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ECOLOGY OF *XANTHOMONAS PHASEOLI* AND
XANTHOMONAS PHASEOLI VAR. *FUSCANS* IN
NAVY (PEA) BEANS (*PHASEOLUS VULGARIS* L.)

presented by

David M. Weller

has been accepted towards fulfillment
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Major professor

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ECOLOGY OF *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS* IN NAVY (PEA) BEANS (*PHASEOLUS VULGARIS* L.)

By

David M. Weller

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ABSTRACT

ECOLOGY OF *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS* IN NAVY (PEA) BEANS (*PHASEOLUS VULGARIS* L.)

By

David M. Weller

Common and fuscous bacterial blights of bean (*Phaseolus vulgaris* L.), incited by *Xanthomonas phaseoli* (E.F. Sm.) Dows and *X. phaseoli* var. *fuscans* (Burkh.) Starr and Burkh. (*Xanthomonas campestris*), respectively, are serious diseases of Michigan Navy (pea) beans. Rifampin-resistant mutants of *X. phaseoli* (Xp) and *X. phaseoli* var. *fuscans* (Xpf) were screened for similarities to wild-type isolates and their utility in ecological studies. A mutant (Ra) isolate of Xp and one (R10) of Xpf were similar to wild-types in numerous bacteriological tests, grew *in vivo* at rates identical to, and were as virulent as the wild-types in bean leaves. The doubling time for R10 and Ra was about 11% longer than that for the wild-types in buffered yeast-extract liquid medium. A mutant (R10-S6) resistant to both rifampin and streptomycin was similar

in virulence to its R10 parent and original Xpf wild-type. The rifampin-resistance marker permitted selective isolation of Ra and R10 from field-grown bean plants; growth of all phyllosphere bacteria was inhibited on media with rifampin (50 µg/ml).

Population trends of R10 and Ra in inoculated leaves of field-grown Navy beans were similar to a standard bacterial growth curve. Mean doubling times of R10 and Ra were 19.4 and 18.8 hours, respectively during the exponential growth phase. Leaf populations of R10 and Ra peaked during the stationary phase and remained stable until leaf abscission; a slow death phase accompanied leaf decomposition on the soil surface. Symptom development on leaves required minimal populations of 5×10^6-2×10^8 bacteria per 20 cm^2 leaf tissue and usually corresponded to the early stationary phase.

The ecology of Xp and Xpf was studied in field-grown Navy beans by monitoring populations of R10, Ra, or R10-S6 in plants grown from infected seeds or sprayed with the bacteria. All above and below ground portions of seedlings grown from infected seeds were colonized by the blight bacteria immediately after germination. Primary leaf colonization initiated vertical bacterial spread into the expanding leaf canopy. By continually sampling leaves from the main stem of R10 or Ra inoculated plants, population profiles were established

which described the multiplication and spread of the bacteria in the canopy from seedling to reproductive phases of plant development. The profiles were characterized as a series of growth curves displaced over time with each curve representing bacterial multiplication on an individual leaf relative to the primary leaf node. Correlation of the sequence of symptom expression with the population profile explained the phenomenon of late disease development which is characteristic of common and fuscous blights. The spread of R10 and Ra was facilitated by rain, bud colonization, and systemic movement. Ten to 50% of the R10 or Ra leaf population was removed as secondary inoculum during rainfall. Vegetative buds were continually colonized by up to 10^3 bacteria per bud and leaves from the buds were usually infected. Isolates R10 and Ra displayed a significant systemic phase with doubling times of 22.8 and 23.8 hours in stems. The population profiles of R10 and Ra in stems were characterized as a series of growth curves, each representing bacterial multiplication in a section of stem relative to the cotyledon node. Isolates of R10 and Ra were associated with Navy bean roots and occupied the rhizosphere throughout the growing season.

Seeds externally infested with blight bacteria were shown to be an important source of primary inocula for

common and fuscous blights and 14% of commercial Navy bean seed lots were contaminated. Surface populations of Xp and Xpf ranged from 0-4 x 10^4 bacteria per seed, however, minimal populations of 10^3-10^4 bacteria/seed were required for infected plants to be produced under natural growing conditions. Symptomless seed internally bearing Xp or Xpf was identified as a potential primary inoculum source. Seeds with symptoms were always associated with visibly infected pods; when pod infection results from systemic-borne bacteria, hairline suture lesions may be produced which are difficult to detect.

This dissertation is dedicated to my wife

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TABLE OF CONTENTS

PART II

POPULATION TRENDS AND DISTRIBUTION OF *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS* IN FIELD-GROWN NAVY BEANS (*PHASEOLUS VULGARIS* L.)

PART III

PRIMARY INOCULA SOURCES OF COMMON AND FUSCOUS BACTERIAL BLIGHTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

GENERAL INTRODUCTION AND LITERATURE REVIEW

Common blight incited by *Xanthomonas phaseoli* (E. F. Smith) Dowson (Xp) and fuscous blight incited by *Xanthomonas phaseoli* var. *fuscans* (Burkh.) Starr and Burkh. (Xpf) are important diseases of most commercial bean (*Phaseolus vulgaris* L.) cultivars. Common blight was first reported by Beach (4) in 1892, about the same time, Halsted (27) reported a similar disease of bean pods and seeds. Erwin F. Smith in 1897 described the etiology of common blight and named the causal bacterium *Bacillus phaseoli* E. F. Smith (47, 48). Smith (49) further characterized the pathogen and transferred it into the genus *Pseudomonas* in 1901 then into the genus *Bacterium* in 1905 (50). Bergey's Manual ed II (5) renamed the blight bacteria *Phytomonas phaseoli* (E. F. Smith) Bergey *et al.*; Dowson (22) in 1938 placed the bacterium in the currently accepted genus of *Xanthomonas*.

Fuscous blight was discovered by Burkholder (11) in 1924 on an unknown bean cultivar from Switzerland. Disease symptoms were identical to common blight and the only means of distinguishing the diseases was by

culturing the causal bacterium. Xpf was initially known as *Phytomonas phaseoli* var. *fuscans* but later it was placed in the currently recognized genus of *Xanthomonas*.

Xp and Xpf are obligately aerobic, gram negative, straight rods, which produce a yellow nondiffusible pigment and are motile by a single polar flagellum. Both bacteria produce hydrogen sulfide, liquefy gelatin, proteolize milk, hydrolize starch and Tween 80 and produce an alkaline reaction in phenol red dextrose agar (7). Both bacteria are differentiated on the basis of a brown diffusible pigment produced by Xpf (2, 3, 28) and are otherwise physiologically and biochemically identical. Bergey's Manual 8th ed. currently recognizes that Xp and Xpf are nomen species of *Xanthomonas campestris* (7). Nomen species terminology will be used in this paper.

Xp and Xpf produce visible disease symptoms on all plant parts except the roots; symptomatology of the two diseases is essentially identical. However, Zaumeyer (58) reported that Xpf may cause a slight hypertrophy in tissue around a stem lesion and in seedlings, a darkening of the stem around the point of inoculation.

Leaf symptoms are the most diagnostic feature of common blight or fuscous blight. Infection begins as minute water-soaked spots on the abaxial surface of the leaf with yellow discoloration opposite the spot on the adaxial side. Lesions then enlarge irregularly, dry out

and become brown and brittle; occasionally a slight crust of bacterial exudate is present. The necrotic area is surrounded by a distinctive yellowish halo-like zone. Several lesions on a leaf may coalesce, and eventually occupy most of the leaf area and cause premature leaf drop (11, 57).

Stem lesions begin as water-soaked dark green spots which eventually become dry, sunken and reddish brown in color. The lesion usually extends longitudinally up the stem but shows little downward movement. Stem symptoms are most common on seedlings and less evident as the plant matures (11, 58).

Pod symptoms appear as dark green water-soaked spots which later become dry and sunken. Drying begins at the outer edge of the lesion and extends inward; a yellow crustation of bacterial exudate may cover the lesion (11). Pod infection occasionally results in seed infection. On white seeded bean cultivars, Xp and Xpf cause a shiny yellow spotting of the seed coat, the extent of which varies from small blotches to complete seed discoloration. Infection of dark-seeded bean cultivars is harder to detect due to seed coat pigmentation; symptoms appear as a darkening of the seed coat. Seeds which are infected early in development may be completely shrivelled or badly wrinkled, whereas, those infected near pod maturity are only darkened at the hilum (11). Seed

infection is most probable when the dorsal pod suture is infected. The bacteria invade the funiculus, pass through the raphe, and finally into the seed coat (55). Seeds may also be infected through the micropyle if the bacteria invade the pod cavity (56).

Common and fuscous bacterial blights have been reported in all bean producing areas of the world (6, 19, 53, 58). The diseases often are major limiting factors in bean production; in lesser-developed countries losses are especially severe due to inadequate control practices. Common and fuscous blights historically have been important in the United States and especially in Michigan where approximately 35% of all dry edible beans and 85% of all U.S. Navy (pea) beans are produced. According to the USDA's disease surveys the two diseases were recognized as widespread and serious threats soon after the discovery of common blight in 1892 (4). In 1919 common blight was prevalent in 75% of New York's bean fields and caused serious damage; one year later, Colorado suffered a 40% crop loss. The most severe loss in the nation due to common blight in 1921 was in Michigan where yields were reduced by 25%; in 1927 the average loss to bean blight throughout the U.S. was 1.4% (56). Andersen (1) estimated that bacterial blight caused a 3.5 million dollar loss in 1949-1950 to growers in three Michigan counties; in Nebraska 1953 losses

were estimated at greater than $1,000,000 due to blight. In 1967 (33) at least 75% of Michigan's 650,000 acres of Navy beans were damaged by common and fuscous blights with yield reduction of 10-20%. In 1962, Sutton and Wallen (51) reported 60% of the Navy bean fields in Southwest Ontario, Canada infected with fuscous blight. Wallen and Jackson (52) estimated 4.60%, 6.60%, and 0.70% of the bean acreage in Ontario infected with the two blights and losses of 1.7%, 2.5%, and .25% in 1968, 1970, and 1972, respectively. Yield losses attributable to either Xp or Xpf are difficult to estimate due to the similarity of symptoms produced; damage resulting from halo blight may also alter the above estimates.

The large volume of research conducted on common and fuscous blights reflects the historical importance of these diseases to the American bean industry. The early research conducted on common and fuscous blight, especially that by Burkholder and Zaumeyer (9, 11, 56, 57) focused on the basic aspects of the disease life cycle and symptomatology; their research provided a foundation of data for understanding the field ecology of Xp and Xpf. Detailed field studies which would have expanded Burkholder's and Zaumeyer's concepts and increased the understanding of how blight bacteria act in the field have never been conducted. Lack of such basic information has prevented research on other

aspects of common and fuscous blights.

Research to develop control measures for bean blight has received priority among bean researchers. The greatest emphasis has been on developing blight resistant or tolerant bean cultivars. Burkholder in 1924 (10) was the first to conduct extensive screening of bean germplasm for resistance to common blight; he concluded that none of the cultivars tested were immune, however, some differed in the disease severity. In 1946 Burkholder (12) reported varietal susceptibility trials to Xpf; all cultivars tested were susceptible except two, which were slightly resistant. Major programs for breeding resistance to common and fuscous blights in dry edible beans were initiated 10-15 years ago in many areas throughout the world (39). Coyne and Schuster (17) tested 1080 PI accessions, cultivars, and breeding lines of *P. vulgaris* for reaction to Xp; high tolerance was detected in some lines, and correlated with late maturity (16). Susceptibility or tolerance of beans to common blight also depends upon the stage of development of the plant (18). Using Great Northern Nebraska No. 1 selection 27 as a source of resistance to common and fuscous blights, Coyne and Schuster developed the tolerant Great Northern varieties Tara and Jules (14, 15). Resistance to common and fuscous blights appears to be polygenic, quantitative, and highly heritable (16, 36).

Pathogenic variation exists among Xp and Xpf isolates in their interaction with various bean cultivars and is considered an important factor in developing tolerant varieties. Pathogenic variation was first suggested by Smale (46) who detected differences in virulence in individual colonies of stock Xp cultures. Schuster and Coyne presented definitive evidence for variation among geographic isolates of Xp (43, 44, 45); Colombian and Ugandan isolates of Xp were more virulent on the tolerant Great Northern selection 27 than the standard Nebraska Xp isolate. Ekpo and Saettler (25, 40) confirmed these findings and extended the existence of pathogenic variation to Xpf; generally, Xpf isolates were somewhat more virulent than Xp isolates.

Much research has been directed toward maintaining bean seed stocks free of Xp and Xpf contamination and developing methods to detect seed-borne infection. Most bean producing states maintain seed certification programs which oversee the quality of commercial seed. The Michigan seed certification program is administered by the Michigan Crop Improvement Association under authority delegated to it by the Michigan Department of Agriculture (34). 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 diseases. Seed from fields which pass visual inspection for the presence of common and fuscous blight symptoms, and which show no contamination in laboratory tests for Xp and Xpf can be sold as certified. By using certified seed a grower minimizes the probability of blight in his crop.

Numerous assay methods have been developed or adapted to detect the presence of seed-borne Xp and Xpf. Katznelson (29) detected internally-borne Xp by measuring the increase in phage titer after incubation with seed. Schuster's leaf water-soaking method (42), designed to test resistance in beans to blight, has been adapted for use in seed testing. Saettler (38) developed the seedling injection technique for assaying Michigan seed lots for blight. Serological techniques are used by Guthrie (26) in Idaho to detect blight bacteria. None of the seed assaying methods have proven entirely satisfactory for detecting blight bacteria and all suffer from the inability to detect low levels of the blight bacteria.

Numerous chemicals have been tested throughout the years for control of Xp and Xpf. Dimond and Stoddard (21) reported control of common blight of kidney bean in the greenhouse with several systemic compounds applied as soil drenches. Although streptomycin inhibited symptom expression of common blight of kidney

beans in the greenhouse (31, 32); it has provided only limited blight control in the field (30, 37, 54). Copper-containing compounds have been the most widely studied chemicals for control of common and fuscous blights. Bordeaux mixture was tested over many years with variable results (8, 13, 24). More recent work with modern copper formulations and non-metallic organic bactericides has yielded promising results. In several studies, Dickens and Oshima (20, 35) reported excellent control of blight with applications of copper sulfate, copper ammonium carbonate and NEMA. Similar chemicals produced only marginal control of blight in tests by Saettler (37, 41), however, recently good control of common and fuscous blights was obtained by Weller and Saettler (54) with NEMA and copper hydroxide.

The purpose of this study was to increase our understanding of how common and fuscous blights develop naturally in the field. Three objectives were involved in the overall study, and each will be presented separately. The first objective was to develop a selective system whereby Xp and Xpf could be selectively isolated and studied in the field. The second objective was to monitor Xp and Xpf population trends in field grown Navy (pea) beans and relate bacterial multiplication and spread to the pattern of disease development. The third objective was to evaluate the relative

importance of different sources of primary inoculum in the establishment of bean blight in the field.

GENERAL MATERIALS AND METHODS

Bacterial storage and culture. Isolates of *Xanthomonas phaseoli* (Xp) and *Xanthomonas phaseoli* var. *fuscans* (Xpf) used in this study were stored in 40% aqueous glycerol at -10 C and in pulverized diseased leaves at 4 C. Bacteria were grown on yeast extract calcium carbonate agar (YCA: 10 g yeast extract, 15 g agar, and 2.5 g calcium carbonate per 1000 ml glass distilled water), or in buffered yeast extract (BYE: 10 g yeast extract per 1000 ml 0.01 M phosphate buffer, pH 7.2). The bacteria were subcultured no more than five times past the stock culture in order to maintain genetic stability. All isolates were periodically assayed for changes in virulence by seedling injection technique (38).

Bean culture. Navy (pea) beans (cultivars Seafarer, Sanilac, and Tuscola) and Kidney beans (cultivar Manitou) were grown in the greenhouse and the field. In the greenhouse the plants were grown in the standard greenhouse soil mix in 5-cm diameter clay pots. Day-light was supplemented with 14 hours of fluorescent

lighting from 0600 and 2100 hours. The temperature generally was maintained at 24-30 C and the plants were watered alternately with Rapid-Gro (1 teaspoon per 2 liters of water) and tap water.

Field experiments were conducted at the Botany and Plant Pathology Research Farm, Michigan State University and the Saginaw Valley Bean and Beet Research Farm, Saginaw, Michigan. Beans were seeded using standard planting techniques or by hand with 28 inches between rows and 2 inches between plants in the row.

Plant inoculation. Greenhouse-grown plants were inoculated with blight bacteria by one of three methods: (i) seedling injection (38), in which a bacterial suspension (10^8 cells per ml) was injected into the primary node of a 10-day-old kidney bean seedling (cultivar Manitou); (ii) leaf water-soaking (42), in which the undersurface of bean leaves was sprayed to a water-soaked appearance with a bacterial suspension (10^8 cells per ml) with a DeVilbiss sprayer attached to a compressed air line at a pressure of 1.2 kg per cm^2; and (iii) leaf misting, in which a bacterial suspension (5×10^7 cells per ml) was lightly sprayed from a DiVilbiss sprayer to runoff on the leaf with no visible water soaking. Plants in the field were sprayed to runoff with a bacterial suspension (10^8 cells per ml) using a Knapsack sprayer with no visible watersoaking.

Disease rating. Inoculated plants were rated for disease symptoms by one of three methods depending on the type of inoculation. Plants inoculated by seedling injection were rated on a 0-3 scale: 0 = no symptoms; 1 = stem lesion and primary leaves partially collapsed; 2 = stem lesion and primary leaves completely collapsed; and 3 = stem lesion, primary leaves completely collapsed, and apical meristem dead. Leaves from greenhouse grown beans inoculated by water-soaking or leaf misting were rated on a 0-10 scale where 0 = no symptoms and 10 = total leaf necrosis. The amount of disease on leaves from field-grown plants was rated by counting lesions on individual leaflets.

Bacterial isolation and identification. Bacteria were isolated by homogenizing plant tissue with 0.01 M phosphate buffer, pH 7.2, with mortar and pestle, glass tissue grinder, or Waring Blendor; sometimes plant tissue was shaken or soaked in buffer. Plant homogenates or washings were serially diluted in phosphate buffer and 0.1 ml portion of appropriate dilutions spread onto 48 hour-old plates of agar media. The number of colony forming units was used as an estimate of the number of bacteria in a sample. Yellow bacteria suspected as being Xp or Xpf were identified based on growth rate, odor and color on YCA, production of brown soluble pigment, production of hydrogen sulfide, and color

reaction in phenol red dextrose agar. Occasionally other standard tests for *Xanthomonas* spp. were employed (23). Seedling injection provided positive identification if the above physiological tests were inconclusive, however, such cases were rare. Xp and Xpf require 72 hours growth on YCA for the formation of distinct colonies, whereas, most yellow saprophytic, phyllosphere bacteria produce colonies in 24-48 hours. Phenol red dextrose agar was especially useful in screening large numbers of yellow isolates; all blight bacterial produce a characteristic red (alkaline) reaction whereas most yellow saprophytes produce a yellow (acid) reaction.

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PART I

ISOLATION AND SCREENING OF RIFAMPIN-RESISTANT MUTANTS OF *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS*

INTRODUCTION

The lack of suitable culture medium selective for Xp and Xpf has limited basic ecological studies of blight bacteria under field conditions. Kado's medium D5 (11) reported selective for *Xanthomonas* spp. and Schaad's (16) medium for *Xanthomonas campestris* are not suitable, due to the lack of specificity and low plating efficiency for Xp and Xpf. To isolate Xp and Xpf from field-grown bean tissue, dilution plating of homogenates on nutrient medium is necessary. Field-grown beans support a large, diverse microflora of bacteria, yeasts, and fungi; blight bacteria grow slowly and, therefore, are easily overgrown by faster-growing saprophytic bacteria on nutrient media. Only when disease symptoms are apparent is isolation of blight bacteria assured because of the high bacterial populations in the tissue. Indeed, *in vivo* population studies of bean blight bacteria are impossible by this technique.

Incorporation of antibiotic-resistance into Xp and Xpf could provide a system for the selective isolation and quantitation of blight bacteria from field grown

plants whether or not visible disease symptoms are apparent. Antibiotic-resistant mutants could then be inoculated in the field and reisolated by plating homogenates on antibiotic supplemented media.

Antibiotic-resistance has been used in the study of some plant pathogenic bacteria but not extensively in quantitative field studies. Gowda and Goodman (5) and Lewis and Goodman (14) used *Erwinia amylovora* resistant to streptomycin in greenhouse studies of fire blight. Hsieh used streptomycin resistance in the study of the ecology and epidemiology of *Xanthomonas oryzae* (6, 7, 8). Hsu (9) studied mixed infection of *Xanthomonas phaseoli*, *X. vesicatoria*, and *X. campestris* in host and non-hosts plants with streptomycin-resistant mutants. Rifampin and neomycin resistant *Erwinia rubrifaciens* have been used in the study of deep bark canker of Persian walnut (4, 10). Rifampin resistance has recently been used in ecological studies of *Agrobacterium tumefaciens* (1, 15).

This portion of the paper: (i) describes a system for selective isolation of Xp and Xpf based upon bacterial resistance to the antibiotic rifampin, (ii) compares some characteristics of rifampin-resistant Xp and Xpf mutants with those of their respective wild types, and (iii) documents the usefulness of rifampin mutants as tools for studying Xp and Xpf under field conditions.

