THE TOXEMIA OF HALO BLIGHT OF BEAN

(Short title)

THE TOXEMIA OF HALO BLIGHT OF BEAN

By

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A thesis presented to the Faculty of Graduate Studies and Research of McGill University in partial fulfilment of the requirements for the degree of Master of Science.

Department of Plant Pathology,
Macdonald College of McGill University. June, 1964

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Professor J.G. Coulson and Professor R.L. Pelletier, for their sustained interest and helpful suggestions during the course of this work.

To the members of the staff of the Department of Plant Pathology, Macdonald College, who, in ever so many ways, offered me help and encouragement, I acknowledge my thanks.

The author is also indebted to Dr. M.D. Sutton and Dr. John J. Natti for their helpful suggestions and for supplying cultures of the pathogen.

The financial support given by the National Research Council of Canada is gratefully acknowledged.

Last but not least, I would like to extend my thanks to my colleague, Dr. M.A. Viswanathan, for his help in taking pictures presented in this thesis.

TABLE OF CONTENTS

Table of Contents (continued)

Table of Contents (continued)

Table of Contents (continued)

Page

I. INTRODUCTION

The toxigenic theory of plant disease explains the occurrence of pathological disturbances in advance of the invading pathogen as the effect of a toxin produced in the course of host-pathogen interaction. A considerable amount of work has been done on such diseases, which are called toxemia; however much of it is still a matter of controversy. Much more research must be done on this subject before many of the concepts involved can be accepted. At present, there is need for studies to show that pathogens can produce the same toxins both in axenic culture and in infection, and that injection with toxins can reproduce the physiological disturbances and the symptoms characteristic of the disease. Furthermore, it needs to be shown that environmental factors and chemical treatment have a similar effect on infection and on injection with the toxins. Finally, the toxin must be obtained in pure form so that its effects can be studied in the absence of possibly interfering substances.

The toxemia of halo blight of bean was chosen for studies of this kind. Its characteristic symptom is a leaf spot with a necrotic centre surrounded by a

pathogen-free chlorotic area. The latter is referred to as a halo and is presumably induced under the action of a toxin.

This thesis reports results of work carried out in the Department of Plant Pathology, Macdonald College of McGill University, Quebec, from May, 1962, to June, 1964.

II. LITERATURE REVIEW

A. INTRODUCTION

In 1924 in New York State, halo blight of bean was reported for the first time. Two years later Burkholder (1926) isolated the pathogen, which is now known as Pseudomonas phaseolicola (Burk.) Dows., a facultative anaerobe, and described the typical symptoms on leaves as small necrotic spots frequently surrounded, halo-like, by a yellow-green border. This latter characteristic has led Harter and Zaumeyer (1944) to suggest, although without any experimental evidence, that a toxin produced by the pathogen was the cause of the yellow border.

This toxigenic theory of disease provides a simple and plausible explanation for the occurrence of symptoms in tissues neighbouring those invaded by the parasite and in remote parts of the plant, for diffusion through tissues or translocation by the transpiration stream of phytotoxic metabolites could readily account for derangements occurring in tissues both near and far from infection sites. However, to prove this, experimental evidence had to be produced.

B. CRITERIA FOR ESTABLISHING IN VIVO PRODUCTION OF A TOXIN WITH CULTURE FILTRATES

A relatively large number of findings have been submitted as evidence for the toxigenic theory of disease, although not so much for halo blight as for other diseases. Among these findings is the common occurrence in culture filtrates of the pathogen of substances toxic to the host. However, the elaboration of toxin in axenic culture is found to be conditioned both qualitatively and quantitatively by the composition of the nutrient medium. Hence, if a pathogen elaborates a toxin in axenic culture, it does not necessarily follow that it will do so when growing in its host.

Results reported by Heitefuss et al. (1960) illustrate this point. They found that the Fusarium responsible for cabbage yellows produced fusaric acid in Richard's solution, but they could not find this compound in Fusarium-infected plants.

Because of this and other types of difficulty in proving the toxigenic theory of disease pathologists have felt the need for a set of criteria that would have to be fulfilled to prove this type of causation. At least three groups of authors have discussed this matter.

First Dimond and Waggoner (1953) suggested that

specific criteria are needed to establish the role of a compound in producing disease symptoms. "The compound must be (a) isolated from the diseased but not present in the healthy host, (b) characterized chemically, and (c) re-introduced in pure form into a healthy host where it must produce the symptoms of disease or a portion of the syndrome". They suggested the name vivotoxin for such a substance to distinguish it from a toxic compound isolated from pure culture filtrates,but not proven to be produced and active in a diseased plant. Then Braun and Pringle (1959) pointed out that it may be very difficult to apply the above criteria. The toxin may be formed or accumulate only in very low, hard-to-detect, yet effective amounts or its occurrence may be transitory due to it being constantly formed and broken down. According to Gäumann (1958) fusaric acid is formed as a vivotoxin in tomatoes infected with Fusarium oxysporum f. lycopersici (Sacc.) Snyder and Hansen, but its presence in the host can only be established by extremely sensitive methods as it is metabolized by the plant and being highly reactive unites readily with various compounds in the plant. It is obvious that available methods of detection may fail to reveal the presence of significant amounts of toxin and on the other hand a toxin might be detected in such low concentrations that it might not be able to play a sitnificant role in the causation of disease. Braun and

Pringle suggest further that certain other types of information can indicate an etiological role for a toxic metabolite in culture. "In some cases the pathological lesion is so characteristic there can be little doubt that the toxin elaborated in culture by a pathogen is the same substance as that produced by the organism in the host. A second criterion that may be used to establish this point is the selective action of a toxin". A toxin may show selectivity with respect to species, variety, tissue, and physiologic process. Apparently the most recent discussion of criteria for the demonstration of the toxin theory is that by Wheeler and Luke (1963). They report that the clearest and most valid evidence for a toxin to be a disease producing entity has been obtained with studies on victorin. They state the following: "The criteria of Dimond and Waggoner are inadequate to establish that a given compound functions as a causal agent of disease, their procedures will serve to demonstrate that the material occurs in the diseased plant and they were rightly concerned about the many materials produced by plant pathogens in artificial culture, but, without adequate evidence they ascribed roles in plant diseases".

To establish satisfactory criteria Wheeler and Luke (1963) suggested that all the symptoms of a given disease

result from the direct action of a toxic product of the pathogen involved in that disease. The criteria were that: (a) the toxin, applied at concentrations which could be reasonably expected in or around the diseased plant, produces in a susceptible host all the symptoms characteristic of the disease; (b) the pathogen and the toxin exhibit similar suscept specificity; (c) the ability of the pathogen to produce the toxin varies directly with its ability to cause disease; (d) a single toxin is involved. For those toxins which have been shown to play an important causal role in disease, they proposed the term "pathotoxin".

C. THE TOXEMIA OF HALO BLIGHT

Muller (1950) made a physiological study of the host-parasite relationship in halo blight of bean. He produced a culture filtrate that lost its capacity to induce chlorosis in bean plants upon standing overnight. The toxic principle appeared to be very unstable. However, Skoog (1952), reported that toxin production by Pseudomonas phaseolicola was demonstrated by growing pure cultures in glucose broth from 10-14 days at temperatures of 10°C and 15°C. Little or no toxin was obtained at growth temperatures of 20°C or higher. The toxin was thermostable and readily detected by the needle puncture method on young bean leaves. He also reported that the temperature range for toxin pro-

duction in pure culture corresponds closely with the development of typical halos in natural infection. Waitz und Schwartz (1956) reported that the organism would elaborate the toxin only when grown on "bean press sap" for 5-8 days. They suggested that the pathogen required some substance in the bean tissue for the production of the halo-inducing principle.

