STUDIES ON THE DEVELOPMENT OF THE CULTURES AND THE SUSCEPTIBILITY OF ALFALFA TO LEAF SPOT, *PSEUDOPEZIZA MEDICAGINIS*

by

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INTRODUCTION

One of the most common and one of the more destructive foliage diseases of alfalfa is common leaf spot caused by the fungus, Pseudopeziza medicaginis (Lib.) Sacc. The damage caused by this disease varies from year to year depending upon the prevailing climatic conditions. In favorable seasons this disease will cause partial defoliation of the stand which results in reduction of the yield, a decrease in the quality of the forage produced, decreased vigor, and a shortening of the life of the stand. Old stands whose growth is retarded and young seedlings which have not become established are especially susceptible to attacks by this fungus.

At the present time there is no satisfactory method for controlling the disease. Premature mowing of the crop has been suggested as a method of control as this will prevent excessive loss of leaves, which serve as a source of inoculum. Stands which have considerable vigor may be able to grow rapidly enough to keep ahead of the fungus and also escape infection to some extent. The burning of the infected areas also have been suggested as a method of control. However, the use of resistant varieties would be a more desirable and satisfactory method of control. This is possible as field observations and greenhouse tests have revealed that some alfalfa plants carry more resistance to this disease than others.

The Kansas Agricultural Experiment Station is particularly interested in this disease because a considerable portion of the

Kansas grown seed is sold to growers in the Eastern and Southeastern regions of the United States where this disease is of considerable importance.

Some of the plants which carry leaf spot resistance do not possess all of the desirable agronomic characters. As a result it is necessary to combine the leaf spot resistance of one line with as many desirable characters as possible of another into a single line. The factors which control the inheritance of resistance to leaf spot must be determined before any appreciable advancements are made in the development of a desirable resistant line. First, it had to be determined whether the factors which governed resistance were recessive or dominant. Secondly, the number of such factors must be determined.

The cultural characteristics of the fungus had to be studied thoroughly before an adequate supply of inoculum could be maintained. It took approximately one month for the fungus to mature on the ordinary types of cultural media. Because of this characteristic it would be very desirable to find some media which would shorten the time required for maturity.

Several methods of attack were used in attempting to determine the inheritance of resistance. The methods used were the selfing of resistant lines, reciprocal crosses between susceptible lines and resistant lines and crossing between resistant lines .

Selfed seedlings of three resistant lines and of one susceptible line were inoculated with bacterial wilt (Corynebacterium insidiosum (McC.) Bergey et al.) the purpose being to determine

whether or not any plants carry factors for resistance to both bacterial wilt and common leaf spot.

The final objective of these studies is to combine into a single variety the desirable agronomic characters with resistance to two major alfalfa diseases, bacterial wilt and common leaf spot. Desirable agronomic characters would include yield, quality of forage, longevity of stand and adaptation to Kansas conditions as well as adaptation to the Eastern and Southeastern regions where a large portion of Kansas seed is sown.

REVIEW OF LITERATURE

Leaf spot belonging to the genus Pseudopeziza was first collected on Medicago lupulina wildenowii and described by Libert (19) of France in 1832. This fungus was classified under the name of Phacidium medicaginis. In 1841 Desmazieres (10) found Pseudopeziza upon alfalfa but assumed it to be identical with the species described on Medicago lupulina wildenowii.

Saccardo (22) in 1883 transferred this species to the genus Pseudopeziza in which P. trifolii had been established as the type species. Consequently the question arose as to whether or not these fungi were identical or two different species of fungi. The two fungi were compared by Briesi (2) in 1888 as they occurred on several species of Trifolium and Medicago and no morphological difference was found, so it was suggested that Pseudopeziza on alfalfa be called Pseudopeziza trifolii forma medicaginis. Jones (16) in 1919 provided sufficient evidence to show that these

were two distinct species. He found the two species to differ in host specificity and in several morphological characteristics. Host specificity was demonstrated by inoculating Medicago sativa, M. lupulina, Melilotus alba, Trifolium pratense and T. hybridium with P. medicaginis. Within a week small brown spots were observed on alfalfa and these spots developed apothecia. Some minute brown lesions were formed on sweet clover but when paraffin sections were made of these areas, they were found to consist of dead shrunken cells. No macroscopic evidence of infection was observed upon Medicago lupulina, Trifolium pratense or T. hybridium. Jones concluded that P. medicaginis will infect sweet clover only under ideal conditions. Davis (9) considered sweet clover a host to alfalfa leaf spot in Wisconsin. Medicago sativa, M. lupulina, Melilotus alba, Trifolium hybridium and T. pratense were also inoculated with P. trifolii. No macroscopic evidence of infection was observed on Medicago sativa; however, some yellowed cells were observed upon M. lupulina. Germ tubes were observed inside the epidermal cells of Melilotus alba but had advanced only slightly after three days. Abundant spotting was produced upon Trifolium pratense and many leaves died and dropped off. Even though abundant spotting was observed upon T. hybridium, no fruiting bodies were obtained, which demonstrated that alsike clover was a less congenial host than red clover.

Jones (16) found that the two species differed morphologically in that the mycelia of P. medicaginis branched earlier than that of P. trifolii in culture and the branches came off at acute angles while the branches of P. trifolii came off at right angles

or occasionally at an obtuse angle. Spores of the two fungi also differ in size as those of P. trifolii were 11 microns or more in diameter while those of P. medicaginis were 10 microns or less. Also the spores of P. trifolii were sometimes slightly flattened on one side. There were an abundance of conidium-like structures formed on the mycelium of P. medicaginis while they were rarely observed on mycelium of P. trifolii when growing in artificial culture.

As a result of many observations, Jones (16) found that a viable spore lying upon a leaflet of a host immediately sent out a germ tube which penetrated the cuticle and passed directly into an epidermal cell. Only extreme minute openings occurred in the cell wall. The germ tube immediately expanded to normal size after it passed through the cell wall. Usually the germ tube divided into 2 or 3 branches upon reaching the center of the cell. These branches passed into the adjoining epidermal cells or down into the palisade layer and the cell walls apparently did not offer any resistance to the advancing hyphae. There was no marked disorganization of the cell contents until the hyphae became quite numerous.

