

# Effect of Pure and Mixed Suspensions of Virulent and Heterologous Isolates of *Xanthomonas campestris* on the Infectivity of the Inoculum on Two Species of *Phaseolus*<sup>1</sup>

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## ABSTRACT

The virulence of *Xanthomonas campestris* pv. *phaseoli* (Xcp) in mixed suspensions with a closely related heterologous soybean pathogen of *X. campestris* pv. *glycines* (Xcg) was studied. Differences produced on pods by inoculation of mixed suspensions of Xcp depended on host susceptibility and on the relative proportion in the suspension. Suspensions of Xcp mixed with Xcg showed decreased symptom development which did not depend on host susceptibility, or on the relative proportion of the pathogens in the suspension.

## INTRODUCTION

Many inoculation techniques have been utilized to identify sources of resistance to *Xanthomonas campestris* pv. *phaseoli* (E. F. Smith) Dowson (Xcp) (= *X. phaseoli*), but the wide range of strain virulence on beans complicated such studies. For this reason, horizontal resistance seems to be one of the best measures of control for this pathogen. In order to identify germplasm with this resistance, suspensions containing strain mixtures of the pathogen have been used for inoculation by several workers (4, 11). However, there has been a lack of information about the effect on the inoculum infectivity when several strains are used in mixed suspensions in the laboratory.

A first step of defense in a plant might be the capacity to maintain an epiphytic flora antagonistic to the pathogen. Teliz and Burkholder (18) found that the Xanthomonads were more sensitive than the Pseudomonads to *P. fluorescens* obtained in dilution plates as contaminants during isolation of *X. fuscans* from diseased bean plants. In addition, various forms of antagonism, such as bacteriocin production (17) may affect the viability of different strains in the inoculum.

The objectives of this study were 1) to determine the effect on virulence of the inoculum when two of the most virulent isolates, *X. c.* pv. *phaseoli* (Xcp) and/or *X. c.* pv. *phaseoli fuscans* (Burkh.) Starr and Burk. (Xcpf)

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(=*X. phaseoli* var. *fuscans*) were mixed in suspension in different proportions and inoculated on pods of the two species, *Phaseolus vulgaris* L. (common bean) and *P. coccineus* L. (scarlet runner bean), and 2) to determine the effect of *X. c.* pv. *glycines* (Xcg), an isolate heterologous on bean but closely related to Xcp and Xcpf, on infectivity of Xcp and/or Xcpf, pods of *P. vulgaris* and *P. coccineus*, and on leaves of *P. vulgaris*.

## MATERIALS AND METHODS

### BACTERIAL ISOLATES

The isolates used belong to the bacterial collection at the USDA Tropical Agricultural Research Station (TARS), Mayagüez, Puerto Rico. Isolates *X. campestris* pv. *phaseoli* (Xcp) 820 and *X. campestris* pv. *phaseoli fuscans* (Xcpf) 144, were selected for their high virulence on *P. vulgaris* (21). Isolate *X. campestris* pv. *glycines* (Xcg) 907 was selected because it is avirulent on *P. vulgaris* and is closely related to Xcpf.

The pathogens were cultured on YDC agar medium containing 10 g dextrose, 2.5 g calcium carbonate, and 20 g agar per liter. The cultures were maintained at 26°C for 48 h. The cell suspension was prepared with sterilized distilled water and standardized to 10<sup>9</sup> cells/ml with a spectrophotometer at 625 nm and 10% transmission.

### PLANT MATERIAL

*Phaseolus vulgaris* pods. The cultivars used were La Vega (black seed) and W-117 (white seed). The seeds were treated with streptomycin powder and planted in 15-cm plastic pots containing a sterile soil mixture. Twenty-five plants were maintained in the greenhouse until six to 11 uniform green pods were collected for each treatment.

The four bacterial suspensions tested were pure suspensions of Xcp and Xcg, and mixed suspensions of Xcp/Xcg in proportions of 1:4 and 4:1 (v:v).

*Phaseolus coccineus* pods. Seven selections of *P. coccineus* grown under greenhouse conditions, which showed relatively good pod set in the second cycle of a recurrent selection program, were used for testing. These selections were 5a-1, 11b-3, 3d-1, 3a2P, 19a-3, 12b-1, and 22L-1. *Phaseolus vulgaris* cv. La Vega was included as the check.

