Studies on Fertilisation in *Pteridium aquilinum* (bracken).

A thesis presented for the degree of Doctor of Philosophy of the University of Glasgow.

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Contents.

Introduction.

Genetical investigations in ferns have been comparatively few in the past, the only comprehensive work having been that carried out by Andersson-Kotte (1938). She was able to demonstrate mendelian segregation and recombination in Polystichum angulare and Phyllitis Scolopendrium and also the genetic control of chloroplast characters in these two plants as well as in Adiantum cuneatum and Lastrea atrata. Lang (1953) first found mendelian segregation in ferns from naturally occurring heterozygotes of Phyllitis Scolopendrium and the extent to which hybridisation takes place in ferns has been clearly shown by the cytological studies of Manton (1950).

Of great advantage in genetical studies in ferns is the fact that the independent gametophytic phase of the life-cycle is comparatively well developed and may be vegetatively propagated indefinitely. It is possible to establish clones of prothalli by regeneration from cuttings (present work) and large numbers of such genetically identical individuals have been produced and have constituted a valuable tool in subsequent investigations. The main disadvantage for genetic investigations in ferns lies in the difficulty of growing young sporophytes which may take upwards of two years before sporulating.

When the present work was undertaken, the aim was to explore the possibilities of genetical analysis in Pteridium aquilinum (bracken). A search was started in the

field for sporophytes showing morphological variation of a possible genetic origin. In the laboratory it was proposed to study gametophytes in the hope of finding genetic variation in morphological and nutritional properties. Preliminary work on the breeding mechanism however, indicated the presence of incompatibility and subsequent work was concerned mostly with a detailed investigation of this phenomenon.

The discovery of self-incompatibility in P. aquilinum is the first example of this situation in pteridophytes. It is also the first example of incompatibility at the gametophyte level in any vascular plant.

PART I.

1. Material and cultural methods.

Spore samples were collected from different populations of bracken. Of the populations sampled, one differed morphologically from the others in that the apices of the pinnae tended to branch giving rise to the so-called 'crested' form common in cultivated ferns. This form, somewhat rare in bracken, was listed by Moore as *P. aquilinum* var, *cristata* and is illustrated in Lowe's 'Our Native Ferns' (1867). Also spores of this variety were very much darker in colour than those of the other populations, a difference apparent both in the mass and under the microscope. These differences were constant over a period of three years. The location of the crested variety is at Mugdockbank near Milngavie, Dumbartonshire.

Culture of prothalli was carried out in petri-dishes in daylight on an agar medium containing standard Knop's solution. Where it was necessary to control the number of spores plated in each petri-dish, the density of spore suspensions was estimated from haemocytometer counts and the appropriate volume of suspension added in each case. The amount of contaminent microorganisms in these cultures varied greatly, the most troublesome being unicellular algae. It was possible to get rid of most contaminants by incubating a spore suspension overnight in sterilised

distilled water at 64° C., bracken spores being unaffected by this treatment.

Germination of bracken spores was generally of the order of 70%.

3. Sterile culture of prothalli.

To investigate the possible occurrence in nature of non-autotrophic strains, it was necessary to grow prothalli under sterile conditions. The development of sterile techniques was also undertaken with a view towards the production of nutritional mutants among prothalli by treatment of spores with mutagenic agents, the whole process being similar to that carried out in microorganisms.

Various methods of sterilising spores were tried out, the most suitable being a modified version of that used by Knudson (1940) for spores of Polypodium aureum. A spore suspension in sterilised distilled water was incubated overnight at 64°C. The spores were then centrifuged and the supernatant decanted. To the spores was added 1ml. of a 1% bleaching powder solution freshly made up and filtered. After one minute the chlorine constituent was inactivated by addition of a drop of a weak potassium iodide solution. The spores were then immediately taken up in a pipette and transferred to sterilised distilled water. The viability of treated spores was 40% or thereabouts, and the germination period was increased from 3-4 days to 5-7 days. Subsequent growth of prothalli in sterile culture

was normal in spore samples from two populations (one from Ballochraggan near Stirling, the other from Killearn near Loch Lomond), but with spores from the crested population, although germination took place there was no subsequent growth of germ-tubes beyond a two or three-celled condition in uncontaminated petri-dishes. If, however, these plates were inoculated with any one of a number of fungi or bacteria, further growth of germ-tubes proceeded and gave rise to prothalli at the region of contamination.

On the assumption that a substance (or substances) not present in Knop's agar was required by the prothalli of the crested population and was supplied by the contaminants, an attempt was made to grow the prothalli on a contaminant-free medium supplemented by a variety of growth factors, singly and in combinations. Vitamins, dextrose, hydrolised casein, hydrolised ribo- and desoxyribonucleic acid, yeast extract and Seitz-filtered extracts of contaminated media were tried out. Experiments were also carried out in which CO_2 tension and pH were varied but in no case were prothalli of the crested population able to grow in the absence of microorganisms.

Microscopic examination of the deliberately contaminated plates showed that, as a filamentous fungus such as Penicillium or Aspergillus spread slowly across the agar, the germ-tubes continued their growth only after the

hyphal tips had reached them. If a piece of agar were cut out before any hyphae had reached it and kept sterile in a separate dish, then germ-tubes on this agar did not develop into prothalli but remained in the two or three-celled condition.

One particular fungus was found (an Actinomycete) which was ineffective in enabling the prothalli to grow but this was a single exception and all other contaminants used, whether fungal, bacterial or algal were effective.

Discussion.

In this investigation of the nutritional requirements of prothalli of the crested population, the evidence suggests that the contaminant microorganisms supply the prothalli with a substance (or substances) which is very unstable and which must be taken up as soon as produced. Diffusion of the hypothetical substance across the agar does not take place, the stimulating effect on the prothalli being apparent only in those areas in which the contaminant is growing.

Strains of prothalli requiring this type of nutritional association with microorganisms may be widespread in bracken and pteridophytes generally since in nature there would be no selection against this condition, microorganisms being abundant in most soils. In ecological

studies these findings have some significance.

Morel-Py (1950) found abnormal growth of fern prothalli in sterile culture and showed that normal growth was restored by addition of glucose to the medium.

Prothalli of Osmunda cinamomea cultured under sterile conditions (Morel and Wetmore, 1951) gave rise to undifferentiated 'callus' outgrowths which, when detached required a medium supplemented by vitamins of the B1 complex.

3. Morphological variation in the prothallus.

Accounts of the development from the spore to the typical heart-shaped prothallus are given in the literature for many ferns and by Orth (1936) and Conway (1949) for bracken. The development of an apical meristem in the young prothallus is a necessary preliminary to the production of the female reproductive organ, the archegonium. The meristematic cells in the apical notch alone are capable of division in the vertical plane to give rise to a 'cushion' of tissue in which the archegonia arise. In bracken, the male organs (antheridia) develop simultaneously with the archegonia so that sperm and egg are mature at the same time on one prothallus (Fig.1A). If a meristem does not develop, the prothallus remains a plate of cells and growth is generally arrested at the 20-30 cell stage. Numerous antheridia are produced on this ameristic prothallus but no archegonia form (Fig.1B). Thus with the ameristic and the meristic prothallus is associated maleness on the one hand and the hermaphrodite condition on the other.

The occurrence of the small male form was of relatively high frequency in all cultures (Fig.2) and highest in those cultures where there was a very dense sowing of spores.

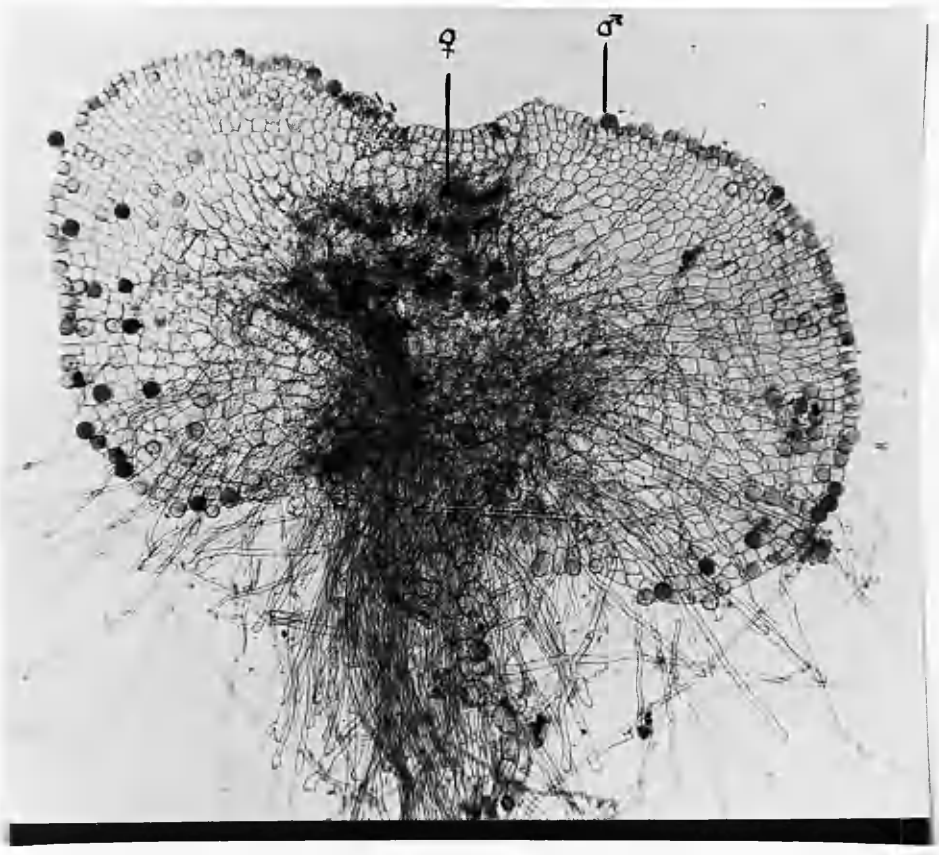
20 of these small males were transplanted to fresh medium and kept under observation and it was found

Fig.1 A. Meristic, cordate prothallus with antheridia (♂) and archegonia (♀).

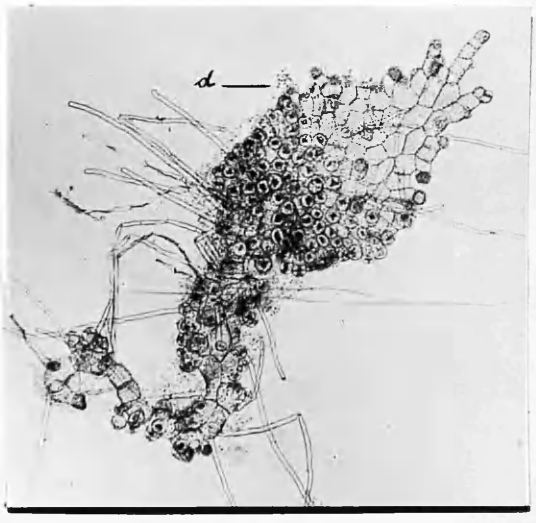
B. Ameristic prothallus with antheridia only.

d, antheridium dehiscing.

Both x30.

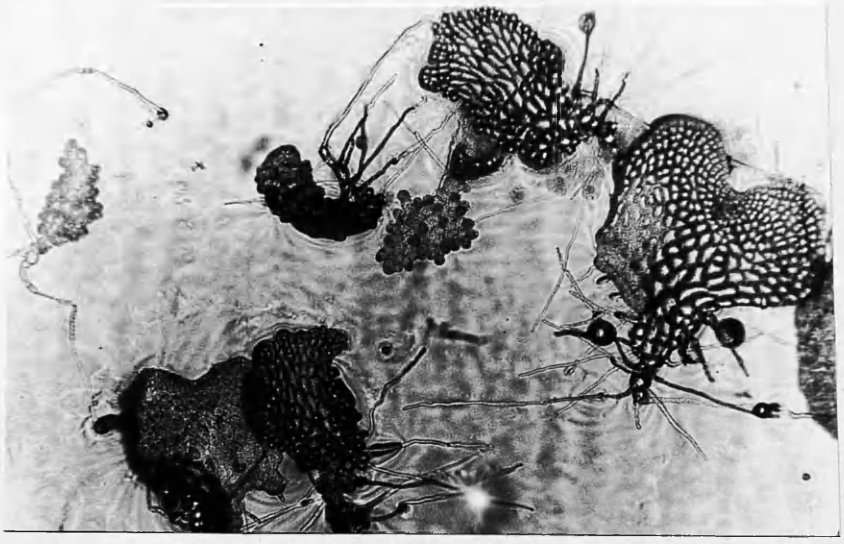


A.