These reports indicate that the pathogen can elaborate a toxin in axenic culture that reproduces the features of the halo upon introduction into the host tissue. The mode of action of the toxin appears to have much in common with the wildfire toxin as reported by Braun (1955) and Braun and Pringle (1959). They found that, although the wildfire toxin is not specific, in that it acts as a general protoplasmic poison severely damaging the cells of many plant species, it is highly specific in its mode of action within a cell, for there it acts to block a clearly defined reaction in a metabolic sequence of events. When the wildfire bacterium, Pseudomonas tabaci (Wolf and Forster) Stevens, loses its capacity to elaborate the toxin in axenic culture, it then produces symptoms indistinguishable from the angular leaf spot disease of tobacco caused by Pseudomonas angulata (Fromme and Murray) Holland, which forms no halo and elaborates no halo inducing toxin when grown in axenic culture.

Braun and Pringle based their conclusions on the production of the characteristic pathological lesion by injection with the culture filtrate. However, other features of the halo could be used to establish toxin production by the pathogen. Chlorosis is the outstanding feature of the halo. Richmond and Lang (1957) reported that when a leaf is detached from a plant its protein content undergoes a prompt and rapid decline, the chlorophyll content decreases in close proportion and the life-span of the leaf is markedly reduced. In an attempt to control experimentally the survival and protein balance in detached leaves, the effect of some plant regulators on their processes was studied. Kinetin, 6-furfuryl-aminopurine, reduced protein loss in a consistent and striking manner and prevented chlorophyll breakdown.

Farkas and Lovrekovitch (1963) reported that the wildfire toxin does affect the protein level of the host leaves. Their results seem to support the idea that the action of the toxin is based on competitive antagonism resulting in a damage to protein metabolism. However, the activity of the toxin in damaging the protein metabolism and induction of chlorosis could be counteracted if whole leaves that had been injected with the toxin were sprayed immediately with 10^{-5}

M kinetin. The spray was repeated three times at one-day intervals. It is not known whether kinetin has the same effect on the halo inducing principle of halo blight of beans. An investigation of this type might supply evidence for the mode of action of this principle.

According to Shaw et al. (1954), Shaw and Samborski (1956) and Tomiyama (1963) accumulation of starch and increase in proteins, phenolics and respiration in tissue surrounding the infected cells often occurs. Lovrekovich et al. (1963) studied the effect of Ps.tabaci on the metabolism of starch in tobacco leaves. The toxin induced chlorotic halo in infections failed to show any starch reaction. The decrease in starch content could also be induced by injection of bacteria free culture filtrate. It is not known how the starch metabolism in halos induced by Ps. phaseolicola is affected.

D. FACTORS AFFECTING THE WIDTH OF HALOS

Ever since the disease was first described by Burkholder (1926), it has been known that the width of halos in infections varies. Several workers have investigated the nature of this difference. Temperature was found to be an important factor in determining the width of a halo. However, it has also been reported that various isolates differ in

their capacity to induce halos in infections.

Goss (1940), worked out the relation of air temperature to common and halo blight of bean. Six to ten days after spray inoculation at 24 - 28°C symptoms appeared. At lower temperatures symptoms appeared 2-3 days later. The relative humidity did affect the development of symptoms until 24 hours after inoculation. Halos occurred at 12-16°C and at 20-24°C small halos were noticed as a light green yellow border around the brownish necrotic spots; at 28-32°C no halos were induced. In addition, Patel and Walker (1963) found that the widest halos were induced at 16°C continuously but at 16°C day temperature and 28°C night temperature they were slightly narrower; however, the number of lesions at these temperatures was larger. At this high temperature the organism also grew faster in axenic culture. When the plants were kept at 28°C day temperature and 16°C night temperature the halos were narrower than in the reverse combination.

Jensen and Livingston (1943) studied 13 isolates of halo blight and found that on the basis of pathogenicity they fell into the following main groups. One group producing only halo-less lesions at 28°C gave rise to a typical halo at 16°C and 22°C; another group of isolates produced exclusively halo-less lesions at all three

temperatures. In an attempt to study the toxemia of halo blight of beans an isolate should therefore be selected that induces wide halos in infections.

E. PURIFICATION OF THE TOXIN

The preparation of pure toxin from a culture filtrate can be very difficult and easily leads to misinterpretations. A reliable quantitative method for judging the amount of toxin in a solution has to be found that can be used as a bioassay.

Woolley et al. (1952) isolated the phytopathogenic toxin of Ps. tabaci. They took the diameter of a halo induced by injection of a culture filtrate as a measure for its potency. Once a reliable bioassay is available a method of production of a filtrate can be worked out. Higher concentrations of toxin may be produced in shake-culture than in still-cultures. Burkholder (1926) reported that Ps.phaseolicola is a facultative anaerobe; aeration of the culture by means of shaking might thus affect the elaboration of toxin.

The toxic principle present in the culture filtrate may be stable or unstable. During the process of isolation the temperature may have to be lowered or raised so that it

is important for the experimentor to be aware of the capacity of the principle under investigation to resist these temperature fluctuations.

Reports in the literature on the thermostability of the toxin elaborated in culture by Ps.phaseolicola differ widely. Muller (1950) described a toxin that lost its activity in culture upon standing overnight at room temperature. Skoog (1952) reported that the toxin elaborated in glucose broth was thermostable. These reports are not conclusive as to the thermostability of the toxin and should therefore be looked into.

Woolley et al. (1952), and Woolley (1959) reported that the wildfire toxin was difficult to isolate as it was very unstable. Their method of isolation was basically as follows: Norit A was used to decolorize the filtrate. The toxin was then adsorbed on aluminum oxide, from which it was eluted with water. After concentration under reduced pressure, and addition of propanol, the solution was fractionated on a powdered cellulose column. The fraction containing the toxin was then separated on Whatman No.1 filter paper and crystallized out after concentration to dryness.

Muller (1950) reports that the toxin elaborated in culture by Pseudomonas phaseolicola was inactivated upon

dialysis. He could not recover its activity from the dialysate nor from the dialysis residue. About the adsorption of the toxin to Norit A and its passage through separation columns nothing has been reported. These characteristics of the toxin should be looked into for the purpose of its isolation and purification.

The literature reviewed above, pictures the significance of studies on the elaboration of toxins by pathogens in axenic culture in obtaining further information about the role of toxins in disease causation.

III. MATERIALS AND METHODS

Only those materials and methods generally used throughout this work are described here. Those used in only one experiment are described along with the results of that experiment.

A. ISOLATES OF Ps.PHASEOLICOLA AND BEAN VARIETY USED

Cultures of Ps.phaseolicola referred to herein as isolates P_3, P_{33}, P_{56}, P_{129} and P_{130} were obtained from Dr. Sutton*, and isolate P_1 was obtained from Dr. Natti**. The pathogenicity of the isolates when received was not known.

