STUDIES OF THE BLACK-STEM DISEASE OF ALFALFA

by

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	3
MATERIAL AND METHODS	4
Development of Pure Cultures	4
Sources of *Ascochyta imperfecta*	5
Preparation of Inoculum	7
Method of Inoculation	8
Disease Readings	10
Use of Check Plants	12
Sample Calculation	13
EXPERIMENTAL RESULTS	15
Laboratory Studies	16
Studies Concerning the Relative Pathogenicity of Strains	29
Correlation of Colony Characteristics on Artificial Media with Differences in Pathogenicity	31
Proof of Pathogenicity	35
Life History of *Ascochyta imperfecta* Peck	35
Inheritance Studies	39
Reaction of Inbred Lines	41
Hybrid Studies	52
Miscellaneous Experiments	54
DISCUSSION	56
SUMMARY	58
ACKNOWLEDGMENT	59
LITERATURE CITED	60

INTRODUCTION

Alfalfa is commonly referred to as the "Queen of Forage Crops." The title is an appropriate one, for the crop possesses forage value and soil building abilities that are unsurpassed by any other plant used in agricultural practices today. Its prominence is emphasized by the fact that 35 million acres were devoted to hay production throughout the world in 1929, and a proportionately large acreage was devoted to seed production (2).

Alfalfa is subject to attacks by diseases and insects which, during the past twenty years, have become increasingly serious. This problem has been recognized by state experiment station and United States Department of Agriculture workers as among the most important with which they must deal in their alfalfa breeding and improvement programs.

Leaf and stem diseases have been ranked second in importance only to bacterial wilt caused by Phytomonas insidiosum (11). These diseases are particularly prevalent in wet seasons and in humid regions. The urgent need for varieties resistant to these foliage-robbing diseases is of special significance to Kansas, which has long been the seed producing center for the alfalfa grown in eastern states where humid conditions prevail. If the state is to retain its position as a major seed producer, it must be able to furnish disease resistant

varieties.

Among the more serious of the leaf and stem diseases that attack alfalfa is the black-stem disease caused by Ascochyta imperfecta Peck. In the opinion of the workers at the Kansas State Agricultural Experiment Station and the Division of Forage Crops and Diseases this disease was of sufficient economic importance to warrant a more comprehensive and detailed study than had been made previously. The studies were initiated by Peterson (4) in 1939-40 and continued in the present study in 1940-41.

The final goal of the problem was to develop strains of alfalfa resistant to the disease. Many related problems had to be solved, however, before this goal could be attained. Thus, it was found necessary to develop methods for laboratory inoculation of plants in order that the disease might be observed under carefully controlled conditions; the life history of the pathogen in the field had to be determined; the possibility of the occurrence of strains in the causal organism had to be studied; and, most important, the fact that resistance to the disease was an inherited characteristic had to be ascertained. Only when these preliminary questions were answered could an intelligent program for the development of resistant strains of alfalfa be undertaken.

Therefore, it was the purpose of this study to solve some of the problems relative to developing strains of alfalfa resistant to the black-stem disease.

REVIEW OF LITERATURE

Peterson (4) made a detailed review of the literature concerning the taxonomy of the causal organism, and concluded that the black-stem disease of alfalfa was caused by Ascochyta imperfecta Peck. He thoroughly described the symptoms of the disease, and listed several common varieties and species of alfalfa in the order of their relative degrees of resistance. He also made observations on the life history of the pathogen in the field and reported that the removal of all the above ground material from the field in the fall prevented the development of the disease during the following spring.

No reports have appeared in the literature concerning the breeding of alfalfa for resistance to black-stem, and it is evident that little work has been done on the causal organism, itself. Remsberg and Hungerford (5) presented evidence that the perfect stage of Phoma medicaginis Malbr. and Roum., considered by Toovey, Waterston and Brooks (10) and Peterson (4) to be Ascochyta imperfecta, was Pleospora rehmiana. (Staritz) Sacc. Stevens (8) describing the Ascomycete, Pseudopeziza medicaginis Libb. Sacc., which also causes a serious leaf spot disease of alfalfa, referred to Fron (1), who stated, "A Phyllosticta thought to be its (Pseudopeziza's) conidial stage has been reported." Phyllosticta medicaginis was also listed by Peterson as synonymous with Ascochyta imperfecta. Thus, it appears entirely possible that a perfect, or sexual stage,

might occur in the inperfect fungus which causes the black-stem disease.

MATERIAL AND METHODS

Excellent facilities were provided for the work on this problem. Considerable greenhouse space and equipment were allotted by the Department of Agronomy for the inoculation studies, and laboratory space and equipment were furnished by the Department of Botany and Plant Pathology.

Development of Pure Cultures

Pure cultures of Ascochyta imperfecta were essential for the studies undertaken. Thus, single spore isolations were made, and all cultures were developed from the isolates.

The technique employed in the isolation of single spores involved the use of a Fink micromanipulator with a glass microneedle as the isolating instrument. A droplet of water containing conidia was placed on the underside of a cover glass on a Van Tieghem cell. From this droplet a single spore was removed on the tip of the microneedle. A second Van Tieghem cell replacing the first was moved into position on the microscope stage, and the single spore was deposited on the surface of sterile water agar on the cover glass. The cover glass and the cell were then removed to a sterile Petri dish for incubation.

Single spores isolated in this manner were allowed to

germinate and produce a small vegetative growth before they were transferred aseptically to sterile potato-dextrose agar slants. The colonies were examined periodically for purity, and contaminated slants were discarded as soon as discovered. During the course of the investigations, all cultures were maintained on potato-dextrose agar slants. Transfers of growth to new media were made at regular intervals of three weeks in order to keep the colonies viable.

Sources of Asochyta imperfecta

A rather representative study of the organism was made possible by the utilization of several cultures which had entirely different points of origin. Most of these cultures were available in the stock culture collection maintained by the Department of Botany and Plant Pathology. They were supplemented by cultures developed during this study.

The history of each source is listed below.

1. A pure culture of Ascochyta imperfecta isolated by Dr. D. B. Creager at Manhattan, Kansas, on April 30, 1938, from leaf lesions found on a series of infected alfalfa plants in greenhouse flats. The plants, from which the culture was obtained, had overwintered out of doors in flats from which the fall growth had not been removed. At the time the culture was made, typical black-stem disease symptoms had appeared on the lower halves of the plants, and defoliation had commenced.

2. A pure culture developed from the same material as No. 1.

3. A pure culture isolated by Dr. Creager from infected plant material received from B. L. Richards of Utah on June 22, 1938.

4. A pure culture of Ascochyta imperfecta received from Dr. F. R. Jones on July 15, 1938. This culture had been developed from isolations made from infected alfalfa plants at Monroe, Wisconsin, on June 1, 1931.

5. A pure culture received from Dr. Jones on July 15, 1938. It had been isolated from infected alfalfa at Geneva, New York, on August 5, 1935.

6. The pure culture used by Peterson (4) in his studies. It was isolated from the stems of diseased alfalfa plants at Manhattan, Kansas, in the summer of 1938.

7. A pure culture developed from isolations made from alfalfa stubble at the Agronomy Farm, Manhattan, Kansas, December 10, 1940.

8. A pure culture of Ascochyta imperfecta isolated at the Agronomy Farm January 16, 1941. This culture was also developed from infected stubble.

As stated before, pure cultures were developed from all of these various sources by the single spore method. Several single spore cultures were developed from each source.

It was considered that a composite of these pure cultures would furnish an inoculum that would preclude the possibility of a single strain or race reaction on the plants tested.

Preparation of Inoculum

Potato-dextrose agar slants afforded a convenient method for keeping cultures in a pure, viable state, but due to the inaccessibility of the growth, another method had to be devised in order to facilitate the preparation of spore suspensions. Peterson (4) found that three to four inch sections of second year sweet clover stems furnished a very satisfactory type of medium for this purpose. The stem sections were softened in boiling water and then dropped into test tubes containing about one inch of plain water agar, which served to keep the stems moist during their use. The test tubes were plugged and the medium sterilized by autoclaving at fifteen pounds of pressure for twenty minutes.

The sterile stems were inoculated by streaking them with hyphal fragments transferred from the culture slants on a sterile wire needle. They were then stored at room temperature until the organism attained abundant mycelial and pycnidial growth.

The accessibility of the growth in this type of culture made the preparation of spore suspensions a rather simple matter. Sterile water was added to the tubes containing the pycnidia laden stems, and the growth was scraped from the stems into the water by a sterile wire needle. A period of three hours was allotted to permit the spores time to escape from the fruiting bodies into the surrounding water.

The excess growth was then strained from the suspension by passing the liquid through cheese cloth. Preliminary trials had shown that six tubes of suspended spores diluted to four hundred cubic centimeters gave a concentration of spores which produced the optimum amount of infection. Microscopic examinations were made of each suspension, and the amount of water added was more or less regulated by the concentration of the spores revealed by this method. Thus, in some cases more than four hundred cubic centimeters were found necessary to bring the suspension down to the proper concentration.

