A MULTIPLE INOCULATION TECHNIQUE FOR SELECTION OF BEAN SEEDLINGS WITH RESISTANCE TO THREE PATHOGENS[1]

J. C. DE FARIA AND D. J. HAGEDORN

Centro Nacional de Pesquisas de Arroz e Feijão (CNPAF-EMBRAPA)
Caixa Postal 179, 74000 Goiânia, GO, Brazil, and Department of
Plant Pathology, University of Wisconsin, Madison WI 53 706, USA

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ABSTRACT

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A multiple inoculation technique to evaluate resistance of bean plants to *Fusarium solani* f. sp. *phaseoli* (Fsp), *Uromyces phaseoli* var. *typica*, and *Xanthomonas capestris* pv. *phaseoli* (Xcp) was developed. Differences in disease reaction were highly significant among cultivars inoculated with the three pathogens. Disease reactions in the multiple inoculation experiments were not affected to the extent of changing a cultivar reaction from susceptible to resistant or vice-versa as compared to single inoculation experiments. Inoculation with Fsp at high inoculum level caused overall reduction of symptom severity brought about by inoculation with Xcp, but this effect was cultivar non-specific.

The technique should be of importance to evaluate beans for multiple disease reaction especially in tropical areas of the world where those diseases occur simultaneously. It can also be used in situations of limited space, or to obtain faster results of bean germplasm evaluation for resistance to more than one of the pathogens.

[1]Portion of a Ph.D. Thesis submitted by the senior author to the University of Wisconsin – Madison USA.

RESUMO

Técnica de inoculação múltipla para seleção de feijoeiro com resistência a três patógenos

Uma técnica de inoculação múltipla foi desenvolvida objetivando avaliar a resistência do feijoeiro a *Fusarium solani*. f. sp. *phaseoli* (Fsp), *Uromyces phaseoli* var. *typica* e *Xanthomonas capestris* pv. *phaseoli* (Xcp). As diferenças quanto as reações às doenças entre as cultivares inoculadas com os três patógenos foram altamente significantes. As reações das cultivares às doenças nos experimentos de inoculação múltipla não foram alteradas a ponto de alterar sua classificação de suscetível para resistente, ou vice-versa, quando comparada à inoculação com os mesmos patógenos individualmente. A inoculação com alta concentração de inóculo de Fsp causou redução geral da expressão de sintomas após inoculação com Xcp, mas este efeito não foi específico para nenhuma das cultivares testadas.

A inoculação múltipla poderá ser de grande importância para as avaliações de feijoeiro para resistência múltipla a diversos patógenos especialmente nas áreas tropicais onde várias doenças são importantes, simultaneamente. A técnica poderá também ser usada em situações onde existam limitações de espaço, ou para obter resultados mais rápidos em avaliações de germoplasma para resistência a várias doenças.

INTRODUCTION

Among the limitations to yield increase of beans (*Phaseolus vulgaris* L.) in the tropics is the crop susceptibility to an impressive array of diseases (Schwartz & Galvez, 1980). Techniques for inoculation and selection of resistant plants have been individually developed for all major bean pathogens (Zaumeyer & Meiners, 1975; Meiners, 1981). A seedling test to evaluate reaction to several diseases simultaneously could save time by the early determination of disease reaction and, therefore, expedite the use of resistant germplasm in breeding programs. The detection of promising interactions between the host and several pathogens (Kilpatrick *et al.*, 1981) would be of great value when scientists have available only small quantities of seeds of certain types of materials (plant introductions, crosses etc), or in a program to breed for multiple resistance.

Work on multiple inoculation has been initiated in beans by Hubbeling (1961) and Lopes (1977), in cucumber by Abul-Hayja (1975), and in barley by Kilpatrick (1981).

The purpose of the present work was to develop a reliable, simple, and effective multiple inoculation technique to evaluate the reaction of young bean plants to several bean pathogens. Efforts were made to detect possible interactions among pathogens *in vivo,* so that the reaction to single pathogens would not be changed in multiple inoculations.

