

AN EXPERIMENTAL INVESTIGATION OF SEX IN  
THE RUST FUNGI.

A Thesis

submitted to the University of Manitoba

- by -

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in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy.

April, 1930.

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I. Introduction.

The Rusts comprise a distinct group of the Basidiomycetes. They are all parasitic, and some of them may under favorable conditions cause destructive epidemics. On account of their obligate parasitism, they present considerable difficulties to cultural studies. Owing to this fact, possibly, certain aspects of their developmental cycle have remained obscure. They have, however, been the subjects of a great deal of investigation. The black stem rust of cereals has been studied more than any other rust fungus, and, in general, it may be said that the amount of study given to a particular species has been about proportional to its economic importance.

This paper is another contribution to our knowledge of the Rust Fungi, particularly in respect to their sexuality; and the observations about to be recorded have been made on Puccinia graminis, P.

helianthi, P. coronata, P. Fringsheimiana, and a Gymnosperangium.

## II. Historical Summary.

Our knowledge of the life-history of Rust Fungi made a great advance in the middle of the nineteenth century. Previous to that time each spore-form was regarded as belonging to a distinct genus. The pycnial stage of the various rusts was grouped under the genus Aecidiolum; the aecial, under Aecidium; the uredinial, under Uredo; and the telial, under Puccinia. Tulasne (78), in 1854, showed that the uredinial and telial sori that are found on stems of wheat are not derived from two rust species as had previously been believed, but that they are products of the mycelium of one and the same species, namely, Puccinia graminis. In 1865, De Bary (16) established experimentally the genetic connection of the two stages of the rust fungus on wheat with the aecial stage (Aecidium berberis Pers.) on the barberry. He inoculated barberry plants with sporidia from germinating

teliospores. From these inoculations arose pycnia and aecia. Wheat plants inoculated with the aeciospores produced uredinia and telia. Thus he demonstrated that Puccinia graminis produces five spore-forms which appear in regular sequence; pycniospores in pycnia; aeciospores in aecia; urediniospores in uredinia; teliospores in telia; and sporidia on the basidium derived from a germinating teliospore.

Pycnia were discovered by Ungar (80) in 1833 and were considered by him to be the fructification of a distinct fungus. Since that time the pycnia have been the object of much speculation. Meyen (49) noticed the close association of pycnia and aecia and suggested that they represented the sexual organs of one and the same fungus. This view was supported by Tulasne (79). As the pycniospores were apparently lacking in power of germination and as the pycnia bear a striking resemblance to structures in collemaeous lichens which were believed to be sexual in nature, Tulasne (77) called the pycnia spermogonia. De Bary (15) also regarded the pycnia as spermogonia, but he records that aecia are sometimes formed in the complete

absence of pycnia and, consequently, in these cases, fertilization can not take place. In nutrient solutions, the germination of pycniospores was observed by Cornu (10) and the germination of the spermatia of lichens by Möller (50). The fact that pycniospores had been observed to germinate and that aecia are sometimes unaccompanied by pycnia led Brefeld (7) to oppose the idea of sexuality in the rusts, and to consider the pycnia not as spermagonia but as asexual reproductive organs to which the name pycnidia should be applied. Klebahn (32), for similar reasons, opposed the view that the pycnia are male organs. From a review of the literature and from his own experimental results, Plowright (60) arrived at the same conclusion; but he pointed out that if the pycniospores are conidia they should be able to infect the host plant, although he had never been able to prove such infection experimentally. However, he observed the germination of pycniospores in sugar solutions.

The opinion of Brefeld was generally accepted until 1904, when Blackman (5) revived the theory of the sexual nature of the pycnia. He supposed that

the pycniospores were once functional like the spermatia of the red sea-weeds, but that they became functionless when the trichogynes disappeared. In support of his view that pycniospores are functionless male cells, he referred to the density of their nuclei, the paucity of their cytoplasm, and the thinness of their cell-wall. In addition, he pointed out that pycniospores possess but feeble power of vegetative development and are (as far as known) incapable of producing infection. Christman (9) inclined to the view that the pycnia once produced functional asexual gametophytic spores - conidia - as do the pycnidia of Ascomycetes. He questioned the belief that the pycniospores are functionless, although he admitted that their nature was not very evident. It was McAlpine's (46) belief that the pycnia are isolated organs and that, whatever their original function may have been, they are now quite functionless. Grove (24) advanced eight reasons in support of the assumption that the pycniospores represent functionless male cells. Later investigators accepted one or other of these theories, but the opinion was fairly unanimous that the pycnia, whether

they were originally asexual gametophytic reproductive organs or sexual organs producing spermatia, they are no longer functional. The view that the pycniospores are functionless was very definitely expressed by Gwynne-Vaughan and Barnes (25) in their text-book published in 1927.

Cytological investigations have made important contributions to our knowledge of the Rust Fungi. In 1880, Schmitz (66) discovered paired nuclei in the mycelium and urediniospores of Coleosporium campanulae. Peirault and Raciborski (61) introduced the term "conjugate nuclei" for the paired nuclei, and made some observations on the chromosomal behaviour during nuclear fusion and reduction. Two nuclei were seen by Rosen (63) in the aeciospores and urediniospores of Uromyces pisi and Puccinia asarina, but only one in the mature teliospores. A comprehensive study of the mycelia and fructifications of a large number of rust species was made by Sappin-Trouffy (65). He found that the pycniospores and the hyphae which produce them are uninucleate; that the aeciospores, urediniospores, and the hyphae arising from them are binucleate, as

are also the cells of the immature teliospores, but that before germination the cells of the teliospores become uninucleate through the fusion of their conjugate nuclei. Two divisions occur in the basidium, one of which is a reduction division, so that the four basidiospores are uninucleate and produce uninucleate mycelia. He considered that the fusion of the two nuclei in the teliospore represents a true sexual process, but paid little attention to the origin of the binucleate condition in the asciospores.

The origin of the binucleate condition was investigated by Blackman (5) who found that in Phragmidium violaceum the nucleus of one cell migrates through a pore into a neighboring cell. He interpreted this as the beginning of a sexual act which culminates in the fusion of the two nuclei in the teliospore. Christman (9), in a similar study on Phragmidium speciosum, found that fusion occurs between two neighboring cells, and suggested that the nuclear migration observed by Blackman might have been due to a pathological condition. Nuclear migration has also been observed by Welsford (68), Kursanov (37), and several others, but most of the later workers, Olive (57),



Kursanov (36), Fromme (22), Maire (45), Lindfors (41), etc., have found that the conjugate condition of nuclei in the spore-bed of the aecia of many rust species is due to cell-fusion, although both types have been found in the same species (37, 41, 57). Very recently, Hanna (28) has shown cell-fusion occurs in Puccinia graminis.

The phenomenon of heterothallism is known to occur in the Phycomycetes, the Ascomycetes, and the Basidiomycetes. It has been demonstrated experimentally: in the Phycomycetes, by Blakeslee (6) in Mucor and other Mucorineae, and by Couch (11) in Dictyuchus; in the Ascomycetes, by Dodge (19) in Ascobolus magnificus, by Betts (4) in A. carbonarius, by Derx (17) in Penicillium, by Wieben (89) in Taphrina, and by Shear and Dodge (68) in Neurospora sitophila and N. crassa. Dodge (20) secured fertile hybrids from a cross between N. sitophila and N. tetrasperma.

In the Hymenomycetes heterothallism has been shown to occur: by Mlle Bensande (3) in Coprinus finetarius; by Kniep (33, 35) in Schizophyllum commune and Aleurodiscus polygonius; by Miss Mounce (52, 53) in Coprinus lagopus, C. niveus, and Fomes pinicola; by

Vandendries (81,82,83) in Collybia velutipes,  
Hypoheloma fasciculare, Panaeolus campanulatus, P.  
separatus, P. fimicola, and Coprinus radians; by  
Brunswik (8) in a number of species of Coprinus;  
by Miss D. E. Newton (54) in Coprinus Restrupianus;  
and by Miss Gilmore (23) in Psilocybe coprophila.

Among the Smut Fungi, Kniep (34) found  
evidence of heterothallism in Ustilago violacea;  
Stakman and Christensen (70), in Ustilago zaeae;  
Dickinson (18), in Ustilago levis; and Hanna (27),  
in Ustilago zaeae and Sorosporium reiliana.

Until 1894, Puccinia graminis was con-  
sidered to be a single species capable of attacking  
all of the common cereals and grasses, but in that  
year Eriksson (21) showed that it consisted of several  
pathogenic strains or forms, each of which is specific  
for certain gramineous hosts. These strains he  
designated as "formae speciales", and classified them  
according to their infective capabilities as follows:  
Puccinia graminis tritici on wheat, P. graminis avenae  
on oats, P. graminis secalis on rye, P. graminis airae  
on Aira, P. graminis agrostis on Agrostis, and P. graminis  
poae on Poa. All had the common barberry as an aecial

host. Results of a confirmatory nature were obtained by Rostrup (64), Magnus (42), Hitchcock and Carleton (29), Klebahn (31), and Ward (86).

A careful study by Stakman and Piemeisel (75) in 1917 revealed that Fuccinia graminis tritici is not a simple form, but consists of at least two pathogenic strains. In the following year, a third strain was identified by Melchers and Parker (48) and a fourth one by Levine and Stakman (39). By using twelve standard varieties of wheat, called "differential hosts", Stakman and his co-workers (71, 74, 76) have been able to distinguish a large number of pathogenic forms by their reaction on these hosts. Newton, Johnson, and Brown (55) have reported eight additional forms. As the morphological characters of all these strains are more or less identical, the dissimilarity of their reactions is considered to be due to physiological differences in the strains, and hence the strains are now referred to as "physiologic forms."

Not only has physiologic specialization been found in Fuccinia graminis tritici but it has been demonstrated by Stakman, Levine, and Bailey (72) in Fuccinia graminis avenae, and by Levine and Stakman (40) in Fuccinia graminis secalis. It has also been shown to

occur in other rusts. Mains and Jackson (44) distinguished twelve forms in Puccinia triticina; Mains (43), four forms in Puccinia sorghi and two in P. anomala; Hoerner (30), four forms in Puccinia coronata; and Bailey (2), at least three forms in Puccinia helianthi. These citations are sufficient to indicate that the phenomenon of physiological specialization occurs more or less generally among Rust Fungi.

From the historical summary just concluded, it is evident that prior to 1926, at which time the investigation here recorded was begun, many fundamental facts concerning the life history of Puccinia graminis had been brought to light; it was known that this rust is heteroecious; that fusion of the conjugate nuclei occurs in the teliospores prior to germination; that reduction takes place in the basidium; that the sporidia give rise to uninucleate mycelia; and that the aeciospores are binucleate. By inference from what was known to occur in other rusts, the binucleate condition of the aeciospores was supposed to arise either by cell fusion or nuclear migration in the aecial spore-bed. It was known also that physiological specialization in Puccinia graminis is very pronounced. And, finally, it was generally agreed that,

whatever the original function of the pycnia might have been, that function had been lost.

No one up to the year 1927 had ever studied the problem of sex in Puccinia graminis or any other rust experimentally; but, in that year, the writer was able to announce that Puccinia graminis is heterothallic and that the pycnia are functional (12,13). A further communication from the writer (14) in 1928 contained additional observations on sex in the Rust Fungi.

### III. Problem Stated.

The Dominion Rust Research Laboratory was organized for the purpose of finding some means by which the ravages of black stem rust in western Canada might be prevented, or, at least, mitigated. It seemed possible that the end in view might be attained by breeding rust-resistant varieties of cereals. It was hoped to produce varieties of wheat and oats which would be resistant to all the physiologic forms of P. graminis tritici and P. graminis avenae, or

at least resistant to their most prevalent forms. To attempt to do this seemed sufficiently difficult, even if there was no possibility that other physiologic forms might arise which would be more virulent, or capable of attacking a wider range of host plants, than the forms already known. Whether or not new physiologic forms arise from time to time under natural conditions was not known, but it was argued theoretically that, if Puccinia graminis is heterothallic, any two physiologic forms of this organism may hybridize on the barberry and so produce new forms which may render breeding for rust resistance more or less futile. Stakman, Levine, and Leach (74), as early as 1919, mentioned the possibility of hybridization of physiologic forms occurring on the barberry. Whether or not hybridization does take place is a question of very practical importance, as well as one of considerable scientific interest. Dr. D. L. Bailey, formerly Officer in Charge of the Rust Research Laboratory, proposed to the writer that he investigate the problem.

