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M.Sc. THESIS.

A STUDY OF PROBLEMS IN THE LIFE-CYCLE OF PUCCINIA MALVACEARUM, FROM MONOSPORIDIAL INOCULATIONS, AND CULTURES MADE UNDER CONTROLLED CONDITIONS.

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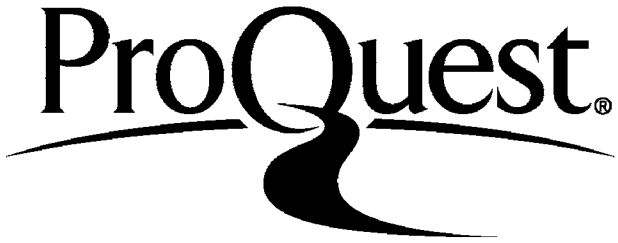

Abstract of Thesis to be presented for the Degree of M.Sc. in Botany

by

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A study of the problems in the life-cycle of Puccinia malvacearum from monosporidial inoculations and cultures made under controlled conditions.

The work began with an endeavour to find a technique for inoculating the host plants of the Uredineae with single sporidia of their specific rust, with precision and certainty. This has been achieved, and single sporidia have been isolated by means of the microdissection apparatus of Dr. Chambers. The most favourable conditions for sporidial infection were then determined, and cultures kept under controlled conditions.

The rust used was Puccinia malvacearum, chosen because its teleutospores germinate immediately they are ripe so that a constant supply is available over a long period. This faciliated preliminary experimentation.

Results show that this fungus is homothallic: a single sporidium being sufficient to cause infection, and the production of teleutospores. This was to be expected since there is no pycnidium or aecidium in the life cycle. The method of inoculation has universal application for the rusts and it is hoped to demonstrate this at once in a long cycled form.

Reviewing the literature on Puccinia malvacearum several problems in the life-cycle were found to be as yet unsolved. A study of the complete life history by means of examination of infections of known age, made under controlled conditions, has helped to elucidate these points.

Infected plants in the field have also been under observation day by day throughout the winter, to solve the problem of hibernation. The results from these experiments show that

there is a retardation in the cycle consequent on the less frequent occurrence of suitable conditions for germination and infection, but new infections do occur whenever these favourable conditions prevail.

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INDEX.

	Page.
INTRODUCTION	1.
MATERIAL AND METHODS.	
Source of material	4.
Inoculation	5.
Methods of examination	11.
HOMOTHALLISM	13.
LIFE-CYCLE.	
Period of development	16.
Mycelium in haplophase	18.
Development in diplophase	26.
The return to the haplophase	33.
HIBERNATION	37.
Diary of observations	37.
Discussion of hibernation	42.
Biological note	47.
SUMMARY	49.
LITERATURE CITED	51.
DESCRIPTION OF PLATES	54.

A STUDY OF PROBLEMS IN THE LIFE-CYCLE OF PUCCINIA MALVACEARUM, FROM MONOSPORIDIAL INOCULATIONS, AND CULTURES MADE UNDER CONTROLLED CONDITIONS.

INTRODUCTION.

There is already accumulated so wide a literature dealing with Puccinia malvacearum, that some justification must be made for yet another contribution. During recent years the attention of botanists has again been drawn to the rusts by the suggestive results of Craigie's experiments with Puccinia Helianthi and Puccinia graminis. These results, stimulating and suggestive though they be, call for more precise experimentation, and there seems to be a crying need for single spore cultures made from the uninucleate spores of the rusts: from pycniospores or from sporidia. In an endeavour to help to supply this need the present investigation was undertaken. The material used was determined chiefly by the fact that in the botanical garden in connection with the laboratory of Royal Holloway College, there appeared to be a continuous supply of teleutospores of Puccinia malvacearum which have the valuable character, from the point of view of an investigation such as this, of being able to germinate without a period of rest, so that sporidia can be obtained in great numbers at any time of the year. This is a "short cycled" rust and there are no pycniospores in the life cycle. Sporidia of Puccinia malvacearum were therefore chosen for single spore cultures. They are very small and very delicate but with a Chamber's micro-manipulator it has been found possible to transfer them, without exposure to desiccation, on to a host. The host plants, species of Malva and Althea which were used for the inoculation experiments, were grown from

seed and kept in a greenhouse leading off the physiology room in the laboratory, far removed from the garden where the naturally infected plants were growing.

Whilst consulting the literature already contributed on this fungus it was apparent that there are several gaps in our knowledge of the life cycle, and also several points upon which further evidence is desirable for their more complete solution. These points are emphasised in the following short historical account of our knowledge of the fungus up to the present time.

As early as 1882 (27) Plowright published a paper in the Gardener's Chronicle in which he considers the general facts of the life cycle. A sudden epidemic of the hollyhock disease in Manchester led Robinson (28) to study the relations of the fungus to the host plant, the pathology of the invaded cells, and the extent of invasion of the tissues. So that in England the disease has been studied more from an economic than a purely scientific standpoint.

In America, Taubenhaus, (31) Dandeno, (16) and Duggar (17) have all made contributions. The former interested himself in the problem of overwintering of the fungus, and is opposed to some of the suggestions brought forward by the other two American writers on this point. But perhaps the name most readily associated with Puccinia malvacearum is that of Eriksson (18,19) who claims to solve the hibernation problem by his mycoplasm theory. He considers evidence called from the behaviour of Puccinia malvacearum, to be in complete agreement with his mycoplasm hypothesis. In England this work has been most convincingly refuted by Baily (2).

The results of these observations on overwintering are all so confusing and in some cases contradictory, that it was thought probable that the rust might behave differently in different climates with regard to overwintering. That it is

affected by external conditions is evident from the fact that Professor Buller finds it difficult to cultivate in Winnipeg. It therefore seems possible that the difference in behaviour reported by Dandeno, and Eriksson, may be explained by the difference in climate. In this study the fungus has been found to be most dependent on correct moisture relations, and the non-occurrence of these might lead to some slight difference in behaviour. So far no one has consistently observed the method of survival from autumn to spring in England, and so experiments have been made to test the behaviour of the host and parasite during this period.

Again Puccinia malvacearum is one of the rusts to which attention has turned for a satisfactory elucidation of the mode of origin of the diplophase in short cycled forms. From comparison with events in the aecidium of Phragmidium, Blackman and Fraser[6] suggested a similar migration at the base of the teleutosorus, whereby this might be effected, but were unable to produce any evidence. Werth and Ludwig[33] however claim to have seen this migration of nuclei although they also report the presence of binucleate cells in the vegetative mycelium. A recent paper on short cycled rusts by Walker,[34] records a variety of ways in which the initiation of the diplophase is effected.

There thus appear to be three main problems requiring further investigation

(1) the occurrence of homothallism.

(2) the origin of the diplophase,

(3) the problem of hibernation.

MATERIAL AND METHODS.

Source of material.

The investigation of the life history of a rust is hampered considerably by the impossibility of growing the fungus independently of the host. The only method for a detailed study is therefore the inoculation of plants day by day, followed by fixation at known intervals and examination of the stages of definite age so obtained. Further, Craigie's work on Puccinia Helianthi, and Puccinia graminis suggests that a different development may ensue according as to whether these inoculations are the result of monosporidial or of multisporidial infection. Ideally then inoculations with both one and with a number of sporidia should be made. In the present study both these methods have been used to elucidate the life-history of Puccinia malvacearum.

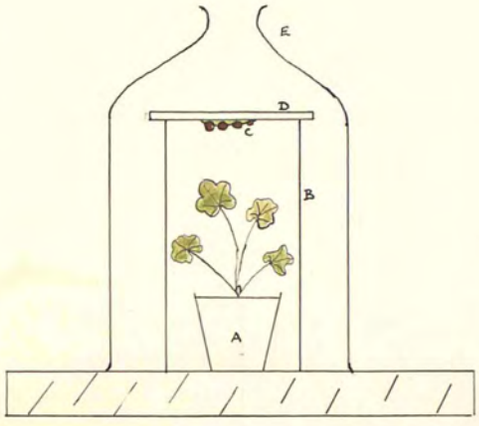
The diseased plants which were used as a source of Puccinia malvacearum for the inoculation experiments were found growing in the Botany Garden of Royal Holloway College, and were of the following species: Althea rosea, Althea officinalis, Malva borealis, Malva rotundifolia, and Malva sylvestris.

The hosts selected for the inoculation experiments were Althea rosea, Althea officinalis, and Malva borealis. The seed from which these plants were grown was not specially sterilised but freedom from the disease was evident since in no case during the whole study did infection occur without inoculation. The plants when used were about six months old, and were kept in a special greenhouse. This was not heated and the same range of temperature was experienced in the house as in the field.

Inoculation.

a) By many sporidia.

From field observations it is evident that the presence of moisture is one of the essential conditions for infection, since the disease is most prevalent during periods of damp weather. This fact was borne in mind when making the inoculation experiments in the greenhouse. The plant to be inoculated was therefore first sprayed with tap water and then placed in a damp chamber. At first a bell jar was considered sufficient, but it was found that moisture was not retained for a sufficiently long period. The use of an inner inoculation chamber was then tried. The form selected was that of a cylinder the roof of which could be formed by a glass plate bearing the source of infection. A sample of "Windolite", — a form of safety "glass" consisting of a thin plate of mica-like substance re-inforced by a loose mesh of fine wire seemed to possess the properties required., for it was pliable and yet sufficiently strong to support the glass plate. Strips of this material were sewn into cylinders, lined with damp filter paper, and placed round the plant. Unfortunately the mica-like substance proved to be some compound of acetone and when damp the smell of this had a poisonous effect on the plant, causing the leaves to turn brown and become unhealthy. Other methods were tried and finally the most successful inoculation chamber was formed by wrapping a length of corrugated cardboard round the plant pot to form a cylinder. The cardboard was especially useful as it retained water, when it was saturated, for a considerable period, and also the size of the chamber could be easily regulated according to the size of the plant pot used. The form of the apparatus is seen text figure I.



TEXT FIGURE 1. INOCULATION APPARATUS.

