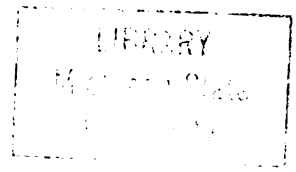

A MACROSPORIUM LEAF SPOT DISEASE OF RED CLOVER.

(MACROSPORIUM SARCINAEFORME CAV.)

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Thesis for the degree of Master of Science.

Michigan Agricultural College

Department of Botany

L. J. Krakover

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INTRODUCTION

The diseases of red clover are so common that little attention seems to be given to them. Rarely is a specimen sent in to the Botanical Dept. for identification. These diseases are are widespread wherever clover is grown, and tho the loss they cause has not been definitely determined, it appears to be large. The culture of red clover is rapidly being discontinued because of fungus diseases and other troubles, in favor of other legumes. The general abandonment of a crop of such agricultural importance has given rise to apprehension. The questions to be settled are (1) whether red clover with its wide agricultural utility can be profitably replaced by other legumes,- alfalfa for example; (2)whether the troubles which are making the crop unprofitable can be remedied. The very important hindrance to the culture of red clover known as "clover sickness" may eventually be eliminated by proper soil management. The fungus diseases of the crop, in order to be controlled,at once open up a big field of endeavor. The possibility of breeding resistant strains, the introduction and the testing of foreign strains which in themselves might be resistant, the study of the methods by means of which the diseases are disseminated, and the modification of cultural methods, suggest themselves when considering the problem of control.

This paper deals with a leaf spot disease of red clover, caused by Macrosporium sarcinaeforme Cav. This disease was very serious in the vicinity of East lansing, Mich. during the entire growing season of 1915. The various phases which enter into the study of a plant disease will be taken up more or less in their usual order.

THE RED CLOVER CROP

The importance of the red clover crop is too well known to require an extended discussion here. The following brief summary is given because of its importance, distribution and commoner cultural methods are given because of their bearing on the disease and methods of control:

Importance and distribution:- Red clover is by far the most important leguminous crop grown in America. The area devoted to it, according to Piper*, is about five times that devoted to alfalfa. The total acreage in 1909 reached almost 12,000,000. The greater part of this is grown in the northern states west of the great plains and on the Pacific coast, but it is of little importance in southern and semi-arid states. It is essentially a crop for humid regions without excessive summer or winter temperatures. Dry atmospheric conditions are very unfavorable as is high temperature combined with humidity.

Utility and culture:- Red clover is really a short lived perennial but as a crop is treated as biennial. It has a very wide agricultural adaptability: as a forage crop, hay, pasture, silage, green feed, and as a cover crop for orchards; as a nurse crop it is especially valuable when sown with wheat, barley rye, or oats, and it also does well with corn or a root crop. If sown alone, a good stand may often be secured but this is seldom done unless all other methods prove a failure.

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* Piper, p. 362.

If sown in a mixture with timothy or other grasses a good hay crop may be secured. Being a biennial it is adapted to a short rotation. A five-year rotation of corn, oats, wheat, clover, and timothy is commonest in the central states. Corn, clover, or corn, corn, clover are the simplest rotations, but the clover is repeated too often to give the best results.

Sowing is best done early in the fall in order to avoid excessive winter-killing, or in the spring by broad casting upon the fall-sown grain. The seed should be planted shallow,- 1 - 1-1/2 in. deep,- in order to get the best results. The first cutting yields a heavy hay crop, while the second is usually allowed to mature for seed. "If cut for hay, different authorities recommend cutting in full bloom, in young bloom, and when the heads are half brown. The contents of the digestible nutrients is greatest in full bloom. Later cuttings, however, cure more easily than the earlier ones, and it is possible that the better curing counterbalances largely the lower content of nutrients."* If used for seed the clover should be cut when all of the heads have turned brown and the seed is firm and shiny. The commonest method of harvesting is by mowing and raking into windrows. Under favorable conditions the clover is ready to store within about four days, if cut in the late dough stage. It is then piled into cocks or best kept under cover, as during wet weather there is danger of the seeds sprouting if kept continuously damp.

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* Piper, p. 380.

Clover diseases in general: The most common diseases of clover are those which attack the foliage. Since the leaves constitute the most valuable part of the hay, the defoliation which results from these disease may cause considerable loss. Perhaps the most widespread of all diseases on plants of the genus Trifolium , is the rust caused by species of Uromyces. Uromyces is cosmopolitan, and the most susceptible hosts are important forage plants. Red clover, (T. pratense) alsike clover, (T. hybridum) white clover, (T. repens) and crimson clover, (T. incarnatum) are the most commonly infected. There is no exact knowledge of the relation of the disease to climatic conditions. It varies with the season, and is to a considerable extent determined by spring conditions. Stevens and Hall* state that it is most injurious to the second crop, to which it may cause a damage of 20-50% if the weather is cool and damp.

A very common leaf spot is caused by Pseudopeziza trifolii(Pers.) Fckl,, and is at times the cause of much damage. This fungus is closely related to Pseudopeziza medicaginis(Lib) Sacc., the leaf spot of alfalfa., with which it may be identical. The sooty spot caused by Polythrincium trifolii Kze. (Phyllachora trifolii(Pers.) Fckl.) is distributed upon species of clover in many parts of the world, especially on red clover. Another leaf spot which is widely disseminated, but seems to have attracted little attention, is that caused by the black mold, Macrosporium sarcinaeforme Cav,, the disease to be discussed in this paper.

The most serious disease of the clover stem and petiole is the anthracnose caused by Colletotrichum trifolii Bain.

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* Bibliography given on page

Bain and Essary,(1908) in investigating the cause of the failure of the red clover crop in Tennessee found this to be the chief agent. It is found mostly on the stems near the ground or just below the flower cluster., and but rarely on the leaves. The elongated, sunken spots which are formed, result, eventually, in the death of the plant. The fungus also attacks alfalfa, but alsike clover seems to be nearly immune. Thus far the disease has already been found in Tenn., Ohio, W. Va., Ky., Del., Ark., Va., and My. Another anthracnose caused by Gloeosporium cauli-vorum is said to cover nearly all of Europe where red clover is g grown. Plant grown from European and American seedsuffer alike. The brown to black sunken spots on the stems and petioles cause the death of the more distal parts of the plant. The disease is said to be carried by the seed and wet weather is favorable to the spead. Seed tratment with one percent Bordeaux has been recommended as a control. The Gloeosporium may be identical with the Colletotrichum above mentioned, while both may eventually connected with Glommerella cingulata (Stonem) S& v. S.

The stem rot of clover, a decay near the base of the s stool, resulting from an attack of Sclerotinia trifoliorm Eriks., has several times been reported as epidemic in the United States. It is not widely distributed, nor is occurence so frequent that it can be considered a serious disease. The fungus is occasionally destructive to various species of clover in Europe, and has been reported upon clover and alfalfa in Michigan and Ohio.

The damping off fungus, Pythium de Baryanum Hesse, and Peronospora trifoliorum de Bary have been reported on clover but are not ofmuch importance.

Clover diseases in E. Lansing Mich. during 1915: The most serious disease of red clover was that caused by Macrosporium sarcinaeforme Cav. The next in importance was rust, Uromyces fallens Desm., which caused considerable damage during the late summer and early fall months, Pseudopeziza trifolii first became common in August, but did little damage. The sooty spot, Polythrincium trifolii, was quite common during the rainy periods, upon many species of Trifolium, especially on red clover (T. pratense) and white clover (T. repens) but not persistent. In one instance, a second crop was noticed which was practically free from the disease, altho the first crop was badly infected. This was perhaps due to the decreased rainfall. Anthracnose, stem rot, and the other clover diseases were not found in this vicinity.

Macrosporium diseases of other plants: This genus contributes toward the diseases of several economic plants of importance. One of the most important is the early blight of potat caused by M. solani E. & M. This disease is common throuout the United States, Canada, Europe, Asia, and Australia. Jones (1899), has conclusively proven the parisitism of the fungus, this depending upon sufficient moisture to allow germination and vigorous growth. The fungus occurs also upon tomato and jimson weed (Datura stramonium). The control has been found possible by means of spraying.

M. sarcinula Berk. var. Parisiticum Thum, commonly associated with Peronospora Schlaideniana De Bary on onion, is of secondary importance. It may be a wound parasite, while in other cases it may be the direct cause of a spot involving the seed stalks. In such cases it is very injurious, since the

seed stalks affected seldom mature their product. Several other species have been reported upon onion.

M. brassicae Berk. is the cause of a leaf spot on cabbage, and is sometimes very destructive.

M. cucumerinum is the cause of the so-called leaf blight or rust of the muskmellon. It attacks the older leaves and destroys the foliage of the central part of the hill. In Colorado, Georgia, and Delaware the disease has been very serious but is controlable by spraying with Bordeaux.

Other Macrosporium diseases which are more or less common, but have not yet become of economic importance, are M. nigricanthum Atkinson on cotton, M. tabacinum Ell. & Ev. on tobacco, and M. iriidis C. & E. on Iris.

Anumber of the foregoing species exhibit a catenulate arrangement of the spores when grown in culture, and are, therefore, sometimes referred to the genus Alternaria.

Field observations: During the spring months a close watch was kept on all clover growing on, and in the vicinity of, the M.A.C. Campus. Since fungi of all kinds were unusually abundant, it was expected that the Macrosporium disease would appear. Nothing was found, however, until June 12, when it was observed in a large field of red clover, situated on the north side of the railroad spur which runs by the collegee forestry nursery. The plants were 32-45 cm. high, with a few beginning to bloom. only the lower leaves were infected, i.e. these leaves close to the ground and shaded by the upper leaves. When the plants were looked at from above, no diseased leaves could be seen. The lower leaves bore characteristic spots, with mature spores present. The number of spots varied from one to five per leaflet, andin some cases the spots were run together. The older diseased leaflets were dried, shriveled, and lying upon the ground, but still clinging to the petiole. In the light of later knowledge, the disease must have been present at least two weeks. At this time no other fields of red clover , or volunteer plants were found to be infected.

The field was revisited from time to time. By June 21 the disease had spread to the uppermost leaves, not a single plant escaping infection. Up to this time the plants had been practically free from other fungi, but now the rust(Uromyces fallens (Desm)) was beginning to appear.

The disease later spread to every clover field in the vicinity of East Lansing, and is still common at the time of this writing (Nov. 15). It is possible that the field first observed was the original source from which the disease spread to other fields.

The correlation between the spread of the disease to toward the upper parts of the plants in the field above described, and the precipitation during the month of June, is striking, and corroborates what was lafer proven by experiment. The total precipitation from June 1 to June 10 inclusive was 0.46 inch, or a daily average of 0.046 inche. The total precipitation from June 11 to June 21 inclusive was 3.28 inches, or a daily average of 0.298 inch. The fieald observations showed that the spread upward was most rapid between the last mentioned dates. This relation will be further discussed under the subject of dissemination.

HISTORICAL

The first mention of this disease made in the literature was made by Cavara, F., (1890) who discovered it in the vicinity of Pavia, Italy. He gives a brief description of the causal organism and names it Macrosporium sarcinaeforme Cav. As the symptoms he gives "a leaf spot." Tubeuf and Smith (1897) in their text book are the next to record the disease, but add nothing new. Malkoff, v. K., (1902) found the disease at Göttingen, Germany. He inoculated the organism upon red clover leaves kept damp under a bell jar, and observed characteristic spots within five to seven days. His description of the fungus differs in certain details from that of Cavara and the present author. A discussion of these differences will be taken up later.

Volkart, A., (1903, 1904), first records the presence of the disease in Switzerland, remarking that heretofore the disease was known only in Germany and in Italy.

Orton, W. A., (1904), in discussing the American plant diseases for 1903, states, "Comment was also caused by the presence of clover leaf spot (Macrosporium sarcinaeforme Cav. and Phyllachora trifolii (Pers.) Fckl .) in Connecticut and New York."

Bain, S, M. and Essary S. H., (1905), note the disease in Tennessee thus: "A rather destructive disease caused by Macrosporium sarcinaeforme Cav. is very frequent and widely distributed. It often appears on stray alsike plants associated with red clover. The Macrosporium disease appears to capable of destroying the plants unassisted tho the statement is made only on field observation."

Under the name of "Macrosporiose" it is feferred to as widely disseminated in America by Stevens and Hall (1910).

Milburn, F.,(and Bessey, E. A.) (1915), states that the fungus causes considerable damage on the leaves and stems of clover and lucerne, and that it " has been found inside the seed causing non-germination. Such diseased seed is shrunk and wrinkled, and much darker than healthy seed . The mode of infection is not known, but in all probability it spreads from the stems and leaves to the seed." Dr. E. A. Bessey informs the author that he has confirmed the presence of the fungus within the seed sent to him from England by Mr. Milburn.

ECONOMIC IMPORTANCE.

Since this disease has been so little noticed, little or nothing has been recorded concerning the loss which it causes. However, during seasons which are favorable to its spread the damage caused may be very great. In East Lansing, during the past season fields have been observed where the damage ranged from a loss of 15 to 40 percent of the crop. The loss may be especially great when, as is often the case, the young crop is attacked. The nature of the damage caused in the United States is to a certain extent given in the report as to its dissemination given below.

DISTRIBUTION.

The disease seems to be widely disseminated on red clover, in Europe and the United States. In Europe it has been reported from Italy, (Cavara, 1890), Germany, (Malkoff, 1902), Switzerland, (Volkart, 1903, 1904), and England, (Milburn, 1915). In the herbarium of the U. S. Dept. of Agriculture there are specimens collected in Moravia, Austria, and Saxony, Germany. The same herbarium also contains specimens collected in 1889 in Manhattan, Kansas, - probably the first specimens collected in this country. The M. A. C. herbarium contains specimens collected in the local Botanical Garden in 1898. Other specimens contained in the U. S. Dept. of Agriculture herbarium were collected at Houlton, Me., (1906), Arlington Farm, Va., (1907), Germantown, Md., (1908), and Philadelphia, Pa., (1909), besides one specimen on Trifolium sp. collected at Potomac Flats, Va., (1890).

