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THE USE OF SEROLOGY AND SEMI-SELECTIVE MEDIA AS AIDS IN THE DETECTION OF XANTHOMONAS BEAN BLIGHT BACTERIA

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1979

ABSTRACT

THE USE OF SEROLOGY AND SEMI-SELECTIVE MEDIA
AS AIDS IN THE DETECTION OF XANTHOMONAS
BEAN BLIGHT BACTERIA

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Seed-borne common (Xanthomonas phaseoli, Xp) and fuscous (X. phaseoli var. fuscans, Xpf) bacterial blights continue to affect dry bean production in many areas of the U.S., Canada and Latin America. While planting of pathogen-free seed remains effective for control of the Xanthomonas diseases, techniques for detection of internal seed contamination by blight bacteria are often time-consuming (seedling injection) or require large bacterial populations (serology).

A combined serological and semi-selective media technique for the detection of internally-borne Xp and Xpf in dry bean seed was developed.

Antisera of Xp and Xpf at titres of 1:2000-1:5000 were produced in rabbits by intravenous injfection of formalin-killed cells suspended in buffered saline (10^9 cells/ml). Injections were made at 0 (0.1 ml), 4 (0.3 ml), 8 (0.5 ml), 11 (1.0 ml), and 14 (2.0 ml) days; sera were collected 7, 14, and 21 days after the last injection.

Agar gel double diffusion tests were found more reliable than micro- and tube-agglutinin tests in serological studies of Xp and Xpf. Live cells of Agrobacterium, Bacillus, and Corynebacterium sp. did not react in agar gel double diffusion tests; when steamed cells (60 min. at 100°C) of other bacterial isolates (Erwinia or Pseudomonas) were tested, the reaction was Xanthomonas specific.

In agar double diffusion tests, Xp and Xpf antisera reacted positively to steamed cells of 20/20 Xp and 29/29 Xpf isolates, but did not react to steamed cells of 19 internal bacterial contaminants obtained from surface-sterilized bean seeds.

In absorption tests it was shown that Xp and Xpf possess species-specific heat stable antigens. Xp and Xpf concentrations near 10^7 cells/ml were sufficient to be detected in agar gel double diffusion tests.

A semi-selective media (SSM), highly selective for Xanthomonas, was developed and contained: 1.0 gm yeast extract, 25 mg cycloheximide, 2 mg nitrofurantoin, 1 mg nalidixic acid, and .05 mg gentamicin in 1000 ml .01 M phosphate buffer pH 7.2.

Utilizing XpfR10, resistant to 50 ppm rifampin, and selective plating on media with and without rifampin, we obtained maximum XpfR10 and minimum bacterial contaminant populations by incubating R10-infected seed (1 infected seed:4 non-infected seed) in the SSM.

SSM was inhibitory to all of the Gram positive bacterial idolates tested, including different isolates of Corynebacterium and Bacillus sp., and inhibited the growth of most of the Gram negative bacteria tested.

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The Michigan Department of Agriculture (MDA) test for internal blight contamination of bean seed currently involves: (1) surface sterilization of 1.9 kg seed for 10 minutes in 2.6% NAOcl; (2) rinsing in sterile H_2O; (3) incubation of seed for 18-24 hours in sterile H_2O containing 10 gm/litre yeast extract; and (4) injfection of a sample of liquid surrounding seed into primary leaf node of young kidney bean seedlings.

Samples of 1 ml of the surrounding liquid obtained from the MDA were individually incubated in 25 ml SSM for 24-36 hours in a rotary shaker. Bacteria were then sedimented by 15 minutes centrifugation at 5000 x g, resuspended in 1 ml buffered saline, steamed 60 min. at 100°C, and tested serologically (SSMS). Sixty-one of sixty-five bean seed samples found to carry internal blight contamination in the Michigan Department of Agriculture test reacted positively in the serological test.

Thirty-seven of the ninety-nine navy bean samples obtained from the MDA showed positive results for internal blight contamination with the SSMS procedure. Of these 37 the MDA seedling injection detected blight in only 25. The SSMS also consistently detected Xanthomonas blight in other plant tissues infected with blight (stems and leaves). Pseudomonas phaseolicola and Ps. syringae were found to be internally seed-borne in navy (pea) beans.

To my father, Juan, and my friend, Salomon

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. A. W. Saettler who provided counsel, guidance and encouragement throughout the entire investigation, and during the preparation of this manuscript.

Appreciation is likewise extended to Drs. W. M. Adams, E. J. Klos and W. J. Hooker who served as members of my guidance committee and who reviewed this manuscript.

The Facultad de Agronomia and the Consejo de Desarrollo Cientifico y Humanistico, Universidad Central de Venezuela Venezuela) provided financial assistance to the author during this graduate program.

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GENERAL INTRODUCTION

Common blight caused by Xanthomonas phaseoli (E. F. Smith) Dowson (Xp) and fuscous blight caused by Xanthomonas phaseoli var. fuscans (Burkh.) Starr and Burkh. (Xpf) are bacterial diseases of major importance to dry bean (Phaseolus vulgoris L.) production.

Xp and Xpf are considered two of the most important seed-borne diseases of dry edible and green beans in many production areas throughout the world (15, 22) and have been reported from Australia (2, 11), Russia (5), Yugoslavia (19), Michigan, U.S.A. (1) and Venezuela (14).

The pathogens are seed-borne, both internally and externally (16, 22) and are capable of being transmitted long distances with seed. Xpf can survive in seeds for three years at 20-35°C and seed transmission is the primary means for dissemination of these bacterial diseases.

Much research has been directed toward maintaining bean seed stocks free of Xp and Xpf contamination and developing methods to detect seed-borne infection (21). Disease control is based on a seed certification program to maintain clean seed stocks. The Michigan seed certification program is administered by the Michigan Crop Improvement Association under authority delegated to it by the Michigan Department of Agriculture (12).

Copeland et al. (4) have described the process of producing Michigan certified bean seed from breeder and foundation seed stocks. The first step in certified seed production is to plant foundation seed supplied by the Michigan Foundation Seed Association. Such seed is usually grown in the semi-arid or arid west where conditions are unfavorable for seed-borne bacterial diseases. Seeds from fields which pass visual inspection for the presence of blight symptoms, and which show no contamination in laboratory tests for Xp and Xpf, can be sold as certified.

Numerous assay methods have been developed or adapted for detecting the presence of seed-borne Xp and Xpf and other bacterial diseases.

Direct plating of seed in agar has been used (7, 20). The cotyledon method (13, 18) has been used for the detection of Ps. glycinea in soybean seeds (13). Another method used is the phage plaque count (8, 9). Schuster has used a leaf water-soaking method (17). Serology has been used by several workers (6, 10), and, in Michigan, the Michigan Department of Agriculture Bean Seed Testing Program uses a laboratory blight test developed by Saettler (16).

There is no doubt that such tests have been successful in reducing seed infection by the bacterial pathogens; nevertheless, outbreaks of common and fuscous blights persist and some fields are rejected annually for certification. This suggests that present methods for assaying seed are not entirely satisfactory for detecting internal blight contamination of bean seed.

Objectives

1. Production and characterization of antisera to *Xanthomonas phaseoli* and *X. phaseoli* var. *fuscans*.
2. Development of a semi-selective medium for *Xp* and *Xpf*.
3. To determine the possible utility of combined serological and semi-selective media techniques for the detection of *Xanthomonas* blight bacteria in seeds and other plant tissues.

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CHAPTER I

SEROLOGY OF *XANTHOMONAS PHASEOLI* (BEAN COMMON BACTERIAL BLIGHT) AND *XANTHOMONAS PHASEOLI* VAR. *FUSCOUS* (BEAN FUSCOUS BACTERIAL BLIGHT)

Literature Review

The genus *Xanthomonas* was proposed in 1939 by Dowson (9) and and was defined as follows: "*Xanthomonas*, n.g., non-sporing rod-shaped bacteria, gram negative, motile by means of one polar flagellum (rarely two present) or non-motile, yellow in the (mass) of nutrient agar and on potato, on which abundant slimy growth is formed. Most species digest starch and produce acid in lactose. None produce acid in salicin; they are all pathogenic for plants, and there seems no doubt that these organisms form a definitive group." On the basis of morphology and biochemical properties, the individual species have few distinguishing features (3).

In 1962 Dye (10) performed a comparative study of 209 phytopathogenic *Xanthomonas* species comprising 57 recognized species, using numerous standard identification methods. He concluded that they formed a remarkably uniform group which could easily be distinguished from some other yellow pgimented organisms.

Dye and Lelliot described the genus *Xanthomonas* in the 1974 Bergey Manual of Determinative Bacteriology (4). Up until this

time, serological work with Xanthomonas species was scattered and incomplete (11).

St. John-Brooks, Nain and Rhodes in 1925 (38), working with 33 strains received from Dr. Erwin Smith and using tube agglutination tests, found that of three strains of Xanthomonas campestris, two appeared to act alike, while one was unique in its agglutinative reactions in ten antisera prepared with bacterial plant pathogens, and that two strains of Xanthomonas malvacearum showed identical cross reactions. They also observed a close relationship between X. campestris and X. malvacearum and between X. phaseoli, X. phaseoli var. sojense, X. pelargoni and X. vitians.

In 1927 Sharp (36) produced sera against Corynebacterium flaccumfaciens, X. phaseoli and X. phaseoli var. sojense, with which these bacteria could be differentiated in tube agglutination tests with live cells as antigen.

Link and Link in 1928 (21) found that the agglutination tests could differentiate Xanthomonas malvacearum from X. campestris, X. phaseoli, X. citri, X. cucurbitae and X. pruni. These workers found that direct agglutination tests were of little use in distinguishing between X. malvacearum and X. phaseoli sojense. They concluded that not all of the yellow organisms tested form a single serological group.

In 1929 Link, Edgecombe, and Godkin (20) utilized agglutinin absorption as well as agglutination tests. They stated that serological studies apparently gave promise in grouping and classifying

at least some of the closely related species of phytopathogenic bacteria.

In 1931, William and Glass (42) and Horgan (17) independently established the serological homogeneity of Xanthomonas malvacearum by agglutinin absorption tests.

In 1940, McNew and Braun (27) and Braun and McNew (5) concluded that X. stewartii was not serologically homogenous; we know now that X. stewartii corresponds to the genus Erwinia (3).

In 1947, Elrod and Braun reported serological studies in the genus Xanthomonas (13, 14, 15). They found that when phytobacteria were grown in sugar-rich media for 72 hours at room temperature, the bulk of the growing mass was composed of extracellular mucoid material and that the mucoid material interfered with antigenic patterns. Mucoid material extracted with warm saline and harvested according to the method of Morgan and Beckwith (28) yielded a large quantity of polysaccharide. In agglutinin tests the semi-purified polysaccharide acted as a common component giving strong reactions at high serum concentrations. They stated that the common mucoid material, produced by many bacteria, was responsible for the cross reaction obtained by earlier workers in the field. Elimination of the mucoid exudate resulted in cellular antigens which, on cross agglutination, reacted more specifically than gummy suspensions. They defined five immunological groups: "vascularum," "phaseoli," "campestris," "translucens," and "pruni." The X. phaseoli group contained X. phaseoli, X. phaseoli var. fuscans, X. geranii, X. pelargoni, and X. malvacearum. The phaseoli block

showed strong antigenic ties to one another and to X. campestris and X. barbarbareae.

The "fuscans" bacteria agglutinated in mahy "vascularum" antiserum but the reciprocal case was not true. They stated that members of the Xanthomonas phaseoli group had common group components and absorption of any of the individual antisera by a heterologous organism (of the same group) removed all group components and left specific factors.

The next report on serology of Xanthomonas appeared in 1959, when Mushin et al. (31) mentioned the use of slide and tube agglutination and agglutinin absorption tests. "O" antisera were prepared with bacteria grown 24 hours on carbohydrate-free medium (meat infusion or brain heart agar); both steamed and Roschka's antigens (alcohol-acetone treated suspensions) were used for antisera production. Mucoid was removed from Xanthomonas cultures using trypsin digestion. "H" antisera (flagella) were prepared by passing bacteria several times through semi-solid (0.4%) agar in Craigie tubes until active motility was induced. Preliminary agglutination tests were performed with steamed, Roschka's, alcoholized, and autoclaved bacterial suspensions as antigens. The first two antigens proved to be the most sensitive. Antisera to Xanthomonas phaseoli reacted non-specifically with X. campestris, X. carotae, X. vesicatoria, X. incanae, X. juglandis, and X. albineans.

Until 1959, then, the only information relative to the serology of Xanthomonas was that various species cross agglutinate and that some species agglutinate in some sera prepared against

other bacterial genera. Only short notes and abstracts on serological techniques applied to the genus Xanthomonas have been presented in recent years.

In 1964, Morton (30) detected Xanthomonas vesicatoria in leaves of Capsicum frutescens by bentonite flocculation. The same author (29) suggested the use of antibodies conjugated with fluorescent iso-thiocyanate and ultraviolet examination to detect X. vesicatoria on pepper leaf sap.

In 1970, R. Charudatan and R. E. Stall (7) used agar gel double diffusion tests and examined 72 isolates of X. vesicatoria from pepper and tomato against antisera prepared with sonicated cells; two serotypes were described based on the presence or absence of specific precipitin bands.

In 1971, Orellana and Weber (33), working with X. cyamopsidis races 0 and 1 and using immunodiffusion analysis, found that the two races differed antigenically.

In 1972, Saaltink et al. (35) prepared antisear to X. hyacinthi and X. gummisudans and used a microdroplet agglutination method under liquid paraffin, described by van Slogteren (37).

In the same year Namekata and Oliveira (32) reported a comparative gel diffusion and immuno-electrophoresis study of X. citri and a bacterium causing a canker disease on Mexican lime; the authors used bacterial extracts obtained by heating suspensions for 45-60 minutes at 100°C.

Saalting and others (35) present procedures for serological identification of bacteria in tissues of leaves or bulb scales of

infected hyacinths. X. hyacinthi was identified with the micro-droplet agglutination method (37). Erwinia and Corynebacterium species have been identified using agar gel double diffusion (18, 41).

Recently, a new bacterial disease attacking onions was identified serologically as being caused by a Xanthomonas sp. (1).

A thorough literature search, including a computer search in 1977 and 1978, disclosed no additional work on the relationship between X. phaseoli and X. phaseoli var. fuscans or any other Xanthomonas nomen species. However, there are a number of papers on similar other genera, in particular the genus Pseudomonas.

Pertinent papers include: in 1969 Lucas and Grogan (24, 25) reported on serological variation and identification of Pseudomonas lachrymans and other Pseudomonas nomen species. They used gel diffusion tests with antigens from different plant pathogenic Pseudomonas and found that all shared common antigens but each possessed at least one specific antigen. Common antigen bands were eliminated by heating antigen preparations one hour at 100°C. The same workers also found that specific antigens from smooth serotypes I and II of Pseudomonas lachrymans and smooth isolates of other nomen species could be extracted with trichloracetic acid or phenol water; antigens of rough isolates could not be extracted with these procedures. Antigens from smooth isolates were serologically active, heat stable with properties similar to "O" antigens of other Gram negative bacteria.

Otta and English in 1970 (34) divided 450 isolates of Ps. syringae into ten distinct serotypes based on the reaction of their

heat stable antigens in gel diffusion tests. Rough and smooth isolates possessed serologically identical heat-stable antigens. In the same year, Taylor (39) showed that the heat labile antigen possessed by Ps. phaseolicola was common to 12 other plant pathogenic Pseudomonas species. The heat stable antigen was more specific and was detected only in Ps. morspronorum and Ps. primilae, neither of which occur on bean. Coleno et al. (8) in 1971 studied several phytopathogenic Pseudomonas by the complement fixing technique and showed the existence of several antigenic determinants of which some were common, and others were specific. The specificity could be used in taxonomy or detection of seed-borne bacteria (with the possibility of estimating contamination rates). In 1975, Tunstall and Gowland (40) found that three different antigens were associated with the cell walls of Pseudomonas species: (1) a highly specific antigen of high molecular weight conposed of protein or lipoprotein in nature as an envelope around the cell; (2) another antigen common to all Pseudomonas sp. was studied and was situated below the first antigen, and composed of polysaccharide or lipopolysaccharide in nature; and (3) a heat-labile antigen which was common to all Pseudomonas sp. and consisted of the mucopeptide of the cell wall, and which was dependent on both the carbohydrate and polypeptide components of the macro-molecule.

Objectives

The objectives of this study were threefold: (1) the production and characterization of antisera to Xanthomonas phaseoli

and X. phaseoli var. fuscans using O antigen (steamed) and H antigen (formalized); (2) to determine the specificity of the antisera obtained against Xanthomonas phaseoli and X. phaseoli var. fuscans; and (3) to examine the use of microagglutination and agar gel double diffusion techniques for studying antisera to Xanthomonas phaseoli and X. phaseoli var. fuscans.