Cunningham (8) preformed studies on the histologic changes in the alfalfa leaf due to infection by Pseudopeziza medicaginis. The lesions on the leaf were small holonecrotic areas which were dark purple in color and were surrounded by a yellowish plesio-necrotic border which blended into the normal green of the leaf. The holonecrotic area was characterized by the palisade cells being almost entirely replaced by the stroma of the fruiting

body and the cells of the spongy parenchyma were either collapsed or filled with hyphae. Some epidermal cells in the plesionecrotic zone were collapsed also. Frequently the hyphae was seen in one or more cells beyond this point. Occasionally the hyphae was seen in the mesophyll cells of this region and was observed to extend within two or three cells of normal tissue. In this zone the contents of the cells were more or less disorganized and occasionally a palisade cell was found which was filled with some dense brownish substance; however, there was little, if any, collapsing of the cell walls.

Many of the early European mycologists and plant pathologists have reported associated conidial stages of P. medicaginis. At one time it was thought that Sporonema phacidioides (Desm.) was the conidial stage; however, Jones (16) found evidence which indicated that Sporonema was the conidial stage of P. jonesii (Fckl.) Nannf. Voges (28) in Germany attempted to demonstrate that Phyllostricta was the conidial stage of P. medicaginis, but the description of his results were quite inadequate. Jones observed associated conidial stages in culture but never in nature; consequently, only the sexual stage is known to occur in nature.

In 1889 Chester (5) reported that the leaf spot fungus attacked several plots of alfalfa seedlings which were located in different parts of Delaware. The seedings were made with seed all of the same source. From these experiments Chester concluded that the disease was seed born and proceded to try a method of disinfecting the seed in order to determine if the disease was actually seed born. The seed was treated with copper sulphate and

was planted in heat-sterilized soil in cans. All seedlings derived from the plantings became infected with common leaf spot. Therefore, Chester concluded that the disease was wind born. In 1897 Coombs (6) reported that alfalfa plants were attacked by the fungus anytime after they had attained a height of 4 to 6 inches from the seed. He stated that infection was due to wind blown spores and that infection was local as the mycelium did not extend beyond the area occupied by the brown spot.

Stewart (26) reported that leaf spot not only reduced yield and quality of forage through defoliation, but also the quality was reduced further by a decrease in percentage of food nutrients. It was found that leaf spot caused a reduction of 10 percent in the fat content, 12 percent in protein, 11 percent in carbohydrates and 18 percent in fiber.

Reported losses from this disease ranged from complete to only slight defoliation of the plants. Pammel (21) in 1891 estimated the loss of alfalfa due to defoliation in the Ames, Iowa, neighborhood at 50 percent. Haskell (12) in 1918 reported that the disease was more severe on the first crop. This disease was also very destructive over the Cape Girardeau County, Missouri region in 1918 and Tennessee reported 90 percent of the crop affected. In Texas the disease was very severe as the susceptible varieties were completely defoliated. In 1923 Haskell and Wood (14) reported that 70 percent of the foliage was destroyed in Missouri in one instance. A report from Wisconsin indicated that the disease was not as severe in fields which had received sufficient lime in seed bed preparation (14). Illinois and New

Mexico reported a loss of 15 percent due to leaf spot and Iowa reported a loss of 10 percent in 1924 (13). Horsfall (15) in 1927 estimated the loss to be five percent of the potential yield or 30,704 tons.

Burlison et al. (4) believed that early mowing should not be used to control the disease unless very heavy infection occurred. In North Dakota Weniger (30) finds that more damage is caused by leaf spot than is usually supposed. Brown and Streets (3) reported that even though moist conditions favored the development of the disease, weak plants growing in dry soil were attacked severely. Workers in Michigan (1) found that plants whose vigor was reduced when grown in slightly acid soil were especially susceptible to leaf spot attacks. Weimer and Madson (29) of California reported that leaf spot was most severe during the cooler parts of the year while during the summer months it almost entirely disappeared, at least in the interior valleys. Spragg and Down (25) found that showers interspersed with high temperatures were especially favorable for the development of leaf spot in Michigan. Melchers (20) found that leaf spot was sometimes more severe during dry seasons than wet ones in Kansas. Second and third cuttings were reported to be most severely attacked by the leaf spot fungus (1, 3, 4, 12, 20, 26).

Varietal resistance has been known for sometime but little has been done about the use of resistant varieties as a method of control. Spragg and Down (25) reported that the variety Hardigan was able to reset leaves when leaf spot defoliated the

the plant and still produce a seed crop. Weniger (30) noted varietal resistance in North Dakota. Haskell (12) reported that the variety Hairy Peruvian carried some resistance to leaf spot in Texas. Smith (24) found that certain strains of Turkistan origin were usually more susceptible than common and variegated varieties. Jones (18) and Jones et al. (17) found that clonal lines and varieties showed rather marked differences in disease reactions when growing in a nursery at Madison, Wisconsin. All varieties contained some plants which were resistant. For instance, 50 percent of the plants in the variety Ladak were resistant; however, when these plants were tested under field conditions, only scattered plants were found to be free of spots. Some of the clonal lines which showed difference in infection in the field were inoculated in the greenhouse with pure culture and differences in disease development were obtained corresponding to those in the field.

MATERIALS AND METHODS

Since there were no alfalfa lines of known leaf spot resistance in the breeding program at the Kansas Agricultural Experiment Station, it was expedient to obtain material of known resistance. Of the several alfalfa clons obtained from Dr. R. L. Davis, of Purdue University and Dr. W. M. Myers, formerly of the United States Regional Pasture Laboratory, Pennsylvania State College, Findley (11) found C192, C193, C194, C198[1], 1573F, and 1705 x 1699 to be highly resistant to leaf spot under greenhouse

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1 Alfalfa improvement conference "C" number assigned to clonal lines.

conditions. Nemastan, C200, Buffalo and Kansas Common were found to be susceptible under the same conditions and were used as susceptible checks.