A report from the Plant Disease Survey of the Bureau of Plant Industry, from records kept between 1903 and 1912 reads as follows:

"1903, Connecticut: Disease reported by G. P. Clinton. Common and injurious in certain fields.

1905, Tennessee: Reported by S. M. Bain as widely distributed in Tennessee.

1906, West Virginia: 'The cause of great loss of young clover.'

1910, Minnesota: Reported by E. C. Stakman, locally prevalent in the neighborhood of Minneapolis. Earliest reported, July 5. First time reported in this territory.

1910, West Virginia: Reported by J. L. Clinton, Monongalia and Ritchie Counties, occasionally serious in the northern part of the State.

1911, West Virginia: Unimportant.

1912, West Virginia: 'Rather destructive in patches at Morgantown this year.' "

The department of the herbarium of the U. S. D. A. also reports the disease on four specimens of alfalfa. Slides from some of this material were sent for examination. It at once became evident that the fungus on alfalfa is not the same as that on red clover. Slide mounts of spores on material collected in Philadelphia, Pa., and Arlington Farm, Va., (Turkestan alfalfa) contained spores which in shape and color are the same as the spores of Macrosporium sarcinaeforme Cav.

on clover, but they are smaller in size and decidedly warty (echinulate). On the strength of these morphological differences, the author believes that the fungus on alfalfa is a new species of Macrosporium, and is surely not identical with M. sarcinaeforme Cav. on red clover. A search of the literature has so far failed to reveal a species of Macrosporium which conforms to that described here on alfalfa. A more complete description of this fungus will be given at another time.

ETIOLOGY.

Macrosporium sarcinaeforme was isolated from the leaf spot several times during the course of this study. In a demonstration of Koch's rules of proof, the fungus from one of these isolations was inoculated upon red clover leaves, and reisolated from one of the resulting disease spots. The fungus from the second isolation was inoculated upon another plant, the disease produced, and the fungus again isolated, thus proving its constant association with the disease.

The causal organism: The spores are muriform, sarcina-like (packet) in shape, constricted in the middle, and separated at that point into two distinct parts by a cross wall. These parts are subdivided by transverse and longitudinal partition walls which are ordinarily not as thick as the outer or the median division wall. A spore viewed from the point of attachment shows a small circular scar where it was attached to the conidiophore. The contents of the spore consists of a dense, hialin protoplasm with many globules (mostly oil) which oozes out when the spore is crushed. The color ranges from hialin in the very young to dark brown or fuliginous in the older spores. Occasionally a yellow-colored spore may be seen fully developed in form and size, but this darkens with age. The surface of the spore is smooth and no roughenings or prominences of any sort have ever been seen. The size ranges from 22.4 - 37.7 X 19.1 - 27.4 microns, with an average of 28.9 X 22.4 microns.

The conidiophores are usually borne at an approximate right angle to the mycelium which gives rise to them. They

are dark brown to fuliginous in color, and on the host are 23.2 - 74.7 microns long by 5.0 - 5.1 microns wide. The tip cell which is darkest in color is swollen and flattened, resembling in shape the knob of a pestle. The two or three cells nearest the tip of the conidiophore are more homogenious and finely granular than the basal cells. These basal cells frequently bear knobs (Plate III, Fig. 2). Some times on the host, but rarely in culture, the tip cell of the conidiophore instead of at once giving rise to a spore, sends out another cell which is similar to it in shape and structure. The spore is then produced upon this secondary tip cell (Plate III, Fig. 3).

The young mycelium is more or less vacuolate and finely granular in structure, sparingly septate, branched, and 2.5 - 4.0 microns in diameter. As it grows older it darkens to a brown or fuliginous color and attains a diameter of 4.0 -5.1 microns. Within the tissues of a well developed spot the mycelium is sparingly branched and does not exhibit the modifications which appear in culture. The type of mycelium found when the fungus is grown on culture media of the various sorts will be described later.

A comparison of the type of M. sarcinaeforme studied by the author with those described by Cavara (1890) and Malkoff (1902):-

In comparing the organism studied with those described by Cavara and Malkoff, several differences may be noted, especially with reference to the size of spores and the length

of conidiophores. The following is a comparative table:

	Spores	Conidiophores
Cavara	24-28 x 12-18μ	14-18μ long
Malkoff	25.2-33.6 x 16.8-22.4μ	95.2-142.8 x 4.2μ
Author	22.4-37.7 x 19.1-27.4μ Avg. 28.9 x 22.4μ*	30-154 x 4.9-5.1μ Avg. 77 x 5.0μ** On host: 23.2-74.7x5-5.1μ

* These figures are the average of 217 measurements made upon spores grown on seven different media; age of cultures 1 - 3 weeks.

** Measurements are the average of 58 made from four of the above cultures.

Since Cavara makes no mention of having cultured the fungus his measurements were evidently made from that growing on the host. It will be noted that his spore and conidiophore sizes are smaller than those of both Malkoff and the author, especially in regard to the width of the spores. A specimen issued by Briosi and Cavara* was available for examination. The few spores which were found when measured agreed fairly well with the size he gives. The measurement

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* Briosi and Cavara - I Funghi parassiti delle piante coltivate od utili.
116. - Macrosporium sarcinaeforme Cav., in Difesa dai Parassiti N. 4. Milano 1890.

of only a few very old spores cannot be considered as a criterion, but there is no reason to believe that Cavara was mistaken in his measurements. It is possible, however, that he measured the very young spores. The figure which accompanies the herbarium specimens looks very much like that of a young spore.

The measurements of Malkoff and those of the author agree closely enough, so that at least in this respect they may be considered the same. The author has measured the spores from material collected in various parts of the U. S. and finds that they agree in size with those found in East Lansing, Mich.

Malkoff believed that the fungus he observed was perhaps not exactly the same as that described by Cavara, for, he remarks, "If my diagnosis is compared with the original diagnosis (Cavara), one may see that they entirely agree except for the size and form of conidia, which are not entirely sarcina-like.

Just what he meant by "not entirely sarcina-like" or whether his conception of the term sarcina was different, can not be determined. That the fungus studied by the present author, may, by anology with the coccus bacteria grouped in packets , (Sarcina lutea, for example) be considered as sarcina-form, is entirely within the common acceptation of this term. This is evidently what Cavara had in mind when he named the fungus.

While the average length of the conidiophores when the fungus is grown on culture media is more than those growing on the host , the length of the conidiophores as

given by Cavara is much less than that given by the author. According to the former's measurements, they are only a little over one half the length of the spore(12-18:24-28), while his figure represents the ratio as about 2:1, an apparant contradiction.

One important point, wherein Malkoff differs with both Cavara and the author, would indicate that he was perhaps dealing with another species. He states that the spores are somewhat warty. Cavara does not mention this (a point he could hardly have overlooked) and the author has not found this to be the case with spores from amterial from various parts of the U. S. In this connection the warty spores on alfalfa already referred to, is of interest.

Ther is also considerable difference in the appearance of the leaf spots on Cavara's material, (herbarium specimen) and on his figure which accompanies it. The spots as compared with American specimens, are smaller, far more numerous, irregular in shape, and do not bear the concentric markings so typical of the spoton the latter specimens. The small size and greater number of the spots may be due to the smoothness of some European varieties of red clover. A smooth leaf surface has a better chance of retaining many small droplets of moisture than a hairy surface, whereon the droplets have a tendency to collect in one or several large drops on the hairs. Aphotograph of a diseased leaf accompanies the description given by Malkoff, but it is too blurred to be of any use for comparison.

The specimens collected in Manhattan, Kas. in 1889

and identified by G. H. Hicks, evidenly after Cavara's publication (1890) bear a question mark after the name, (Macrosporium sarcinaeforme Cav. ?) indicating that he had some doubt as to whether it was the same fungus .

On the basis of these differences, the conclusion that the author is dealing with a fungus other than those previously described, may not be justified. Only by a comparative series of cultural and infection studies could this be determined. Again, these differences might be entirely within the limit of variation of a single species.

INFECTION EXPERIMENTS.

In performing these experiments three method of inoculation were used. These will be referred to by number in the subsequent discussions, where convenient.

Method 1 - The plants are first drenched with water (watering can) and then sprayed with a suspension of spores in sterile water. The plants are then kept in a humid atmosphere under a bell jar. It was found that the droplets containing the spores adhere much better if the leaves are first drenched with water. By this method, characteristic spots develop within five to seven days.

Method 2 - Small masses of fungus growth (spores and mycelium) are placed upon the upper surface of the leaflets, the inoculum covered with a tuft of cotton, and the plants kept under a bell jar as above.

Method 3 - A drop of spore suspension is carefully placed upon the leaflets by means of a capillary pipette and then covered with a tuft of cotton. This retains the moisture and prevents the droplet from rolling off the leaf. The plants are also kept under a bell jar.

When inoculations are made by either of the last two methods, typical spots are not produced. The leaf tissue surrounding the inoculum is progressively killed and the spores produced upon the dead area. When young leaves are so inoculated, the entire leaflet may be killed within twelve to fifteen days.

During this study red clover plants were inoculated many times under a variety of conditions, using all the methods above described. Successful infection was

almost invariably secured.

The following experiment was undertaken to determine the relative susceptibility of red clover plants in the various stages of their development:

Group 1:- A lot of healthy, green, plants, about 5 cm. high, grown in the greenhouse in a large flower pot. Age 3 weeks.

Group 2:- Healthy plants, about 28 cm. high, were transplanted to the greenhouse, care being taken not to injure the roots.

Group 3:- Two large, leafy plants, about 45 cm. high, transplanted to the greenhouse together with some soil from the field in which it was growing.

All diseased or otherwise weakened leaves were removed from the plant. All were drenched with water and sprayed with a suspension of spores. An effort was made to make the amount of spray as nearly as possible proportional to the quantity of foliage in each group. The plants of Group 3 were sprayed with 30 cc. of the spore suspension, those of Group 2 with 20 cc., and those of Group 1 received only 6 "squirts" from the atomizer.

After eight days the plants showed infection in about the following proportion:

Group 1:- All but a few leaflets infected.

Group 2:- About one-half of the total number of leaves infected, with the heaviest infection on the lower and central leaves.

Group 3:- About one-third of the total number of leaflets infected, with the heaviest infection on the lower and central leaves.

From these and similar infection experiments, besides observation in the field, it is concluded that the very young plants are more susceptible to the attack of the fungus than are the older plants. This is perhaps due to the more tender and succulent condition of the young leaves. In West Virginia, in 1906, it was reported as causing a great loss of young clover.

Stem and petiole infection:- Although numerous infection experiments by spraying a suspension of spores upon the plant were made, in no case was the infection of the stems or petioles observed as the result of such a method of infection. This may be due to the fact that a drop of spore-containing water adheres with difficulty to these parts of the plant, and may roll off before the fungus has a chance to penetrate. Infection of the petioles, however, is readily obtained by inoculating with a small mass of fungus growth and then tieing cotton around the part inoculated. Within five to seven days dark brown to black, linear streaks are developed. Similar attempted inoculations made upon the stems have not been successful. In another experiment, longitudinal slits about 1 cm. in length, were made upon four old, woody stems, and the fungus inserted in the wound. Even after two weeks there was no sign of infection. Some of the inoculum was removed and

examined: the spores had merely germinated but seem to have been incapable of entering the tissue of the stem. This may be due to the comparative dryness of these older stems.

Only in a few rare cases have infected petioles been seen in the field. Young leaflets when inoculated by Method 2 may be eventually destroyed and the fungus spread from them to the petiole. Petioles so infected have a black, pinched appearance near the base of the leaflets (Plate I, Figu 4, a).

Floral infection:- Floral infections were undertaken with the idea of infecting the seed. Reference to the statement of Milburn (1915) concerning the presence of the fungus in the seed, has already been made. At various times during the summer flower heads of red clover in early, full, and late bloom respectively were inoculated by Method 1, but in no case was the infection of any floral part or of the seed obtained. Flowers from plants in the field whose leaves were badly infected with the disease were often examined but the fungus was never found growing upon them.

Seed infection:- It was found that the fungus grows readily upon sterilized seed ($HgCl_2$, etc.) with just enough water present to allow the seed to germinate. The growth of the fungus was rapid and abundant. Within two weeks the entire mass of seed was well overrun, and an abundance of spores produced. The seed coats were almost completely covered by the fungus and eventually destroyed. The cotyledons of germinating seeds could be seen emerging from a black fungus shell of what had been the seed coat. The cotyledons were not

observed growing in the midst of badly diseased red clover, but neither were these diseased.

We may conclude, therefore, that the strain of alsike clover tested under East Lansing conditions is not so susceptible to the strain of M. sarcinaeforme on the red clover growing in this vicinity. Information from Mr. Bain as to the nature of this disease on alsike clover in Tennessee was not forthcoming.

Inoculations upon other hosts:- Similar inoculation experiments were performed upon crimson clover (T. incarnatum), white clover (T. repens), sweet clover (Melotus alba), alfalfa (Medicago sativa), and vetch (Vicia villosa), without success.*

Among the non-legumes the following plants were tested and found to be not susceptible to the disease: Potato, tomato, cucumber, muskmelon, cabbage, rape, and lettuce.

While there may be some conditions under which alsike clover and other legumes are susceptible to attack by the fungus, M. sarcinaeforme, for the present must be considered a parasite restricted to red clover.**

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* Pea (Pisum sativum) and also bean (Phaseolus vulgaris) were also found to be unsusceptible to the disease.

** A discussion of the Macrosporium found on specimens of alfalfa have already been referred to on page 13.

attacked, though some of them were slightly discolored, but the tip of the radical, which in some cases attained the length of 10 mm. was attacked by the fungus and killed, thereby preventing further growth. It is possible that as the seed dried out somewhat, the growing tip died and was attacked saprophytically. However, some of the radicals were attacked just as they were emerging from the seed. The large bulk of the seed was so completely enveloped by the fungus that they could not even germinate. Some of these seeds were planted but failed to grow.

INOCULATIONS UPON OTHER HOSTS.

Alsike Clover:- Bain and Essary,(1905) state that M. sarcinaeforme was often found upon stray Alsike plants associated with red clover in Tennessee. Repeated attempts were made to infect alsike clover, but without success.

