Rifampin-Resistant Xanthomonas phaseoli var. fuscans and Xanthomonas phaseoli: Tools for Field Study of Bean Blight Bacteria

David M. Weller and A. W. Saettler

Graduate Student, Department of Botany and Plant Pathology, and Research Plant Pathologist, Bean Disease Investigations, Agricultural Research Service, U.S. Department of Agriculture, Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824.

Cooperative Investigations of Agricultural Research Service, U.S. Department of Agriculture, and Michigan State University, East Lansing.

Michigan Agricultural Experiment Station Journal Series Article 8010.

This paper reports the results of research only. Mention of a pesticide or proprietary product in this paper does not constitute a guarantee, warranty, or recommendation by the U.S. Department of Agriculture nor does it imply registration of the pesticide under FIFRA (the Federal Insecticide, Fungicide, and Rodenticide Act).

Accepted for publication 18 November 1977.

ABSTRACT

WELLER, D. M., and A. W. SAETTLER. 1978. Rifampin-resistant Xanthomonas phaseoli var. fuscans and Xanthomonas phaseoli: Tools for field study of bean blight bacteria. Phytopathology 68: 778-781.

Rifampin-resistant mutants of *Xanthomonas phaseoli* (Xp) and *X. phaseoli* var. *fuscans* (Xpf) were screened for similarity to wild-type isolates and their utility as tools for field study of bean blight bacteria. A mutant (R10) isolate of Xpf and one (Ra) of Xp were similar to wild types in numerous bacteriological tests, grew 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. The rifampin-resistance marker permitted selective isolation of R10 and Ra from field-grown inoculated bean leaves; growth of all phyllosphere bacteria was inhibited on media with rifampin (50 μg/ml). Addition of cycloheximide at 25 μg/ml reduced growth of resident yeasts and fungi. The rifampin resistance marker was stable when the bacteria were grown in culture or beans; no reversions to rifampin sensitivity have been detected.

Common blight and fuscous blight of bean (*Phaseolus vulgaris* L.), which are incited by *Xanthomonas phaseoli* (E. F. Sm.) Dows. and *X. phaseoli* var. *fuscans* (Burkh.) Starr and Burkh. (*Xanthomonas campestris*) (2), respectively, are serious diseases of dry edible beans in many areas of the world. *Xanthomonas phaseoli* (Xp) and *X. phaseoli* var. *fuscans* (Xpf) essentially are identical pathogens and are distinguished only on the basis of brown pigment production by Xpf in certain culture media. The lack of culture media for the selective isolation of Xp and Xpf from field material has limited basic epidemiological studies on bacterial blights.

Incorporation of antibiotic resistance into Xp and Xpf, which would allow isolation of the bacteria from inoculated plant tissue plated on antibiotic-containing media, would be useful for studying the population dynamics of Xp and Xpf under field conditions. Antibiotic resistance occasionally has been used in ecological studies of plant pathogenic bacteria. Gowda and Goodman (5) and Lewis and Goodman (8) used a strain of *Erwinia amylovora* which was resitant to streptomycin in greenhouse studies of fire blight. Hsieh et al. (6) used streptomycin resistance to follow infection of rice seed by *Xanthomonas oryzae*. Stall and Cook (12) studied the multiplication of *Xanthomonas vesicatoria* in susceptible and resistant peppers with a streptomycin-resistant mutant. Rifampin resistance recently has been used in ecological studies of *Agrobacterium tumefaciens* (1, 9).

This paper: (i) compares some characteristics of rifampin-resistant Xp and Xpf mutants with those of the wild types, and (ii) documents the usefulness of a mutant as a tool for studying Xp and Xpf under field conditions. A preliminary account of this work was published previously (14).

MATERIALS AND METHODS

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 of methanol and diluted with distilled water to 10 ml; the solution was passed through a fritted-glass bacterial filter and added to 190 ml of autoclaved yeast extract-calcium carbonate agar (YCA: 10 g yeast extract, 15 g agar, and 5 g $CaCO_3$ per 1,000 ml of water). The RAM sometimes was supplemented with filter-sterilized cycloheximide at 25 μg/ml in distilled water to reduce fungal growth. 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. The rifampin-resistant isolates were subcultured twice on RAM and then stored in 40% aqueous glycerol at −10 C.

00032-949X/78/000 131$03.00/0
Copyright © 1978 The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, MN 55121. All rights reserved.

Physiological tests were performed as described by Dye (4), with the following modifications: (i) pigment production by the 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 of yeast extract, 10 g of dextrose, 15 g agar, and 5 g of $CaCO_3$ per 1,000 ml water).