B.

Fig.2 Photomicrograph of a young prothallial culture on agar showing ameristic males and meristic forms. x20.



that all eventually gave rise to the meristic form and produced archegonia (Fig.3). In this experiment prothalli were of the crested population.

In another experiment in which spores of the Killearn population (K) were used, single spores were picked up from a dilute suspension by means of a finely drawn-out pipette and single-spore cultures set up. Developing prothalli then had presumably, optimal conditions for growth, the object in mind being to see if small males would develop under such conditions. This experiment was carried out in sterile and non-sterile culture and the results are recorded in table 1.

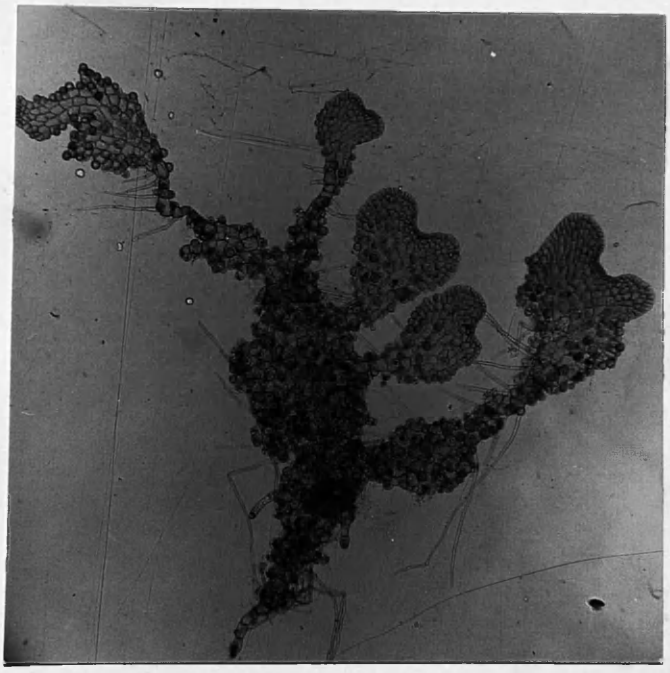
Table 1.

Incidence of male prothalli in single-spore cultures of population K.

Cultural conditions	No.of spores isolated	No.which germinated	No.of hermaphrodite prothalli	No.of male prothalli
Sterile	40	31	19	12
Non-sterile	40	36	21	15

These data for one population show the incidence of the ameristic male form to be of the order of 40% both in sterile and non-sterile dishes.

Fig.3 Proliferation of marginal cells of small male to give cordate forms. x20.



The incidence of males under various cultural conditions is given in table 2, again using spores of population K.

Table 2.

Incidence of male prothalli under different cultural conditions (population K).

No.of spores per petri-dish	Cultural conditions	No.of prothalli examined	No.of male prothalli	Incidence of males
5,000	Sterile	96	65	66%
	Non-sterile	85	52	60%
1,000	Sterile	50	27	54%
	Non-sterile	100	38	38%
500	Sterile	82	30	37%
	Non-sterile	90	19	22%

It can be seen that the incidence varies considerably, the lowest being 22% in a non-sterile culture with 500 spores per petri-dish.

Discussion.

Maleness and femaleness in the prothalli of homosporous ferns was the subject of much investigation by early botanists. Strasburger (1873), Bauke (1878), Jonkman

(1877) and Kny (1880) among others, expressed the opinion that there was a tendency to dioecism. Their conclusions may have been biased in an attempt to establish a link between homosporous and heterosporous ferns for which there was expectation at the time. Prantl (1881) disagreed with these authors and held that the occurrence of the ameristic male was due entirely to cultural conditions and that if the correct cultural conditions could be set up this form of prothallus would never arise. However, he himself was unable to find such ideal conditions and always had small male in his cultures.

Numerous workers have collected information as to the influence of external conditions on the development and form of the fern prothallus. Prantl (1881) has shown that the development of the prothallus may be arrested at the stage of a cell plate without meristem by cultivation on a substratum devoid of nitrates. A similar result was obtained by growing prothalli under conditions of reduced light intensity (Mehra, 1928; Stephan 1929). From such investigations it appears that each growth form, in the presence of an adequate supply of water and oxygen, is the result of the interaction of a number of factors of which the most significant are the quantitative and qualitative nature of the light, the supply of mineral salts and temperature. The nature of the

mechanism intervening between the stimulating factors and the response is, however, entirely unknown.

In the present work an attempt has been made to determine whether or not the different end products (meristic versus ameristic) correspond to a difference in the intervening mechanism (the action of the genes) or to differences in the stimulating factors (the environment).

From the results of the experiments described it is obvious that the problem is a complex one and would require much more extensive treatment than it has been given here. It could be the case that genetic factors control the time lag in the development of a meristem, the penetrance of these factors being raised by conditions of growth other than optimal. To test this hypothesis, experiments along the following lines might be carried out.

Isolated male protnalli after proliferation and the establishment of the hermaphrodite condition, would be selfed. The sporophytes so produced would be homozygous for all genes and the spores they produced would all be genetically identical. This proceedure with male protnalli would also be carried out with meristic protnalli and identical spores obtained in this case also.

In experiments using homogeneous spores, variability would be due to the environment and could not be attributed to genetic differences in the protnalli.

In this way a comparison could be made between the variation in cultures in which homogeneous spores were used and those using heterogeneous spores. Any differences between the two would be presumably, of genetic origin. It would also be possible in this way to estimate the penetrance of the hypothetical factor controlling the production of a meristem.

Whatever the mechanism controlling the production of a meristem, in nature the occurrence of these males would ensure an adequate supply of sperms at all times. This is important in view of the fact that prothalli produce relatively few archegonia in their lifetime.

It is of interest in this connection to note that recent investigations of Galan (discussed by Mather, 1949) show a genetic control of a protandrous condition in the cucurbitous plant Ecballium.

4. Summary.

1. Spores were collected from different populations of bracken, one of the populations being a 'crested' variety.

2. Techniques for the sterile and non-sterile culture of prothalli have been developed.

3. Prothalli of the crested population were unable to grow in sterile culture and media had to be inoculated with microorganisms for growth to take place. Media were supplemented with a variety of growth substances in an attempt to find out the growth requirements of the prothalli but without success. It was concluded that a labile substance (or substances) was liberated by the microorganisms and taken up by the prothalli thereby enabling them to grow.

4. Two forms of prothalli, one with and one without a meristem, were of constant occurrence in all cultures and under a wide variety of cultural conditions. Ameristic prothalli (which developed only male organs) eventually became meristic after a period of cultivation and it is possible that the difference in time in the development of a meristem is genetically controlled.

5. References.

1. Andersson-Kotto, I. 1938. Manual of Pteridology. The Hague.

2. Conway, E. 1949. The autecology of the bracken (Pteridium aquilinum); the germination of the spore and the development of the prothallus and the young sporophyte. Proc.Roy.Soc.Edin., B, 63, 325-343.

3. Hurel-Py, Mme. 1950. Recherches preliminaires sur la culture aseptique des prothalles de filicinees. Rev.Gen.Bot., 57, 637-694.

4. Knudson, L. 1940. Permanent changes of chloroplasts induced by x-rays in the gametophyte of Polypodium aureum. Bot.Gaz., 101, 721-758.

5. Lowe, E.N. 1867. Our Native Ferns. II. London.

6. Lang, W.H. 1923. On the genetic analysis of a heterozygotic plant of Scolopendrium vulgare. J.Genet., 13, 753-770.

7. Manton, I. 1950. Problems of Cytology and Evolution in the Pteridophyta. Cambridge.

8. Mather, K. 1949. Genetics of monoecy and dioecy in Ecballium. Nature, 163, 926.

9. Mehra, P.N. 1923. Factors influencing the development and form of the fern prothallus. Current Science, Bangalore. 1, 6.

10. Morel, G. and Wetmore, R. H. 1951. Fern callus tissue culture. Am.J.Bot., 38, 141-143.

11. Orth, R. 1936. Morphologische und physiologische untersuchungen an farnprothallien. Planta, 25, 637-684.

12. Prantl, K. 1881. Beobachtungen uber die ernahrung der farnprothallien und die verteilung der sexualorgane. Bot.Zeit., 39, 753-770.

13. Stephan, J. 1929. Entwicklungsphysiologische untersuchungen an einigen farnen. Jahrb.fur wiss Bot., 453-469. (earlier literature fully quoted here)

Part II.

The movements of spermatozoa of bracken.

The movements of spermatozoa in the vicinity of mature archegonia were investigated in some detail in connection with the fertilisation studies described later. The particular problem of whether sperms are chemotactically attracted to mature archegonia formed part of this investigation and the techniques employed and the results obtained in this part of the work formed the basis of a paper now in the press (Experimental Cell Research) and of which the following is a copy.

The Movements of Spermatozoa of Bracken (Pteridium aquilinum).

The assumption has been made repeatedly in the literature that fern spermatozoa are chemotactically attracted to mature female organs (archegonia) since (a) sperms in a suspension are seen to accumulate around the necks of mature archegonia; (b) sperms are attracted by certain organic substances, particularly malic acid as shown by Pfeffer (6), Bruchmann (1), Rothschild (7) and others and (c) most text books (e.g. Fritsch and Salisbury (4)) make the statement that some of these substances are present in archegonia. A thorough search of the literature has provided no clue as to where this statement originated.

The archegonium of bracken consists of a ventral portion embedded in the prothallus, and a neck portion. The neck, which projects above the surface of the prothallus, consists of a wall composed of four rows of cells; it encloses a single elongated neck canal-cell. The ventral portion contains a large egg-cell and a ventral canal-cell immediately above it. As the archegonium matures both canal-cells become disorganised and fill the canal with a mucilaginous substance. This swells on the admission of water, and, rupturing the neck at the apex, is discharged from the archegonium which is now ready for fertilisation. Hanstein (5) was first to report that sperms accumulated in

this extruded mucilage and this undoubtedly happens in bracken (Fig.4).

As found at the start of the present work, the accumulation of sperms in the mucilage is due to the fact that on entering it they stop swimming. This change is irreversible since such sperms, on removal from the mucilage, never recover their original motility. Thus, sperms will accumulate in the mucilage without there being an attraction in the same way as flies would accumulate on a fly-paper. The object of the present investigation has been to find by quantitative methods whether or not there is an attraction of sperms to mature female organs.

Statistical approach.

The method of investigating the movements of spermatozoa in tap-water was to mark out a grid on the surface of a glass slide using a diamond objective on a microscope fitted with mechanical stage. Squares on the grid had 0.5mm. sides and the grid was enclosed by strips of glass cut from a glass plate about 1mm. thick and fastened to the slide with wax (Fig.5). The sperm suspension at 18oC was introduced into this compartment and the rectangular components x and y of the distance travelled by a sperm in a given time estimated under the dissecting microscope. The observations are summarised in table 3. Since the mean square displace-

Fig. 4.

A. Photomicrograph of spherical mass of spermatozoa in the mucilage of a mature archegonium. x60.

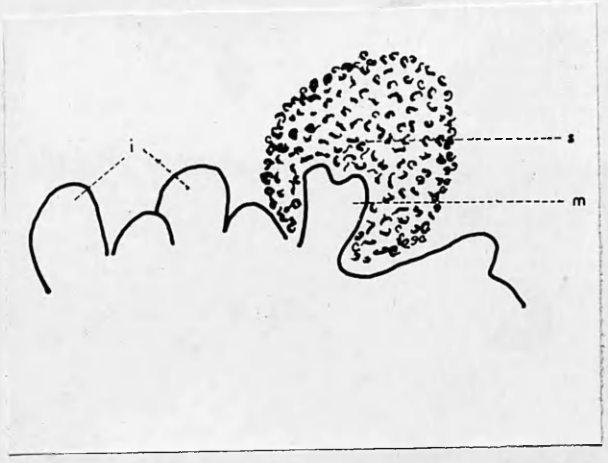
B. Diagrammatic representation of A.

s, the sperm mass.

m, open neck of mature archegonium.

