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CULTURE EXPERMENTS

WITH RUSTS

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THESIS FOR DEGREE OF M. S.
GEORGE R. GAGE

1915

Uredinales

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THESIS

Culture Experiments with Rusts

The Uredinales, also spoken of as the Uredineae or rusts, form a group of fungi which insofar as we understand them represent the extreme of obligate parasitism.

The purpose of this work has been to find out if it is possible to grow these rusts in artificial culture, that is to say, apart from their hosts. Little need be said of the importance of any successful efforts.

Incidentally several experiments have been made on the germination of rust spores, the procedure and results of which have been included in this paper.

Previous Work

In some of the later text books on fungi the following statement, or one similarly worded, has been found:- "In no case has it been possible to grow these organisms upon artificial media, or apart from their hosts beyond the stage of mere germination or promycelial production." (1). Nowhere in the literature on rusts, however, can be found any work the results of which would warrant such a conclusion. Furthermore only one publication whatsoever on artificial rust culture can

(1). Duggar, B. M. Fungus Diseases of Plants. 384. 1909.

be found. Ray (2) makes the following statements: "Sur trente especes d'Uredinees on d' Ustilaginees recueillies, nous avons pu obtenir des cultures pures pour neuf d'entre elles; Charbons du Ble, de l'Avoine, du Mais, de la Saponaire, du Lychnis; Rouilles du Ble, de la Clematite, du Rosier, du Fusain. Dans tous ces cas, il s'est manifeste un developpement vegetatif abondant; les Charbons ont donne une forme levure, seule au debut accompagnee plus tard de mycelium: les Rouilles ont immediatement produit un mycelium, et le Rouille du Rosier a forme une quantite considerable de spores noires cloisonnees." As for media and methods employed:- "Le milieu de culture employe le premier est un bouillon prepare avec des echantillons sains des divers hotes et additionne de gelose. Mais sur d'autres milieux, tels que la carotte cuite a 115°, le lait sterilise, il est possible, pour les charbons surtout, d'obtenir des cultures tres developpees, sinon physiologiquement identiques aux premiers."

Summary of Results

1. A duplication of the methods of Julian Ray, who claims to have succeeded in growing rusts in artificial culture, insofar as they could be carried out from his description, failed to produce rust development

(2). Ray, J. M. Etude biologique sur le parasitisme.
Comptes Rendus De L'Academie Des Sciences.
136:568. 1903.

beyond spore germination. Page 5.

2. The mycelial development of *Puccinia malvacearum* could not, at ordinary temperatures, be produced upon sterilized and sterile cut plugs of vegetables usually used for such purposes. Page 7.

3. The mycelial development of *Puccinia malvacearum* could not be produced at ordinary temperatures, upon media made from decoctions of ordinary vegetables. Page 8.

4. Mycelial development of *Puccinia malvacearum* could not be produced, at ordinary temperatures, upon substrata of filter paper, elder pith, and mallow stems, supplied with a nutrient of sterile (filtered) *Malva rotundifolia* juice. Page 10.

5. Mycelial development of *Puccinia malvacearum* could not be produced, at ordinary temperatures, upon mallow stems rendered sterile by removal of the epidermis, the stems being supplied with nutrients of sterilized mallow juice, (autoclaved), sterile filtered mallow juice (Chamberlain filter), and sterile distilled water. Page 11.

6. Mycelial development of *Puccinia valvacearum* could not be produced, at ordinary temperatures, upon the following media:- Plain agar, glucose agar, nutrient agar, Ashby's synthetic agar, and nutrient gelatin. Page 12.

7. Mycelial development of *Puccinia coronata* could not be produced, at ordinary temperatures, upon oat seedlings, sterilized in the autoclave and also by exposing

to 60° for 1 hour on 5 consecutive days. Page 14.

8 and 9. Mycelial development of *Puccinia malvacearum* could not be produced, at ordinary temperatures, upon various agar media, constantly washed with water (distilled) to carry off by-products of the fungus which might be injurious to itself. Page 15,16.

10. Mycelial development of *Uromyces caryophyllinus* could not be produced, at ordinary temperatures, upon a porous cup atmometer constantly supplied with a nutrient solution and in a slightly greater concentration of Carbon Dioxide than in ordinary air. Page 17.

Germination experiments:-

1. Carnation rust urediniospores exhibited no heliotropic curvatures when subjected to unilateral diffused daylight. Equilateral light, unilateral light, and total darkness, seemed to have no effect upon the percentage of germination or upon the incubation period. Page 18.