The following experiment was repeated at various times during the study: A pot of red clover and a pot of alsike clover plants in about the same stage of development were sprayed thoroughly with a suspension of spores, and both pots kept under the same bell jar. Within the usual time the red clover plants were infected, but never the alsike. None of the other methods of inoculating, using a heavy inoculum, produced infection, except for an occasional slight discoloration of the leaf, but never infection. Wounded leaves also failed to take the disease. Other inoculations were made upon young seedlings and plants in other stages of growth, without success. In the field alsike plants have often been

SYMPTOMS OF THE DISEASE

On the leaves:- Usually within 24-36 hours after inoculation, a minute brown spot just visible with a hand lens, appears on the leaf surface as a result of the penetration of the fungus. After three days, the spot has attained a size of 2-3 mm. diameter but has no definite shape. After this the spot enlarges rapidly, and the typical concentric markings begin to form. The center of the spot (where the spore has entered) is darkest and very distinct. Around this appear the alternately lighter and darker concentric rings. The darker rings are sepia to dark brown in color, the lighter ones ochre to light brown. Toward the center the dark rings form ridges and are narrow, while towards the margin of the spot they are broad and not raised. The color contrast between the two outermost rings is very sharp. Conidiophores and spores usually appear first on the underside of the leaf, and can be seen with a hand lens as tiny black clusters, densest nearest the center of the spot.. Spores on the upper side of the spot may appear simultaneously with those on the lower side, or later. The shape of the spot is oval to round, and after seven days attains the size of 4 - 7 mm. x 3 - 5 mm. The spot if isolated, may increase in size, the maximum noted being 13 x 8 mm.

The spots resemble in general other Macrosporium or Alternaria leaf spots. They are easily distinguished from the spots caused by Pseudopeziza trifolii, which do not have well marked concentric rings and bear the characteristic central apothecium. In the early stages of infection, however,

it is difficult to distinguish between them.

Spots seem to be most frequent toward the edge of the leaf. Those adjacent to each other, soon run together and lead to the death of the tissue lying beyond them, especially that near the edge of the leaf (Pl. I, Fig. 1). Sometimes a large area is completely covered with small spots which have run together and shriveled up the leaf. (Pl.I, Fig,3) The spots become dry and brittle with age and eventually cause the death and drying out of the entire leaf. If the weather is dry such leaves may hang on for a few days and then fall to the ground. During wet weather they cling to the plant and in the case of the lower leaves, the petioles droop and leaves soon rot in the wet soil.

On the petioles:- This form is very uncommon, and is restricted to the young, succulent petioles. Upon them the fungus appears in the form of dark brown to black linear streaks one to three mm. long. Little black clusters of spores may be seen on the surface of these streaks. (Pl. I, Fig. 5) The nature of the petiole infection which may spread from the leaves has already been described on page 24. (Pl. I, Fig. 4a)

THE MORBID HOST

1. Morbid Anatomy.

For a cytological study of the disease tissue the material was treated in the following manner:

Gilson's fixing fluid, 6-8 hrs.
Washed in 70% alcohol until odor of acetic acid disappeared.
70% alcohol 24 hrs.
From this point, dehydration, embedding, until the sections were ready to stain, were proceeded with in the usual manner.
Staining:- Delafield's haemotoxylon, 4 - 6 hrs.; Wash in water 20 - 30 min. Eosin, 30-45 sec.; Clear in phenol-turpentine; mount in balsam.

The fixing and subsequent treatment failed to remove the brown color from either the dead tissues or the spores and conidiophores. Peroxide of hydrogen likewise failed to bleach this color. By this staining process the mycelium within the tissue was stained deep purple, the healthy tissue, red to light purple, and the diseased tissue either a reddish brown or not at all (retaining its original brown color).

The diseased spots are completely collapsed and only about one-third to one-half as thick as the normal tissue. Within the spot proper the cells are almost entirely disorganized and permeated with the older mycelium. This mycelium is brown in color, septate, branching, and of a more or less homogenious structure without any vacuoles. From both sides of the leaf, at irregular intervals, project conidiophores, their length varying from 1-3 times that of the spore. Upon these conidiophores are borne the spores.*

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* The fixing fluid did not, as it is reputed, serve to retain the spores upon the conidiophores.

Near the edge of the spot, the young mycelium may be seen spreading intercellularly and to a certain extent intracellularly, into the adjacent healthy tissue. The mycelium does not as a rule, grow straight up through the leaf, but ordinarily spreads laterally among the cells and may grow for a considerable distance immediately underneath the epidermis. (Pl.I, Figs. 6 & 7). Usually the hyphae begin to come up through the leaf surface on about the fourth day after inoculation. The hyphae underneath the epidermis turn up and penetrate between the epidermal cells and in some cases a hypha may be seen coming up through a stoma. (Pl. I, Fig. 6) The former method is much more common. The hyphae once out side the leaf begin to swell at the tip and produce spores. The process of spore formation will be described later.

2. Physiology:- Experiment to determine whether or not the disease spots transpire.

Because of their dried and collapsed structure, the old and fully developed disease spots would not be expected to transpire. In order to determine this the following experiment was performed:

A simple potometer (modification of Ganong's) the construction of which may be easily understood by referring to the diagram (Pl. XI, Fig.) was used. Into this was fitted a red clover leaf bearing 6 - 8 large, old disease spots, the place where the petiole enters the tube being sealed by the application of lanolin. By careful manipulation, an air bubble was obtained in the horizontal arm of

the tube. A paper centimeter scale was then glued to the outside of the tube so that the zero point was opposite the bubble. Three such potometers were set up. As water evaporates from the leaf, the bubble moves forward, and the distance traversed being read on the scale. By this means the relative transpiration may be determined. After three hours the distance traversed by the bubble was recorded. The disease spots were then coated with lanolin on their upper and lower surfaces in order to prevent the evaporation of water from these parts. After three hours the reading was again taken. The following table gives the results.

Leaf No.	Distance traversed by the bubble.	
	Spots before covering	Spots after covering
1	21 mm.	22 mm.
2	17 "	17 "
3	24 "	23 "
Avg.	20.67 "	20.67 "

Hourly readings of the temperature were taken. This ranged from 19° to 20° C.

While this experiment was conducted upon a small scale, yet from the fact that the relative transpiration of the leaves with the spots uncovered checked so closely with that from the leaves with the spots covered, it is fairly certain that the disease spots, at least when old and dried, do not transpire.

Whether the transpiration of the diseased leaves is less than that of healthy leaves, was not determined. If all other factors are eliminated, the transpiration of the diseased leaves would be less because of the reduction in the transpiration surface by the non-transpiring disease spots.

INFECTION PHENOMENA

The following method was used in determining how the fungus enters the host: A leaf attached to the living plant was inserted through the opening in the stage of the microsoppe from which the sub-stage had been removed. One of the leaflets was clamped in place by means of clips and a small drop of spore suspension placed upon its surface. The germination of the spores could thus be observed under the low power of the microscope. A fresh drop of water was added from time to time to prevent drying.

Usually germination begins within two to three hours after inoculation. Within ten to fourteen hours the germ tubes have attained a length of four to six times that of the spore, after which, penetration begins. This is best observed by focusing the high power directly into the drop, using for illumination a micro-asc focused on the mirror.

The germ tube appears to enter between the epidermal cells. A few cases of stomatal entrance have been observed, but this is not characteristic.

Where it was desirable to examine the means of penetration more closely the leaf was treated in the following

manner: The spores were permitted to dry down on the leaf and a piece of the infected tissue fixed in 95% alcohol for one hour. This treatment removes the chlorophyll and makes the tissue almost transparent. The material was then stained in eosin, 5-10 minutes, cleared in phenol-turpentine, and mounted in balsam. The germ tubes are stained a deep pink while the leaf tissue is stained a lighter shade of the same color. Stained in this manner, the place where the germ tube has entered, and the mycelium which has already begun to grow through the leaf tissue, may be plainly observed.

The conditions which favor infection are discussed under the headings of humidity and light relations.

GERMINATION STUDIES

The various studies in the germination phenomena of the fungus were made in the usual van Tieghem cells kept at room temperature (20° - 24° C.).

The spores of M. sarcinaeforme germinate readily in either tap or distilled water. If taken from a young culture, they begin to germinate within an hour. Usually within six hours every viable cell of the spore has sent out a germ tube. These are at first hyalin and finely granular, but become much vacuolated as the tube elongates. The limit of growth in water is reached after 36-48 hours. At this stage, the mycelium is somewhat branched, septate, vacuolate, with a length of 500 to 700 microns. Spore formation in water has not been observed. Malkoff (1902) mentions observing the formation of new spores in a hanging drop, but does not state what medium he used.

In clover juice,* the germ tubes are at first hyalin but more coarsely granular than those growing in water. They begin to swell at the base very early, and darken in color. This basal swelling is soon cut off by a cross wall and rounds up into a cell containing one or more large central vacuoles. These vacuoles enlarge much faster than the cells containing them(Pl. IV). From this time on the growth of the tubes is more rapid; the mycelium becomes coarsely granular, turning to a brown color which is darkest near the spore and shades off to hyalin at the growing tip. As the mycelium

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* Method of making this and other media given on page

becomes older, the cell walls thicken, and oil globules appear. Within three days the colony attains a diameter of 2-4 mm.

Spore formation:- The conidiophores begin to differentiate about the fourth day. A short branch usually at right angles to the main thread, begins to swell at the tip, This branch may or may not elongate as the swelling progresses. The swollen cell darkens, and is further differentiated from the vegetative cells in that it is finely granular and contains no oil drops or vacuoles. The first sign of the spore, which usually begins to appear on the fifth day, is a small, hyalin protuberance from the swollen tip cell. This enlarges rapidly, until it emerges as an oval, hyalin cell perched on the tip of the conidiophore. A horizontal cross wall divides the cell in the middle, and soon the first longitudinal divisions appear. As the spore matures the color gradually darkens to a dapp brown or fuliginous color, the constriction at the middle becomes more prominent, and the remaining subdivisions are produced. As a rule, the outer cell wall and the median division wall become more thickened than the partition walls of the spore. Sometimes the end of the mycelium which gives rise to the conidiophores, or even a primary branch becomes modified into a conidiophore and bears a spore. This is the source of some of the abnormally long conidiophores. The process of spore formation is deliniated on Plate II, Figs. 1-IX.

The general method of spore formation is analogous to the process among many of the Alternarias, where a spore

sends out a little swelling from the beak end, which later enlarges, becomes muriform, and develops into a spore like that which gives rise to it.

The method of spore formation as observed by the author does not agree with that described by Cavara or Malkoff, who state that the apical cell of the conidiophore swells, and itself becomes differentiated into the spore. The author has never witnessed this proceedure.

In a 5% dextrose solution, growth proceeds to the formation of spores, but the germination process develops abnormal swellings. In a few instances, a cell budding from the spore was seen to enlarge to a size equal to that of the spore which gave rise to it, and become divided by one or two lateral cross walls, giving it the appearance of a young spore. (Pl. III, 5) Though this develop into a mature spore, it might possibly have done so under the proper conditions. This may be evidence of a tendency towards an Alternaria-like habit. Some Macrosporiums are known to develop the Alternaria habit in culture. The cause of these abnormalities is not due entirely to the nature of the medium, since they did not appear in a series of 5% dextrose sol. cultures prepared at another time. The mature mycelium also germinated readily. In water, thin hyalin threads, similar to the germ tubes from the spores, are produced. Old conidiophores from which the spore has fallen often germinate from the tip cell, sending out a long vacuolate tube with occasional swellings. (Pl. III, Fig. 6).

The percentage of germination of spores from young

cultures is practically 100%. The following table gives the time and amount of germination of five series of van Tieghen cells (6 to a series). The spores were sown in sterile tap water and kept at room temperature. Age of culture from which spores were taken was eight days.

♀ Series	6 hours	13 hours	24 hours
1	90%	99% plus	same
2	85	99 "	"
3	95	99 "	"
4	90	95 "	"
5	70	95 "	99% plus

In cases where old spores were used, the germination percentage was found to vary from 70 to 90%.

CULTURAL STUDIES

The original culture of the fungus was obtained from the dried material, which at the time wa s 18-20 months old. Dilution plates were poured, using corn meal agar. A single spore was marked, and when germination occurred it was removed together with a small block of agar containing it and transferred to prune juice agar. From the colony which developed, transfers were made, originating the stock cultures.

The comparative cultural study of M. sarcinaeforme has not revealed any striking morphological divergencies. The fungus grows readily on a large variety of media. Three groups of media were used: agars, vegetable plugs, and liquids. All cultures in each group were run simultaneously, and growth observed over a period of three weeks.

In general growth proceeds as follows: The spores germinate within a few hours and after 24-28 hrs., small white tufts of aerial mycelium, 1-3 mm. long, appear. During the next two or three days the mycelium spreads gradually over the surface of the medium, the rate of spread depending upon the amount of moisture present and the amount of inoculum used. Beginning with the third day, the mycelium usually darkens in color, at first a dull gray woolly appearance which gradually darkens. Spore formation begins on the fifth or sixth day, and as the spores mature and increase in number, the culture assumes a black, felt-like appearance. On agar media, the fungus usually begins to grow down below the surface after the first week. This submerged growth which may extend down

5-10 mm., consists of a dark mycelium and spreads out more or less like the roots of a plant. The maximum amount of growth is reached within 16-20 days.

Of the various agar media used, oat meal agar produces the most abundant growth of mycelium and spores. Plain nutrient and the synthetic agars used produced the smallest amount of growth. With the addition of glucose or dextrose to the nutrient agar, growth is materially increased.

Of the vegetable media used, it is noteworthy that on potato, while a dense mycelial growth is produced, spores are few. The presence of sugar seems to favor spore production. Of these media sugar beet and red table beet rank first in the amount of spores formed, carrot second, parsnip third, and potato last, which also represents the relative order of their sugar content. If glucose is added to potato, as in the case of hard potato agar, spores grow in abundance. On the other hand, wheat starch paste (Kahlbaum's) is an excellent medium for spores. A test of the substrat upon which the fungus had been growing for two weeks showed that much dextrose had been formed. The sterile, uninoculated starch paste check gave no such test. Evidently the fungus secretes the enzyme invertase.