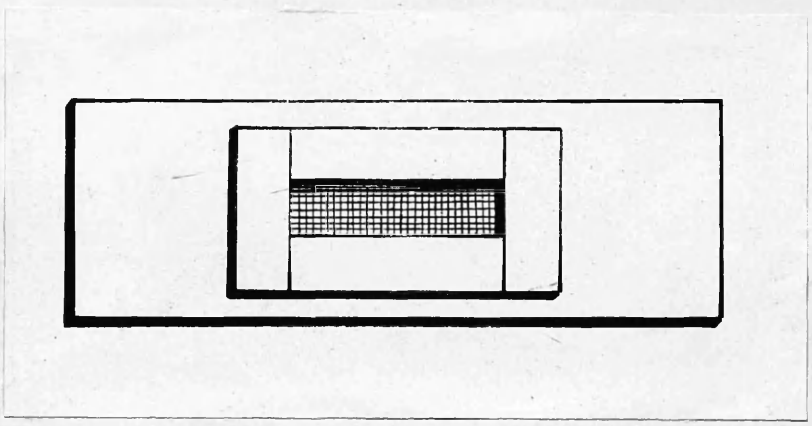
i, closed necks of immature archegonia.

A



B

Fig.5. Slide with grid enclosed by glass strips. Sperm suspensions are introduced into this compartment and the rectangular components of the distances travelled by sperms in given times measured on the grid.



ment in unit time is constant then the movements of the sperm can be regarded as truly random and the behaviour of a suspension characterised by a diffusion coeffecient $D = \frac{\overline{x^2 + y^2}}{4t}$ (2,3). From table 3, D averages 0.0055 (x and y in mm.).

Table 3.

Movement of spermatozoa in tap water at 13°C.

Time interval t.	Mean and standard deviation for 100 measurements of co-ords.of displacement				Mean of $x^2 + y^2$
	x	σ_x	y	σ_y	
5 sec.	0.38	0.22	0.41	0.24	0.44 ± 0.03
10 sec.	0.53	0.40	0.54	0.37	0.86 ± 0.08
20 sec.	0.76	0.49	0.72	0.56	1.77 ± 0.10

Suppose the initial concentration of such a suspension is C_0 (number per unit volume) and that organisms arriving on a fixed sphere of radius a are caught, then at times t from the start of the experiment the concentration at a point r away from the centre of the sphere will have fallen to a value C given by

$$C_0 - C = \frac{a C_0}{r}\left\{1 - \operatorname{erf}\frac{(r-a)}{2\sqrt{Dt}}\right\}.$$

Then $-\frac{dC}{dr} = -\frac{aC_0}{r^2}\left\{1 - \operatorname{erf}\frac{(r-a)}{2\sqrt{Dt}}\right\} - \frac{aC_0}{r}\cdot\frac{d}{dr}\cdot\operatorname{erf}\frac{(r-a)}{2\sqrt{Dt}}$.

i.e. $\frac{dC}{dr} = \frac{aC_0}{r^2}\left\{1 - \operatorname{erf}\frac{(r-a)}{2\sqrt{Dt}}\right\} \frac{aC_0}{r\sqrt{\pi Dt}}\cdot\exp\left[-\frac{(r-a)^2}{4Dt}\right]$

The rate of arrival on the surface is then $4\pi a^2 D\left(\frac{dC}{dr}\right)_a$

$$= 4\pi a^2 D C_0\left(\frac{1}{a} + \frac{1}{\sqrt{\pi Dt}}\right).$$

And the number which has arrived since time zero (start of the experiment) will be

$$4\pi a^2 D C_0\left(\frac{t}{a} + 2\sqrt{\frac{1}{\pi D}}\right).$$

If in an actual experiment we find that the numbers of sperms arriving on the surface of the mucilage are greater than this then it can be taken that there is an attraction.

In a first experiment where the density of sperm suspension was 10^4/ml. (estimated from haemocytometer counts) the initial rate of arrival at the mucilage was 1.2/sec. increasing steadily to a maximum of approximately 10/sec., the time taken to reach this maximum being about 5 minutes. Counting sperms at this stage presented a difficulty and the rate of 10/sec. was arrived at after independent estimates were made by colleagues. This rate of arrival was maintained for a further 5 minutes and thereafter steadily decreased to a minimum of 1 in 12 sec. The mucilage was then filled with sperms and it was at this stage that the photograph in Fig.1 was taken.

There was no further increase in this mass of sperm since those now arriving moved off again. The diameter of the mass then measured 0.5mm.

Putting in numerical values for a, D, and C_0 in the expression for the rate of arrival, we find that the term $\frac{1}{\sqrt{\pi D t}}$ may be neglected for observations taken later than 1 minute from the start of the experiment so that the rate expected after 5 minutes (when actual rate of arrival was at a maximum in the experiment) can be taken as $4\pi a D C_0$.

In the course of these experiments, samples of sperm suspensions were withdrawn and diffusion coefficients calculated for sperms in these samples. These did not differ significantly from those found for sperms in tap water (Table 4). It may also be mentioned that the number of sperms in the mucilage was small in comparison with the total number of sperms in the suspension, so that the change in the concentration of sperms in the suspension during the experiment could be neglected. The results of these experiments are summarised in Table 4.

These results show that sperms are actually attracted to mature archegonia since their rate of accumulation in the mucilage is much greater than would be expected on the basis of a fly-catching mechanism. They also indicate that the attraction operates on the direction of motion rather than on its velocity. Rothschild (7) concluded that the effect of malic acid on bracken sperms was to change the direction of motion

Table 4.

Rate of arrival of sperms at sphere of mucilage.

Experiment	Sperm density C_o	Diffusion coefficient (D)		Radius of sphere of mucilage a.	Rate of arrival at mucilage	
		In tap water	During experiment		Expected ($4\pi a D C_o$)	Measured
1	10^4/ml.	0.0056	0.0051	0.25mm.	0.2/sec.	10/sec.
2	10^3/ml.	0.0055	0.0052	0.23mm.	0.02/sec.	1.2/sec.

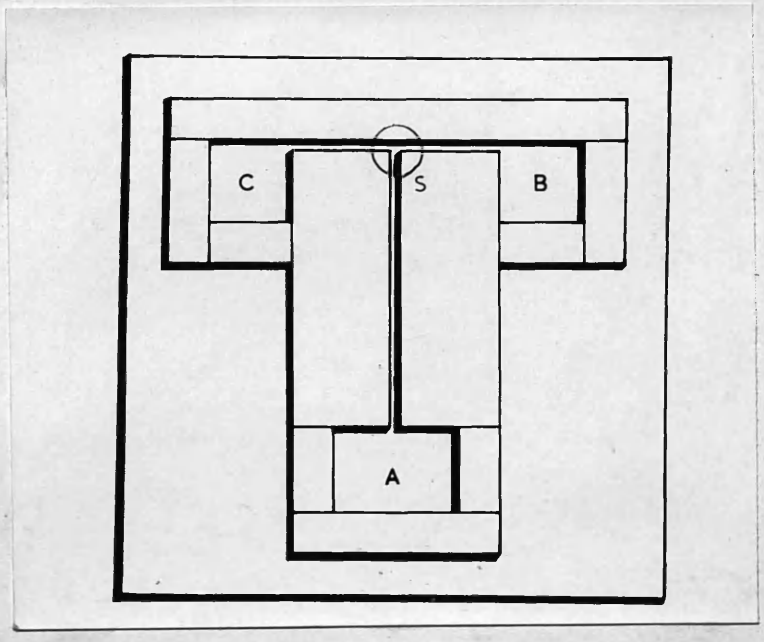
and not to increase its rate.

Qualitative approach.

In a further set of experiments the apparatus used was that shown Fig.6. Again strips of glass were fastened to a glass plate with wax. In compartment A was placed approximately 0.5ml. of a sperm suspension of density 10^5/ml. shut off from channel CB by a rubber stopper at S. Compartments C and B and the connecting channel were filled with water. Into B was placed a prothallus with a mature archegonium and into C a prothallus with immature archegonia. These prothalli were previously rinsed in several changes of water to rid them of mature spermatozoa and also to examine them for mature archegonia. After 15 minutes (to allow any diffusion gradients to be set up) the stopper was removed. The flow of water was in the direction of A, the level of water in the channel CB being slightly higher than in AS. The field of the microscope was then focussed on the area at the junction of the two channels indicated by a circle in the diagram. Of the first 20 sperms observed, 2 moved into the channel leading to C, the others having moved in the general direction of the right hand channel. After 5 minutes there were about 100 sperms in the right hand channel while 10 were counted in the left hand channel. After 10 minutes a mass of sperms had built up in the mucilage of the mature archegonium. At the same time there were no sperms to be seen in compartment C and 6 were counted in the left hand channel.

This type of experiment was repeated with several

Fig.3 Glass plate with channels and compartments A, B and C, enclosed by glass strips.
S, rubber stopper.
A sperm suspension is introduced into compartment A and water into the others. A prothallus with a mature archegonium is placed in B, and one with immature archegonia in C. The stopper is removed and the movements of sperms into B and C noted.


C
S
B
A

variations but the final result was the same in that the overwhelming majority of sperms swam towards the mature archegonium giving strong evidence of an attraction presumably of a chemotactic nature.

To investigate the hypothesis of a substance released by the archegonium being responsible for this attraction, a few prothalli were each placed in 1ml. of water. After 15 minutes the water was taken up in a pipette from one of the prothalli showing a mature archegonium and left for about 2 hours so that any sperms present would have died off. 0.1ml. of this liquid was introduced into compartment B, compartments B and C having been previously filled with water and A with a sperm suspension. After 10 minutes the stopper at S was removed. Sperms were attracted to the solution, many more sperms moving into B than into C. When the experiment was repeated with the liquid diluted ten times, sperms did not show a preference for compartment B but moved freely between C and B. Obviously, the concentration of the stimulating substance or substances cannot be much reduced (say by more than 10^{-2}) without losing its activity. This finds a parallel with Pfeffer's (6) experiments in which sperms were not attracted to sodium malate solutions of less than 0.001 per cent.

Behaviour of spermatozoa towards ruptured cells.

Following the chance observation that spermatozoa are attracted to the broken cells of the prothallus, a series of experiments was carried out. First it was shown that a

single somatic cell of the prothallus when punctured with a needle, was effective in attracting spermatozoa. Sperms were also shown to be attracted to the ruptured cells of the bracken sporophyte. The attraction was demonstrated for the ruptured cells of the leaves of Senecio vulgaris, Poa annua, Fuchsia, and Taraxacum, as well as for the cells of the fruit fly Drosophila melanogaster, an annelid and a mollusc.

Thus the attraction of sperms to archegonia is not specific but includes the cells of a variety of plants and animals, provided these cells are ruptured. Since the archegonium is able to bring about the disintegration of the neck cells, this property alone might account for the attraction, the hypothesis being that sperms are attracted to a substance or substances common to the cells of plants, animals and neck cells of archegonia. A simple organic acid would be such a substance. It is also possible that the neck cells of the archegonium contain a specific substance which attracts sperms and which is not to be found in other cells. However, this latter hypothesis is the more unlikely of the two.

Discussion.

Rothschild (7) investigating the block to polyspermy in sea-urchin eggs, estimated the number of sperm-egg collisions by assuming that a suspension of spermatozoa could be treated as an assemblage of gas molecules in which the displacement is proportional to time. In the case of fern spermatozoa, this

assumption is erroneous (Rothschild himself expressed doubt as to its validity (7)), the square of the displacement being proportional to time. However, it was his quantitative approach to the problem which stimulated the present investigation.

Both the simple statistical and experimental techniques which have been described here seem to be applicable to animal sperms.

Summary.

1. The movements of bracken sperms in tap water is random, the mean square displacement being proportional to the time.
2. It is known that when sperms arrive at the mucilage at the necks of mature archegonia, they become immobilised; it has been found in the present work that this immobilisation is irreversible.
3. Sperms are attracted to mature archegonia since the number of sperms arriving in unit time at these archegonia is enormously greater than the number expected to arrive by chance. This increase in the rate of arrival is not due to an increase in the diffusivity but, presumably, to a superposed directed attraction.
4. When sperms are presented with two routes along which they may swim, they take preferentially that route which leads to a mature archegonium, indicating that they are attracted in that direction. Sperms are also attracted to the water in which a mature archegonium has been previously immersed.

