

## A Rapid Technique for Inoculation of *Phaseolus vulgaris* with Multiple Pathotypes of *Uromyces phaseoli*

J. R. Stavely

Research plant pathologist, Plant Pathology Laboratory, Plant Protection Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705.

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### ABSTRACT

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Modification of a readily available, inexpensive, hand-held, pressurized spraying device was made by attaching a 5-cm-long, 12-mm-diameter, section of Plexiglas tubing over the spray nozzle. This modification permitted inoculation of bean plants with each of many pathotypes of *Uromyces phaseoli*. Negligible differences in numbers of uredinia among pathotypes were obtained by standardizing the concentrations of viable urediniospores. Each unifoliolate leaf was inoculated with four pathotypes

per leaf 6-8 days after seeding and the first trifoliolate leaf was inoculated with up to six pathotypes 6-8 days later. Resistant and susceptible host reactions to specific pathotypes were not affected by simultaneously or sequentially inoculating other leaf areas with other pathotypes. The rapid technique described here is potentially useful for determining linkage relationships among host resistance genes and in breeding beans for resistance to multiple pathotypes of *U. phaseoli*.

*Additional key words:* bean rust, resistance screening.

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Rust, caused by *Uromyces phaseoli* (Reben) Wint., is among the most destructive of the leaf diseases of common bean, *Phaseolus vulgaris* L. (14). Losses of up to 78% occurred in pinto beans in the United States in 1981 (9). The bean rust fungus has many pathotypes (physiologic races) (6,14). Field collections of urediniospores are often composed of more than one pathotype (6). Germplasm of *P. vulgaris* varies greatly in reactions to these pathotypes. There are usually many cultivars or accessions resistant to any one pathotype. However, only three of the several hundred bean accessions tested are either moderately or highly resistant to all presently available continental United States pathotypes (12). An efficient method is needed to inoculate many plants with multiple pathotypes of *U. phaseoli*, one pathotype at a time, to facilitate breeding bean cultivars with resistance to several pathotypes and to determine the genetics of resistance so that linkages among resistance genes can be identified.

Many methods have been used to inoculate bean leaves with *U. phaseoli* (1,3,4,6-8,10). Forty years ago, Harter and Zaumeyer (6) inoculated by painting a water suspension of urediniospores onto the surfaces of one-half to two-thirds expanded primary leaves with a camel's hair brush or by dusting dry urediniospores onto dry leaves. The plants were then placed in chambers with high humidity for 24 hr. Recent studies (8) have shown that at 17.5-22.5 C, most urediniospores on bean leaves germinate during the first 6-8 hr of wetness. Davison and Vaughan (3,4) inoculated primary leaves by spraying them with a suspension of  $2 \times 10^4$  *U. phaseoli* urediniospores per milliliter of an aqueous solution containing Ivory soap, Triton B1956, and carboxymethyl cellulose.

Schein (10) developed a sophisticated quantitative inoculator that was designed to deliver precise numbers of urediniospores to a small leaf area, but it lacks portability, requires suspension of urediniospores in 0.125% water agar to prevent settling, and is not as well suited for use with multiple pathotypes and large numbers of plants as the method described here. Imhoff et al (7,8) have shown that variability in rust intensity on inoculated bean leaves is primarily due to variable urediniospore germination caused by the conditions under which the spores were produced. They inoculated young trifoliolate leaves by brushing them with dry urediniospores.

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Groth and Urs (5) and Imhoff et al (8) have shown the critical effects of leaf age on receptivity and germinability of subsequently produced urediniospores. When Schein's device (5) was used, most of the variability came from the among-plant component.

The objective of this research was to develop a rapid, simple, and dependable method to inoculate many individual bean plants with several pathotypes of *U. phaseoli*, one at a time. Use of the technique described has enabled the testing of F<sub>1</sub>, F<sub>2</sub>, F<sub>3</sub>, and backcross populations from several crosses of *P. vulgaris* for their reactions to several pathotypes (11).