MATERIALS AND METHODS

Rifampin agar medium. Rifampin (99.9% active) was obtained from Calbiochem, San Diego, CA 92112. To prepare rifampin agar medium (RAM) 10 mg of rifampin was dissolved in 0.4 ml methanol and diluted to 10 ml with distilled water. The solution was passed through a fritted-glass bacterial filter and added to 190 ml of autoclaved YCA. The amount of rifampin in RAM sometimes was increased from the normal up to 250 µg/ml. RAM was usually supplemented with filter-sterilized cycloheximide in distilled water at 25 µg/ml; at times the concentration was increased up to 10 fold. RAM was occasionally supplemented with penta-chloro-nitro-benzene (PCNB) or streptomycin sulfate at 100 and 250 µg/ml, respectively. The PCNB was added prior to autoclaving the YCA; streptomycin sulfate was passed through a fritted glass bacterial filter.

Isolation and culture of rifampin-resistant mutants. Naturally-occurring rifampin-resistant mutants were selected from wild type isolates Xpf 16, Xp 11 (isolated from Michigan bean seed) and Xp 21 (isolated from

Colombian bean seed) by spreading 10^9 cells on RAM. These wild type isolates were selected because they were highly virulent relative to other isolates. The selection of mutant colonies which developed on RAM was based upon colony size, shape and vigor of growth. Naturally-occurring mutants resistant to both rifampin and streptomycin were selected from rifampin resistant isolates of Xpf by spreading 10^9 cells on YCA supplemented with 250 µg/ml streptomycin sulfate. The rifampin resistant isolates and the rifampin-streptomycin resistant isolates were subcultured twice on RAM and RAM + streptomycin sulfate, respectively, and then stored in 40% aqueous glycerol at -10 C.

Physiological tests. Physiological tests were performed as described by Dye (3) with the following modifications: (i) pigment production by Xp and Xpf isolates was observed on YCA, (ii) lead acetate paper was used to detect hydrogen sulfide produced by the isolates on YCA, and (iii) slime production was determined on yeast extract dextrose calcium carbonate agar (YDC: 10 g yeast extract, 10 g dextrose, 15 g agar, and 2.5 g calcium carbonate per 1000 ml water).

Greenhouse and field inoculation. To compare virulence and growth of wild type and mutant isolates greenhouse-grown bean plants were inoculated by seedling injection, leaf water-soaking, or leaf misting. Six

circular spots (1 cm in diameter) were water-soaked and the symptoms developing outward were rated on successive days according to the scale previously described. A randomized block design was used for all experiments.

In 1976 the virulence of isolates R10 and Xpf 16 were compared under field conditions; isolates Ra and Xp11 were compared in 1977. Xpf and Xp isolates were inoculated to runoff to Navy beans (cultivar Seafarer) 49 and 45 days after planting, respectively; in both years plants were in the flat green pod stage. Individual treatment plots were three rows of beans four meters long and arranged in a randomized block design, with each isolate replicated three times. Xpf and Xp inoculated plants were rated 24 and 32 days after inoculation by counting the number of leaflets and pods bearing lesions on 12 plants in each replication.

RESULTS

Comparison of rifampin-resistant mutants of Xp and Xpf and the wild-type parents. The seedling injection test was used to test the virulence of each rifampin-resistant mutant relative to its wild type; each injection was replicated three times with three seedlings per replication. Two of five mutant isolates of Xpf 16, two of three of both Xp 11 and Xp 21 produced the same virulence rating as the wild types. One of the rifampin-resistant isolates of Xpf 16 (designated R10), one of Xp 11 (designated Ra), and one of Xp 21 (designated Rd) were selected for further studies. All three isolates were resistant to greater than 500 µg/ml rifampin. Five mutant isolates resistant to rifampin and streptomycin sulfate were selected from a population of R10; two of the five double-marked mutants produced the same virulence rating as Xpf 16 and R10 in the seedling injection test. Isolates R10 and Ra produced as much disease as their wild types in greenhouse-grown kidney bean leaves, whereas, isolate Rd produced less disease relative to its wild parent (Table 1). One

TABLE 1. Disease severity in leaves of greenhouse-grown kidney beans (cultivar Manitou) inoculated with wild-type *Xanthomonas phaseoli* var. *fuscans* (Xpf 16), and *X. phaseoli* (Xp 11), (Xp 21) compared to that produced by their respective rifampin-resistant mutants (R10, Ra, and Rd).[a]

Isolate	Post inoculation disease rating at[b]			
	14 days	20 days	25 days	34 days
Xpf 16	4.0	5.9		
R10	3.9	6.1		
LSD (P = 0.05)	1.1	1.0		
Xp 11			2.6	4.2
Ra			2.9	5.0
LSD (P= 0.05)			0.7	1.5
Xp 21			2.6	4.8
Rd			1.2	1.8
LSD (P = 0.05)			0.7	1.0

[a]Beans were grown in 15-cm diameter clay pots, two plants per pot, and the trifoliate leaves were inoculated with an aqueous suspension of 10^8 bacteria per milliliter sprayed at a pressure of 1.2 kg/cm^2. Isolates Xpf and Xp were used to inoculate 32 and 50-day-old plants, respectively.

[b]Disease rating scale: 0-10, with 0 = no symptoms and 10 = complete yellowing and necrosis. Data of Xpf and Xp inoculations are averages of three and four replications, respectively, of 12 leaflets each.

rifampin-streptomycin resistant mutant designated R10-S6 produced as much disease as Xpf 16 and R10 in greenhouse-grown kidney bean leaves (Table 2); a second isolate designated R10-S2 produced more disease, however the symptoms were somewhat atypical. The virulence of R10 and Ra was compared to that of the wild types in field-grown Navy beans (cultivar Searfarer). Twenty-four days after inoculation with R10, 16.9% of the leaflets and 5.2% of the pods were visibly infected, whereas, 15.4% of the leaflets and 4.6% of the pods from plants inoculated with Xpf 16 were infected. Thirty-two days after inoculation with Ra, 37.1% of the leaflets and 10.1% of the pods were visibly infected, whereas 36.4% of the leaflets and 11.5% of the pods from plants inoculated with Xp 11 were infected. In no case were there significant ($P = 0.05$) differences in the amount of disease produced by rifampin-resistant mutants and their respective wild types. All Xp and Xpf rifampin-resistant mutants and double marked mutants retained cultural characteristics such as colony size, colony shape, slime formation, and yellow and brown pigment formation similar to that of their respective wild types. The responses of R10 and Ra to standard physiological tests were identical to their wild types.

The *in vitro* doubling times of R10 and Ra were approximately 11% longer than those of the wild types

TABLE 2. Disease severity in leaves of greenhouse-grown kidney beans (cultivar Manitou) inoculated with wild type *Xanthomonas phaseoli* var. *fuscans* (Xpf 16), and rifampin-resistant mutant (R10) compared to that produced by rifampin-streptomycin-resistant mutants (R10-S2 and R10-S6).[a]

Isolate	Post inoculation disease rating at[b]	
	12 days	17 days
Xpf 16	2.6	5.8
R10	2.9	5.3
R10-S2	3.0	7.6
R10-S6	2.7	6.0
LSD (P = 0.05)	0.5	1.5

[a] Beans were grown in 15 cm diameter clay pots, two plants per pot, and the trifoliolate leaves were inoculated with an aqueous suspension of 10^8 bacteria per milliliter sprayed at a pressure of 1.2 kg/cm^2. Plants were inoculated 33 days after planting.

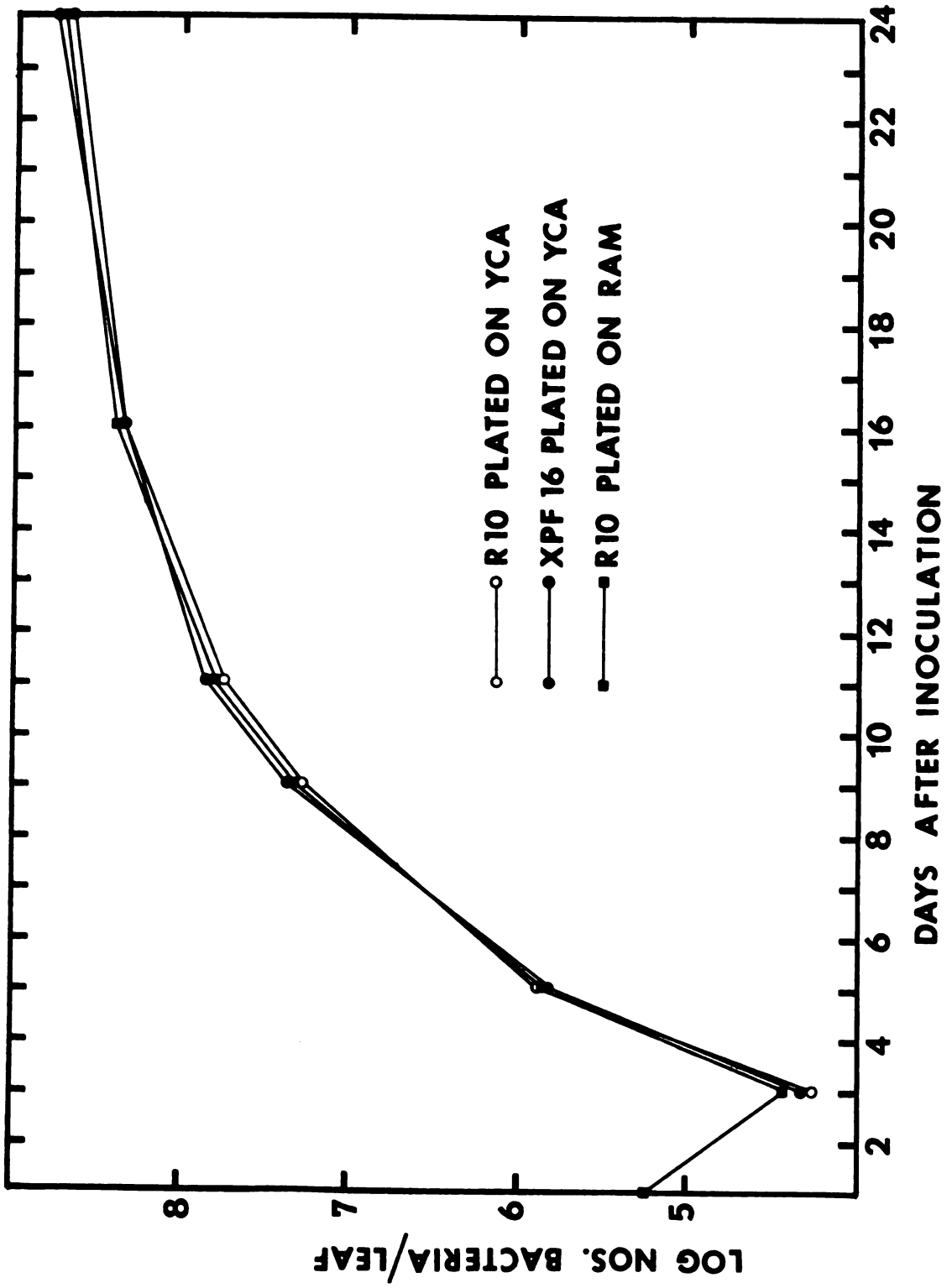
[b] Disease rating scale: 0-10, with 0 = no symptoms and 10 = complete yellowing and necrosis. Data are averages of four replications.

in BYE shake culture at 25 C, however *in vivo* growth rates of R10 and Ra in primary leaves of greenhouse-grown Navy beans (cultivar Seafarer) were identical to those of the wild types (Figures 1 and 2).

Rifampin-resistance in R10 and Ra was stable; no revertants to rifampin sensitivity were detected by replica plating (13) after 16 and 11 consecutive transfers of R10 and Ra, respectively, on YCA; after each transfer ten plates with 100-150 colonies per plate were tested for revertants. Moreover, all Xpf and Xp bacteria isolated on YCA from 11 and 15 leaf lesions of field grown Navy beans 60 days after inoculation with R10 and Ra in separate plots were rifampin resistant. No revertants to rifampin-streptomycin sensitivity were detected after ten consecutive transfers of R10-S6 on YCA or in R10-S6 isolated from lesions of greenhouse-grown plants.

Recovery of rifampin-resistant mutants of Xp and Xpf from field-grown beans. The growth of fungi, yeasts, and bacteria from symptomless leaves of field-grown Navy beans inoculated with R10 was compared on YCA, RAM, and RAM + cycloheximide. Three replicates of two leaflets each were homogenized in 10 ml of phosphate buffer and 0.1 ml of a dilution series was plated on each media to yield 10^{-1} to 10^{-4} dilution of the homogenate (Table 3). Phyllosphere bacteria were

Figure 1. Growth of rifampin-resistant *Xanthomonas phaseoli* var. *fuscans*, R10, and wild type Xpf 16 on primary leaves of greenhouse-grown Navy (pea) beans (cultivar Seafarer). Fourteen-day-old plants were lightly sprayed to runoff with an aqueous suspension (5×10^7 cells/ml) of R10 or Xpf 16. The bacterial populations were sampled by vigorously shaking six leaves (average leaf area, 30 cm^2) in 100 ml of phosphate buffer. Data are averages of three replications. The samples were plated on YCA or RAM.


LOG NOS. BACTERIA/LEAF
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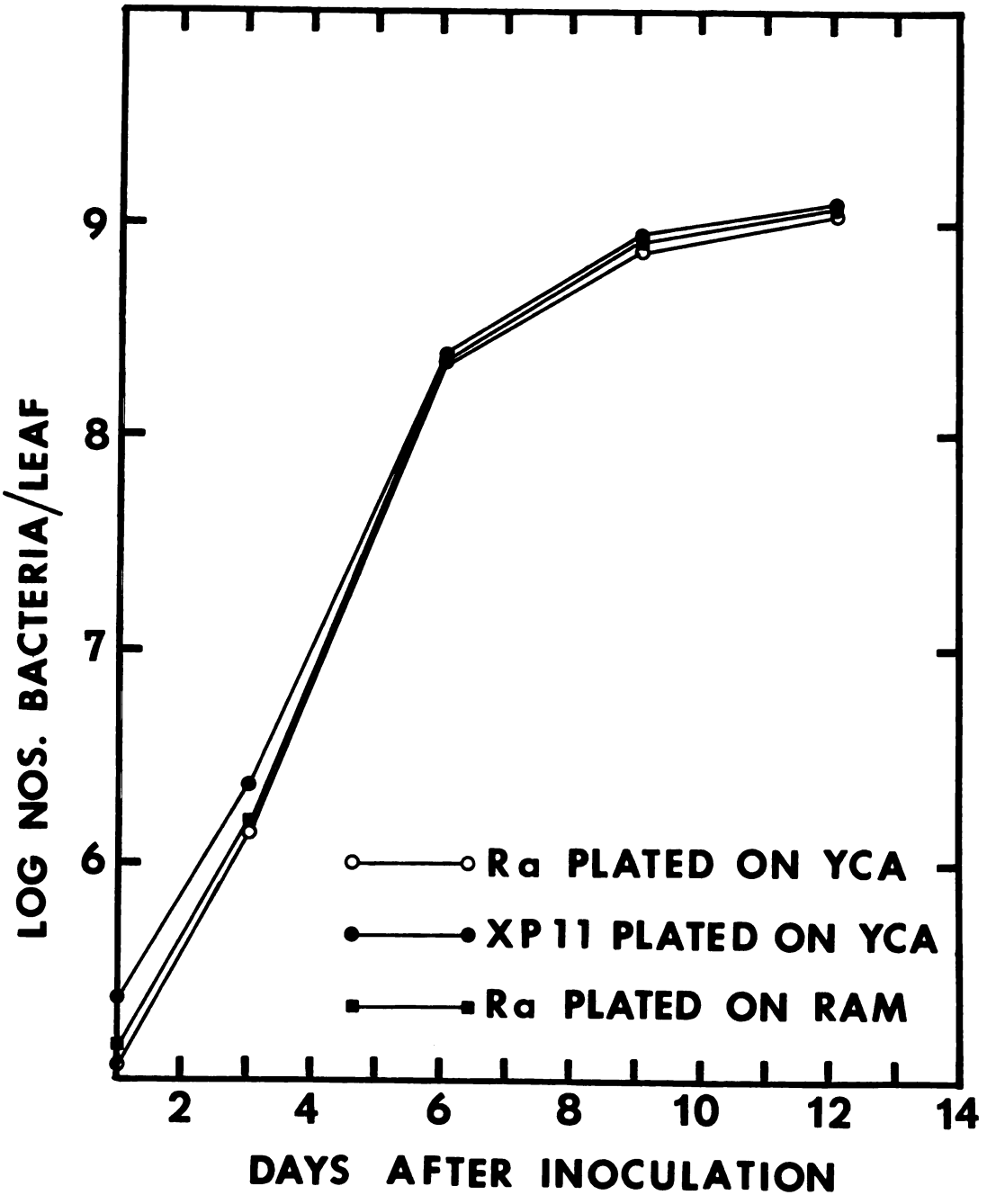
DAYS AFTER INOCULATION
R10 PLATED ON YCA
XPF 16 PLATED ON YCA
R10 PLATED ON RAM

Figure 2. Growth of rifampin-resistant *Xanthomonas phaseoli*, Ra and wild type Xp 11 in primary leaves of greenhouse-grown Navy (pea) beans (cultivar Seafarer). Thirteen-day-old plants were lightly sprayed to run-off with an aqueous suspension (5×10^7 cells/ml) of Ra or Xp 11. The bacterial populations were sampled by homogenizing six leaves (average leaf area, 30 cm^2) in 75 ml of phosphate buffer. Data are averages of three replications. The samples were plated on YCA or RAM.


LOG NOS. BACTERIA/LEAF
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DAYS AFTER INOCULATION
Ra PLATED ON YCA
XP11 PLATED ON YCA
Ra PLATED ON RAM

TABLE 3. Comparative growth of fungi, yeasts, rifampin-resistant isolate R10 and other (presumably nonpathogenic) bacteria from bean leaf tissue[a].

Dilution of Homogenate	No. of Colonies[b]								
	YCA			RAM			RAM + cycloheximide		
	Fungi + yeasts	R10	NP[c] bacteria	Fungi + yeasts	R10	NP bacteria	Fungi + yeasts	R10	NP bacteria
10^{-1}	0	0	>500	>500	0	0	0	>500	0
10^{-2}	0	0	>500	106	0	0	0	>500	0
10^{-3}	0	0	>500	11	165	0	0	220	0
10^{-4}	0	5	63	2	20	0	0	25	0

[a] Two Navy bean (cultivar Seafarer) leaflets from field grown plants inoculated with R10 were ground in a mortar and pestle with 10 ml of phosphate buffer; 0.1 ml of serial dilutions of the homogenate was plated.

[b] Data are means of three replications; YCA = yeast extract-calcium carbonate agar, RAM = YCA + rifampin (50 µg/ml), RAM + cycloheximide contains 25 µg/ml cycloheximide.

[c] NP = non-pathogenic.

completely inhibited on the RAM and the R10 were recovered. Colonies of phyllosphere bacteria prevented detection of R10 colonies on YCA except at the 10^{-4} dilutions of the homogenate; the number of R10 colonies was 30% less than on RAM. At 10^{-1} and 10^{-2} dilutions fungi and yeasts overgrew RAM plates; the addition of cycloheximide reduced the growth of fungi and yeasts and allowed detection of R10 colonies. Cycloheximide was neither antagonistic to rifampin activity nor toxic to R10. Pure suspensions of R10 were plated on YCA, RAM, RAM + cycloheximide, and YCA + cycloheximide; there were no statistical differences in the number of colonies developing on each media. Results were similar when Ra or R10-S6 were substituted in the above tests.

The usefulness of the rifampin-resistant mutants for field study of Xp and Xpf population dynamics was tested with R10. Nineteen-day-old field grown Navy bean plants (cultivar Seafarer) were sprayed to runoff with an aqueous suspension of R10 (5×10^7 cells per ml). Bacteria were isolated from first and second trifoliolate leaves homogenized with 75 ml of phosphate buffer; each sample was replicated four times. The following populations of R10 were detected per leaflet (average leaflet area, 20 cm^2) 1, 6, 11, and 17 days after inoculation: 4.2×10^4, 9.2×10^6, 9.8×10^7, and 2.8×10^8, respectively.

The efficiency of R10 recovery from leaf tissue was determined. Fifteen healthy leaves were homogenized with 75 ml of phosphate buffer and 10^8 R10 cells in a Waring Blendor and serial dilutions of the homogenate were plated on the RAM. Controls consisted of 10^8 cells not homogenized but diluted and plated, and 10^8 cells shaken in 75 ml of phosphate buffer. The same number of R10 cells was recovered after blendor treatment as in the controls; thus mechanical damage or release of toxic substances by the leaf does not occur with the blendor method.

RAM + cycloheximide was sufficient to inhibit the growth of all bacterial and most fungal residents of the phyllosphere. However, this media was not selective enough for rhizoplane isolations of R10 or Ra because of the natural resistance in soil bacteria to these antibiotics. Isolate R10 was easily isolated from root samples only when the population of the bacteria was above approximately 10^2-10^3 bacteria per 0.3 g root tissue; by using R10-S6, populations of the bacteria were detected at levels of near 10^1 per 0.3 g root tissue when root homogenates were plated on RAM (250 µg/ml rifampin) supplemented with 250 µg/ml streptomycin sulfate, 250 µg/ml cycloheximide and 100 µg/ml PCNB.