On plain corn meal, growth is abundant, but the hypothetical ascus stage has thus far failed to appear on this medium. (Oldest culture, nine months). Clover stems, bean pods, and sorghum stems are very good media for obtaining an abundance of spores.

The following tables give a comparison of the growth on the various media, under as uniform conditions as it was

possible to obtain. As a quantitative standard of growth, oat meal agar was used for the various agar media, sugar beet for the vegetables, and clover juice for the liquids.

LIQUID MEDIA

	3 days	6 days	21 days
Clover juice	Many floating colonies; white aerial mycelium; many small colonies beginning to form on the bottom of the flask.	Colonies dark gray, 2-8 mm. diameter. A ring of colonies has formed where the liquid touches the glass. Bottom of flask completely covered by white mycelial mat consisting of many small colonies. Spores being formed.	Most of the colonies have grown together, forming a heavy, black spore covered mat. Mycel on bottom also dense but bears no spores. Best of the liquid media used.
Full Nut. Sol.	Few colonies 1-3 mm. diam., floating on the surface.	Good surface growth but light in color. Dark ring of colo's around edge of liquid. Spore production slight.	Heavy growth of mycel. thruout the liquid, but spores are not abundant.
Coon's Synthetic Med. same as Full Nutrient Sol.			
Dunham's Peptone Sol. same as Full Nutrient Sol.			

	3 days	6 days	18 days
Oat meal agar	White, aerial mycel. 1-3 mm. high; also resupinate mycel. on surface of agar.	Agar well covered with a dark growth. Spores being formed in abundance. Some dark gray aerial mycel.	Dense growth covering entire surface of the agar. Mycel. growing out on walls of tube.
Prune juice agar	Center of colonies slightly darker than those on oat agar. otherwise the same.	Growth not as heavy as above; spores being formed; aerial mycel. dark gray.	Growth entirely black; medium in amount as compared with oat agar.
Corn meal agar	Same as prune agar.	Good growth about same as on prune ag. Color greenish bl'k. Mycel. growing down about 3 mm. into ag.	Same as prune agar.
Clover juice agar	Same as prune agar.	Spores not as abundant as on prune ag. Color greenish bl'k.	Same as prune agar.
Hard potato agar	Growth rapid col. 2-4 mm. diam.	Color deep bl'k; abundance of spores; submerged mycel.	Growth abundant;
Plain nutrient agar	Same as prune agar.	Color dark gray; spores just beginning to form.	Growth lightest of all compared with oat agar.

AGAR MEDIA (CONT'D)

	3 days	6 days	18 days
Nut. ag. plus 5% dextrose	Growth rapid, much aerial mycel.	Heavy bl'k growth; spores abundant; second only to oat agar.	No increase in amount of growth. May be due to drying of agar.
Nut. glucose agar.	Same as dextrose agar.	Same as dextrose agar.	Growth has spread somewhat; otherwise same as dextr. ag.
Sodium asparaginate agar.	Growth slight.	Colony small, not run together. Spores in process of formation.	Amount of growth slight; more submerged than surface growth.
Coon's Syn. ag.	Ditto	Ditto	Ditto
Litmus lactose agar	Same as dextrose agar.	Fair amount of growth. Spores present. Litmus beginning to turn blue.	The blue color has diffused thru the ag. a considerable distance from region of growth.

VEGETABLE MEDIA

Medium	3 days	6 days	21 days
Potato	Mycel. beginning to spread; colo's 1-3 mm. in diam.	About 1/2 of surface covered by a dark gray growth. very few spores mostly immature. Mycel. toruloid and coarsely granular.	Growth is Blk almost completely enveloping the plug. Growth is somewhat brittle, forming a crust about 3 mm. thick and can be "scaled" off with a scalpel. Interior of plug is disorganized and permeated with a hyalin mycel. Spores very few.
Parsnip	Like potato.	General appearance, texture, amount of growth, and mycelium like that on potato, but spores are far more numerous.	
Carrot	Like potato.	About two-thirds of surface covered by dark gray growth. Spores abundant.	Blk brittle growth similar to that above.
Red table beet	Like potato.	Blk dense growth; spores abundant.	Like carrot: Spore production best of series.

Medium	3 days	6 days	21 days
Sugar beet	Like potato	Like table beet	Slight greenish tinge; otherwise like red table beet.
Clover stem.	A little white aerial mycel.	Bl'k scattered growth. Spores abundant.	Surface growth mostly of short conidiophores covering entire stem; superficial mycel. slight. Interior prevaded by hyalin threads.
Sorghum stem	Ditto	Bl'k growth; spores abundant; more vegetative mycel. than on clover stem.	Ditto except for more mycel.
Corn meal	Growth is rapid; mycel. spreading.	About two-thirds of surface covered by bl'k growth. Spores abundant.	Growth has ramified thruout the meal, blackening it. Much better than on corn meal agar.

There are no striking differences in the general appearance of the fungus on the various media studies, all look more or less alike and can be distinguished from each other only in a comparative series of cultures. The gross amount of growth and the abundance of spores varies to a certain extent even on the same medium. Again organic media are not always alike even if prepared under the same formula. For example, in one lot of corn meal agar pre - pared by another individual the submerged mycelium extended down to a much greated depth than on the same medium prepared by the author. The dearth of spores on potato seem to be constant on several lots of this medium.

On culture media the fungus exhibits many variations in the form and structure of the mycelium not found when it is growing within the host. In cultures which are well developed (2-3 weeks), the cells of some of the mycelium are closely packed with highly refracted glabules,- mostly oil.* Other cells contained only a few larger globules, and an occasional cell may be seen completely filled by a single oil globule. Individual cells may swell up and assume a globular form, with a diameter of 8-11 microns. The submerged mycelium differs from that growing on the surface in that it is toruloid or beaded. Some of these cells form internal cross walls and appear almost like the beginning of a spore, but further development has not

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* Test for oil made with Sudan III; oil globules are stained light red by this preperation.

been found. These cells may perhaps be chlamydospores. The branching habit is poorly developed in this mycelium, anything more than a primary branch seldom appearing. (Pl.II,Figs.6-7)

Upon agar media kept under humid conditions, newly formed spores may germinate directly. In the center of the colony where the growth is very dense, many of the germ tubes sent out at once differentiate into conidiophores and bear spores (Pl. III, Fig. 4). These newly formed spores are somewhat smaller than the spore from which they arise, ranging in size from 18-24 microns x 11-18 microns. As many as six of these conidiophores varying in length from 12-58 microns have been observed arising from a single spore. This direct spore formation, i.e., from spore to spore without any vegetative mycelium intervening may be due to the inability of the germ tube to penetrate the dense mass of growth which lies below it, so that the agar,- the source of food, - cannot be reached. If this is the case such direct spore formation is evidently the result of starvation. Near the edge of the colony the germ tubes have ready access to the fresh,unoccupied agar, hence vegetative mycelium is produced.

It was noted that in the foregoing spores giving rise directly to the new spores were not attached to the conidiophore upon which they were originally borne. It was thought that the humidity of the surrounding atmosphere may have something to do with the falling of these spores. To determine this, the following experiment was performed:

Some Petri dish cultures containing numerous, small, closely crowded colonies, and in which the agar was completely dried out, were used. These were kept in an inverted position for a day so that any spores which might fall naturally might be removed. One of these dishes was then suspended in an inverted position over a freshly poured dish of agar, so that any falling spores would be caught. This arrangement was then placed in a deep culture dish in the bottom of which was a little water. A similar arrangement was placed in a deep culture dish without any water, that is, in a dry atmosphere. When examined after ten days the agar underneath the dish in the moist chamber, bore colonies corresponding almost exactly with the location of the colonies in the inverted dish suspended above it, indicating that the spores had been thrown off. The lower dish of agar in the dry chamber contained no growth. The results of this experiment indicate that the humid atmosphere is one of the factors involved in the release of the spores from their conidiophores.

METABOLISM

When cultured upon a medium containing either organic or inorganic nitrogen, one of the end products of metabolism is ammonia. The reaction of the medium which is at first acid is slowly changed to alkaline. Thus in a series of clover juice cultures (100 cc. in a liter flask) the reaction changed, viz.,

Cultures inoculated 10-11-15.

	Reaction		Reaction of sterile medium	
10-17.....	3-1/2°*	acid	7°	acid
10-20.....	1°	alk.		"
11-3	4°	"		"
11-15.....	5°	"		"
11-30.....	5°	"		"

* Fuller's scale. Titrations made cold, using phenolphthalein as an indicator.

In another series of clover juice cultures the reaction proceeded as far as 9° alkaline. A quantitative analysis of the ammonia content gave .0130%. If the 9° alkalinity is calculated as being entirely due to ammonia, it would be equivalent to .017%, the difference between this and the quantitative determination being within the limit of experimental error. The presence of CO_2 was tested for with HCl, but none was found.

Cultures growing on litmus lactose agar caused the litmus to begin to turn blue within four days. After two weeks the blue coloration of the litmus had permeated the agar for a considerable distance beyond the region of

fungus growth, indicating the presence of an extracellular protein splitting enzyme resulting in the formation of ammonia which causes the color of the litmus to turn blue; also that this enzyme can diffuse to a considerable degree through the agar.

After about a month the fungus usually stops growing in the clover juice. In order to determine the cause of this the following experiments were performed:

Clover juice liquid upon which the fungus had been growing for five weeks was made sterile by passing first through paper and then through a sterile Berkfeld filter. The reaction of this liquid was 5° alkaline. To a 20 cc. portion of this was added 5 cc. of a sterile 10% glucose solution, thus making a medium with a concentration of 2% sugar. This was then heavily inoculated with spores from a twelve day old culture. A similar inoculation was made into the liquid to which no glucose had been added. By the addition of sterile normal NaOH solution the reaction of portions of the original uninoculated medium was adjusted to 5° and 12° alkaline respectively. Twenty-five cc. of each of these was inoculated from the same culture as the other two. The following table gives the results:

	7 days	12 days	21 days
Culture liquid plus 2% glucose.	-	-	-
Culture liquid no glucose.	-	-	-
Sterile clover juice medium, 5° alkaline.	+	++	++
Same 12° alkaline.	+	++	++

-, no growth; +, fair growth; ++, good growth.

From the above results it is evident that neither the alkalinity nor the absence of feed are the causes of the discontinuance of growth. The accumulation of metabolic by-products is the probable cause of the retardation of growth.

Neither acid nor gas is formed in fermentation tubes when the fungus is grown in 2% glucose, 2% maltose, or 2% saccharose. The quantity of growth in these solutions is fair, and if any acid is formed, the amount is so small that its presence cannot be detected with litmus paper.

Anerobic growth:- A Lavaran tube was used for growing the fungus in the absence of oxygen. In one arm of the tube clover juice agar which had been boiled for several minutes to drive out the air was poured and slanted. When hardened this was at once inoculated. In the other arm of the tube pyrogallic acid and KOH solution were placed, and the mouths of both tubes quickly sealed with rubber stoppers which were then heavily coated

with paraffin. Within two weeks the first signs of growth usually appear. From that time on growth proceeds slowly with light spore formation between the fourth and fifth week. This experiment was repeated several times with the same results. Just whether a perfect anerobic condition was present inside the tube cannot be known. It is possible that the agar upon cooling absorbed sufficient air for the growth of the fungus.

TEMPERATURE RELATIONS

This series of temperature studies was made by inoculating two tubes each of corn meal agar, clover agar, and clover juice medium and keeping incubators at the various temperatures.

The lowest temperature at which observations were made was 6° C. A light aerial mycelium appears with four days. Some spores though comparatively few in number are produced within 7-9 days. At the same time a healthy plant inoculated with the fungus was placed in the incubator beside the culture tubes. The first signs of the disease appeared on the fourth day, and typical spots bearing a few spores were observed on the eight day. The humidity in this incubator varied from 85-98% of saturation. The light was very diffused entering the incubator through a glass door facing a north window. Although the temperature is low, conditions favor the fungus more than the plant, which is practically dormant. The high humidity favors the germination of the spores, hence, infection may be expected.

At 13° C. (Exp. Sta. icebox), the fungus grows slowly, making about the same amount of growth as at 6° C.

At room temperature, (20-24° C.) the fungus makes it optimum growth. At this temperature all cultural studies were made.

At 30° C., growth is a little retarded. The spore development is fair.

At 33-34° C., growth appears like that at 30° C.,

but microscopic examination shows that no spores have been formed. An occasional young conidium appears at this temperature, but on the whole spore formation is retarded. If cultures kept at this temperature are removed to room temperature, spores develop within 1-3 days, even after they had been kept at the higher temperature for 19 days.

At 37° C., growth proceeds no farther than the germination of the spores. Removal to room temperature brings about normal growth even if the cultures are kept in the incubator for 11 days. This temperature may be considered as the inhibiting one.

The clover juice cultures act as a check upon the low humidity associated with the higher temperature. Even at 37° C. growth does not proceed beyond germination, showing that the low humidity associated with this temperature is not the inhibiting factor.

Thermal death point:* The technique used in determining the thermal death point of fungus spores is as follows: The constant temperature bath consists of an iron kettle of water heated by a large gas burner and stirred by an electrically driven paddle. The temperature is adjusted by a thermel gas regulator suspended in the water. A small wire basket serves as a recepticle for the test tubes and also a tube of clover juice containing a thermometer reading in tenths of degrees. Two tubes of clover juice, one of full nutrient solution, and a tube of melted corn meal agar are placed in the basket besides the thermometer tube. The water is heated until

the desired temperature is reached and then held constant by adjusting the regulator. Each of the media tubes is then inoculated with 0.1 cc. of a sterile water suspension of spores, using a sterile 1cc. pipette. The tubes are heated at the desired temperature for ten minutes, after which those containing the liquid media are plunged into ice water, and the agar tube emptied into a Petri dish. The cultures are kept at room temperatures and the presence of growth recorded after seven or eight days.

