NITED STATES DEPARTMENT OF AGRICULTURE

BULLETIN No. 759

Contribution from the Bureau of Plant Industry
WM. A. TAYLOR, Chief

Washington, D. C. PROFESSIONAL PAPER June 19, 1919

THE LEAF-SPOT DISEASES OF ALFALFA AND RED CLOVER CAUSED BY THE FUNGI PSEUDOPEZIZA MEDICAGINIS AND PSEUDOPEZIZA TRIFOLII, RESPECTIVELY

By

FRED REUEL JONES, Pathologist, Cotton, Truck, and Forage Crop Disease Investigations

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By FRED REUEL JONES, *Pathologist,*
Cotton, Truck, and Forage Crop Disease Investigations.

CONTENTS.

SCOPE OF THE INVESTIGATION.

Among the diseases of the foliage of the alfalfa plant, the one which is most widely known and is reported to cause the greatest loss is the leaf-spot caused by the fungus *Pseudopeziza medicaginis.* The disease is commonly called the alfalfa leaf-spot. This name is not distinctive, and its continued use is open to the objection that it promotes the present tendency to apply it inclusively to all the several leaf diseases that may be present with the true leaf-spot. However, the usage is so prevalent that it appears likely to persist.

Although the importance of the disease has caused it to be mentioned widely and frequently, little careful study has been devoted to it. A great number of scattered and conflicting observations have left the life cycle of the fungus causing the disease in doubt; the

method of overwintering of the fungus has not been conclusively demonstrated; and the oft-considered question whether this fungus is identical with or merely similar to one which causes a corresponding leaf-spot on red clover has never been decisively answered. It is because of the last consideration that the two diseases have been studied together. The leaf-spot of red clover caused by the fungus *Pseudopeziza trifolii* occurs over a wide range of territory, but usually not so abundantly as that on alfalfa. Mention of it occurs frequently in literature. No distinctive common name has been applied to it. Much of the later interest in this disease is in connection with the question whether or not red clover is a source of infection, producing destructive outbreaks of leaf-spot on neighboring alfalfa when this plant has been introduced into new localities.

Leaf-spots of a nature very similar to the two already mentioned and commonly reported to be caused by the same fungi are known on a long list of clovers, alfalfas, and closely related plants. All of these diseases should be studied together and the relationship of the causal organisms determined. However, most of them do not occur in America or only in restricted localities, and none of them causes great damage to the host plants. The only one of these of which any living material has been available for study is that caused by *Pseudopeziza medicaginis* on *Medicago lupulina*. The incomplete notes on this disease have been included.

THE DISEASES.

ECONOMIC IMPORTANCE.

As has already been stated, the assertion has been made again and again that leaf-spot is the most common and destructive of the foliage diseases of alfalfa. That it is the most common is beyond question. But in the estimates of the loss which it has caused it appears highly probable that damage from other causes than the conspicuous leaf-spot has been included. Nevertheless, even if proper deductions for these inclusions could be made it might still be true that leaf-spot causes greater loss than any other foliage disease.

The highest estimate of loss from this disease is that of Pammel (1891) [1] from Iowa. In 1890 he attributes to this cause a loss of half the crop. Stewart and others (1908, pp. 384–387) report from New York that young stands are often ruined and that old stands are killed outright. Chester (1891) reports that some plats at the Delaware station in 1889 were attacked severely before the plants were large, and some of them were completely destroyed. Voges (1909) in Germany and Ivy Massee (1914) in England note the sickly appearance of diseased fields.

[1] The dates in parentheses refer to "Literature cited" at the end of this bulletin.

The amount of damage which the fungus may cause appears to depend on several circumstances relating to the development of the crop and the weather. Under ordinary conditions the incubation period of the disease is more than a week. If for any reason the plant is growing slowly, the stand is thick, and the weather is frequently wet, only a few of the upper leaves reach full development before they are covered with the disease. Thus young stands which grow slowly before becoming firmly rooted and old stands which are retarded for any reason are likely to show bad attacks, while stands which are growing rapidly keep most of the upper leaves well above the rising invasion of the fungus and show little harm. Thus, in most cases where the fungus is found in great abundance, apparently defoliating plants, it will be found that some condition has reduced the normal rate of growth of the plants and is in part responsible for the resulting damage. When plants are vigorous, infection must be heavy indeed to cause extensive yellowing and falling of leaves, though this may occasionally occur.

Nevertheless, the fungus is present in almost every alfalfa field, if not in all fields, taking a small toll of the foliage under even dry conditions and a large toll under more humid conditions. Since it rarely produces great loss at one time it has come to be regarded as one of the unavoidable evils to which the alfalfa plant is subject.

The leaf-spot of red clover caused by the fungus *Pseudopeziza trifolii* has not frequently been reported as occasioning great loss. In Russia Jaczewski (1912, p. 98) speaks of it as causing appreciable damage. Blasdale (1902, p. 75) states that it injures nearly all the clovers of the stock ranges of northwestern California. Freeman (1905, p. 309–310) notes that it causes local epidemics in clover fields in Minnesota. In fields in northern Wisconsin and in Maine in the summer of 1915 it was observed by the writer to be so abundant as to cause appreciable loss of foliage. From the evidence at hand it appears that the disease is not of great significance to clover and that this significance is only in northern regions. However, the destroyed foliage is so much less conspicuous than that on alfalfa that the amount of damage is more likely to be underestimated than overestimated.

DESCRIPTION OF THE DISEASE ON ALFALFA.

There are two characteristics of the leaf-spot caused by *Pseudopeziza medicaginis* which usually serve to distinguish it from spots caused by other parasitic fungi. The first of these is the circular shape and limited size of the spot. (Pl. I, *A*.) The second is the presence of a small raised disk (Pl. II, *B*) that appears in the center of the spot when it has reached full development. The edge of the spot may be smooth and definite, especially if the leaf has been much

exposed to the sun, or it may be more or less dendritic, with a fringe of olive-colored rays. No marked killing or sinking of the leaf tissue occurs. In size, the spot rarely exceeds 2 or 3 millimeters in diameter.

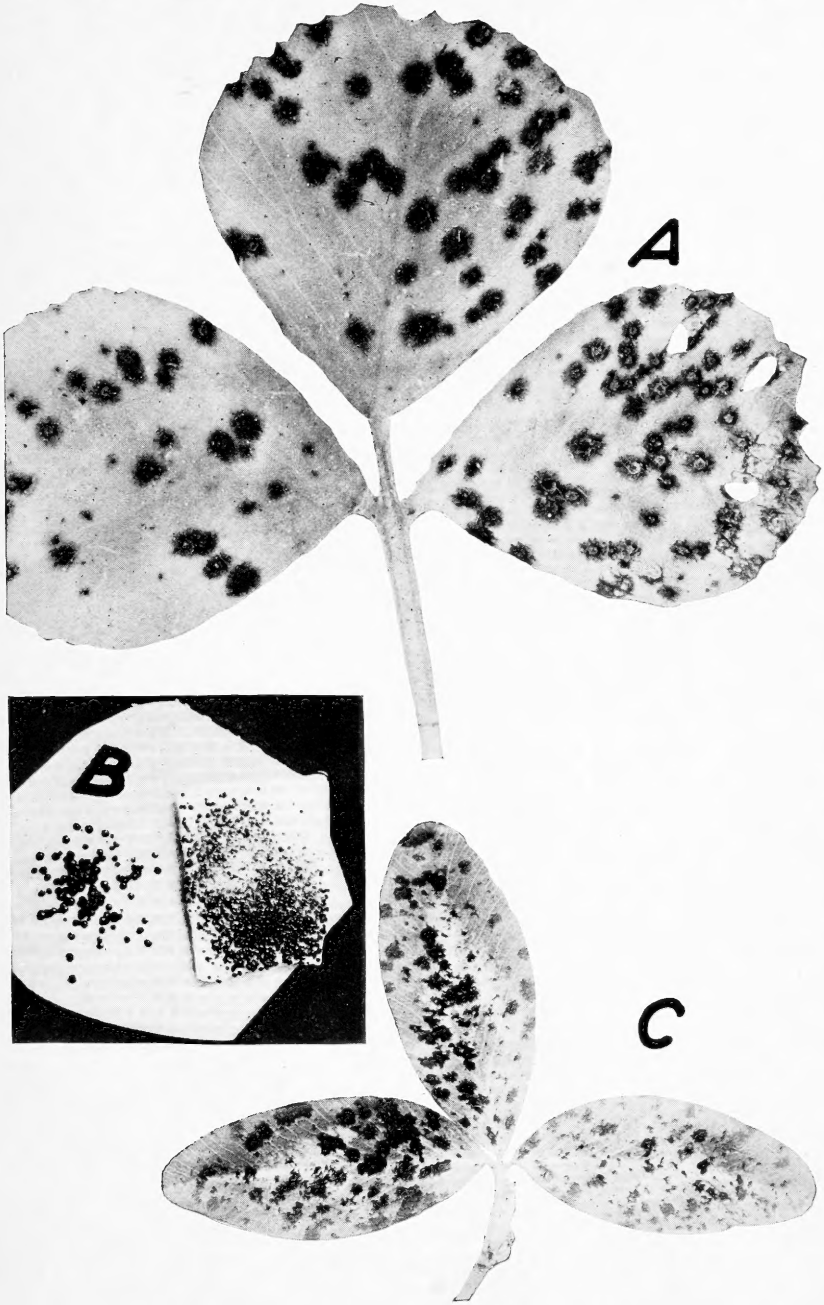
The disk at the center of the spot is the fruiting structure of the fungus and consists of a mass of asci which discharge large numbers of spores when sufficient moisture is present. These disks usually occur on the upper side of the leaf, sometimes on the lower side, and rarely on both sides from the same spot. Typically they are 1 to 1½ millimeters in diameter, slightly raised, and when fully developed surrounded by the torn edges of the epidermis of the leaf. Rarely the central disk is found surrounded with several smaller disks at its margin. Under very moist conditions the disk may appear as a jellylike drop of exudate at the center of the spot. Under arid conditions it becomes very dark in color, often almost black.

There is not usually a striking difference in color between the diseased tissue and the disk at the center. This color varies from dark brown to almost black. If the leaf has begun to yellow, the green color is sometimes retained longest around the diseased area.

The disease often occurs on succulent stems, where it has an appearance so characteristic that it can hardly be confused with injury from any other cause. The spot is elliptical in shape, with perfectly smooth edges. In size it is about 1½ by 3 mm. It is not abundant, and rarely bears a fruiting disk.

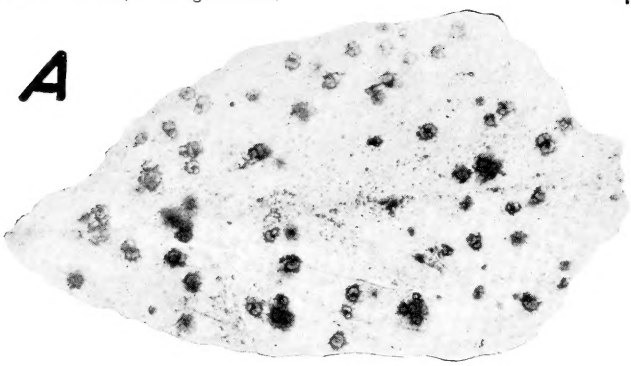
DESCRIPTION OF THE DISEASE ON RED CLOVER.

