Factors Affecting the Production, Dispersal and Infectivity of Ascospores of Leptosphaerulina briosiana (Poll.) Graham and Luttrell, a Leaf Pathogen of Alfalfa

by

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ABSTRACT

An investigation was made of the effects of some environmental factors on the discharge and germination of ascospores of Leptosphaerulina briosiana (Poll.) Graham and Luttrell. Optimum germination was obtained at 20° C. and 100% relative humidity. A reduction of the relative humidity to 98% was associated with a significant reduction in the spore germination and a significant increase in the production of appressoria by germinating spores. Spore germination and appressorium formation were negligible at all temperatures under a relative humidity of 95% or less. Spore discharge from V-8 agar cultures of L. briosiana kept in a dry atmosphere fell to a low level before drying out of the medium became evident, but continued at a moderate intensity from those kept in a relative humidity of 100%.

Studies on penetration indicated that an estimate of the extension of fungal hyphae in the host tissue, or of the lesion size can be used as a measure of resistance. An inoculation technique, in which L. briosiana cultures grown on filter paper soaked in 20% V-8 juice were supported over

alfalfa plants and allowed to discharge spores onto them, was developed and compared with previously described inoculation methods. The new inoculation method was found to be superior to other methods, and compared to the V-8 agar method which it resembles possessed the advantages of ease of preparation, simplicity of applicability on a large or small scale, avoidance of drying out problems, and suitability for storage in a frozen state between inoculations. An examination of several hundred alfalfa seedlings inoculated by this method revealed a very low percentage of resistant plants.

INTRODUCTION

Leafspots of forage legumes caused by species of Leptosphaerulina have become of increasing importance since 1950. On alfalfa in particular, the disease has reached epidemic proportions in parts of the U.S.A. during a number of wet, cool summers in the last ten years (5, 6, 7). Forage crop breeders have been unable to incorporate resistant sources into their breeding programs because available inoculation methods are laborious to perform and give erratic results.

This study was undertaken to investigate the effects of various environmental factors on the discharge and germination of ascospores and the penetration and development of the pathogen within the host tissues. The information gained was used to develop a reliable and reasonably rapid method of artificially inoculating alfalfa with Leptosphaerulina briosiana (Poll.) Graham and Luttrell. and of determining a reliable index of resistance to the pathogen.

LITERATURE REVIEW

Leafspot diseases of forage legumes caused by members of the genus Leptosphaerulina have been known for many years. Because of considerable variation in morphologic and pathogenic characteristics, isolates obtained by several workers (1, 2, 7, 10, 16) from different hosts have been identified as belonging to several closely related genera. Allison, Hemerick and Nelson (1) discussed briefly the binomials which have been applied to this group of fungi. They considered that the name Pleosphaerulina hyalospora (Ell. and Ev.) Berl. had priority, and they reduced all other binomials to synonymy. More recently, Graham and Luttrell (7) investigated the taxonomic relationships of the group, and assigned the name Leptosphaerulina to the genus. They divided members pathogenic to leguminous forage plants into two species on the basis of differences in the morphology of the ascocarp, ascus, and spores, and in growth characteristics in culture. The two species are L. trifolii (Rost.) Petr. which causes pepperspot of Trifolium species, and is also capable of producing severe infection of

Medicago and Melilotus species; and L. briosiana (Poll.) Graham and Luttrell which produces the most severe infection on Medicago species, and causes only minor infections on other legumes.

Until 1953, species of Leptosphaerulina were believed to reproduce by means of ascospores only. In that year, Nelson and Kernkamp (18) reported that single ascospore cultures of L. briosiana produced conidia of Stemphylium botryosum when grown on alfalfa leaves or on artificial medium. Conversely, single conidial cultures of S. botryosum produced ascocarps of L. briosiana. Allison et al. (1) also reported finding Stemphylium type conidia in their Leptosphaerulina isolates. Other workers (5, 6, 7, 13, 22) have been unable to find an imperfect stage of either L. trifolii or L. briosiana.

The disease symptoms produced by the two species of Leptosphaerulina on the leaves and petioles of their respective hosts are identical in all respects. Individual spots are small, 1-3 mm. in diameter, with a pale grey sunken centre surrounded by a dark brown or black ring. In the early stages of development, examination of a spot under a

dissecting microscope reveals a large phragmosporous or muriform spore near the centre of the spot. Later, perithecia develop there. The spots may or may not be surrounded by a chlorotic area of greater or lesser extent. The disease on alfalfa is sometimes called Brown Leafspot (4), and on clover, the descriptive name, Pepperspot, is used when the leaf is covered with small uniform dark spots. In Britain, the disease is called Burn (20).

Since L. briosiana was first reported by Pollacci, who isolated it from alfalfa in Italy in 1901 (Miles 1925), the pathogen has been found in most areas of the world where alfalfa is grown (9). The disease was first found in the U.S.A. by Melchers (15) in Kansas in 1914. In 1916, Jones (10) isolated it from diseased alfalfa samples sent to him from several states. The first report of Leptosphaerulina (presumably L. briosiana) leafspot on alfalfa in Britain was not made until 1957 (2) although L. trifolii, found on white clover in 1922 (20), is prevalent. Miles (16) reported the disease on young stems and petioles of bur clover and found that sclerotia-like structures on seeds of infected plants gave him viable cultures of the

pathogen.

For the first half of this century, spasmodic, local epidemics of pepperspot occurred on clovers, especially on Ladino clover in the southern U.S.A. It was a minor disease of other forage legumes. Since 1952, a series of cool, wet seasons has coincided with a great increase in the incidence of the disease on alfalfa (5, 22), and in some parts of the U.S.A. it is now considered to be one of the most important leaf diseases of the crop. Considerable defoliation may result in spring and fall when weather conditions are optimum for the development of the disease. The aftermath growth in particular is susceptible and, in the Northeastern U.S.A., such defoliation in late fall can lead to winterkill of the hardiest alfalfa varieties (5). Williams (22) reported that L. briosiana reaches epidemic proportions in Virginia in certain years, and Elliott (4) rated the disease in West Virginia as of major importance in 1953.

The disease seems to be limited to the cooler, wetter parts of the year. Overwintering occurs on older leaves or on dead plant matter on the ground (9). Early infection

comes from spores ejected from these leaves. Graham and Luttrell (7) suggested that the seasonal occurrence of the disease is due to the effect of temperature on spore discharge. They found that the severity of the disease reflects mainly the number of spores available.

There appears in the literature no method of preventing the disease other than by the development of resistant varieties. Until such varieties are developed, crop sanitation might help to prevent epidemics by removing much of the primary inoculum.

SPORE DISCHARGE

Introduction

The initial infection of alfalfa leaves by L. briosiana originates only from ascospores discharged from perithecia, and to produce artificial inoculations with this pathogen, the factors affecting this process of ejection must be known. The most important of those investigated are temperature, light, and moisture supply. Miller (17) found that spore ejection from diseased leaves reached a maximum between 9 and 10 A.M. There was little discharge during the remainder of the day. He suggested that early morning sunlight dried off night dew and caused the asci to shrink and eject spores. Elliott (3) observed that more spores were ejected from asci at 30° C. than at 20° or 10° C. Frosheiser (5) reported spore discharge at all temperatures between 4° C. and 30° C., and indicated that spore discharge at low temperatures continued until the host leaves decayed. Graham and Luttrell (7) found that spore discharge of L. briosiana was not greatly affected by

temperature in the range 15° C. to 25° C. and that the majority of their cultures produced ascospores only when exposed to light. Kilpatrick (12) found a similar effect in L. trifolii.

The above investigations reveal that under normal growing temperatures, light and available moisture control spore discharge in L. briosiana. Investigations of spore discharge under alternating light and dark periods were made to further elucidate the effects of light on sporulation. An experiment was also conducted to determine if a relative humidity of 100% led to an increase in spore discharge.

Materials and Methods

The cultures used for spore ejection studies were obtained by inverting a sporulating culture of L. briosiana over petri plates of V-8 agar for 24 hours, and incubating the inoculated plates at 70° F. in a day length of 15-16 hours. The cultures commenced to discharge spores three days after inoculation. They were then placed in an apparatus (described in appendix A) which was designed to measure the

number of spores ejected through a small aperture over a 24 hour period. At the end of 24 hours, the number of spores ejected during each half hour interval was determined and the totals were graphed against time. The spore discharge characteristics of each culture were studied by the above method for four consecutive days under the same conditions of light and temperature as had been used during the incubation period.