No growth developed in any of the cultures heated at 60° C. for ten minutes. At 56° C., growth in all media was normal. Between 57 and 59° C., growth was present but much retarded. Microscopic examination of the plates showed many ungerminated spores. The thermal death point, therefore, must lie between 59 and 60° C., but many spores are killed between 57 and 59° C., though some always seem to survive. This experiment was twice repeated with the same results.

No standard method has as yet been evolved for the determination of the thermal death point of fungus spores. The above method is similar to that used by bacteriologists, and is not very applicable to the phytopathologic fungi. The thermal death point at high temperatures of short duration is of value only as a means of sterilization. The thermal death point at a low temperature of longer duration, and especially the temperature at which growth is inhibited is of more importance. Furthermore, the use of nutrient media in determining the thermal death point introduces unknown chemical and physical factors.

In order to determine the effect of dry heat a large test tube was used, with a thermometer inserted through a rubber stopper so that the bulb was suspended about 5 mm. above the bottom of the tube. The tube was heated in the water bath until the air within reached the desired temperature as was indicated by the thermometer. Three cover slips upon which a drop of spore suspension had been previously placed and dried over $CaCl_2$, were dropped into the tube and heated at the constant temperature for ten minutes. It was found that the spores survived even when heated at 75° C.

Thermal death point of the germinated spores:- Spores in the process of germination are supposed to be more sensitive to high temperatures than ungerminated spores, because of their weaken condition. This is the principle involved in the Tyndal method of fractional sterilization.

Clover juice cultures 34 hours old were heated (in triplicate) for ten minutes in the manner before described. An additional thermometer immersed in tube of clover juice was kept in the basker along with each set of culture tubes, and the time required to raise their temperature from that of the room to that of the bath, determined. This varied from 2 - 2-1/2 minutes, and was not considered. After heating, the tubes were immediately cooled by plunging into ice water. The thermal death point of these germinated spores was found to lie between 45 and 48-1/2° C. Within 34 hours, it is quite certain that all the viable spores have germinated,

thus eliminating the possibility of growth after heating, of any ungerminated spore. In another experiment it was found that ungerminated spores heated in clover juice at 47-1/2° C. for one hour are not killed.

Aside from their physiological interest these thermal relations fail to reveal anything of pathologic value. Under field conditions a high temperature might perhaps act as a disease inhibiting factor, especially because of the low humidity which ordinarily goes with it. However, high temperatures under humid conditions are known to be very unfavorable to successful clover growing.

HUMIDITY RELATIONS

Lesage, (1895), gives a method for determining the minimum humidity requirements for the germination of the spores of Penicillium glaucum. This method has a wide adaptibility, and should prove valuable in dealing with parasitic fungi. A similar method was used for determining the humidity requirements of M. sarcinaeforme.

The principle involved is that the saturation of air above a given solution varies inversely as the concentration of the salts dissolved therein. For NaCl the formula $l - n\,a$ has been deduced, where l equals 100% of saturation, n the number of grams of salt, dissolved in 100 cc. of water, and a is a constant, which for NaCl is .00601. The humidity is said to remain constant no matter how much the temperature may vary.

NaCl solutions of various concentrations were placed in dishes 5 cm. in diameter and 4 cm. deep (with the cover on). One-tenth of one cc. of a sterile distilled water spore suspension was dropped on the under side of the cover, and then dried in vacuo over $CaCl_2$. The cover was then placed over the dish of salt solution and sealed with vaseline, the distance between the surface of the solution and the dried spores being two cm. The dishes were kept in an incubator at 25° C. in order to prevent condensation of water upon the spore bearing surface, such as might take place with fluctuations in temperature. Examinations were made from time to time under the low power of the microscope, germination being

thus readily observed. The following table gives the results of four sets of experiments:

No. gms. NaCl dis. in 100 cc. Water.	0	5	10	11	12-35
% Humidity	100%	97%	93.4%	92.8%	92.2-79%
Set 1	12 hr.	18 hr.	36 hr.	--	--
" 2	10	12	48	6 da.*	--
" 3	16	24	76	--	--
" 4	--**	--**	58	--	--
Avg.	12.67	18	54.5	--	--

* Only a few spores germinated. Probably accidental.
** Spores acidentally wet by solution; discarded.
No germination after five weeks in the case of spores kept over solutions with a concentration of 11-35 gms. per 100 cc.

From the above results it is evident that the minimum amount of moisture required for germination lies between 92.8 and 93.4% of saturation, and that the time required for germination varies inversely as the percentage of humidity.[1]

1 The results obtained by Lesage for P. glaucum are of interest for the sake of comparison. It is at once evident that this common mold has a humidity requirement far below that of M. sarcinaeforme.

Gms. NaCl/100 cc.	0	21.5	23.5	30-33.5
Interval	1 da.	6 da.	11 da.	No germ after 171 da.

The spores germinating in the more humid air appear to be hygroscopic, judging from the thin enveloping ring of water visible under the microscope. The germ tubes were also hydrotropic, projecting out into the moist air rather than adhering to the surface of the glass as is the case when they are germinated in a hanging drop.

As a corallary to the preceding results, an experiment was performed to determine whether infection can occur in a relatively dry atmosphere. Red clover plants were inoculated by placing small masses of air dry fungus growth (culture 10 days old) upon the surface of many of the leaflets. The plants were then well watered and the soil and outside of the pots completely covered with paraffin to prevent the evaporation of water from any source but the plant. These plants were placed under a sealed bell jar, through which air, previously dried by passing through H_2SO_4 and $CaCl_2$, was drawn by means of a suction pump. The air was passed through continuously for seven days at the rate of about forty bubbles per minute (in H_2SO_4). This constantly moving stream of air besides a dish of $CaCl_2$ under the bell jar served to carry off the transpiration moisture. A polymeter was suspended in the bell jar for the purpose of determining the amount of humidity. Humidity and temperature (in the room) were recorded at about eight hour intervals. The former ranged from 50 to 53% and the latter from 17 to 21° C. during the entire seven days. There were no signs of infection at the end of this period, though one of the

plants was badly wilted. An inoculated check plant kept under a humid bell jar developed the disease within the usual time.

From these experiments it is evident that the moisture requirements for germination are high. While this has not, so far as the author is aware, been determined for fungi similar to M. sarcinaeforme, fungi of analagous structure would probably have similar high moisture requirements.

It would seem that a parasite, once having entered the host would be independent of the humidity of the atmosphere, because it has already reached a more or less saturated environment. To determine this relation, plants were inoculated and kept under a humid bell jar for 28 hours. A few of the leaflets were then killed in 95% alcohol, cleared in phenol-turpentine, mounted in balsam and examined under the microscope. The fact of the penetration of the host being established the plants were then placed under a bell jar containing $CaCl_2$ and a polymeter. In spite of the dryness of the atmosphere which contained 54-57% relative humidity, disease spots developed within six days after being removed to a dry atmosphere. This and the previous experiment would indicate that the humidity of the atmosphere is an inhibiting factor only until the fungus has entered the host. Once having entered the external humidity is immaterial.

RESISTANCE TO DESSICATION

The spores of M. sarcinaeforme can withstand a long period of dessication. As previously remarked, the material from which the fungus was first isolated was 18 to 20 months old, Furthermore, it had been kept in a drawer where the temperature for hours at a time sometimes reached 65° C. (drawer was over a steam radiator). In November 1915 the fungus was again isolated from material collected in the fall of 1914. Dessicated spores kept on a cover glass for nine months were found to be viable.

The multicellular structure of the spore and its large size give it more opportunity to withstand dessication. There is always a possibility of at least one of the many cells of the spore to survive the dessication process, thus providing for the further propogation of the fungus.

In the case of bacteria, Staphlococci (in clusters) are known to be far more resistant to dessication than Diplococci or Streptococci. Again the presence of Sarcina in dust would indicate that its packet-like structure (to which M. sarcinaeforme is analagous) is an important factor in its resistance to dessication.

LIGHT RELATIONS

The fungus grows as well in complete darkness as in light, provided all other conditions are the same. To determine this, four different kinds of media were inoculated in duplicate and wrapped in black paper, and put away in a dark cupboard. A similarly inoculated set of cultures were kept in the light near an east window. Two weeks later the tubes were examined and the amount of growth in both was found to be about the same.

As has been noted, under field conditions, the lower leaves of the plant are usually infected first, and suffer the most destruction. Aside from their favorable location near the ground, (i.e., favorable with regard to the possibility of infection) this is due for the most part, to the fact that the lower leaves retain more moisture. To determine this relation, the following experiment was performed:

Four healthy, well-developed, potted plants were thoroughly sprayed with a heavy suspension of spores. Each plant was placed under a separate bell jar, and the lower half of two of these was covered with black paper, so as to prevent any lateral light from reaching the lower leaves. The air in the bell jars was kept saturated during the entire experiment. Thus all conditions were equal, except that the lower leaves of the half darkened plants received only what light filtered through the upper leaves, whereas the other two plants received light from the side as well.

After eleven days a comparison between the two sets of plants showed that the lower leaves of the half darkened plants, besides being partially etiolated, bore more and larger spots than the lower leaves of the fully lighted plants. Twenty leaflets of the former picked at random, bore fifty-one spots as compared with thirty-seven spots on a like number of the latter.

In the case of plants kept in total darkness, disease was found to infect and spread very rapidly. The spots besides being large caused the death of considerable tissue beyond them. While darkness does not affect the growth of the fungus alone, it may be expected that the plant, because of its weakened condition, would be more susceptible.

Effect of direct sunlight:- The usual method of determining the effect of sunlight upon a fungus is to place a Petri dish containing the freshly cpoured culture, in direct sunlight for various lengths of time. This method has its defects, as for example, retention of heat by the glass, diffusion of light by the agar possible chemical and physical changes in the medium, etc. The following method was used by the author:

Discs of filter paper, 1 cm. in diameter were cut with a cork borer, a pin run through each, near the edge, and then dry sterilized. A drop of spore suspension was then placed upon the upper side of each disc and the water permitted to evaporate rapidly in vacuo in a dessi-

cator containing $CaCl_2$. The discs were then mounted in a horizontal position by sticking the pins into a cardboard box, and then placed in the direct sunlight coming through an east window. At intervals of ten minutes three of these discs were removed and each dropped into a tube of clover juice medium. The discs were constantly shifted at times, so as to be constantly in the direct rays of the sun. This experiment was run June 28, '15, between 9:30 and 11:40 A.M. The temperature in the direct rays of the sun varied from 29 to 34° C. Within the usual time growth appeared in all the tubes showing that the fungus spores can tolerate direct sunlight for at least a period of two hours and ten minutes. Several cases of contamination occurred, but these did not interfere with the results, as the experiment was run in triplicate.

This method has its advantages in that paper is a poor conductor of heat, the disturbing influence of a nutrient medium with its attendent moisture is eliminated, and it is also readily workable. Furthermore, it simulates more nearly the natural conditions to which the spores are subjected when inhabiting the host.

EXPERIMENTS DEALING WITH THE TOXIC NATURE OF THE FUNGUS.

Historical Introduction

Our knowledge of that biological function which makes an organism parasitic, and that, quite often, on only one host, is as yet very indefinite. The search in this vast field is only in the embryonic stage, though some brilliant studies have already been made.

DeBary's(1886) epoch-making researches with Sclerotinia libertiana, was one of the first successful attempts at determining the nature of parasitism. Those who followed him proceeded along the trail he blazed. Most of the investigations seem to have been directed toward the cytolytic activities of the fungus upon the host. While the demonstration of the destruction of the cell wall by cellulose destroying enzymes may be considered the first step in parasitism, this fails to explain the subsequent killing and disorganization of the protoplast. It must also be remembered that the cellulose walls are not supposed to be living, and again, these walls being porous would not be an impediment to any osmotic activity of fungus secretions upon the plasma membranes. The fact that other enzymes have been demonstrated both within the cells of certain fungi and in the dead tissue of the hosts which they parasitize, does not explain what actually causes the killing of the cells. De Bary has shown for S. libertiana that the fungus must kill the host cells in advance of growth, and that it is the disintregation products of the dead cells which provide food for the

fungus. He also suggests that the oxalic acid found in connection with the fungus might be the toxic principle. Among the contributions which followed De Bary's we find Marshall Ward's dealing with a Botrytis disease of lily (1888). He confirmed De Bary as to the presence of a cytolytic enzyme, but contributed nothing further toward our knowledge of the toxic principle. Later, follow in quick succession, the work of Behrens, (1898), Nordhausen (1899), and Smith (1902) dealing with the parasitic nature of Botrytis cinerea. Smith concludes with certainty that oxalic acid is the toxic principle. However, Brown (1915) in a very recent paper, has proven conclusively that neither oxalic acid nor oxalates contribute to the lethal principle of the fungus extract. He has also made and advance in that he has established a quantitative basis of experimentation.

In the realm of the bacterial diseases of plants, similar work has been done. Jones (1909), Potter (1908), and Van Hall (1903) have demonstrated the presence of toxic enzymes in liquid culture media in which the bacteria had been growing.

The series of experiments which follow were undertaken to determine whether the metabolic by-products produced by M. sarcinaeforme in the culture medium, have any toxic effect upon the leaf tissue of red clover; and, if such a toxine were present, to determine its nature. Along with this, tests were made, using extracts from the

fungus whose by-products were studied.

Concerning the technique involved, the author believes, that in at least one respect, he has made an advance. In studying the toxic activities of various extracts most of the experimentors have used sections of tissue or entire organs detached from the living plant. While there may be no doubt that the tissue remains alive during the entire experiment, yet the cells may be subject to serious organic disturbances as a result of the separation from the main plant or organ.

The author has devised a means of applying the extract to be tested directly to the leaves of the living plant without detaching them. The method used is as follows: A card-board disc about 5 cm. in diameter, with a slit extending from a small hole in the center to the circumference, is slipped around the petiole near the base of the leaf. This disc is then supported upon a horizontal wire ring bent at right angles to a vertical piece of wire stuck in the ground. As a retainer of the liquid to be tested a van Tieghen cell is sealed with vaseline upon the upper surface of the leaflet. The weight of the cell serves to hold the leaflet in a flat horizontal position upon the card-board disc. The liquid is then dropped into the cells with a pipette, and cover slips smeared with vaseline pressed down upon the upper edges in order to prevent evaporation. (Pl.VI, Fig.) This arrangement, if carefully prepared, will retain the

liquid for a long time without drying out. In order to examine, macroscopically from time to time, the effect upon the leaflet, (where the liquid is dark colored or non transparent) the liquid may be drawn up into a pipette with a fine point, replaced if necessary, and the cell again sealed. That the vaseline has no effect upon the leaf tissue nor upon the liquids tested, has been determined by the use of proper controls with each experiment.

