The Parasitism of *Colletotrichum lindemuthianum* (Sac. and Mag.) Bri. and Cav.

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Introduction

It has been a matter of common knowledge for many years that some species and varieties of plants are more resistant than others to certain diseases. The discovery of the causes for these differences in resistance has been the object of numerous investigations, and, while much has been learned, the question is still far from solved. It is quite evident from what is known that no single factor can explain resistance in all cases and it is indeed probable that several factors, working together or independently, may be involved in most cases of disease resistance. It is also obvious that a more thorough knowledge of the factors underlying normal parasitism and of the relations normally existing between host and parasite must be obtained before much progress can be made in the solution of this difficult problem. The investigations reported here constitute the first steps in a study of the parasitism of Colletotrichum lindemuthianum (Sac. and Mag.) Bri. and Cav. They were made with the purpose of obtaining information which might be applied to the solution of the general problem of disease resistance in plants.

Historical

The literature on disease resistance in plants has been so thoroughly reviewed by Butler (14) that it will be necessary here only to indicate the trend of the development of our knowledge of

the subject. Early workers, basing their conclusions chiefly on observation, often confused disease-escaping and disease-enduring varieties with disease-resistant varieties. Ward (50), Orton (37) and others soon pointed out and clearly distinguished between these phenomena. Further study of the question has demonstrated that true resistance must depend either on some anatomical character of the host tissue or on the physiology of its protoplasm. As an example of the former, Hawkins and Harvey (31) recently have proved that the resistance of the McCormick potato to Pythium deBaryanum is due to the thickness of the cell walls of the tuber which is sufficient to withstand the mechanical pressure exerted by the fungus. Also Valleau (48) has shown that certain varieties of plums are more resistant to Sclerotinia cinerea because the stomata become plugged with masses of small corky cells.

Ward (50), Gibson (30) and Marryat (36), however, proved for several different species of rust fungi that resistance could not be due to anatomical characters of the host as had been claimed for Puccinia graminis by Anderson (1) and Cobb (17). On the other hand it was shown to depend on the "physiological reactions of the protoplasm of the fungus and of the cells of the host".

Resistance to certain fungous parasites has been correlated with a greater degree of acidity of the sap of host plant tissues. Averna-Sacca (4) found that the resistance of grapes to species of Oidium and Peronospora was correlated with a relatively high acidity of the cell sap.

Comes (18) has correlated the rust-resistance of a variety of wheat known as Rieti with a high acid and low sugar content of the sap. With the discovery by Stakman and his co-workers(43,46) of the numerous biologic forms of stem rust (Puccinia graminis) attacking wheat it became very evident that the degree of acidity of the cell sap cannot explain all the facts of resistance to this rust. The writer (34) pointed out in an earlier paper that the factors responsible for the resistance to the biologic forms of stem rust of wheat must be specific in relation to each form of rust and suggested a tentative hypothesis, based on a possible specific food requirement on part of the fungus and a corresponding specific food substance within the host. It was very difficult to secure any direct evidence for or against this hypothesis as we were unable to culture the rust fungi on artificial media. For further work on the subject it was desirable to secure a fungus, specialized in its relation to host varieties and at the same time capable of growth on artificial media. Since Colletotrichum lindemuthianum appeared to meet these requirements it was selected for study.

Colletotrichum lindemuthianum, the fungus causing the pod spot or anthracnose of beans, was first observed in Popplesdorf, Germany in 1875 by Lindemuth. It was described by Saccardo and Magnus (38) and named Gloeosporium lindemuthianum. A few years later Briosi and Cavara (8) discovered the presence of setae and changed the generic name to Colletotrichum. Since this time the fungus has been the subject of numerous investigations, most of which have dealt principally with its life history and control. Barrus (5), however, in

1911 reported the discovery of strains of the fungus which differed in their ability to attack certain varieties of beans. Edgerton and Moreland in 1916 (27) reported the result of inoculation experiments with eleven collections of the fungus, concluding that there were "a number of different strains, affecting bean varieties differently". Barrus in 1918 (6) published results of additional inoculations with ten collections on 137 varieties of beans, but distinguishes only two biologic forms. In a still more recent publication, Barrus (7) has summarized fully previous literature dealing with this fungus recording also some additional observations and experiments of his own. This paper and other work treating particular phases of the parasitism of the fungus will be referred to in more detail later.

Materials and Methods

All of the inoculation experiments reported here were made in the greenhouse on pure line varieties of field beans (Phaseolus vulgaris L.) furnished either by Mr. F. H. Steinmetz of the Division of Agronomy and Farm Management of the University of Minnesota or by Dr. J. T. Barrett of the California Agricultural Experiment Station. Eight collections of Colletotrichum lindemuthianum were used. These were obtained during the summer of 1920 from the sources indicated below.

Culture No. 2. Collected from the Agronomy plots, University Farm, St. Paul, Minn.

Culture No. 3. Isolated from bean pods obtained from the markets, Minneapolis, Minn.

Culture No. 5. Collected from a market garden, Duluth, Minn.

Culture No. 6. Collected from a garden on the University of

Minnesota Fruit Breeding Farm, Excelsior, Minn.

Culture No. 7. Obtained from the University of Minnesota mycological herbarium, originally obtained from Dr. G. H. Coons, Michigan Agricultural College, East Lansing, Michigan.

Culture No. 9. Obtained from the University of Minnesota mycological herbarium, originally collected on the University Farm, St. Paul, November 1919 by F. J. MacInnes.

Culture No.10. Collected from a garden at Grand Rapids, Minn.

Culture No. 13. Isolated from diseased bean pods sent by Dr. W. D. Valleau from Lexington, Ky.

Culture No. 15. Isolated from a bean seedling that came up infected in the Plant Pathology Greenhouse.

Before comparative inoculations were made, single-spore cultures were started by picking up single spores with a platinum needle from poured agar plates. Stock cultures were grown on green-bean agar and on sterilized bean pods. These were kept either at room temperature or in an incubator at from 20°-25°C. The fungus rarely formed spores on the agar but it fruited abundantly on the bean pods.

Inoculation was effected by atomizing the plants thoroly with a suspension of spores obtained by washing a sporulating bean pod in from 50 to 100cc of distilled water. The inoculated plants were then kept in a moist chamber for 48 hours, after which they were removed and placed on a bench.

Signs of infection appeared in from four to six days, depending upon the age of the plant. Notes were taken, whenever possible, ten days after inoculation. Since the age of the plant has a marked effect on the symptoms, as will be pointed out in more detail later, plants of approximately the same age were used in all comparative inoculations. Any differences in age were taken into consideration in taking notes.

The first series of inoculations were made with culture 2 on sixty-nine varieties of beans. Various degrees of resistance and susceptibility appeared. A brief description of these symptoms with the symbols used in recording them are given below. Representative types also are illustrated in Plate 1 Fig. 1.

Types of anthracnose infection produced by artificial inoculation on bean seedlings in the greenhouse

Susceptible	S+	Plants wilt and fall over in from 5 to 6 days with profuse and extensive production of acervuli. Red color may be entirely lacking.
	S	Large sunken reddish-brown lesions, numerous acervuli.
	S-	Small to medium, slightly sunken reddish-brown lesions - few or no acervuli.
Resistant	R	Small to very small slightly sunken reddish-brown lesions, no acervuli.
	R-	Extremely small reddish-brown lesions no acervuli.

No completely immune plants were found. Very small lesions were produced on the most resistant plants.

The Effect of Age on Symptoms

It was soon observed that the age of the plant influenced greatly the type of lesions produced. Old plants, as well as the old tissue of comparatively young plants, appeared to be more resistant than younger tissue or younger plants. All types of lesions from complete wilting to very small lesions were produced on certain plants. The severity of infection in all cases, however, was inversely proportional to the age and hardness of the tissue. In order to more carefully verify these observations two varieties, 20 and 21, susceptible and highly resistant respectively to culture 15, were planted at intervals of several days. These were then inoculated at the same time with culture 15. The results of this inoculation are given in Table 1. Plate 1 (Figs. 2 and 3) illustrates the effect of the age of tissues on the types of lesions produced in this experiment.

These results show very clearly that the age of the tissue involved determines largely the type of lesions produced on a susceptible variety. A resistant variety, however, is resistant regardless of the age of the tissue. It should be stated here that the resistant variety, (Ruby Horticultural Bush, Minn. Selection 3 B18), used in this experiment is very susceptible to culture 5 as illustrated in Plate II, Fig. 1. The reasons for this effect of age on the development of the disease will be discussed in more detail later. On account of this influence of age on susceptibility, a variety manifesting only a slight degree of resistance was always carefully checked with the specific purpose of eliminating error due to the age factor.

Table 1. Effect of age of host on susceptibility to Colletotrichum lindemuthianum

Variety Number	Age from time of planting	Stage of development at time of inoculation	Results of Inoculation
20	11 days	Just emerged	Complete susceptibility throughout.
21	11 days	Just emerged	Highly resistant throughout, numerous minute brown lesions.
20	18 days	Second pair of leaves unfolding	Complete susceptibility from cotyledons upward-numerous small lesions on hypocotyl. (See Plate I, Fig.3)
21	18 days	Second pair of leaves unfolding	Highly resistant throughout, numerous minute brown lesions.
20	27 days	Third pair of leaves unfolding	Susceptible only on upper node above second pair of leaves - small to minute lesions below.
21	27 days	Third pair of leaves unfolded	Highly resistant throughout, numerous minute brown lesions.
20	35 days	Blossoms not open	Susceptible on very young tissue only.
21	35 days	Blossoms not open	Highly resistant throughout, numerous minute brown lesions.
20	53 days	In full blossom	Susceptible on very young tissue only.
21	53 days	In full blossom	Highly resistant throughout, numerous minute brown lesions.
20	67 days	Bearing several small immature pods.	Young pods badly affected - rest of plant free from large lesions.
21	67 days	Bearing several small immature pods.	Highly resistant throughout, numerous minute brown lesions.
20	83 days	Bearing both old and young pods.	Young pods badly affected, small lesions on older ones.
21	83 days	Bearing both old and young pods.	Highly resistant throughout, numerous minute brown lesions.

From the varieties inoculated with culture 2, nine susceptible and seven resistant ones were selected for use as differential hosts on which comparative inoculations were made with the remaining seven cultures. The results of these inoculations are summarized in Table II.

An analysis of these data shows clearly that there are at least four different biologic forms among the eight cultures tested. Cultures 3 and 5 are identical insofar as could be determined with the differential hosts used. The same is true of 7,10,12 and 15. Cultures 5 and 6 are each distinctly different from any of the others. The biologic form represented by cultures 7,10,12 and 15 differs from the remaining three on the basis of the reaction on ten of the differential hosts. Cultures 3, 5 and 6 are distinguished on the basis of differences in reaction on two hosts in each case with the exception of culture 5 which differs in reaction on three hosts. These differences are distinct and constant and are deemed sufficient for designating the cultures as separate biologic forms. Barrus (6, p.597) presented similar data from his inoculation experiments but concludes "that we must regard each of these several cultures as similar either to A or to B and that we have been concerned here with only two strains of the pathogene". The writer does not consider this justifiable because if we consider difference in parasitism a sufficient basis for distinguishing biologic forms, a difference cannot justly be disregarded even if manifested on one host only. Furthermore these forms may act differently on many other untried bean varieties.

