An In Vitro Screen for Detecting Resistance in *Pelargonium* Somaclones to Bacterial Blight of Geranium

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ABSTRACT

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Resistance to *Xanthomonas campestris* pv. *pelargonii* was detected among somaclones of *Pelargonium* species with an assay of plantlet reactivity to inoculum titer, strain, and pathovar. Three weeks after plantlets growing in culture tubes were inoculated with a 10^7 cfu/ml cell suspension of *X. c.* pv. *pelargonii*, seed geranium (*P.* × *hortorum*) somaclones from susceptible cultivars had a significantly lower survival rate than did those from the more resistant regal geranium (*P.* × *domesticum*). Symptoms did not develop among plantlets treated with *X. c.* pv. *campestris* or sterile water. Somaclones of *P. scabrum, P. betulinum, P. grandiflorum, P. multicaule*, and *P. hispidum* had significantly higher levels of resistance to *X. c.* pv. *pelargonii* than did those of *P.* × *hortorum*. This assay holds promise as a means of rapidly identifying bacterial blight-resistant somaclones.

Hybrid seed geranium (*Pelargonium* × *hortorum*:n=18) and cutting geranium (*P.* × *hortorum*:n=36) (4) compose the major portion of more than $49 million in annual potted geranium sales in the United States (11). Bacterial blight caused by *Xanthomonas campestris* pv. *pelargonii* (Brown) Dye is one of the most serious diseases of geranium (13,19). Localized epidemics occur throughout the United States each year (7,21). Once introduced, this pathogen often spreads throughout the greenhouse, causing great losses (21). Currently, the only controls for bacterial blight are to exclude the bacterium from greenhouses and to practice strict sanitation (18). In spite of good management practices, this disease continues to cause losses.

In general, the most effective and least expensive control for plant disease is the use of resistant cultivars. Useful resistance to bacterial blight has not been reported for cutting geranium (7,9,20,21) or hybrid seed geranium (19). Somaclonal variation has been used successfully as a source of novel resistance to plant disease in a number of crop species (1,14,22,23). A high degree of somaclonal variation has been reported for the genus *Pelargonium* (6,16), making somaclonal variation a promising source of resistance to bacterial blight. The frequency of a particular somaclonal variant trait has been estimated to be between 0.2 and 3% (1); therefore, a large number of plants must be regenerated in order to select for a particular variant trait. Procedures were recently developed that facilitate regeneration of large numbers of hybrid seed geraniums from callus culture (3). The purpose of this investigation was to develop a procedure to rapidly evaluate resistance to bacterial blight in geranium somaclones soon after shoot regeneration.

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MATERIALS AND METHODS

Regeneration of hybrid seed geraniums. To eliminate surface populations of microbes, seeds from hybrid geranium cultivars Red Orbit, Scarlet Orbit, Appleblossom Orbit, and White Orbit were submerged first in 95% ethanol for 1 min, then in 20% (v/v) household bleach (5.25% sodium hypochlorite) solution containing 0.5 ml of Tween 20 per liter for 30 min. The seeds were rinsed three times in sterile distilled water, then germinated on moist filter paper in 100 × 25 mm petri dishes (15 seeds per dish) sealed with Parafilm M. After the seedlings were 10–14 days old, 4-mm shoot tip and hypocotyl explants were removed with a sterile surgical blade and placed on Murashige and Skoog (MS) medium (12) that had been supplemented with 11.0 μM indole acetic acid, 9.3 μM zeatin (*trans* isomer), and 2.0% sucrose, adjusted to pH 5.8 with 1.0 N KOH, and solidified with 0.9% agar. After 30 days, calli with shoot primordia were transferred to the same medium with no auxin and with one-tenth the cytokinin. Thirty days later, well-developed shoots were transferred into culture tubes (25 × 150 mm) containing 15 ml of Hoagland's solution (2) that had been solidified with 0.7% agar (HSS) for rooting. All plant material was maintained at 24 C with 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ light and a 16-hr photoperiod provided by cool-white fluorescent lamps.