2. Carnation rust urediniospores exhibited no thermotropic curvatures when subjected to unilateral low or high temperatures. Page 19.

3. *Puccinia coronata* urediniospores exposed to a variation of temperatures act as follows:- Spores at all temperatures from 16° to 36° begin to swell at the germ pores at the same time. The germ tube emerged also

at the same time. At a temperature of 18°, however, not only did a higher percentage continue their growth, but the rate of growth was somewhat increased. Page 20.

4. Alternate wetting and drying urediniospores of *Puccinia coronata* had no effect upon the incubation period. The incubation period was the same for spores in a strong glucose solution as in double distilled water, likewise the emergence of the germ tube, but the rate of growth of the tubes was somewhat increased in the sugar solution. Page 22.

Procedure

1. Ray's methods were duplicated insofar as they could be ascertained from his description.

Stems and leaves of *Malva rotundifolia* were collected from non-infected parts of susceptible plants. These were ground up and the juice strained through cheese cloth. Half of the juice was then filtered through filter paper and the other half through a Chamberlain filter. To the former distilled water and agar were added in the proportions: 500 cc. mallow juice, 500 cc. water, 20 grams of shredded agar. This was steamed for two hours, filtered through cotton, tubed and sterilized in the autoclave 20 minutes at 20 pounds pressure. To the second lot, water and agar

were added as follows:- 500 cc. water and 20 grams of agar were steamed, filtered through cotton, and sterilized twenty minutes at 20 pounds. This was cooled to 45° and mixed with the mallow juice which had been warmed to 45°. The mixing was done in a culture room, all precautions against contamination being taken.

Thus two different media were obtained, the first with the mallow juice subjected to heat and the second with it in its natural condition as far as temperature was concerned.

Teliospores of *Puccinia malvacearum* were brought in directly from the field and used in making both poured and sprayed plates with each agar. Tubes of each agar were also inoculated with spores and with the rust mycelium itself. The mycelium was obtained from stems on which the flecks were just appearing as yellow spots. These were washed in tap water, immersed in HgCl_2 (1-1000) and rinsed in sterile distilled water. The mycelium was picked from the flecks with a sterile needle.

The plates and tubes were examined for a month or so, but nothing more than mere germination of the spores resulted. In a few tubes and plates there was present contamination which proved to be either a *Fusarium*, *Alternaria*, *Mucor*, or *Penicillium*.

Since Ray's description of his methods is so brief, we can only assume that it has been duplicated.

However, it is rather difficult to refrain from drawing the conclusion that his "developpement vegetatif abondant" and "quantite considerable des spores noires cloisonnees" were rather illusionary or, better, the mycelium and spores of some other fungus than rust.

2. Vegetables used as media:-

Carrots, white potatoes, sweet potatoes, pumpkins, celery, sugar beet, turnips, cabbage stalks, bean stems, bean pods, and mallow stems were prepared in two different ways, as follows:-

First set. The vegetables were scrubbed in tap water, placed in HgCl_2 (1-1000) for 15 minutes, and rinsed in distilled water. Plugs were then cut with a sterile (flamed) knife and placed in tubes containing a little moist cotton. These were sterilized for 20 minutes on each of three consecutive days in the Arnold steamer.

Second set. The vegetables were scrubbed in tap water, placed in HgCl_2 (1-1000) for 15 minutes, and rinsed in sterile distilled water. The plugs were cut in a culture chamber with a sterile (flamed) knife, the knife being sterilized after each direct cut. These were then placed in sterile tubes containing moist cotton. They were not steamed.

Tubes of both kind of plugs, sterilized and sterile

cut, were inoculated with *Puccinia malvacearum* teliospores and mycelium from young flecks of the same rust.

The tubes were examined for a month or more but nothing developed which gave any indication of rust. Eleven of the sixty-six tubes inoculated were contaminated with either *Fusarium* or *Mucors*. All the plugs were finally macerated and examined microscopically, but no internal mycelial development of rust was found.

3. Plant decoctions used as media:-

Prune, oatmeal, cornmeal, potato, cucumber, bean, celery, carrot, pumpkin, beet, cabbage, and turnip were used. They were prepared as follows:

Prune:- 120 grams dried prunes

1000cc double distilled water

Steamed in Arnold for 2 hours

Filtered through cheese cloth

Restored to original volume

12 grams shredded agar added

Steamed in Arnold for 2 hours

Cleared with egg white

Filtered and adjusted to +12 (Fuller's scale)

Tubed and autoclaved 20 minutes

at 20 pounds.