It seemed obvious to the writer that the first step in the investigation was to discover whether Puccinia graminis is homothallic or heterothallic. Both homothallic

and heterothallic species are known among the Basidiomycetes. No sexual differentiation was found by Miss Mounce (51) to exist in the basidiospores of Coprinus sterquilinus or of C. stercorarius, or by Brunswik (8), in the basidiospores of Coprinus narcissicus or of C. ephemeroideus. On the other hand, Kniep (34) showed that the basidiospores of Ustilago violacea are divisible into two sexual groups, and a similar condition was demonstrated by Vandendries (33) in Coprinus radians, and by Miss D. E. Newton (54) in Coprinus Bostrupianus. Four sexual groups of spores were found by Kniep (33, 35) in Schizophyllum commune and Aleurodiscus polygonus, and by Hanna (26) in Coprinus lasopus. It was therefore possible that one of these three conditions might exist in P. graminis.

If the sporidia of this rust are not differentiated for sex, or in other words, if the species is homothallic and its sporidia are all sexually alike, an infection of a barberry leaf by a single sporidium should produce a pustule in which normal aecia would arise. If the sporidia were differentiated for sex, or, in other words, if the species is heterothallic and its spores are divisible sexually into 2 or 4 groups, an

infection by a single sporidium should produce a mycelium which would remain in the haploid condition. Accia would not be expected to develop, therefore, in such a pustule, unless, indeed, a spontaneous change from the haploid to the diploid condition occurs, similar to that found by Vandendries (84,85) in Coprinus radians and C. micaceus, and by Miss D. B. Newton (54) in Coprinus Restrupianus. Accia might be expected, however, to arise in a compound pustule formed by the coalescence of two monosporidial pustules of opposite sex, but not in a compound pustule formed by two coalescing pustules of the same sex or of two sexes incapable of interacting sexually.

In attempting to elucidate the sexual condition of Puccinia graminis from the point of view of the possibilities just discussed, it was necessary to obtain two kinds of pustules: (1) simple pustules, each derived from the sowing of a single sporidium, and (2) compound pustules, each derived from the sowing of two sporidia very close to one another and from the eventual coalescence of the two simple pustules resulting therefrom.



IV. Materials.

As the primary object of the work was a study of the sexual behaviour of Puccinia graminis, this rust was used throughout the investigation, and exclusively in 1926. For reasons that will be mentioned later, Puccinia helianthi Schw. was used extensively in the experiments of 1927. The source of the telial material of Puccinia graminis was heavily infected culms of wild barley (Hordeum jubatum), and that of P. helianthi, withered leaves of the cultivated sunflower (Helianthus annuus). Collections of both rusts were made at the Agricultural College, Winnipeg, Manitoba.

For convenience in handling, only small plants of the respective hosts were employed. The barberry plants varied from 6 to 12 inches in height, and grew singly in 6-inch flower pots. Sunflower seedlings were grown in similar pots, usually four or five to a pot. They were inoculated as soon as the first foliage leaves were one-inch in length.

Owing to the fact that barberry leaves become very highly resistant to infection after they are twelve days old, only young leaves of this plant were successfully inoculated. Moreover, as a leaf

grows older, it appears to offer progressively greater resistance to the spread of the mycelium through its tissues. The older the leaf when infected, the smaller usually is the pustule that arises from the infection.

#### V. Methods.

Inoculations were made by two methods, A and B. By method A, sporidia were picked off their sterigmata and deposited, either singly or in pairs, in a drop of water on the leaf of the host plant. By method B, sporidia were sown sparsely over the leaves of the host plant.

Method A was employed exclusively during 1926. It was not, however, very productive of results, but as it was the method by which the first results were obtained and as, in the opinion of the writer, the comparative failure attending its employment was not inherent in the mechanism or manipulation of the spore-picking apparatus, but attributable to another factor, a description of the

apparatus and its operation will be given in detail.

(1) Method A.

Before inoculations could be made by this method, it was necessary to construct a spore-picking device which was amenable to rather quick manipulation. A drop of water begins to form at the base of a sporidium just about 15 seconds before the sporidium is discharged. The formation of this drop indicates that the sporidium is mature. Only mature sporidia were desired for inoculating barberry leaves. Contact had to be made with a sporidium while its drop was being excreted, i.e. within a period of 15 seconds. Also a moist chamber was required in which an optimum humidity for teliospore germination could be maintained and still permit ready access to an instrument to pick off the sporidia.

Apparatus. The iris diaphragm was removed from a Leitz microscope and to the projection which supported the diaphragm was clamped in an inverted position a mechanical stage (Fig. 1). The swing arm of the stage

was disjoined and at its place of attachment was erected a wooden pillar, A, as shown in Fig. 2. At the top of A, a short wooden arm, B, was fixed at right angles. On the upper side of B, a spring clip, C, was firmly secured.

A short piece of glass tubing, 5 mm. in diameter, was drawn out to a fine capillary bore. The unreduced part was fitted into the end of a piece of soft rubber tubing about 8 inches in length and of the same diameter as the glass tube. Thus connected, the two were mounted as seen in Fig. 3. The part of the glass tube covered by the rubber tubing was placed in the notch (Fig. 2) cut on the upper side of B and was held in position by the spring clip, C. The capillary part of the tube reached to the proximal edge of the field of view of the microscope; and the free end of the rubber tubing, closed by a spring clamp, rested on the table (Fig. 3).

It was then possible to raise or lower the capillary tube by means of the rack and pinion which served to adjust the substage of the microscope; and to move it from right to left, or from front to rear, within limits, by the inverted mechanical stage.

To form a moist chamber, a heavy strip of Bristol board was cut to the pattern shown in Fig. 4 and folded along the dotted lines; so that, when completed and cemented to a glass slide, it presented the appearance shown in Fig. 5. Before being cemented to the slide, it was dipped in liquid paraffin to prevent water absorption when in use. The rectangular opening in the front (Fig. 5) afforded access to the interior. A filter-paper pad lined the interior of the other three sides. Midway across the chamber lay a small wooden pin (Fig. 6), supported at one end by a small hole in one side-wall of the chamber and, at the other end, by the notch cut in the opposite side-wall. The pin projected about a half an inch beyond the side of the chamber so that it could easily be reached by the hand and be rotated by the thumb and finger.

Preparation of inoculum. A straw bearing numerous telial sori was soaked in water for about an hour. From it was cut off a piece just long enough to reach across the moist chamber. The wooden pin was passed through the hollow center of the piece of straw and was then replaced in the chamber as shown

in Fig. 6. Sufficient water was added to the filter paper pad to wet it thoroughly. Thus both ends of the piece of straw were in contact with water, and, so long as the filter paper remained wet, the telial sori were kept damp. To prevent the mount from drying during the ensuing night, the moist chamber (all the apparatus shown in Fig. 6) was set in a petri dish, the bottom of which was covered with water.

To secure basidia which projected sufficiently from the straw, it was necessary to keep the telial sori covered with a thin film of moisture. No exact data on the optimum moisture requirement were taken. Experience alone served as guide. But when a certain thickness of film was present, the basidia were somewhat longer and stood well out from the sori, in which case it was easy to bring the capillary tube into contact with individual sporidia. If too much moisture was present, the basidia were distorted and produced abnormal sporidia.

Manipulation. When the teliospores in the sori began to germinate, the moist chamber was removed from the petri dish and covered with a cover-glass. The chamber, thus completed, was then mounted under the microscope on the regular mechanical stage which had previously been

moved somewhat to the right to prevent the chamber, when being mounted, from coming into contact with the end of the capillary tube. The capillary tube was then removed from its position and filled about two-thirds full with distilled water. This was done by first releasing the end of the rubber tubing from the spring clamp and then by drawing the thumb and index finger of the right hand along the tubing, thereby creating a partial vacuum in the tubing while the capillary end of the tube was held by the left hand under water. When the desired amount of water was drawn up, the spring clamp was replaced and the tube mounted again in its former position, the capillary end of the tube appearing at the edge of the microscopic field (Fig. 7).

By means of the mechanical stage, the moist chamber was moved to the left until the piece of straw bearing the telia appeared at the side of the field of view opposite to that occupied by the end of the capillary tube, the capillary tube having been adjusted for height so that it passed freely through the rectangular opening in the side of the chamber (Fig. 7). Search was then made for a somewhat isolated basidium bearing

a sporidium which was approaching maturity. When one was found, it was brought directly opposite the end of the capillary tube (Fig. 6), but not close up to it, so that other sporidia which might be discharged while this one was maturing would not come into contact with the tube. If by any chance an unwanted sporidium did come into contact with the tube, the tube was removed, sterilized in boiling water, refilled with distilled water, and put back into position again.

Whenever the drop of water which forms at the base of the sporidium first became visible, the mount and capillary tube were moved towards one another so that the sporidium and the end of the tube were separated by a very small distance. A light pressure on the rubber tubing caused the water in the tube to bulge out in hemispherical form, just far enough to establish contact with the sporidium. On release of the pressure, the water was drawn back into the tube, carrying with it the sporidium.

The mount was drawn back as quickly as possible by a reverse movement of the mechanical stage to prevent contamination of the tube by other sporidia. The tube was removed, and, by a light pressure on the



rubber tubing, a droplet of water containing the sporidium was deposited on a barberry leaf. The plant was then placed in an incubation chamber for forty-eight hours. At the end of that period, it was set on a bench in the greenhouse. After each inoculation, the capillary tube was sterilized in boiling water.

If a bisporidial inoculation was to be made, the process was repeated, and the two sporidia were deposited on the leaf in the same drop.

Generally the second and third sporidia on a basidium mature first, and at about the same time; later, the one towards the tip; and finally, the one situated nearest the teliospore. The latter one not infrequently aborts and collapses before it reaches maturity.

The comparative failure of this method of inoculation seemed to be due chiefly to physical causes. The waxy cuticle of a barberry leaf has little or no affinity for a droplet of water, so that, when one was placed on a leaf, it remained in almost spherical form, as if placed on an oily surface. It seems probable that the sporidium was held in the drop of water and either

did not germinate, or, if it did germinate, its germ tube had little opportunity of establishing contact with the leaf.

(2) Method B.

This method was much simpler. Petri dishes containing sufficient water to cover the bottoms were used as germination chambers for the teliospores of P. graminis and P. helianthi.

Inoculations with P. helianthi were made as follows. Pieces of rusted sunflower leaves were soaked in water for an hour. Some petri-dish covers were then lined with thin pads of filter paper. The leaves were placed flat in the petri-dish covers so that their upper sides were in contact with the filter paper while their lower sides, which bore the telia, were turned upwards and exposed to the air. The moisture caused the filter paper to adhere to the covers, and the leaves, in turn, to the filter paper; so that, when the covers were set on their respective dishes, both the filter paper and the pieces of leaves remained in position.

If inoculations with P. graminis were to be

made, the procedure was similar, except that the rusted pieces of culms were supported by short pieces of twine (Fig. 9), the ends of which were made fast to the covers by means of sealing wax.

With both P. graminis and F. helianthi, the same method of inoculation was employed. The plant to be inoculated was first covered with a fine film of water. Over it was inverted a hollow cylinder (Fig. 10), at the upper end of which was held a petri-dish cover bearing the leaves on which the sporidia were developing. The cover was kept continually in motion by a circular movement of the hand. The cylinder prevented air currents from carrying the sporidia away from the plant, and the movement of the cover insured an even distribution of the sporidia over the leaf-surface. After inoculation, the plant was placed in an incubation chamber for forty-eight hours. At the end of that period, it was placed on a bench in the greenhouse.