The number of resistant lines used in the breeding program were decreased to four except for a few instances so that more progress could be made in a given period of time. C192, C193, C194, and C198 were the four resistant lines chosen, as they seemed to carry as much or more resistance as did any of the other lines tested. C192 was a vigorous, leaf spot resistant, blackstem (Ascochyta imperfecta Peck) resistant, clonal line selected from an old field in New Hampshire. The variety was not known. C193 was a vigorous, leaf spot resistant, blackstem resistant selection from Ranger, a synthetic variety produced by the Nebr. Agr. Expt. Sta. C194 was a very vigorous, leaf spot resistant, moderately blackstem resistant selection from Atlantic. C198 was a medium vigorous, blackstem resistant, leaf spot resistant selection from Atlantic.

Reciprocal crosses were made between three susceptible and three resistant lines. C192, C193, and C194 were used as the resistant parents and Nemastan, C200 and Buffalo were used as the susceptible parents. Crosses between resistant lines such as C192, C193, C194, and C198 and selfs of C192, C193, and C194 were also made.

In the early stages of the experiment seeds from the crosses were planted in flats and the seedlings transplanted to six inch pots. Later the method was changed and the seeds were planted in

flats which were lined with roofing paper and filled with vermiculite. A two inch pot was placed in the center of each flat through which water was added. The flats were leveled in order to facilitate equal distribution of water. This latter method was used to lessen possible damage caused by damping off.

The technic used in transferring the seedlings from the vermiculite to flats containing soil mixed with sand was as follows: Six randomized rows of plants were planted in each flat and 30 seedlings were planted per row. After the seedlings were transplanted, a small amount of commercial fertilizer (6-10-4) was added to the soil.

When using the alcohol emasculation method of crossing developed by Tysdal and Garl (27), all but five or six of the desired flowers were removed from the raceme of the female parent used in the cross. The standard petals were clipped and the flowers were tripped with forceps. The tripped flowers were immersed in a beaker of 57 percent ethyl alcohol for a period of not less than 10 seconds and then rinsed in a beaker of tap water. An atomizer bulb was used to dry the flowers. Pollen from the desired male parent was collected on a small piece of emery paper which was attached to an end of a toothpick and then applied to the stigma of the emasculated flower. The raceme was tagged and then covered with a glassine bag. All tools including the transferer's hands were sterilized in 95 percent ethyl alcohol before making a cross in which different parents were involved. All crossed plants growing in 6 inch

EXPLANATION OF PLATE I

Alfalfa seedings as they appeared growing in flats containing vermiculite.

PLATE I

pots were made by this method.

Later the technic in crossing was changed to increase the population. The flowers were not emasculated because foreign pollen was more vigorous in fertilization than pollen from the same plant (7). Approximately 90 percent of the seeds produced by applying foreign pollen without emasculation will be crossed seeds. In using this method more crosses and different crosses can be made in a given period of time than by the alcohol emasculation method.

All immature and withered flowers were removed from the racemes of the plants used for crossing. A small piece of stiff paper was folded and cut so that a point was produced on one end. The pointed end of this paper was pressed against the keel of the flower, which caused the flower to trip. Pollen was collected on the paper and transferred to the flower of the other parent. In transferring the pollen from the first parent to the second, pollen from the second parent was also collected on the paper. This procedure was continued, going from one parent to the other and back again, until all flowers on both racemes were tripped. The first flowers tripped were removed from the raceme as no pollen from the other parent was on the paper at that time. A different piece of paper was used when making a cross whose parents were different from the preceding cross. The hands of the transferer were washed in tap water and dried. Tags with the necessary information were placed on the crossed racemes, however, the racemes were not bagged because there were no insects such as bees in the greenhouse which might transfer un-

desirable pollen to the crossed flowers.

As yet there have been no definite methods devised by which the accidental selfed plants can be distinguished from the crossed plants. Vigor and flower color might possibly serve as a basis for classification.

In selfing alfalfa all of the old and immature flowers were removed and the remaining flowers were tripped by forceps. The raceme was tagged and a glassine bag was placed over the selfed flowers. Before moving to the next plant the forceps and the operator's hands were sterilized in 95 percent alcohol. Glassine bags were not placed over the flowers which were selfed during the fall and winter months because of the reason mentioned previously.

Cultures of the fungus were descendants of the original cultures supplied to Findley (11) by Dr. F. R. Jones, formerly of the Division of Forage Crops and Diseases, United States Department of Agriculture and the Department of Plant Pathology, University of Wisconsin. The fungus was grown on oatmeal agar and was kept in a refrigerator in which the temperature was maintained at approximately 70 degrees F. which is the optimum for growth and fruiting.

During the summer months no inoculations were made because there was not sufficient inoculum and also it would have been virtually impossible to distinguish between natural and artificial infection as there was no glass cover on the greenhouse. During the early fall months inoculations were made in the large electric cold storage unit in the basement of the Plant

Research Laboratory where the temperature could be controlled at 70 degrees F. and a high relative humidity maintained. As the plants were seldom left in this unit for a long period of time, artificial lighting was not necessary.

Some inoculations were made in galvanized cylinders which were 12 inches high and 14 inches in diameter. Two such cylinders were required to make an inoculation as one was placed on top of the other. This was necessary to provide sufficient room for the plants during inoculation. The bottom cylinder was embedded in sand and a damp cotton cloth was placed in the areas between the two cylinders. After inoculation a pane of glass was placed over the cylinders.

Practically all inoculations were made in an inoculating chamber which was constructed in the greenhouse during the fall. This chamber was very similar to the one Findley (11) constructed. Unbleached muslin was stretched tightly over a wooden frame. The chamber was 60 inches long, 34 inches wide, 32 inches high in front and 28 inches high in the rear. This difference in height provided a slope which enabled the excess water to drain from the top of the chamber. The muslin was dampened thoroughly at the time of inoculation and kept moist at all time during the incubating period.

Two thicknesses of muslin were applied on all sides except the north. The purpose of the double thickness of muslin was to keep evaporation caused by the sun to a minimum. The front of the chamber was constructed so that it served as a portal of entry. Within the chamber there was a galvanized metal pan,

6 inches deep, which was filled with sand and kept saturated with water during inoculation.