The plant A is surrounded by the cylinder of cardboard B. The roof of the chamber is formed by the glass plate D. To the underside of this an infected leaf C from the garden is attached by means of vaseline on the upper surface, so that the pustules of the fungus on the lower side of the leaf are hanging into the chamber. The height of the chamber was always so arranged that the source of infection was four or five inches away from the plant to be inoculated in order to ensure a fairly thin scattering of sporidia. The glass plate was placed in position on top of the moist cardboard cylinder, and the whole apparatus was then enclosed in the bell jar E to prevent unnecessary evaporation.

To obtain the earliest stages of infection for examination the source of the disease was placed nearer to the plant to ensure a denser scattering of the sporidia so that less leaf surface had to be examined for these stages.

Preliminary experiments showed that germination of the teleutospores began in three hours and continued in any one sorus for a day or more. The plant was therefore labelled

and left in this moist chamber on the greenhouse bench for 48 hours ~~when~~ before observations began.

More than a hundred inoculations in this manner have been made day by day during the months October 1930 to June 1931 except for the University vacations at Xmas and Easter. This period includes the winter months and it has therefore been possible to compare the behaviour of the fungus in the greenhouse during this time with events out of doors. On some days in January and February the minimum temperature of the house was as low as 31 F. whilst the maximum for the same day was not more than 40 F., showing that the temperature was little different from that out of doors and yet infection was obtained. A winter temperature therefore is not the cause of absence of germination out of doors, but rather unsuitable moisture conditions.

(6) Monosporidial inoculations.

In making monosporidial inoculations the precedure was as follows. A small leaf fragment A containing a teleutosorus of the fungus was fixed by means of vaseline on to the base of a moist chamber formed by a glass ring B (diameter ½ inch) mounted on a microscope slide C. A drop of water D was placed near the sorus to ensure a moist atmosphere. The chamber was enclosed by a coverslip coated with a film of plain agar. The whole was inverted and supported by two glass rods E, E in a Petri dish. The apparatus is similar to that used by Robinson, in his experiments on the germination of the sporidia, of this fungus. The outer chamber formed by the Petri dish however was found useful in preventing evaporation.

TEXT FIGURE 2. COVERSLIP CULTURE
FOR MONOSPORE INOCULATIONS.

After two or three hours the culture was examined. During this time the teleutospores had germinated and sporidia were being abstricted. These fell on the agar film and could be seen on examination under the low powers of the microscope. This first scattering was always discarded. A second coverslip was substituted for the first and the culture re-inverted for a period of 10 - 15 minutes. This ensured a fresh scattering of sporidia of known age. These were used for infection experiments.

Isolation of these sporidia wasveffected by means of a Chambers micromanipulator fitted with a micro-pipette coaser than those used for micro-injection as described by Chambers.(12) These pipettes were made from hard glass tubing of 5 mm. external diameter. This was first drawn out into a capillary, about 1 - 1.5 mm. in diameter in an ordinary flame. For the second process a minute flame was utilised and the capillary passed through the flame in a direction away from the worker as recommended by Chambers. The capillary separated with a slight tug, a feeling Bobles Lee describes as that "experienced when a taut thread held in the fingers is parted in the flame." After practice most attempts were successful. The

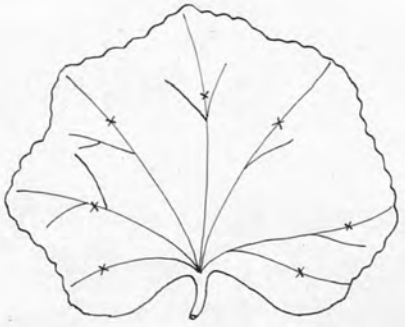
last operation required the most skill, i.e. the bending of the tip of the micro-pipette to a suitable angle.

Preliminary experiments next had to be made with the pipette to ensure successful isolation of the sporidia. The needle was clamped into position in the manipulator and adjusted for focussing. A film such as that described above was placed in position above the chamber and the scattering of sporidia brought into focus. Adjustments were then made according to the height of each chamber which varied with the depth of the agar film, and a particular sporidium well isolated from the rest was selected. Slowly the mouth of the pipette was adjusted until the sporidium in its drop of water, with which it is abstricted from the sterigma, was in the centre. The vertical lever was then moved until the pipette was in contact with the agar film and then quickly taken out of contact. Generally the sporidium had vanished. Was it within the pipette or had it adhered to the side? To determine this the pipette was removed from the clamp of the manipulator and the liquid taken up, blown on to a slide. If the sporidium had been sucked up it was ejected from the pipette with the drop of water. The preliminary experiments showed that this was usually the case. Later this ejection of the sporidium was facilitated by first allowing the tip of the pipette to come in contact with and suck up a further drop of water.

It was thus apparent that sporidia could be successfully isolated by this means. Would they however germinate after such treatment? To test this several were isolated as described above and kept in a moist chamber and if, the moist conditions were maintained germination ensued. These preliminary experiments were repeated with each new pipette made.

The next step was to apply this method to plant inoculations. The plants to be inoculated were first sprayed with tap water and covered with a bell jar for 4 - 6 hours.

At first this was thought to be long enough to produce the required moist and receptive condition of the host, but experiment showed that a period of 24 - 48 hours gave better results. A film scattering of sporidia was then placed over the moist chamber of the manipulator. The first few sporidia isolated, and the last few were transferred to a moist chamber (see text fig. ~~II~~ 2.) and kept as controls. The middle isolations were used as inoculations, each sporidium being placed as near as possible on to a vein . (see text fig. 3. ~~III.~~) This fixed the position for further observation.



TEXT FIGURE 3. LEAF SHOWING POINTS OF INOCULATION ON THE MAIN VEINS.

Between each isolation, the pipette was thoroughly washed out in water. Even if the pipette failed to isolate a sporidium it was cleaned in the same way. That the sporidium had been deposited on the leaf could be demonstrated by re-isolating it from the surface of the leaf and depositing on a microscope slide where it c ould be identified. This was done at intervals The bell jar was replaced over the plant after each monospore inoculation. ~~made.~~

After each plant had been fully inoculated it was placed in an inner chamber for a further 48 hours, exactly as for the cultures described above.

One plant every day was set aside as a control. In no case did any of the control plants become infected.

Methods of examination.

The first stages of infection were most easily studied by stripping off the upper epidermis of the leaf, fixing it in 70% alcohol, and staining either with cotton blue in dilute glycerine, erythrosin, or Guegen's reagent, (a mixture of cotton blue and Sudan III in glycerine).

For examination of later stages of development, three methods were found useful. The first is described by Mangin (23) in his researches on the Peronosporales. The method is as follows. Fresh material is first boiled for a few minutes in methylated spirit to remove air. The material thus treated is placed in hydrochloric acid, of ordinary strength or diluted by an equal volume of water. To this liquid are added crystals of potassium chlorate (about 1 gramme for 20 cubic centimetres of the liquid). The tissue is left in this solution overnight when it attains a white colour. If it is still yellow more chlorate is added. After this the material is washed in water and preserved in alcohol. Tissues thus treated can be successfully stained with cotton blue in dilute glycerine. The method is useful for determining the stage of development and limits of the pustule.

The second method is one formerly used by Dr. Wager and was recommended to this Botanical department for the investigation of hyphal branching in Agaricus. It was found to be of useful application in the present case. Moderately thin hand sections of the fresh infected leaf were made and fixed in acetic acid for a few minutes. After washing with water these were stained

with 5% Delafield's haematoxylin. The stain was allowed to act until the sections were of a deep brown colour and they were then rinsed in water. Later they were transferred for a few minutes to 1% potassium hydroxide, to loosen the hyphae, and mounted, either in dilute glycerine or glycerine jelly. The coverslip was tapped in order to loosen the hyphae. This method was useful since it isolated the fungus from the host tissue and the distribution of the mycelium and the exact stage of development were easily seen. It was thus of value in determining the state of material before more careful fixation.

The third method used was that of embedding in paraffin wax and the preparation of microtome sections. The most useful fixatives were found to be Allen's modification of Bouin and Medium-Chrom-Acetic. Sections were cut 8μ - 15μ in thickness. Haidenhain's iron-alum-haematoxylin, with Congo red as a counter stain as recommended by Blackman, (5), Gram's iodine gentian violet, and Flemming's triple stain gave satisfactory results.

HOMOTHALLISM.

The short cycled life history of Puccinia malvacearum, and the absence of pycnidia and aecidia suggests its homothallic nature. By means of the precise and certain method of inoculation described above it has been definitely proved that this rust is homothallic i.e. infection follows from the germination of a single sporidium and teleutospores are formed just as rapidly as they are in nature from mass inoculation. During the course of this work some thousand single sporidium cultures have been made. Of these 18 have been successful. This may seem slight evidence on which to base the assumption of homothallism, but there are two points to be remambered; Firstly the delicacy of the sporidium itself, and secondly the amount of handling to which it is subjected during the operation, But all of the precautions taken seemed essential to ensure definitely the isolation of the single sporidium. Moreover the results represent the whole of the experiments made and naturally the first were less successful than the last. In fact conditions of inoculation became so precise at the end of the work that 9 out of 20 isolated sporidia caused infection on one plant. On the shoot shown in Plate 1 Fig. everyone of the 6 sporidia put on the leaf A, germinated and produced teleutosori. The other leaves were not ~~inc~~ inoculated. The precise position of the sori on the leaf is due to the fact that these points on the main veins were always selected for inoculation. The occurrence of homothallism is further supported by the following facts. Firstly, in no case was there evidence for delayed development such as the sterile aecia described by Allen (/) in monosporidial cultures of Puccinia graminis. Secondly no more time elapsed between inoculation and the production of the teleutospores in single

sporidium cultures than in the mass inoculations. In Puccinia Helianthi Craigie (14,15) found that although heterothallism was the rule, some monosporidial cultures would become spontaneously diploid, but development took longer.

It is hoped to use this technique for the investigation of a long cycled rust, and to demonstrate heterothallism and if possible the distribution of the + and - sporidia on the promycelium. This is possible in the following way. Teleutospores are first isolated and placed on the agar film on a coverslip at the base of a moist chamber. Their germination can be watched under the microscope. When the sporidia are abstricted the path of their ejection is noted and their final position on the agar film. This coverslip film is then removed from the moist chamber and inverted over the moist chamber of the micromanipulator. The sporidia are then transferred to the leaf in different combinations, according to their position on the promycelium. In this way the distribution of the + and - sporidia on the promycelium can be demonstrated.