As a rule, the sporidia settled on a leaf at some distance apart; but, sometimes, two of them settled close together. The sporidia were not actually seen on the leaves after they had settled, but their

location was inferred from the position of the infections to which they gave rise (Fig. 11).

The time required for each inoculation varied inversely with the number of sporidia which were being produced. An index of this number was obtained just before the inoculations began. A glass slide was placed in each petri dish directly below the telia and was left there for ten minutes. Thereafter, each slide was examined under the microscope, the number of sporidia which settled on it was noted, and then the time necessary for an inoculation was estimated and marked on the outer surface of the cover of the petri dish. If the sporidia were relatively numerous, the time during which it was necessary to expose the leaves to the falling sporidia was short; if relatively scarce, the time was longer. Usually the time varied from two to five minutes.

#### VI. Experimental Results in 1926.

During the year 1926, inoculations were made exclusively by Method A. By it two mono-

sporidial pustules of P. graminis were obtained on barberry leaves. One of these appeared on a young leaf on July 9. It developed rapidly and eventually attained a diameter of 6 millimeters. The other one occurred on a comparatively old leaf on August 3. It grew less rapidly and scarcely attained half the diameter of the first one. Both pustules, however, developed pycnia which exuded nectar containing numerous pycniospores. But, although both pustules remained healthy for five weeks, neither of them produced aecia. A cytological examination of each pustule, when five weeks old, showed that its mycelium was still in the haploid condition.

Three of the bisporidial inoculations by this method were successful. On one of the leaves inoculated on August 5, two neighboring infections occurred. These appeared first as tiny, pale-yellow pustules, approximately 2 mm. apart. About six days later, the two pustules coalesced. Five days after coalescence, aecia began to appear in the compound pustule thus formed. On August 17, two other barberry leaves were successfully inoculated. Two pustules, about 2 mm. apart, developed on each leaf

and coalesced seven to eight days later. Aecia appeared in one of the compound pustules so formed within six days, but none appeared in the other one within that time or thereafter.

These results, although few in number, were of considerable significance, for they at least indicated that the mycelia of the pustules, and consequently the sporidia from which the mycelia originated, were of two sexes and that in all probability Puccinia graminis is heterothallic. They also were indicative of the behaviour that might be expected of pustules in further experimentation.

#### VII. Experimental Results in 1927.

So far in the investigation, Puccinia graminis was employed; but, as the teliospores of this rust begin to germinate rather late in the spring - towards the end of April in Canada - it was decided to select another rust, one in which teliospore germination occurs earlier, for the preliminary investigational work of 1927. Puccinia helianthi was chosen. Its telio-

spores were known to germinate earlier in the spring than those of P. graminis, and an abundance of telial material was easily procurable. Moreover, as the writer wished to try inoculation Method B, this rust seemed well adapted to his purpose. In order to have telial material available, a liberal supply of rusted sunflower leaves was collected late in the autumn of 1926 and stored in the basement of the laboratory.

Towards the end of February, 1927, the teliospores of P. helianthi began to germinate. Sunflower seedlings were inoculated by sowing sporidia sparsely over the leaves, as already described. Infections became manifest about eight days after inoculation as tiny red pustules on the leaves. Very frequently the pustules were isolated, often only one on a leaf, or two or three rather widely separated from one another on the same leaf. Less frequently, two pustules arose relatively close together, from 1 to 4 mm. apart, and later coalesced to form a compound pustule. Occasionally three or four pustules occurred in a cluster, but these were discarded forthwith.

As soon as the pustules became visible, the position of the pustule, or pustules, on each leaf was

mapped on a label which was then attached to the leaf. In this way it was possible to determine, when the pustules became older, which pustules were simple in origin (monosporidial), and which were compound in origin (bisporidial). If the distance between two neighboring pustules was greater than 4 mm. each one was usually considered as a simple pustule. Usually two neighboring pustules were sufficiently far apart when they first appeared to be readily distinguishable from one another (Fig. 11); but, occasionally, it was difficult to decide whether or not the infection giving rise to a pustule was of monosporidial or of bisporidial origin. In these cases, which fortunately were not numerous, the pustules were considered as monosporidial in origin.

In the course of the investigation, many sunflower seedlings were inoculated and, as a result of these inoculations, a large number of pustules were made available for study. On account of the uniformity of technique and the similarity of results in all the earlier experiments, it is unnecessary to describe the experiments, in consecutive order, or to record separately the data for each one. It will suffice to give



a general descriptive statement concerning the development of both simple and compound pustules, and a summary of the experimental results obtained with each type.

(1) Evidence of Heterothallism in  
Puccinia helianthi.

The simple (monosporidial) pustules of Puccinia helianthi developed vigorously. A few of them finally attained a diameter of 12 mm., but usually the diameter was much less, from 6 to 8 mm. Pycnia developed plentifully on the upper side of all the pustules, and rather rarely (Fig. 12), or not at all on the under side. When they were present on the under side, they were generally most numerous in the peripheral region of the older pustules, many of which were beginning to die at the center. Nectar containing numerous pycniospores was exuded by the pycnia of both sides of the pustules. If the pycnia were relatively close to one another, as on the upper side of the pustules, the globules of nectar, as they grew larger, came into contact and fused, so that eventually the whole surface of the pustule was covered

with a layer of nectar. The process is illustrated diagrammatically in Fig. 13.

The majority of the simple pustules never produced aecia (Fig. 14), although some of them remained alive for upwards of five weeks. Aecia, however, appeared in a minority of them. Out of a total of 2,153 simple pustules, 1,641 went through the whole course of their development without producing aecia. The remainder, 512 in all, at one time or another developed aecia.

No regularity or periodicity marked the appearance of aecia in these pustules. The time of their appearance in a pustule seemed to bear no relation to the age of the pustule. The sudden and spontaneous change from the haploid to the diploid condition took place without any apparent cause. Reference has already been made to a parallel phenomenon in the Hymenomycetes.

Each compound pustule came into being as a result of the coalescence of two neighboring simple pustules. The time necessary for two such pustules to accomplish coalescence depended largely on the distance they were separated. If the two pustules were about 1 mm.

apart, they coalesced early, within two or three days after they appeared; if they were more widely separated, they coalesced later, possibly from 8 to 10 days after they appeared; but if they were more than 4 mm. apart, they rarely coalesced. Prior to coalescence, each of the two pustules developed independently as a simple pustule. Following coalescence, one of two things happened. Eitheraecia appeared in the compound pustules thus formed within from five to six days (Fig. 15) or they did not appear (Fig. 16). Of the 246 compound pustules of P. helianthi that came under observation, 108 developedaecia within from five to six days, while, in the 138 others, noaecia appeared within that time. However, some of these 138 pustules producedaecia at one time or another afterwards, so that, by the time all of the 246 pustules had died, those that had developedaecia had increased from 108 to 145, leaving 101 that had failed to produceaecia.

(2) Evidence of Heterothallism  
in Puccinia graminis.

Experiments similar to these just described

were made with Puccinia graminis, and similar results were obtained. As the barberry plants that were suitable for inoculation purposes were somewhat limited in number, the pustules available for observation were fewer than those of Puccinia helianthi. Both simple and compound pustules appeared. Each pustule arose as a tiny pale-yellow spot on the leaf. The difficulty in determining whether certain pustules were of monosporidial or of bisporidial origin was again encountered. Possibly owing to the greater resistance offered by the leaf tissue of the barberry to the radial advance of the mycelium, the pustules of P. graminis did not grow so rapidly, or become so large, as those of P. helianthi. Very few of them exceeded 5 mm. in diameter. On account of this rather restricted growth, coalescence of pustules more than 4 mm. apart rarely occurred. On the other hand, the pustules of P. graminis produced nectar more copiously than did the pustules of P. helianthi, and they possessed greater longevity. The sunflower leaves aged more quickly than the barberry leaves, and the earlier aging of the P. helianthi pustules seemed to be attributable more to the decreased vigor of the leaves than to any lack of vitality on the part of the organism.

The data concerning the simple pustules of P. graminis may be summarized as follows. Ten days after the first appearance of the pustules, the number of simple pustules in whichaecia had developed and the number in whichaecia had not developed were recorded. Out of a total of 174 simple pustules on which observations were made, 11 producedaecia within that time, while 163 were withoutaecia (Fig. 17). From time to time, one or another of these pustules developedaecia, so that, when practically all the pustules had died, it was found that altogether 37 of the simple pustules had producedaecia, and 117 had failed to produceaecia. The apparently spontaneous change from a haploid to a diploid condition of the mycelium, as evidenced by the production ofaecia in a certain number of the pustules of monosporidial origin thus was observed in P. graminis, just as in P. helianthi.

In the compound pustules five or six days after coalescence had been effected, a record was made of those pustules that had producedaecia and of those that had not producedaecia. In a few of these pustules, coalescence was not thoroughly accomplished at that time. The number in whichaecia were present was 24, and the

number in which aecia were not present was 35. At the termination of the experiments, when most of the pustules had died, the proportion was 36 with aecia and 23 without aecia.

Figure 18 shows the under side of a barberry leaf which was photographed 20 days after it was inoculated. On the left-hand side of the midrib appears a compound pustule bearing aecia. On the right-hand side are three simple pustules in which no aecia developed. The pocked appearance of these three pustules is due to the formation of wefts of haploid hyphae which develop in simple pustules just beneath the epidermis and simulate in general contour a young aecium.

Mention was made of these structures by Olive (58) in 1911. He believed them to be sterile aecia. They were observed by the writer (14) in 1928. Hanna (28), in 1929, referred to them as being "evidently haploid rudiments of aecial cups waiting to be stimulated into further developmental activity." In the same year, Miss Allen (1) stated that they resemble aecia but "consist of haploid mycelium only." As this paper is not primarily concerned with the cytological

aspect of the problem, no further reference will be made to these haploid hyphal wefts. It might be stated, however, that they were present in practically all the older simple pustules, and also in all the older sterile compound pustules of P. graminis and P. helianthi.

The results thus far enumerated indicate that Puccinia graminis and Puccinia helianthi are heterothallic and that the mycelium of an individual pustule is either (+) or (-) in its sexual nature, so that, when a (+) pustule and a (-) pustule coalesce, a sexual interaction takes place between the two mycelia which results in the production of aecia; but that, when two pustules of the same sex coalesce, i.e. a (+) pustule with another (+) pustule or a (-) pustule with another (-) pustule, no sexual interaction occurs and no aecia are produced.

Although heterothallism accounts for the behaviour of the majority of the simple and compound pustules in P. graminis and P. helianthi it gives no explanation of the apparently spontaneous change from the haploid to the diploid condition in simple pustules, or in compound pustules which have long remained sterile.

A partial explanation at least of this behaviour was brought to light when the function of the pycnia was discovered.

### (3) Function of the Pycnia.

The discovery of this function came by way of a fly which Prof. A. H. Reginald Buller saw alight on a sunflower leaf and sip up the nectar from one of the pustules. It flitted about from one pustule to another, sipping nectar, thus imitating the bee on his beneficent mission of promoting cross-fertilization in flowers. With characteristic intuition Professor Buller concluded that the nectar produced by the pycnia attracted flies, and that through their agency the pycniospore-containing nectar of (+) pustules was carried to (-) pustules, and of (-) pustules to (+) pustules, and that very possibly the diploid phase of the mycelium was induced by the germination of the pycniospores. A series of experiments was devised during the summer months to test the accuracy of his interpretation.

Before treating of the individual experiments



which deal with the function of the pycnia, it might be pointed out that in each experiment the treated pustules and those kept as a control were of the same age and of comparable vigor. All of the pustules were of monosporidial origin: they bore numerous pycnia, but aecia were entirely absent from them. At the beginning of each experiment, the minimum age of the P. graminis pustules, was 16 days, and of the P. helianthi pustules, 14 days. Wherever the experimental technique demanded it, the instruments used were thoroughly sterilized in an alcohol flame.