A similar technique was used to study the effect of high humidity on sporulation. Four V-8 agar cultures of L. briosiana were used. Two of them (Nos. 1 and 2) were kept on the laboratory bench as in normal practice. The other two (Nos. 3 and 4) were suspended by wire netting over water in a small, clear plastic container. The free water surface in the bottom of the container maintained a saturated atmosphere, as indicated by a hydrometer, throughout the experiment. The spore discharge of the four cultures was examined in rotation, with cultures from the two differing conditions being placed in the spore discharge apparatus alternately. The experiment continued for eight days.

Results

A. Light. A typical example of the spore discharge rate found at first is given in Figure 1. On the first day, a fairly steady, low number of spores were discharged during each half hour period until about seven hours after the start of the light period. Then the discharge rate suddenly increased fourfold until the onset of darkness when it returned to the original low rate at which it remained throughout the dark period. Discharge during the second day was similar to that during the first day, except that such a high discharge rate was not reached in the evening. On the third and fourth days of discharge, the rate remained at a low level throughout the 48 hour period. Later trials (Figure 2) showed no significant differences in numbers of spores discharged during dark and light periods.

B. Humidity. The rate of spore discharge was similar under both conditions of humidity during the early stages of the experiment. After the trial had been running for five days, the cultures which had been kept on the laboratory bench (1 and 2) discharged significantly fewer

Figure 1

The rate of spore discharge, during the third to the sixth day following incubation, from a V-8 agar culture of L. briosiana incubated at room temperature and with a sixteen hour daylength (dark period stippled). A typical result from an early trial.

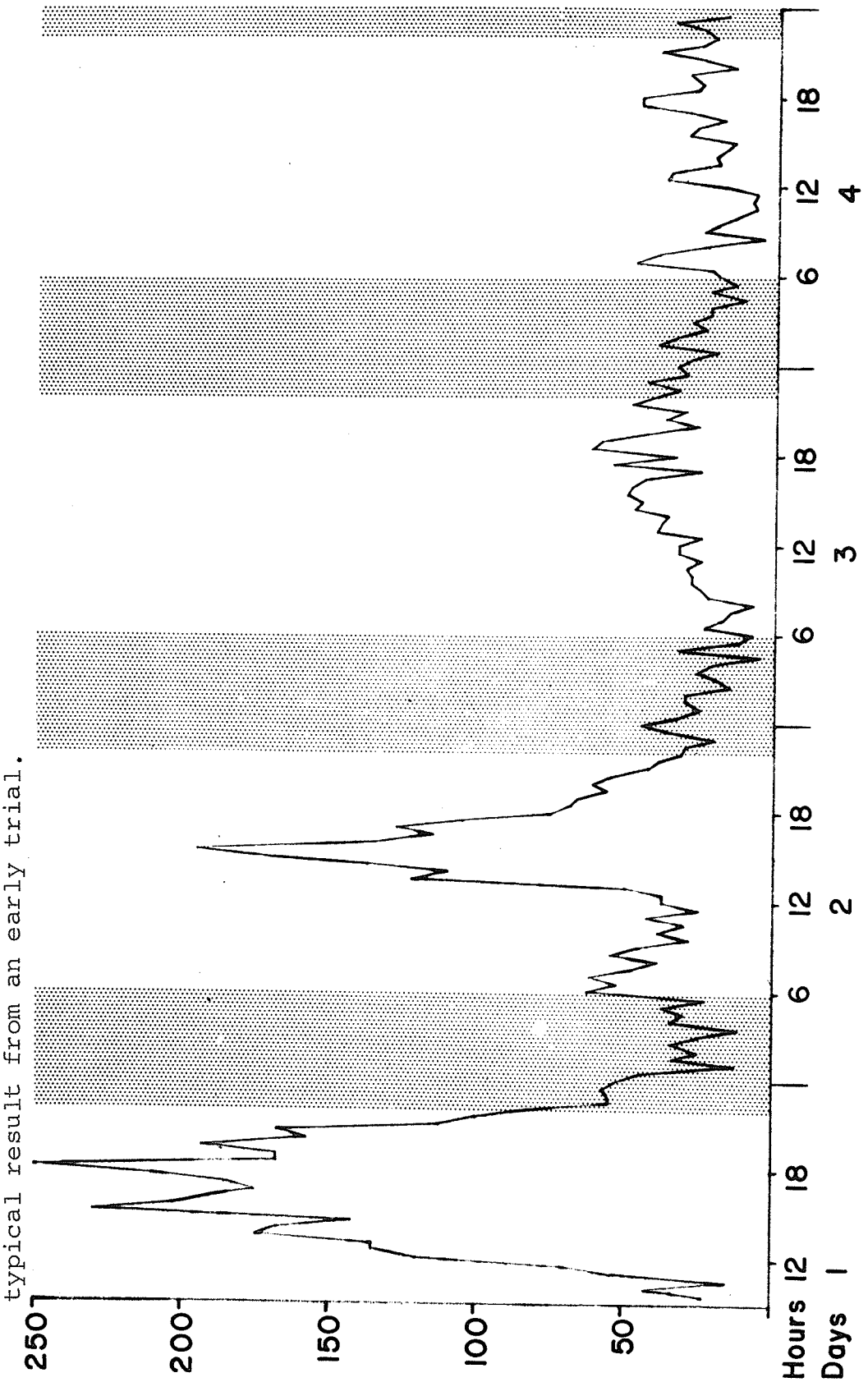


Figure 2

The rate of spore discharge, during the third to sixth day following inoculation, from a V-8 agar culture of L. briosiana incubated at room temperature and with a sixteen hour daylength (dark period stippled). A typical result from a later trial.