The significance of homothallism in the life history, will be more suitably dealt with in the discussion on the origin of the diplophase. Its relation to the phylogeny of the group may be briefly discussed here.

A study of homothallism in autoecious rust like Puccinia malvacearum suggests the question as to whether there is any relation between autoecism, and homothallism, heteroecism and heterothallism. Then follows a variation of the old vexed question, are autoecious or heteroecious forms more primitive? Blackman supports the view that heteroecism is the more primitive, and the micro and lepto forms came last in evolution by way of apogamy. Christman considers, that as the cell fusions are pushed further and further back from the teleutospore, the sporophytic generation becomes longer and

longer and so an aecidial stage has been interealated.

The idea that heteroecism is primitive was derived from the conception of the spermatia as male organs and therefore forms without spermogones should apogamous development. Since the discovery of the function of these spermatia as conidia (Craigie) the conception of autoecism as the more primitive condition is considerably strengthened.

Although it has not yet been definitely proved in all cases, from the results obtained with Puccinia graminis, and Puccinia Helianthi, it seems safe to assume that rusts with pycnidia are heterothallic, and those without are homothallic. But heterothallic forms include both autoecious and heteroecious forms, i.e. in the one genus Uromyces, and Endophyllum; forms without pycnidia are sometimes heteroecious. Clearly then no parallel can be drawn between autoecism and homothallism, and heteroecism and heterothallism. The teleutospore is the important spore in the life history of all rusts, for in it the complete fusion is effected and later the reduction division. It stands for the basidium, the common characteristic of the Basidiomycetes. It seems probable that the short cycled forms are the more primitive.

LIFE CYCLE.

Period of Development. The length of time required for the development of ripe teleutospores in Puccinia malvacearum was found to differ considerably according to the time of the year at which the inoculation was made. The first visible sign of infection is a tiny white spot or pustule on the under surface of the leaf, which is found on examination to indicate the grouping of the hyphae at the lower epidermis. The change of these spots to a yellow tint marks the change to the diplophase. The following table has been compiled from evidence gathered by fixation and observation of material at known times.

TABLE I.

PERIOD OF DEVELOPMENT IN MASS INOCULATIONS.

DATE OF INOCULATION.	FIRST APPEARANCE OF PUSTULES.	INITIATION OF THE DIPLOPHASE.	TELEUTOSPORES RIPE.	PERIOD OF OBSERVATION.	AVERAGE MAXIMUM TEMP IN THIS PERIOD	AVERAGE MINIMUM TEMP IN THIS PERIOD.	MEAN TEMP.
*Oct 28th - Nov 3rd	10 DAYS AFTER INOCULATION	13 DAYS AFTER INOC	18 DAYS AFTER INOC	Oct 28th - Nov 21st	—	—	—
Nov 30th	18 " " "	>18 " " "	———	Nov 30th - Dec 4th	50°F.	40°F.	45°F.
*Jan 24th - Feb 3rd	15 " " "	<20 " " "	22 " " "	Jan 24th - Feb 25th	65·5°F.	31·5°F.	48·5°F.
Feb 3rd	15 " " "	<20 " " "	22 " " "	Feb 3rd - Feb 24th	62·5°F.	31·5°F.	51·0°F.
*Feb 17th - 18th	16 " " "	19 " " "	22 " " "	Feb 17th - March 11th	60·5°F.	38°F.	49°F.
May 16th	8 " " "	10 " " "	12 " " "	May 16th - May 25th	74°F.	51°F.	62·5°F.

NOTE. THE DAYS MARKED WITH ASTERISKS ARE THE PERIODS OF INOCULATIONS MADE ON CONSECUTIVE DAYS AND LATER FIXED ALL ON THE SAME DAY. THUS STAGES OF DIFFERENT AGE WERE OBTAINED. THE OTHER DATES ARE THE DAYS OF INOCULATION FIXATION BEING EFFECTED LATER ON CONSECUTIVE DAYS. THIS GAVE THE SEQUENCE OF EVENTS IN THE MASS INOCULATIONS.

Similar results to these just recorded were obtained from observations of monospore inoculations, as may be seen from the second table.

TABLE II.

PERIOD OF DEVELOPMENT IN MONOSPORE INOCULATIONS.

DATE OF INOCULATION.	FIRST APPEARANCE OF PUSTULES.	INITIATION OF THE DIPLOPHASE.	TELEUTOSPORES RIPE.	PERIOD OF OBSERVATION	AVERAGE MAXIMUM TEMP.	AVERAGE MINIMUM TEMP.	MEAN TEMP.
Feb 23rd	14 DAYS AFTER INOCULATION	<20 DAYS AFTER INOC.	28 DAYS AFTER INOC.	Feb 23rd - Feb 16th	70·5°F.	38·5°F.	54.5°F.
Apl 19th	10 " " "	12 " " "	18 " " "	Apl 19th - May 7th	72° F.	44°F.	58°F.
Apl 20th.	10 " " "	12 " " "	18 " " "	Apl 20th - May 6th	73°F.	44°F.	58°F.

Clearly there is a marked variation of the rate of development with the temperature, and this again varies with seasonal change. The period of development in these inoculation experiments is shorter in the spring and autumn than in the winter. In the field, infection is found to be most severe at the former periods, and its severity may partly be explained by this quickening of the life cycle, consequent on the higher temperatures experienced in these months. In the winter and summer there is a decrease in the virulence of the disease. The decrease in the former period is considered in another section of the paper, under hibernation. The summer decrease requires a further explanation. It may be due to lack of moisture or to an increase of temperature, above the optimum temperature suitable for development. Had time allowed the inoculation experiments would have been continued indoors in order to discover whether there is such an optimum temperature for development and how far the period of development decreases with an increase of temperature.

At the beginning of the work it was thought that there might be some difference in the rate of development of

the rust on different hosts. An experiment was made to determine this. From February 15th until March 4th, a plant of Althea rosea, and a plant of Althea officinalis were each inoculated every day. These two hosts were selected, as the leaves show a marked difference of texture. The leaf of Althea rosea is thick and has a thick cuticle, whilst that of Althea officinalis is much thinner and has little or no cuticle. In spite of this considerable difference, the pustules appeared on the same day on each corresponding pair of plants. This period varied from 16 days at the beginning of the experiment, to 13 days at the end. Further development also took place at the same time in each case. Observation of the behaviour of the fungus on other hosts supports this conclusion.

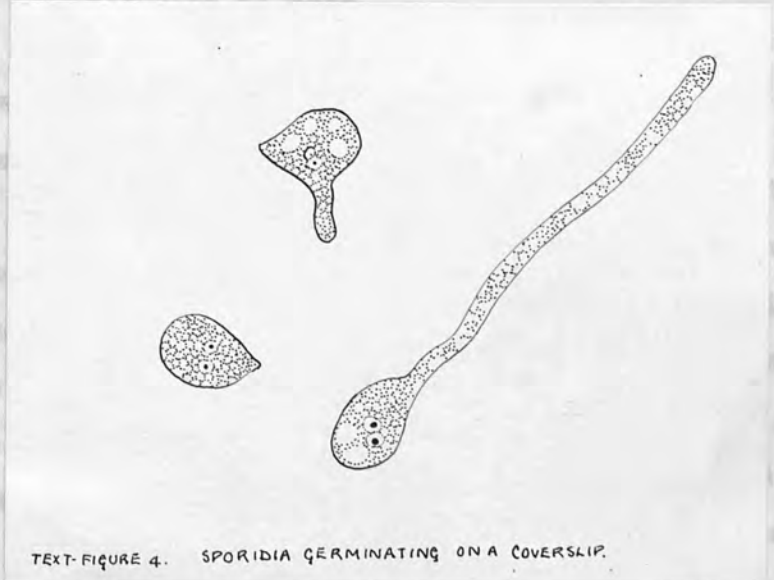
From these considerations it is apparent that the rate of development of Puccinia malvacearum, is independent of the host, but varies appreciably with the temperature.

Entrance of the mycelium and its further development in haplophase.

An examination of a one day old inoculation, prepared as described above (page 6), shows sporidia scattered on the surface of the epidermis. Some of these have just started to germinate by putting out a beak-like protuberance from the side as figured (~~no.~~ TEXT FIGURE 4), or from the hilum. No record has been obtained of more than one germ tube emerging from a single sporidium although in Puccinia graminis Waterhouse (32) reports the presence of two or even three germ tubes. Under abnormal conditions the germ tube of the sporidia of Puccinia malvacearum may branch irregularly. Robinson's (29) found this to occur when pieces of Primula or Geranium leaf were placed near the sporidia. The age of the sporidia in a one day old inoculation varies from four to twenty four hours, and those which were abstricted first are seen to have effected a successful

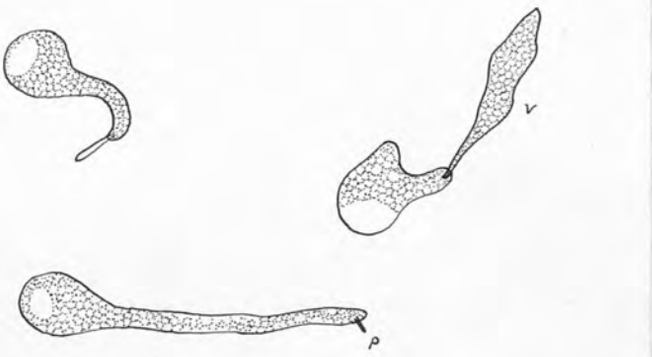
entry,whilst the younger ones have not started to germinate.