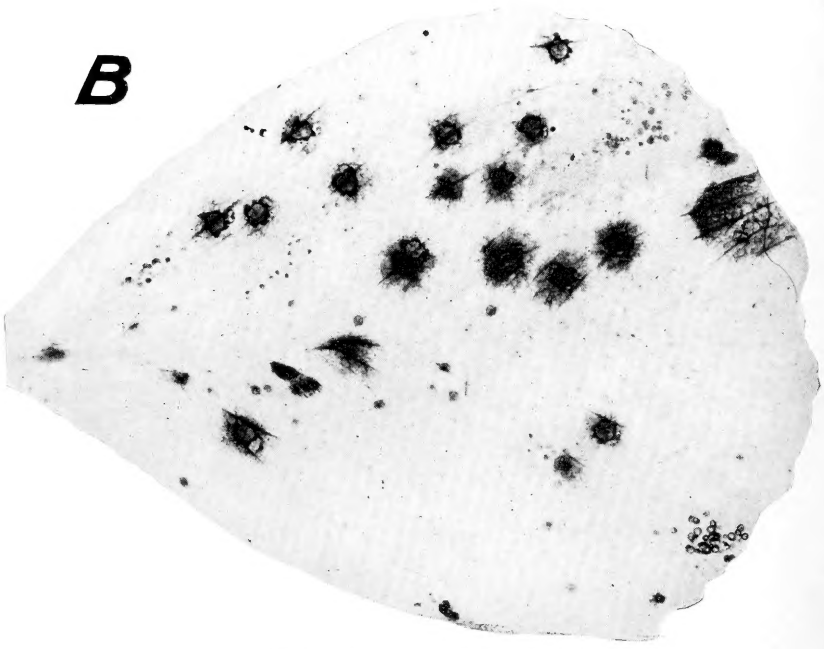
The leaf-spot on red clover caused by Pseudopeziza bears a very close resemblance to the similar disease on alfalfa. The spots are limited in size, usually slightly larger than on alfalfa, in early stages tending to be angular (Pl. I, *C*). The border of the spot is more frequently dendritic in outline. In early stages the color is dark olive, becoming brown or almost black in later stages. After the death of the entire leaf, the spot usually becomes almost indistinguishable. Fruiting disks are not as frequently found abundantly on the spots while the leaf is still alive as in the case of the leaf-spot of alfalfa, but they may develop abundantly after the death of the leaf. In early stages they are brownish or dirty yellow, but later they become almost black. They are more frequently found on the under side of the leaf than on the upper side, and occasionally occur on both sides from the same spot. On dead leaves they appear as amber drops of jelly in wet weather, but when dried they shrink to bodies so small and inconspicuous that it is practically impossible to find them. The disease has not been noted on any other part of the plant than the leaves.



PSEUDOPEZIZA ON HOST PLANTS AND PURE CULTURES.

A, Leaf-spot of *Medicago lupulina* caused by *Pseudopeziza medicaginis* (Lib.) Sacc. *B*, *Pseudopeziza medicaginis* developing in pure culture from spores discharged on plaster of Paris (right) and filter paper (left). *C*, Leaf-spot of red clover caused by *Pseudopeziza trifolii* (Biv.-Bern.) Fckl. at an early stage of development. Apothecia have not yet appeared.





PSEUDOPEZIZA ON ALFALFA.

A, Apothecia of Pseudopeziza developing on old leaf-spots on overwintered leaves. In many cases the apothecia are clustered. × 3, approximately. *B*, Apothecia of Pseudopeziza on an alfalfa leaflet. The leaf has been decolorized to show the fungus more clearly. The small dark circles near the lower right-hand edge of the leaf are early stages of apothecia of *Pyrenopeziza medicaginis*. × 5, approximately.

HOST PLANTS.

Although these fungi occur extensively in America on alfalfa and clover only, each of them has been reported in mycological literature on a number of related plants.

The names of these host plants are shown in Table I.[1]

TABLE I.—*List of host plants of Pseudopeziza trifolii and Pseudopeziza medicaginis.*

Hosts of Pseudopeziza trifolii.		Hosts of Pseudopeziza medicaginis.	
Name of host plant.	Authority cited.	Name of host plant.	Authority cited.
Trifolium:		Medicago:	
alpestre L	Saccardo, D., 1903, no. 526, 1319.	carstiensis Jacq	Saccardo, D., 1903, no. 526, 1319.
arvense L	Krieger, 1892, no. 781, 794.	fulcata L	Cavara, 1892, p. 243.
cyathiferum Lindl	Blasdale, 1902.	hispida denticulata (Wild.) Urban.	Massee, 1914.
fragiferum L	Massee, 1914.	lupulina L	
hybridum L	Bivona-Bernardi, 1816, man. 4, p. 27, pl. 6, fig. 5.	lupulina wildenowii (Boenn.) Aschers.	Libert, 1832, fasc. 2, no. 176.
incarnatum L	Saccardo, P. A., 1889, pp. 723–724.	minima Link	Fuckel, 1870, p. 290, 236.
medium L	Lambotte, 1880, p. 264.	prostrata Jacq	Jaap, 1916.
macrodon Hook and Arn.	Blasdale, 1902, p. 75.	sativa L	Desmazieres, 1847, pp. 182–183.
nigrescens Viv	Briosi, 1888.	Melilotus alba Desv	Tracy and Earle, 1895, p. 106.
pallescens Schreb	Massee, 1914.	Onobrychis sativa Link	Berthault, 1913.
pratense L	Saccardo, P. A., 1889, pp. 723–724.	Trigonella:	
pallidum W. and K.	Maire, 1913, no. 119.	coerulea (L.) Ser	Mazerius, 1875, fasc. 33, no. 1645.
repens L	Briosi, 1888.	corniculata L	Jaap, 1916.
resupinatum L	Massee, 1914.	foenum-graecum L	Massee, 1914.
spadiceum L	Saccardo, P. A., 1897, p. 623.	Vicia villosa Roth	Tracy and Earle, 1895, p. 106.

THE FUNGI.

SYNONOMY OF PSEUDOPEZIZA TRIFOLII.

Pseudopeziza trifolii was first described by Antonio Bivona-Bernardi (1816, Mar. 4, p. 27, pl. 6, fig. 5) on *Trifolium hybridum* from Sicily under the name of *Ascobolus trifolii*. When Boudier (1869) revised the genus Ascobolus he listed this species among those which he believed should be excluded, and suggested that it be placed in the genus Phacidium. The following year Fuckel (1870) made this species the type of his new genus Pseudopeziza.

Other early synonyms as given by Rehm (1892, p. 597–598) are as follows: *Peziza trifoliorum* Libert, *Trochilia trifolii* DeNot., *Molliscia trifolii* Phill., *Phyllachora trifolii* Sacc.

The following three names have been included in the synonomy by Ivy Massee: *Pseudopeziza* (*Phacidium*) *divergens* (Desmaz.) Sacc.; *Peziza dehnii* Rab., a common parasite of Potentilla; *Pyrenopeziza medicaginis* Fckl. The last of these three species has already been shown by the writer (Jones, 1918) to be the ascigerous stage of

[1] In compiling this host list, the fungus found on species of the genus Trifolium is regarded as *Pseudopeziza trifolii* (Biv.-Bern.) Fckl., while the fungus on species of the genus Medicago or closely related genera is regarded as *P. medicaginis* (Lib.) Sacc. Owing to the fact that the two fungi have frequently been regarded as one, the fungus on Medicago and its relatives has often been reported as *P. trifolii*.

Sporonema phacidioides Desmaz. The writer has not been able to discover any adequate reason for the inclusion of the other two names.

SYNONOMY OF PSEUDOPEZIZA MEDICAGINIS.

The first collection and description of Pseudopeziza upon a species of the genus Medicago were made by Madam Libert (1832, fasc. 2, no. 176) under the name of *Phacidium medicaginis*. The host was *Medicago wildenowii*, now known as *Medicago lupulina wildenowii*. Later, when Desmazieres (1841) found Pseudopeziza upon alfalfa he assumed that it was identical with the species described on *Medicago wildenowii*. His assumption has not been seriously questioned.

In 1883 Saccardo (1883, no. 1390, 1391) transferred this species to the genus Pseudopeziza which Fuckel (1870) had established with *Pseudopeziza trifolii* as the type species. As soon as the two fungi were brought together in the same genus their similarity raised the question whether they were not identical. Briosi (1888) compared the fungi as they occurred on several species of Trifolium and Medicago and failing to find sufficient morphological difference between them to justify retaining them as distinct species advised that Pseudopeziza on alfalfa be called *Pseudopeziza trifolii* forma *medicaginis*. This usage has been followed by Rehm (1892, p. 597–598) and appears to have been generally accepted by mycologists, many of whom drop the form name altogether. Plant pathologists, on the other hand, have found it more convenient to retain the two names, though in most texts it is noted that possibly or even probably the two species are identical. The writer believes that the following pages present adequate evidence that the fungi on the two hosts are separate and distinct species.

COMPARATIVE MORPHOLOGY OF THE FUNGI.

The apothecia of both these species of Pseudopeziza arise in a delicate stroma beneath the epidermal layer of the leaf. The apothecia on alfalfa are usually solitary, except on overwintered leaves, where several clustered apothecia may develop on a stroma. On red-clover apothecia are sometimes clustered. The hymenial layer when first developed is covered with a thin stromatic stratum of small rounded cells, the outer layer of which may develop thick dark-colored walls. This stroma usually remains adherent to the epidermis when this is ruptured by the developing asci.

As the hymenial layer develops, the stroma from which it arises becomes thicker, forming in and among the collapsing leaf cells. The epidermis is ruptured, the hymenium is raised above the surface of the leaf, and after the spores have been largely discharged and the hymenium has shrunken the recurved flaps of the torn epidermis become conspicuous around the apothecium.

Under favorable conditions apothecia may reach 1½ mm. in diameter, but are usually 1 mm. or less. Asci are 60 to 70 microns long, and about 10 microns in diameter. Paraphyses are slightly longer than the asci, nonseptate, and swollen at the ends.

Ascospores of the two species (figs. 1 and 2) show slight differences in size, those of *Pseudopeziza trifolii* being larger. The spores of each species have shown small variations in measurement when they were obtained under different conditions affecting their discharge. The most important of these variations has occurred when spores are obtained from apothecia which are drying rapidly. Under these conditions discharge is greatly accelerated, and the number of spores of smaller size is increased. Therefore, in order to obtain comparable measurements certain precautions were always taken to obtain spores of the same degree of maturity. Fruiting cultures of the fungus or leaves bearing apothecia were placed in the cover of a Petri dish over a layer of clear agar. After about 10 hours, when the discharge of spores was apparently proceeding at a uniform rate, the cover of the dish was turned about so that the spores now fell on a new portion of the agar surface. After half an hour a considerable number of spores were usually found on the agar. The cover was then removed from the dish, a small drop of water and a cover glass were placed on the area on which the spores were scattered, and measurement was made as rapidly as possible.

FIG. 1.—Ascospores of *Pseudopeziza trifolii*. × 600.

FIG. 2.—Ascospores of *Pseudopeziza medicaginis*. × 600.

When a large number of spores have been measured to the nearest micron and the spore lengths arranged, as shown in Table II, it has always been found that the number of spores of *Pseudopeziza medicaginis* which measure 10 microns and less constitute more than half the total, while in the case of *P. trifolii* the number of spores which are 11 microns and longer constitute more than half the total. Table II presents a typical comparison of the measurement of 100 spores of each species.

TABLE II.—*Comparison of the lengths of 100 spores each of Pseudopeziza medicaginis and Pseudopeziza trifolii, measured to the nearest micron.*

Spores of—	Length (microns).						
	8	9	10	11	12	13	14
Pseudopeziza medicaginis.....number..	7	26	33	24	10	.....	.....
Pseudopeziza trifolii.....do....	.....	3	26	46	18	6	1

By careful comparison in this manner it has been found possible to distinguish between the two species on the basis of spore measurement alone.

