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Reaction of Certain Diploid and Tetraploid Alfalfas to Some Phytopathogens Including the Blackstem Disease

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REACTION OF CERTAIN DIPLOID AND TETRAPLOID ALFALFAS TO SOME PHYTOPATHOGENS INDUCING THE BLACKSTEM DISEASE

By

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REACTION OF CERTAIN DIPLOID AND TETRAPLOID ALFALFAS
TO SOME PHYTOPATHOGENS INDUCING THE BLACKSTEM DISEASE

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

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INTRODUCTION

Alfalfa in South Dakota as in other parts of the United States and the world is adversely affected by a disease known as Blackstem. This disease is induced by one or more fungi or bacteria whose abundance depends as much on the high susceptibility of the current alfalfa varieties as on the weather. Differences in plant susceptibility to one of these microorganisms, Ascochyta imperfecta, has already been shown to occur within and between alfalfa varieties or strains. Reitz, et al.(18) working in Kansas obtained F_1's between highly resistant and highly susceptible plants that were intermediate in reaction to this fungus, and in a few crosses they noted a tendency of the resistant reactions of plants to be dominant over the susceptible. By inbreeding and hybridizing selected plants, they obtained a plant population with a higher resistance to this pathogen than the current varieties. Similar possibilities undoubtedly could exist for the other microorganisms inducing Blackstem, but no attempt has been made to date to determine this. Even for Ascochyta imperfecta, however, the preliminary work of Reitz, et al.(18) has never been extended or applied to the development of a commercial variety resistant to the disease.

The purpose of the present study was to find out to what extent alfalfa plants differed in their reaction to the Blackstem disease induced by the various pathogens and to determine to what extent the reactions to these pathogens were heritable. If such plant differences could be shown to be heritable, an opportunity would thus be afforded to develop an alfalfa variety that resists the disease to a greater extent than any

of those currently used. Field and greenhouse screening tests accordingly, were conducted to measure plant reactions to three of the pathogens and the results obtained are presented herein.

DESCRIPTION OF THE BLACKSTEM COMPLEX

The alfalfa Blackstem disease, or Blackstem complex, of the North Central United States is induced by one or more of four organisms, of which three are fungal, and one bacterial. Ascochyta imperfecta Peck and Cercospora zebrina Pass. appear to be of major importance, while Colletotrichum trifolii Bain and Essary, and Pseudomonas medicaginis Sack. appear to be minor. The disease is complex for the reason that the various organisms produce similar symptoms at the same or different times.

The disease complex may be found at three different periods of the growing season. During the early spring and late fall of cool, humid weather, Ascochyta imperfecta and Pseudomonas medicaginis thrive most abundantly, while during the summer and early fall of hot weather, Cercospora zebrina and Colletotrichum trifolii thrive best. The common names of the diseases produced by these pathogens are Spring Blackstem, Stem Blight, Summer Blackstem, and Southern Anthracnose, respectively.

Ascochyta imperfecta has been known to cause Spring Blackstem of alfalfa since 1908, when it was first described. Peterson and Melchers(17), showed from field studies that its infectivity depended upon high humidity and low temperature. Infection by it usually is heaviest during early spring and late autumn. It attacks any part of the plant, including the roots, producing the most severe damage when on young shoots. It may also cause considerable losses in seed and forage production, due to girdling of the peduncles and petioles. Cormack(3, 4) and Reitz et al.(18) reported the fungus to cause severe defoliation, and Kernkamp and Hemerick(12) described it as being the most destructive foliage pathogen of alfalfa in Minnesota. The latter

workers reported in 1952(11) that the seed crop loss in Minnesota from the disease ranged from two to twenty-eight percent. Aamodt(1) reported the fungus to be one of two most limiting factors of alfalfa culture in Argentina. Smith(23) and Henderson(7) included Ascochyta in their list of major alfalfa pathogens for Nevada and Virginia, respectively. The pathogen also was reported for Ontario by Baylis(2), for Manitoba by McDonald(13), and for Saskatchewan by Mead(14), but it had not become a serious problem in alfalfa culture in those areas, except where seed was being produced.

The symptoms produced by Ascochyta imperfecta on alfalfa have been described by many authors (3, 5, 11, 12, 16, 17, 18). In the early stages of infection, small black lesions appear on the leaves and stems. As the lesions enlarge on the leaves, they form irregular dark brown spots that continue enlarging until the infected leaves turn yellow and drop off. Stem lesions usually enlarge and coalesce until the entire stem becomes surrounded by a black sheath of fungus mycelium in discolored host tissue.

The symptoms produced by the other Blackstem pathogens are very similar to those produced by Ascochyta imperfecta. The symptoms of Stem Blight, induced by Pseudomonas medicaginis Sack., are diffuse olive-green areas on the leaves and stem that may show a bacterial exudate. The symptoms produced by Phoma medicaginis Malbr. and Roun. as reported by Johnson and Valleau(9) are the same as those for Ascochyta imperfecta. This is also true for the symptoms produced by Pleospora rehmina, Staritz, as described by Remsberg and Hungerford(19).

One sure way to identify the diseases produced by the different fungi is to recognize the fungus fruiting bodies produced in or on the

diseased tissues while on the plant or detached and placed in a moist chamber or on an agar medium. Pleospora rehmina forms perithecia, while Phoma medicaginis and Ascochyta imperfecta form pycnidia. Phoma medicaginis and Ascochyta imperfecta can only be distinguished from one another by spore morphology. Phoma medicaginis has one-celled, hyaline, spherical or oval, conidia while Ascochyta imperfecta has two-celled, hyaline, conidia. This distinction has been challenged by Schenck and Gerdemann(22) who claim that the fungi are similar and should be known as Phoma herbarum var. medicaginis West.

Cercospora zebrina, the causal agent of Summer Blackstem, attacks alfalfa plants regardless of age, causing lesions on stems, petioles, leaves, inflorescences, and/or seed. Horsfall(8) described the lesions on leaves as being small, round, and sunken, and somewhat less than five millimeters in diameter. He also described stem lesions as being elongated and occurring usually on one side of the stem. The lesions on the leaves were described as ranging from ashy-gray to tawny, and those on the stems, petioles, and inflorescences were described as ranging from reddish-brown to dark brown, depending on the tissues involved. The stem lesions usually were found to be sunken, as the result of excessive necrosis.

In moist weather, the center of the lesions develops a whitish cast which is due to the conidia. Dickson(5) described the conidia as cylindrical fusoid, hyaline to light yellow, three to six septate, and averaging three by fifty microns in size.

Colletotrichum trifolii, or "Southern Anthracnose", was first recognized in 1905, when Monteith(15) described its presence on the leaves

of alfalfa in Tennessee. Jones(10) substantiated the importance of this pathogen when he reported that alfalfa seedlings in the greenhouse at Wisconsin were killed by it. As with Ascochyta imperfecta, it may occur on any green part of the plant, including the upper portion of the tap root. It may attack the plant at any time from the seedling to the mature stage(15).

The chief type of damage the pathogen produces is stem girdling. With severe infections, stem breaking may occur at the points where the lesions form. Leaf lesions, as described by Monteith(15) are dark brown to black, of irregular shape and size, varying from minute spots to large areas of coalesced spots. The lesions on leaves are more common near the margin than away from it, and are angular in shape, according to the venation of the leaf. The short stalk of the leaflet is the point of most frequent attack by the pathogen. The health of petioles and peduncles are most important to the well-being of the plant than are leaf lesions.

As described by Dickson(5), C. trifolii produces conidia in acervuli, with numerous dark brown setae. The acervuli develop as scattered erumpent bodies of varying diameter, in mature sunken lesions. The conidia are hyaline, to pink, straight, rounded at the ends, and without septations. The setae are numerous, uniseptate, and much longer than the conidia.

Pseudomonas medicaginis produces Stem Blight that is usually common in areas where frost cracks appear in the host stems. It was first reported in Colorado in 1910 by Sackett(21) and since then the disease has been noted in many widely separated areas. Gardner(6) re-

ported the disease in Indiana in 1926, and Smith(23) reported it in Nevada in 1950. In Utah in 1933, Richards(20) reported decreased alfalfa yields of forty to fifty percent from the disease.

The bacterium, as described by Sackett(21), produces light yellow colonies in culture. The cells are short, thick, rods with rounded ends, are actively motile, and have one to four polar flagella. They occur either singly or in pairs.