Red Kidney bean seed was obtained from the Genetics and Plant Breeding Research Institute, Canada Department of Agriculture, Research Branch, Central Experimental Farm, Ottawa, Ontario. This variety has been reported to be highly susceptible to infection of Ps.phaseolicola and to produce uniform plants.

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B. CULTURE OF THE PLANTS

Red Kidney seed was planted in a greenhouse at regular intervals of three weeks, so as to have a supply of plants of comparative age and stage of development for use in a sequence of experiments. The greenhouse temperature was 16 - 27°C and the relative humidity 50 - 80%. The normal daylight was supplemented by a bank of Coolwhite, reflector fluorescent lights suspended two feet above the plants from 9 a.m. - 11 p.m.

C. PRODUCTION AND PREPARATION OF INOCULUM

Cultures from which inoculum was prepared were grown on yeast dextrose carbonate agar, abbreviated hereafter as Y.D.C.A. When a broth was required the agar was omitted. The composition of Y.D.C.A. was as follows:

Difco yeast extract	10 gr.
Dextrose	20 gr.
$CaCO_3$	20 gr.
Bacto-agar	20 gr.
Distilled water to make	1000 ml.

Four days after streak inoculation and incubation at room temperature (20 - 22°C) plates were flooded with saline (0.85% NaCl) and the resulting suspension was

washed twice with sterilized tap water to remove toxic metabolites that might have been released into the medium. After the last centrifugation the cells were suspended in water at the rate of about 25 ml. per plate used in preparing the original suspension.

D. SPRAY INOCULATION OF THE PLANTS

Four days before inoculation the number of plants required for the experiment were selected for uniformity and then placed in an artificially illuminated growth chamber providing controlled light, temperature and relative humidity conditions. This four-day period served to acclimatize the plants to their new environmental conditions.

In order to improve the chances of successful inoculation, the plants were kept well watered and the relative humidity was gradually increased to 90 - 99% which was maintained for a 24-hour period immediately preceding and following inoculation. This treatment presumably caused a certain degree of water-soaking of tissues previous to and after inoculation, and thus increased the proneness of the plants to infection as reported by Goss (1940).

Plants were inoculated by spraying with the bacterial suspension, using a throat atomizer (De Vilbiss), until

drain-off. The spray was directed mainly at the abaxial surface of the first trifoliate leaf. After inoculation the lights were kept on for 16 hours in order to assure open stomata, which provide an easy entrance for the bacteria. Incubation temperature varied with the experiments.

E. PREPARATION OF STERILE CULTURE FILTRATES

For this purpose, the pathogen was grown in Y.D.C. broth either in still-cultures or in shake-cultures. The shaker used was of the wrist-action type, operating at 300 strokes per minute. The amplitude was adjusted at the lowest setting required to keep the $CaCO_3$ in suspension. After 14 days of incubation at the desired temperatures, the broth was centrifuged at about 1000 g for five minutes to remove the $CaCO_3$ from the culture medium. The supernatant was then filtered through a Millipore filter (Millipore Filter Corporation, Bedford, Mass., U.S.A.) with a pore size of 0.45 micron.

The sterility of the filtrate was tested by adding a small portion of it to Difco Micro Inoculum Broth. If proven sterile, the remainder was stored in quantities of 10 ml. in 20 ml. vials, at a temperature of -15°C until use, this to prevent thawing of large quantities each time that a small sample was required.

A batch of two liters of sterile culture filtrate of isolate P_{56} that was prepared from shake-cultures incubated at 18 - 19°C will be referred to hereafter as Stock Culture-Filtrate.

F. INJECTION WITH CULTURE FILTRATE

Injection was accomplished by depositing a 0.05 ml. droplet on the adaxial surface of just fully unfolded leaflets of the first trifoliate leaf with a micrometer syringe. The leaflet was then punctured with a dissecting needle passing through the droplet. Following injection, the relative humidity in the growth chamber was kept at 90 - 95% for a period of 24 hours, unless specified differently, so as to prevent rapid evaporation of the droplet and provide a favourable humidity for the filtrate to penetrate the leaf.

G. OPTICAL DENSITY OF THE LEAF TISSUE

The degree of chlorosis of the halo was determined by calculating the difference in optical density of the halo region and that of the non-chlorotic surrounding region.

The optical density was measured with a Photovolt photometer (Photovolt Corporation, New York City) using a

Jena OG 2-2, orange red glass filter. The photometer was adjusted to 2.0 when no light entered the photoreceptor and to 0.2 with light passing through the yellowest halo.

H. STARCH IODINE REACTION

The starch test was performed according to the method of Johansen (1940). Two gr. of KI were dissolved in 100 ml. of water and in this solution 0.2 gr. of iodine was then dissolved. Starch appears blue to black in a few minutes. Newly formed starch can appear red to purple.

For our purpose leaflets of plants to be tested for starch were kept in the dark for two days before they were killed in boiling water and then kept in boiling 80% ethanol until all of the chlorophyll had been extracted. The leaflets were then placed into the iodine potassium iodide solution for five minutes, after which starch was detected by its blue appearance. To remove the excess of iodine the leaflets were washed with water until the starch free areas were light yellow.

IV. EXPERIMENTAL RESULTS

SECTION 1. PRELIMINARY EXPERIMENTS ON HALO INDUCTION BY VARIOUS ISOLATES

A. INTRODUCTION

Different isolates of the organism have been reported to vary in the width of halos that they typically induce. Also the proneness to infection varies with the incubation temperature at the time of inoculation. Evidently an isolate and incubation temperature should be selected that causes the development of wide halos in infection. The purpose of the experiments in this section is to obtain results that will help in making such a selection.

B. HALO PRODUCTION BY THE VARIOUS ISOLATES OF PS. PHASEOLICOLA

In the process of selecting an isolate that induces large halos in infections an experiment was carried out in which Red Kidney bean plants were spray-inoculated with isolates P_1, P_3, P_{33}, P_{56}, P_{129} and P_{130}.

1. Materials and Methods

Twenty-four hours before inoculation fourteen plants readied for leaf spray-inoculation were taken from the greenhouse and placed in an inoculation chamber providing a temperature of 18 - 19°C, a relative humidity of 90 - 95% and a length of day of 14 hours. Two plants were spray-inoculated with each isolate and two plants were sprayed with an aqueous suspension prepared from plates that were kept as a check. Isolate P_{129} was a fast grower, Isolate P_{33} and P_{130} were slow growers in culture. During the first 24 hours after inoculation the relative humidity was kept at 95 - 100%, the temperature remained at 18 - 19°C and lights were kept on for 16 hours after inoculation. During the next two days the relative humidity was lowered to 70 - 80%.

Four days after inoculation, the plants were removed from the inoculation chamber and placed in a growth chamber providing 18 - 19°C, 65 - 85% relative humidity and a length of day of 14 hours.

2. Results and Observations

The plants were kept under observation as long as significant changes in symptoms were taking place, which was for a period of 21 days. Symptoms first appeared five days after inoculation with isolates P_1 and P_{129} as small

chlorotic spots on the lower leaf surface. These spots were especially distinct when examined in transmitted light which also revealed a translucent area in the center of each lesion.

On the sixth day isolates P_3 and P_{56} induced similar symptoms. As the leaves expanded a malformation of the leaf was observed especially if a lesion developed at the margin of the leaf.