Materials and Methods

Antisera Preparation

Antisera were prepared against formalized and steamed cells of several isolates each of Xanthomonas phaseoli (bean common blight) and Xanthomonas phaseoli var. fuscans (bean foscous blight).

Formalized cells.--Cells were obtained by inoculating the surface of YCA plates (10 gm yeast extract, 2.5 gm $CaCO_3$, 20 gm of agar in 1,000 ml of distilled water), with the desired isolate of Xanthomonas. The agar cultures were incubated at room temperature (23°C) for 24-48 hours. The growth then was removed with formol saline. The cell suspension was allowed to stand at room temperature for 48 hours to kill the cells but maintain the flagella intact (Formalin = 40% v/v formaldehyde in psysiological saline. Formol-saline = .6% v/v Formalinin Physiological saline. Saline = 8.5 gm NaCl in 1,000 ml distilled water).

Cells were then centifugated twice in a Sorvall centrifuge SS-1 set at 30 for 20 minutes and resuspended in saline. The cells were adjusted to a density of 1×10^9 cells/ml using McFarland nephelometer tube no. 3 (2).

Five ml volumes of the suspension were aseptically stored in sterile serum bottles; 2-3 drops were removed and transferred to YCA plates to check for sterility.

Steamed cells.--The somatic antigens were prepared by removing the 24-48 hr. growth from YCA plates with saline.

Cell suspensions were washed twice by centrifuging as in Formalized Cells and resuspended in a buffer saline. Cell suspensions standardized to 10^9 cells/ml were steamed at 100°C for one hour. This treatment killed the cells as well as destroyed the flagella (19). Volumes of 5 ml cell suspension were stored in sterile serum bottles and checked for sterility as before.

The antigens prepared in Formalized Cells and Steamed Cells were stored in 4°C and used when needed.

Immunization of rabbits.--Young female rabbits were injected at 0 (0.1 ml), 4 (0.3 ml), 8 (0.5 ml), 11 (1.0 ml) and 14 (2.0 ml) days; blood for sera was collected 7, 14, and 21 days after the last injection (6). The marginal ear vein was used for injection. The first injection of the series was always made as near the tip of the ear as possible; succeeding injections were made closer to the animal's head, so the scar tissue would not interfere with the injections.

Rabbits were bled for normal sera, prior to the first injection; titre of the normal sera were determined using standard tube agglutinin tests.

The marginal ear vein was used for bleeding. The vein is located at the outer edge of the dorsal side of the ear. One ear was used for bleeding and the other one for injections. The first bleeding was made from a site near the middle of the ear; succeeding punctures were made distal to the head.

Antisera collection.--Blood was collected in a sterile, dry glass test tube and allowed to stand undisturbed until a firm clot formed. The clot was carefully detached from the inside surface of the container using a sterile wooden applicator. The sample was left at room temperature for 3 hours and placed in a refrigerator overnight. The fluid serum was carefully removed, centrifuged 2,000 x g for 5 minutes to remove cellular components and stored at -5°C in serum bottles.

Serological Techniques

Agglutination.--The clumping of particulate antigen--in our case bacterial cells, mediated by antibodies (agglutinin). Agglutination is the specific combination of agglutinin with its homologous antigen or a closely related one, followed by aggregation of the particles and antibody (6, 12).

a. General Procedure

1. 12 x 75 mm test tubes were placed in a rack.
2. 0.8 ml of saline was added to tube 1 and 0.5 to the remaining tubes.
3. 0.2 ml of serum was added to tube 1.

4. 0.5 ml from tube 1 was transferred and mixed with tube 2; 0.5 ml from tube 2 was transferred and mixed into tube 3; continuing until contents of the next to the last tube had been mixed; 0.5 ml was then discarded from the next to the last tube.
5. 0.5 ml of antigen (adjusted to 1 x 10^8 cells/ml) was added to all tubes and mixed. The last tube contained no serum and represented the control.
6. The tubes were incubated 24-48 hr. in a water bath at 37°C.

b. Agglutination Reactions

Each tube was examined for settled aggregates of cells and the cell patterns on the bottom of the titration tubes were compared with the pattern of the cells in the control tube. Tubes were marked 4+, 3+, 2+, 1+ or negative, depending on the amount of agglutinated cells on the bottom of the tube relative to the control.

Microagglutination.--One disadvantage of the tube agglutinin test is the requirement for large volumes of antigen and antiserum (12). On the other hand, the use of a grid titration system in the microagglutination test would utilize only 0.2 ml total volume of antiserum and antigen.

a. Procedure

A square plastic petri dish (9 cm square) was divided in a checkerboard pattern with wax pencil; each check

was 1.5 cm square. A small drop of the serial dilution of tne antisera was placed in each square. A drop of antigen adjusted at 10^8 cells/ml was placed over each drop of the dilution. A check consisting of a drop of saline plus a drop of antigen was always used.

The petri dishes were incubated at room temperature and were carefully sealed with two layers of parafilm paper. The reaction was read after two hours and again in the morning using obliquely-transmitted light in a stereoscopic microscope.

Gel diffusion tests.--These tests utilize the precipitation of antigens and antibodies in a gel medium rather than a fluid medium. Lines of precipitation will form when specific antigen and antibody come together in equivalent proportions by diffusing through the agar media.

a. Ouchterlony (Immunodiffusion in agar plates)

The media contained 8.5 gm, purified agar, 10 ml of solution containing 1% w/v Orange G, 30 ml of a solution containing 10 mg/ml NaN_3, and 1000 ml of buffered saline. NaN_3 was added to prevent bacterial and fungal contamination (16); Orange G was added to increase contrast during photography (18).

Petri dishes of 9 cm diameter were filled with 20 ml of melted medium, and 2 serological tests were routinely performed in each plate. Each serological test

consisted of one central hole of 5 mm diameter surrounded by 6 holes of 5 mm diameter.

Normally, the central hole contained the antisera and the surrounding holes contained the antigens. All plates were maintained inside plastic bags to avoid loss of humidity, and readings were taken every two days.

Radial immunodiffusion.--This procedure employs the precipitation of antigen-antibody complex in a gel for quantification of one of the reacting components (19). Antibody is incorporated into a buffered agar medium, and the soluble antigen is placed in wells cut in the medium. Antigen diffuses out from the well and precipitates with the antibody in the medium in a radial pattern. The diameter of the ring of precipitation directly reflects the concentration of antigen in the well.

a. Procedure

1. 1.7 gm of purufied agar (Difco Co.); 100 ml Borate buffer (pH 8.4); 6 ml of NaN_3 solution containing 10 mg/ml. The media was melted at 100°C and equilibrated to 55°c in water bath.
2. Antisera were diluted 1:15 or 1:20 with borate buffer and maintained at 55°C in a water bath.

Equal volumes (10 ml) of (1) and (2) were mixed in a petri dish and allowed to cool. Equally spaced wells (6) of 5 mm diameter were cut in each petri plate.

In each petri plate two of the wells were filled with reference antigens, the remaining wells were filled with test antigens. Rings were measured one week later.

Preparation of the absorption-antisera.--Agglutinin-absorption is used to prepare "typing serum" which contain the antibodies desired to identify (by agglutination or precipitation) only those major determinants as desired (19).

a. Procedure

1. 1 ml of the desired antiserum was diluted 1:10 with saline and mixed with 1 ml of packed cells of the selected antigen in a 12 x 75 mm test tube.
2. Tubes were incubated for 4 to 5 hours in 45°C water bath and then refrigerated overnight.
3. Contents of the tube were centrifuged at 2000 x g, the supernant was carefully removed to a clean test tube. Efficiency of the absorption was determined by a standard agglutination test against the homologous antigen, and the procedures in (1) and (2) were repeated as necessary.

Results

Titres of Different Antisera to Xanthomonas Using the Tube and Microagglutinin Tests

Antisera titres obtained when formalized cells (H antigen) were used as antigen were equal to or greater than titres obtained when steamed cells (O antigen) were used (Tables 1, 2). Titres

TABLE 1.--Titres of Different *Xanthomonas* Antisera as Determined by Agglutinin Tests with Homologous Antigen.[a][b]

Antisera Prepared Against	Antisera to	Antisera from 1st Bleeding	2nd Bleeding	3rd Bleeding	4th Bleeding	Nomal Sera
Formalized cells	*Xpf*16	4096	4096	512	256	16
	*Xp*11	4096	2048	256	128	32
	*Xp*15	4096	4096	256	128	0
	*Xpf*1085	2048	2048	256		0
Steamed cells	*Xpf*16	1024	256	256	128	8
	*Xp*15	2048	512	256	128	8
	*Xp*11	2048	512	256	128	8

[a]Antigen represents steamed cells adjusted to an optical density of h = 0.3.

[b]Data represent the greatest dilution of the antisera at which agglutination still occurs.

TABLE 2.--Titres of Different Xanthomonas Antisera as Determined by Microagglutination Tests with Homologous Antigen.[ab]

Antisera Prepared Against	Antisera to	Antisera from 1st Bleeding	2nd Bleeding	3rd Bleeding	4th Bleeding	Normal Sera
Formalized Cells	Xpf16	8192	4096	512	256	16
	Xp11	4096	2048	256	256	32
	Xp15	8192	4096	512	128	0
Steamed Cells	Xpf16	1024	256	256	128	8
	Xp15	2048	512	256	256	8
	Xp11	2048	512	512	256	8

[a]Antigen represent steamed cells adjusted to an optical density of h = 0.3.

[b]Data represent the greatest dilution of the antisera at which agglutination still occurs.

were determined against steamed cells (1 hour) of the homologous antigen adjusted to an optical density of h = 0.3, and were added to the corresponding dilution of the antiserum. Titres of the first bleeding were always greater than 1:1024 and decreased with time; the rabbits were bled at least three times. With microagglutinin tests the results were quite similar to agglutinin tube tests, but microagglutination seemed a little more sensitive; in some cases higher titres were found (Table 2).

Antisera Titres as Determined by Immunodiffusion in Agar Plates (Ouchterlony)

Antisera from the first and second bleeding diluted 1:2 showed no differences in band formation compared with undiluted antisera. At antisera dilutions of 1:4, 1:8, and 1:16 numbers of bands decreased and time required for band formation generally increased (Table 3). With some antisera, bands were still formed with 1:32 dilutions in physiological saline.

Excellent band formation (3 or more bands) at 48 hours were obtained with sera from the first, second and third bleedings with titres ranging from 256-8192 (in microagglutinin tests).

Weaker band formation was accompanied by fewer bands (2 or 1) and more time to appear (greater than 3 days), when antisera of titres less than 256 were used (Table 4).

TABLE 3.--Agar Gel Double Diffusion Tests of Different Dilutions of Xanthomonas Antisera Against the Homologous Antigen.[a]

Antisera Prepared Against	Antisera to	Micro-agglutinin Titre	Undiluted Antisera	Band Numbers with Different Dilution of the Antisera[b]					
				1:2	1:2	1:8	1:16	1:32	1:64
Formalized Cells	Xpf16	8192	>4[c]	>4	2	2	2	2	0
	Xp11	4096	>4	>4	3	2	2	1	1
	Xp15	8192	4	3	2	2	2	1	0
Steamed Cells	Xpf16	1024	3	3	3	2	1	0	0
	Xp15	2048	>4	3	2	1	1	0	0
	Xp15	2048	>4	3	2	1	1	0	0

[a]Antigen represent formalized cells adjusted to about 10^{10} cells/ml.

[b]Antisera was diluted with physiological saline.

[c]>4 = 4 or more bands.

TABLE 4.--Agar Gel Double Diffusion Tests of *Xanthomonas* Antisera with the Homologous Antigen.[a]

Antisera Prepared Against[b]	Antisera to	1st Bleeding	2nd Bleeding	3rd Bleeding	4th Bleeding
Formalized Cells	*Xpf*16	++[c]	++	++	+
	*Xp*11	++	++	++	+
	*Xp*15	++	++	++	+
Steamed Cells	*Xpf*16	++	++	++	+
	*Xp*11	++	++	++	+
	*Xp*15	++	++	++	+

[a]Antigen represent steamed cells adjusted to about 10^{10} cells/ml.

[b]Antisera from different bleedings.

[c]++ = clear band formation, 3 or more bands, 48 hours required for formation;
+ = weak band formation, 1 or 2 band, 72 hours required for formation.

The Effect of Antigen Concentration on Band Formation in Agar Gel Diffusion Tests

All experiments were performed using steamed bacterial cells as the antigen. In preliminary studies it was noted that antigen adjusted to an optical density, h = 0.1, resulted in excellent band formation against the homologous antisera (Table 5).

Antigen at h = 0.1 represents 0.45 x 10^7 Xp cells/ml or 0.6 x 10^7 Xpf cells/ml; antigen adjusted to h = 0.05 failed to give band formation; h = 0.05 represents 0.2 x 10^7 Xp cells/ml or 0.3 x 10^7 Xpf cells/ml.

In another series of experiments, a range of antigen concentrations between h = 0.1 and h = 0.05 was studied. Bands were consistently formed with antigen at h = 0.07 but not at h = 0.06 (Table 6). Antigen at h = 0.07 represents 0.45 x 10^7 Xp cells/ml or 0.35 x 10^7 Xpf cells/ml; antigen at h = 0.06 represents 0.38 x 10^7 Xp cells/ml or 0.29 x 10^7 Xpf cells/ml.

Reactions of Xanthomonas Antisera Against Live and Steamed Cells of Various Bacteria

In tube and microagglutination tests with live cells as antigen, Xanthomonas antisera reacted strongly with the homologous antigen; however, the antisera also cross-reacted with other Xanthomonas species as well as with bacteria from the genera Pseudomonas, Erwinia and Corynebacterium (Table 7).

When antigen preparations were washed two times and steamed for one hour, the antisera reacted strongly with the homologous and

TABLE 5.--Agar Gel Double Diffusion Analysis of *Xanthomonas* Antisera Tested Against Various Concentrations of Homologous Antigen.[a]

Antisera Prepared Against	Antisera to[b]	Heavy Suspension	Optical Density (h) of the Homologous Antigen					
			0.4	0.3	0.2	0.1	0.05	0.025
Formalized Cells	*Xpf*16	++[c]	++	++	+	+	-	-
	*Xp*11	++	++	++	+	+	-	-
	*Xp*15	++	++	++	+	+	-	-
Steamed Cells	*Xpf*16	++	++	++	+	+	-	-
	*Xp*11	++	++	++	+	+	-	-
	*Xp*15	++	++	++	+	+	-	-

[a]Antigen represent steamed cells.

[b]Titres of antisera used were 512 or 1024 (undiluted).

[c]++ = Clear and strong band formation, always 3 or more; + = clare band formation, no more than 2 bands; - = no band formation.

TABLE 6.--Agar Gel Double Diffusion Analysis of Different Xanthomonas Antisera Against Different Concentrations of the Homologous Antigen.[a]

Antisera Prepared Against[b]	Antisera to	Optical Density (h) of the Homologous Antigen					
		0.1	0.09	0.08	0.07	0.06	0.05
Formalized Cells	Xpf16	++[c]	++	+	+	+	-
	Xp11	++	++	+	+	-	-
	Xp15	++	++	+	+	+	-
Steamed Cells	Xpf16	++	++	+	+	+	-
	Xp11	++	++	+	+	-	-
	Xp15	++	++	+	+	-	-

[a]Antigen represent steamed cells.

[b]Titres of antisera used were 512 or 1024 (undiluted).

[c]++ = clear band formation, no more than 2 bands, formation starts after 48 hours;
+ = band formation no more than one band, after 72 hours;
- = no band formation.

Figure 1.--Serologic reactions of X. phaseoli var. fuscans (16) antisera at different dilutions with homologous antigen. The center well contained 10^{10} steamed Xpf cells/ml. Antisera dilutions were: L = undiluted, 2 = 1:2, 3 = 1:4, 4 = 1:8, 5 = 1:16, 6 = 1:32.

Figure 2.--Serologic reactions of X. phaseoli var. fuscans (16) antisera at different dilutions with homologous antigen. The center well contained 10^{10} live Xpf cells/ml. Antisera dilutions were: 1 = undiluted, 2 = 1:2, 3 = 1:4, 4 = 1:8, 5 = 1:16, 6 = 1:32.

