). Recently, studying the effect of 4 common pathogens on leaves of Ginkgo, Adams, Sproston, Tietz and Major (1962) found that although there was extensive mycelial development with appressorial formation on the epidermal surface, no infection pegs were formed and non-lignified local swellings were formed in the epidermal walls beneath the appressoria. In some cases the thickenings may take on a definite form such as the collar-shaped formations around the haustoria of certain Erysiphales at the points of entry into the cells (Corner, 1935) or the protuberances surrounding the infection pegs of other fungi. These sheath-like formations have been variously named callosities, lignitubers or papillae and have been reported by many authors including De Bary (1863), Kusano (1911 and 1936), Young (1926), Fellows (1928), Ikata and Yoshido (1940), Craig and Hooker (1961) and Struckmeyer, Nichols, Larson and Gabelman (1962). The nature of the papilla has been discussed and Corner (1935) suggested that it was a wall growth as did Smith (1900) who called it a cellulose collar. Aronescu (1934) and Kusano (1936), on the other hand, considered it to be a deposition of cytoplasmic material. Struckmeyer et al.(loc. cit.) have stated that the pegs found in the cortical cells of onion infected by the pink root fungus (Pyrenochaeta terrestris) were the stretched

cell wall. The sheaths may be composed of cellulose, callose or lignin. Craig & Hooker (1961) tested the callosities found in Diplodia rot of corn for lignin but their phloroglucinol tests were negative.

Normally the hypha grows through the callus and assumes its normal diameter; however failure to penetrate the callus has been recorded by Young (1926) and Fellows (1928). Similarly, in hosts susceptible to Olpidium viciae the papillae were penetrated by the fungus, while in non-susceptible hosts the callus prevented penetration.(Kusano, 1936). Akai (1959), however, has suggested that no close connection exists between the resistance of a plant and callus formation.

In considering mechanical resistance to penetration it must be remembered that a plant resistant to penetration may in fact be susceptible to the fungus and its resistance to penetration may not reduce its disease proneness (Gäumann, 1950). The factors which exist in the plant prior to infection and are essentially passive or static have been called 'axenic' (Gäumann, loc. cit.). This term, 'axeny', refers to the lack of fitness of an organism to act as a host in contrast to its dynamic defence reactions which begin to operate after infection.

It has been reported (Ward, 1905; Newton, Lehmann & Clarke, 1929; and Böhme, 1959) that resistant and

susceptible plants were penetrated with the same frequency but in resistant plants the development of the pathogen falls behind that in the susceptible plants. One of the groups of factors involved here is the group concerned with static resistance to spread which exists prior to infection as opposed to the dynamic reactions released after infection (Gäumann, 1950). At this stage the mechanical barriers involved in resistance to spread will be considered. The varying resistance to spread of the hyphae of Pythium debaryanum in potato tubers has been found to depend on the resistance of the cell walls to penetration (Hawkins and Harvey, 1919). Using a Joly balance and a fine needle, these authors found that the force required to penetrate the tissue of resistant tubers was greater than that required to penetrate susceptible tissue. Using similar methods, Willaman, Pervier and Triebold (1925) measured the resistance of the flesh of plums to puncture and found that the firmness of the flesh was a factor related to resistance of varieties to brown rot (Sclerotinia cinerea). More recently, Struckmeyer et al (1962), investigating the onion pink root fungus, found that the hyphae were restricted to the outer cortical cells in resistant varieties by the swelling of the cell walls to form callosities.

Although the authors reviewed above have shown that

morphological characters are involved in some cases of disease resistance, other authors have found that, in other diseases, no mean morphological differences such as hairiness or wall thickness could be correlated with resistance or susceptibility (Mackie, 1928; and Smith & Blair, 1950). Chester (1946) has reviewed and discussed the role of morphological factors in rust resistance and has concluded that, while mechanical protective structures may play some part in determining the resistance of wheat varieties toward leaf rust, this is a minor part and the main answers to the complex resistance phenomena must be sought in the chemistry and physiology of the host plant. There is mounting evidence (Allen, 1959) that the health of many plants is preserved, not by virtue of mechanical barriers but through an active metabolic initiative which destroys or immobilises the pathogen at some stage before it can produce serious disease. One of the ways in which this can happen is the inhibition of germination or germ-tube growth.

It has been shown (Brown, 1922, 1936) that the moisture on the surface of the host may contain organic and inorganic solutes, released from the underlying cells. Hafiz (1952) showed that malic acid was secreted from glandular hairs on gram (Cicer arietinum L.) and this inhibited spore germination of Mycosphaerella rabei.

Newton et al (1929) have suggested that, concerning the exodiffusion of stimulatory or inhibitory substances from the cell into the infection drop, plants may chemically resist or stimulate fungal attack, subject to mechanical modification of the permeability of the cuticle. Walker (1923) found that pigmented varieties of onion owed their resistance to onion smudge (Colletotrichum circinans) to inhibition of spore germination of the pathogen by diffusible substances which passed from the coloured outer scale leaves into the infection drops. When the pigmented outer scales were removed, the resistance was lost and these varieties became susceptible as were the white-skinned varieties. Extracts of the pigmented scales, but not of the colourless ones, inhibited C. circinans spore germination and also caused the spores and young hyphae to rupture with liberation of the protoplasmic contents. The active inhibitor was identified as a phenolic compound, protocatechuic acid (Angell, Walker & Link, 1930; Walker, Link and Angell, 1929; Link, Angell and Walker, 1929; and Link, Dickson and Walker, 1929). Catechol was also identified as being responsible for part of the activity. Although inhibition was associated with pigmentation of the scales, neither the pigments themselves, nor the scales when the component cells were living, inhibited germination. This property was only found when the cells were dead and

dry, in which condition a colourless substance was released. Other fungi, for example Aspergillus niger, were found to be unaffected by this resistance mechanism. Allen (1959) has reviewed the investigations into the role of root exudates in relation to parasitism. Instances of positive correlations between resistance to wilt diseases and the toxicity of root diffusates are reported.

The host surface may contain substances capable of affecting spore germination (Martin, 1961). Leaf-surface waxes extracted from leaves of apple varieties resistant to powdery mildew (Podosphaera leucotricha) prevented conidial germination on the surfaces of susceptible leaves which had the aqueous extracts deposited on their surfaces before inoculation (Martin, Batt and Burchill, 1957). The fungitoxic substance was identified chromatographically as a phenol, and Topps and Wain (1957) found a similar substance in exudates of leaves and roots of Vicia faba. Kovacs (1955) found that poor spore germination of Cercospora beticola Sacc. on resistant leaves of sugar beet was correlated with inhibition of germination and germ tube growth by leaf extracts obtained by simply washing the leaves in water. The effect was still present with considerable dilution. In similar work (Kovacs and Szeöke, 1956) Botrytis cinerea was found to be the most sensitive species to the exosmotic excretions from leaves

of various plants which exerted stimulatory or inhibitory effects upon spore germination. The active components were considered to dissolve slowly from the leaves and to be possibly present, under normal conditions, in the dew or raindrops on the leaf surfaces in sufficient concentration to inhibit conidial germination of Botrytis cinerea. More recently, Mower (1962) found that in situ inhibition of conidial germination and irregularities in the germ tubes of Helminthosporium sativum were caused by a toxic principle on the leaves of a resistant variety of Poa pratensis L. The resistance of this grass variety was attributed in part to this substance. In connection with the chemical nature of the wall, Roberts, Martin and Peries (1961) and Martin (1961) have reported that comparisons of the levels of waxes and cutin in resistant and susceptible leaves of several plants showed no relationship with their reaction to mildew.

One of the main features normally associated with penetration is the formation of appressoria and the effects of various factors on appressorial development has been considered by Flentje (1959). Van Burgh (1950) has reported that inorganic nutrients can inhibit their formation in Colletotrichum.

When the pathogen has overcome the mechanical and chemical barriers to penetration, it may yet be unable to

overcome the chemical and physiological resistance of the cell to spread. The natural immunity of some plants, says Wingard (1953), depends on the properties of the cells' cytoplasmic contents and an active resistance of the host plant's cells, usually accompanied by a complicated physiological reaction in response to penetration. There is mounting evidence (Allen, 1959) that the health of many plants is preserved, not by virtue of mechanical barriers but through an active metabolic initiative which destroys or immobilises the pathogen at some stage before it can produce serious disease. There have been reviews of the literature on this aspect of the defence system of plants (Wingard, 1941 and 1953; Gaumann, 1950; Sempio, 1950; Allen, 1954 and 1959; Brown, 1955 and Walker and Stahmann, 1955).

There are two main factor groups co-operating in the chemical or physiological resistance to spread. Firstly, there are the factors which exist prior to infection and secondly there are the dynamic defence reactions released after infection.

When the fungus reaches the cells of the host it may be prevented from setting up a pathogenic relationship due to the presence in the host cells of preformed toxic substances. There are many cases where such substances have been demonstrated, usually by maceration and extraction

of the host tissues and then studying the effect of the extracts on spore germination, germ tube growth and appressorial formation (Gilliver, 1947; Whitney and Mortimore, 1959; Ludwig, Spencer and Unwin, 1960; and Valenta and Sisler, 1962). Ewing (1959) and Clauss (1961) attributed resistance in peas to pre-emergence damping-off (Pythium ultimum) and foot rot (Ascochyta spp.) to the presence in the testas of phenolic substances such as leuco-anthocyanins. From resistant rye seedlings substances toxic to Fusarium nivale and Sclerotinia trifoliorum have been isolated (Virtanen and Hietala, 1955 and Wahlroos and Virtanen, 1959). Little, Sproston and Foote (1948) attributed the resistance of Impatiens balsamina to Monilinia fruticola to the naturally-occurring quinone, 2-methoxy-1,4-naphthoquinone which they extracted from balsam. From lower plants, Major, Marchini and Sproston (1960) isolated an inhibitor of fungi, including M. fruticola, by steam distillation of leaves of Ginkgo biloba and Maruzzella (1961) has claimed antimicrobial properties for extracts of 33 of 34 different ferns. There was, however, no inhibition of fungi.

Mothes (1955) has reviewed the evidence for the role of alkaloids in disease resistance and has concluded that they generally offer no protection against parasitic fungi.

An unfavourable chemical environment in a potential

host may consist of a deficiency of an essential substance rather than a toxic level of an inhibitor, and this aspect has been reviewed and considered by Allen (1959). Attempts have also been made to correlate the amino acids in the host cells with their resistance to invasion and spread (Hardwiger and Hall, 1961; Kuc, Barnes, Daftsios and Williams, 1959; Rubin and Ivanova, 1959; Siebert, 1961; Lewis, 1962 and Holowczak, Kuc and Williams, 1962) and also host reaction and host sugar content (Lyles, Futrell and Atkins, 1959 and Krog, Le Tourneau and Hart, 1961).

Hurd (1923 and 1924) was unable to find any correlation between the hydrogen-ion concentration of the host fluids and rust resistance at any stage in the growth of wheat plants. On the other hand Taha and Sharabash (1960) found that the susceptibility of tomato fruits to Fusarium semitectum and Alternaria tenuis was influenced by the pH of the tomato tissues. The hydrogen-ion concentration was one of the main factors affecting the destructive pectolytic action of the fungi. For plant pathogenic fungi, however, the acidity of the cell sap is, as a rule, probably without significance (Gäumann, 1950).

The second factor-group operating in the resistance of the plant to the establishment and spread of the pathogen is concerned with the cell's physiological and chemical

response to infection. There have been many reports of the formation of toxic substances in infected cells and these have mainly involved the identification of phenolic compounds. This aspect has been reviewed recently by Allen (1959) and Farkas and Kiraly (1962). Allen (loc. cit.) concluded that the most important factor in relation to the effect of phenols is the metabolic changes in which these substances are involved as opposed to the actual levels present in the cells. Further, the presence of a toxic substance in a host extract does not necessarily signify that it plays an important role in the mechanism of resistance of that plant. Newton, Lehmann and Clarke (1929), for example, related phenols to rust resistance in wheat without demonstrating the part played by these substances. Kirkham (1959) acknowledged the fact that phenols were potential resistance factors but stated that their function has not yet been fully elucidated. There is a greater probability of a given toxic substance playing a role in resistance if this can be correlated with the distribution of the substance (Allen, 1959). Johnson and Schaal (1952) have shown such a correlation between the distribution of chlorogenic acid in potato tubers and resistance to scab. The same authors (Johnson and Schaal, 1957) and also McLean, Le Tourneau and Guthrie (1956,1961) correlated the resistance of potato to scab and to

Verticillium wilt respectively with the distribution of phenols, including chlorogenic acid, using the ferric chloride spot test. Increased toxicity occurred in potato inoculated with Helminthosporium carbonum (Kuc, Ullstrup and Quackenbush, 1955 and Kuc, Henze, Ullstrup and Quackenbush, 1956) but this increase could not be attributed to the chlorogenic and caffeic acid contents of the infected tissue (Kuc, 1957). The same author (Kuc, 1960) showed that an inhibitory substance was produced, in response to infection, in maize plants resistant to H. carbonum while susceptible plants produced it in a lesser concentration. Both resistant and susceptible tissues contained little or none of the inhibitor before inoculation. Buxton (1957) found that pea seedlings inoculated with Fusarium solani f. pisi yielded extracts toxic to F. oxysporum although neither extracts of uninoculated seedlings or culture filtrates of the pathogen were toxic when tested separately. Buxton and Perry (1959) obtained water extracts from pea stems with foot rot lesions and found that one fraction of the extract stimulated and another inhibited germination of spores of F. oxysporum. Dilution to $1:10^4$ nullified the stimulatory effect and spore germination was inhibited. Inoculation of detached, opened pods of Pisum sativum L. with a spore suspension of Sclerotinia fruticola, produced a substance which was

fungistatic to this fungus and the name pisatin has been proposed for the substance (Cruickshank and Perrin, 1960). The same substance was produced on inoculation with other fungi and analogous extracts from Phaseolus vulgaris and Capsicum frutescens showed similar antifungal properties, but this was not due to pisatin. Other authors have reported the formation of antifungal substances in the host after inoculation (Spencer, Topps and Wain, 1957 and Gäumann and Hohl, 1960) and changes in the phenolic content of the cells (Condon and Kuc, 1959 and 1960; Gubanov and Bredikhina, 1960; and Hampton, 1962).

It has been found, however, that changes in susceptibility to Gloeosporium perennans, in detached Cox's Orange Pippin apple fruits, were not accompanied by a significant change in gross phenolic content and no extracts so far obtained had any marked effect on the germination of conidia at a pH where hydrogen-ion concentration had no effect (Hulme and Edney, 1960).

The mode of action of phenolic substances in resistance is thought to be due, not to their direct toxicity, but to substances produced under the influence of the host polyphenol oxidase system (Sokolova, Savel'eva and Solov'eva, 1960, 1961; and Schaal and Johnson, 1955) and some authors have shown that infected tissues had greater polyphenol oxidase activity than had healthy tissues (Solymosy, Farkas

and Kiraly, 1959; and Khandobina and Ozoretskovskaya, 1959).

There is some evidence that tannins play a part in the defence system of plants (Maranon, 1924). Cook and Taubenhaus (1912) found instances of disease resistance apparently correlated with the rate of tannin formation in the host cells. More recently Babaev and Bagirov (1961) have reported that large quantities of tannins were produced by cotton varieties in response to invasion by Verticillium dahliae and that these tannins played a protective role. In comparatively resistant cotton varieties infected with Verticillium sp. the concentration of tannic substances was found to increase sharply, resulting in a restriction of the flow of nutrients to the parasite (Avetisyan, 1960).

The relationship between the metabolic state of the host and its resistance to disease has been reviewed by Sempio (1950) and more recently by Scheffer and Gothoskar (1953) and Uritani and Akazawa (1959). High respiratory activity was found to promote resistance to infection (Takahashi and Ōishi, 1958; Khandobina and Ozovetskovskaya, 1959; and Dvoretskaya, Pȳrina and Feoktistova, 1959), and a close connection has also been found between resistance and peroxidase activity (D'Yachenko, 1959 and Kedar, 1959).

THE MORPHOLOGY OF INFECTION

Introduction

The work reviewed in the introduction to this part of the thesis has shown that resistance may involve differences in the nature of the cell walls, or differences in the physiological reaction of the suscept to the pathogen.

This section records an investigation of the morphology of infection in prothalli which are resistant or susceptible to Botrytis cinerea. It was carried out mainly to see if the visible characters of infection would give any indication of the nature of the resistant property.

Methods

Prothalli of clone B (resistant) and clone N (susceptible) were placed on agar blocks and were inoculated with drops of a spore suspension of Botrytis cinerea. The blocks were placed in the thermo-controlled room under normal culture conditions. The development of the disease was observed by mounting prothalli in water under coverglasses and watching the progress of the disease at intervals under a microscope. In some experiments the prothalli were removed from the agar for examination and in others the complete agar block with the prothallus was taken, a drop of water at room temperature placed on the surface of the prothallus, and then a coverglass. In the latter cases, the same prothalli could be observed over a period of days. All observations were made in the thermo-controlled room.

The observations of the living material were supplemented by a study of sectioned material. This was prepared by fixing selected prothalli in formalin-acetic-alcohol (formula 70% ethylalcohol:40% formalin: 45% acetic acid = 1:1:1) for 18 hours, embedding in paraffin wax after alcohol/chloroform dehydration and sectioning at 10-12μ thickness on a rotary microtome. The sections were stained in orange G, safranin, tannic acid and iron alum

(Sharman, 1943).