Oatmeal:- 50 grams oatmeal

350 cc double distilled water

Steamed in Arnold 2 hours

Filtered through cheese cloth

10 grams of shredded agar added

Double distilled water added to
make 500 cc.

Steamed in Arnold 2 hours

Filtered and adjusted to +12

Tubed and autoclaved 20 minutes
at 20 pounds.

Cornmeal:-1000cc double distilled water

3 tablespoons of cornmeal

Steamed in Arnold for 2 hours

Decanted and restored to original
volume

15 grams shredded agar added

Steamed in Arnold for 2 hours

Filtered and adjusted to +12

Tubed and autoclaved 20 minutes
at 20 pounds.

Cucumber, bean, celery, carrot, pumpkin,
beet, turnip, and potato:-

250 grams of ground up vegetable

1000 cc double distilled water
Steamed in Arnold for 2 hours
Decanted and restored to original
volume
20 grams of glucose added (except beets)
• 35 grams of shredded agar added
Steamed in Arnold 2 hours
Filtered and cleared with egg white
Adjusted to +12
Tubed and autoclaved 20 minutes
at 20 pounds.

Plates were made by the dilution method and also by spraying. Tubes were inoculated directly. Both spores and mycelium of *Puccinia malvacearum* were used in each case, the infected plants having been brought into the greenhouse.

The plates and tubes were examined daily for a month or so, but no sign of rust development appeared. A contamination of *Mucors* was present in a few cases, especially in those tubes inoculated with spores.

4. Special decoction of mallow leaves:-

A special decoction of mallow leaves was made as follows:-

Enough leaves were ground up and pressed

to give 20 cc. of juice. This was diluted 5 times and filtered through a Chamberlain filter. Tubes containing as substrata filter paper, elder pith, and mallow stems and leaves, were sterilized and 3 cc. of the decoction poured into each.

The tubes were then inoculated with *Puccinia malvacearum* spores and mycelium as in the previous experiment. These were examined externally for a month but no trace of rust development appeared. At the end of the month the substrata were macerated and examined, but no internal development could be found.

5. Special substratum of mallow stems:-

Mallow stems were washed in tap water and placed for 15 minutes in HgCl_2 (1-1000). After rinsing in double distilled water the epidermis of each was carefully skinned off. This process was carried on in a culture chamber and with sterile (flamed) instruments. A decoction of mallow juice was prepared as in the previous experiment. A similar decoction but autoclaved instead of filtered was also prepared. Using the stems as plugs and the two decoctions and sterile distilled water as nutrients, tubes of each were inoculated with spores and mycelium of the same rust, *Puccinia malvacearum*.

At the end of a month, no external development

of rust appearing, the stems were macerated and examined. These, however, were also free of internal mycelial development.

6. Special agar media used:-

Plain agar:- 1000cc double distilled water

15 grams of agar

3 grams of beef extract

10 grams of peptone

Titrated to +12 (Fuller's Scale)

Cleared with egg white

Filtered and tubed

Autoclaved 20 minutes

at 20 pounds

Glucose agar:-

1000cc double distilled water

15 grams of shredded agar

3 grams of beef extract

10 grams of peptone

20 grams of glucose

Titrated to +12

Cleared with egg white

Filtered and tubed

Autoclaved for 20 minutes

at 20 pounds.

Nutrient glucose agar:-

1000cc double distilled water
40 grams of glucose
3 grams of NaCl
20 grams of peptone
5 grams of beef extract
30 grams of shredded agar
Cleared with egg white
Filtered and tubed
Autoclaved for 20 minutes
at 20 pounds.

Ashby's synthetic agar:-

1000cc double distilled water
20 grams of mannite
0.2 grams K_2HPO_4
0.2 grams H_2SO_4
0.2 grams NaCl
0.1 gram $CaSO_4$
5.0 grams $CaCO_2$
15 grams powdered agar
Tubed and autoclaved for
15 minutes at 15 pounds.

Nutrient gelatin:-

1000cc of boullion
110 grams of gelatin.
Titrated to +10 (Fuller's scale)
Tubed and steamed 30 min.
on each of three consecutive
days.

Tubes of each of the above media were inoculated with spores and mycelium of *Puccinia malvacearum* as before. Plates were also poured and sprayed with spores and mycelium. Again no rust development took place. A contamination of *Fusarium* was present in several tubes.