#### (4) The Effect of Mixing Nectar.

In one experiment with Puccinia helianthi, the nectar of 184 simple pustules was intermixed by means of a small scalpel, so that the nectar of each pustule was distributed over the surface of several other pustules. This procedure may have caused a slight irritation of the upper surfaces of these pustules; and, therefore, to procure comparable conditions in 174 other similar pustules, which served as a control, the nectar of each of these

pustules was stirred separately with the scalpel but not mixed with any other nectar.

Five days after the beginning of the experiment, the condition of the pustules was as follows: of the 184 pustules in which the nectar had been mixed, 176 had produced aecia, 4 no aecia, and 4 had wilted and died through leaf-injury; of the 174 pustules in which the nectar had been stirred but not mixed, only 20 had produced aecia, while 154 were entirely free from aecia (cf. Fig. 19).

An experiment similar to that just described was made with Puccinia graminis. The nectar on the upper surface of 116 simple pustules was intermixed; while, as a control, the nectar of 85 other similar pustules was stirred separately but not mixed with any other nectar.

Six days after the experiment had begun, the condition of the pustules was as follows: of the 116 pustules in which the nectar was mixed, 102 had produced aecia and 14 no aecia; whereas, of the 85 pustules in which the nectar was stirred but not mixed, 17 had produced aecia, while 68 were free from aecia (cf. Fig. 20).

(5) The Role Played by Flies.

Proof that flies mix the nectar of separate monosporidial pustules and so cause the mycelia of the pustules to change from the haploid to the diploid phase, as shown by the appearance of aecia, was obtained in an experiment with Puccinia helianthi.

Fifteen to twenty flies were enclosed in a large screen-wire cage with twelve pots of sunflower seedlings on the foliage leaves of which there were 98 simple pustules bearing pycnia but no aecia. As a control, 159 similar pustules on the foliage leaves of sunflower seedlings were protected by a screen-wire cage from the visits of flies.

Eight days after the beginning of the experiment, 96 of the 98 pustules to which flies had access had produced aecia and only 2 no aecia; whereas, only 5 of the 159 pustules to which flies had not had access had produced aecia.

It seems very probable that many other insects are active agents in bringing about the transfer of nectar from one pustule to another. Rathay (62) states that he observed one hundred and thirty-five different species of insects, belonging to the Coleoptera, Hymenoptera,

Hemiptera, and Diptera, visiting pustules of two *Gymnosporangium* species and also pustules of the common cereal rusts. Shear (67), Grove (24), Meineke (47) and Spaulding (69) also have pointed out that insects are attracted by the nectar.

That insects visit rust pustules is not surprising. It is known that the pycnial nectar of many rusts is sweetish to the taste, and that in some rusts the pycnia have a distinct odor. Rathay (62), by chemical tests, showed that the nectar of *Gymnosporangium sabinae* contained dextrose and laevulose. The odor of the pycnia of certain rusts have been mentioned by Persoon (59), Tulasne (79), Léveillé (38), and Rathay (62). Plowright (60), pointed out that these odors were possibly to attract insects, mimicking as they do the perfume of flowers. No odor has been detected by the writer in *P. graminis* and *P. helianthi*, but he has found that the nectar of the former is distinctly sweetish to the taste.

#### (6) Effect of Heating the Nectar.

By another experiment it was found that heating the nectar of *P. graminis*, or of *P. helianthi*, to a

temperature of 70°C. for three hours to kill the pycniospores rendered the nectar ineffective in inducing the production of aecia when it was applied to the pycnia of individual simple pustules.

Nectar was collected from approximately fifty pustules of Puccinia graminis by means of short capillary tubes. One tube was used for each pustule. The nectar was then deposited in a single drop, mixed thoroughly, and then sucked up into two fine glass tubes, each tube taking up approximately the same amount of nectar. One end of each tube was stopped with sealing wax and the other, by a plug of cotton wool. One of the tubes was placed in an oven and kept for three hours at 70°C., the other was kept at room temperatures for the same length of time. The heated nectar was then allowed to cool to room temperature, and a small quantity of it was then deposited on each of 25 simple pustules. The droplet on each pustule was mixed thoroughly with the nectar of the pustule to which it was applied. The unheated nectar was similarly distributed among 20 other similar pustules of Puccinia graminis.

Six days later, the condition of the pustules

was as follows: of the 25 pustules to which the heated nectar was applied, only 1 developed aecia, while 24 were entirely free from aecia; of the 20 pustules to which unheated nectar was applied, 17 developed aecia, whereas only 3 were free from aecia.

Fig. 21 illustrates the results. The pustules on the left of the midrib, to which heated nectar was applied, have produced no aecia; those to the right of the midrib, to which unheated nectar was applied, have produced aecia.

A similar experiment was made with P. helianthi. Heated nectar was added to 27 simple pustules, and unheated nectar to 25 other similar pustules. At the end of five days, aecia were completely absent in 23 of the 27 pustules to which the heated nectar was added, 4 only having formed aecia; while, of the 25 pustules to which the unheated nectar was added, all produced aecia.

These two experiments indicate that it is the pycniospores which are the effective agents in inducing the formation of aecia and not the nectar. The evidence, however, is not absolute. It is recognized that if the agent were an enzyme in the nectar the heating might destroy its activating property.

(7) Sex of Pycniospores.

Another experiment was designed to show whether or not the pycniospores of P. graminis are of two kinds, (+) and (-). The nectar of one mono-speridial pustule of P. graminis was drawn off by means of a capillary tube and divided into several drops, and then the drops were applied singly to the pycnia of as many pustules as there were drops. The nectar of other simple pustules was divided in like manner and distributed among other pustules. Altogether, 74 individual pustules were thus treated. Six days later, 30 of the 74 pustules had developed aecia, while 44 were free from aecia. As a control, 26 similar pustules were left untreated. Aecia appeared in only one of these.

Fig. 22 is illustrative of the results. Nectar from one pustule was applied to each one of the four pustules. Aecia arose in two of them but not in the other two. Usually, however, among pustules on the same leaf, the ratio of those with aecia to those without aecia was less evenly balanced.

The experiment just described was repeated

with P. helianthi. Five days after the drops of nectar were applied, 15 of the 48 pustules treated had developed aecia, whereas, 33 of them were without aecia. In a control of 66 similar pustules, only 5 produced aecia within that time.

Theoretically, if each basidium bears two (+) sporidia and two (-) sporidia, the number of pustules with aecia and the number without aecia should be equal in each experiment. The ratio obtained with the pustules of P. graminis is possibly as near the theoretical ratio as could be expected with such a small number of pustules. With the pustules of P. helianthi, there is a much greater divergence, yet not so great a one as appears in the control. The rather indifferent ratio given by the pustules of P. helianthi is very probably attributable, in some way or other, to the meagre quantity of nectar which was being produced by pustules at the time the experiment was performed.

(8) Aecia in Monosporidial Pustules.

It will be observed that in all the experiments so far discussed aecia developed apparently in a spontan-



eous manner in at least some of the monosporidial pustules. When the effect of mixing the nectar of pustules of opposite sex was discovered, it was thought that possibly mixing of nectar had taken place by some unknown means and that, under carefully controlled conditions, few or none of the pustules would develop aecia. It is to be noted that in the earlier experiments: (1) the pustules were examined frequently, and there was the possibility that, in the repeated handling of the leaves bearing pustules, nectar was transferred from one pustule to another; and that (2) no protection was afforded the pustules from the visits of flies or other insects which might by chance have been in the greenhouse.

A more critical experiment than any of those already recorded was devised with a view to preventing the accidental intermixing of nectar.

Sunflower seedlings were inoculated in the greenhouse by allowing sporidia of P. helianthi to fall sparsely on to the upper surface of the first two foliage leaves when they were about one inch in length. Usually about six seedlings grew in a pot; but, whenever the minute pustules appeared, each plant showing infection was

planted by itself in a separate pot and covered with a screen-wire cage. By keeping each plant in an individual cage, the opportunity for the transfer of nectar from one pustule to another was reduced to a minimum. All uninfected leaves were removed, and no new ones were allowed to develop.

Only pustules which were entirely free from aecia at 17 days of age were selected for observation. Of these there were 228, distributed as follows: 93 pustules were borne singly on as many plants; the remaining 135 pustules were borne on 59 other plants all of which bore at least two pustules, usually one on each leaf; but a small number of the plants bore three pustules, and a very few, four pustules.

Not all of the pustules persisted for the same length of time. Some of them died when about four weeks old and many of them before they were six weeks old, but a few were still living and exuding nectar in their peripheral region when seven weeks old - the time when the final data were recorded. Within the seven weeks, aecia developed in 11 of the 228 pustules. Of the 93 pustules, each of which was borne on a separate plant, only 2 produced aecia. Of the 135 pustules, of which two or

more were borne on individual plants, 9 produced aecia. Altogether 217, or 95 per cent, remained free from aecia during the whole course of the experiment.

From the time the pustules first appeared, daily inspections were made of the plants in order to destroy any insects which might have gained access to them. White flies (Aleyrodes) and thrips (Heliothrips) were the only ones discovered, and then only infrequently. The former were rarely found in contact with the pustules, but the latter seemed to be attracted by the nectar and fed upon it in preference to the leaf tissue. Consequently, in spite of the precautions taken, there was the possibility that thrips carried the nectar of one pustule to the pycnia of another pustule, especially on leaves which bore two or more pustules. This assumption is supported by the fact that nine of the pustules which produced aecia occurred on leaves bearing two or more pustules, and only two on plants bearing but a single pustule.

If, in the experiment just described, there had been an absolute exclusion of insects and a complete absence of any means by which the transfer of nectar could have taken place, it is possible that no aecia whatever would have developed in any of the pustules. At present, it is impossible to say that aecia never develop spon-

taneously in simple pustules of P. helianthi; but it has been clearly shown that, under the conditions of the experiment, the percentage of pustules that actually produced aecia was extremely small, not more than 5 per cent. The experiment was not repeated with pustules of P. graminis, but later observations showed that, where monosporidial pustules of this rust were protected from the visits of insects, very few of them developed aecia.

(9) Occurrence Under Natural Conditions  
of Pycnia Unaccompanied by Aecia.

So far as the writer is aware, it is not recorded that pustules of monosporidial origin occur in nature, although undoubtedly such pustules have been seen by other observers and mistaken by them for young pustules in which aecia had not yet developed. Observations made during the summer of 1927 and 1928, in the grounds of the Agricultural College at Winnipeg, Manitoba, have revealed that, in nature, pycnia are frequently unaccompanied by aecia in the following rusts: Puccinia graminis on Berberis vulgaris var.

purpurea; P. coronata Cda. on Rhamnus cathartica;  
P. Pringsheimiana Kleb. on Ribes grossularia  
(cultivated and wild); and a Cymnosporangium sp.  
(possibly corniculans Kern.) on Amelanchier  
alnifolia (Fig. 23).

Leaves of these hosts bearing young and apparently monosporidial pustules were marked by means of a small tag as soon as the pustules were noticed. When the pustules were about 14 or 15 days old, those which showed no signs of aecia were selected for further observation. It was thought that pustules of this age could not include any compound pustules of two mycelia of opposite sex, for compound pustules of this type would have produced aecia when several days younger.

At intervals during the next three or four weeks, the tagged pustules were examined. Within that time, some of the pustules produced aecia, but others did not. A few of the pustules persisted for a week or more longer, but among these there was none which bore aecia. The results for 1927 are summarized in Table I.

It should be noted that none of these pustules were protected in any way from the visits of insects,

Table I.

Summary of Observations made in 1927 on the Occurrence in Nature of Aecia  
in Monosporidial Pustules.

Name of rust	Period of observation (in weeks)	Total number of pustules	Pustules at end of period	
			Number with aecia	Number with- out aecia
<u>Puccinia graminis</u>	4½	50	37	13
<u>P. coronata</u>	2½	61	45	15
<u>P. Pringsheimiana</u>	3	60	16	44
<u>Gymnosporangium</u> sp.	3	60	52	8

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and there is little doubt that insects did actually transfer nectar from one pustule to another, as insects of various kinds were seen flying about, or crawling over, the leaves of the host plants, and some of them were even observed in contact with the pustules. However, it should also be mentioned that, on account of the excessive precipitation, the pustules were frequently washed, and only very seldom was there any noticeable amount of nectar available for transfer. Perhaps the reduction in the amount of available nectar accounts for the failure of so many of the pustules to produce aecia, despite the activity of the insects.