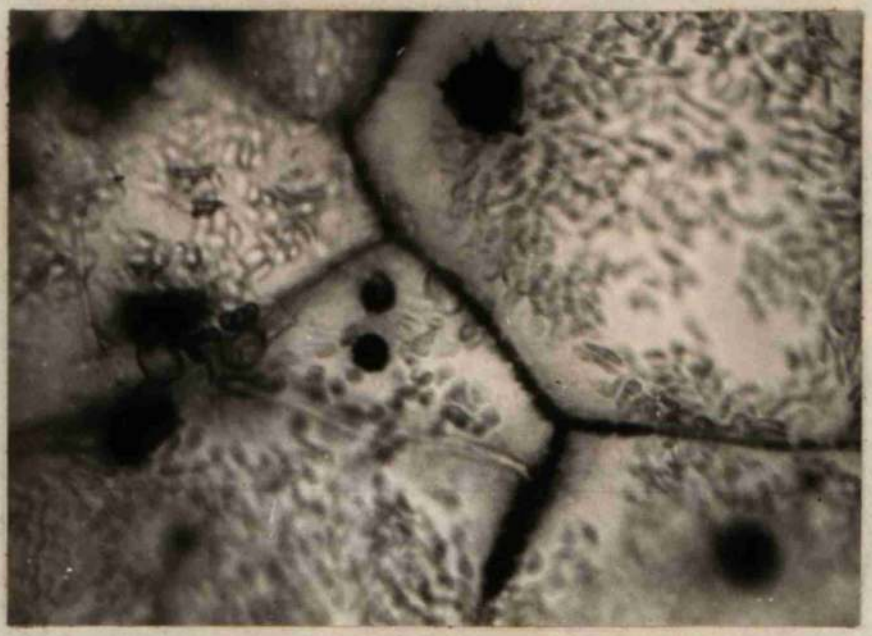
Results

In the first 24 hours after inoculation, germination and the production of germ tubes and appressoria took place. It appeared to be similar in infection drops on both resistant and susceptible prothalli. All the spores present in each infection drop were seen to have germinated. A brown pigment developed in the prothallial cell walls which were in contact with spores, appressoria and superficial hyphae (Plate 6a). Infection pegs could be seen in both types of prothalli as translucent spots in the middle of the brown appressorial regions (Plate 7a) but at this stage no difference in reaction to the fungus was observed between the two types of prothalli.

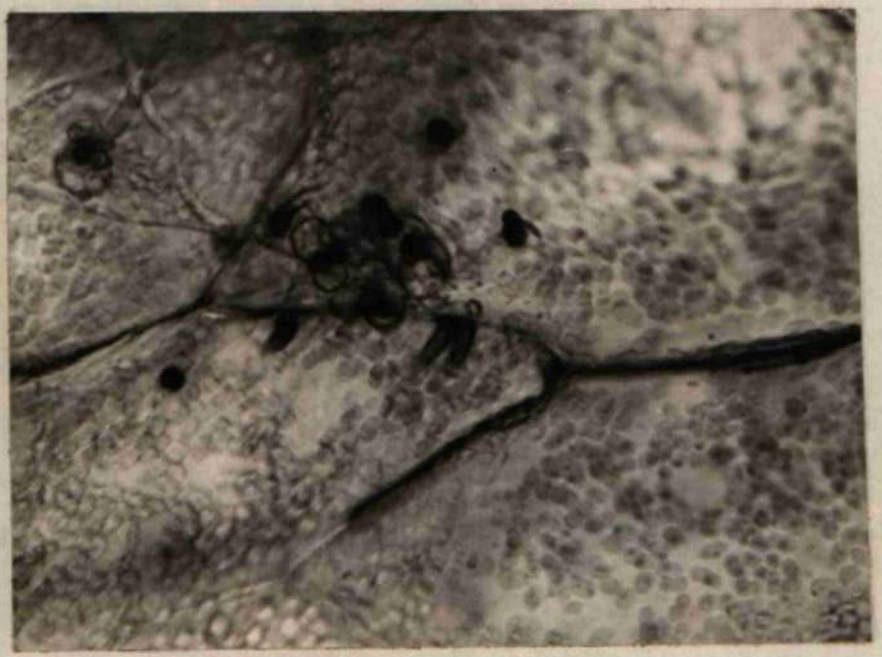
On the second day after inoculation, many infection pegs were visible on prothalli of both clones. Hyphae had grown into some of the cells of susceptible prothalli and these cells showed some signs of disorganisation. Despite the presence of numerous infection pegs, no hyphae were seen in cells of the resistant prothalli and disorganisation in these cells was limited to a movement of the chloroplasts away from the area of the infection peg.

After four days there was a noticeable difference in the reaction of the two clones. Susceptible prothalli had developed large lesions which, in the most advanced state,

PLATE 6

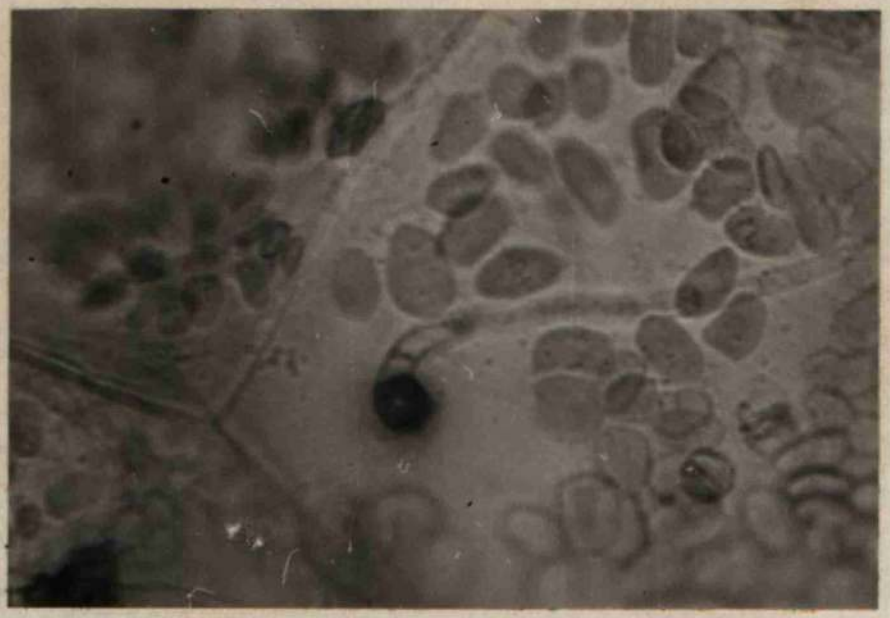


a. Part of infected, susceptible prothallus showing swollen, brown cell walls. (x 540)

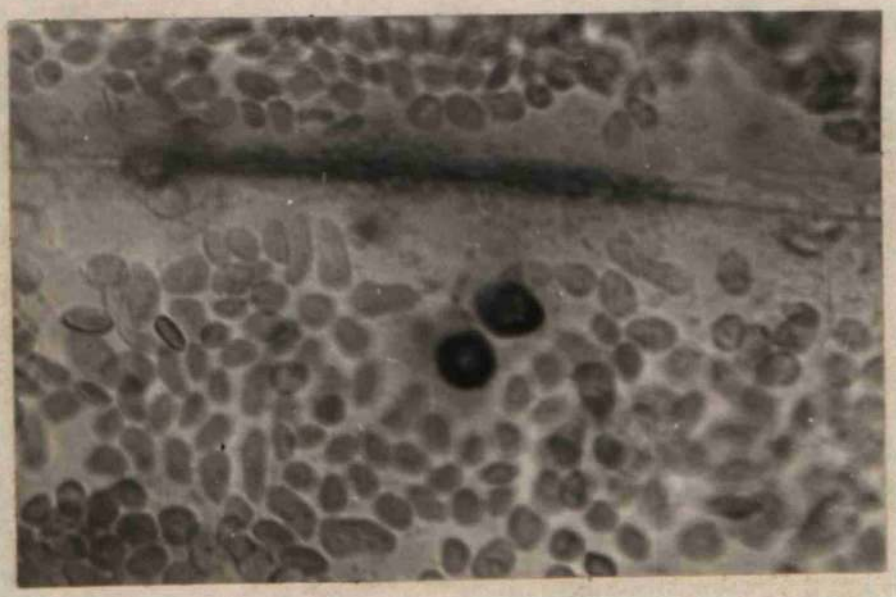


b. Group of spores on the surface of a susceptible prothallus showing brown cell walls and infection peg papillae. (x 425)

PLATE 7



a. Infection peg visible in the centre of the brown pigmented appressorial region of a resistant prothallus. The chloroplasts have moved away from the point of penetration. (x 1,120).

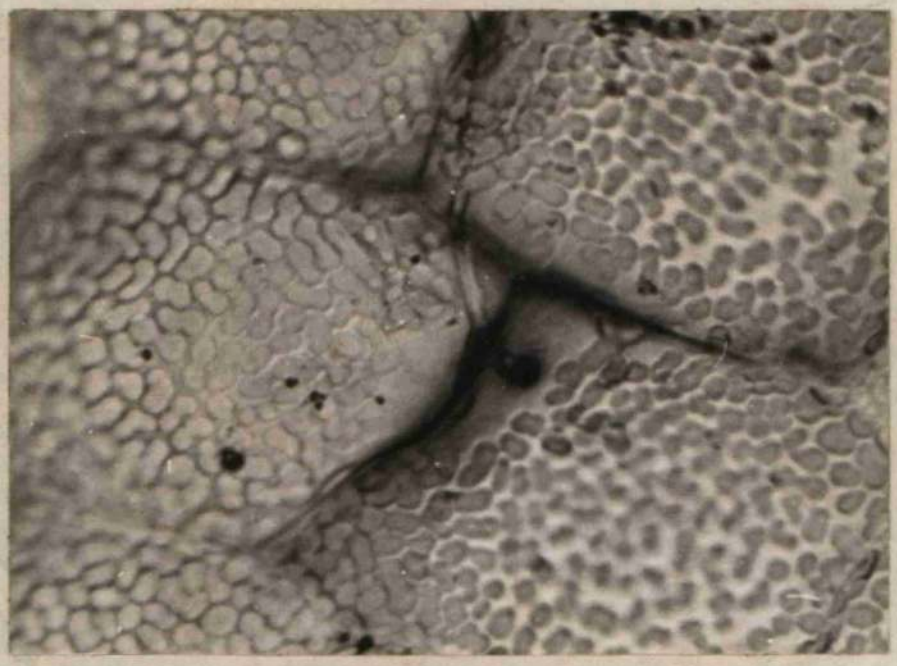


b. Development of the brown pigment in the wall adjacent to two infection peg papillae in a resistant prothallus. (x 940).

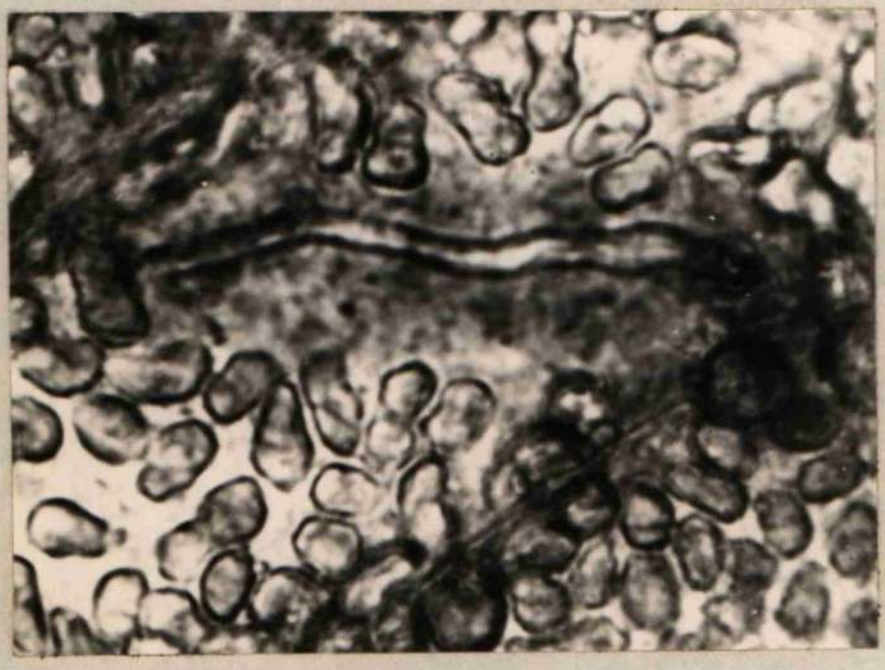
appeared as areas of cells whose walls were mainly colourless and fell apart when touched. Their contents had completely disintegrated and they were permeated with fungal hyphae. Adjacent to the lesions, but in a less disorganised state, the cells were brownish and they contained scattered disintegrating chloroplasts and numerous hyphae. In some places the intercellular walls showed brown pigmentation (Plate 6b) and these walls were usually swollen like those in Plate 6a. In a few cases, parts of the lesions were delimited by brown cell walls, but in the susceptible prothalli the hyphae normally spread rapidly through the cells without such walls forming to an extent sufficient to restrict lesion development.

Resistant prothalli, after the same period, showed very little cellular disorganisation and rarely any lesion development. Where a lesion was present it was very small (less than ten per cent of the surface area of the prothallus). Browning had occurred, mainly in the intercellular walls adjacent to infection pegs (Plate 7b), or where superficial hyphae crossed these walls (Plate 8a). Apart from the browning reaction of the walls and scattering of the chloroplasts in the region of the infection peg (Plate 8a), cells with these intrusions showed no sign of infection, and no hyphae were seen inside the cell. The other cells rarely showed hyphae present and many of

PLATE 8



a. Resistant prothallus showing formation of brown pigment in wall under hypha and the absence of chloroplasts from the infection peg region. The rest of the cells appear normal. (x 640)



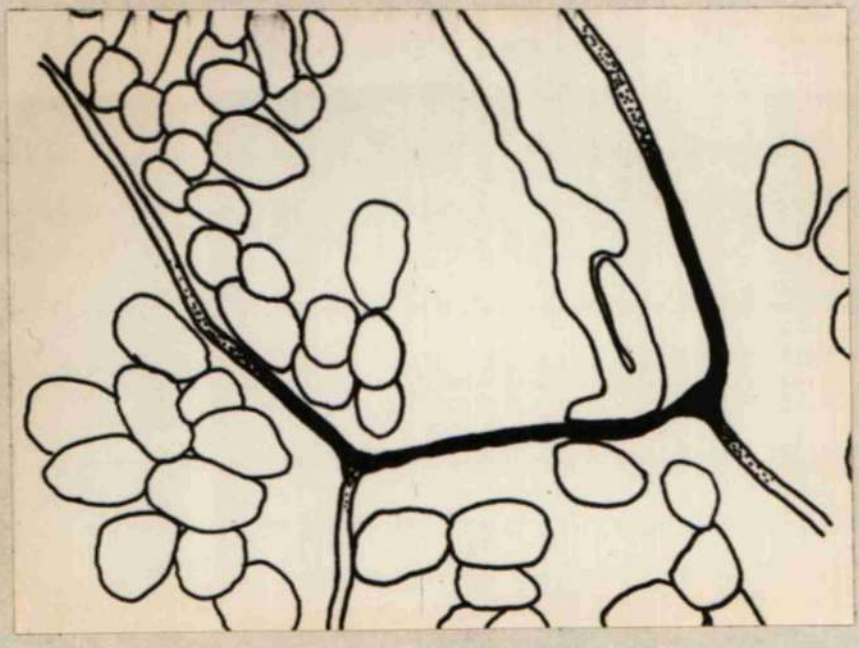
b. Part of an infected cell in a resistant prothallus containing a moribund hypha surrounded by a brown colouration. (x 1,500)

the prothalli examined had only one or two infected cells. The typical appearance of these cells is illustrated in Plates 8b and 9. In the cell shown in Plate 8b, the chloroplasts had moved from around the hypha which was surrounded by a brown colouration, apparently due to pigmentation of the outer wall. Typically, all other cells in the surrounding tissue appeared normal and showed no trace of this hypha. The effect of the browning of the cell wall in preventing hyphal penetration is shown in Plate 9. This shows a hypha whose branched tip was pressed against the intercellular wall. Evidence of the pressure exerted by the hypha is demonstrated by the indentation in the wall. One hyphal branch had grown back from the tip, in the opposite direction to the main growth, while the other branch had grown along the brown wall. Pigmentation had extended completely round the hyphal tip (Plate 9b) and there was scattering of the chloroplasts, both in the infected cell and in those parts of the neighbouring cells bordering on the hyphal tip region.

On the sixth day after inoculation, the susceptible prothalli presented a similar appearance to that described above for the fourth day but lesion development was much greater, almost the whole of each prothallus being infected. In one particular case (Plate 10a) a hypha was seen penetrating a brown cell wall, but this was the only instance

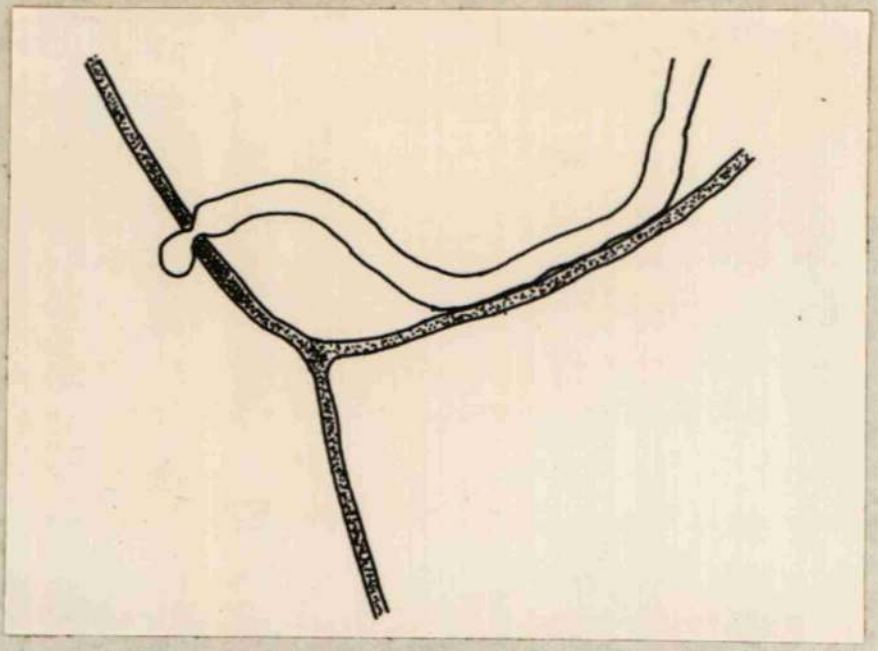
PLATE 9

a. Part of an infected cell of a resistant prothallus containing a hypha which has been prevented from penetrating the adjacent cell by a brown cell wall. (x 1,500)



b. Camera lucida drawing of part of the above cell and hypha showing the extent of the browning reaction. (x 1,500)

PLATE 10



a. Camera Lucida drawing of a hypha which has penetrated a brown cell wall of a susceptible prothallus.
(x 1,500)

b. Transverse section of a susceptible prothallus showing two infection pegs enclosed within papillae.
(x 1,200)

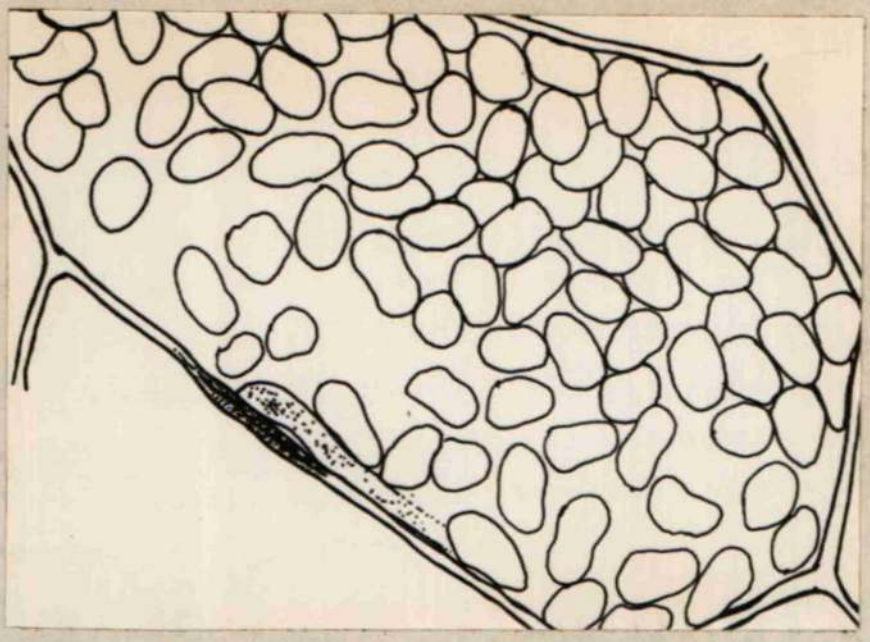
of such penetration that was observed in any prothallus of either clone.