An abstract of this report has been published previously (Faria & Hagedorn, 1981).

MATERIAL AND METHODS

The pathogens used were: *Fusarium solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans. (Fsp), *Uromyces phaseoli* var. *typica* Arth (Upt), and *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye (Xcp), incitants of *fusarium* root rot, rust, and common blight, respectively.

Inoculum preparation. The Fsp isolate W-9 was supplied by J. M. Kraft, USDA, Prosser, Washington. Inoculum was increased by flooding the surface of PDA plates with a spore suspension prepared from PDA slants. After seven-day incuba-

tion at 24°C, in the dark, the spores were scraped off the medium surface with a transfer needle in 100 ml of sterile distilled water. The spore concentration was determined using a Spencer bright line hemacytometer following filtration through a double layer of cheesecloth and adjusted to desired level, according to the experiment.

The Upt isolate FA-2 was collected at the Hancock Experimental Station, Wisconsin. The fungus was single-spored and increased in isolation on Pinto UI-111 beans. A spore suspension of 2×10^4 uredospores/ml made in water and Tween 80 at 50 ppm was sprayed over the plants in all cases.

The isolate EK-11 of Xcp was obtained from M. L. Schuster, Department of Horticulture, University of Nebraska, Lincoln, Nebraska. The bacterium was grown in nutrient dextrose broth for 20 h in a rotary shaker at 28°C in the dark. Cells were pelleted, washed twice in phosphate buffered saline (PBS) at pH 7.0 and 0.001 M phosphate and resuspended in PBS. The final concentration of bacteria was adjusted based on the turbidity of the suspension as measured in a Spectronic 20 colorimeter. A ten-fold dilution series was plated in duplicate to estimate the number of colony forming units (cfu) used in each experiment.

Inoculation procedures. Bean seeds were germinated in vermiculite for 6-8 days under greenhouse conditions for all experiments. Seedlings of uniform size were carefully pulled and their roots washed before using. After dip inoculation in a suspension of macroconidia of Fsp for 10 min, the seedlings were transplanted into 10 cm clay pots filled with silica sand. They were watered with half strength Hoagland's solution as needed. To inoculate with Upt the uredospore suspension was sprayed over the plants using a DeVilbiss No. 15 atomizer. Plants were then incubated for 24 h at 20°C in a mist chamber; To inoculate with Xcp a multiple needle florists' frog which had been dipped in the bacterial suspension was pricked on the leaves.

In the first experiment, three plants of 'Top Crop' bean were inoculated with either Upt at 10^4 uredospores/ml or Xcp at 10^7 cfu/ml or both, in the primary or in the trifoliolated leaves, for a total os six combinations in addition to controls singly inoculated with rust. A singly inoculated control for Xcp was not maintained because the cultivar Top Crop was known to be susceptible to it, but rather a treatment using bacteria on opposite leaves with Upt was adopted. The treatments were organized in a completely randomized design. Leaves were first prick-inoculated with Xcp, and immediately spray-inoculated with the Upt uredospore suspension. The experiment was repeated. Rust pustule sizes were measured in both blighted and non blighted areas of Xcp-inoculated plants and in the controls using a filar drum micrometer (Baush & Lomb) replacing the ocular of a stereo microscope. The diameter of the epidermal eruption was measured rather than the mass of uredospores protruding from it.

The second experiment was set to test the possible interaction between disease readings for Xcp and Fsp when sequentially inoculated on bean plants. The experimental design used was a factorial in a randomized block design with three inoculum levels of Fsp (0, 10^4, and 3×10^5 conidia/ml), three inoculum levels of Xcp (0, 10^7, and 10^9 cfu/ml), and eight bean cultivars or lines, with three replications. The plants were inoculated with Xcp two days after they had been dip-inoculated in the Fsp spore suspension. Heretofore the above inoculum levels of Fsp and Xcp are referred to as 0, 1, and 2.