7. Oat seedlings as media:-

Oat seedlings were grown and when they had reached the height of five or six inches were placed in tubes kept moist with wet cotton and sealed with wax. Half of the tubes were sterilized in the autoclave at 20 pounds for 20 minutes. The other half were placed in a water bath at 60° for one hour on each of five consecutive days. These tubes were then unsealed and inoculated with uredinio-spores and mycelium of *Puccinia coronata*, infected oat plants having been introduced into the greenhouse.

These tubes were kept for two months and very carefully examined from time to time. No signs of rust

appeared externally and when the seedlings were macerated nothing was found to have developed internally. In some of the checks and also in a few inoculated tubes a *Sporotrichum* was found to have developed.

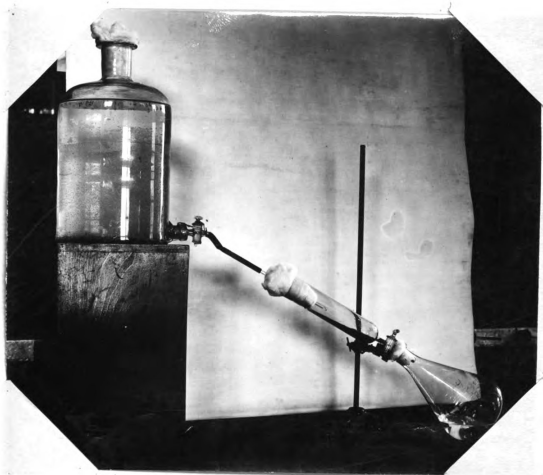
8. Constantly washed media:-

On the assumption that the rust mycelium produces a by-product injurious to itself but carried off by the susceptible host the following experiment was made:

Several of the various media used previously were put on glass slides in drops. They were inoculated with *Puccinia malvacearum* spores and mycelium. Each slide was then clamped between two strips of filter paper and the whole used as a siphon in double distilled water. Thus the media were kept constantly moist and any by-produce of the fungus was washed away.

Again negative results only were obtained. Contamination of course was very great, so a similar experiment in which contamination would be prevented was planned.

9. Tubes of agar made from decoctions of oat leaves and mallow leaves were used as shown in the photograph below.

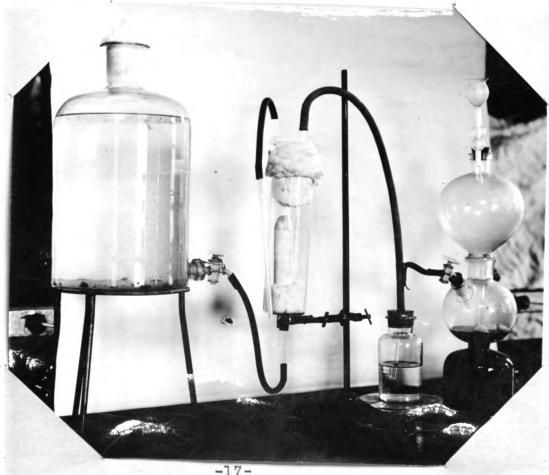


The tubes were sterilized and sterile water was allowed to drip constantly over the inoculated surface of the agar. No development of rust resulted, nor was there any contamination.

10. Medium of CO_2 increased:-

Thinking that slightly greater concentration of CO_2 in the intercellular leaf spaces may have some influence upon fungus development, this factor was introduced as follows:

A porous cup atmometer was placed in a glass cylinder and connected to a feed tube through the bottom of the same. Cotton was used in both ends of the cylinder to prevent contamination. Through the upper plug two small glass tubes were fitted, one for the entrance of CO_2 from a Kipp generator and the other tube for an exhaust. See photograph. The feed tube



of the porous cup was connected to a large flask containing the following solution: 2000 cc. double distilled water, 1 gram glucose, and 1 gram of asparagin. The whole was then sterilized in the autoclave. The CO₂ was allowed to flow very slowly into the cylinder and a circulation was accomplished by connecting the exhaust tube to a suction pump. Urediniospores of *Uromyces caryophyllinus* were spread freely upon the surface of the atmometer and the apparatus was then run for 18 days. At the end of that time the atmometer was examined but no rust development was noticeable. A contamination of *Penicillium*, however, was present.

It is the intention of the writer to carry on a series of similar experiments with different nutrient solutions when time permits.