During the summer of 1928, young pustules which were apparently monosporous in origin were again marked, but instead of leaving them exposed, as was done in the previous year, most of the leaves bearing them were covered with one layer of white cheese-cloth in order to prevent insects from visiting the pustules. This protection excluded most of the winged insects, but was less effective against ants and small spiders.

Not all the pustules marked were found as soon as they appeared. At the time the coverings were

applied, some pustules were at least one week old; most of them, however, were just appearing or were not more than two or three days old. Unfortunately, the pustules available for study were much less numerous this year than in the previous year. There were 20 pustules of P. graminis on Berberis vulgaris var. purpurea, 2 pustules of P. coronata on Rhamnus cathartica, and 51 pustules of P. Pringsheimiana on Ribes grossularia. No pustules of the Gymnosporangium sp. occurred on Amelanchier alnifolia.

About one-third of the pustules on Ribes were left unprotected to serve as a check for the protected ones and for comparison with the data collected in 1927. The results for 1928 are summarized in Table II.

It will be seen from Tables I and II and that: (1) a much smaller percentage of the covered pustules in 1928 produced aecia than the uncovered ones in 1927; and (2) that, in 1928, less than 20 per cent of the covered pustules of P. Pringsheimiana developed aecia, as compared with 50 per cent of the ones which were not covered. Apparently, by preventing the free access of insects to the pustules, the



Table II.

Summary of Observations made in 1928 on the Occurrence in Nature of  
Aecia in Monosporidial Pustules.

Name of rust	Covered or uncovered	Period of observation (in weeks)	Total number of pustules	Pustules at end of period	
				Number with aecia	Number with- out aecia
<u>Puccinia graminis</u>	covered	3	20	4	16
<u>P. coronata</u>	covered	3	2	0	2
<u>P. Fringsheimiana</u>	covered	3	33	6	27
<u>P. Fringsheimiana</u>	uncovered	3	18	6	12

opportunity for transferring the nectar of one pustule to another was reduced, and consequently the number of simple pustules which remained free from aecia was augmented. It is not probable that the cheese-cloth covering intercepted sufficient sunlight to inhibit, or retard materially, the formation of aecia. This supposition is supported by the fact that aecia formed in a small number of the covered pustules. The development of aecia in these pustules was very probably induced through the transfer of nectar to them by insects before the coverings were applied.

From the fact that, under natural conditions, monosporidial pustules of P. coronata, of P. Pringsheimiana, and of the Gymnosporangium sp. behave like monosporidial pustules of P. graminis, and, from the fact that P. graminis has been shown experimentally to be heterothallic, it may be inferred that P. coronata, P. Pringsheimiana, and the Gymnosporangium sp. are also heterothallic.

(10) Aecia Limited to One Part of a Pustule.

While the data recorded above were being collected in the field, it was noticed that, occasionally in a pustule, aecia developed only on one side of a pustule and not all over it. This phenomenon was observed in pustules of P. coronata, P. Fringsheimiana, and P. graminis (Fig. 25). A plausible explanation of this behaviour is that nectar from a pustule of opposite sex had been deposited on the upper surface of the pustule but on one side and only over the area under which the aecia had developed.

An attempt was made to reproduce experimentally similar results in monosporidial pustules of P. graminis. Nectar was collected from about a dozen pustules and deposited on a glass slide in one drop. As (+) and (-) pustules were known to occur in about equal numbers, it was anticipated that in the drop both (+) and (-) pycniosperes would be present. The drop was then stirred to distribute the two kinds of pycniosperes evenly through it, and a small portion of this composite nectar was spread over one-half of the

upper surface of a pustule that was twenty-five days old. Several other pustules of the same age were similarly treated. Before this nectar was added to a pustule, however, as much as possible of its own nectar was drawn off by means of a capillary tube in order that the nectar which was being added might be more definitely restricted to the area for which it was intended. Within five days, aecia developed in each pustule on the under side of the treated parts, but not of the untreated parts (Fig. 25).

The experiment was repeated with monosporidial pustules of P. helianthi, and similar results were obtained.

In most of the pustules, particularly in those of P. helianthi, the aecia remained localized in the manner just described, but, in two or three pustules of P. graminis, there appeared to be a tendency for the development of aecia to extend subsequently from the treated half of the pustule, first to the adjacent untreated part, and finally to the remainder of the pustule.

The extension of aecial development just referred to may be explained by assuming that, in

the course of a few days after the composite nectar was applied to the pustules, the pycniospores on the treated parts were carried slowly across the untreated parts by a flowing movement of the nectar; for the upper surfaces of the pustules were usually convex or concave, rarely flat, and day by day, as the pustules secreted more nectar, a state of flux was set up, and thus the composite nectar was brought into contact with the pyenia of the untreated parts. In support of this assumption, it may be mentioned that in pustules of P. helianthi the amount of nectar exuded is much less than in pustules of P. graminis, and that in P. helianthi aecial development was confined to the parts which received the composite nectar. Another possible explanation is that the mycelium of the treated part may have advanced gradually into the untreated part and there in some way or other have caused the development of aecia.

From the above it will be seen that the phenomenon of the occurrence of aecia in one part of a monosporidial pustule, as observed under natural conditions for P. coronata, P. Pringsheimiana, and P. graminis, has been successfully reproduced in the

laboratory in experiments made upon P. graminis and P. helianthi.

(11) Origin of a New Physiologic Form.

During the investigation, some of the aecia which had been caused to develop by the application of nectar to the pycnia of pustules were given to Dr. Newton and Mr. Johnson, of the Rust Research Laboratory, in order that they might identify the physiologic forms represented in these aecia. It was thought that possibly in the telial material that produced the sporidia which led to the development of the pustules two forms of rust might have been present and that, in some of the experiments, hybridization of two forms might have taken place. The form (or forms) present in the parental teliospores was not known; but it was argued that, if a physiologic form hitherto unknown could be identified by sowing the aeciospores on wheat and making cultural studies on the differential wheat varieties, its identification would be partial evidence at least that hybridization had occurred. On the other hand,

there was the possibility that, if a new form was found, it might have been present in nature previously without having been discovered. However, as the survey for physiologic forms had been quite thorough in the vicinity of the Agricultural College where the telial material was collected, it was not very probable that an existing form had escaped detection. The likelihood was that, if any new form could be isolated, it would be the product of a cross, or the result of segregation in a physiologic form, which of itself would be evidence of the hybrid nature of physiologic forms.

From one of the aecial cultures, Dr. Newton and Mr. Johnson (55), in 1928, isolated a new and distinct physiologic form of stem rust. This form has been assigned the number, No. 57, by Stakman and Levine.

#### VIII. Recent Work of Others on Hybridizing Physiologic Forms.

Since the investigation here described was carried out, confirmatory evidence that physiologic

forms of Puccinia graminis do hybridize on the barberry has been obtained by several investigators. The crossing of physiologic forms was effected by the various investigators by transferring pycnial nectar in the manner described by the writer in 1927 and in an earlier part of this paper. Waterhouse (87), in 1929, reported that he had obtained a new form of rust in Australia from a cross made between two other forms on the barberry. Newton, Johnson, and Brown (56), in 1929, working at Winnipeg, found that, when certain physiologic forms are selfed on the barberry, segregation takes place with the production not only of some old forms but also some new ones. The identification of these old and new forms indicated that the original forms must have been heterozygous for pathogenicity and therefore hybrid in their nature. Newton, Johnson and Brown found one form that was homozygous. Furthermore, by making reciprocal crosses between forms, they obtained other forms which were apparently the products of direct crosses. Stakman, Levine, and Cotter (73), in 1929, working at the University of Minnesota, reported successful crosses between Puccinia graminis tritici form 36 and Puccinia graminis agrostis, from



which they obtained forms which were different, in respect to pathogenicity, from both forms.

### IX. Discussion.

Experimental evidence from (1) observations on simple (monosporidial) and compound (bisporidial) pustules and from (2) observations on the effect of transferring pycnial nectar has been presented which shows that simple (monosporidial) pustules of Puccinia graminis and P. helianthi are of two kinds, (+) and (-), and that these two kinds are about equal in numbers. As a deduction, it follows that the mycelia of the pustules, and, therefore, the sporidia from which they originate, are of two different kinds, (+) and (-), and are about equal in numbers. This suggests that segregation of the (+) and (-) factors takes place in the basidium during the nuclear division in the same manner as it was found to take place in Coprinus Rostrupianus by D. E. Newton (54) and in Coprinus radians by Vandendries (83).

A cytological study of the mycelia of P. graminis and P. helianthi has not been made; but, from

the results obtained by sowing two sporidia of the same species close together on a leaf of the appropriate host, a theoretical explanation of what takes place may, for the present, be offered. It is as follows: When a (+) sporidium and a (-) sporidium are sown close together on a leaf so that the pustules arising from the two infections coalesce, the two monosporous mycelia come into contact, fuse together, and give rise to binucleate aeciospores, each conjugate pair of nuclei formed in the spore-bed consisting of a (+) and of a (-) nucleus derived from a (+) and a (-) mycelium respectively. On the other hand, when two sporidia of the same sex, either two (+) sporidia or two (-) sporidia, are sown close together on a leaf so that the two pustules arising from the two infections coalesce, the two monosporous mycelia come into contact but do not interact sexually and consequently do not give rise to aeciospores.

The investigation has not finally disposed of the age-long question regarding the real nature of the pycniospores, yet it has produced some tangible evidence from which certain inferences may be drawn. In the first place, it has shown that pycnia develop in

every pustule of monosporidial origin and that they all produce pycniospores; secondly, that (+) pycnia produce (+) pycniospores, and (-) pycnia (-) pycniospores; thirdly, that the application of pycniospore-containing nectar taken from a (+) pustule to the pycnia of a (-) pustule, or vice-versa, leads to the production of aecia in the pustule to which the nectar is applied; and, lastly, that, when the pycniospores in the nectar are killed by heat, the nectar loses this activating property.

Since pycniospores are produced by the pycnia of every monosporidial pustule, it is clear that, if the pycniospores are male gametes (spermatia), Puccinia graminis and P. helianthi are not dioecious. In other words, the monosporidial mycelia are not of two kinds: (a) male, bearing spermatia, and (b) female, not bearing spermatia.

Assuming that the pycnia are male conceptacles and that the pycniospores are gametes, it is logical to expect that female counterparts would also be present. Such counterparts, however, have not been discovered. Their absence might be explained by their complete degeneration; but, in the evolutionary

development of other fungi, in those forms where degeneration of the sexual organs occurs, it is the male elements that first undergo degeneration. If in the pycnium-producing rust fungi the female structures have entirely disappeared, these fungi occupy a wholly anemalous position among the fungi. Particularly is this true if the pycniospores perform some definite function. The evidence advanced in this paper indicates quite clearly that they do. Complete degeneration has then overtaken the female elements, while the male elements have retained their pristine vigor.

That the pycniospores are not male gametes seems most probable. The writer is inclined to regard the pycniospores as conidia corresponding to the uninucleate oidia of such heterothallic Hymenomycetes as Coprinus lagopus, C. niveus, Stropharia semiglobata, and Collybia velutipes. In these species, oidia are produced by both (+) and (-) monosporous mycelia, and cell fusions between (+) and (-) mycelia initiate the diploid phase.

The pycnia may then be regarded, not as spermagonia producing non-functional spermata, but as active organs which develop either (+) or (-) pycniospores and attract flies and other insects by

means of which the pycnospores of one sex are carried to the pycnia of another sex. They occur chiefly on the upper side of leaves where they are readily accessible to insects, and they are usually red or yellow in color by which means they are made conspicuous. Odors are emitted by the pycnia of some rusts and in many species the nectar is sweetish. These properties, no doubt, are advantageous to the species that possess them in that they attract flies and other insects and thus ensure that the function of the pycnia is fulfilled.