Figure 1

Figure 2

TABLE 7.--Reactions of Several Plant Pathogenic Bacteria in Agglutination Tests Against *Xanthomonas* Antisera.[a]

Antigen[b]	Antisera Prepared Against					
	Formalized Cells			Steamed Cells		
	*Xpf*16	*Xp*15	*Xp*11	*Xpf*16	*Xp*15	*Xp*11
Corynebacterium fascians	+[c]	+	+	+	+	+
C. flaccumfaciens 9A	++	++	++	++	+	+
C. flaccumfaciens 6887	+	++	+	+	+	+
C. flaccumfaciens CFA	+	++	++	+	+	+
C. michiganense	+	+	+	+	+	+
Erwinia amylovora	+	+	+	++	+	+
E. carotovora var. *atroseptica*	+	+	+	++	+	+
E. herbicola	++	+	+	++	+	+
Pseudomonas fluorescens	+	+	++	++	++	++
Ps. glycinea	++	++	++	++	++	++
Ps. syringae	+	+	++	+	++	+
Ps. phaseolicola 301	++	++	++	+++	++	++
Ps. phaseolicola 302	++	+++	++	+++	++	++
Xanthomonas campestris	++++	++++	++++	++++	++++	++++
X. juglandis	++++	++++	++++	++++	++++	++++
X. pelargoni	++++	++++	++++	++++	++++	++++
X. phaseoli 11	++++	++++	++++	++++	++++	++++
Xp 15	++++	++++	++++	++++	++++	++++
X. phaseoli var. *fuscans* 16	++++	++++	++++	++++	++++	++++

[a]Titres of the antisera with the homologous 1024.

[b]Antigen, live cells adjusted to an optical density of h = 0.3.

[c]+ = titres 32 or less; ++ = titres between 64-128; +++ = titres equal to 245; ++++ = titres equal to or greater than 512; - = no cross agglutination (equal to or less than normal sera agglutination).

other Xanthomonas species but did not cross-react with bacteria from most of the other genera as before. Xanthomonas antisera always reacted with Pseudomonas sp., particularly with Ps. phaseolicola when tested against steamed antigen (Table 8).

Specificity of Xanthomonas antisera was tested against six isolates of Xanthomonas phaseoli and ten isolates of X. phaseoli var. fuscans. In general, there were higher titre end points in reactions between homologous antigen/antisera combinations than in reactions between heterologous antigen/antisera combinations.

Xanthomonas antisera reacted with all Xp and Xpf isolates, and dilution end points obtained were between 256-1024 (Table 9).

Low or no cross-reaction was found with all antisera to Xp and Xpf when tested against steamed cell preparations of 15 bacterial contaminants isolated from surface-sterilized navy bean seeds (Table A3).

Agar Gel Double Diffusion Tests of Xanthomonas Antisera Against Whole and Steamed Cell Antigens

Xanthomonas antisera reacted strongly with homologous and other siolated of Xanthomonas phaseoli (20 isolates) and Xanthomonas phaseoli var. fuscans (29 isolates) when live cells were used as antigens. However, in similar tests against live cell antigen, the Xanthomonas antisera reacted with X. campestris, X. juglandis, and X. pelargoni (2-3 bands) (Table 11). The Xanthomonas antisera also reacted with live cell antigen of Pseudomonas fluorescens, Ps. glycinea, Ps. morsprunorum, Ps. phaseolicola (2 isolates),

TABLE 8.--Reactions of Several Plant Pathogenic Bacteria in Agglutination Tests Against *Xanthomonas* Antisera.[a]

Antigen[b]	Antisera Prepared Against					
	Formalized Cells			Steamed Cells		
	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
Corynebacterium fascians	-[c]	-	-	-	-	-
C. flaccumfaciens 9A	+	-	-	-	-	-
C. flaccumfaciens 6887	+	+	-	+	-	-
C. flaccumfaciens CFA	+	-	-	-	-	-
C. michiganense	-	-	-	-	-	-
Erwinia amylovora	+	+	-	-	-	-
E. carotovora var. *atroseptica*	+	+	-	-	-	-
E. herbicola	+	-	-	-	-	-
Pseudomonas fluorescens	+	-	+	+	-	+
Ps. glycinea	+	+	-	+	-	+
Ps. syringae	+	-	-	+	-	-
Ps. phaseolicola 301	+	++	++	+	++	+
Ps. phaseolocioa 303	+	++	+	+	++	+
Xanthomonas campestris	++++	++++	++++	++++	+++	+++
X. juglandis	+++	+++	++++	+++	+++	+++
X. pelargoni	++++	+++	+++	++++	+++	+++
X. phaseoli 11	++++	++++	++++	++++	+++	++++
Xp 15	++++	++++	++++	++++	++++	++++
X. phaseoli var. *fuscans* 16	++++	++++	++++	++++	++++	++++

[a]Titres of the antisera with the homologous 1024.

[b]Antigen, steamed cells adjusted to an optical density of h = 0.3.

[c]- = no reaction or equal to or less than the normal sera titre; ++++ = equal to or greater than 640; +++ = between 320-640; ++ = between 1/40 to 1/160; + = 1/40.

TABLE 9.--Cross Agglutination Reactions of Various Xanthomonas phaseoli (Xp) and Xanthomonas phaseoli var. Fuscans (Xpf) Isolates Against Xanthomonas Antisera.[a]

Antisera Prepared Against[b]	Dilution End Point Isolate[c]															
	Xp 11	Xp 15	Xp 47	Xp 57	Xp 101	Xp Neb24	Xpf 107	Xpf 108	Xpf 109	Xpf 110	Xpf 111	Xpf 113	Xpf 114	Xpf 115	Xpf 116	Xpf 16
1) Xpf16	512	512	512	512	256	512	512	512	512	1024	512	512	1024	512	512	1024
2) Xp15	512	1024	512	512	512	1024	512	512	512	512	512	512	512	512	512	512
3) Xp11	1024	1024	512	512	512	1024	256	256	512	512	512	512	512	512	512	1024
4) Xpf16	256	512	512	256	256	512	512	512	512	512	512	512	1024	512	512	1024
5) Xp15	512	1024	512	1024	512	1024	512	512	512	512	512	1024	256	256	512	512
6) Xp11	1024	1024	512	1024	512	512	512	512	256	256	512	512	512	256	256	1024

[a]Antigen was steamed for 1 hour and adjusted to an optical density of h = 0.3.

[b]Antisera 1, 2, and 3 produced injection formalized cells; Antisera 4, 5, and 6 produced with the steamed cells.

[c]Greatest dilution at which agglutination still occurred.

Ps. syringae (4 isolates), Ps. tomato (2 isolates), Erwinia amylovora, E. carotovora var. atroseptica, and E. herbicola. Xanthomonas antisera did not react to live cell antigen of: Agrobacterium tumefaciens, Bacillus megaterium, Corynebacterium flaccumfaciens (4 isolates), C. fasciens, C. michiganense (Table 10).

In agar gel double diffusion tests against steamed cell antigens, Xanthomonas antisera still reacted strongly to the homologous and to other Xp and Xpf isolates but fewer bands were produced (Tables A1, A2, 11). No bands were formed by Xanthomonas antisera against steamed cell antigens of Pseudomonas fluorescens. Corynebacterium fasciens; C. flaccumfaciens (3 isolates), C. michiganense, Erwinia amylovora, E. carotovora var. atroseptica, E. herbicola, Bacillus megaterium and Agrobacterium tumefaciens.

Steamed cell antigens of several Pseudomonas species resulted in band formation with the Xanthomonas antisera. However, these bands did not occur when the steamed cell antigen was diluted.

No cross-reaction was found with all antisera to Xp and Xpf when tested against steamed cell preparation of 19 bacterial contaminants isolated from surface-sterilized navy bean seeds (Table A4).

Agar Double Diffusion Test of Xanthomonas Antisera Against Cell Free Steamed Supernant as Antigen

One to two bands were formed against Xanthomonas antisera, when antigen consisted of Xanthomonas phaseoli or Xanthomonas phaseoli var. fuscans (5 isolates each) as well as Xanthomonas pelargoni. No bands were formed when Xanthomonas antisera were

TABLE 10.--Reactions of Different Plant Pathogenic and Non-Pathogenic Bacteria to *Xanthomonas* Antisera in Agar Gel Double Diffusion Tests.

	Antisera Prepared Against					
	Formalized Cells			Steamed Cells		
Antigen Used[a]	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
Agrobacterium tumefaciens	-[b]	-	-	-	-	-
Bacillus megaterium	-	-	-	-	-	-
Corynebacterium fascians	-	-	-	-	-	-
C. flaccumfaciens 9a	-	-	-	-	-	-
C. flaccumfaciens NE23	-	-	-	-	-	-
C. flaccumfaciens 6887	-	-	-	-	-	-
C. flaccumfaciens a	-	-	-	-	-	-
C. michiganense	-	-	-	-	-	-
Erwinia amylovora	+	+	+	+	+	+
E. carotovora var. *atroseptica*	-	-	-	-	-	-
Pseudomonas fluorescens	+	+	-	+	-	-
Ps. glycinea						
Ps. morsprunorum	+	+	+	+	+	+
Ps. phaseolicola 1	+	+	+	+	+	+
Ps. phaseolicola 2	+	+	+	+	+	+
Ps. syringae 40	+	+	+	+	+	+
Ps. syringae D-3-3	+	+	+	+	+	+
Ps. syringae Y30	+	+	+	+	+	+
Ps. tomato 1	+	+	+	-	+	+
Ps. tomato 2	+	+	+	+	+	+
Xanthomonas juglandis	++	++	++	++	++	++
X. campestris	++	++	++	++	++	++
X. pelargoni	++	++	++	++	++	++
X. phaseoli 11	++	++	++	++	++	++
X. phaseoli var. *fuscans*	++	++	++	++	++	++

[a]Antigen consisted of live bacteria cell adjusted to about 10^{10} cells/ml.

[b]- = no band formation; + = 1 or 2 bands; ++ = 2 or 3 bands.

TABLE 11.--Reactions of Different Plant Pathogenic and Non-Pathogenic Bacteria to *Xanthomonas* Antisera in Agar Gel Double Diffusion Tests.

	Antisera Prepared Against					
	Formalized Cells			Steamed Cells		
Antigen Used[a]	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
Agrobacterium tumefaciens	-[b]	-	-	-	-	-
Bacillus megaterium	-	-	-	-	-	-
Corynebacterium fascians	-	-	-	-	-	-
C. flaccumfaciens 9a	-	-	-	-	-	-
C. flaccumfaciens NE23	-	-	-	-	-	-
C. flaccumfaciens 6887	-	-	-	-	-	-
C. flaccumfaciens a	-	-	-	-	-	-
C. michiganense	-	-	-	-	-	-
Erwinia amylovora	-	-	-	-	-	-
E. carotovora var. *atroseptica*	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-
Ps. glicinea	-	-	-	-	-	-
Ps. morsprunorum	-	+	+	+	+	+
Ps. phaseolicola 1	+	-	+	+	+	+
Ps. phaseolicola 2	+	-	+	+	+	+
Ps. syringae 40	+	-	-	+	-	+
Ps. syringae D-3-3	-	-	+	+	-	-
Ps. syringae Y30	+	+	-	-	+	+
Ps. tomato 1	-	+	+	-	+	-
Ps. tomato 2	-	-	+	-	+	-
Xanthomonas juglandis	++	++	++	++	++	++
X. campestris	++	++	++	++	++	++
X. polargoni	++	++	++	++	++	++
X. phaseoli 11	++	++	++	++	++	++
X. phaseoli var. *fuscans* 16	++	++	++	++	++	++

[a]Antigen consisted of steamed cells adjusted to about 10^{10} cells/ml.

[b]- = no band formation; + = 1 or 2 bands; ++ = 2 or 3 bands.

were tested against: Pseudomonas fluorescens, Ps. syringae, Ps. phaseolicola, Erwinia herbicola, Agrobacterium flaccumfaciens, C. fascians and B. megaterium (Table 12) in similar tests.

Absorption Tests

Antisera prepared against whole cells of Xpl5 did not cross react with the following bacteria after absorption with Xpfl6 or of Xpfl085: X. juglandis, X. pelargoni, X. phaseoli var. fuscans (2 isolates), Bacillus megaterium, E. herbicola, Agrobacterium tumefaciens, Ps. fluorescens, Ps. syringae and Ps. phaseolicola. The antisera did react with 3 isolates of X. phaseoli (Table 13).

After absorption, final antisera titres were reduced from 2560 to 320.

Antisera prepared against whole cells of Xpfl6 did not cross-react with the following bacteria after absorption with Xpl1 or Xpl5: X. juglandis, X. pelargoni, X. phaseoli (isolates 11 and 15), B. megaterium, E. herbicola, A. tumefaciens, C. michiganense, C. fascians, Ps. fluorescens, Ps. syringae or Ps. Phaseolicola. However, the absorbed antisera did react only to Xpf isolate 1085 and to the homologous isolate Xpfl6. Absorption reduced titres from 2560 to 320.

Antisera prepared against whole cells of Xpl5 or Xpfl6 reacted only with the Xanthomonas group after absorption of the antisera with Ps. phaseolicola; absorption reduced antisera titres from 2560 to 640.

TABLE 12.--Reactions of Various Bacterial Isolates Against *Xanthomonas* Antisera in Agar Gel Double Diffusion Tests.

Antigen used[a]	Antisera Prepared Against					
	Formalized Cells			Steamed Cells		
	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
Bacillus megaterium	-[b]	-	-	-	-	-
Corynebacterium fascians	-	-	-	-	-	-
C. flaccumfaciens 6884	-	-	-	-	-	-
C. michiganense	-	-	-	-	-	-
Erwinia herbicola	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-
Ps. glicinea	-	-	-	-	-	-
Ps. syringae	-	-	-	-	-	-
Xanthomonas phaseoli 15	+	+	+	+	+	+
X. phaseoli var. *fuscans* 16	+	+	+	+	+	+
X. pelargoni	+	+	+	+	+	+

[a]Antigen = precipitin obtained by centrifugation of bacterial cultures 15 min. at 3,0000xg, resuspension in buffer saline, steaming 60 min. at 100°C. and filter sterilization.

[b]- = band formation; + = 1 or 2 bands present.

TABLE 13.--Tube Agglutinin Absorption Tests with Different Xanthomonas Antisera.[a,b]

Test Antigen[c]	Antisera Prepared Against Formalized Cells: Absorbed with Formalized Cells	Xpf16: (Check) Not Absorbed	Xpf16: Xp15	Xpf16: Xp11	Xpf16: Ps. phaseolicola	Xp15: (Check) Not Absorbed	Xp15: Xpf16	Xp15: Xpf1085	Xp15: Ps. phaseolicola
Agrobacterium tumefaciens		20	0	0	0	20	0	0	0
Bacillus megaterium		20	0	0	0	20	0	0	0
Corynebacterium fascians		20	0	0	0	40	0	0	0
C. michiganense		40	0	0	0	80	0	0	0
Erwinia herbicola		40	0	0	0	40	0	0	0
Pseudomonas fluorescens		80	0	0	0	160	0	0	0
Ps. phaseolicola 301		160	0	0	0	160	0	0	0
Ps. syringae		160	0	0	0	160	0	0	0
Xanthomonas juglandis		320	0	0	160	320	0	0	80
X. pelargoni		640	0	0	320	640	0	0	160
X. phaseoli 11		1280	0	0	640	1280	160	320	640
X. phaseoli 15		2560	0	0	640	1280	160	160	640
X. phaseoli Neb 24		1280	0	0	640	1280	160	160	320
X. phaseoli var. fuscans 16		2560	320	320	640	1280	0	0	640
X. phaseoli var. fuscans 1085			320	320	640		0	0	640

[a]Titre of the antisera unabsorbed with the corresponding homologous 2560.

[b]Data are presented as dilution end point.

[c]Antigen represent steamed cell adjusted to an optical density of h = 0.3.

Figure 3.--Serologic reactions of X. phaseoli 11 antisera (central well) with live cells 48 hours old of different Xanthomonas isolates adjusted at 10^{10} cells/ml. Well 1 = Xp11, 2 = Xp Neb 24, 3 = Xp15, 4 = Xpf16, 5 = Xpf17, and 6 = Xpf28.

Figure 4.--Serologic reactions of X. phaseoli var. fuscans antisera (central well) with live cells 48 hours old of different bacterial isolates adjusted at 10^{10} cells/ml. Bacterial antigen were 1 and 2 = Ps. phaseolicola, 3 and 4 = Corynebacterium michiganense, 5 and 6 = Xpf16.

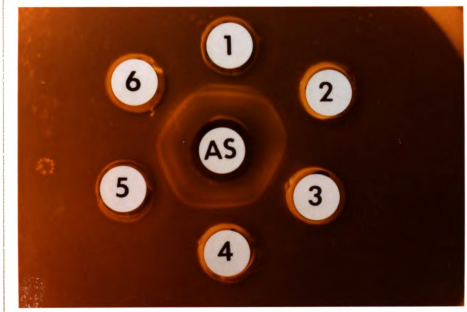

Figure 3

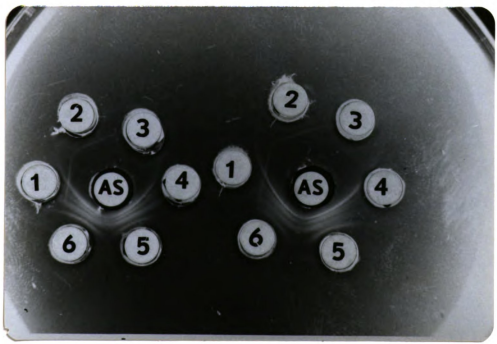

Figure 4

Figure 5.--Serologic reactions of X. phaseoli (15) antisera (central well) with steamed cells of different bacterial isolates adjusted at 10^{10} cells/ml. Wells 1 and 2 Ps. phaseolicola, 3 and 4 E. amylovora, 5 and 6 Xp15.