Nine treatments with six repetitions were tested in three replications. The treatments included pure suspensions of Xcp, Xcpf and Xcg, and mixed suspensions of Xcp/Xcpf, Xcp/Xcg, Xcpf/Xcg, Xcg/Xcp, and Xcg/Xcpf (1:4, v:v).

## INOCULATION OF PODS

The pods were washed by agitation with water in plastic bags and placed on moist paper towels on sterile aluminum trays. Three replications per plant selection were used.

The needle-scratch-inoculation method was used for this study because it gave the most uniform and rapid results according to previous research (22). The inoculation method consisted of depositing 0.01 ml of bacterial suspension on the distal part of a pod and then scratching the pod surface with two dissecting needles 2 mm apart. For control, the proximal section of the pod was scratched in a similar manner but without the inoculum. The pods were incubated for 7 days in environmental chambers at 27°C. Daylength was maintained for 12 hours with fluorescent lamps at 3228 lux.

## EVALUATION OF SYMPTOMS ON PODS

Water-soaking and callous formation. The scale used for the water-soaked symptom was 1 = no water-soaking, 2 = narrow border of water-soaked tissue  $\pm$  1 mm, 3 = water-soaked area of 2-3 mm wide, 4 = water-soaked in round spot, 5 = water-soaked reaching the pod suture.

The scale used for callous formation was 1 = no callous, 2 = spongy tissue  $\pm$  1 mm, 3 = spongy tissue 2-3 mm wide, 4 = spongy tissue 3-4 mm, 5 = callous tissue opens 3 mm or more and raises the tissue (20).

INOCULATION ON *P. VULGARIS* LEAVES

Bacterial blight resistant germplasm from CIAT (13 lines) and Cornell University (12 lines) were tested and compared with La Vega (P R.). Four bacterial suspensions were tested: pure suspensions of Xcp, Xcpf and Xcg, and a mixture of Xcp, Xcpf and Xcg at 1:1:1 (v:v). The youngest and full expanded trifoliolate leaf of plants at the flowering stage was inoculated using the multineedle technique (22). Ten repetitions per replicate (3) were used. The inoculation technique consisted of a multi-needle perforator on a foam cushion, and then rubbing of the leaves while they were submerged in the bacterial suspension. Each suspension was standardized to contain  $10^9$  cells/ml. Data were recorded at 7 and 14 days after inoculation.

The scale of symptom expression was 1 = resistant, 2 = slightly susceptible, 3 = moderately susceptible, 4 = susceptible, and 5 = highly susceptible.

## RESULTS

The water-soaking and callous symptoms induced by Xcpf on inoculated pods of *P. vulgaris* cv. La Vega and W-117 were clearly expressed

and showed significant differences when compared with pods inoculated with Xcg, Xcpf/Xcg and Xcg/Xcpf, which were not significantly different between each other (table 1). This indicates an antagonistic effect in relation to the symptoms developed on pods when Xcfp was mixed with Xcg in the proportions 4:1 and 1:4 (v:v). There was no significant difference in the infection index between the treatment containing a pure suspension of Xcg and the mixed suspension of Xcg with Xcpf (table 1).

There were no significant differences between Xcp and Xcpf with

TABLE 1.—Reaction of pods of *Phaseolus vulgaris* inoculated with four different suspensions of *Xanthomonas campestris*

Cultivars	Treatment <sup>2</sup>	Reactions <sup>1</sup>	
		Water-soaked	Callous
		$\bar{x}$	$\bar{x}$
La Vega	Xcpf	1.64** <sup>3</sup>	1.64**
	Xcpf/Xcg	1.06	1.06
	Xcg	1.06	1.06
	Xcg/Xcpf	1.06	1.06
W-117	Xcpf	1.42**	1.50**
	Xcpf/Xcg	1.08	1.08
	Xcg	1.06	1.06
	Xcg/Xcpf	1.07	1.07

<sup>1</sup> Reactions 7 days after inoculation: Water-soaked: The water-soaked area bordering the scratch was rated as follows: 1 = no water-soaking; 2 = narrow border of water-soaked tissue  $\pm$  1 mm; 3 = water-soaked area of 2-3 mm wide; 4 = water-soaked in round spot; 5 = water-soaked reaching the pod suture. Callous: 1 = no callous; 2 = spongy tissue  $\pm$  1 mm; 3 = spongy tissue 2-3 mm wide; 4 = spongy tissue 3-4 mm; 5 = callous tissue opens 3 mm or more and raises the tissue.