5. The attraction of sperms is not specific for mature archegonia since sperms are attracted to ruptured cells of prothalli and sporophytes. Ruptured cells of a variety of animals and plants are also effective.
6. It is suggested that, since the maturation of the archegonium involves the rupture of two neck cells, this process alone might account for the attraction.

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References.

1. Bruchman, H. Flora, 99, 193 (1909)
2. Chandrasekhar, S. Revs.Mod.Phys, 15, 1 (1943)
3. Einstein, A. Ann.Physik., 17, 549 (1905)
4. Fritsch,F.E. and Salisbury, E.J., Plant form and function. Bell, London, 1944.
5. Hanstein,R., Jahrb.wiss.Bot., 4, 356 (1865).
6. Pfeffer, W., Unter.Bot.Inst.Tubingen., 1, 363 (1884).
7. Rothschild, Lord. Internatl.Rev.Cyt., 1, 257 (1952)

Part III.

Incompatibility in bracken.

1. Introduction.

A result of sexual reproduction in living organisms is to recombine in the offspring hereditary differences of the parents. Such recombination is of primary importance in evolution. Recombination is increased in genetic systems in which outbreeding is prevalent, that is, when mating takes place more often between unrelated than between related individuals of a species.

In nearly all animals, cross-fertilisation is the rule since the sexes are generally separate. In plants, on the other hand, the hermaphrodite condition is the usual one and species which are self-fertile are not uncommon. This is particularly true of the grass family in which a relatively large number of species have been studied genetically (Beddows, 1931; Jenkin, 1931, 1933; Nilsson, 1933 and others). But species which regularly fertilise themselves form a minority (Stebbins, 1950) and although there seems to be no ill-effects from continued close inbreeding in these species, the resulting elimination of genetic variability is believed to be a serious disadvantage from the point of view of evolution. Mechanisms promoting

outbreeding in plants are in fact widespread and their mode of action has been the subject of much investigation particularly in the flowering plants and in the fungi.

Many plants have developed morphological barriers to self-fertilisation and these include such devices as protandry, protogyny and the extreme case of complete separation of the sexes as seen for example in the dioecious members of the Labiatae and Cariophyllaceae among the flowering plants. In nearly all other cases the barrier is a physiological one and the term self-incompatibility has been applied to barriers of this kind.

The genetics of incompatibility in angiosperms has been widely investigated, the control of mating between pollen and egg of the same plant being achieved by the intervention of somatic stylar tissue. This tissue, acting as a sieve, generally inhibits the growth of pollen tubes of similar genetic constitution while permitting others to grow successfully. Where this reaction depends on the genotype of the pollen, the system may be termed haplo-diploid (Mather, 1944) as for example in Nicotiana (East and Mangelsdorf, 1925) and Oenothera (Emerson, 1938; Lewis, 1942). When the action of the pollen is that impressed on it by the diploid tissue from which it came, the term diplo-diploid may be applied as for example in Linum grandiflorum (Lewis, 1943) and Forsythia intermedia (Moewus, 1950). The diplo-

diploid system occurs generally in heterostyled plants and the haplo-diploid relationship in homostyled plants. This distinction formed the basis of a classification of incompatibility in angiosperms put forward by Lewis (1944) and which may be summarised as follows.

I. Homostyled plants. (With the exception of Capsella)
   1. One gene with multiple alleles.
   2. Independent gene action in the style but not in the pollen.
   3. Haploid control of the pollen.
   4. No dominance.

II. Heterostyled plants. (Including Capsella).
   1. One or two genes with two alleles.
   2. Co-ordinated gene action in style and pollen.
   3. Diploid pollen control.
   4. Dominance.

A novel incompatibility system combining features of both these classes has been described recently for two species of Compositae, Crepis foetida (Hughes and Babcock, 1950), + Parthenium argentatum (Gerstel, 1950) while other systems are claimed by Bateman (1952) to exist among the Cruciferae. This auther has proposed a third classification dividing all known incompatibility systems in the plant kingdom into two categories: 1. complementary types, in which there is

stimulation of unlike genotypes, and 2, oppositional types in which there is inhibition of like genotypes, this second category including nearly all systems found in the angiosperms.

In the fungi, barriers to self-fertilisation are seen in those cases where sexual reproduction takes place only between two differing thalli (heterothallism). Since the reaction is between two haploid thalli, the system may be classed as a haplo-haploid type of incompatibility (Mather, 1944).

Some heterothallic species show sex differentiation between thalli, i.e., thalli are either male or female as for example in Dictyuchus monosporus (Couch, 1938) and Achlya bisexualis (Raper, 1936). These may be said to show morphological heterothallism. To those species in which there is no morphological difference between mating thalli, the term physiological heterothallism may be applied (Whitehouse, 1949).

Genetical analysis of species with physiological heterothallism has shown the following systems to be in operation:

1. One gene, two alleles (often referred to as plus and minus), e.g. Ascobolus magnificus (Gwynn-Vaughan and Williamson, 1932), and Nurospora sitophyla (Shear and Dodge, 1927).

2. One gene, multiple alleles (the bipolar type), e.g. Coprinus rostrupianus (Newton, 1926).

3. Two genes, multiple alleles (the tetrapolar type) e.g. Coprinus fimetarius (Brunswick, 1924).

Species have been described which are not typically homothallic or heterothallic but intermediate between the two and these are said to show partial heterothallism. In the homothallic Ascomycete Aspergillus nidulans, strains exist which are self-fertile, but which when crossed may produce (1) only crossed asci, (2) a mixture of selfed and crossed asci, and the type of asci produced seems to depend entirely on which strains are crossed (Pontecorvo, 1953). This situation (termed 'relative heterothallism') is more subtle than any of those considered previously. Its mechanism is at present unknown.

In the Pteridophyta, incompatibility has not been demonstrated although its existence was suspected by Czaja (1921) in some species of ferns. His inability to demonstrate the phenomenon may have been due to the inadequate techniques which , in the author's opinion, he employed. Morphological barriers to self-fertilisation however, are widespread, the majority of ferns showing a well-defined protandrous condition in the gametophyte. The

ferns investigated by Andersson-Kotto for example, all showed this type of out-breeding mechanism (1938 and personal communication) and in these cases it was only after prolonged cultivation of prothalli that the simultaneous presence of mature antheridia and archegonia on the same prothallus was brought about. One would not expect to find incompatibility in ferns with a morphological barrier to self-fertilisation since each is an outbreeding mechanism and a combination of the two would not increase the efficiency of outbreeding.

In bracken, on the other hand, sexual organs develop at the same time and no case has been seen by the author where archegonia reached maturity without there being mature sperms available on the same prothallus. Thus there would appear to be ample scope for self-fertilisation. Preliminary tests, however, suggested that an incompatibility system was in operation and the experiments and techniques outlined below were devised to establish the existence of incompatibility and to elucidate its mechanism.

2. Tests for Incompatibility.

(a) Preliminary tests.

In priliminary experiments spores used were taken from a single frond of the crested (C) population. From a petri-dish culture on Knop's agar, a random sample of young prothalli, before development of sexual organs, were isolated into phials. The transfer was effected by means of a platinum wire under the dissecting microscope. After a few weeks the isolated prothalli were examined under the microscope and those that were sexually mature (i.e. had developed both sexual organs) were flooded with water. At the same time those prothalli remaining in the petri-dish from which the isolates were taken, were also flooded and these numbered several hundred. Flooding of the isolated and massed prothalli was repeated every three days until sporophytes began to appear when flooding was discontinued. The numbers of prothalli tested in this experiment and the results obtained are given in table 5.

Table 5.

Results of simultaneous flooding of isolated and massed prothalli of population C with water.

	No.of prothalli fertilised	No.of prothalli not fertilised	Total
Isolated prothalli	9	85	94
Massed prothalli	189	11	200

In a second experiment young prothalli were again isolated, this time in pairs as well as singly. At maturity flooding with water was carried out as in the first experiment. The results are recorded in table 6.

Table 6.

Results of flooding paired and isolated prothalli of population C with water.

	No.of phials with sporophytes	No.without sporophytes	Total
Paired prothalli	9 (both members of pair)	9	18
Single prothalli	1	18	19

Thus half of the pairs of prothalli produced sporophytes. In the nine pairs which produced sporophytes,

both prothalli of each pair did so. Only one out of nineteen single prothalli produced a sporophyte. These results are suggestive of a simple plus and minus incompatibility system as seen in certain fungi where 50% of random pairs would be compatible.

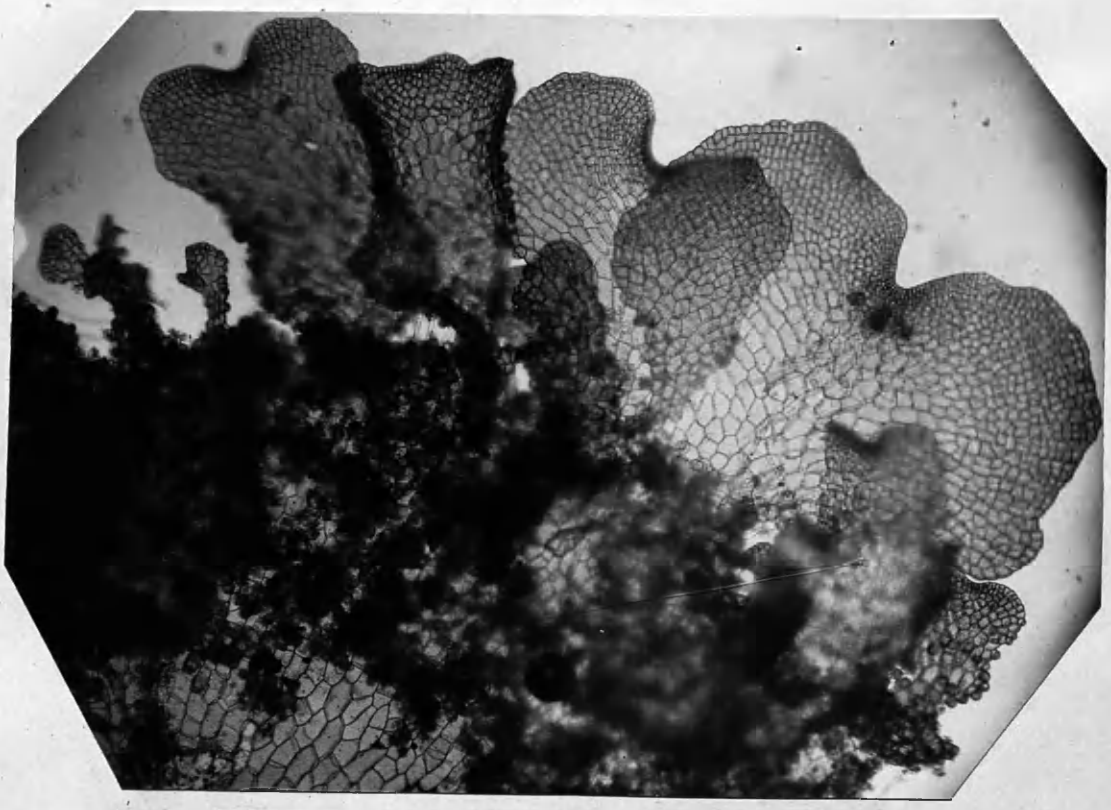
(b) Production of clones and their use.

Clones of prothalli were produced by sectioning individual prothalli of population C. It was found that regeneration of numerous new prothalli took place from the edges of segments cut from parent prothalli (Fig.7). Proliferation of this kind has been reported for other ferns (Albaum, 1938). These new prothalli were detached, each one into a separate phial and in this way clones were built up. The number of individuals in the clones could be augmented indefinitely by repeating the segmentation process. Fifteen clones of population C were established in this way, each originated from a single prothallus and therefore from a different product of meiosis.