It should perhaps be stated here, that sporidia caught on a dry coverslip enclosing a moist chamber germinate rapidly. Robinson (29) investigated the germination of these sporidia, and found that the germ tubes always grew out of a water drop into the moist atmosphere around. They were also found to be negatively phototropic, and although fragments of Hollyhock or Mallow leaf excited no chemotropism the presence of Geranium or Primula leaves inhibited germination. When grown in a coverslip culture in a moist atmosphere development only proceeds for a short time. The sporidium is at first uninucleate, but a nuclear division may occur immediately it is formed so that even before abstriction the sporidium is binucleate. (Text fig. 4) On germination a fairly thin hypha is first put out. The contents of the sporidium slowly pass into this tube. If the germ tube is formed from the hilum, the opposite end of the spore becomes empty but the nuclei remain almost at the surface of the retreating protoplasm and do not pass into the tube. If germination is lateral, two large vacuoles are formed and the nuclei remain in the central band of cytoplasm. This can be seen by reference to the accompanying text figure. (no.4)



TEXT-FIGURE 4. SPORIDIA GERMINATING ON A COVERSLIP.

The events just described are the more usual. In some coverslip cultures however, development of a fine penetration hypha occurs. (text fig. 5) Just at the tip of the germ tube a very fine hypha (p) can be seen coming out at right angles.



TEXT FIGURE 5. SPORIDIA PUTTING OUT INFECTION VESICLES.

In relatively fewer cases still, this penetration hypha swells out again into an infection vesicle (v) similar to that formed within the epidermis of the host plant. It was impossible to determine what were the particular conditions which controlled this further development. The penetration hypha always is put out on the side in contact with the coverslip so that there may be a contact stimulus at work

Infection of the epidermis of the host plant proceeds as follows. The beak-like protuberance grows a little in length and from the tip of this a fine penetration hypha is put out, just as has been described in the coverslip cultures. The penetration hypha pierces the epidermal wall and slowly works itself in. Once the cell is entered the contents of the sporidium pass through this fine penetration hypha, to form an infection vesicle. In preparations stained with cotton blue, this perforation can easily be seen, (plate II. fig. 2 and 3)

Eriksson (19) describes the entry of the germ tube of the sporidium into the epidermal cell thus: "wahrscheinlich infolge einer auflosinden Einwirkung des Korpers selbst auf die Epidermiswand bilder sich an den Kontaktpunktchen einer sihr keines kaum sichtbares Loch durch das sich dere Inhalt des Korpers hineingiesst".

Describing the entry of the germ tube of the sporidium in Puccinia graminis Allen (1) reports a thickening of the cell wall of the host at the point of perforation but this has not been seen to occur in infection by Puccinia malvacearum.

A later stage of infection is seen in plate II. figure 1. This is still within the first twenty four hours after inoculation. Entry has been effected through the narrow neck (a), the infection vesicle is just forming, and the contents are passing in. The sporidium is lying on the surface of the epidermis, but the vesicle is inside the cell, and is not clearly defined at this focus. By making a slight adjustment to the microscope this is soon brought into focus. The host nucleus, is more centrally placed in the cell, and so is at a still different focus. The position is indicated in the drawing. Not all the sporidia which germinate gain entry as is evidenced by the presence of these early stages in older infections.

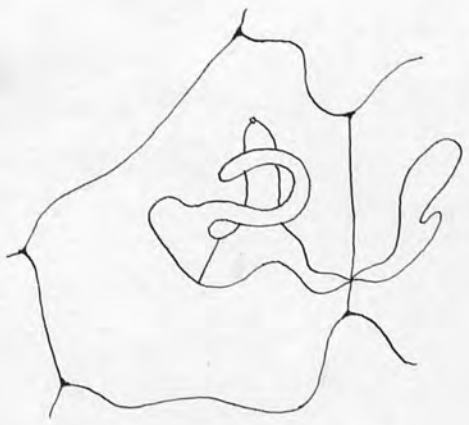
Infection never occurs through a stoma although one may be near at hand. Sometimes a considerable depth of cell wall is penetrated before entry into the cell is effected as shown in plate II. figure 2. Infection through the lower epidermis is rare.

<u>The primary infection hypha.</u> Once the penetration tube has entered the cell the tip swells to form what will now be termed the primary infection hypha. Two early stages in the development of this are seen in plate II. figure 3. The hypha is at

first binucleate, but most frequently a division wall is formed a short distance from the tip dividing the swollen hypha into two uninucleate cells. The development of this wall must take place without nuclear division. At this stage in Puccinia graminis Allen reports the frequent presence of two nuclei in the cell nearer the point of entry. This suggests the division of one of the two nuclei of the aseptate vesicle and the formation of a wall, between the daughter nuclei. It was thought probable that this was the case in Puccinia malvacearum, but there is no evidence for this.

The primary infection hypha grows in length, frequently in the direction of the nucleus of the host cell. The full significance of this if any, is difficult to interpret, except that the nucleus is the centre of the most active metabolism of the cell and as such offers the best nutrition to the invading hypha. (Plate II. figure 5.)

The individual cells of the primary infection vesicle have well marked nuclei, but as the cells become older, the protoplasm becomes more vacuolate. The cells are quite characteristic in form for they are much wider than those of the mycelium to which they give rise. The primary infection hypha most commonly becomes only three or four cells long. It may attain a length of five or six cells, ~~but~~ and does not always remain confined to the one epidermal cell (Text fig. 6)



TEXT FIGURE 6. PRIMARY INFECTION HYPHA INVADING A NEIGHBOURING CELL.

This same figure shows how the infection of a neighbouring cell has been accomplished. The hypha in the cell to the right is still broad and irregular and appears to be a continuation of the primary infection hypha, with all its characteristics. It cannot be confused with the haustoria which are later found in the neighbouring cells of the epidermis. There is quite as marked a difference in the rate of development of the primary infection hypha as in the complete development of the pustule. In May this 3 celled stage was achieved in twenty four hours. after inoculation, whilst in the winter months it was not until two or three days after inoculation that this stage was reached.

After this, development takes place rapidly, and within the next few days marked progress is made in the invasion. The cells of the primary infection vesicle are seen to be sending out finer branches, the beginnings of which can be seen in plate II. figures 4 and 7, 8. Generally the first cell remains unbranched, but the younger ones branch freely. These branches grow in all directions. At first they are confined to the epidermal cells immediately surrounding the point of the primary infection. They pierce the walls of the host cells through fine perforations always becoming constricted there, but swelling out on the far side. This vegetative mycelium becomes both intercellular and intracellular. As infection usually occurs on the upper epidermis of the leaf the palisade parenchyma with its few intercellular spaces allows very little development of this type of mycelium. The hyphae generally enter the palisade cells, form haustoria and then pass on. Once free of this compact tissue the mycelium becomes chiefly intercellular, ramifying in the mesophyll, and putting out large haustoria into the cells of all tissues. Each pustule

always develops in relation to one or more vascular strands, and haustoria are formed abundantly in the phloem region. Although entry to the vessels is sometimes achieved, little further development occurs in these. Robinson (28) reported a marked diminution in starch in the regions invaded by the fungus. A good deal of confusion has existed in regard to the haustoria of Puccinia malvacearum. Taubenhaus (31) reports them to be infrequent but other workers, Sappin Trouffy (30) Eriksson (19) and Robinson (28) found them to be of universal occurrence, as found in the present study. They are most variable in form and are well described by Sappin Trouffy: "Les suçoirs sont très variables. Ils peuvant être fourchus, spiralés, rameux, utriculaires ou même pelotonnés à l'intérieur des cellules hospitaliers". They can easily be seen in the fresh condition but are especially well shown in material stained with gentian violet. Each haustorium is uninucleate and commonly becomes associated with the nucleus of the host cell, in the same way as does the primary infection hypha. By means of plasmolysis experiments, Robinson (28) has shown that the cells of the host plant remain alive for a considerable time after the entry of the haustoria although various changes consequent on the entry of the haustoria can be demonstrated: the chloroplasts become aggregated round the nucleus, lose their colour and disintegrate; and the nucleus moves from a peripheral position, towards the centre of the cell increasing in size and showing a diminution in chromatin content.

Thus at this time the infection consists of an intercellular vegetative mycelium composed of long uninucleate segments traversing the spaces of the surrounding tissue, and an intracellular growth, with well developed haustoria, tapping the food supply of the host tissues. Meanwhile the primary infection hypha is dead and appears in the epidermal cell discoloured with the yellow products of disintegration (plate [illegible])

The point of entry of the infection can be seen in the epidermal cell (c). From this point, hyphae are seen to ramify in the palisade cells immediately below the primary infection, and to radiate out through all the leaf tissues. Typical and well marked haustoria can be seen at the extremities of the intercellular hyphae. Although the hyphae are septate, septation is often difficult to see in microtome sections, for the cells of the mycelium are very long, and the interlacing makes it difficult to see a good length of the mycelium at once. The cells are uninucleate at this stage, the nuclei possessing well marked nucleoli. The mode of division of these vegetative nuclei is characteristic of the rusts, the chromatin (~~is not divided into chromosomes, but is~~ appears to be drawn apart as undifferentiated masses, between which a kinoplasmic thread represents the spindle.

A little later stage than the one figured shows the mycelium to have invaded the lower epidermis and formed haustoria in these cells. As a response to this check in invasion a second development seems to take place. (plate III fig a) The intercellular mycelium begins to branch repeatedly, the branches grow in the direction of the centre of the pustule, i.e. in a reversed direction to their previous development. Each of these branches assumes a broader form than the earlier branches of the vegetative mycelium, and becomes club-shaped. A single nucleus is seen at the tip of each, this appears larger than the nuclei of the vegetative mycelium. This massing of the mycelium takes place with great rapidity. In fact material examined one day, in which the mycelium was seen to be invading the lower epidermis, was shown to be in this later condition by the next morning. The pustules still appeared white to the naked eye.

Development in diplophase.

The change in colour of the pustule from the white tint of its first appearance to a pale yellow tint, marks the initiation of the diplophase. The colour becomes deeper and deeper in shade until finally the epidermis is ruptured, and the rusty brown teleutospores are exposed.

Unfortunately the cytological details of the change from haplophase to diplophase, are not at all easily observed, and although attempts to discover how the diplophase is initiated have been made, the problem still remains unsolved.