Experiment 1.

A clover juice culture 75 days old was used. The fungus growth, which consisted of a heavy mycelial mat was removed by first passing the culture liquid through a filter paper and then through a sterile Berkfeld filter. Transfers were at once made to agar in order to determine later whether or not the filtrate was sterile, although microscopic examination at the time failed to reveal the presence of any fungus or bacterial cells.

With a sterile pipette, a small quantity of the filtrate was added to each cell arranged as above described, (0.4-0.5 cc. reaching to within a few mm. of the top of the cell) and sealed with a cover slip. The test was made upon both wounded and unwounded leaves. In wounding, the epidermis was lightly scratched with a fine-pointed sterile needle, care being taken not to puncture the leaf. Two or three of these scratches, 3-4 mm. long, were made in the center of the area enclosed by the cell. Such scratches could barely be seen with the naked eye. In the table below each number corresponds to the three

individual leaflets of a single leaf:

No.	Treatment	Liquid added
1-5	Wound	Filtrate from fungus
6-8	No wound	" " "
9-11	Wound	Uninoc. clover juice *
12-14	No wound	" " "
15	Wound	Nothing added

* This was taken from the same lot of media which was used for the above culture. Controls.

Thirty-four hours after the liquid was added, those parts of the leaflets inclosed by the rings on leaves 1, 2, 3, and 5 showed slight signs of blackening around the wound. After 56 hours, the entire area enclosed by the cells on these leaflets were discolored. Number 4 showed a less degree of discoloration, but sufficient to be considered positive action. The discolorations varied from brown to black in color. The unwounded leaves showed nothing to indicate that there had been any action by the liquid. Except for a slight dead area around the borders of the wounds, the controls containing the sterile clover juice medium were unchanged (No. 9 to 14). Number 15, the wounded check without any liquid added, had the same appearance as the other wounded controls. These control tests are sufficient to prove that neither the vaseline nor the wounding contributed toward the discoloration.

It may be stated here that while contamination of the liquid in the cells was to be expected, there was no evidence of fungus growth at the end of the 56 hour period.

A hanging drop of the sterile clover juice used as a check showed numerous bacteria,,but these apparently had no effect even upon the wounded leaflets. The filtrate which caused the discoloration contained only a few bacteria. It is therefore reasonable to suppose that the contaminating organism played no part in the discoloration of the tissue. Microscopic examination of the discolored tissue did not reveal any fungus growth and no growth appeared on the agar to which transfers had been made from the filtrate, Four days after the first series of tests were started a similar series was set up, using the same filtrate as before, which in the meantime had been kept sterile in the filter flask. In this series, practically the same results as before, were obtained.

Experiment 2.

The purpose of this experiment was, (1) to determine whether the toxic substance is an enzyme; (2) whether any toxic substance is secreted within the fungus itself; and (3) a repetition of Experiment 1.

For this purpose a clover juice culture 32 days old was used. The superficial fungus growth was removed by passing through a filter paper, thereby obtaining a filtrate of 80 cc. volume. To this was added 300 cc. of 95% alcohol, and the precipitate thus formed allowed to settle for one hour. This flocculent, brown precipitate was filtered off and more alcohol added to the filtrate. It was washed several times with alcohol, dried in a dessicator, and redissolved in 60 cc. of distilled water. Alcohol was again

added to make a concentration of 80% and the resulting precipitate together with that formed in the first filtrate, filtered off. After washing and drying it was again dissolved by repeatedly passing 30 cc of distilled water through the filter. To this was added 0.6 cc. of toluol (2%) as a preservative. For convenience in tabulating the data which follows this will be called preparation "A".

The bulk of the alcoholic precipitate probably consists of protein and brown pigment matter. It is supposed *a priori* that any enzyme which may be present in solution will be precipitated by the alcohol. If any white enzymic precipitate was present, there could be no macroscopic method of determining this, because of the presence of the brown colored impurities, which the above treatment failed to remove.

Preparation "B":- The fungus growth from a clover juice culture of the same age as the preceding was filtered out through paper and then through a sterile Berkfeld filter. To this filtrate 2% toluol was also added.

Preparation "C":- The fungus growth which had been removed from the above cultures and that from two others of the same age was washed in a little running tap water and the superficial moisture removed by pressing gently between sheets of filter paper. In order to insure rapid and thorough drying, the fungus growth was then placed in a dessicator containing $CaCl_2$ and the air exhaust attached. When dessicated it was groung with clean quartz sand until practically no entire spores or mycelial cells

remained. This ground mass together with 60 cc. of water plus 2% toluol was put into a bottle and agitated for one hour in the shaking machine. The extraction was prolonged for 18hrs. more with an occasional shaking by hand. The sand and fungus particles were removed by filtering through cotton, and the filtrate used for the experiment.

Preparation "D":- Sterile clover juice medium plus 2% toluol.

These preparations were tested upon a lot of clover plants all of which had been planted in the greenhouse at the same time, from the same lot of seeds. They were all in normal healthy condition, and well watered during the entire experiment. The van Tieghen cell arrangement, above described, was used; likewise all other technique was the same. Each number again refers to a leaf with each of the three leaflets bearing a cell containing the same preparation.

° The following table explains the proceedure and the results after 48 hours:

1	A, no wound	-	14	B, boil.** no wound	-
2	A, wound	-	15	B, boil. no wound	-
3	D, no wound (ck)	-	16	C, boil. no wound	-
4	B, no wound	-	17	C, boil. wound	-
5	B, wound	++	18	D, wound (ck)	-
6	B, wound	++	19	D, " "	-
7	B, wound	++	20	D, " "	-
8	C, no wound	-	21	A, wound	-
9	C, wound	-	22	B, wound	+
10	C, wound	-	23	C, no wound	-
11	D, wound (ck)	-	24	C, wound	***
12	A,, boiled, *no w'nd.	-	25	B, wound	++
13	A, boiled, wound	-	26	B, wound	+

-, no change; +, partial discoloration; ++ complete discoloration.
* Liquid merely heated to boiling which was then discontinued.
** Boiled before toluol was added.
*** More than ordinary discoloration than is usually due to injury.

A glance at the results in the above table will show that if any enzyme was precipitated by the alcohol, it had no effect upon the leaves, wounded or unwounded. The same holds true for the extract of the fungus growth. However, the results of the preceding experiment are confirmed by the action of the filtrate "B". It is also evident from the controls that the addition of toluol as a preservative has no injurious effect upon either the host or the filtrate, but that boiling inactivates the toxic principle in the filtrate.

When the filtrate was titrated, it was found to be 9° alkaline. (Fuller's scale). A test with Nessler's solution gave ~~the test~~ indication of the presence of ammonia, which upon quantitative analysis was determined as .013% NH_3. It was thought that the ammonia might be the cause of the discoloration of the tissue. To determine this, leaves both wounded and unwounded were subjected to the action of concentrations of ammonia equaling .01%,.02%, and .05% respectively, using the same set up as in the preceding experiments. No signs of discoloration appeared within 3 days , proving that at least the ammonia alone is not the toxic principle.

This series of experiments was later repeated, For this purpose, clover juice cultures similar to those tested before, were used. These were inoculated Oct.11, just six weeks after the cultures used in the preceding experiment were inoculated. The filtrate was prepared as before, at intervals when the ~~fifth~~ cultures were 4, 5, and 7 weeks old. In no case, however, were the leaves in any way discolored as a result of their contact with these filtrates. Likewise the filtrate from a culture in Dunham's peptone solution had no effect upon the leaves, altho it contained considerable ammonia.

A probable cause for this apparent loss in toxicity was later discovered. It was observed that the inoculations which were made from time to time during the months of October and November failed to produce positive infection. Inoculations were made repeatedly under a variety of conditions but

without success. It was therfore evident that the fungus had lost much if not all of its virulence. In order to make certain of this, a fresh strain of the fungus was isolated from material collected during the summer. Four sets of red clover plants an the same stage of growth were selected. These were then inoculated with both the old and the new cultures as shown in the following table:

No.1...Old culture..... Sprayed with suspension of spores.

No.2... " " .....Mass of fungus growth placed upon leaves and covered with cotton.

No.3...New culture..... Sprayed with suspension of spores.

No.4... " " .....Mass of fungus growth placed upon leaves and covered with cotton.

All the plants were kept under bell jars where the atmosphere was saturated. After 7 days plants number 3 and 4 showed typical infection on many of the leaves. Number 1 showed no signs at all,while on number 2 the leaf surface directly underneath the inoculum, in some cases, was discolored. Microscopic examination revealed a large number of germinated spores, some of which had barely entered the host tissue but seeemed to have stoppeed growing at that point.

This comparative test of the two cultures was repeated under more exact conditions as follows: Two pots of red clover, each containing numerous plants , all planted at the same time from the same lot of seed were used. One pot of plants was sprayed thoroly with a spore suspension from the old culture, and the other was sprayed with a suspension of spores from the new culture, in both cases an effort being made to

make the quantity of spores sprayed upon each plant, as nearly as possible the same. Each pot was kept under a separate bell jar, and the atmosphere maintained in a saturated condition. The plants sprayed with spores from the new culture began to develope the disease on the fifht day after inoculation. After ten days, these plants were heavily infected, while those inoculated with spores from the old culture showed only a few isolated cases of poorly developed spots. Examination of some of the uninfected leaflets of the latter plants revealed many spores on the surfacewhich had not entered the host. The difference between the degree of infection caused by the old culture and the new culture is well illustrated by plates IX and X.

Fom the results of such a comparative experiment, we can not but conclude that the old culture has lost much, if not all of its virulence. This attenuation may be due to the prolonged saprophytic habit which the fungus has been subject to while growing upon culture media. It is not uncommon for parasitic fungi to loose their virulence when kept in culture for a long time.

In view of the apparent attenuation of the fungus virulence, the failure of the filtrate from its culture liquid to have any effect upon the leaf tissue, may be explained. Time has not permitted a comparative study of the filtrate from both the old and the new cultures. Such a test would be the only way of determining definitly the relation between the loss in virulence and the toxic activity of the by-products of the fungus.

Perhaps conclusions are not justified on the basis of

a few experiments which are only preliminary. The following is a summary of what these experiments indicate:

1. That the fungus secretes a substance in the culture medium, which discolors and finally kills the leaves.

2. That this toxic substance is inactivated by boiling.

3. That the ammonia present in the culture liquid is not, at least by itself, the toxic principle.

4. That any enzyme which can be separated from the culture liquid by precipitation with alcohol, etc. is not the toxic principle.

5. That an extract from the fungus which produces the toxic substance in the culture liquid, has no toxic effect upon the leaves.

6. That the failure of the culture liquid to affect the leaves is in some manner connected with the attenuation of the virulence of the fungus.

METHODS OF DISSEMINATION.

The possible methos of the dissemination of the fungus were deduced from extensive field observations and experiments under control conditions. Therd are two phases to the process by which a plant pathogen may be disseminated: First the translocation of the causal agent; second, the presence of those conditions which are conducive toward the entranee of the host by the organism. In the experiments whih follow, these two factors were considered wherever possible.

Soil:- In the dissemination of any disease, the soil merely plays the part of a harboring agent. To determine whether the plants can be infected directly from the soil which has grown a diseased crop, the following experiment was performed:

The upper two or three inches of spil from a field in which badly diseased plants were growing, was selected. This soil, upon examination, was found to contain bits of dried, diseased leaves. Alittle of the soil was also shaken in water, which was then removed and centrifuged. Spores of M. sarcinaeforme were found in the sediment, thus making positive the presencd of the fungus. In this soil, red clover seed which had been previously sterilized by trating with 1:1000 $HgCl_2$ for 10 minutes, and washing in three changes of sterile water, were planted. The soil was watered every day from a beaker. The seedlings which appeared above were not infec ted even after they had reached a heighth of 6 cm. The plants at this stage were then separated into two groups. The first group was watered as before, (from a beaker) while the second group was watered from

above with a hose, the water simulating more or less the falling of rain. The soil was thus splashed up onto the lower leaves. One week later the plants so watered were infected, while those watered from below were not.

The result of this experiment agrees with field observations. The correlation between the amount of rainfall and the appearance of the disease in an uninfected field, was noted under the following circumstances: A second crop of red clover following a first crop which had been badly diseased, reached the heighth of 7-9 cm. without becoming affected. Oct. 15, this field was carefully examined for the presence of the disease but nothing was found. On Oct. 25, many of the lower leaves were found to be infected with the fungus. The weather reports for October indicate the following precipitation:

Total precip'n Oct. 1-15,.....0.34 in.
Daily average " " .....0.021 in.
Precipitation " 17 ..... 0.06 in.
" " 18 .....0.30 in.

The disease was found in the field Oct. 25, seven days from the last date. The heaviest rainfall between Oct.1 and 15 occured on Oct. 1, but at this time the plants were hardly out of the ground. The heaviest rainfall of the month occured on Oct. 18, and it will be noted that the disease appeared one week later. The heavy rain of the 18th was probably the cause of the splashing of the infected soil upon the plants, with the resulting infection.

Seed:- The seed was next considered as a possible carrier of the disease. Reference has already been made to

the statement of Milburn (1915) as to the presence of the fungus on the seed. Seed samples from this, and last year's crop were examined for the presence of the fungus. It was not found growing upon any of the seed, as a parasite. However, by centrifuging for one hour at a high rate of speed, spores of M. sarcinaeforme were found in four out of nine samples so treated. Incidentally, a large number of Rust (Uromyces) and other fungus spores were found. Some of the seed from samples containing diseases spores were planted in sterile soil, the pot containing it being covered with a bell jar. None of those plants which came up developed any signs of the disease, even after 6 weeks. This negative result is not conclusive, inthat the experiment was not conducted upon a large enough scale. The spore containing seed may be a means of trahsfering the disease to the soil from which point , the possibility of infection has already been demonstrated.