## MATERIALS AND METHODS

**Cultivars and plant propagation.** Cultivars representative of several types of beans and having variable reactions to the pathotypes in the Beltsville collections of *U. phaseoli* were used to compare inoculation methods. These included BBL 47, BBL 94, and Eagle snap beans; Olathe and Pinto 111 pinto beans; and B-190 and Compuesto Negro Chimaltenango (CNC), broadly rust-resistant, black seeded Latin American beans (12). The plants were kept in a rustfree greenhouse until they were inoculated. All seeds were sown 2.5 cm deep in a friable, moist potting mixture containing one part each of peat moss and perlite to four and one-half parts of Plant Industry Station soil (13). Two seeds were planted per 10-cm-diameter Kord plastic pot. The pots were placed in a greenhouse kept at 24 ± 3 C, covered with light-impervious plastic, and not uncovered or watered until the plants began to emerge from the soil. Unifoliate leaves were inoculated when they were 35–65% expanded, which was usually 6–8 days after seeding, depending upon the season.

**Pathotypes and inoculum preparation.** The urediniospores used for inoculum were from collections or cultures of *U. phaseoli* kept at Beltsville. The collections were obtained during the past 10 yr from individual bean fields at many locations in the United States. Included are many unique pathotypes of the fungus. None of these pathotypes is identical to the races previously described (6,14), although one collection, 73-16, resembles Zaumeyer's race 32 (15). Each collection has been assigned a three- or four-digit number in which the first two digits refer to the year originally collected. Single uredinium cultures of many unique pathotypes from these collections have been isolated and tested on the appropriate differential cultivars. Each culture so obtained carries the original collection number followed by a capital letter, for example, 73-16A. For the inocula used in this study, the urediniospores were increased every 4 mo on isolated young plants of an appropriate bean cultivar. Urediniospores were collected 12–14 days after inoculation, placed in screw-cap bottles, and stored at -18 C or in a liquid nitrogen tank. Percent germination of urediniospores was determined as described by Baker et al (2).

To prepare inoculum suspensions, approximately 0.03 g of frozen urediniospores were placed in 50 ml of 0.01% Tween-20 in tap water in a 250-ml Erlenmeyer flask. A magnetic stirring bar was added and the mixture was stirred at top speed (approximately 800 rpm) on a Fisher Flexa-Mix Stirrer for at least 2 min while adding another 50 ml of the Tween-water suspension to wet and disperse the spores. Urediniospore concentration was determined with a hemacytometer and adjusted to the desired level by appropriate dilution.

**Inoculum application methods.** In the search for an efficient method for testing rust reactions of each plant in large populations to multiple pathotypes of *U. phaseoli*, three methods of applying inoculum were tested. In the first method, which will be referred to as the *brush method*, aqueous urediniospore suspensions of each of several pathotypes were painted onto a predesignated portion of the two monofoliate leaves with a small camel's hair brush. In the second method, the *spray method*, three-quarters of the monofoliate leaf was covered with a piece of thin cardboard and the exposed portion was sprayed with a urediniospore suspension of one pathotype from a distance of 5–8 cm. Upon drying inoculum of the next pathotype was applied in the same manner to the next quarter leaf.

Spray inoculum was applied with a Crown Sprā-Tool (Crown

Industrial Products, Hebron, IL 60034 or the Fisher Scientific Co., Pittsburgh, PA 15219) or a similar device, the Badger Propel (Badger Air-Brush Co., Franklin Park, IL 60013). The propellant container and a 237-ml (8-oz) plastic jar for the urediniospore suspension are attached to a sprayer head containing the spray nozzle. A fine spray is released by depressing a valve on the sprayer head. These devices are light, portable, usable with one hand, and those parts that contact the inoculum can be easily sterilized between uses for different pathotypes.

For the third method of applying urediniospores, the *confined spray method*, Plexiglas tubing (12 mm in diameter) was cut to various lengths, notched to fit, and glued over the nozzle of a Crown or Badger spray head (Fig. 1). This device, with propellant and jar of inoculum attached, was then used for inoculating. Inoculum was applied by lightly pressing the open end against the leaf and depressing the spray valve for 0.5 sec to apply a light spray over a 12-mm-diameter circular area. Four pathotypes could be applied per primary leaf. Six to 8 days after inoculating primary leaves, the number of days depending upon the time of the year, the first trifoliolate leaf could be inoculated with up to six additional pathotypes. Between uses for different pathotypes, the equipment was sterilized by immersion in 0.6% sodium hypochlorite or 70% ethanol for 10 min.

Whole plants (both leaves) of each cultivar were also inoculated with each pathotype singly at the same times as multiple pathotypes were inoculated onto other similar plants. This was done to determine if there was any effect upon host reaction from inoculating single leaves or plants with multiple pathotypes. Unmodified Crown Sprā-Tools were used to apply the inoculum.