It has long been remarked that, in those rust fungi which possess them, the pycnia are the first spore-producing organs to appear. Since they play such an important part in changing the haplophase into the diplophase and in inducing the formation of aecia, their appearance on the mycelium in advance of the aecia can be readily understood: pycnia precede aecia because by their action aecia are formed.

Under natural conditions, there are at least two ways in which pustules of monosporidial origin may change from the haploid to the diploid condition: (1) by a (+) sporidium and a (-) sporidium settling on a leaf close together so that they form pustules which

coalesce in such a way that the (+) mycelium and the (-) mycelium come into contact directly; and (2) by means of flies and other insects which carry the pycniospore-containing nectar of a (+) pustule to the pycnia of a (-) pustule or, conversely, carry the pycniospore-containing nectar of a (-) pustule to the pycnia of a (+) pustule.

Whether or not a spontaneous change from the haploid to the diploid condition occurs in the mycelia must be further investigated. A phenomenon of this kind occurs in certain Hymenomycetes, but in P. graminis and P. helianthi, the apparently spontaneous change may be tentatively attributed to the fortuitious intermixing of nectar.

The crossing of two physiologic forms of Puccinia graminis, P. helianthi, or of any other heterothallic rust species which behaves like P. graminis and P. helianthi may take place in two ways: (1) by the union, within the tissue of one and the same host plant, of the (+) mycelium of a pustule belonging to one form with the (-) mycelium of a pustule belonging to a different form, or vice versa; and (2) by the application of the pycniospore-containing nectar of a (+) pustule of one physiologic form to the pycnia of a (-) pustule of another physiologic

form, or by the application of the pycniospore-containing nectar of a (-) pustule of one physiologic form to the pycnia of a (+) pustule of another physiologic form. The second method is much more simple than the first, and it was used by all those investigators who have recently succeeded in crossing physiologic forms of rust.

X. Summary.

(1) A study was made of the sexual behaviour of two rusts, Puccinia graminis Pers. and Puccinia helianthi Schw.

(2) An apparatus is described by which single sporidia can be picked off their sterigmata.

(3) Two methods of inoculation are described which make it possible to obtain monosporidial and bisporidial pustules.

(4) Pycnia developed on the upper side of each and every pustule of P. graminis and P. helianthi, and, occasionally, on the lower side, particularly in the peripheral regions of the older pustules.

(5) The pycnia of each and every pustule of P. graminis and P. helianthi exuded nectar containing numerous pycniospores.

(6) Both Puccinia graminis and Puccinia helianthi are heterothallic. Some evidence was secured which indicates that Puccinia coronata, P. Pringsheimiana and a Gymnosporangium sp. (corniculans?) are also heterothallic.

(7) A large majority of the monosporidial pustules of P. graminis and P. helianthi failed to produce ascia.



(8) A small minority of the monosporidial pustules of P. graminis and of P. helianthi produced aecia at some time during the course of their development, possibly owing to a fortuitious mixing of their nectar by handling or through the agency of insects.

(9) Approximately half of the compound (bisporidial) pustules of P. graminis and of P. helianthi produced aecia, while the remainder did not produce aecia.

(10) The monosporidial pustules of P. graminis and of P. helianthi are of two sexes, (+) and (-). The (+) pustules are about equal in number to the (-) pustules. This fact indicates that, in all probability, the sporidia which are produced by the basidia and to which the pustules owe their origin are divisible into two groups, (+) and (-), which are about equal in number.

(11) Intermixing the nectar of monosporidial pustules of P. graminis, or of P. helianthi, induces the formation of aecia in the pustules so treated within from five to six days.

(12) Flies are active agents in carrying the nectar of one pustule to another and in thus effecting the transfer of pycniospores from (+) pustules to (-) pustules, and of (-) pustules to (+) pustules.

(13) Nectar heated to 70°C. for three hours to kill the pycniospores does not induce the formation of aecia in the pustules to which it is applied.

(14) Some monosporidial pustules of Puccinia coronata, Puccinia Pringsheimiana, and a Gymnosporangium sp. (corniculans?), found in nature, failed to produce aecia, thereby indicating that these species are heterothallic.

(15) Nectar of a (+) pustule applied to the pycnia in one part of a (-) pustule induces the production of aecia in that part to which it is applied, and rarely beyond that part.

(16) The origin of a new physiologic form of Puccinia graminis is reported.

(17) Two methods are described by which a cross may be made between two physiologic forms of a heterothallic rust which produces pycnia and aecia.

Acknowledgements.

The writer wishes to acknowledge his indebtedness to Dr. D. L. Bailey, formerly Officer-in-Charge of the Rust Research Laboratory, who was a constant source of help and inspiration to him throughout the investigation. He wishes also to thank Professor A. H. Reginald Buller, of the University of Manitoba, for his unfailing interest in the work, and for many valuable suggestions and much helpful criticism.

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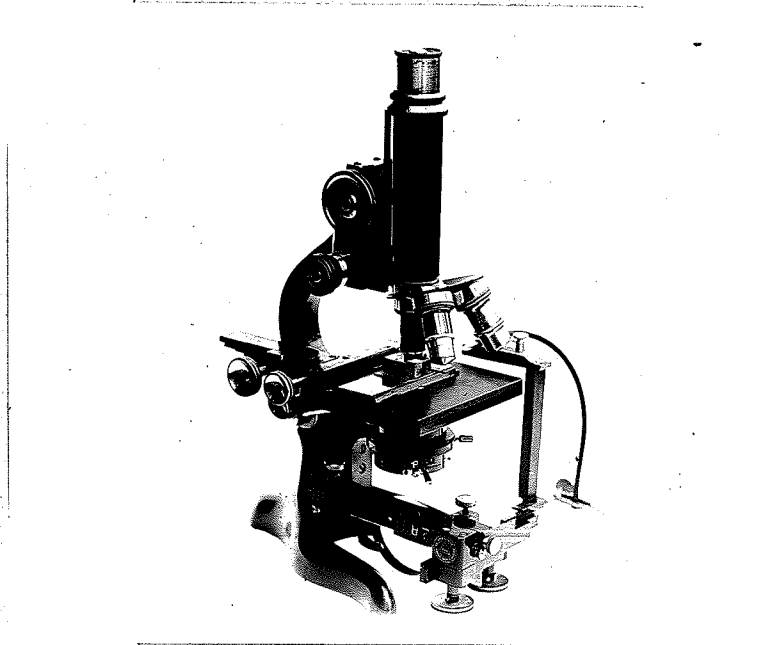


Fig. 1. - View of right-hand side of microscope showing one mechanical stage in the usual position and another one inverted and clamped to the projection on the substage which supported the iris diaphragm.

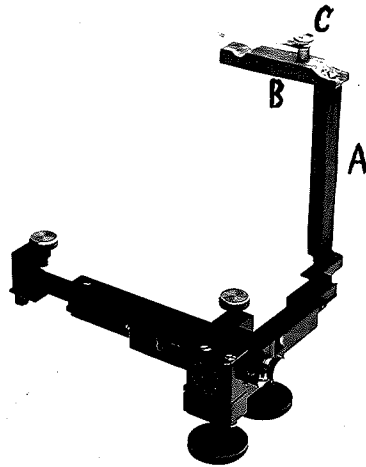


Fig. 2. - View of mechanical stage in inverted position showing: the upright pillar, A; the horizontal arm, B, with its curved notch (towards the left end); and the spring clip, C. The stage is ready to be attached to the microscope.

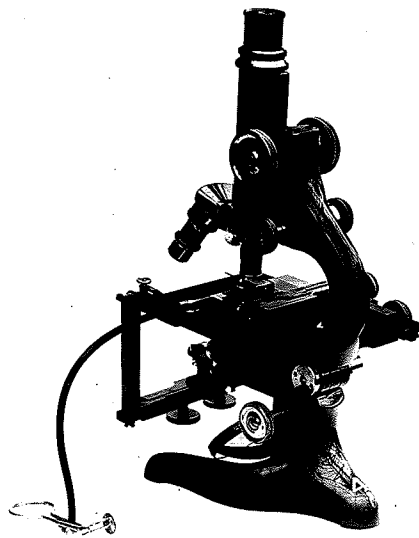


Fig. 3. - View of left-hand side of microscope showing capillary tube with attached rubber tubing in position and ready for use.

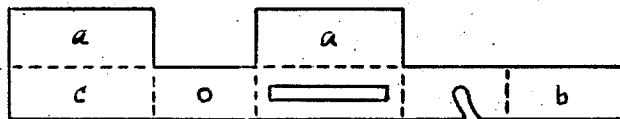


Fig. 4. - Pattern to which a piece of Bristol board was cut to form the walls of a moist chamber. The Bristol board was folded along the dotted lines so that the two wings, marked a, projected outward and lay flat on the surface of the glass slide. The narrow end, marked b, overlapped the corresponding portion of the other end, marked c, and was glued to it. Slightly reduced.

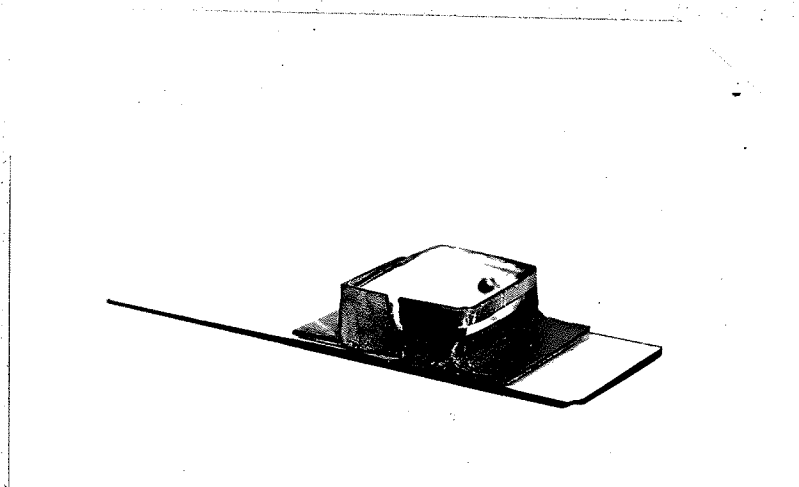


Fig. 5. - Moist chamber showing rectangular opening in front to the right, the notch and hole in the two side-walls, and the filter-paper pad. Slightly reduced.

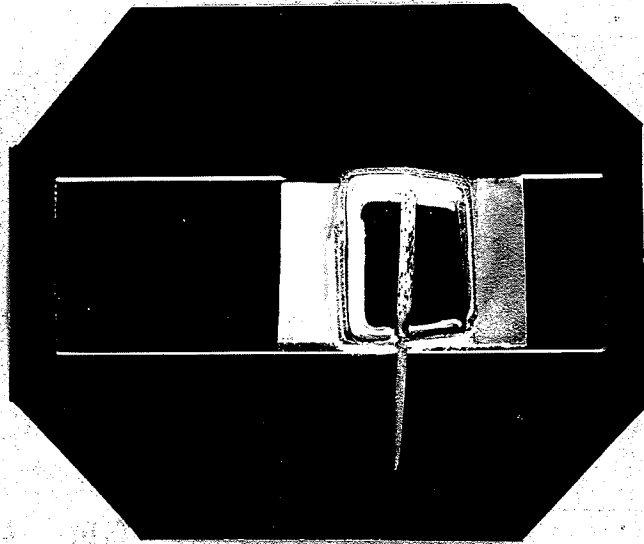


Fig. 6. - Top view of moist chamber  
showing the filter-paper lining (white)  
and the piece of straw bearing telia  
mounted on the pin and ready for use.  
Actual size.