The discovery of nuclear migration at the base of the aecidium of Phragmidium violaceum, Puccinia poarum, and Uromyces poae led Blackman and Fraser to investigate the mode of origin of the diplophase in the short cycle rusts, and Puccinia malvacearum was one species they chose for their study. They obtained no definite evidence of migration although young teleutospores were binucleate and indeed frequently trinucleate. They had also found trinucleate teleutospores in Puccinia poarum where they had demonstrated nuclear migration at the base of the aecidium. From this similarity they concluded that the method by which the binucleate condition is achieved is similar in Puccinia malvacearum, but takes place at the base of the teleutosorus. In 1906 they reported as follows "very careful search failed to reveal the exact method by which the transition from the single to the conjugate nuclear state is brought about. The smallness of the cells and nuclei and the absence of any regular group of cells - such as are found in the aecidia- on which attention may be focussed renders the task of aleucidating such a point almost hopeless". (6)

In 1912 Werth and Ludwig (33) attempted to solve this problem, and claimed to have established nuclear

migration.at the base of the teleutosorus as Blackman and Fraser had described for other rusts at the base of the aecidium They reported cell fusions between the tips of club-shaped hyphae. The fusing cells were usually of unequal size, and the nucleus of the smaller passed over into the larger. The satisfaction of this explanation is spoilt by the further report that binucleate cells were occasionally found in the vegetative tissue. This occurrence they were unable to explain.

In 1912 Moreau (25) found the origin of the diplophase to be effected, not by nuclear migration, but by dissolution of the common wall between two contiguous cells. "Les cellules proches de l'epiderme s'allongent, se meltant bientôt par paires et se fusionnent La cloison qui separe deux cellules se dissout à la partie superieur. Il en resulte une cellule binuclée". This is the method described by Christman for Phragmidium speciosum, Caeoma nitens and Uromyces Caladii, and by Blackman and Fraser for Melampsora Rostrupi.

In 1927 Miss Allen (1) in a cytological study of the long cycled rust Puccinia graminis reported yet another mode of origin of the diplophase. She found that after pycniospores are transferred to an infection "the number of nuclei in the sporophytic cells in this region increases, apparently by nuclear divisions. Cells with three to six nuclei are common. On the forth day after fertilisation these multinucleate cells push down into the sterile space making tissue of the aecium to produce spore chains; just before division a multinucleate cell contains 8 or 10 nuclei. These extra nuclei are utilised in forming the bi-nucleate spore-mother cells so that the number in the basal cell steadily decreases. In well established spore chains basal cells are regularly binucleate".

Writing in 1929 (32) Hanna reported nuclear association in the aecidium of this same fungus. In a monosporodial

pustule haploid rudiments of aecidia are formed. When nectar, containing pycniospores of one sex is applied to a monosporidial culture, the nuclei in this weft of hyphae become enlarged, and neighbouring hyphae fuse in pairs as described by Christman for Phragmidium speciosum. The two nuclei become associated and this fusion cell initiates the diplophase. Hanna suggests that when pycniospores of one sex are applied to a pycnidium of opposite sex they are stimulated to germinate and to produce haploid hyphae which pass down and fuse with the hyphal wefts at the lower epidermis.

In the same year Walker (34) again turned attention to the mode of origin of the diplophase in the short cycled rusts. She found that not only may the diplophase originate in a variety of ways in different rusts, but may even be effected by different methods in the same rust. In Puccinia asteris she found no cell fusions and concluded that the diplophase must have come about by simple mitotic division without wall formation. In Puccinia cryptotaeniae, this was also the most frequent method, but nuclear migration also occurs. In Puccinia Xanthi the absorption of the walls of two contiguous uninucleate cells initiates the binucleate condition, whilst in Puccinia fusta, there is cell fusion, and also nuclear division not followed by cell division.

It seems then that one must assume that any method may be followed by Puccinia malvacearum, nevertheless a genuine attempt has been made to collect further evidence for the mode of origin of the diplophase in this species and in the process of collecting this evidence one has been unavoidably impressed by the possibility of different interpretations of the same phenomena.

Taking first the evidence of possible nuclear migration. Several instances of this have been seen but only

one case of a nucleus apparently migrating into a second uninucleate cell. This is figured in plate IV fig. 1. The nucleus in the lower cell is passing through the wall. At a lower focus the nucleus of the upper cell is seen. A convincing case of nuclear migration is figured in plate IV fig. 2b. Judging by the clear space round the nucleus in the upper cell migration is taking place from the upper to the lower one but the nucleus of the lower cell could not be found. Plate IV fig. 2a shows a similar case. At one focus the nucleus appears as two chromatin masses in separate cells. At a lower focus, these two masses coalesce, and appear to pass through a transverse wall.

No case of initiation of the diplophase by the fusion of two contiguous cells has been seen.

In some sori multinucleate cells are of common occurrence (plate IV fig. 3). This reminds one of the state of affairs described by Allen (1) as occurring in Puccinia graminis. It has also been described by Olive (26) and other workers, and is responsible for the suggestion made long ago that there was an ascogonium at the base of the aecidium, (or teleutosorus in the case of micro-forms.)

Evidence for the origin of the diplophase by nuclear division not accompanied by wall formation is seen in plate IV fig. 4 . Here the terminal cell of the club-shaped hypha is binucleate, and the second cell uninucleate. It was not possible to find another nucleus belonging to the second cell in the neighbouring sections. It appears then that the terminal cell has become binucleate simply by nuclear division. Again, there is no nucleus in the hypha in contact, which seems to suggest that its nucleus has passed into the upper cell, and the diplophase thus initiated by migration, ~~On examination of the next serial section however, two nuclei are seen to be~~ So that two explanations of the case seem possible.

~~present so that the former explanation of the case seems more probable.~~ Other points in favour of this mode of initiation of the diplophase are firstly, its spontaneous appearance: one day the hyphae are all uninucleate, the next bi-nucleate. Secondly, the nuclei of the club-shaped hyphae frequently appear larger than those of the vegetative mycelium as though preparing for division.

At the International Botanical Congress, 1930, Professor Buller reported his discovery of a new phenomenon of nuclear migration in heterothallic Coprini which he terms 'diploidisation' and he emphasised the part played by oidia in bringing about the diplophase in Hymenomycetes. Further contributions on this subject have been made by him in a letter to Nature (11) and in a paper read to the British Mycological Society in October 1930 and by his student Brodie in the April number of the Annals of Botany 1931. The results are also reported in some detail in his fourth volume of the "Researches on Fungi" (10). They are mentioned here because of his application of them to other branches of the Basidiomycetes, notably the rust fungi. For the origin of the diplophase in the heterothallic Uredineae, he suggests the following two hypotheses.

Hypothesis No. 1. "The two haploid mycelia (A) and (a) as their growth proceeds, intermingle so that, eventually, two kinds of hyphae, (A) and (a) come to be arranged almost alternately in the spore bed of the young aecidium. Then in the spore-bed, pairs of (A) and (a) cells combine to form conjugate nuclei, either as in Phragmidium violaceum, or Puccinia poarum by a cell wall becoming perforated to allow of an (A) nucleus migrating into an (a) cell or vice versa, or as in a number of other rust species, by the wall between an (A) and an (a) cell breaking down so that the (A) and (a) cells combine to form a single cell. In support of Hypothesis No. 1 , one may cite the illustrations of two cells in a spore-bed uniting laterally to form a single binucleate basal cell, as given amongst others by Christman for Phragmidium speciosum.

Hypothesis No. 2. "The two haploid mycelia (A) and (a) on coming into contact do not intermingle appreciably, but each remains in the leaf territory which it has grown through and more or less exhausted. Hyphal fusions take

place between the two mycelia, with the result that one or more (A) nuclei move into the (a) mycelium and one or more (a) nuclei move into the (A) mycelium. The (A) nuclei move along the hyphae of the (a) mycelium, and the (a) nuclei move along the hyphae of the (A) mycelium, and the moving nuclei undergo division and make their way to the places where the rudiments of the aecidia are being formed; so that, in the hyphae which form the aecidial rudiments, both (A) and (a) nuclei are present. These hypha now form a series of uninucleate cells, some of which contain an (A) nucleus, and some an (a) nucleus. Then the co-operation of (A) and (a) cells by means of nuclear migration or cell fusion sets in, and this leads to the formation in each aecidium, of a spore-bed in which the basal cells contain the conjugate nuclei (A) (a)".

With regard to homothallic forms he offers no such hypothesis, but since he draws a parallel between the part played by the oidia of the heterothallic Coprini and the pycnidiospores of the heterothallic rusts, events in the homothallic rusts may be considered comparable to those in the homothallic Hymenomycetes. Here the diplophase arises spontaneously, independently of hyphal fusions, whilst in heterothallic forms hyphal fusions must occur before the binucleate condition is attained. Thus the difference in nuclear constitution is accompanied by a difference in the mode of origin of the diplophase. Are we therefore at liberty to assume a simpler mode of origin in homothallic rusts from the more complicated nuclear migration or cell fusion in the heterothallic forms and so give support to the theory of the initiation of the diplophase by nuclear division unaccompanied by wall formation.

On the other hand, the cases of nuclear migration must be considered. Their value here is difficult to assess for they appear to occur infrequently. In homothallic Hymenomycetes, hyphal fusions may occur, and indeed they increase in number long after the whole mycelium has become diploid, thus proving the occurrence of spontaneity in the origin of the diplophase and yet the possibility of hyphal fusions.

For the solution of the problem of the mode of origin of the diplophase, in both long and short cycled rusts, more evidence is needed, and evidence drawn from many species. Until this is available, the problem of the initiation of the diplophase in short cycled rusts at least, cannot be definitely solved. At present one of the weakest spots in its solution is the possibility of a variety of methods. This is probably due to the difficulties of observation, and the different interpretations possible.

Development of the teleutospores. The further development of the binucleate cells was not specially followed. Werth and Ludwig (33) report that the bi nucleate cell first divided to form a stalk cell, and an upper cell. This upper cell divides again to form the two celled teleutospore. No deviation from this development was apparent. Whilst the cells of the spore are still binucleate a thick yelowish brown wall is deposited. Later the two nuclei in each cell fuse (30).