In the third experiment the treatments were set in a factorial in a completely randomized design with two inoculum levels of Fsp (10^4 and 3×10^5 conidia/ml), one of Xcp (10^7 cfu/ml), one of Upt (2×10^4 uredospores/ml), and 10 bean cultivars or lines, repeated four times. Plants were prick inoculated with Xcp and spray inoculated with Upt two days after they had been dip-

inoculated with the Fsp inoculum levels. For this experiment singly inoculated control plants for each disease were not included.

The statistical analysis were done using the Statistical Analysis System (SAS), in an IBM computer.

Throughout the experiments the greenhouse temperature was kept at 24 + 3^0C with a relative air humidity range from 40 to 80%, and supplemental fluorescent lighting on a 12 h period. Light readings ranged from 105 to 140 MEs^{-1}. cm^{-2} for Lambda LI-185 meter, 20 cm from the light bank.

Disease ratings. For Fsp a five grade scale was used, being 1 = healthy or very slightly discolored hypocotyl and root system; 2 = moderate hypocotyl damage, limited to small reddish spots and root damage; 3 = hypocotyl discolored and slightly shrunken, and roots discolored; 4 = hypocotyl discolored and severely shrunken, root system discolored and greatly reduced; and, 5 = dead or dying plants with hypocotyl rotten and roots pruned. For Upt, five reaction types or pustule sizes were considered for disease grading, as described in Davison & Vaughan (1963), being 1 = immune or with no visual evidence of infection; 2 = necrotic flecking of variable sizes and shapes without sori or spores; 3 = pustules with diameter of 300*u*m or smaller; 4 = pustules with diameter of 300 of 500 *u*m; and 5 = pustules with diameter of 500 *u*m or larger. For Xcp, the disease severity rating system used was as follows; 1 = healthy, with no apparent symptoms, and 2 to 6 representing, respectively, 1 to 5%, 6 to 25%, 26 to 50%, 51 to 75%, and 76 to 100% of the multiple needle inoculated area showing wilting or necrosis (Valladares-Sanchez *et al.,* 1979).

RESULTS AND DISCUSSION

Rust pustules developed both in leaf tissues infected or not with Xcp. In some cases their sizes were significantly reduced in the areas showing Xcp symptoms (blighted areas) both in primary and in trifoliolated leaves (Table 1). Pustule sizes did not differ among treatments when measured in the non blighted areas and controls singly inoculated with Upt, either within primary or trifoliolated leaves. These results indicated that if the blighted leaf areas are avoided for rust pustule evaluations, the two diseases do not interact and can be tested in simultaneous or sequential inoculations. There were good symptoms of common blight in all Xcp inoculated leaves regardless of inoculation with rust.

In the second experiment, the analysis of variance using Xcp as the dependent variable indicated significant differences (P = .05) for the variables cultivars or lines, levels of Fsp inoculum, levels of Xcp inoculum, the interaction cultivars/lines-Fsp, and cultivars/lines-Xcp, but not the triple interaction or between Fsp and Xcp (Table 2). There was a significant reduction in the severity rating for Xcp at the higher Fsp inoculum level but not at the lower one. The above change in disease ratings was, however, observed for all cultivars or lines tested and was not high enough to preclude the separation between a resistant and a susceptible germplasm.

When Fsp disease readings were used as the dependent variable, the analysis of variance indicated significance (P = .05) for the triple interaction cultivars/lines-Fsp-Xcp, besides cultivars/lines-Fsp, and cultivars/lines-Xcp. The other sources of variation were not significant. The degrees of freedom for the triple interaction were partitioned for inoculum levels of Fsp within cultivars/lines. It was found that line G-700 and cultivar Cornell 49-242 showed decreased Fsp readings at higher Xcp inoculum levels. These two bean accessions, however, had the highest disease ratings among the eight tested (Table 3). None of the tested bean accessions possessed double resistance, but they rather differed widely in their reaction to these two diseases.

Table 1. Effect of *Xanthomonas campestris* pv. *phaseoli* (Xcp) symptoms on the *Uromyces phaseoli* var. *typica* (Upt) pustule size on 'Top Crop' inoculated by different methods.