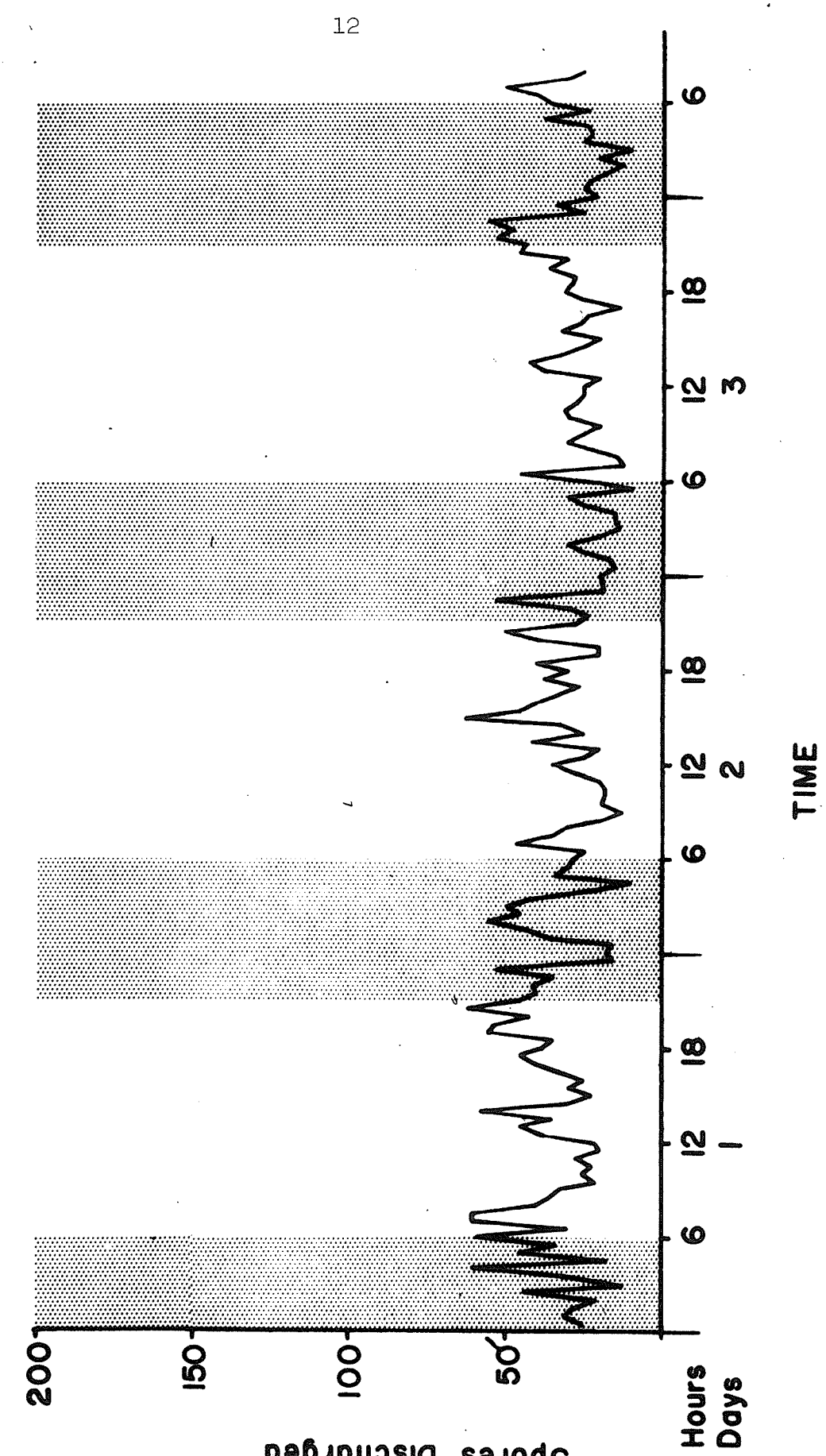
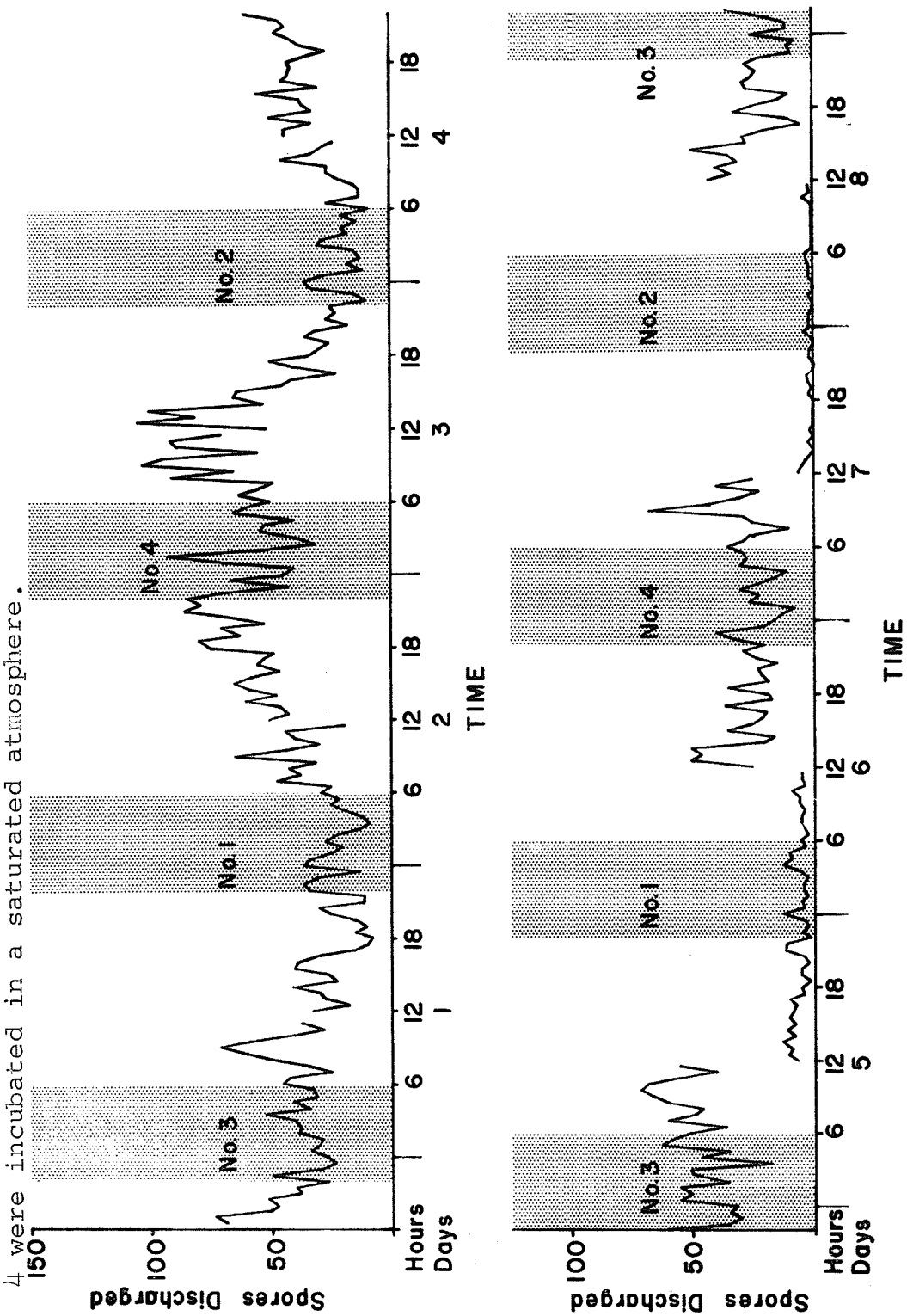


Figure 3

A comparison of the rates of spore discharge, from the third to the tenth day after inoculation, from four V-8 agar cultures of L. briosiana incubated at room temperature and with a sixteen hour daylength (dark period stippled). Cultures 1 and 2 were incubated without a humidity control. Cultures 3 and 4 were incubated in a saturated atmosphere.



spores than the cultures kept in a saturated atmosphere. After eight days, cultures 1 and 2 had almost ceased spore discharge, whereas spore discharge from cultures 3 and 4 was only slightly lower than at the beginning of the trial (Figure 3).

Discussion and Conclusions

The conflicting results make it impossible to ascribe to light a definite effect on spore discharge of L. briosiana cultures. The results in Figure 1 show a maximum spore discharge in the evening similar to the one found in Taphrina deformans by Yarwood (23). This is in distinct contrast to the observation by Miller (17) that his cultures of L. briosiana on leaves discharged the maximum number of spores in the morning. A maximum spore discharge in the evening, allowing spore germination and penetration of the host leaf during the night when favorable conditions (cool temperatures and high humidities) prevail, would be expected on the basis of natural selection.

Later results as typified by Figure 2, show no significant increases in spore output: spore discharge was

at a low level throughout the four days of the experiment. The cultures used to obtain these results were direct descendants a few generations removed of the cultures used to produce the results shown in Figure 1. The loss of the light effect on spore discharge could be explained by assuming an inadvertent selection pressure for heavily sporulating cultural types over types showing the light effect brought about by using a mass spore inoculation over a period of 24 hours. Under this theory, spore transfer during the period when spore discharge was at its highest level would ensure a continued selection of spores showing the light effect. This hypothesis was checked by returning to the original culture which had been stored in a refrigerator. When this was retested under the same conditions as had been used for the other experiments, a fairly steady, low level spore discharge similar to that shown in Figure 2 was obtained. The only certain conclusions that can be drawn are that no endogenous spore discharge rhythm is present in the culture of L. briosiana used for this work, and that any light effect picked up or normally present in the isolate before it was brought into artificial culture

can be easily lost when cultures are transferred by mass spore inoculation.

The results presented in Figure 3 suggest that a plentiful supply of water is necessary for sporulation. The experiment does not differentiate between water used for spore development and that used for spore discharge. It shows only that more spores are discharged from cultures of L. briosiana kept in a saturated atmosphere than from cultures kept under drier conditions, and indicates that a high humidity must be maintained if plants are to be inoculated successfully by direct spore discharge.

SPORE GERMINATION

Introduction

A knowledge of the conditions under which spore germination and appressorium formation takes place is desirable for a full understanding of the problem of penetration and initiation of the disease caused by L. briosiana. Frosheiser (5) reported that spore germination occurred in the range 4° C. to 37° C. Graham and Luttrell (7) compared the germination of naturally discharged ascospores on agar and of spore suspensions on glass slides in moist petri dishes. After the spores had been incubated at 15° - 30° C. for 3-4 hours, it was found that in terms of percentage germination or of germ tube length, the optimum temperature for germination was between 20° C. and 25° C. Kilpatrick (12) found that spores from cultures of L. trifolii exposed to a 24 hour day germinated poorly, while spores from cultures exposed to a ten hour day germinated well. No investigation of appressorium formation has been reported in the literature.