The Morphology of the Biologic Forms

From the data presented above it is quite obvious that these biologic forms are distinct entities as indicated by the reactions on differential hosts. It is desirable also to know if there are

Table II. Infection capabilities of eight collections of Colletotrichum lindemuthianum on sixteen differential hosts*

Host Acc. No.	Variety Inoculated	Culture used for inoculating							
		2	3	5	6	7	10	12	15
2	Brown Swedish Minn.Selection 132	S 2/8	S 2/20	S 5/55	S 2/30	S 1/6	S 4/27	S 1/5	S 3/34
3	Zebra Minn.Selection 135	R 2/23	R 1/4	R 5/49	R 2/19	S 1/5	S 3/13	S 1/6	S 5/31
4	Brown Swedish Minn.Selection 134	R 2/18	R 1/4	R 5/48	R 2/19	S 1/7	S 1/6	S 1/6	S 3/23
7	White Kidney Minn.Selection 1077	S 4/19	S 1/9	S 1/3	S 1/9		R 1/1	R 1/5	R 1/12
13	Navy type Minn.Selection 1083	R 3/27	R 1/6	R 4/19	S 2/10	S 1/5	S 1/1	S 1/6	S 1/3
14	Ruby Horticultural Bush Minn.Selection 98	S 3/28	S 1/10	S 3/22	S 2/22	R 1/6	R 1/5	R 1/6	R 1/5
15	Improved yellow Eye Minn.Selection 1096	S 3/21	S 1/12	S 1/2	R 4/18	R 1/6	R 2/8	R 1/6	R 1/1
16	Red Indian Minn.Selection 1101	S 2/12	R 1/8	R 4/55	R 2/18	S 1/6	S 2/15	S 1/4	S 3/24
20	Large Garaypata Minn.Selection 119	R 2/9	R 1/5	R 4/39	R 2/13	S 1/6	S 1/2	S 1/10	S 10/81
21	Ruby Horticultural Bush Minn.Selection 3B-18	R 2/23	S 1/9	S 5/48	R 3/27	R 1/4	R 1/7	R 2/16	R 10/92
27	Improved Goddard Minn.Selection 1098	S 2/15	S 1/3	S 5/50	S 2/18	R 1/6	R 1/5	R 1/6	R 4/29
28	Red Kidney Minn.Selection 156	S 2/16		S 5/61	S 1/9	R 1/6	R 1/5	S 1/6	R 4/26
43	Long Yellow Minn.Selection 142	S 3/38	S 1/6	S 4/38	S 1/7	S 1/7	S 1/6	S 1/6	S 1/5
65	Navy type Minn.Selection 69	S 1/7	S 1/7	S 1/1	S 1/11	S 1/5			S 1/5
80	Pink	R 1/10	R 1/10	R 10/124		R 1/5			
81	Red Mexican	R 2/16	S 1/6	S 2/10	R 2/17		S 1/6	S 1/6	S 1/5

* The numerator of the fraction indicates the number of separate inoculations, and the denominator indicates the total number of plants inoculated.

R = Resistant

S = Susceptible

(See Page 6)

differences in morphology. Levine (43 p.484-486, 35) has shown that the biologic forms of Puccinia graminis Erikss. and Henn. can be distinguished on the basis of spore size, provided that sufficient spores are measured and that they are produced under normal and uniform conditions. Burger (13) found a great difference in size of spores of several strains of Colletotrichum gloeosporioides Penz. The mean spore size in microns for the several strains ranged from 11.54 x 5.52 to 30.34 x 6.45. LaRue and Bartlett (33) also have distinguished on the basis of spore size numerous distinct strains within the species Pestalozzia guepini Desm. A series of comparative spore measurements were made to determine if there were any such differences in the size of spores of these biologic forms. Cultures 6 and 15, two forms differing widely in parasitism were used. The data from these measurements are summarized in Tables II and III. All measurements were made with a No. 6 Leitz objective using a screw micrometer. With this combination one space on the rotating drum was equal to .25 microns. It was found after a number of trial measurements that the biometrical mode could readily be determined by measuring one hundred spores for length and fifty for width. The data presented here are based on the measurement of one hundred spores for length and sixty-four for width with the exception of three cases where fifty spores only were measured for width.

The comparative spore measurements of the two forms were made as nearly as possible under the same conditions. Thus the spores taken from sterilized bean pods were from two normal cultures of the same age which had been incubated at the same temperature. Also those spores taken from susceptible hosts were from the same variety inoculated the same day and kept in the same greenhouse. It can be seen from an

analysis of the data in Tables III and IV that, although there seems to be an appreciable difference in spore size when measured from sterilized bean pods, this difference does not hold when the spores are taken from susceptible host plants. In fact the relation of size is completely reversed. The spores of culture 15 which were smaller than those of culture 6 on sterilized bean pods become larger when developed on the host plant. The effect of the medium in this case on the modal spore length is graphically illustrated in Fig. 1. The data indicate that the medium has a marked influence on spore size and that the medium does not necessarily affect both strains in the same manner. Burger (13) found the same to be true for certain strains of C. gloeosporioides.

Since the medium may affect differently the size of spores of different biologic forms, it would appear that, in order to be significant, any differences in spore size must be consistently greater than the variations in size due to the influence of media. It seems, therefore, that the differences in morphology of the two strains measured, as indicated by spore size, are so small and are so readily and specifically influenced by the medium that they are of little or no practical significance.

The Physiology of the Biologic Forms

The effect of temperature

Edgerton (26) was the first to study the temperature relations of C. lindemuthianum. He found that the fungus was very sensitive to high temperatures. He gave the optimum and maximum temperatures for growth as 22° to 23°C. and 30° to 31°C. respectively. Barrus (7) has recently corroborated Edgerton's results. The minimum, optimum

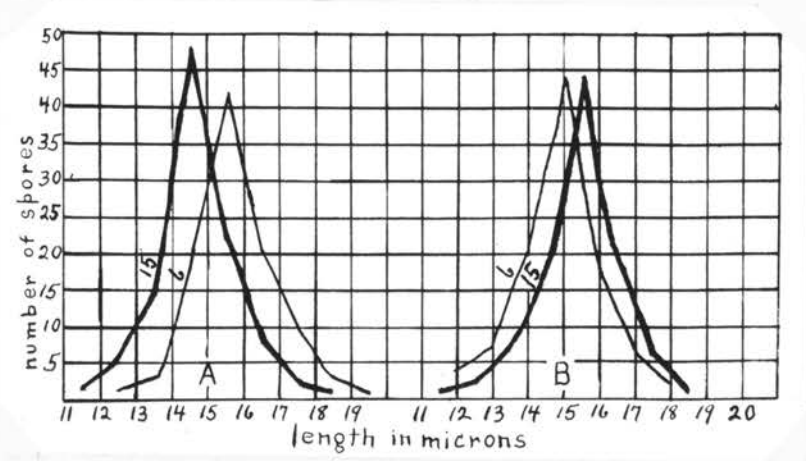

Fig. 1. Graphs showing the influence of the medium on the length of spores of two biologic forms of Colletotrichum lindemuthianum.

Table III. Table of Spore Measurements

Culture No.	Source of spores	Mean length in microns	σ	Mean length in microns	σ
15	Sterilized bean pod. Room temperature.	14.70 ± 0.075	1.12 ± 0.053	5.11 ± 0.036	0.545 ± 0.026
6	Sterilized bean pod. Room temperature.	15.72 ± 0.077	1.14 ± 0.054	5.58 ± 0.036	0.428 ± 0.025
15	Susceptible host 3-2-22	15.01 ± 0.075	1.26 ± 0.053	5.49 ± 0.057	0.678 ± 0.040
6	Susceptible host 3-2-22	14.89 ± 0.080	1.19 ± 0.056	5.41 ± 0.047	0.562 ± 0.033
15	Green bean agar Room temp.	14.31 ± 0.063	0.95 ± 0.044	5.87 ± 0.055	0.655 ± 0.039
15	Susceptible host 3-6-22	15.44 ± 0.077	1.141 ± 0.054	5.86 ± 0.065	0.776 ± 0.046
15	Green-bean agar 13°C	14.63 ± 0.070	1.04 ± 0.050	5.69 ± 0.046	0.550 ± 0.033

Table IV. Calculated differences in spore sizes under various conditions

Culture No.	Source of spores	Variable factor	Difference in mean length	Difference in mean width	Difference in mean length divided by probable error of the difference	Difference in mean width divided by probable error of the difference
6 and 15	Sterilized bean pod	fungus	1.02± 0.107	0.53± 0.050	9.65	10.60
6 and 15	Susceptible host;variety No. 2.	"	0.12± 0.109	0.08± 0.073	1.10	1.23
15	Sterilized bean pod and green bean agar	Medium	0.39± 0.097	0.76± 0.065	4.00	11.66
15	Green bean agar; room temperature and 13°C.	Temperature	0.38± 0.094	0.18± 0.071	3.40	2.53
15	Susceptible host;variety No.2. 3-2-22 3-6-22	Theoretically none.An attempt to duplicate conditions	0.43± 0.107	0.37± 0.086	4.02	4.30

and maximum temperatures were given as 0°, 22° and 34°C. respectively. Edgerton (l.c), although reporting no differences in temperature relations between biologic forms of C. lindemuthianum, was able to distinguish between two biologic forms of Glomerella cingulata (Stonem.) S. and V.S., by means of temperature relations. To determine if the biologic forms used in this work could be distinguished on the basis of reaction to temperature a series of tests were made as follows. Petri dishes of uniform size were poured with uniform amounts of the same preparation of green-bean agar and were allowed to stand for 48 hours. Duplicate plates were then inoculated in the centre with fresh spores of cultures 5,6 and 7, taken from sterilized bean pods. The plates were incubated at room temperature for 48 hours before distribution to the various temperatures. The diameter of each colony was measured at the end of ten days. The results of the experiment are illustrated graphically in Fig. 2. The differences in the amount of growth made by the three biologic forms at the various temperatures were very slight and without a doubt fall within the limits of experimental error. The difference in growth of two colonies of the same strain was often much greater than the average difference between the strains. With loosely fitting petri dishes, evaporation from the agar takes place rapidly, and often unevenly, especially at the higher temperatures. The rather low optimum obtained in this experiment can no doubt be accounted for by the drying of the agar in the 23° incubator near the end of the experiment. Several non-comparative tests indicated an optimum of 22° to 23°C. which agrees closely with the results of Edgerton and Barrus given above. (Plate III.)

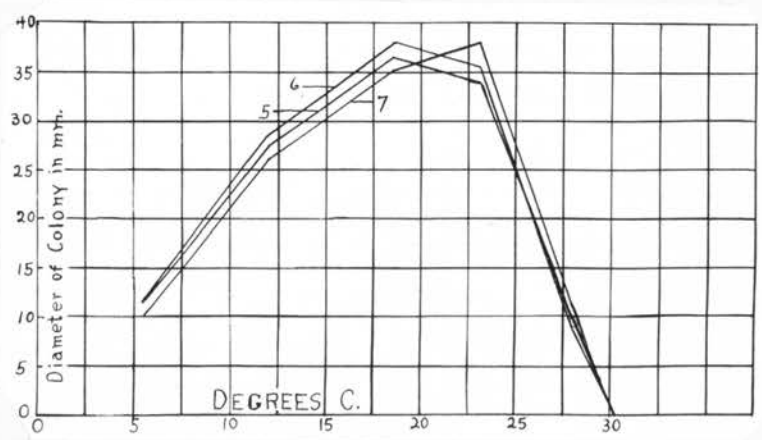

Fig. 2. Graph showing the influence of temperature on the growth of three biologic forms of Colletotrichum lindemuthianum on plates of green-bean agar.

The effect of the medium

The cultural characters of microorganisms on differential media have been used by various workers to distinguish between species or forms which could not readily be differentiated on a morphological basis. Appel and Wollenwebber (2) and Sherbakoff (40) have demonstrated clearly the value of this method of identification for species of Fusaria. Thom (47) also has been able to distinguish morphologically similar species by cultural characteristics on different media. The use of differential media is universal in bacteriology. It seems reasonable, therefore, to suppose that if sufficiently uniform conditions could be maintained, the biologic forms of C. lindemuthianum might be distinguished by their reactions on various media. All attempts, however, to do this with the usual nutrient agars have been unsuccessful. Several of the biologic forms have been grown side by side on several different agars in test tubes and petri dishes with this purpose in view. In several instances apparent differences were observed, but in as many cases there were greater differences in duplicate cultures of the same biologic form on the same medium. (Plate IV.) Nor could any constant differences be detected in the character of growth of the various forms on sterilized bean pods. Careful and long continued observations have shown that slight changes in moisture content of a medium will often produce much greater differences in growth characters in the same biologic form than have ever been observed between any of the different forms on any of the agars used. For example, three tubes of freshly prepared green-bean agar were inoculated with culture 15, and five days later the culture was sporulating profusely. Seven days after the first inoculation other tubes of the same lot were inoculated with spores from the same original culture but a very different type of growth was produced

with no sporulation whatever.