Nine days after inoculation the central translucent area of the lesions appeared brownish. With time, isolate P_{56} induced a broad halo around a small necrotic spot with a diameter of approximately 2 mm. Isolate P_{129} induced a narrower halo and a larger necrotic area of about 3 mm. in diameter.

Fifteen days after inoculation no symptoms of disease were noticed on plants inoculated with isolates P_{33} and P_{130}.

Twenty-one days after inoculation the most severely infected leaves had shed. Isolates P_1 and P_3 induced narrow halos around small necrotic spots. On plants inoculated with isolate P_{129} the veins near the infection court had a brownish discolored appearance which was noticed at first as translucent tissue. This was especially notice-

able on the lower side of the leaves. The brownish necrotic lesions eventually turned purplish.

C. EFFECT OF INCUBATION TEMPERATURE ON PATHOGENICITY AND SYMPTOM EXPRESSION

In the previous experiment isolates P_{33} and P_{130} did not cause infection, and were found to be slow growers in axenic cultures. From the literature it is known however that the organism grows best in culture when incubated at 20 - 25°C. It was thought that an incubation temperature of 25°C might alter the invasiveness of the isolates. To test for this an experiment was carried out in which the pathogenicity and symptom expression of the various isolates at 25°C were determined.

1. Materials and Methods

The experiment was similar to the preceding one except that during the first 36 hours after inoculation the temperature was raised to 25°C instead of 18 - 19°C.

2. Results and Observations

The plants were kept under observation until no further significant changes in symptom expressions took place, which was for a period of five weeks. Four days after spray-

inoculation numerous small chlorotic spots were noticed on leaves of plants inoculated with isolate P_{33} . Isolates P_1 and P_{129} induced only a few chlorotic spots per leaf.

Six days after inoculation spots on plants inoculated with isolate P_{33} were translucent, and with time the spots became brown and surrounded by a yellow border about 1 mm. wide as illustrated in Figure 1.

Ten days after inoculation on plants inoculated with isolate P_{129} several small chlorotic spots appeared. Isolate P_1, P_3 and P_{130} induced narrow halos with a width of 1 - 5 mm. and isolate P_{129} produced some halos with a width of 5 - 10 mm. Isolate P_{56} induced the broadest halos, some of which had a width of 30 mm. as illustrated in Figure 2.

Five weeks after inoculation, on some plants inoculated with isolates P_{129} and P_{56}, a systemic chlorosis was noticed especially on the third and fourth set of trifoliate leaves (Figure 3).

The widths of the halos were measured 10 days after inoculation with the various isolates, and are presented in Table I.

The results and observations presented indicate that an incubation temperature of 25°C is most suitable to determine the width of halos that the isolates induce in infection.

Fig. 1. Red Kidney bean leaflet infected with isolate P_{33}.
Note the small central necrotic spot surrounded by a narrow yellow border.

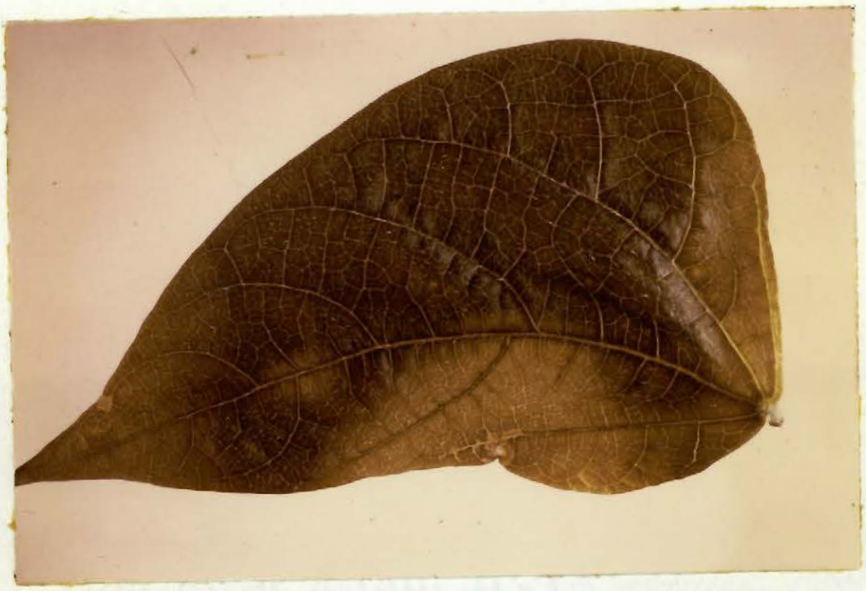

Fig. 2. Red Kidney bean leaflet infected with isolate P_{56}.
Note the small central necrotic spot surrounded by a broad yellowish green halo that may reach a width of 30 mm.

Fig. 3. Red Kidney bean plant infected with isolate P_{56}.
Note the single lesions on the first set of trifoliate leaves and systemic chlorosis especially on the third and fourth set.

TABLE I

Width of halo induced by the various isolates in infections.

Isolate	Width of halo
P_{33}	< 1 mm
P_1, P_3, P_{130}	1 - 5 mm
P_{129}	5 - 10 mm
P_{56}	> 10 mm

SECTION 2. THE PRODUCTION OF A VIVOTOXIN

A. INTRODUCTION

Reports in the literature indicate that the typical symptoms induced in infections can be reproduced by injection with culture filtrates when incubated at a suitable temperature. It is not known however whether isolates that vary in the width of halos that they typically induce in infections also vary in the amount of toxin that they elaborate in axenic culture. The capacity of a culture filtrate to duplicate some of the physiological changes brought about in infections could be used as a criterion for production of a vivotoxin. Toxins generally are highly specific in their mode of action. It is not known whether the activity of the halo-inducing principle is host-specific. An altered starch metabolism and the effect of kinetin might be valuable criteria for establishing the production of a vivotoxin by the pathogen.

In this section the similarities in these effects between halos induced by infection and those induced by injection will be studied.

B. ELABORATION OF A TOXIN IN AXENIC CULTURE BY VARIOUS ISOLATES

The object of this experiment is to test the capacity of various isolates to elaborate a toxin in axenic culture and to compare the widths of halos induced by injection with their culture filtrates with the widths of halos that they typically induce in infections.

From these results an isolate is then to be selected that is most suitable for further studies.

1. Materials and Methods

Shake-culture filtrates of the isolates P_1, P_3, P_{33}, P_{56}, P_{129}, and P_{130} were prepared from cultures grown in a growth chamber set to provide 18°C and 65 - 85% relative humidity. Plants grown in the greenhouse were acclimatized for four days before inoculation to the conditions at which they were kept subsequently, namely, 18°C, 70 - 85% relative humidity and a length of day of 14 hours at 650 foot candles. The filtrate of each isolate was injected at one point into the leaflets of the first set of trifoliate leaves of four plants. As a check, a droplet of the Y.C.D. broth and a droplet of water were injected at different loci on the same leaflets.

2. Observations and Results

Plants were kept under observation for a period of four weeks after injection. Needle punctures produced by the injection of water appeared as small holes in the leaves and no halos were induced. The injection with the Y.C.D. broth resulted in a narrow necrotic border around the locus of injection as shown in Figure 4.