The following work was undertaken to compare the rates

of germination of L. briosiana spores over a range of temperatures and humidities.

Materials and Methods

Petri dishes were used as germination chambers. A perforated plastic shelf was placed inside the dish to carry coverslips on which spores were deposited. The petri dishes were filled with solutions to a level at which the shelves just floated. This ensured that the coverslips bearing the spores were in air of maximum relative humidity, and reduced the error due to zonation of humidity layers in the dish to a minimum. Lids were sealed to the dishes with vaseline to prevent evaporation and the dishes were placed in constant temperature cabinets, one dish at each of the temperatures 0°, 10°, 20°, and 30° C. Spores were collected by inverting sporulating V-8 agar cultures of L. briosiana grown at a temperature of 70° F. and a 13 1/2 hour day, over clean, dry coverslips for half an hour. The slips were then put in the humidity dishes. The spores were exposed to 24 hour illumination from fluorescent lights in all experiments.

In a second experiment, spores were incubated at 20° C. in three levels of relative humidity: 100%, 99%, and 98%. A relative humidity of 99% was attained by using a solution of 1.81% by weight of sodium chloride in water[1]. A relative humidity of 98% was produced by using a saturated solution of lead nitrate in water in the presence of excess crystals (8). A relative humidity of 100% was obtained by using distilled water.

The coverslips were removed from each humidity dish at intervals and germinated spores, total spores, and number of spores with appressoria, were counted using a magnification of 120x. The criterion of germination was taken to be the production of a germ tube of sufficient length to show parallel sides under the magnification used in counting. The ends of the germ tubes of a number of spores ceased growth, became swollen and rounded off, and adhered closely to the glass. These structures were considered to be appressoria.

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[1]International Critical Tables. Vol. III, page 370.

Results

The effects of temperature on germination are presented in Figure 4. The optimum temperature for germination was 20° C. Percentage germination was poor at both 0° C and 30° C.

The analysis of variance presented in Table 1 indicates that humidity has a significant effect ($P = 0.01$) on spore germination. The average germination after transformation of percentages to degrees was 64.7, 62.9, and 42.8 at relative humidities of 100%, 99%, and 98% respectively. The least significant difference was 12.0, indicating that spore germination at a relative humidity of 98% was significantly lower than germination at higher humidities. The effect of time on germination, as expected, was significant at the 1% level. Differences significant at the 5% level were present between replications. As the spores for each replication were obtained from different generations of the isolate of L. briosiana used, this result is not surprising.

The analysis of variance presented in Table 2

Figure 4

The effect of temperature on the percentage germination of spores incubated on glass slides at a relative humidity of 100%.

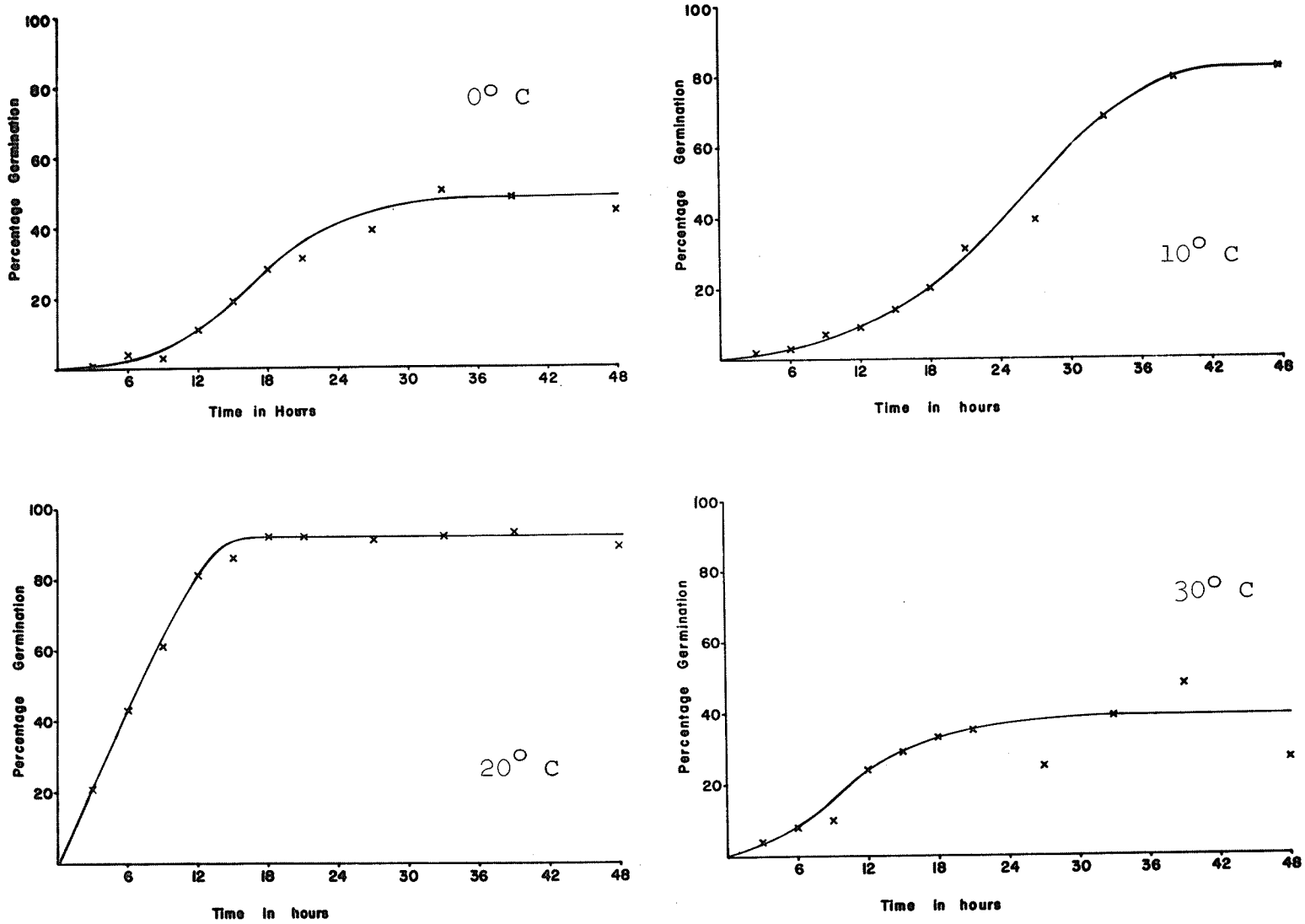


Table 1

An analysis of variance of the effect of humidity on spore germination after a transformation of the original data to angles

Source of Variance	Degrees of Freedom	Sums of Squares	Mean Square	F	L.S.D.
Total	47	17393.12			
Replications	3	1531.86	510.62	3.35*	
Time	3	5561.60	1853.87	12.21**	
Humidity	2	4729.06	2364.53	15.57**	12.0
Interaction of time & humidity	6	558.96	93.16	0.61	
Error	33	5011.64	151.87		

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

Table 2

Analysis of variance of the effects of humidity on the formation of appressoria by germinating spores on glass

Source of Variance	Degrees of Freedom	Sums of Squares	Mean Square	F
Total	47	5259.19		
Replications	3	76.32	25.44	< 1
Time	3	100.65	33.55	< 1
Humidity	2	3069.36	1534.68	34.42**
Interaction of time & humidity	6	450.56	75.09	1.59
Error	33	1562.30	47.34	

**Significant at the 1% level. Analysis after transformation of percentages to degrees.

indicates that humidity has a significant effect on the production of appressoria by germinating spores. At relative humidities of 100% and 99% respectively only 2.8 and 8.5 appressoria were formed. Appressorial formation at 98% relative humidity was 21.9, and was significantly greater (L.S.D. 1% = 6.7) than that at the higher humidities used in the investigation. The period of spore incubation had no significant effect on the formation of appressoria.