It might be argued that this change in growth characters was due to a loss of the ability to sporulate. Such variations have been reported by Dastur (19) for certain species of Glomerella. That this was not the case is indicated by the fact that abundant sporulation occurred when the culture was transferred to sterilized bean pods.

Since Pasteur in 1861 first demonstrated the specific action of Penicillium glaucum towards the dextro-and levo-tartaric acids, numerous cases of specific relationships between microorganisms and carbohydrates containing assymetric carbon atoms have been reported. (21,24) A specific reaction towards the common sugars constitutes an important means of identification of bacteria. (28) To find whether or not biologic forms of C. lindemuthianum differed in their action on such compounds, three biologic forms were cultured on a modified Czapek's solution in which the sucrose was replaced by .8 percent of a given sugar or related compound. The carbohydrates used were specially purified biological products prepared by the Speciality Chemical Company, Highland Park, Illinois. These were dissolved in water and sterilized by filtration thru Mandler filters. Then the required amount was added by means of sterile pipettes to previously sterilized flasks of Czapek's solution from which the sucrose had been omitted. Duplicate 200 cc flasks, containing 50 cc of the solutions prepared in this manner, were inoculated with fresh spores and incubated at 30° to 35°C. for two months. At the end of this period the dry weight of the mycelium, produced by each biologic form on the various media, was determined. Table V shows the results of the experiment.

Table V. Growth of three biologic forms of *Colletotrichum lindemuthianum* on different carbohydrates

Carbohydrate	Dry weight of mycelium in mg.		
	Culture No. 5	Culture No.6	Culture No.15
Mannose	136	132	100
Maltose	76	66	66
Raffinose	116	88	92
Rhamnose	78	54	50
Xylose	52	56	28
Levulose	120	104	132
Mannitol	96	122	50

Although some growth was made by all three biologic forms on all the carbohydrates used, there was a remarkable difference in the amount of growth of the different biologic forms on certain of the solutions. The differences were especially marked on xylose and mannitol where the amounts of mycelium produced by cultures 5 and 6 were approximately twice that produced by culture 15. There was also a decided difference in the type of growth produced by the two forms on these solutions. (Plate V.)

The effect of the concentration of hydrogen ions in the medium

It has been demonstrated in numerous cases that microorganisms react strongly to very slight changes in the concentration of hydrogen ions in the culture medium. (52) Furthermore, the reaction of different organisms to different hydrogen-ion concentrations shows great variation. C. lindemuthianum according to Edgerton (25) is very sensitive to acids and grows best in an alkaline medium. Edgerton's tests were made on agar titrated to various degrees of acidity and alkalinity as indicated by Fuller's scale. Since the reliability of Fuller's scale as an index of the true acidity of a medium is somewhat questionable (52,15), it was thought desirable to repeat these tests using the concentration of hydrogen ions as a criterion. Since the fungus grew readily on Czapek's solution, this medium was chosen for the work. A series of solutions of different pH value was made up by adding to Czapek's solution various amounts of N/5 HCl and N/20 KOH, following approximately the curbe given for this medium by Karrer and Webb. (32) The pH value of each member of the series was first tested colorimetrically by the method given by Clark (16) and determined more accurately on a potentiometer just before being inoculated.

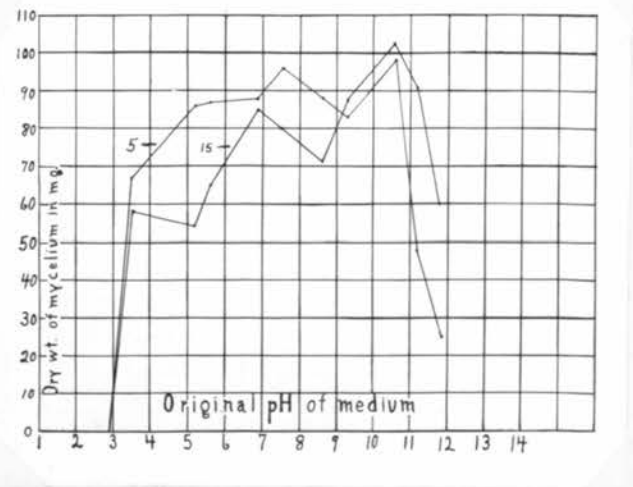

Fig. 3. Graph showing the growth of two biologic forms of Colletotrichum lindemuthianum, cultures 5 and 15 on Czapek's solution at various hydrogen-ion concentrations. Plotted on pH value of the medium at the time of inoculation.

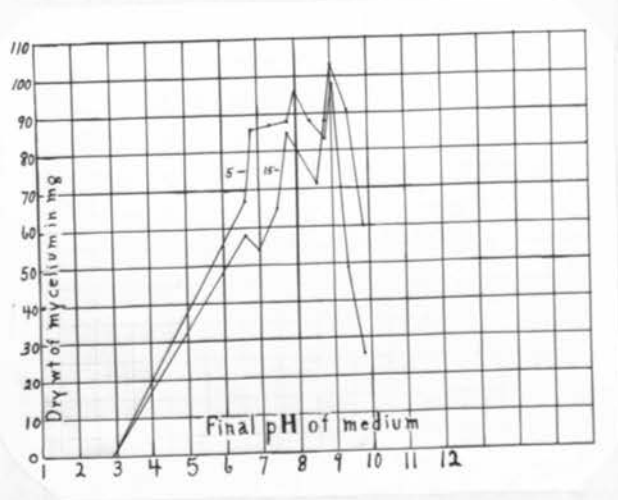

Fig. 4. Graph showing the growth of two biologic forms of Colletotrichum lindemuthianum, cultures 5 and 15 on Czapek's solution at various hydrogen-ion concentrations. Plotted on the pH value of the medium after incubation for two months.

The cultures were made in triplicate on 50 cc of the medium in 150 cc Erlenmeyer flasks. A comparative test was made of two biologic forms, cultures 5 and 15. The cultures were incubated at from 20° to 25°C. At the end of two months the dry weight of the mycelium produced was determined. The results are summarized in Table VI and are illustrated graphically in Figs. 3 and 4.

The fungus obviously grows best in an alkaline medium. No growth was made by either biologic form in a medium with a greater concentration of hydrogen ions than pH 3.5, although some growth occurred in the most alkaline medium used, pH 11.8. In a preliminary experiment in which the mycelium produced was not weighed a few small colonies were formed at pH 11.9 but none at pH 12.2. Not only did the fungus grow better in an alkaline medium but where growth in an acid medium occurred the acidity of the medium was decreased. There was also a decrease in alkalinity of the more alkaline members of the series. This latter change might have been due to the absorption of CO_2 from the air as no precautions were taken to prevent this and no blanks were run.

The curves of growth of the two forms were, in general, similar. There appears to be a "double optimum" for both forms, but one crest did not occur at exactly the same point for both forms.

The Plasticity of the Biologic Forms

It is important practically as well as interesting scientifically to know whether the parasitic capabilities of these biologic forms are stable and constant or whether they are plastic and variable. Eriksson (29) is of the opinion that the parasitic capabilities of biologic forms of *Puccinia graminis* are more or less modified by

Table VI. Growth of Colletotrichum lindemuthianum on Czapek's solution at various pH values.

Culture 5			Culture 15		
pH of medium		Dry wt. of mycelium in mg.	pH of medium		Dry wt. of mycelium in mg.
Original	Final*		Original	Final	
1.2	1.2	0	1.2	1.2	0
1.5	1.4	0	1.5	1.4	0
1.9	2.0	0	1.9	2.0	0
2.0	2.2	0	2.0	2.2	0
2.9	3.0	0	2.9	3.0	0
3.5	6.6	87	3.5	6.6	58
5.2	6.8	86	5.2	7.0	54
5.6	7.3	87	5.6	7.5	65
6.9	7.8	88	6.9	7.8	85
7.5	8.0	96	7.5	8.0	95
8.6	8.4	88	8.6	8.6	71
9.3	8.8	83	9.3	8.8	88
10.6	9.0	98	10.6	9.0	103
11.2	9.4	48	11.2	9.4	91
11.8	9.8+	26	11.8	9.8+	60

*Determined by colormetric method only.

association with various hosts as well as by environmental conditions. Ward (49,51) also thought he was able to change the parasitic capabilities of the biologic forms of the brome rusts (P. dispersa) by growing them for some time on the proper host. It has been conclusively proven, however by Stakman et al,(44,45) that the biologic forms of Puccinia graminis are constant and are not easily modified. But since C. lindemuthianum is a facultative saprophyte it would be only reasonable to suppose that it might more readily be modified than a strictly obligate parasite. Experiments were made, therefore, to determine the point.

There are two ways by which, theoretically, such a change might be brought about. First, by consecutive transfer of the pathogene from a susceptible variety to increasingly resistant ones, i.e. the use of the so-called "bridging hosts". Second, by growing the fungus for several consecutive generations on a resistant host.

The conception of bridging hosts was first introduced by Marshall Ward (51). He concluded from inoculation experiments that a biologic form of P. dispersa which would not attack a given species of Bromus, when transferred directly to it from another given species, could be made to do so, if it was first transferred to a third one, intermediate taxonomically between the other two. The material used in this investigation offers nothing strictly analogous to the above. A problem, however, which is fundamentally similar exists. Thus variety 20 is highly resistant to culture 5 and very susceptible to culture 15, while variety 21 is very susceptible to culture 5 and highly resistant to culture 15. Variety 43 is very susceptible to both culture 5 and 15, and theoretically might serve as a bridging host for either of these biologic forms. Numerous attempts were made to infect resistant varieties, by first transferring the fungus to such theoretical bridg-

ing hosts. Several combinations of varieties were used, but all efforst were unsuccessful. The virulence of the parasite never was increased as a result of its development on such hosts.

In testing the second method, in as much as the fungus is known to live over in the seed, it was realized that to avoid chances of error it would be necessary to have absolutely clean seed. For this experiment a variety (Pink), the seed of which was obtained from California*, was used. In order to avoid other chances of error the experiment was made in a greenhouse in which no other beans were grown. The procedure was as follows: The plants were inoculated and incubated in the usual manner. When numerous small to medium brown lesions had appeared, a stem was cut into sections about one half inch in length. These were immersed from three to five minutes in mercuric bichloride (1-1000), then removed and washed in sterile distilled water and plated in agar. After about 48 hours the fungus had begun to grow thruout the tissue of most of the segments of bean stem which were soon covered with spores. A distilled water suspension of these spores were made and used for inoculating another set of plants. This process was repeated for six generations without the slightest increase in the virulence of the pathogene. This experiment indicates that a biologic form of C. lindemuthianum does not increase in virulence by constant association with a resistant host.

The Constancy of Host Resistance

Since all efforts to increase the virulence of the fungus failed attempts were made to break down the resistance of the host. Salmon (39) reports experiments in which the resistance of certain species of Bromus to Erysiphe graminis was broken down by injuring mechanically

*This seed was very kindly furnished by Dr. J. T. Barrett.

the surface layers of the plant tissue. Stakman (41) was unable to break down the resistance of various hosts to Puccinia graminis.

Since C. lindemuthianum is a facultative saprophyte, it seemed possible that injury of the host tissue might predispose the plant or tissue to infection. It also seemed possible that an abundance of nutrients in the inoculation drop might give the fungus added infective power. A few experiments were made to test these points. The results are given in Table VII. These experiments were sufficient to convince one that such treatment would not overcome the resistance of a host plant. Subsequent histological studies corroborated this fact.