In addition to the difference in size, there is a difference in shape that is discoverable by the examination of many spores—a difference that does not significantly appear in measurement. Some of the spores of *Pseudopeziza trifolii* are slightly flattened on one side. When the flattened side is seen in profile the spore has a somewhat pointed appearance. The occurrence of occasional pointed spores (fig. 1) is a distinguishing feature of this species.

Spores from dried specimens have not been found satisfactory for comparative measurement. Unless the collection is made just before the apothecium is completely mature, nearly all of the spores are unavoidably discharged during drying. The few remaining are likely to be found much shrunken.

MORPHOLOGICAL CHARACTERS IN CULTURE.

MYCELIUM.

In culture these fungi preserve the same general characteristics that they show on the host plant. The mycelium radiating from the germinating spore or group of spores soon produces a stroma at the center. Thereafter this stroma is surrounded with a narrow fringe of hyphæ, which never advance far beyond the stroma. When the fungi are grown from spores on the same nutrient substratum, differences in the character of the mycelium can be noted. That from *Pseudopeziza medicaginis* branches earlier than that of *P. trifolii;* most of its branches come off at an acute angle, while those of *P. trifolii* come off somewhat regularly at a right angle, or occasionally at an obtuse angle.

CONIDIUMLIKE STRUCTURES.

Although no conidia have been found in nature, conidiumlike structures occur regularly in culture and are a feature by which cultures of the two species can be most easily distinguished. They arise from the ends of branches or from the distal ends of somewhat swollen cells. They measure 5 to 8 by 3 to 5 microns. They occur most abundantly when the ascospores are germinated on clear agar to which no nutrient has been added, appearing in about three days in the case of *Pseudopeziza medicaginis* and somewhat later on mycelium of *P. trifolii.* On mycelium of the first fungus they are produced in great abundance before the end of the first week, though the mycelium from different spores or groups of spores produces them in varying amount. (Fig. 3.) The mycelium may grow but little, becoming thickly covered with the conidia, or it may grow more freely with but a few conidia at the ends of short branches. Rarely are they

absent. In striking contrast is the scarcity of these spores on mycelium from spores of *P. trifolii.* (Fig. 4.) Never are they produced in great numbers, and frequently they are entirely absent from all but a few fungous colonies. Thus, the striking abundance of these structures on mycelium of *P. medicaginis* and their scarcity on

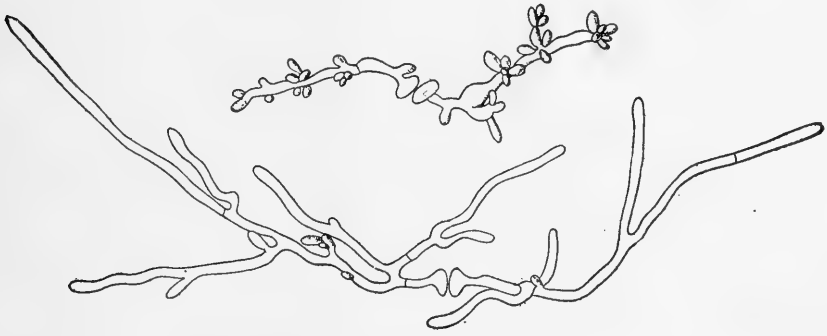


FIG. 3.—Mycelium and conidiumlike structures developing from ascospores of *Pseudopeziza medicaginis* on agar agar. × 400.

mycelium of *P. trifolii* furnish an easy morphological distinction between the two species.

REPORTED CONIDIAL STAGES OF THESE FUNGI.

It is a matter of some interest to note that all the studies of Pseudopeziza on alfalfa and clover which have been made by European mycologists and pathologists with but a single exception (Briosi, 1888) have contained a discussion of an associated conidial stage. Thus at least three, perhaps four, imperfect fungi have been assigned to this rôle in addition to the conidiumlike structures which are produced in culture. A summary of the evidence on the basis of which the association of these conidial stages has been made is here given.

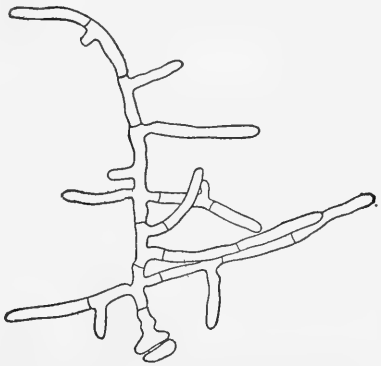


FIG. 4.—Mycelium developing from an ascospore of *Pseudopeziza trifolii* on agar-agar. × 400.

The first of these fungi to be regarded as a conidial stage of *Pseudopeziza medicaginis* was *Sporonema phacidioides* Desm. Since the writer has a previous article (Jones, 1918) traced the development of the purely observational evidence on which this association was based and has shown that this Sporonema is the conidial stage of *Pyrenopeziza medicaginis* Fckl., no further discussion is necessary here.

The second suggestion that Pseudopeziza produces conidia comes from the work of Brefeld (1891, p. 325). In the course of his study of Pseudopeziza on both clover and alfalfa he germinated the ascospores. The cultures thus obtained of these fungi behaved alike. Very little mycelium was produced. After about 14 days conidia were cut off laterally from certain threads and from the ends of side branches. These conidia were not observed to germinate. After describing them, Brefeld refers them to the conidia described by Tulasne (probably referring to the conidia of *Sporonema phacidioides*), but he does not state whether he regards his conidia identical with those described by Tulasne or not. The structures which Brefeld describes and figures as conidia appear to be identical with those already described as occurring in culture.

The third and most extended reference to a conidial stage of *Pseudopeziza medicaginis* is that of Voges (1909). In the course of a study of an outbreak of the disease in fields under his observation in Germany, he reports that he finds closely associated with the fruiting disks of Pseudopeziza on living leaves the pycnidia of a Phyllosticta which does not appear to him to belong to a previously described species. Unfortunately, his own description of this Phyllosticta is so meager that it does not serve to identify it. He states that the spores are differentiated into two forms—a smaller 1-celled spore and a larger, often 2-celled spore. No mention is made of any attempt to determine whether or not the two types really belong to the same fungus or not, nor does he explain why such a fungus should not be called Ascochyta rather than Phyllosticta.

Voges next attempts to identify the Phyllosticta with Pseudopeziza by cultural methods. He places fragments of Pseudopeziza fruit disks on a nutrient substratum. When this is done in March and October, no results are obtained; but in June he gets a fungus on his plates which first produces aerial conidia and later pycnidia like those previously found on the leaves. Inoculations made on alfalfa leaves with these leaf-spot cultures produced typical Phyllosticta spots. Inoculation of alfalfa leaves with fragments of Pseudopeziza fruiting disks gave like results. Consequently he concludes that the Phyllosticta and Pseudopeziza are identical and that Pseudopeziza has three spore forms—aerial conidia, conidia in pycnidia, and ascospores. Finally he inoculates clover leaves with fragments of his Phyllosticta culture and finds that typical spots bearing Phyllosticta spores are produced. Hence he concludes that the Pseudopeziza on alfalfa must be identical with that on clover.

Even if these results of the few experiments which he performed are accepted at their full value, the conclusions which he draws are manifestly not justified. In the first place, the fact that he was unable to get cultures of his Phyllosticta from Pseudopeziza spots

except at a certain period in the summer is not adequately explained by his extraordinary theory of a periodicity in the vegetative vigor of the fungus. In the second place, inoculations with this Phyllosticta whether upon alfalfa or upon clover produced lesions which bore only the pycnidia of the Phyllosticta, never the apothecia of the Pseudopeziza. These cultures obtained under doubtful conditions produced ascospores neither in culture nor as a result of inoculation. Thus, the evidence which Voges presents, judged entirely by itself, does not prove or even clearly indicate that he ever had Pseudopeziza in culture. It does not appear, however, that the work of Voges has been widely accepted, at least not in America, even though the report of his work as presented in the Experiment Station Record [1] is incorrect or misleading in almost every detail, causing his conclusions to appear much more justifiable than when they are read in the original article.

In the same year that the article by Voges was published Voglino (1909, pp. 226–228) in Italy presented evidence which he believed indicates that *Gloeosporium caulivorum* Kirch. or *G. trifolii* Pk., which in his opinion may be identical with it, is the conidial stage of Pseudopeziza on *Trifolium pratense*. His evidence was obtained both from observation and from cultures. In a certain field considerably injured by Gloeosporium he finds apothecia of *Pseudopeziza trifolii* developing in close association with the acervuli of the Gloeosporium. Later he makes cultures from conidia obtained from stems on a clover decoction with gelatin, and in a single culture he found after 30 days three apothecia of a fungus which he assumes to be a Pseudopeziza. On the basis of this evidence he decides that the Gloeosporium must be the conidial stage of Pseudopeziza.

The account which Voglino gives of his work is very brief and bare of details. No mention of inoculations is made. No description of the Pseudopeziza which he regards as *Pseudopeziza trifolii* is given. It is not clear from his account that he obtained a pure culture. Therefore his results can hardly be regarded as having more than a suggestive value.

This review of European literature brings us to the conclusion that, with the possible exception of the description by Brefeld of conidia in culture, there is no conclusive observational or experimental evidence that either of these Pseudopezizas has an associated condidial stage.

PHYSIOLOGY OF THE FUNGI.

ISOLATION OF THE FUNGI.

Efforts to isolate these fungi by ordinary methods were continued for a long time without avail. The first success was obtained by

[1] Experiment Station Record, v. 22, no. 7, p. 648.

taking advantage of the fact that when the ascospores are discharged from the ascus they are thrown several millimeters vertically. Repeated trials showed that if fresh leaves were used, spores could sometimes be obtained in considerable numbers on an agar surface placed over the apothecia without bacterial or fungous contamination. This work of collecting spores was best carried out in a Petri dish in which a layer of very clear agar had been poured. The dish was inverted and the leaf bearing the apothecia supported 2 or 3 millimeters below the agar. After a short period the area over the apothecia was marked, the dish turned, and examination made with the low power of the microscope to determine whether the requisite number of spores were present. The difficulty would have been lessened had a few spores been sufficient for the development of a culture, but experience soon showed that a large number of fungous colonies crowded together developed better than a few. When several areas on the plate had been scattered with spores, the leaf bearing the apothecia was transferred to another plate. By using the utmost care and exposing a large number of plates a few could be obtained without contamination or with so small a number of foreign organisms that they could be cut out with a sterile needle. After a plate had been observed until it appeared certain that no foreign organism was present, it was found advisable in order to prevent drying to cut out the area bearing the developing fungous colonies and transfer them to agar slopes in test tubes.

In the course of experiments with cultures made in this way the first culture of Pseudopeziza from alfalfa to produce apothecia was obtained. The spores were discharged on an alfalfa-agar plate on October 5, and the agar was transferred to a water-agar slope on October 22. The ascospores were being produced on November 6. At first it was assumed that the fungus had been starved into fruiting by this process, but later work does not indicate that this was the case. Fruiting cultures can be obtained most readily by transferring the developing fungous colonies as soon as they become macroscopic from the water-agar plates to oatmeal-agar slopes. In this way the fungus was isolated six times in the autumn of 1914 and once in the autumn of 1916.

Pseudopeziza was isolated from red clover in the same way as from alfalfa. Two isolations were made of this fungus in 1915, one from clover leaves collected by Prof. H. H. Whetzel at Ithaca, N. Y., and one from clover collected in Door County, Wis.