The disease is primarily a stem disease, but lesions may occur on the foliage of young plants and they may extend into the crown and roots of older ones. Infected plants tend to grow more spindly than healthy plants. The stems lose their succulent appearance, and take on a watery, semi-transparent, yellowish to olive-green appearance along one side. The lesion gradually girdles the stem and may extend for a length of one to three internodes. The stems later take on a dark, olive-green, watery appearance and an exudate begins to form on the lesions and collect in bead-like droplets. As the exudate dries, the stem assumes a glistening finish, as though varnished. Infected stem parts become very brittle and break easily.

Leaves are first attacked near the petiole, and exhibit a watery appearance. They become dwarfed, narrow, light green, and show a tendency to remain partly closed. A single leaf lesion is an irregular, small spot. If the petioles become infected they become watery and pale yellow, and they will droop. Later the lesions will become coated with an exudate and turn black.

MATERIAL AND METHODS

Decription and Source of Plant Material

Since the purpose of the present study was to determine the range and heritability of reaction to Blackstem, plant materials from widely separated sources were used. Diploid source materials (2N=16) having relatively simpler genetic and cytological behavior were used primarily and in conjunction with one tetraploid stock.

The plant materials were derived from ten geographic sources. Nine sources provided diploid plants, and of these, six sources provided plants of the species Medicago falcata, and three of Medicago sativa. The tenth source provided tetraploid plants of the cross M. falcata by M. sativa. The identifying name or other designation, the accession number[1], the degree of ploidy, the species, the source and the origin of these materials are given in Table 1.

Plants of each of the ten sources were selected initially at Brookings on the basis of crown diameter and observed field reaction to certain leaf and stem diseases. These characters are shown in Table 2. The initial material consisted of forty plants which were chosen to cover the range for these characteristics. The plants, whose origins are shown in Table 1, were as follows: one selection each of Iran, Turkey, Caucasus, Alaskan, and Don, three selections each of Don-S and S33-1, seven of MF, eight of S2128, and fourteen of C1Ck.

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[1]Alaskan and Don were originally introduced from this station, therefore the designation S. D. 42 and S. D. 46 refer to the original type material and is not used at this time.

Table 1. Identification of Plant Materials

IDENTITY	ACCESSION NUMBER	DEGREE OF PLOIDY	SPECIES	SOURCE OF MATERIAL	ORIGIN
Alaskan	S.P.I. 24452 (S.D. 42)	2N= 16	Medicago falcata	Alaska Agr. Exp. Sta.	Collected by N. E. Hansen near Obb, Tomsk Province, Siberia
Caucasus	Ultuna #109	2N= 16	Medicago falcata	Univ. of Alberta	Collected by Dr. Vasiljtzenka from Northwest Caucasus region of the U.S.S.R.
Don	S.P.I. 20725 (S.D. 46)	2N= 16	Medicago falcata	Univ. of British Columbia	Collected by N. E. Hansen in Don Prov. of Lower Volga region of Southeast Russia
Don-S	-------	2N= 16	Medicago falcata	S. Dak. Agr. Exp. Sta.	Seed obtained from herbarium specimen of Don
HF	-------	2N= 16	Medicago falcata	S. Dak. Agr. Exp. Sta.	Seed collected by D. D. Harpstead near Hohenfels, Bavaria, in Southern Germany
S33-1	-------	2N= 16	Medicago falcata	Univ. of Saskatchewan	Origin unknown
Iran	Ultuna #206	2N= 16	Medicago sativa	Univ. of Alberta	Collected near Kashan, Iran
S2128	Saskatoon #2128	2N= 16	Medicago sativa	Univ. of Saskatchewan	Collected at Botanical Gardens, Acad of Sci., Armenian S.S.R. at Erevan, Kanaku, U.S.S.R.
Turkey	Iowa #1976 Edmonton #252	2N= 16	Medicago sativa	Univ. of Alberta	Collected originally in Turkey
ClCk	-------	4N= 32	From crosses involving M. sativa and M. falcata	Dominion Exp. Sta. Swift Current, Saskatchewan	Selections of crosses involving Siberian and Ladak

Table 2. Characteristics for which Initial Plants were Selected

Plant Selection	Crown Diameter in Inches	Cercospora Blackstem (1-10 score)***	Cercospora Leafspot (1-10 score)***	Pseudopeziza Leafspot (1-5 score)***
Tetraploid selections				
C1Ck 1	34	2.3	4.2	4.4
C1Ck 2	34	2.3	3.4	2.8
C1Ck 4	26	3.1	5.0	3.3
C1Ck 6	30	2.7	3.4	1.2
C1Ck 11	30	3.0	3.5	1.0
C1Ck 13	35	1.8	3.8	5.0
C1Ck 14	32	2.4	3.4	4.7
C1Ck 16	32	2.0	2.6	4.0
C1Ck 18	31	2.4	3.3	1.0
C1Ck 19	34	2.1	3.6	1.0
C1Ck 20	41	1.1	4.3	1.0
C1Ck 21	42	1.2	3.3	3.6
C1Ck 22	44	4.0	4.1	3.8
C1Ck 23**	--	5.0	4.2	1.4
Diploid selections				
Iran*	--	--	--	
Turkey*	--	--	--	
Caucasus*	--	--	--	
Alaska*	--	--	--	
Don	20	1.0	1.0	
Don S-1	9	4.0	5.0	
Don S-3	8	8.0	1.0	
Don S-4	9	9.0	2.0	
S2128-1	9	8.0	5.0	
S2128-2	7	8.0	5.0	
S2128-4	7	9.0	2.0	
S2128-6	7	10.0	1.0	
S2128-8	6	9.0	5.0	
S2128-9	8	9.0	3.0	
S2128-10	8	9.0	5.0	
S2128-11	10	10.0	5.0	
S33-1-7	10	8.0	2.0	
S33-1-12	13	7.0	3.0	
S33-1-14	20	1.0	1.0	
HF-1	14	1.0	5.0	
HF-2	13	1.0	6.0	
HF-6	13	1.0	1.0	
HF-7	16	3.0	1.0	
HF-9	16	2.0	2.0	
HF-11	15	1.0	3.0	
HF-12	10	4.0	3.0	

*No previous information available on these lines

**This plant is not a spreading type

***Score of 1 indicates least infection, Score of 5 or 10 indicates greatest infection

Blackstem disease reaction of these plants, their self or their open-pollinated progeny were evaluated in the greenhouse and field by handling them in the following ways. Vegetative cuttings of these plants were established during the fall of 1954 in the greenhouse in ten replications of three cuttings each. At the same time the parent plants were cross- and self-pollinated to establish F_1 and S_1 progenies. A high degree of self-sterility was encountered in certain lines and this resulted in S_1 progenies of various sizes. The progenies and four replications of the parent plants were established in the field during the spring of 1955.

Open-pollinated seeds of HF, S2128, S33-1, and Alaskan alfalfa were collected from plants in a field nursery in the fall of 1955 and plants from these were established in the greenhouse during the ensuing winter.

Vegetative cuttings of S_1 progenies in the field were also established in the greenhouse.

All plants in the greenhouse were grown in soil, either on a large greenhouse bench or in wooden flats. The plants were allowed to make several inches of growth before inoculation, to insure a leaf area suitable for disease evaluations, since leaf lesions are the dominant type of infection under greenhouse conditions.

Determination and Isolation of Blackstem Microorganisms

Two methods were used to isolate and determine the microorganisms associated with the Blackstem disease under field conditions. In the first, the plant materials were placed on moist filter paper in petri

plates and after several days the mature spores were transferred directly to sterile media. From the colonies that developed on this medium, pure cultures of fungi were established.

In the second method, the plant parts were surface sterilized in 95% ethyl alcohol, rinsed in sterile water, and placed on a carrot-agar medium. Pure cultures of fungi were established from the mycelial growths that developed from the leaf or stem lesions.

Increase of Pathogens for Inoculum Purposes

Isolates of the various fungi were maintained in culture tubes until they were needed for inoculation purposes. At such times a small portion of their mycelial mats and/or spores from a culture tube were transferred to a tube containing a small amount of sterile water, whereupon it was thoroughly mashed or agitated and several loops of the suspension transferred to plates containing carrot-agar medium.[2] The material was then allowed to grow for several days and at that time the mycelium on the plates was again streaked to insure coverage of the medium surface.

Inoculation Procedure in the Greenhouse

The inoculum so prepared was macerated and diluted in a Waring blender, and sprayed on the plants. In the first spraying attempt, a vibrator paint sprayer was used for applying the inoculum, but in later sprayings this was replaced with a DeVilbiss sprayer connected to an

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[2]Carrot-agar medium was prepared by mixing the water extract of 40 grams of autoclaved carrot with a two percent agar suspension.

electrically driven pressure pump. The latter method of spraying proved more satisfactory than the first for the reason that a large amount of inoculum could be placed in a one liter flask making it unnecessary to stop to replenish the supply.