Bean plants were grown in a greenhouse at 23 C under 14 hr of supplemental lighting each day from 0600 to 2100 hours. These plants were inoculated by one of three methods: (i) a bacterial suspension (5×10^8 cells/ml) was injected into stems of 10-day-old kidney bean seedlings (cultivar Manitou) (10), (ii) the undersurfaces of kidney bean leaves were sprayed (at a pressure of 1.2 kg/cm^2) to a water-soaked appearance with a bacterial suspension (10^8 cells/ml (11), and (iii) a bacterial suspension (5×10^7 cells/ml) was lightly sprayed to runoff on the leaf. The virulence of each isolate was tested by seedling injection and rated after 7 days on a 0-3 scale: 0 = no symptoms; 1 = stem lesion and primary leaves partially collapsed; 2 = stem lesion and primary leaves completely collapsed; 3 = stem lesion and primary leaves collapsed and apical meristem dead. Leaves that were water-soaked were rated on a 0-10 scale where 0 = no symptoms and 10 = total leaf necrosis.

Navy (pea) beans (cultivar Seafarer), planted in the field, were sprayed to runoff with a knapsack sprayer containing an aqueous suspension of 5×10^7 cells/ml.

To reisolate bacteria from greenhouse-and field-grown plants, leaves either were homogenized for 2.5 min in 0.01 M phosphate buffer (pH 7.2) with a Waring Blendor, or shaken for 2.5 min in phosphate buffer. Serial dilutions of these suspensions were spread on agar plates, and colonies were counted after 4-days of incubation at room temperature.

RESULTS

The seedling injection 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, and two of three of both Xp 11 and Xp 21 received the same virulence rating as the wild types. One rifampin-resistant isolate of Xpf 16 (designated R10), one of Xp 11 (designated Ra), and one of Xp 21 (designated Rd) were selected for further screening. All three isolates were resistant to greater than 500 μg/ml rifampin. 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). Isolates R10 and Ra also produced as much disease as the wild types on leaves and pods of field-grown Navy beans. All 11 Xp and Xpf rifampin-resistant mutants retained cultural characteristics such as colony size, colony shape, slime formation, and yellow and brown pigment formation typical of their respective wild types. The responses of R10 and Ra to 14 standard physiological tests were identical to those of their wild types.

The in vitro doubling times of R10 and Ra were approximately 11% longer than those of the wild types in buffered yeast extraxt (10 g of yeast extract per liter of phosphate buffer) shake culture at 25 C; however, in vivo growth rates of R10 and Ra in primary leaves of greenhouse grown Navy (pea) beans (cultivar Seafarer) were identical to those of the wild types (Fig. 1 and 2).

Rifampin resistance in R10 and Ra was stable; no revertants to rifampin sensitivity were detected by replica-plating after 16 and 11 consecutive transfers of R10 and Ra, respectively, on YCA; after each transfer 10 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 (pea) beans 60 days after inoculation with R10 and Ra in separate plots were rifampin-resistant.

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 were homogenized in 10 ml of phosphate buffer and 0.1 ml of a dilution series was plated on each medium to yield a 10^{-1} to 10^{-4} dilution of the homogenate. Phyllosphere bacteria were completely inhibited on RAM and the R10 bacteria were recovered. Colonies of phyllosphere bacteria prevented detection of R10 colonies on YCA except at the 10^{-4} dilution 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. The concentration of rifampin and cycloheximide could be increased 10- and 20-fold, respectively, without decreasing R10 recovery.

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 Seafarer bean

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[b] at			
	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)			.7	1.5
Xp 21			2.6	4.8
Rd			1.2	1.8
LSD (P = 0.05)			.7	1.0

[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. 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.

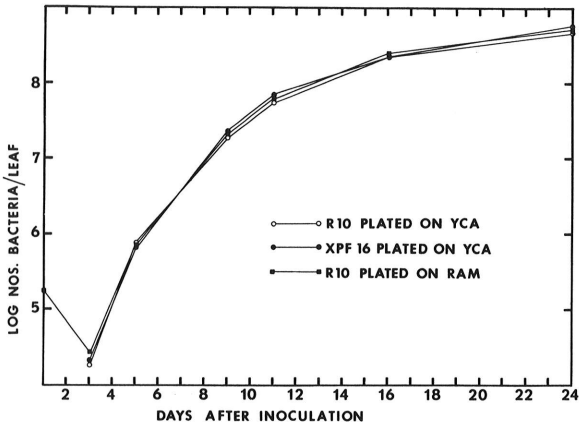

Fig. 1. Growth of rifampin-resistant *Xanthomonas phaseoli* var. *fuscans*, R10, and wild-type Xpf 16 on primary leaves of greenhouse-grown Navy (pea) beans (cultivar Seafarer). Fourteen-day-old plants were lightly sprayed to runoff with an aqueous suspension (5×10^7 cells/ml) of R10 or Xpf 16. The bacterial 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.