Regeneration of regal geraniums. Regal geranium leaves were washed with a 0.01% (w/v) Alconox detergent solution (Alconox Inc., New York), rinsed for 3 min under tap water, surface-washed in 95% ethanol for 1 min, submerged in a 20% (v/v) household bleach solution with 0.5 ml of Tween 20 per liter for 20 min, and rinsed three times with sterile water. The surface-disinfected leaves were cut into 1-cm^2 sections, which were plated on MS medium supplemented with 9.3 μM 6-benzylaminopurine (6-BAP), 11.0 μM naphthalene acetic acid (NAA), and 2.0% sucrose, adjusted to pH 5.8 with 1.0 N KOH, and solidified with 0.9% agar. Callus tissue with shoot primordia was transferred to the same medium with no NAA and with one-tenth the 6-BAP to induce shoot development. After 30 days, well-developed shoots were placed in culture tubes containing 15 ml of HSS medium. Regeneration procedures for *P. scabrum, P. betulinum, P. grandiflorum, P. multicaule*, and *P. hispidum* were the same as for regal geranium. Regeneration procedures for *P. denticulatum* were the same as for regal geranium except that 5-mm stem sections replaced leaf tissue as the explants. After 2–3 mo on HSS medium, rooted plants were transferred to Bacto Professional Planting Mix (Michigan Peat Co., Houston, TX) in plastic cell packs (9.0 × 12.5 cm) and covered with a plastic bag for 1 wk.

Bacterial strains. The four strains of *X. c.* pv. *pelargonii* used in the investigation were X-1, X-4, X-5, and X-7. Strain X-1 was collected in Kansas and X-5 was collected in Michigan. Strains X-4 (5-2-4 from Israel) and X-7 (5-1-7 from New York) were provided by M. Daughtrey, Cornell University. The strain of *X. c.* pv. *campestris* used was collected in Michigan. Strains of *X. c.* pv. *campestris* and *X. c.* pv. *pelargonii* stored in saline (0.85% NaCl) at 4 C were streaked onto Difco nutrient agar in petri dishes and incubated at 23 C. After 3–5 days, single colonies were transferred to 25 ml of Lederberg's complete broth (10) modified by elimination of glucose. Broth cultures were incubated at 25 C on a rotary shaker at 125 rpm. After 48 hr, bacteria were pelleted by centrifugation at 900 *g* for 15 min. Pellets were resuspended in 25 ml of saline and centrifuged as before. The pellet was then resuspended in sterile water and diluted to the desired concentration of colony-

forming units based on standard dilution-plating and turbidimetric techniques.

Inoculations. Thirty days after the regenerated shoots had been placed onto HSS medium in culture tubes, the upper surface of individual leaves was gently rubbed with a sterile cotton swab that had been moistened with inoculum, and the tubes were sealed with Parafilm M for 2 days. Then, 14 or 21 days later, the plants were rated for disease as follows: 1 = no symptoms, 2 = <20% tissue blighted, 3 = 20–50% tissue blighted, 4 = 51–75% tissue blighted, 5 = >75% tissue blighted, and 6 = plant death.

To determine the effect of inoculum concentration on disease development in plants growing in tubes, seedlings of hybrid White Orbit were inoculated as described above with 0, 10^3, 10^5, 10^7, and 10^9 cfu/ml. Seeds were disinfected and germinated as described above. Germinated seeds were transferred to HSS medium and inoculated 30 days later. This experiment had a completely random design with six single-plant replicates (one plant per culture tube) per treatment. The experiment was repeated once.

To determine the effect of inoculum concentration on disease development in plantlets regenerated from calli, White Orbit plants growing in tubes were inoculated with 0, 10^5, 10^7, and 10^9 cfu/ml. This experiment had a random design with six single-plant replicates per treatment. The experiment was repeated once with White Orbit and twice with Appleblossom Orbit.

To test the reaction of plants growing in tubes to another plant-pathogenic bacterium, White Orbit somaclones and 30-day-old seedlings of cabbage (*Brassica oleracea* var. *capitata*) were inoculated with 10^7 cfu/ml of *X. c.* pv. *pelargonii* or *X. c.* pv. *campestris* or with sterile water. Seeds of cabbage hybrid H (Harris Seeds, Rochester, NY) were disinfected and germinated (30 per petri dish) as described above for hybrid geranium seeds. After 1 wk on filter paper, seedlings were placed onto 15 ml of HSS medium in 25 × 150 mm culture tubes. The experimental design was random and factorial, with plant species and inoculum as independent variables. There were 10 single-plant replicates per treatment, and the test was repeated.

To determine if the use of different strains of *X. c.* pv. *pelargonii* might lead to different disease severities, geranium hybrids White Orbit, Red Orbit, Appleblossom Orbit, and Scarlet Orbit were inoculated with four different strains, each at 10^7 cfu/ml. This experiment was a random, two-factor factorial with five replicates per treatment and was repeated once.