**Incubation conditions.** After the leaf surfaces had dried, the plants were placed in the dark in a Percival model I-35DL dew chamber set to regulate an air temperature of approximately 19 C and programmed to deliver a consistent, very light dew deposition with negligible runoff. The plants were kept in the dew chamber for 16 hr, then allowed to dry and placed in a greenhouse for 15 days before host reactions were recorded. The Davison and Vaughan (3) bean rust grading scale, to which an 800- $\mu$ m size category has been added, was used for estimating uredinium size.

## RESULTS AND DISCUSSION

Rust pustules developed from compatible host-pathogen combinations, regardless of the method used to apply inoculum. However, the confined-spray method employing the Crown or Badger type spray device with the added Plexiglas tube (Fig. 1), gave the most consistent results and was easiest to use (Fig. 2). When the urediniospore suspensions were applied by the brush method, application was slow and, on certain cultivars with less wettable leaves, the spore suspension did not disperse evenly and large droplets formed and often ran down to an area reserved for another pathotype. With the spray method, that involved covering three-quarters of the leaf and spraying the inoculum onto the remaining quarter, more time was required than for the brush method, but the leaves were more easily wetted. This greater ease of wetting with a

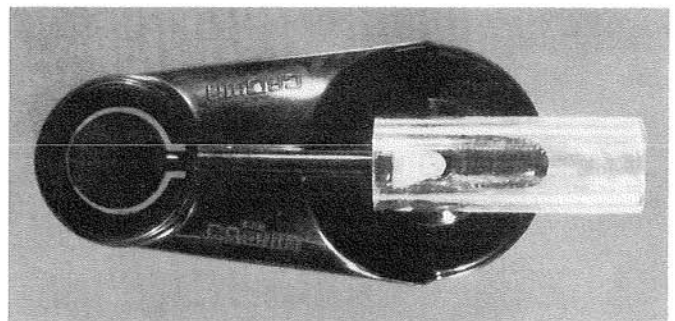


Fig. 1. Appearance of a Crown sprayer head after modification by attachment of Plexiglas tubing to restrict the area for spray inoculation of bean leaves to a 12-mm-diameter circle.

spray suggested the alternative of modifying the sprayer head.

The optimal length for the 12-mm-diameter tubing that was attached to the sprayer head was 5 cm. This allowed 4 cm between the outlet of the spray nozzle and the distal end of the tubing. With less than 5 cm of tubing, too great a volume of spore suspension was deposited on the leaf, resulting in formation of droplets. When the tube was longer than 5 cm, insufficient spray reached the leaf and too much of it was deposited on the walls of the tubing. Accumulation also occurred on the walls with the 5-cm length, but this was overcome by wiping the inside of the tube with a rolled paper towel after every second or third inoculation. With the 12-mm-diameter tube mounted as shown in Fig. 1, an open space was left behind the nozzle around the end of the tube. This space had to be loosely covered with tape or toweling to block dispersal of spores into the air. Completely sealing this space was undesirable due to creation of back-pressure when spraying.

Inoculum was applied by holding the leaf with one hand, and the sprayer with the other hand. The distal end of the tube was placed against the abaxial surface of a predesignated quarter or half of a unifoliolate leaf or trifoliolate leaflet, respectively. More uredinia developed if the abaxial, rather than the adaxial, surface was inoculated. The spray valve is depressed with the thumb for 0.5 sec. The spray device was shaken to keep the spores in suspension as the applicator proceeded to the next plant. Upon inoculation of all plants with one pathotype, the equipment was immersed for sterilization as already described, and a duplicate, previously sterilized device was used to apply the next pathotype. For convenience, six to eight modified sprayer heads should be made up for regular use. Two people can inoculate 100 plants with eight pathotypes in 2 hr or less.

Rate of plant development was variable by season. In December and January, the unifoliolate leaves reached 35–65% expansion 8 days after seeding; in June this stage was reached in 6 days.

Variation within cultivars was not as great as variation among cultivars, and greatest variation in rate of plant development occurred within certain  $F_2$  populations. Therefore, the stage of development and not days from seeding must be used to determine which plants are to be inoculated in a particular day.