Figure 6.--Serologic reaction of X. phaseoli (15) antisera (central well) with steamed cells of different bacterial isolates adjusted at 10^{10} cells/ml. Wells 1, 2, 3, 4, and 5 internal bacterial bean contaminants, 6 Xp15.

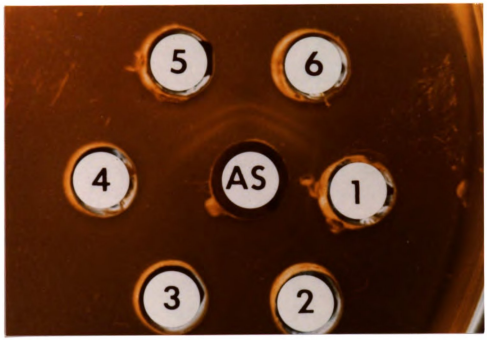

Figure 5

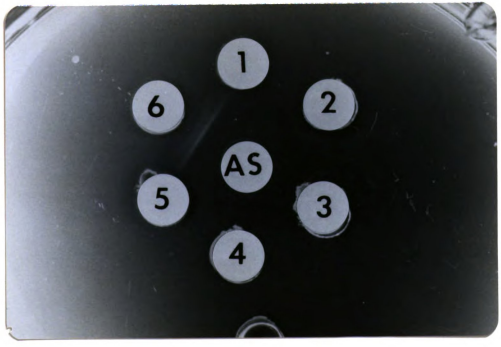

Figure 6

Figure 7.--Serologic reaction of X. phaseoli (11) antisera with different concentrations of the homologous (steamed) antigen (antigen adjusted at a wave length of 620 nm). Antigen concentrations were 1 = h = 2.5; 2 = 1.5; 3 = 1.0; 4 = 0.5, 5 and 6 = 0.25.

Figure 8.--Serologic reaction of X. phaseoli (15) antisera with different concentrations of the homologous steamed antigen. Supernant was taken after centrifugation passed through glass filter well 1 and 2 = X. phaseoli (11); 3 and 4 = X. phaseoli 15; 5 and 6 = Xpf16.

Figure 7

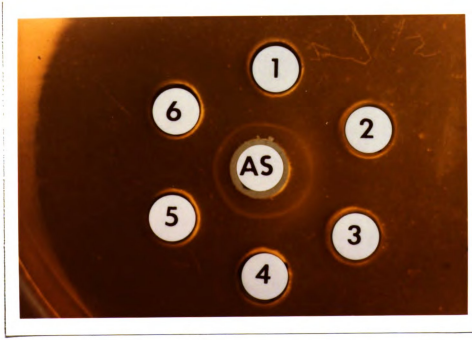

Figure 8

Figure 9.--Radial immuno difusion reactions of X. phaseoli (11) antisera with several pathogenic and non-pathogenic bacteria. Antisera incorporated into agar medium at a final dilution of 1:30. Wells contain: 1, 2, 3 through 10, steamed cells at 10^{10} cells/ml of different internal bacterial bean contaminants, Well 11 and 12 checks, 10^{8} cells/ml of Xp11 and Xpf16 respectively.

Figure 10.--Radial immunodifusion reactions of X. phaseoli (15) antisera incorporated into agar medium at a final dilution of 1:30. In the center of the plate a navy bean seed suspected to be infected with Xanthomonas blight.

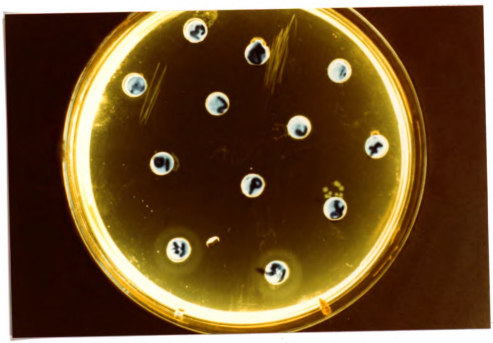

Figure 9

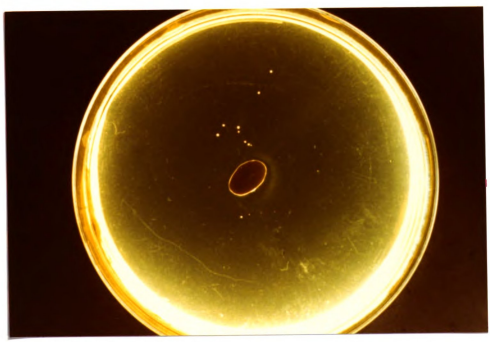

Figure 10

Discussion

Two methods were used to obtain antisera to Xanthomonas phaseoli and to X. phaseoli var. fuscans in rabbits. Antigen injected in the form of formalized cells always gave higher titres than antigens in the form of steamed cells. These results are in agreement with other workers (31, 39).

In this study we used yeast extract-calcium carbonate agar as a basal medium, and removed the growth from the cultures before 48 hours. To remove any mucoid fraction, bacterial cells were washed two times with buffered saline by centrifugation and resuspension. The Xanthomonas group is notorious for producing mucoid substances whose polysaccharide nature was investigated by various workers (13, 14, 15). These exudates were found to give non-specific serological reactions, but such antigens have been found to be heat-labile for most Pseudomonas tested (8, 22, 24, 40), and for some Xanthomonas (7, 40) as well. We obtained similar results when live and steamed cells were used as antigen. Xanthomonas antisera cross-reacted to different degrees with all of the different bacterial isolates when live cells were used as antigen; however, the titres were always higher with bacterial isolates from the same genus as used to immunize the rabbits. When steamed cells were used as antigen, tube and microagglutinin tests became more specific but some cross-reaction was still observed, mostly with Pseudomonas sp.

The fact that antisera to X. phaseoli and X. phaseoli var. fuscans reacted against all the other isolates of the Xanthomonas

group when steamed cells were used as antigen is not new. In 1959, Mushin et al. (31) reported that antisera produced against X. phaseoli reacted with X. campestris, X. carotae, X. vesicatoria, X. incanae, X. juglandis, X. albilineans; in the same paper they reported that antisera produced against X. carotae and X. juglandis seem to be more specific and did not agglutinate with X. campestris, X. vesicatoria, X. incanae, X. juglandis, X. albilineans.

In 1977, Mahanta and Ady (26) showed in agglutinin tests that antisera against X. oryzae reacted with steamed antigen of X. pruni, X. vesicatoria, X. phaseoli, X. citri, several Pseudomonas sp., and to a lesser extent, with several Erwinia sp. Our results indicate that Xanthomonas phaseoli and X. phaseoli var. fuscans antisera agglutinate with all of the Xp and Xpf isolates tested, suggesting that Xp and Xpf are closely related. Our results also suggest that Xp and Xpf share heat-labile antigens with most members of the genera: Pseudomonas, Erwinia and Corynebacterium. This was clearly demonstrated when antisera to Xp and Xpf was absorbed with Pseudomonas phaseolicola. All of the group components common to other genera were removed and only the antigenic factors against Xanthomonas remained. When sera against Xp were absorbed with Xpf, specific factors to Xp remained, and the opposite was true also. These results indicate that Xp and Xpf have a species-specific factor (or antigen), which was shown for the first time in 1947 by Elrod and Braun (13).

Agar gel double diffusion tests were found more reliable than agglutinin tests in serological studies of Xanthomonas phaseoli

and X. phaseoli var. fuscans. Live cells of Agrobacterium, Bacillus, and Corynebacterium sp. did not react in agar gel double diffusion tests. When very heavy suspensions of steamed cells were used, no band or weak band formation was obtained against Erwinia isolates and Pseudomonas isolates respectively. Other workers have found that steaming bacterial cells for 45-60 minutes does not alter antigen specificity (25, 32); and (1) may increase the speed of reaction in gel diffusion tests; (2) the precipitation lines in the gel diffusion tests become very clear; and (3) a distinct difference between heterologous and homologous is obtained (32). All Xp (19) and Xpf (28) isolates tested as steamed cells in agar gel double diffusion tests gave good reactions with antisera against Xanothomonas; also, more bands were formed when antisera-antigen combinations were closely related (Xp-Xp or Xpf-Xpf). Of 19 internal bean seed contaminants tested as steamed cells, none reacted against Xp or Xpf antisera. Agar gel double diffusion has been accepted as a reliable technique for many workers in the field (22, 23, 26, 1, 41).

Kiraly, et al. (18) in 1974 stated that the antigen to be used in agar gel double diffusion tests must contain 10^{10} cells/ml. However, in our studies with antisera prepared against Xanthomonas phaseoli and Xanthomonas phaseoli var. fuscans, we obtained excellent band formation with both steamed and live cell antigen preparations containing between 6×10^6 and 1×10^7 x cells/ml.

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CHAPTER II

DEVELOPMENT OF A LIQUID MEDIUM SELECTION FOR *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS*

Introduction

In recent years there have been numerous attempts to find selective media for rapid isolation of plant pathogenic bacteria (11, 19, 21, 26).

Semi-selective media which allow and promote growth of suspected pathogens, but inhibit or eliminate many common saprophytes and secondary invaders, have been developed for several bacterial plant pathogens (10, 14, 25). Such media may play an important role in the future for diagnostic purposes (17).

Media are available for the selective isolation of certain *Erwinia* sp. (13, 5, 6, 21), fluorescent *Pseudomonas* (22), and for *Agrobacterium* (24, 20).

The relative availability of pure antibiotics during the past three decades has permitted studies of specific bacteriostatic and bactericidal effects on plant pathogenic bacteria. Most of the results vary considerably due to differences in methods of testing, different antibiotics used, different conditions of growth and evaluation, and the use of different test organisms (15).

Gulliver (9) in 1946 tested 14 antibiotic substances on representative species of plant pathogens by a dilution method and found that Gram positive pathogens were more sensitive than Gram negative bacteria to proactynomycin, mycophenolic acid, helvolic acid, penicillin, tyrothricin, and gliotoxin. Both Gram positive and Gram negative bacteria were equally sensitive to penicillic acid, aspergillic acid, and clavacin; Xanthomonas species were among the most sensitive of the Gram negative bacteria to all of these antibiotics.

In 1951, Katznelson and Sutton (15) reported that for Xanthomonas species, aureomycin was the most bacteriocidal agent, followed by terramycin and polymyxin; 0.1 to 0.05 ppm aureomycin completed inhibited growth of most Xanthomonas cultures at 24 hours.

In 1974, I. M. Szabo (27), working with 12 different antibiotics, found that Xanthomonas sp. were resistant to nitrofurantoin but susceptible to penicillin. No inhibition zones developed when discs containing 300 ug. nitrofurantoin were placed in agar plates seeded with Xanthomonas species. At this concentration nitrofurantoin was strongly inhibitory to: Bacillus cereus, B. circulans, B. megaterium, B. mycoides, B. mesentericum, B. pumilis, B. polymixa, B. subtilis, Enterobacter aerogenes, Flavobacterium aurantiacum, and many unidentified bacteria. The same concentration of nitrofurantoin was inhibitory, but to a lesser extent, to: Chromobacterium sp., Micrococus agilis and Rhizobium sp.

In 1972, Kado's medium D5 (14) was reported selective for Xanthomonas spp. In 1974, Shaad and White (26) reported a selective

medium for soil isolation and enumeration of Xanthomonas campestris. The organisms were distinguished by colony morphology and starch digestion. According to these workers, however, the media only recovered about 10 percent of the X. campestris population added to the soil, but still was superior to medium D5.

In 1975, Shaad and Kendwick (24) reported a new selective medium, nutrient-starch-cycloheximide agar (NSCA); cycloheximide was added to eliminate fungal contaminants (4, 31).

Neither medium is suitable for our studies due to low plating efficiencies for Xp and Xpf. At present there is no efficient culture medium sufficiently selective for Xp and Xpf to be used for identification of these important bean pathogens.

Objective

The objective of this study was to develop a medium selective for Xanthomonas phaseoli and X. phaseoli var. fuscans in the presence of other contaminating microorganisms.

Materials and Methods

Bacterial Storage and Culture

Bacterial isolates used in this study were stored: (a) in 40% v/v aqueous glycerol at -10°C, and (b) in the case of bean plant pathogens, in dried, infected leaf and stem tissues.

Most bacteria were grown on yeast extract-calcium carbonate-agar (YCA): 10 gm yeast extract, 2.5 gm calcium carbonate, 15 gm agar per 1000 ml glass distilled water. Bacteria belonging to the genus Pseudomonas were grown on King's medium B (KMB): 15 ml

glycerol, 3 gm $MgSO_4H_2O$, 2 gm KH_2PO_4, 20 gm Peptone, 15 gm agar, per 1000 ml of distilled water.

Selection of a Basal Medium

Media were prepared to contain: 0.0, 0.1, 1.0, 5.0 gm of yeast extract per litre of 0.1 M phosphate buffer pH 7.2. Two hundred and fifty disease-free seeds of the Tuscola been cultivar were surface sterilized for two minutes in a 1:1 dilution of sodium hypochlorite (2.6% NaOCl) and rinsed twice with sterile distilled water. Seafarer navy bean seeds internally infected with XpfR10, resistant to 50 ppm rifampin (32), were separately surface-sterilized and rinsed in a similar manner.

Samples of 250 disease-free seeds plus one internally infected seed were placed in flasks containing 150 ml of media and put on a rotary shaker. Control flasks contained only 250 disease-free seeds. Ten ml liquid samples were removed from the flasks after 10, 24, 48, and 72 hours incubation and centrifuged at 300 x g for 15 minutes. Pellets were resuspended in 1.5 ml of sterile buffer saline, steamed for one hour at 100°C and used as antigen in Ouchterlony agar gel double diffusion tests and microagglutination tests.

Preparation of Bean Seed Flour Containing XpfR10

Eighty disease-free Tuscola bean seeds were surface sterilized as before, dried on a sterile paper towel and added to 20 surface sterilized seeds internally infected with XpfR10 (obtained

from D. M. Weller). The 100 seed sample was ground to a flour in a G. E. Mill using a 40-mesh screen: all parts of the Mill on contact with the seeds were previously sterilized.

The resulting flour was placed in a sterile bottle, shaken briefly, and kept in the refrigerator at 4°C. Dilution plate tests of the flour on rifampin containing media (RAM) and on YCA resulted in populations of 3×10^5 Xpf R10 bacteria per mg dry weight (RAM) and 5.5×10^2 bacterial contaminants per mg dry weight (YCA).

Experiments with Liquid Media

In other experiments, plant pathogenic and non-pathogenic bacteria were grown in liquid basal medium (1.0 gm yeast extract, 25 mg cycloheximide in 100 ml of .01 M phosphate buffer pH 7.2) for 48 hours, and adjusted to an optical density of h = 0.25 at wavelength 620 nm. Samples of the adjusted suspensions were used to inoculate different liquid medium-antibiotic combinations.

In all experiments controls consisted of basal medium without antibiotics. Growth was determined by measuring the optical density at wavelength 620 nm. at 24 or 48 hours of shaker incubation.

Experiments Using Antimicrobial Discs

Plates of YCA or King's medium B were seeded with 0.1 ml of bacterial suspensions adjusted to h = 0.25. An antibiotic containing disc was placed in the center of the seed plate; zones of inhibition were measured in mm. diameter after 72 hours incubation. There were at least three repetitions for each antibiotic.

Origin of Isolates

a. The source of all bacterial isolates used in the present study are summarized in the Appendix (Table B2). In all cases bacteria pathogenic to bean were tested regularly for pathogenicity.

b. Bacterial contaminants used in this study were isolated from numerous seed lots obtained from Michigan seed growers or from seed certification agencies. The contaminants generally were isolated from surface-sterilized bean seeds according to methods mentioned elsewhere. Several of the contaminants were characterized to genus and are summarized in the Appendix (Table B3).

Methods of Antibiotic Preparation

Recommendations given by Bailey and Scott (3) were used to prepare the antibiotic solutions. Cycloheximide was filter sterilized and added to the sterilized media.

Most of the antibiotics were weighed out and multiplied by "activity standard" provided by the manufacturer. The material was added to 200 ml of sterile distilled water in the case of penicillin G, tetracycline, kanamycin, gentamicin, polymixin B, methicillin. After preparing the stock solutions, the necessary amount of each antibiotic was aseptically added to the basal media to obtain a desired concentration.

. Chloramphenicol was weighed out and dissolved in 1 ml of ethyl alcohol. Sterile distilled water was added to obtain the desired concentration.

. Nalidixic acid was added to 2 ml 1 M NaOH, allowed to stand until dissolved, and diluted with sterile distilled water.

. Nitrofurantoin was dissolved in sterile distilled water and the stock solution was passed through a sintered glass bacterial filter. The corresponding volume was added to sterilized basal media.