A later successful reisolation of this fungus from plants inoculated in the greenhouse suggests that it may not always be necessary to employ this tedious process. In the instance referred to, diseased leaf fragments were cut from clover leaves two weeks after inoculation. The fragments, each bearing from one to three infections, were

dipped in 50 per cent alcohol and placed in a solution of 1 part of bichlorid of mercury in 1,000 parts of water for 1 to 1½ minutes. After washing, the fragments were placed separately on slopes of 2 per cent water agar. After these cultures had been kept three weeks at 17° to 19° C., small tufts of mycelium emerged from the fragments which had remained free from contamination. The fragments were then transferred to oat agar. Apothecia appeared two weeks later, and cultures were started. Success in this instance seems to be due to the relative freedom of these greenhouse plants from fungi which quickly enter the host tissue that has been killed by the parasite.

CULTURAL CHARACTERS OF SPECIAL MEDIA.

Only a few of the more common media on which the fungi grow most readily and show the most striking differences are selected. In connection with these descriptions the following facts regarding the method of making cultures and their habit of development should be kept in mind:

(1) New cultures are started by placing near the top of an agar slope a fragment of a culture which is producing and discharging ascospores abundantly. The position of the slope should be changed from time to time to insure a somewhat uniform distribution of the spores over its entire length. After from one to four days the original transfer may be removed to another slope and thus serve to start a number of cultures successively.

(2) The small fungous colonies which arise fruit better and earlier when closely crowded together. Yet excessive crowding may delay fruiting.

(3) Apothecia appear in three to five weeks at favorable temperatures. After a period of active spore production lasting from one to two weeks, further spore production takes place only occasionally. Transfer of the stroma to new media increases the likelihood of its occurrence but does not insure it. The stroma itself continues to grow very slowly.

Oatmeal agar.—As a culture medium oatmeal agar has proved to be the most useful for general culture work, because upon it ascospores are produced in greatest abundance. The following description applies to cultures kept at 20° to 22° C.

Pseudopeziza medicaginis: The first evidence of growth appears about one week after spores are discharged upon the medium. At this time the surface of the substrate appears roughened as though pushed up into a multitude of minute flat cones. In about two weeks the cones are increased in size and show a rusty brown color at the center. The color becomes darker at the center of the cone, surrounded by a rusty rim. Soon the dark-brown color covers the entire slope. If the colonies are not sufficiently close to touch each other in three weeks, the color is darker than if the colonies merge. At the end of three or four weeks apothecia appear at the center of the stromata as small grayish white, often glistening gelatinous

masses, very small at first, but often extending until they merge. The apothecium is merely a rounded, sometimes flattened, firm mass of asci and paraphyses which crumbles when crushed (Pl. III, *B*). The gray color of the mass becomes darker with the increased age of the culture, and finally is dark brown or almost black. The rusty brown color that is seen early in the development of the culture is usually found to have permeated the agar slightly by the time the fruiting stage is reached. A greater amount of color is usually found in cultures which have relatively few colonies and which fruit but little. Retardation of the growth of the cultures by low temperature appears to allow the color to diffuse farther through the agar. In cultures which grow rapidly, the yellow color may be diffused only in the upper part of the slope where the layer of agar is thin.

Pseudopeziza trifolii: The development of *Pseudopeziza trifolii* on this culture medium differs from that described for *P. medicaginis* only in the details here stated. The color which develops in the stromata as they develop is dark gray, becoming black, with no trace of brown. The fungous colonies appear a little more vigorous and coalesce into a more solid crust on the surface of the medium than those of *P. medicaginis*. The fruiting structures appear more typical (Pl. III, *A*), being flat on top, but are not surrounded by a wall. The substrate never becomes discolored.

Potato-dextrose agar.—Cultures of both fungi grow very rapidly on potato-dextrose agar. When the colonies are much crowded the leathery surface growth becomes crumpled.

Pseudopeziza medicaginis: Color at the end of four weeks brown, sprinkled with a few black stromata; substrate decidedly colored; apothecia produced in small numbers.

Pseudopeziza: Culture coal black, with slight amount of frosty mycelium. There is a slight staining of the substrate.

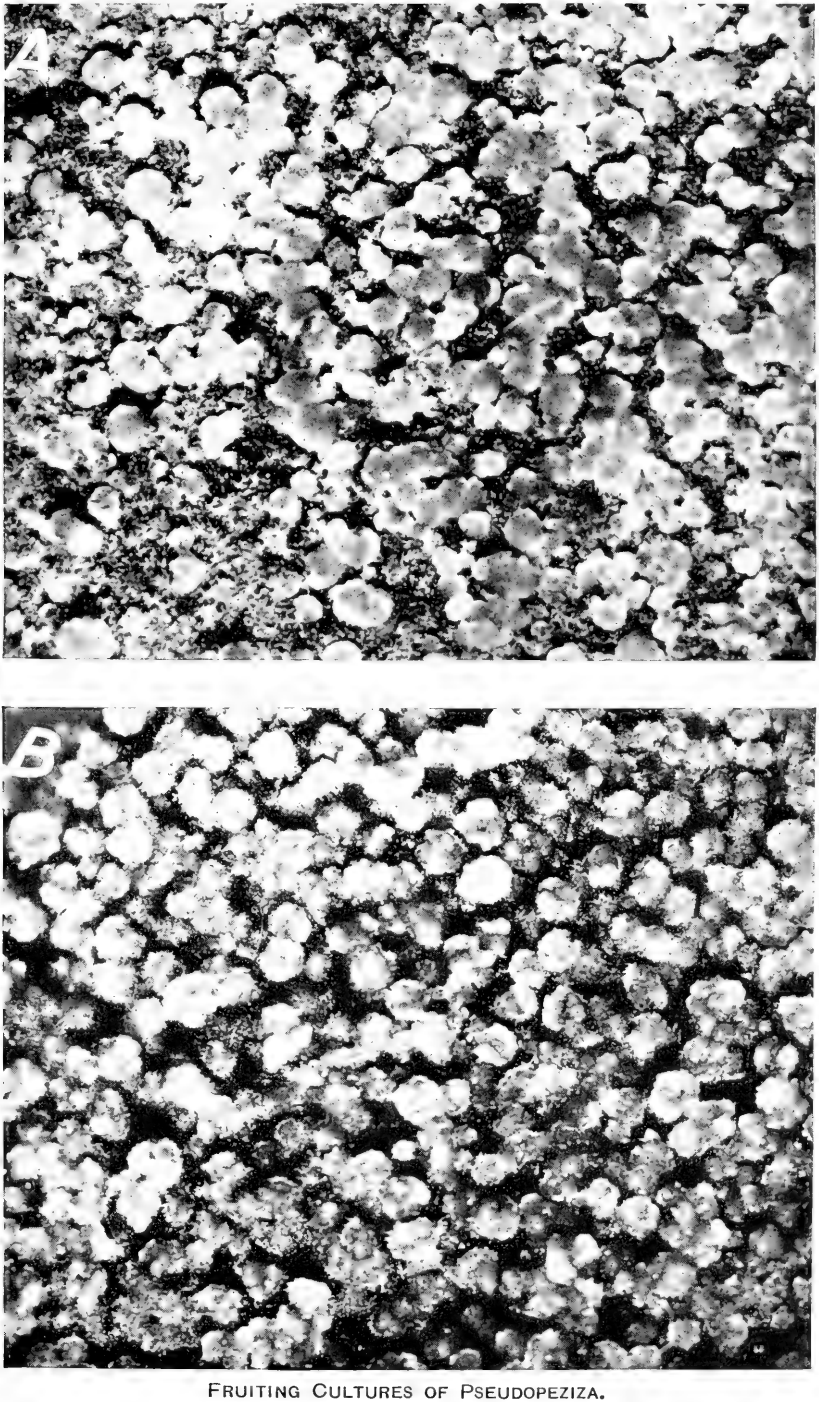
Lima-bean agar.—Pseudopeziza medicaginis: Growth yellowish in varying degree; no fruiting observed; substrate shows discoloration.

Pseudopeziza trifolii: The growth is a rough black mat with a little white mycelium. The substrate shows slight discoloration.

Corn-meal agar.—Pseudopeziza medicaginis: Growth not vigorous; colonies scattered, black, and distinctly raised from the surface, which appears as though sprinkled with coarse black pepper; apothecia minute and scattered.

Pseudopeziza trifolii: Growth black, almost submerged in the substrate; colonies tend to remain separate, though the slope appears a solid black color; scattered minute apothecia occur.

Liquefaction of gelatin.—Both fungi cause rapid liquefaction of gelatin.



FRUITING CULTURES OF PSEUDOPEZIZA.

A, Fruiting culture of *Pseudopeziza trifolii*. × 15, approximately. *B*, Fruiting culture of *Pseudopeziza medicaginis* from alfalfa. × 15, approximately.

SPORE DISCHARGE.

The discharge of ascospores has been observed frequently under the microscope when apothecia have been crushed in water. The ascus slowly becomes longer and of greater diameter, forcing the spores forward in a more or less oblique biseriate position. The increase in diameter of the ascus of *Pseudopeziza medicaginis* appears to be somewhat greater than in the case of *P. trifolii*, thus allowing the spores to come more nearly into a biseriate position. When the limit of resistance of the ascus wall has been reached, the end ruptures, allowing the spores to be expelled in a column. When discharge takes place under water the spores show no tendency to remain together, but when they are discharged in air they show a tendency to remain in pairs. This pairing of the spores is probably due to the adhesive quality of the spore wall, a quality which is also shown by the tenacity with which the spores adhere to the cuticle of a leaf.

It is interesting to note that the tendency to remain in pairs is not shown equally by spores of the two species of Pseudopeziza. It is somewhat more marked in the case of *Pseudopeziza medicaginis*. This may be due to the fact already cited that the spores of this species are brought more clearly into biseriate position before discharge takes place. In order to determine this difference, spores were collected on water agar as though for measurement. The spores in each group in selected miscroscopic fields were counted until the grouping of 1,000 spores had been determined. The results obtained at various times are shown in Table III.

TABLE III.—*Grouping of ascospores of Pseudopeziza medicaginis and Pseudopeziza trifolii when caught on an agar surface after being discharged normally from cultures.*

Spores of—	Spores in group—							
	1	2	3	4	5	6	7	8
Pseudopeziza medicaginis:								
First lot.....number..	60	238	19	43	7	19	2	9
Second lot.....do....	66	342	8	30	4	9	.....	4
Third lot.....do....	58	356	6	33	6	4	.....	1
Total.....	184	936	33	106	17	32	2	14
Pseudopeziza trifolii:								
First lot.....number..	133	215	39	28	16	11	2	6
Second.....do....	272	189	48	22	7	4	5	3
Third lot.....do....	242	185	54	19	10	9	2	4
Total.....	647	589	141	69	33	24	9	13

It will thus be seen that while in the case of *Pseudopeziza medicaginis* each spore group containing an even number of spores is greater than the preceding or following group, in the case of *P.*

trifolii there is almost a regular decrease of the number of groups from those containing one to those containing eight. Sufficient evidence has not been collected to determine whether the same clean-cut difference in the behavior of the two species appears when the spores are discharged from apothecia on living leaves.

SPORE GERMINATION.

In all the more obvious features of germination, the ascospores of the two fungi behave alike. They germinate readily when they have been discharged naturally upon a suitable moist surface; but germination is infrequent if the spores have been crushed out of the asci or if they are submerged in water. A single germ tube emerges from any point in the circumference of the spore, except that germination from the end has been observed only in the case of a few spores of *Pseudopeziza trifolii.* Under the best conditions that have been found, the proportion of germination is usually from 30 to 50 per cent. The vigor of germination varies greatly. Many of the spores which push out short germ tubes cease growth promptly, while a few develop vigorous germ tubes.