Method of Inoculation

Peterson (4) developed the method of plant inoculation followed in these studies. He determined that conditions of high humidity and moderate temperatures were essential for infection of alfalfa by Ascochyta imperfecta. These conditions were obtained by the use of a moist chamber constructed of canvas stretched over all sides of a wooden frame which was six feet long, four feet wide, and five feet high to the peak of an ordinary gable type roof with a half pitch. The canvas at one end of the chamber was left free as a flap through which the plants were transferred to and from the chamber. Water was allowed to run down the roof and all sides of the chamber into a galvanized tin tray equipped with an outlet drain. The water was supplied from small holes drilled into a pipe

which extended along the peak of the roof and across the two ends. A raised wooden platform inside the chamber kept the plants above the level of the water which collected in the tray.

Another chamber similar to this, but constructed of metal, was also utilized. Hygrothermographic records in both chambers showed that the relative humidity remained fairly constant between 90 and 100 percent and that the temperature varied from 60 to 70 degrees Fahrenheit at all times.

Plants selected for inoculation were placed in the chambers and moistened by a fine mist spray. The spore suspension was then sprayed on the damp plants with an atomizer manipulated by air pressure. After the inoculation period had elapsed, the plants were allowed to dry slowly in the chamber. This prevented dehydration damages incurred by the removal of plants directly to greenhouse atmosphere.

Peterson (4) reported that an incubation period of three to five days in the moist chamber was necessary to secure good infection. Preliminary trials showed that three days furnished ample time for optimum infection. After this period, lesions became barely visible as small water soaked areas which later developed a yellow brown color. As the disease progressed, lesions coalesced and darkened to a brownish black color.

In order to make accurate observations, a stage of infection had to be chosen which presented the sharpest differences between plants. This point was reached, in most cases,

about 10 days after inoculation, at which time the lesions were dark colored in all parts and defoliation had proceeded to a degree which made contrast between plants sharply evident. All disease readings, therefore, were made after ten days of infection.

Disease Readings

A complete picture of the total amount of infection in every plant had to be ascertained in order that reliable comparisons between infected plants could be made. This picture had to be drawn in terms which described the total level of plant infection, and which, at the same time, adapted themselves to statistical treatment.

An estimate of the extent of infection was made according to a numerical scale, for each of the following characters: Number of leaf lesions, size of leaf lesions, stem infection, petiole infection, and percentage defoliation.

Number of Leaf Lesions. The average number of lesions on the leaves of the plants was estimated on the basis of a scale ranging from 0 to 10. Complete absence of lesions was represented as class 0 with increasing numbers of lesions designated as higher class numbers grading up to class 10 which denoted the largest possible number of leaf lesions. A pictorial scale made by Peterson (4) representing the grades of infection was utilized as a guide in making the readings.

Size of Leaf Lesions. The estimation of classes for size

of leaf lesions was more difficult than for numbers of lesions due to the lack of distinctness in size differences. Thus, an average size lesion had to be estimated for each plant. A scale grading from 0 to 5 was used, and here, again, the smaller numbers represented the lesser degrees of infection. A picture scale employed by Peterson (4) was also used in making size of leaf lesion readings.

Stem Infection. Lesions on stems often became very marked after the ten day infection period. This condition furnished valuable information on the plant's ability to resist the disease, for a great deal of the loss in the field in early spring arises from the girdling of stems. Differences in severity of infection were great enough, however, to warrant the estimation of only 5 classes of infection. Class 0 indicated no lesions and class 5 designated the most severe type of stem infection. The various classes of infection were judged on the basis of a series of mounted stems representing the classes prepared by Peterson (4).

Petiole Infection. The degree of petiole infection in the plants was, in many cases, difficult to determine, but its importance in cases of severe defoliation was so prominent that estimations of its abundance were made. Estimation was based on a 0 to 5 scale much the same as stem defoliation was judged.

Percentage Defoliation. The final, and perhaps most important, reading made was the estimated percentage of de-

foliation due to the disease. Defoliation is the most serious damage that this disease produces in alfalfa. Although it was difficult to ascertain precisely, any estimate of the extent of defoliation was considered to be of value. This reading was made on the basis of a 0 to 10 scale, 0 representing no defoliation and 10 denoting the most severe type of defoliation.

Each of the readings was converted into a percentage figure and the average of the five criteria was derived and designated as the "plant score."

Use of Check Plants

Preliminary studies of clonal lines of several varieties indicated that wide variations in severity of infection were introduced by inoculations made at different times. Peterson (4) encountered the same difficulty in his work. This condition, obviously, had to be overcome if large numbers of plants were to be compared directly in regard to their reaction to the disease.

A method involving check plants, therefore, was devised. A clonal line of plants was developed from a fairly resistant Ladak plant which Koepper (3) discovered and utilized in his studies of alfalfa rust. Two of these check plants were placed in the moist chamber with each group of plants inoculated. Readings were made on the check plants and the plant score for each was calculated. In most cases the plant scores of the check plants were identical. This plant score was arbitrarily

designated as 100 percent infection. From the 100 percent infection figure the rest of the plants were given an "index number" derived in terms of percent of check. All analytical treatment of the data was made on this "index number of infection."

Variations between dates, otherwise uncontrollable, became an insignificant factor as the result of the use of check plants. It was found, for example, that when severe infection occurred on one particular date, the check plants were affected as much as the rest of the plants. All plant scores, consequently, were high, but, when converted into terms of index numbers, using the checks' plant scores as 100 percent infection, the general trend of the group was highly comparable with the other dates of inoculation. All plants inoculated during the course of these studies, therefore, were considered to be directly comparable regardless of the date of their inoculation.

Sample Calculation

A sample calculation is presented at this point to clarify the procedure employed in deriving the "index of infection numbers" by which all plants were compared. As already pointed out, readings of number of lesions and percentage defoliation were on a scale ranging from 0 to 10, and the other three characters, size of lesions, stem infection, and petiole infection were graded only from 0 to 5. The following computa-

tions illustrate the conversion of these scores for one plant from the actual reading to a 100 percent basis and the averaging of the five readings in the derivation of the "plant score."

Table 1. Sample calculation of plant score and index of infection number.

Character	Reading		Reading converted to percent for averaging plant score
Number of leaf lesions	7	x 10	70
Size of leaf lesions	2	x 20	40
Stem infection	2	x 20	40
Petiole infection	3	x 20	60
Percent defoliation	3	x 10	30
			Plant score = $\frac{240}{5}$ = 48

This plant score was then converted to an "index of infection number" calculated on the basis of its value in terms of percent of the plant scores of the Ladak check plants, which were inoculated the same date and whose scores were compiled by the same method.

Example's plant score 48
Average Check plant score 40 $\frac{48}{40}$ = 120

Index of infection number = 120. Henceforward, this plant was represented by this number in all statistical treatments.

EXPERIMENTAL RESULTS

The major portion of the work herein reported concerned studies involving the inheritance of resistance in alfalfa to the black-stem disease. This work was considered to be the most important phase of the problem, for it promised to furnish the most readily applicable information in terms of a breeding program. These studies were so inseparably associated with pathological work, however, that one phase of the problem of necessity involved the other. Thus, considerable time was devoted to laboratory and field studies of the pathogen, and the results obtained represented a large portion of the experimental results.

The appearance of colonies of the pure cultures of Ascochyta imperfecta varied somewhat among the respective isolates. This condition of variability suggested the possibility of the existence of "strains" or "races," and made further studies imperative. The probability of the occurrence of a sexual stage in the organism has been reported by Remsberg and Hungerford (5), and emphasizes the possibility of such strains, for they commonly occur in the perfect fungi. The importance of determining the presence or absence of strains and the role that they might play in their effects on alfalfa, therefore, was of great significance, for a breeding program would have to be developed along lines that would include testing of new lines of alfalfa for each of the known strains

of the organism rather than simply testing their resistance to the disease as though there were no strain differences.

Laboratory Studies

The differentiation of strains was accomplished in the laboratory by growing the various single spore isolates on selected media in Petri dishes, and comparing the colonies on the basis of their outstanding differences. Some difficulty was encountered in securing a medium which would sharply contrast these differences. The following media were tested before a suitable one was discovered:

1. Czpak's medium - an inorganic medium

Formula -	$MgSO_4$ -	.5 g.
	KH_2PO_4 -	1.0 g.
	KCl -	.5 g.
	$FeSO_4$ -	.01 g.
	$NaNO_3$ -	2.0 g.
	Agar -	17.0 g.
Distilled water -		1000 ml.

This formula was taken from Thom and Church (9).

2. Bacto-wort agar
3. Carrot infusion agar
4. Carrot plus $CaSO_4$ infusion agar
5. Alfalfa infusion agar
6. Alfalfa plus $CaSO_4$ infusion agar
7. Plain water agar
8. Potato-dextrose agar
9. Cornmeal agar
10. Oatmeal agar - neutral

11. Oatmeal agar - acidified to pH 5.0

The formulae of the media were adapted from Riker and Riker (6).

Czpak's medium, Bacto-wort agar, and plain water agar proved to be very unsatisfactory as differentiating substrata, for the colonies produced on them were abnormally small and slow growing, with no variation observable. The alfalfa and carrot media, though slightly better, did not produce enough contrast to warrant their use. The rest of the media, including potato-dextrose agar, corn meal agar, and oatmeal agar neutral and acidified, furnished fairly good conditions for differentiating between colony characteristics.