Spore Germination

The process of spore germination in C. lindemuthianum has been described by Atkinson (3), Edgerton (25), Dey (20), Barrus (7) and others. It is not necessary, therefore, to describe the process at length. Under the most favorable conditions spores may germinate within 8 or 9 hours, but it usually requires somewhat longer; and some may not germinate for days, although, to all appearances, conditions are favorable. One or more germ tubes (seldom more than two) are sent out from any portion of the spore, although they arise most commonly from the side and near the end. The size and length of the germ tubes differ on different media. In nutrient media the germ tubes commonly grow directly into profusely branching mycelium, although appressoria may be formed. In non-nutrient solutions appressoria are formed if the germ tube comes into contact with a hard surface; otherwise the germ tube disintegrates without further development. The appressoria are apparently thick walled and dark brown in

Table VII. Results of efforts to break down host resistance

Host Acc. No.	Variety	Previous Reaction*	Treatment	Result
25	Black and White (1099)	R	Spores sprayed on in Czapek's solution	No appreciable increase in virulence
26	Yellow Indian (1100)	R		
33	Shoe Smith	R		
34	Small White (27-1918)	R		
39	Colorado Wonder	R		
40	Black Turtle Soup (104c)	R		
43	Long Yellow (142)	S		
44	Newport Navy (1084)	R		
37	State Fair Navy (59)	R	Numerous pin pricks over surface of stems	R some browning R around pricks but no typical lesions-no fructification
38	White Wonder (26c-1918)	R		
37	State Fair Navy (59)	R	Checks not inoculated	Tissue around pricks slightly browned
38	White Wonder (26c-1918)	R	"	"
42	Flageolet (1088)	R	Stems split lengthwise	R browning of exposed tissue -no lesions
42	Flageolet (1088)	R		Check similar to above.
79	Arikora	R	Epidermis scraped off one side of stem	R lesions slightly larger where epidermis was removed but no normal infection.
81	Mexican	R		

* Culture No. 2 was used throughout the experiment.

color. The shape is more or less spherical and the diameter is from 6 to 10 microns. The spores may or may not become septate on germination. Under unfavorable conditions germination may be abnormal.

All of the writers mentioned above commented on the difficulty of securing a high percentage of germination, especially in distilled or tap water. Webb (53) was forced to discard C. lindemuthianum from the fungi selected for study of spore germination as affected by hydrogen-ion concentration, because of his inability to secure germination in control solutions. Edgerton (25) and Barrus (7) state that the spores germinate better in nutritive solutions than in distilled or tap water.

In the first spore germination experiments made by the writer, spores were taken from a sterilized bean pod and placed in hanging drops of distilled water. Less than 2 percent of the spores germinated. It was observed that in placing the spores in the hanging drops of water some of the gelatinous matrix surrounding the spores also was introduced. This substance readily dissolves in the water. Since spores never germinate while still imbedded in this matrix, it was thought that some inhibiting substance might be carried over into the hanging drop. If this were true, the substance should be removed if the spores were washed sufficiently. Therefore, a dense spore suspension was made in 15 cc of distilled water, placed in a centrifuge which was run for 2 minutes at 1800 revolutions per minute. The liquid was then poured off. A quantity of spores were then transferred from the bottom of the centrifuge tube to hanging drops of distilled water. Distilled water was again added to the spores in the centrifuge tube. The spores were thoroughly agitated and centri-

fuged for two minutes as before. This process was repeated five times, a mount of spores being made after each washing. For the results of the germination tests see Table VIII. It will be observed that washing did not appreciably increase germination, although a lower percentage of the unwashed spores germinated. Apparently the matrix is not responsible for the poor germination in distilled water. If there is an inhibiting substance in the matrix its action must be specific because it did not inhibit the germination of spores of Fusarium lini when they were sown in the liquid decanted after the first washing.

In an effort to secure a medium in which a uniformly high percentage of germination could be obtained, many germination tests were made with various media and under various conditions. Too much space would be required to give these in full. A few of the essential and representative ones are presented in Table IX.

A number of interesting facts were discovered in these tests. The addition of a small bit of leaf tissue stimulates spore germination to a remarkable degree. In a number of the tests positive chemotropism of the germ tubes was stimulated by the leaf tissue. (Fig. 5.) This is not always evident, due probably to a more rapid diffusion of the stimulating substance throughout the drop. In a few of the tests fresh tissue did not stimulate germination. These cases, however, were rare. Boiled bean decoction did not stimulate germination. Fresh bean juice, on the other hand, was as effective as the piece of tissue. The synthetic nutrient solutions used did not stimulate germination. The fungus, however, will grow luxuriently on Czapek's solution. The best germination was obtained when the spores were plated in nutrient agar.

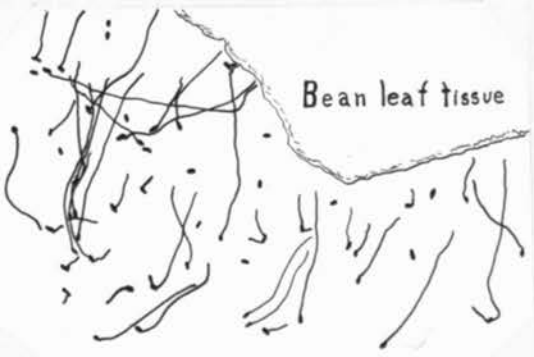

Fig. 5. A camera lucida sketch of germinating spores of Colletotrichum lindemuthianum manifesting chemotropic response under the stimulus of a piece of fresh bean leaf tissue. Less than 1 percent of the checks in distilled water germinated.

Table VIII. The effect of washing on spore germination.

Treatment of Spores	Solution	Percent Germination
Not washed	Distilled water	<1
Washed once	Do.	2-3
Washed twice	Do.	2-3
Washed three times	Do.	2-3
Washed four times	Do	2-3
Washed five times	Do.	2-3
Washed five times	Liquid decanted after 1st. wash	2-3
Fusarium lini	Do.	95-100
" "	Distilled water	95-100

Table IX. Effect of various media on spore germination

Medium	Percent Germination*
Distilled water	6
Tap water	8
Czapek's solution	11
Czapek's solution 1 part Bean decoction 1 part	10
Bean decoction	8
Bean decoction 1 part Distilled water 1 part	5
Bean decoction 1 part Distilled water 5 parts	7
Distilled water plus a small piece of fresh bean tissue	83
Green bean agar	97
Potato dextrose agar	98

* An average of three or more separate tests replicated not less than three times in each test.

In order to learn more about the nature of the stimulating substance found in fresh bean tissue a series of germination tests was made in distilled water in which the effects of fresh tissue of various plants were compared. Tissues from resistant and susceptible bean varieties also were compared. The results, summarized in Table X, indicate that sunflower, corn and wheat tissue can stimulate spore germination but not as much as bean tissue does. Tissue from resistant and susceptible bean varieties apparently stimulates germination equally well. In one test in which spores of two biologic forms were germinated in contact with tissue of one variety there were indications of a difference, but subsequent tests showed that this particular lot of spores would not germinate even in contact with tissue of a susceptible variety. This difference in germination of different lots of spores of the same age under apparently identical conditions has been observed on several occasions. The writer can offer no explanation of the phenomenon.

The fact that boiled bean decoction failed while fresh bean extract succeeded in stimulating germination to a marked degree would indicate that the stimulating substance was destroyed by heat; yet it should be remembered that very good germination was obtained in green-bean agar made from a similar decoction. A fairly high percentage of germination has been obtained also in non-nutrient agar. It is possible that two distinct stimulating factors are involved.

Brown (11) has observed that Botrytis spores germinate readily in "infection drops" on the surface of host tissue, while they germinate very poorly in distilled water. He has demonstrated that the stimulation is due to substances passing into the "infection drop" by exosmosis from the host tissue. The stimulation has been correlated

Table X. Effect of fresh tissue of various plants on spore germination

Medium	Percent germination		Remarks
	24 hrs.	40 hrs.	
Distilled water	<1	3	
Distilled water plus tissue of resistant bean	±95	±95	Normal germination
Distilled water plus tissue of susceptible bean	±95	±95	Normal germination
Distilled water plus sunflower tissue	<1	5	Considerable abnormal budding
Distilled water plus wheat tissue	<1	12	Do.
Distilled water plus corn tissue	<1	10	Do.
Distilled water plus tomato tissue	<1	2	Do.
Czapek's solution	<1	5	Do.
Czapek's solution plus susceptible bean tissue	±95	±95	Normal germination
Czapek's solution plus resistant bean tissue	±95	±95	Do.

with a high conductivity of the solution in the infection drop. The spores, however, do not germinate in tap water which has a relatively high conductivity.

Such exosmosis of stimulating substances into the infection drop may play an important part in infection by C. lindemuthianum. Heavy infection is easily obtained when a distilled water suspension of spores is atomized on to the plant. No extensive experiments along this line, however, have yet been made.

An attempt was made to determine the effect of the hydrogen-ion concentration of the medium on spore germination. The necessity of adding fresh bean tissue or juices to the medium in order to get a high percentage of germination made it difficult to obtain accurate results. Such procedure of course alters the pH value of the medium. Czapek's solution minus the sugar was made up to various pH values with N/5 HCl and N/5 KOH. Spores were planted in hanging drops of these solutions. Fresh bean tissue was added to one series of mounts. In order to avoid bacterial contamination the tissue added was taken from plants grown from sterilized seeds in sterile test tubes and was handled under aseptic conditions. The results are summarized in Table XI. It will be observed that no germination occurred in a more acid medium than pH 3.4 with the exception of less than one percent at PH 2.6 when the tissue was added. A trace of germination was obtained in a solution as alkaline as pH 9.6. The stimulating effect of the tissue was noticeable only between pH 3.4 and pH 7.0.

Germination tests to determine the effect of temperature were made by plating the spores in green-bean agar and incubating at various temperatures. The results are given in Table XII. It is

Table XI. Effect of hydrogen ions on spore germination

pH	Percent Germination		pH	Percent Germination	
	No tissue added	Tissue added		No tissue added	Tissue added
1.2	0	0	5.8	5.	93
1.5	0	0	6.0	<1	78.
2.0	0	0	6.5	<1	58
2.6	0	<1	7.0	<1	11
3.4	<1	76	8.0	3	<1
4.6	<1	75	9.6	<1	<1
4.8	1-3	73	9.6+	0	<1
5.0	4	92	Dist. H_2O	2	90

Table XII. Effect of Temperature on spore germination.

Temperature °C	Percent Germination*			Remarks
	20 hrs.	40 hrs.	100 hrs.	
5	0	0	2	Germinated 95 percent when changed to 17°.
12	7	8	51	Bud-like secondary spores formed.
17	18	51	85	Normal germination for most part but numerous bud-like secondary spores also present.
22	40	47	92	Normal germination.
27.5	46	96	96	Normal germination at first but later becoming abnormal.
32	71	97	97	Abnormal germ tubes; short and twisted.
35	0	0	0	
37	0	0	0	

* An average of several tests

interesting to note that germination takes place more rapidly at temperatures higher than the optimum for growth but that germ tubes do not develop normally at 27.5°C. or higher. The critical temperature evidently lies somewhere between 32° and 35°C.

Infection and Development of the Fungus in the Host

Dey (20) has carefully worked out and described the mechanism of penetration of C. lindemuthianum into a susceptible host. Further development of the fungus was not studied. Nor was the process of penetration into a resistant host observed. Edgerton (25) and Barrus (7) each have described in more or less detail the development of the fungus within the tissue of a susceptible host.

Dey (l.c) concluded that both the spore and the appressorium became attached to the cuticle, the latter by means of a mucilaginous sheath. No such sheath was observed around the spore. This fastened the germ tube at both ends, so that its further growth caused it to curve up, thereby exerting pressure sufficient to cause a slight indentation of the epidermal cell wall. On the under side of the appressorium a slight protuberence was then formed which pushed in the cuticle still farther. From this protuberence a very fine infection hypha developed which ruptured the cuticle. No dissolution of the cuticle was observed indicating that the process was of a purely mechanical nature. After passing thru the cuticle the infection hypha gradually enlarged at the same time causing a dissolution of the cellulose layers. After growing a short distance into the cell the infection hypha formed a vesicle from which one or more branches emerged. These observations were made on stained sections of pods from material imbedded in parraffin.

The writer has endeavored to trace the development of the fungus in both susceptible and resistant varieties. Observations were made on both fresh and imbedded material. Infection was first observed by examining strips of the epidermis, peeled from bean stems which had been previously inoculated and incubated in the usual manner. The further development was observed in imbedded material and in fresh sections cut on a freezing microtome, on a sliding microtome or free hand. The imbedded material was killed at various intervals in medium chromacetic, picric acid (sat. sol. in 70 percent alcohol) or formalin alcohol. This material was imbedded in paraffin in the usual manner, cut in sections ranging from 3 to 13 microns in thickness and stained with light green and diamant fuchsin. A splendid contrast was obtained with this combination of stains. The cuticle, lignified tissue, and host nuclei, as well as the fungous mycelium were stained a deep purplish red, while the cellulose tissues were stained a bright green. The host protoplast also stained red, but it was not sufficiently dense to decrease, to any appreciable extent, the contrast between mycelium and cell wall.