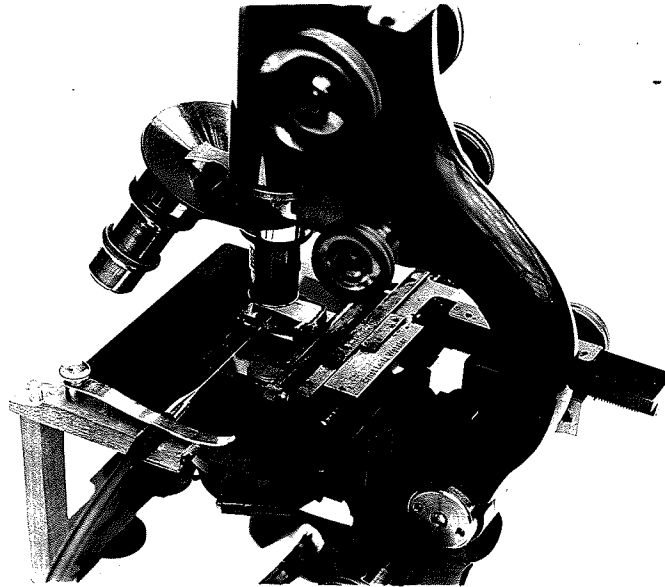


Fig. 7. - View of middle portion of microscope showing moist chamber (cover-glass removed) and capillary tube in position. The end of the capillary tube has passed through the rectangular opening of the moist chamber and has approached the mount of telium-bearing straw which is supported by the wooden pin.

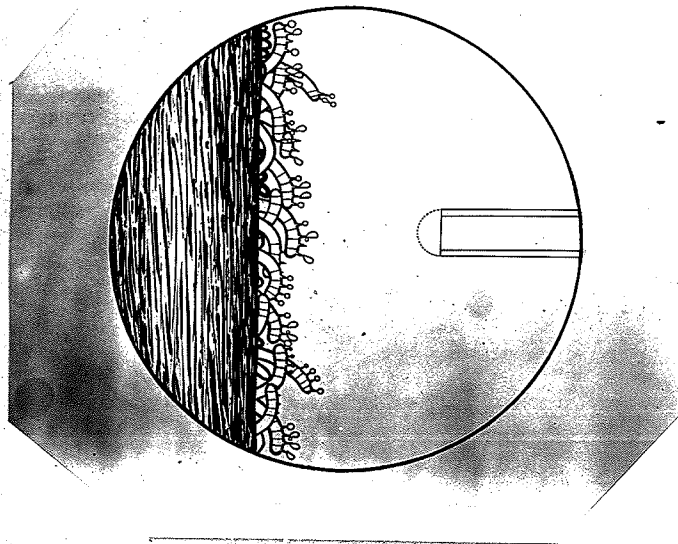


Fig. 8. - Diagrammatic representation of a microscopic field showing the end of the capillary tube approaching a mature sporidium. The dotted half-circle represents the hemispherical globule of water that comes into contact with the sporidium.



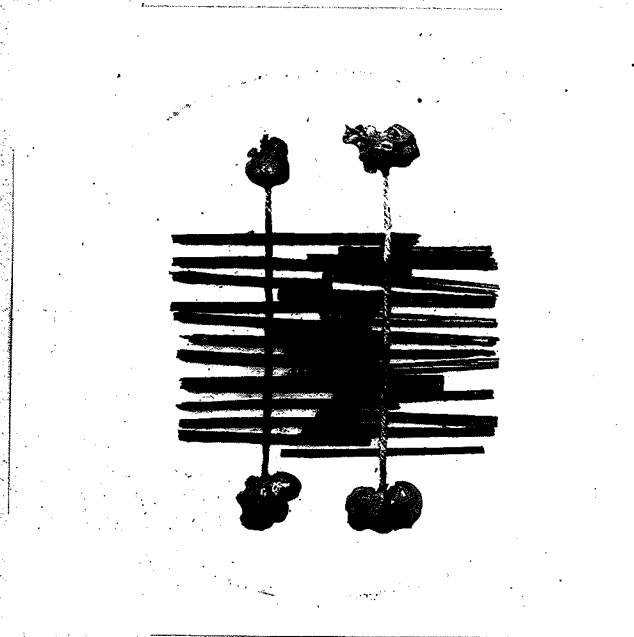


Fig. 9. - Inside of a petri-dish cover showing the method of mounting telial material of Puccinia graminis for inoculation purposes. Reduced.

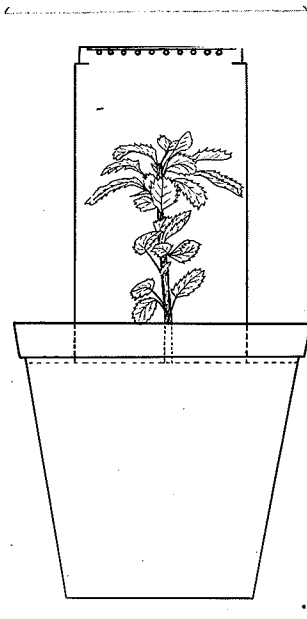


Fig. 10 - Semi-diagrammatic drawing to show a barberry plant being inoculated by method B. The small circles at the top represent short pieces of straw bearing rust spori. The pieces of straw are attached to the inner side of a petri-dish cover which rests on the end of the hollow cylinder surrounding the plant.

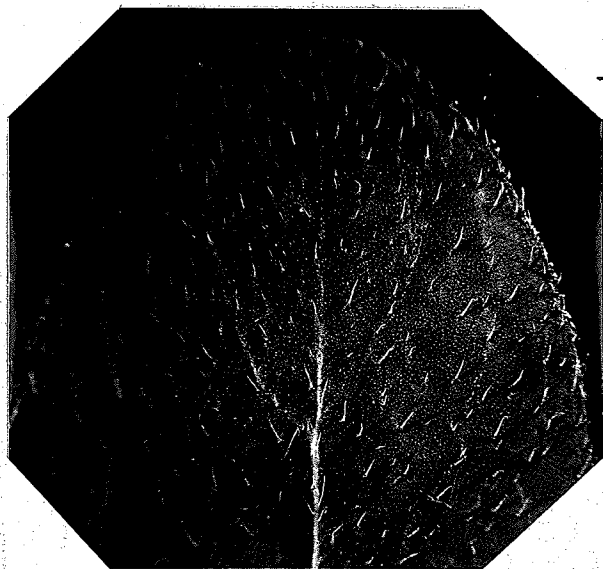


Fig. 11. - Under side of a portion of a sunflower leaf showing two neighboring monosporidial pustules shortly after they appeared. These two simple pustules will soon coalesce to form a compound pustule. Magnification, 2.5.

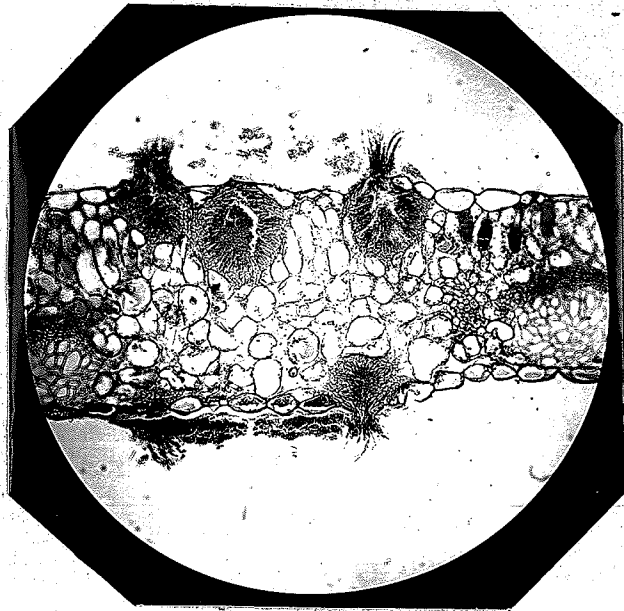


Fig. 12. - Section through a part  
of a monosporidial pustule of  
P. graminis on a barberry leaf  
showing pycnia present on both  
sides of the pustule. Magnification,  
100. Microphotographed by W. F. Hanna.

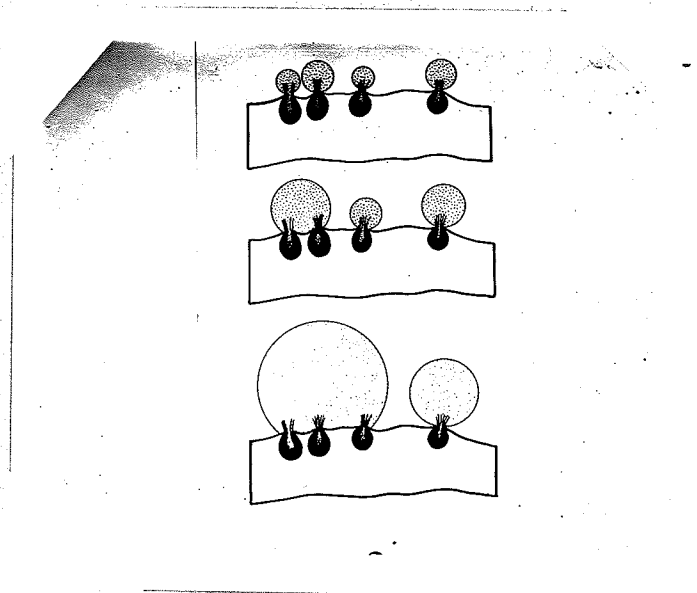


Fig. 13. - Diagrammatic representation of a vertical section through a barberry leaf to show how the globules of nectar produced by the pycnia enlarge and finally fuse to form a layer of nectar over the whole surface of the pustule. The small dots in the globules represent pycniospores.



Fig. 14. - Under side of a portion of a sunflower leaf showing a monosporidial pustule of P. helianthi which has not produced aecia. A few pycnia (dark specks) are seen scattered over the surface of the pustule. Photographed 33 days after inoculation. Magnification, 5.

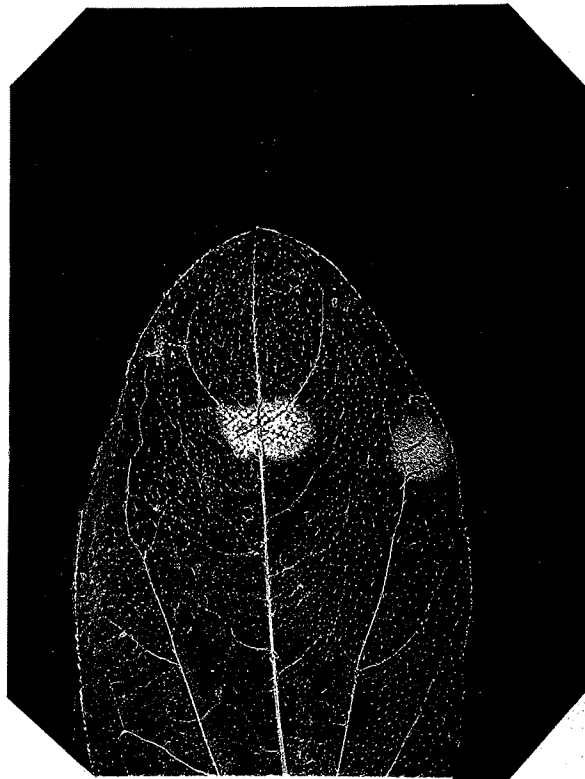


Fig. 15. - Under side of a sunflower leaf showing a compound pustule of P. helianthi astride the midrib, with aecia, and a monosporidial pustule without aecia at the right-hand edge. Photographed 16 days after inoculation. Magnification, 1.75.



Fig. 16. - Under side of a portion of a sunflower leaf showing a compound pustule of P. helianthi withoutaecia. Photographed 24 days after inoculation. Magnification, 4.