Germination Experiments

1. Light effects:-

A search of the literature brought forth three instances in which a phototropic reaction was noted in germination of rust spores. Ward (1) states that in some of his work the germ tube of urediniospores

(1). Ward, H. M. Ann. Bot. 16:267. 1902.

of *Puccinia dispersa* exhibited phototropic curvatures. Robinson (1) has shown recently that the sporidial germ tubes of *Puccinia malvacearum* react negatively to daylight. Fromme (2), working with urediniospores of *Puccinia Rhamni*, has likewise shown that the germ tubes of these spores exhibit negative phototropism. The writer using methods similar to those of Fromme has tested out urediniospores of *Uromyces caryophyllinus*. Spores were dusted over the surface of plain gelatin in plates containing moist filter papers. Checks were maintained in total darkness and equilateral diffused daylight. Those exposed to unilateral daylight were placed on a window ledge in dark boxes with an aperture of 1 cm. in diameter toward the window. This experiment was repeated at least 12 times, but in no case did those spores subjected to unilateral light show any phototropic curvatures whatsoever. It was noted also that the time for germination and percentage of same was practically the same in total darkness, unilateral light, and equilateral light.

2. Thermotropic influences:-

In view of the fact that evaporation from a

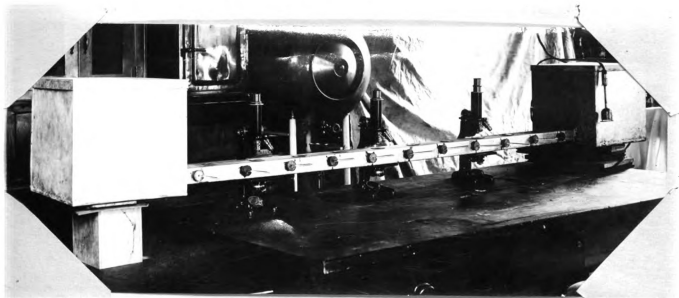
(1). Robinson, W. Ann. Bot. 28:331-340. 1914.

(2). Fromme, F. D. Journal Bot. 2:82-85. 1915.

leaf is greater at the stomata, it was thought that the lower temperature due to this greater evaporation may have some effect upon the direction of germ tube growth. The surface of plain gelatin in plates with moist filter paper was dusted with urediniospores of *Uromyces caryophyllinus*. These plates were kept in boxes with bags of cracked ice at one end and with the other end in contact with an incubator of 37°. No thermotropic curvatures, however, resulted.

3. Temperature effects:-

To test out accurately the effects of temperature without varying other factors a piece of apparatus as shown in the photograph was constructed and used.



This consists of a water-tight tube the ends of which

are enclosed in two large tanks. One tank is kept constantly filled with ice and the other with hot water, heated with an electric immersion boiler. The tube is fitted with thermometers at regular intervals. Along the top and alternating with the thermometers are small holes just large enough to admit Van Tieghem cells. These cells are held in place by means of rubber washers. Under each cell there is fitted in the tube a glass window to admit light in order that microscopes can be used in watching germination. When in operation a quite regular variation in temperature is obtained along the tube. This is not as constant as is preferred but with careful manipulation the change is never more than one degree.

Urediniospores of *Puccinia coronata* were used. Samples were always taken from the same fleck in order to get them of the same age and produced under the same conditions. The temperature range used was 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36 degrees C. At all temperatures the swelling of the germ pores seemed to take place at about the same time and with about an equal percentage. The germ tubes emerged also at the same time at all temperatures but it soon became noticeable that at a temperature around 18° not only did a higher percentage continue their growth but that the rate of growth was also increased.

4. Alternate wetting and drying of rust spores:-

The purpose of this experiment was to note any effect of alternate wetting and drying upon the incubation period of rust spores. The actual wetting and drying process is hardly possible without changing other factors so another method had to be employed. Since spores contain some moisture in their natural condition, it was therefore concluded that wetting and drying is no more or less than changing the concentration of the spore content. Taking advantage, then, of osmotic forces, the spores were subjected alternately to double distilled water and a strong sugar (glucose) solution, the former being the wetter, the latter the dryer. The following method was used:-

Gooch crucibles were fitted with filter paper discs which had been cut out with a cork drill. Urediniospores of *Puccinia coronata* taken from one fleck were divided into three lots. The first was placed immediately in Van Tieghem cells in drops of double distilled water. The second was placed in Van Tieghem cells in drops of the strong sugar solution. The third lot was placed in one of the crucibles and was subjected alternately to distilled water and sugar solution by means of wash bottles. The solution was changed every minute, quickly

changes being accomplished by blowing out the solution through the filter paper discs. The spores were subjected 25 times to each solution in as many minutes. These were then also placed in Van Tieghem cells in drops of double distilled water.