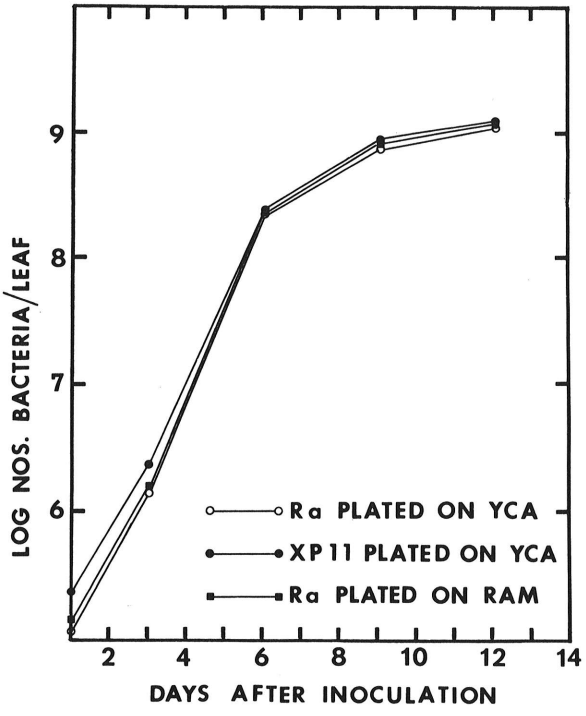

Fig. 2. Growth of rifampin-resistant *Xanthomonas phaseoli*, Ra, and wild-type Xp 11 in primary leaves of greenhouse-grown Navy (pea) beans (cultivar Seafarer). Thirteen-day-old plants were lightly sprayed to runoff with an aqueous suspension (5×10^7 cells/ml) of Ra or Xp 11. The bacterial populations 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.

plants were sprayed to runoff with an aqueous suspension of R10 (5×10^7 cells/ml). Bacteria were isolated from first and second trifoliolate leaflets homogenized with 75 ml of phosphate buffer; each sample was replicated four times. The following populations of R10 per leaflet (average leaflet area: 20 cm^2) were detected 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. Isolate R10 was isolated easily and in large numbers from the surface of symptomless inoculated and noninoculated leaves by pressing them on RAM. No R10 revertants were detected by replica plating at the end of the sampling period when tissue with distinct lesions was plated on YCA.

DISCUSSION

Rifampin was selected for use in an antibiotic-resistance selection system because of its wide spectrum of antibacterial activity and high toxicity (3, 7, 13). 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 usefulness 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 that R10 and Ra adequately model several important aspects of wild-type activity and they should behave similar to Xpf 16 and Xp 11 under natural conditions. Multiplication and disease production by R10 and Ra were identical to those of the respective wild types in bean leaves. The mutation in R10 and Ra was stable when the bacteria were grown in culture or bean leaves. Mutation stability assures the usefulness of the mutants throughout season-long studies.

That the rifampin mutant can be selectively isolated and its growth in field-grown Navy beans monitored over several weeks indicates its potential as a tool 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.

LITERATURE CITED

1. ANDERSON, A. R., and L. W. MOORE. 1976. Survival of Agrobacterium in soil and on pea roots. Proc. Am. Phytopathol. Soc. 3:258 (Abstr.).
2. BUCHANAN, R. E., and N. E. GIBBONS, (eds.). 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams and Wilkins, Baltimore. 1,268 p.
3. CORCORAN, J. W., and F. E. HAHN. 1975. Antibiotics III: Mechanisms of action of antimicrobial and antitumor agents. Springer-Verlag, New York-Heidelberg. p. 252-268.
4. DYE, D. W. 1962. The inadequacy of the usual determinative tests for the identification of Xanthomonas spp. N. Z. J. Sci. 5:393-416.
5. GOWDA, S. S., and R. N. GOODMAN. 1970. Movement and persistence of Erwinia amylovora in shoot, stem, and

root of apple. Plant Dis. Rep. 54:576-580.

6. HSIEH, S. P. Y., I. W. BUDDENHAGEN, and H. E. KAUFFMAN. 1974. An improved method for detecting the presence of Xanthomonas oryzae in rice seed. Phytopathology 64:273-274.
7. KUNIN, C. M., D. BRANDT, and H. WOOD. 1969. Bacteriological studies of rifampin, a new semisynthetic antibiotic. J. Infect. Dis. 119:132-137.
8. LEWIS, S. M., and R. N. GOODMAN. 1965. Mode of penetration and movement of fire blight bacteria in apple leaf and stem tissue. Phytopathology 55:719-723.
9. MOORE, L. W. 1977. Prevention of crown gall on prune roots by bacterial antagonists. Phytopathology 67:139-144.
10. SAETTLER, A. W. 1971. Seedling injection as an aid in identifying bean blight bacteria. Plant Dis. Rep. 55:703-706.
11. SCHUSTER, M. L. 1955. A method for testing resistance of beans to bacterial blight. Phytopathology 45:519-520.
12. STALL, R. E., and A. A. COOK. 1966. Multiplication of Xanthomonas vesicatoria and lesion development in resistant and susceptible peppers. Phytopathology 56:1152-1154.
13. WEHRLI, W., and M. STAEHELIN. 1971. Actions of the rifamycins. Bacteriol. Rev. 35:290-309.
14. WELLER, D. M., and A. W. SAETTLER. 1976. Rifampin-resistant Xanthomonas phaseoli var. fuscans: A tool for selective study of bean blight bacteria. Proc. Am. Phytopathol. Soc. 3:269 (Abstr.).