The media were prepared and sterilized by autoclaving at 15 pounds pressure for 20 minutes. They were then poured into sterile Petri dishes to a uniform depth of 1 centimeter and allowed to harden.

The technique of plate inoculation required great care, for the success of the tests depended on the uniformity of the amount of fungus planted in each plate. Further precautions were taken to insure the uniformity of age in all the pure cultures from which inoculum was obtained. This was done by transferring growth to fresh slants twice during the week preceding plate inoculation. From these fresh cultures, small 3 mm. square sections were removed with a sterile wire needle and were transferred to the center of the respective plates.

The characteristics which provided the greatest contrast

Table 2. Reaction of eight cultures of Ascochyta imperfecta to different media.

Culture No.	Medium	Characteristic: Zonation of colony	Color of colony	Size of pycnidia	Relative abundance of pycnidia	Radial arms	Aerial hyphae	Relative rank on rate of growth: Medium	Total
1	Oatmeal agar, neutral	++	Dark outer ring, brown green center	+	+	+	+	6	
	Oatmeal agar, acid	++	Dark outer ring, brown green center	+	+	-	+	6	
	Cornmeal agar	++	Dark outer ring, brown green center	++	+	-	+	6	
	Potato dextrose agar	++	Dark outer ring, brown green center	+	++	+	+	6	6
2	Oatmeal agar, neutral	++	Olive green	++	+	+	++	1	
	Oatmeal agar, acid	++	Olive green	+	+	+	++	1	
	Cornmeal agar	++	Olive green	++	++	+	++	1	
	Potato dextrose agar	++	Olive green	++	+++	+	++	3	1
3	Oatmeal agar, neutral	-	Brown green	+	++	-	++	3	
	Oatmeal agar, acid	-	Brown green	+	++	-	++	2	
	Cornmeal agar	-	Brown green	+	++	-	++	1	
	Potato dextrose agar	+	Brown green	+	+++	-	+	1	2
4	Oatmeal agar, neutral	-	Dark outer ring, white center	+	++	-	+	8	
	Oatmeal agar, acid	+	Dark outer ring, white center	+	++	-	+	8	
	Cornmeal agar	+	Dark outer ring, white center	+	+	-	+	8	
	Potato dextrose agar	+	Dark outer ring, white center	+	+++	-	-	8	8
5	Oatmeal agar, neutral	-	Light grey-green	++	++	+	++	3 & 4	
	Oatmeal agar, acid	-	Light grey-green	++	++	-	++	3	
	Cornmeal agar	-	Light grey-green	+++	++	-	++	3	
	Potato dextrose agar	+	Light grey-green	++	+++	-	++	2	3
8	Oatmeal agar, neutral	+	Dark brown-green	++	++	+	-	2	
	Oatmeal agar, acid	+	Dark brown-green	+	+++	+	-	2	
	Cornmeal agar	+	Dark brown-green	++	++	-	-	5	
	Potato dextrose agar	+	Dark brown-green	++	++	+	-	4	4
7	Oatmeal agar, neutral	-	Olive green	++	+	++	+	3 & 4	
	Oatmeal agar, acid	-	Olive green	+	+	++	+	4	
	Cornmeal agar	-	Olive green	+++	+	++	+	4	
	Potato dextrose agar	-	Olive green	+++	+	++	+	4	5
6	Oatmeal agar, neutral	-	Dark outer ring, white center	++	+	-	+	7	
	Oatmeal agar, acid	-	Dark outer ring, white center	+	+	-	+	7	
	Cornmeal agar	-	Dark outer ring, white center	+++	+	-	+	7	
	Potato dextrose agar	-	Dark outer ring, white center	++	+	-	+	7	7

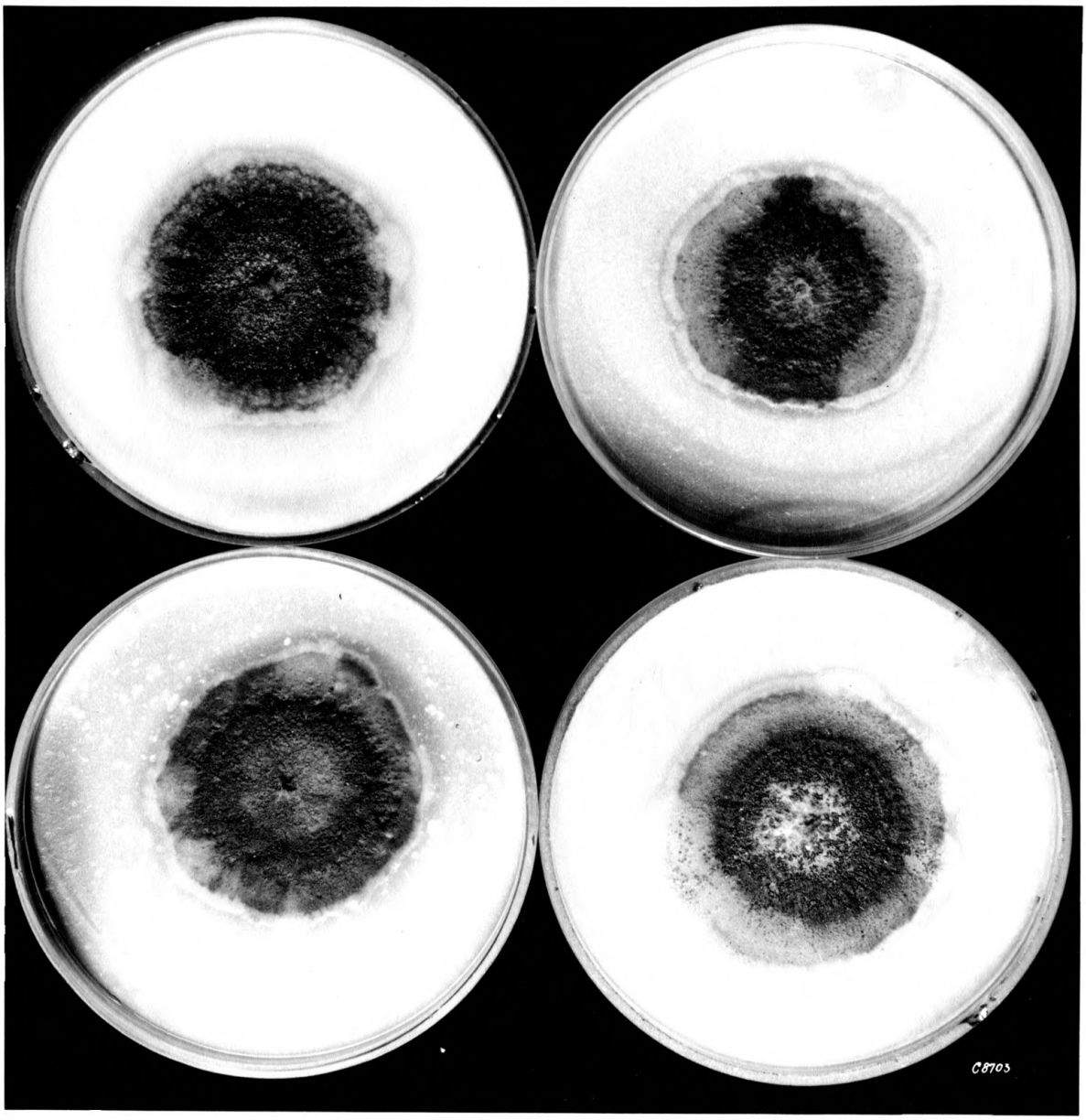
between colonies were observed and recorded daily. Certain of these contrasting features were more striking and constant than others, and Table 2 shows a summary of the characteristics and differences as they occurred on several of the media. One character, the rate of growth of the various colonies, was strikingly constant throughout all of the tests. The average diameter of the flat growing Ascochyta imperfecta colonies on the surface of the various media was measured each day with a millimeter rule. Acidified oatmeal agar proved to be the most effective differentiating medium.

Each single spore isolate was considered an individual strain and tested as such, no matter from what source it had arisen. Thus, it was of interest to observe whether the single spore isolates from the same source were similar in colony characteristics, or whether they varied as much as single spore isolates taken from different sources. Plates I and II show rather clearly that little, or no, variation existed between single spore isolates from the same source. Plate I shows the colonies developed from four different single spore isolates in source No. 5, the culture from the isolations made at Geneva, New York by Dr. Jones. Plate II illustrates four single spore isolates from source No. 8, an isolation made during the winter of 1940-41 at the Agronomy Farm, Manhattan, Kansas. Each colony illustrated in the plates represents one of a triplicate of Petri dishes inoculated with each isolate. Triplication of all single spore isolate cultures was practised

EXPLANATION OF PLATE I

Four different single spore isolates from the same source (Geneva, New York, 1935) are illustrated. The striking lack of variation between single spore isolates from the same source is typical of all the different sources studied.