It was found that penetration is accomplished in the same manner and equally well on susceptible and resistant varieties. The writer did not observe the attachment of the spores to the epidermis as reported by Dey (30). Nor was there observed any upward bending of the germ tube due to growth after the appressorium and spore had become fastened. As a matter of fact, in a large majority of the cases observed, the germ tube was extremely short and apparently did not increase in length after the appressorium was formed. (Plate VI. Figs. 1, 2 and 9.) In rare instances a well developed germ tube was observed but in such cases the spore was easily dis-

placed. The appressorium, however, was very firmly attached by a gelatinous matrix. (Plate VI. Figs. 3,4 and 10. Plate VIII. Figs. 3 and 5.) The actual penetration of the cuticle by the infection hyphae could not be seen with sufficient detail to judge whether or not it was accomplished by mechanical pressure. This is very probably the case as no solvent action could be detected and since the appressorium is fastened to the epidermis it is reasonable to suppose that sufficient pressure could be developed to effect an entrance in this way. The infection hypha at the point of penetration is without doubt extremely small.

The development of the fungus in a susceptible host

After penetration of the epidermal cell wall of young susceptible tissue the infection hypha rapidly enlarges, often filling the entire epidermal cell as seen in cross section. (Plate VI. Figs. 2 and 4. Plate VII. Fig. 4.) Further development shows that this enlargement is not of the nature of a vesicle as assumed by Dey (20). It is, on the other hand, merely thickened mycelium, due partly to an abundant food supply and partly to retardation of apical growth, caused by the resistance of the cell wall to penetration. If a thin cross section is cut from an infected stem about four days after inoculation, the nature of the early stages of development can readily be observed. Practically all of the mycelium which develops during the first 48 hours after penetration is of this thickened type and for the most part adheres closely to the cell wall for approximately one-half of its inner circumference. (Plate VI. Figs. 2 and 4. Plate VII. Figs. 1, 2 and 3.) In the rare cases in which the mycelium does extend directly across the cell cavity a still greater enlargement occurs at the point of cell-wall penetration. (Plate VI. Fig. 5. Plate VII. Fig. 1.) This does not agree with the ideas of Barrus (7)

who illustrates small strands of mycelium uniform in size, radiating from a point near the epidermis and passing directly across the cell cavities. Since this mycelium differs greatly from that which is developed later the term primary mycelium has been applied to distinguish it from the latter which will be called the secondary mycelium.

In the penetration by the primary mycelium only a very small hole is made in the cell wall. The hole is usually less than one tenth the diameter of the mycelium. (Plate VI. Figs. 2 and 5.)

Approximately fifty hours after penetration the primary mycelium has made its way thru the cortex, involving a relatively small number of cells and with very little effect on their structure. At about this time, however, many smaller hyphae develop and spread rapidly in all directions. The tissues of the bean stem at this time have a decided water soaked appearance around the point of infection, indicating that the intercellular spaces have become filled with liquid. This secondary mycelium commonly passes directly across the cells and penetrates the cell wall with little or no permanent enlargement or bending. (Plate VI, Figs. 6, 7 and 8. Plate VII. Fig. 5.) A much larger hole is made in the cell wall and there is very little constriction in the mycelium at the point of penetration. Of course all gradations between the two types of penetration occur but the change in type is rapid and complete. The secondary mycelium penetrates the tissue in all directions but in the stems and leaf petioles it advances more rapidly upward and downward. This is due to the fact that the cells of the cortex are much longer in this direction and the mycelium has fewer cell walls to penetrate.

It is quite evident that the differences in character of the primary and secondary mycelium are due to the difference in resistance offered by the cell walls. It has not been possible to determine conclusively the exact means by which cell-wall penetration is accomplished. The mechanical pressure of the growing mycelium undoubtedly is an important factor. (Plate VI. Fig. 3. Plate VII, Fig. 3.) This is especially true of the primary mycelium as indicated by the thickening and bending of the hyphae. The peculiar adherence of the primary mycelium to the cell wall (Plate VI. Figs. 2, 3 and 4. Plate VII. Figs. 1 and 2.) plainly is due to bending of the hypha after the apex has reached the opposite side of the cell.

In the later stages, however, the cell walls have in some way become softened. In sections of material fixed 126 hrs. after inoculation small thread-like mycelium can be seen penetrating the cell walls as if they were extremely soft and gelatinous. (Plate VI. Figs. 6,7 and 8. Plate VII. Fig. 5.). The immediate cause of the softening of the cell walls has not been determined. The fungus very probably secretes an enzyme or some other substance which might have such an effect but as yet no efforts have been made to identify it. If the softening is due to the secretions of the fungus the substance diffuses very rapidly for there is no localization of the softening effect in the immediate vicinity of a mycelial strand. It is also possible that a certain amount of autolysis might take place after an initial number of host cells are attacked by the fungus. Such substances if set free on the penetration of the cell

by the fungus would spread rapidly with the sap which, obviously, flows into the intercellular spaces as has been previously described. Cases have been observed in which the middle lamella quite clearly has been dissolved but this occurs chiefly in the later stages of development and is by no means general.

The growth of the mycelium within the tissues was observed in free hand sections of the living material, made at various intervals after inoculation. The progress of a mycelial strand through two cell walls is illustrated in Plate VI. Fig. 3. The section from which this sketch was drawn was cut free hand 72 hours after inoculation. At the time the section was cut the thickened primary mycelium was abundant but there was very little mycelium of the secondary type. The section was mounted in water, left for three hours, and examined again. Small strands of mycelium had begun to grow in all directions through the tissue and out into the water. When the mycelial strand which is illustrated here was first observed, it had not yet reached the point of penetration a. It reached a at 8:09 o'clock. At 8:30 penetration at a was complete. In the meantime the mycelium had slowly bent over to the cell wall at the side where it remained. There was a slight swelling at the tip, most of which disappeared as soon as penetration had occurred. The distance from a to b was 55 microns. At 9:29 b was reached. This time the mycelium did not bend to one side but became slightly wavy. As soon as penetration was accomplished (9:47) it again became straight. Here, the two cell walls were pulled apart for some distance before the second one was penetrated at c (10:04). The observation of such phenomena convinces one that mechanical pressure is indeed a very important factor in cell-wall penetration by the fungus. The process here outlines resembles very much that described by Hawkins and Harvey (31) for Pythium deBaryanum on the

potato tuber. The rate of growth, however, was only about one tenth of that of P. deBaryanum; and the time required for penetration of a cell wall in the cases observed was approximately twenty minutes, or about four times as great. There is apparently another difference in the greater increase in diameter of the mycelium of C. lindemuthianum and the permanence of this character, as well as that of the bending of the mycelium. Moreover, no softening of the cellulose layers of the cell walls was observed in the potato tubers, while in the bean, such softening is very pronounced in the later stages of development.

There is very little discoloration of the tissue of a susceptible host during the early stages of infection. Cell after cell may be almost completely filled with the large primary mucelium, and yet show no discoloration. And moreover the host protoplasm does not seem to be injured appreciably. At least there is no apparent disorganization or disintegration. Sometimes the fungus almost filled the cell cavities with large thick primary mycelium which apparently developed between the protoplasmic membrane and cell wall, crowding the protoplast into a small compact mass against the cell wall. The organization of the protoplast however was not destroyed. (Plate VI. Fig. 4.) (The above applies, of course, only to young, susceptible tissue. The effect on tissue of a susceptible plant which has become resistant due to age is quite different as will be described later.) There is very little destruction of tissue until the secondary mycelium begins to develop, about 100 hours after penetration. When this happens there is a rapid collapse and discoloration of parasitized cells. About 125 hours after infection the mycelium has a tendency to grow towards the surface and aggregations form just beneath the epidermis. The sporophores arise in palisade layers from these,

rupture the epidermis and produce a slimy mass of spores.

In old tissue the primary mycelium advances much more slowly, the rate depending upon the age of the tissue. If the tissue is sufficiently old, only a very few cells are penetrated. If the fungus has not advanced more than three or four cells beneath the epidermis, about 72 hours after inoculation, discoloration is very pronounced and the mycelium shows signs of disintegration. Later the parasitized cells are completely killed and then they collapse. The mycelium also disintegrates without the production of secondary mycelium or spores. All gradations between the two conditions occur, depending upon the age of the tissues. It is hardly to be doubted that the failure of the mycelium to develop is due to the increased resistance to penetration by the cell wall. Whether it is due to resistance to mechanical penetration or to an increased resistance to an enzyme secreted by the fungus cannot be stated with certainty. The former undoubtedly is of much importance. More extensive biochemical studies would very probably throw some light on the subject.

The characteristic lesions produced by C. lindemuthianum on a susceptible host are typical of what is termed local necrosis. The dependence of the fungus on mechanical pressure for its advance through the tissue and the increased resistance of the tissue as it becomes older very probably explains the cause of the local necrosis. Thus, a young rapidly growing bean pod is infected, but before the fungus has had time to make more than a limited development the tissue has become sufficiently old to check its further advance. That the extent of the necrosis depends largely on the age of the tissue is well illustrated in Plate 1. Figs. 2 and 3. Some varieties of beans

undoubtedly develop such resistance much earlier than others, and it is very probable that some varieties that are resistant to all of the biologic forms owe their resistance to this fact.

The development of the fungus in a resistant host

When a highly resistant host such as variety 21 (Ruby Horticultural Bush, Minn. Selection 3B 18) is inoculated with culture 15, rarely more than one or two cells are attacked. The infection hypha seems to meet an unfavorable environment as soon as it has penetrated the outer epidermal cell wall. Sometimes the mycelium may be checked before it reaches the opposite wall of the epidermal cell; (Plate VIII. Fig.5.) again it may succeed in penetrating this wall but seldom advances farther. (Plate VIII. Figs. 1 and 4. Plate VI. Fig. 10.) At the same time the cell walls and protoplast are stained reddish brown. Cell walls so discolored lose their affinity for light green and take up the diamant fuchsin instead. (Plate VI. Figs. 10 and 11. Plate VIII. Figs. 2,3 and 5.) These cells soon collapse completely. The mycelium and host protoplast both disintegrate and stain a homogeneous deep red with diamant fuchsin. (Plate VI. Figs. 9, 10 and 11. Plate VIII. Figs. 2,3,4,5 and 6.) The host protoplast in this case seems to be extremely sensitive to the invading mycelium, while in a susceptible host the mycelium apparently passes through without any immediate detrimental effects. (Plate VI, Fig. 4.) In the tissues of less resistant varieties the fungus may advance through a considerable number of cells but the end result is the same.

The retarding of the fungus here undoubtedly is due to some quality of the protoplast and not to the resistance of the cell walls although the final result and the discoloration phenomena are somewhat similar to those which occur in the older tissues of

susceptible varieties. The resistance here also is specific, for when this same variety is inoculated with culture 5, complete susceptibility results. (Plate II. Fig. 1.) Here then we have two types of resistance, each due to a different factor; in one case, a non-specific factor i.e. age of tissue, and in the other, a specific factor lying within the protoplast. A recognition of this fact should be of some significance in the breeding of beans for resistance to anthracnose, since in all probability it would be much easier to deal with a single non-specific factor controlling resistance to all biologic forms of the fungus than with a specific factor for each one. Barrus (6) has discovered one variety of merit (Wells' Red Kidney) which seems to be resistant to all the known biologic forms of the fungus and Burkholder (12) has succeeded, by crossing, in uniting the resistance of this variety with the desired characteristics of the White Marrow. It would be interesting to know which factor controls resistance in this case.