Streptomycin-resistance has been used in some studies as the basis of an antibiotic-selective system for plant pathogenic bacteria. The toxicity of streptomycin and rifampin to bean phyllosphere bacteria was compared (Table 4). Rifampin at 50 µg/ml inhibited the growth of all bacteria, whereas, about 6% of the bacteria were resistant to streptomycin at the same concentration.

TABLE 4. Growth of phyllosphere bacteria on antibiotic-supplemented media[a].

Antibiotic added	Concentration (µg/ml)	No. of phyllosphere bacteria detected per 0.07 g dry wt. of bean leaf tissue[b]	Growth (%) of colonies on control medium
None	0	37.7×10^5	100
Streptomycin sulfate	50	2.19×10^5	5.8
Streptomycin sulfate	100	1.66×10^5	4.4
Streptomycin sulfate	250	0.51×10^5	1.3
Streptomycin sulfate	500	0.42×10^5	1.1
Rifampin	50	0	0

[a] Yeast extract-calcium carbonate agar + cycloheximide at 25 µg/ml.

[b] Tissue was ground in 10 ml of phosphate buffer in a glass tissue-grinder and plated after serial dilution. Colonies were counted after four days incubation at room temperature.

DISCUSSION

Rifampin was selected for use in an antibiotic-resistance selective system for bean blight bacteria because of its wide spectrum of antibacterial activity and high toxicity (2, 12, 18). No bacteria from the bean phyllosphere showed resistance to rifampin; natural resistance to other antibiotics, particularly streptomycin, is quite common. Moreover, rifampin has only limited use in human chemotherapy, thus there is little concern about long-term effects of any transfer of resistance from blight bacteria to other phyllosphere residents.

We conclude for several reasons that R10 and Ra adequately model several important aspects of wild-type activity and they should behave similarly to Xpf 16 and Xp 11 under natural conditions. Multiplication and disease production of R10 and Ra were identical to that of the respective wild types in bean leaves. Isolate R10-S6 is expected to act similar to the wild parents based upon the comparison of virulence in bean

seedling and leaves. The mutation of R10, Ra, and R10-S6 was stable when the bacteria were grown in culture or bean leaves. Mutation stability assures the usefulness of these mutants throughout season-long studies.

That the rifampin mutants of Xp and Xpf can be selectively isolated and their growth in field-grown Navy beans can be monitored over several weeks indicates their potential as tools for study of bean blight ecology. Use of the mutants should permit monitoring the sequence of seedling infection by Xp and Xpf originating from various sources of primary inocula, such as internally infected seed, externally infected seed, and infected plant refuse. Finally, use of R10 and Ra will permit a quantitative study of the build up and dispersal of secondary inoculum.

The use of antibiotic-resistant mutants of plant pathogenic bacteria to enhance selective isolation is not a technique unique to this study. Mutants resistant to streptomycin, aureomycin, and rifampin have been described. However, none of the studies which have utilized mutants have presented data indicating extensive prescreening of the isolates relative to the wild parents. Gardner (4) reported that his rifampin-neomycin-resistant isolate of *Erwinia rubrifaciens* was "equivalent" in virulence to the wild type. Hsu (9) reported that population changes of streptomycin-resistant mutants of

Xanthomonas phaseoli, *X. vesicatoria*, and *X. campestris* were similar to the wild types in leaves of host plants. Hsieh (6) reported a streptomycin-resistant *Xanthomonas oryzae* "identical with its parent isolate in virulence and in 30 physiological and biochemical characters". The above reports provided only sparse evidence for the claims of wild type-mutant similarity and gave no details of the techniques involved in the screening procedure. The results of this study indicate a need for comprehensive screening of isolates, since reduction in virulence accompanied rifampin-resistance in some isolates. Further, the contrasting reaction of isolate Rd in seedlings and leaves suggest the need for a series of pathogenicity tests in the screening procedure.

A change in wild type cell physiology or structure occurs when a bacterium becomes resistant to an antibiotic. In the case of rifampin-resistance the B subunit of the DNA dependent RNA polymerase is altered so that the antibiotic cannot bind. Normally, changes in the wild type reduce the efficiency of the bacterium; with an altered RNA polymerase RNA transcription is less efficient and bacterial growth is reduced; other secondary changes might also occur. Small reductions in the *in vivo* growth rate or virulence of a mutant may be unimportant over a short period of time but in a season long study such differences are greatly magnified.

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PART II

POPULATION TRENDS AND DISTRIBUTION OF *XANTHOMONAS PHASEOLI* AND *XANTHOMONAS PHASEOLI* VAR. *FUSCANS* IN FIELD-GROWN NAVY BEANS (*PHASEOLUS VULGARIS* L.)

INTRODUCTION

The studies of Burkholder (6, 7) and Zaumeyer (49, 50, 51) form the foundation for present concepts about the disease cycles of bean common and fuscous blights. Infected seed is the main source of primary inocula; blight bacteria are located under the seed coat and do not enter the cotyledons until germination. Imbibition of water swells the seed resulting in a pulling apart of the epidermal cells of the cotyledon. Bacteria enter rifts in the epidermis and multiply in intercellular spaces; lesions eventually develop on the cotyledons. Initial seedling symptoms usually appear on the primary leaves which become infected while folded between the cotyledons (50). As lesions enlarge, bacterial exudate may accumulate on the surface; rain blown by wind splashes the bacteria to other parts of the plant or to uninfected plants, where new infections start under favorable conditions (52). Bacteria invade leaves through stomata or wounds, enter the intercellular spaces, cause a gradual dissolution of the middle

lamella and eventually host cells begin to disintegrate with the formation of bacterial pockets. Under proper conditions the bacterial mass will ooze from the lesion and become available for secondary spread, thus continuing the secondary infection cycle (50, 52). With the onset of the reproductive phase of the bean, pod and seed infection complete the disease life cycle.

Blight bacteria may move systemically in an infected plant. Systemic movement of Xp was first noted by Barlow (2) in 1904 and later studied by Burkholder (6) and Barass (4) in 1921. Zaumeyer's histological studies (49, 50, 51) revealed the systemic pattern of Xp during early disease development. The bacteria may enter the stem through the stomata of the hypocotyl and epicotyl, through the vascular elements leading from the leaf and from infected cotyledons. Under favorable temperature and moisture conditions, the bacteria move rapidly upward in the stem. Drooping of one or both primary leaves is the first indication of systemic invasion. The bacteria concentrate in the pulvinus region and subsequent invasion of the parenchyma results in loss of tissue turgidity. If the bacteria spread extensively, apical meristem or buds in the primary axils may be killed. Leaf xylem eventually become invaded and lesions may occur when bacteria break through the vessels and invade surrounding parenchyma.

The bacteria may also break through the stem xylem and cause stem lesions. In seedlings, lesions usually occur at the cotyledon attachment or the primary leaf node and the stem becomes girdled and lodges. When the bacteria invade systemically, symptoms may not be detected until flowering or the plant may be slightly dwarfed. An important feature of systemic infection is the ability of the bacteria to move from stem xylem through the pod suture and into the seed with no trace of pod infection (6).

Appearance of bacterial blight in bean fields is closely related to the stage of plant development. Blight symptoms sometimes are apparent during the seedling phase on cotyledons and primary leaves; however, during the vegetative phase, when the foliage is rapidly expanding, symptoms generally are not seen. Typical field symptoms only appear with the onset of the reproductive phase, which is initiated by flowering (6, 7, 16, 41). In Michigan, Navy bean fields appear blight-free until late July and early August at which time the fields suddenly become blighted. Because of this type of disease development, common and fuscous blights are considered to be late season diseases. To explain this phenomena, Gloyer (16) suggested that bean plants are not as blight susceptible during the vegetative stage as during the seedling and reproductive stages; more

recent studies have confirmed slightly greater susceptibility of bean plants during the reproductive stage of growth (8, 13). Burkholder suggested that late symptom expression is due to environmental conditions unfavorable for disease development during the first month and a half of the growing season (6, 7). Menzier (34) suggested the use of overhead irrigation on beans grown in hot, arid areas during vegetative growth, since blight did not appear to spread readily during that time.

While the life histories of Xp and Xpf have received considerable qualitative study; the epidemiologies of common and fuscous blights are still poorly understood. The poor understanding of blight is especially apparent in the lack of a suitable explanation for the phenomenon of late symptom expression. The first studies of blight were based primarily upon field and histological observations. More recent research on blight development has usually measured lesion formation but not considered bacterial populations. *In vivo* studies of Xp and Xpf multiplication have been confined to controlled conditions mainly to study the physiological and genetic controls of resistance to the bacterial blights. A quantitative study of the population dynamics of Xp and Xpf under field conditions has never been conducted; such a study would contribute to an understanding of blight disease development under natural conditions.

Absence of selective media for Xp and Xpf has been responsible. The recent development of a selective system based upon rifampin-resistant mutants of Xp and Xpf now permits field study of blight bacteria (46).

In this section: (i) the population dynamics of Xp and Xpf in field-grown Navy beans are described during seedling, vegetative, and early reproductive phases of plant growth, and (ii) the multiplication and spread of the bacteria are related to the pattern of disease development.

MATERIALS AND METHODS

Inoculation and isolation of R10, Ra, and R10-S6. Several rifampin-resistant blight isolates were studied in eight separate plots of Seafarer (planted 6/4/76, 6/21/76, 7/7/76, 7/13/76, 7/25/76, 7/19/77, 8/20/77, and 6/15/78), one plot of Sanilac (planted 6/10/77), and one plot of Tuscola (planted 6/22/77) Navy (pea) beans. All plots were located at the Botany and Plant Pathology Farm, East Lansing, MI. Generally, rifampin-resistant mutants were introduced into experimental plots via a Knapsack sprayer. Isolates R10 and R10-S6 were also introduced by planting internally infected seed. The mutants were isolated from field-grown bean tissue by homogenizing leaves with 0.01 M phosphate buffer, pH 7.2, in a Waring blendor, mortar and pestle or by shaking the leaves in phosphate buffer; serial dilutions of the homogenate or washate were plated on RAM + cycloheximide occasionally supplemented with streptomycin sulfate or PCNB. Rifampin, cycloheximide, and PCNB in the media varied depending on the populations of resident bean microflora. Plating efficiencies of R10, Ra or R10-S6 were not altered by

such changes. Approximately 5 ml of phosphate buffer was used for each leaflet homogenized; no less than 75 ml of buffer was used for a sample. In 1976, samples consisted of 12 primary leaves or 15 trifoliolate leaves and were replicated four times. In 1977, samples consisted of 14 primary leaves, 21 or 42 trifoliolate leaflets and were replicated two to three times. Leaf populations of blight bacteria are expressed on the basis of 20 cm^2 leaf tissue; this area is considered the average size of a trifoliolate leaflet or a primary leaf.

To study bacterial blight spread within a Navy bean (cultivar Sanilac) canopy, ten-day-old seedlings possessing fully expanded primary leaves and half expanded first trifoliolate leaves were inoculated with isolates R10 and Ra; successive leaves of the main stem were subsequently assayed for the presence of the mutants until the mid-reproductive phase. During the vegetative phase, leaves expanded from the main stem at approximately two to three day intervals. Twenty-eight days after inoculation the plants were in bloom and by 33 days 1-5 cm long, flat, green pods were present; little vegetative expansion continued past bloom. Isolate Ra was assayed for up to the eight trifoliolate leaf until 13 days after bloom; isolate R10 was assayed for up to the seventh trifoliolate leaf until 15 days after bloom.

Multiplication and spread of R10 and Ra in Navy bean (cultivar Seafarer) stems were studied in 1978 by inoculating a bacterial suspension (1 x 10^8 cells/ml) into the cotyledon scar of 20-day-old plants with a syringe and subsequently assaying portions of the main stem. Each stem section consisted of a node and the internodal region up to the next higher node; the root portion consisted of both tap and lateral roots; all bacterial populations were based on a 0.35 g average stem or root fresh weight.

To isolate internally borne R10 and Ra, stems were washed in running distilled water for five minutes, soaked in 70% ethanol for five minutes, soaked in 50% bleach for ten minutes and rinsed in sterile distilled water before homogenizing.

Effect of simulated washing and rain on blight bacterial populations on Navy bean leaves. Loss of blight bacteria from leaves due to rain water runoff was studied by several methods: 1) leaves from field-grown plants inoculated with R10 or Ra were gently washed in 0.01 M phosphate buffer for two minutes; 2) 13-day-old greenhouse-grown seedlings were inoculated with R10 and misted for one to two hours with a Herrmidifier model 500 humidifier. Water drippings from the primary leaves was collected in plastic petri plates. Water on leaf surfaces was removed by a two second dip

into a beaker of sterile distilled water. Total runoff water was considered the sum of the runoff and the leaf dip populations. In the field, 11-day-old Navy bean seedlings (cultivar Seafarer) were inoculated with isolate Ra and runoff water was collected after each rainfall. Two or three wax cups (11.5 cm diam.) were placed around the base of a plant with the lip of the cup resting against the stem and the bottom anchored in the soil. Runoff water in the cups was assayed for bacteria within four hours after each rain. Total populations of Ra on all leaves usually were assayed no more than six hours prior to rainfall.

Detection of surface-borne blight bacteria. Direct leaf prints were made by gently pressing leaves onto 48-hour-old plates of RAM + cycloheximide for one minute. Indirect leaf prints (40) were made by pressing a replica plater covered with parafilm (American Can Co.) onto a leaf and then to RAM + cycloheximide. Leaves were surface-sterilized by immersion and gentle agitation for 30 seconds in a 50% bleach solution or by UV irridation [253.7 nm at 3.5×10^3 ergs $sec^{-1}cm^{-1}$ for 20 minutes (3)].

Production of bacterial blight infected Navy bean seeds. Half-filled green pods were inoculated along the dorsal suture with a syringe containing an aqueous

suspension (10^8 cells/ml) of R10, Ra, or R10-S6. Mature pods were hand-harvested and visibly infected seeds collected.

RESULTS

Multiplication of R10 and Ra in leaves of field-grown beans. R10 and Ra populations were monitored in inoculated primary leaves or first and second trifoliolate leaves during 1976 and 1977. Population trends for the isolates resembled a standard bacterial growth curve with a one to three day lag phase and a six to nine day exponential growth phase (Figs. 1 and 2). The mean doubling time for R10 on Seafarer beans in five separate experiments in 1976 was 18.3 hours (range, 13.6-23.8 hours) and on Sanilac bean in one experiment in 1977 it was 20.1 hours. In 1977, the mean doubling time for Ra in one experiment on Sanilac beans was 19.8 hours and on Seafarer it was 17.7 hours. The R10 doubling times were negatively correlated with temperature (Fig. 3); higher mean temperatures resulted in greater bacterial multiplication. Populations of R10 and Ra peaked during the stationary phase and remained fairly stable until leaf abscission; a very slow death phase accompanied decomposition of infected leaves on the ground. In a study with Ra (Fig. 2), 13 and 23 days after abscission

Figure 1. Population trends of Xpf isolate R10 and resident bacteria and yeasts in first and second trifoliolate leaves of field-grown Navy beans (cultivar Seafarer). Nineteen-day-old plants were sprayed to runoff with an aqueous suspension (1 x 10^8 cells/ml) of R10. Bacterial populations were sampled by homogenizing 15 leaflets (average leaflet area, 20 cm^2) in 75 ml of phosphate buffer. Samples were plated on RAM + cycloheximide, and YCA. Data are means of four replications $\pm$ standard error.

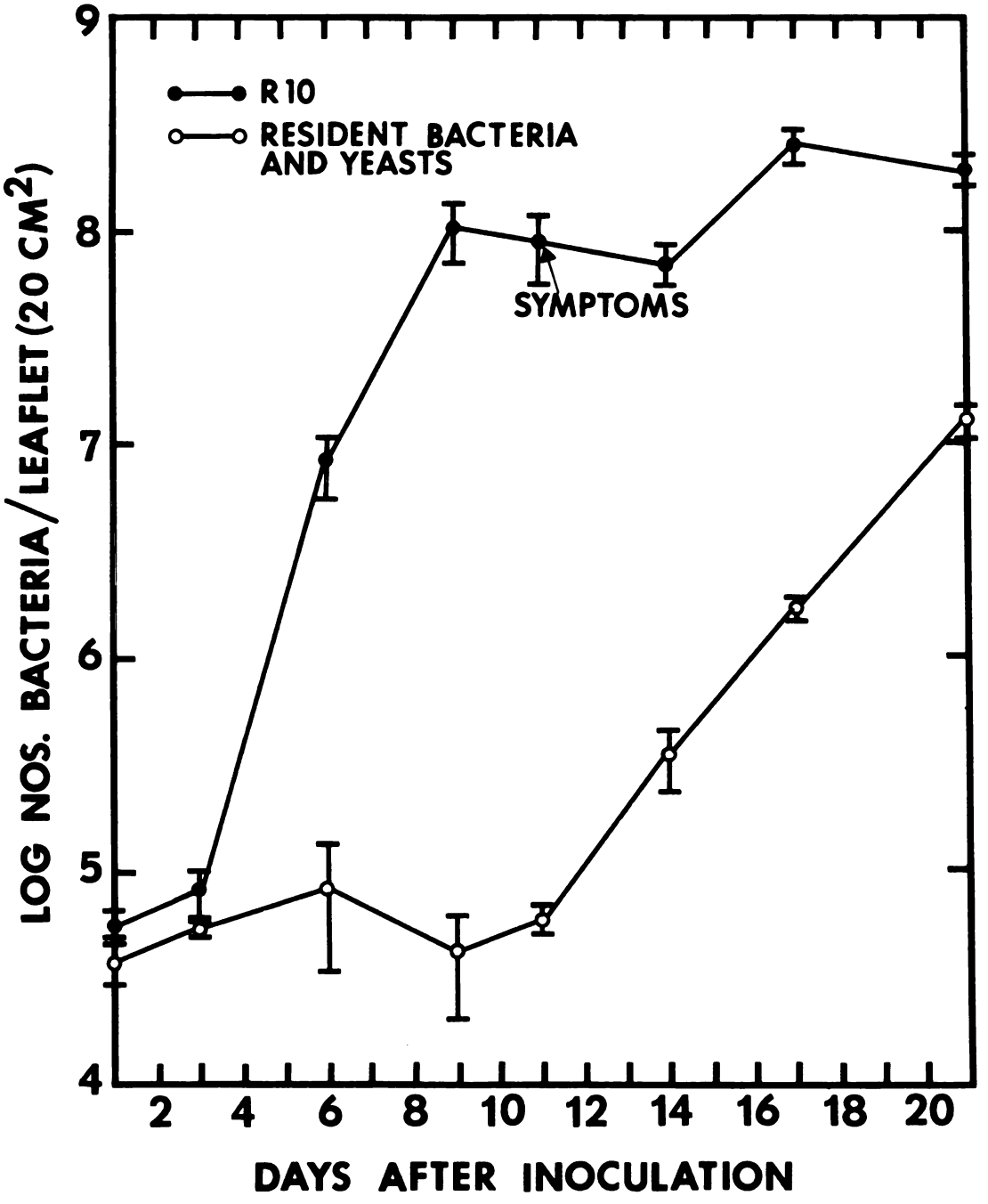

R 10
RESIDENT BACTERIA AND YEASTS
SYMPTOMS
LOG NOS. BACTERIA/LEAFLET (20 CM²)
DAYS AFTER INOCULATION
9
8
7
6
5
4
2
4
6
8
10
12
14
16
18
20

Figure 2. Population trends of Xp isolate Ra in primary leaves of field-grown Navy beans (cultivar Seafarer). Eleven-day-old plants were sprayed to runoff with an aqueous suspension (1 x 10^8 cells/ml) of Ra. After abscission of primary leaves, groups of 21 brown and dry leaves on the soil surface were covered with a double layer of cheese cloth to aid in recovery. Populations of Ra were sampled by homogenizing 21 leaves (average area, 20 cm^2) in 105 ml of phosphate buffer. Samples were plated on RAM (100 µg/ml rifampin) + cycloheximide (100 µg/ml); PCNB (100 µg/ml) was added to the medium for leaf samples from the ground. Data are means of two replications ± standard error.