To determine if different *Pelargonium* species and cultivars varied in susceptibility to *X. c.* pv. *pelargonii*, somaclones were obtained from *P.* × *hortorum*, *P.* × *domesticum*, *P. scabrum*, *P. betulinum*, *P. grandiflorum*, *P. multicaule*, *P. hispidum*, and *P. denticulatum* and inoculated with strain X-1 in the first test and with strain X-7 in the second test. The experiment had a random design, with five single-plant replicates per treatment.

Greenhouse inoculations. Plants were grown and inoculated in the greenhouse using the procedures described by Stephens and Tuinier (19). Eight cultivars of *P.* × *domesticum* (Grand Slam, Lavender Grand Slam, Olga, Pink Gardener's Joy, Virginia, Parisienne, Melissa, and Tiny Tot) and two cultivars of *P.* × *hortorum* (Red Orbit, a seed geranium, and Mrs. Parker, a cutting geranium) were inoculated with strain X-1. The experiment had a random design with four single-plant replicates and was repeated.

RESULTS

In vitro inoculations. Symptoms of bacterial blight observed on regenerated geraniums grown on HSS medium and inoculated in vitro included circular water-soaked lesions, leaf blight, leaf wilt with erect petiole, and plant death. Water-soaked lesions were observed within 7 days after inoculation. Leaves with lesions often became completely blighted within 1–2 wk. Occasionally, V-shaped lesions were observed.

In preliminary experiments with 5-wk-old hybrid geranium seedlings, use of 10^3 cfu/ml of *X. c.* pv. *pelargonii* for inoculations led to variable disease severity, so this concentration was not used to inoculate plantlets. Cell suspensions of 10^7 and 10^9 cfu/ml led to death of all plantlets within 3 wk (Fig. 1). In contrast, with 10^5 cfu/ml, 84% of the plantlets survived for 3 wk but were dead within 6 wk. Symptoms of disease were not observed on somaclones inoculated with sterile water, although the lower leaves became senescent within 2–3 wk, which led to a disease rating of 3.0 (Fig. 1). An inoculum concentration of 10^7 cfu/ml was chosen for successive experiments because it was the lowest inoculum level that allowed rapid screening of geranium somaclones for resistance to bacterial blight. Similar results were obtained in the second test

Table 1. Bacterial blight in somaclones of different *Pelargonium* species and cultivars 2 wk after inoculation of plantlets grown in tubes[x]

Pelargonium species/cultivar[y]	Disease rating[z]
P. × *hortorum* Red Orbit (s)	6.0 a
P. × *hortorum* Scarlet Orbit (s)	5.6 ab
P. × *hortorum* Appleblossom Orbit (s)	5.2 ab
P. denticulatum	4.6 bc
P. × *domesticum* Melissa (r)	4.3 cd
P. hispidum	3.0 d
P. multicaule	3.0 d
P. grandiflorum	2.6 d
P. × *domesticum* Virginia (r)	2.4 d
P. × *domesticum* Olga (r)	2.2 d
P. betulinum	2.2 d
P. scabrum	2.0 d

[x]Thirty days after shoots regenerated from calli were transferred to HSS medium, plantlets were inoculated by gently rubbing the upper leaf surface with a cotton swab moistened with 1 × 10^7cfu/ml of *Xanthomonas campestris* pv. *pelargonii*.

[y](r) = Regal geranium, (s) = hybrid seed geranium.

[z]1 = No symptoms, 2 = <20% tissue blighted, 3 = 20–50% tissue blighted, 4 = 51–75% tissue blighted, 5 = >75% tissue blighted, and 6 = plant death. Means with the same letter are not significantly different according to Duncan's multiple range test ($P = 0.01$).