In the first experiment with these clones, the isolated prothalli of one clone were tested against sperm suspensions from each of four other clones and against a suspension of 'mixed' sperms. To obtain a suspension of 'mixed' sperms, a petri-dish culture containing a large number of mature prothalli was flooded with water. Each

Fig.7. Proliferation from a segment of a prothallus.

x30.



prothallus in the culture had originated from a single spore, the spores being taken from the same spore sample as the clones under test.

In these tests a departure was made from the procedure outlined in the preliminary tests. Instead of periodic flooding, prothalli were flooded once with the appropriate sperm suspension and placed under the dissecting microscope. Only those showing mature archegonia were taken into account, the criterion for maturity of archegonia being the massing of sperms in the mucilage as described before. This was thought necessary in view of the number of self-fertilisations which took place in the preliminary tests, the number of such selfings obviously depending on the number of times prothalli were flooded with their own sperms. This procedure has been adopted in all the tests which follow so that all prothalli listed in the tables were flooded once and at this flooding have shown at least one mature archegonium. In a few cases more than one flooding was required to obtain conclusive results and these cases are clearly indicated as such.

The results of the first experiment are recorded in table 7.

Table 7.

Results of flooding isolated prothalli of one clone with sperm suspensions from four other clones of poulation C with appropriate controls.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 1C	Clone 1C sperm	1	23	24
	" 2C "	1	20	21
	" 3C "	2	22	24
	" 4C "	1	25	26
	" 5C "	0	21	21
	mixed "	18	4	22

All the prothalli in the totals column were flooded with the appropriate sperm suspension once and showed mature archegonia. Prothalli were then kept under observation until the sporophytes listed had developed. The table was compiled when it was apparent that no more sporophytes were going to be produced.

From the results obtained it would appear that clones 2C, 3C, 4C and 5C were incompatible with clone 1C.

Prothalli of the remaining ten clones were used in the next experiment and tested against sperm

suspensions from clone 1C. The results obtained and the controls used are given in table 8.

Table 8.

Tests of clone 1C sperms against prothalli of ten other clones also of population C.

		No.of prothalli fertilised	No.not fertilised	Total
Clone 6C prothalli	Clone 1C sperm	12	1	13
	" 6C "	1	15	16
	mixed "	15	2	17
Clone 7C prothalli	Clone 1C sperm	1	11	12
	" 7C "	0	15	15
	mixed "	8	2	10
Clone 8C prothalli	Clone 1C sperm	1	17	18
	" 8C "	1	6	7
	mixed "	15	3	18
Clone 9C prothalli	Clone 1C sperm	0	9	9
	" 9C "	1	15	16
	mixed "	8	1	9
Clone 10C prothalli	Clone 1C sperm	11	1	12
	" 10C "	1	8	9
	mixed "	9	1	10
Clone 11C prothalli	Clone 1C sperm	0	20	20
	" 11C "	0	6	6
	mixed "	9	3	12
Clone 12C prothalli	Clone 1C sperm	8	1	9
	" 12C "	1	9	10
	mixed "	14	3	17
Clone 13C prothalli	Clone 1C sperm	10	0	10
	" 13C "	0	8	8
	mixed "	8	1	9
Clone 14C prothalli	Clone 1C sperm	1	10	11
	" 14C "	0	8	8
	mixed "	9	0	9
Clone 15C prothalli	Clone 1C sperm	11	2	13
	" 15C "	1	14	15
	mixed "	7	1	8

These results indicate that when clones 6C - 15C are used as female and clone 1C as male, clones 3C, 10C, 12C, 13C and 15C are compatible and clones 7C, 8C, 9C, 11C and 14C incompatible with clone 1C.

These tests were repeated with seven out of the fifteen clones of population C but with the order of the sexes changed. The results are recorded in table 9.

Table 9.

Tests of clone 1C prothalli against sperm suspensions from six other clones of population C, the prothalli of which were previously tested with clone 1C sperms.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 1C	Clone 1C sperm	2	17	19
	" 6C "	9	2	11
	" 7C "	0	10	10
	" 9C "	1	9	10
	" 10C "	11	2	13
	" 11C "	1	10	11
	" 12C "	15	1	16
	mixed "	12	3	15

Thus clones 3C, 10C and 12C are compatible

with clone 1C when used either as male or female parent, and clones 7C, 9C and 11C incompatible.

In a final experiment with clones of population C, two clones compatible and two incompatible with clone 1C were tested against one another although not in all possible combinations. The results are given in the next table (table 10).

Table 10.

Results of testing four clones of population C against each other in various combinations, two of the clones being compatible and two incompatible with clone 1C

		No.of prothalli fertilised	No.not fertilised	Total
Clone 9C prothalli	Clone 9C sperm	1	10	11
	" 11C "	1	17	18
	" 12C "	16	1	17
Clone 11C prothalli	Clone 9C sperm	0	15	15
	" 11C "	2	14	16
Clone 6C prothalli	Clone 6C sperm	1	12	13
	" 12C "	1	16	17

These results show that clones 6C and 12C which are compatible with clone 1C, are incompatible when tested against each other.

Clones 9C and 11C which are incompatible with clone 1C are also incompatible when tested against each other.

Clone 9C, compatible with clone 1C, and clone 12C, incompatible with clone 1C, are compatible when tested against each other.

A summary of the results obtained in tests of clone 1C against the other clones of population C is given in table 11.

Table 11.

Summary of results of tests with clone 1C against the other clones of population C.

	Clone 1C as ♂	Clone 1C as ♀
Clone 2C	−	
3C	−	
4C	−	
5C	−	
6C	+	+
7C	−	−
8C		−
9C	−	−
10C	+	+
11C	−	−
12C	+	+
13C		+
14C		−
15C		+

Thus five clones were compatible with clone 1C and nine were incompatible.

These findings, together with the results from paired prothalli, are not inconsistent with a one-locus, two-allele system of incompatibility comparable to the plus and minus situation in certain fungi. The fact that the spores used were taken from a single frond, however, would preclude the possibility of finding more than two alleles at that locus.

From the relatively high frequency of selfing and of crossing between incompatible types in these experiments with the crested population (a summary of which is given in table 12), it would appear that the block to self-fertilisation is not very strong.

Table 12.

The number of sporophytes produced by selfing as against those produced by crossing in population C.

Prothalli flooded with compatible sperm		Prothalli flooded with own sperm.	
Total	No.fertilised	Total	No.fertilised
457	412 (90%)	297	22 (7%)

Selfing then is of the order of 8%.

Since, in nearly all cases, the fertilisations listed in

the tables were obtained after a single flooding, it is apparent that continuous selfing of prothalli would give rise to a high proportion of self-fertilisations.

In these experiments where sperm suspensions were used, the sperm density, ascertained by haemocytometer counts, was standardised so that all prothalli in the tests were presented with approximately the same number of sperms thereby eliminating a possible source of error in the results obtained. However, an experiment was carried out using the prothalli of one of the clones of population C against suspensions of mixed sperms (obtained as indicated previously by flooding a petri-dish culture containing many prothalli grown from a random sample of spores) also from the crested population and ranging in density from 10^4/ml. to 50/ml., without any difference in the final results.

(c) Tests of incompatibility in the Ballochraggan (B) and Killearn (K) populations.

The tests carried out so far were confined to prothalli derived from one frond of the crested population. The investigation of incompatibility was extended to two other populations (B and K respectively). The geographical location of these populations is mentioned in an earlier part of the work and also mentioned is the fact that the

morphology of the fronds was normal for both unlike the fronds of population C. Again the spores used in the tests were taken from a single frond in each of the populations. Initial tests were carried out with prothalli isolated at an early stage in development from mass cultures in petri-dishes. The results of these experiments are recorded in table 13. As in the experiments with population C, the term 'mixed sperm' denotes a sperm suspension obtained by flooding a random sample of prothalli in a petri-dish, these prothalli having been grown from spores taken from the same spore sample as the prothalli under test, i.e. from a single frond.

Table 13.

Results of flooding isolated prothalli of populations B and K with water and with suspensions of mixed sperm.

Population	Treatment of isolated prothalli	No. fertilised	No. not fertilised	Total
B	Flooded with water	8	46	54
	" " mixed sperm	40	8	48
K	Flooded with water	7	44	51
	" " mixed sperm	41	8	49

As in the tests with population C,

prothalli were flooded once and observed under the microscope for mature archegonia. Those with mature archegonia were put aside and kept under observation and the number which ultimately produced sporophytes recorded in the table. In the various tables of results that follow, again those cases requiring more than a single flooding for conclusive results, will be indicated as such.

The results of the preliminary tests (table 13) clearly indicate that prothalli have a preference for sperm other than their own.

In the next experiment with populations B and K, prothalli were isolated in pairs and flooding with water carried out when prothalli reached maturity. Each pair was flooded once and observed under the microscope amd only those cases in which both members of a pair showed the simultaneous presence of mature archegonia were taken into account. These were put aside and the number of sporophytes produced were recorded (table 14).

Table 14.

Results of flooding isolated pairs of prothalli of populations B and K with water.

Population	No.of compatible pairs	No.of incompatible pairs	No.of single fertilisations	Total No.of pairs
B	17	8	2	27
K	19	12	3	34

In those cases where only one of the members of a pair produced a sporophyte, it was concluded that the combination was incompatible. Thus in population B, 17 pairs were compatible, each member of a pair having produced a sporophyte, and 10 incompatible. In population K, 19 pairs were compatible and 15 pairs incompatible.

In a single gene, two-allele system of incompatibility, half of the pairs isolated at random would be compatible. The deviations from a 1:1 ratio of compatible to incompatible pairs found here are not significant.

Clones were produced from prothalli of populations B and K by the method outlined for the crested population and used in tests similar to those previously described. Nine clones were produced in population K, and seven in population B.

The first experiment was carried out with clones of population K in which prothalli of eight of the nine clones were tested against sperms of the remaining clone. The results are recorded in table 15.

In these tests the numbers of fertilisations listed were obtained after a single flooding with the exception of the test with clone 9K. In this test the sporophytes produced from a single flooding were as follows:
4 out of 16 prothalli of clone 9K produced sporophytes after a single flooding with sperms from clone 1K
1 out of 19 prothalli produced a sporophyte after a single

flooding with clone 9K sperms (selfing).

On the second flooding the number of self-fertilisations rose from 1 to 2 and of cross-fertilisations from 4 to 13.

Table 15.

Results of flooding prothalli of clones 2K-9K with their own sperm, with sperm of clone 1K and with mixed sperm.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 2K	Clone 1K sperm	15	2	17
	" 2K "	1	10	11
	mixed "	10	2	12
Prothalli of clone 3K	Clone 1K sperm	17	1	18
	" 3K "	2	10	12
	mixed "	9	4	13
Prothalli of clone 4K	Clone 1K sperm	1	18	19
	" 4K "	2	10	12
	mixed "	12	1	13
Clone 5K prothalli	Clone 1K sperm	13	2	15
	" 5K "	0	8	8
Clone 6K prothalli	Clone 1K sperm	1	12	13
	" 6K "	4	10	14
Clone 7K prothalli	Clone 1K sperm	9	1	10
	" 7K "	1	12	13
Clone 8K prothalli	Clone 1K sperm	0	17	17
	" 8K "	2	12	14
Clone 9K prothalli	Clone 1K sperm	13	3	16
	" 9K "	2	17	19

From the results recorded in table 15, clones 4K, 6K and 8K are incompatible with clone 1K, and

clones 2K, 3K, 5K, 7K and 9K are compatible.

In the next series of tests, four of the nine clones of population K were again tested against clone 1K but with the order of the sexes changed i.e. with clone 1K as female. The expected results were obtained (table 16).

Table 16.

Results of flooding prothalli of clone 1K with sperms from four other clones of population K.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 1K	Clone 1K sperm	2	14	16
	" 2K "	10	1	11
	" 3K "	15	2	17
	" 4K "	1	14	15
	" 6K "	1	12	13

Thus clones 2K and 3K are compatible with clone 1K and clones 4K and 6K incompatible when used either as male or female parent.

In a further experiment, clones 2K and 3K (compatible with clone 1K) and clones 6K and 8K

(incompatible with clone 1K) were tested against clone 4K (incompatible with clone 1K). The results obtained (table 17) were as expected although the number of 4K prothalli fertilised by 8K sperm was rather high. In a previous test (table 15), selfing of 8K prothalli was also higher than expected. (See also further experiments with clone 8K below).

