Use of Detached Soybean Cotyledons for Testing Pathogenicity of *Xanthomonas campestris* pv. *glycines*

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ABSTRACT

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A rapid technique soybean (*Glycine max*) cotyledon assay was developed to test the pathogenicity of *Xanthomonas campestris* pv. *glycines*, which causes bacterial pustule. Detached soybean cotyledons from 10- to 14-day-old seedlings grown in the greenhouse were surface-sterilized with 0.5% sodium hypochlorite for 5 min and washed with sterile distilled water. The center of the cotyledons were wounded with multiple pins attached to the end of a wooden stick (six pins spaced evenly in an area 6 mm in diameter). Wounds were rubbed gently with cotton swabs dipped in a suspension of pathogenic and nonpathogenic strains of *X. c. glycines*, and inoculated cotyledons were placed in moist trays and kept in a lighted incubator at 30 C. Within 2–3 days after inoculation, areas of the cotyledons inoculated with pathogenic strains turned yellowish, whereas those inoculated with nonpathogenic strains did not show any discoloration. Pathogenic and nonpathogenic responses based on detached cotyledon assay were not distinguishable from those based on intact cotyledon assay. Lack of symptom induction on detached cotyledons was equivalent to lack of pathogenicity on leaves. Repeated experiments with this technique resulted in consistent detection of nonpathogenic mutants from 2,000 colonies of the bacterium treated with *N*-nitro-*N*-methyl-*N'*-nitrosoguanidine.

Xanthomonas campestris pv. *glycines* (Nakano) Dye is the causal agent of bacterial pustule of soybeans (*Glycine max* (L.) Merr.), which is one of the most prevalent bacterial diseases in many areas where soybeans are grown (1). The bacterium infects through stomata and wounds on soybean leaves and causes hypertrophy of host cells (7).

Forcible spraying of inoculum to leaves commonly is used to inoculate soybean plants with this pathogen (6). Development of pustule symptoms is affected by host resistance, inoculum concentration, temperature, and relative humidity, and results are not always consistent (4). Testing large numbers of bacterial strains for pathogenicity requires a rapid and reliable assay technique. Large numbers of plants can be screened for resistance to bacterial pustule in a small space by infiltrating soybean seedlings with dilute cell suspensions of *X. c. glycines* (2). In addition, a rapid inoculation method using turnip seedlings grown on agar plates has been used successfully to screen large numbers of colonies of mutagenized *X. c. campestris* (Pammel) Dowson strains for pathogenicity (3). In this article, a rapid, reliable assay technique using detached soybean cotyledons to test large numbers of colonies of *X. c. glycines* for pathogenicity is described.

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MATERIALS AND METHODS

Bacterial strains. A strain of *X. c. glycines*, 8ra, was obtained from E. J. Braun, Department of Plant Pathology, Iowa State University, Ames, IA. Strain 8ra is resistant to 50 μg/ml of rifampicin. Strains of *X. c. glycines* (xp29, 1717, 1124, and S-9-4) were obtained from W. F. Fett, Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, PA. These strains are avirulent to soybean cultivars carrying gene *rxp*. A strain of *X. c. glycines* 8601 was isolated from the Agronomy-Plant Pathology South Farm at Urbana, IL. This strain is virulent to cv. Williams. *X. c. campestris* XCC1 (resistant to 50 μg/ml of rifampicin), pathogenic in cabbage but not in soybean plants, was obtained from S. M. Ries, Department of Plant Pathology, University of Illinois, Urbana, IL. Bacteria were routinely cultured on YDC (10 g of yeast extract, 5 g of dextrose, 20 g of calcium carbonate, and 15 g of agar per liter of distilled water) agar (10) at 30 C for 24 hr.

Strain 8ra was mutagenized with *N*-nitro-*N*-methyl-*N'*-nitrosoguanidine (NTG) to obtain nonpathogenic mutants as follows. One milliliter of an overnight culture of *X. c. glycines* grown in L broth at 30 C on a shaker was centrifuged at 14,000 *g* for 1 min. The pellet was suspended in 0.1 M sodium citrate/citric acid buffer (pH 5.5), centrifuged, and suspended in 1 ml of the same buffer. The NTG was added to a final concentration of 10 μg/ml, and the suspension was incubated for 10–50 min at room temperature. Fifty to 70% of the bacterial cells were killed as determined by a survival curve. The bacterial suspension treated with NTG was centrifuged at 14,000 *g* for 1 min. The bacterial pellet was washed twice with 0.1 M sodium phosphate buffer (pH 7.0), resuspended in 5 ml of L broth (8), and incubated for 24 hr at 25 C. Cells were diluted serially, transferred to plates of YDC agar, and incubated overnight at 30 C. Individual colonies were isolated and replica-plated onto YDC agar and M9 minimal agar medium (9) to distinguish prototrophic from auxotrophic mutants. Colorless mutants were identified on YDC agar. The pathogenicity of colorless mutants was determined by inoculating leaves of susceptible soybean cv. Pella. Two thousand prototrophic colonies of *X. c. glycines* were obtained from a cell suspension that had been exposed

to NTG. The prototrophs were maintained on YDC agar and tested for pathogenicity.

Growth of soybean plants. Seeds of Pella were planted in 18 × 30 cm metal flats filled with sterilized sand or in 20-cm-diameter clay pots filled with sterilized soil/sand (1:1, v/v). Ten to 14 days later, before primary leaves were fully expanded, cotyledons were detached from the seedlings.