Resistant prothalli, on the other hand, showed little perceptible change from their appearance on the fourth day. There were few infected cells, and such cells contained, as before, single moribund hyphae in contact with the cell wall which had become pigmented at the point of contact (Plate 11a).

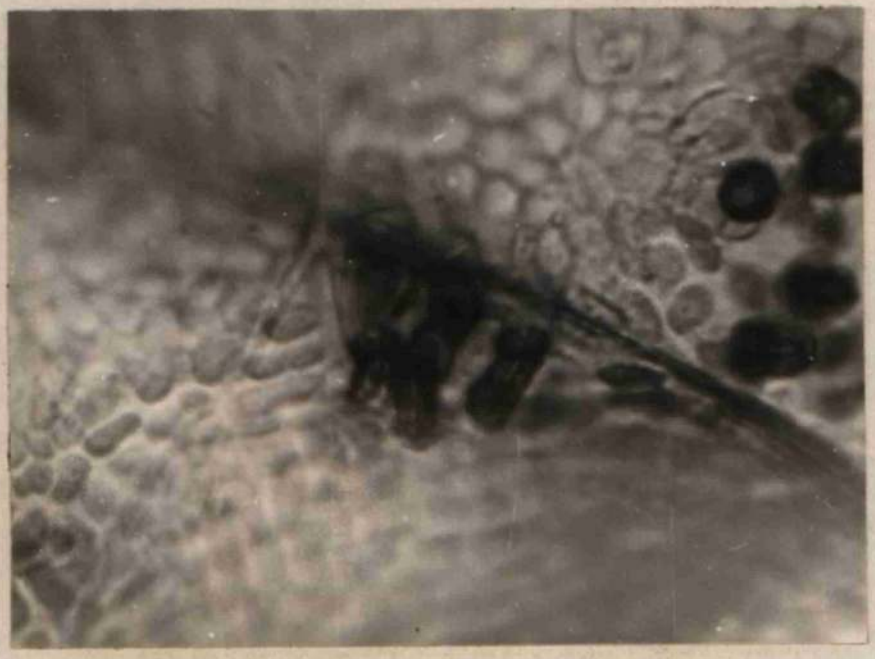
It was noted, in all the stages examined, that the infection pegs were enclosed within brown pigmented papillae (Plates 6b and 11b). In many cases the infection peg was not readily discernible, but in others it was clearly seen to have stopped short of the tip of the papilla (Plate 12a). Infection pegs were readily seen in the sectioned material (Plates 10b and 12b), although in many cases it was difficult to determine if the infection peg did completely penetrate the papilla. No gross morphological differences in papilla structure or arrangement were observed in the two types of prothalli. They appeared equally frequent in both types.

At the magnifications available, it was not possible to study any structural details in the wall or to determine if a cuticle were present. No visible mechanical features (e.g. wall thickness) which could account for resistance were present.

PLATE 11

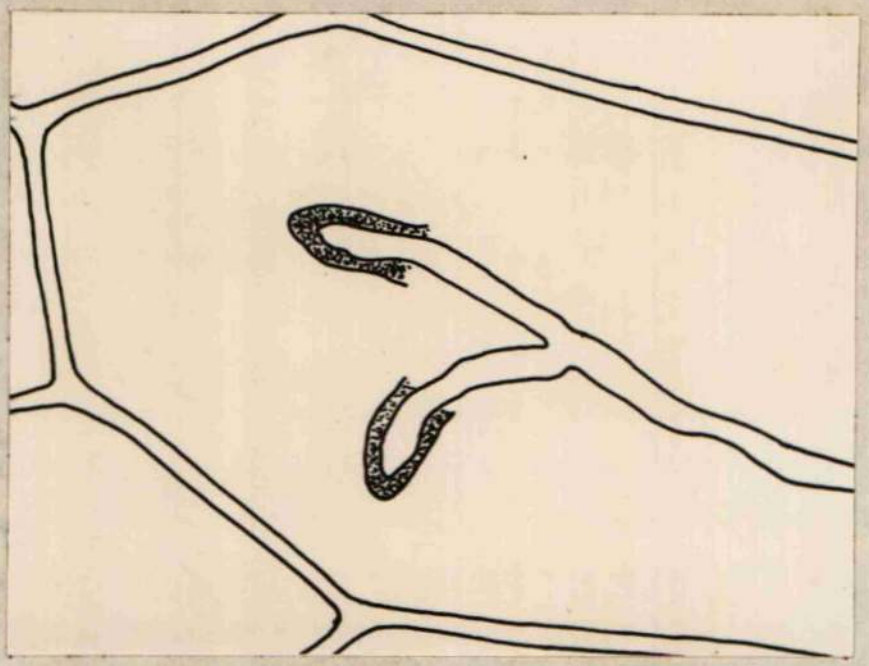


a. Part of an infected cell in a resistant prothallus showing the formation of the brown pigment where the hypha touched the cell wall. (x 1,500)

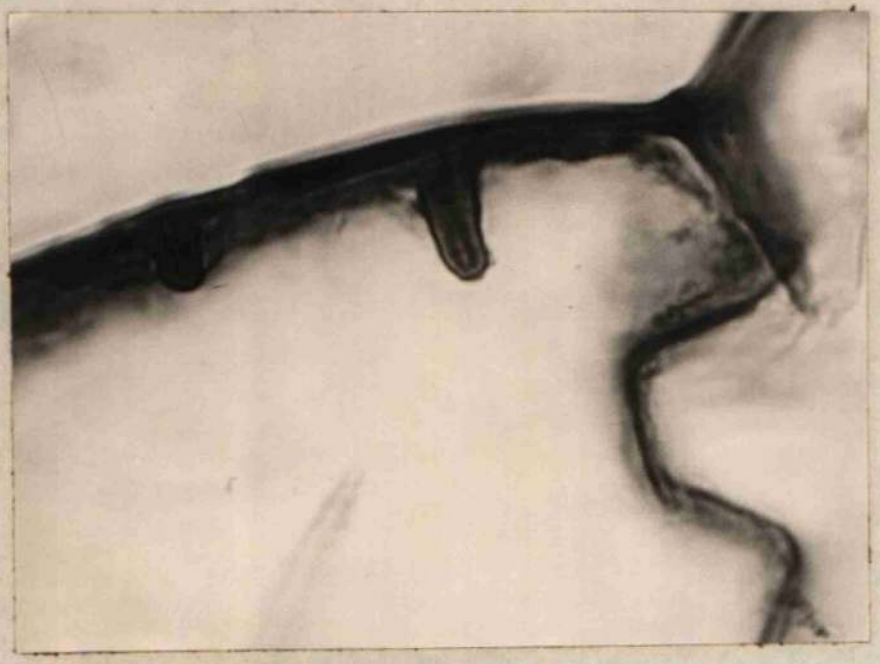


b. Group of papillae showing infection pegs in a resistant prothallus. (x 1,100)

PLATE 12



a. Camera lucida drawing of a cell of a resistant prothallus showing incomplete penetration of two papillae by a branched hypha. (x 1,500)



b. Transverse section of a resistant prothallus showing two infection pegs enclosed by papillae. (x 1,200)

Discussion

Considering, firstly, the process of penetration, it was found that infection pegs were formed on both clones. The cells of both resistant and susceptible prothalli formed papillae around the infection pegs and no hyphae were seen passing through the outer wall without formation of these papillae. In many cases, in both types, the papilla appeared to have prevented the infection peg from penetrating the cell. Hyphae were abundant in the cells of lesions on susceptible prothalli, however. The origin of these hyphae was not seen in any case, but it seems likely that they had come from infection pegs which had penetrated some of the papillae. The infection peg was very small in many cases, and it could not always be traced throughout the papilla. These observations support the hypothesis that resistance to infection can be attributed, in part, to a qualitative difference in the cell wall reaction.

Resistance to the spread of infection seems to be commonly associated with a difference in the rate of browning of the intercellular walls. In some cases, however, the growth of hyphae within the cells of resistant prothalli seemed to be checked by the activity of the cytoplasm.

From these observations it seems reasonable to put emphasis on the study of the differences in wall structure and reaction. It may also, however, be profitable to examine the possibility of cytoplasmic characters affecting the reaction in other ways.

STUDY OF THE CELL WALL

ELECTRON MICROSCOPE INVESTIGATION

Introduction

From the observations made in the preceding section it was noted that the cell wall of the prothalli was relatively thin and thus, even at the highest magnifications available, the light microscope did not reveal any structural details which could account for a difference in susceptibility. Since the cell wall had been found to be involved in resistance, it was decided to use the higher magnifications available with the electron microscope to study the wall structure, and the wall reaction after inoculation.

No records of previous electron microscope studies of this type, involving the attempted correlation of resistance with structure and reaction, were found and there was no record of the study of the cell wall structure of prothalli.

The investigation is separated into (a) the nature of the morphology of the wall and (b) the nature of the walls reaction.

Methods

It was decided to use the two common fixatives, osmium tetroxide and potassium permanganate, in these investigations since it was thought that the use of two fixatives would increase the chance of finding any differences in wall structure which existed between the two clones. Osmium tetroxide reacts with a variety of different cell constituents including phospholipids and unsaturated fats (Bahr, 1954) and this was the first fixative employed. The formula used was that devised by Rodin and Zetterqvist (Zetterqvist, 1956) and is given in Appendix B. Small pieces, approximately 1mm. square, were cut from the wings of prothalli and were immediately placed in 5ml. of cooled fixative in a glass weighing bottle which was then kept at $0^{\circ}C$ for 2 hours. After this period, the fixative was poured off and the pieces were washed in running tap water for 15 minutes, and dehydrated by passing through a graded alcohol series (Appendix B).

Fixation in Luft's Permanganate (Luft, 1956) required a shorter time since the permanganate is a vigorous oxidising agent. Consequently, $1mm^2$ pieces of prothalli were placed in 5ml. of the buffered permanganate solution (Appendix B) for between 30 minutes and 1 hour at $0^{\circ}C$. Washing and dehydration were carried out as in osmium fixation.

The embedding medium used was the epoxy resin, Araldite (Glauert and Glauert, 1958) and the composition of the mixture used is given in Appendix B. The absolute ethanol containing the specimens was replaced by a mixture of equal volumes of absolute ethanol and Araldite mixture and the specimens incubated for two hours at 48°C. The ethanol-Araldite mixture was then removed, and pure Araldite without the amine accelerator added before a further two hours incubation. After this period this was replaced by a fresh quantity of the same mixture and the specimens incubated for between 15 and 18 hours. A final two hours incubation in the complete Araldite mixture followed. The specimens were then placed singly in number 00 gelatine capsules which were filled with fresh Araldite and polymerization of the resin took place within 48 hours at 48°C. It was necessary to maintain the correct orientation of the pieces of prothalli during the early stages of polymerization by the occasional use of a needle since the resin initially became less viscous and the specimens settled in the wrong position for sectioning. When the blocks had polymerized, the gelatine capsules were removed by soaking in warm water.

The specimens in the blocks were trimmed to about 0.1mm^2 and sections were cut using a Porter Blum ultra-microtome (Porter and Blum, 1953) and glass knives

prepared by the method of Latta and Hartmann (1950). The sections were floated on to a ten per cent solution of acetone in water and were flattened by chloroform vapour. Sections showing grey and silver interference colours, that is, ones of approximately 600Å thick and 600-900Å thick respectively (Peachey, 1958), were mounted on 0.1% Formvar films supported on specimen grids.

The sections were examined on a Phillips E.M.100B electron microscope and also on a Siemens Elmiskop I with accelerating voltages of 60kV and 80kV respectively.

Results

a) The morphology of the wall

Plates 13 and 14 show parts of typical sections of the outer wall of material fixed in osmium tetroxide. Several distinct layers were evident. The thickest layer of the wall was the cellulose layer and this appeared relatively undifferentiated but for a slightly more electron-dense outer edge. No fine structure was visible in the cellulose, even at quite high magnifications (Plate 14). The outermost layer appeared as a thin faint line and was generally found to have separated from the cellulose (Plate 13). In Plate 14, this layer appears more darkly-stained than normal but this did not appear to be significant.

The potassium permanganate-fixed material was examined to investigate if any further details were visible. This fixative was found in general to produce better fixed sections and the remainder of the investigation of the wall morphology of uninoculated prothalli was carried out using this fixative. The typical appearance of the outer wall is shown in Plate 15. The same layers which were shown in Plate 13 were visible but the outermost layer, which for convenience will be called the limiting layer, was more electron-dense in the permanganate-stained sections and it

PLATE 13

Outer wall of prothallus of Clone B (resistant).
Osmium tetroxide fixed. (x 23,000).

PLATE 14

Outer wall of prothallus of Clone N (susceptible).
Osmium tetroxide fixed. (x 270,000).

PLATE 15

Outer wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 24,000).

showed up as a discontinuous, granular layer. This character can be seen more clearly in Plate 16.

The staining properties of the limiting layer suggested that it had a waxy or lipid composition and it may therefore be a cuticle. To investigate its true morphological nature more thoroughly, it was decided to compare its appearance with that of a known cuticle. Thus, sections of the leaf of privet (Ligustrum vulgare L.) were prepared in the same manner as the prothallial sections and were examined as before. The cuticle of the privet leaf appeared different in structure from the limiting layer of the prothalli. As shown in Plate 17, the cuticle was approximately six times thicker than the limiting layer, and it was less electron-dense. It formed a continuous layer, as opposed to the granular prothallial layer, and was closely attached to the cellulose of the epidermal wall. However it has been found (Lee and Priestley, 1924) that in the Ferns, the cuticle remained thin and was often discontinuous or absent, so that it is possible that the electron-dense limiting layer in the prothalli may in fact be a layer of wax such as is found on the surfaces of many plants (Esau, 1953). Cuticle is typically a continuous layer as shown in Plate 17 (Esau, loc. cit.). The waxy limiting layer was found to be more closely attached to the cellulose of the walls in some cases (Plate 18), but

PLATE 16

Outer wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 80,000).

PLATE 17

Epidermal wall of privet (Ligustrum vulgare L.).
Potassium permanganate fixed. (x 21,000).

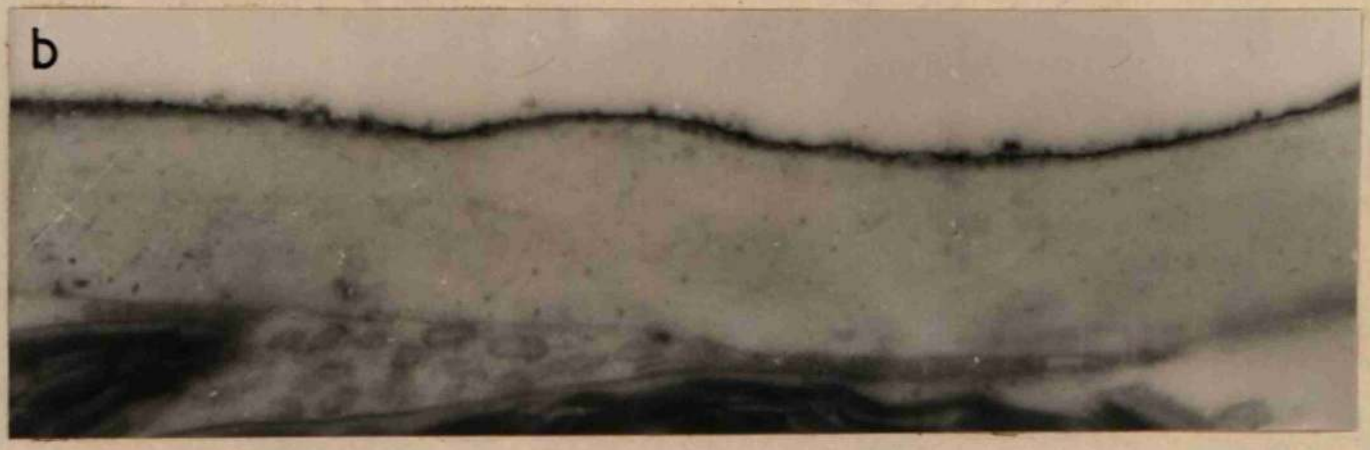
PLATE 18

a. Outer wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 19,000).

b. Outer wall of prothallus of Clone N (susceptible).
Potassium permanganate fixed. (x 24,500).

c. Outer wall of prothallus of Clone N (susceptible).
Potassium permanganate fixed. (x 27,000).


a


b


c

more generally there was a gap between them. Close examination of this gap at high magnifications has suggested that the limiting layer is only loosely attached and no distinct morphological layer exists between it and the cellulose.

It was not possible with uninoculated prothalli to determine which surface was dorsal, since the proper orientation was lost in the preparation of the specimens. The wall, however, was found to have the same structure on both surfaces. Plate 19 shows the form of the wall on the opposite side of the same cell shown in Plate 15. No visible difference was evident other than a slightly more irregular deposition of waxy material on the wall.