Treatment	Xcp disease severity[a]	Upt mean pustule size (*um*)[b]
Upt alone on primary leaves	–	470 bc
Upt and Xcp applied on opposite leaves	6	550 cde
Upt and Xcp on primary leaves. Pustule size measured in the non-blighted area	6	573 cde
Upt and Xcp on primary leaves. Pustule size measured in the blighted area	6	387 ab
Upt on primary and Xcp on trifoliolated leaves	6	484 bcd
Upt alone on trifoliolated leaves	–	637 e
Upt and Xcp on trifoliolate leaves. Pustule size measured in the non-blighted area	6	609 de
Same as above, with pustule size measured in the blighted area	6	317 a
Upt on triofoliolate and Xcp on primary leaves	6	540 cde

[a]Disease severity rating for Xcp: see the text.
[b]Means followed by the same letters are not significantly different based on Tukey's studentized range (HSD) test at P = .05.

Table 2. Effect of inoculum concentration on common blight ratings for bean cultivars or lines inoculated with *Xanthomonas campestris* pv. *phaseoli* (Xcp) following inoculation with *Fusarium solani* f. sp. *phaseoli* (Fsp)[W].

Cultivar or line	Fsp inoculum concentration[X]						Mean[Z]
	0		1		2		
	Xcp1[Y]	Xcp2	Xcp1	Xcp2	Xcp1	Xcp2	
R-275	5.4	6.0	6.0	5.4	5.3	5.7	5.6 a
Olathe	4.0	6.0	5.0	5.6	3.3	5.7	4.9 ab
Mexico 309	4.4	6.0	3.6	6.0	2.0	4.4	4.4 bc
Black Turtle Soup	3.8	6.0	3.3	5.7	1.0	4.2	4.0 bcd
Cornell 2114-12	2.0	5.6	2.3	5.7	1.7	5.7	3.8 cd
G – 700	3.4	6.0	4.0	5.0	2.0	2.6	3.8 cd
Cornell 49-242	2.4	6.0	2.4	4.0	1.3	2.7	3.1 d
Great Northern Nebraska 1 Sel. 27	1.4	2.0	1.0	1.6	1.0	1.0	1.3 e
Mean	4.4 a		4.2 a		3.1 b		

[W]Rating system: see the text.
[X]Fsp inoculum concentrations were: 0 = check uninoculated; 1 = 10^4 conidia/ml; and, 2 = 3 x 10^5 conidia/ml of isolate W-9.
[Y]Xcp1 and Xcp2 are, respectively, inoculum concentrations of 10^7 and 10^9 cfu/ml of isolate EK-11.
[Z]Means followed by the same letters are not significantly different based on Tukey's studentized range (HSD) test at P = .05.

Table 3. Disease ratings of bean cultivars or lines to *Fusarium solani* f. sp. *phaseoli* (Fsp) in plants also inoculated with *Xanthomonas campestris* pv. phaseoli (Xcp)[W].

Cultivar or line	Xcp inoculum concentration[X]						Mean[Z]
	0		1		2		
	Fsp1[Y]	Fsp2	Fsp1	Fsp2	Fsp1	Fsp2	
G – 700	4.4	5.0	5.0	5.0	3.0	5.0	4.6 a
Cornell 49-242	4.0	5.0	3.0	5.0	4.6	5.0	4.4 a
Great Northen Nebraska 1 Sel. 27	3.7	5.0	3.7	5.0	3.4	5.0	4.3 a
Black Turtle Soup	3.6	5.0	3.4	5.0	3.3	4.7	4.2 a
México 309	3.4	5.0	3.0	5.0	3.0	5.0	4.1 a
Olathe	2.7	3.7	2.6	4.0	3.0	4.3	3.4 b
Cornell 2114-12	1.7	3.3	2.7	3.5	1.3	3.7	2.7 c
R-275	1.3	3.0	1.0	3.0	1.3	3.3	2.2 c
Mean	3.7 a		3.7 a		3.7 a		

[W]Rating system: see the text.
[X]Xcp inoculum concentrations were: 0 = check uninoculated; 1 = 10^7 cfu/ml; and, 10^9 cfu/ml of isolate EK-11.
[Y]Fsp1 and Fsp2 were, respectively, inoculum concentrations of 10^4 and 3 x 10^5 conidia/ml of isolate W-9.
[Z]Means followed by the same letters are not significantly different based on Tukey's studentized range (HS) test at P = .05.