The ripe teleutospore. At maturity the teleutospore consists of a two celled spore, supported by a fairly long stalk, the wall of which is slightly thickened, but not so markedly as that of the spore. One germ ~~tube~~ pore is present in each cell. In the upper cell this is terminally placed and consists of a break in the wall just enclosed by a thinner cap. In favourable cases, this cap can be seen pushed aside on germination. The germ pore of the lower cell is laterally placed just to one side of the cross septum dividing the two cells. The teleutospores are now ready for germination whenever favourable conditions intervene.

The return to the haplophase.

The teleutospores are ready for germination about three weeks after the inoculation has been made for they do not require a period of rest as do the teleutospores of the majority of the rusts. Dame Helen Gwynne Vaughan considers the basidium of the rust fungi to comprise two phases, a thick walled protected phase — the teleutospore cell, germinating to give a thin walled phase — the promycelium. It is in this later thin walled stage that there is a return to the haplophase. Before this is described however the conditions necessary for germination must first be recorded.

The conditions for germination. In practice it was found that the only condition that need be supplied for germination was moisture. This ~~was~~ is the experience of horticulturists who find the disease is most prevalent in damp rainy seasons. In the field, germination chiefly occurs after a shower of rain or at night when the dew is falling. The latter must be the most valuable period, for Robinson in his experiments with sporidia found that the germ tubes were directed away from the light. This is probably one reason why the corrugated cardboard was so useful in the inoculation experiments, since it ensured a dark inoculation chamber.

Teleutosori kept dry in the greenhouse at 55 F. or even lower would remain ungerminated for weeks or even months. A very pretty instance of this and of the immediate germination as soon as moisture should be supplied, was given by a badly infected plant. It had been kept in the greenhouse for some weeks and no germination occurred. One day however during watering, (always directly on to the soil), the edge of the pot accidentally received a good deal of water. One of the infected leaves was overhanging this. About three hours afterwards a line of sori had germinated corresponding to the arc of mois-

ture given by the damp pot, whilst all the others further from the edge of the pot still remained ungerminated.

A high temperature is not necessary for germination. Teleutospores germinated far more successfully on the bench at 15° C. than in the incubator at 22° C. Indeed germination was found to occur out of doors in winter during suitable moist periods.

Germination of teleutospores. Eriksson (18) recognised two biological forms of teleutospores according to their mode of germination. One is the autumn spore formed from August to October on young plants of the first year, and old plants of the second year. These might germinate in either of the two ways, according to the presence of a water film or a saturated atmosphere. The other is the summer spore formed on hibernated plants of the second year, which germinate by end conidia (oidia) from May to July. In the present study conidia have been formed only in relation to a water film, not to differing teleutospores.

Formation of sporidia. A most excellent account of the germination of teleutospores to form sporidia is given by Buller in his "Researches on Fungi" vol. III. The process can be very conveniently observed by watching the germination of the spores in a vertical section of a leaf taken through a teleutosorus and mounted in a moist chamber on a small block of plain agar or gelatine. The wonderful precision and regularity of the process defies description. Under moist conditions such as those supplied in the inoculation experiments the contents of each cell of the teleutospore, enclosed in the endospore, emerge as a straight stout hypha, the promycelium or thin walled stage of the basidium. Later the promycelium becomes curved. Meanwhile according to the work of Sappen Trouffy (30) the fusion nucleus of the basidium has been

entering on meiosis, and as a result of this 4 haploid nuclei are formed. These become separated by three septa to form four uninucleate cells. On the convex side of the promycelium each of the cells puts out a tiny projection which grows to form a sterigma. Buller (9) shows how this curving of the basidium and formation of the spores on the upper surface leads to a more effective method of dispersal. At the tip of each sterigma a sporidium begins to form as a tiny globose swelling. As it enlarges the nucleus and most of the cytoplasm of the promycelial cell pass through, but a small portion of the protplasm remains. At first the sporidium appears globose, but as it enlarges it becomes constricted at the point of attachment by the hilum. Since germination of the teleutospores takes place in situ there is always a great crowd of sporidia to be successfully expelled. The method of expulsion is that characteristic of the whole of the Basidiomycetes. When the sporidia are fully formed, a drop of moisture is secreted from the hilum. This drop increases in size until it is almost the size of the sporidium. Suddenly the sporidium is ejected forcibly from the sterigma, together with the drop of water, a considerable distance from the leaf. Once free of the leaf surface the sporidia are borne away by air currents. Although they are all capable of germination in culture, in nature a large percentage of them must be sacrificied to incipient drying and other damage. The tiny drop of liquid with which each is expelled seems to be mucilaginous in nature, and stains easily with gentian violet. This probably helps the sporidium to adhere to the leaf on which it falls. Although the sporidia are uninucleate when formed, the nucleus may undergo division as soon as ejection occurs, so that sporidia often appear binucleate, Plate IX fig 6. Sappan Trouffy (30) reports the presence of two nuclei whilst the sporidia ~~is~~ are still on the sterigma.

Development by conidia or oidia. If the condition for germination is such that a water film is present, a series of events is seen similar to that described by Blackman (3) for Phragmidium, Gymnosporangium and Uromyces. The promycelium grows straight and continues to grow until it reaches the surface of the water film. This type of promycelium is generally much thinner than the other. The contents collect at the end, nuclear division takes place and septa are formed as before, but the cells so formed instead of putting out a sterigma and expelling sporidia become detached from one another as broadly elliptical bodies, termed end-conidia or oidia.

HIBERNATION.

The teleutospores of the rust fungi are often termed "winter spores" because although they may be formed as early as August, yet they remain dormant until the following Spring and so ensure the survival of the rust over the winter. The teleutospores of Puccinia malvacearum however differ, in that, provided conditions are favourable they are always capable of germination immediately they are formed. The problem of overwintering then is evidently solved in a different way. There are only the two kinds of spore in the life history viz. teleutospores and sporidia, and the latter are essentially delicate and unsuited to survive desiccation for more than a few minutes. The question of hibernation thus must rest with either the teleutospore or the mycelium.

There has been a good deal of interest shown in the hibernation of this rust and as a result, several methods have been suggested but since the evidence brought forward in their support is often contradictory clearly the whole story has not been told. Moreover as already suggested above the fungus may behave differently under different climatic conditions, and therefore the sequence of events in America and Sweden may be different from those in England where it has not so far been consistently observed. During the present study the behaviour of the fungus has been watched from day to day from March 1930 - June 1931, in an attempt to establish some definite facts concerning the problem of overwintering.

DIARY OF OBSERVATIONS.

At the end of March 1930, the Mallows and Hollyhocks in the Malvaceae bed of the R.H.C. Botany garden were found to be quite badly infected with the Hollyhock rust, Puccinia malvacearum. From the fresh appearance of the infected shoots it seemed probable that the teleutosori were the result of an early Spring infection. The germination of the teleutospores

was tested and was found to occur in either of the two ways described above according as to the moisture conditions present: in a saturated atmosphere by sporodia, in a water film by conidia. This was well illustrated by the following experiment. Fairly thin hand sections through a ripe pustule were placed on the surface of a plain agar plate in a Petri dish. The surface of the section in contact with the agar lay in a water film whilst the upper surface was in a damp atmosphere. The teleutospores in the water film germinated to give straight promycelia and conidia, whilst those on the upper surface, formed curved promycelia and abstricted sporidia.

During the months of April, May and June teleutospores were germinated practically every day, and no difference could be seen in the germination of the teleutospores to give sporidia or conidia.

In the long vacation two attempts were made to establish diseased plants in the writer's own garden near Manchester, but both attempts were unsuccessful, in that the plants first shed the leaves already formed, and then formed new uninfected leaves, and recovered from the disease. The weather at the time of planting was hot, but damp weather followed within 3 or 4 days. In this period the pustules germinated. As the leaves were withering however, there was no suitable tissue left for infection by the sporidia so formed and consequently the fungus was unable to establish itself.

Early in October the observations were continued at Royal Holloway College. These are of the greatest importance since they record the behaviour of the fungus during the winter months. In the early autumn infection was heavier than during the summer. At this time plants were being inoculated in the greenhouse by means of diseased leaves brought from

plants in the garden. Subsequent examination of the leaf surfaces of the plant under inoculation showed dense scatterings of sporidia. Together with these experiments, two sori ~~every day,~~ every day were germinated in coverslip cultures when films of sporidia were obtained as described above.

New infection was still occurring in the bed as evidenced by the presence of young sori. That this infection was caused by sporidia was shown in the following way. Two plants of Althea rosea were taken from the greenhouse, and placed overnight amongst the diseased plants in the garden. Next morning they were removed, labelled and taken into the greenhouse. For two days they were enclosed in bell jars to provide the requisite moist atmosphere. After twelve or thirteen days young sori appeared on the leaves and matured within three weeks. Considerable new infection was found to occur in the bed until January 16th in the following year. The infection by this time however was lighter than in the autumn months. After this only occasional new pustules were seen.

Germination still occurred when sori were brought indoors from the garden. In fact on some days although the leaves were stiff with frost when gathered, germination of the teleutospores occurred within five hours. On January 22nd four particular leaves on which pustules were just reaching maturity were labelled to discover their subsequent fate. The next day one of the pustules was removed and when brought indoors the teleutospores germinated showing that they were ripe. Those left in the field did not germinate. By the beginning of February however the pustules no longer appeared rusty brown, but were a greyish white indicating that they had given rise to promycelia. When examined with the microscope these promycelia were seen to have abstricted sporidia.

A characteristic of the disease at this time was the heavy infection of the petioles. Bearing in mind the suggestion of other workers of the presence of a perennating mycelium, it was thought probable that this petiolar infection was the result of such a mycelium in the shoot tips and rudimentary leaves, renewing growth and passing upwards to the leaves. Fixation and examination of the shoot tips however gave no grounds for this assumption. In fact although in one case a ripe pustule was found on one of the bud scales the infection was seen to be quite local, and the mycelium did not approach the growing point. Badly infected seedlings were ~~forced~~ fixed in October in an attempt to find the perennating mycelium, as it was thought that at this time the mycelium might be passing down to the shoot apex there to remain dormant for the winter. On examination no mycelium was seen.