The typical appearance of the intercellular wall is shown in Plate 20. In most cases this wall was convoluted, due possibly to the state of turgidity of the cells when fixed. The middle lamella was frequently visible and plasmodesmata were often seen (Plates 20 and 21). The layer which occurred on the outside of the outer wall was never found associated with the intercellular wall.

From comparison of the sections and plates, no obvious differences in thickness or in gross morphology of the wall were found between the two clones. It is possible, however, that differences which were not readily seen, even at the highest magnifications, might exist; for example,

PLATE 19

Outer wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 24,000).

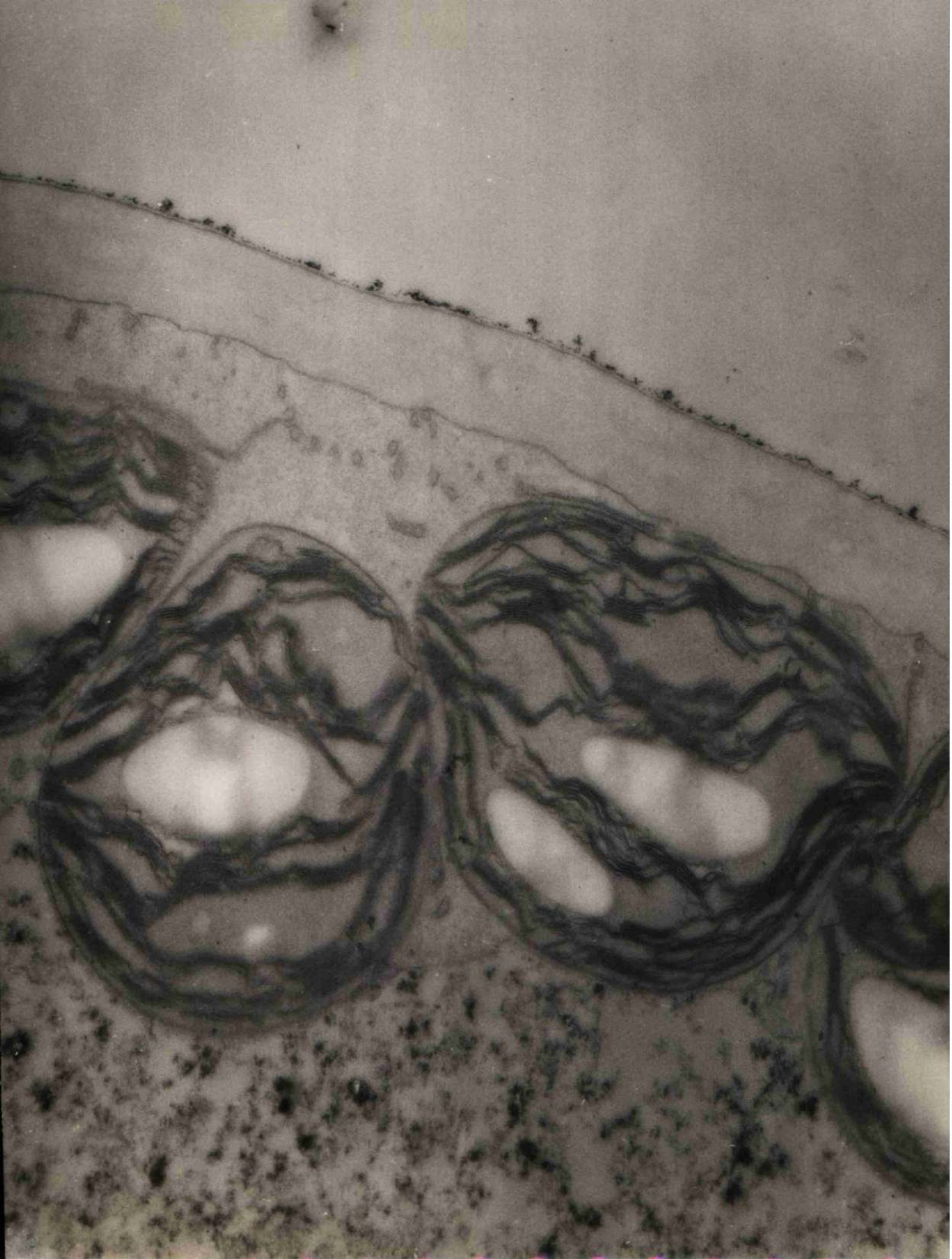
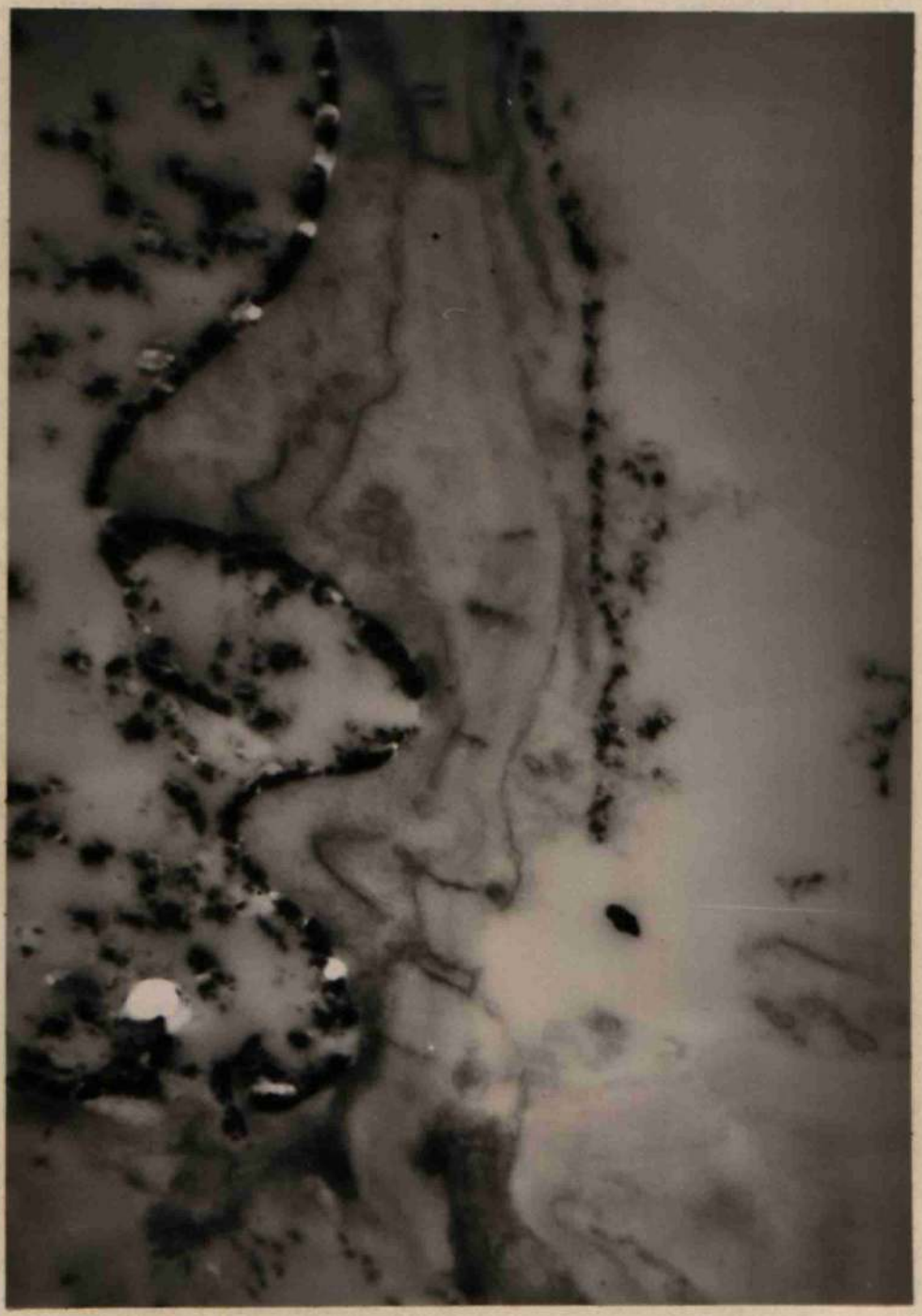


PLATE 20

Intercellular wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 24,000).

PLATE 21



Intercellular wall of prothallus of Clone N (susceptible).
Potassium permanganate fixed. (x 17,750).

the degree of compactness of the fibrils in the cellulose layer. Only one difference in structure was found between the two types of prothalli and this is shown in Plates 18a and 22a (arrowed). On one surface of a resistant prothallus the outer wall showed a broad electron-dense layer at the outer edge (Plate 22a). The other surface of the same section showed no clearly defined similar layer (Plate 22b). One other section which only had part of one surface present showed a similar layer (Plate 18a). No layers such as these were seen in any other sections of prothalli of either clone so no definite conclusions can be drawn from these two cases.

Pores resembling the ectodesmata of some authors were frequently found in sections of prothalli of both clones (Plate 23) and their possible relationship with disease resistance is discussed below. They occurred on both surfaces of the prothalli and were similar in appearance and distribution in both clones. In some cases the pores had well defined edges at their extremities while the central part was very electron-dense, due possibly to densely stained contents or unremoved fixative (Plate 24). In other cases, there was no electron dense region and the outline of the pore could be seen clearly, although in many instances it did not completely traverse the wall (Plate 25). This may have been the result of the angle of sectioning.

PLATE 22

a. Outer wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 27,000)

b. Outer wall of prothallus of Clone B (resistant).
Potassium permanganate fixed. (x 27,000).

PLATE 23

Outer wall of prothallus of Clone N (susceptible).
Potassium permanganate fixed. (x 19,500).

PLATE 24

Outer wall of prothallus of Clone B (resistant).
Osmium tetroxide fixed. (x 62,000).

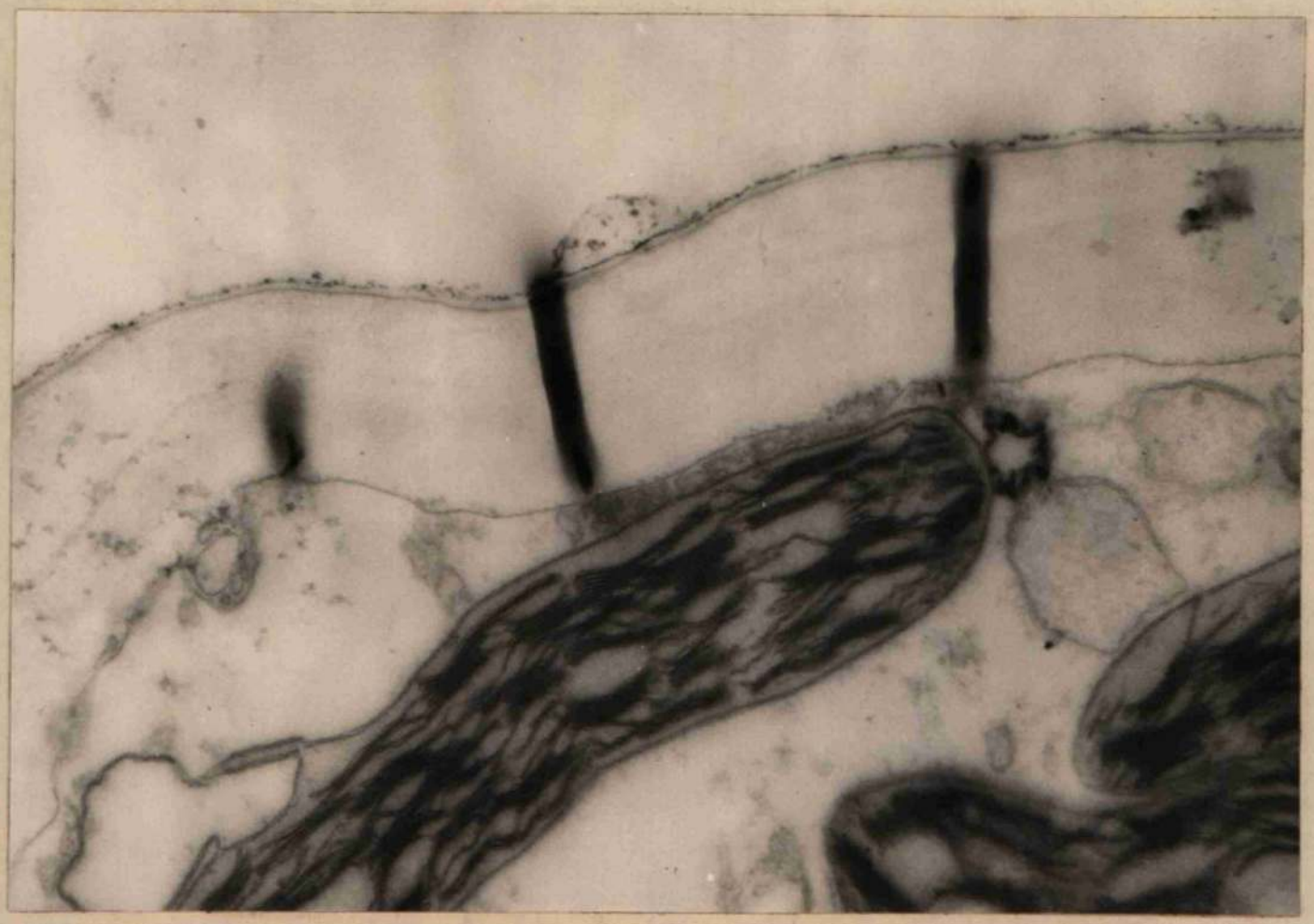


PLATE 25

Outer wall of prothallus of Clone B (resistant).
Osmium tetroxide fixed. (x 58,500).

PLATE 26

Outer wall of prothallus of Clone N (susceptible).
Potassium permanganate fixed. (x 24,500).

Since the pores were approximately 0·1μ to 0·2μ in diameter and the section thickness was around 600 Å, a non-median section through a pore would also result in the appearance of the pores shown in some sections (e.g. Plate 25). A median section would be required to show a whole pore. Evidence that the structures observed were pores in section was afforded by examples such as those shown in Plate 26. This section showed a group of pores which were electron-dense, either due to the presence of waxy or lipid contents, or unremoved fixative. When the microscope condenser was turned up, the electron-dense regions in the pores were broken up in places by the electron beam. The wall around the pore remained intact. Further evidence was obtained from a comparison of the suspected pores or ectodesmata with known folds occurring in the sections. Folds occurred at random in the sections and varied greatly in size while the pores described above were located in the outer cell walls only and were always of similar size. In a few cases ectodesmata were found with well-defined contents (Plates 27 and 28). In the section shown in Plate 27, the contents of the very electron-dense pore had apparently been partly extruded from the outer surface, producing a mound of particles of a waxy composition. The section in Plate 28 shows a similar situation. Two well-defined pores are visible and these contain electron-dense objects which appear

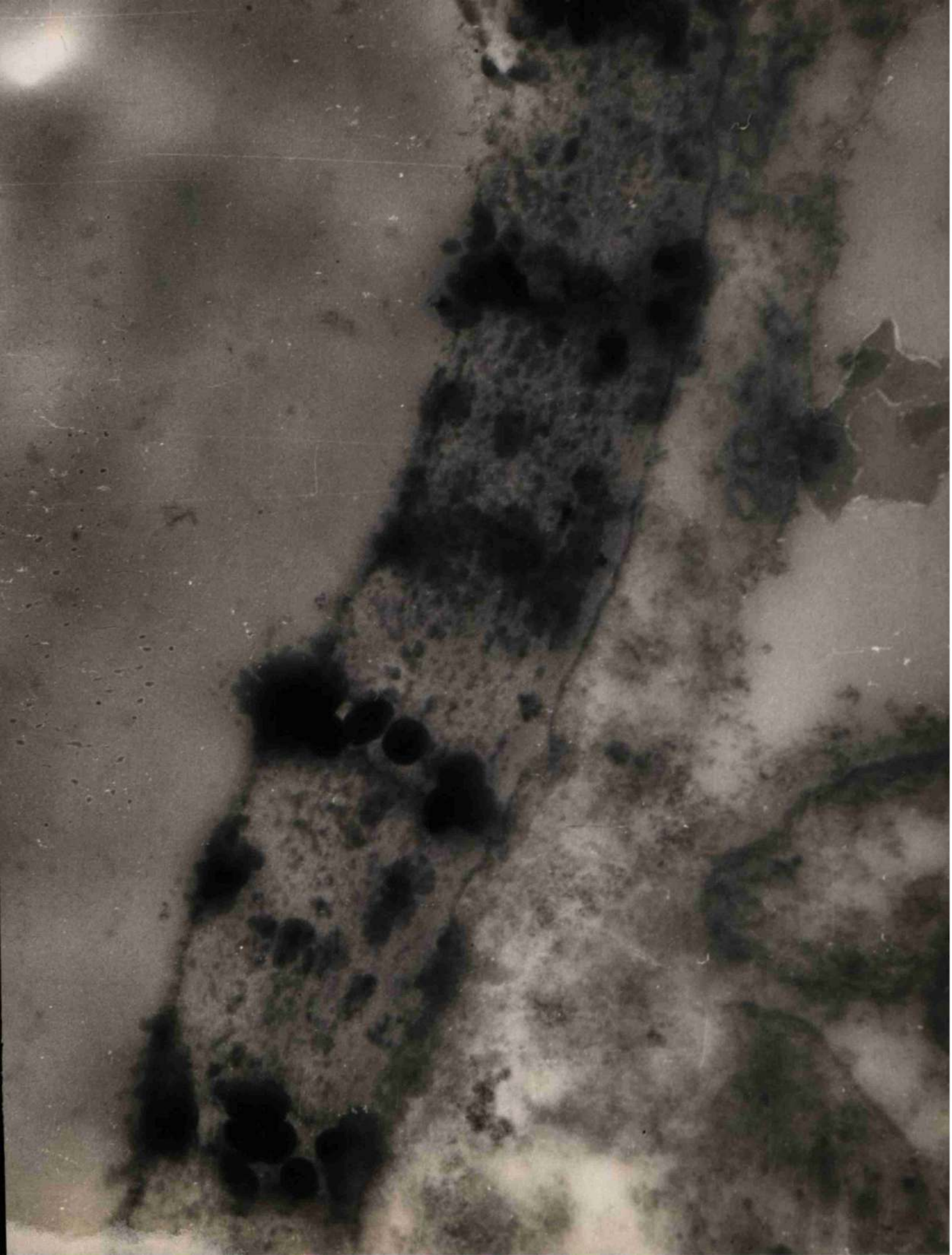
PLATE 27

Outer wall of prothallus of Clone N (susceptible).