The sequence of results described above, namely cell penetration and absorption of food without immediate death and disorganization of host protoplast, in susceptible tissues, and the death and disintegration of both the invading mycelium and a limited number of cells in resistant tissues is strikingly similar to that described by Marryat (36) for Puccinia glumarum on two immune varieties of wheat and by Stakman (42) for Puccinia graminis on hosts highly resistant to its attack. This similarity suggests the possibility of an explanation similar to that offered by Marryat (l.c) for the death and disintegration of the invading mycelium, namely, starvation. But C. lindemuthianum is a facultative saprophyte and should be able to utilize the food products set free by the dead

host cells. This in all probability would be the case provided the host cells were killed before the death of the mycelium. We have no proof, however, that such is the case. In fact all visual evidence tends to indicate that the fungous mycelium disintegrates first and that it is the products of such disintegration that kill the host cells and stain the cell walls. Intact mycelium is never found in a collapsed cell of a resistant host.

Of interest in this connection is the work of Dox and Maynard (22) in which it was shown that when molds were grown upon a fluid synthetic medium the nitrogen was almost completely taken up by the mycelium during the vegetative period and then gradually returned to the medium after growth had ceased. This was accompanied by a corresponding loss in weight of the mycelium. "During this time the mycelium lost its turgidity and the medium became dark in color, although it retained its original clarity. This phenomenon was ascribed to the autolysis of the fungus".* Further work along this line by Dox (23) showed conclusively that the autolysis was due to the exhaustion of the carbohydrate from the medium.

All the evidence obtained from the writer's histological studies indicates that the mycelium on entering the cell of a resistant host is unable to get sufficient food for its nourishment and promptly undergoes autolysis and that the products of this autolysis are fatal to the host cell. If this is true it would seem that there must be some specific difference in the nature of the food materials elaborated in the cells of a resistant plant from those elaborated in the cells of a susceptible plant and a corresponding specificity

*Quoted from a second paper on the subject by Dox (23). The italics are mine.

of assimilation capabilities of the fungus.

Numerous cases of specificity of fungi in regard to assimilation have been reported. The classic work of Pasteur who demonstrated the relation of Penicillium glaucum to the isomeric tartaric acids has been referred to in a previous paper by the writer (34) and is too well known to be repeated here. Dox and Roark (24) have shown a striking difference in the rate of utilization of alpha- and beta-methyl glucoside by Aspergillus niger. The fungus grows readily on the beta-form but makes extremely scant growth on the alpha-form. Furthermore the writer has presented evidence, in this paper, of a difference in ability of the biologic forms of C. lindemuthianum to assimilate certain carbohydrates.

An effort was made to determine whether or not there was a specific food substance in the resistant host which could not be assimilated by the particular biologic form of the parasite. The sap was expressed with a screw press from about 200 seedlings of variety 21, which is highly resistant to culture 15 and very susceptible to culture 6. The plants were frozen prior to the extraction. The extracted sap was sterilized by filtration thru Mandler filters. Duplicate flasks were inoculated with the above mentioned cultures. Both cultures grew equally well on the extract. This at first seems to indicate that no such specific food substance occurs in the resistant plant, but it does not necessarily do so. If, in the process of extraction, a substance capable of assimilation, not found in an available form in the living cell, is set free, the fungus would utilize it and leave the other just as Penicillium glaucum on racemic tartaric acid uses the dextro-form and leaves the levo-form. It is entirely possible that the food substances

are held by the living protoplasm in a particular state which is not available to a given biologic form of the fungus but is made available on the death of the protoplasm. This is a point, however, which is very difficult either to prove or disprove. The mechanism of nutrition and assimilation by protoplasm is a phenomenon about which we know very little and it probably will not be until we know more about this that the problem of disease resistance will be solved.

It should be pointed out that the parasitism of C. lindemuthianum as here described differs fundamentally from that of other non-obligate parasites which have been carefully studied. It has been assumed generally that parasitic fungi, other than the strictly obligate parasites, first killed the host cells then obtained their nourishment from the products of the dead cells. The ability to obtain nourishment from living host cells has been attributed only to the obligate parasites. Brown (10, p.125) expresses this conception as follows: "With highly specialized "obligate parasites" the reactions between host and parasite are of a very recondite nature, and as yet there seems no possibility of subjecting them to experimental analysis.....In the case however of less specialized forms such as Botrytis, the reactions of host and parasite are comparatively simple in virtue of the phenomenon of 'action in advance'. On this account the host plant can be treated as a special kind of culture medium. Thus the problem entails simply the investigation of the growth of the parasite in a culture medium together with that of the enzymic process by which the living plant is converted into a dead culture medium". Such "action in advance" by means of which the living protoplasm is transformed into a dead culture medium has been demonstrated for Botrytis by Brown (9) and for Pythium

debaryanum by Hawkins and Harvey (31). The latter authors conclude from this that for the fungus in question "it seems hardly probable that resistance to fungus attack can be due to the living protoplasm".

C. lindemuthianum, however, does not normally kill the host cell in advance of its growth but, on the other hand, penetrates a large number of cells without any immediate lethal effect and quite obviously obtains nourishment from the living cells. All evidence seems to indicate that resistance is due to the inability of the fungus to obtain nourishment from the living protoplasm.

SUMMARY

1. At least four distinct biologic forms of Colletotrichum lindemuthianum were found as a result of comparative inoculations made with eight cultures of C. lindemuthianum on fourteen varieties of field beans.

2. The size of the lesions produced on a susceptible bean plant, when inoculated with C. lindemuthianum, was inversely proportional to the age of the tissue inoculated; the older the tissue, the smaller the lesion. For this reason the age of the plant must be taken into consideration when interpreting the results of comparative inoculations. The age of the tissue had no effect on the size of the lesions produced on a highly resistant variety.

3. The size of the spores of C. lindemuthianum was influenced by the medium on which they were produced. Different biologic forms, however, were not influenced in the same way by the same medium. The variations in size due to the influence of the medium were sometimes as great or greater than the difference in size of the spores of two forms on the same medium. Therefore the differences in size of spores of the biologic forms are not considered

of any practical significance.

4. The cardinal temperatures for the growth of C. lindemuthianum on agar plates are approximately as follows: 0°C, 22.5°C, and 32°-34°C. The reaction to temperature as indicated by rate of growth on agar plates was approximately the same for three biologic forms.

5. The common nutrient agars were not useful as differential media. In some cases there were apparent differences in growth of two biologic forms on the same agar but more often there were greater variations within replicate cultures of the same biologic form. Such variations were not permanent.

6. Some biologic forms of C. lindemuthianum grew better than others on certain modifications of Czapek's solution. This difference was the greatest when xylose or mannitol replaced the sucrose. The differences on these media were manifested both by the amount and character of mycelium produced.

7. C. lindemuthianum grew best in an alkaline medium. No growth occurred on Czapek's solution more acid than pH 2.9. When growth occurred on an acid medium it was made alkaline. The fungus grew fairly well on Czapek's solution, as alkaline as pH 11.8 at the beginning of the experiment. Such solutions, however, were less alkaline two months after inoculation. The growth of the two biologic forms tested was, in general, similar at the various pH values. There appeared to be a "double optimum", both optima falling on the alkaline side.

8. Efforts to change the parasitic capabilities of a biologic form of C. lindemuthianum were unsuccessful. Also all efforts to break down host-resistance failed.

9. Spores of C. lindemuthianum germinated poorly in distilled or tap water and in certain other solutions either with or without nutrients. Fresh bean tissue or fresh bean juice stimulated spore germination to a remarkable degree. Such tissue also often stimulated positive chemotropism of the germ tubes. The nature of the stimulating substance has not been determined. Good germination was obtained in green-bean agar.

10. The concentration of hydrogen-ions in the medium was not the cause of the poor germination. The effect of various concentrations of hydrogen-ions in the medium was not accurately determined on account of the necessity of adding tissue to induce normal germination. The stimulation of germination, by the addition of fresh tissue, was obtained only between pH 3.4 and pH 7.0.

11. Spore germination occurred between 0° and 32°C. Most rapid germination was obtained at the higher temperatures but at 27.5°C. or above, the germination was abnormal from the first or shortly after. Bud-like secondary spores were formed at 17°C. or lower.

12. Penetration of the epidermal cell wall is accomplished in the same manner and equally well on susceptible and resistant varieties of beans. The spore, when germinating on the host, forms an appressorium almost immediately, which becomes attached to the cuticle by means of a muscilaginous sheath. A small infection thread is sent into the epidermal cell, apparently by mechanical penetration.

13. In young tissue of a susceptible variety the infection hypha rapidly enlarges into a normal mycelium and continues its growth without bringing about the immediate destruction of the host protoplast. When the inner cell wall is reached a resistance is met which retards apical growth, causing the mycelium to become greatly

enlarged and often causes it to bend outward and become closely appressed to the cell wall for approximately one-half its circumference. Penetration finally takes place through a very small hole without any staining or swelling of the cell wall. This process is continued throughout numerous cortex cells until about 100 hours after penetration when the cell walls seem to become softened throughout the region of infection. The mycelium then resumes its normal size and pushes its way through the cell walls with little or no constriction. At about this time the tissue begins to collapse and is usually stained brown. Strands of mycelium collect beneath the epidermis, produce acervuli and rupture the epidermis with a mass of spores.

14. In old tissue of susceptible varieties, the mycelium is retarded in its development by increased resistance of the cell walls to penetration. Such retarded mycelium disintegrates killing the host protoplast and staining the cell wall and its contents reddish brown.

15. Growth of the mycelium and cell-wall penetration was observed in fresh cut sections of living material. The fungus penetrates through numerous cells without killing the host protoplast. The bending and swelling of the hyphae during cell-wall penetration indicate that mechanical pressure is an important factor in the process, especially during the early stages of development.

16. In the early stages of normal infection of a susceptible host C. lindemuthianum obtains its nourishment from the living host protoplast.

17. C. lindemuthianum will grow equally well on expressed juice of resistant and susceptible hosts.

18.In a highly resistant variety, seldom more than one or two cells are attacked. The fungus hyphae soon disintergrate and during this process bring about the death and disintergration of the host protoplast, at the same time staining the entire cell contents, as well as the cell walls, reddish brown. In a less resistant variety more cells may be attacked but the mycelium always disintergrates sooner or later with the same destruction and staining of the host protoplast. This process is interpreted as a nutrition phenomenon, the mycelium being destroyed by autolysis induced by starvation and the resulting products killing and staining the host cells. This is in keeping with what is known to take place when fungi are starved in artificial culture.

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PLATE I.

Lesions produced by Colletotrichum lindemuthianum.

Fig. 1 Representative types of lesions produced on stems of bean seedlings in the greenhouse. See page 6 for explanation of symbols.

Fig. 2 Bean pods of different ages, all of the same susceptible variety, inoculated at the same time with one biologic form of Colletotrichum lindemuthianum. Very small brown lesions only were produced on oldest pod although it was still green and succulent. The specimens were taken from the experiment described in the text.

Fig. 3 A plant of variety 20, inoculated with culture 15 when eighteen days old. Note the complete susceptibility of epicotyl, while on the hypocotyl very small lesions only are produced. The same variety, inoculated when eleven days old, was completely susceptible.

Fig. 1.

Fig. 2.

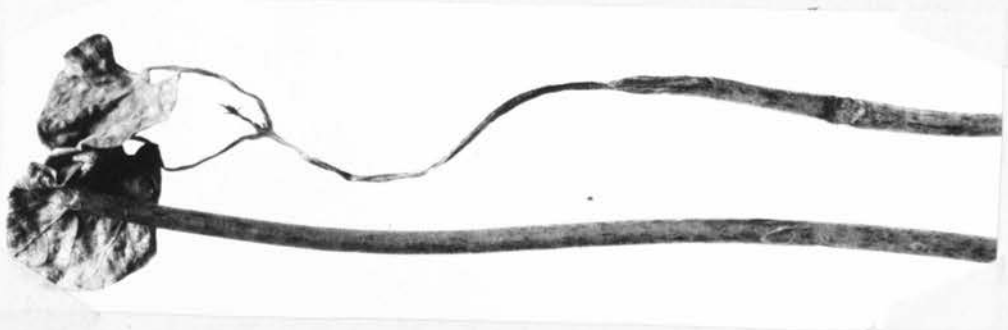


Fig. 3.

PLATE II

The effect of two different biologic forms of Colletotrichum lindemuthianum on two varieties of beans.

Fig. 1 Varieties 20 and 21 inoculated with culture 5.

Fig. 2 The same varieties inoculated with culture 15.

Fig. 1.

Fig. 2.