TABLE IV.—*Time required for the germination of ascospores of Pseudopeziza medicaginis and Pseudopeziza trifolii and rate of growth of the germ tube for three days at constant temperatures.*

[A plus sign indicates that the germ tube could be seen emerging from the spore, but that it did not reach a length equal to half the length of the spore. The figures in the body of the table represent the estimated length of the germ tube in terms of the length of the spores.]

Temperature (°C.).	Pseudopeziza medicaginis.						Pseudopeziza trifolii.					
	4 hours.	8 hours.	12 hours.	24 hours.	48 hours.	72 hours.	4 hours.	8 hours.	12 hours.	24 hours.	48 hours.	72 hours.
2.5 to 3	.....	.....	.....	0.5	1	2	.....	.....	.....	+	1	2
6 to 7	.....	.....	+	.75	1.5	2	.....	.....	+	0.75	1.5	2
9 to 10	.....	+	+	.75	1.5	2	.....	+	+	.5	1.5	2
12	.....	+	0.5	1.5	3	4	.....	+	0.5	1.5	3	4
16	+	0.5	1	1.5	3	4	+	0.5	1	1.5	3.5	5
21 to 22	+	.5	1	1.5	5	.....	+	.5	1	2	5	.....
24 to 25	+	.5	1	2.5	5	.....	+	.5	1	2.5	5	.....
27 to 28	.....	+	.5	2.5	4	.....	.....	.....	+	1	(1)	(1)
29	.....	.....	+	1	2	(1)	.....	.....	.....	+	.....	.....
30	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)

[1] Spores disintegrate. [2] No germination.

The one significant difference that they show is found in the fact that the spores of *Pseudopeziza medicaginis* will continue to germinate at a slightly higher temperature than those of *P. trifolii.* In order to test this relation of temperature to germination, spores were discharged naturally upon an agar surface with all the precautions necessary to secure mature spores previously described in obtaining spores for measurement. The agar used in all cases consisted of 2 per cent of agar-agar in water carefully cleared and filtered. In order to insure identical conditions for the two species in each test,

transfers of both were made to the cover of the Petri dish containing the agar. The spores were discharged simultaneously for 1 to 1½ hours. If at the end of this time sufficient spores were present, the cover containing the cultures was transferred to another dish and the spores placed at the desired temperature. Petri dishes to the number of 72, nearly all of them containing spores of the two fungi, were incubated in this way. The results are given in Table IV.

This table shows that the two species germinate with almost exactly the same degree of vigor at each temperature, except that the spores of *Pseudopeziza trifolii* cease to germinate, or, perhaps more exactly, to make growth after the initial stages of germination, at a slightly lower temperature than those of *P. medicaginis*. Although the difference here may seem slight—almost within the limit of experimental error—it has been found constant and definite in repeated tests. The possible significance of this fact in connection with the difference in geographic range of the two fungi will be considered later.

EFFECTS OF TEMPERATURE ON SPORE PRODUCTION.

When cultures of the two fungi are placed at a series of constant temperatures they appear to show constant differences in the time required for spore production and in the abundance of spore production at each temperature. At the outset it must be stated that limitations inherent in the method of starting cultures cause wide variation in the behavior of the cultures of each species. This difficulty is partly overcome by using several cultures and by repeating the work. Even then the results must be regarded as being suggestive rather than accurate. The time required for the production of ascopores as obtained in two trials is given in Table V. Three cultures of each fungus were used, and the earliest time at which the discharge of ascospores could be demonstrated in any of the three cultures was recorded. The three highest and the lowest temperature rarely varied more than one degree, and the remaining temperatures varied only half a degree.

TABLE V.—*Time required for the production of ascospores of Pseudopeziza medicaginis and Pseudopeziza trifolii at constant temperatures.*

Ascospores of—	Temperatures (°C.).											
	8	9	11	12.5	13	14	15	16	18.5	20	21.5	24
Pseudopeziza trifolii.....days..	82	53	52	30	43	30	36	30	20	16	16	21
Pseudopeziza medicaginis.....do....	82	-----	-----	58	-----	52	49	36	16	14	14	20

Table V shows that below 14° C. the behavior of *Pseudopeziza medicaginis* was erratic, rarely fruiting at all. Only one culture

fruited at 8° C. in one of the trials. At and below 16° *P. trifolii* requires a shorter time for the production of spores than *P. medicaginis*, while above this temperature the condition appears to be reversed. But the most striking differences in the behavior of the two fungi are not shown in the table. These differences lie in the length of the spore-producing period and in the abundance of the spores produced. Although *P. trifolii* is under most circumstances the more prolific spore producer, this preponderance is greatly increased at and below 18° C. Here abundant fruiting may occur for two weeks and longer, while cultures of *P. medicaginis* fruit very meagerly and only for a short period at these temperatures. Above 16° *P. medicaginis* begins to fruit more abundantly, reaching its maximum at temperatures a little above 20° C. Thus, the optimum temperatures for the fruiting of these fungi may be judged roughly as about 13° to 22° C. for *P. trifolii* and 16° to 24° for *P. medicaginis*.

RESISTANCE OF THE SPORES TO DESICCATION.

In order to determine whether ascospores discharged from the ascus might be able to live over winter on seed or débris carried with the seed, it was desirable to test the resistance of discharged ascospores to periods of drying of such duration as they would be obliged to endure on the seed. Owing to the slow growth of the fungus after germination and the limitations of conditions under which spores will germinate at all, it is obvious that the spores must be dried and germinated under such conditions that all other organisms will be excluded; that is to say, the entire process must be carried out under conditions of pure culture.

Obviously, the preferable method would be to dry the spores on the seed itself. In order to do this, sterile seeds were necessary. The difficulty of obtaining such seeds which were certainly free from any residual effect of the sterilizing agent on the seed coat was so great that it was finally abandoned.

Preliminary tests with plaster of Paris blocks as the conveyor for the spores during desiccation were so satisfactory that they were used exclusively. Thin blocks small enough to slip into a test tube easily, were sterilized by heat and placed beneath cultures which were discharging spores actively. After a period of 8 to 12 hours the blocks were placed in sterilized test tubes. These were stored, some in a glass case in the laboratory and some outside a north window.

From time to time one or more of these blocks were placed on water agar to which a small amount of alfalfa-leaf decoction had been added. The amount of the decoction appeared to make no material difference up to any amount that could be added to 3 per cent agar without causing it to lose its ability to solidify upon cooling. Other culture media were tried, but none gave as prompt a result

as this. The red-brown color of the Pseudopeziza stroma produced by this medium on these white blocks (Pl. I, *B*) could be recognized at an earlier date than the pale-colored growth which developed when other media were tried.

Spores of *Pseudopeziza trifolii* were tested in the same way with those of *P. medicaginis*, though not as extensively. The results are given in Table VI.

TABLE VI.—*Viability test of ascospores of Pseudopeziza medicaginis and Pseudopeziza trifolii viable when dried on plaster of Paris blocks in the laboratory and out of doors.*

Place and date of ending desiccation.	Time (days).	Results.	Place and date of ending desiccation.	Time (days).	Results.
PSEUDOPEZIZA MEDICAGINIS.			PSEUDOPEZIZA MEDICAGINIS—continued.		
In laboratory:			Out of doors—Continued.		
Feb. 1, 1915	32	+	May 20, 1916	120	+
Jan. 23, 1915	36	+	Feb. 15, 1916	108	+
Dec. 3, 1915	37	+	Apr. 7, 1916	159	+
Feb. 1, 1915	38	+	May 20, 1915	212	0
Dec. 9, 1916	39	+	Sept. 1, 1916	303	+
Dec. 16, 1915	48	+	Do	329	0
Feb. 20, 1915	49	+			
Mar. 26, 1915	63	+	PSEUDOPEZIZA TRIFOLII.		
Jan. 5, 1916	66	0			
Jan. 12, 1916	72	+	In laboratory:		
Jan. 17, 1916	76	+	Jan. 17, 1916	36	+
Jan. 23, 1915	76	+	Mar. 7, 1916	63	+
Jan. 22, 1915	79	+	Feb. 15, 1915	64	+
Jan. 1, 1915	85	0	Apr. 6, 1916	112	0
Aug. 26, 1915	93	0	May 15, 1916	113	+
Do	94	0	Apr. 20, 1916	120	0
Apr. 11, 1915	100	0	Sept. 1, 1916	258	0
Feb. 12, 1916	104	+	Do	256	0
Sept. 1, 1916	210	0	Out of doors:		
Jan. 15, 1916	357	0	Jan. 17, 1916	28	+
Out of doors:			Do	25	+
Dec. 9, 1915	27	+	May 4, 1916	104	+
Dec. 16, 1915	34	+	Apr. 7, 1916	107	0
Dec. 22, 1915	52	0	Sept. 2, 1916	265	0
Jan. 17, 1916	77	+	Sept. 1, 1916	265	0
Jan. 13, 1916	78	+			
Feb. 15, 1916	106	+			

From these results it appears that drying alone can not be depended upon to kill all the spores of either Pseudopeziza in less than one year. Severe freezing during drying had no apparent effect. If conditions for survival are as favorable on the seed as on plaster of Paris blocks, the spores should be able to live from one season to the next on the seed. But unless conditions on the seed are more favorable than on the block, they should not be able to survive and germinate during a second year.

PATHOGENICITY OF THE FUNGI.

METHOD OF MAKING INOCULATIONS.

In all inoculations that have been made, ascospores alone have been used as the inoculum. The conidiumlike structures which have been described are produced almost wholly in the substrate, and since only rarely will one of them separate from the mycelium, no

practicable method for using them has been developed. Since no evidence has been found indicating that they occur in nature or that they germinate, no great importance attaches to them.

In the first inoculations an attempt was made to obtain the ascospores in water suspension, but when the apothecia were crushed in water usually only a few spores became separated from the ascus. The few attempts to make inoculations with such meager spore suspensions failed. The method finally adopted and used with minor modifications in all inoculations reported takes advantage of the natural discharge of spores from cultures. If a Pseudopeziza culture is removed from the test tube carefully it may be cut into fragments and placed on a support, where it will continue to discharge spores for several days provided it is not exposed to direct sunlight or high temperatures. A culture may thus be removed from a test tube and placed over a plant upon which the spores will fall. If the whole plant is to be inoculated, the culture may be placed in the top of a bell jar which is set over the plant and turned from time to time to insure a uniform distribution of the spores.

If single leaves are to be inoculated, the culture or fragments of the culture may be placed for a short time over these leaves in succession. A more uniform discharge of spores for long periods is obtained in a dark room at 16° to 20° C. If inoculations are made in the field, they should be made at night or on a cloudy day. The plants may be wet with a fine spray before the spores are discharged, or if time permits this may be deferred until the spores are on the leaf. In the latter case, a larger number of infections are usually secured, due apparently to the fact that spores falling on large drops of water are held from sinking by surface tension and germinate too far from the leaf surface to effect penetration. After the inoculated plants are sprayed, they should be kept in a moist chamber for at least 12 hours.

This method has been employed in all inoculations made, unless otherwise stated.

CONDITIONS UNDER WHICH INOCULATIONS WERE MADE.

If inoculations are to be entirely conclusive in result, the control plants must remain free from the disease. It has been found impossible to keep plants free from leaf-spot for infection experiments during the summer at Madison, Wis., where the work was done. This has been due to the fact that alfalfa fields are located so close to the greenhouse that spores are easily blown in through the ventilators. But it has been found that if all diseased alfalfa foliage was removed from the greenhouses in the autumn after the ground outside froze, it was possible to keep alfalfa plants free from infection with leaf-spot during the winter and spring. Therefore all inoculations have been made or at least repeated during the winter months. This precaution

has not been necessary in the case of inoculations with *Pseudopeziza trifolii*, since the Pseudopeziza leaf-spot has not occurred on any species of Trifolium about Madison during the time this work was in progress. The following host plants have been inoculated with pure cultures of both species of Pseudopeziza.