<sup>2</sup> Bacterial suspensions of approximately  $10^9$  cells per ml. Proportion of mixed suspension: 1:4 (vol:vol), each figure representing the bacterial isolates, respectively; Xcpf = *X. campestris* pv. *phaseoli* (fuscans); Xcg = *X. campestris* pv. *glycines*.

<sup>3</sup> In each variety of the Xcpf treatment was compared with the other treatments using the *t* test at probabilities of 0.01\*\*.

respect to the infection index produced on inoculated pods, but at least one or both isolates differed significantly from Xcg on *P. vulgaris* and *P. coccineus* pods. Neither Xcg nor the check treatment produced any visible symptoms in inoculated pods.

The mixed suspension of Xcp and Xcpf in proportions 4:1 and 1:4 (v:v) on pods showed no synergistic or antagonistic effect when they were compared with the pure suspensions of Xcp and Xcpf.

The mixed suspensions of Xcp/Xcg and Xcpf/Xcg in the proportions 4:1 and 1:4 (v:v) were not significantly different from the pure suspension of Xcg with respect to the infection index developed on the pod, but were different from those of Xcp/Xcpf (table 2).

Symptoms produced by the inoculation of mixed suspensions of Xcp/Xcpcf apparently depended on host susceptibility to each pathogen and on the relative proportion of each pathogen in the suspension (table 2, IIIa and IIIb). No synergistic or antagonistic effect was found to be related to the combination of the agents. On the other hand, mixed suspensions of Xcp/Xcg and Xcpcf/Xcg showed an antagonistic effect on

TABLE 2.—*Bacterial treatment comparison for the determination of an antagonistic effect of mixed suspensions of Xanthomonas campestris on pods*

	<i>P. vulgaris</i> LV <sup>1</sup>	<i>P. coccineus</i>						
		5a-1	11b-3	3d-1	3a-2P	19a-3	12b-1	22L-1
I. Xcp/Xcg <sup>2</sup>	1.00** <sup>3</sup>	1.00**	1.00**	1.00**	1.00**	1.00	1.00	1.00
Xcg/Xcpcf	1.00**	1.00**	1.00**	1.00**	1.00**	1.00	1.00	1.00
Xcp	3.00	2.00	2.00	1.88	1.83	1.50	1.33	1.33
II. Xcpcf/Xcg	1.00**	1.00**	1.00**	1.00	1.00**	1.00**	1.00**	1.00**
Xcg/Xcpcf	1.00**	1.00**	1.00**	1.00	1.00**	1.00**	1.00**	1.00**
Xcpcf	2.00	2.00	2.00	1.50	2.00	2.50	1.67	2.17
IIIa. Xcp/Xcpcf	3.00	1.75	2.00	2.50	1.50	1.50	1.17	2.00
Xcpcf/Xcp	1.75*	2.00	2.00	2.17	1.83	2.90*	1.83	2.00
Xcp	3.0	2.00	2.00	1.88	1.83	1.50	1.33	1.33
IIIb. Xcpcf/Xcp	1.75	2.00	2.00	2.17	1.83	2.90	1.83	2.00
Xcp/Xcpcf	3.00	1.75	2.00	2.50	1.50**	1.50*	1.17*	2.00
Xcpcf	2.00	2.00	2.00	1.50	2.00	2.50	1.67	2.17
IIIc. Xcp	3.00**	2.00**	2.00**	1.88**	1.83**	1.50	1.33	1.33
Xcpcf	2.00**	2.00**	2.00**	1.50	2.00**	2.50**	1.67*	2.17**
Xcg	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

<sup>1</sup> LV = La Vega.

<sup>2</sup> Treatment: Bacterial suspensions approximately of 10<sup>9</sup> cells per ml. Proportion of mixed suspensions 1:4 (vol/vol); each figure represents the bacterial isolate. Xcp = *X. campestris* pv. *phaseoli*, Xcpcf = *X. campestris* pv. *phaseoli* (*fuscans*), and Xcg = *X. campestris* pv. *glycines*.

<sup>3</sup> In each section the first two treatments were compared with the third using the *t* test at  $p = 0.05^*$  and  $p = 0.01^{**}$ . The water-soaked area bordering the scratch was rated as follows: 1 = no water-soaking, 2 = narrow border of water-soaked tissue  $\pm 1$  mm, 3 = water-soaked area of 2-3 mm wide, 4 = water-soaked in round spots, 5 = water-soaked reaching the pod suture.

symptom development when compared with pure suspensions of Xcp and Xcpcf, which did not depend on host susceptibility or on the relative proportions of the pathogen in the suspension (table 2).