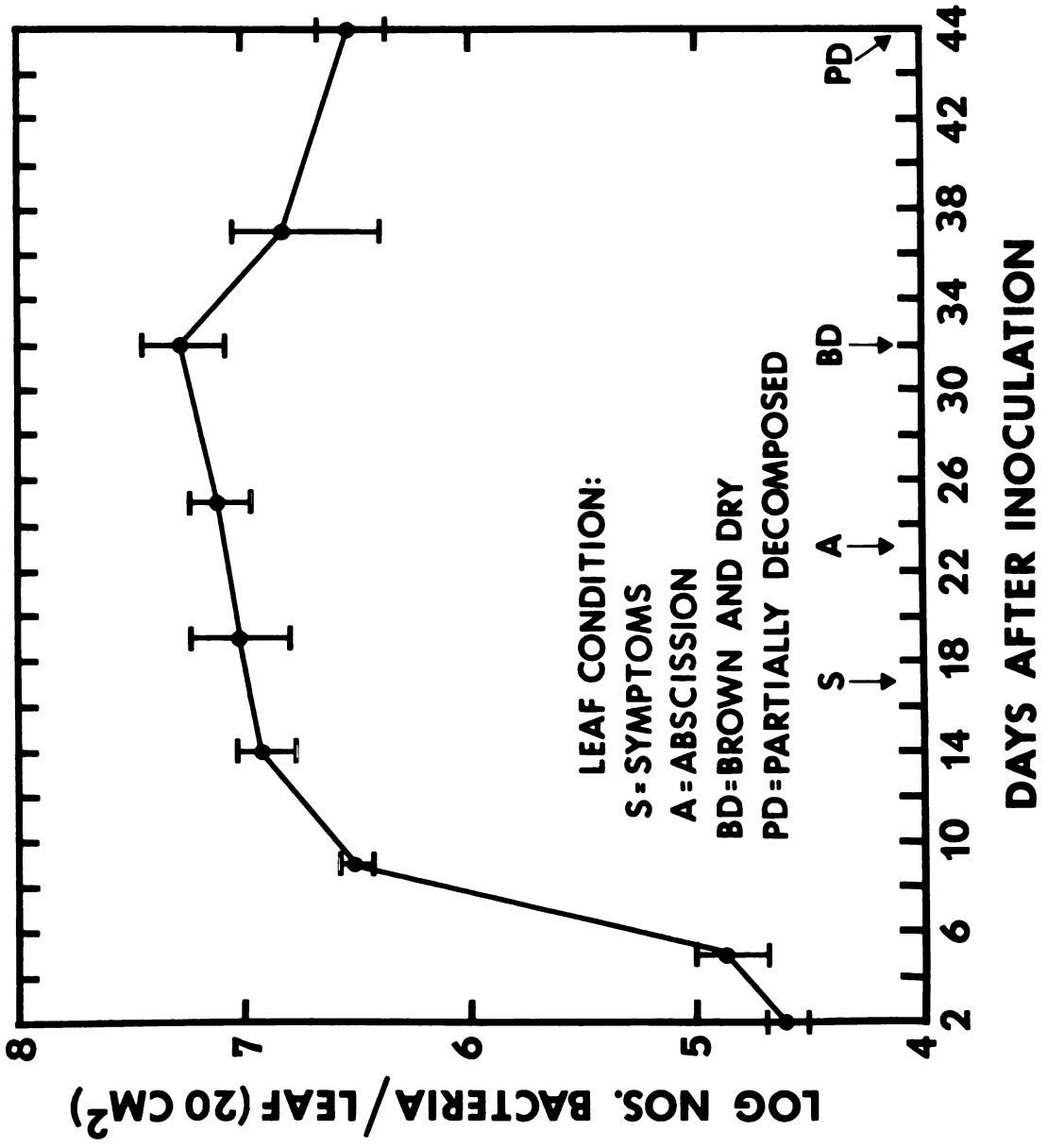
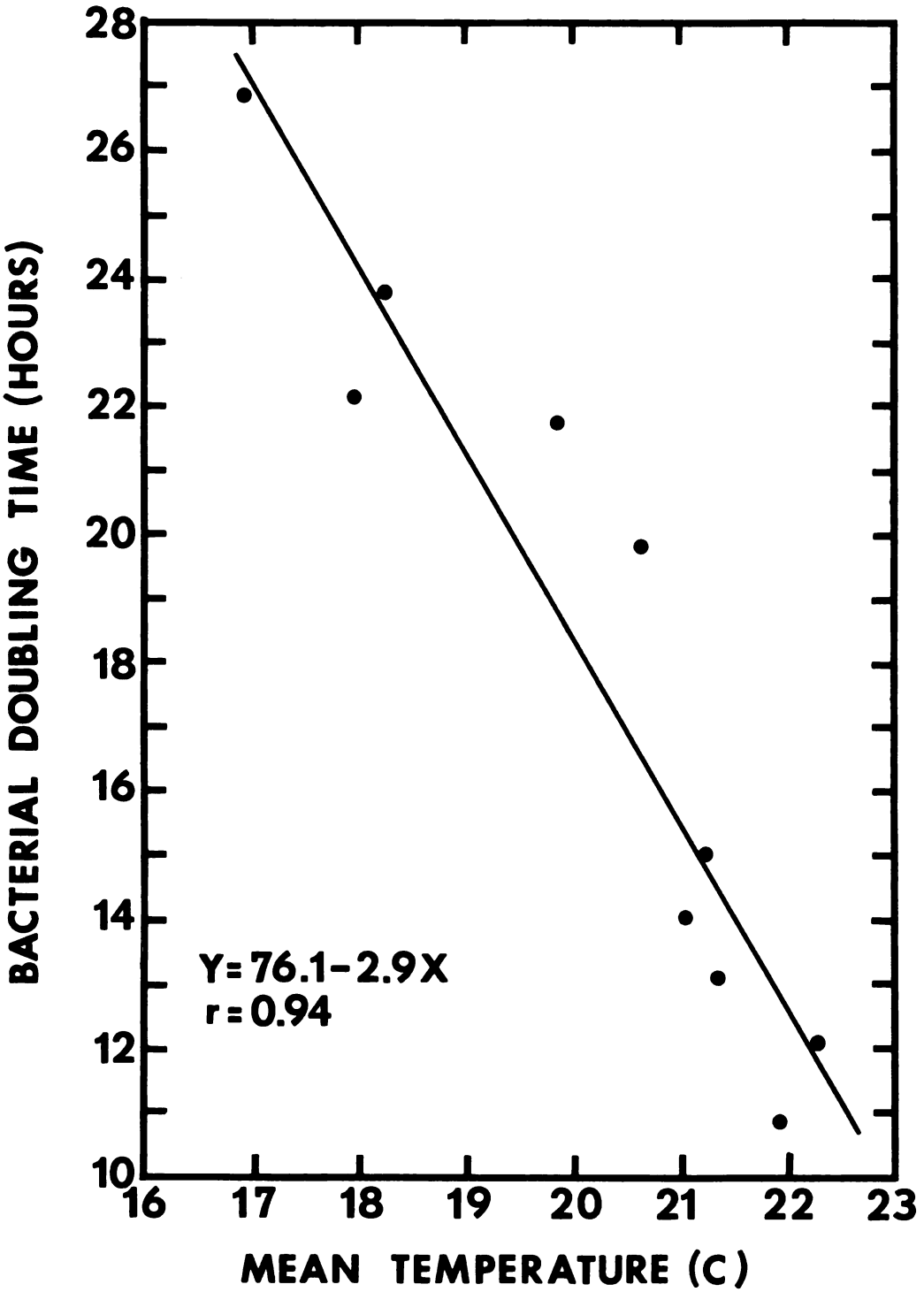

LOG NOS. BACTERIA/LEAF (20 CM²)
8
7
6
5
4
2
6
10
14
18
22
26
30
34
38
42
44
DAYS AFTER INOCULATION
S
A
BD
PD
LEAF CONDITION:
S=SYMPTOMS
A=ABSCISSION
BD=BROWN AND DRY
PD=PARTIALLY DECOMPOSED

Figure 3. Effect of temperature on the doubling times of R10 in primary leaves or first and second trifoliolated leaves of field-grown Navy beans (cultivar Seafarer). Five plots of beans were planted between June 5 and July 25, 1976 and inoculated with an aqueous suspension (1×10^8 cells/ml) of R10 when primary leaves or first and second trifoliolate leaves were expanded. Four replicates of 12 primary leaves of 15 trifoliolate leaflets (average area, 20 cm^2) were homogenized in 75 ml of phosphate buffer and plated on RAM + cycloheximide. Doubling times were calculated for population changes during the exponential growth phase between each sample. Mean temperature is the average of daily maximum and minimum temperatures for days between sampling times. Temperatures were measured 200 meters away.

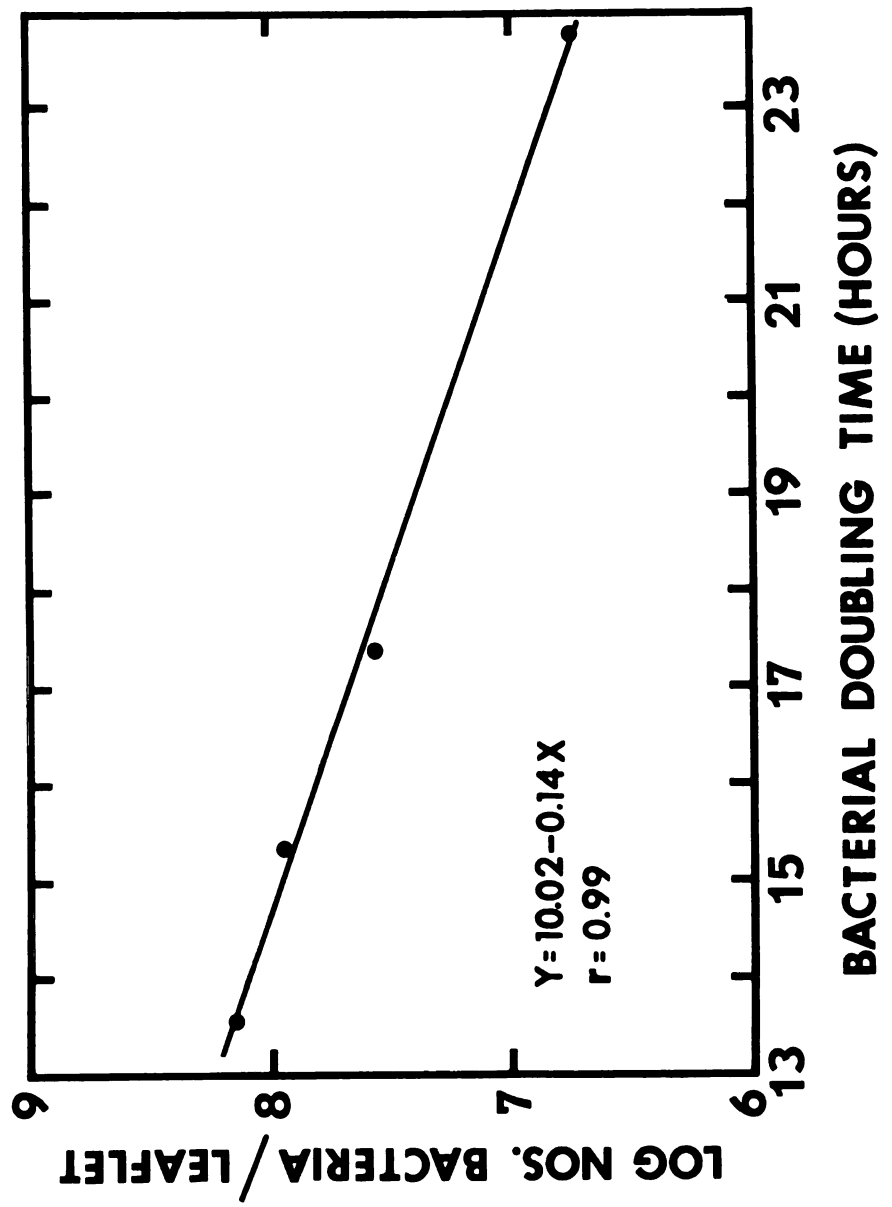

BACTERIAL DOUBLING TIME (HOURS)
28
26
24
22
20
18
16
14
12
10
Y=76.1-2.9X
r=0.94
16
17
18
19
20
21
22
23
MEAN TEMPERATURE (C)

of inoculated primary leaves, 58% and 30% of the population detected at first symptoms were still viable; the leaves disintegrated after the last sample and prevented further sampling. In a similar experiment, 55% of the R10 population at first symptoms were viable 30 days after first trifoliolate leaf abscission.

Symptom development on leaves inoculated with R10 or Ra required a minimal population of approximately 5×10^6-2×10^8 cells per 20 cm^2 leaf tissue and usually corresponded to the early stationary growth phase. The yield of bacteria per infected leaflet was affected by the bacterial doubling time, the initial inoculum density, and the physiological age of the leaf. In 1976 the population of R10 accompanying first symptoms on primary leaves or first and second trifoliolate leaves was negatively, linearly related to the exponential phase doubling time (Fig. 4). Also, greater initial inoculum levels of Ra and R10 resulted in higher bacterial yields. Finally, leaves in the physiological stage of senescence were unable to sustain R10 and Ra populations in the exponential growth phase.

Resident bacteria and yeasts associated with bean leaves. Populations of resident saprophytic bacteria and yeasts associated with bean leaves generally ranged between 10^4-10^7 cells per 20 cm^2 leaf tissue. The

Figure 4. Effect of doubling times of R10 in primary leaves or first and second trifoliolate leaves of field-grown Navy beans (cultivar Seafarer) on the bacterial yield at first symptoms during the stationary growth phase. Five plots of beans were plated between June 5 and July 25, 1976, and inoculated with an aqueous suspension (1×10^8/ml) of R10 when primary leaves or first and second trifoliolate leaves were expanded. Four replicates of 12 primary leaves or 15 trifoliolate leaflets (average area, 20 cm^2) were homogenized in 75 ml phosphate buffer and plated on RAM + cycloheximide. The mean doubling time was the average of the doubling times calculated between each sample during the exponential growth phase.


LOG NOS. BACTERIA / LEAFLET
9
8
7
6
13
15
17
19
21
23
BACTERIAL DOUBLING TIME (HOURS)
Y=10.02-0.14X
r=0.99

resident population generally was stable while blight bacteria were in the exponential growth phase but increased as blight bacteria entered stationary growth phase and symptoms appeared (Fig. 1).

Multiplication and spread of R10 and R10-S6 in Navy bean seedlings. Hilum-spotted Navy bean seeds (cultivar Seafarer), containing an average of 10^6 R10 or R10-S6 cells per seed, were used as inocula sources for studies of multiplication and spread of blight bacteria during the seedling stage. In a preliminary study, isolate R10 was found associated with all seedling parts (leaves, hypocotyl, cotyledons, terminal bud and roots) very soon after seedling emergence (Table 1). Bacterial populations varied among seedling parts, indicating that infected seedlings are not uniformly colonized; no symptoms were observed on these seedlings. In a second study, seeds infected with R10-S6 were used so that blight bacteria associated with the roots could be sampled without interference from rifampin-resistant bacteria that exist naturally in the rhizosphere. Due to cool weather following planting, seedlings emerged in 12 days as opposed to the usual five days and subsequent growth was also slowed. Bacteria of isolate R10-S6 were isolated from all plant parts except the cotyledons which were not sampled (Fig. 5). Bacterial populations

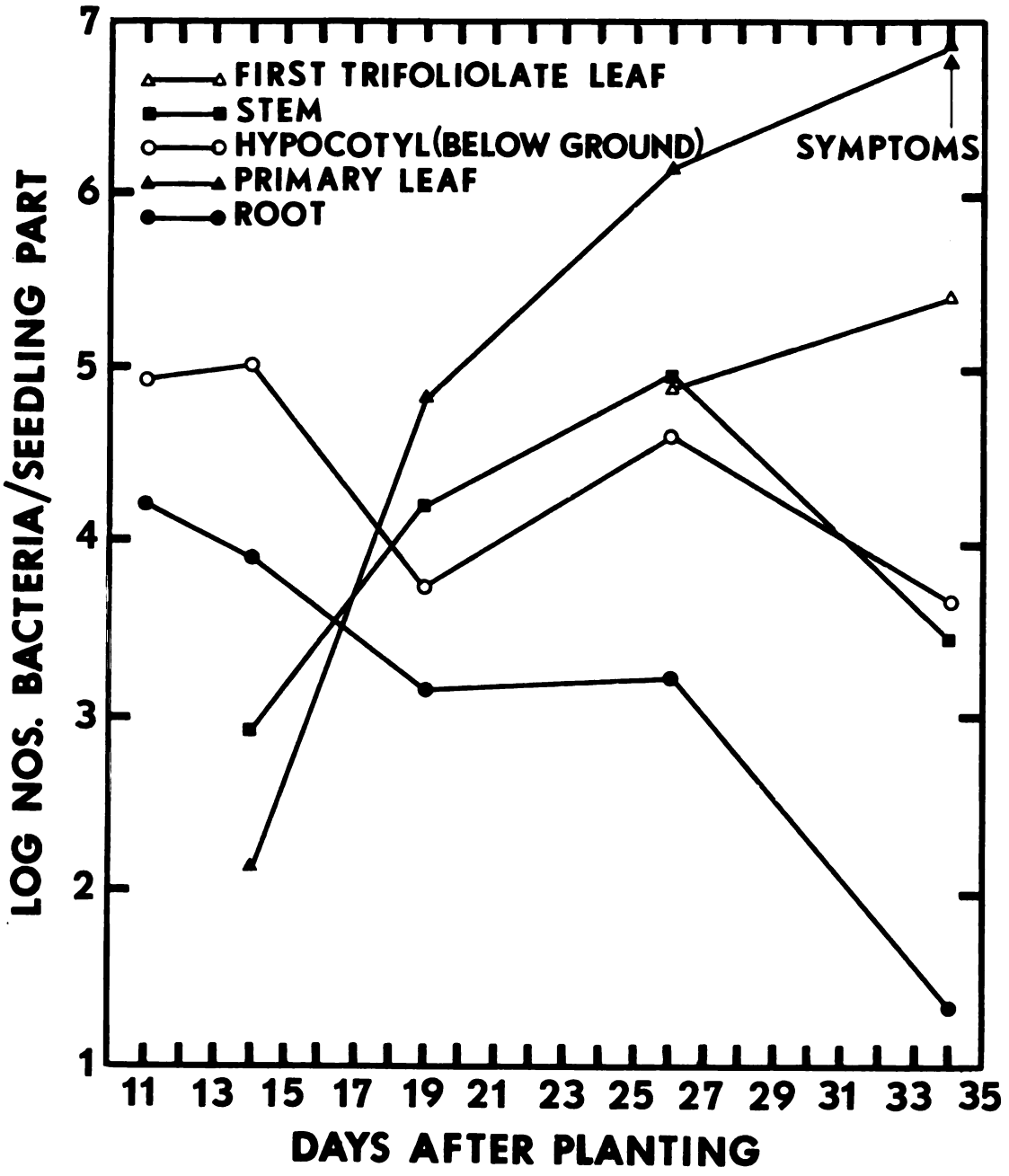
TABLE 1. Distribution of isolate R10 over Navy bean seedlings (cultivar Seafarer) grown from hilum spotted seeds.

Seedling part[a]	Number of bacteria at indicated time after planting[b]	
	6 days	9 days
Cotyledon	$7.41 \times 10^{1} \pm 3.05 \times 10^{1}$	$4.88 \times 10^{3} \pm 2.82 \times 10^{3}$
Hypocotyl + epicotyl	$1.03 \times 10^{6} \pm 7.22 \times 10^{5}$	$3.29 \times 10^{6} \pm 3.14 \times 10^{6}$
Primary leaf	$7.30 \times 10^{3} \pm 6.87 \times 10^{3}$	$9.88 \times 10^{3} \pm 1.79 \times 10^{3}$
Root mass	$7.44 \times 10^{4} \pm 6.44 \times 10^{4}$	$2.20 \times 10^{5} \pm 1.91 \times 10^{5}$
Terminal bud	<20	<20

[a] Hypocotyl + epicotyl included tissue from the terminal bud to the roots, average fresh weight 0.3 g; primary leaf, average area 20 cm^2; root mass included root tissue with associated soil particles, average weight 0.3 g.

Six-day-old seedlings were 3.0 cm high with expanded primary leaves; nine-day-old seedlings were 4.0 cm high with the first trifoliolate leaf expanding. Values indicate the average of three replications ± the standard error of the mean, with each consisting of 12 cotyledons, or six hypocotyls or 12 primary leaves, or six root masses or six terminal buds. All tissue samples were ground in a mortar and pestle with phosphate buffer, serial dilutions of the homogenate were plated on RAM (100 µg/ml) + cycloheximide (100 µg/ml).

Figure 5. Population trends of isolate R10-S6 in Navy bean seedlings (cultivar Seafarer). Hilum-spotted seeds, infected with R10-S6, were planted and seedlings emerged after 12 days. Bacterial populations were determined by homogenizing the various parts of the seedling in a mortar and pestle with phosphate buffer and plating aliquots on RAM (100 µg/ml rifampin) + cycloheximide (100 µg/ml) + PCNB (100 µg/ml) + streptomycin sulfate (250 µg/ml). Primary leaf and first trifoliolate leaflet, average area, 20 cm^2 area; the stem, average weight 0.3 g, included the above ground portion of the hypocotyl and the epicotyl up to the terminal bud; below ground portion of the hypocotyl, average weight 0.1 g, later constituted portion of tap root; root mass, average weight 0.3 g, included all fibrous roots and adhering soil. Data are means of two replications with ten primary leaves, 15 trifoliolate leaflets, and five of other parts per replication.


FIRST TRIFOLIOLATE LEAF
STEM
HYPOCOTYL(BELOW GROUND)
PRIMARY LEAF
ROOT
SYMPTOMS
LOG NOS. BACTERIA/SEEDLING PART
DAYS AFTER PLANTING
1
2
3
4
5
6
7
11 13 15 17 19 21 23 25 27 29 31 33 35

were the greatest on the primary leaves, and the first trifoliolate leaf became colonized while expanding. The population of R10-S6 on the above-ground portion of the hypocotyl and stem increased slightly and later declined. Below-ground seedling parts were colonized throughout the entire sampling period but declined slowly on the roots. Nearly the same number of R10-S6 associated with root and hypocotyl tissue could be removed by washing as by homogenizing.