Inoculation. Three different inoculation procedures were evaluated, i.e., wound inoculation of detached cotyledons, wound inoculation of intact cotyledons, and spray inoculation of leaves. For the detached cotyledon assay, cotyledons were washed with sterile distilled water, soaked in 0.5% sodium hypochlorite for 5 min, and washed again with sterile water. They were allowed to air-dry on a clean bench. Each cotyledon was pricked once with sterilized needles attached to the end of a wooden stick (six pins were spaced evenly on a surface 6 mm in diameter). The wounded area then was rubbed gently with a cotton swab that had been dipped into a bacterial suspension (10^8 cfu/ml) of *X. c. glycines* or of the nonhost pathogen *X. c. campestris* XCC1. After inoculation, cotyledons were placed in moist trays, sprayed lightly with sterile water, and kept in a lighted incubator at 30 C. For controls, wounded cotyledons were rubbed with cotton swabs dipped in sterile water. The same technique was used in the attached cotyledon assay, except for cotyledon removal from the plants. In the spray inoculation, bacterial cells were resuspended in sterile water and adjusted turbidimetrically to a final concentration of 10^8 cells per milliliter. Seedlings of Pella at the V3 growth stage (5) were sprayed with bacterial suspensions at a pressure of 5.3 kg/cm^2 with an airbrush until runoff. The airbrush was held approximately 15–20 cm from the leaves. Sterile water was sprayed on leaves for controls. Inoculated plants were kept at 25–30 C in the greenhouse. Symptom development was evaluated 7–10 days after inoculation.

Fig. 1. Symptoms on 10-day-old soybean cv. Pella cotyledons 5 days after inoculation with water (left), *Xanthomonas campestris* pv. *glycines* parent strain 8ra (middle), and nonpathogenic mutant NP1 (right).

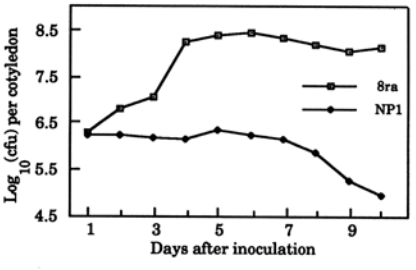

Fig. 2. Growth of *Xanthomonas campestris* pv. *glycines* parent strain 8ra and nonpathogenic mutant NP1 in soybean cotyledons. Values of Log_{10} (cfu) per cotyledon are the mean of three cotyledons. Individual cotyledons were macerated in 5 ml of sterile distilled water and diluted serially. One-tenth of a milliliter of each diluted cell suspension was transferred onto YDC agar. Bacterial colonies were counted 3 days after inoculation at 30 C.

Growth of bacteria in cotyledons. Pathogenic strain 8ra and nonpathogenic strain NP1 of *X. c. glycines* and *X. c. campestris* strain XCC1 were grown overnight on YDC agar at 30 C. The cells were pelleted by centrifugation, suspended in 5 ml of sterile distilled water, and adjusted to 10^8 cfu/ml. Wounds were made on detached cotyledons of Pella as described. Ten-microliter portions of the cell suspensions were placed on the surface of the wounded area. Nine cotyledons, three from each of three replications, for each treatment were sampled each day after inoculation for 10 days. Each cotyledon was macerated in 5 ml of sterile distilled water with a sterile mortar and pestle. Suspensions were diluted serially with sterile distilled water, and three 0.1-ml samples of each diluted tissue suspension were transferred to three culture plates containing YDC agar supplemented with 50 μg/ml of rifampicin. Three days after incubation at 30 C, colonies formed in the plates were counted. This test was repeated three times.

RESULTS AND DISCUSSION

Yellow chlorotic areas developed on the wounded detached cotyledons of Pella inoculated with all pathogenic strains of *X. c. glycines* 2–3 days after inoculation (Fig. 1). Sixteen nonpathogenic mutants were obtained from the 2,000 prototrophic colonies of the mutagenized strain 8ra with NTG. They were designated as NP1, I3, I8, I9, I10, I11, I13, I14, III1, III2, III3, III5, III6, IV3, IV6, and V5. No yellow chlorotic symptoms were observed in the wounded detached cotyledons of Williams when inoculated with strains xp29, 1717, 1124, and S-9-4. However, strain 8601 caused yellow chlorotic symptoms in the wounded detached cotyledons of Williams. Yellow chlorotic symptoms did not develop in cotyledons inoculated with *X. c. campestris* XCC1 or water (Fig. 1).

When the intact cotyledons of seedlings were inoculated with the pathogenic parental strain 8ra in the greenhouse, symptoms identical to those on the detached cotyledons developed 2–3 days after inoculation. No symptoms, however, developed on intact cotyledons inoculated with the 16 nonpathogenic strains, *X. c. campestris* XCC1, or water. When pathogenic and nonpathogenic strains were sprayed onto soybean leaves, the 16 nonpathogenic strains did not produce symptoms and the pathogenic strain produced pustules. Thus, the production of chlorotic areas around the wounds was associated with pathogenicity. Four colorless mutants identified on YDC agar caused yellowing in the wounded cotyledons and produced pustules in intact plants.

The pathogenic strain 8ra multiplied in the cotyledons, reaching maximum populations of 1.5 × 10^8 to 4.0 × 10^8 cfu per cotyledon by 5 days after inoculation (Fig. 2). The nonpathogenic strain tested, NP1, did not multiply and the bacterial populations exhibited a gradual decrease. Both NP1 and the nonhost bacterium, *X. c. campestris* XCC1 (*data not shown*), survived during the 10-day period after inoculation. This experiment was repeated three times, and similar growth patterns were obtained from each experiment.

The detached cotyledon assay in the laboratory is an efficient method for testing the pathogenicity of a large number of strains of *X. c. glycines*. The assay provides rapid, reliable, and consistent results.

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