In *P. vulgaris* leaves, 72% of the lines which were significantly less susceptible to the Xcp inoculation than the check La Vega, were significantly resistant to the mixed suspensions containing Xcp:Xcpcf:Xcg (1:1:1 v:v). The average comparisons of the virulent strains (Xcp and Xcpcf) versus the mixed suspensions containing virulent and avirulent Xcg showed significant differences in the intensity of the symptoms developed on the leaves (table 3).

TABLE 3.—Responses of bean (*Phaseolus vulgaris*) lines obtained from CIAT and Cornell inoculated with three isolates of *Xanthomonas campestris* from Puerto Rico on trifoliolate leaves at flowering stage

Identity	Pedigree	Bacterial isolate <sup>1</sup>			
		Xcp	Xcpf	Xcg	Mix
<i>CIAT Lines</i>					
BAC 13	P458 × P698	1.8**2	2.0	1.0	1.0**
BAC 16	P436 × P698	1.0**	2.0	1.0	1.0**
BAC 30	XAN 47 × G11894	2.7	2.0	1.0	1.5
BAC 80	XAN 449 × XAN 93	3.3	2.2	1.0	1.3
BAC 87	XAN 450 × (P436 × P698)	1.5**	1.0**	1.0	1.0**
BAC 88	ICA L23 × XAN 93	3.7	2.0	1.0	2.0
BAC 90	XAN 930 × XAN 93	3.0	2.3	1.0	2.0
BAC 112	BAC 18 × ((P566 × P698) × P566)	1.2**	2.2	1.0	2.0
BAC 105 = G11509 × XAN 93	XR 6237-8-1-CM-CM	2.5	2.3	1.0	1.0**
BAC 137 - G5147 × ICA L23	XR 6957-2-1-CM	1.7**	2.2	1.0	1.3
XAN 93	((P538 × P684) × (P459 × P567))	2.7*	2.8	1.0	2.5
XAN 1272	ICA L23 × (P 11 × P635)	1.5**	2.0	1.0	1.0**
XAN 1318	ICA L23 × XAN 93	3.2	2.3	1.0	1.5
<i>Cornell Lines</i>					
80-4498-4	(79-3865-1 × 79-3763-1)	1.5**	1.5**	1.0	1.5
80-4499-5	(79-3772-2 × 79-3764-1)	1.5**	1.6*	1.0	1.0**
80-4512-8	(79-3774-1 × 79-3812-1)	1.3**	1.5**	1.5**	1.0**
80-4527-6	(79-3775-1 × 79-3835-1)	1.3**	1.3	1.0	1.0**
80-4530-6	(79-3774 × 79-3841-1)	1.8**	2.3	1.0	1.0**
80-4531-6	(79-3772-2 × 79-3843-1)	1.8**	1.0**	1.0	1.0**
80-4533-5	(79-3836-1 × 79-3850-1)	2.0**	1.2**	1.3	1.0**
80-4535-6	(79-3764-1 × 79-3863-1)	1.7*	1.0**	1.3	1.0**
80-4543-4	(79-3889-2 × 79-3886-3)	2.2*	2.2	1.5**	1.0**
80-4548-5	(79-3899-1 × 79-3895-2)	2.8	2.0	1.0	1.3
80-4552-5	(79-3770-1 × 79-3903-2)	2.0*	2.3	1.3	1.0**
80-4774-5	(78-1678-1 × 78-1633-2)	1.7*	1.8	1.0	1.5
<i>Control Line</i>					
La Vega		2.1**	1.9**	1.1*	1.3 $\bar{x}$ <sup>3</sup>

<sup>1</sup> Xcp = *Xanthomonas campestris* pv. *phaseoli*, Xcpf = *X. campestris* pv. *phaseoli* (fuscan), Xcg = *X. campestris* pv. *glycines*, Mix = Xcp + Xcpf + Xcg (1:1:1).

<sup>2</sup> Readings were compared with the standard La Vega in the columns using the *t* test at P = 0.05\* and P = 0.01\*\*. Scale: 1 = resistant, 2 = slightly susceptible, 3 = moderately susceptible, 4 = susceptible, and 5 = highly susceptible.

<sup>3</sup> Average comparisons between the bacterial isolates with the mixed suspension using the *t* test at P = 0.05\* and P = 0.01\*\*.