HOST PLANTS INOCULATED.

Medicago sativa with Pseudopeziza medicaginis.—In seven or eight days, under greenhouse conditions, infections begin to show as minute brown spots scattered over the foliage. If infection is very abundant these leaves quickly die. If only four or five infections are scattered over each leaflet, apothecia begin to appear in about two weeks. For instance, one inoculation made on December 2, 1915, produced abundant apothecia on the spots by December 15. On December 20 spores discharged from apothecia on one of the leaves of this plant were cultured, and the fungus was recovered. An inoculation made on April 17, 1915, on plants in the field before the natural infection developed showed abundant spotting 11 days later. The weather turned cold after this date and no fruiting was observed.

Leaves of all ages are attacked. Leaves which have grown to full size appear to develop more abundant infections than leaves which are not full grown, but leaves which are yellow and weak do not seem to become infected as easily as those which are more vigorous.

None of the infection experiments performed during three winters has failed to develop a greater or less amount of typical leaf-spot.

Medicago sativa with Pseudopeziza trifolii.—Inoculation experiments have been repeatedly conducted parallel with those already cited on alfalfa, using plants of different ages. No infections visible to the naked eye have been produced.

Medicago lupulina with Pseudopeziza medicaginis.—Plants of this host have never been very thrifty under greenhouse conditions, and therefore not a large number have beeen available for inoculation. In no case has any infection been obtained. Attempts to secure infection by setting *Medicago lupulina* plants in the garden among alfalfa plants which were heavily infected with *Pseudopeziza medicaginis* also failed to produce infection.

Medicago lupulina with Pseudopeziza trifolii.—Only two inoculations have been tried. No infections resulted.

Melilotus alba with Pseudopeziza medicaginis.—On March 28, 1915, several leaves of a vigorous sweet-clover plant were placed beneath fragments of a culture of *Pseudopeziza medicaginis* which was discharging spores. At the end of 24 hours the culture was placed over the entire plant, which was kept in a moist chamber 48 hours longer. After four days the leaves first inoculated showed minute brownish spots. These did not increase in size. After two weeks the portions of the leaves bearing the minute spots were embedded in paraffin and sectioned. In these sections the brown spots were found to consist of dead shrunken cells in which traces of mycelium could be found. But this mycelium appeared to be shrunken and dead and not advancing into the living cells of the host. From this, it appears possible that under favorable conditions *P. medicaginis* may be able to cause a very slight spotting of sweet-clover leaves.

Trifolium pratense with Pseudopeziza medicaginis.—Inoculations of red-clover plants in the greenhouse with pure cultures failed to produce any infection. Red-clover plants grown in the garden in close proximity to badly dis-

eased alfalfa plants have never shown a trace of this leaf-spot during the two years that these diseases have been under observation.

Trifolium pratense with Pseudopeziza trifolii.—Infection from inoculation with spores from pure cultures has been at all times easy under greenhouse conditions. Frequently such abundant infection has been obtained that the leaves are killed before the spots develop to a fruiting stage. The incubation period of the disease appears to be slightly longer than that of *Pseudopeziza medicaginis*, varying somewhat with the abundance of the infection. If a leaf is heavily infected, the individual infections appear to develop more rapidly and produce small characteristic killed areas earlier than if infections are few. Numerous infections appear in 11 or 12 days, and in the greenhouse the leaf frequently dies a few days later. Fruiting bodies have not been observed on such leaves, but a reisolation has been made from infections two weeks after inoculation.

Less numerous infections may not show for two weeks or even longer, but at the end of three weeks or more they may produce typical apothecia if the air of the greenhouse is sufficiently moist. These apothecia have been found on leaves nearly or quite dead. In this respect the fungus behaves differently than does *Pseudopeziza medicaginis*. Apothecia on dead leaves are very difficult to discover unless the leaf has been in a moist atmosphere for some time, when they appear as dark-amber gelatinous masses on the leaf surface. The exposure of these gelatinous masses of asci to dry air for even a few minutes causes them to discharge the larger part of the spores present and to shrink to a minute mass only a little darker in color than the leaf and therefore difficult to identify. The best development of apothecia has been obtained by placing infected plants outside the greenhouse during protracted periods of rainy weather.

Trifolium hybridum with Pseudopeziza medicaginis.—No success in obtaining visible infections has been attained.

Trifolium hybridum with Pseudopeziza trifolii.—Of the several plants inoculated only one survived in a vigorous condition for a sufficient period to show infection. This showed an abundant spotting, which was in every way characteristic of the Pseudopeziza spot on red clover except that the spot appeared to be somewhat limited in development by the veins, thus showing a slight tendency to become angular. No fruiting bodies were produced. Apparently infection takes place only under the most favorable circumstances. This plant appears to be a much less congenial host than red clover.

GERMINATION OF THE SPORES ON THE LEAF.

While study was being made of the leaf-spot fungus in the host tissue it was found that the mode of penetration could be observed very readily by decolorizing the leaf soon after inoculation was made. This method of study was used, not only to determine the normal penetration of these fungi into their own hosts, but to determine the relation of these parasites to other closely related plants reputed to be hosts of these fungi but upon which infection had not been obtained. In case preliminary inoculations failed to give visible results it was more simple and rapid to determine whether the spores of that fungus could penetrate the host in question and develop after penetration had taken place than to conduct other extensive inoculations. Thus, a study of penetration has formed a part of all inocu-

lation trials. The results of the two methods of attacking host relationships should be considered together.

METHOD OF STUDY.

The most of the data given here have been obtained by the following simple procedure: Leaves which have just reached full development are selected for inoculation. A culture of Pseudopeziza known to be discharging spores abundantly is supported over the leaf or one of the leaflets so that the spores as they are discharged will all fall upon it. The leaf may be removed from the plant for studies which do not involve a period of more than two days, since results obtained from such leaves have always been found by comparison to agree with results obtained from leaves attached to the plant. The leaf may be sprayed with very fine spray before the spores are discharged upon it, but more abundant penetrations are usually obtained if the spores are allowed to stick to the leaf before it is wet. When the leaf has been kept moistened for at least 12 hours, usually longer, it is removed, dropped into a mixture of equal parts of acetic acid and alcohol and promptly heated to the boiling point. Leaves which are killed promptly in this fashion decolorize in better condition than when slower killing takes place in cold acetic alcohol. The acetic alcohol is changed until all color has been removed from the leaf.

The leaf may then be mounted in this liquid on a slide under a cover glass and examined under the microscope. The epidermal cells should be perfectly clear, and the entire structure of the leaf to its very center should be visible. The spores remain attached to the leaf during the treatment, and the method of entry and the mycelium within the leaf can be clearly seen. No method of staining has been found to improve the visibility of the fungus.

Although this method works best in the case of alfalfa leaves, it works well enough with the various clovers to give entirely satisfactory results.

METHOD OF PENETRATION.

In all of the hundreds of penetrations observed the method of entry has invariably been as here stated. The spore is found stuck fast to the leaf. The germ tube emerges from the spore either within or at the margin of the area of contact of the spore with the leaf and passes directly through the cuticle into the epidermal cell. Occasionally a spore sends out its germ tube along the surface of the leaf, but such a germ tube has never been observed to enter the leaf. Apparently the germ tube must enter the leaf at the moment of emergence from the spore, if at all. Ordinarily there is no perceptible thickening or alteration of the wall in consequence of this penetration. The actual opening appears to be extremely minute.

After passing through the wall, the germ tube quickly expands to normal size. When it reaches the center of the cell it usually divides (fig. 5) into two or three branches, which pass into the adjoining epidermal cells or down into the palisade layer. Cell walls do not appear to offer any obstruction to advancing hyphæ. No marked disorganization of the cell contents appears to result from this invasion until hyphæ become very numerous.

The actual time required for a spore to germinate and transfer its contents to the germ tube inside the leaf has not been determined accurately, but in most cases it must be less than 12 hours at 18° to 22° C.

This description applies to the method of penetration of *Pseudopeziza medicaginis* and *P. trifolii* in their respective hosts. The following notes have been made of the penetration of germinating spores of these fungi in other reported hosts that have been available:

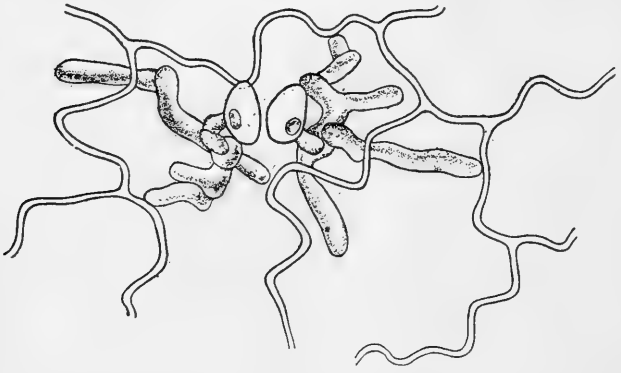


FIG. 5.—Penetration of the epidermis of an alfalfa leaf by the germinating ascospores of *Pseudopeziza medicaginis*. × 800.

Trifolium pratense by Pseudopeziza medicaginis.—Inoculated leaves were decolorized in two, three and five days after inoculation. After three days the host cells beneath many of the germinated spores had yellow granular contents. The yellow color made the exact relation of the germ tube to the cell impossible to determine. In five days it could be observed that in the case of at least a part of these yellowed cells the germ tube had passed through the epidermal cell wall, but had not advanced far into the cell.

Medicago sativa by Pseudopeziza trifolii.—A leaf inoculated on the plant on March 28, 1915, appeared to show penetration in 60 hours. Five leaves inoculated on December 4, 1916, and decolorized four days later showed many yellowed cells beneath germinated spores. Suitable fragments were embedded and penetrations found in sections. The germ tube had not advanced beyond the first cell which it entered.

Medicago lupulina by Pseudopeziza medicaginis.—Four series of leaves removed from the plant were tried. Penetrations were noted in 23 hours at 22° C. Penetrated cells always show yellow granular contents. Even after 75 hours it was doubtful whether the germ tube had advanced beyond the first cell penetrated.

Medicago lupulina by Pseudopeziza trifolii.—One series of leaves was removed from the plant. Penetrations were abundant and the penetrated cells yellowed,

but even after 52 hours the germ tubes had not advanced beyond the cell first penetrated.

Melilotus alba by Pseudopeziza trifolii.—Three days from inoculation a few germ tubes were found distinctly inside epidermal cells, but they had advanced but slightly. Penetrated cells were not yellowed.

Melilotus alba by Pseudopeziza medicaginis.—The cultures used for inoculation in this series did not produce many spores. Nevertheless, after four days two penerations were found. The penetrated cells were very slightly yellowed.

Trifolium hybridum by Pseudopeziza trifolii.—No examination was made until two days after inoculation. At this time penetrations were abundant and easily seen. In a few cases germ tubes had advanced into cells adjoining those first penetrated. No yellowing of penetrated cells was observed.

Trifolium hybridum by Pseudopeziza medicaginis.—In 24 hours penetrations were abundant, but the germ tubes had not advanced far into penetrated cells. Penetrated cells showed no yellowing.