In preparing the media 17 grams of agar-agar dissolved in 500 cc of distilled water were added to a mixture of 500 cc of distilled water and 50 grams of finely ground oatmeal which had been heated to 70 degrees C. and cooled slightly. This mixture was thoroughly stirred and was then added to the test tubes. Approximately 20 to 25 cc of media were added to each tube. A cotton plug was inserted into the mouth of each tube. Media was not allowed to get smeared on the mouth of the tubes because organisms may gain entrance to the sterile tubes by growing down through the cotton plug on the media.

The tubes were autoclaved for 45 minutes at 16 pounds pressure. After autoclaving the pressure was allowed to fall to zero and the temperature to 90 degrees C. before opening the autoclave so that the media would not boil upon the cotton plugs. The tubes were then slanted and the media solidified.

Cultural Characteristics

The method of starting cultures was the same as described by Findley (11). A mature culture will shoot ascospores several millimeters into the air and this property was of considerable value when making transfers. A small piece of fruiting culture which was approximately one-half inch wide and three-fourths inch long was transferred to a new agar slant and allowed to remain from one to four days, generally two to three days. At the end of this period the fragment was transferred to a new slant

and left for a corresponding length of time, then discarded. Frequent transferring provided cultures which mature at approximately the same time. If the cultural fragments were allowed to dehisce spores for a period of one week or more upon one agar slant, colonies of many different ages would be produced.

The first evidence of growth appears about one week after spores were dehisced upon the agar slant. Many small white glistening cone-like structures appeared upon the media which gave a somewhat roughened appearance. When the cultures were 2 weeks of age, a small brown or buff colored area appeared in the center of the cone-like structures. In about 3 weeks the brown color had diffused throughout the culture. The cone-like structures tended to coalesce during this period. At the end of 4 weeks the cultures were darker in color, especially if the colonies were not merged or crowded. Also during this period a small grayish white glistening gelatinous mass developed in the center of the stroma. This mass was the apothecium. As the cultures advanced in age their color changed to a very dark brown or black. Eight ascospores were found in each ascus which was produced in a hymenium of a delicate stroma. Jones (16) found that mature spores measured approximately 10 microns in diameter and many contain 2 oil droplets, one in each end. The spores were oblong in shape.

Two methods were used to determine if a culture was mature. In one method a small section of a culture was removed and placed in a drop of water on a slide and then crushed with a cover slip. The slide was examined under the microscope and if there were a

a multitude of spores present, some containing 2 small oil droplets, the culture was mature. In the other method a fragment of a culture was placed a few millimeters below the surface of clear water agar in a Petri dish. Several hours later the surface of the agar was examined with the low power objective of a microscope for the presence of ascospores. The spores are quite easily seen if present. The latter method was used very little as it was more time consuming than the former method and it was also felt that the former method was just as accurate.

Methods of Inoculation

Seven or more tubes containing mature cultures were used in each inoculation. The cultures were removed from the tubes by use of a spatula. Special care was taken so that as little media as possible was removed. On removal the cultures were placed in a wareing blender with 200 to 500 cc of distilled water and allowed to run for 3 minutes to insure uniform distribution of spores. Inoculum was sprayed by use of a hand sprayer on the plants which had previously been randomized in the inoculating chamber. All such inoculations were made between 5 and 6 p. m. because a high humidity favored infection and by inoculating in the evening a high humidity could be maintained easily.

Another method of inoculation was used which employed the ability of the fungus to dehisce spores several millimeters into the air. Cultures which were observed to be shooting spores were removed from the test tube and inverted on a wire screen which

was placed over the plants. From time to time these cultures were moved to different positions on the screen. At the end of 2 to 3 hours the cultures were removed and the plants were atomized with water. The plants were not atomized until after inoculation because this procedure eliminated the possibility of spores being trapped in droplets of water and germinating too far from the leaf surface to effect penetration of the epidermis by the germ tube while the spore was still viable.

On other occasions a leaf was clamped between a piece of wire screen and held in place with paper clips and this apparatus was supported by a piece of number 9 wire. A culture which was observed to contain mature spores was inverted upon the screen and allowed to dehisce spores for 2 to 3 hours. The culture and wire apparatus were removed and the leaves tagged and atomized with a fine mist of water.

Mode of Spore Penetration

The method used in studying the germination of the spores on the leaf and mode of penetration was similar to the method described by Jones (16). A culture which was known to be dehiscing numerous spores was placed over an individual leaf which was held between a piece of screen wire and left for a period of 2 to 3 hours. The leaf was tagged and atomized with water after removing the culture. Several hours later the leaf was removed and placed in equal parts of alcohol and acetic acid and brought to a boil. The acetic alcohol mixture was changed several times until no further decolorizing occurred.

The leaf was mounted in this liquid and observed under a microscope. The spores remained attached to the leaf during the treatment, and the method of entry and the mycelium within the leaf could be clearly seen. At the present no method of staining has been found which will improve the visibility of the fungus.

Classification of Disease Reactions

The classification of plants for disease reaction ranged from 0 to 5, depending upon the number and kind of lesions present. A score of 0 was given to plants which had no macroscopic evidence of spotting due to Pseudopeziza medicaginis; one, plants which had only a few nonsporulating lesions per leaflet; 2, when the leaves had several nonsporulating lesions; 3, both sporulating and nonsporulating lesions upon the same plant; 4, a few sporulating lesions per leaflet; 5, many sporulating lesions per leaflet.

It was rather easy to determine the difference between sporulating and nonsporulating lesions as the sporulating lesions were larger and often had a halo surrounding them. Many of the nonsporulating lesions were darker in color than the sporulating lesions. Lesions on susceptible plants were usually larger than those on resistant plants. Most accurate readings were obtained after the plants had been incubated for 4 weeks.

About one week after inoculation under greenhouse conditions, small brown spots became scattered over the surface of the leaf.

Usually the spots were found only on the upper surface of the leaf, but they can occur on the under surface as well. Occasionally a lesion was found on a succulent stem. It was, however, elliptical in shape with smooth edges and rarely bore a fruiting disk.