Two days after injection with the culture filtrates halos appeared as small chlorotic spots. The halos gradually increased in width until the sixth day after injection. Halos induced by injection with culture filtrates of isolate P_{33} were narrow, and those induced by the culture filtrates of isolates P_1, P_3, and P_{130} had a width of about 2 - 3 mm. Halos induced by injection with the culture filtrate of isolate P_{129} increased in width until some reached a width of 20 mm. Halos induced by injection with the culture filtrate of isolate P_{56} covered a large area of the leaf and attained a width of up to 40 mm. The interveinal tissue appeared yellowish green and chlorosis of the veinlets was less obvious (see Figure 4).

Four weeks after injection with the filtrates, halos could no longer be distinguished, as plants were then in senescence and appeared yellow. Leaves injected with culture

Fig. 4. Red Kidney bean leaflet injected with water, Y.C.D. broth and the culture filtrate of isolate P_{56}.
Note the small hole in the leaflet (to the lower right hand corner) caused by injection with water. Also the narrow necrotic border at the site of injection (to the lower left hand corner) with Y.C.D. broth and wide yellowish green halo (upper right) induced by injection with the culture filtrate.

filtrates of isolate P_{56} had a malformed appearance.

The width of a halo induced by injection is taken as a measure of the amount of toxin elaborated in axenic culture. In Table II the width of halo induced by injection with the culture filtrate of each isolate is presented.

These results suggest that isolate P_{56} elaborates the greatest amount of toxin in axenic culture and it is therefore selected as the isolate used for further studies.

TABLE II

Width (mm) of halos induced by injection with culture filtrates of various isolates

Isolate	Width of halo in injection
P_{33}	< 1
P_1, P_3, P_{130}	1 - 5
P_{129}	5 - 20
P_{56}	10 - 40

C. EFFECT OF TEMPERATURE ON THE ELABORATION OF TOXIN IN AXENIC CULTURE AND ON THE REACTION OF THE HOST PLANT TO INJECTION WITH THE CULTURE FILTRATE

It has been reported that at high temperatures of 28 - 32°C no halos are induced in infections. It is not known whether this is due to inactivation of the toxin or whether the pathogen no longer elaborates it at this temperature. To investigate this problem an experiment was designed where bean plants were kept at both 18°C and 28°C. Culture filtrates were produced from cultures that had been incubated at 18°C and at 28°C.

1. Materials and Methods

Sixteen Red Kidney bean plants readied for injection were kept in each of two growth chambers providing 18° - 19°C, 80 - 95% relative humidity, a length of day of 14 hours; and 27 - 29°C, 80 - 95% relative humidity, and a length of day of 14 hours.

Cultures of isolate P_{56} were incubated for 14 days as still cultures at 18°C and 28°C. The filtrates of these cultures were then injected into the first set of trifoliate leaves of plants kept at both temperatures.

2. Results and Observations

The plants were kept under observation as long as significant changes in the symptom expression were taking place which was for a period of two weeks. Six hours after injection with the culture filtrate, produced from cultures that had been incubated at 18°C, chlorosis became evident on plants that were kept at 27 - 29°C. With time a small area became necrotic. On plants that were kept at 18° - 19°C halos appeared after one day and no necrosis was observed.

Eight hours after injection with the culture filtrate produced from cultures that had been incubated at 28°C, a small necrotic spot was induced on plants that were kept at 27 - 29°C. On plants kept at 18° - 19°C, a small necrotic spot was observed on the second day after injection. Halos induced by the filtrates had irregular margins. In some lesions the chlorosis was stopped by veins.

From the observations presented we may conclude that the host plant is more sensitive to the activity of the toxin at high temperature than at low temperature. A temperature of 28°C inhibits the elaboration of toxin by the pathogen when grown in axenic culture. Furthermore the absence of a halo in natural infections at 28 - 32°C suggests that the

pathogen does not elaborate the halo-inducing principle in host tissues at this temperature.

D. HOST SPECIFICITY OF THE TOXIN

It is not known whether the toxin elaborated in culture by Ps.phaseolicola has a host specific reaction. To test this characteristic various bean varieties of reputed resistance, and also several non-host plants, were injected with the culture filtrate and others were inoculated with isolate P_{56}.

1. Materials and Methods

The bean varieties used are Red Kidney, Kentucky Wonder Wax, Pencil Pod Black Wax, Stringless Greenpod, and Clipper. The non-host plants were tomato, soybean and tobacco.

Five plants of each were spray inoculated with Isolate P_{56} and five of each were injected with the Stock Culture-Filtrate of Isolate P_{56}. The incubation temperature was kept at 25°C; later the temperature was lowered to 18 - 19°C. Five days after inoculation and injection the plants were moved to a greenhouse that provided conditions of 18 - 25°C, 60 - 75% relative humidity and a length of day of 14 hours.

2. Results and Observations

On the varieties Kentucky Wonder Wax, Stringless Greenpod, and Clipper narrow halos were induced in infections. Injection with the culture filtrate induced halos with a width of 5 - 10 mm. On the variety Pencil Pod Black Wax halos with a width of 5 - 10 mm. were induced in infections and injections. On the variety Red Kidney both in infections and injections halos were wider than 10 mm.

Inoculation of the non-host plants did not result in infections. However injections with the culture filtrate resulted in wide halos. Halos induced on tobacco by injections with the filtrate are shown in Figure 5.

In Table III a comparison is presented of the relative width of halos induced in infections and by injection with a culture filtrate into various host and non-host plants.

E. THE EFFECT OF KINETIN ON THE WIDTH OF HALOS

In the preceding experiments halo induction by injection with culture filtrate was compared under various aspects and experimental conditions, with halo induction in infection. Evidence for the presence of a vivotoxin was thus obtained. However, this evidence was not conclusive. There was a need for further comparison under different

Fig. 5. Tobacco plant injected with the culture filtrate.
Note the wide halos induced by injection.

TABLE III

Comparison of widths of halos (mm) induced in infections and by injections into host and non-host plants.

Host Non-Host	Width of Halo in Infections	Width of Halo in Injections
Kentucky Wonder Wax	< 1	5 - 10
Stringless Greenpod	< 1	5 - 10
Clipper	< 1	5 - 10
Pencil Pod Black Wax	5 - 10	5 - 10
Red Kidney	>10	>10
Tomato	0	>10
Tobacco	0	>10
Soybean	0	>10

conditions. Therefore plants were treated with kinetin, which is known to reduce chlorosis in wildfire of tobacco.

1. Materials and Methods

Thirty Red Kidney bean plants acclimatized at 18 - 19°C, 80 - 90% relative humidity and a length of day of 14 hours at 750 foot candles were spray-inoculated with isolate P_{56}. Another set of 30 plants was injected with the Stock Culture-Filtrate. These plants as well as healthy control plants, were sprayed until drain-off with 10^{-5} M kinetin (Sigma Chemical Co., St.Louis, Missouri) according to the schedule presented in Table IV. The width of halos was measured two weeks after injection and inoculation. The whole experiment was performed twice.

2. Results and Observations

On plants that had been inoculated with isolate P_{56} and sprayed five times, narrow halos were induced. On plants that were inoculated and had been sprayed only on the fifth and seventh day after inoculation halos were induced that were as broad as those on plants that had not been sprayed with kinetin.

Halos induced by injection with culture filtrate were

TABLE IV

Schedule of spray applications of kinetin to various treatments.