Table 17.

Results of testing clone 4K prothalli (incompatible with clone 1K) against sperms from clones 2K, 3K, 6K and 8K (2K and 3K compatible, 6K and 8K incompatible with clone 1K).

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 4K	Clone 2K sperm	14	2	16
	" 3K "	10	5	15
	" 6K "	10	12	22
	" 8K "	1	13	14
	" 4K "	2	12	14

Thus two clones, both compatible with a third clone, are incompatible when tested against each other. Two clones, one compatible, the other incompatible with a third clone, are compatible when tested against each other. As already pointed out, only the result of clone 4K versus

clone 6K is of a doubtful nature. Since both clones were incompatible with clone 1K, they would be expected to be incompatible when tested against each other, whereas in a single flooding 10 prothalli of clone 4K were fertilised out of a total of 22.

Clones 4K and 6K were tested against each other in a further experiment with the results as shown in table 18.

Table 18.

Results of tests of clone 4K against clone 6K, both clones being incompatible with clone 1K.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 4K	Clone 4K sperm	1	14	15
	" 6K "	10	9	19
	mixed "	12	2	14
Prothalli of clone 6K	Clone 6K sperm	7	12	19
	" 4K "	2	13	15
	mixed "	8	2	10

It appears that the mechanism of incompatibility has broken down to a large extent in the sperms of clone 6K since a high proportion both of selfing and of crossing with an incompatible clone is able to take

place. However, when clone 8K is used as female against sperms of clone 4K, there is incompatibility.

Most of the sporophytes produced in these tests with clone 8K were examined cytologically (see below in the chapter on cytology) and all were diploid and of normal morphology. It may be assumed, then, that they were formed as a result of sexual fusion and not due to any abnormality in the life-cycle.

The seven clones of population B were next used in a series of tests, the first of these being to test six clones against the sperms of the remaining clone with the results as shown in table 19.

Table 19.

Results of testing prothalli of six clones against sperms of a seventh clone all of population B.

		No. of prothalli fertilised	No. not fertilised	Total
Prothalli of clone 2B	Clone 1B sperm	24	3	27
	" 2B "	2	19	21
Prothalli of clone 3B	Clone 1B sperm	2	21	23
	" 3B "	3	20	23
Prothalli of clone 4B	Clone 1B sperm	18	3	21
	" 4B "	3	12	15
Prothalli of clone 5B	Clone 1B sperm	14	2	16
	" 5B "	1	14	15

Table 19 (contd.).

		No. of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 6B	Clone 1B sperm	13	2	15
	" 6B "	1	12	13
Prothalli of clone 7B	Clone 1B sperm	2	18	20
	" 7B "	3	14	17

Thus, clones 2B, 4B, 5B and 6B were compatible and clones 3B and 7B incompatible with clone 1B.

Some of these tests were repeated this time using clone 1B prothalli against sperms from four other clones, two of which were compatible and two incompatible with clone 1B when tested with sperms from this clone. The results which are given in table 20 were as expected.

Table 20.

Results of testing prothalli of clones 2B, 3B, 4B and 7B against sperms from clone 1B.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 1B	Clone 1B sperm	2	14	16
	2B	12	2	14
	3B	1	13	14
	4B	13	4	17
	7B	2	13	15

The prothalli of clone 2B (compatible with clone 1B) were tested against sperms from clone 4B (also compatible with clone 1B), and also against sperms from clones 3B and 7B (both incompatible with clone 1B) with the results as shown in table 21.

Table 21.

Results of testing prothalli of clone 2B against sperm from clones 3B, 4B and 7B, clones 2B and 4B being compatible with clone 1B, and 3B and 7B incompatible.

		No.of prothalli fertilised	No.not fertilised	Total
	Clone 2B sperm	2	12	14
Prothalli	3B	10	2	12
of	4B	1	12	13
clone 2B	7B	7	5	12
	mixed	9	4	13

Again the expected results have been obtained.

In a final test the two clones which were found to be incompatible with clone 1B (i.e. 3B and 7B) were tested against each other and once more the expected results were obtained (table 22).

Table 22.

Results of testing two clones against each other, both of which were incompatible with a third clone (population B).

		No. of prothalli fertilised	No. not fertilised	Total
Prothalli of clone 3B	Clone 3B sperm	2	11	13
	" 7B "	18	4	22

The flooding of prothalli with the appropriate sperm suspensions had to be carried out twice before the numbers of fertilisations listed in table 22 were obtained.

From the results recorded in tables 22 and 21, it can be seen that

(1) two clones, both compatible with a third clone, are incompatible when tested against each other,

(2) two clones, both incompatible with a third clone, are also incompatible when tested against each other, and

(3) two clones, one compatible, the other incompatible with a third clone, are compatible when tested against each other.

From these and previous data, the existence of two mating types can be postulated for the prothalli of population B. It must be borne in mind, however, that these

prothalli originated from spores taken from a single frond of this population.

In populations K and B, the mechanism of incompatibility appears to be even less rigid than in the crested population from the amount of selfing which has taken place. The incidence of selfing in populations B and K in the various tests described are summarised in table 23 and are compared with selfing in the crested population.

Table 23.

Comparison of the number of sporophytes produced by selfing with the number obtained from compatible crosses in populations C, B and K.

Population	Prothalli flooded with compatible sperm		Prothalli flooded with their own sperm.	
	Total	No.fertilised	Total	No.fertilised
B	195	160 (82%)	188	25 (13%)
K	246	208 (85%)	218	33 (15%)
C	457	412 (90%)	297	22 (7%)

If the numbers of fertilisations obtained with compatible sperm are taken as the maximum possible under the experimental conditions used, then the frequency of selfing is of the order of 16% in population B, 17% in population K and 8% in the crested population.

(d) Tests for compatibility between mating types of populations B, C, and K.

Two mating types have been distinguished in each of the three populations tested and a single gene, two-allele system of incompatibility is postulated in each case. A single gene, multiple-allele system could be in operation in each case but the fact that spores were taken from one frond in each population, would allow for the detection of only two alleles and hence of only two mating types in each case.

To determine whether or not these two alleles were the same in all three populations, clones of each mating type in the populations were tested against each other. The first of these tests involved two compatible clones of population K and two of the crested population with the results given in table 24.

In this experiment, the prothalli of clone 3K were flooded twice with the appropriate sperm suspensions before the results recorded in the table were obtained.

From these results, there seems to be complete cross-fertility between the two mating types of population K (3K and 4K) and those of the crested population (4C and 6C).

Table 24.

Results of tests between the mating types of populations C and K.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 3K	Clone 3K sperm	5	17	22
	" 4C "	21	2	23
	" 6C "	24	1	25
Prothalli of clone 4K	Clone 4K sperm	2	11	13
	" 4C "	19	1	20
	" 6C "	17	2	19

Prothalli of two compatible clones of population B (3B and 4B) were next tested against sperms from each of the two mating types of populations C and K, with the results as shown in table 25. From these results, the three populations are cross-compatible in all combinations.

If incompatibilty is controlled by a single gene as appears to be the case, there must be multiple alleles and each of the three populations carries at least two alleles and these are different from those carried by the other two populations.

Table 25.

Results of testing prothalli of the two mating types of population B against sperms of the mating types of populations C and K.

		No.of prothalli fertilised	No.not fertilised	Total
Prothalli of clone 3B	Clone 3B sperm	2	9	11
	" 4C "	9	2	11
	" 6C "	8	2	10
	" 2K "	10	5	15
	" 4K "	11	3	14
Prothalli of clone 4B	Clone 4B sperm	2	10	12
	" 4C "	12	2	14
	" 6C "	7	3	10
	" 2K "	9	1	10
	" 4K "	8	2	10

(3) Cytology.

Developing sporangia on the fronds of populations B, C and K were examined cytologically. Sections of sporangia (Fig.8) showed the chromosomes in meiotic division in which pairing and disjunction appeared to be normal.

The chromosomes of the gametophyte were studied in dividing antheridia, and those of young sporophytes produced in mass culture, in root tips and frond apices. In all three populations, a regular alternation of the n (=52) and 2n number of chromosomes took place (Figs.9, 10, and 11) indicating a normal sexual cycle. Manton (1950) also lists bracken as having n=52 from her studies of developing sporangia.

The sporophytes produced as a result of selfing in populations B and K were all of normal morphology. Nearly all were examined cytologically and had the diploid number of chromosomes. Unlike populations B and K, self-fertilisation in the crested population gave rise to sporophytes all of which were weaklings to a greater or lesser degree. Two distinctly abnormal forms occurred and were the subject of special study.

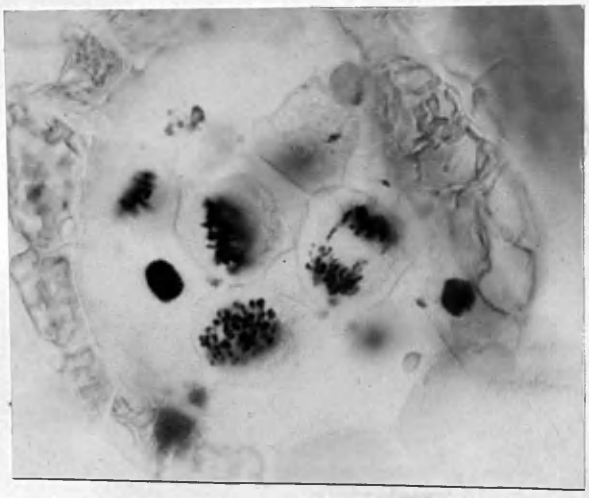
One of these forms (Fig.12B) was confined to a single clone and selfing in this clone produced only this form of sporophyte. These sporophytes did not develop fronds but produced a cylindrical shoot variously branched.

Fig.8. Meiosis in sporangia of

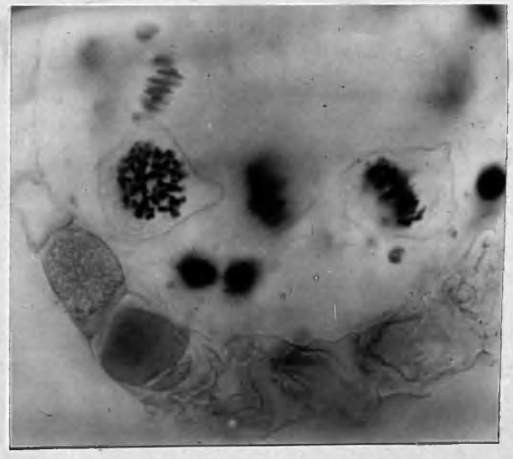
A, population K,

B, the crested variety.

Sections stained with Feulgen. Both x600 approx.

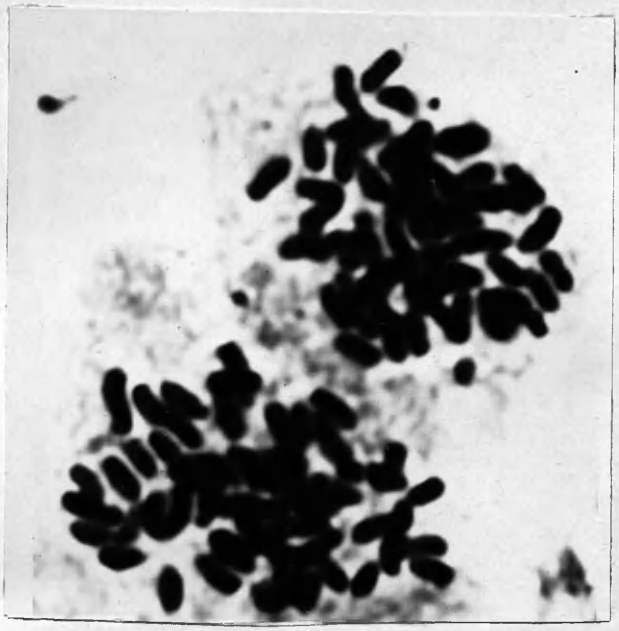


A

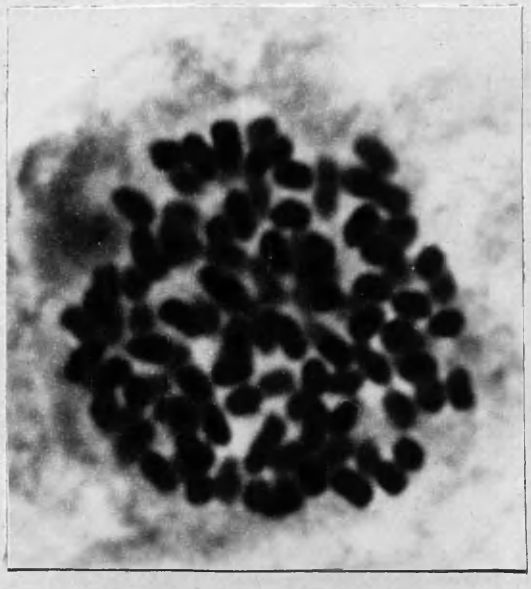


B.