. Rifampin was dissolved in 0.4 ml methanol and diluted to 10 ml with distilled water (32).

Results

The Effect of Yeast Extract Concentration on the Detection of Internal Xanthomonas phaseoli Contamination of Navy Bean Seeds

Xanthomonas blight bacteria were consistently detected in agar gel double diffusion tests using antisera to Xpf16 at yeast extract concentrations of 0, 0.1, 1.0, and 5.0 gm/litre. Antisera prepared against Xp15 gave inconsistent results when tested against the same preparations (Table 14).

The results suggest that a minimum period of 24 hours shaker incubation is necessary before detecting Xanthomonas blight bacteria in bean seeds incubated in the basal media. Yeast extract levels of 0.1 and 1.0 gm/lt consistently gave positive tests for Xanthomonas blight at 72 hours in agar gel double diffusion tests (Table 14).

Xanthomonas blight bacteria were consistently detected by microagglutinin tests at antisera dilutions of 1:640 in basal media containing 0, 0.1, 1.0, and 5.0 gm of yeast extract per

TABLE 14.--The Effect of Media Composition on the Detection of Internal *Xanthomonas phaseoli* Contamination in Navy Bean Seed in Agar Gel Double Diffusion Tests.[a]

Yeast Extract Added gm/l	Number of Blighted Seeds	Antisera Prepared Against Formalized Cells[b]							
		*Xpf*16				*Xp*15			
		10	24	48	72	10	24	48	72
0.0	1	-	+	+	-	-	±	±	-
0.1	1	-	+	+	+	-	±	±	-
1.0	1	-	+	+	+	-	±	±	-
5.0	1	-	+	+	±	-	±	+	-
0.1	0	-	-	-	-	-	-	-	-
5.0	0	-	-	-	-	-	-	-	-

[a]Basal media contained (25 mg of cycloheximide in 1 litre 0.01 M buffer phosphate pH = 7.2).

[b]Titre of the antisera with the homologous antigen was 1:1280.

[c]Blighted seed added to 250 seed samples. Samples were surface-sterilized prior to shaker incubation in media. Bacteria were sedimented by centrifugation, resuspended in buffer saline and steamed 60 minutes at 100°C.

- = no reaction; + = band formation; ± = band formation in one or two replications but not in all three.

litre (Tables 15, 16); seed samples were shaker incubated for 24 hours. However, after 48 hours of shaker incubation, Xanthomonas blight bacteria were detected at antisera dilutions of 1:1280 in basal media containing 0, 0.1, 1.0 gm of yeast extract for litre. Based on these results all subsequent studies were performed with basal media containing 1.0 gm yeast extract per litre.

Sensitivity of Various Plant Pathogenic and Non-Pathogenic Bacteria to Several Antibiotics in Bioassay Tests

The following antibiotics were inhibitory to several Xanthomonas blight bacterial isolates when bioassayed as commercially available impregnated discs: aureomycin, carbenicillin, chloromycetin, dihydrostreptomycin, erythromycin, kanamacyn, polymyxin, and tetracycline (Table 17).

Growth of Several Xanthomonas Bacterial Blight Isolates in Liquid Media with Various Antibiotics

Dose response studies were conducted against six Xanthomonas blight isolates in liquid media supplemented with nine different concentrations, each of eleven antibiotics (Table 18).

In general, the Xanthomonas isolates were able to tolerate up to 0.05 mg/ml of chloramphenicol, gentamicin, neomycin, novobiocin, and terramycin; up to 0.1 ug/ml methicillin; up to 1.0 ug/ml nalidixic acid; and up to 2 ug/ml nitrofurantoin. Streptomycin sulphate, rifampin and tetracycline were extremely inhibitory to the Xanthomonas isolates at very low concentrations.

TABLE 15.--The Effect of Media Composition on the Detection of Internal Xanthomonas phaseoli var. Fuscans Contamination in Navy Bean Seed in Microagglutinin Tests.[a]

Yeast Extract Added gm/l	Number of Blighted Seeds	Antisera Dilution[b]									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Check
0.0	1	+	+	+	+	+	+	±	-	-	-
0.1	1	+	+	+	+	+	+	±	-	-	-
1.0	1	+	+	+	+	+	+	±	-	-	-
5.0	1	+	+	+	+	+	+	-	-	-	-
0.1	0	+	+	-	-	-	-	-	-	-	-
5.0	0	+	+	-	-	-	-	-	-	-	-

[a]Basal media contained (25 mg of cycloheximide in one litre of 0.01 M buffer phosphate). Experiment involved 24 hour shaker incubation.

[b]Antisera prepared against formalized cells of Xpf16, titre of the antisera with the homologous antigen 1:5120.

- = no agglutination; + = agglutination; ± = agglutination in one or two replications, but not in all.

[c]Blighted seed added to 250 seed samples were surface-sterilized prior to shaker incubation in media. Bacteria were sedimented by centrifugation, resuspended in buffer saline and steamed 60 minutes at 100°C.

TABLE 16.--The Effects of Media Composition on the Detection of Internal Xanthomonas phaseoli var. Fuscans Contamination in Navy Bean Seed in Microagglutinin Tests.[a]

Yeast Extract Added gm/l	Number of Blighted Seeds	Antisera Dilution[b]								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
0.0	1	+	+	+	+	+	+	+	-	-
0.1	1	+	+	+	+	+	+	+	-	-
1.0	1	+	+	+	+	+	+	+	-	-
5.0	1	+	+	+	+	+	+	-	-	-
0.1	0	+	+	-	-	-	-	-	-	-
5.0	0	+	+	-	-	-	-	-	-	-

[a]Basal media contained (25 mg of cycloheximide in one litre of 0.01 M buffer phosphate). Experiment involved 48 hours shaker incubation.

[b]Antisera prepared against formalized cell of Xpf16, titre of the antisera with the homologous antigen 1:5120.

- = no agglutination; + = agglutination.

[c]Blighted seed added to 250 seed samples were surface sterilized prior to shaker incubation in media. Bacteria were sedimented by centrifugation, resuspended in buffer saline and steamed 60 minutes at 100°C.

Figure 11.--Serologic reactions of X. phaseoli var. fuscans (16) (central well) with the antigen from one blighted seed added to 250 seed samples (media contain 25 mg cycloheximide in one litre buffer phosphate pH = 7.2). Sample surface-sterilized prior to shaker incubation for 24 hours in media, bacteria sedimented by centrifugation, resuspended in buffer saline and steamed 60 minutes at 100°C. Different levels of yeast extract were used: 1 and 2 = 5.0 gm/l, 3 and 4 = 1.0 gm/l, 5 = .1 gm/l, 6 = no yeast extract used.

Figure 11

TABLE 17.--Sensitivity of Various Bacteria to Several Antibiotics in Bioassay Tests.

Antiobiotic[b]	Concentration	Diameter (mm) of Inhibition Zone when Tested Against[a]				
		Bacillus megaterium	Cornynebacterium michiganense	Erwinia herbicola	Pseudomonas fluorescens	Xp + Xpf combination
Aureomycin	10 cg	10	10	0	8	8
Carbenicillin	50 mcg	12	15	0	0	30
Chloromycetin	10 mcg	20	15	25	20	30
Dihydrostreptomycin	10 mcg	25	20	0	22	30
Erythromycin	5 mcg	27	30	10	28	26
Kanomacyn	30 mcg	24	14	25	18	30
Polymyxin	100 u	8	0	8	0	10
Tetracycline	10 mcg	15	10	12	0	12

[a]YCA plates were seeded with 0.1 ml bacterial suspension adjusted at h = 0.25.

[b]Commercially available samples containing the indicated concentration.

TABLE 18.--Growth of Six Xanthomonas Bacterial Blight Isolates in Liquid Media Supplemented with Various Antibiotics.[a]

Antibiotic	Growth[b] at Indicated Concentrations of Antibiotic (ug/ml.)									
	0	.01	.02	.05	.1	.5	1.0	2.0	4.0	8.0
Chloramphenicol	.29			.21	.18	.10	.03	0	0	0
Gentamicin	.29		.22	.21	.12	.02	0	0	0	0
Methicillin	.29			.23	.22	.15	0	0	0	0
Nalidixic acid	.24					.23	.22	.18	.05	.0
Neomycin	.24			.23	.15	0	0	0	0	0
Nitrofurantoin	.24					.24	.22	.23	.17	.10
Novobiocin	.24	.24	.24	.26	.17	.13	0	0	0	0
Streptomycin sulphate B			.20	.08	0	0	0	0	0	0
Rifampin		.21	0	0	0	0	0	0	0	0
Terramycin				.22	.18	.04	0	0	0	0
Tetracycline	.24	.19	.13	.09	.04	0	0	0	0	0

[a]Basal media composition (1.0 gm yeast extract, 25 mg of cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate) was supplemented with the indicated antibiotic, and inoculated with 0.1 ml of the bacterial suspension adjusted to h = 0.3.

[b]Growth is expressed as the OD (h = 620 nm) of the flasks after 48 hours of shaker incubation, each value is the average of 18 observations (6 isolates by 3 replications).

Twelve antibiotics were evaluated relative to their effects on various seed borne contaminants and on several Xanthomonas isolates in liquid media. Concentrations of antibiotics that were used were those that were shown to be inhibitory to contaminants in previous studies but not to Xanthomonas phaseoli or to Xanthomonas phaseoli var. fuscans (Table 19).

Of the contaminants tested, Gram positive C_4 and C_4 were both effectively inhibited by chloramphenicol, nalidixic acid, nitrofurantoin, penicillin, and streptomycin sulphate.

Nalidixic acid at 1.0 ug/ml inhibited six of the seven contaminants tested. Penicillin, rifampin and terramycin, however, were inhibitory to the two Xanthomonas control isolates. Antibiotics showing good activity against seed borne contaminants were then evaluated relative to their effect on internal bean seed microflora of disease-free seeds incubated in liquid media (Table 20). All of the antibiotics used were able to significantly reduce bacterial contaminants in the liquid media. Gentamicin at 0.05 ug/ml, methicillin at 0.5 ug/ml, nalidixic acid at 0.5 and 1.0 ug/ml, and nitrofurantoin at 2.0 ug/ml provided the greatest degree of control of the growth of internal bean seed bacterial contaminants.

Combinations of various antibiotics at effective concentrations as determined in previous studies were then evaluated as to their inhibitory effect upon the growth of internal bean seed bacterial contaminants in liquid media (Table 21).

The following antibiotic combinations gave statistically more significant control of the bacterial contaminants: nalidixic

TABLE 19.--Growth of Xanthomonas Blight Isolates and Internal Bean Seed Contaminants[c] in Liquid Media Supplemented with Various Antibiotics.[a]

Antibiotic	Concentration ug/ml	Bacterial Isolate[b,c] C1	C4	C5	C8B	C11	C15	C18	Xp11	Xp15	Xpf16
None		.27	.45	.45	.39	.35	.35	.45	.17	.18	.18
Chloramphenicol	.1	.27	0	.20	.30	.35	.12	.45	.17	.16	.19
Gentamicin	.05	0	.03	.45	.37	.35	0	.50	.18	.17	.17
Methicillin	.1	.26	.38	.41	.39	.25	.28	.42	.17	.16	.17
Nalidixic acid	1.0	.06	0	.02	.16	.24	.02	.04	.16	.16	.17
Neomycin	.05	.04	.36	.37	.37	.31	.30	.45	.15	.16	.16
Nitrofurantoin	4.0	.25	.15	.19	.36	.18	.20	.41	.15	.15	.16
Novobiocin	.1	.15	.39	.38	.37	.31	.32	.32	.16	.16	.16
Penicillin (u/ml)	4.0	.26	0	.02	.40	.37	.25	.02	.08	.09	.10
Rifampin	.02	0	.04	.39	.39	.31	.30	0	.05	.05	.05
Streptomycin Sulphate B	.05	0	.02	0	.15	.35	.02	.03	.15	.16	.15
Terramycin	.1	.26	0	.43	.45	.36	.25	.47	.09	.08	.10
Tetracycline	.01	.12	.35	.38	.37	.28	.30	.40	.16	.15	.18

[a]Basal media composition (1.0 gm yeast extract, 25 mg of cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate) was supplemented with the indicated antibiotic, inoculated with 9.1 ml of the bacterial suspension adjusted to h = 0.3 at wavelength of 620 nm.

[b]Growth is expressed as the OD (h = 620 nm.) of the flasks after 36 hours shaker incubation, each value is the average of three observations.

[c]See Table for contaminant identification.

TABLE 20.--Growth of Internal Bean Seed Bacterial Contaminants in Liquid Media Supplemented with Various Antibiotics.[a]

Antibiotic	Concentration ug/ml	Repetitions[b] 1	2	3	4	5	
None		.52	.72	.65	.24	.53	b
Chloramphenicol	.1	.05	.39	.08	.12	.275	a
Gentamicin	.05	.04	.02	.06	.03	.038	a
Methicillin	.5	.01	.01	.03	.02	.0175	a
Nalidixic acid	.5	.07	.08	.10	.06	.0775	a
Nalidixic acid	1.0	.06	.04	.03	.02	.0375	a
Nitrodurantoin	2.0	.01	.01	.21	.03	.065	a
Penicillin (u/ml)	4.0	.07	.04	.25	.08	.11	a
Streptomycin Sulphate B	.05	.07	.09	.35	.05	.14	a

[a]Basal media composition (1.0 gm yeast extract, 25 mg of cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate) was supplemented with the indicated antibiotic and 20 surface-sterilized navy bean seeds were added.

[b]Growth is expressed as the O.D. (h = 620) of the flasks after 24 hour shaker incubation. Mean with the same letter are not different at α = 0.01 level by Duncan multiple range test.

TABLE 21.--Growth of Internal Bean Seed Bacterial Contaminants in Liquid Media Supplemented with Various Antibiotics.[a]

Antibiotic	Concentration ug/ml	Repetitions[b] 1	2	3	4	$\bar{X}$
None	--	.55	.52	.40	.28	.44[c*]
Nalidixic acid	1.0	.07	.20	.08	.11	.11[b]
Nalidixic acid + Nitrofurantoin	1.0 2.0	.04	.10	.12	.04	.07[ab]
Nalidixic acid + Gentamicin	1.0 .05	.07	.06	.06	.15	.08[ab]
Nalidixic acid + Chloramphenicol	1.0 .1	.07	.20	.04	.04	.08[ab]
Nalidixic + Nitrofurantoin + Gentamicin	1.0 2.0 .05	.01	.02	.02	.02	.017[a]
Nalidixic + Gentamicin + Chloramphenicol	1.0 .05 .1	.04	.02	.03	.02	.027[ab]
Nalidixic acid + Nitrofurantoin + Chloramphenicol	1.0 2.0 .1	.03	.05	.05	.04	.04[ab]
Nalidixic acid + Gentamicin Chloramphenicol + Nitrofurantoin	1.0 .05 .1 2.0	.02	.01	.02	.03	.02[a]
Chloramphenicol + Gentamicin + Nitrofurantoin	.1 .05 2.0	.05	.04	.03	.09	.05[ab]

[a]Basal media composition (1.0 gm yeast extract, 25 mg of cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate) was supplemented with the indicated antibiotics, and 20 surface-steralized navy bean seeds were added.

[b]Growth is expressed as the O.D. (h = 620) of the flasks after 24 hour shaker incubation.

[*]Values followed by any common letter are not statistically different at $\alpha = 0.05$.

acid plus nitrofurantoin plus gentamicin, nalidixic acid plus gentamicin plus chloramphenicol plus nitrofurantoin, and chloramphenicol plus gentamicin plus nitrofurantoin.

The Effect of Antibiotics and Antibiotic Combinations on Populations of XpfR10 and Bacterial Contaminants

Studies were initially conducted on the effect of individual antibiotics on population levels of XpfR10 and bacterial contaminants when samples of bean seeds containing both types of bacteria were incubated in basal liquid media supplemented with the antibiotics (Table 22).

Populations of XpfR10 were greatest in liquid media containing chloramphenicol, gentamicin and nitrofurantoin. Populations of internal bacterial contaminants were least in liquid media containing nalidixic acid.

When the ratio of XpfR10 to contaminants was calculated, the following antibiotics were the most effective at increasing the ratio: Nalidixic acid 1.0 ug/ml, gentamicin .05 ug/ml, methicillin .5 ug/ml, chloramphenicol .1 ug/ml and nitrofurantoin at 2.0 ug/ml.

Studies were then conducted to determine the effects of various antibiotic combinations on XpfR10 and contaminant populations in bean flour samples (Table 23).