Each bench of plant material was sprayed twice with inoculum to provide satisfactory coverage. To insure maximum coverage of plant area a wetting agent was added to the suspension. A one percent solution of dreft was used in first inoculations but since burning was noticeable, this detergent was replaced by Tween-20 in later sprayings.

Incubation Procedure

Immediately following the application of inoculum the bench or a series of wooden flats containing the plants was covered with plastic sheeting supported by a framework of wooden laths. The space between the plastic sheet and plants was made large enough to allow for a free circulation of air and water vapor.

The relative humidity of the space over the plants was maintained between ninety and one hundred percent by the use of two humidifiers,[3] one at each end of the bench. Occasionally the machines were transferred to different points along the bench to equalize the humidity and to prevent an accumulation of moisture in any one place.

To further insure a relatively constant high humidity the humidifiers were run intermittently through an electric time clock. The clock

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[3]The humidifiers were manufactured by the Standard Engineering Works, Pawtucket, Rhode Island. These were designated as Standard Humidifiers, Model 42L.

was adjusted so that the machines would be ON for one hour and OFF for three, making a total of six cycles every twenty-four hours.

Temperatures during the incubation period were controlled for the most part between twenty-one and twenty-seven degrees Centigrade. This covered the range for sporulation of the fungi since the optimum is twenty-two degrees Centigrade for Ascochyta imperfecta and twenty-five for Cercospora zebrina. Colletotrichum sp. incubation was kept near the upper limit of the temperature range.

During late mornings and early afternoons greenhouse temperatures sometimes rose above the desirable limits.

The plastic hood covering the plants was kept over the plants for periods of from three to five days, the usual period being from three and a half to four days. Undesirable stem elongation and etiolation resulted when longer periods were used. The excessive growth reduced the accuracy of taking disease readings because the infected leaves would drop when adjacent plants were being untangled.

Scoring of Infection

A numbering system to indicate the amount of leaf area infected was used to score the individual plants for disease. At first, the plants were scored for lesion size and lesion abundance. Later they were scored subjectively only for the amount of leaf area infected.

The first type of scoring used is illustrated in Plate I for Ascochyta imperfecta. The lesions were divided into three size classes, small, medium, and large and into three degrees of abundance, few, several and many. This resulted in nine classes of infection plus a score of no

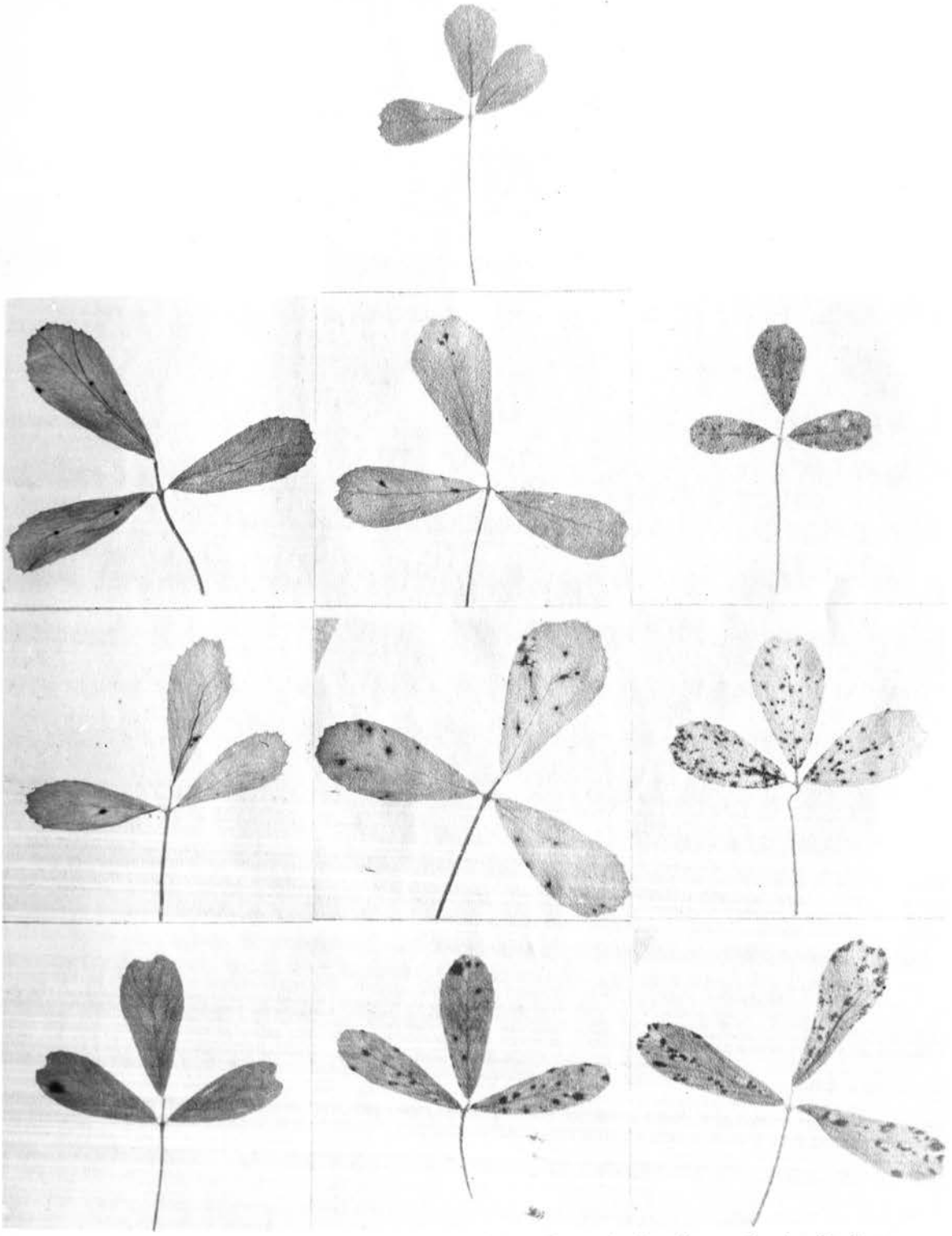

PLATE I. Alfalfa Leaf Lesions Induced by Ascochyta imperfecta Under Greenhouse Conditions. Top Center-No Infection (0), Lesion Quantity Score From Left to Right (1,2,3), Lesion Size From Top to Bottom (1,2,3).

infection giving a total of ten classes.

This method of scoring was discontinued for the reason that (1) different leaves on any one plant would show different sizes and numbers of lesions, (2) zero classes complicated analysis, and (3) lesion size was different between field and greenhouse tests, as may be seen in comparing lesion sizes in Plates I and II.

The second method of scoring on the basis of leaf area infected is illustrated in Plate III for Ascochyta imperfecta. A score of one was given to plants that were completely free of infection. A score of two was given to plants with only a few lesions per leaf, while three was given to plants with a considerable number of lesions. Class four was applied for heavy infection, while five was applied only to plants that were badly infected on all leaves, or where there had been an extremely large amount of leaf drop. Scores of 1.5, 2.5, 3.5, and 4.5 were applied to borderline cases where the plant could be classed in either of two whole number classes. These same types of classes were used for scoring Pseudomonas medicaginis leaf infection, as shown in Plate IV, and for Colletotrichum trifolii leaf infection as shown in Plate V.

Stem infections were scored similarly to leaf infections with a scale divided into five parts. A score of one indicated no infection, two light infection, three moderate infection, four heavy, and five extremely heavy. Scores of 1.5, 2.5, 3.5, and 4.5 were also used for intermediate cases. The five main classes of stem infection are illustrated for Ascochyta imperfecta in Plate VI and for Pseudomonas medicaginis in Plate VII. Unclassified stem infections caused by Cercospora zebrina and Colletotrichum trifolii are illustrated in Plates VIII and IX, respectively.