The three sets were carefully watched through a microscope with the following results: The germ pores in all cases commenced swelling at the same time; the germ tubes emerged at the same time; and the percentage of germination was equal. However, it was noted that the rate of growth was greatest from spores which had been subjected only to the sugar solution, next from the spores which had been wetted and dried, and least from the spores which had been subjected only to double distilled water.

The experiment was repeated several times with similar results. It was concluded, therefore, that wetting and drying, or at least, changing the concentration of spore contents has no effect upon the incubation period, but that the concentration of the media has some effect upon the rate of growth of the germ tube.

Conclusions

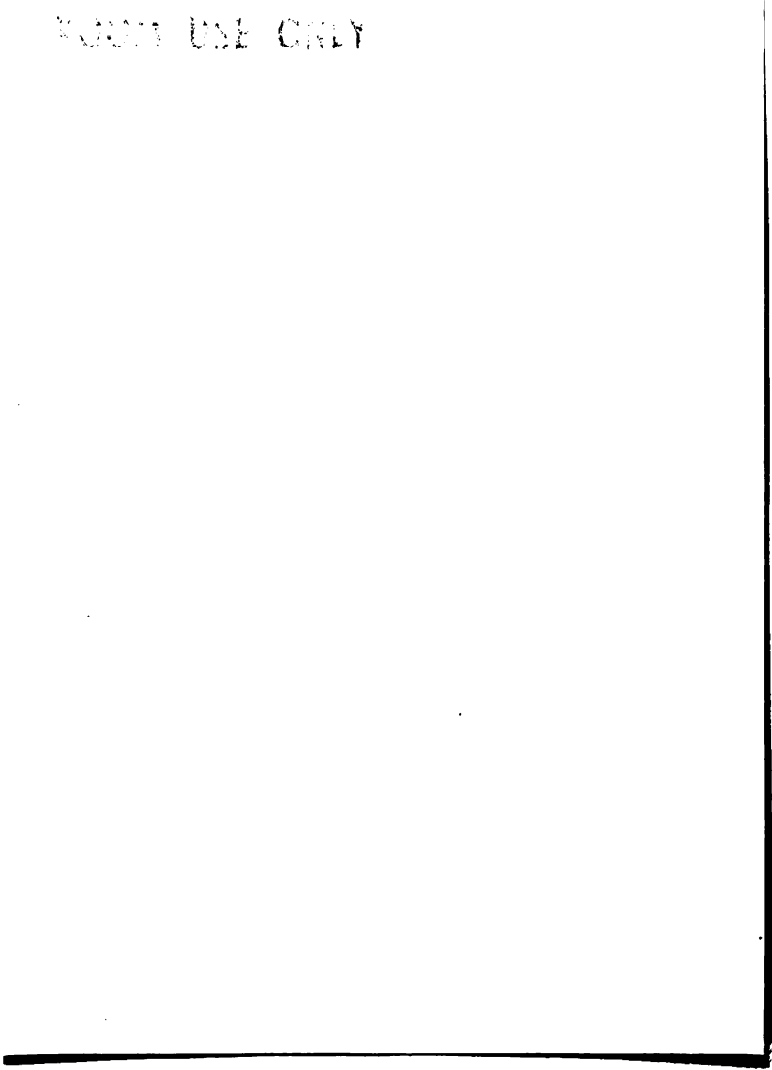
Although time permitted the testing of little more than ordinary methods of artificial culture, we can at least conclude that rusts are extremely parasitic, that is, restricted to a very high degree to living substrata. The fact that in nearly all methods used, contamination was present, substantiates the above conclusion, in that the contamination indicated favorable conditions for the growth of various other forms of fungi. The work was hardly extensive enough to go so far as to state that rusts cannot be grown in artificial culture, but it nevertheless leads one to believe that in the present state of our knowledge of the symbiotic nature of rusts and hosts the former cannot be grown separately.

The results of the experiments on spore germination, especially those on the effect of temperature and concentration of medium, bring forth a new aspect of the problem. Since the mere emergence of the germ tube must be called germination, then the above factors have, within certain limits, no effect upon the ability of the spores to germinate. On the other hand, however, the results plainly show that these factors do influence the germ tube after its emergence by restricting it entirely or by regulating its rate of growth.

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