Although all antibiotic concentrations resulted in a significantly greater ratio of XpfR10 to contaminants as compared to the check (without antibiotic), the most effective antibiotic concentration was: nalidixic acid, gentamicin, and nitrofurantoin at the

TABLE 22.--Populations of Xpf10 and Bacterial Contaminants in Liquid Media Supplemented with Various Antibiotics.[a]

Antibiotic	Concentration ug/ml	Replications $\bar{X}$ Cont.	Replications $\bar{X}$ Xpf	Ratio Xpf/cont.
None		3.5×10^{10}	2.1×10^{7}	.008 c*
Chloramphenicol	.1	3.3×10^{9}	2.3×10^{8}	.3525[a]
Gentamicin	.05	3.2×10^{9}	5.7×10^{8}	.2284[ab]
Methicillin	.5	7.9×10^{9}	6.7×10^{7}	.090 bc
Nalidixic Acid	.5	3.3×10^{9}	6.0×10^{7}	.2577[ab]
Nalidixic Acid	1.0	8.6×10^{7}	8.8×10^{7}	.3786[ab]
Nitrofurantoin	2.0	1.5×10^{9}	1.0×10^{8}	.3786[ab]
Penicillin G (u/ml)	4.0	2.4×10^{9}	9.4×10^{7}	.0298[abc]

[a]Basal media composition (110 gm yeast extract, 25 mg of cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate) was supplemented with the indicated antibiotics, inoculated with 20 mg of bean flour containing 2×10^5 XpfR10 per mg, XpfR10 were determined with rifampin selective media (32). Bacterial contaminants were detemined on YCA.

*Values followed by any common letter are not statistically different at 0.05. Duncan Multiple Range Tests.

TABLE 23.--Populations of Xpf R10 and Bacterial Contaminants in Liquid Media Supplemented with Various Antibiotics.[a]

Antibiotic	Concentration ug/ml	Xpf	Cont.	Ratio Xpf/cont.
None		1.2×10^7	2×10^{10}	1/1.666[c*]
Nalidixic acid	1.0	9.5×10^7	3×10^8	1/3[ab]
Nalidixic acid + Gentamicin	1.0 .05	1.0×10^8	1.2×10^8	5/6[a]
Nalidixic acid + Nitrofurantoin	1.0 2.0	1.4×10^8	2.9×10^8	1/2[ab]
Nalidixic acid + Chloramphenicol	1.0 .1	2.2×10^8	3.1×10^8	2/3[a]
Nalidixic acid + Gentamicin + Chloramphenicol	1.0 .05 .1	9.6×10^7	2×10^8	1/2[ab]
Nalidixic acid + Gentamicin + Nitrofurantoin	1.0 .05 2.0	3.9×10^8	3.2×10^8	10/8[a]
Nalidixic acid + Chloramphenicol + Nitrofurantoin	1.0 .1 2.0	1.0×10^8	8×10^8	1/8[b]
Nalidixic acid + Chloramphenicol + Nitrofurantoin + Gentamicin	1.0 .1 2.0 .05	$3,5 \times 10^8$	8×10^8	1/2[ab]

[a]Basal media composition (1.0 gm yeast extract, 25 mg cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate) was supplemented with the indicated antibiotics, inoculated with 20 mg of bean flour containing 2×10^5 XpfR10 per mg., XpfR10 were determined with rifampin selective media (32), bacterial contaminants were determined on YCA.

*Values followed by any common letter are not statistically different at $\alpha = 0.01$. Duncan Multiple Range Test.

concentrations indicated in Table 22 as being effective. Therefore, in all subsequent studies the semi-selective media (SSM) contained: nalidixic acid 1.0 ug/ml, gentamicin .05 ug/ml, and nitrofurantoin 2.0 ug/ml. The SSM was then tested against various plant pathogenic and non-pathogenic bacteria in liquid culture (Table 24). SSM was very inhibitory to Agrobacterium tumefaciens, Bacillus megaterium and to species in the genera Corynebacterium and Erwinia. SSM was somewhat effective against certain Pseudomonas species and generally ineffective when tested against several other plant pathogenic Xanthomonas species.

Minimal Number of Bacterial Cells Detected by SSM

A serial dilution of X. phaseoli and X. phaseoli var. fuscans cell suspensions was prepared: one ml of each dilution was added to 125 ml flasks containing 25 ml SSM. All dilutions led to turbidity of the SSM. Turbidity at 48 hours contained 10^4 cells (therefore, the SSM initially contained 400 cells/ml).

Internal Bacterial Contaminants of Navy Bean Seeds

More than 56 internal bacterial contaminants were isolated from various lots of navy bean seeds which differed, on the basis of colony type, color, rate of growth, and Gram reaction. The contaminants were divided into 19 representative groups and these groups were selected for further characterization. All of the bacterial contaminants were injected into Red Kidney bean plants

TABLE 24.--Growth of Various Plant Pathogenic and Non-pathogenic Bacteria in Liquid Media with and without Added Antibiotics.

Bacteria[a]	Growth in[b] Basic Media	Basic Media + Antibiotic
Agrobacterium tumefaciens	.36	0
Bacillus megaterium	.46	0
Corynebacterium fasciens	.40	0
C. flaccumfaciens NE23	.24	0
C. flaccumfaciens 6887	.22	0
C. flaccumfaciens	.25	0
Erwinia amylovora	.21	.06
E. carotovora var. atroseptica	.30	0
E. herbicola	.35	0
Pseudomonas fluorescens	.45	.21
Ps. glycinea	.27	.19
Ps. phaseolicola	.26	.19
Ps. syringae	.29	.22
Ps. syringae Y30	.23	.19
Xanthomonas campestris	.19	.17
X. juglandis	.20	.16
X. pelargonii	.21	.18
X. pruni	.18	.16

[a]Flasks were seeded with 0.1 ml of the bacterial suspension adjusted at h = 0.3. Growth is expressed as the O.D. (h = 620) of the flask after 36 hours shaker incubation, each value is the average of three observations.

[b]Basal media composition (1.0 gm yeast extract, 25 mg cycloheximide in one litre of 0.01 M pH 7.2 buffer phosphate), basal media plus antibiotic (basal media, 1.0 ug/ml nalidixic acid, 2.0 ug/ml nitrofurantoin, .05 ug/ml gentamicin).

to test for pathogenicity. None of the contaminants were pathogenic to bean.

Of the 19 contaminants characterized to genus or species, 4 were found to be Pseudomonas fluorescens on the basis of: Gram negative reaction, the production of a green fluorescent pigment on King's B medium, positive for catalase activity and oxidase activity, and negative in inducing hypersensitive reaction when infiltrated into tobacco leaves (Table B1).

Isolates 3, 13 and 15 were identified as Bacillus sp. because they were Gram positive, catalase positive and possessed endospores; isolates 1 and 10 were identified as Erwinia sp. because they were Gram negative, with production of a yellow non-diffusible pigment and did not reduce nitrates.

Discussion

From the initial results an optimum shaker incubation period of 48 hours was necessary to obtain a Xanthomonas population large enough to detect in agar gel double diffusion tests (from 10^4 cells/ml to 10^7 or more cells/ml).

Yeast extract concentrations of 0, .1, and 1.0 were all equally capable of supporting Xanthomonas growth to levels sufficient for serological detection.

In 1974, Szabo (27) reported that X. silvestris strains were unresponsive to nutrient concentrations but that the contaminant population tended to grow faster with increasing nutrient concentration. We selected a basal medium containing 1.0 gm yeast

extract/litre for use in antibiotic studies primarily because of its ease of preparation and its efficiency in encouraging Xanthomonas growth to levels high enough to detect serologically.

The basal medium consistently permitted the detection of Xanthomonas in seed internally infected with this pathogen.

Our preliminary bioassay results with commercially available impregnated discs are in agreement with previous reports (1, 2, 15, 30), which found that the antibiotics aureomycin, carbenicillin, chloromycetin, dihydrostreptomycin, erythromycin, kanamacyn, polymyxin and tetracycline are inhibitory to Xanthomonas, as well as to other Gram negative and Gram positive bacteria.

The antibiotic concentrations selected for further detailed studies were those which did inhibit the growth of several internal bean seed contaminants (Table 19). The internal bacterial contaminants chosen for our experiments represented the same types of microflora known to inhabit bean seed, such as Bacillus, Erwinia herbicola and Pseudomonas fluorescens (12, 16, 18, 23).

The combination of nalidixic acid at 1.0 ug/ml, gentamicin at .05 ug/ml and nitrofurantoin at 2.0 ug/ml was chosen for use in the semi-selective media (SEM) because of its superior performance in increasing Xanthomonas contaminant ratios.

Taylor (28) found that Pseudomonas phaseolicola were readily obtained from white bean seeds if the dry seeds were ground to a flour, dispersed in sterile water, and sampled. Although there was considerable variation in the number of bacteria present in

individual infected seeds, 80% of the seeds contained 1 x 10^5 or more bacteria.

Ednie and Needham (7) used in their experiments an average number of 7.8 x 10^7 X. phaseoli var. fuscans cells per infected seeds.

SSM is highly selective for Xanthomonas. Nalidixic acid is a relatively simple synthetic antibacterial compound used in the treatment of the genitourinary tract (8). Its mode of action is unusual since it appears to depend on selective inhibition of DNA synthesis in pathogenic micro-organisms, especially in Gram negatives (28).

Nalidixic acid was used by Grant and Hold (10) in a selective medium for Pseudomonas. The author suggested that the primary function of nalidixic acid was the inhibition of enteric bacteria and of Gram negative cocci, especially Acinetobacter.

Gross and Vidaver (11) recently developed a medium selective for Corynebacterium nebraskense which included nalidixic acid, lithium chloride and Bravo 6F. The medium inhibited the growth of Corynebacterium fascians; and reduced the growth of C. flaccumfaciens. In our studies, Corynebacterium sp. did not grow in SSM.

Gentamicin is an aminoglycoside antibiotic that affects protein biosynthesis and acts in a manner similar to streptomycin; gentamicin produces higher levels of M-RNA misreading than streptomycin (8).

Many synthesis antibacterial compounds are based on the 5-nitro-2 furfurylidene structure. One of the best known is

nitrofurantoin, which has a wide sprectrum of antibacterial activity covering both Gram positive and Gram negative organisms (8). Our results are in agreement with the paper of Szabo (27) who used disc bioassays and showed that nitrofurantoin was highly inhibitory to many bacterial isolates but not to Xanthomonas sp.

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CHAPTER III

A LABORATORY TEST FOR THE DETECTION OF *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS* IN BEAN SEED

Introduction

Nearly half of the United States' supply of dry edible be beans (*Phaseolus vulgaris* L.) is produced in the Upper Great Lakes region, particularly Michigan and New York (1).

Common and fuscous bacterial blights, incited by *Xanthomonas phaseoli* (E. F. Sm.) Dows (*Xp*) and Burk h. (*Xpf*) respectively, are major diseases of Michigan navy beans (2, 4, 5, 15) and, according to Zaumeyer (23) and Zaumeyer and Thomas (25), internally infected seeds are the main source of primary inoculum. In addition, surface contamination of bean seed by *Xp* and *Xpf* may also be important (13, 22).

Weller, in 1978 (22), showed that seeds externally contaminated with *Xp* or *Xpf* can serve as primary inocula sources and that inoculum levels of 10^3-10^4 cells per seed were required for transfer of the bacteria from seed to seedling. However, treatment of bean seed with a slurry containing streptomycin sulfate effectively eliminates external contamination by *Xp* and *Xpf*. Internal seed infection, therefore, is considered the important inoculum source (3, 24) for disease disemination.

"Seed certification is a program to maintain and make available to the public high quality seeds and propagating material of genetically distinct crop varieties" (4). In Michigan, seed certification includes Breeder seed, Foundation seed, and Certified seed classes.

> The Breeder seed of Michigan certified bean varieties has been produced and maintained in irrigated regions of California and Idaho where climatic conditions, rigid quarantine and blight prevention programs minimize risk of infestation by bacterial organisms. In Idaho any field in which blight is found by the Department of Agriculture is required to be completely plowed under and destroyed (4).

In the case of Pseudomonas phaseolicola infection of bean seed, a minimum acceptable level of infection has not been defined. Walker and Patel, in 1964 (21), reported that an infection level of 0.02% in seeds is capable of promoting a general epidemic, but Guthrie, et al., in 1965 (7), reported that 0.006% is sufficient contamination to result in a complete crop loss under epiphytotic conditions. In epidemiological studies it was demonstrated that even 0.5% seed infection could cause serious outbreaks of fuscous blight (6).

The most common method suggested to control bacterial blights of beans is to ensure that seed used be as free of infected seed as possible (6, 15, 17, 20). Numerous assay methods have been developed or adapted to detect the presence of seed borne Xp and Xpf.

Direct plating of seeds on agar have been used (8, 20). Taylor described the dry grind extraction method for detecting Ps. phaseolicola, causal agent of bean halo blight (18).

The cotyledon method (12, 16) has been used for the detection of Ps. glycinae in soybean seed (12), and in the detectionof Xanthomonas vesicatoria in chili seeds (16) (Capsicum annum L. and C. Frutescens L.). Seeds were collected from diseased plants and sown in autoclaved soil. Seedlings in the cotyledonary stage were then transferred to humidity chambers (24-30°C; RH 95-100% for 3-5 days and returned to the greenhouse; and scored for disease symptoms).

Recently, Venette (19) reported a Dome test, consisting of vacuum infiltration of liquid obtained from soaked bean samples into seedlings. The seedlings were obtained from surface sterilized seeds of the same seed lot germinated on filter paper in the laboratory. Disease symptoms appeared as water-congested spots in the left tissue after 7-10 days in the laboratory under fluorescent light.

Another method used is the phage plaque count (9, 10, 17). Katznelson, in 1950 (9), detected internally borne Xp by measuring phage titres after incubation of phage with seed. Ednie and Needham (6) modified the method in 1973, and wet ground 3.5 lb. (1.6 kg) samples of surface-disinfected seed. A dilution series was prepared from a sample of the slurry produced, and an aliquot of each dilution placed on agar plates and incubated five to ten days. Xanthomonas-like colonies were then isolated, phage-typed and tested for pathogenicity.

Serology has been used in some states to detect blight bacteria in bean seed. Guthrie, et al., in 1965, used serological techniques to detect Pseudomonas phaseolicola (7). They used

slide and tube agglutinin tests and agar gel double diffusion tests. Lovrekovich and Kelment (21) combined plating techniques and serology in the identification of bacterial infection of bean seeds: they surface sterilized the seeds, prepared a suspension, an aliquot of which was spread on nutrient agar. Colonies characteristic for blight bacteria were tested against a series of antisera in agglutinin tests.

In Michigan, the Michigan Department of Agriculture Bean Seed Testing Program uses a laboratory blight test developed by Saettler in 1971 (14). A 2.21 kg sample of surface sterilized seed is incubated in dilute yeast extract solution (10 gm/l) for 24 hours, after which a sample of the liquid is injected into the primary leaf node of young Manitou kidney bean seedlings.

It is apparent that such tests for detection of bacterial blight organisms have played an important role in reducing and often eliminating seed infection by bacterial pathogens (13), but most of them have some limitations. Serological tests, in order to be used with accuracy, must rely on two important aspects: (a) specificity of the antisera used, and (b) a given concentration of bacterial cells is necessary in order to have clear results.

Tests using plating techniques and plant material are not always clear, and retesting and reisolation is time consuming. Furthermore, there is indication that the sensitivity of any seed test system would be decreased as the incidence of saprophytic bacteria increased above their normal level of occurrence (6); according to some workers this could occur with seed samples

harvested under adverse weather conditions wherein the pods are in contact with the soil longer than normal.

In 1978-79, the seed testing program of the Michigan Department of Agriculture released the following data: of 640 bean seed lots from public sources, 34% were found to carry internal blight infection, and of 404 samples from the Michigan Crop Improvement Association, 15% were found to carry internal blight infection (W. J. Young, personal communication).

Although the Michigan bean seed testing program has reduced the incidence of seed borne Xp and Xpf, outbreaks of common and fuscous blight persist, and some fields are rejected annually for certification.

It was therefore important to develop an accurate and rapid technique to detect internal Xanthomonas contamination of bean seed.

Objective

To determine the possible utility of combined semi-selective media and serological techniques for the detection of Xanthomonas blight bacteria in seed and other plant tissue.

Materials and Methods

Samples

a. The Michigan Department of Agriculture (MDA) test for internal blight contamination of bean seed currently involves: (1) surface sterilization of 2.2 kg seed for ten minutes in 2.6% NaOCl; (2) rinsing in sterile water; (3) incubating for 24 hours in a liquid media containing about 10 gm/l of yeast extract, and

(4) injfection of a sample of liquid surrounding the seeds into the primary leaf node of young kidney bean seedlings.