PLATE III

Colonies of *Colletotrichum lindemuthianum* on plates of green-bean agar, after incubation for ten days at various temperatures as indicated.

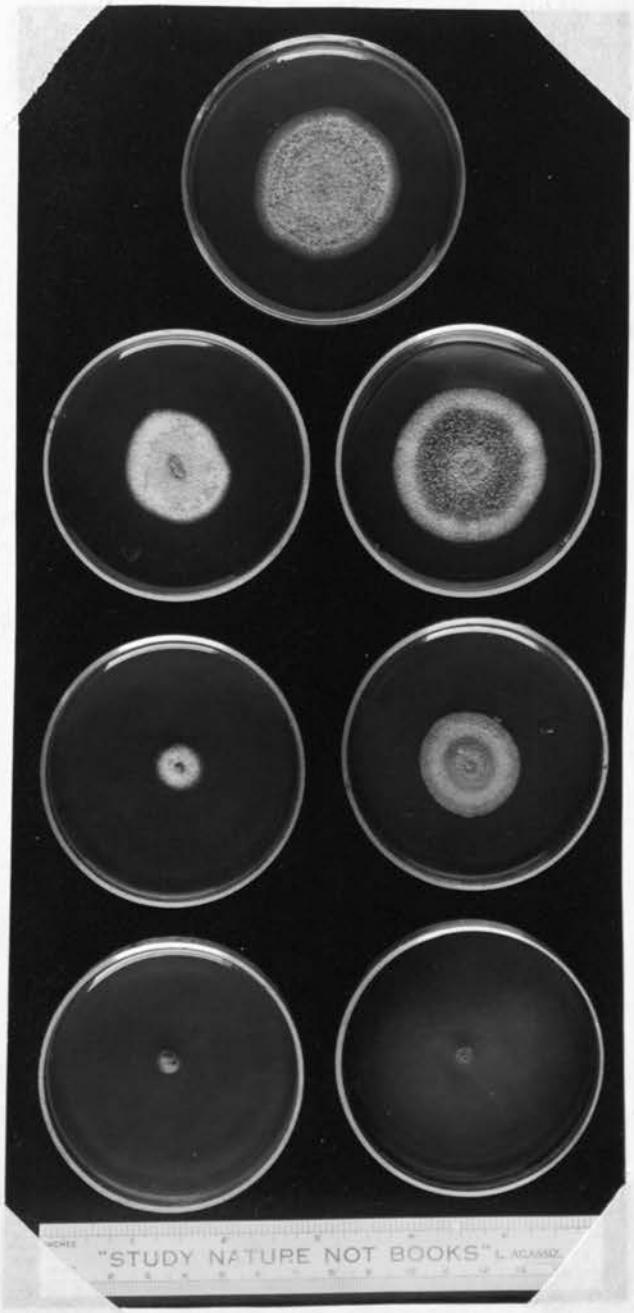
Plate III.

17°-22°C

13°-15°C

6°-8°C

0°-3°C



21°-24°C

29.5°-32°C

34°-35°C

PLATE IV

Variability in cultural characters of *Colletotrichum lindemuthianum* on plates of green-bean agar.

Fig. 1 Three biologic forms on green-bean agar incubated at 11°C. There is apparently a difference between the character of growth made by the three forms.

Fig. 2 The same three biologic forms on the same medium and inoculated at the same time but incubated at 19° C. There is a greater difference between two colonies of the same biologic form than between any two biologic forms.

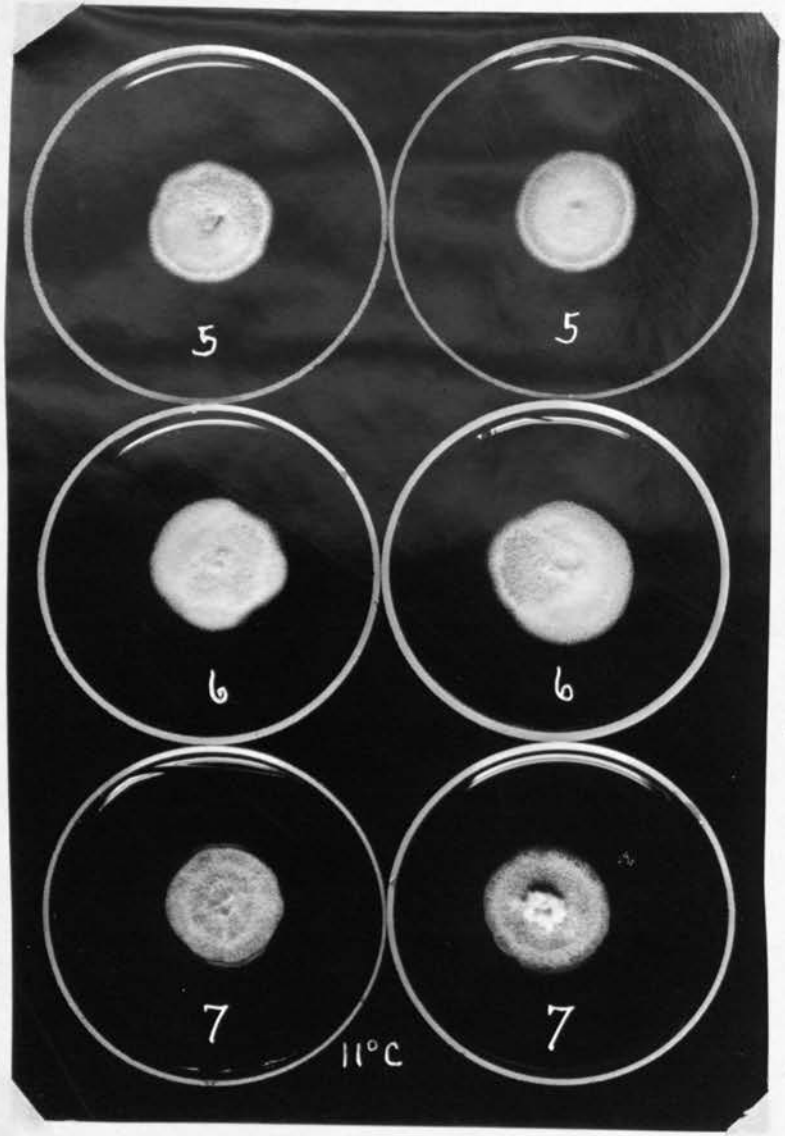


Fig. 1.

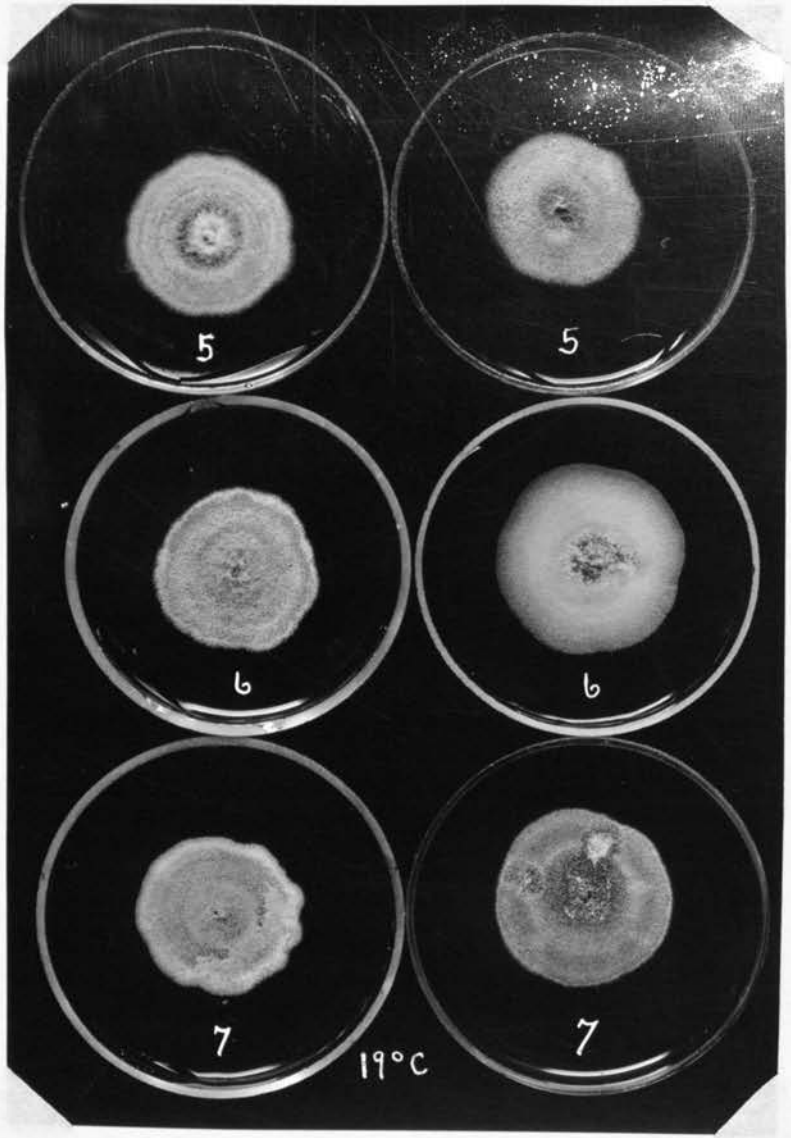


Fig. 2.

Plate IV.

PLATE V

Two biologic forms of Colletotrichum lindemuthianum growing on flasks of modified Czapek's solution containing carbohydrates as indicated. Culture 6 grew fairly well on both solutions, while culture 15 produced only a small quantity of mycelium which was abnormal in appearance.

Plate V.

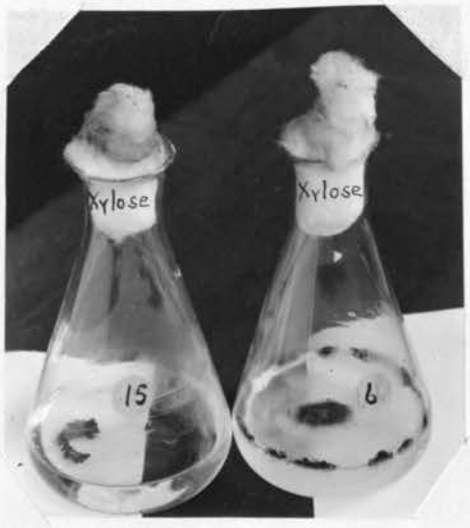


PLATE VI

Camera lucida drawings illustrating stages in the development of Colletotrichum lindemuthianum in susceptible and resistant hosts.

Fig. 1 A surface view of a piece of epidermis, stripped from a susceptible bean stem, showing stage of infection 52 hours after inoculation. The appressorium has been formed very close to the spore, penetration has occurred and the large primary mycelium can be seen in three epidermal cells. No detrimental effects on the host protoplast or cell walls can be detected. x 420 (approx.)

Fig. 2 Infection and subsequent development of the primary mycelium as seen in cross section of a susceptible bean stem 99 hours after inoculation. Observe the thickness of the mycelium in the epidermal cell and its adherence to the walls of the cortex cells. This is very probably due to the retarding of apical growth during the process of cell-wall penetration. Note also the very small hole through which penetration was effected. No swelling or discoloration of the cell walls could be detected. A composite drawing of three sections from a stained slide. A photomicrograph of one of the sections is shown in Plate VII, fig. 2. x 1050 (approx.)

Fig. 3 A sketch showing the successive stages of cell-wall penetration as observed from living material in a cross section of a stem of a susceptible variety. The section was cut free hand, 72 hours after inoculation and mounted in distilled water. The position of the mycelium when it reached the point a is indicated by the dotted line. During penetration it bent slowly over to the wall of the cell at the same time becoming slightly enlarged. It retained this position after penetration was accomplished and there was a distinct indentation of the cell wall that only partially disappeared. While the mycelium was penetrating at b it became distinctly wavy, as indicated by the dotted line, but straightened again after penetration had been effected. The two cell walls were split apart along the middle lamella during the process of penetration at c. For a more detailed description see the text. x 500 (approx.)

Fig. 4 Mycelium in two host cells of a susceptible variety as seen in a cross section of a stem 99 hours after inoculation. The large primary mycelium has almost filled the cells in cross section, crowding the protoplasts to one side. Notice that the organization of the host protoplast is yet intact. From a stained section 12 microns thick. x 1000 (approx.)