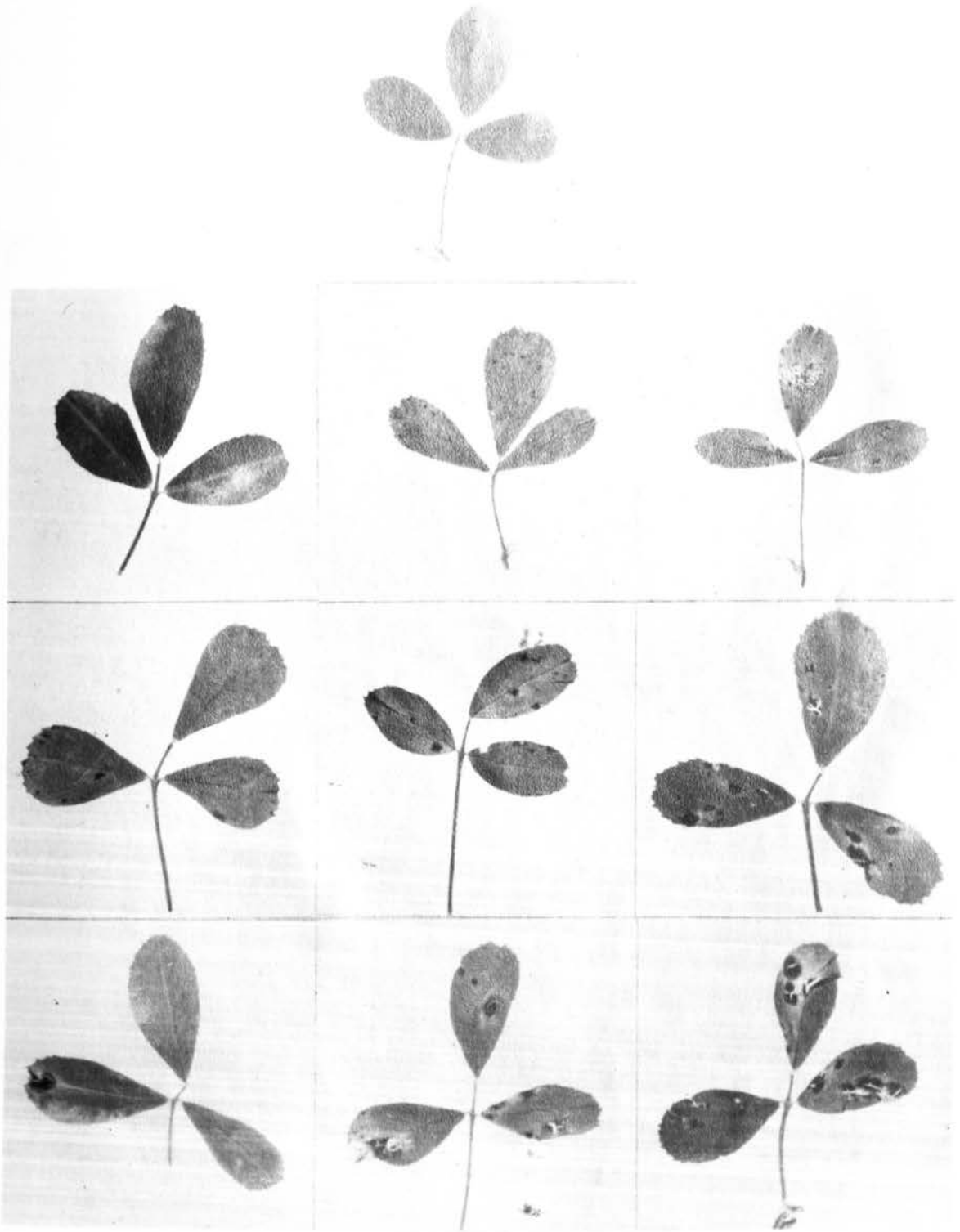

PLATE II. Alfalfa Leaf Lesions Induced by Ascochyta imperfecta Under Field Conditions. Top Center-No Infection (0), Lesion Quantity Score From Left to Right (1,2,3), Lesion Size From Top to Bottom (1,2,3).

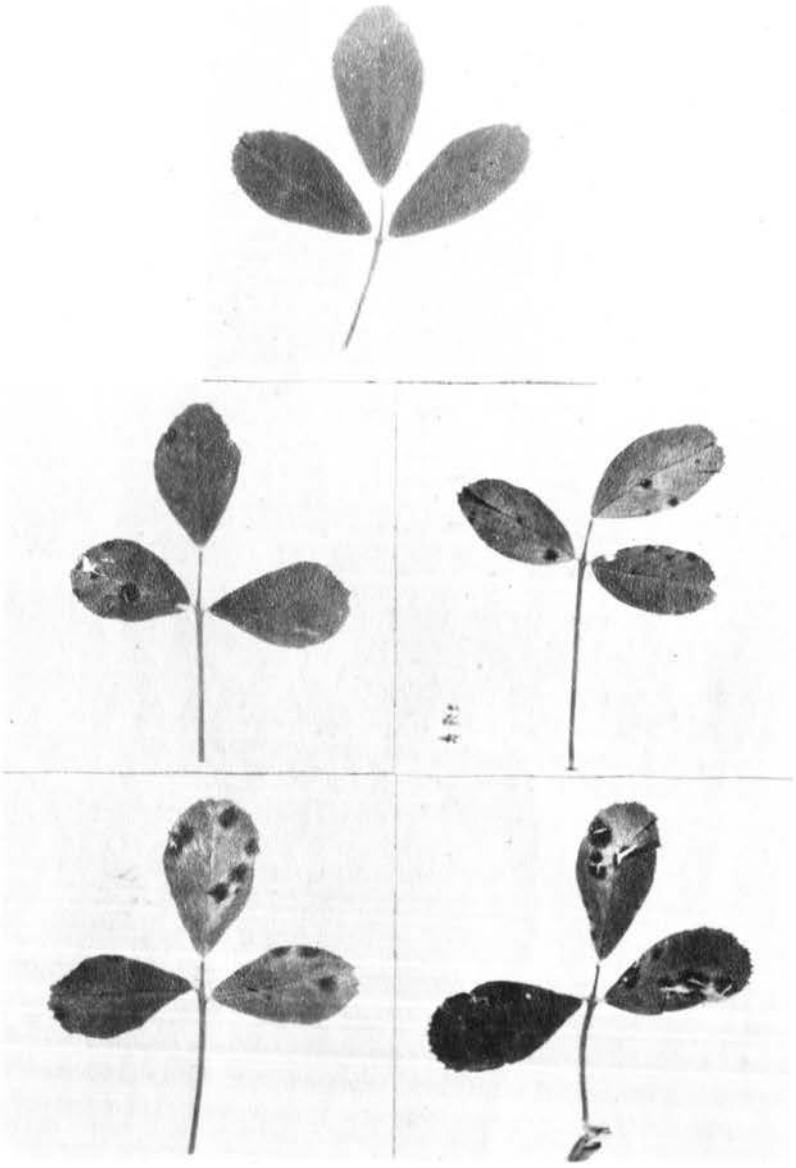

PLATE III. Scale (1 to 5) Used to Read Leaf Infection of Alfalfa Induced by Ascochyta imperfecta. Top Center-No Infection (1), Upper Left (2), Upper Right (3), Lower Left (4), and Lower Right (5).