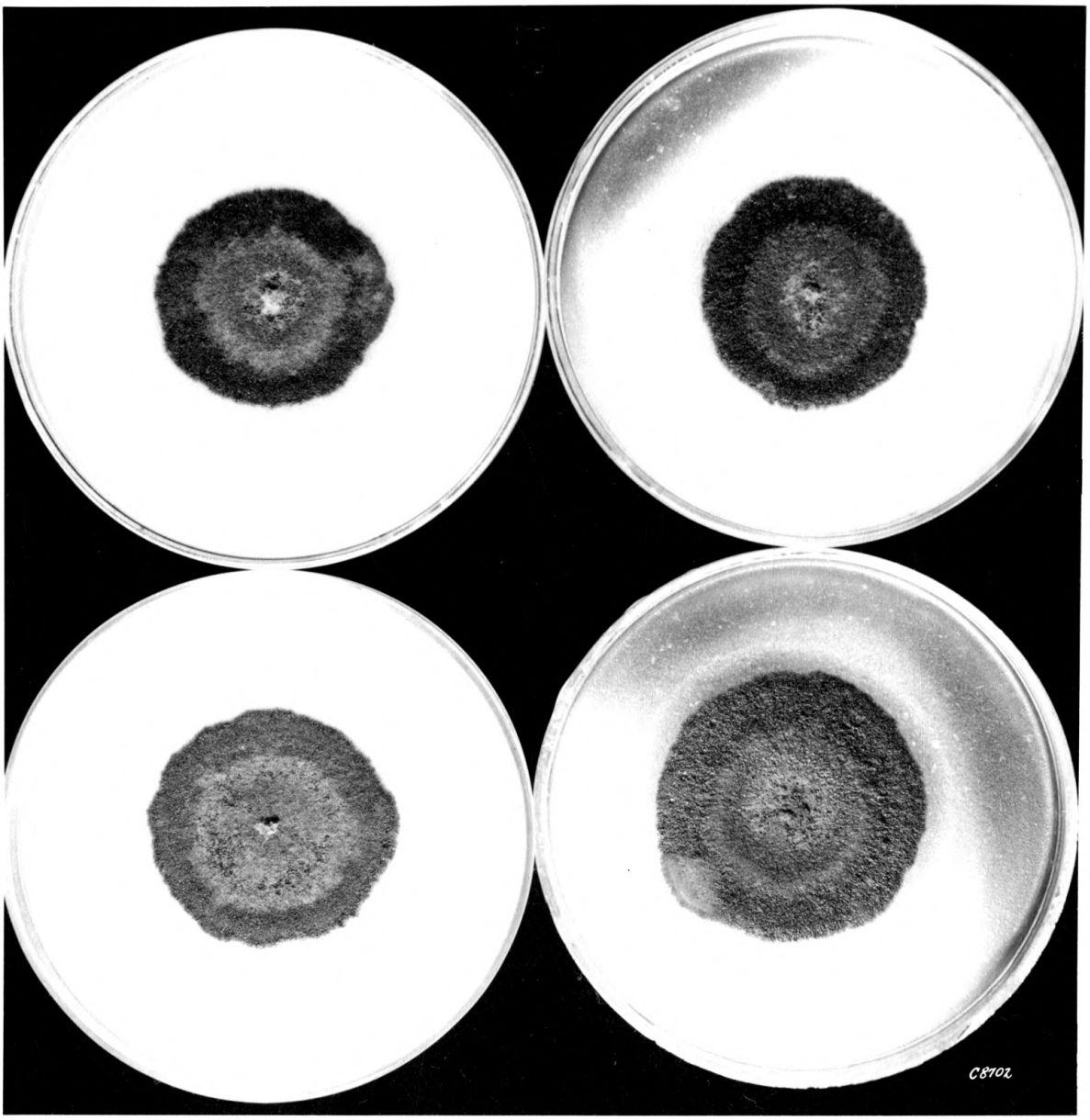
Plate I



EXPLANATION OF PLATE II

This plate shows four single spore isolates from the same source (Manhattan, Kansas, 1941), and, as in Plate I, the similarity between isolates from the same source is manifest.

Plate II



throughout the studies in order to give a check on the similarity of the colonies that arose from each single spore isolate, and also to allow for contamination. At no time were any differences observed within a triplicate of cultures. Plates I and II are merely typical examples of the condition that existed in all of the sources worked with in the studies. Thus, it appears that any differences that occurred were not between single spore isolations from the same source.

Plates III and IV demonstrate the differences that existed between sources. The pictures show variations in colony characteristics which, when coupled with rate of growth data, furnish conclusive evidence that there is variation in Ascochyta imperfecta. The features seen in the plates were fairly constant in all colonies observed, and also, repeated trials showed a consistent ranking of the various sources in regard to their respective rates of growth. This ranking is seen in Table 3.

Table 3. Relative rank of the various sources of Ascochyta imperfecta with respect to rate of growth.

Rank	Source No.	Origin
Fastest growing	2	Kansas by Dr. Creager
Second	3	From Utah by Dr. Creager
Third	5	New York by Dr. Jones
Fourth	6	Manhattan, Agronomy Farm
Fifth	7	Manhattan, Agronomy Farm
Sixth	1	Manhattan by Dr. Creager
Seventh	4	Wisconsin by Dr. Jones
Slowest growing	8	Kansas by Peterson

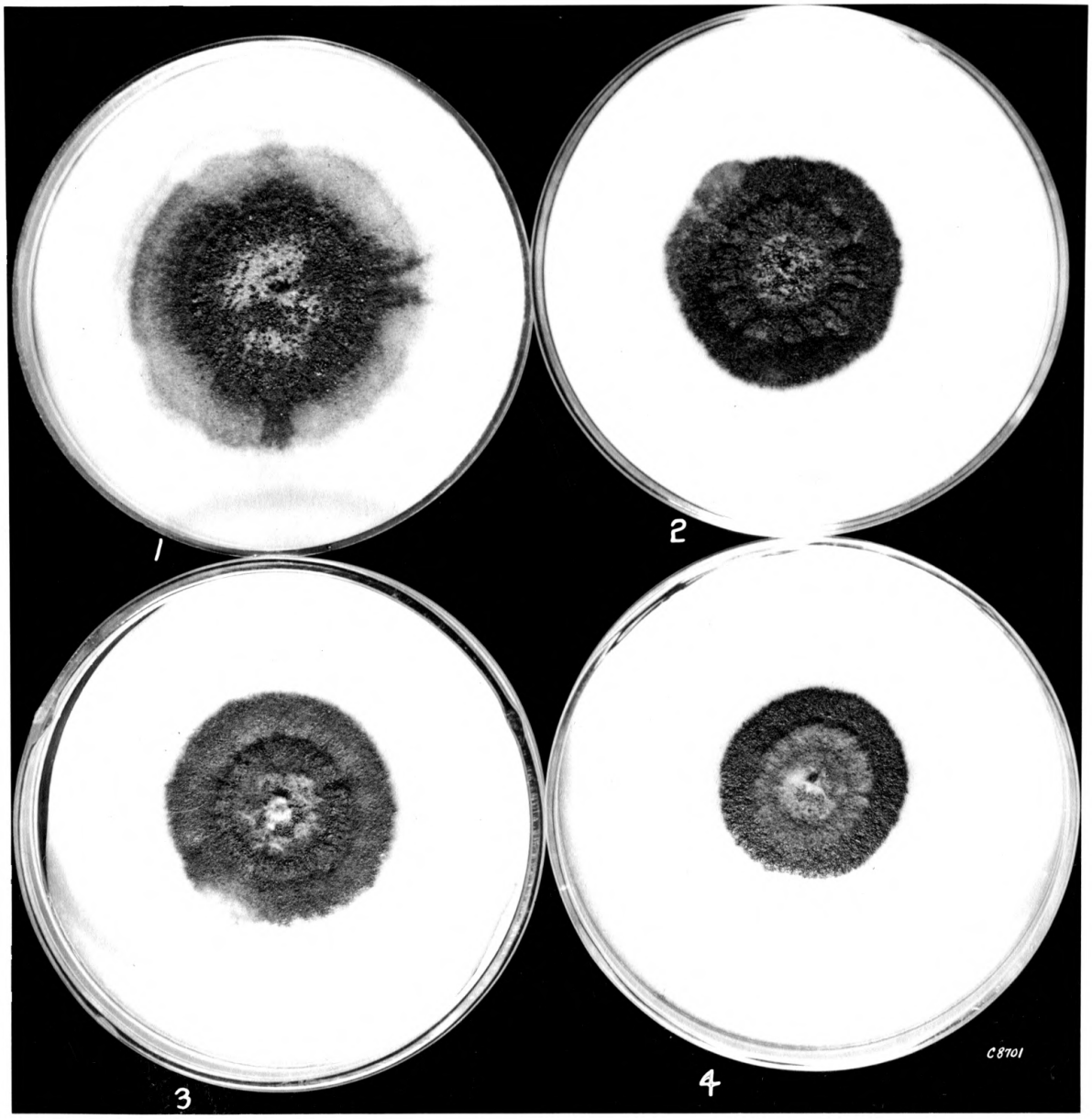
EXPLANATION OF PLATE III

Single spore isolates from four of the eight sources are shown.

Petri dish 1 - Geneva, New York, 1935 (Jones)
Petri dish 2 - Manhattan, Kansas, 1940
Petri dish 3 - Manhattan, Kansas, 1938 (Creager)
Petri dish 4 - Manhattan, Kansas, 1938 (Peterson)

The differences between the colonies are marked and proved to be constant throughout the studies.