Plant condition	Number of plants	Time of spray application (days before and after inoculation or injection)				
		1 before	0	3 after	5 after	7 after
Healthy	5	+*	+	+	+	+
Infected	5	+	+	+	+	+
or	5	-	+	+	+	+
Injected	5	-	-	+	+	+
	5	-	-	-	+	+
	5	-	-	-	-	+
	5	-	-	-	-	-

* + sign indicated that the spray was applied.

of the same size in all treatments. The healthy plants that had been sprayed five times appeared normal.

It is concluded that kinetin can reduce the width of halos in infections but not the width of those induced by injection with the culture filtrate.

F. ACCUMULATION OF STARCH IN HALO TISSUES

Starch accumulation is a well-known physiologic response of the host to invasion by parasites. It is not known how the starch metabolism in the host-parasite relationship of halo blight is affected. The capacity of the culture filtrate to duplicate these effects, if any, would supply further evidence for production of a vivotoxin by the pathogen.

To investigate this problem, an experiment was performed in which halos, induced by inoculations with isolate P_{56} and by injection with its culture filtrate, were analyzed for the presence of starch.

1. Materials and Methods

Red Kidney bean plants that were infected with isolate P_{56} and were showing broad halos, together with another set of plants on which halos had been induced by injection

of the Stock Culture-Filtrate, were placed in the dark for 48 hours to deplete the healthy leaves of starch. Healthy and diseased leaves were then tested for the presence of starch using the starch iodine test as described in Materials and Methods. The experiment was performed three times.

2. Results and Observations

Starch was found to accumulate throughout the chlorotic area of halos induced by infection and by injection. See Fig. 6. The central necrotic spot did not contain starch. Similar results were obtained each time that the experiment was performed.

It is concluded that it is possible to reproduce the effect of infection on starch accumulation by injection with the culture filtrate.

Fig. 6. Accumulation of starch in halos induced by infection (bacteria) and by injection (filtrate) with the culture filtrate.
Note the presence of starch indicated by the blue color (deep) in chlorotic tissues that had been cleared and stained with iodine.

SECTION 3. BIOLOGICAL, PHYSICAL AND CHEMICAL PROPERTIES OF THE TOXIN IN THE CULTURE FILTRATE

A. INTRODUCTION

Results of the preceding experiments showed that injection with the culture filtrate could reproduce to a considerable extent the effect of infection on the host. Therefore, it appeared that further information on the biological, physical and chemical properties of the culture filtrate could be useful in interpreting its action on the plant.

B. THE EFFECT OF THE CULTURE FILTRATE ON THE GROWTH OF THE PATHOGEN IN VITRO

A typical lesion of halo blight consists of a small central necrotic area surrounded by a yellowish green halo. The pathogen is generally confined to this small central area. It is not known what prevents the spread of the pathogen to other regions. One possible explanation is that the bacterium is inhibited in its growth by an inhibitor released in the host tissues which might also be present in the culture filtrate. To test this possibility, an experiment was carried out in which the effect of the culture filtrate on the growth of the pathogen *in vitro* was determined.

1. Materials and Methods

The disk-plate technique, normally used for testing antibiotics was employed.

Eight petri dishes, each containing ten ml. of Difco Micro Inoculum Agar, were seeded with one ml. of an aqueous suspension of isolate P_{56} and similarly ten plates were seeded with isolate P_{33}.

Forty sterile disks of Whatman No.1 filter paper of 6 mm. in diameter were infiltrated with 0.04 ml. of culture filtrate of isolate P_{56} and the same number of disks with the culture filtrate of isolate P_{33}. Disks with the filtrate of isolate P_{56} were placed, four per plate, on the surface of five plate cultures of each isolate. Disks with the culture filtrate of isolate P_{33} were used similarly. The plates were then incubated for 48 hours at 22°C. At the end of this period the zone of inhibition around the disks was measured. The experiment was performed twice.

2. Results and Observations

The width of the zone of inhibition was about the same, i.e., less than 2 mm., in all cases. It is concluded that

isolate P_{33} and P_{56} did not produce self-inhibitors in their cultures under the conditions used in this experiment.

C. BIOASSAY

To reach a better understanding of the toxemia, it is important to characterize the toxin chemically and to study the action of the pure compound on the host. However, before purification can be attempted a bioassay must be available that will yield a reproducible indication of the potency of any particular preparation. Furthermore, with a bioassay the most suitable method of preparation, storage, and the stability of the toxin in the culture filtrate could be determined.

Some easy-to-measure and reproducible characteristic of the halo, indicative of the potency of the culture filtrate was looked for in developing the bioassay. Woolley *et al.* (1952) took the diameter of the halo as an indication of the potency of the wildfire toxin in culture filtrates.

Halos induced by injections vary in width but appeared to vary also in degree of chlorosis. The width of the halo by itself might therefore not be sufficiently accurate indication of the potency of the toxin preparation.

An experiment was carried out in which the culture filtrate was diluted 0, 2, 4, 8, 16, 32, and 64 times. These solutions were injected into leaflets of the first set of trifoliate leaves. When the halos had reached full size their widths were measured and the degrees of chlorosis were determined by means of differences in optical density as outlined in Materials and Methods. Readings on the optical density varied too much to be of any significance; however, the size of the halo appeared to vary much less. Therefore a more elaborate experiment was performed, to determine more precisely the value of the degree of chlorosis, and at the same time the value of the size of the halo as measures of the potency of the culture filtrate.

1. Materials and Methods

Fourteen Red Kidney bean plants of similar age and stage of development and which had just unfolded their first set of trifoliate leaves were kept in a growth chamber providing 18 - 19°C, 75 - 95% relative humidity and an illumination of 14 hours at 750 foot candles per day. The culture filtrate was injected at double its normal concentration (concentrated in a rotating flask at 35°C) and at the following dilutions of 0, 2, 4, 8, 16, and 32 times. The volume of the droplet placed on the leaf was 0.05 ml.

The experiment was carried out according to a Balanced Incomplete Bloc design as outlined in Cochran and Cox (1957). Randomized blocks of three were replicated five times. A block consisted of three injections on a set of trifoliate leaves. The whole experiment was duplicated and both duplications were run at the same time.

Ten days after injection, when the halos had fully developed the area of the halo was determined by means of an Ott Compensating Planimeter with a unit range of 0.016 sq. inch, and their degree of chlorosis was determined by means of their optical density as described previously in Materials and Methods.

2. Results and Observations

As in the preliminary experiment, optical density readings varied so much between replicates that no accurate or precise evaluation of the potency of the solution applied could be obtained from these; moreover the method was relatively time-consuming.

The analysis of variance of the various measurements on the area of halos induced by the treatments are presented in Appendix I. In Table V the means of the size of the halos induced by the various treatments are presented. Because the

TABLE V

Area (cm^2) of halos induced by various concentrations of culture filtrate.

Filtrate concentration	Area	Adjusted area
2	4.26	4.39*
1	2.48	2.61
1/2	1.93	2.06
1/4	1.34	1.47
1/8	0.39	0.535
1/16	0.31	0.441
1/32	0.00	0.129

* Adjusted means of six observations S.E. = $\pm$ 0.34 cm^2. The bars indicate groups of means within which it is not possible to demonstrate significant differences.