Experiments in Wind Dissemination of Spores

That the wind is more or less a factor in the dissemination of fungus spores, has always been assumed, but little experimental work has been done to demonstrate this. The carrying of the spores of Endothia parasitica (Murr) And. has been suggested by several authors, but it remained for Heald, Studhalter, and Gardiner (191) to prove this. They obtained spores in large numbers at a distance of 300-400 feet from the source of supply, and justifythe conclusion that they may be carried much greater distances.

The only accurate way of determining the spore carrying capacity of the wind of known velocity is by outdoor experiment. This was the method used by the above mentioned author. In the case of M. sarcinaeforme, however, this was impractical during the summer because of the general distribution of the disease. Any experiment under laboratory conditions is necessarily inacuurate, because of the difficulty of creating a wind similar to that out doors; also the absence of altitude which out doors, gives an opportunity for upward play of the wind. Nevertheless the following experiments were undertaken:

Experiment 1.

Two pieces of glass tubing 1.5 meters long and 4.8 cm. inside diameter were sterilized within by rinsing several times with 95% alcohol. Melted clover agar was poured into each tube which was then tilted a few degrees from the horizontal. In this manner, a uniform strip of agar about 3 cm. wide was obtained down the entire length of each tube. The tubes were then placed end to end in a horizontal position, and the joint sealed by winding a strip of paper dipped in melted paraffin, thus making it air tight. In the meantime the ends were plugged with cotton. A paper bag was then attached at its mouth, to one end of the tube (A) by means of a piece of string. Through the bottom of the bag a compressed air tube was thrust and likewise fastened with a piece of string(B). This arrangement provided a flexible extension of the glass tube. At the opposite end of the

tube, an anemometer was placed close to the opening. A paper funnel was so arranged as to cover the end of the tube and the anemometer, in order to prevent, in a measure, the lateral dispersal of the air as it struct the anemometer. Several preliminary trials were made to determine the possible velocities obtainable with this arrangement. The air tube was then removed, and the bag closed near the mouth of the glass tube by tying tightly with string(C). Three old, dried agar cultures were introduced into the bag (D) and the air tube returned to its original position. By manipulating the bag, the cultures were grasped from without and brought close to the air tube. A few short blasts of air served to blow many spores from the culture. In order to remove the remains of the cultures, the bag was closed at (E) so that by removing the air tube the cultures could be ejected without permitting any of the spores to escape. The air tube was again replaced, and the constriction at (E) removed. The chamber now contained the spores which were to be carried into the tube. The air pressure was t turned on very slightly in order to get the spores in circulation. The string at (C) was removed and the bag at this point constricted with the thumb and forefinger. Simultaneously with the increase in the air pressure the digital constriction at (C) was released, so that when full pressure was reached, the opening at (C) was complete. This entire manipulation required a few seconds. The air blast was continued for five minutes, the pump running

steadily at about 25 pounds pressure. The anemometer recorded 480 meters during this time, which is equivalent to a velocity of 3.58 miles per hour. The tubes were then taken apart and the ends plugged with cotton. Within 3 days many colonies of M. sarcinaeforme developed throughout the entire length of the tubes, somewhat more numerous at the initial end, but numerous enough at the opposite end to indicate that some spores had passed completely through the tube.

The velocity registered by the anemometer was not as high as it should have been because the outer part of the wind-receiving device extended beyond the mouth of the tube. However, the velocity could not have been very much higher than that recorded for when the hand was placed near the mouth of the tube the force of the blast did not feel very strong.

Experiment 2.

This experiment was conducted on a much larger scale. For this purpose a tube 48 ft. long, 14 in. high, and 12 in. wide was constructed thus: The framework was made in three 16 foot sections, using strips of yellow pine one inch square (Pl. VI, Fig. 3). Each frame was then covered on the outside with wrapping paper and the seams thoroughly glued. The sections were set up out doors in a horizontal position three feet above the ground and the ends joined together by covering with more paper. Since the wind at the time was blowing from the east, the tube was set up in an east and west direction. Petri

dishes containing agar were then placed at two foot intervals on three boards 16 ft. long, 6 in. wide, and 1/2 in thick, and held in place by driving 2-penny nails into the wood. The boards with the dishes of agar were then shoved into the tube so that the rested on the bottom framework. In this manner a means of catching spores at two foot intervals along the entire 48 ft. was obtained.

The spores used in this experiment were prepared in the following manner: The growth from a clover juice culture was removed and shaken with a little water in order to wash off many of the spores. The spores were then precipitated from suspension by centrifuging. They were then dried over $CaCl_2$ in vacuo, and kept in a tightly stoppered tube until ready for use. Just before using those which were massed together in small dried lumps were ground lightly in a mortar.

The dried apores were slowly released into the east end of the tube through a small funnel inserted through a hole on the top side. This was done gradually, by rubbing a little at a time between the fingers and dropping into the apex of the funnel. Just inside of the west end of the tube, an anemometer was placed so that the center of the wind-receiving device was in line with the axis of the tube. The wind was permitted to blow 33 minutes from the time the first spores were released and then both ends of the tube were closed.

The anemometer registered 34476 meters during this time, which is equivalent to a velocity of 3.93 miles per hour. Here too, the reading given by the anemometer cannot

be considered accurate, because the wind was constantly changing direction and the wooden framework inside the tube impeded to a certain extent the velocity of that wind which passed through the tube.

The dishes were later examined microscopically and all except those located at the 12, 14, 22, and 28 foot mark were found to contain spores. The distribution of spores was very uneven, but the fact that some fell into the dishes at the farthest end of the tube, is a positive indication that they were carried at least 48 feet. Minute, black deposits of spores were also found scattered along the board on which the dishes were placed. It is not probable that the spores remained suspended in the air over the entire distance which they were blown. It is more likely that they fell to the bottom of the tube several times while in transit, to be lifted again by the wind and carried farther.

This assumption is not based entirely upon the uneven distribution of spores within the tube but also upon the result of the following experiment, whose purpose was to determine the velocity of spores falling in still air. For this purpose a glass tube 1.5 meters long and 4.8 cm. inside diameter was used. This tube was set up in a vertical position so that the lower opening rested about one cm. above the top of the table. The windows and doors of the room were closed, so as to exclude, as far as possible, any disturbing air drafts. A Petri dish containing agar was slipped under

the lower opening of the tube. Dried spores were then released at the upper opening. Eight seconds after the release of the spores, the agar dish was quickly removed and another immediately slipped into its place. This second dish was no sooner in place when it was replaced by another. The second dish was removed ten seconds, the third, 12 seconds, the fourth, 15 seconds, and the fifth, 20 seconds after the release of the spores. Other dishes were placed under the tube at 5 second intervals after the 20th second. Only in the first two dishes were spores found,- a large number in the first, and only a few in the second. It requires, therefore, a maximum of about 10 seconds for the spores to fall a distance of 1.5 meters,- that is, the velocity of a falling spore is about 0.15 meter per ~~.5 feet~~ second. A velocity of 3.93 miles per hour is equivalent to 5.8 feet per second. In the 48 foot tube used in the preceding experiment two seconds would therefore be required to fall from the top to the bottom,- a distance of one foot. A wind with a velocity of 5.8 feet per second will carry the spore a maximum horizontal distance of two x 5.8 ft. or 11.6 ft., before it fell. Once having fallen, there is no reason why a slight upward trend of the wind could not again lift the spore and carry it farther, provided of course, it were not already trapped or impeded by some obstacle.

From these experiments, no definite assertion can, of course, be made as to how far the spores can be carried

the wind. However, the indications are that the spores of M. sarcinaeforme, though comparatively large and heavy, might be carried a sufficient distance from their source to reach and infect other fields of red clover. Out doors, where a much higher elevation is obtainable, this distance should be much greater than in the tube experimented with.

This point should be considered, however. Under field conditions, especially in damp weather, many spores fall to the ground and are caught by the wet soil or between the lower parts of the plants. Spores so situated would not, therefore, be in a favorable position for dissemination by the wind.

CONTROL

Any method of controlling the disease by the application of sprays is impracticable. Cavara's suggestion that the field be watched and the diseased leaves be removed as they appear, would, to say the least, be very difficult to follow where clover is grown on a large scale. The mowing of the young crop as soon as the disease appears, has been suggested as a remedy for several diseases of red clover, However, in the case of M. sarcinaeforme, the presence of the cut diseased leaves fn the field would be a source of infection for the new crop, especially during rainy weather.

The ultimate solution of the problem of the control of this disease, and for that matter, the control of all field crop diseases, is the breeding of resistant varieties. In the case of Anthracnose of clover (Colletotrichum trifolii), this has been done. Bain and Essary (1906), in looking for a means of controlling this disease in Tennessee, began by selecting uninfected plants growing in a badly diseased field. From these selections, a strain of clover has been bred which is fairly resistant to the disease. Other important work has been done in breeding disease resistant varieties, as for example, that of Orton in the case of cotton and watermelon.

The method of selection could probably be used in producing a resistant strain of red clover against any of its diseases. The author has seen occasional plants during the summer which were unaffected though growing in the midst

badly diseased plants.

It is noteworthy that alsike clover is far more resistant to most of the common diseases than red clover. The possibility of hybridizing red and alsike clover in order to produce a disease resistant strain suggests itself. Certain cultural characters of alsike clover, as for example, the ability to grow upon "clover sick" soil, might also be contributed to the improvement of the red clover strain.

SUMMARY

1. Because of fungus diseases and other troubles, the culture of red clover,- a crop of great economic importance,- is being discontinued in favor of other legumes. This is alarming and more investigation along the lines of disease control and disease resistance, is necessary.

2. Red clover is affected by diseases which cause considerable loss. Among the more important ones are rust (Uromyces fallens(Desm.) Kern.), leaf spot (Pseudopeziza trifolii), and anthracnose (Colletotrichum trifolii). During the past season M. sarcinaeforme was the cause of much loss in East Lansing.

3. The disease caused by M. sarcinaeforme has so far not been carefully investigated. It is widely distributed in U. S. and Europe. The loss it causes has not been estimated, but it has been responsible for local damage in various parts of the United States.

4. The fungus studied by the author may not be the same as the one first described. Cavara, (1890).

5. Red clover is readily infected by the fungus. Inoculation made upon local alsike clover have not been successful, although Bain and Essary claim to have seen it on alsike clover associated with badly diseased red clover plants in Tennessee.

Inoculation experiments have also been unsuccessful on other legumes. The fungus on alfalfa called M. sarcinaeforme (U.S.D.A. Herb. specimens) has been found

to be a different form from the M. sarcinaeforme on red clover from various parts of the U. S.

6. The fungus affects both the leaves and the petioles,- on the former causing a typical concentrically marked leaf spot, on the latter brown to black linear streaks.

7. The fungus enters the host usually between the epidermal cells of the leaf and attacks at first the parenchyma, proceeding intercellularly. The cells are eventually killed and invaded by the fungus, which finally sends up hyphae between the epidermal cells (sometimes through stomata) on both sides of the leaf and produces spores. Typical spots develop within five to seven days. As a result of infection the leaves eventually shrivel and fall to the ground.

8. Inoculations have not been successful upon either the old stems or the flower. The fungus readily attacks germinating seeds.

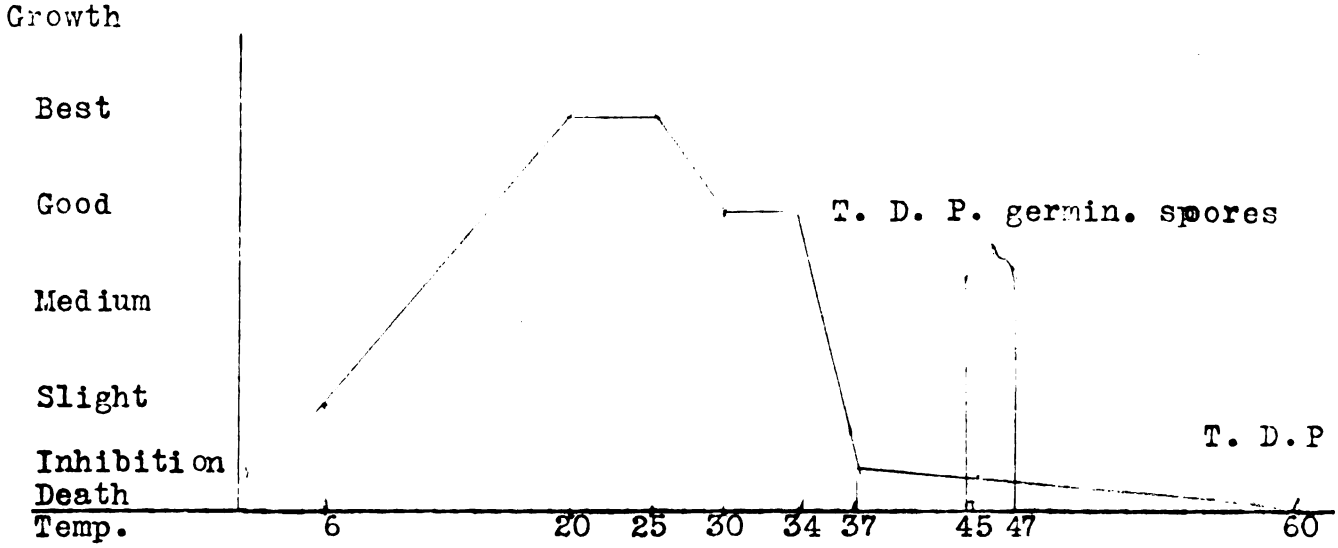
9. The spores germinate readily in water. In clover juice large swellings at the base of the germ tube appear, and new spores are formed within five to seven days. In 5% dextrose solution the spores in some cases produce large, bud-like swellings resembling a young spore. This may be a trend toward an Alternaria habit.

10. The fungus grows well upon a large variety of culture media, without striking modification,- except, perhaps, the dearth of spores on potato plugs on which growth is otherwise well developed. Sugar seems to favor

spore production.