Plate III



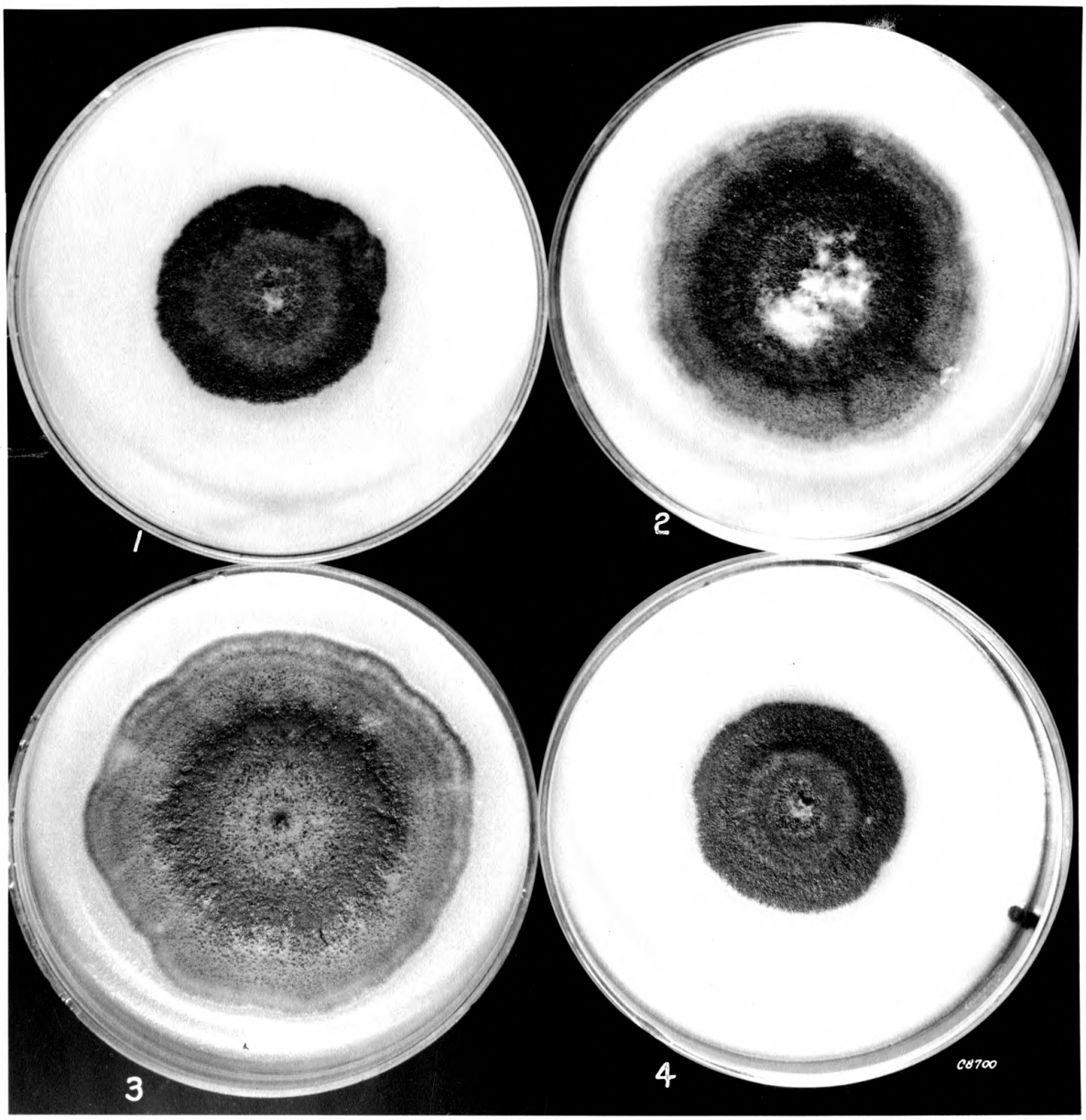
EXPLANATION OF PLATE IV

This plate shows the remaining four of the eight sources tested.

Petri dish 1 - Manhattan, Kansas, 1941
Petri dish 2 - Manhattan, Kansas, 1938 (Creager)
Petri dish 3 - Ogden, Utah, 1938 (Richards)
Petri dish 4 - Monroe, Wisconsin, 1931 (Jones)

Variations between single spore isolates from different scources is very striking in this picture.

Plate IV



On the basis of these data, it was concluded that strains do occur in Ascochyta imperfecta.

Studies Concerning the Relative Pathogenicty of Strains

The occurrence of variations in the pure cultures of Ascochyta imperfecta as exhibited on artificial media pointed to the possibility of an even more important type of difference, namely, one of pathogenicity. Marked variations in the virulence of strains obviously would have a significant modifying influence on the type of breeding program undertaken. An attempt, therefore, was made to discover whether differences in pathogenicity did occur among the several strains utilized in these studies.

A study designed to test the variation of the organism, of necessity, required genetically uniform plants. The plants used in the inheritance studies, therefore, were also employed in these tests. Clonal lines were established from each of the plants in the inbred lines in order to furnish enough plants of constant genetic make-up on which to test all of the strains.

Each strain was used separately to inoculate clonal lines and two methods of comparing the relative virulence of each strain were devised. The methods of inoculation, disease reading, and scoring plants was the same as described in Material and Methods. Time did not permit testing all of the inbred plants or their clones, but an attempt was made to include as

many of each line as possible in each strain test.

The first method of comparing the relative virulence of each strain was to compare the reaction of the clones when tested with the single strain of the organism with the reaction that had occurred on the same clones in the inheritance studies when a mixture of the strains was utilized. Table 4 shows the results of this type of comparison in terms of the F values from analysis of variance as described by Snedecor (7). In the table, the mixture of strains is represented in the column to the left and the single strains are listed across the top. The square beneath each strain represents the comparison of that strain with the mixture, and the more virulent of the two appears in that square with asterisks representing the level of significance of the difference between the two. The number of clones and the means of the two types of inoculum are also shown in the table in the respective squares. Thus, there was only one strain more virulent that the mixture of strains, strain No. 7. Three strains were significantly less virulent than the mixture of strains, strains 1, 3, and 4. Four strains, 2, 5, 6, and 8 were just as virulent as the mixture of strains.

The other method of comparing the virulence of the various strains was to compare the reactions of each single strain with each of the other strains on the common clones on which they were tested. The significance of the differences were tested by the analysis of variance of virulence of the various

strains on clonal lines. The results are summarized in Table 5. In Table 5, as in Table 4, the more virulent of the two strains compared is marked in the square representing the comparison, and the level of significance is designated by the asterisk. Squares in which the word "same" appears indicate that there was no difference between the two strains in terms of virulence.

Thus, strain No. 7 appears to be the most virulent by this method of comparison, also. Strains 1, 3, and 4 rank lowest in virulence, and strains 2, 5, 6, and 8 are the intermediate ones. The differences between the strains was not great, however.

It was concluded, from these studies, that, though differences do occur within Ascochyta imperfecta both on artificial media and in terms of pathogenicity, these variations are only of minor importance. The alfalfa breeder, therefore, probably need not worry about the complicating factor of strains in breeding alfalfas resistant to black-stem.

Correlation of Colony Characteristics on Artificial Media with Differences in Pathogenicity

An attempt was made to correlate the most constant colonial feature, rate of growth, with the virulence of the several strains. Table 6 shows the comparison of the rank held by each strain for these characters.

Table 4. A comparison of mixture of strains and individual strains as tested on clonal lines.

	Strain 1	2	3	4	5	6	7	8
Mixture	Mix. **	Same	Mix. **	Mix. *	Same	Same	#7 **	Same
No. of clones tested	101	101	99	110	89	76	78	106
Means of 2 groups	Mix 122 #1 99	Mix 121 #2 115	Mix 123 #3 104	Mix 119 #4 109	Mix 118 #5 121	Mix 121 #6 115	Mix 119 #7 138	Mix 117 #8 116

Table 5. A direct comparison of the virulence of strains tested on common clonal lines of alfalfa.

	Strain							
	1	2	3	4	5	6	7	8
Strain 1		Same	Same	Same	#5 **	Same	#7 **	#8 *
Strain 2	Same		Same	#2 **	Same	Same	#7 **	Same
Strain 3	Same	Same		Same	#5 **	#6 **	#7 **	#8 *
Strain 4	Same	#2 **	Same		Same	Same	#7 **	Same
Strain 5	#5 **	Same	#5 **	Same		Same	Same	#8 *
Strain 6	Same	Same	#6 **	Same	Same		Same	Same
Strain 7	#7 **	#7 **	#7 **	#7 **	Same	Same		Same
Strain 8	#8 *	Same	#8 *	Same	#8 *	Same	Same	
Totals								
More virulent	0	1	0	0	2	1	4	3
Less virulent	3	1	4	2	1	0	0	0
Same virulence	4	5	3	5	4	6	3	4
Relative rank in virulence	7	5	8	6	3	4	1	2

Table 6. A comparison of the rate of colony growth of each strain with that strain's pathogenicity.

	Strain							
	1	2	3	4	5	6	7	8
Rank for rate of growth	6	1	2	7	3	8	4	5
Rank for pathogenicity	7	5	8	6	3	4	1	2

Obviously there is no correlation between the two features. It would be impossible, therefore, to predict a strain's pathogenicity on the basis of its cultural characteristics on artificial media in the laboratory.