It appears that the results which are shown above are exactly parallel to the results obtained from inoculations. In every case where infection in varying degree has been attained, penetration has occurred abundantly and the growth of the hyphæ within the host cells has been rapid without causing discoloration of the cell contents. In cases where visible infection has not been obtained, the relative number of penetrations is usually reduced, but in any case growth of the germ tube ceases promptly upon entering the epidermal cell. Thus, the resistance which the plants that can not be infected offer appears to be due not to any mechanical obstruction to entry, but to something within the epidermal cell which prohibits growth.

These infection experiments supported by penetration studies have failed to produce a completely successful infection of any of the hosts tried except those from which the fungi were isolated. This result is very different from that which was anticipated from a consideration of the host lists. Have we here a group of closely similar fungi highly specialized in their host relationships?

It is noteworthy that in both host lists only two or three of the species of Trifolium are native to America. All the rest have been introduced if they occur here at all. Moreover, some of these introduced hosts which are widely distributed do not appear to be attacked by this parasite except in certain limited areas. For example, *Pseudopeziza medicaginis* has been found on *Medicago lupulina* only in New York; *P. trifolii* has been reported on *Trifolium repens* only by McClatchie (1895) on the Pacific coast[1] and not at all on *T. hybridum*. It is not likely that these fungi have been overlooked on these hosts in other localities, and therefore the conclusion that they do not always pass to these hosts can hardly be escaped. Thus, there is reason to suspect that these two species, as now re-

[1] An excellent collection of Pseudopeziza on *Trifolium repens* now in the possession of the writer was made by Mr. C. W. Hungerford at Olga, Wash., on Sept. 3, 1916.

garded, are made up either of several closely similar species or of a group of specialized races.

Especially in the case of the Pseudopeziza on *Medicago lupulina* the fragmentary evidence indicates a species distinct from that on alfalfa. In addition to the evidence already given, the measurements of 113 ascospores collected under standard conditions is adduced. (Table VII.)

TABLE VII.—*Lengths of 113 ascospores of Pseudopeziza medicaginis on Medicago lupulina, measured to the nearest micron.*

Ascospores of—	Length (microns).				
	9	10	11	12	13
Pseudopeziza medicaginis.....number..	1	14	59	31	8

When Table VII is compared with Table II, it will be noted that these spores are even larger than those of *Pseudopeziza trifolii*. Unfortunately, the cultures which were made of this fungus were destroyed, and complete evidence of the relationship of these fungi was not obtained.[1] Of the other hosts of *P. medicaginis*, less can be said. Field observation indicates that the fungus in *Medicago falcata* and *M. hispida denticulata* occurs by infection from Pseudopeziza in alfalfa. A collection of the fungus has been found in only one instance each on *Melilotus alba* and *Vicia villosa*. An examination of a portion of the collection on *Melilotus alba* reveals a lesion that is in all respects similar to that which the fungus causes on other hosts, but no asci and spores by which the fungus could be identified were found. Certainly the occurrence of the fungus on these hosts is not common or of economic importance. No material of Pseudopeziza on *Onobrychis sativa* or any species of Trigonella has been available for study.

Of the host list of *Pseudopeziza trifolii* only a few species have been available for study. The fungus from *Trifolium pratense* has not infected any other species to produce fruiting of the fungus on that host. Furthermore, it has not been found fruiting on any other host in America except in the one instance already mentioned. Yet reports well supported by herbarium specimens indicate that *Trifolium repens* and *T. hybridum* are abundantly infected in Italy. Whether this infection is by the same species of the fungus or not can not be determined at present.

[1] It is of interest to note that *Pseudopeziza medicaginis* was first described on what is now known as the variety *wildenowii* of *Medicago lupulina*. If it should be shown that Pseudopeziza on *Medicago lupulina wildenowii* is a distinct species from that on *Medicago sativa* the name *Pseudopeziza medicaginis* will be restricted to the fungus on the original host, and a new species name will be required for the fungus on alfalfa.

LIFE HISTORY OF THE CAUSAL ORGANISM IN RELATION TO THE HOST PLANTS.

AMERICAN STUDIES BEARING ON LIFE HISTORY.

The great economic importance of the alfalfa crop in America has inspired the small amount of work which has been done upon this disease with a point of view quite different from that shown in the European studies already reviewed. Life history has been studied with a view to the possible control of the disease.

The first work was done by Chester (1891) at the Delaware Agricultural Experiment Station. In 1889 twenty plats of alfalfa were seeded in different parts of Delaware, with seed from the same source. The disease appeared on all of these plats at about the sixth week after planting. The plat under closest observation at Newark showed a yellowing of the leaves accompanied by black spots before Pseudopeziza was found fruiting on some of the dark spots. Evidently some other disease was associated with Pseudopeziza. Chester concludes from this experiment that the disease is carried by the seed and next tries a method of disinfecting the seed in order to prevent such conveyance. Seed was treated with copper sulphate and planted in heat-sterilized soil in cans. The diseases appeared on all the plants from these cans. Therefore, Chester concludes that the source of the disease must be a general atmospheric infection. Unfortunately, in none of his experiments does he give any details regarding the proximity of his plats or plants in cans of sterile soil to alfalfa which was infected with the leaf-spot and which might have been a source of wind-borne spores.

The only other attempt to study the disease which has been reported was made by Coombs (1897a) in Iowa. On August 20 alfalfa seedlings 3 weeks old grown under bell jars were treated as follows: One was left as control, one sprayed with germinating Pseudopeziza spores, and one sprinkled with powdered diseased leaves showing disease. Next, plants growing in the field were cut back, and after the débris was removed, the roots were protected by large bell jars. In the next six weeks the plants outside the bell jars became diseased, while those inside were healthy. However, when the jars were removed the plants immediately became diseased. As a result of this work Coombs concludes that two things are established: (1) That plants are infected by spores from the air and (2) that the disease is strictly local and not systemic.

It will be seen at once that these conclusions are based on a very small amount of experimental evidence. Such important factors as the high temperature and absence of dew or rainfall inside the bell jars do not appear to have been considered as possible conditions

which might have prevented infection even had the spores been present.

At this point, it is convenient to mention an English discussion of this disease by Ivy Massee (1914) in which an effort is made to throw light on the method by which the fungus is conveyed from one locality to another. In this article Miss Massee states that so far as England is concerned the dissemination of the disease is due to diseased seed which is badly cleaned. She says, "I have recently examined a sample of commercial seed and found the fungus present in abundance on minute fragments of leaves and calyces, and rarely on the seed itself." Unfortunately, Miss Massee does not state the methods by which she was able to make the identification of the fungus with such certainty on this single sample of commercial seed. Even if she was correct in this observation, it still remains to be proved that the fungus which she found was alive and capable of infecting the plants grown from this seed.

The many scattered observations of the disease merely contribute data regarding distribution, seasonal occurrence, and environmental factors. Most of these reports indicate that the disease is worse on plants during the first summer of their growth than later, but Coombs (1897) speaks of attacks as being worse after the first year. Most reports indicate that the disease usually gains headway slowly in the spring and becomes worse later in the season, but this is not always the case. Stewart, French, and Wilson (1908, p. 384–387) speak of the disease as being worse in dry years while most reports, especially from drier regions, indicate that the disease is worse in wet seasons.

The facts bearing upon the overwintering of the fungus in the field are surprisingly meager. The only definite bit of observational evidence is that of Chester (1891), who states that he found live asci on leaves in midwinter. Voges (1909) suggests in addition that the fungus survives the winter in living leaves.

After surveying these scattered references in American literature we find that there is a general belief that *Pseudopeziza medicaginis* is distributed with the seed and survives the winter on dead leaves. This opinion has been reached, not so much as the result of careful experimental evidence, which in fact is meager, but more as the cumulative effect of the expressed opinions of competent observers who have watched its development during a series of years.

METHOD OF OVERWINTERING.

The first evidence of the method of overwintering of the leaf-spot fungus in the field was obtained in the spring of 1916. On April 11 overwintered alfalfa leaves showing abundant Pseudopeziza spotting were brought into the laboratory and placed in a moist chamber.

Two days later, when one of the apothecia was crushed out in water, asci containing spores apparently mature were found. The leaf was then supported over an agar surface. In two hours a large number of spores were discharged, which germinated promptly. At this time the young alfalfa shoots had hardly emerged from the mulch of the débris of the previous season's growth.

On May 6 spots began to appear on the alfalfa foliage of some of the plats under observation. When the spotted leaves were decolorized, characteristic Pseudopeziza spores with germ tubes penetrating the epidermal cells were found in nearly all the spots.

A search of the overwintered foliage discovered a large number of fresh-appearing apothecia developed on leaf areas that had been diseased the previous year. When the overwintered leaves were placed over agar plates, a large number of viable ascospores were caught.

Fragments of overwintered leaves bearing apothecia were placed over ten marked leaves of a healthy alfalfa plant in the greenhouse, and the plant was kept in a moist chamber for 24 hours. On May 17 seven of the ten marked leaves showed more or less of the characteristic Pseudopeziza spotting.

Five or six of these overwintered leaves bearing apothecia were placed on the ground under a rank growth of alfalfa plants in the greenhouse. The plants were then sprayed, but not covered to prevent evaporation of water from the foliage. On May 21 the alfalfa foliage in the vicinity of these overwintered leaves was found infected with leaf-spot.

On March 31, 1917, overwintered alfalfa leaves bearing leaf-spot lesions were collected in an alfalfa plat. At this time no spores could be found in the apothecia. These leaves were kept in a moist chamber for a week, care being taken to soak them in water twice a day to remove the products of decomposition. At the end of the week asci with mature spores had developed in the old apothecia.

Thus, it appears evident that *Pseudopeziza medicaginis* survives the winter on diseased foliage which escapes decay. When the weather becomes sufficiently warm in the spring and moisture is provided by protracted rains or the shelter offered by the young growing foliage, new asci develop apparently in the old apothecia and, in addition, new apothecia are produced around the old one (Pl. II, *A*). The spores thus produced furnish the primary infection in the spring.

Apothecia producing spores indistinguishable from those of *Pseudopeziza trifolii* were found on overwintered clover leaves in the spring of 1916 in northern Wisconsin, but since no inoculations were made with these spores, their identity was not determined. However,

it does not seem unlikely that this fungus winters in the same way that has been demonstrated for its close relative on alfalfa.[1]

METHOD OF DISTRIBUTION.

SUGGESTIONS FOUND IN THE LITERATURE.

Since the method of overwintering of these fungi has been traced, it is now possible to see their entire annual cycle in a field in which they have once been introduced. But thus far no information has been gained which serves to indicate how they are conveyed into new localities in which the host plants are grown for the first time. This phase of the problem is of special importance in connection with the alfalfa leaf-spot. A large amount of recorded experience indicates that this disease appears wherever alfalfa is grown, regardless of environment. A knowledge of the source of infection in these new localities might suggest feasible control measures.

Scattered through the various discussions of the alfalfa leaf-spot are found four suggestions that have been put forth to explain the constant appearance of Pseudopeziza on alfalfa in newly seeded fields: It is suggested (1) that the fungus is carried with the seed, (2) that it is conveyed in soil that is used to inoculate the new field with the bacteria producing nodules on the roots, (3) that the fungus spores are generally distributed in the air, and (4) that other host plants near by furnish the source of infection. Since none of these suggestions are supported by carefully controlled experimental evidence, they must be subjected to examination before they are used as working hypotheses in experimental work.

The first suggestion, that the fungus is carried with the seed, deserves careful attention. This might happen in three ways. Spores might adhere to the seed coat, spores or fragments of the fungus might accompany the seed, and living mycelium of the fungus might occur within the seed. When the conditions under which seed is produced are examined it is found that the fungus spores are practically all discharged and blown away before the seed is thrashed, thus making it highly improbable that spores are attached to the seed except as a rare occurrence. Commercial seed is so well cleaned that there appears to be small chance that fragments of the fungus are often conveyed with the seed.