11. In media containing nitrogen, ammonia is one of the end products of metabolism. No acid or gas is produced in glucose, maltose, and saccharose solution.

12. The temperature relations are best summarized by the following curve:



13. The spores require an air humidity of 92.8-93.4% saturation before they can germinate. The spores will not germinate if the saturation is below 92.8%. The spores can withstand dessication for a long period of time,- at least for 18 months, and probably very much longer.

14. The fungus grows equally as well in light and darkness. The shaded lower leaves of the plant are more readily attacked than those well lighted.

15. In culture, the fungus produces a toxic substance. This subject has already been summarized on page 77.

16. The disease may spread from the soil to the plant as a result of splashing by rain. The ascent of the

disease upward from the lower leaves seems to be coordinate with the amount of precipitation. The spores of M. sarcinaeforme have been found in the red clover seed but not growing upon it. This is a possible means of disseminating the disease. Experiment has shown that the spores can be carried at least 48 feet by a wind velocity of 3.93 miles per hour, and it is very probable that out doors, because of the higher altitude obtainable, they may be carried a greater distance,- sufficiently far to reach and infect other fields. The fungus probably lives over winter on the dead trash in the field.

17. The ultimate method of controlling this, and other clover diseases, must be by the breeding of resistant varieties.

MEDIA FORMULAE.

Clover juice

200 g. of finely cut clover plants (leaves and stems)
1000 cc. tap water.
Boil one hour, adding water occasionally to make up for loss by evaporation.
Filter through cheese cloth and let stand a few hours until fine settlement settles to bottom.
Filter through paper.
Autoclave 15-20# for 15 min.

Clover juice agar

Add 1.2% agar to above liquid.
Clear and filter.
Autoclave 15-20# for 15 min.

Prune juice agar

120 g. prunes
1000 cc. water
Boil in steamer 2 hr.
Filter thru cheese cloth.
Restore to original volume
12.g. agar.
Boil in steamer 2 hrs.
Clear and filter
Autocl. 20#, 20 min.

Corn meal agar

3 tablespoonsfull corn meal.
1000 cc. water
Cook in double boiler at 60° C. for 3 hrs.
Decant, restore to original volume.
15 g. agar.
Clear and filter
Autocl. 15#, 20 min.

Oat agar

75 g. Quaker oats
1000 cc. water
Cook in double boiler 2 hrs.
Strain thru cheese cloth, squeezing out the slimy matter.
10 g. agar.
Boil 1 hr.
Autocl. 15#, 20 min.

Thaxter-Hard potato agar

250 g. sliced potato
1000 cc. water
Cook one-half hour in steamer
Decant and restore to original volume.
20 g of glucose.
30 g. of agar.
Cook in steamer 3 hrs.
Clear and filter.
Autocl. 15#, 20 min.

Nutrient glucose agar

40 g. glucose
1000 cc. dist. water
3 g. NaCl
20 g. peptone
5 g. beef extract
30 g. agar
Clear and filter
Autocl. 20#, 20 min.

Plain nutrient agar

1000 cc of water
15 g. agar
3 g. beef extract
10 g. peptone
Titrate
Clear and filter
Autocl. 15#, 15 min.

Nutrient dextrose agar

Plain nutrient agar
plus 5% dextrose.

Full nutrient sol. Knudson

1000 cc. water
1 g. KNO_3
0.5 g. KH_2PO_4
0.25 g. $MgSO_4$
0.002 g. Fe_2Cl_6
5 g. cane sugar

Coon's synthetic Sol.
Stock m/5

$MgSO_4$, 2.466 g. + 50 cc H_2O
KH_2PO_4 1.36 " " "
Asparagin 1.33 " " "
Maltose 3.60 " " "
For 100 cc. Synthet. sol.;
1 cc. of m/5 $MgSO_4$
5 cc. of each of the others.
84 cc of water.
Steam 3 consecutive days.

Coon's synthetic agar

Above solution plus
1.2% agar.
Steam 3 consecutive days.

Rice

Add 3 volumes of tap water
to 1 volume of rice.
Autocl. 15#, 20 min.

Corn meal

Corn meal with about equal
volume of tap water
Autocl. 15#, 20 min.

Potato plugs

Wash and peel a firm
tuber.
Cut into wedge shaped
plugs, and place in
tubes containing short
piece of glass rod and
few cc. of water.
Autocl. 20#, 20 min.

The following other
vegetable plugs were
prepared in the same
manner as the potato:
carrot, parsnip,
sugar beet, red table
beet.

Sorghum stems

Cut stems into pieces
2-3 in. long.
Put into tubes dontain-
ing little water.
Autocl. 15#, 20 min.

Clover stems

Same as sorghum.

Bean pod

Mature pod of wax bean
put in tube with little
water.
Autocl 15#, 20 min.

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EXPLANATION OF PLATES

Plate I.

Fig. 1, Clover leaf, showing typical spots, X 1/2.

" 2 Leaf spot, showing concentric rings, X 7.

" 3 Leaf in the last stage of the disease, showing the shriveled condition of the leaflets (b,c). Note aggregation of small spots at a. X 1/2.

" 4 Young leaflet with infection spreading to pedicle at (a). X 1/2/

" 5 Showing linear streaks on portion of pedicle. X 1.

" 6 Germinating seed showing seed coat completely enveloped by fungus growth; also tip of radical attacked by fungus. X 4.

" 7 Section through leaf spot, somewhat diagrammatic, showing how the tissue is completely disintegrated and invaded by the fungus. Note fungus spreading into healthy tissue at the edge of the spot. X 150.

" 8 Early stage of infection. Note the intercellular mycelium, also the hyphae coming up thru stoma. X 300.

Plate II.

Fig. a-i Showing various shapes of spores. Note scar of attachment on Fig, (i). X 800.

" 1. Young mycelium. X 500.

" 2, 3, 4, 5, 9, 10. Various types of mycelium found in culture. Note oil globules. X 500.

" 6, and 7. Submerged mycelium. X 500

" 8 Showing how the conidiophores are borne. Note long conidiophore on the end of the main thread. X 500.

" 1-IX. Showing spore formation. X 500.

Plate III.

Fig. 1 Typical conidiophore bearing spore.

" 2 Showing the knob-like projections on the cells of some conidiophores.

Plate III Cont'd

Fig. 3 A conidiophore with double tip cell swellings.

" 4 Spore giving rise directly to conidiophores bearing new spores.

" 5 Type of germination in 5% dextrose solution. Note large partition cell budding from spore. This looks like the beginning of a new spore, perhaps a tendency to an Alternaria-like habit.

" 6 Germination of tip cell of conidiophore after spore has fallen off.

" 7 Spore germinating in distilled water. Note narrow, uniform mycelium. Growth proceeds no farther than this.

Plate IV.

Germination of a spore in clover juice. Note swelling of basal cells of germ tubes and hyalin cells at end of tubes.

Plate V.

Same spore as above after 48 hours.

Plate VI.

Fig. 1-3 Apparatus used in first wind dissemination expr.

" 4-5 " " " second " " " .

" 6 Van Tieghen cell arrangement used in toxin exper.

" 7 Potometer used in transpiration experiment.

Plate VII.

Typically infected red clover leaves.

Plate VIII.

Showing infection produced by spraying red clover with suspension of spores from new culture.

Plate IX.

SHowing infection produced by spraying red clover with suspension of spores from old culture.

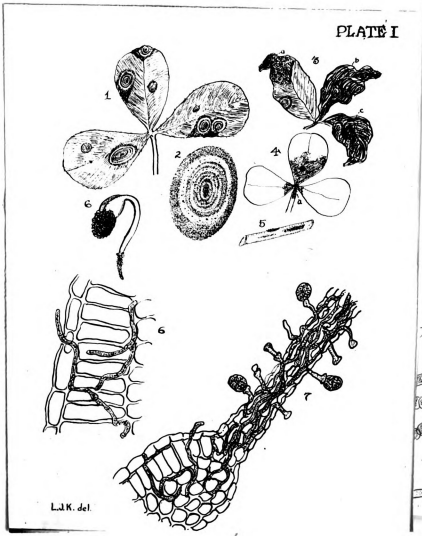
PLATE I
L.J.K. del.

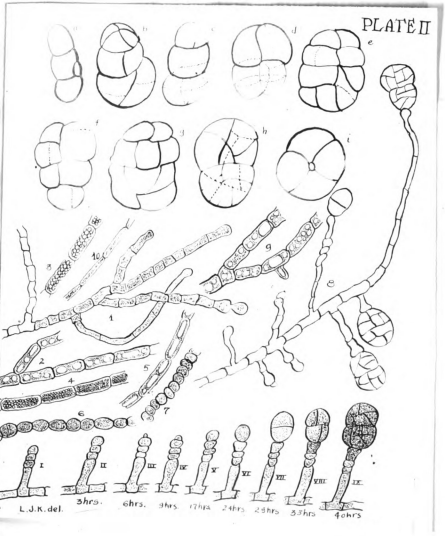
PLATE II
L.J.K. del.
3hrs.
6hrs.
9hrs.
17hrs.
24hrs.
29hrs.
33hrs.
40hrs.

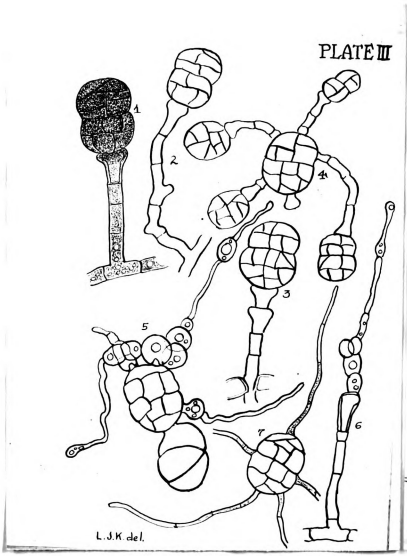
PLATE III
1
2
3
4
5
6
7
L.J.K. del.

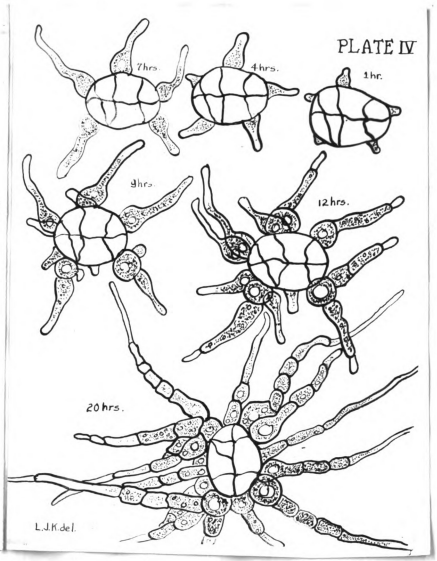
PLATE IV
7hrs.
4hrs.
1hr.
9hrs.
12hrs.
20hrs.
L.J.K. del.

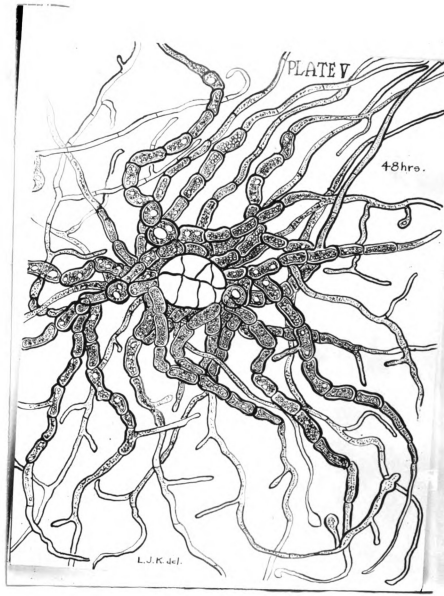
PLATE V
48hrs.
L. J. K. del.

PLATE VI.

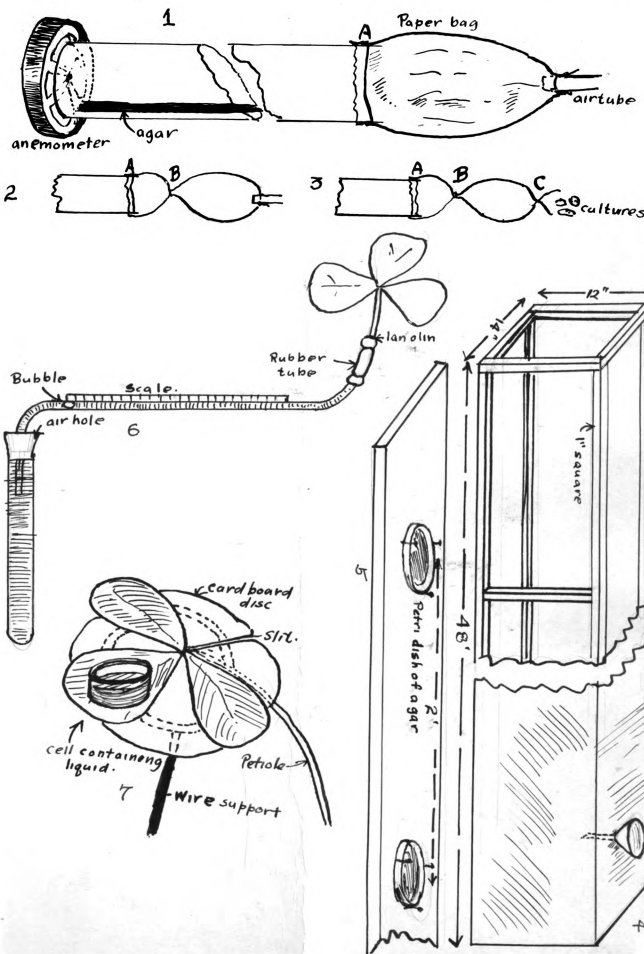
1
Paper bag
A
airtube
anemometer
agar
2
A
B
3
A
B
C
20 cultures
lanolin
Rubber tube
Bubble
scale.
air hole
6
12"
14"
1" square
5
48'
Petri dish of agar
2'
4
cardboard disc
slit.
cell containing liquid.
Petiole
7
Wire support
Wire

PLATE V11

PLATE V111.

PLATE IX