Based upon many comparisons, the optimum urediniospore concentration for the recommended method for multiple inoculation of bean populations segregating for resistance is 25,000 spores per milliliter when using urediniospores having 70% germination. The urediniospore concentration should be proportionally decreased if the spores have a higher percent germination or increased if germination is lower. At 17,500 viable urediniospores per milliliter, infection and subsequent symptom development is assured if the host-pathogen combination is compatible, and leaf development, inoculum preparation, and incubation conditions are as specified above. With the confined-spray multiple inoculation technique, those host-pathogen combinations resulting in a small uredinium or a necrotic lesion were unaffected, having identical reactions to those that developed on entire plants inoculated with only one pathotype (Table 1). In host-pathotype combinations that normally result in uredinia larger than 500  $\mu\text{m}$ , there can be some reduction in predominant uredinium size, due to crowding. This occurs if the valve on the sprayer is depressed somewhat longer than the desirable 0.5 second or the concentration of viable urediniospores has been underestimated. A somewhat higher inoculum concentration would cause no problem if the resistance being studied is expressed as necrotic spots or small uredinia, less than 300  $\mu\text{m}$  in diameter. The method recommended here could be used for studies on the genetic control of the intermediate-size, 300–800  $\mu\text{m}$ , uredinia, but the concentration of viable urediniospores should then be meticulously adjusted to the recommended level and depression of the sprayer valve carefully controlled to avoid obtaining