Discussion and Conclusions

The results of the experiment on the effect of temperature on spore germination agree with the reports of previous investigators (5, 6, 7) that the optimum temperature is approximately 20° C. (Figure 4). In all cases, the graph produced by plotting germination percentage against time is sigmoidal in form. This is the expected form in the absence of inhibiting or accelerating factors. At 20° C., germination is so rapid that the initial acceleration period is too short to appear. The irregularities which appear in the 30° C. curve are probably

due to the killing of many spores by heat.

There is no significant difference between the average germination of spores at 100% R.H. and at 99% R.H. A further drop of 1% in relative humidity produces a very considerable drop in germination. This is in agreement with the results of a preliminary experiment in which germination was negligible at all temperatures when the relative humidity was 95% or less.

The significant increase in the number of spores which produced appressoria on a glass surface as the humidity was lowered to 98% suggests that inoculation of plants would be more reliable if the humidity could be held steadily at a level slightly less than 100%.

GERM TUBE PENETRATION AND MYCELIAL DEVELOPMENT IN HOST TISSUE

Introduction

Much of the previous work on L. briosiana has been concerned with cultural studies and with methods of inoculating alfalfa with the pathogen. Few investigations have been made on the differentiation of resistant and susceptible plants. Renfro and Sprague (19) used the percentage of leaf area affected by the pathogen as their criterion for rating diseased plants in their search for resistant Medicago species. In view of the known irregularity of sporing of L. briosiana, the use of this criterion of disease reaction could lead to incorrect estimates of the degree of resistance of a plant. In 1953, Jones (11) reported that differences existed in the extent of growth of Pseudopeziza medicaginis (Lib.) Sacc. mycelium in leaf tissue of infected plants. Hyphae ramified through susceptible plant leaves apparently unimpeded, whereas the penetrating germ tube remained short and did not branch in

leaves of resistant plants. He also found all intermediate types between the two extremes and was able to correlate the degree of mycelial extension with the resistance or susceptibility of strains of alfalfa to common leafspot. Graham and Luttrell (7) included the size of the lesion as part of their criterion for differentiating resistant and susceptible host species to infection by species of Leptosphaerulina.

Studies on germ tube penetration and mycelial extension in host tissue inoculated with L. briosiana and measurements of lesion diameter in hosts of differing susceptibility were made to determine whether either or both of these techniques could be used successfully to differentiate between resistant and susceptible clones of alfalfa.

Materials and Methods

Penetration studies were made on leaves of two alfalfa plants selected from preliminary tests for their apparent susceptibility or resistance and on a red clover plant known to be very resistant. A number of leaves were

removed from each of the three plants and were floated on water in a petri dish. The leaves were inoculated by inverting a sporulating culture of L. briosiana over them for one hour. After incubation at 70° F. for 24 hours pieces of leaves were treated with hot potash solution, followed by aniline blue stain in acetic acid to clear and stain (Appendix B). Transverse leaf sections were cut by hand and treated in a similar manner. The extent of mycelial growth from the point of germ tube penetration was observed under a microscope using a x45 objective lens.

Variation in the diameter of lesions on a diseased leaf made it necessary to obtain a reliable estimate of average lesion diameter for scoring plants for their resistance or susceptibility. The number of spots which would have to be measured in order to obtain a reliable estimate was calculated from data obtained from a preliminary test. Leaves of alfalfa plants which were believed to be susceptible, moderately susceptible, and resistant were inoculated by the floating leaf technique described in the preceding paragraph and incubated for a week at 70° F. Then the diameters of 1380 spots taken in

approximately equal numbers from each of the three plants were measured. The estimation indicated that a sample of about 54 measurements would be adequate to give a mean lesion diameter which would not differ from the true mean by more than two units.

In an experiment to evaluate the reliability of the estimation of disease resistance obtained from spot diameter measurements, six leaves were picked at random from a number of alfalfa plants and inoculated by the floating leaf technique. They were incubated at 70° F. for seven days. The diameters of nine spots, taken at random from each leaf were measured after this time, giving a total of 54 measurements for each plant. The experiment was repeated using different cultures of the same isolate and the results were subjected to an analysis of variance. Because of the close relationship of the cultures used in both trials, the two were treated as replicates.

Results

The observations of Miles (16) and McDonald (13) that penetration of the leaf epidermis usually takes place

directly below the spore is here confirmed. On the leaves examined, appressorium formation generally took place as soon as the germ tube contacted the leaf surface. Some germ tubes grew over the leaf surface for a distance of a few epidermal cell diameters. Such germ tubes occasionally formed appressoria over a stomatal opening and sent an infection peg into the substomatal cavity, but normally they penetrated an epidermal cell. Generally, it would appear that germ tubes were not affected by stomatal openings since several penetrations were found to have taken place through guard cells.

In all sections investigated, the mycelium remained entirely intracellular. No case of hyphae growing across intercellular spaces was observed. Hyphal cross walls invariably were produced where the hyphae had passed from one host cell into the next. Walls were also observed to form across the hyphae slightly beyond a point of branching.

After the initial penetration of susceptible leaves, hyphae grew quickly in all directions, branching frequently. In 24 hours, the mycelium had spread through the leaf

almost to the lower epidermis (Figure 5) and had spread a similar distance radially from the point of infection. Growth of the majority of hyphae penetrating into resistant alfalfa leaves was observed to be similar to but much slower than growth in the susceptible leaves. Although a few spores seemed to be able to overcome the leaf resistance and to spread through the resistant leaf a similar distance to the spread through susceptible leaves, the majority of penetrations produced hyphae which grew at only one-half to one-third of the speed of the hyphae in susceptible tissue (Figure 6). Most of the spores deposited on the red clover leaves produced infection hyphae which branched only once or not at all inside the leaf, and formed only one or two cells. Transverse sections indicated that penetration rarely went beyond the epidermis.

Table 4 shows that the mean lesion size varies from 12.8 units in the resistant M. falcata clone to 23.8 units on leaves of the susceptible Grimm clone. The analysis of variance of the lesion sizes of the above clones is given in Table 3. The differences in size of disease spots on

Table 3

Analysis of variance of the diameters of lesions of L. briosiana on leaves of a number of alfalfa clones

Source of Variance	Degrees of Freedom	Sums of Squares	Mean Square	F
Total	863	87,203		
Replications	1	69	69	< 1
Varieties	7	13,175	1,882	21.76**
Error	855	73,959	86.5	

**Significant at the 1% level

Table 4

Mean diameters of lesions of L. briosiana on leaves of eight alfalfa clones

Clone number	Source	Mean lesion diameter*
R.L. 43	M. falcata	12.8
3 B	Cossack	14.7
6 A	Ile de France	15.5
3 A	Cossack	18.0
7 D	Ladak	19.6
17 A	Ferax	22.3
3 D	Cossack	23.2
-	Grimm	23.8

*Each unit of measurement is equivalent to 6.6 microns. The mean diameter is based on 108 lesions.

Figure 5

Transverse section of a susceptible alfalfa leaf made 24 hours after inoculation with L. briosiana, showing extensive mycelial development from the point of penetration at A.