Proof of Pathogenicity

During the course of the plant inoculation experiments, lesions were isolated from infected plants, and the organism responsible for these lesions was developed in pure culture in the laboratory. The organism thus isolated from the plants inoculated with pure cultures of Ascochyta imperfecta proved to be identical in all respects to the pure cultures used in the original inocula. This was considered to furnish ample proof of the pathogenicity of Ascochyta imperfecta.

Life History of Ascochyta imperfecta Peck

Peterson (4) made several important observations concerning the life history of Ascochyta imperfecta in his work. He reported that the organism overwinters on stubble left standing in alfalfa fields. Fruiting bodies are formed on the stubble during the fall and winter, and when moist, humid conditions prevail in the spring, spores are released from these bodies and splashed onto the young growth by falling rain. Under such conditions, primary infections are established which develop and often cause great damage to the first alfalfa crop. He also noticed that alfalfa escapes infection, to a great

degree, during the summer months, but becomes diseased again in the fall. It appears that the moist conditions occurring in the spring and fall seasons are essential for infection to occur.

Observations made during the course of these studies corroborated those made by Peterson. Collections of plant material were made every two weeks from late August, 1940, until May, 1941, and again during the fall of 1941. The collections gathered in the fall of 1940 were all made from alfalfa fields which had been cut regularly during the summer. Thus, at the time of collection, the growth was always of the same age and comparatively young. Pycnidia were not discovered in these fields until mid-November following a severe freeze which killed all of the above ground parts of the alfalfa plants. Soon after this freeze, fruiting bodies covered the standing stubble, and laboratory examinations revealed an abundance of spores to be present in the pycnidia. The organism overwintered in this stage.

These observations seemed to indicate that the organism did not fruit on living plants. In fact pycnidia were never observed on live plants until subsequent examinations on more mature plants were made during the fall of 1941. Collections were made in late September in severely diseased fields from which only one cutting of alfalfa had been removed in late July. The plants were still green, but had been standing for three months. Microscopic examinations of the lesions on the

stems of the plants revealed that some pycnidia had developed and spores were present. Later in the fall, in the latter part of October, collections were made from several other fields on which alfalfa had stood for approximately three months without having been cut. Pycnidia and spores were found in the stem lesions of these plants, also.

Thus, it appears that the formation of pycnidia on infected plants required a period of maturation. The process is probably one involving the starvation of the fungus, for the pycnidia found on living plants occurred in extensive lesions which were more or less isolated from the live plant tissues. Undoubtedly, the killing of the plant by freezing hastens this starvation process, although attempts in the laboratory to induce pycnidial formation by freezing infected stem sections from live plants were not effective.

Careful observations were made during the spring of 1941 in an effort to discover more exactly the environmental conditions under which the disease developed in the field. Several weeks of intermittent rains occurred during the early part of the spring, and during this period daily collections were made. Young alfalfa shoots had just begun to appear when the rainy weather set in, and temperatures for the first week that collections were made averaged 46 degrees Fahrenheit. Only .01 inch of rain fell during this week, from March 26 to April 2, and the relative humidity averaged only 55 percent. No disease was observed during this seven day period.

Temperatures rose to an average of 55 degrees Fahrenheit the following week, April 2 to 8, and light showers fell on four of the seven days. The relative humidity for this period was 83 percent as an average. The first lesions became apparent on April 9. Thus, conditions of moderate temperatures, light rains, and high humidity seem to have furnished ideal conditions for infection.

The organism was identified by isolating lesions from infected leaves and developing plate cultures from them. The lesions were surface sterilized in a .35 percent solution of calcium hypochlorite, and then were planted in sterile potato-dextrose agar medium in Petri dishes. Typical Ascochyta imperfecta colonies developed, and the production of spores, made it possible to be certain of the identity of the organism.

Severe infection occurred in the first alfalfa crop during the spring of 1941. Continuous rains and high humidity throughout the season provided optimum conditions for infection. When the first crop was harvested, it was estimated that at least 50 percent of the leaves had dropped, and many of the stems had become completely girdled. A great deal of the defoliation was, undoubtedly, caused by other diseases, but Ascochyta imperfecta was of major importance in this respect.

Little or no infection was evident during the summer months in regularly harvested fields. The approach of cooler weather and numerous rains during the fall, however, brought

a return of leaf spot diseases. Black stem was of relatively minor importance during the fall, while other diseases, especially leaf rust, Uromyces striatus Schroet., and leaf blotch Pyrenopeziza medicaginis Fekl. assumed the major role in leaf destruction.

Inheritance Studies

Differences in varietal reactions to infection by Ascochyta imperfecta had strongly indicated that resistance or susceptibility of alfalfa to the disease might be a problem involving inheritance. Proof of such inheritance was, naturally, of prime importance if a breeding program for developing resistant types of alfalfa were to be undertaken.

Two factors entered the problem which complicated inheritance studies a great deal. In the first place, alfalfa is an often cross pollinated plant. Thus, a condition of heterozygosity for many characters, including disease resistance, was considered highly probable. Since stocks known to be homozygous for resistance to the black-stem disease were not available, the possibility of utilizing hybridization as a method of investigation, of necessity, had to be discarded. Secondly, alfalfa has a perennial habit of growth, and, as is the case with most perennials, it is slow growing under greenhouse conditions. Therefore, one generation a year was all that could be developed. With such heterogeneous, slowly developing material, a plan had to be evolved which would furnish the most possible information in a short time.

The most applicable method of proving the inheritance of resistance appeared to be a simple problem of inbreeding. Thus, if it were possible to establish resistant lines from resistant plants and susceptible lines from susceptible plants of the same variety, it was considered safe to conclude that resistance was inherited.

Variation, presumably due to the heterozygosity of the parent plants, was foreseen. Large numbers of the progeny, therefore, had to be used in order to give a correct picture of the general level of resistance in an inbred group.

The varietal tests conducted by Peterson clearly demonstrated that, though there were marked variations within varieties, the greatest variations existed between varieties of alfalfa when the varieties were considered as a whole. Parental stocks for inbreeding were selected from plants used in his variety tests. At least one resistant and one susceptible plant was chosen from each variety. The terms "resistant" and "susceptible" plants, as used here, refer to the relative resistance of plants within each variety. Thus, a "resistant" plant from a susceptible variety might have been more susceptible to the disease than a "susceptible" plant from a more resistant variety.

The selected plants were self pollinated in the greenhouse in an insect-free room. Enough seed was gathered from each plant to insure a progeny class of at least 27 plants. The seeds were scarified and planted in flats. When seedlings

had attained about four inches of growth, they were transferred to six-inch pots, where they were left throughout the tests.

Inbred plants were allowed to develop a vigorous growth and were selected for inoculation when they were 10 to 12 inches in height. Plants were chosen at random throughout all of the varieties used with the size of the plant as the only characteristic considered in making selections for inoculation. This randomization offered the advantage of decreasing variation due to different dates of inoculation, for a cross section of all the varieties was usually present in each inoculation. Thus, if the method of utilizing check plants did not correct entirely for variation due to date, this variation was dispersed at random through all of the varieties tested. Large numbers were used in order to give representative results by overcoming these smaller variations.

Reactions of Inbred Lines

As stated heretofore, several plants from the variety tests conducted by Peterson were utilized as breeding material. A detailed description of the results obtained in testing the inbred plants from the selected parents follows:

Kansas Common Variety. Two plants were used as parents from which inbred lines were established in the variety Kansas Common. Kansas Common No. 6 was a resistant plant and Kansas Common No. 18 was susceptible. Twenty-seven inbred plants from each of these parents were tested. Their plant

scores were derived and each plant was given an index of infection number in terms of percent of the check plants' score, as described in Material and Methods. The two progenies were compared by the use of Analysis of Variance and the results are presented in Table 7.

The means of each group clearly indicate the probability that the progeny of the resistant Kansas Common No. 6 plant was significantly more resistant than the progeny of the susceptible plant Kansas Common No. 18. The analysis of variance substantiated this assumption, for it showed a highly significant difference to exist between the two inbred lines. The standard deviations of the two groups showed that considerable variation occurred in each population, illustrating the fact that resistance in each line was a general level which tended to be higher for one line than the other.

Ladak Variety. Four inbred lines of twenty-seven plants each were developed in the variety Ladak. Of the four parent plants, one, Ladak No. 11 was resistant, and the other three plants, Ladak Nos. 20, 5, and 15 were only medium in their resistance. On the basis of the inheritance hypothesis, the inbred lines of these four plants were expected to react as their parents had, and the resistant plant's progeny should have been significantly more resistant than any of the other three lines. Also, no differences were expected to occur between the latter three lines.

Table 8 shows the differences that occurred between the progeny of the resistant plant and the progenies of each of

Table 7. Analysis of variance of disease resistance in the inbred progenies of Kansas common plants. Resistant K.C. 6 vs. Susceptible K.C. 18.

Source	Degrees of freedom	Sum of squares	Mean squares	F value	Level of significance		Means of inbred progenies		Standard Deviations
Between lines	1	28,843	28,843	25.32**	1%	7.17	K.C. No. 6	101.3	37.9
Within lines	52	59,274	1,139		5%	4.03	K.C. No.18	147.3	29.0
Total	53								

Table 8. Analysis of variance of disease resistance in the inbred progenies of four Ladak plants.