Multiplication and spread of R10 and Ra in leaves and buds of field-grown beans during the vegetative and early reproductive stages. Population profiles of Ra and R10 in beans from seedling until early reproductive stages could be characterized as a series of bacterial growth curves displaced over time with each curve representing bacterial multiplication on individual leaves relative to the primary leaf node (Figs. 6 and 7). As each leaf differentiated from the main stem it became colonized by Ra or R10 and a gradient of bacterial populations was established in the leaf canopy of the infected plants with the oldest leaves closest to the primary leaf node supporting the highest bacterial populations. The sequence of symptom development followed a similar gradient; symptom appeared first on the primary leaves and sequentially on the

Figure 6. Population trends of isolate R10 in the leaves and buds of field-grown Navy beans (cultivar Sanilac). Sixteen-day-old plants with expanding first trifoliolate leaves were inoculated with a suspension (1 x 10^8 cells/ml) of R10. Bacterial populations were individually sampled from the primary to the seventh trifoliolate leaves of the main stem by homogenizing 14 primary leaves or 21 trifoliolate leaflets (average area, 20 cm^2) in 105 ml phosphate buffer. Seven terminal buds from the main stem and 10-50 axillary buds from both main and lateral stems were homogenized with a mortar and pestle with 10 ml of phosphate buffer. Samples were plated on RAM (100 µg/ml rifampin) + cycloheximide (50 µg/ml). Data are means of three replications. Symptoms were initially detected as leaf chlorosis; flower buds were first noted as clusters of swollen buds at the growing tip; bloom represents the stage where all lower canopy flowers were open and some upper canopy flowers were closed; flat green pods indicate pods with no visible seed filling; 1-12 indicates the number of trifoliolate leaves expanded along the growing tip of the main stem.

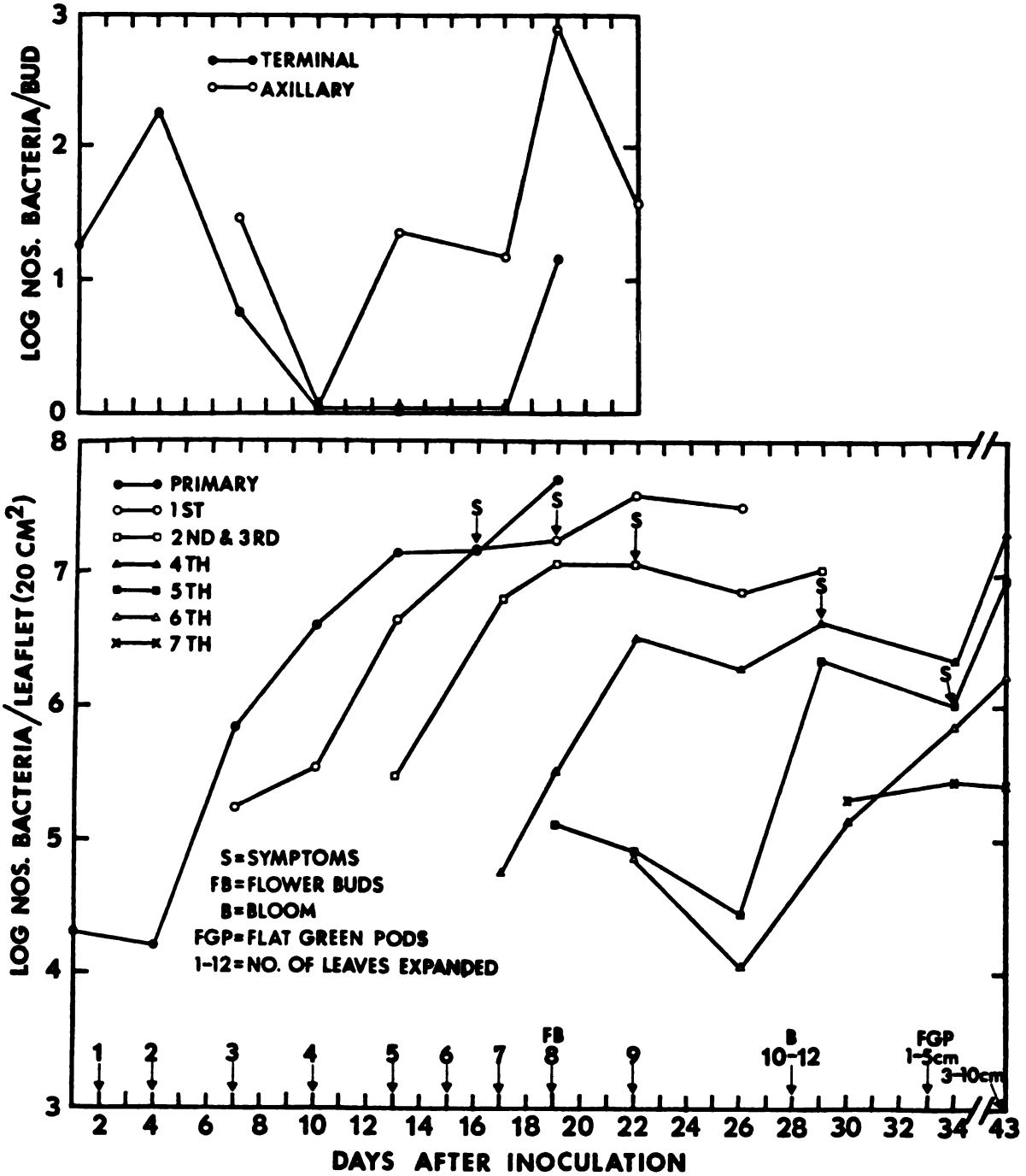
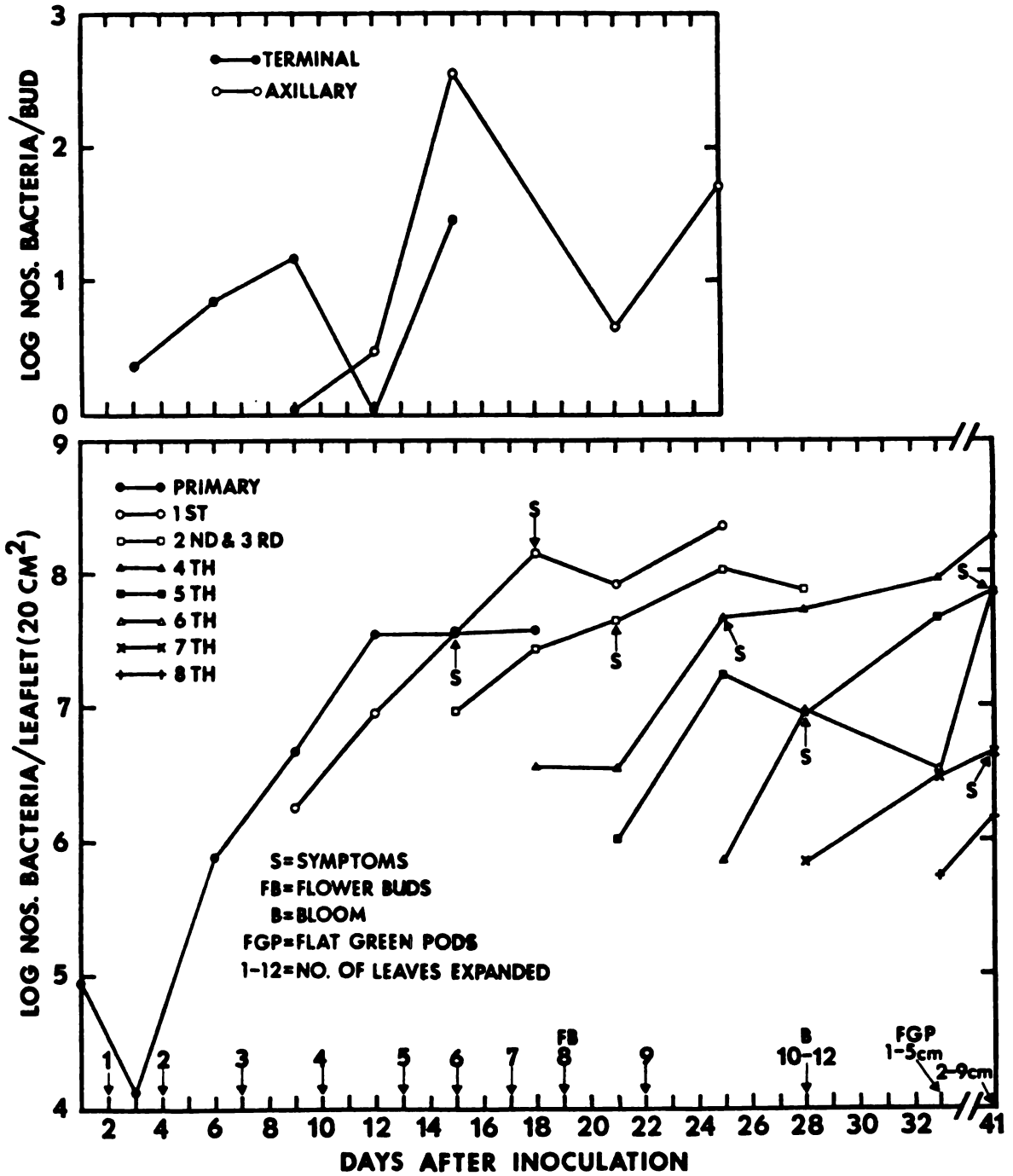

LOG NOS. BACTERIA/BUD
TERMINAL
AXILLARY
LOG NOS. BACTERIA/LEAFLET (20 CM²)
PRIMARY
1ST
2ND & 3RD
4TH
5TH
6TH
7TH
S=SYMPTOMS
FB=FLOWER BUDS
B=BLOOM
FGP=FLAT GREEN PODS
1-12=NO. OF LEAVES EXPANDED
FB
B
10-12
FGP
1-5cm
3-10cm
DAYS AFTER INOCULATION

Figure 7. Population trends of isolate Ra in the leaves and buds of field-grown Navy beans (cultivar Sanilac). Sixteen-day-old plants with expanding first trifoliolate leaves were inoculated with a suspension (1 x 10^8 cells/ml) of Ra. Bacterial populations were individually sampled from the primary leaves to the eighth trifoliolate leaves of the main stem by homogenizing 14 primary leaves or 21 trifoliolate leaflets (average area 20 cm^2) in 105 ml of phosphate buffer. Seven terminal buds from the main stem and 10-50 axillary buds from both main and lateral stems were homogenized with a mortar and pestle with 10 ml of phosphate buffer. Samples were plated on RAM (100 µg/ml rifampin) + cycloheximide (50 µg/ml). Data are means of three replications. Symptoms were initially detected as leaf chlorosis; flower buds were first noted as clusters of swollen buds at the growing tip; bloom represents the stage where all lower canopy flowers were open and some upper canopy flowers were closed; flat green pods indicates pods with no visible seed filling; 1-12 indicates the number of trifoliolate leaves expanded along the growing tip of the main stem.


LOG NOS. BACTERIA/BUD
TERMINAL
AXILLARY
LOG NOS. BACTERIA/LEAFLET(20 CM²)
PRIMARY
1 ST
2 ND & 3 RD
4 TH
5 TH
6 TH
7 TH
8 TH
S=SYMPTOMS
FB=FLOWER BUDS
B=BLOOM
FGP=FLAT GREEN PODS
1-12=NO. OF LEAVES EXPANDED
FB
B
10-12
FGP
1-5cm
2-9cm
DAYS AFTER INOCULATION

oldest to the youngest trifoliolate leaves. Symptom expression on each leaf corresponded to the period of stationary phase bacterial growth and required a minimal bacterial population as previously described. The same sequence of symptom expression described for the main stem was also associated with leaves of lateral stems. As pods developed they were colonized similar to expanding leaves. By bloom, symptoms in the R10 and Ra inoculated plants were detected only up to the fourth and sixth trifoliolate leaves, respectively, even though bacteria were detected by plate counts or leaf prints up to the top trifoliolate leaf. Thus, the bacteria were spreading more rapidly upward and outward into the leaf canopy than the symptoms indicated. Generally, isolate Ra colonized the plants more rapidly, produced higher stationary phase populations and produced more symptoms than isolate R10.

Isolates R10 and Ra were consistently detected in terminal and axillary buds throughout the monitoring period (Figs. 6 and 7). Bacteria in either bud type displayed a considerable variation in population between samples which ranged from near zero to almost 10^3 bacteria per bud. Newly expanded leaves less than 1 cm long consistently were colonized by R10 and Ra. Flower buds were consistently colonized with Ra and R10

levels comparable to the vegetative buds. Up to 1 x 10^4 bacteria were detected in both unopened and opened flowers from plants used in this study and those from 1976, however, the populations were generally 10^1-$2x10^2$ bacteria per flower.

Field observations of disease development. Overhead visual ratings of plants inoculated with R10 and Ra (Figs. 6 and 7) were taken periodically in 1977. These observations simulated what a grower inspecting his field or a researcher inspecting experimental plots might observe. Typical field symptoms were not detected by overhead observation taken throughout the entire vegetative stage. Only when the outer leaf canopy was cut away could the lower leaves with typical symptoms be seen. The rapidly expanding outer foliage continued to camouflage blight symptoms in the leaf canopy until ten days after bloom when symptoms developed on the outer trifoliate leaves. This pattern of symptom appearance was identical to that normally seen in commercial fields.

Multiplication and systemic spread of R10 and Ra in stems of field-grown Navy beans. Ten days after inoculation isolates R10 and Ra initially were detected internally in stems of Navy beans which were used for the study shown in Figures 6 and 7. A steady increase in the number of plants systemically colonized was

observed. Thirteen, 19, 26, and 31 days after inoculation 20, 64, 71, and 85% of the stems were infected with R10; at 12, 21, 28, and 31 days after inoculation 19, 43, 62, and 80% of the stems were infected with Ra. Studies in 1976 indicated that 75% of Seafarer plants were systemically infected with R10. Tuscola plants were infected to a similar degree.

Isolates R10 and Ra, which were introduced into the cotyledon node to study systemic colonization, moved rapidly upward in the main stem and established a population gradient inversely related to the distance from the cotyledon node. The population profile of R10 and Ra in the main stem during the vegetative and reproductive stages was characterized by a series of growth curves with each representing bacterial multiplication in a section of stem relative to the cotyledon node (Figs. 8 and 9). Similar patterns of bacterial multiplication and movement was detected in lateral stems and leaf petioles. The average doubling time of R10 and Ra in stem tissue up to the fourth trifoliate node was 22.8 hours (range 14.6-37.6 hours) and 23.8 hours (range 12.3-31.0 hours), respectively. Stem lesion formation required minimal populations of R10 and Ra of approximately 10^7-10^8 bacteria per 0.35 g stem tissue; symptoms appeared first at the injection

Figure 8. Population trends of isolate R10 in Navy bean stems and roots (cultivar Seafarer). Twenty-day-old plants with third trifoliate leaves just expanding were inoculated by jabbing the cotyledon scar with a syringe containing 1×10^8 cells R10 per ml. Bacterial populations were sampled from the roots up to the eighth trifoliate leaf node of the main stem by homogenizing stem portions or roots from five plants with phosphate buffer and plating on RAM + cycloheximide. All data are based on an average node + internode fresh weight of 0.35 g and are means of two replications. ROOT, included both the tap and the fibrous roots; COT, included the cotyledon node and internodal region from the soil line to the primary leaf node; PRI, included the primary node and the internodal region up to the first trifoliolate leaf node; 1ST, included the first trifoliolate leaf node and the internodal region up to the second trifoliolate leaf node; 2ND-3RD, included the second and third trifoliolate leaf nodes and the internodal region up to the fourth trifolielate leaf node; 4TH-5TH, included the fourth and fifth trifoliolate leaf node and the internodal region up to the sixth trifoliolate leaf node; 6TH-7TH, included the sixth and seventh trifoliolate leaf node and the internodal region up to the eighth trifoliolate leaf node. Symptoms were noted as the first appearance of a redding of the node or internode; flower buds were first noted as a cluster of swollen buds at the growing tip; bloom represents the stage where all lower canopy flowers were open and some upper canopy flowers were closed; flat green pods indicate pods with no visible filling; 3-9 indicates the number of trifoliolate leaves expanded from the main stem.

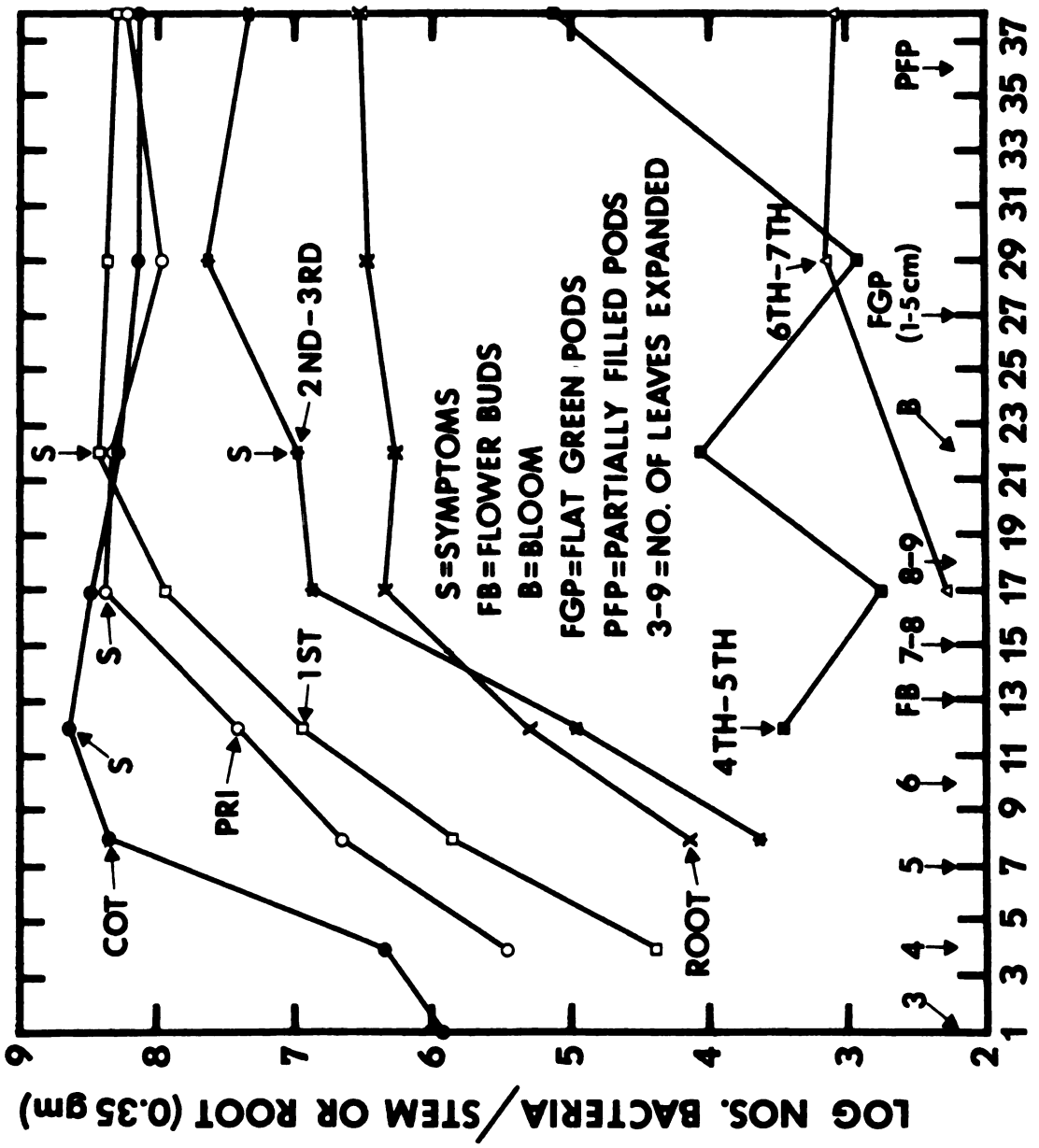
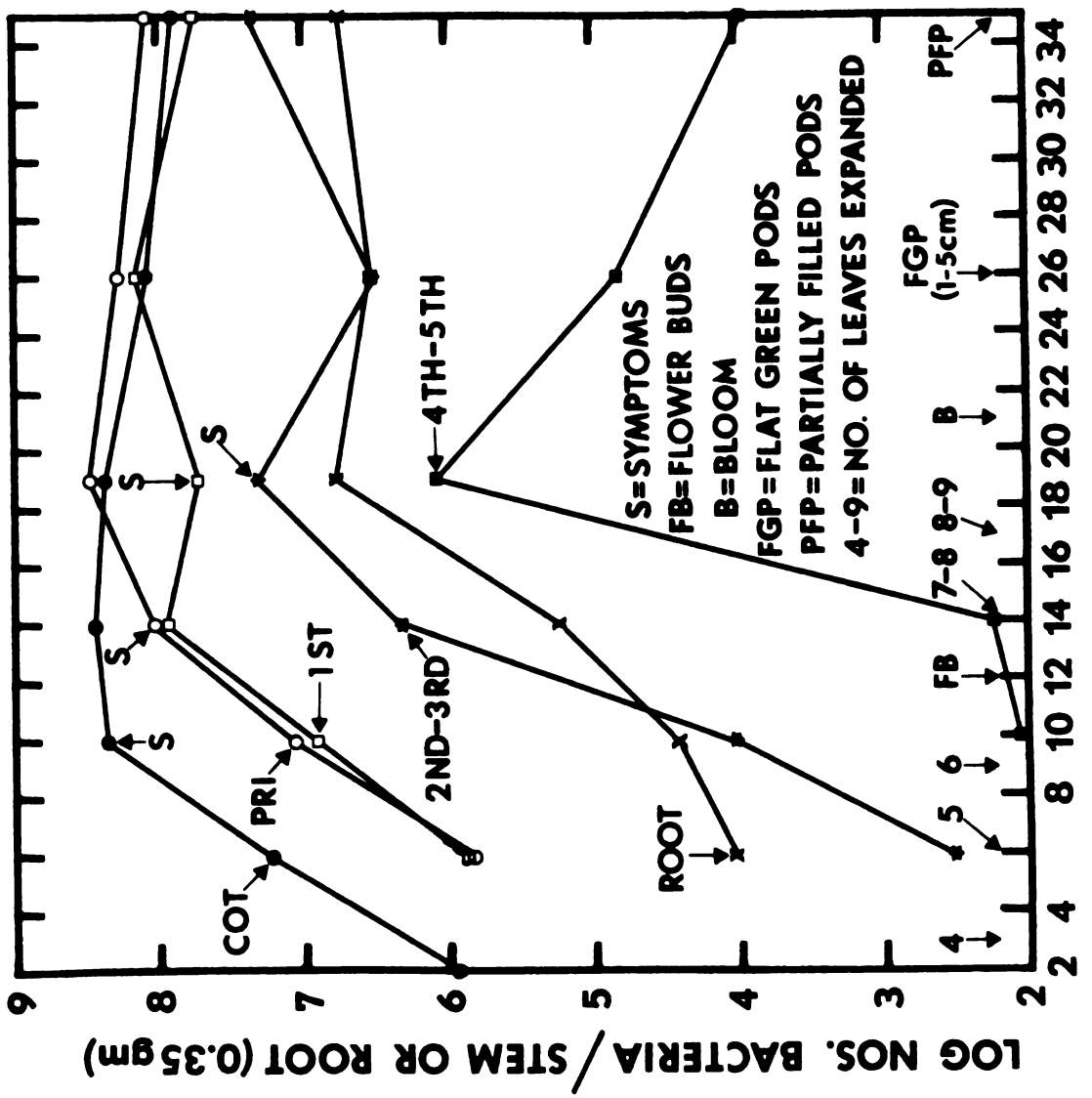