## DISCUSSION

The heterologous isolate Xcg reduced the infectivity on pods of *P. vulgaris* and *P. coccineus* when mixed with the virulent isolates Xcp and Xcpf. This is the first report of such antagonism on pods inoculated with mixed suspensions of virulent and heterologous isolates of *X. campestris* pv. *phaseoli*. These results partially agree with findings of Leben (7) and Sequeira (13) who showed that the inoculation of a plant with a mixed inoculum of virulent and avirulent or nonpathogenic bacteria usually results in less disease. Mixed suspensions of the virulent isolates were compatible.

The mechanisms by which an isolate of low virulence favors a resistant reaction is not well understood. Possible explanations may be related to bacteriocins or other inhibitor factor production, bacterial attachment to the cell wall and plant products, such as lectins and phytoalexins or inducible compounds (5, 8, 9, 12, 15).

In this study, both low and high proportions of the heterologous isolate mixed with the virulent isolates gave some effects on infectivity, indicating that the proportion of the heterologous cells in the suspensions was not involved in the antagonism.

Another explanation for the results obtained may be that Xcg produces a bacteriocin or an inhibitor factor that affects Xcp and Xpf. Abo-El Dahab and El-Goorani (1) studied the antagonism between virulent and avirulent strains of *Pseudomonas solanacearum* on agar. They found that indoleacetic acid was partially responsible for the antagonism since virulent cells were inhibited at concentrations in which the avirulent continued to grow.

The mechanism of attachment (5, 6, 10, 13) could be different on Xcg and on Xpc and/or Xcpf because mixed suspensions with Xcg showed significant reduction in symptom development on pods but no differences with mixed Xcp/Xcpf. In this way, antagonism might be related to the degree of attachment or specificity of attachment of homologous (Xcp and Xcpf) and heterologous (Xcg) strains of the bacteria on the host. Attachment and envelopment of bacteria occur in many different plant species, such as tobacco leaves infiltrated with *P. pisi* (5), avirulent or heterologous strains of *P. solanacearum* (14), heat killed bacteria (14), or saprophytic bacteria (2, 5, 14); potato leaves infiltrated with avirulent strains of *P. solanacearum* (15); cucumber leaves inoculated with saprophytic bacteria (14); corn leaves infiltrated with *Erwinia carotovora* subsp. *carotovora* (19); bean leaves inoculated with saprophytic bacteria (16); and bean pods tissues with *P. syringae* pv. *syringae* (3).

The reasons why the isolate of low virulence Xcg can favor a resistant reaction on pods of *P. coccineus* and *P. vulgaris* and leaves of *P. vulgaris*

should be fully investigated. For example, correlations between normal lectin content in healthy plants of *P. vulgaris* and *P. coccineus* (leaves and pods) versus plants resistant to bacteria should be clarified.

#### RESUMEN

El patógeno bacteriano *Xanthomonas campestris* pv. *phaseoli* (E. F. Smith) Dowson, agente causante del tizón común de la habichuela común, *Phaseolus vulgaris* L., coexiste mundialmente con su cultivo y limita seriamente los rendimientos de la habichuela en los climas tropicales. Se han desarrollado varias técnicas de inoculación para identificar fuentes de resistencia a la infección bacteriana, pero la amplia variedad de cepas ha complicado el trabajo. Algunos investigadores, sin considerar el efecto en la infectividad del inóculo, han usado suspensiones de cepas mezcladas del patógeno.

El objetivo de este estudio fue determinar el efecto en la virulencia de *X. campestris* pv. *phaseoli* (virulento) cuando se mezcla con un cultivo heterólogo de *X. campestris* pv. *glycines* (baja virulencia) sobre dos especies de *Phaseolus*. Se encontró que las diferencias producidas por la inoculación de suspensiones mezcladas de *X. campestris* pv. *phaseoli* dependen de la susceptibilidad del huésped y de la razón o proporción del patógeno en la suspensión. No se encontró efecto sinérgico o antagónico asociado a la combinación de diferentes aislamientos del patógeno. Sin embargo, las suspensiones mezcladas de *X. campestris* pv. *phaseoli* con el cultivo heterólogo de *X. campestris* pv. *glycines* mostraron efecto antagónico en el desarrollo de síntomas en las vainas. Estos síntomas no dependen de la susceptibilidad del huésped ni de la proporción de cada patógeno en la suspensión bacteriana. Los resultados indican que en una suspensión bacteriana mezclada con diferentes aislamientos no siempre predomina la reacción al de más alta virulencia.

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