Fig. 5 A type of cell wall penetration by the primary mycelium occasionally found in which no bending occurs, but in which there is a permanent enlargement just over the point of penetration. x 1000 (approx.)

PLATE VI (Cont'd)

Figs. 6, 7 and 8. Three typical cases of cell wall penetration by the secondary mycelium as seen in sections of material killed 126 hours after inoculation. The cell walls have become softened and the small mycelium meets very little resistance. It penetrates through a large hole with almost no constriction and very little enlargement. x 1000 (approx.)

Fig. 9 A surface view of a piece of epidermis stripped from a resistant bean stem 72 hours after inoculation. Two cells have been penetrated. The walls and contents of both have been stained reddish brown. Both the mycelium and host protoplast show signs of disintegration. Compare with Plate VI, fig. 1. x 500 (approx.)

Fig. 10 A portion of a cross section of a stem of a resistant variety killed 83 hours after inoculation. The cell directly beneath the appressorium has collapsed and its cell walls and contents stain a homogeneous red with diamant fuchsin. In fresh material they are stained reddish brown. Two fragments of partly disintegrated mycelium can be seen in the second and third cells below the epidermis. These cells have not yet collapsed but the walls are becoming discolored, as indicated by their affinity for diamant fuchsin. x 1000 (approx.)

Fig. 11 A section similar to fig. 10 with the exception that only one cell was penetrated by the fungus. x 1000 (approx.)

Plate VI

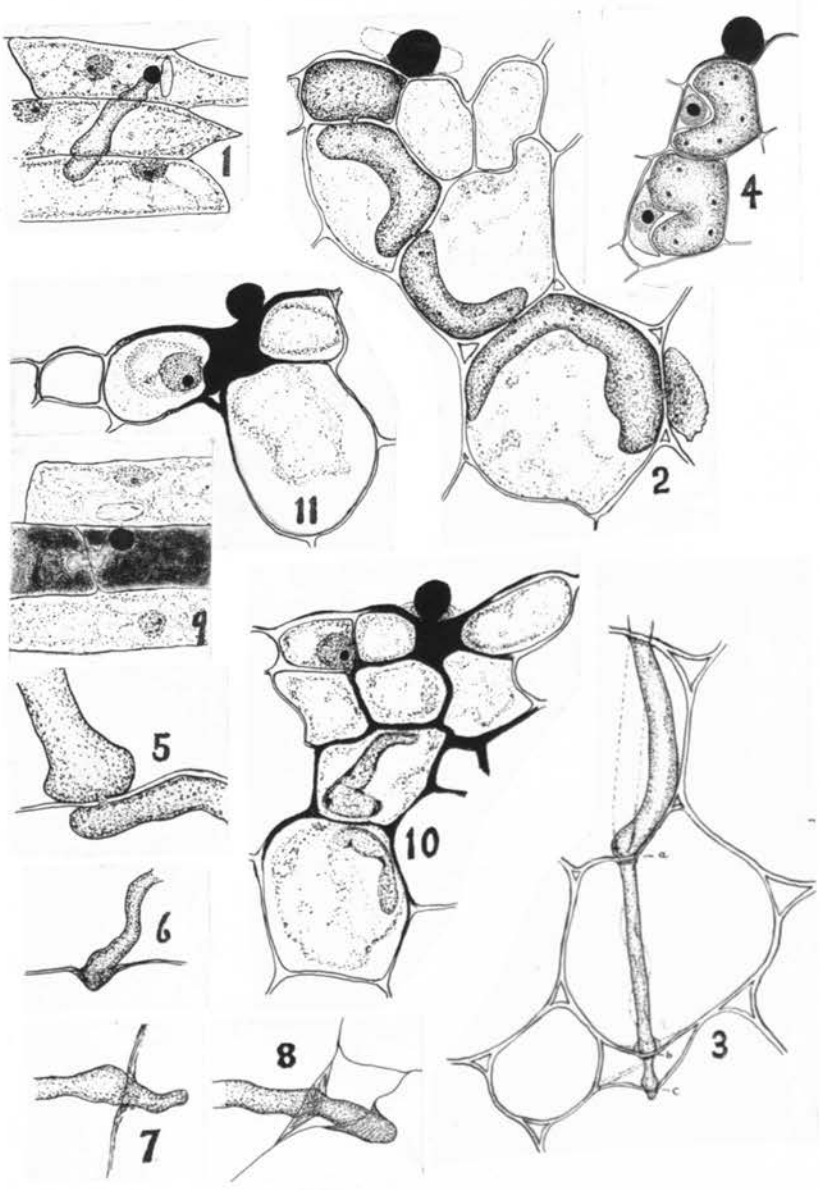


PLATE VII

Photomicrographs of stages in the development of *Colletotrichum lindemuthianum* in the tissues of a susceptible variety of bean.

Fig. 1 A free hand section of a susceptible bean stem cut about 75 hours after inoculation. Note the large primary mycelium lying against the cell walls. No discoloration or disintegration of host protoplast or cell wall could be detected at this point. The fungus was evidently taking its nourishment from the host protoplast which was yet alive. x 500 (approx.)

Fig. 2 Characteristic development of the primary mycelium. The adherence of the hyphae to the cell wall is undoubtedly due to the bending brought about by the resistance of the cell wall to penetration. From a stained section slide. x 500 approx.

Fig. 3 A free hand section of a susceptible bean stem, showing a hypha bending during the process of cell wall penetration. x 250 (approx.)

Fig. 4 Infection of a susceptible stem and development of the primary mycelium as seen in cross section. From a stained slide of material killed 99 hours after inoculation. The large primary mycelium is found always in the early stages of infection of a susceptible variety. x 600 (approx.)

Fig. 5 A typical example of cell-wall penetration by the secondary mycelium. There is no constriction or enlargement of the mycelium at the point of penetration. The wall has been penetrated at the corner of the cell and the wall of the adjacent cell has been pushed aside, indicating that the middle lamella has probable been dissolved. From a stained longitudinal section of a stem killed 126 hours after inoculation. x 350 (approx.)

Plate VII

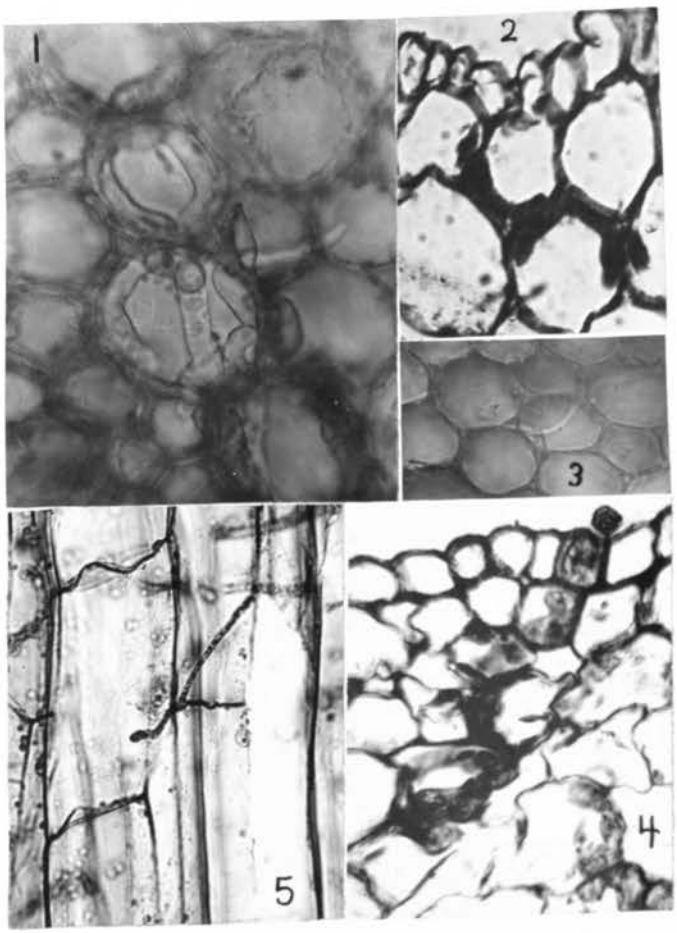


PLATE VIII

Photomicrographs of stages in the development of *Colletotrichum lindemuthianum* in the tissues of a resistant variety of bean.

Fig.1 A cross section of a stem of a highly resistant variety 83 hours after inoculation. The mycelium has advanced into two cells only, and is beginning to disintegrate. The walls of the epidermal cell have become slightly discolored and are beginning to collapse. From a stained slide. x 1200 (approx.)

Fig.2 A section similar to that in fig. 1 but more advanced. The mycelium and the protoplast of the attacked cells have completely disintegrated and the cell walls have collapsed. Both the walls and cell contents have been stained a homogeneous red by the diamant fuchsin. x 1000 (approx.)

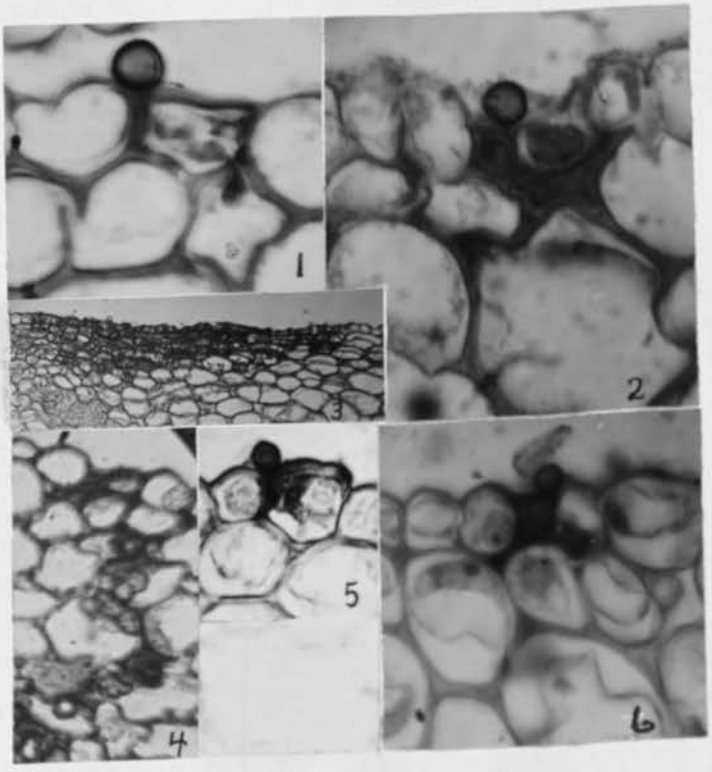
Fig.3 A cross section of a lesion on a less resistant variety. The mycelium advanced some distance from the point of infection, but it has entirely disintegrated, causing also the destruction of the host cells. From a stained slide of material killed 160 hours after infection. x 100(approx.)

Fig.4 Disintegrating mycelium in a resistant variety 70 hours after inoculation. From a stained slide. x 350 (approx)

Fig.5 A cross section of a stem of a resistant variety, 83 hours after inoculation, in which the fungus has penetrated only one cell. The mycelium has disintegrated, staining the host-cell wall and protoplast. The cell wall has not yet collapsed. x 750 (approx.)

Fig.6 A section showing the result of infection on a highly resistant variety. From a stained slide of material killed 83 hours after inoculation. The mycelium has disintegrated and the host cell has collapsed. The walls and entire contents have been stained a homogeneous red by the diamant fuchsin. The protoplasts of adjacent host cells are uninjured. x 750 (approx.)

Plate VIII



Julian Gilbert Leach was born at Somerville, Tennessee, November 16, 1894. He attended the public schools of Somerville graduating from the Fayette County High School in June 1913.

In September 1913 he entered the College of Agriculture of the University of Tennessee receiving the degree of Bachelor of Science in Agriculture in the spring of 1917. He entered the Graduate School of the University of Minnesota in October 1917 where he was Shevlin Fellow in Agriculture for the scholastic year, 1917-1918. He was granted the degree of Master of Science from this institution in June 1918. From July 1, 1918 to March 1919 he was Assistant in Plant Pathology at the University of Minnesota and Agent in the Office of Cereal Investigations of the United States Department of Agriculture.

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