LOG NOS. BACTERIA/STEM OR ROOT (0.35 gm)
DAYS AFTER INOCULATION
S=SYMPTOMS
FB=FLOWER BUDS
B=BLOOM
FGP=FLAT GREEN PODS
PFP=PARTIALLY FILLED PODS
3-9=NO. OF LEAVES EXPANDED
COT
PRI
ROOT
1ST
2ND-3RD
4TH-5TH
6TH-7TH
FB
FGP (1-5cm)
B
PFP
S

Figure 9. Population trends of isolate Ra in Navy bean stems and roots (cultivar Seafarer). Twenty-day-old plants with third trifoliolate leaves just expanding were inoculated by jabbing the cotyledon scar with a syringe containing 1×10^8 cells R10 per ml. Bacterial populations were sampled from the roots up to the eighth trifoliolate leaf node of the main stem by homogenizing stem portions or roots from five plants with phosphate buffer and plating on RAM + cycloheximide. All data are based on an average node + internode fresh weight of 0.35 g and are means of two replications. ROOT included both the tap and the fibrous roots; COT included the cotyledon node and internodal region from the soil line to the primary leaf node; PRI included the primary node and the internodal region up to the first trifoliolate leaf node; 1ST included the first trifoliolate leaf node and the internodal region up to the second trifoliolate leaf node; 2ND-3RD included the second and third trifoliolate leaf nodes and the internodal region up to the fourth trifoliolate leaf node; 4TH-5TH included the fourth and fifth trifoliolate leaf node and the internodal region up to the sixth trifoliolate leaf node. Symptoms were noted as the first appearance of a redding of the node or internode; flower buds were first noted as a cluster of swollen buds at the growing tip; bloom represents the stage where all lower canopy flowers were open and some upper canopy flowers were closed; flat green pods indicates pods with no visible filling; 3-9 indicates the number of trifoliolate leaves expanding from the main stem.


LOG NOS. BACTERIA / STEM OR ROOT (0.35gm)
DAYS AFTER INOCULATION
COT
PRI
1ST
2ND-3RD
4TH-5TH
ROOT
S
FB
B
FGP (1-5cm)
PFP
7-8
8-9
S=SYMPTOMS
FB=FLOWER BUDS
B=BLOOM
FGP=FLAT GREEN PODS
PFP=PARTIALLY FILLED PODS
4-9=NO. OF LEAVES EXPANDED

point and progressively upward. Lesion formation was most pronounced at the nodes, however, internodal regions were also diseased. By bloom, stem lesions appeared only up to the second and some third trifoliolate leaf nodes, and lesions were not widely spread throughout the stem system until near pod maturity. Leaf lesions, which were primarily initiated by vascular-borne R10 and Ra; appeared first on the primary leaves and progressively upward in succession on the trifoliolate leaves. By bloom, symptoms appeared only up to the third trifoliolate leaf. Symptoms on the lower leaves were not readily apparent due to camouflaging from the upper canopy and typical field symptoms were not visible until after bloom. The pattern of symptom expression in the leaf canopy was identical to that detected in leaf studies shown in Figures 6 and 7.

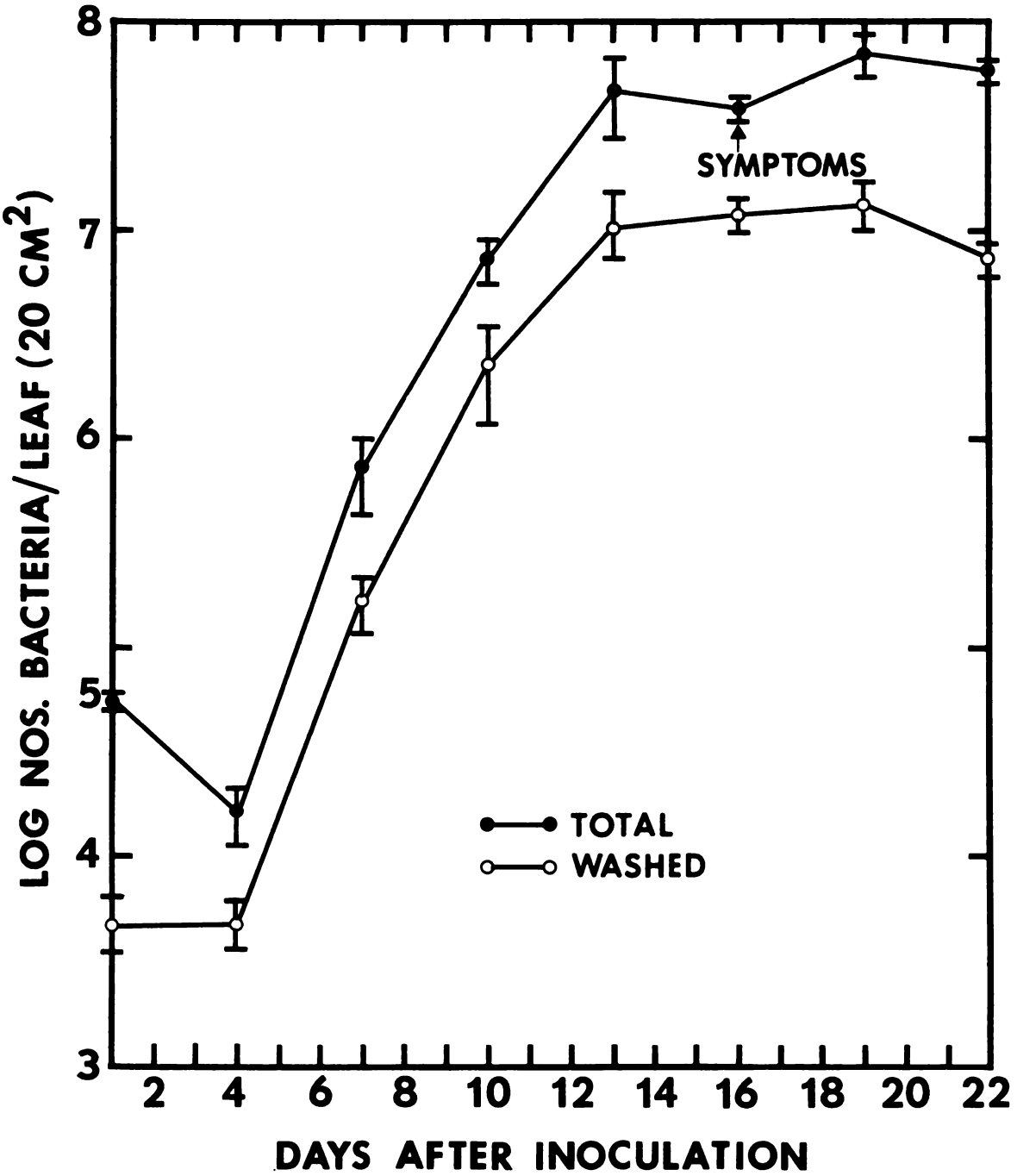
Both R10 and Ra also displayed strong downward mobility into the roots; substantial population increases occurred but no root lesions were detected (Figs. 8 and 9). A population gradient of R10 and Ra, similar to that in the stem, was established in the root system with the lowest population in the lateral roots. Although the vast majority of the root population was internal, approximately .03-.8% of the total population of R10 or Ra could be consistently

isolated from the root surface by shaking the roots for 30 seconds in distilled water.

Effect of washing on blight bacterial populations of Navy bean leaves. Primary, first and second trifoliolate leaves were inoculated with R10 and Ra and the bacteria were washed from the leaves at various intervals. Number of bacteria recovered by washing was proportional to the total leaf population present at the time of sampling; population changes of bacteria washed from the leaf followed a growth curve similar to that of the total population (Fig. 10). An average of 27% and 21% of the total R10 population on primary leaves (cultivar Seafarer) were removed during the exponential and stationary phases of bacterial growth, respectively (Fig. 10); 35% and 22% of the total R10 population were washed from first and second trifoliolate leaves during the exponential and stationary phases, respectively. An average of 46% and 22% of the total population of Ra in primary leaves (cultivar Sanilac) was removed during the exponential and stationary phases, respectively.

To more carefully simulate natural washing of infected leaves by rain water, infected Navy bean seedlings were misted in the greenhouse. Two, six, and 13 days after inoculation 64%, 46%, and 32% of the total R10 population was removed compared to non-misted plants. In the runoff water, however, only 35%, 26% and

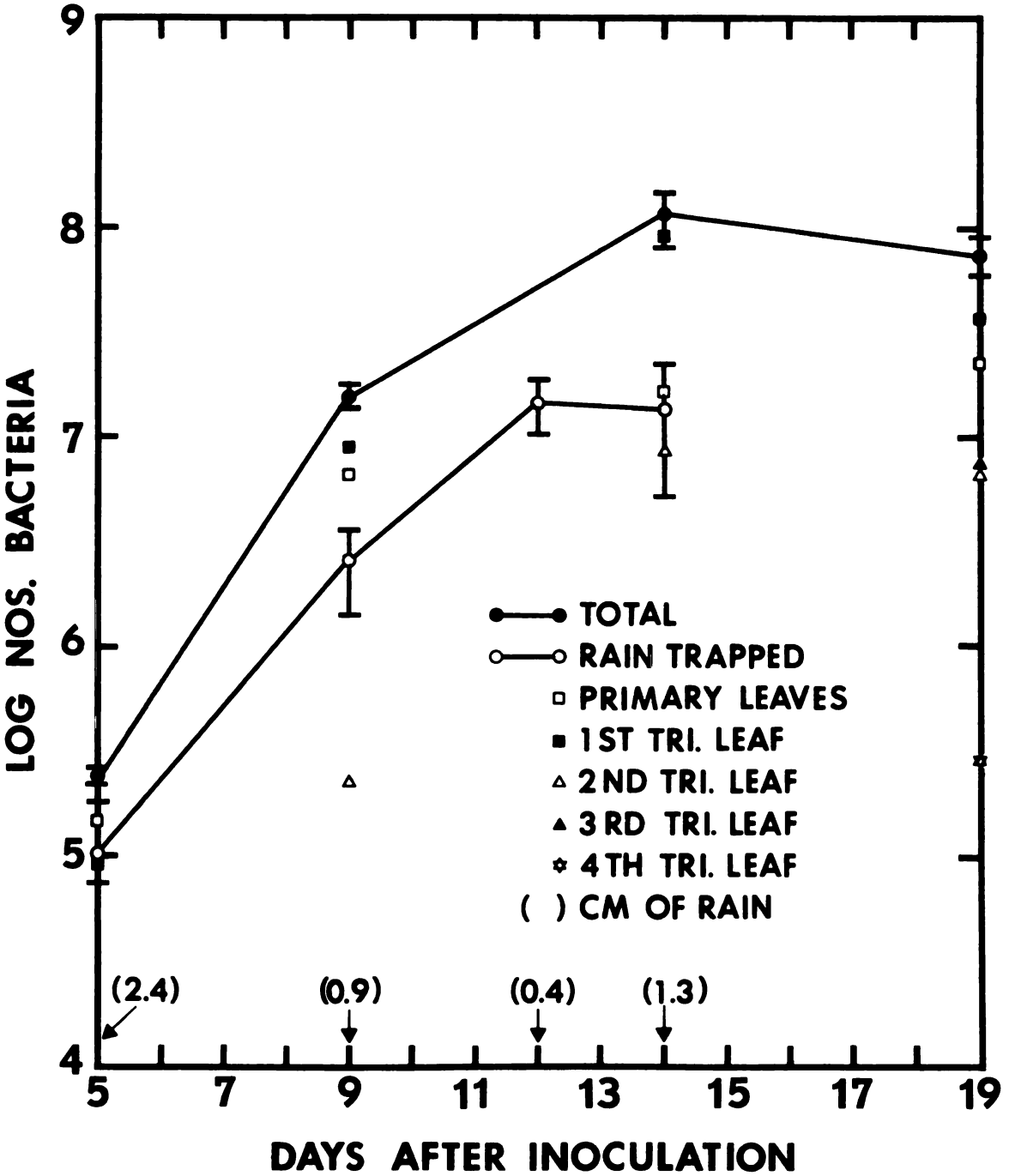
Figure 10. Comparison of the total R10 populations in field-grown leaves of Navy beans (cultivar Seafarer) and R10 populations readily removed by leaf washing. Eleven-day-old plants were sprayed to runoff with a aqueous suspension (1 x 10^8 cells/ml) of R10. Total bacterial populations were sampled by homogenizing 12 leaves (average leaf area 20 cm^2) in 75 ml of phosphate buffer, pH 7.2; washed populations were sampled by gently washing 12 leaves in 100 ml of phosphate buffer for 2.5 minutes. Samples were plated on RAM + cycloheximide. Data are averages of four replications $\pm$ standard error.


LOG NOS. BACTERIA/LEAF (20 CM²)
8
7
6
5
4
3
SYMPTOMS
TOTAL
WASHED
2
4
6
8
10
12
14
16
18
20
22
DAYS AFTER INOCULATION

20% of the total population was recovered. The number of R10 in the runoff water was lower than expected because of spillage during the collecting process; also some bacteria may have died before plating. The primary leaves were symptomless during the first two samples and definite symptoms were present during the third sample. In both the washing and misting studies, a higher proportion of the bacteria were removed during the exponential than the stationary phase.

Rain-trapped Ra from field-grown Navy beans. Runoff water was collected after rains which occurred 5, 9, 12, and 14 days after inoculation; populations of rain trapped Ra increased logarithmically over time in proportion to the total plant population (Fig. 11). Five days after inoculation 50% of the total blight bacteria from primary and first trifoliolate leaves were washed off, however, this figure is artificially high since total leaf population were not assayed until after the wetting period. Runoff water sampled nine and 14 days after inoculation contained 16% and 12% of the total leaf population of Ra. These samples provided the best estimates since total populations were determined immediately before the wetting period began. While total leaf populations were not sampled 12 days after inoculation, the number washed from leaves followed the same trend as established on other sampling dates. Rain

Figure 11. Comparison of total Ra populations in field-grown Navy beans (cultivar Seafarer) and rain trapped populations in leaf runoff water. Eleven-day-old plants were sprayed with an aqueous suspension (1×10^8 cells/ml) of Ra. Bacterial populations were sampled by homogenizing 14 primary leaves or 21 trifoliolate leaflets (average area, 20 cm^2) in 105 ml 0.01 M phosphate buffer, pH 7.2. Total Ra populations were determined by summing populations on primary through fourth trifoliolate leaves present at the time of rainfall. Rain trapped bacteria were collected in wax cups placed under single bean plants. All samples were plated on RAM (100 µg/ml) + cycloheximide (50 µg/ml). Total plant populations are averages of two replications $\pm$ standard error and rain trapped populations are averages of six replications $\pm$ standard error.


LOG NOS. BACTERIA
9
8
7
6
5
4
5
7
9
11
13
15
17
19
DAYS AFTER INOCULATION
TOTAL
RAIN TRAPPED
PRIMARY LEAVES
1ST TRI. LEAF
2ND TRI. LEAF
3RD TRI. LEAF
4TH TRI. LEAF
( ) CM OF RAIN
(2.4)
(0.9)
(0.4)
(1.3)

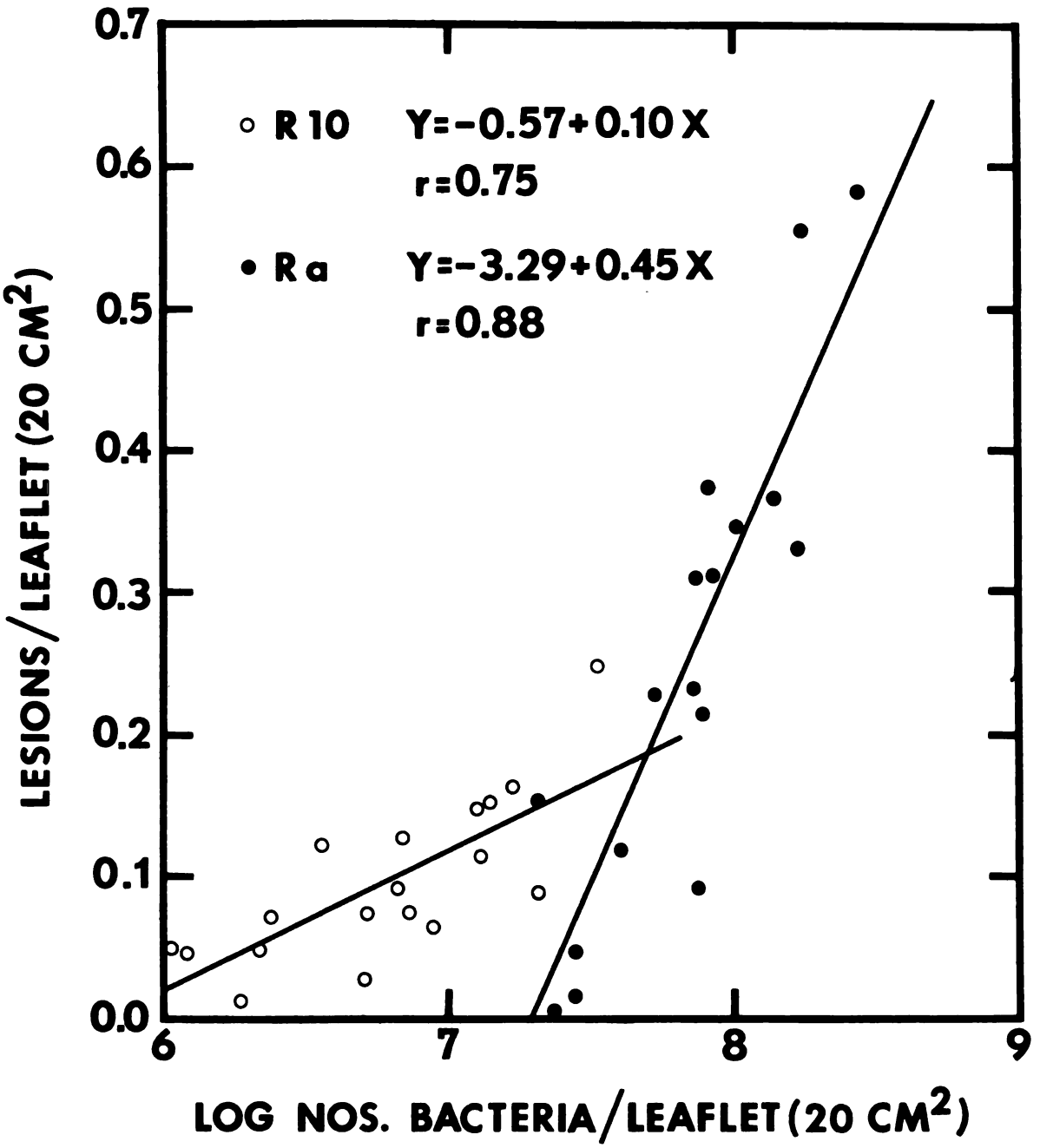
runoff water was also collected from Sanilac beans inoculated with R10 or Ra (Figs. 6 and 7). Sampling was not initiated until the leaf canopy was well expanded, thus limiting the amount of water collected. Because exact comparisons of bacterial numbers in runoff water to total populations were impossible, data are expressed as numbers of bacteria per ml runoff water. At 19, 23, and 26 days after inoculation Ra was detected in runoff water at a level of 7.12×10^4, 8.13×10^4, and 2.76×10^4 cells/ml, respectively; isolate R10 was detected at 6.86×10^4, 7.38×10^4, and 3.00×10^3 cells/ml runoff water, respectively. Data are means of four replications.