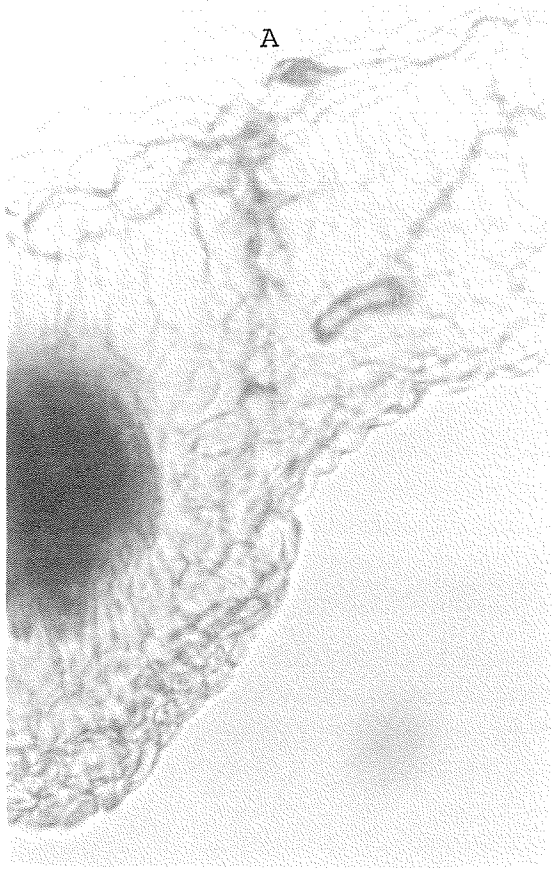
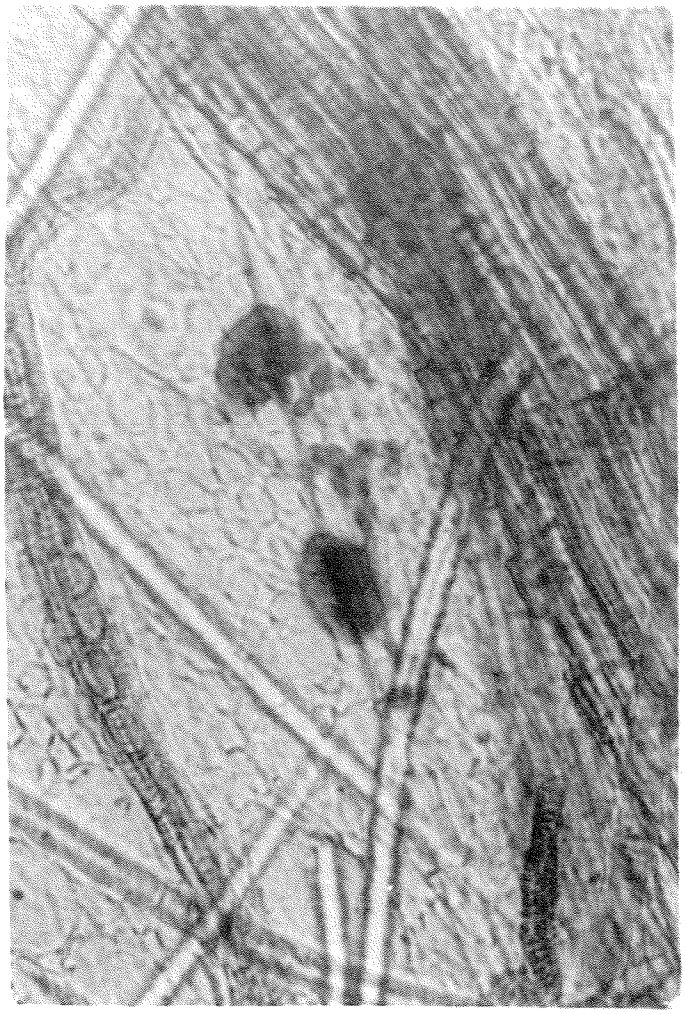


Figure 6

Surface view of a resistant alfalfa leaf made 24 hours after inoculation with L. briosiana showing limited mycelial development from the point of penetration.



clones is significant at the 1% level (L.S.D. = 3.3).

Discussion and Conclusions

The observations made on leaves of alfalfa infected with L. briosiana and subsequently cleared, indicated that differences in resistance could be based on the degree of mycelial extension in host tissue. The results of the lesion measurements agreed with these observations, showing that the size of spot produced depends on the extension of mycelium in the host around the point of infection. The variations in mycelial extension from different infection points on the same leaf could have resulted from two causes. First, there was the time lag from the start to the end of the period of inoculation. In the cleared leaf studies, this was a difference of one hour in 24, and would have resulted in small differences only. In the lesion size estimation, this factor was reduced to negligible proportions by inoculating for a half hour only, and incubating the infected leaves for seven days before taking measurements. The second, and probably the most important

cause of variation could have been caused by genetic variability of the spores which are produced by a sexual process. The observation by Frosheiser (5) that different cultures of the same isolate behave differently under the same conditions supports this possibility.

The estimated means (Table 4) show a continuous variation from resistant to susceptible between the plants tested. This was expected on the basis of the results which Jones (11) obtained with Pseudopeziza medicaginis. This makes it impossible to draw a line between resistant and susceptible plants on other than an arbitrary basis. In spite of this, the method can be used as a measure of resistance to L. briosiana provided that it is not applied too rigorously in an attempt to isolate resistant plants. Because of the variation found when adult leaves are used, it might be better to use seedling leaves, all of an equal age. The method could be used in a genetic study of resistance only if distinct categories of lesion size are produced on the different host genotypes.

PLANT INOCULATIONS

Introduction

Early workers failed to find a satisfactory method of inoculating alfalfa with L. briosiana. The appearance of disease symptoms in the field and penetration of leaves by germ tubes left Jones (10) in no doubt that the organism was pathogenic, but he was unable to get positive results with greenhouse inoculations. Miller (17) could get leaf infection only when the leaf had been killed, and he concluded that the fungus was a saprophyte. Miles (16) found that his pure cultures would not give him satisfactory infections because they produced no spores. His successful inoculations were obtained by using infected leaves which discharged spores down onto the leaves of plants below. Frosheiser (5) had difficulty in obtaining consistent sporulation in cultures. He observed much variation between cultures of the same isolate under identical conditions. Graham and Luttrell (7) also found that the results of their inoculations were erratic. The

reasons they gave for these results were: insufficient inoculum, inferior method of inoculation, and the use of older plants, which were more resistant than seedlings. They developed a method of inoculation using vigorously sporulating petri plate cultures of the fungus on V-8 agar supported over the plants and left for several hours. They sprayed the plants with water and covered them with plastic bags to keep the relative humidity at a high level.

McDonald (14) tried more indirect methods of inoculation. Agar cultures comminuted in a Waring Blendor and sprayed on healthy plants produced no symptoms. Ascospores collected in talc and dusted on the plants produced very few spots, but this proved to be a good method of inoculating petri plates. He found that ethylene glycol was toxic to spores. The use of glycerol as a suspending medium and spray gave promising results. Esso Co's "Bayol D" mineral oil was slightly phytotoxic, but spores collected in it germinated well when sprayed on plants.

Frosheiser (5) felt that progress in selection of resistant plants to include in a breeding program was

hindered by lack of a good method of inoculation. As none of the methods reported in the literature have been entirely successful, a new method was developed and compared with three others previously described.

Materials and Methods

An inoculation method using ascospores ejected from cultures of L. briosiana grown on filter paper was developed. The filter paper medium was prepared by autoclaving 2 qt. preserving jars each containing a 6 x 13 inch sheet of #3 Whatman filter paper moistened with 20% V-8 juice. Sterile 20% V-8 juice was added later to any jars which had dried out during autoclaving. The papers inside the jars were inoculated by dusting them heavily with talc containing ascospores of the pathogen. After incubation for eight days at 60° F., the cultures were examined for spore production by suspending a strip of paper inside the jar for about an hour and then examining it under a binocular microscope for discharge spores.

The above method was compared with the Bayol and glycerol inoculation techniques reported as showing promise

by McDonald (14), and with the method using talc as the spore carrier. Spores were collected in Bayol over a period of two weeks by inverting sporulating V-8 agar cultures of L. briosiana over the oil in a petri dish. Spores were collected in a 50% glycerol and water mixture by the same method for a period of a week only, to avoid the mortality encountered in spores kept for longer periods in glycerol (14). Sporulating cultures were inverted over talc for several weeks to get a spore concentration high enough for inoculation purposes.

The Bayol and glycerol (plus gelatin to make a concentration of 2%) suspensions were sprayed on the plants with a DeVilbiss atomiser attached to a small compressor. The talc and spores mixture was dusted on plants previously wetted with a 2% gelatin solution. All inoculated plants were placed for 48 hours in a cabinet in which a high humidity was maintained by periodic fogging. The plants sprayed or dusted with inoculum were grouped at the opposite end of the growth cabinet to those receiving the spore discharge method, to avoid contamination. Each treatment was applied to a plate of water agar at the time of inoculating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the plants to provide an estimate of the spore deposit on the leaves and of spore germination. Data for the comparison of the methods was obtained from the following material:

Test I. One plant of each of five clonal lines was inoculated by each method. After 48 hours incubation, six leaves were picked at random from each plant. The leaf areas were measured and the number of disease lesions counted. Water agar plates inoculated at the same time were incubated at room temperature for 24 hours and then examined for the number of spores deposited and their percentage germination.