1/32 x concentration did not induce a halo, 0.02 sq. inch (0.129 cm^2) was added to each measurement for the purpose of statistical analysis. The Duncan Multiple Range test was applied to these means.

These results show that at the higher concentrations the halo area is roughly proportional to, and is a sensitive measure of the potency of the culture filtrate. However, at lower concentrations changes in potency do not affect proportional halo areas. It is concluded that the area of a halo induced by injection with a culture filtrate can be used as a measure of the potency of the toxin in the culture filtrate.

D. TOXIN-PRODUCTION IN STILL AND IN SHAKE-CULTURES

Isolation of a substance from a mixture usually involves several fractionations in each of which part of the total amount of the substance sought is lost. Consequently it is frequently necessary to use as rich a source as possible of that substance. It was not known whether filtrates of still-cultures contain more of the toxin than those of shake-cultures. Therefore an experiment was performed to obtain information on that point.

1. Materials and Methods

Eight 250 ml. Erlenmeyer flasks containing 50 ml. of Y.D.C. broth were seeded with one loopful of a 2-day old Y.C.D.A. slant culture. Four cultures were kept still and four were placed on a shaker (See Materials and Methods) for 14 days at 18 - 19°C and 60 - 75% relative humidity in the dark. The potency of the sterile culture filtrates of the cultures was then assayed using the halo-area method.

2. Results and Observations

Halos induced by shake-culture filtrates had larger areas than those induced by still culture filtrates. It is concluded that the pathogen elaborates more toxin in shake-cultures than in still-cultures.

E. EFFECT OF HEAT ON THE STABILITY OF THE TOXIN IN THE CULTURE FILTRATE

For the purpose of knowing the possibility of sterilizing the culture filtrate by heat and also to give some information of the chemical nature of the toxin it is necessary to determine the thermostability of the toxic principle. Therefore an experiment was carried out on the effect of heat on the halo inducing capacity of the culture filtrate.

Several samples of the Stock Culture-Filtrate were heated to 60, 80, and 100°C for ten minutes. Another sample was heated in the autoclave at 121.5°C for ten minutes (15 lbs. per sq. inch steam pressure). Unheated Stock Culture-Filtrate served as control. The samples were assayed by the halo-area method.

The area of the halos induced by samples from the different treatments did not differ. It is concluded that the toxin is thermostable.

F. EFFECT OF STORAGE AT LOW TEMPERATURE ON THE STABILITY OF THE TOXIN

To study the effect of low temperature, a sample of the Stock Culture-Filtrate that had been stored at -15°C for one year was bioassayed for its potency. Freshly prepared culture filtrate served as control.

Areas of halos for the two kinds of filtrates did not differ to any considerable extend. It is concluded that cold storage at -15°C over a period of one year does not affect the potency of the culture filtrate.

G. ATTEMPTS TO PURIFY THE TOXIN

Adsorption of a desired compound on an adsorbent from which it can be eluted in purification procedures is often

the fastest and easiest technique available. For these reasons the first attempt to purify the toxin consisted of testing its adsorption on wood charcoal. When it was found that purification could not be achieved by this technique ion-exchange resins and dialysis were tried.

1. Norit A as an adsorbent

One hundred ml. of Stock Culture-Filtrate (pH 6.5 - 7.5) were heated to 80°C, and then one gr. of Norit A was added. The resulting suspension was stirred for 15 minutes and filtered through Whatman No.1 filter paper with a Buchner funnel. The Norit A on the filter paper was washed with 50 ml. of water twice. This water extract was concentrated rapidly to 50 ml. under vacuum in a rotating evaporator at a temperature below 50°C. Norit A filtrate and concentrated water extract of Norit A were kept under refrigeration until bioassay. One portion (½ gr.) of the Norit A was extracted with 100 ml. methanol and the other portion (½ gr.) with 100 ml. ethanol. These alcoholic extracts were concentrated to very small volume, and water was then added to make 50 ml. All the fractions were thus bioassayed by the halo-area method. The whole experiment was performed twice.

Only the injections with the Norit A filtrate induced halos, which suggests that the toxin was not adsorbed. However, Norit A decolorized the culture filtrate.

2. Ion-exchange column separation

Baker Chemical Co. described the laboratory requirements for Dowex ion-exchange resins. In passing the Stock Culture-Filtrate through columns of Dowex resins the recommendations in the technical publication dealing with these resins were followed. As a cation-exchange resin, Amberlite I.R.-120 (H), of the nuclear sulfonic-acid type was used, and as anion-exchange resin Dowex 1-x8, 50-100 mesh, a quarternary ammonium type; the latter column was charged with 10% KOH.

Forty ml. of culture filtrate at pH 7.0 were passed through the columns each time with a flow rate of one ml. per minute. After passage through the cation-exchange column the pH of the effluent solution was adjusted from 2.5 to 7.0 with 0.1 N NaOH. The anion-exchange column increased the pH up to 10.9 which was brought back to pH 7.0 with 0.1 N HCl. The whole experiment was repeated four times. Effluent solutions were assayed for the presence of the toxin by the halo-area method.

Passage of the culture filtrate through the cation-exchange resin did not remove the toxin. However, out of four runs the anion-exchange resin retained the toxin once as no halos were induced by injection with that effluent solution.

3. Dialysis

In a final attempt to purify, at least partially, the toxin dialysis was used. Fifty ml. of the Stock Culture-Filtrate were placed in a Fisher Seamless Cellulose tubing 1 1/8" in diameter. The tubing was then submerged for 24 hours at 15°C in one liter of stirred water, which was replaced three times during the interval. There were three such attempts.

Bioassay of the dialysed culture filtrate showed that it had no toxicity.

V. DISCUSSION AND CONCLUSION

The interpretation of results obtained with culture filtrates on toxemia is difficult and some of these difficulties should be pointed out before a discussion of the results reported herein is presented.

The culture filtrate contains, in addition to the toxin, several other metabolites of the pathogen. Some of these metabolites most likely are different from those normally produced in the course of host-parasite interactions. Consequently, injection with a culture filtrate creates a biochemical environment in host tissues which can be quite different from that during disease development. The metabolism of the toxin in the infected host can therefore be altered in the injected host through the presence of various metabolites of the pathogen in the culture filtrate.

Studies on injection with the culture filtrate reported herein were limited to the reaction of the first set of trifoliate leaves of Red Kidney bean plants. Other parts of the plant or a different bean variety could give different results. Another limitation is that elaboration of toxin was followed in only one culture medium. Different media might very well induce the pathogen to elaborate a different toxin. Furthermore, the pathogen has been reported to vary

considerably in its capacity to induce halos in infection. It is not known whether this variation is caused by quantitative or qualitative differences in toxin production. Most studies were based on characteristics of the culture filtrate of only one isolate, which tends to limit the significance of these studies to some extent.

Preliminary experiments showed that the incubation temperature of 25°C was the most favourable of the temperatures used for infection. Muller (1950) reported that the growth rate of the pathogen in axenic culture at this temperature was faster than at other temperatures. This suggests that the effect of temperature on the growth rate of the pathogen in the host during incubation is similar to that in axenic culture. The evidence, however, is not conclusive.