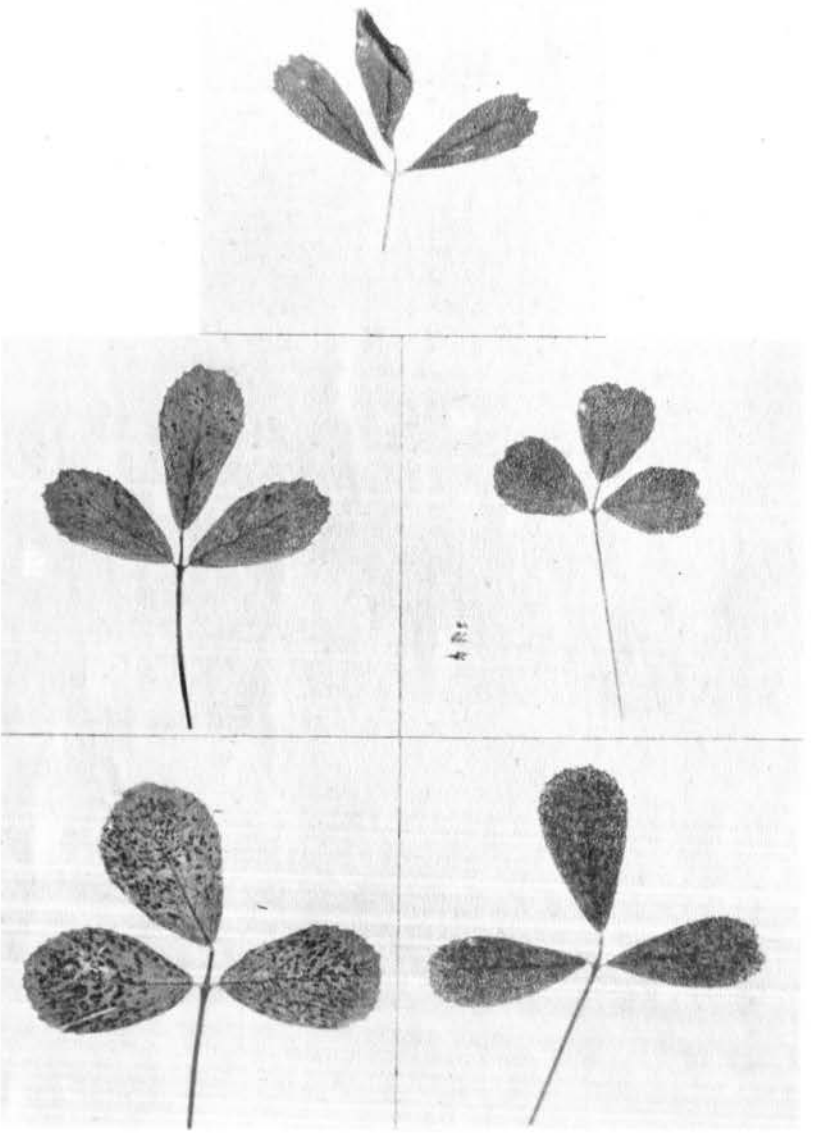

PLATE IV. Scale (1 to 5) Used to Read Bacterial Leafspot or Stem Blight of Alfalfa Induced by Pseudomonas medicaginis Sack. Top Center-No Infection (1), Upper Left (2), Upper Right (3), Lower Left (4), and Lower Right (5).

PLATE V. Typical Leaf Lesions of Southern Anthracnose on Alfalfa Caused by Colletotrichum trifolii Bain and Essary. Infection is Scored Right to Left (2,3,4).

After the disease notes were taken the plants were cut off directly above the crown, the dead leaves were removed from the soil surface by use of a vacuum cleaner, and growth was allowed for a subsequent inoculation with the same or a different pathogen.

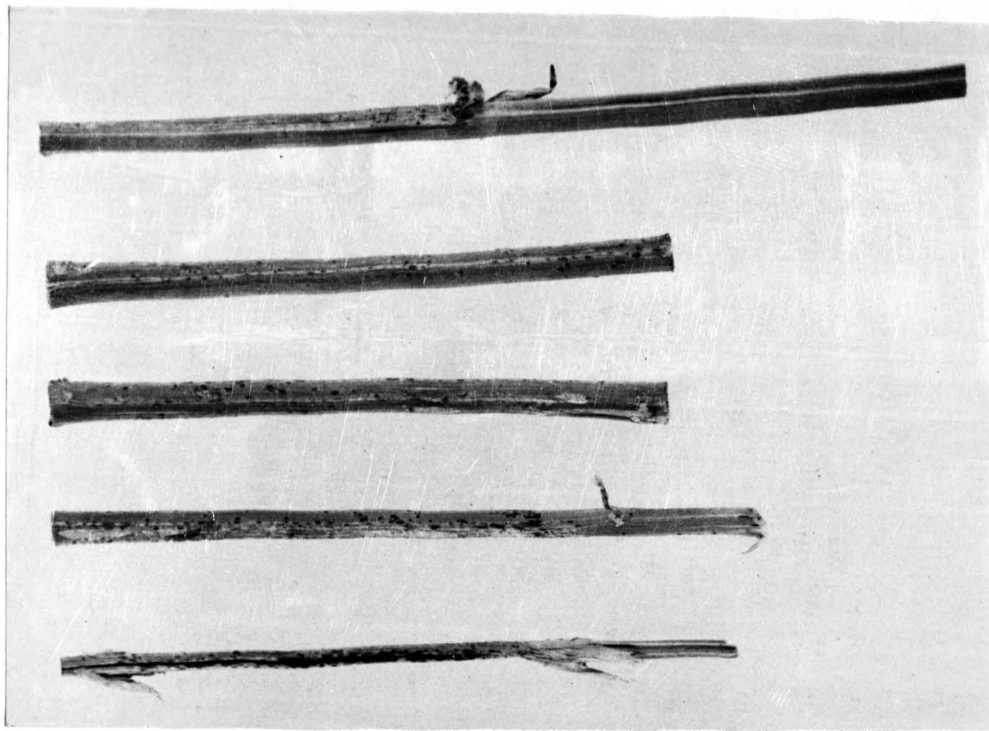

PLATE VI. Typcial Stem Lesions of Spring Blackstem on Alfalfa Caused by Ascochyta imperfecta Peck. Infection is Scored from Top to Bottom (1,2,3,4,5).

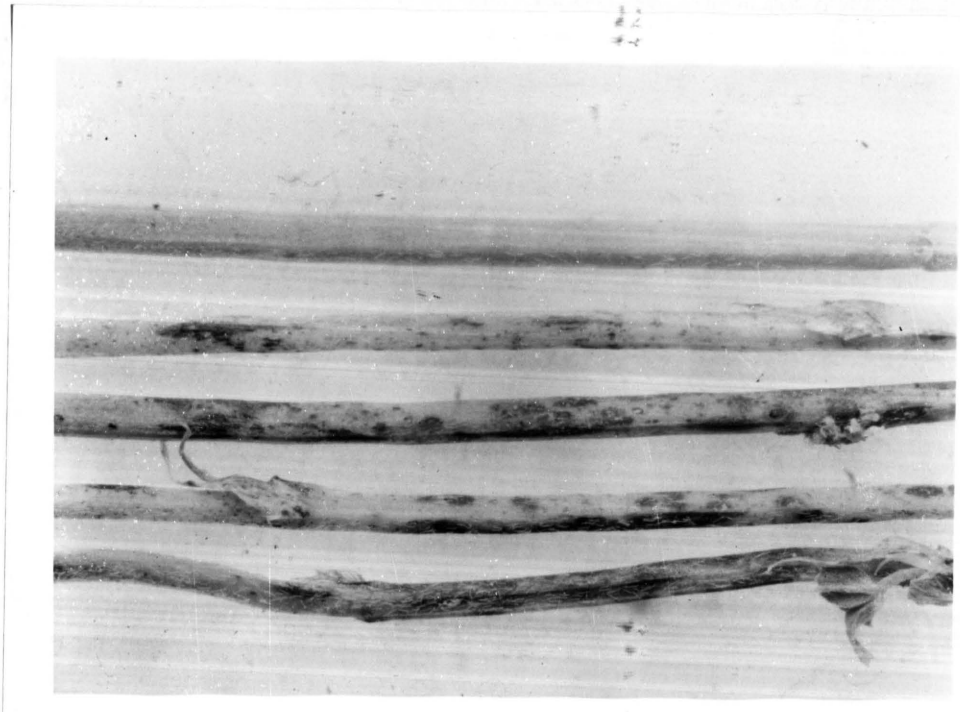

PLATE VII. Typical Stem Lesions of Stem Blight, or Bacterial Blackstem Caused by Pseudomonas medicaginis Sack. Infection Scored from Top to Bottom (1,2,3,4,5).

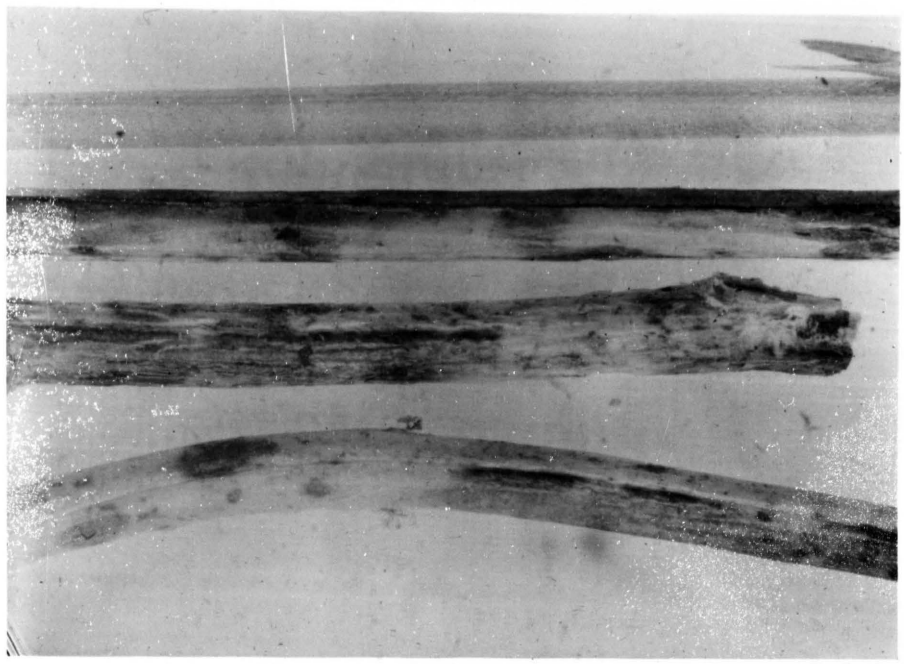

PLATE VIII. Typical Stem Lesions of Summer Blackstem on Alfalfa Caused by Cercospora zebrina Pass. Top-No Infection, the Remaining are Various Quantities of Infection.