Test II. One plant of each of eight different clonal lines was inoculated by three methods (talc excluded). The glycerol concentration in which spores were collected was reduced to 30% and further reduced to 15% when the gelatin was added just prior to spraying. Water agar plates incubated for 24 hours, and six leaves removed at random from each plant immediately after inoculation, were examined to determine the number of spores deposited per unit area. Six leaves were also removed at random from

each plant 48 hours after inoculation to determine the number of lesions on each.

In both tests, spore deposits on agar and on leaves, and lesion totals were compared on the basis of their frequency on equal areas. The criterion of infection was the production of a distinct brown spot by a spore as seen with the aid of a binocular microscope with a magnification of x50.

Test III. Eleven varieties of alfalfa were screened for resistance in the seedling and adult plant stage using the filter paper method. Selection among the seedlings grown in flats was made five days after inoculation and after three days among the adult plants. Selection was made on the basis of the following three groups.

Group 0. Resistant plants or disease escapes. These plants had no spots on them. Many were found around the edges of the flats, and this suggested that they were disease escapes.

Group 1. Moderately susceptible. These plants had small lesions which were otherwise typical of L. briosiana infection.

Group 2. Susceptible. Plants in this category showed large, brown spots which were often surrounded by a chlorotic area. In badly affected cases, the leaves were shrivelled.

Results

The density of spores deposited and the percentage germination of spores applied to alfalfa plants by the four inoculation methods compared in each of two tests are presented in Table 5. Good spore germination was obtained only from the filter paper and Bayol methods of inoculation in the first test. In the second test a more dilute solution of glycerol was used and spore germination was high with all three methods of inoculation. The table also shows that for the filter paper and Bayol methods of inoculation, measurement of spore numbers by the use of agar plates gave close estimates of the spore numbers found on the leaves. On the other hand, the glycerol method gave a very poor correlation between spores on agar plates and spores on leaves. It must be pointed out here that the tendency of spores collected in Bayol to stick together made

Table 5

Percentage germination and density of spores deposited on agar plates and leaves, and the numbers of lesions produced by each of the four methods of inoculation used in the two comparison tests

	Agar Plates				Leaves		
	Germination %		Germinated Spores per sq. in.		Total Spores per sq. in.	Lesions[1] per sq. in.	
	1st	2nd	1st	2nd	2nd	1st	2nd
Filter Paper	96.6	98.0	1858	787	510	214.4	292.1
Bayol	100.0	88.3	13[2]	93[2]	93[2]	12.0	52.9
50% Glycerol	9.0		69			14.8	
15% Glycerol		99.0		1107[2]	77[2]		20.8
Talc			33			2.4	

[1] Average of readings on five clones in the 1st test and eight clones in the 2nd test.

[2] Numbers of spore clusters.

it necessary to consider each clump as an individual spore for germination and degree of infection estimates.

The average numbers of lesions per square inch on the leaves of the five alfalfa clones inoculated in the first test and on the eight clones inoculated in the second test are also presented in Table 5. The filter paper method of inoculation is by far the best method on the basis of lesion numbers per square inch. The average lesion numbers on each plant inoculated may be found in Appendix C.

The results of seedling and mature plant inoculations are shown in Table 6. Of 57 mature plants tested, 52 were susceptible; 5 were moderately susceptible; none were resistant. A total of 1439 seedlings were inoculated. Of these, 1303 were in Group 2 and were discarded; 136 were retained for further testing. Medicago falcata and the varieties Ladak, Provence, and Ranger appeared to be the best sources of resistance to L. briosiana among the varieties examined.

Figure 7 shows the large numbers of lesions formed on alfalfa leaves one week after inoculating them with

Figure 7

A typical degree of infection obtained by inoculating alfalfa with L. briosiana using the filter paper method.

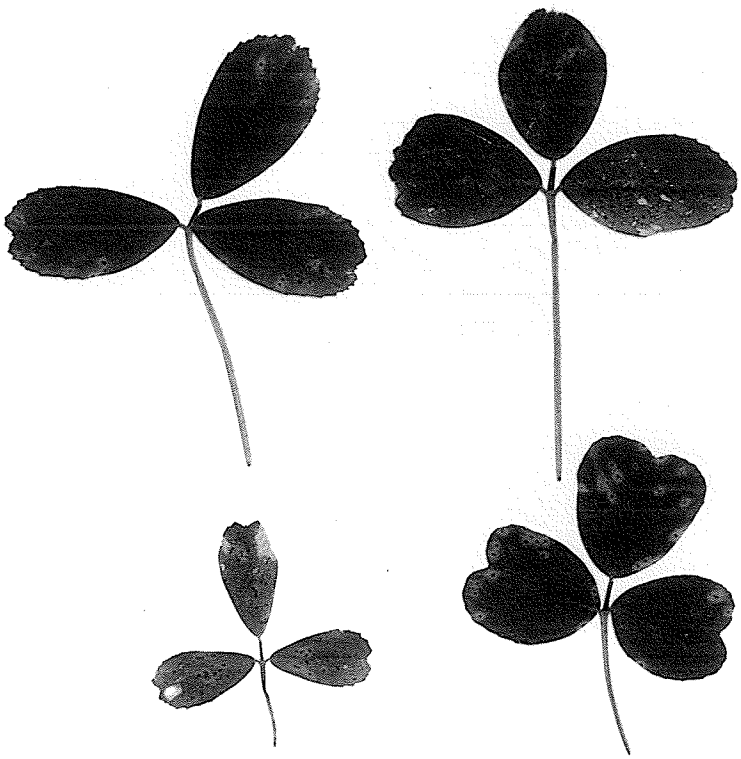

Table 6

Reactions of alfalfa plants to L. briosiana

Variety	Mature Plants Tested*			Seedlings Tested*			Total
	2	1	0	2	1	0	
Atlantic	4	0	0				4
Buffalo	4	0	0	42	0	5	51
Canauto	4	0	0				4
Cossack	1	3	0				4
Du Puits	3	0	0				3
Eynesford				80	2	4	86
Ferax	4	0	0				4
Grimm	4	0	0				4
Ile de France	4	0	0				4
Ladak	3	0	0	27	1	5	36
Narragansett	3	1	0				4
Nomad	3	0	0				3
Provence	4	0	0	48	11	7	70
Rambler				781	29	25	835
Ranger	3	1	0	18	16	3	41
Rheinheissiche	4	0	0				4
Rhizoma				52	0	5	57
Vernal				50	0	0	50
Viking	4	0	0				4
M. falcata				102	13	9	124
M. lupulina				45	0	1	46
M. glutinosa				58	0	0	58

* 2 = very susceptible; 1 = susceptible; 0 = no lesions

L. briosiana by the filter paper method. The leaves are typical of those obtained from Test II.

Discussion and Conclusions

A comparison of the numbers of lesions produced on a unit area of the plants inoculated, indicates that the amount of spotting is no reflection of the disease resistance (Appendix C). The most resistant plant available, R.L. 43, had far more spots than had 17. A, which is susceptible, based on size of lesion as a criterion (see Table 4). A large number of small lesions could infect a larger area of leaf than a small number of large lesions. This makes the use by Renfro and Sprague (19) of the percentage of leaf infected by the pathogen as an estimate of disease resistance somewhat suspect. Their measurement is more a reflection of the sporing ability of their L. briosiana cultures than a measure of resistance of their Medicago species. Their method of inoculation - spreading infected alfalfa debris over plants growing in the field - is itself liable to lead to erratic results.

The use of Bayol oil as a spore carrier for

inoculating alfalfa plants with L. briosiana has been attended by seedling root decay (14). In the two experiments on adult plants reported here, Bayol inhibited internode expansion and caused an increased growth from axillary buds, with the resulting production of a bushy plant. The roots of mature plants appeared to be unharmed. A further disadvantage of Bayol as a spore carrier is the marked tendency of spores to clump together in groups of up to 30 spores when collected in it. In Table 5, the numbers of spores given for the Bayol treatment is actually the number of spore clumps. This change was made because the spore clump is the unit of infection in this case and the spore number has no relationship to the number of lesions produced. A reduction in infection because of this was expected, although there is some compensation owing to infection by a large spore group being almost a certainty. In the first test, a very low infection unit number was reflected in the low spot number. The much heavier spore concentration used in the second experiment led to a better infection. The method appears to be usable if a sufficiently high concentration of spores can be collected

in the oil but cannot be used for reading size of lesion. Clumps of spores produce larger spots than single spores.