One ml of the surrounding liquid from 175 seed samples obtained through the MDA were individually incubated in 25 ml of the semi-selective media (SSM) (1.0 gm yeast extract, 25 mg cyclohexiмide, 2 mg nitrofurantoin, 1 mg nalidixic acid, 0.05 mg gentamicin in 1,000 ml of 0.01 M phosphate buffer pH 7.2). After 48 hours shaker incubation, bacteria were sedimented by 15 minutes centrifugation at 5,000 x g, resuspended in 1 ml buffer saline, steamed 60 minutes at 100°c, and tested using agar gel double diffusion. The results of these serological tests were then compared with the MDA test for internal blight detection.

b. Navy bean samples from the MDA were tested. Eighty gms of navy bean seed lots (400 seeds) were weighed, surface-sterilized two minutes with 2.6% NaOCl (1:1 dilution of commercial bleach), rinsed twice with double distilled water, and divided into two parts. Samples were taken, each of approximately 40 gms and separately placed in flasks containing 120 ml SSM. After 48 hours shaking, bacteria from 30 ml of the SSM were sedimented by 15 minutes centrifugation at 500 x g, and resuspended in 2 ml of buffer saline. One portion of the bacterial suspension was used for seedling injection, and the other was steamed 60 minutes at 100°C and tested serologically as before, in agar double diffusion tests (SSMS).

c. Stem Samples: A number of stems suspected of being infected with bacterial blight (Xp or Xpf) were received from the

MDA. A number of other stem sections from injfected plants in our greenhouse, suspected of being infected with bacterial blight (Xp or Xpf), were taken five weeks after plant infection. Sections of the stems at the injection point were excised, rinsed with distilled water, and placed in 25 ml of SEM, following the same steps as in (b).

d. Leaf Material: When Xp and Xpf symptoms were atypical or were doubtful, three leaves were taken from the plants, rinsed with distilled water, cut in pieces with sterilized scissors, and treated as in (a).

e. Stored material and seeds: Dry infected tissue samples and seed were kept in the refrigerator or stored at room temperature for different periods of time (six months to 30 years) and tested for blight following the same procedure as in (a). Most of this material had been collected by Dr. A. Saettler.

Serological Test

Agar gel double diffusion tests were used in all these experiments. Two antisera were consistently employed; one prepared against Xanthomonas phaseoli isolate 11 or 15, and one prepared against Xanthomonas phaseoli var. fuscans isolate 16 or 1085.

Seedling Injection

Disease-free seed of Manitou light red kidney bean were planted 2 cms deep in sterile soil plus vermiculite (1:1) in clay pots (3 plants per pot). Plants were grown in the greenhouse and watered alternately as needed with deionized water and Rapid Gro

(1 tspn/gal). When ten to fourteen days old, plants were injected at the primary leaf node with bacterial suspensin using a hypodermic needle.

Plants were maintained in the greenhouse at least six weeks after injection. When symptoms were unclear or were doubtful, isolations were attempted with (a) selective enrichment, (b) dilution plate methods, and (c) plant injection. Plants were considered infected with blight when at least one plant showed the typical disease symptoms.

Results

Michigan Department of Agriculture Liquid Samples

Ninety of the 1975 liquid samples obtained from the MDA Plant Diagnostic Laboratory were found to contain Xp or Xpf when tested with the combined semi-selective media plus agar gel double diffusion technique (SSMS) (Table 25).

MDA tests reported 65 of the 175 samples containing internal blight infection. Of these 65, 61 were found to carry internal blight contamination using the combined selective enrichment media-serology technique. Therefore, the MDA testing procedure did not detect blight in 29 samples, which were found positive for blight with the combined technique.

TABLE 25.--The Use of Serology, Semi-Selective Media, and Seedling Injection for the Detection of *Xanthomonas* Blight Bacteria in Bean Seed.

Sample	Number	Detected by Serology	Detected by Seedling Injection	Free of Blight with Both Methods	Blight with Both Methods
MDA[a]	175	90	65 (MDA)	80	61
Stems[b]	32	32	32	0	32
Stems[c]	40	35	35	0	35
Leaves[d]	40	30	27	10	27
Dried[e]	10	10	10	0	10

[a]1 ml of the surrounding liquid from each sample used in the MDA blight test was placed in 25 ml SSM. After 48 hours of shaker incubation bacteria were sedimented by centrigutation, resuspended in 1 ml buffer-saline, and steamed for 60 minutes at 100°C. Agar double diffusion tests were performed on the bacterial suspensions.

[b]Stems suspected of being infected with blight bacteria were received from the MDA, rinsed with sterile distilled water, and tested with SEM and serology as in (a).

[c]Stems suspected of being invected with *Xanthomonas* from greenhouse experiments were rinsed with sterile distilled water and treated as before; however, a sample of the bacterial suspension was used for seedling injection prior to steaming.

[d]Leaves from plants injected with blight (but without typical blight symptoms) were rinsed with sterile distilled water and treated as before; however, a sample of the bacterial suspension was used for seedling injection piror to steaming.

[e]Four-year old dried infected plant material stored at 4°C. was suspended in buffer saline, and treated as in (a); however, a sample of the bacterial suspension was used for seedling injection prior to steaming.

Michigan Department of Agriculture Seed Samples

Thirty-seven of the 99 navy bean samples obtained from the MDA showed positive results for internal blight contamination with the SSMS procedure (Table 26). Of these 37, the MDA seedling injection procedure detected blight in only 25. In our own seedling injection tests, Xp or Xpf were detected in 34 (4 of the seedling injections were reisolations and rechecked because the symptoms were doubtful with the first seedling injection). The remaining 15 seedling injection tests were not rechecked for internal blight contamination.

In just five cases was blight contamination found in the MDA seedling injection procedure but not found in the combined SSMS procedure. From these five cases we detected blight contamination in two of our seedling injections, and after isolation of the organisms, host range study and numerous physiological tests (Table 26) we concluded that these two isolates were Pseudomonas phaseolicola (Tables C1, C2 and C3).

In our seedling injections we found another two cases where serology was negative and the plant gave blight symptoms after seedling injection; after isolation, host range study and physiological tests (Table 26) we concluded that one of the isolates was Pseudomonas syringae and the other one, Ps. phaseolicola.

Of the 12 test samples that showed visible evidence of seed carrying blight infection, 10 tested positive with the SSMS procedure and 9 with the MDA testing procedure.

TABLE 26.--Detection of Internal Bacterial Blight Contamination in Bean Seed Lots by Several Procedures.

MDA Seed Lot	Procedure			
	SSMS[a]	Seedling Injection[a]	MDA Test[b]	Appearance of Seed After Disinfection[c]
10607	+	+	+	HS
10608	+	+*	-	HS
10614	-	-	-	C
10615	+	+	+	C
10616	+	+	+	BS
10617	-	-	-	C
10640	+	+	+	HS

[a]80 grams of navy bean seed lots (400 seeds) were weighed, surface-sterilized two minutes with NaOCl (1:1 v/v), rinsed twice with double distilled water, and divided into two parts. Samples were taken, each of approximately 40 grams and separately placed in flasks containing 120 ml SEM. After 48 hours shaker incubation, bacteria from 30 ml of the SSM were sedimented by 15 minutes centrifugation at 5000 x g, and resuspended in 1 ml of buffer saline. One portion of the bacterial suspension was used for seedling injection, and the other was steamed 60 minutes at 100°C and tested serologically as before, in agar double diffusion tests (SSMS).

*= bacteria (Xp or Xpf) isolated from injected plants showing no symptoms; ** = bacteria other than Xp or Xpf were recovered from injected plants showing symptoms; + = blight infection; - = no infection.

[b]The Michigan Department of Agriculture (MDA) test for internal blight contamination of bean seed currently involves: (1) surface sterilization of 2.2 kg seed for ten minutes in 2.6% NaOCl; (2) rinsing in sterile water; (3) incubating for 25 hours in a liquid media containing about 10 gm/l of yeast extract; and (4) injection of a sample of liquid surrounding the seeds into primary leaf node of young kidney bean seedlings.

[c]C = clean seeds, no visible blight symptoms; HS = hilum-spotted seeds; most of these seeds carry internatl blight bacteria; BS = typical symptoms of internal blight infection.

TABLE 26.--Continued.

MDA Seed Lot	Procedure			
	SSMS[a]	Seedling Injection[a]	MDA Test[b]	Appearance of Seed After Disinfection[c]
10641	+	+	+	BS
10862	+	+	+	BS
10863	+	+	+	C
10944	-	-	-	C
10945	-	-	-	C
10946	-	-	-	C
10947	+	+*	-	C
10948	+	+	-	BS
10949	-	-	-	C
10950	+	+	-	HS
10951	+	+	-	HS
10952	+	+	-	C
10953	-	-	-	C
10954	-	-	-	C
10955	-	-	-	C
10956	-	-	-	C
10957	-	-	-	C
10958	-	-	-	C
10959	-	-	-	C
10960	-	-	-	C
10961	+	+	+	HS
10985	+	+	+	BS
11100	+	+	+	BS
11101	+	+	+	HS
11212	+	+	+	HS
11213	-	-	-	HS
11089	-	-	-	BS

TABLE 26.--Continued.

MDA Seed Lot	Procedure			
	SSMS[a]	Seedling Injection[a]	MDA Test[b]	Appearance of Seed After Disinfection[c]
11090	+	+	-	BS
11091	-	-	-	HS
11092	-	-	-	HS
11093	+	+	+	BS
11094	-	-	-	C
11095	+	+	-	C
11096	+	+	+	C
11097	-	-	-	C
11098	-	-	-	C
11099	+	+	+	C
11210	-	-	-	C
11211	+	+	+	C
11214	-	-	-	C
11215	-	-	-	HS
11216	-	-	-	C
11217	-	-	-	C
11218	-	-	-	C
11219	+	+	-	C
11220	-	-	-	C
11221	+	+	+	C
11222	-	-	-	C
11223	-	-	-	C
11224	+	+	-	C
11225	-	-	-	C
11226	-	+**	-	HS
11227	-	-	-	C
11228	+	+	-	HS

TABLE 26.--Continued.

MDA Seed Lot	Procedure			
	SSMS[a]	Seedling Injection[a]	MDA Test[b]	Appearance of Seed After Disinfection[c]
11229	-	-	-	C
11230	-	-	-	C
11231	+	+	+	C
11232	-	-	-	C
11233	-	-	-	C
11234	-	+**	-	HS
11235	-	-	-	C
11236	-	-	-	C
11237	-	-	-	C
11238	-	-	-	C
11239	-	-	-	C
11241	-	-	-	C
11242	-	-	-	C
11243	-	-	-	C
11251	-	+**	+	C
11252	-	-	-	C
11255	-	-	-	C
11310	+	+	+	HS
11329	+	+	+	C
11330	+	-	+	HS
11331	-	-	-	C
11332	-	-	-	HS
11338	+	-	+	C
11339	+	+	+	HS
11340	-	-	-	C
11341	-	-	-	C
11342	-	+**	+	BS

TABLE 26.--Continued.

MDA Seed Lot	Procedure			
	SSMS[a]	Seedling Injection[a]	MDA Test[b]	Appearance of Seed After Disinfection[c]
11481	-	-	-	C
11494	-	-	+	C
11495	+	+	+	BS
11693	-	-	-	HS
11694	-	-	-	C
11695	-	-	-	C
11780	-	-	-	C
11829	-	-	-	C
11952	-	-	+	C

Nineteen of the seed samples also showed evidence of hilum-spotted seed which is another characteristic symptom of blight contamination. Of 19 samples containing hilum-spotted seed, 12 tested positive with the SSMS procedure and 8 with the MDA testing procedure.

Symptomless seeds carrying internal blight contamination by the SSMS procedure were found in 15 of 68 seed lots.

MDA Stem Sections

All 32 stems obtained from the MDA (Table 26) and showing typical blight symptoms tested positive with both SSMS and seedling injection procedures.

Stems Suspected of Being Infected with Blight Bacteria from Greenhouse Experiments

Forty stems (Table 26) in which blight symptoms were doubtful in greenhouse tests were tested with the SSMS procedure, and 35 were found to contain blight both serologically and in the seedling injection test.

Leaves from Plants Suspected of Being Infected with Blight Bacteria

Forty leaf samples from different plants (Table 25) in which blight symptoms were unclear were tested with the SSMS procedure; 30 were found to contain blight serologically, and 27 were found to contain blight with the seedling injection method.

Dried Material

Ten separate samples of four-year-old infected plant material stored at 4°C were tested with the SSMS procedure, and all ten were found to contain blight serologically, as well as by seedling injection (Table 26).

Stored Seeds Infected with Blight

Navy bean seeds with visible blight symptoms and of different ages (6 months, 1, 2, 4, 12, 29, 30 years old) were tested using the SSMS procedure. Bacterial blight was found in all samples; seedling injection showed that the bacteria were still pathogenic.

Discussion

The use of semi-selective media combined with agar gel double diffusion tests (SSMS) resulted in a highly reliable procedure for the detection of internal *Xanthomonas* blight infection of bean seed and for the detection of *Xanthomonas* in various other plant tissues.

The SSMS procedure was more efficient in detecting blight than seedling injection. In addition, the SSMS procedure is more rapid, does not require a large amount of space (greenhouse or plants) or special equipment.

A population of 10^4 *Xanthomonas* blight bacterial cells/ml added to 25 ml of the semi-selective media resulted in turbidity after 48 hours shaker incubation. This means that SSM will allow turbidity (and therefore serological detection) after 48 hours shaker incubation with an initial concentration of 400 cells/ml.

Figure 12.--Symptoms of internal seed infections by Xanthomonas blight bacteria. A = heavily blighted seeds, B = moderately blighted seeds, C = hilum-spotted seeds, D = symptomless seeds.

Figure 13.--Seeds from the same sample: A = after surface sterilization, B = before surface sterilization.

Figure 12

Figure 13

Figure 14.--Symptoms in red kidney bean plants after injection of Xanthomonas blight bacteria.

Figure 14

Figure 15.--Red kidney bean plants injected with isolate 59 (*Ps. syringae*). Note red necrosis extending through the stem and top necrosis.

Figure 16.--Red kidney bean plant injected with isolate 59 (*Ps. phaseolicola*). Note red necrosis and vein clearing of the leaves.

Figure 15

Figure 16

Figure 17.--Typical symptoms of Ps. Phaseolicola. Note the halo blight formation.

Figure 17

Several workers have reported populations greater than 4×10^5 Xp or Xpf cells per seed in internally infected bean seeds (6, 18). Recently, Weller (22) reported 1.8×10^5 Xanthomonas blight bacteria/seed as the lowest population in hilum-spotted seed. Visibly blighted seeds have an average internal population of Xanthomonas blight bacteria ranging from 10^7-10^9 bacterial cells/seed.

Our study supports Weller's findings (22) that symptomless seed internally infected with Xp or Xpf are potential primary inoculum sources. Fifteen of sixty-eight samples containing symptomless seeds were found to contain internal blight infection with the SSMS procedure.

The SSMS procedure decreases the growth rates of internal bean seed bacterial contaminants, which makes the procedure preferable to other tests since accuracy of the test is influenced by contaminant populations (6, 9, 11, 18).

Navy beans are susceptible to halo blight (Ps. phaseolicola) in the greenhouse (8), but the disease has not been observed in Michigan navy bean fields (15). The present study is in agreement with Weller's findings (22) that navy beans are a potential source of Ps. phaseolicola inoculum. In addition, Ps. syringae was found to be internally infecting navy beans, but according to our results, the percentage of seeds infected was low (1-3% of the seed samples tested).

One disadvantage of the SSMS procedure is that it is primarily selective for Xanthomonas blight bacteria and cannot

consistently detect other seed borne bacterial pathogens. However, this disadvantage is not particularly important for Michigan since Xanthomonas bacterial blights are the most important navy bean diseases (2, 5, 15).

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APPENDICES

APPENDIX A

TABLE A1.--Reactions of Xanthomonas phaseoli (Xp) Isolates Against Xanthomonas Antisera in Agar Gel Double Diffusion Tests.

Isolates Used	Antigen - Live Cells[a]						Antigen - Steamed Cells					
	Antisera Prepared Against						Antisera Prepared Against					
	Formalized Cells			Steamed Cells			Formalized Cells			Steamed Cells		
	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
1) Xanthomonas phaseoli 11	+++[b]	+++	+++	+++	+++	+++	+	++	++	+	++	++
2) Xp 15	+++	+++	+++	+++	+++	+++	+	++	++	++	++	++
3) Xp Neb24	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++
4) Xp 101	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
5) Xp 103	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
6) Xp 104a	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
7) Xp 105	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++
8) Xp 109	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
9) Xp 110a	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
10) Xp 115B	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++

[a]Antigen adjusted to about 10^{10} cells/ml.

[b]+++ = 4 or more bands; ++ = 2 or 3 bands; + = 1 or 2 bands.

TABLE A1.--Continued.

Isolates Used	Antigen - Live Cells[b]						Antigen - Steamed Cells					
	Antisera Prepared Aganist						Antisera Prepared Against					
	Formalized Cells			Steamed Cells			Formalized Cells			Steamed Cells		
	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
11) Xp 116B	+++	+++	+++	+++	+++	+++	+	++	++	++	++	++
12) Xp 121B	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
13) Xp 122B	+++	+++	+++	+++	+++	+++	+	++	++	+	++	++
14) Xp 124	+++	+++	+++	+++	+++	+++	+	+	++	+	++	++
15) Xp 134a	+++	+++	+++	+++	+++	+++	++	++	++	+	++	++
16) Xp 135b	+++	+++	+++	+++	+++	+++	++	++	++	+	++	++
17) Xp 147	+++	+++	+++	+++	+++	+++	++	++	++	+	++	++
18) Xp 150	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++
19) Xp 157	+++	+++	+++	+++	+++	+++	+	++	++	++	++	++

TABLE A2.--Reactions of Xanthomonas phaseoli var. fuscans (Xpf) Isolates Against Xanthomonas Antisera in Agar Gel Double Diffusion Tests.