There were two important characteristics which served to distinguish common leaf spot from other parasitic leaf spots of alfalfa. First, the lesions were circular in shape and limited in size, rarely exceeding 2 or 3 millimeters in diameter. The size was partially determined by the number of lesions per leaf, the fewer the lesions the larger their size. Secondly, a small raised disk occurred in this spot in 2 or 3 weeks after inoculations which ranged from 1 to 1½ millimeters in diameter. The margin of the lesion was usually smooth and definite; however, it may be more or less dendritic surrounded by a fringe of olive colored rays. The latter type of margin was most prevalent under greenhouse conditions.

The small raised disk in the lesion was the apothecium or fruiting structure of the fungus. A mass of asci was contained within the disk and when there was sufficient moisture present, many spores were discharged. Usually the disks occurred on the upper surface of the leaf; occasionally, they were found on the lower surface and rarely were found on both sides from the same spot. When the disk was fully mature, it was surrounded by the torn edges of the epidermis.

There was very little difference in the color of the diseased tissue and the disk. The color usually ranged from a reddish brown to black. If heavily infected, the leaf turned

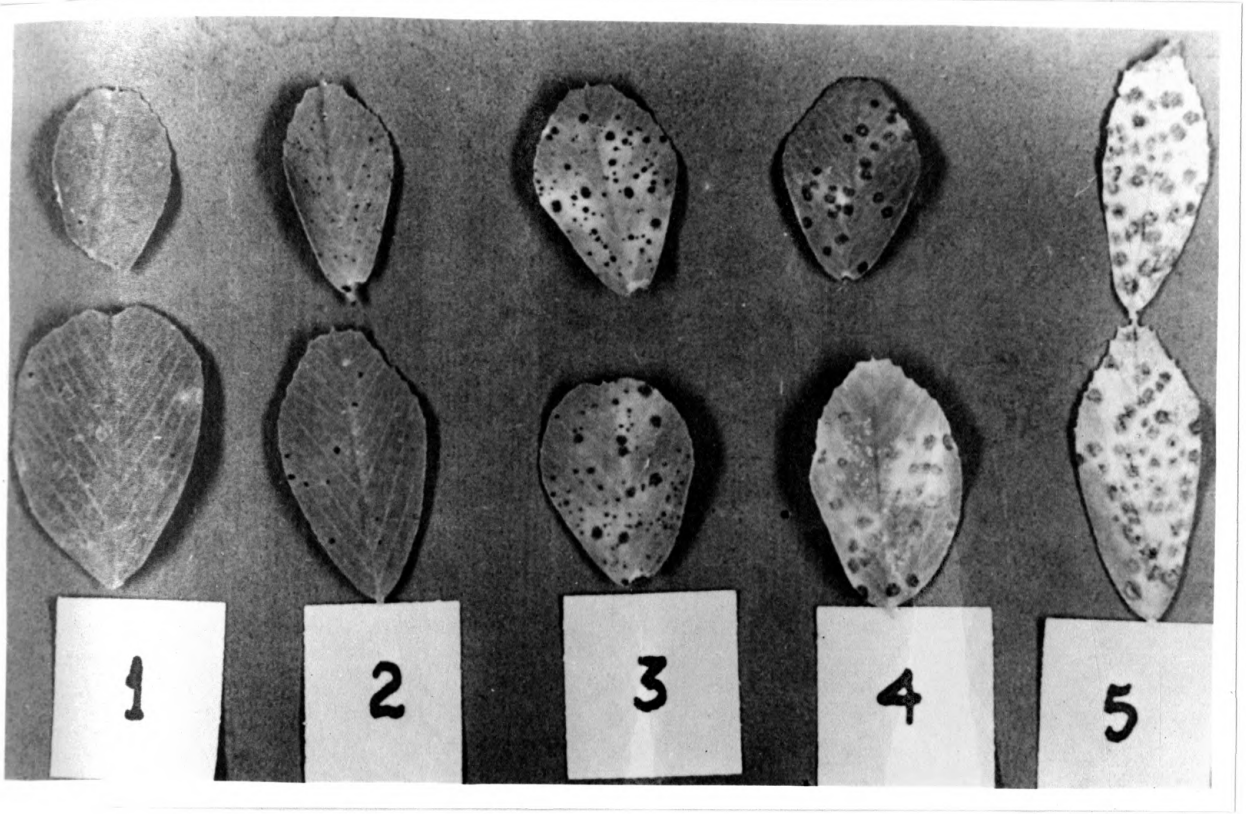
EXPLANATION OF PLATE II

Leaflets infected with *Pseudopeziza medicaginis* showing infection type characteristic of that appearing on plants scored from 1 to 5.

No. 1. Nonsporulating lesions - very few spots.
2. Nonsporulating lesions - some spotting.
3. Both sporulating and nonsporulating lesions.
4. Sporulating lesions - few spots.
5. Sporulating lesions - many spots.

Photo courtesy of Dr. R. L. Davis, Purdue University.

PLATE II



yellow and dropped off. When there were just a few spots per leaf, the only discoloration that occurred was around the margin of the lesion.

EXPERIMENTAL RESULTS

The results reported herein are concerned with the studies on the inherent nature of alfalfa in respect to resistance to Pseudopeziza medicaginis, susceptibility of alfalfa to P. medicaginis with respect to age of top growth, the influence of various vitamins upon maturity of P. medicaginis when grown under artifical conditions, and the relationship between resistance to leaf spot and bacterial wilt of alfalfa plants.

Inheritance Studies

The first inoculations were made in galvanized metal cylinders on 6 plants. The plants were Buffalo #9, Kansas Common #9 x C193, C200 x C194, Kansas Common #9 x C194, C200 X C193 and Nemastan #6. None of the 6 plants showed evidence of infection following the inoculation. It is possible that 5 of the 6 plants were resistant; however, Nemastan would be expected to show some spotting because of its known susceptibility. Later 30 plants were inoculated in the cold storage refrigerator. As in the previous inoculation none of these plants showed any macroscopic evidence of infection. Possibly infection did not occur because sufficient mature spores were not present or because the fan in the refrigerator caused the plants to dry too quickly, thereby decreasing the humidity of the atmosphere surrounding

the plants. All remaining inoculations were made in the moist chamber constructed in the greenhouse. The plants inoculated and the results obtained are found in Table 1.