Osmium tetroxide fixed. (x 21,000).

PLATE 28

Outer wall of prothallus of Clone J.
Osmium tetroxide fixed. (x 81,000).



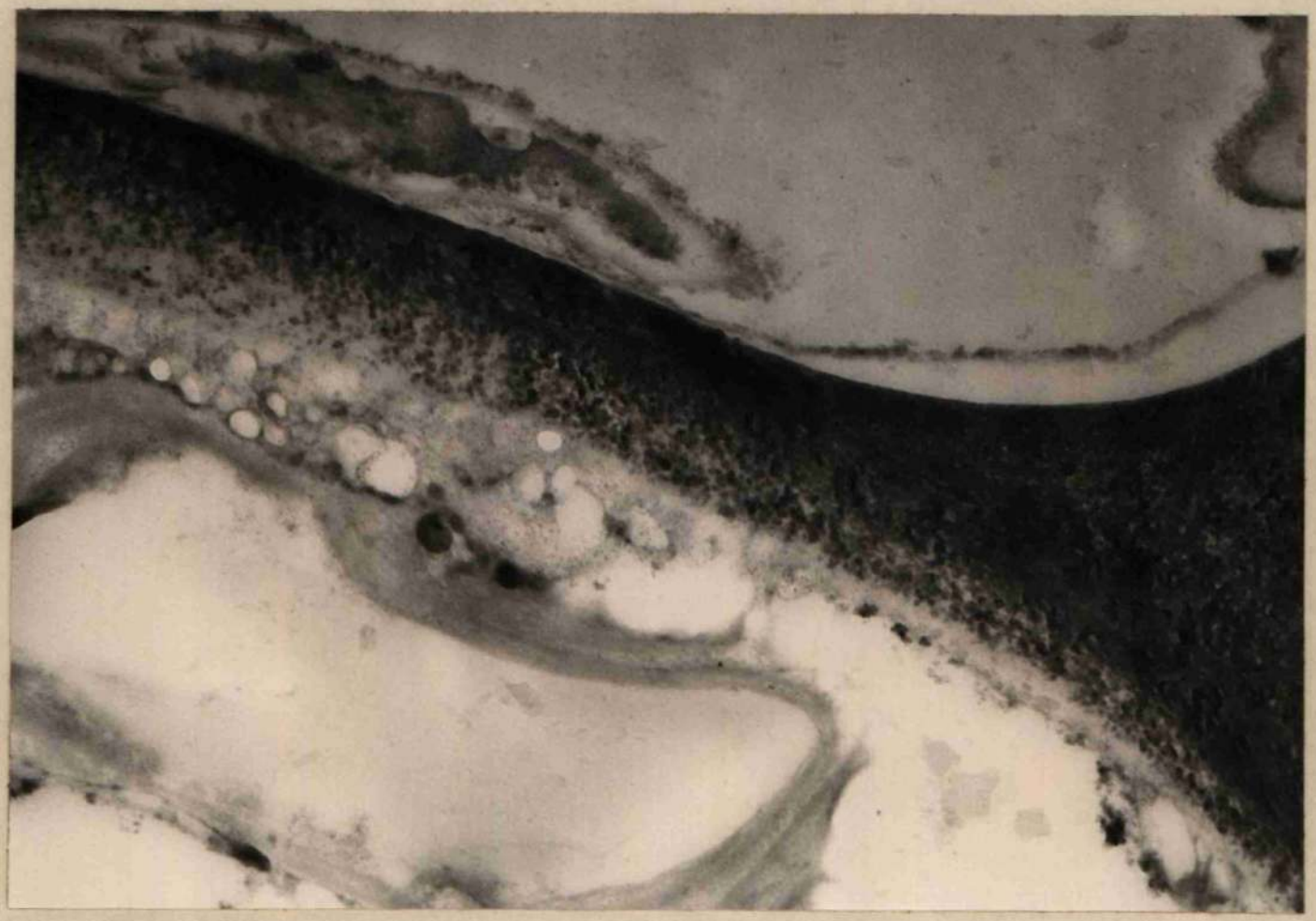
globular. At the outer surface these objects are much smaller and have the appearance of having been extruded. At other places on these are similar small groups of electron-dense particles on the surface while the actual cellulose layer appears to contain large amounts of similar material.

The distribution of the ectodesmata was not uniform. In some cases they occurred in groups in large numbers of sections examined while other sections showed a few isolated ectodesmata or none. Both uninoculated and inoculated prothalli of the two clones examined contained them and no obvious difference in their distribution was evident. Prothalli of a third clone were examined and this clone also contained ectodesmata (Plate 28). As mentioned above, the intercellular walls contained plasmodesmata.

b) The reaction of the wall.

Two distinct types of wall structure were found in sections of infected prothalli. The first type was that described above for uninfected prothalli. In the same sections, however, walls having an entirely different appearance were found (Plate 29). From the occurrence of these walls in material known to contain brown-pigmented walls and their presence under spores and hyphae which are known to stimulate the production of the brown pigmentation, it seemed reasonable to equate the wall reaction described below with brown pigmentation. The walls were more electron-dense than normal and this was due to the presence, in the cellulose layer, of numerous electron-dense particles (Plate 30). In many instances, the wall appeared more electron-dense towards the outer edge and this was due to the greater density of particles and also to apparent coalescing of these particles (Plate 30). In other cases there was no visible gradation (Plate 31). Many walls examined did not appear particulate but were extremely electron-dense (Plate 32). Evidence of the hard nature of this type of wall was given by the large number which showed splitting, caused by the microtome knife (Plate 32). It seems probable that this represents a later stage in development of the wall reaction than that shown in Plates 29 and 31.

PLATE 29



Outer wall of prothallus of Clone B (resistant)
after inoculation with Botrytis cinerea.
Osmium tetroxide fixed. (x 30,000).

PLATE 30

Outer wall of prothallus of Clone B (resistant) after inoculation with *Botrytis cinerea*. Osmium tetroxide fixed. (x 69,250).

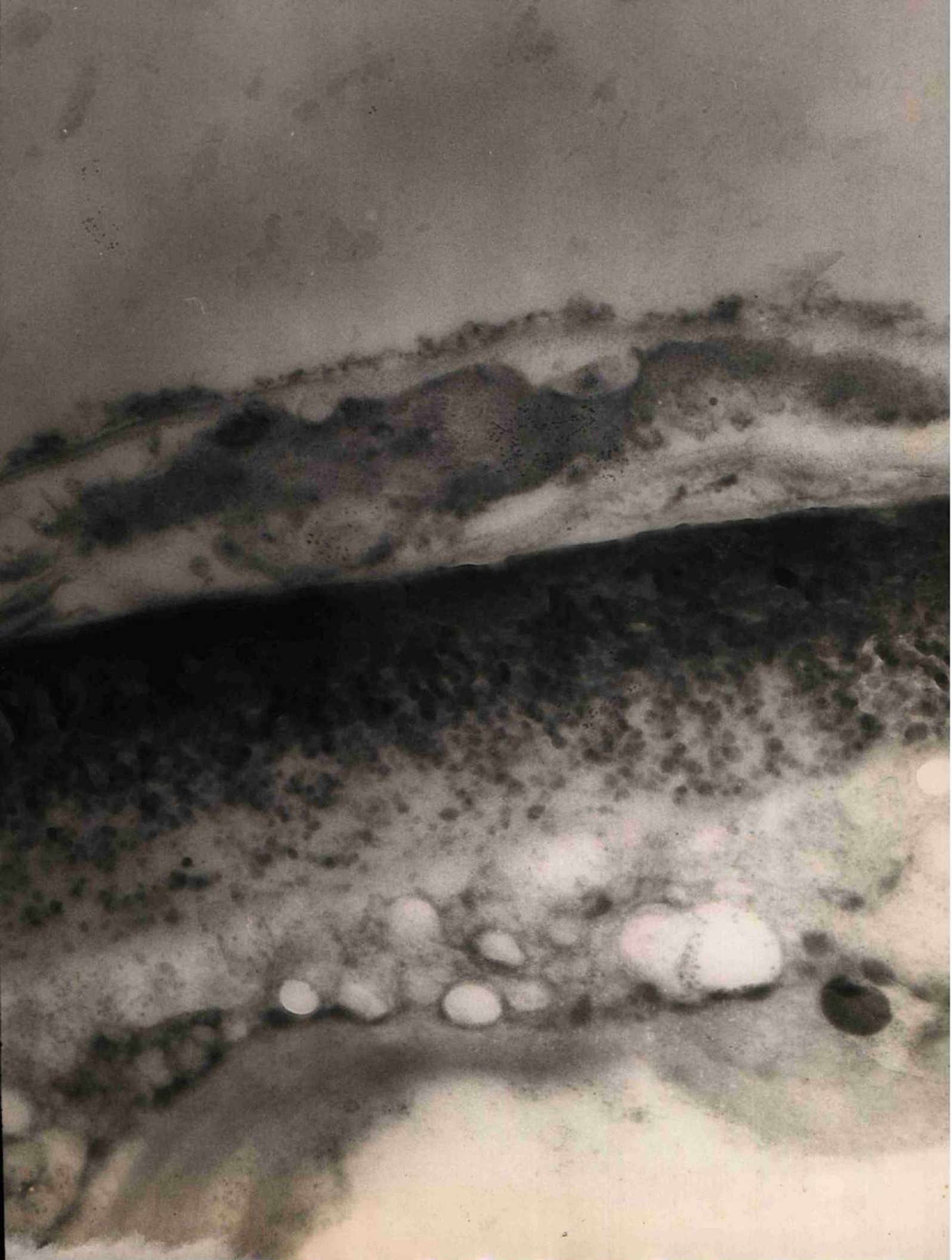
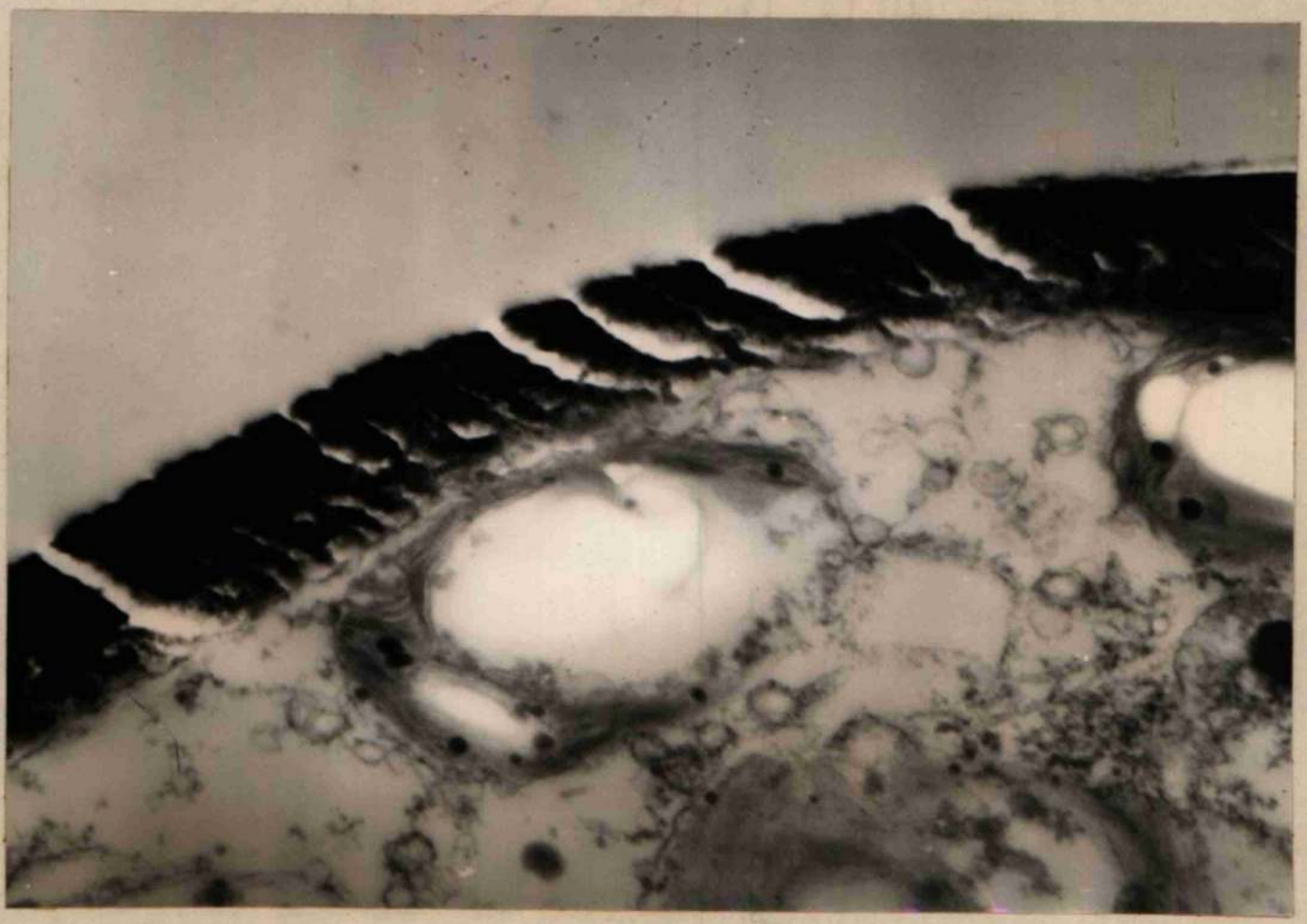


PLATE 31

Outer wall of prothallus of Clone N (susceptible) after inoculation with Botrytis cinerea. Osmium tetroxide fixed. (x 15,300).

PLATE 32

Outer wall of prothallus of Clone N (susceptible) after inoculation with Botrytis cinerea. Osmium tetroxide fixed. (x 14,250).



Spores and hyphae of Botrytis were frequently found on the walls (Plate 31) but no obvious signs of intercellular hyphal development were seen. No penetration of the wall was seen, nor were there any infection papillae found. Plate 33 shows a germ tube and spore of Botrytis in section, however. The germinating spore was closely attached to the limiting layer and the underlying wall was very electron-dense. In most sections examined, the fixing of the spore to the limiting layer was very evident (Plate 31) and there were many cases found of spores detached from the wall, but still closely fixed to the limiting layer (Plate 34).

The intercellular wall showed a similar reaction to that produced in the outer wall.

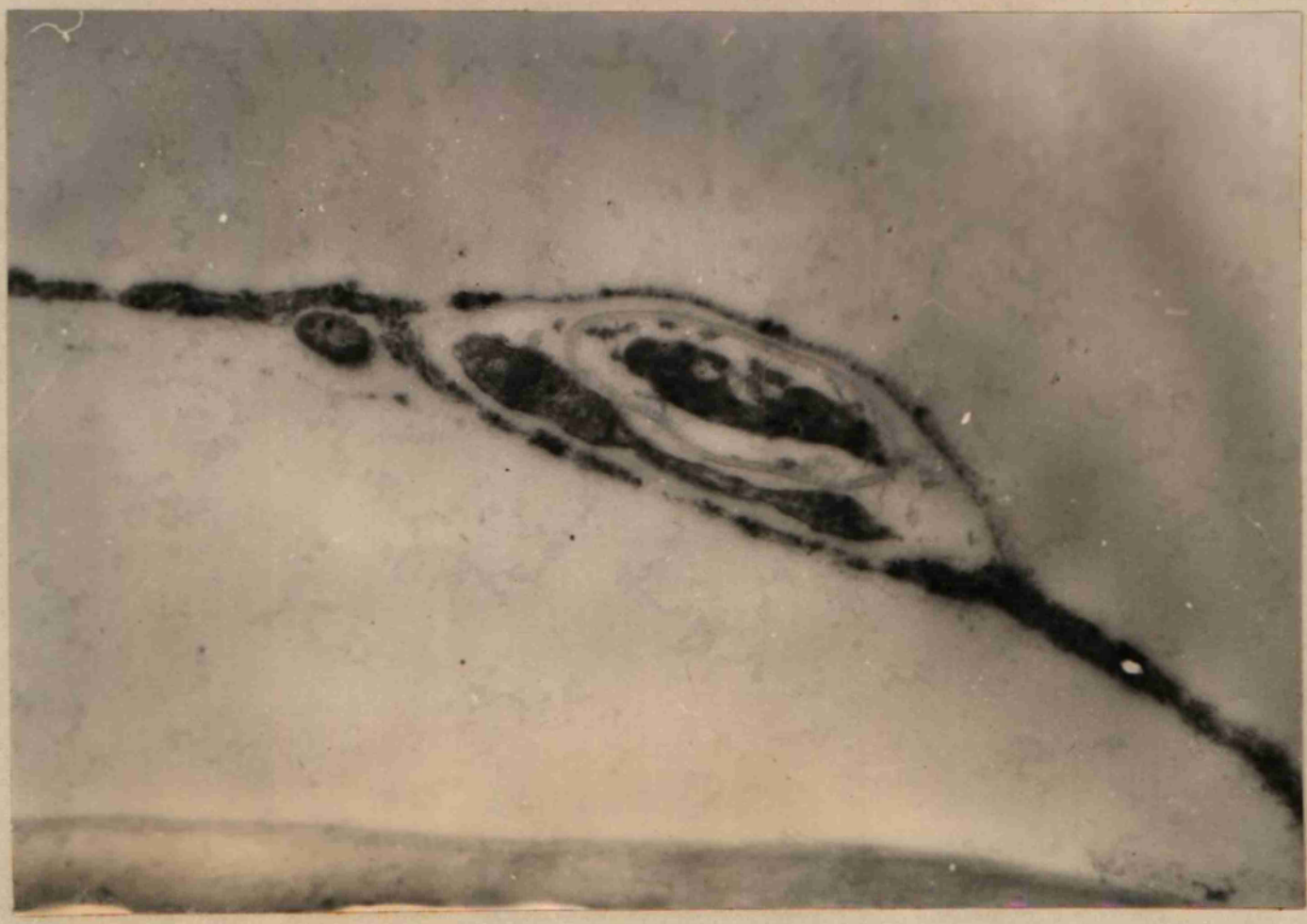
No differences in distribution, structure or development of the electron-dense walls were found between the two clones.