Isolated Used	Antigen - Live Cells[a]						Antigen - Steamed Cells					
	Antisera Prepared Against						Antisera Prepared Against					
	Formalized Cells			Steamed Cells			Formalized Cells			Steamed Cells		
	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
1) Xanthomonas phaseoli var. fuscans	+++[b]	+++	+++	+++	+++	+++	++	+	+	++	+	+
2) Xpf 17	+++	+++	+++	+++	+++	+++	++	+	+	++	++	++
3) Xpf 20	+++	+++	+++	+++	+++	+++	++	+	++	++	++	+
4) Xpf 28	+++	+++	+++	+++	+++	+++	++	++	+	++	+	+
5) Xpf 107	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
6) Xpf 108	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
7) Xpf 109a	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
8) Xpf 110b	+++	+++	+++	+++	+++	+++	++	+	++	++	+	+
9) Xpf 111	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
10) Xpf 112	+++	+++	+++	+++	+++	+++	++	++	+	++	+	+
11) Xpf 113	+++	+++	+++	+++	+++	+++	++	++	+	++	+	++
12) Xpf 114	+++	+++	+++	+++	+++	+++	++	++	+	++	+	++
13) Xpf 134B	+++	+++	+++	+++	+++	+++	++	++	++	++	+	++
14) Xpf 135	+++	+++	+++	+++	+++	+++	++	+	++	++	+	+
15) Xpf 146	+++	+++	+++	+++	+++	+++	++	+	+	++	+	++

[a]Antigen adjusted to about 10^{10} cells/ml.

[b]+++ = 4 or more bands; ++ = 2 or 3 bands; + = 1 or 2 bands; nt = not tested.

TABLE A2.--Continued.

Isolates Used	Antigen - Live Cells[a]						Antigen - Steamed Cells					
	Antisera Prepared Against						Antisera Prepared Against					
	Formalized Cells			Steamed Cells			Formalized Cells			Steamed Cells		
	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11	Xpf16	Xp15	Xp11
16) Xpf 147	+++	+++	+++	+++	+++	+++	++	+	+	++	+	++
17) Xpf 148	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
18) Xpf 149	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
19) Xpf 150	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
20) Xpf 115A	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
21) Xpf 116A	+++	+++	+++	+++	+++	+++	++	+	+	++	++	++
22) Xpf 117	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
23) Xpf 118	+++	+++	+++	+++	+++	+++	++	+	+	++	+	++
24) Xpf 119	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
25) Xpf 120	+++	+++	+++	+++	+++	+++	++	+	+	++	+	+
26) Xpf 121	+++	+++	+++	+++	+++	+++	++	++	+	++	+	+
27) Xpf 122	+++	+++	+++	+++	+++	+++	++	nt	nt	nt	nt	nt
28) Xpf 123	+++	+++	+++	+++	+++	+++	++	++	+	++	+	nt

TABLE A3.--Cross Agglutination Reactions of Various Seed Borne Contaminants of Navy Bean Seed and of Different *Xanthomonas* Isolates Against *Xanthomonas* Antisera.

Antisera Prepared Against	Dilution and Point Isolate[b]																	
				Pseudomonas fluorescens				Erwinia herbicola		Bacillus			Gram +			Gram -		
	Xpf 16	Xp 15	Xp 11	C7a	C8b	C11	C16	C1	C10	C3	C13	C15	C2a	C9	C14	C5a	C6	C17
1) *Xpf*16	512	512	256	32	16	64	-	32	32	-	32	-	-	-	-	-	16	64
2) *Xp*15	512	512	256	16	16	64	-	32	64	-	-	-	32	-	-	-	16	-
3) *Xp*11	256	256	512	32	16	32	-	32	32	-	32	32	16	-	-	-	16	-
4) *Xpf*16	512	512	256	16	-	32	-	-	16	16	-	32	-	-	-	-	-	-
5) *Xp*15	256	512	512	32	16	-	-	-	-	-	32	-	-	-	-	-	16	-
6) *Xp*11	256	512	256	16	16	16	16	16	16	16	16	-	16	-	-	-	-	16

[a]Antisera 1, 2, and 3 produced with formalized cells; antisera 4, 5, and 6 produced with steamed cells.

[b]Antigen was steamed for 1 hour and adjusted to an optical density of h = 0.3.

- = no reaction.

TABLE A4.--Reactions of Various Bacterial Contaminants Isolated Internally from Navy Bean Seeds Against *Xanthomonas* Antisera in Agar Gel Double Diffusion Tests.

Isolate Used	Antisera Prepared Against					
	Formalized Cells			Steamed Cells		
	*Xpf*16	*Xp*15	*Xp*11	*Xpf*16	*Xp*15	*Xp*11
1) C1	-[b]	-	-	-	-	-
2) C2a	-	-	-	-	-	-
3) C3	-	-	-	-	-	-
4) C4	-	-	-	-	-	-
5) C5	-	-	-	-	-	-
6) C6	-	-	-	-	-	-
7) C7a	-	-	-	-	-	-
8) C8b	-	-	-	-	-	-
9) C9	-	-	-	-	-	-
10) C10	-	-	-	-	-	-
11) C11	-	-	-	-	-	-
12) C12	-	-	-	-	-	-
13) C13	-	-	-	-	-	-
14) C14	-	-	-	-	-	-
15) C15	-	-	-	-	-	-
16) C16	-	-	-	-	-	-
17) C17	-	-	-	-	-	-
18) C18	-	-	-	-	-	-
19) C19	-	-	-	-	-	-
20) *Xpf*16	+	+	+	+	+	+
21) *Xp*11	+	+	+	+	+	+

[a]Antigen steamed cells adjusted to an optical density of h = 0.3.

[b]- = no reaction; + = band formation.

APPENDIX B

TABLE B1.--Various Characteristics of Bacterial Contaminants Isolated from Surface Sterilized Bean Seeds.[a]

Contaminant Number	Gram Reaction	Colony Color on YCA	Fluorescence on KM B.	Hypersensitivity on Tobacco	Seedling Injection	Phenol RED	Oxidase Test	Endospores Present	Presumed Genus
1	-	yellow	-	-	-	-		-	Erwinia[b]
2A	+	yellow	-	-	-	-		-	
3	+	cream	-	-	-	-		+	Bacillus
4	+	yellow (pale)	-	-	-	-		-	
5A	-	white (cream)	-	-	-	-		-	
6	-	cream	-	-	-	-		-	
7	-	cream	+	=	=	=	+	-	Pseudomonas
8	-	cream	+	-	-	-	+	-	Pseudomonas
9B	+	yellow	-	-	-	-		-	Erwinia

[a] + = positive reaction; - = negative reaction.

[b] With isolates suspected to be Erwinia, additional tests were conducted: Starch hydrolysis, growth on yeast dextrose Agar and nitrates reduction.

TABLE B1.--Continued.

Contaminant Number	Gram Reaction	Colony Color on YCA	Fluorescence on KM B.	Hypersensitivity on Tobacco	Seedling Injection	Phenol RED	Oxidase Test	Endospores Present	Presumed Genus
10	-	yellow	-	-	-	-		-	Erwinia
11	=	cream	+	-	-	-	+	-	Pseudomonas
12	-	yellow	-	-	-	-		-	
13	+	cream	-	-	-	-		+	Bacillus
14	+	yellow (pale)	-	-	-	-		-	
15	+	cream	-	-	-	-		+	Bacillus
16	-	cream	+	-	-	-	+	-	Pseudomonas
17	-	yellow (pale)	-	-	-	-		-	
18	-	yellow (strong)	-	-	-	-		-	
19	-	white	-	-	-	-		-	

TABLE B2.--Bacterial Isolates and Their Sources.

Laboratory I.D. Number	
200	Agrobacterium tumefaciens: U.S., Upjohn Company
201	Bacillus megaterium: MSU, Microbiology Dept., 1976.
202	Corynebacterium fasciens: ATCC 13000.
203	C. flaccumfaciens: a. Nebraska. Schuster.
204	C. flaccumfaciens: NE21. Wisconsin. Vidaver.
205	C. flaccumfaciens: 6887. Wisconsin. Vidaver.
206	C. flaccumfaciens: 9A. Navy bean. Michigan, 1978
207	C. michiganense: Tomato fruit. Michigan, 1976.
220	Erwinia amylovora: Jonathan canker, Carpenter, Paw Paw, Michigan, 1975.
221	E. carotovora var. atroseptica: SR 8, Wisconsin Potato, 1969, Kelman.
222	E. herbicola: A-E from apricot, MSU.
250	Pseudomonas fluorescens: MSU, Microbiology Dept., 1975
251	Ps. glycinea: Soybean. Michigan, 1977.
252	Ps. morsprunorum: Source unknown.
253	Ps. phaseolicola L.: Michigan, 1977.
254	Ps. phaseolicola 2 (35): Michigan, 1978.
255	Ps. syringae DH24: Cherry canker. Michigan, 1976.
256	Ps. syringae D-3-3: Cherry canker. B. La Torre.
257	Ps. syringae, Y30: bean, Wisconsin, D.J. Hagedorn, 1978.
258	Ps. tomato 1, tomato: southwest Michigan, 1977.
259	Ps. tomato 2 (525-1): U. Cal., R. Grogan.
1	Xanthomonas campestris: cabbage. Michigan, 1974.
2	X. juglandis: diseased walnut, California, 1975.
3	X. pelargoni: geranium, Michigan, 1977.
11	X. phaseioli 11: Navy bean, Michigan, 1971.
15	X. phaseoli 15: Navy bean, Michigan, 1971.
24	X. phaseoli Neb24: from Nebraska, Colombia isolate.

TABLE B2.--Continued.

Laboratory I.D. Number	
101	X. phaseoli 101: Navy bean, Michigan isolate, 1977.
103	X. phaseoli 103: Red kidney bean, Michigan isolate, 1977.
104a	X. phaseoli 104a: Red kidney bean, Michigan isolate, 1977.
105	X. phaseoli 105: Red kidney bean, Michigan isolate, 1977.
109	X. phaseoli 109: Navy bean, Michigan isolate, 1977.
110a	X. phaseoli 110a: Navy bean, Michigan isolate, 1977.
115b	X. phaseoli 115b: Navy bean, Michigan isolate, 1977.
116b	X. phaseoli 116B: Navy bean, Michigan isolate, 1977.
121B	X. phaseoli 121B: Navy bean, Michigan isolate, 1977.
122B	X. phaseoli 122B: Navy bean, Michigan isolate, 1977.
124	X. phaseoli 124: Navy bean, Michigan isolate, 1977.
134a	X. phaseoli 134a: Navy bean, Michigan isolate, 1977.
135b	X. phaseoli 135b: Navy bean, Michigan isolate, 1977.
147	X. phaseoli 147: Navy bean, Michigan isolate, 1977.
150	X. phaseoli 150: Navy bean, Michigan isolate, 1977.
157	X. phaseoli 157: Navy bean, Michigan isolate, 1977
16	Xanthomonas phaseoli var. fuscans 16: Navy bean, Michigan, 1970.
17	Xpf17: Navy bean, Michigan, 1970.
20	Xpf20: Navy bean, Michigan, 1970.
28	Xpf28: Idaho.
107	Xpf107: Red kidney bean, Michigan, 1977.
108	Xpf108: Navy bean, Michigan, 1977.
109a	Xpf109a: Navy bean, Michigan, 1977.
110b	Xpf110b: Navy bean, Michigan, 1977.
111	Xpf111: Navy bean, Michigan, 1977.
112	Xpf112: Navy bean, Michigan, 1977.
113	Xpf113: Navy bean, Michigan, 1977.
114	Xpf114: Navy bean, Michigan, 1977.

TABLE B2.--Continued.

Laboratory I.D. Number	
115A	Xpfl15A: Navy bean, Michigan, 1977.
116A	Xpfl16A: Navy bean, Michigan, 1977.
117	Spfl17: Navy bean, Michigan, 1977.
118	Xpfl18: Navy bean, Michigan, 1977.
119	Xpfl19: Navy bean, Michigan, 1977.
120	Xpfl20: Navy bean, Michigan, 1977.
121	Xpfl21: Navy bean, Michigan, 1977.
122	Xpfl22: Navy bean, Michigan, 1977.
123	Xpfl23: Navy bean, Michigan, 1977.
134B	Xpfl34B: Navy bean, Michigan, 1977.
135	Xpfl35: Navy bean, Michigan, 1977.
146	Xpfl46: Navy bean, Michigan, 1977.
147	Xpfl47: Navy bean, Michigan, 1977.
148	Xpfl48: Navy bean, Michigan, 1977.
149	Xpfl49: White haricot bean, Ethiopia, 1977.
150	Xpfl50: Navy bean seeds from 1950, Michigan, 1979.
151	Xpfl850: Navy bean seeds from 1967, Michigan, 1978.
152	Xpfl949: Navy bean seeds from 1949, Michigan, 1949.

APPENDIX C

TABLE C1.--Host Range of *Pseudomonas* Species and Several Baceterial Isolates Obtained from Navy Bean Seed Lots.[a]

	Pseudomonas phaseolicola[b]	*Pseudomonas syringae*[b]	*Pseudomonas phaseolicola* 3S (Mich. isolate)	Isolate 59	Isolate 67	Isolate 77
Phaseolus vulgaris L.						
var. Manitou	+	+	+	+	+	+
var. Seafarer	+	+	+	+	+	+
var. Tuscola	+	+	+	+	+	+
Pisum sativum						
var. Selected Wales	-		-	+	-	-
Black Cowpea	-	+	-	+	-	-
Glycine max.						
var. Hark	-	+	-	+	-	-
Phaseolus coccineus						
var. Scarlet Runner	+*		+	+**	+*	+*
Phaseolus lunatus						
var. Fordhook Bush	+	+	+	+	+	+

[a]Pathogenicity tests were conducted by injecting bacterial suspensions (10^7 cells/ml) into the primary leaf node of 14-day-old plants on each host.
+ = systematic reaction; +* = slow reaction; +** = only stem reaction; - = no reaction.

[b]Host range of *Ps. phaseolicola* and *Ps. syringae* taken from W. J. Zaumeyer, and H. Rex Thomas, 1957. A monographic study of bean diseases and methods for their control. Tech. Bull. No. 868, 255 pp.

TABLE C2.--Physiological and Biochemical Characteristics of Pseudomonas Species and Several Isolates Obtained from Navy Bean Seed Lots.[a]

	Pseudomonas phaseolicola[b]	Pseudomonas syringae[b]	Pseudomonas phaseolicola 3A (Mich. isolate)	Isolate 59	Isolate 59	Isolate 59
Gram stain	-	-	-	-	-	-
Diffusible pigment on KMB	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-
Denitrification	-	-	-	-	-	-
Hydrolysis of starch	-	-	-	-	-	-
Gelatin	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-
Production of hydrogen sulfide	-	-	-	-	-	-
Hypersensitivity in tobacco	+	+	+	+	+	+

[a]Tests performed according to: Jean F. MacFaddin, 1976. Biochemical tests for identification of medical bacteria. The Williams and Wilkins Company, Baltimore, 312 pp.

[b]Information from "Bergey's Manual of Determinative Bacteriology," 1957, 7th ed., R.S. Breed, E.G.D. Murray, and N.R. Smith (eds.). The Williams and Wilkins Co., Baltimore, 1094 pp.

TABLE C3.--Utilization of Carbon Compounds by Several Pseudomonas Species and Several Bacterial Isolates Obtained from Navy Bean Seed Lots.[a]

	Pseudomonas syringaeb	Pseudomonas phaseolicola[b]	Isolate 59	Isolate 67	Isolate 77
Arabinose	+	+	-	+	+
Fructose	not reported	+	+	+	+
Galactose	+	+	+	+	+
Glucose	+	+	+	+	+
Glycerol	+	+	+	+	+
Lactose	-	-	-	-	-
Maltose	-	-	-	-	-
Mannitol	+	-	-	-	-
Mannose	+	-	-	-	-
Raffinose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Salicin	-	-	-	-	-
Sucrose	+	+	+	+	+
Xylose	+	+	+	+	+

[a]Tests performed according to: Jean F. MacFaddin, 1976. Biochemical tests for the identification of medical bacteria. The Williams and Wilkins Co., Baltimore, 312 pp.

[b]Data from "Bergey's Manual of Determinative Bacteriology." 1957. 7th ed. R.S. Breed, E.G.D. Murray, and N.R. Smith (eds.). The Williams and Wilkins Co., Baltimore, 1094 pp.