Table 4. Disease ratings for bean cultivars or lines inoculated with *Xanthomonas campestris* pv. *phaseoli* (Xcp) and *Uromyces phaseoli* var. *typica* (Upt) following inoculation with *Fusarium solani* f. sp. *phaseoli* (Fsp).

Cultivar or line	Fsp1[W]	Fsp2	Mean for Fsp	Xcp[X] at Fsp1	Xcp at Fsp2	Mean for Xcp	Upt[Y] at Fsp1	Upt at Fsp2	Mean for Upt
G – 700	4.0	4.5	4.3 a	6.0	4.5	5.3 a	3.0	3.0	3.0 a
Cornell 49-242	3.0	4.5	3.8 ab	4.8	4.0	4.4 a	3.0	3.0	3.0 a
Pinto UI 111	3.0	4.3	3.6 ab	5.3	4.5	4.9 a	5.0	5.0	5.0 a
Top Crop	3.0	4.3	3.6 ab	5.5	4.0	4.8 a	5.0	5.0	5.0 a
Black Turtle Soup	3.0	4.0	3.5 b	6.0	4.8	5.4 a	1.0	1.0	1.0 d
801397	2.5	4.0	3.3 b	6.0	4.8	5.4 a	3.0	3.0	3.0 b
Great Northern Nebraska 1 Sel. 27	2.3	4.0	3.1 b	2.3	2.3	2.3 b	5.0	5.0	5.0 a
Olathe	1.8	3.0	2.4 c	5.5	4.8	5.1 a	2.0	2.0	2.0 c
R-275	1.0	2.0	1.5 d	6.0	5.3	5.6 a	5.0	5.0	5.0 a
Cornell 2114-12	1.0	2.0	1.5 d	6.0	5.3	5.6 a	5.0	5.0	5.0 a
Mean[Z]	2.5 a	3.7 b		5.3 a	4.5 b		3.7 a	3.7 a	

[W]Fsp1 and Fsp2 were, respectively, inoculum concentrations of 10^4 and 3 x 10^5 conidia/ml of isolate W-9 – Rating system: see the text.
[X]Xcp inoculum concentration was 10^7 cfu/ml of isolate EK-1. Rating system: see the text.
[Y]Upt inoculum concentration was 2 x 10^4 uredospores/ml of isolate FA-2. Rating system: see the text.
[Z]Means followed by the same letters are not significantly different based on Tukey's studentized range (HSD) test at P = .05.

For the third experiment, the analysis of variance indicated cultivars/lines interaction with each of the diseases studied. There was a significant reduction in common blight severity at the Fsp inoculum level of 3 x 10^5 conidia/ml (Table 4). This type of interaction was not evident in relation to Upt.

Even though the inoculation with Fsp at the 3 x 10^5 conidia/ml consistently caused reduction in Xcp severity ratings, the cultivars or lines ranking did not change. This fact indicates that progress can be made to select for multiple disease resistance in a breeding project. Rust readings were not affected by inoculation with Fsp (Table 4), as they had not been affected by Xcp (Table 1) when taken from the leaf areas not expressing symptoms of common blight.

Besides to illustrate similarities and differences between multiple and single inoculation results, Tables 2, 3, and 4 provide information on the cultivars or lines reactions to the three diseases studied. Disease reactions in the multiple inoculation experiments were not affected to the extent of changing a cultivar ranking from resistant to susceptible, or vice-versa, as compared to single inoculation experiments. Multiple inoculation of beans is therefore feasible and reliable, and can be used in screening for resistance provided that control cultivars for each of the diseases are included in the tests.

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