The plants in the field were most severely affected by a high east wind on March 6th and 7th and as a result, most of the leaves wilted and decayed in the wet weather that followed. It was still possible however to find pustules for germination tests and ~~this was done~~ these were made throughout March and April. On April 18th only three pustules were visible on some twenty or thirty plants. During the next few weeks occasional sori appeared, and by May 18th, there was quite a considerable infection. The petioles and main veins of the leaves seemed most heavily infected and shoot tips were again fixed and examined for perennating mycelium. No trace of such a mycelium was found.

The history of the fungus in the field throughout the year has now been followed but before considering the evidence derived therefrom it would be well to add certain observations made on the inoculated plants in the greenhouse as they throw considerable light on the factors determining

teleutospore germination.

In October a set of these greenhouse grown plants, as soon as the teleutospores had reached maturity, were removed to another greenhouse where they could be kept at a temperature of 60° - 65° F. indefinitely. They were watered carefully without wetting the leaves. Under these conditions the teleutospores did not germinate but remained viable. Tests were made at intervals from October to June and it was found that immediately moisture was supplied to the teleutospores they germinated.

One fact became increasingly evident, that the time required for germination varies with the age of the teleutospore. This germination period can be conveniently

TABLE III.

AGE OF SPORE	TIME OF EXPERIMENT IN HOURS.					
	1 HOUR.	2 HOURS	3 HOURS.	7 HOURS.	21 HOURS.	27 HOURS.
20 DAYS	—	—	—			
22 DAYS	—					
24 DAYS						
27 DAYS.						
33 DAYS.						
37 DAYS.						
49 DAYS						
54 DAYS.	—					
4 MONTHS.	—	—	—			
5 MONTHS.	—	—	—	—		
6 MONTHS.	—	—	—	—		

divided into two parts, first the time required for the promycelium to emerge and second, the time required for meiosis and the abstriction of sporidia. This latter period was the same in all cases, it was the length of the first period that altered. Teleutospores of all ages were germinated and the period of germination noted. The results of one of these experiments can be seen from the accompanying table, (III).

The following explanation may be tentatively put forward. It is well established that the stimulus for germination is the presence of moisture. It is possible that this enters the teleutospore wall by differential imbibition which causes swelling of the wall and sets up a pressure in the cell. As a result of this the thin caplike membrane over the germ-pore yields, and the contents of the teleutospore emerge as the promycelium, enclosed in the extending endospore. The time taken for this imbibition will differ accordingly to the age of the teleutospore. If lately matured the spore will possess a higher water content than one which has undergone desiccation for some months. Consequently the first period: the period required for the absorption of water, will be longer in the older spore and the time needed to complete abstriction of sporidia consequently longer too.

From these considerations the following facts emerge:

1. that the summer and autumn spores do not differ biologically,
2. that germination depends on the correct moisture conditions,
3. that the life cycle is retarded by the less frequent occurrence of conditions favourable for germination,
4. that new infections occur during the winter so that all the pustules present in spring are not the result of a late autumn infection,
5. that the teleutospores remain viable for months,
6. that a perennating mycelium has not been demonstrated.

DISCUSSION OF HIBERNATION.

In the case of a rust like Phragmidium violaceum the

teleutospores resist all efforts to encourage germination during the winter. Apparently they need a long period of time in order to attain maturity, and cannot germinate until the following spring. But for Puccinia malvacearum the winter conditions do not occasion a period of obligate dormancy except by drought. For as soon as moisture is supplied this assumed dormancy is interrupted by germination.

Plowright (27) and Taubenhaus (31) accept this overwintering of teleutospores as a method of hibernation for Puccinia malvacearum. Taubenhaus kept infected leaves which he had collected at Cornell University in November, both indoors at a low temperature, and out of doors, and by testing spores taken from them at intervals from December to April he found that they still remained viable though more and more slowly as time went on. The important difference between this result and that of the present study is that, in the latter, leaves have been gathered from the plant daily and not kept stored, and also the germination of the spores has been watched in the garden.

Dandeno (16) however thought this overwintering of teleutospores improbable. He writes as follows. "The idea of teleutospores wintering over had to be abandoned. A large amount of dead leaves was strewn the following spring among certain patches of mallows which were to all appearances free from rust. The infected litter did not seem to produce any effect whatever." Dandeno is the only other worker who notes the development of sori during the winter. He reports that plants growing in Ithaca N.Y. became infected late in November, and by December white spots had appeared on the leaves, which spots appeared yellow during February and finally matured in April. There has been no such extensive retardation of development observed in the present case, for the slowing down of the life cycle seems dependent on the longer period which intervenes

Before the appearance of favourable conditions.

Plowright[27] suggested that Puccinia malvacearum passed the winter as pustules fallen to the ground,* and Taubenhaus[31] asserted there was no evidence for this. Results of the present work are in agreement with this conclusion too. Healthy plants potted in soil from the diseased bed showed no signs of infection. It seems probable that infection would be more easily caused by whole leaves falling to the ground rather than sihgle pustules.

The presence of a wintering mycelium in young leaf rudiments is considered by Grove "to be extremely likely although one can say it has hardly been proved". Taubenhaus and Dandeno in America report its occurrence. Eriksson in Sweden has found no evidence for this. Young shoot tips of Althea rosea, and Malva borealis have been fixed and stained, and in no case has the presence of a wintering mycelium been indicated. Even in shoots in which the bud-scales were infected the mycelium was found to be quite local there.

That there is no such resting mycelium present in the summer, was well illustrated in the case of the heavily infected plants which were sent by post to Manchester. On arrival there, the plants were planted immediately, and watered at the soil level. During the next few days the leaves withered and fell to the ground. New growth started from axillary buds and the plants grew into most healthy specimens quite free from disease and the fact that they never became re-infected provides further evidence for the non transference of the disease by sori fallen to the ground.

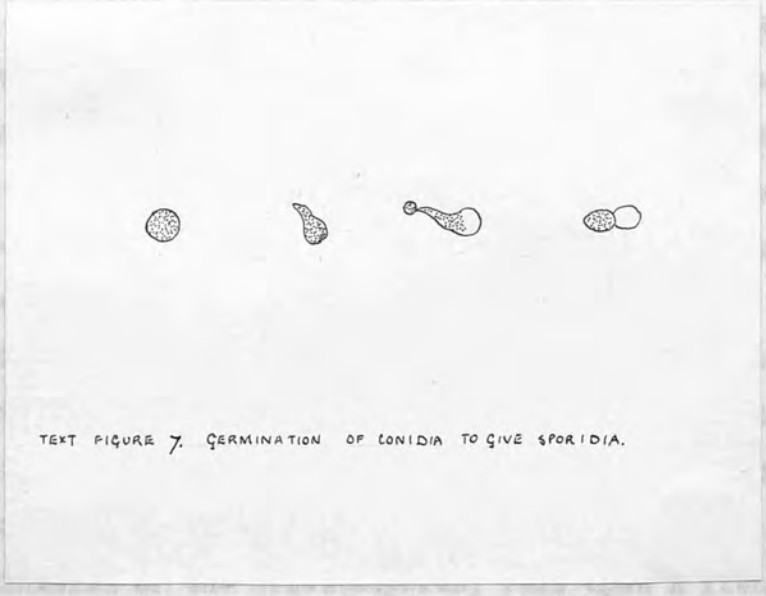
As reported above the fixation of shoot tips has been effected in October, February and ~~June~~ May, and in no case was there any evidence for the presence of mycelium. The material was fixed at this period because it was felt that in the early autumn, preparations might be occurring for overwintering, and

* Foot note
See Biological Note, page 47.

the mycelium might be passing down to the shoot apex. In February it was expected to have reached the tips, whilst in May it was felt that the re-appearance of the disease in quantity might be due to this, rather than the germination of the few pustules remaining. It therefore seems safe to assert that in this particular study hibernation has not been effected by a wintering mycelium.

Eriksson (9) explained the hibernation of the rust by his mycoplasm theory. He held that those teleutospores formed from May to July germinated to give conidia. These he considered to cause a different kind of infection from the sporidia. Instead of putting out a penetration tube and forming an infection vesicle inside the cell the conidia "pour forth their protoplasm, as it seems, without the formation of an opening, through the plasma connections of the outer wall of the epidermis of the host, into an epidermal cell", and so into the tissues where it vegetates until required. This protoplasm he terms the mycoplasm which infects the autumn buds. This mycoplasm divides every time the cell divides and so is distributed throughout the plant. This latter statement is open to the criticism that a cell such as that into which a conidium gains entry is a mature cell, and as such undergoes no further division. Its ultimate fate seems to be daath and therefore no future for the mycoplasm. In England careful experiments by Bailey (2) have disproved the mycoplasm theory. He grew plants from sterile seed under absolutely sterile conditions and in no case did infection occur, thus showing the absence of mycoplasm in the seed. Moreover experiments have shown that conidia may develop further to form sporidia. As soon as the water film in which they are formed evaporates, they continue their development in the saturated atmosphere. First a tiny projection is put out

to form a sterigma and through this the contents of the conidium pass to form a sporidium at the tip of the sterigma. This is seen in the accompanying figure 7. A drop of water is secreted and abstriction occurs in the usual way.



TEXT FIGURE 7. GERMINATION OF CONIDIA TO GIVE SPORIDIA.

A more feasible method of hibernation is with the seed of diseased plants. Rust pustules are very commonly formed on the flowers or even the carpels. Unless seed from such a source is properly disinfected there is a chance of contamination of the seedlings. Seed grown from infected plants of Althea rosea, Althea officinalis, Malva rotundifolia, and Malva borealis, has been sown and in two seedlings each developed one pustule on the first leaf. It is interesting to note in passing that this is evidence against the statement that infection of seedlings less than six months old rarely occurs. Seedlings from self-~~set~~ sown seed in the garden became heavily infected before they were three months old. This method can be included in the overwintering by teleutospores.

There is another suggestion, that the embryo itself contains the fungus, but this has been disproved by Baily's work, and also by other experiments on seed sterilisation.

In England, at least therefore, Puccinia malvacearum seems to persist from season to season, because of the power of its teleutospores to retain their viability for considerable periods, and yet germinate quickly whenever favourable conditions may occur.