Two lots of seedlings grown in flats were inoculated, one containing 2 flats and the other 3 flats, but it was very difficult to obtain leaf spot readings on these plants. By the time readings could be made, the plants had attained considerable growth and many of the lower leaves which would be expected to show leaf spotting had turned yellow and dropped off. Also there was considerable infection due to alfalfa rust (Uromyces striatus Schroet) which further complicated the readings. Those plants which had readings of 0, 1, and 2 were classified as resistant while those plants which had readings of 3, 4, and 5 were classified as susceptible. Most of the plants fell in the classes of 0, 3, and 4. The results are shown in Table 2.

Susceptibility of Alfalfa Top Growth at Different Ages

Plants of the variety Buffalo were used in the studies which were designed to determine the susceptibility of alfalfa to Pseudopeziza medicaginis with respect to age of top growth. The required number of seedlings were dug from a field on the agronomy farm which was seeded in August, 1950. The seedlings were packed in shingle toe and placed in a cold storage unit at a temperature of 38 degrees F. until time to plant. On the desired planting date approximately 50 seedlings were transplanted to 6 inch pots which were filled with a soil mixed with manure

EXPLANATION OF PLATE III

An alfalfa plant resulting from the cross C198 x C193 as it appeared 45 days after inoculation by spraying a spore suspension upon it.

PLATE III

Table 1. The classification of F_1 alfalfa plants according to their reaction to artificial inoculation of *Pseudopeziza medicaginis*.

Cross	Number of plants according to classification*						Total	Percent resistant
	0	1	2	3	4	5		
C193 x C200	7	0	0	0	0	1	8	87.5
C200 x C193	4	0	0	1	0	1	6	66.6
C193 x Buffalo	0	0	1	0	0	0	1	100.0
Buffalo x C193	8	0	0	1	0	1	10	80.0
C193 x Nemastan	11	1	0	1	0	0	13	92.3
Nemastan x C193	3	0	0	0	0	0	3	100.0
C193 x Kansas Common	5	1	0	2	1	0	9	66.6
Kansas Common x C193	10	0	0	1	0	0	11	90.9
C192 x Buffalo	8	1	0	0	1	1	11	81.8
Buffalo x C192	2	1	0	0	0	0	3	100.0
C192 x Nemastan	7	2	0	0	1	2	12	75.0
Nemastan x C192	5	0	0	1	0	2	8	62.5
C193 x Kansas Common	2	0	0	0	0	0	2	100.0
C194 x C200	1	0	0	1	0	0	2	50.0
C200 x C194	5	1	1	0	1	2	10	70.0
Buffalo x C194	16	4	0	0	0	2	22	90.9
Nemastan x C194	4	2	0	1	0	0	7	85.7
Kansas Common x C194	5	2	0	2	1	1	11	63.6
C198 x C192	15	2	0	0	4	1	22	77.3
C192 x C198	1	0	0	0	1	0	2	50.0
C193 x C198	2	0	0	0	0	2	4	50.0
C198 x C193	8	0	0	0	0	0	8	100.0
C198 x C194	2	0	0	0	0	0	2	100.0
C192 x C193	6	0	0	0	0	0	6	100.0
C192 x C194	1	0	0	1	0	0	2	50.0
C193 x C192	3	0	0	0	1	0	4	75.0
C193 x C194	3	0	0	0	0	0	3	100.0
C198 x Nemastan	0	1	0	1	0	1	3	33.3

* Classes 0 to 2 inclusive are resistant.
Classes 3 to 5 inclusive are susceptible.

Table 2. Reaction of seedlings of several lines of alfalfa to Pseudopeziza medicaginis.

Line	Number of plants			Percentage resistance
	Resistant	Susceptible	Total	
C192 x C200	67*	3	70	95.7
C200 x C192	13	0	13	100.0
C193 x C200	58	2	60	96.7
C200 x C193	68	3	71	95.8
Buffalo #51 x C193	36	2	38	94.7
C193 x Buffalo #51	54	1	55	98.2
C192	71	1	72	98.7
C193	97	3	100	97.0
C194	93	1	94	98.9

* Reaction of 0 to 2 were considered resistant.
Reaction of 3 to 5 were considered susceptible.

and sand. It takes approximately 30 days for the leaf spot fungus to mature on cultural media, therefore, it was necessary to coordinate the planting as described previously with the expected date of spore maturity. Four such plantings were made at 10 day intervals.

Four inoculations were made in all. Forty-four plants, 11 from each set, were randomized in the moist chamber and inoculated. The plants were remoistened at 8 o'clock the following morning and they were still moist when they were removed 24 hours after inoculation. On removal from the inoculating chamber the plants were placed in a larger chamber, similar to the inoculating chamber, and allowed to remain there for a period of 36 hours, after which they were placed on a greenhouse bench for further observation. Within the latter chamber the temperature ranged from 60 to 75 degrees F. with a 98 to 99 percent relative humidity.

The average disease reaction of the four replication of plants with different ages of top growth are shown in Table 3. In running the analysis of variance test for significance on this data, a transformation was used as described by Snedecor (23). A significant difference between plants of different sets or ages was obtained by the analysis of variance test as shown in Table 4. An F value of 6.47 was obtained while an F value of 3.91 is required for significance at the one percent level. The least significant difference at the 5 percent level showed that there was a significant difference in the susceptibility of the top growth of alfalfa 16 days of age and the top growth which was 26, 36, and 46 days of age, the oldest being more susceptible.

Table 3. Ranking of the ages of the top growth to the total average disease reactions.

Age	Disease reaction mean replication 1	2	3	4	Grand mean
16 days	1.25	1.43	0.86	1.02	1.14*
26 days	1.44	1.58	1.46	1.48	1.49
46 days	1.69	1.83	1.50	1.33	1.59
36 days	1.62	1.73	1.75	1.78	1.72

* L.S.D. at the 5 percent level is 0.27.
L.S.D. at the 1 percent level is 0.36.

Table 4. Analysis of variance of the reactions of the top growth of alfalfa plants of different ages to *Pseudopeziza medicaginis*.