PLATE 33

Spore and germ tube of *Botrytis cinerea* on the outer wall of a prothallus of Clone B (resistant). Osmium tetroxide fixed. (x 26,400).

PLATE 34

Detached spore of *Botrytis cinerea*. Osmium tetroxide fixed. (x 15,500).



Discussion

The results of this investigation have shown that the morphology of the cell wall and also its reaction do not differ between resistant and susceptible prothalli. There was no sign of any obvious structural feature such as wall thickness which may have contributed to resistance. The possibility of a difference in the cellulose still exists, of course, as the fine structure of this layer was not revealed by the techniques employed here.

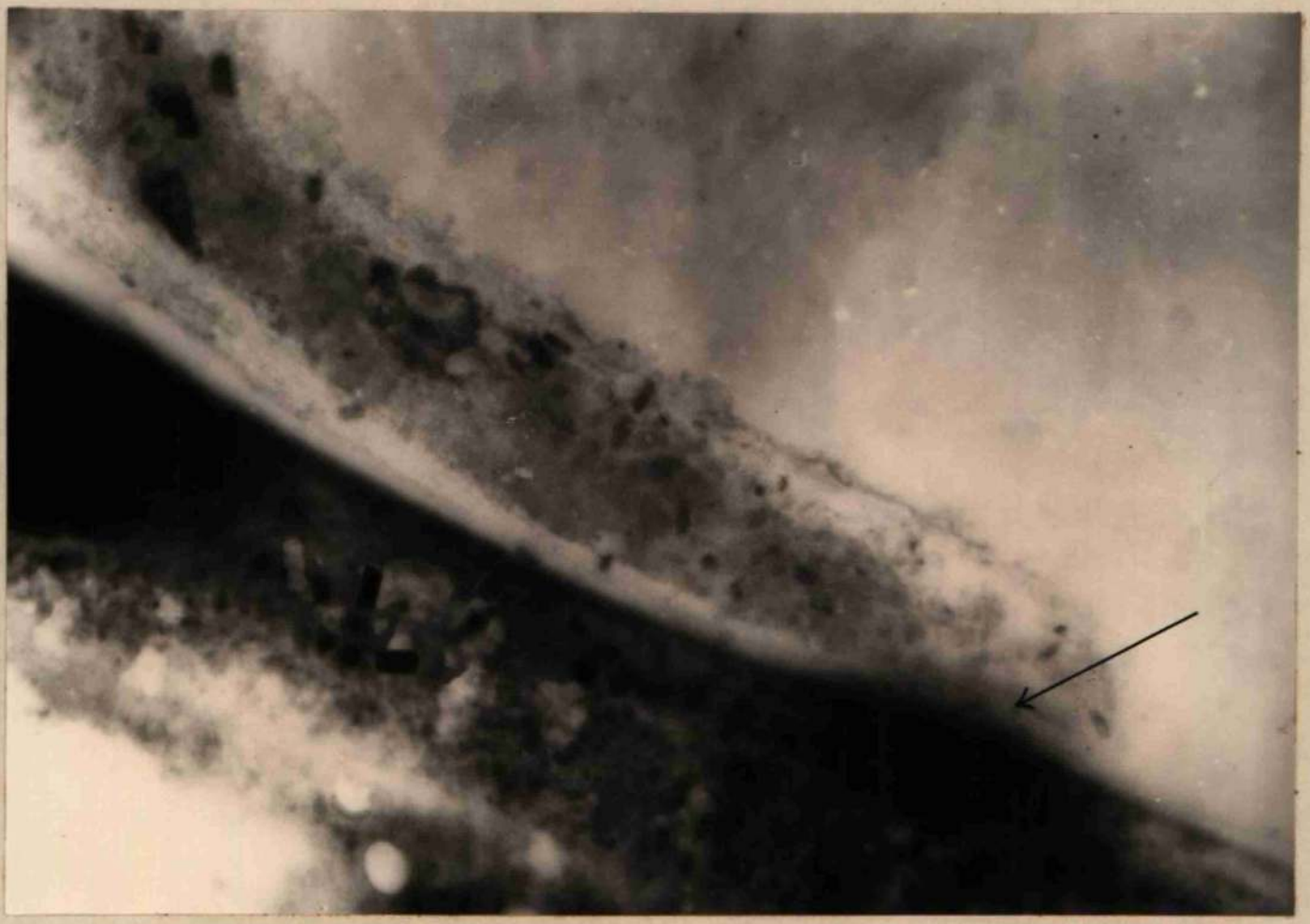
The nature of the reaction of the host cell wall as shown by the electron microscope is interesting although the reaction appeared to be similar in both clones. It seems reasonable to identify the electron-dense walls with the brown walls found in infected prothalli when examined by light microscopy. From the staining reactions of the walls with the two fixatives used, it appears that the browning reaction may be associated with the deposition of fatty, waxy or lipid materials in the cellulose layer of the wall. The distribution of the particles or globules suggests in many cases that deposition may commence at the outer edge of the wall. The very electron-dense walls which did not show an obvious particulate structure may be considered to show the extreme wall reaction and this view was substantiated by the hardness of such walls (Plate 31)

and the extreme electron-density of parts in contact with hyphal tips (Plate 35, arrowed).

An interesting feature shown in the sections was the close attachment of the spores and hyphae to the limiting layer which was frequently found separated from the cellulose and taking the spores with it.

There have been reports of pores and plasmodesmata in the outer wall of epidermal cells of plants (Gardiner, 1897 and Schumacher and Halbsguth, 1939). It was suggested that these pores were artifacts (Meeuse, 1941) but it now seems possible that plasmodesmata do exist in outer cell walls (Schumacher, 1942; Lambertz, 1954; and Schumacher and Lambertz, 1956). Further evidence concerning the presence of ectodesmata has been furnished by the electron microscope studies of Huber, Kinder, Obermüller and Ziegenspeck (1956), Schumacher (1957) and Schnepf (1959). The ectodesmata have been found to be protoplasmic (Franke, 1962) and they are claimed to be direct protoplasmic connections between the cell and the external surface (Franke, 1961a). It has been suggested (Franke, 1961a and b) that ectodesmata may serve as pathways for the transport of liquids and dissolved solutes from the epidermal cells to the outer surface. Bearing in mind that cells may inhibit or stimulate spore germination and germ tube growth by the exudation of substances in solution

PLATE 35



Tip of germ tube shown in Plate 33.

Osmium tetroxide fixed. (x 36,000).

then the channels through which these solutions pass may be the ectodesmata. Thus their presence or distribution may be indirectly involved in disease resistance of a plant. Franke (1961a) has suggested the possibility of ectodesmata serving as entrances for viruses in suscepts which have these organisms on the surface. There are obvious problems involved in obtaining ultra-thin sections through infection pegs when the sections are approximately 0·1μ in thickness and the face of the material being sectioned is only 0·1mm. square. Thus, even although large numbers of sections were examined no infection pegs were found so that there was insufficient evidence obtained for drawing any conclusions concerning ectodesmata serving as initial sites of penetration of the infection pegs of Botrytis, but they were frequently found in the walls of infected material. The possibility of ectodesmata being involved in the stimulation of appressorial formation by serving as the pathways for stimulatory exudates seems worthy of future investigation. Similarly the pores may furnish at least part of the answer to the question of the factors which cause a germ tube to produce an infection peg at the particular point where this happens, after growing haphazardly over the surface.

The pores shown in Plates 27 and 28 show an interesting feature which, although not entirely relevant to the discussion on disease resistance, is worthy of consideration.

The origin of the wax deposits on leaf surfaces has interested many workers since De Bary (1871) postulated the extrusion of wax through the cuticle. There has been much speculation concerning the source of migrating fatty substances and their path of movement (Esau, 1953). Priestley (1943) has suggested that the movement of the fatty substances occurs through special channels and this idea has been investigated by electron microscopy (Mueller, Carr and Loomis, 1954). These authors postulated that wax is not extruded in liquid form but in a softened form under some pressure and that the wax is extruded through preformed pores. Juniper and Bradley (1958) used a carbon replica technique to study leaf surface waxes but they did not demonstrate the presence of pores. Scott, Hamner, Baker and Bowler (1957), in their studies on the onion epidermis, have suggested that wax, presumably in liquid form, extrudes through pits in the outer wall. More recently, Hall and Donaldson (1962) have shown that pores were present beneath each particle of wax on the leaf surfaces of several plates. All the above work has been carried out with surface replica techniques and so it is interesting to compare these results with the sections in Plates 27 and 28. These sections suggest that the contents of the pores are fatty substances (by virtue of their staining reaction) and that these substances are being

extruded on the outer surface of the wall in fine particles or globules. The wall also shows much electron-dense material dispersed in the cellulose layer. Thus the pores in the prothallial wall may be the pathways for the secretion of the waxy limiting layer which was found on the wall surface.

STUDY OF THE CELL WALL

THE EFFECT OF THE MACERATING ENZYME PRODUCED BY BOTRYTIS CINEREA ON THE WALL

Introduction and Literature Review

Since the early work of Brown on the part played by the pectic enzymes in pathogenesis (Brown, 1915), much has been published, especially on the action of these enzymes and their production *in vitro*. This section reports on a test of the hypothesis that resistance to *Botrytis cinerea* in clonal populations of bracken prothalli is correlated with resistance of the tissues to maceration by the enzyme produced by the fungus. The enzyme protopectinase, which has as its substrate the substance protopectin, has been thought to be responsible for this maceration, but recently, Wood (1960b) has stated that although maceration may include the action of protopectinase, it may also include other activities so that until maceration has been established as being essentially due to the degradation of protopectin, then it seems better not to equate the term protopectinase with maceration. In this report, however, the term protopectinase activity is used to describe the whole macerating activity of the culture filtrates of *B. cinerea*. Wood (1959, 1960b) has reviewed the work on the role of pectolytic enzymes in plant pathogenesis.

Wood (1960b) has suggested that the composition of the middle lamella probably varies a great deal between different plants and between different parts of the same plant at different stages of development, and that these differences undoubtedly affect the susceptibility of the tissues to degradation. The middle lamella is thought to consist mainly of the calcium and magnesium salts of pectic acid and so the calcium and magnesium nutrition of plants during the formation of the middle lamella may affect the susceptibility of the tissue to attack (Wood, 1959). It has been found, for example, that calcium deficiency in tomato resulted in increased susceptibility to wilt (Edgington and Dimond, 1959 and Edgington, Corden and Dimond, 1961) and an increase in resistance was correlated with an increased supply of calcium (Edgington and Walker, 1958). It has been shown by Ginzburg (1958) that the middle lamella may contain other substances as well as pectates. He showed that the middle lamella of the cells in pea root tips contained protein. The presence of protein, evenly dispersed in the middle lamella, could profoundly alter the susceptibility of the pectates to pectic enzyme activity since the pectate would be inaccessible until exposed by the proteolytic enzymes which, although present in the organism, may be absent from culture filtrates of the pectic enzymes (Wood, 1959).

There is also evidence that the susceptibility of tissues to enzyme action may be affected by the presence of specific inhibitors of pectic enzymes in the host (Cole, 1956; Echandi and Walker, 1957; Byrde, 1957; Ramaswamy and Lamb, 1958; and Grossman, 1958). Phenolic substances in the hosts are important in pathogenesis in that they may be resistance factors giving rise to specific enzyme inhibitors upon oxidation (Wood, 1959).

Method

An active preparation of protopectinase was prepared by a modification of Brown's method (Brown, 1915) as described by Hutchinson and Fahim (1958). A spore suspension was prepared by scraping the spores from the surface of each of five 12-day-old Roux flask cultures of Botrytis cinerea a potato extract agar medium into 15 ml. of sterile Wood's solution.* The suspension was filtered through sterile muslin and 5 ml. aliquots were used to inoculate 10 conical flasks of 250 ml. capacity, each containing 35 ml. lots of sterile Wood's solution. After 6 days incubation at 20°C in the dark, the contents of the flasks were filtered through sterile muslin and centrifuged for 5 minutes at 3000 r.p.m. to remove mycelium and spores. The filtrate thus obtained contained a solution of the enzyme protopectinase.

Prothalli which were to be tested for resistance to maceration were injected under vacuum with water to remove

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* Formula of Wood's Solution:

Glucose 20 g.; Asparagine 2·5 g.; Potassium dihydrogen phosphate 1·0 g.; Magnesium sulphate 0·3 g. and Distilled Water 1000 ml.

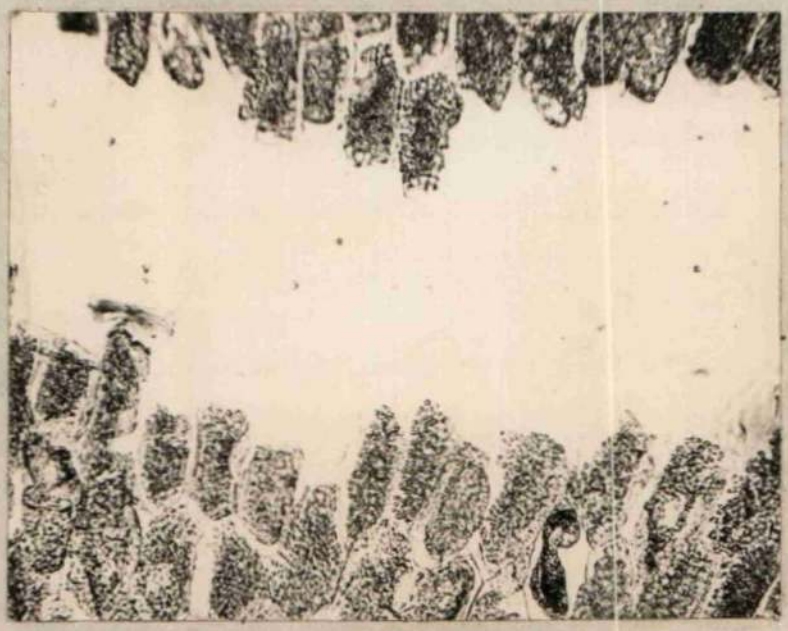
intercellular air and render all cells equally turgid. This procedure also ensured that the prothalli remained submerged in the test solution. After removing the surplus water from the prothalli by draining them over clean filter paper, they were placed in watchglasses of the filtrate. Macerated prothalli were identified by lifting the plants on a needle when those which were macerated collapsed and tore easily, the line of cleavage being along the region of the middle lamella (Plate 36a) while unaffected plants remained stiff and broke irregularly across the cells (Plate 36b). The time for maceration to occur was noted.

In these experiments four prothalli of each clone were placed in the culture filtrate. A control was set up using unfermented Wood's solution.

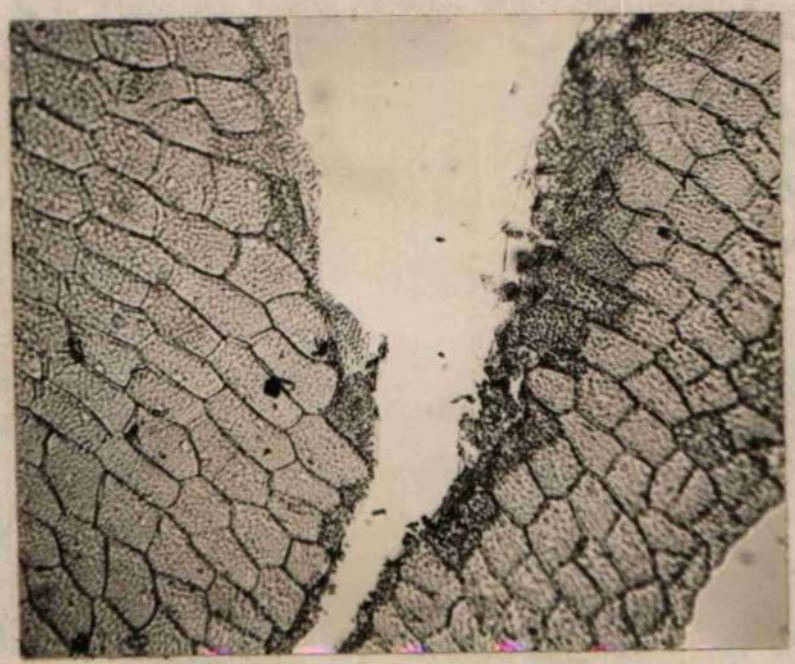
To enable comparison of the results of different experiments to be made, the relative macerating activity of the filtrate used in each case was determined by the method of Brown (1915) involving potato discs. Sections (0•5 mm. thick) of potato tuber were prepared from a cylinder of tissue, 1•5 cm. in diameter and these discs were evacuated under water as were the prothalli. After thorough washing in distilled water, they were surface-dried and placed in the filtrate. The activity of the protopectinase was estimated as the inverse of the time taken for the tissue to lose coherence as determined by pulling the discs

PLATE 36

The effect of pulling upon macerated and non-macerated bracken prothalli.



a. Tearing of a macerated prothallus along the line of the middle lamella. (x 70)



b. Tearing of a non-macerated prothallus across the cells. (x 70)

by hand. When no perceptible resistance was felt, it was considered that coherence was lost.

Results

The results of three replicate experiments are summarised in Table XVI. In addition to prothalli of clones B (resistant) and N (susceptible), plants of clone M (susceptible) were used in the first two experiments since they were readily available at this time.

In all three experiments, potato discs were macerated in between 2 and 2¼ hours. The control discs in unfermented solution were unaffected and were as stiff and firm as at the time of immersion.

The prothalli became affected after ½ to 1¼ hours after immersion, the cell contents becoming disorganised with scattering of the chloroplasts and with less clearly seen walls (Plate 37 a & b). Cellular disorganisation of almost all cells in the prothalli occurred between 3 and 3¾ hours in the three experiments. After this time, the prothalli were completely limp when lifted on the point of a needle and fell apart at the slightest touch, the line of cleavage being along the line of the middle lamella (Plate 36a). Control prothalli appeared unaffected and, when pulled, they tore across the cell walls and cytoplasm (Plate 36b).

There was no perceptible difference in time taken for maceration of the prothalli of the different clones involved.

At the point of maceration, all prothalli showed similar amounts and type of cell disorganisation.