PLATE IX. Typical Stem Lesions of Southern Anthracnose on Alfalfa Caused by Colletotrichum trifolii Bain and Essary.

EXPERIMENTAL RESULTS

This work consisted of nine greenhouse disease screening tests for three microorganisms, and seven field screening tests for four microorganisms. The results of these experiments were analyzed statistically to determine the differences, if any, between diploid and tetraploid alfalfas, between parental clones at a given level of ploidy, between open-pollinated progenies, between S_1 progenies, and for correlations between parents and open-pollinated progenies, and between parents and S_1 progenies.

Fungi Isolated

Fungi belonging to eight different genera were isolated. The genera as shown in Table 3, were Cercospora, Ascochyta, Colletotrichum, Pleospora, Phoma, Fusarium, Rhizoctonia, and Alternaria. The bacterium Pseudomonas medicaginis was isolated on another occasion.

Fungi of the genera Phoma and Pleospora were less commonly isolated than those of any other genera listed. Those of the genera Cercospora and Ascochyta were isolated more commonly from stems than from leaves, those of the genera Colletotrichum and Alternaria were isolated more commonly from leaves than from stems, while those of the genera Fusarium and Rhizoctonia were isolated as frequently from leaves as from stems. Representatives of the genus Pleospora were isolated only from leaves, while those of the genus Phoma were isolated only from stems.

All of the parental alfalfa plants shown in Table 3 yielded fungi representing two or more genera. Fungi of the genera Fusarium, Rhizoct-

Table 3. Genera of Fungi Isolated From Stems or Leaves of Sixteen Diploid and One Tetraploid Alfalfa Grown in the Field, 1954.

GENUS OF FUNGI	CERCOSPORA (3)		ASCOCHYTA (4)		COLLETOTRICHUM		PLEOSPORA		PHOMA		FUSARIUM		RHIZOCTONIA		ALTERNARIA	
Plant Identity	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf
Don	x		x	x	x	x					x	x			x	x
Don-S-1	x										x		x		x	x
Don-S-4	x		x	x	x	x					x	x		x		x
S2128-1	x		x			x					x		x	x		x
S2128-4	x	x	x	x												
S2128-6					x	x		x								x
S2128-10	x	x	x													
S2128-11	x	x	x		x						x				x	x
S33-1-7	x		x		x	x					x	x			x	x
S33-1-14			x			x		x			x	x		x	x	x
HF-1			x			x						x	x			x
HF-6			x		x	x		x			x		x			x
HF-7	x	x														
HF-12	x	x	x		x	x		x				x			x	x
Caucasus		x								x						
Iran			x	x						x						
C1Ck(2)			x	x						x						

Notes: (1) The presence of x's does not imply complete susceptibility, nor does the absence of x's imply immunity.

(2) C1Ck is tetraploid (4N=32)

(3) Cercospora zebrina

(4) Ascochyta imperfecta

onia, and Alternaria were generally associated with fungi of the other genera and were considered saprophytic or semi-parasitic contaminants that aggravated the Blackstem disease. Fungi of the other genera, which are considered phytopathogenic, were found on three or more of the seventeen parental lines tested but their absence on some of the lines cannot be attributed at the present time to resistance for the reason that the isolation tests were not extensive enough.

Screening Tests

The results of screening studies with three of the pathogens of the Blackstem Complex are presented in the following tables. In tables 4 and 9, the Pooled Error, which included all interactions involving blocks, was used to decrease any bias which may have resulted from differences in scoring, time of scoring, or quantity of inoculum applied.

Ascochyta imperfecta

The significant F value obtained in Table 4 indicates that real differences exist among both diploid and tetraploid alfalfas, in their reactions to this pathogen. Significant differences were also found between the two groups of selections representing these two levels of ploidy. The two isolates used in the screening test were strikingly different in pathogenicity, but there were no differential reactions of the clones with the isolates.

The regression analysis of S_1 progeny performance on diploid parents (Table 5), which was significant at the 1% level, indicated that the differences found in the diploids were highly heritable.

The regression analysis of open-pollinated progeny performance on

diploid parents for reaction to A. imperfecta Blackstem (Table 6) also indicated that the reactions were highly heritable.

An analysis of variance for reaction of sixty open-pollinated families of diploid alfalfa to Blackstem, under greenhouse conditions, (Table 7) indicates that significant genetic differences in reaction exist among the families. These families were derived from clones belonging to both diploid Medicago falcata and M. sativa originating from several sources.

An analysis of variance for reaction of an additional forty open-pollinated families of diploid alfalfa to A. imperfecta leafspot (Table 8) indicated that real differences existed among individual open-pollinated families derived from a single source (Medicago falcata var. Alaskan). The heritability estimate which is the ratio between the additive genetic variance and the total phenotypic variance indicated that 35.07% of the mean superiority of selected families would be transmitted to their open-pollinated progeny.

Table 4. Analysis of Reaction of 40 Clones of Alfalfa to Leafspot Induced by Ascochyta imperfecta. (Greenhouse 1954-1955)

Source of Variation	D.F.	S.S.	M.S.	F
Clones	-	-	-	-
2N vs 4N	1	130.00	130.000	14.93**
Among Diploids	25	389.46	15.578	1.79*
Among Tetraploids	13	453.53	34.887	4.01**
Isolates	1	236.60	236.600	27.18**
Clones x Isolates	39	297.65	7.632	.88
Blocks	1	0.75	0.750	.09
Pooled Error	79	687.75	8.706	-
Within Plots	320	227.33	0.710	-
Total	479	2423.08	-	-

** Significant at 1% level * Significant at 5% level

Table 5. Analysis of Regression of S_1 Progeny Performance on Diploid Parents for Reaction to Blackstem Induced by Ascochyta imperfecta. (Field, August 1956)

Source of Variation	D.F.	S.S.	M.S.	F
Regression	1	3.233	3.2330	14.76**
Deviations from Regression	15	3.287	.2191	
Total	16	6.520		
b = .5954**		r = .7042**		

** Significant at 1% * Significant at 5%

Table 6. Analysis of Regression of Open-Pollinated Progeny Performance on Diploid Parents for Reaction to Blackstem Induced by Ascochyta imperfecta (Field, August 1956)

Source of Variation	D.F.	S.S.	M.S.	F
Regression	1	1.822	1.822	22.58**
Deviation from Regression	11	.888	.0807	
Total	12	2.710		
b= .5714**		r = .8201**		

** Significant at 1% level * Significant at 5% level

Table 7. Analysis of Variance for Reaction of Sixty Open-Pollinated Families of Diploid Alfalfa to Blackstem Induced by Ascochyta imperfecta. (Greenhouse, 1955-1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	59	149.49	3.2286	17.6715**
Within Families	1698	310.22	.1827	
Total	1757	500.71		

** Significant at 1% level

Table 8. Analysis of Variance for Reaction of Forty Open-Pollinated Families of Diploid Alfalfa (Alaskan) to Leafspot Induced by Ascochyta imperfecta. (Greenhouse, 1955-1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	39	249.49	6.397	29.34**
Within Families	1151	251.56	.218	
Total	1190	501.05		

** Significant at 1% level H^2 = .3507

Cercospora zebrina

The analysis of reaction of forty clones (Table 9) to infection by this pathogen reveals that the differences in reaction were significant only between degree of ploidy and among tetraploids. There were no significant differences between pathogenicity of the isolates or interactions of the isolates with clones.

A separate analysis of the data used in Table 9, wherein the isolates were pooled and treated as replications (Table 10), indicated that significant differences do actually exist between the diploid clones for their reaction to C. zebrina.

The analysis of regression of the S_1 progeny performance on diploid parents for reaction to this pathogen indicated that the parental differences were highly heritable.

The analysis of variance for reaction of fourteen tetraploid alfalfa clones to infection by C. zebrina (Table 12), indicates that the differences between the clones are highly significant when isolate sums of squares were pooled as replications.

The analysis of variance for reactions of twelve S_1 tetraploid families to Blackstem induced by C. zebrina, (Table 14), indicated that real differences existed between the families.