Sources of variation	Degrees of freedom	Sum of squares	Variance	Calculated F	Table readings of F (P - 0.05)	Table readings of F (P - 0.01)
Between inoculations	3	1.8488	.6163	0.2277		
Between sets	3	8.1213	2.7071	6.4686**	2.666	3.904
Set x inoculations	9	2.1018	.2335	0.5579		
Between individuals within same set and inoculation	160	66.9541	.4185			
Total	175	79.026				

** Highly significant.

The least significant difference at the 1 percent level showed that there was a significant difference in the susceptibility of the top growth of alfalfa 16 days of age and the top growth 36 and 46 days of age. The difference between the grand means of the plants with top growth 16 and 26 days of age was 0.35 while a value of 0.36 was required for significance at the 1 percent level. Thus the probability of obtaining this difference purely by chance is about one in one hundred times. There was no significant difference between the susceptibility of the top growth of the 3 older sets at either level of significance. These data indicate that the young top growth of alfalfa is less susceptible to leaf spot than the older top growth.

Vitamin Studies

Oatmeal media was prepared in the same manner as previously described. On removal of the media from the autoclave, the vitamin solution was added to the media by use of a sterile pipette. The vitamin solution was prepared by T. E. Brooks, formerly of the Department of Botany and Plant Pathology, Kansas State college, and contained the following constituents:

Constituent	Micrograms per Milliliter
Thiamin	100.0
Pyridoxine HCl	100.0
I-inositol	5000.0
Hypoxanthine	500.0
Ca partothenate (d)	100.0
Nicotinamide	100.0
Biotin (crystalline)	2.5

Some of the vitamins such as thiamin are destroyed by heat, therefore, they had to be added after autoclaving. The first time this experiment was carried out, the media in some of the tubes solidified before slants could be made; consequently, the tubes had to be reheated in order to liquify the media so that slants could be formed. The second time this experiment was undertaken, the tubes of media were kept in a pan of water at 60 degrees C. to prevent solidification. The following concentrations of vitamins were used: 2 cc of vitamin solution per 1000 cc of media, which was recommended by Brooks, 4 cc of vitamin solution per 1000 cc of media and 8 cc of vitamin solution per 1000 cc of media. As there was approximately 20 cc of media in each tube, only .04 cc of vitamins could be added per tube when using the 2 cc in 1000 cc concentration; therefore, some method of dilution was necessary. Two cc of the original vitamin solution was added to 50 cc of sterile distilled water, in turn 1 cc of this dilution was added to each tube of media. One cc of the diluted solution per 20 cc of media is equivalent to .04 cc of the original concentration. A similar method of dilution was used in adding the vitamins of the other 2 concentrations. Ten tubes of each vitamin concentration and ten tubes of oatmeal agar which served as checks were used in both experiments. After the vitamins had been added to the media, the tubes were slanted and allowed to remain in the laboratory at room temperature for a period of 3 to 4 days. If any contamination had occurred during the process of adding the vitamin solution, it would have been visible by the end of 3 days. No

tubes were found to be contaminated in either of the 2 trials. At the end of the 3 day period cultural fragments which were observed to contain mature spores were transferred to 5 of the 10 tubes in each of the 4 sets. Three days later the cultural fragments were transferred to the 5 remaining tubes in each set and allowed to remain for 3 days before being discarded.

The cultures were examined each day from the time they were beginning to mature until all observations were made. Cultures were considered mature when they appeared ready for inoculating purposes. Even at best the maturity date of the cultures was difficult to determine. The results of the first experiment were not given as the media solidified before the agar slant could be made and as a result they had to be reheated. It was thought that the reheating might have destroyed some of the vitamins; however, the time required for cultural maturity of the various concentrations was very similar in both experiments. The results of the second experiment are given in Table 5. There was a very small difference in time of maturity of the fungus when grown on media of various vitamin concentrations and the checks and the difference is probably not significant. Because of the rather large number of misses the data were not analyzed statistically.

Bacterial Wilt Test

Selfed seedlings of the clonal lines C200, C192, C193, and C194 were inoculated with bacterial wilt (Corynebacterium insidiosum (McC.) Bergey et al.). Buffalo was used as the

Table 5. The effect of different concentration of a vitamin mixture on the rate of spore maturity of Pseudopeziza medicaginis.

Tube No.	Numbers of days			
	2cc per 1000cc	2cc per 1000cc	8cc per 1000cc	Check
1	**	**	34	29
2	**	34	**	33
3	**	34	34	28
4	36	34	35	29
5	34	36	33	33
6	**	30	32	30
7	34	31	**	**
8	**	31	32	29
9	30	31	32	30
10	35	30	32	30
Mean	33.8	32.3	33.0	30.1

** Fruiting time not observed.

resistant check and Kansas Common served as the susceptible check.

The two cultures of wilt organisms obtained from Dr. F. R. Jones were contaminated, therefore, a series of isolations had to be made. A sterile cultural loop was inserted into a tube containing the contaminated culture and a loop of the organisms was removed from the least contaminated area and streaked on a new agar slant. Two days later a third isolation was made from the second isolates. No contaminates were observed to be growing on any of the third isolates; however, some of the second isolates were contaminated.

Potato dextrose agar was the media upon which the wilt bacteria were grown. The media was prepared by cooking 300 grams of potatoes in 500 cc of distilled water in an Arnold sterilizer until the "crumbling or breaking stage". The potato juice was drained from the crumbled potatoes but it was not strained. Twenty grams of agar-agar was dissolved in 500 cc of distilled water and added to the potato juice, making approximately 1000 cc of media. Twenty grams of dextrose and 20 grams of calcium carbonate were added to prevent the media from becoming so acid that bacterial development would be prohibited. Approximately 45 cc of the media were added to a 150 cc capacity bottle. Cotton plugs were inserted into the bottles and the media was autoclaved for 45 minutes at 16 pounds pressure. The pressure was allowed to decrease slowly after autoclaving and to fall to zero before the autoclave door was opened. This procedure was followed to prevent the agar from boiling upon the cotton plugs. On re-

moving from the autoclave the bottles were tilted so that an agar slant with a large surface would be produced.

The bacteria were increased by streaking a loop of wilt organism over the surface of a large agar slant. Approximately 3 to 4 weeks after transferring to the large agar slants, the cultures were used for inoculating purposes.