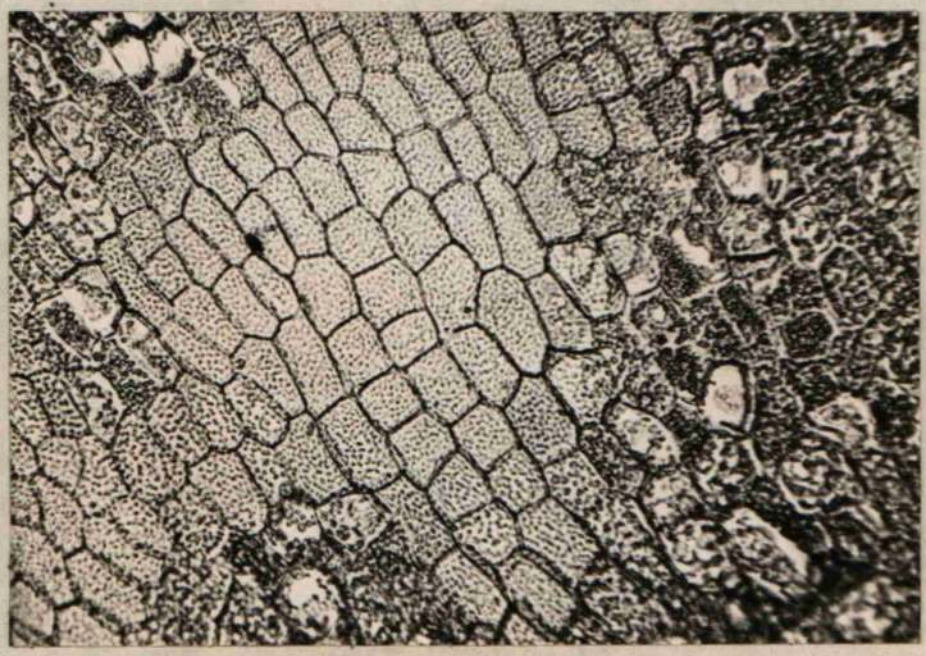
Table XVI

The effect of filtrates from cultures of Botrytis cinerea on prothalli of three clonal populations of bracken

Experiment	I			II			III	
Time taken to macerate potato discs	2 hours			2¼ hours			2 hours	
Clone	B	M	N	B	M	N	B	N
Time taken to macerate prothalli	3½	3½ hours	3½	3¾	3¾ hours	3¾	3 hours	3
Proportion of disorganised cell in prothalli at point of maceration	88%	88%	88%	100%	100%	100%	100%	100%

PLATE 37

The effect of the culture filtrate of Botrytis cinerea on prothalli of bracken.



a. Part of the surface of a macerated prothallus showing a reas of disorganised cells with scattered chloroplasts. (x 110)

b. Photograph of disorganised cells with contorted cell walls. (x 280)

Discussion

The above results show that the resistance of prothalli of Clone B to *Botrytis cinerea* cannot be correlated with resistance of the tissue to maceration by the enzymes produced by the fungus *in vitro*. The possibility exists, however, that the enzymes responsible for maceration may behave differently *in vivo*, but further study would be required to verify or reject this. Essential active factors of the fungal enzymes may be absent from the culture filtrate and this may affect the prothallial resistance. There is no obvious inhibition of the macerating activity in the resistant prothalli nor inhibition of the toxic effect of the filtrate on the cells since the results showed that disorganisation occurred to the same extent in prothalli of each clone.

STUDY OF THE CELL SAP

Introduction

The possibility of resistance to *Botrytis cinerea* in bracken prothalli being related to properties of the cell sap will be examined in this section. The importance of the presence of toxic substances in the plant cell, before or after infection, or of the absence of an essential substance, has already been discussed in the introduction to this part of the thesis.

Method

Approximately one gramme of living prothalli of the clone to be tested was placed in a sterile test-tube and frozen in solid carbon dioxide for 5 hours to facilitate sap extraction. After freezing, the prothalli were slowly thawed and ground up with 0·5 ml. of sterile distilled water in a sterile homogenizer, a separate one being used for each clone. After grinding, the contents of the homogenizers were centrifuged at 3000 r.p.m. for 5 minutes to remove the solid material, the supernatant was removed and the volume obtained from each clone was measured with a hypodermic syringe. The extracts were then diluted 1:1 with a spore suspension of Botrytis cinerea, prepared in the usual manner. A control suspension was prepared with sterile distilled water.

Sterile glass slides, each bearing one drop of each spore suspension, were kept in a humid atmosphere by placing them on moistened filter papers in Petri dishes. These dishes were placed in an incubator at 17°C. This temperature had been found to be convenient in preliminary experiments as 18 hours incubation in these conditions allowed the amount of germination to be determined and the germ tubes had grown to a suitable size for observation. The percentage spore germination was determined by counting

the numbers germinated in ten random high power microscope fields for each drop. A minimum of 100 spores was counted in each drop and the germ tube growth was examined qualitatively.

Two series of experiments were carried out:

1) Using uninoculated prothalli to investigate the hypothesis that resistance could be due to some different extractable property of the cell sap of the different clonal populations.
2) Using uninoculated prothalli and prothalli inoculated with a spore suspension of Botrytis cinerea four days before extraction. This was carried out to investigate the hypothesis that some different extractable property could develop postinfectionally in the two clones, and that this could affect resistance.

Results

The amounts of germination are shown in Tables XVII and XVIII, and the germ tube growth is shown in Plates 38 and 39.

The results shown in Table XVII were obtained using extracts of uninoculated prothalli while Table XVIII summarises the results obtained in three replicate experiments of the second series, using inoculated and uninoculated prothalli. An experiment involving uninoculated and inoculated prothalli of one clone only is included in Table XVIII.

The appearance of the germ tubes shown in Plates 38 and 39 was similar in each experiment in each series.

Table XVII

The percentage spore germination of Botrytis cinerea in water extracts of two clonal populations of bracken prothalli

Experiment	Source of Extract		
	Clone B	Clone N	Distilled Water
I	97·7	98·7	98·7
II	98·8	97·2	95·5

PLATE 38

Photographs of spores and germ tubes of Botrytis cinerea after germination in distilled water and extracts of prothalli of different clonal populations of bracken.

a. Germination in extract of Clone B (resistant).

b. Germination in extract of Clone N (susceptible).

c. Germination in distilled water.

Magnification in all cases = x 200.

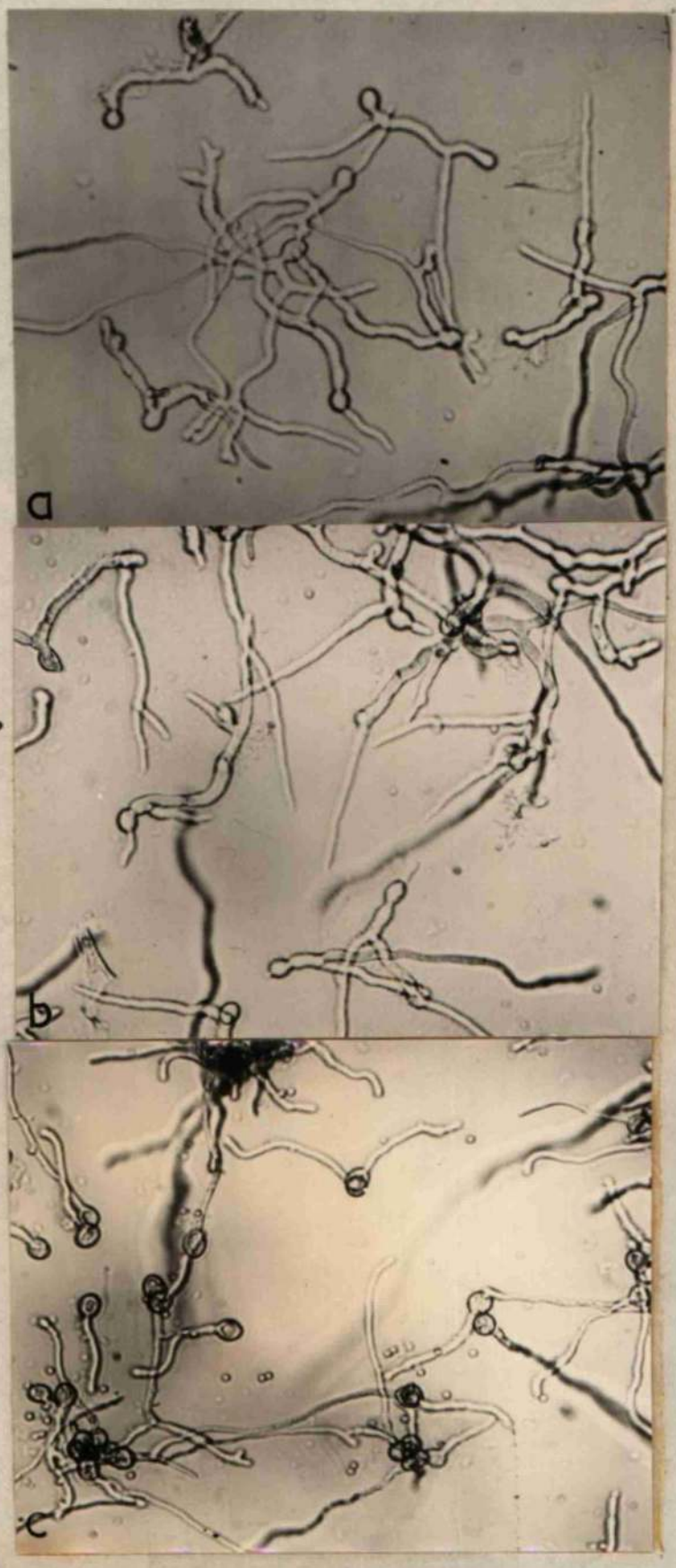


Table XVIII

The percentage spore germination of Botrytis cinerea in water extracts of two clonal populations of bracken prothalli

Experiment	Source of Extract				
	Clone B uninoculated	Clone B inoculated	Clone N uninoculated	Clone N inoculated	Distilled Water
I	95·2	94·9	*	*	96·9
II	98·5	98·1	98·8	98·6	98·5
III	97·5	98·7	96·5	98·3	98·2
IV	99·1	99·1	99·4	98·1	93·9

* - not used in this experiment.

PLATE 39

Photographs of spores and germ tubes of Botrytis cinerea after germination in distilled water and in water extracts of prothalli of different clonal populations of bracken.

a. Germination in extract of Clone B (resistant), inoculated.

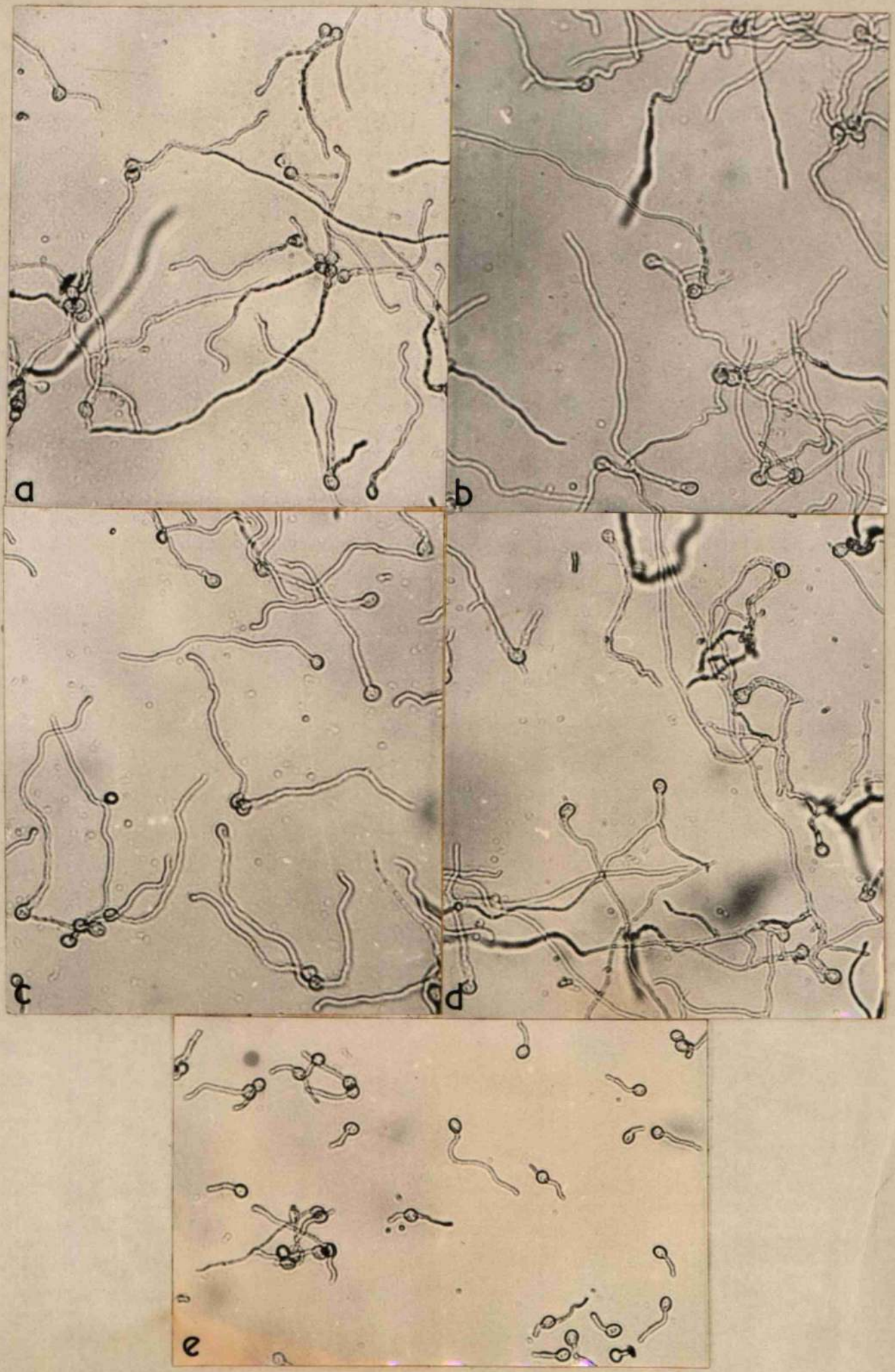
b. Germination in extract of Clone B, uninoculated.

c. Germination in extract of Clone N (susceptible), inoculated.

d. Germination in extract of Clone N, uninoculated.

e. Germination in distilled water.

Magnification in all cases = x 200.


a
b
c
d
e

Discussion

It appears that the amount of germination is similar throughout and there is no evidence to support the hypothesis of resistance being associated with any extractable property of the host cell sap or that any differences in cell sap properties develop after inoculation.

Plates 38 and 39 show that hyphal growth is stimulated by the presence of prothallial extracts for any clone (compare the difference between the growth in distilled water, and that in any prothallial extract). In the second series of experiments (Plate 39) the hyphae in extracts of uninoculated prothalli showed more branching (Plate 39 b&d) than those in extracts of inoculated prothalli (Plate 39 a & c) but this difference was slight and the possibility of it being significant would require that further experiments of a quantitative nature be carried out.

The results, of course, do not preclude the possibility of the existence of some difference in reaction of substances in the intact living cells. It was thought however that investigation of this possibility would be a complex matter and it should not be investigated until other simpler alternatives had been examined. It is also possible that differences, which could not be demonstrated by the present method of extraction, may be present.

SUMMARY

SUMMARY

Part One of this thesis records the work involved in the development of suitable methods for this investigation. This included

a) the development of a suitable cultural technique for the establishment and maintenance of clonal populations in pure culture,

b) the development of a reproducible and consistent inoculation technique, and

c) the examination of methods of disease assessment.

Part Two records the analysis of differences in resistance between clonal populations. This work showed that significant differences could be demonstrated.

Part Three records the investigation of the nature of the resistance property using two clones selected as a result of the work in Part Two. This work involved four phases.

a) A detailed study of the morphology of the host-parasite interaction during infection.

b) A study of transverse sections of the wall of the host by electron microscopy to see if this demonstrated any difference in structure which could be correlated with resistance. No such differences were found.

c) A study of the reaction of the wall to the macerating enzyme produced by Botrytis cinerea in culture. No

differences were found between clones.

d) A study of the effects of cytoplasmic extracts of uninfected and infected prothalli on the fungus *in vitro*. No differences were seen.

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APPENDIX A

TABLES IV TO XV

APPENDIX A

This appendix contains the detailed results of the experiments discussed in Part Two of this thesis. The figures in each table record the percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of different clonal populations of bracken with Botrytis cinerea.

The analyses of variance recorded below were based on the description given in Brownlee (1949). Unless stated, the level taken for significance is the five per cent level, and the degrees of freedom are indicated by the terms n_1 and n_2.

Table IV

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone D_1				Clone M				Clone N			
	Days after inoculation											
Replicate	2	3	4	6	2	3	4	6	2	3	4	6
1	20	78	100	100	20	55	70	70	50	70	70	70
2	10	33	40	40	20	68	60	60	50	65	70	70
3	60	70	80	80	20	68	70	70	45	48	50	70
4	20	48	50	50	40	90	90	90	40	70	70	85
5	15	65	75	90	10	20	35	60	35	40	40	50
6	15	35	60	80	30	78	90	90	50	70	70	70
7	40	68	75	80	25	80	80	80	10	48	75	90
8	50	60	60	80	10	55	85	90	80	100	100	100
9	85	100	100	100	10	18	40	100	40	40	55	80
10	60	88	90	90	10	38	60	60	80	100	100	100

Results of an analysis of variance. Table IV

Second day after inoculation.

Clones D_1, M and N

Between clones variance ratio = 8·6, $n_1 = 2$, $n_2 = 18$.

Significant at the 1% level.

Between plates variance ratio = 1·04, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones M and N

Between clones variance ratio = 24·1, $n_1 = 1$, $n_2 = 9$.

Significant at the 0·1% level.

Between plates variance ratio = 0·85, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones D_1 and M

Between clones variance ratio = 5·3, $n_1 = 1$, $n_2 = 9$.

Significant at the 5% level.

Between plates variance ratio = 0·73, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones D_2 and N

Between clones variance ratio = 1·0, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 1·9, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table V

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone J			Clone M			Clone N		
	Days after inoculation								
Replicate	2	3	4	2	3	4	2	3	4
1	0	10	20	30	100	100	40	90	100
2	10	30	40	40	100	100	50	100	100
3	0	10	20	30	80	100	50	100	100
4	20	40	90	30	70	90	50	100	100
5	0	0	10	40	100	100	20	60	90
6	0	0	10	40	90	100	50	100	100
7	0	0	10	20	100	100	0	0	10
8	0	0	20	40	100	100	50	100	100
9	0	0	0	40	90	100	40	100	100
10	0	0	10	50	90	90	40	90	100

Results of an analysis of variance — Table V

Second day after inoculation.