Detection of blight bacteria on leaf surfaces. Isolates R10 and Ra were consistently isolated from upper and lower leaflet surfaces of inoculated and uninoculated leaves by direct and indirect printing on plates of RAM + cycloheximide. Blight bacteria were detected on all parts of the leaf but occurred most frequently along the veins. Frequency of upper and lower leaflet colonization was compared by direct printing of symptomless first and second trifoliolate leaflets 30 days after 11-day-old seedlings with half expanded primary leaves were inoculated with R10. Sixty leaves were divided into four reps, one distal leaflet

was printed with the upper surface down and the other outer leaflet printed in the opposite way. A significantly (P = 0.05) greater number (49%) of bottom surfaces were colonized by R10 compared to top surfaces (32%); the number of colonies developing from lower surface prints also was usually greater. Similar results were obtained with leaves higher in the leaf canopy and when Ra was the inoculum. Bleaching and UV irridation of symptomless leaves infected with R10 and Ra from the field resulted in a reduction of about 20-40% in leaf populations compared to untreated controls.

Effect of R10 and Ra populations on the severity of leaf symptoms. In 1977, there was a significant (P = 0.01) correlation between the number of lesions and the R10 or Ra population per leaflet (average area, 20 cm^2) (Fig. 12). The data were collected from plants used for experiments shown in Figures 6 and 7; only the second through fifth trifoliolate leaves were considered to insure that infection resulted from natural bacterial spread. Isolate Ra produced higher bacterial populations per leaflet, required greater populations prior to initial symptoms and incited greater levels of disease than R10.

Figure 12. Effect of R10 and Ra on the severity of disease in the second through fifth trifoliate leaves of the main stem from field-grown Navy beans (cultivar Sanilac). Beans were planted on 6/10/77 and inoculated 17 days later with an aqueous suspension of (1×10^8 cells/ml) of R10 or Ra when first trifoliolate leaves were partially expanded. Twenty-one trifoliolate leaflets (average area, 20 cm^2) from the same level in the canopy were homogenized in 105 ml of 0.01 M phosphate buffer, pH 7.2, and plated on RAM (100 µg/ml rifampin) + cycloheximide (50 µg/ml). Disease in each sample was determined by counting individual lesions on 21 leaves per replication.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6
7
8
9
○ R 10 Y=-0.57+0.10 X
r=0.75
• R a Y=-3.29+0.45 X
r=0.88
LESIONS/LEAFLET (20 CM²)
LOG NOS. BACTERIA/LEAFLET (20 CM²)

DISCUSSION

This study examined the population dynamics of Xp and Xpf in Michigan Navy (pea) beans. Blight bacteria were monitored in field-grown beans from seedling emergence until pod formation and population profiles were correlated with the appearance of typical field symptoms. The basic tools of the study were the rifampin-resistant mutants of Xp and Xpf, Ra and R10, respectively; the isolates were easily selected even in the presence of a large and diverse phyllosphere microflora by plating plant tissue on rifampin-containing medium.

Growth curves of R10 and Ra in leaves of field-grown plants were similar to those of Xp and Xpf in previous greenhouse studies (1, 13, 23, 24, 42) and to those for bacteria inciting other plant diseases (14, 18, 22, 30, 38, 44, 48). In artifical inoculation studies the lag phase was probably longer than expected for natural inoculum due to the difference between bacteria grown in culture and in the plant. The exponential growth phase

consistently occurred for six to ten days and R10 and Ra had similar average doubling times of 19.4 and 18.8 hours, respectively. The doubling times are somewhat larger than values calculated from previous greenhouse data (13, 24, 42). During the exponential phase the bacteria responded to varying environmental conditions by changes in doubling time; this is evident in the direct effect of temperature. Populations of R10 and Ra showed little fluctuation after exponential growth ceased and population decline began only after leaves abscissed. Symptom production by R10 or Ra in bean leaves was a function of bacterial multiplication; minimal populations were required for symptoms to be produced. This is consistent with the findings of Ercolani (14). That lesion numbers significantly increase with higher peak bacterial levels per leaflet indicates the importance of bacterial population in the expression of disease severity.

The seedling phase of common and fuscous blights is the most critical phase of disease development since bacterial populations must become established to insure secondary spread. All above and below ground portions of seedlings grown from infected seeds were colonized immediately after seed germination and the primary leaves were the most important site for early bacterial multiplication. Bacteria on the hypocotyls and

cotyledons provided a reservoir of inocula to insure primary leaf infection. Abscission of Navy bean cotyledons can be rapid, thus decreasing their importance as a source of secondary inocula. Initiation of the secondary disease cycle has previously been linked to the appearance of oozing lesions on cotyledons and primary leaves (52), however, in this study secondary spread began prior to any seedling symptoms. In fact, typical symptoms were never detected on cotyledons of seedlings developing infected seed and primary leaf lesions occurred only during the early vegetative phase.

The population profile of R10 and Ra during the vegetative stage was described by a series of bacterial growth curves (Figs. 6 and 7), each curve described the state of the bacterial population at a given position in the leaf canopy. The profile indicates stepwise spread of Xp and Xpf into the bean leaf canopy. A correlation of the population profile to observations of symptom appearance provides an explanation for late disease development of common and fuscous blights in the field. Primary leaf infection initiates the sequence of upward and outward spread. As each trifoliolate leaf develops from the apical meristem, it is colonized by blight bacteria and a population gradient is established in the canopy. The prerequisite for a minimum bacterial population of at least 5×10^6 cells/20 cm^2 leaf tissue

for symptom development places a latent period requirement on the appearance of symptoms after a leaf is initially colonized. Symptoms appear first on the primary leaves and subsequently on the oldest to the youngest trifoliolate leaves. By the time bacterial populations in the lower leaves reach critical levels and symptoms appear, these leaves are hidden by the newly expanded foliage. This umbrella effect continues throughout the vegetative stage of plant growth since rapid leaf expansion (one leaf expanded per two to three days) continues until bloom. The pattern of disease development is evident in Figures 6 and 7 in that bloom, blight lesions caused by R10 and Ra were present only up to the fourth and fifth trifoliolate leaves, respectively, whereas 10-12 trifoliolate leaves were expanded. The same pattern was repeated in the series of leaves from the lateral stems. Field inspection of the plots from an overhead perspective indicated that the plants were disease-free, since only newly expanded leaves were observed; despite the absence of disease in the upper leaves they were heavily colonized by blight bacteria. Because Navy beans are determinate in growth habit, differentiation of new leaves from growing tips ceases at bloom; bacterial populations in the outermost portion of the leaf canopy reach minimal levels only after bloom and symptoms which develop are no longer camouflaged. The

high visibility of disease after bloom creates the effect of a sudden common blight or fuscous blight invasion. It is obvious, however, that the phenomena of late disease development is an artifact of how beans are inspected and rated for disease.

The results of this study contradict past assertions that unfavorable environmental conditions for blight early in the growing season account for late disease development (6, 7). Isolates R10 and Ra multiplied, spread, and induced disease symptoms in the Navy beans throughout all phases of plant growth in the field and bacterial growth rates were similar before and after bloom. Reports that beans are less susceptible during the vegetative phase compared to the reproductive phase also cannot account for late disease development since such reports represent differences in disease severity and not the presence or absence of symptoms. In this study all infected leaves were equally diseased before and after bloom.

Several methods of bacterial spread appeared most likely to account for the stepwise spread of Xp and Xpf into the bean canopy. Rain-splash dispersal of blight bacteria has traditionally been considered the most important dispersal mechanism. Definite blight lesions have been considered necessary for secondary spread to occur (50, 52); slimy masses of bacterial ooze from

blight lesions are washed away from leaves by rain water. In contrast, however, this study indicates that blight bacteria are available for secondary spread immediately after Xp and Xpf colonize a leaf and well before symptom appearance. Presymptomatic spread of other plant pathogenic bacteria is apparent (29, 30, 36). Isolates R10 and Ra were isolated several leaves higher in the leaf canopy substantially before lower inoculated leaves showed symptoms. The number of blight bacteria which can be washed from an infected leaf is directly proportional to the total leaf population. The greatest numbers of leaf-borne bacteria are washed-off during the stationary growth phase, however, a higher proportion of the total population is removed during the exponential phase. Laboratory and greenhouse measurements of leaf washing correlated fairly well with direct field measurements of rain-trapped bacteria; approximately 10-50% of the leaf population of Xp and Xpf can be removed by run-off rain water from rain. This corresponds to measurements of rain-splash and washing removal from plants of several other plant pathogenic bacteria (11, 15, 21, 30). The continued availability of large amounts of bacterial inocula emphasizes the importance and effectiveness of the rain-splash dispersal mechanism in the epidemiologies of common and fuscous blights. In general then, the blight population on leaves should not be viewed in a

traditional sense as a "closed dividing" population but rather as a "stem type" population (5) where sloughing of cells during rain-fall accounts for substantial emigration of bacteria from the leaf.

The greater availability of Xp and Xpf inocula during the exponential growth phase compared to the stationary phase may be explained by Leben's (30) suggestion, relative to *P. glycinea* in soybeans, that the bacteria are "glued down" in older lesions. Xp and Xpf can produce copious amounts of extracellular polysaccharide *in vitro* (26) and *in vivo* such polysaccharide comprises a portion of the bacterial ooze covering lesions. In leaves polysaccharide levels would be greatest at the population peak and as the infected tissue dried the bacteria would become more firmly bound in and on the leaf. The presence of mucoid substances has been suggested to play a role in the ease of removal of *P. lachrymans* from cucumber leaves (20, 21). Because mucoid material appears to enhance the survival (26, 47) of blight bacteria as well as other pathogenic bacteria (43), polysaccharide production might prepare Xp and Xpf populations for the harsh conditions experienced after leaf abscission. The hyperbolic state (29, 33) which Xp and Xpf enter after tissue necrosis, appears quite stable as indicated by the high survival of Ra and R10 in partially decomposed leaves.

Epiphytic survival and multiplication on surfaces of host and non-host plants has been described for several plant pathogenic bacteria (9, 10, 11, 27, 28, 29, 32, 35, 37) but not conclusively demonstrated for Xp and Xpf in relation to bean. The results of this study indicate that a portion of the blight population which is easily washed from the leaf appears to exist on the surface. This is indicated by the isolation of R10 and Ra by direct and indirect leaf prints on RAM and the consistent reduction in leaf population by treatment with bleach and UV light.

The ability of some pathogenic bacteria to multiply and spread from buds of annual plants to unfurling leaves is well documented for some disease (12, 31, 32) but not for common and fuscous blights. In this study bud colonization was an important mechanism for spread of Xp and Xpf into the leaf canopy. Both terminal and axillary buds were continually colonized by R10 and Ra (Figs. 6 and 7) over the entire sampling period; subsequently blight bacteria were usually isolated from leaves less than 1 cm long soon after expanding from the buds. Multiplication of R10 and Ra in the buds was detected; however, constant recolonization by blight bacteria in rain and dew water which runs down the petioles and into the axils appears more important in maintaining the buds infected. Blight bacteria were also consistently

isolated from flower buds and flowers; these bacteria no doubt play a role in the early colonization of pods as they develop from the flowers.

Burkholder (6) and Zaumeyer (50, 51) characterized the systemic phase of Xp, however the relative importance of systemic infection in the development of common and fuscous blights especially in Navy beans, is not fully understood. Haas (19) previously suggested that Xp does not systemically colonize Navy bean cultivars Sanilac, Seaway, and Seafarer. The results of the present study with R10 and Ra indicate that systemic colonization is an important component in the life histories of Xp and Xpf in Michigan Navy beans. Greater than 75% of the stems of field-grown Navy beans (cultivars Seafarer, Sanilac, and Tuscola) became colonized with R10 and Ra during 1976 and 1977. Vascular invasion occurs rapidly after a seedling is initially colonized; for example R10 and Ra were detected inside Sanilac stems only ten days after seedlings were spray inoculated. In addition to wounds and stomata, leaf and cotyledon scars appeared to be important points of entry.

The population profile of stem-borne (Figs. 8 and 9) blight bacteria was similar to the profile in the leaf canopy. The blight bacteria moved rapidly upward after they were injected into the stem and establish a population gradient with highest levels closest to the

ground and lowest near the growing tip. The average bacterial doubling times of 22.8 and 23.8 for R10 and Ra, respectively, were slightly greater than doubling times calculated for leaf populations and lesion formation required minimal populations of approximately 1×10^7 cells per 3.5 g tissue. Correlation of the population profiles of R10 and Ra in stems to symptom expression in the plant canopy supports the explanation for late disease development suggested by data from monitoring bacterial populations in leaves. Stem and leaf symptoms developing from systemic bacteria remained hidden during the vegetative stage of plant growth. By bloom, symptoms appeared only up to the third trifoliolate leaf and node; stem lesions were not widespread in the canopy until near pod maturity whereas, typical leaf symptoms were initially apparent by early pod fill.

The extent to which bean roots are colonized by blight bacteria has not been fully appreciated. Burkholder (6) observed masses of bacteria in the tap root and lateral roots, however, Zaumeyer (50) confirmed their presence only in the region where xylem development from exarch to the endarch condition. Stanek and Lasik (45) reported that Xpf colonized the bean rhizosphere for two weeks. The present study with R10 and Ra confirms a strong association between blight bacteria and the roots of Navy beans during natural

disease development. During the seedling phase most of the bacteria are epiphytes on the root surface, and over time the population is gradually reduced, possibly by competition from rhizosphere residents; isolate R10-S6 was detected for more than three weeks after emergence of infected seedlings. A second phase of root colonization occurs when Xp or Xpf moves systemically into the stem and subsequently downward into the root. The blight bacteria become distributed throughout the entire length of the root system and considerable population increase occurs (Figs. 8 and 9). Although most of the root population is internally borne throughout the vegetative and reproductive phases, a small portion (.03-0.8% of the total) resides epiphytically throughout the growing season. Possible sources of the epiphytic population are survivors from the seedling phase, bacteria washed from the leaf canopy and bacteria excreted from internal root populations.

The overall rate at which a Navy bean plant is colonized by blight bacteria is strongly influenced by the specific growth rate of the blight population on each infected leaf. Because the bacterial doubling time directly affects the leaf population during the exponential growth phase and the bacterial yield during the stationary phase (Fig. 4), the amount of inocula available for secondary spread, and the severity of leaf

symptoms will be indirectly influenced. Thus, if blight bacterial populations are multiplying slowly, there will be a reduction in amount of secondary inocula and initial inocula established on new tissue, as compared to higher multiplication rates. The lower initial inocula also will reduce the eventual bacterial yield on an infected leaf. The influence of inoculum levels on bacterial yield is apparent in several studies of *in vivo* multiplication of Xp and other pathogenic bacteria (14, 25, 30). The bacterial doubling time ultimately is under the control of environmental parameters and host physiology. Although environmental factors were not studied in detail, temperature strongly influenced doubling times, such that higher temperatures yielded lower doubling times. This supports previous reports that common and fuscous blights are more severe at higher temperatures (17, 39). Leaf physiology was equally important since senescent leaves were unable to sustain exponential phase populations regardless of population level. Thus, if the initial inoculum level on a leaf is too low or doubling time too large then symptoms might not be seen. In such a case the blight bacteria would appear to be in a resident phase (29).

In 1977, isolate Ra was more aggressive in colonization of Sanilac bean foliage than isolate R10. This is apparent in the higher leaf populations attained

(Figs. 6 and 7) and the greater amount of disease produced throughout the canopy. However, direct inoculation studies indicate that the virulence of the two isolates and *in vivo* doubling times are similar. The difference might be in a greater availability of secondary inocula of Ra than R10; consistently less R10 bacteria became rain-trapped than Ra bacteria. As indicated previously, lower inocula levels resulted in lower bacterial yield, thus less inocula for upward spread.

Although R10 was less aggressive in the leaf this was not seen relative to systemic colonization; R10 moved into and through stems of Seafarer and Sanilac beans as well as isolate Ra. The difference between the two isolates relative to foliage and stem colonization suggests ecological variation among blight isolates, where differences in bacterial survival on, spread to and colonization of healthy tissue would affect the apparent severity of disease in the field.

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PART III

PRIMARY INOCULA SOURCES OF COMMON AND FUSCOUS BACTERIAL BLIGHTS

INTRODUCTION

Common and fuscous bacterial blights, incited by *Xanthomonas phaseoli* (E.F. Sm.) Dows (Xp) and *X. phaseoli* var. *fuscans* (Burkh.) Starr and Burkh. (Xpf), respectively, are major diseases of Michigan Navy beans. Internally-infected seed is the main source of primary inoculum (15, 16) and at times Michigan seed stocks have been heavily contaminated with Xp and Xpf (2, 10). Disease control is based on seed certification programs to maintain clean seed stocks. Epiphytotics of common, fuscous and halo blights in the 1960's prompted expansion of the seed certification program in Michigan to its present form (2, 5, 6). Certification involves both a visual field inspection for blight symptoms and a laboratory seed test for internal bacterial contamination. Although this program has reduced seed-borne Xp and Xpf in Navy bean seed lots, outbreaks of common and fuscous blights persist and some seed fields are rejected annually for certification. The chronic occurrence of blight in Michigan suggests the

certification program is not detecting a high enough level of infection or other sources of infection have been overlooked.

Likely additional sources of primary inocula include externally-contaminated seed and plant refuse. Although the epidemiological significance of Xp and Xpf as seed surface contaminants has never been critically investigated, field observations and research on halo blight (3) suggest a possible role for such inoculum. Whether Xp or Xpf overwinters in Navy bean refuse in Michigan is unknown. Early circumstantial evidence suggested a possible role for overwintering in common blight epidemiology (7) and Schuster and Coyne (11, 12, 13, 14) reported overwintering of Xp and Xpf in bean refuse in Nebraska.

The purpose of this paper is to investigate the relative importance of various sources of primary inocula in the epidemiology of common and fuscous bacterial blights in Michigan Navy beans.

MATERIALS AND METHODS

Surface sterilization of bean seed. To study methods of surface sterilization, Navy bean seeds (cultivar Seafarer) were autoclaved for 2.25 hours, to eliminate internal microflora, and then infested with pulverized bean leaf dust. One hundred grams of seed were shaken in a flask with 0.1 g dry wt. leaf dust infected with three wild type isolates each of Xp and Xpf or rifampin resistant mutants R10 and Ra, and 0.1 ml of water to promote sticking. Infested seeds were shaken in a 1:1 commercial bleach-distilled water solution for varying times, rinsed in sterile distilled water, and individually incubated in test tubes with 3 ml of BYE on a rotary shaker for 96 hours. Growth in the tubes was used to indicate the effectiveness of the surface sterilization. Bleaching times of 30 seconds consistently eliminated all surface-borne blight bacteria, and 45 seconds usually eliminated all surface microorganisms; a 60 second treatment with bleach was selected as a standard throughout the study. Of 300

tubes containing individual infested seeds bleached for 60 seconds none became turbid, whereas, all tubes with untreated seeds did. When seeds were bleached for periods longer than 60 seconds, the seed coats often became wrinkled indicating that the bleach solution was being imbibed.

Externally contaminated bean seed. Seeds externally infested with bean dust containing Xp or Xpf each were assayed for their ability to serve as primary sources of inoculum. Double rows, six meters long, of infested or uninfested seeds were arranged in a randomized block design with five rows of clean seed separating the two treatments and double rows of corn separating blocks. The amount of disease was rated 49 and 56 days after planting using the Horsefall-Barret (4) scale. Minimal surface population of blight bacteria on seeds needed for successful transfer to seedlings was determined by infesting seeds with varying levels of R10 and Ra from zero to 1.6×10^5 bacteria seed. One hundred seeds were planted in a 0.4 m^2 plot and treatments were arranged in a randomized block design with at least 2:1 m between each plot. To determine if seed transmission occurred, leaves and stems were monitored beginning after bloom for R10 and Ra. Experiments were conducted at the Botany and Plant

Pathology Farm, MSU, East Lansing, Michigan, and the Saginaw Valley Bean and Beet Farm, Saginaw, Michigan. Samples of commercial seed lots were obtained from the Michigan Department of Agriculture (MDA), Michigan Crop Improvement Association (MCIA), or were personally collected from Michigan bean growers to assay for external contamination by Xp and Xpf. To assay the samples, healthy seeds (125 g) were shaken in 40 ml of BYE for one minute, the washings were incubated in 125 ml flasks on a rotary shaker at 25 C for 48-72 hours before centrifuging at 10,000 g for 15 minutes, and the pellet was resuspended in 0.25 of the original volume and injected into 15-day-old Manitou seedlings. Isolations were made from all plants 30-50 days after injection regardless of the presence of symptoms. All seed samples were also tested by the MDA Seed Testing Laboratory for internal blight contamination.

Experimental seed lots became externally infested with blight bacteria by mechanically threshing mature plants infected with common or fuscous blight. Populations of Xp or Xpf on the surface of bean seed were determined by shaking the seed in 0.01 M phosphate buffer (pH 7.2), 1 seed/ml buffer, and plating on YCA + cycloheximide (25 µg/ml) or RAM + cycloheximide (25 µg/ml). For samples with low surface populations,

the washings were passed through a 0.22 µm millipore filter and resuspended in 1 ml buffer before plating.