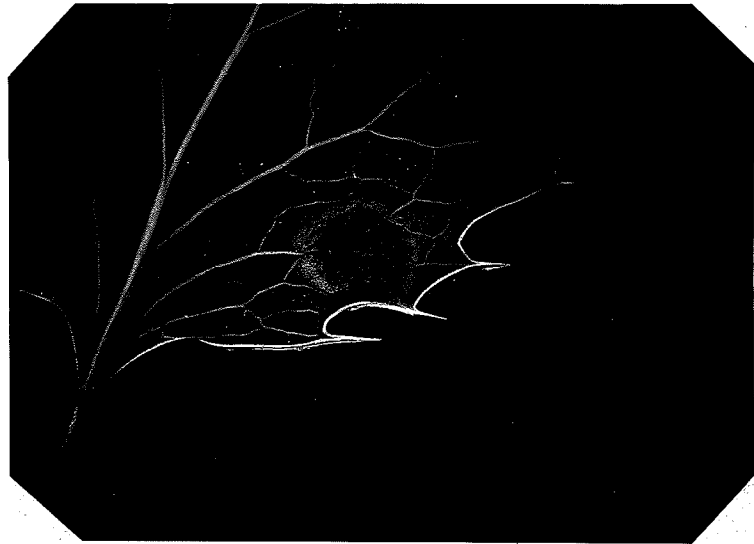


Fig. 17. - Under side of a portion of a  
barberry leaf showing a monosporidial  
pustule of P. graminis without aecia.  
Photographed 23 days after inoculation.  
Magnification, 3.

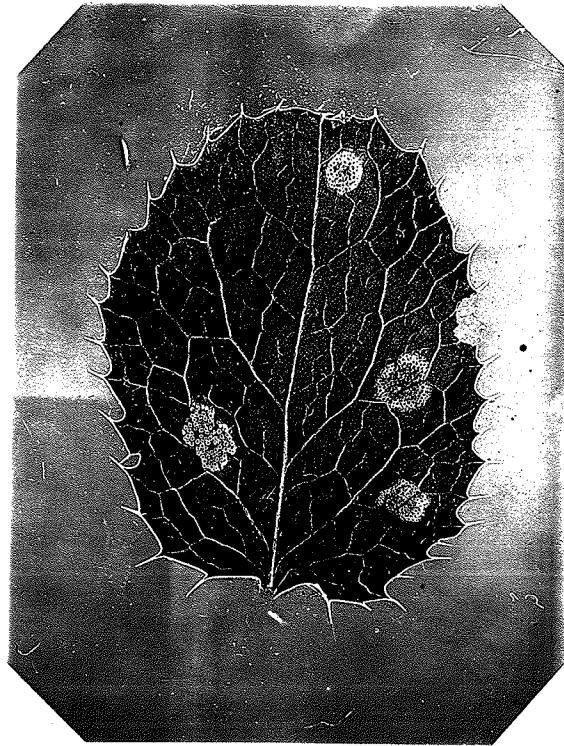


Fig. 18. - Under side of a barberry leaf showing one compound pustule of P. graminis with aecia on the left of the midrib, and three monosporidial pustules without aecia on the right of the midrib. For the peculiar pocked appearance of the three right-hand pustules vide the text. Photographed 20 days after inoculation. Magnification, 2.

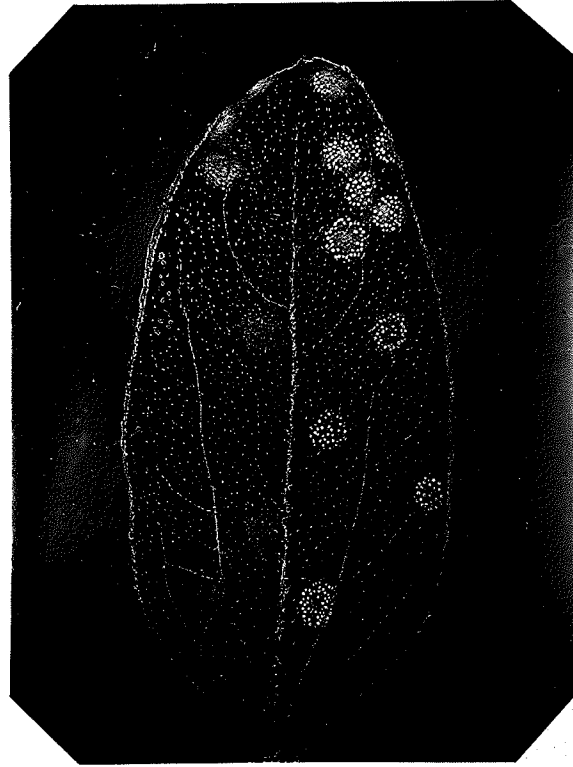


Fig. 19. - Under side of a sunflower leaf showing monosporidial pustules of P. helianthi on either side of the midrib. Twenty days after inoculation, all the pustules were free from aecia. At that time, the nectar of the pustules on the right of the midrib was well mixed; while, as a control, the nectar of the pustules on the left of the midrib was stirred separately but not mixed. The right-hand pustules have all developed aecia, but the left-hand pustules show no aecia whatsoever. Photographed 6 days after the mixing was done. Magnification, 1.5.



Fig. 20. - Under side of a barberry leaf showing mono-  
sporidial pustules of P. graminis on either side of the  
midrib. Thirteen days after inoculation, one pustule, on  
the right of the midrib, produced aecia. All of the others  
were free from aecia seventeen days after inoculation. At  
that time, the nectar of the pustules on the right of the  
midrib was well mixed; while, as a control, the nectar of  
the pustules on the left was stirred separately, but not  
mixed. All the pustules on the right-hand side of the leaf  
have developed aecia, but the left-hand pustules show no  
aecia whatsoever. Photographed 9 days after the mixing was  
done. Magnification, 2.

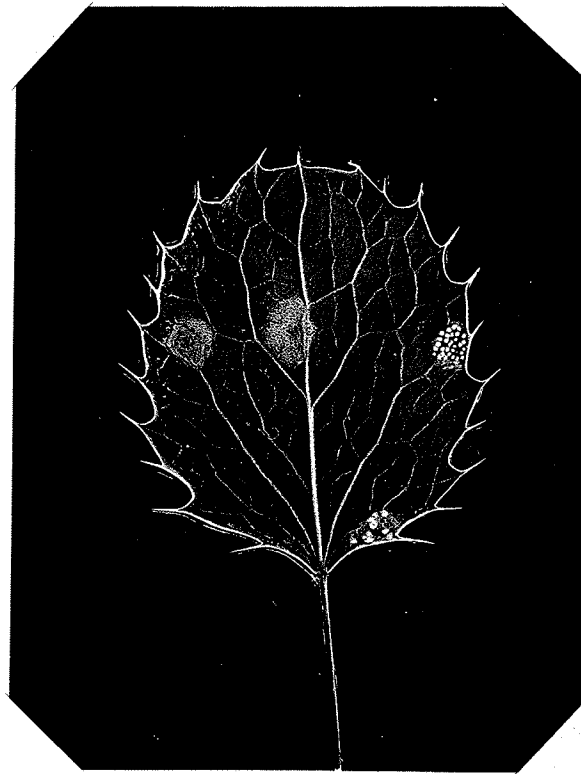


Fig. 21. - Under side of a barberry showing four monosporidial pustules of P. graminis. Sixteen days after inoculation, the pustules were free from aecia. At that time nectar heated for three hours at 70°C. was applied to the upper surface of the two pustules on the left of the midrib; while unheated nectar was applied to the upper surface of the two pustules on the right of the midrib. The right-hand pustules alone have developed aecia. Photographed 8 days after the nectar was applied. Magnification, 1.5.



Fig. 22. - Under side of a barberry leaf showing four monosporidial pustules of P. graminis. The nectar from a monosporidial pustule on another leaf was divided into four small drops and added to the four pustules, one drop to a pustule, when they were 17 days old. Within five days accia arose in two of them but not in the other two. The photograph was taken ten days after the nectar was added. Magnification, 2.

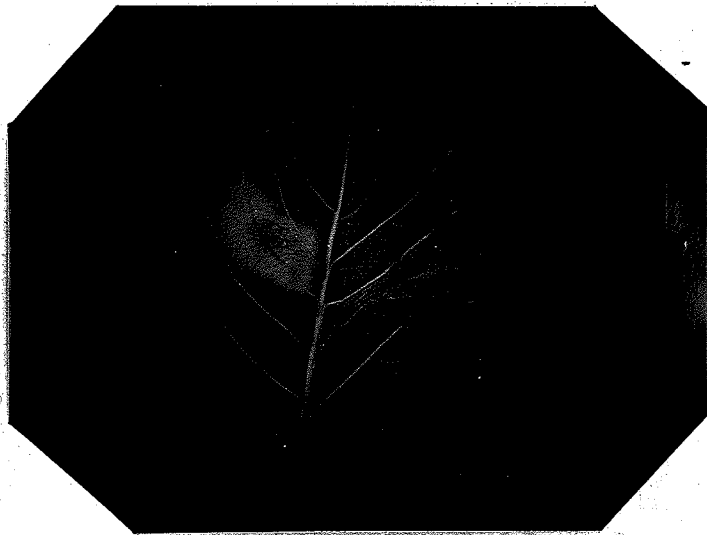


Fig. 23. - Under side of a leaf of Amelanchier alnifolia showing a pustule of a Gymnosporangium sp. (corneculans?) which was six weeks old at the time the photograph was taken. The pustule failed to develop aecia. Magnification, 2.5.

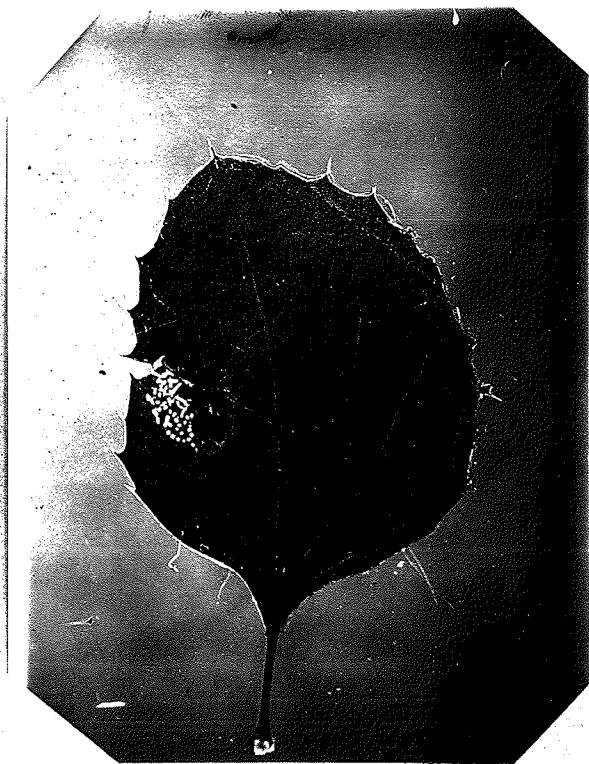


Fig. 24. - Under side of a barberry leaf showing aecia present on the left-hand side, but not the right-hand side of a pustule of *P. graminis*, which arose from an infection by natural inoculation. Magnification, 2.



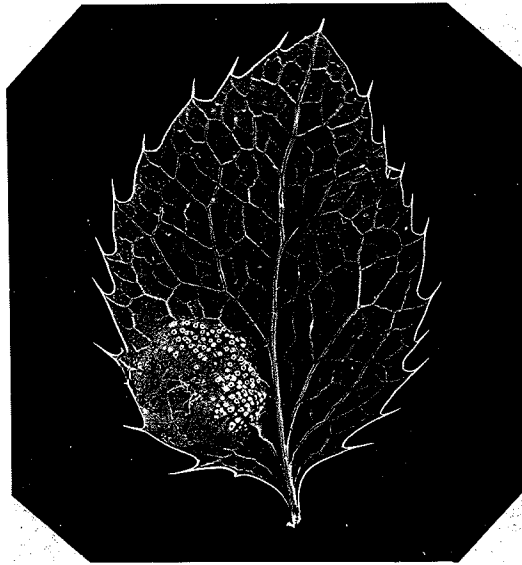


Fig. 25. - Under side of a barberry leaf showing a monosporidial pustule of P. graminis to which, when twenty days old, a small quantity of composite nectar was applied to the upper surface of that part in which aecia have developed, but the other part was left undisturbed. Photographed 7 days after the nectar was applied. Magnification, 2.5.