With a view to substantiating the conclusions here drawn, regarding the overwintering of this rust, a short questionaire was sent to some dozen well-known seedsmen who were good enough to reply in some detail. The disease, it appears, is not treated very seriously since the majority have discovered that by the removal of diseased leaves it can be readily controlled; many do not consider it necessary either to sterilise the seed or to change the seed bed. This is in practice, exactly what one would have suggested from theoretical considerations. Unless the sporidia, formed on germination of the teleutospores, fall upon a fresh leaf, where they can germinate at once there is no danger of infection. Teleutospores that have fallen into the soil have little chance of causing infection. The report of an old custom in the North of England has peculiar significance. It was the practice, it appears, to pile a heap of coal ashes over the crowns of the plants through which the new growth made its way free from disease. This would have the effect of pressing down and burying the old leaves. It is true the ashes might absorb moisture, and so cause the teleutospores to germinate; but their sporidia would be abstricted into the soil and could not cause infection of the new growth.

Biological note. Often when material of Puccinia malvacearum was brought in from the garden for the inoculation experiments, a large proportion of the rust pustules were found to have been eaten, and in a large number of cases a slime track could be seen passing precisely from one damaged pustule to the next.

This slime track suggested the presence of slugs. Slugs were watched for and found in the act of eating the pustules, and progressing from one pustule to another. The slug was identified as Limax flavus. It is obvious that here is no question of spore digestion as is required in some of the Coprophilous fungi, since the teleutospores germinate so easily without such aid nor can this phenomenon be compared with the part played by flies in mixing pyonidia since this rust is homothallic. But it suggests that slugs may be instrumental in spreading the disease by un eaten teleutospores adhering to their mouth parts. One experiment only was made to discover if this were so. A slug which had just finished feeding on a diseased leaf, was transferred to an uninffected plant in the greenhouse, and kept under suitable conditions for germination of the teleutospores and sporidium. No infection was obtained in this one instance. These holes in the leaves recall the observation of other workers. The explanation usually offered is that given by Plowright (27) that the sori have fallen to the ground. It is possible that slugs are responsible for most of the holes found in diseased leaves.

SUMMARY.

1. A method of monosporidial infection for the rust fungi has been perfected with the help of the micromanipulator.
2. By this means Puccinia malvacearum has been proved to be homothallic.
3. The life history of the rust has been followed day by day both under natural conditions in the garden and under controlled conditions in the greenhouse.
4. The period required for development of both monosporidial and mass inoculations, has been found to vary inversely with the temperature.
5. Shortly after formation and sometimes even before abstriction, the sporidium becomes binucleate.
6. Entry to the host cell is effected through the epidermis by the formation of a fine penetration hypha at the tip of a short germ tube.
7. The contents of the sporidium pass through this to form an infection vesicle: an a-septate binucleate hypha, the primary infection hypha.
8. This primary infection hypha is wide, and becomes septate forming three or four uninucleate cells.
9. After septation each of these cells puts out a thinner hypha which is the beginning of the vegetative mycelium. The vegetative mycelium is both intracellular and intercellular with haustoria in all tissues.
10. The development continues until the lower epidermis is reached.
11. A renewed vigorous growth starts from the centre of the mass of vegetative mycelium. Branches are put out, these ramify repeatedly towards the lower surface.

12. These branches are wider than the early vegetative mycelium and form club-shaped end-cells which however are still uninucleate.
13. The change to the diplophase is now effected and evidence is cited for the occurrence of this both by nuclear migration, and nuclear division not accompanied by wall formation.
14. That the diplophase arises more than once in one sorus is evidenced by the irregular distribution of uninucleate and binucleate cells.
15. Each binucleate cell divided into a lower stalk cell, and an upper cell, which by a further division forms the two celled teleutospore.
16. The epidermis is ruptured and the teleutospores can germinate immediately they are exposed provided moisture is available.
17. In a moist atmosphere germination takes place to give a curved promycelium and sporidia: in a water film, the promycelium is straight and abstricts conidia.
18. The conidia may develop further to give sporidia.
19. The return to the haplophase occurs in the promycelium.
20. There is no biological difference between the teleutospores formed in different seasons.
21. During winter the life cycle is retarded by the less frequent occurrence of conditions favourable for the germination of the teleutospores.
22. New infections occur throughout the winter so that all the pustules present in the spring are not thevresult of a late autumn infection.

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DESCRIPTION OF PLATES.

Plate I. Photograph of infected plant of Althea officinalis. The lower leaf to the right, received 6 inoculations on the veins and shows 6 pustules.

Plate II. fig. 1. Early stage in infection of upper epidermal cell of Malva borealis by a sporidium. The ~~upper~~ infection vesicle is just forming in the cell.

" fig. 2. Slightly later stage. The primary infection hypha consists of two uninucleate cells.

" fig. 3. A three celled hypha.

" fig. 4. The two older cells have put out the finer vegetative hyphae.

" fig. 5. A three celled hypha showing contact with the host nucleus.

" fig. 6. A four celled hypha. The empty sporidium can be seen.

" fig. 7. Three stages of infection in one host cell. The two youngest show the binucleate vesicle. The oldest is putting out the finer vegetative hyphae.

" fig. 8. Two similar stages in one host cell. In the right hand one the nuclei of the primary cells are seen passing up into the finer vegetative hyphae.

Plate III. fig. 1. A later stage of infection. The mycelium is still vegetative and has not reached the lower epidermis.

" fig. 2. A still later stage. Infection of the lower epidermis has been effected. The club-shaped hyphae are seen massing towards the lower epidermis.

Plate IV. fig. 1. Migration of nucleus into a second uninucleate cell.

" fig. 2 a. Migrating nucleus at two foci.

b. Another migrating nucleus.

" fig. 3. Two cases of multinucleate cells.

" fig. 4. Uninucleate mycelium bearing bi-nucleate club-shaped end-cell.

" fig. 5 a, and b. Initiation of the club- shaped hyphae.

" fig. 6. Sporidia showing binucleate condition.

PLATE I

PLATE II

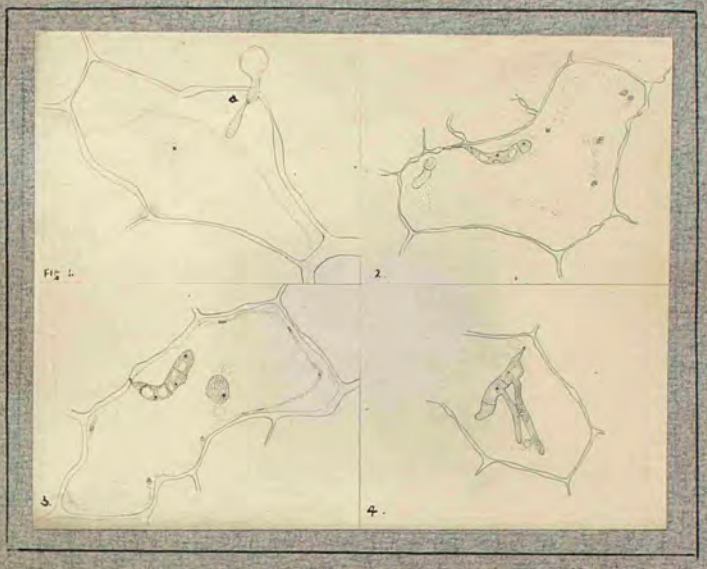
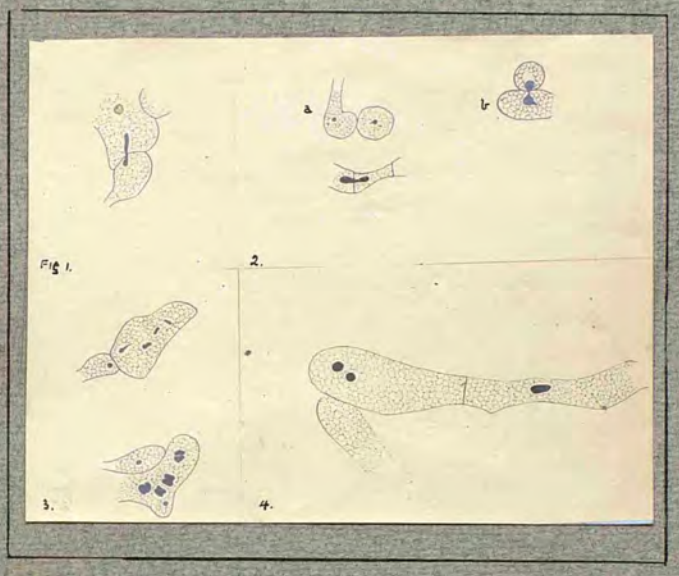


PLATE III

FIG 1.

FIG 2.

PLATE IV



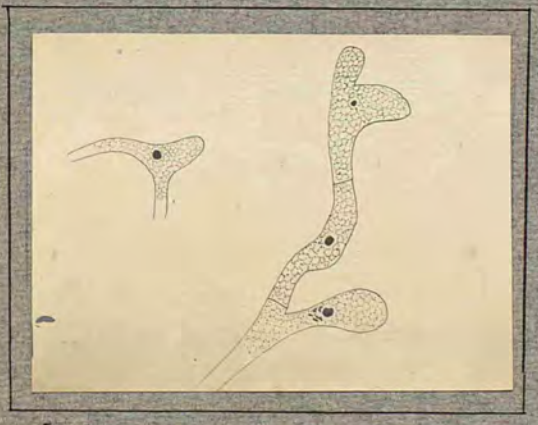


Fig 5

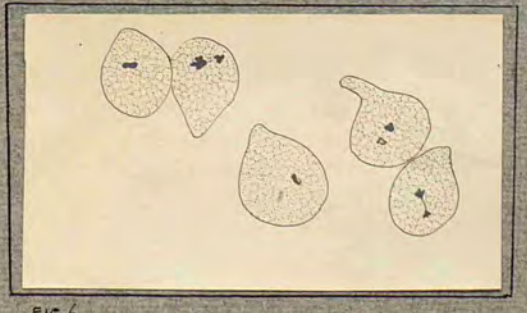


Fig 6.