An analysis of regression (Table 14), of open-pollinated progeny performance on diploid parents for reaction to Blackstem induced by C. zebrina under field conditions, indicated that the differences were highly heritable.

Table 9. Analysis of Reaction of Forty Clones of Alfalfa to Leafspot Induced by Cercospora zebrina. (Greenhouse, 1954-1955)

Source of Variation	D.F.	S.S.	M.S.	F
Clones				
2N vs 4N	1	662.02	662.020	60.82**
Among Diploids	25	339.56	13.582	1.25
Among Tetraploids	13	433.30	34.100	3.13**
Isolates	2	63.02	31.510	2.90
Clones x Isolates	78	461.42	5.916	.54
Blocks	1	24.20	24.200	2.22
Pooled Error	119	1295.22	10.884	
Within Plots	480	28.58	.060	
Total	719	3317.32		

** Significant at 1% level

Table 10. Analysis of Variance for Reaction of Twenty-Six Diploid Alfalfa Clones to Leafspot Induced by Cercospora zebrina. (Greenhouse, 1954-1955)

Source of Variation	D.F.	S.S.	M.S.	F
Clones	25	339.56	13.582	2.715**
Blocks	5	99.22	19.844	3.966**
Error	125	625.34	5.003	
Total	155	1064.12		

** Significant at 1% level * Significant at 5% level

Table 11. Analysis of Regression of S_1 Progeny on Diploid Parents for Reaction to Blackstem Induced by Cercospora zebrina. (Field, 1955)

Source of Variation	D.F.	S.S.	M.S.	F
Regression	1	6.4677	6.4677	13.52**
Deviations from Regression	16	7.6523	.4783	
Total	17	14.1200		
b = .6808		r = .6767**		

** Significant at 1% level

Table 12. Analysis of Variance for Reaction of Fourteen Tetraploid Alfalfa Clones to Blackstem Induced by Cercospora zebrina. (Greenhouse, 1954-1955)

Source of Variation	D.F.	S.S.	M.S.	F
Clones	13	443.30	34.100	3.930**
Blocks	5	93.91	18.782	2.164
Error	65	563.98	8.677	
Total	83	1101.19		

** Significant at 1% level * Significant at 5% level

Table 13. Analysis of Variance for Reaction of Twelve S_1 Tetraploid Families of Alfalfa to Blackstem Induced by Cercospora zebrina. (Field, 1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	12	12.84	2.070	11.07**
Within Families	564	105.67	.187	
Total	576	130.51		

** Significant at 1% level

Table 14. Analysis of Regression of Open-Pollinated Progeny Performance on Diploid Parents for Reaction to Blackstem Induced by Cercospora zebrina. (Field, October 1955)

Source of Variation	D.F.	S.S.	M.S.	F
Regression	1	5.013	5.013	12.66**
Deviations from Regression	23	9.117	.396	
Total	24	14.130		

b = .37** r = .59**

** Significant at 1% level * Significant at 5% level

Colletotrichum trifolii

Real differences were found between sixty diploid open-pollinated families from several sources (Table 15), in their reactions to Blackstem induced by this pathogen. These differences were significant at the 1% level.

Significant differences were also found between forty diploid open-pollinated families from a single source (Alaskan), (Table 16), in their reactions to Blackstem induced by this microorganism.

Significant differences were found to exist between sixty open-pollinated families of diploid alfalfa from several sources (Table 17), in their reactions to leaf infection induced by C. trifolii.

The differences between twelve S_1 tetraploid families of alfalfa in their reactions to leafspot infection induced by C. trifolii (Table 18) were found to be significant at the 1% level.

The differences in the reaction of forty open-pollinated families of diploid alfalfa (Alaskan) (Table 19) were significant at the 1% level. A heritability estimate indicated that about 79% of the mean superiority of selected families would be transmitted to their open-pollinated progeny.

Table 15. Analysis of Variance for Reaction of Sixty Open-Pollinated Families of Diploid Alfalfa to Blackstem Induced by Colletotrichum trifolii. (Greenhouse, 1955-1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	59	77.97	1.3215	8.4171**
Within Families	1701	267.11	.1570	
Total	1760	345.08		

** Significant at 1% level

Table 16. Analysis of Variance for Reaction of Forty Open-Pollinated Families of Diploid Alfalfa (Alaskan) to Blackstem Induced by Colletotrichum trifolii. (Greenhouse, 1955-1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	39	56.00	1.436	4.88**
Within Families	1152	339.11	.294	
Total	1191	395.11		

** Significant at 1% level

Table 17. Analysis of Variance for Reaction of Sixty Open-Pollinated Families of Diploid Alfalfa to Leaf Infection Induced by Colletotrichum trifolii. (Greenhouse, 1955-1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	59	106.62	1.8071	8.1437**
Within Families	1701	377.47	.2219	
Total	1760	484.09		

** Significant at 1% level

Table 18. Analysis of Variance for Reaction of Twelve S_1 Tetraploid Families of Alfalfa to Leafspot Induced by Colletotrichum trifolii. (Greenhouse, 1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	12	3.60	.300	2.27**
Within Families	502	66.29	.132	
Total	514	69.89		

** Significant at 1% level

Table 19. Analysis of Variance for Reaction of Forty Open-Pollinated Families of Diploid Alfalfa (Alaskan) to Leafspot Induced by Colletotrichum trifolii. (Greenhouse, 1955-1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	39	84.91	2.18	8.38**
Within Families	1151	302.19	.26	
Total	1190	387.10		

** Significant at 1% level $H^2 = .7940$

Pseudomonas medicaginis

The analysis of variance for reaction of twelve S_1 tetraploid families of alfalfa (Table 20) indicated that real differences existed between the families in reaction to leafspot induced by the bacterium.

A similar analysis, (Table 21), also indicated that real differences existed between the families in reaction to Blackstem induced by P. medicaginis. In both cases the differences were significant at the 1% level.

Table 20. Analysis of Variance for Reaction of Twelve S_1 Tetraploid Families of Alfalfa to Leafspot Induced by Pseudomonas medicaginis. (Field, 1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	12	24.90	2.080	5.44**
Within Families	505	192.70	.382	
Total	517	217.60		

** Significant at 1% level

Table 21. Analysis of Variance for Reaction of Twelve S_1 Tetraploid Families of Alfalfa to Blackstem Induced by Pseudomonas medicaginis. (Field, 1956)

Source of Variation	D.F.	S.S.	M.S.	F
Between Families	12	21.87	1.822	6.55**
Within Families	504	140.07	.278	
Total	516	161.94		

** Significant at 1% level

Association Between Measurement Studies

The possibility that relationships existed between various plant characters and disease reactions led to a series of notes which could be analyzed to determine if the relationships were real. Growth habit of plants was thought to influence the microclimate of the plant and in this way effect its relative resistance or susceptibility. The correlation coefficient which was obtained between these characters (.0701) indicated that there was no significant relation. This value left some doubt because the experiment contained both diploid and tetraploid plants. These plants differ markedly in both growth habit and disease reaction, and therefore, may be masking the effects of each other. The correlations were then determined between the same two factors for each ploidy level. The "r" value for the twenty-six diploids was -.0453, while that of the fourteen tetraploids was -.5821. The latter value thus indicated that there was some relationship. In the case of the tetraploids the value was found to be significant at the 1% level. In both cases the "r" value was negative which indicated that the more erect the plants, the more re-

sistant they were to Cercospora Blackstem.

Notes were taken for size and number of leaf lesions on plants infected by Cercospora zebrina. The correlation coefficient for these two characters was .3462 which with 119 degrees of freedom was significant at the 1% level. This would indicate that plants which have large lesions on their leaves usually have a greater number of lesions also.

An analysis of regression of lesion size on leaf drop was highly significant and indicated a relationship existed between these two characters. This would mean that for each increase in the size of lesions there is also an increase in the amount of leaves dropped from the plant.

Table 22. Analysis of Regression of Lesion Size and Leaf Drop for 40 Clones of Alfalfa Infected by Cercospora zebrina. (Greenhouse, 1954-1955)

Source of Variation	D.F.	S.S.	M.S.	F
Regression	1	10.02	10.02	21.78**
Dev. from Regression	118	54.77	.46	
Total	119	64.79		
b = .3811**		r = .3932**		

** Significant at 1% level

The analysis of regression of lesion number on leaf drop was also highly significant and indicated that as lesion numbers increase there is also a corresponding increase in leaves dropped.