Source	Degrees of freedom	Sum of squares	Mean squares	F value	Level of significance		Means of inbred progenies		Standard deviations
		Resistant Ladak No. 11 vs. Medium Resistant Ladak No. 20							
Between lines	1	6,359	6,359	25.32**	1%	7.17	Ladak No. 11	89.92	20.4
Within lines	52	27,075	520		5%	4.03	Ladak No. 20	111.6	24.9
Total	53								
		Resistant Ladak No. 11 vs. Medium Resistant Ladak No. 5							
Between lines	1	10,837	10,837	14.41**	1%	7.17	Ladak No. 11	89.92	20.4
Within lines	52	39,110	752		5%	4.03	Ladak No. 35	118.26	32.0
Total	53								
		Resistant Ladak No. 11 vs. Medium Resistant Ladak No. 15							
Between lines	1	3,392	3,392	7.26**	1%	7.17	Ladak No. 11	89.92	
Within lines	52	24,303	467		5%	4.03	Ladak No. 15	105.8	
Total	53								
		Medium Resistant Ladak No. 20 vs. Medium Resistant Ladak No. 5							
Between lines	1	593	593		1%	6,302	Ladak No. 20	111.6	24.9
Within lines	52	44,476	855	1.44	5%	252	Ladak No. 5	118.26	32.0
Total	53								
		Medium Resistant Ladak No. 20 vs. Medium Resistant Ladak No. 15							
Between lines	1	463	463		1%	6,302	Ladak No. 20	111.6	24.9
Within lines	52	29,669	570	1.23	5%	252	Ladak No. 15	105.8	22.7
Total	53								
		Medium Resistant Ladak No. 5 vs. Medium Resistant Ladak No. 15							
Between lines	1	2,103	2,103	2.62	1%	7.17	Ladak No. 5	118.3	32.0
Within lines	52	41,704	802		5%	4.03	Ladak No. 15	105.8	22.7
Total	53								

66

the susceptible plants. This table also demonstrates the similarity of the three medium resistant plants.

Turkistan Variety. Three Turkistan plants were selected for inbreeding. Two of them, Turkistan Nos. 36 and 16 were relatively resistant, whereas the third one, Turkistan No. 40 was very susceptible. Turkistan, as a variety, was more susceptible than either Kansas Common or Ladak, but, as pointed out before, the terms "resistant" and "susceptible" were used to designate the relative degree of resistance within each variety.

The progenies of the two resistant parents maintained that resistance and there was no significant difference between groups, as is seen in Table 9. Table 9 also shows that the progenies from the resistant parents were both significantly more resistant to the disease than the inbred line developed from the susceptible Turkistan No. 40 plant.

The figures in the Degrees of freedom column of Table 9 indicate that the number of plants tested deviated from the customary 27 per inbred line. Difficulty in obtaining seed by self-fertilization in Turkistan No. 36 made it possible to develop only 21 plants in that line. Thirty-three inbreds were established from Turkistan No. 16, and the usual 27 were developed from Turkistan No. 40.

These data indicate that inbreeding in the variety, Turkistan, results in the production of lines that react, as a group, much the same as their parents did.

Table 9. Analysis of variance of disease resistance in the inbred progenies of three Turkistan plants.

Source	Degrees of freedom	Sums of squares	Mean squares	F value	Level of significance		Means of inbred progenies		Standard deviations
			Resistant Turkistan No. 36 vs. Resistant Turkistan No. 16						
Between lines	1	2,843	2,843	3.95	1%	7.12	Turk. No. 36	128.1	10.6
Within lines	53	38,146	719		5%	4.02	Turk. No. 16	113.3	32.9
Total	54								
			Resistant Turkistan No. 36 vs. Susceptible Turkistan No. 40						
Between lines	1	3,002	3,002	12.25**	1%	7.21	Turk. No. 36	128.1	10.6
Within lines	46	11,277	245		5%	4.05	Turk. No. 40	144.0	18.6
Total	47								
			Resistant Turkistan No. 16 vs. Susceptible Turkistan No. 40						
Between lines	1	14,223	14,223	18.69**	1%	7.08	Turk. No. 16	113.3	10.6
Within lines	59	44,899	761		5%	4.00	Turk. No. 40	144.0	18.6
Total	60								

Hairy Peruvian Variety. Three plants were selected from Peterson's stocks for inbreeding from the very susceptible variety, Hairy Peruvian. Two of these plants, Hairy Peruvian Nos. 33 and 5 were relatively resistant, and the third Hairy Peruvian No. 13 was highly susceptible.

No significant difference was observed between the progenies of the two relatively resistant parents, as is shown in Table 10. This table also indicates that the progenies of the two resistant Hairy Peruvian parents were significantly more resistant than the progeny of the susceptible parent, Hairy Peruvian No. 13.

Thus, it appears that resistance was inherited from the parents in the inbred lines in the variety Hairy Peruvian.

Kansas Common Selection 1-3018. Three plants of Kansas Common Selection 1-3018, being tested at Kansas State College, were utilized for inbreeding. Peterson (4) listed these three plants as resistant, medium resistant, and susceptible. An exception to the expected results appeared in these tests. The plant which Peterson listed as susceptible produced an inbred line with a higher level of resistance than either of the progenies of the supposedly resistant parents. Apparently Peterson's (4) classification was in error or this variety did not furnish inbred lines that reacted similarly to the parental reactions. The probability of the latter assumption being true was somewhat lessened by the fact that the progeny of the plant listed as resistant was significantly more resistant than the progeny of the medium resistant plant, demonstrating that in

Table 10. Analysis of variance of disease resistance in the inbred progenies of three Hairy Peruvian plants.

Source	Degrees of freedom	Sum of squares	Mean squares	F value	Level of significance		Means of inbred progenies		Standard deviations
Resistant Hairy Peruvian No. 33 vs. Resistant Hairy Peruvian No. 5									
Between lines	1	58	58		1%	6,032	H.P. No. 33	148.8	20.1
Within lines	52	38,734	744	12.82	5%	252	H.P. No. 5	142.2	32.6
Total	53								
Resistant Hairy Peruvian No. 33 vs. Susceptible Hairy Peruvian No. 13									
Between lines	1	2,889	2,889	6.94*	1%	7.17	H.P. No. 33	148.8	20.1
Within lines	52	21,672	416		5%	4.03	H.P. No. 13	163.4	20.7
Total	53								
Resistant Hairy Peruvian No. 5 vs. Susceptible Hairy Peruvian No. 13									
Between lines	1	6,059	6,059	8.11**	1%	7.17	H.P. No. 5	142.2	32.6
Within lines	52	38,874	747		5%	4.03	H.P. No. 13	163.4	20.7
Total	53								

one instance, at least, this variety behaved as all the other varieties did. On the other hand, some discrepancies were foreseen from the outset, for Peterson (4) employed no method to correct for variations that arose between different dates of inoculation.

Table 11 shows the difference that existed between the progeny of the resistant plant, Selection No. 1-9, and the progeny of the resistant plant, Selection No. 1-6. Also shown in Table 11 are the comparisons of the progenies of the two plants listed as more resistant with the supposedly more susceptible plant's progeny. In both cases, the progeny of the susceptible parent was more resistant than the progenies of the two plants which were listed as more resistant.

Medicago falcata Species. Two strains of the species Medicago falcata were utilized in the inbreeding studies. Both strains were probably hybrids with common alfalfas to some extent, though the M. falcata features were evident in the parental selections, and a high degree of disease resistance, characteristic of the species, prevailed.

Semi-Palatinsk No. 43. Only one Semi-Palatinsk plant produced enough self-fertilized seed to permit the establishment of an inbred line for testing. This plant was rather resistant, and its resistance was carried on in the inbred population, as indicated by the mean, 107.7, of that group. The standard deviation ± 30.4, demonstrated considerable variation to exist within the line. It appears, nevertheless,

Table 11. Analysis of variance of disease resistance in the inbred progenies of three Kansas Selection 1-3018 plants.

Source	Degrees of freedom	Sum of squares	Mean squares	F value	Level of significance		Means of inbred progenies		Standard Deviations
Resistant Selection No. 1-9 vs. Medium Resistant Selection No. 1-6									
Between lines	1	4,471	4,471	7.12*	1%	7.17	Sel. No. 1-9	113.0	20.1
Within lines	52	34,702	667		5%	4.03	Sel. No. 1-6	131.7	30.4
Total	53								
Resistant Selection No. 1-9 vs. "Susceptible" Selection No. 1-13									
Between lines	1	3,536	3,536	9.23**	1%	7.17	Sel. No. 1-9	113.0	20.1
Within lines	52	19,947	383		5%	4.03	Sel. No. 1-13	96.8	18.8
Total	53								
Medium Resistant Selection No. 1-6 vs. "Susceptible" Selection No. 1-13									
Between lines	1	16,467	16,467	25.56**	1%	7.17	Sel. No. 1-6	131.7	30.4
Within lines	52	33,508	644		5%	4.03	Sel. No. 1-13	96.8	18.8
Total	53								

that the high level of resistance of the parent was transmitted to the inbred line.