Clones J, M and N

Between clones variance ratio = 39·5, $n_1 = 2$, $n_2 = 18$.

Significant at the 0·1% level.

Between plates variance ratio = 1·89, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones M and N

Between clones variance ratio = 0·4, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 2·1, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones J and M, and J and N

No analysis necessary since it is apparent from empirical observation that there are differences in the amounts of disease present.

Table VI

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone J				Clone N				Clone U			
	Days after inoculation											
Replicate	2	3	4	5	2	3	4	5	2	3	4	5
1	10	40	60	80	30	90	100	100	30	90	100	100
2	0	0	0	20	50	100	100	100	20	40	50	60
3	10	20	50	70	50	100	100	100	20	30	90	100
4	10	40	60	80	60	80	90	90	0	10	10	20
5	0	0	0	0	50	100	100	100	30	80	100	100
6	0	0	0	10	50	90	100	100	20	60	80	80
7	0	10	10	20	50	90	100	100	20	40	40	50
8	0	20	40	50	50	100	100	100	0	20	20	20
9	10	30	50	60	40	90	100	100	20	40	80	80
10	10	30	60	80	20	60	80	90	20	50	80	80

Results of an analysis of variance — Table VI

Second day after inoculation.

Clones J, N and U

Between clones variance ratio = 34.99, $n_1 = 2$, $n_2 = 18$.

Significant at the 0·1% level.

Between plates variance ratio = 0·29, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones J and U

Between clones variance ratio = 12·0, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 1,0, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones N and U

Between clones variance ratio = 20·2, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 0·38, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones J and N

No analysis necessary since it is apparent from empirical observation that there are differences in the amounts of disease present.

Table VII

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone B						Clone U						Clone O					
	Days after inoculation																	
Replicate	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1	0	0	0	0	0	0	10	20	50	50	50	60	0	0	0	20	50	60
2	0	10	10	10	10	10	0	0	0	10	50	60	0	0	20	80	100	100
3	0	0	10	20	40	50	10	20	40	100	100	100	0	30	70	100	100	100
4	0	10	30	30	30	40	0	10	50	100	100	100	10	30	90	100	100	100
5	0	0	10	10	10	10	0	10	10	10	40	40	0	10	60	100	100	100
6	0	10	10	10	10	10	0	0	10	10	20	20	0	0	20	50	80	80
7	0	0	0	0	0	0	10	20	60	70	80	90	10	40	80	100	100	100
8	0	0	0	0	0	0	10	20	30	60	90	100	0	10	20	80	100	100
9	0	10	20	20	20	20	0	0	10	50	80	80	0	10	40	70	100	100
10	0	10	10	10	10	10	0	10	50	80	100	100	10	40	100	100	100	100

Results of an analysis of variance — Table VII

Second day after inoculation.

Clones B, U and O

Between clones variance ratio = 3·0, $n_1 = 2$, $n_2 = 18$.

Not significant.

Between plates variance ratio = 1·1, $n_1 = 9$, $n_2 = 18$.

Not significant.

Third day after inoculation.

Clones B, U and O

Between clones variance ratio = 7·7, $n_1 = 2$, $n_2 = 18$.

Significant at the 1% level.

Between plates variance ratio = 2·2, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones B and U

Between clones variance ratio = 4·2, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 0·7, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones B and O

Between clones variance ratio = 12·2, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 1·5, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones U and O

Between clones variance ratio = 2·8, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 2·8, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table VIII

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 4 clones with Botrytis cinerea.

	Clone B					Clone J					Clone N					Clone T				
	Days after inoculation																			
Replicate	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	0	0	0	0	0	0	30	60	100	100	10	20	50	100	100	10	10	20	20	20
2	0	0	0	0	0	0	30	70	100	100	10	20	70	80	90	10	20	20	20	30
3	0	0	0	0	10	0	30	90	100	100	0	10	20	40	50	10	10	10	20	100
4	0	0	0	10	10	0	10	20	80	100	10	40	100	100	100	10	20	50	90	100
5	0	10	10	10	10	10	40	60	90	100	20	50	100	100	100	0	30	50	60	60
6	0	0	0	0	0	10	30	80	100	100	10	20	50	70	70	10	40	100	100	100
7	0	0	0	0	0	0	30	60	100	100	0	0	0	0	0	0	20	60	90	90
8	10	10	10	10	10	10	40	40	60	90	10	40	90	90	100	10	10	30	60	60
9	0	0	0	0	0	0	20	60	100	100	0	0	0	10	10	0	10	20	20	20
10	0	10	20	20	20	10	40	60	100	100	10	20	60	100	100	*	*	*	*	*

* Prothallus and agar block contaminated.

Results of an analysis of variance — Table VIII

Second day after inoculation.

Clones B, J, N and T

Between clones variance ratio = 10·6, $n_1 = 3$, $n_2 = 35$.

Significant at the 0·1% level.

Clones B and J

Between clones variance ratio = 182, $n_1 = 1$, $n_2 = 9$.

Significant at the 0·1% level.

Between plates variance ratio = 4·5, $n_1 = 9$, $n_2 = 9$.

Significant at the 5% level.

Clones B and N

Between clones variance ratio = 18·0, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 2·0, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones J and N

Between clones variance ratio = 2·1, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 1·45, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones B and T

Between clones variance ratio = 18·3, $n_1 = 1$, $n_2 = 17$.

Significant at the 0·1% level.

Clones J and T

Between clones variance ratio = 5·9, $n_1 = 1$, $n_2 = 17$.

Significant at the 5% level.

Clones N and T

Between clones variance ratio = 0·25, $n_1 = 1$, $n_2 = 17$.

Not significant.

Results of an analysis of variance — Table IX

Second day after inoculation.

Clones D_1 and M

Between clones variance ratio = 7·9, $n_1 = 1$, $n_2 = 9$.

Significant at the 5% level.

Between plates variance ratio = 1·4, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table IX

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 2 clones with Botrytis cinerea.

	Clone D_1				Clone M			
	Days after inoculation							
Replicate	1	2	3	5	1	2	3	5
1	20	40	100	100	0	20	90	100
2	20	30	100	100	10	20	50	90
3	30	80	100	100	10	20	50	100
4	20	40	100	100	0	0	0	20
5	10	20	100	100	0	0	0	0
6	10	20	80	100	00	20	50	90
7	20	40	90	90	0	20	60	90
8	20	30	90	100	0	10	50	90
9	10	30	100	100	10	50	100	100
10	10	20	90	90	0	0	0	0

Table X

The percentage of the total area of prot

after inoculation of prothalli of 3 clon

	Clone D_1				Clone		
	Period during which lesion						
Replicate	0-2	2-3	3-4	4-6	0-2	2-3	3
1	20	58	22	0	20	35	
2	10	23	7	0	20	48	
3	60	10	10	0	20	48	
4	20	28	2	0	40	50	
5	15	20	10	15	10	10	
6	15	25	25	20	30	48	
7	40	28	7	5	25	55	
8	50	10	0	20	10	45	
9	85	15	0	0	10	8	
10	60	28	2	0	10	28	

Results of an analysis of variance **Table X**

Period between second and third days.

Clones D_1, M and N

Between clones variance ratio = 5·15, $n_1 = 2$, $n_2 = 18$.

Significant at the 5% level.

Between plates variance ratio = 1·37, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones M and N

Between clones variance ratio = 26·0, $n_1 = 1$, $n_2 = 9$.

Significant at the 0·1% level.

Between plates variance ratio = 4·3, $n_1 = 9$, $n_2 = 9$.

Significant at the 5% level.

Clones D_1 and M

Between clones variance ratio = 1·57, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 0·51, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones D_1 and N

Between clones variance ratio = 2·9, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 1·3, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table XI

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone J			Clone M			Clone N		
	Period between the days after inoculation								
Replicate	0-2	2-3	3-4	0-2	2-3	3-4	0-2	2-3	3-4
1	0	10	10	30	70	0	40	50	0
2	10	20	10	40	60	0	50	50	0
3	0	10	10	30	50	20	50	50	0
4	20	20	50	30	40	20	50	50	0
5	0	0	10	40	60	0	20	40	30
6	0	0	10	40	50	10	50	50	0
7	0	0	10	20	80	0	0	0	10
8	0	0	20	40	60	0	50	50	0
9	0	0	0	40	50	10	40	60	0
10	0	0	10	50	40	0	40	50	10

Results of an analysis of variance Table XI

Period between the second and third days.

Clones J, M and N

Between clones variance ratio = 32•72, $n_1 = 2$, $n_2 = 18$.

Significant at the 0•1% level.

Between plates variance ratio = 0•39, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones M and N

Between clones variance ratio = 1•66, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 1•9, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones J and M, and Clones J and N

No analysis necessary since it is apparent from empirical observation that there are differences in the amounts of disease present.

Table XII

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone J				Clone N				Clone U			
	Period during which lesion growth was recorded											
Replicate	0-2	2-3	3-4	4-5	0-2	2-3	3-4	4-5	0-2	2-3	3-4	4-5
1	10	30	20	20	30	60	10	0	30	60	10	10
2	0	0	0	20	50	50	0	0	20	20	10	10
3	10	10	30	20	50	50	0	0	20	10	60	10
4	10	30	20	20	60	20	10	0	0	10	0	10
5	0	0	0	0	50	50	0	0	30	50	20	0
6	0	0	0	10	50	40	10	0	20	40	20	0
7	0	10	0	10	50	40	10	0	20	20	0	10
8	0	20	20	10	50	50	0	0	0	20	0	0
9	10	20	20	10	40	50	10	0	20	20	40	0
10	10	20	30	20	20	40	20	10	20	30	30	0

Results of an analysis of variance — Table XII

Period between the second and third days.

Clones J, N and U

Between clones variance ratio = 14·9, $n_1 = 2$, $n_2 = 18$.

Significant at the 0·1% level.

Between plates variance ratio = 1·3, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones J and U

Between clones variance ratio = 4·4, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 0·86, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones J and N

Between clones variance ratio = 32·0, $n_1 = 1$, $n_2 = 9$.

Significant at the 0·1% level.

Between plates variance ratio = 0·66, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones N and U

Between clones variance ratio = 13·1, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 2·6, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table XIII

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 3 clones with Botrytis cinerea.

	Clone B						Clone U						Clone O					
	Period during which lesion growth was recorded																	
Replicate	0-1	1-2	2-3	3-4	4-5	5-6	0-1	1-2	2-3	3-4	4-5	5-6	0-1	1-2	2-3	3-4	4-5	5-6
1	0	0	0	0	0	0	10	10	30	0	0	10	0	0	0	20	30	10
2	0	10	0	10	0	0	0	0	0	10	40	10	0	0	20	60	20	0
3	0	0	10	10	20	10	10	10	20	60	0	0	0	20	40	30	0	0
4	0	10	20	0	0	10	0	10	40	50	0	0	10	10	60	10	0	0
5	0	0	10	0	0	0	0	10	0	0	30	0	0	10	50	40	0	0
6	0	10	10	0	0	0	0	0	10	0	10	0	0	0	20	30	30	0
7	0	0	0	0	0	0	10	10	40	10	10	10	10	30	40	20	0	0
8	0	0	0	0	0	0	10	10	10	30	30	10	0	10	10	60	20	0
9	0	10	10	0	0	0	0	0	10	40	30	0	0	10	30	30	30	0
10	0	10	0	0	0	0	0	10	40	30	20	0	10	30	60	0	0	0

Results of an analysis of variance — Table XIII

Period between the second and third days.

Clones B, U and O

Between clones variance ratio = 10·2, $n_1 = 2$, $n_2 = 18$.

Significant at the 1% level.

Between plates variance ratio = 2·1, $n_1 = 9$, $n_2 = 18$.

Not significant.

Clones B and U

Between clones variance ratio = 6·5, $n_1 = 1$, $n_2 = 9$.

Significant at the 5% level.

Between plates variance ratio = 1·0, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones B and O

Between clones variance ratio = 22·7, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 1·8, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones U and O

Between clones variance ratio = 4·0, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 2·3, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table XIV

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 4 clones with Botrytis cinerea.

	Clone B					Clone J					Clone N					Clone T				
	Period during which lesion growth was recorded																			
Replicate	0-1	1-2	2-3	3-4	4-5	0-1	1-2	2-3	3-4	4-5	0-1	1-2	2-3	3-4	4-5	0-1	1-2	2-3	3-4	4-5
1	0	0	0	0	0	0	30	30	40	0	10	10	30	50	0	10	0	10	0	0
2	0	0	0	0	0	0	30	30	30	0	10	10	50	10	10	10	10	0	0	10
3	0	0	0	0	10	0	30	40	30	0	0	10	10	20	10	10	0	0	10	80
4	0	0	0	10	0	0	10	10	60	20	10	30	60	0	0	10	10	30	40	10
5	0	10	0	0	0	10	30	20	30	10	20	30	50	0	0	0	30	20	10	0
6	0	10	0	0	0	10	20	50	20	0	10	10	30	20	0	10	30	60	0	0
7	0	0	0	0	0	0	30	30	40	0	0	0	0	0	0	0	20	40	30	0
8	10	0	0	0	0	10	30	0	20	30	10	30	50	0	10	10	0	20	30	0
9	0	0	0	0	0	0	20	40	40	0	0	0	0	10	0	0	10	10	0	0
10	0	10	10	0	0	10	30	20	40	0	10	10	40	40	0	*	*	*	*	*

* Prothallus and agar block contaminated.

Results of an analysis of variance — Table XIV

Period between the second and third days.

Clones B, J, N and T

Between clones variance ratio = 6·85, $n_1 = 3$, $n_2 = 35$.

Significant at the 0·1% level.

Clones B and J

Between clones variance ratio = 18·8, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 0·27, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones B and N

Between clones variance ratio = 20·0, $n_1 = 1$, $n_2 = 9$.

Significant at the 1% level.

Between plates variance ratio = 1·05, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones J and N

Between clones variance ratio = 0·21, $n_1 = 1$, $n_2 = 9$.

Not significant.

Between plates variance ratio = 0·23, $n_1 = 9$, $n_2 = 9$.

Not significant.

Clones B and T

Between clones variance ratio = 10·2, $n_1 = 1$, $n_2 = 17$.

Significant at the 1% level.

Clones J and T

Between clones variance ratio = 0·53, $n_1 = 1$, $n_2 = 17$.

Not significant.

Clones N and T

Between clones variance ratio = 1·27, $n_1 = 1$, $n_2 = 17$.

Not significant.

Results of an analysis of variance — Table XV

Period between second and third days.

Clones D_1 and M

Between clones variance ratio = 9·8, $n_1 = 1$, $n_2 = 9$.

Significant at the 5% level.

Between plates variance ratio = 0·6, $n_1 = 9$, $n_2 = 9$.

Not significant.

Table XV

The percentage of the total area of prothallus occupied by lesion after inoculation of prothalli of 2 clones with Botrytis cinerea.

	Clone D_1				Clone M			
Replicate	0-1	1-2	2-3	3-5	0-1	1-2	2-3	3-5
1	20	20	60	0	0	20	70	10
2	20	10	70	0	10	10	30	40
3	30	50	20	0	10	10	30	50
4	20	20	60	0	0	0	0	20
5	10	10	80	0	0	0	0	0
6	10	10	60	20	0	20	30	40
7	20	20	50	0	0	20	40	30
8	20	10	60	10	0	10	40	40
9	10	20	70	0	10	40	50	0
10	10	10	70	0	0	0	0	0

APPENDIX B

ELECTRON MICROSCOPY FORMULAE

APPENDIX B

The composition of the reagents used in the preparation of specimens for the electron microscopic examination reported in Part Three.

Buffer Solutions

Solution A.

Sodium veronal (Sodium barbitone)	14·7 g.
Sodium acetate.$3H_2O$	9·7 g.
Distilled water	500 ml.

To be stored in refrigerator.

Solution B.

0·1N HCl.

Solution C.

Sodium chloride	40 g.
Potassium chloride	2 g.
Calcium chloride	1 g.
Distilled water	500 ml.

To be stored in refrigerator.

Fixatives

Osmium tetroxide (Zetterqvist, 1956).

Solution A	10·0 ml.
Solution B	3·4 ml.
Solution C	11·0 ml.
Distilled water	50·0 ml.
OsO_4	0·5 g.

pH 7·2 - 7·4.

To be stored in refrigerator.

Potassium permanganate (Luft, 1956).

Solution A	20 ml.
Solution B	22 ml.
$KMnO_4$	2 g.
Distilled water	60 ml.

pH 7·4 - 7·6.

To be stored in refrigerator.

Dehydration Schedule

25% ethanol	.....	.....	15 minutes.
50% ethanol	.....	.....	20 minutes.
75% ethanol	.....	.....	30 minutes.
Absolute ethanol		.....	3 changes in 2 hours.

Embedding Medium

Araldite CY 212	10·0 ml.
Hardener 964 B	10·0 ml.
Dibutylphthalate	1·0 ml.
Accelerator 964 C	0·4 ml.

The resin and hardener were heated in an incubator at 60°C before mixing.

Professor L.W. Shaw

Thesis: Some Effects of Fungi o

by Robert M

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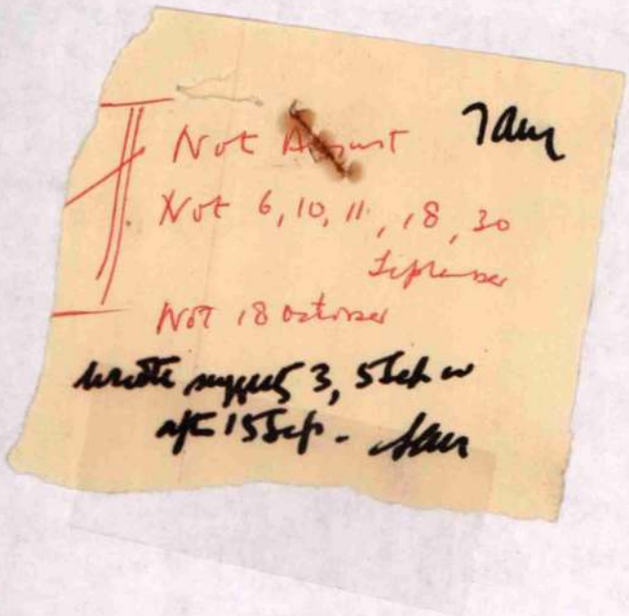
Your Special Committee is Professor

Dr. S.A. A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