Internal seed infection. Several inoculation methods were used to generate internally infected seeds. Half-filled green pods were scratched along part of the dorsal suture or the side of the pods with a syringe containing 10^8 Xp or Xpf cells per ml or the bacteria were injected into the stem at the point of pedicel attachment. R10 and Ra were established systemically in bean stems by injecting a 10^8 cells per ml suspension into the cotyledon node of 20-day-old seedlings. Pods from all inoculated plants were hand harvested and individually opened. Visibly infected seeds were rated for disease severity on a scale of 1-4 where 1 = darkening in the hilum region (hilum spotted seed) and 4 = complete butter-yellow discoloration and shrivelling of the seed. Populations of R10 and Ra in infected seeds were determined by individually grinding five seeds of each infection type in a mortar and pestle with phosphate buffer and subsequently plating serial dilutions on RAM. Data were based on an average seed weight of 0.18 g.

Symptomless internally-infected seed. To assay for Ra or R10 in seed without visible symptoms, pods were hand-harvested, wiped with a damp cloth to remove the dust, individually opened, and the seeds removed with sterile forceps and placed in sterile test tubes.

Individual seeds or all seeds from a single pod were surface sterilized and incubated in 25 x 150 ml culture tubes containing 10 ml of BYE supplemented with rifampin (50 µg/ml) and cycloheximide (25 µg/ml), for five to seven days on a rotary shaker at 25 C. Bacteria from tubes which became turbid were streaked on RAM to confirm the presence of R10 and Ra. During these studies R10 infested seed was used as control to insure the reliability of the surface sterilization technique.

Overwintering of R10, Ra, and R10-S6 in bean refuse. The ability of Xp and Xpf to overwinter in bean straw was examined by wrapping stems or leaves naturally infected with R10, Ra or R10-S6 in double layer, fine mesh nylon. The samples were either buried 30 cm in field soil, or laid on the soil surface from October to June at the Botany and Plant Pathology Farms. To assay for viable bacteria a portion of the tissue was homogenized in phosphate buffer and samples plated on RAM (200 µg/ml rifampin) + cycloheximide (200 µg/ml) + PCNB (100 µg/ml) and sometimes streptomycin sulfate (250 µg/ml). Other portions of the tissue were first incubated in BYE containing the above chemical and then plated on solid media and injected into bean seedlings. In one experiment initiated in 1976, Seafarer beans were grown in a 3 x 7 meter plot and sprayed with R10 12 days after planting. By the end of the summer 85% of the

plants were systemically infected; the dead plants were left standing in the plot and the following June the stems were assayed for R10. Seafarer, Tuscola and Manitous beans were planted the following year in the same plot and later rated for disease.

RESULTS

Surface-infested seed as a source of primary inoculum. In 1975, plants grown from Xp and Xpf surface-infested seeds were significantly (P = 0.01) more diseased, 12% and 19.5% foliage infection, compared to 3.8% and 7.1% foliage infection in plants from uninfested seeds 48 and 55 days after planting, respectively. Similar results were obtained in 1976 using R10-infested seed. In 1977, experiments were conducted at two locations to determine the inoculum load necessary to yield infected plants (Table 1). For blighted plants to develop from externally infested seed, surface populations of 10^3-10^4 bacteria per seed were required. Similar results were obtained in greenhouse studies.

Commercial and experimental Navy bean seed lots were assayed to determine the populations of Xp and Xpf established on seed surfaces by mechanical threshing (Table 2). Populations of surface borne blight bacteria ranged from zero to more than 4×10^4 bacteria per seed; generally the surface population increased with the level

TABLE 1. Relationship between the surface populations of R10 and Ra on Navy bean seed and the development of blighted plants[a].

R10			Ra		
No. of bacteria/ seed[b]	Bean cultivar	Infected plots/ plots planted	No. bacteria/ seed	Bean cultivar	Infected plots/ plots planted
0	Tuscola	0/8	0	Tuscola	0/4
0	Seafarer	0/8	0	Seafarer	0/4
$0\text{-}10^1$*	Seafarer	0/8	5.00×10^1	Tuscola	0/4
$10^1\text{-}10^2$*	Seafarer	0/4	1.28×10^2	Seafarer	0/4
6.25×10^1	Seafarer	0/4	5.52×10^2	Seafarer	0/4
7.23×10^2	Tuscola	0/4	1.03×10^3	Tuscola	1/4
7.90×10^2	Seafarer	0/4	1.48×10^3	Seafarer	1/4
3.15×10^3	Tuscola	1/8	1.04×10^5	Tuscola	2/4
5.70×10^3	Seafarer	1/4	4.45×10^5	Seafarer	3/4
3.39×10^4	Tuscola	1/8			
7.80×10^4	Tuscola	2/4			
1.60×10^5	Seafarer	2/4			

[a]Data of Ra and R10 are pooled from experiments conducted at the Saginaw Valley Bean and Beet Farm, Saginaw, Michigan, and Department of Botany and Plant Pathology Farm, East Lansing, Michigan. One hundred seeds were planted in a 0.4 m^2 plot and treatments were arranged in a randomized block design with at least 2.1 m between each plot.

[b]*indicates seeds naturally infested by mechanical threshing; all other seeds were infested with 0.1 g of R10 or Ra infected bean dust per 100 g seed and 0.1 ml H_2O to promote sticking.

TABLE 2. Surface populations of blight bacteria on mechanically threshed Navy bean seed.

Seed lot	No. bacteria/seed[a]					Average % foliage infection	Isolate
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
Experimental:							
Seafarer (1974)	0.0	2.9×10^4	6.6×10^2	1.3×10^3	0.0	80-90	Xp-Xpf
Seafarer (1975)	4.0×10^4	4.7×10^2	0.0	0.0	0.0	80-90	Xpf
Seafarer* (1976)	0.7	2.4	2.9	2.2	87	10	R10
Seafarer* (1976)	0.6	0.3	3.3	0.1	2.4	5	R10
Sanilac (1977)	2.5×10^3	0.0	0.0	0.0	3.6×10^2	20-40	R10
Sanilac (1977)	2.0×10^2	0.0	1.3×10^4	7.0×10^2	0.0	30-60	Ra
Commercial:							
Seafarer (1976)	5.0×10^3	0.0	0.0	0.0	0.0		Xpf
Sanilac (1977)	0.0	1.4×10^4	9.2×10^2	0.0	0.0		Xp

[a]Surface populations were assayed by shaking 50 seeds in 50 ml phosphate buffer for two minutes and plating the washings on YCA + cycloheximide (25 µg/ml) or RAM + cycloheximide (25 µg/ml); * indicates samples where washings were concentrated by filtering through a 0.22 µm Millipore filter and resuspending in 1 ml buffer.

of disease detected in the field prior to harvest. The bacterial populations in several samples from a single seed lot were quite variable indicating that bacteria are not uniformly distributed over the seeds.

Of 192 commercial seed lots from three growing seasons, 26 were externally infested with Xp or Xpf and seven with *Pseudomonas phaseolicola* (Pp) (Table 3). Results of tests for external blight bacteria in MDA and MCIA samples from 1976 and 1977 were compared to tests by the MDA Seed Testing Lab on the same samples; 14% of the lots were externally infested with Xp or Xpf whereas only 11% were positive for blight by the MDA tests; 2% of the samples reported blighted by the MDA were not externally infested. The MDA seed testing does not distinguish common and fuscous and halo blight infection. Two certified seed samples from 1976 and one from 1977 reported blight-free by the MCIA were externally infested and one of the 1976 samples produced blighted plants when planted in the field in 1977.

Navy bean seeds became externally contaminated during pod infection as well as during mechanical threshing. Firstly, when isolates R10 and Ra were inoculated to the dorsal pod sutures of Seafarer and Sanilac beans, seeds were recovered which appeared healthy except for a faint yellow halo around the hilum region. The yellowing varied in its intensity from

TABLE 3. Frequency of surface blight contamination in commercial Navy bean seed lots.

Year	Source[a]	Total Samples	No. externally infested by			Internally[b] infected	Infected but not[c] infected
			Xp	Xpf	Pp		
1975	Personally collected	24	1	1	0		
1976	MDA	58	8	4	3	11	3
	MCIA	46	2	1	2	1	0
1977	MDA	30	4	2	1	4	0
	MCIA	34	2	1	0	2	1

[a] All samples were from commercial seed lots; MDA = samples submitted to Michigan Department of Agriculture; MCIA = samples collected by Michigan Crop Improvement Association from fields requesting certification.

[b] Results of the Michigan Department of Agriculture's standard test for internal seed borne blight bacteria. Both the MDA and MCIA seed lots were tested by the seed testing lab of the MDA.

[c] No. of samples positive for blight by MDA seed test and negative by assay for external blight bacteria.

quite visible to barely visible under the dissecting microscope. Of 25 and 50 Seafarer seeds with the halo from 1976 and 1977 pod inoculations, R10 was isolated from 11 and 30, respectively. Bleaching surface-sterilization eliminated the bacteria from an equal number of the same seeds. Isolate Ra was isolated from 21 of 30 Sanilac seeds exhibiting the halo and Ra was not isolated from bleached seeds. By pressing the seeds on RAM the bacteria were found to be present only in the halo region; populations of 2.6×10^4-4.5×10^5 bacteria were associated with this region. Secondly, seeds with masses of bacterial ooze adhering to the seed coat were detected from pods inoculated in the suture or side and from commercial and experimental seed lots. The bacterial populations usually ranged from 10^5-10^7 per seed. The seeds became contaminated as a result of inner pod infection. Both of the above types of seed produced infected plants when planted in the field and greenhouse.

Internal seed infection and bacterial populations. Navy bean seeds displayed a variety of symptoms due to internal infection by Xp and Xpf. The severity of symptoms ranges from a slight darkened spot in the hilum region (hilum spot) to complete butter-yellow discoloration and shrivelling of the seed coat. Populations of

Xp and Xpf inside infected seeds increased relative to the severity of seed symptoms. The mean bacterial population ± standard error of the mean per seed with disease ratings of 1-4 was $1.0 \times 10^6 \pm 4.0 \times 10^5$, $3.4 \times 10^8 \pm 1.1 \times 10^8$, $1.5 \times 10^9 \pm 3.6 \times 10^8$, and $4.2 \times 10^8 \pm 1.3 \times 10^8$ for R10, respectively, and $8.5 \times 10^5 \pm 3.1 \times 10^5$, $2.7 \times 10^8 \pm 8.9 \times 10^7$, $9.0 \times 10^8 \pm 2.5 \times 10^8$, and $4.4 \times 10^8 \pm 1.5 \times 10^8$ for Ra, respectively. A population of 1.8×10^5 bacteria was the lowest detected in any hilum spotted seed. Hilum spotted seeds were the predominant type in 30 commercial and experimental seed lots; 123 hilum spotted seeds were detected compared to only 17 seeds with the varnish-yellow seed coat discoloration. R10 and Ra were isolated from 50 of 50 seeds with butter-yellow discoloration, selected from inoculated pods, while R10 and Ra were isolated from 60% and 64%, respectively, of hilum spotted seeds. The germination rate and quality of the seedling produced was affected by the severity of seed infection (Table 4). Hilum spotted seeds (Type 1) were most similar to uninfected seed in germination and the seedlings produced were generally not deformed. Seeds with type 2-3 infection produced mostly deformed seedlings, characterized by reduction in overall seedling size and absence or reduction in size of primary leaves; type 4

TABLE 4. Effect of seed infection by R10 and Ra on seedling growth[a].

Infection type[b]	No. of seeds planted		% germination		% seedlings deformed[c]		Average fresh wt. of seedlings (g)		% seedlings infected[d]	
	R10	Ra	R10	Ra	R10	Ra	R10	Ra	R10	Ra
Uninfected	40	33	100	97	0	0	1.21	1.27	0	0
1	40	30	95	97	3	3	1.06	1.16	60	50
2	34	9	91	100	65	45	0.83	0.68	100	90
3	35	11	63	64	71	57	0.56	0.58	90	100
4	25	8	28	13	100	100	0.33	0.46	100	100

[a] Seeds were planted in flats containing a 1:1 greenhouse soil, vermiculite mix; and data were collected seven days after planting. Data represent pooled data from three separate experiments.

[b] Infection types: 1 = seed with discoloration in the hilum region (hilum spotted seed); 2 = seed with less than 10% butter-yellow discoloration; 3 = seed with 11%-complete butter-yellow discoloration and no shrivelling; 4 = complete butter-yellow discoloration and partial seed shrivelling.

[c] Deformed seedlings were characterized as a stunting of the plants and an absence or reduction in the size of the primary leaves.

[d] % seedlings infected was determined by individually homogenizing ten plants of each infection type and plating on RAM + cycloheximide.

seeds which germinated were always seriously deformed. Isolate R10 was recovered from 43% of 28 seedlings grown from hilum spotted seed planted in the field.

That minimal populations of blight bacteria are required for seed symptoms suggests the existence of symptomless Navy bean seed containing low levels of blight bacteria. Isolates of Ra and R10 were recovered from inside seed showing no visible symptoms or any discoloration of the seed coat (Table 5). Symptomless, but internally infected, seed resulted when pods were inoculated with R10 or Ra along the pod suture or when bacteria invaded the plant systemically; no pod symptoms were present in the latter case.

Relation of pod symptoms and seed symptoms. Bacterial blight lesions on Navy bean pods developed both as a result of infection by external inoculum and by systemic borne bacteria. Visibly infected seeds were always associated with pods bearing some form of lesion (Table 6). Pod infection resulting from external inoculum produced typical irregular water soaked lesion and in the present study this was produced by lightly scratching the pod suture. Only when the lesion included a portion of the dorsal suture were seeds with the butter-yellow discoloration produced. Pods on plants which were systemically-infected developed either water soaked lesions in the suture region or minute hairline lesions

TABLE 5. Detection of R10, R10-S6, and Ra in symptomless Navy bean seeds[a].

Cultivar	Blight isolate	Site of inoculation[b]	No. seeds tested	No. infected
Seafarer (1976)	R10	S	200	3
Tuscola (1977)	R10	S	56	1
Seafarer (1977)	Ra	S	105	0
Sanilac (1977)	Ra	S	150	2
Seafarer (1978)	R10	C	310	1
Seafarer (1978)	Ra	C	250	1

[a] Seeds were assayed by surface sterilizing individual seeds or all seeds from a pod and then incubating the seeds in 25 x 150 ml culture tubes containing 10 ml of BYE + rifampin (50 g/ml) and cycloheximide (50 g/ml) on a rotary shaker for five to seven days.

[b] S = seeds from pods inoculated with R10, R10-S6 or Ra along the suture of half filled green pods; C = seeds from pods of plants inoculated at the cotyledon node with R10 and Ra 20 days after planting.

TABLE 6. Relation between pod symptoms and seed symptoms.

Cultivar	Isolate	No. pods sampled	Site inoculated[a]	No. pods with lesions	No. infected pods with infected seed	No. pods with hair-line pod symptoms[b]	Symptomless pods with visibly infected seed
Seafarer (1975)	Xpf	428	Pedicel	13	6	3	0
	Xp	400	Pedicel	15	4	1	0
	Xpf	431	Suture	431	401	0	0
	Xp	413	Suture	413	396	0	0
Seafarer (1976)	R10	219	Pedicel	5	2	1	0
	R10	186	Suture	180	133	0	0
Tuscola (1977)	Xp	364	Natural infection	15	6	3	0
Seafarer (1978)	Ra	293	Cotyledon scar	20	2	1	0
	R10	375	Cotyledon scar	12	3	1	0

[a] Pedicels were inoculated by injecting with a syringe a 10^8 cell/ml suspension of blight bacteria into the stem where the pedicel attaches to the stem; sutures were inoculated by scratching along the dorsal pod suture with the tip of a syringe containing 10^8 cells/ml; common blight in the 1977 Tuscola beans developed from natural infection; cotyledon scars of 20-day-old plants were inoculated with a 10^8 cell/ml suspension of bacteria using a syringe.

[b] Hair-line symptoms indicate pods with visible symptoms only along the suture which appeared as fine short bands of darkened tissue; some of the lesions required close examination with the dissecting microscope to detect.

confined entirely to the suture (Table 6). The hairline symptoms were characterized by a thin darkened band of tissue several millimeters long, along the side of or in the dorsal suture. When the suture was split the darkening was more evident on the inner portion of the suture. Extremely close visual and sometimes dissecting microscope examination was required to detect the discoloration and with one Tuscola pod no discoloration appeared on the outside of the suture and only a small spot on the inside. The hair-line symptom was confined to the pedicel end of the pod and the hilum spot was on the first or second seed in the pod.

Pod symptoms became camouflaged when mature pods remained in the field for several days during cool moist weather; fungi really colonized the pod surface and caused a darkening of the pod.

Overwintering of R10, R10-S6, and Ra in bean refuse. Isolates R10, R10-S6 and Ra were not detected in bean stem and leaf tissue which was buried in, or laid on, field soil during the winters following the 1975, 1976 and 1977 growing seasons. No R10 was isolated from systemically infected plants left standing in the winter following the 1976 growing season and beans planted in the same plot the following season did not become blighted.

DISCUSSION

Various types of seed-borne Xp and Xpf were evaluated relative to their role in the epidemiology of common and fuscous bacterial blights of Navy beans. Seeds with typical blight symptoms have generally been considered the main sources of primary inocula. This study conclusively demonstrated that seeds externally-infested with Xp or Xpf can serve as primary inocula. Contamination mainly occurs during the threshing process when bacteria from dried bean tissue become air-borne in bean dust. R10 was routinely isolated on RAM when plates of the medium were situated around the thresher during threshing of R10 infected plants. The source of the bacterial contamination is mainly stems and pods and to a lesser extent leaves since most of the foliage drops prior to harvest. Bacterial populations of 10^8 bacteria per g dried tissue are commonly found in stems (Part 2). Populations of blight bacteria on seed surfaces varied over a ten thousand fold range, however, minimal inoculum levels of 10^3-10^4 bacteria per seed

were required for transfer of the bacteria from seed to seedlings. The natural minimal threshold might be somewhat lower, since, in this study, seeds were uniformly coated with bacteria. On the other hand, bacterial populations detected in replicate samples of a seed lot varied considerably, suggesting that the blight bacteria were concentrated over a few seeds and on a smaller area of the seed coat. Besides contamination from mechanical threshing, seed may be contaminated on the surface during pod infection; the infestation is apparent as light yellow halos around the hilum or a crust of yellow bacterial ooze glued to the seed coat. Tests on commercial seed lots indicated that 14% were externally contaminated with Xp or Xpf compared to only 11% reported internally infected by the MDA Seed Testing Laboratory; the MDA's estimates may have also included positive reactions due to Pp. Thus, it is likely that some external-borne blight bacteria are a source of primary blight inoculum in Michigan Navy bean fields.

The detection of Pp as a surface contaminant was of interest in that while Navy beans are susceptible to halo blight in the greenhouse (8) the disease has never been observed in Michigan Navy bean fields (10). The present study indicates that Navy beans may serve as an important reservoir for Pp and the bacteria may be spreading to susceptible Kidney beans.

The Michigan seed certification program assumes a close correlation between pod symptoms and internal seed infection such that rejecting seed fields with visible pod blight eliminates seed infection. On the other hand, Burkholder (1) detected typically common blight-infected seeds in symptomless pods. Analysis of hundreds of Navy bean pods from four separate growing seasons indicates a prerequisite of visible pod infection for production of visibly-infected seed. The relationship between pod and seed infection is most apparent when pods are infected by external inocula, as was done by scratching the pod surface; the infected seeds then appear under the lesion. However, when Xp and Xpf systemically infect the suture, the relation between seed and pod symptoms is much less obvious. Systemically-infected pods may display only a faint hair-line darkening along the suture and normal field inspections would seldom detect these pod lesions. Furthermore, considerable pod molding occurs under cool moist conditions at harvest which eliminates the most distinctive pod blight symptoms. Hilum-spotted seed appears to be the most important type of visible seed infection in blight epidemiology because of its higher frequency in seed lots compared to more severely infected seed. Further, bacteria are transferred from hilum-spotted seed to seedlings with high frequency, and

germination and seedling growth are similar to that of uninfected seed. On the other hand, more severely infected seed (types 2-4) germinate at lower rates or produce deformed seedlings, thus reducing the effectiveness as primary inocula sources.

The present study is the first report that symptomless Navy bean seed contain low populations of blight bacteria. Such seed is produced not only in visibly infected pods but also in symptomless pods as a result of systemic bacterial movement from the stem. Accurate populations could not be determined but it can be assumed that symptomless seed contains less than 10^5 cells/seed. Symptomless seed is almost impossible to detect by current testing methods because of the low frequency in seed lots and low populations within the seed. In the present study such seeds were detected only by assaying seeds from plants inoculated with R10 and Ra.

The results of the present study indicate that the occurrence of common and fuscous blights in Michigan Navy beans may be the result of a cumulative effect of several types of externally infested and internally infected seed. Because of the multiple sources of seed inocula and difficulty in detection of Xp and Xpf in visual field inspection and in seed lots it appears that the perennial outbreaks of blight are inevitable in

Michigan and minimal levels of disease incidence must be expected. In light of this study, perhaps the term certified "disease-free" is misleading and precarious for seed growers who are liable for the purity of their product. Current seed testing techniques (9) can assure only a minimal threshold of infection in any given seed lot.

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