The possibility that fragments of the fungus as well as the spores may be carried with the seed appears unlikely in the case of most commercial seed. Débris consisting of plant parts is so light in comparison with the seed that it is easily removed. Nevertheless, Ivy Massee (1914) states that she has examined commercial seed and

[1] On April 6, 1919, apothecia of *Pseudopeziza trifolii* were found abundantly on living overwintered clover leaves at Madison, Wis., showing clearly that young leaves infected late in the autumn under favorable conditions may carry the fungus over winter.

found the fungus present. This can hardly be a common occurrence in America.

The possibility that the fungus may be present in the seed as mycelium is open to the objection that this involves a larger or smaller amount of systemic infection, both of the plant producing the seed and of the seedling. No evidence of such a relation of these fungi to their host plants has been found, and therefore this method appears highly improbable.

The second suggestion, that the fungus is conveyed with plant débris that accompanies soil which is transported to new fields, may and probably does account for a small amount of the distribution of the fungus. But owing to the restricted extent of this practice, this method must be of minor importance.

The third hypothesis, that spores of Pseudopeziza are generally distributed in the air, has been advanced several times in a vague way either in a discussion of conditions where large areas of diseased alfalfa were growing at no great distance or with an implied belief that some other host plant in the vicinity was the source from which this general infection arose. In the vicinity of areas of diseased alfalfa it is highly probable that spores are borne to a considerable distance by wind, but it is not often that the spores are produced so abundantly that they are likely to be conveyed great distances in large numbers.

The final hypothesis, that other hosts provide the source of infection, has been rendered less probable by results already presented. The only common hosts that can be considered are red clover and yellow trefoil (*Medicago lupulina*). Pseudopeziza on red clover appears to be a distinct species from that on alfalfa, and no evidence has been obtained indicating that the fungus on yellow trefoil can cross to that host.

Thus, a summary of the available evidence does not point clearly to any of these suggestions as the one most likely to contain the truth. However, the suggestion that the fungus is carried with the seed affords most opportunity for experimental study and, if found true, affords the greatest opportunity for the application of control measures. The following experiments in seed sterilization were carried out.

EXPERIMENTAL METHODS AND RESULTS.

Laboratory experiments.—If the fungus spores are carried adherent to the seed they must inevitably germinate upon the seed coat and produce apothecia there, if at all. In order to determine to what extent the fungus is capable of developing upon the seed coat, spores were discharged upon seeds sterilized with formaldehyde. The seeds were then germinated upon agar in test tubes. After a time minute

fungous colonies were found developing upon the seed coats, whether they remained attached to the cotyledon leaves or fell to the agar. In no case were the cotyledon leaves attacked. Subsequent attempts to infect cotyledon leaves of seedlings did not produce macroscopic lesions. The seed coats bearing the minute fungous colonies were subsequently transferred to fresh agar slopes to keep them moist, and finally they developed minute apothecia. This development was so slow, however, that it is doubtful whether it could proceed so far under field conditions, where periods of drying would occur and competition with other fungi would be encountered.

Efforts to grow the fungus on soil sterilized or unsterilized have been entirely unsuccessful.

If the fungus occurs on the outside of the seed or in débris it can easily be destroyed by the surface sterilization of the seed. If such treated seed can then be grown under conditions which will exclude other sources of infection and which will also be favorable for the development of the fungus, the occurrence of the disease will indicate that the fungus is carried within the seed.

A satisfactory method of seed sterilization has been worked out by Mr. A. H. Gilbert (in an unpublished manuscript). He found that treatment with a solution of 1 part of bichlorid of mercury in 1,000 parts of water for five minutes rendered the seed sterile, while treatment for 10 minutes injured the seed. These treatments were repeated, and it was found that treatment for eight minutes was more than sufficient to render the seeds sterile without injury, provided they were washed promptly after treatment. All sterilized seed mentioned in the experiments here described were treated in this way.

Suitable conditions for growing the treated seed were difficult to obtain. Two places were tried—in the greenhouse during the winter months and in the open field in localities as remote as possible from other alfalfa. Experience in the greenhouse in the winter of 1915 showed that unless great care was taken with infected plants, the fungus was likely to occur occasionally on other alfalfa plants in the same house. During the following two winters all inoculated plants were cared for so thoroughly that in not a single instance did the leaf-spot develop upon any other plant in the houses until the disease appeared in the fields outside in the spring. Alfalfa plants grown close to red clover infested with *Pseudopeziza trifolii* remained free from leaf-spot. The following greenhouse-plat trials were made:

(1) Four grams of sterilized alfalfa seed were exposed to a discharge of ascospores of *Pseudopeziza medicaginis* for three days before sowing in the garden greenhouse on March 3, 1916. Thousands of viable spores must have been attached to the seeds at the time they were sown. By April 15 the plants were 6 inches tall and very vigorous. When the experiment was discontinued

on May 10 no trace of Pseudopeziza had appeared. The plants were then 10 to 12 inches tall, very vigorous, and in a dense mat apparently favorable for the development of the fungus. At the end of this experiment the plat was inoculated with Pseudopeziza from overwintered leaves and was quickly overrun with the disease, showing that the greenhouse conditions were favorable for its development.

(2) On January 27, 1917, a plat about 4 feet square was sown in the garden greenhouse with unsterilized Kansas-grown alfalfa seed. This plat developed normally without leaf-spot until May 5, when the disease was present in the field outside.

(3) On February 16, 1917, a plat 2 by 3 feet was sown in a garden greenhouse with sterilized alfalfa seed 3 years old. This plat likewise developed normally with no leaf-spot until May 10, at which date leaf-spot was abundant outside the greenhouse.

Several other plats were started and developed in the greenhouse without leaf-spot, like those referred to above, but owing to insect injury the conditions were not as favorable for the development of the disease as those described. In fact, all greenhouse plats started from seed, whether sterilized, unsterilized, or even treated with spores before sowing, have developed without the appearance of leaf-spot until the disease occurred abundantly in an alfalfa field close outside the greenhouse.

Field plats.—In the selection of locations for plats three conditions were sought: (1) Remoteness from large areas of growing alfalfa; (2) the greatest possible distance from farms where alfalfa has been grown; and (3) accessibility, so that a visit to the plat would be possible. The second condition was very difficult to secure. Small plats of alfalfa are surprisingly abundant even in localities where it is not grown as a farm crop. In consequence of this fact, only one of the eight plats started in 1915 was found upon examination to be sufficiently remote to give results of value.

In 1915 the assistance of the States Relations Service secured the cooperation of several agricultural county agents whose intimate knowledge of local conditions made possible the selection of a larger number of suitable locations. To these men the writer is indebted for any degree of success that was attained in these experiments.

The seed which had been sterilized superficially was furnished to the agricultural county agents, who allotted it to the men on whose farms the plats were to be located. In the autumn all the plats were visited except the one at Bruce, S. Dak., which was under the observation of Dr. A. G. Johnson, and the presence or absence of leaf-spot was determined. In a number of cases alfalfa was found growing nearer the plat than was previously supposed. The results noted on such plats—always an abundance of leaf-spot—are excluded from the summary in Table VIII. However, if, as sometimes occurred, the near-by plants were very few in number and no other

plants were known to exist within a 5-mile radius the results have been included.

TABLE VIII.—*Summary of data of plat tests to determine whether leaf-spot can be prevented on alfalfa sown in isolated localities by the superficial sterilization of alfalfa seed.*

State and town.	Date sown.	Date visited.	Area (square rods).	Distance to nearest alfalfa (miles).	Leaf-spot.
Maine:	1915.	1916.			
Mercer	June 28	June 14	1	6	Present.
	1916.				
Albion	May 6	Sept. 12	20	3½	Do.
Fairfield	June	do	40	5	Do.
Gorham	May 20	Sept. 5	1	½	Do.
Harrison	June 1	do	2	3½	Doubtful.
Vassalboro	May	Sept. 6	40	5	Present.
Windsor	June	Sept. 12	40	5	Do.
South Dakota:					
Bruce	May 20	Aug. 11	40	5	Do.
Wisconsin:					
Doering	June	Oct. 2	40	10	Doubtful.
McConnor	May	Oct. 4	10	½	Present.
Radisson	June	do	40	½	Do.
Merrill	do	Oct. 2	40	5	Do.
Do	do	do	40	8	Do.
Do	do	do	40	12½	Do.
Tomahawk	do	do	40	10	Do.
Do	do	do	40	10	Do.
Weirgor	do	Oct. 5	40	5	Do.

From Table VIII it appears that the one small well-isolated plat started in 1915 did not develop leaf-spot until the following year. Of the 16 plats started in 1916 only 2 failed to show an abundance of leaf-spot in the autumn of the same year. One of these plats, located at Harrison, Me., was in very poor condition, only a few scattering spindling plants about 4 inches high being found. However, other plats in almost as poor condition showed leaf-spot. The second doubtful plat, at Doering, Wis., was in very vigorous condition, but it had been cut just previous to inspection, leaving very little foliage. Unfortunately, it was not feasible to revisit the plat the following year.

These results are in accord with previous experiments and experience. Surface sterilization of seed apparently accomplishes nothing in excluding leaf-spot from alfalfa fields. But these results do point very clearly to one conclusion, that the leaf-spot fungus is not carried on or in débris mixed with the seed. The greenhouse experiments, which are only suggestive because of their limited extent, indicate that the fungus is not carried within the seed.

Thus, in conclusion, it is necessary to say that no positive evidence pointing toward the method by which this disease gains access to remote alfalfa fields has been found. Evidence has been obtained which apparently eliminates other plants previously under suspicion as host plants of the fungus from consideration as sources. A lim-

ited amount of evidence indicates that the fungus is not carried with the seed. Yet the fact of the almost universal occurrence of the disease in remote localities is well established. The explanation of this fact still furnishes a very interesting and apparently very difficult problem.

SUMMARY.

(1) One of the most important diseases, if not the most important foliage disease, of alfalfa is the leaf-spot caused by the fungus *Pseudopeziza medicaginis* (Lib.) Sacc. A similar but less important leaf-spot of red clover is caused by the fungus *Pseudopeziza trifolii* (Biv.-Bern.) Fckl. The morphological differences between these fungi are so slight that doubt has frequently been expressed whether they are not identical. Several conflicting opinions as to the life histories of these fungi are found in mycological literature. This study attempts to determine the relationship of the two fungi here mentioned and to trace as far as possible their life histories in relation to their host plants.

(2) *Pseudopeziza medicaginis* on alfalfa and *Pseudopeziza trifolii* on red clover have been obtained and studied in pure culture. Efforts to cross these fungi from one host to the other have not been successful. Morphological as well as physiological differences have been found which in the opinion of the writer justify retaining the fungi as distinct species.

(3) None of the imperfect fungi which have been regarded as a stage in the development of these fungi have been found to be related. Apparently no other spore form than the ascospore occurs in nature.

(4) Infection is produced by the direct penetration of the germinating ascopores through the cuticle and epidermal cell wall of the leaf. The mycelium developing into a small stroma about the point of entry produces in about two weeks an apothecium.

(5) The fungus lives over winter on dead leaves which escape decay, and ascopores produced in the spring furnish the source of new infection.

(6) Efforts to exclude the disease from alfalfa fields sown in localities remote from other alfalfa by the surface sterilization of the seed have given no degree of success. Evidently, in these experiments at least, the fungus was not carried on the surface of the seed—probably not with the seed at all. The demonstration of the source of infection in such fields still furnishes an interesting problem.

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