The use of glycerol as a spore carrier appears to be limited by a number of factors. Excessive spore mortality occurs when spores are collected in too high a concentration of glycerol. This can be seen in Table 5. In the second test, much better spore germination was obtained by using a lower concentration of glycerol in which to collect the spores (Table 5). A comparison of the spores present on the agar plate and on the leaves (Table 5) indicates that much of the glycerol sprayed on leaves runs off, in spite of the added gelatin. This alone can account to a considerable extent for the low number of spots obtained on leaves using this spray method. The 50% glycerol used in the first experiment was phytotoxic. Many leaf edges on plants sprayed with it turned white after several days and some leaves dried out and died. As the glycerol remained on the leaves as small droplets for over a week, the killing of the leaf tissue may have been due to dehydration. A very heavy concentration of spores collected in glycerol for inoculation in the second comparison test

led to clumping. Spore clumps consisted of six spores at the most; much lower numbers than in clumps in Bayol. A consideration of the disadvantages outlined above, and the low lesion totals per square inch of leaf area obtained by the glycerol inoculation method leads to the conclusion that the use of glycerol as a medium for spore inoculation does not give good results.

The talc method was used only in the first test. Such poor results were obtained that the method was discontinued. The area of leaf infected was very low in all clones given the treatment. It seems likely that when spores are discharged into talc during the collecting period, talc particles stick to the mucilaginous sheath of the spore and prevent it reaching the leaf surface when it is dusted on. A better infection might be obtained if a much heavier dusting were given or if a much greater quantity of spores were collected in the talc before dusting it on the plants.

The filter paper method of inoculation gave satisfactory results on both seedlings and mature plants. It has the added advantage that the inoculum can be stored

in the freezing section of a refrigerator between inoculations. Four inoculations with a week between each were made with the same inoculum before spore discharge fell to too low a level for further use. The technique is a modification of the inoculation method using V-8 agar plates of L. briosiana inverted over alfalfa plants. The present method has advantages over the V-8 plate method in that less work is involved in the preparation of the inoculum, water can be added to the papers to prevent drying out, and the papers can be stored in the refrigerator between inoculations.

The plant screening results indicate that the incidence of resistant plants is very low. Insufficient plants were inoculated to give a reliable indication of the percentage of resistant plants to be found in most of the varieties tested, but M. falcata and the varieties Provence, Ranger and Ladak appear to have a lower incidence of very susceptible plants than the other varieties tested. Of the plants and seedlings retained from the inoculation groups 0 and 1, few can be expected to

be very resistant. Many of these in group 0 were found around the edges of the flats in which they were inoculated and were most probably disease escapes.

SUMMARY

In connection with the development of a method of inoculating alfalfa with Leptosphaerulina briosiana spores, certain aspects of spore discharge and germination were studied. A 16 hour day followed by an 8 hour night at first produced a large increase in spore discharge which started midway through the light period and ended with the onset of the dark period. In further experiments, alternating light and dark periods had no effect on spore discharge rate. It was found that the length of time during which a V-8 agar culture of L. briosiana would sporulate could be considerably increased by placing the culture in a saturated atmosphere. Apparently this effect was due to the prevention of drying out of the agar.

Spore germination was investigated using petri dishes as germination chambers in controlled temperature cabinets. Results indicated that a temperature of 20° C. and a relative humidity of 100% are optimum for germination of L. briosiana spores. Germinating spores formed appressoria at relative humidities of 99% and 98%, but

germination was reduced. At humidities of 95% or less, few spores germinated irrespective of temperature.

Infection studies confirmed previous observations that penetration takes place directly through the epidermis. Mycelium was entirely intracellular, and grew more quickly in susceptible plants. Mycelium in resistant plants remained limited in extent and penetrated only a few cells. Results indicated that an estimate of the extension of mycelium in the host, or of lesion size provides a more accurate assessment of resistance to the disease than the previously used estimate of leaf area affected by the disease.

A new method of inoculating alfalfa with L. briosiana is described. Spores were dusted on filter paper soaked in 20% V-8 juice and incubated at 60° F. Alfalfa plants were inoculated by placing them for 1-2 days in a humid atmosphere beneath wire netting which supported a sporulating culture of the pathogen grown on the filter paper. This method has the advantages of ease of preparation, suitability for storage in a freezer, avoidance of drying out problems, and simplicity of expansion to a large scale,

over the method of growing inoculum on V-8 agar plates which it resembles. The new inoculation method was found to give a much higher number of lesions per unit area than Bayol and glycerol spray methods or the talc dusting method with which it was compared. In a preliminary test, 1500 alfalfa plants of 19 varieties were inoculated by the new method. It was found to give satisfactory results. A very low proportion of resistant plants was found.

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APPENDIX A

A Description of the Design and Use of the Machine used for Spore Collection in the Estimation of Spore Discharge.

Essentially, the machine consists of a platform to support a sporulating culture of L. briosiana, and a revolving table on which is placed a petri dish to collect the discharged spores. The drive mechanism is an electric motor and gear assembly from a 24 hour time switch. A circular piece of perspex was glued to the switch dial to provide a flat table which revolves once in 24 hours, and the assembly was mounted in a small perspex box. A hole 1/4 inch diameter was drilled in the box lid and a sleeve 1/2 inch long was fitted in it so that spores ejected through the hole from a culture inverted on the lid would fall on the revolving table near its outer edge. When a petri dish is centered on the revolving table, a ring of spores is deposited on it in 24 hours. In the experiments reported above, spores were counted under a binocular dissecting microscope by placing the petri dish on a piece of polar coordinate graph paper ruled into half hour sections.

The spore collecting apparatus with a culture in place.

APPENDIX B

The Clearing and Staining Technique used in the Histological Studies.

The clearing and staining method used was a modification of one developed by Noll, as described by Siebert (21). A description of the solutions used and of the technique is given below.

Solution A - 50% Potassium hydroxide in water.

Solution B - 90 ml. Glacial acetic acid.

10 ml. 10% solution of aniline blue in water.

The leaves were heated in solution A on a glycerol bath at 120 degrees C. for 15 minutes. The solution was then drained off and the leaves washed in distilled water for a few minutes. Then the leaves were put in solution B for 20-25 minutes at room temperature. Finally, solution B was drained off and the leaves were washed in distilled water to remove excess stain.

As a result of the above process, the leaf mesophyll is cleared, the xylem is stained blue, and fungal hyphae are stained violet.

APPENDIX C

A comparison of Lesion Numbers per Unit Area on Leaves of Individual Plants Obtained by the Four Inoculation Methods used in Test I

Clone	Inoculation method	Lesions per square inch
R.L. 31 (Ranger)	Filter paper	186
	Bayol	5
	Glycerol	35
	Talc	2
R.L. 38 (Du Puits)	Filter paper	100
	Bayol	9
	Glycerol	14
	Talc	1
R.L. 42 (Narragansett)	Filter paper	361
	Bayol	9
	Glycerol	6
	Talc	4
M. 8 (Ladak)	Filter paper	127
	Bayol	20
	Glycerol	2
	Talc	3
M. 264 (Ladak)	Filter paper	298
	Bayol	17
	Glycerol	17
	Talc	2

A Comparison of Lesion Numbers per Unit Area on Leaves of Individual Plants Obtained by the Three Inoculation Methods used in Test II

Clone	Inoculation method	Lesions per square inch
R.L. 43 (Medicago falcata)	Filter paper	618
	Bayol	22
	Glycerol	11
R. L. 19 (M. falcata X Grimm)	Filter paper	312
	Bayol	29
	Glycerol	28
R. L. 20 (M. falcata X Grimm)	Filter paper	186
	Bayol	15
	Glycerol	21
R. L. 22 (Provence)	Filter paper	384
	Bayol	16
	Glycerol	4
R. L. 25 (Du Puits)	Filter paper	328
	Bayol	45
	Glycerol	5
R. L. 26 (Socheville)	Filter paper	237
	Bayol	74
	Glycerol	21
6 A. (Ile de France)	Filter paper	62
	Bayol	90
	Glycerol	72
17 A. (Ferax)	Filter paper	210
	Bayol	132
	Glycerol	4