In preparing the inoculum the bacteria and the media upon which they were growing were removed from the bottle and placed in distilled water and mixed in a wareing blender. The inoculum was quite concentrated.

Plants were prepared for inoculation by washing and clipping their roots. Clipping of the roots produced and injury through which the bacteria could readily gain entrance into the plant. The plants were placed in the inoculum for a period of 15 minutes, after which they were planted in the wilt bed. The soil was kept extremely moist after transplanting. About one month later the tops of the plants were clipped. By keeping the soil damp and the food reserves of the plant low, environmental conditions were very favorable for the wilt bacteria. Approximately 7 weeks after the first clipping, the plants were clipped again and dug. A diagonal cross-section was made of the roots and if the plant was susceptible to wilt, the cambial region was dark brown in color. Susceptible plants may also develop above ground symptoms such as dwarfing of the plant an the production of small leaves which are light green in color.

Results of the plants inoculated are given in Table 6. Although the population was small, it showed that some plants

Table 6. Reactions of seedlings of several lines of alfalfa to *Corynebacterium insidiosum*.

Line	Number of plants			Percentage resistance
	Resistant	Susceptible	Total	
C192⊗	9	7	16	56.3
C193⊗	0	5	5	0.0
C194⊗	8	11	19	42.1
C200⊗	13	9	22	59.1
Buffalo	7	18	25	28.0
Kansas Common	0	25	25	0.0

which carry resistance to leaf spot may also carry resistance to bacterial wilt. It is quite possible that C193 has some wilt resistance as the number tested was very small. Because of the population size, the data was not analyzed statistically.

SUMMARY

Common leaf spot, caused by the fungus Pseudopeziza medicaginis, is a common foliage disease of alfalfa. This disease causes defoliation and reduction in vigor of the plant and its development is favored by a high humidity.

This fungus is an ascomycete and infection by ascospores takes place directly through the epidermis of the leaf. Infection is local and may occur on both the upper and the lower surfaces of the leaf, however, infection usually occurs on the upper surface. The limited size of the spot and the raised disk within the spot are the two identifying characteristics which serve to distinguish this disease from spots caused by other parasitic fungi.

Reciprocal crosses between resistant lines, C192, C193, and C194, and susceptible lines, C200, Buffalo and Nemastan, yielded F_1 progeny which contained principally resistant but several susceptible individuals. This indicates that resistance is probably dominant to susceptibility.

Plants were inoculated in a moist chamber by spraying a spore suspension upon them in order to determine their resistance. Disease reactions ranged from complete freedom of spotting to abundant spotting.

Results of inoculating Buffalo plants differing in age of top growth by 10 days with Pseudopeziza medicaginis indicated that plants with top growth 16 days of age were more resistant to leaf spot than plants with top growth 26, 36, and 46 days of age.

Bacterial wilt (Corynebacterium insidiosum) resistance determinations were made on selfed seedlings of C192, C193, C194, and C200. Resistance was found in all lines except C193. This shows that some plants which carry resistance to leaf spot may also carry resistance to bacterial wilt.

The length of time required for the leaf spot fungus to mature on artificial media was not affected by the addition of some of the B complex vitamins and vitamin H.

The cultures were grown on oatmeal agar in the laboratory and stored in a refrigerator at a temperature of 70 degrees F.

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STUDIES ON THE DEVELOPMENT OF THE CULTURES AND THE SUSCEPTIBILITY OF ALFALFA TO LEAF SPOT, *PSEUDOPEZIZA MEDICAGINIS*

by

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Common leaf spot, caused by the fungus Pseudopeziza medicaginis (Lib.), Sacc., is a common foliage disease of alfalfa. The severity of this disease varies from year to year, depending upon the climatic conditions. High humidity favors the development of this disease and results in defoliation and reduction in vigor of the plant. The most practical method of control lies in the use of resistant varieties.

This fungus is an ascomycete and infection by ascospores takes place directly through the epidermis of the leaf. Infection is local, that is, the mycelium of the fungus does not occur in any portion of the plant except the area occupied by the lesion. Infection most commonly occurs on the upper surface of the leaf; however, infection may also occur on the lower surface. Two important characteristics are produced by this fungus when growing upon the alfalfa plant which distinguishes it from other parasitic leaf spot fungi. First, the lesion is circular and limited in size and secondly, there is a small raised disk within the lesion which is the apothecium of the fungus.

Reciprocal crosses between resistant lines, selfing of resistant lines, and crosses between resistant lines were made in attempting to determine the inheritance of resistance. C192, C193, C194, and C198 were the resistant lines used and Buffalo, Nemastan and C200 were the susceptible lines used. Evidence indicates that resistance is probably dominant to susceptibility, however, the number of factors involved have not been determined.

Plants were inoculated in a moist chamber by spraying a spore suspension upon them in order to determine their resistance.

Lesions on susceptible plants were larger than those found upon resistant plants and were of the sporulating type. Plants differ considerably in their reaction to the leaf spot fungus as some were completely free of infection while others were severely infected.

It seemed important to know at what stage of development the top growth was most susceptible, therefore, four sets of Buffalo alfalfa plants differing in age of top growth by 10 days were inoculated with Pseudopeziza medicaginis. The data showed that plants with top growth 16 days of age were more resistant to leaf spot than plants with top growth 26, 36, and 46 days of age.

Bacterial wilt (Corynebacterium insidiosum (McC.) Bergey et al.) resistance determinations were made on selfed seedlings of C192, C193, C194, and C200. Resistance was found in all lines except C193. This demonstrates that some plants which carry resistance to leaf spot may also carry resistance to bacterial wilt.

It was thought that some vitamin or vitamins might shorten the time required for the fungus to mature when growing on artificial media. With this in mind, some of the B complex vitamins and vitamin H were added to the cultural media. The vitamins were mixed together into one solution. No difference was found in the time required for the fungus to mature on media with different vitamin concentration than on media without the vitamins.

Cultures were maintained and increased by taking advantage of the characteristic of the fungus to shoot spores several millimeters into the air when mature. A small fragment of a mature culture was placed near the top of an oatmeal agar slant and allowed

to shoot spores for a period of two or three days. The cultures were grown in the laboratory and stored in a refrigerator at a temperature of 70 degrees F.