Fig.9 A, squash preparation of developing antheridium with nuclei showing the haploid number of chromosomes.
B, dividing nucleus in a squash preparation of a frond apex showing the diploid number.
Both population C, and both x2000 approx.

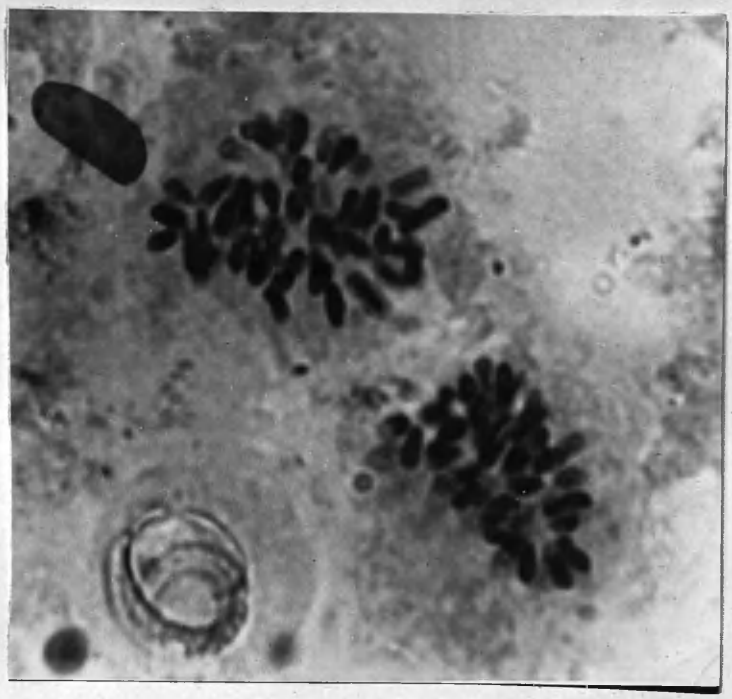


A.

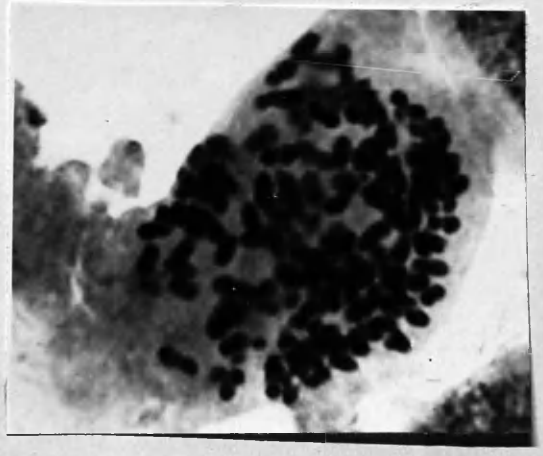


B.

Fig.10 Squash preparations of
(a), developing antheridium with nuclei showing the haploid number of chromosomes.
(b), diploid cell of a frond apex.
Both population K and 1600 approx.



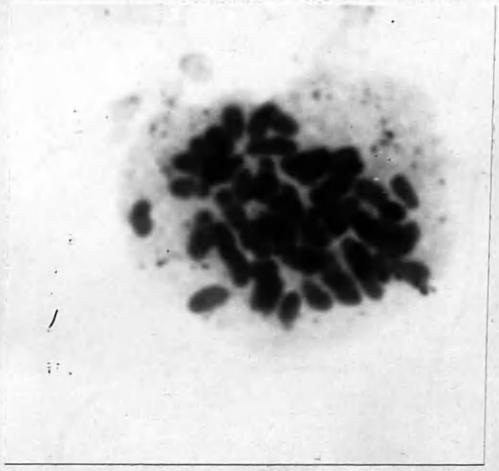
(a).



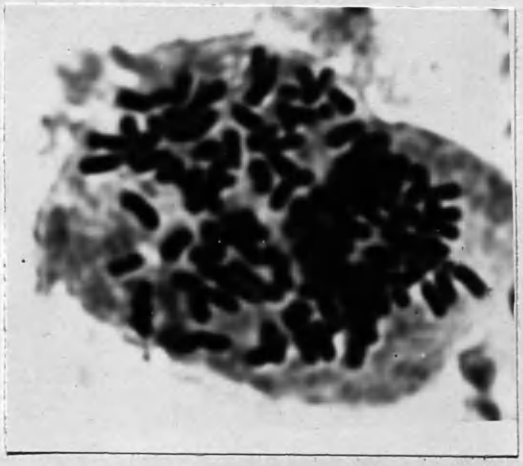
(b).

Fig.11 Squash preparations of

(a), haploid nucleus of an antheridium,
(b), diploid nucleus in the apex of a frond.
Both population B and x1600 approx.



(a).



(b).

The branches did not have apical meristems but a vascular system was present. The root system, on the other hand, was normal and well-developed. Difficulty was experienced in keeping these forms alive and the material available for cytological study was limited. The few successful preparations that were obtained showed these to be diploid (Fig.12C).

It was concluded that prothalli of this clone carried one or more recessive genes which acted only in the sporophyte and when in the homozygous state resulted in a semi-lethal condition.

Abnormal sporophytes have been described in other ferns but have always associated with the phenomenon of apogamy (Lang, 1898; Steil,1939; Manton, 1950). These apogamously produced sporophytes differed from those described here in having no roots at all or at best, an ill-developed system.

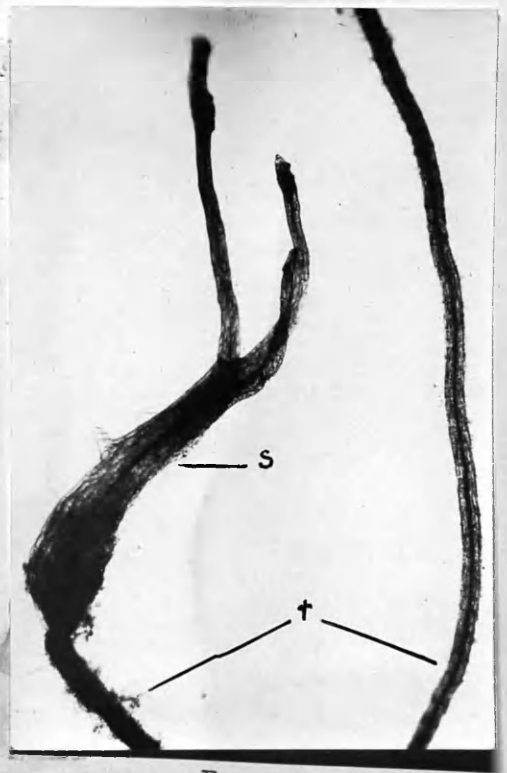
The other abnormal form of sporophyte was not confined to a single clone but occurred in several. The clones which produced this form also produced on selfing, sporophytes, which, though stunted to varying degrees, were of normal morphology and had the diploid number of chromosomes. In this second abnormal form, roots did not develop and the apices of the young shoot, instead of giving rise to fronds as in Fig.12A, developed prothallial tissue with sex organs (Fig.13A). Developing antheridia on these

Fig.12 A. Normal sporophyte with fronds aged about six weeks. x6

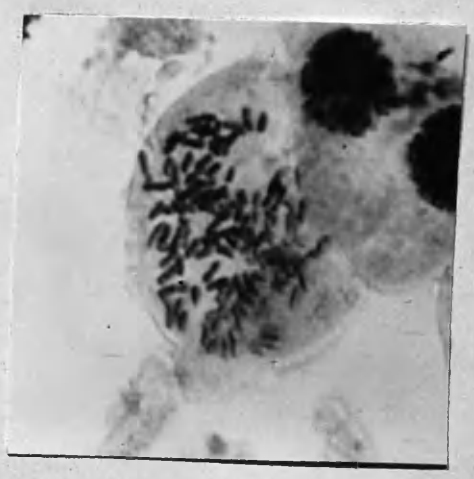
B. Abnormal sporophyte with branched shoot (s) and well developed root (r), aged about three months (the result of self-fertilisation in one particular clone of population C). x6

C. Squash preparation of root-tip of B showing a diploid cell. x1000 approx.

A.



B.

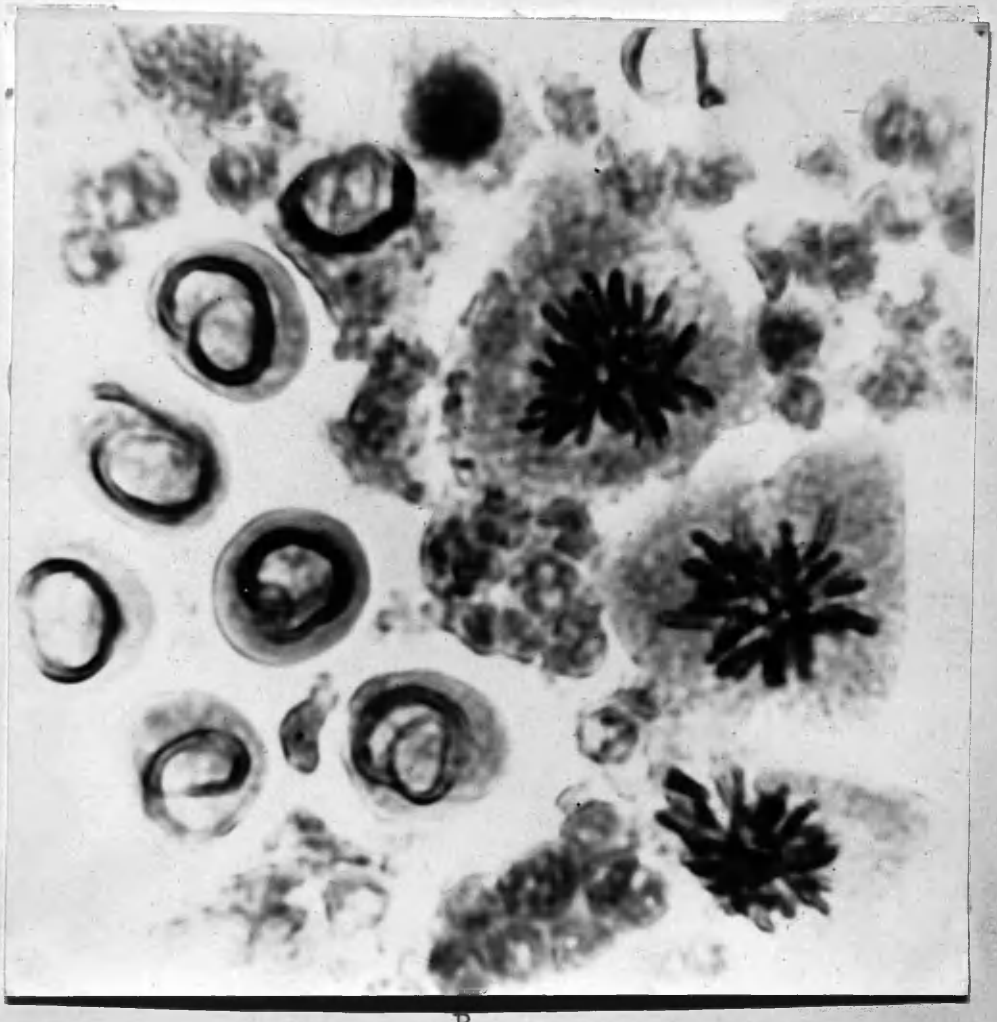


C.

Fig.13 A. Abnormal sporophyte producing prothallial tissue (produced occasionally on selfing in the crested variety). x4.