Table 23. Analysis of Regression of Lesion Number on Leaf Drop for 40 Clones of Alfalfa Infected by Cercospora zebrina. (Greenhouse, 1954-1955)

Source of Variation	D.F.	S.S.	M.S.	F
Regression	1	5.606	5.606	11.17**
Dev. from Regression	118	59.184	.502	
Total	119	64.790		
b = .3037**		r = .2941**		

** Significant at 1% level

Certain plants used in these experiments were found to be highly susceptible not only to Cercospora zebrina, but also to Ascochyta imperfecta. This was the basis for the correlation between reactions of these plants to these pathogens. The correlation coefficient between these reactions was .36 with 39 degrees of freedom. This value was significant at the 5% level.

DISCUSSION

The use of disease readings made in the field on adult plants introduces considerable error in correctly classifying the plant reactions. These errors are due mainly to the overlapping of the infection periods of the various pathogens. Under ordinary circumstances overlapping is not a problem, but in this situation the symptoms produced by several of the microorganisms are so similar that they cannot be positively separated.

Other errors may originate because of the non-uniformity of natural inoculation. Plants under field conditions are dependent on natural factors to carry inoculum from one plant to another. These factors may be wind, rain, insects, animals, etc. If these are limited then true differences between plants may not be manifested. Nevertheless, it is only natural that the proximity of inoculum will influence the degree of infection of any one plant.

Secondary infection of plants is also a criterion which must not be overlooked. Under field conditions inoculation may occur continuously or intermittently, so that at any one time it is impossible to determine the maximum effect on a plant. Leaves which are severely infected drop off and may go unnoticed because of the continuous growing condition of the plants.

The growth habit of a mature plant may also be an influence on degree of infection. Observations of growth habit and disease reaction indicated that the two are inversely related, that is, plants which are more upright in growth habit tend to be more disease resistant. Whether

this is a genetic factor, or an environmental factor, is not known.

The factors indicated above which contribute to incorrect disease scoring are some of the reasons for developing greenhouse screening tests. Artificial inoculation trials under controlled environmental conditions remove all chances of overlapping of the symptoms of the pathogens, provided only one microorganism is used at any one time on the same plant.

When the plants are subjected to an artificial inoculation under controlled conditions it is possible to observe how the complete unit reacts rather than portions of it. Leaf drop can be quickly and accurately observed, and types and numbers of leaf or stem lesions can be seen, because none of the parts are lost.

Under greenhouse conditions, the technician may use individual isolates to determine the relative pathogenicity of each, or he may bulk those of one microorganism to determine the total effects of all isolates.

All of these trials may be conducted in the greenhouse in one season because of the relatively short time required to complete a test. An artificial inoculation may be completed in a month to five weeks, whereas in the field two or three months are required. Also, in the greenhouse, the technician can be certain of what pathogen he is taking disease notes for.

Studies conducted on four pathogens which produce Blackstem symptoms indicated that the reactions were genetically influenced. These reactions varied over a range of highly resistant to susceptible, and appear, from the parent-progeny regression analyses, to be moderately to highly heritable.

The differential reactions were found to exist not only among

plants of a single source but among plants of several other sources. This would indicate that hereditary resistance is characteristic to all of the material and is not confined to an individual, or single gene of a particular introduction or strain.

The widespread availability of genetic differences in the material should allow a distinct improvement in strains. This improvement may be a consequence of single genes or gene complexes which do not necessarily confer complete immunity; with accumulation of these genes in a selected population, a much improved level of resistance should be reached.

The distribution of variability observed within families in these experiments appeared to be of the continuous type, in that at no time was either a completely susceptible or completely resistant family observed. In all families, variability appeared to be unimodal, with the exception of one small selfed family in which the frequency distribution appeared to be bimodal, but even in this situation both groups tended toward the susceptible end of the scale.

In cases where the disease reaction is sharply defined as resistant or susceptible with no intergradations, and the genetic factors are found to be few or singular, the immediate goal of the breeder would be to find clones or lines carrying the gene (or genes) conferring immunity and fix it in his population. This has been the method most widely used in obtaining rust resistance in small grains.

It is certainly not valid, however, to apply this method uncritically to solve disease infection in alfalfa. In the first place, the reproductive biology of the plant itself is such as to render unfeasible any attempt to fix in the homozygous state more than a very few loci

conferring specific immunity to a disease. Secondly, the continuous nature of the family distributions and the heredity significance of the intergrading classes suggests that several genetic factors may be involved and that in no instance are all the favorable genes expected in one genotype. Rather a method of gradual accumulation wherein repeated cycles of selection and interbreeding are employed in order to successively concentrate a high proportion of the favorable genetic effects in a group of selections would appear to suit both the reproductive biology of the plant and the quantitative basis of disease reaction. Such a procedure would allow the breeder to select for other desirable traits in the initially-variable population at the same time. This method is, with justification, known as the recurrent selection method of plant breeding.

One of the studies carried on was the relationship between the reactions of Ascochyta imperfecta and Cercospora zebrina on the same plants. In this case a positive correlation coefficient of .36 was obtained which was significant at the 5% level. This value is too small to assume a priori that selection can be made for both pathogens at the same time, but it does suggest that multiple resistance is not a physiological impossibility.

Most of the information gained in these studies has been obtained with diploid alfalfas. They have been of primary interest because their pattern of inheritance is expected to be simpler than for tetraploids. Inasmuch as resistance appears to be of a quantitative genetic nature, progress toward resistance in a diploid strain would be much more rapid than in a tetraploid strain.

The utilization of disease resistance at the diploid level is not agronomically feasible because diploid varieties are inherently lower in vigor than normal tetraploids.

However, a method of incorporating the resistance of the diploids into agronomically suitable tetraploid varieties may be suggested. This method may be termed polyploid synthesis. This is a method wherein tetraploid alfalfa is reconstituted from selected diploid parents. This may be accomplished by two methods, one being the use of chemical agents which have been known to cause polyploidy.

The other method would entail the use of diploid plants selected for disease resistance, hardiness, vigor, crown development, etc., and used as female parents in crosses with selected tetraploid males. In a small number of cases tetraploid seed would be set that would have resulted from the functioning of unreduced diploid eggs in the female parent. The resulting plants with a tetraploid complement of chromosomes would carry germ plasm for desirable traits for which selection had been practiced in the diploid population. The resulting plants could not be expected to react exactly as the diploid parent had, because only half of their genetic material would have been received from the diploid parent. There would also be the possibility of modification of these diploid genetic effects through dominance and non-allelic interactions. Nevertheless, the favorable alleles would have been introduced at the tetraploid level, and, to the extent that their favorable effects were additive, would contribute to the improved performance of the reconstituted tetraploid variety.

Further selection would undoubtedly be necessary among the synthe-

sized tetraploids; perhaps intercrosses among them would effect desirable recombinations that would result in superior performing clones which could be used in strain synthesis.

This method has not been employed in its entirety by alfalfa breeders, but there are being grown at present two alfalfa varieties which have diploid Medicago falcata in their parentage. Rhizoma, the variegated strain from British Columbia, and Vernal, the new wilt resistant variety from Wisconsin, both derive some of their particular adaptability from diploid parentage.

SUMMARY

Isolation of pathogens of the Blackstem complex of alfalfa yielded fungi of eight genera. These genera are: Ascochyta, Cercospora, Colletotrichum, Phoma, Pleospora, Fusarium, Rhizoctonia, and Alternaria.

Fungi of three of the genera are of most importance in the Blackstem complex, these being: Ascochyta, Cercospora, and Colletotrichum.

One bacterium, Pseudomonas medicaginis, causes blackening of stems of alfalfa; symptoms produced by the microorganism were studied but no isolations were made in this study.

Genetic differences were noted in the reaction of alfalfa to the three genera of fungi and one bacterium.

Genetic differences were noted between the reactions of diploid and tetraploid alfalfas to both Ascochyta imperfecta and Cercospora zebrina.

Significant differences were noted between isolates of Ascochyta imperfecta but no interaction of clones and isolates was observed.

No differences were observed between three isolates of Cercospora zebrina.

Genetic differences in response to the pathogens are found among families from diverse sources.

Analyses of regression of open-pollinated and self-pollinated progeny on diploid parents indicated that the reactions are moderately to highly heritable.

Variability in the reactions of the plants to the pathogens appears to be of a continuous nature.

Regression and correlation values indicated that characters such as leaf drop, lesion size, and lesion number are closely associated.

Correlation coefficients indicated that tetraploid plants which have a more upright growth habit are more resistant to pathogens of the Blackstem complex.

A correlation between the reactions of Ascochyta imperfecta and Cercospora zebrina on the same plant was significant at the 5% level, and indicated that the plants which were susceptible to one pathogen, were also susceptible to the other, and conversely.

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