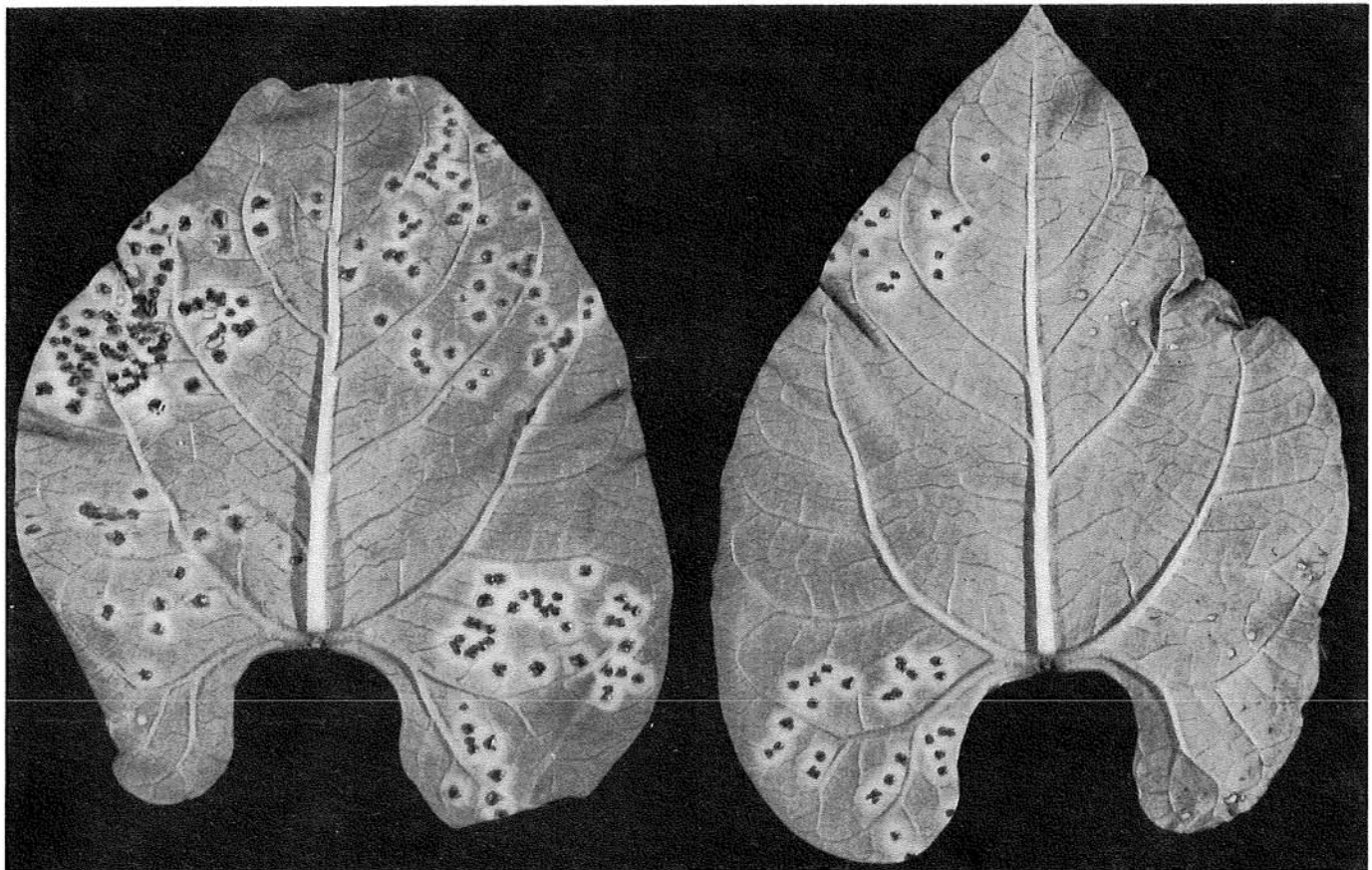


Fig. 2. Unifoliolate leaves from a single plant of *Phaseolus vulgaris* cultivar BBL 94 15 days after inoculation with eight distinct collections or cultures of *Uromyces phaseoli*. Inocula (clockwise from the upper left quadrant) were: left leaf—79-15, 79-6 A, 79-4, and 79-6; right leaf: 73-32, 73-23, 73-16 A, and 75-22.

TABLE 1. Comparison of the effects of two inoculation methods on the reactions of seven cultivars of *Phaseolus vulgaris* to three pathotypes of *Uromyces phaseoli* 15 days after inoculation

Cultivar	Inoculation method <sup>a</sup>	Host reaction to pathotype: <sup>b</sup>		
		73-16 A	79-15 A	75-22
BBL 47	Single	3,4	2,3	2,3
	Multiple	3,4	2,3	2,3
BBL 94	Single	N <sup>+</sup> ,N <sup>++</sup> ,1	2,3,4	2,3,4
	Multiple	N <sup>+</sup> ,N <sup>++</sup> ,1	2,3,4	2,3,4
Eagle	Single	3,4	1,2	2,3
	Multiple	3,4	1,2	2,3
B-190	Single	N	N,1	N,1
	Multiple	N	N,1	N,1
CNC	Single	0	N,1	N,1
	Multiple	0	N,1	N,1
Olathe	Single	N,1	2,3,4	N
	Multiple	N,1	2,3,4	N
Pinto 111	Single	N	3,4	3,4
	Multiple	N	3,4	3,4

<sup>a</sup>All inoculations were done by spraying. Single = plants inoculated with a single pathotype. Multiple = plants inoculated with eight different pathotypes using a sprayer modified to restrict each inoculum application to a 12-mm-diameter area of leaf.

<sup>b</sup>Host reactions: 0 = no symptoms, N = necrotic spots less than 0.3 mm in diameter, N<sup>+</sup> = necrotic spots 0.3 to 1.0 mm in diameter, N<sup>++</sup> = necrotic spots 1-3 mm in diameter, 1 = pustules less than 0.3 mm in diameter, 2 = pustules 0.3-0.5 mm in diameter, 3 = pustules 0.5-0.8 mm in diameter, 4 = pustules larger than 0.8 mm in diameter. When more than one rating is given, the predominant reaction is italicized.

overcrowded uredinia.

A trial test should be conducted on several cultivars having different reactions to the pathotypes being tested prior to testing valuable hybrid plants or populations. If an error has been made in measuring viable spore concentration, it can then be corrected before making the critical inoculations.

The presence of infection by other pathotypes on the same or other leaves of the same plant had no effect on the reactions to any of the pathotypes studied (Table 1 and Fig. 2). This was true with all 14 tested pathotypes. In Fig. 2, uredinia and necrotic reactions are shown in the leaf quadrant inoculated with collection 79-15. This collection contained two pathotypes that gave these same two reactions on BBL 94 leaves inoculated solely with 79-15. When trifoliolate leaves were inoculated on a plant on which the unifoliolate leaves had been inoculated 6-8 days sooner, the reactions were the same as on plants inoculated only once with one pathotype.

The technique described here facilitates breeding beans with

resistance to multiple races of *U. phaseoli* and determination of the genetics of resistance. It has been used successfully on several thousand plants at Beltsville over the past year and has made possible a study of the genetics of resistance that was recently published in abstract form (11).

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