B. Haploid nuclei and spermatocytes in antheridia of A. Squash preparation x1600 approx.

A.



B.

prothallial outgrowths and the apices of the young shoots were investigated and the haploid number of chromosomes was seen in both (Fig.13B). It was concluded that this type of abnormal sporophyte was of apogamous origin.

Apogamy is a permanent feature in the life-history of some ferns and the elimination of nuclear change in these cases is achieved in one of two ways. Apogamy may be followed by apospory as seen for example in the well-known case of Asplenium Filix-foemina var.clarissima and in the present investigation; or unreduced spores may be produced as in Pteris cretica and Cyrtomium falcatum (Manton, 1950) for example. Apogamy has also been produced in some ferns by preventing fertilisation (Lang, 1929; Duncan, 1941) but fertilisation was not prevented in the present case since prothalli were flooded with water at least once.

Cytological techniques.

In preparing squashes, material was treated with a saturated solution of 8-oxyquinoline (Tjio and Levan, 1950) for four hours at 18°C then fixed in acetic-orcein (2% orcein in 45% acetic acid). Normal hydrochloric acid was added, the final mixture being of the composition 1HCl : 9 acetic-orcein. Heating and cooling was carried out three or four times in a watch glass after which the material was mounted in 45% acetic acid and squashed.

The general effect of the pretreatment

with oxyquinoline was to shorten and thicken the chromosomes and also to increase the intensity of staining. Preparations obtained in this way were very much better than those of untreated material, particularly in the case of developing antheridia.

Discussion.

The results obtained from the incompatibility tests in the three populations suggest a single gene, multiple-allele system to be in operation similar to that of the bipolar fungi. To be complete, the investigation would require the use of genetic markers segregating and recombining with the incompatibilty alleles postulated here. However, it has not been possible in the time available either to obtain marked strains or to raise sporophytes to the stage of sporulation.

Due to the relatively high frequency of selfing which takes place, the incompatibility system would appear to be a weak one. As pointed out by Bateman (1952) one would expect to find in nature incompatibility systems continually arising and in all stages of evolution. In systems too weak for the requirements of a species, selection would act to increase the effectiveness of the mechanism and therefore act on its genetic basis. An example of selection for outcrossing (though not for prevention of selfing) is seen in the studies of Pontecorvo (1953) and his co-workers on the genetics of the homothallic Ascomycete Aspergillus nidulans. The original strains of this fungus were completely self-compatible, mixed inoculi of two strains giving crossed asci and selfed asci in proportions usually well below the theoretical maximum for random karyogamy (i.e. 1 : 1). Mutants were utilised in numerous

crosses and new recombinant strains selected. By continuous selection of recombinants, alleles favouring outcrossing (whether present in the original strains or arisen by mutation) were automatically selected. Strains of Aspergillus nidulans now exist which, though self-fertile, will give 100% crossed asci when combined with certain other strains. This behaviour, termed by Pontecorvo (1953) 'relative heterothallism', indicates the existence of positive incompatibility i.e. mechanisms of preferential fertilisation not based on the prevention of selfing ('complementary' systems in Bateman's terminology). It has to be noted, however, that the mechanisms of incompatibility in fungi operate at a level altogether different from those in higher plants (including pteridophytes as shown here). They operate at the level of karyogamy between nuclei in a common cytoplasm.

The condition of partial incompatibility seen in bracken may be adequate in maintaining the necessary interchange of genes and the system become stabilised at the present evolutionary level. Partial self-incompatibility of this type may be widespread in ferns where sexual organs develop simultaneously, but to detect it, critical tests of the kind described here would have to be carried out.

The movements of sperms in the vicinity of mature archegonia were investigated to find out whether

compatible sperms behaved differently from incompatible sperms. As no such difference could be detected, the incompatibility mechanism appears to act somewhere after the time of entry of sperms into the mucilage. That it acts in the zygote is unlikely since too many sporophytes arise on selfing which are perfectly healthy and of normal appearance (at least in populations B and K) for the hypothesis of a lethal condition in the zygote to be tenable. It may be that the barrier arises during the movements of the sperms down through the mucilage in the archegonial neck in analogy to the situation in angiosperms, sperms having to make their way through the mucilage, pollen tubes through the stylar tissue. Lewis (1952) has shown in Oenothera that incompatible pollen tubes carry an antigen the antibody to which is produced in the style so that pollen-tube growth is inhibited. Similarly it is possible that incompatible sperms carry an antigen the antibody to which is present in the mucilage. A very marked change in the mobility of sperms takes place in the mucilage and it is feasible that incompatibility operates at this level. Of some significance in this connection is the work of Hoyt (1910) who showed that crosses between closely related species of ferns did not take place due to the inability of the foreign sperm to reach the egg, although many sperms entered the archegonial necks.

The ecological significance of these findings.

Since, in ferns, the haploid generation has an independent existence and therefore is exposed to selection, the possibilities of adaptation of the sporophyte depend on the action of every gene being restricted with precision to either part of the life-cycle. In bracken, prothalli and sporophytes react very differently to similar environments and the conditions so obviously enjoyed by the sporophyte are not suitable for the growth of prothalli. Thus there seems to be a lack of co-ordination in the requirements of the two phases of the life-cycle.

Records of the occurrence of prothalli and young sporelings are rare (Conway, 1953) and prothalli in fairly large numbers have been seen only in recent years on the rubble of buildings damaged in World War II (Lousley,1943). Sporophytes, on the other hand, occur in a variety of habitats and their rapid spread is a serious problem in agriculture in Scotland. In nature, a prothallus is much more difficult to detect than a young sporophyte and it is probable that numerous isolated prothalli become established each year. The fact that new sporophytes are rare can be attributed to a combination of incompatibility and poor adaptability of prothalli. The chances of an isolated prothallus surviving long enough to produce a sporophyte are slight and the production of new sporophytes would require the establishment of a number of prothalli in close proximity to each other otherwise sperms

travelling from one prothallus to another might be sidetracked by being chemotactically attracted to one or other of the organic substances in the soil humus. However, the fact that bracken is widespread and, presumably, has been so over long periods, suggests that its genetic system is a successful one. It is clear that this genetic system has in store a wealth of interesting problems open to investigation.

Summary.

1. Methods of testing for incompatibility are described and these involve the production of clones and the isolation of prothalli.
2. Incompatibility is demonstrated for three populations of bracken.
3. Two mating types have been detected in each of the populations, spores being taken from a single plant in each case.
4. The three populations were cross-compatible in all combinations of mating types and a single gene, multiple-allele system of incompatibility is postulated.
5. Incompatibility appears to operate between the time of entry of sperms into the mucilage of mature archegonia and their arrival at the egg surface, since there was no difference in the movements of different sperm types up to the point of entry into the mucilage. Incompatibility acting in the zygote is considered unlikely.

6. Incompatibility alleles appear to be relatively weak in their action since selfing was 8%, 16% and 17% respectively in the three populations (C, B and K) tested.

7. The cytology of sporangia of the three populations was investigated as well as antheridia of prothalli and meristems of young sporophytes produced in mass culture. Each population showed a regular alternation of the haploid (n=52) and diploid numbers of chromosomes indicating a normal sexual cycle.

8. Sporophytes produced on selfing in populations B and K were diploid and of normal morphology.

9. Selfing in population C (a 'crested' variety) gave rise to sporophytes all of which were weaklings to a greater or lesser degree. Two distinctly abnormal forms were apparent. One of these forms was confined to a single clone and constantly appeared on selfing in that clone. Cells of the root-tip of this form had the diploid number of chromosomes and it was concluded that prothalli of this clone carried deleterious recessive genes which acted only in the sporophyte.

The second abnormal form occurred in more than one clone and since the cells of this form carried the haploid number of chromosomes, it was concluded that these sporophytes were of apogamous origin.

10. It is suggested that incompatibility may be a factor contributing to the rare occurrence of new populations of bracken in nature.

References.

Bateman, A.J. 1952. Self-incompatibility in angiosperms. Heredity, 6, 285-310.

Beddows, A.R. 1931. Seed-setting and flowering in various grasses, Bull.Welsh Plant Breed.Sta., H, 12, 5-99.

Blakeslee, A.F. 1904. Zygospore formation a sexual process. Science, 19, 864-866.

Brunswick, H. 1924. Untersuchungen uber die Geschlechts und Kernverhaltnisse bei der Hymenomyzeten-gattung Coprinus. Bot.Abh.K.Goesel, 5, 1-152.

Conway, E. 1953. Spore and sporeling survival in bracken. J.Ecol., 41, 289-294.

Couch, J.N. 1926. Heterothallism in Dictyuchus, a genus of the water moulds. Ann.Bot.Lond., 40, 849-881.

Duncan, R.E. 1941. Apogamy in Doodia caudata. Amm.J.Bot., 28, 921-931.

East, E. and Manglesdorf, A.J. 1925. A new inerpretation of the hereditary behaviour of self-sterile plants. Proc.Nat.Acad.Sci., 11, 166-171.

Emerson, S. 1938. The genetics of self-incompatibility in Oenothera organensis. Genetics, 23, 190-202.

Gerstel, D.U. 1950. Self-incompatibility studies in Guayule. II. Inheritance. Genetics, 35, 482-506.

Gwynne-Vaughan, H.C.I. and Williamson, H.S. 1932. The cytology and development of Ascobolus magnificus. Ann.Bot.Lond., 46, 653-670.

Hughes, M.B. and Babcock, E.B. 1950. Self-incompatibility in Crepis foetida subsp.Rhoeadifolia. Genetics, 35, 570-588.

Hoyt, W.D. 1910. Physiological aspects of fertilisation and hybridisation in ferns. Bot.Gaz., 49, 281-297.

Jenkin, T.J. 1933. Interspecific and intergeneric hybrids in herbage grasses, initial crosses. J.Genet., 28, 205-234.

Lewis, D. 1942. The physiology of incompatibility in plants. I. The effect of temperature. P.R.S.B., 131, 13-26.

- 1943. The physiology of incompatibility in plants. II. Linum grandiflorum. Ann.Bot. N.S., 7, 115-132.

- 1944. Incompatibility in plants, its genetic and physiological synthesis. Nature, 153, 575-578.

- 1952. Serological reactions of pollen incompatibility

substances.

P.R.S.B., 140, 127-135.

Lang, W.H. 1898. On apogamy and the development of sporangia upon fern prothalli.

Phil.Trans.Roy.Soc.Lond.B., 190, 187-238.

– 1929. On a variety of Scolopendrium that bears sporangia on the prothallus.

Ann.Bot.Lond., 43, 355-374.

Louseley, J.E. 1947. Flora of the bombed sites in the city of London in 1944.

Bot.Soc.Exch.Cl.Rep., 12, 882.

Mather, K. 1944. Genetic control of incompatibility in angiosperms and fungi.

Nature, 153, 392-394.

Moewus, F. 1950. Zur physiologie und biochemie der selbsterilitat bei Forsythia.

Biol.Z.B., 69, 181-197.

Newton, D. 1926. The bisexuality of individual strains of Coprinus rostrupianus.

Ann.Bot.Lond., 40, 105-128.

Nilsson, F. 1933. Studies on fertility and inbreeding in some herbage grasses.

Hereditas, 19, 1-162.

Pontecorvo, G. 1953. The genetics of Aspergillus nidulans.

Advances in Genetics, 5, 141-238.

Raper, J.R. 1936. Heterothallism and sterility in Achlya and observations on the cytology of A. bisexualis.
J.E.Mitchell Sci.Soc., 52, 347-389.

Shear, G.L. and Dodge, B.C. 1927. Life histories and heterothallism of the red bread mould fungi of the Monilia sitophila group.
J.Agric.Res., 34, 1019-1042.

Stebbins, G.L. 1950. Variation and Evolution in Plants.
London.

Steil, W.N. 1939. Apogamy, apospory, and parthenogenesis in the pteridophytes.
Bot.Rev., 5, 433-453.

Tjio, J.H. and Levan, A. 1950. The use of oxyquinoline in chromosome analysis.
Annales de la Estacion Experimental de aula Dei, 2, 21-64.

Whitehouse, H.L.K. 1949. Heterothallism and sex in the fungi.
Biol.Rev., 24, 411-477.