The effect of temperature on elaboration of toxin in axenic culture and on the reaction of the host plant to injection with the culture filtrate indicates that the host is more severely injured at 28°C than at 18°C. It was also found that culture filtrates prepared from cultures incubated at 18°C induced large halos, whereas those prepared from cultures incubated at 28°C did not induce halos. This effect of temperature on the elaboration of toxin in axenic culture is similar to its effect on size of halos induced in infection. Consequently, it can be concluded that the

absence of halos at 28°C in infections is not due to inactivation of the toxin by the host, but due to interference with its elaboration by the pathogen. Furthermore, similar effects of temperature on the elaboration of toxin in axenic culture and on the development of halos in infection suggests that the halo in infection is caused by a toxin, as reported previously by Skoog (1952).

The discussion on the effects of temperature presented above indicates that the optimum temperature for the growth rate of the pathogen is not the same as the optimum temperature for toxin production.

In the preliminary experiments it was shown also that isolates P_1, P_3, P_{33}, P_{56}, P_{129} and P_{130} differed in the widths of halos that they typically induce in infection. Jensen and Livingston (1944) mentioned a similar type of variation.

Injection with culture filtrates of the isolates induced the characteristic size of halo which each isolate typically induces in infection. These results suggest that the toxin elaborated in axenic culture under the conditions used is the same as that produced in the course of host-parasite interaction. However, many substances and mixtures of substances can induce chlorosis so that these

results are not conclusive.

The results on the effect of the culture filtrate on the growth of the pathogen indicate that isolates P_{33} and P_{56} did not produce self-inhibitors in their cultures. This result and the fact that the filtrate of isolate P_{56} induces wide halos upon injection, means that the halo inducing toxin is not a growth inhibitor for the pathogen in axenic culture. Furthermore, the sudden delimitation of the necrotic spot in infection cannot be explained by the accumulation of an inhibitor at this locus in the lesion.

Results of the experiment on accumulation of starch in chlorotic tissues showed that starch accumulation was induced both by injection and by infection. This finding supplies further evidence that the toxin present in the culture filtrate is similar to that produced in the course of host-parasite interaction. However, several agents can cause starch accumulation. It could be a secondary effect of injection induced by a substance not present in the host-parasite interaction. The significance of this justifies further research.

Kinetin, although it reduced the size of halos in infection, did not reduce the size of those induced by injection. This lack of correlation does not necessarily mean that a different toxin is produced in axenic culture

than in the infected host. It could be that the amount of kinetin supplied in one application is adequate to counteract the relatively small amount of toxin elaborated in infection, whereas it seems inadequate in injection in which case a relatively great amount of toxin is supplied at one time. In addition, various inhibitors of kinetin could be present in the culture filtrate. The mode of action of kinetin on this toxemia could be similar to its mode of action on wildfire of tobacco as reported by Farkes and Lovrekovich (1963). This would mean that the toxin interferes with protein synthesis; however, further studies should be made to make this evidence conclusive.

One of the criteria suggested by Wheeler and Luke (1963) for establishing the toxigenic theory of disease is that the toxin be host specific. Results of the experiment on host specificity of the culture filtrate showed that halos could be induced in various plant species indicating that the toxin is not host specific. This lack of specificity implies that the toxin present in the culture filtrate plays a secondary role in the toxemia of halo blight. Several toxins have been shown to play a secondary role in plant disease. For example in wilts, fusaric acid, lycomarasmin, and other toxins may play a role as reported by Gäumann (1958) and Sadasivan (1961). These toxins, however, are not responsible for the pathogenicity of the pathogen.

The size of a halo induced by injection was a reproducible measure of the potency of the culture filtrate when applied at various dilutions. It was thus demonstrated that culture filtrates prepared from shake-cultures induced larger halos than those prepared from still-cultures which suggests that the pathogen elaborates more toxin in shake-cultures than in still-cultures. However, the possibility that different toxins were produced is not eliminated.

Storage of the culture filtrate in cold temperature over a period of one year did not affect the potency of the culture filtrate. This finding allows one to prepare large quantities of the culture filtrate at one time that can be stored until use. This property of the toxin is important especially for purification purposes because the pathogen is sensitive to small changes in its environment which makes it difficult to prepare culture filtrates of identical composition.

High temperature did not destroy the activity of the culture filtrate, which suggests that the toxin is thermostable as has been reported before by Skoog (1952). This property of the toxin indicates that it most likely is not proteinaceous. Muller (1950) and Waitz und Schwartz (1956) obtained filtrates of unstable toxicity. This difference in stability of toxin preparation could be due to differences in isolates of the pathogen used.

Attempts to purify the toxin revealed that Norit A did not adsorb the toxin but could be useful to decolorize the culture filtrate. Results on the ion-exchange column indicated that the toxin was neither adsorbed on the cation-exchange column nor on the anion-exchange column which suggests that it is a neutral compound; however the evidence is not conclusive. The dialysis residue did not induce chlorosis upon injection which suggests that the toxin diffused through the membrane and has a small molecular size. The dialysate, however, was not tested for toxicity; therefore, it is possible that the toxin lost its activity during the process of dialysis.

From these studies it appears that the toxin elaborated in axenic culture by Ps.phaseolicola is the same as that produced in the course of host-parasite interaction. Except for the effect of kinetin which could be explained, several other criteria were met to establish the toxigenic nature of the halo in halo blight of bean.

The findings discussed above are believed to contribute some basic information about host-parasite relationships and to give a better insight into the toxigenic theory of plant disease.

VI. SUMMARY

Investigations on the toxemia of halo blight of bean were based on the correlation between infection and injection with culture filtrate of Ps.phaseolicola. An attempt was made to determine the nature of the toxin.

Preliminary experiments indicated that the effect of temperature on the growth rate of the pathogen in the host was similar to that in axenic culture.

The absence of halos at 28°C was not due to inactivation of the toxin by the host, but due to interference with its elaboration by the pathogen.

Similar effects of temperature on the elaboration of toxin in axenic culture and on the development of halos in infection suggested that the halo in infection was caused by a toxin.

Injection with culture filtrates of the isolates induced the characteristic size of halo which each isolate typically induced in infection.

Starch accumulated in halos resulting from infection as well as from injection.

Kinetin reduced the size of halos in infection but not the size of those induced by injection; however, this lack of correlation could be explained.

The sudden delimitation of the necrotic spot in infection could not be explained by the accumulation of an inhibitor at this locus in the lesion.

The toxin present in the culture filtrate was not host specific but did induce chlorosis in various plant species.

The size of a halo induced by injection was a reproducible measure of the potency of the culture filtrate when applied at various dilutions.

More toxin was elaborated in shake-cultures than in still-cultures; the toxin was thermostable and its potency was not affected by storage in cold temperature.

Attempts to purify the toxin revealed that Norit A could be used to decolorize the culture filtrate. The toxin was dialysable, and probably, a neutral compound.

The results of this work indicate the toxigenic nature of the halo in halo blight of bean.

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VIII. APPENDIX I

ANALYSIS OF VARIANCE OF MEASUREMENTS ON THE AREA (cm^2) OF HALOS INDUCED BY VARIOUS CONCENTRATIONS OF THE CULTURE FILTRATE

Source	Degrees of freedom	Sum of squares	Mean square	F-value	F-value from Table
Blocs (Unadj.)	13	5.760			
Treatments (Adj.)	6	9.300	1.550		
Intra-block error	22	2.238	0.102	15.19**	3.76
	41	17.298			

** Significant at 1% level.