Medicago falcata No. 39. A very resistant M. falcata plant was successfully inbred and its progeny proved to carry the same high level of resistance. The mean of the group was 81.61, the highest average resistance of any inbred group tested. The variation within this group was also low, as indicated by a standard deviation of ±20.9.

Thus, in all the varieties and species represented in the inbreeding problem, there was a significant tendency for the inbred population from each parent plant to react to the disease in the same manner as the parent had reacted. One exception has been discussed. This tendency in an open-pollinated, heterozygous plant can be explained only on an inheritance hypothesis, for in such a plant, if inheritance were not involved, it is very probable that the observed correlations of resistance in parent and progenies would not have existed.

The validity of the use of the analysis of variance method for determining the significance of differences between the lines had to be tested, for the method is applicable only to data derived from normal populations. The fact that the material represented in these studies was taken from a population which followed the normal distribution curve was tested by determining the correlation between the means and the standard deviations of all the inbred lines.

Table 12 shows the computation of the correlation coefficient for the means and standard deviations. The X values

represent the means and the Y values represent the standard deviations.

Table 12. Computation of correlation between means and standard deviations of inbred lines.

$SX = 2,044$	$n = 17$	$SY = 423$
$SX^2 = 254,470$	$SXY = 50,758$	$SY^2 = 11,331$
$\frac{SX^2}{n} = 245,760$	$\frac{(SX)(SY)}{n} = 50,859$	$\frac{SY^2}{n} = 10,525$
$Sx^2 = 8,710$	$Sxy = -101$	$Sy^2 = 806$

$$r = \frac{-101}{\sqrt{(8710)(806)}} = -.004$$

Level of Significance - 1% = .590, 5% = .468

The correlation coefficient was not largeenough to be significant at the 5 percent level. Thus, there was no correlation between the means and the standard deviations, and it was concluded that normal populations were dealt with in these studies. The use of the analysis of variance method, therefore, was permissible.

Hybrid Studies

Several hybrids were produced in an effort to determine the type of inheritance involved in the resistance of alfalfa to Ascochyta imperfecta. Obviously, no definite conclusions could be drawn from hybridization studies with such heterozygous parents, but it was considered that some valuable clues might be obtained that would be useful in later studies.

The crosses were made in the greenhouse. A technique of emasculation developed by Tysdal and Garl (12) was employed. By this method the flower to be pollinated was tripped, and emasculation was secured by dipping the open flower in 57 percent ethyl alcohol for exactly 10 seconds. The alcohol was then removed immediately by dipping the flower in water. The flower was allowed to dry and pollen was applied from the male parent. According to Tysdal and Garl (12), this method is practically perfect for emasculating, and is far superior to the suction method generally used for this purpose.

Only one cross was made which yielded a large enough hybrid population to warrant discussion. The parents in the cross were the highly susceptible plant Hairy Peruvian No. 33, as the male parent, and the very resistant Medicago falcata No. 39 plant as the female parent. Thirty-three first generation plants were developed from this cross and tested. The mean of the hybrid group was 93.85, denoting a very high degree of resistance. The standard deviation of ±16.8 illustrated the remarkably low variability that occurred in the group. It appears that the resistance of the M. falcata parent completely dominated the cross.

A reciprocal cross was made in order to check on the thoroughness of the emasculation in the first cross. Unfortunately, only four first generation plants were developed and tested in the reciprocal cross. All four plants were resistant, however, and their mean was 101.7. Thus, insofar

as could be judged from these data, resistance also dominate in the reciprocal cross, and the emasculation technique utilized in the original cross was effective.

An interesting comparison was noted between the hybrid population and the inbred groups from each of the parents. The mean of the resistance of all the plants in the Hairy Peruvian No. 1 inbred line was 148.8, whereas the mean of the M. falcata No. 1 line was 81.61. The mean of the hybrid population from these parents was nearly as low as that of the resistant M. falcata No. 39 inbred line, for it was 93.85. Also, the uniformity characteristic of a first generation hybrid population from a cross involving complete dominance was observed, for the standard deviation of the group was very low, ±16.8, as compared with the deviations of the inbred groups from the same parents (Hairy Peruvian No. 33, s = ±20.1; M. falcata No. 39, s = ± 20.9).

Miscellaneous Experiments

Inbreeding and testing of inbreds in the greenhouse showed the probability that resistance of alfalfas to black-stem is inherited. It appeared desirable to obtain data in this regard from actual field conditions to supplement the information secured in the greenhouse. Thus a series of approximately 6000 second generation inbred plants from 56 first generation plants were transferred to the Alfalfa Wilt Nursery at Kansas State College during the spring of 1940. Old stems and pieces of stubble from diseased alfalfa fields were used as sources of

inoculum, and an overhead sprinkling system was employed to furnish conditions of high humidity and moisture essential for infection. The stems and stubble were spread among the plants, and water was added every night for a short period in early June until an infection was well started.

Individual plant notes on the number and size of leaf lesions were taken. Unfortunately, other leaf diseases appeared along with black-stem, and, as a result, the readings were considered to be very inaccurate. Preliminary analyses of the data secured in this study revealed that no differences existed between the inbred lines tested, contrary to results expected on the basis of the greenhouse tests. Little significance was attached to these field tests, therefore, for conditions were not well controlled and complete plant readings were not secured. It appears doubtful that field tests can be successfully applied to careful genetic studies, but the importance of such tests in variety studies cannot be overemphasized, for gross differences are easily discernible in large populations. Thus, a selection program should include some type of field testing of plants on the basis of their resistance to black-stem and other leaf spots.

An experiment of less practical interest was undertaken which involved the inoculation of excised leaves in sterile Petri dishes. A suspension of spores was sprayed on the leaves on moistened filter papers in the sterile dishes. Observations on the progress of the disease were made daily. In general,

there was no correlation of the severity of infection that occurred on excised leaves with that on the plant from which they were removed. This method, however, did provide an ideal medium for studying the development of the pathogen on the host, and several observations were made in this regard.

DISCUSSION

There seemed to be some relation between the type of leaf surface and the resistance that a leaf offered to the organism. Thus, a leaf covered with epidermal hairs, as in Hairy Peruvian, became more severely infected than one with a slick, glossy epidermal coating, such as is true of Ladak leaves. The hairy leaves retained large droplets of spore laden water that struck them, whereas the hairless, slick leaves shed most of the water that fell on them. Thus, the resistance exhibited by certain plants might well be due to a "disease escaping" mechanism rather than true resistance to the disease. This type of resistance has been mentioned by Wingard (13) in a general review of the literature on disease resistance in plants.

An experiment designed to illustrate the fact that resistance was protoplasmic was attempted in which cell sap was extracted from resistant and susceptible plants. The respective saps were sterilized and inoculated with Ascochyta spores, and the resultant mycelial pad weights were compared. The assumption was made that the resistant sap would inhibit the

growth of the organism, but there were no significant differences between the various mycelial growths. This experiment was by no means considered conclusive evidence that true protoplasmic resistance does not occur. The fact remains that resistance, whatever its nature may be, is an inherited characteristic.

Selection seems to offer some possibilities for improving the level of resistance to black-stem in some of the varieties of alfalfa, as is indicated by the variation within varieties. In the more susceptible varieties, however, very little resistance can be gained by a selection program. Hybridization, on the other hand, promises to be a very effective method for increasing the resistance of the susceptible varieties.

Further studies in hybridization should yield valuable information concerning the type of inheritance involved. When more is known about other leaf spot diseases, an intelligent breeding and selection program can be initiated for the development of resistant strains of alfalfa. General field observations have pointed to the fact that to many of these diseases, at least, one variety or strain of alfalfa may show similar resistance. More specifically, a comparison of the levels of resistance of the several varieties and species of alfalfa to Ascochyta imperfecta and rust, Uromyces striatus, as listed by Koepper (3), showed a very similar ranking. This comparison is seen in Table 13.

Table 13. Comparison of the relative resistance of alfalfas to rust and black-stem.

Species or variety	Resistance to rust (Koepper)(3)	Resistance to black-stem (Peterson)(4)
Medicago ruthenica	1	1
Semipalatinsk	2	2
Ladak	3	3
Grimm	8	4
Medicago falcata	4	5
Turkistan	7	6
Kansas Common selection	6	7
Kansas Common	5	8
Hairy Peruvian	9	9

If a correlation such as this could be presented for the resistance to all leaf diseases, the problem could be happily solved simply by the introduction of resistance from one variety or strain in the breeding program.

SUMMARY

1. Resistance in alfalfa to the black-stem disease caused by Ascochyta imperfecta Peck was shown to be an inherited characteristic.

2. Inbred progeny from 17 selections among seven varieties of alfalfa have shown significant inter and intravarietal differences.

3. Different strains of Ascochyta imperfecta were demonstrated on artificial media.

4. The strains varied somewhat in pathogenicity on al-

falfa, but the differences were not considered significant.

5. There was no correlation between the pathogenicity of strains and their cultural characteristics on artificial media.

6. In the field the organism overwinters in the pycnidial stage on alfalfa stubble. Moist, humid conditions are necessary in the spring for primary infection. Secondary cycles follow, but are considerably retarded during the dry periods of the summer.

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