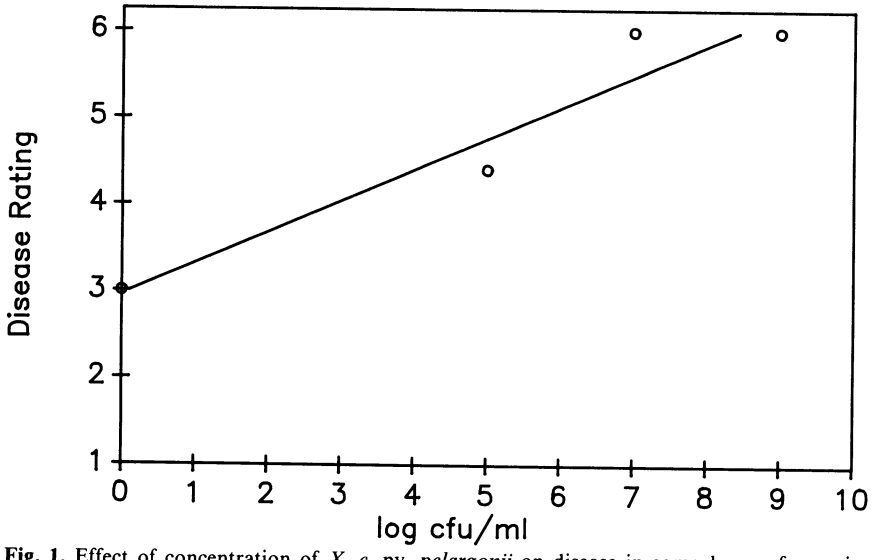

Fig. 1. Effect of concentration of *X. c.* pv. *pelargonii* on disease in somaclones of geranium cultivar White Orbit rated 3 wk after inoculation in vitro: 1 = no symptoms, 2 = <20% tissue blighted, 3 = 20–50% tissue blighted, 4 = 51–75% tissue blighted, 5 = >75% tissue blighted, and 6 = plant death. *r* value = 0.828.

with White Orbit and in the two tests with Appleblossom Orbit (*data not shown*).

Although the environment in the tubes during the 2-wk incubation period was conducive for disease, somaclones of White Orbit inoculated with *X. c.* pv. *campestris* were free from disease (mean disease rating [MDR] = 2.2), as were those treated with water (MDR = 2.6). In contrast, many plantlets inoculated with *X. c.* pv. *pelargonii* were dead (MDR = 5.3). However, *X. c.* pv. *campestris* caused blight symptoms on cabbage seedlings (MDR = 5.1) and *X. c.* pv. *pelargonii* (MDR = 2.5) did not; the MDR of seedlings treated with water was 1.2. The effect of inoculum and the inoculum/host interaction on disease was highly significant (ANOVA $P < 0.001$), but the effect of the host species was not.

The four strains of *X. c.* pv. *pelargonii* differentially affected disease observed 2 wk after inoculation (ANOVA $P < 0.001$). Strains X-1 (MDR = 5.6) and X-7 (MDR = 5.8) appeared to be more aggressive than strains X-4 (MDR = 4.3) and X-5 (MDR = 4.7). However, all somaclones were dead within 2 mo, regardless of the strain. Geranium hybrid and hybrid/strain interaction was not significant.

Two weeks after inoculation, less disease was observed among plantlets of regal geranium than among those of hybrid seed geranium (Table 1). Surviving regal plants were successfully transferred to soil and established in the greenhouse.

Somaclones from *P. scabrum, P. betulinum, P. grandiflorum, P. multicaule*, and *P. hispidum* also had less disease than those of three hybrid seed geranium cultivars (Table 1). Leaf spots were observed on all species within 2 wk. After 4 wk, the percentage of dead plants was 100% for *P.* × *hortorum* and *P. denticulatum*, 33% for *P. scabrum* and *P. multicaule*, 16% for *P. hispidum* and *P. betulinum*, 6% for *P.* × *domesticum*, and 0% for *P. grandiflorum*.

Over 1,000 somaclones regenerated from seed geranium callus cultures have been inoculated in vitro with *X. c.* pv. *pelargonii*. Only two have survived to be rooted in soil and transferred to the greenhouse.

Greenhouse inoculations. The eight regal geranium cultivars inoculated in the greenhouse with *X. c.* pv. *pelargonii* were more resistant than the seed geranium or cutting geranium cultivars tested. Three days after inoculation, water-soaked lesions were observed on all inoculated plants, although the regal had fewer lesions than the seed or cutting geraniums. After 3 wk, plants of *P.* × *domesticum* had necrotic spots and less than 50% of leaf tissue necrotic, whereas those of *P.* × *hortorum* had over 75% necrosis. After 6 wk, all *P.* × *hortorum* plants were dead, whereas *P.* × *domesticum* plants had produced new growth free from disease symptoms.

DISCUSSION

The efficient detection and selection of resistant individuals from populations of somaclones derived from *P.* × *hortorum* requires methods for rapid regeneration and disease screening. A leaf-rub inoculation of plantlets growing in tubes can be used to differentiate between resistant and susceptible plant genotypes. Moreover, the reaction of the plantlets was similar to that observed for mature plants. In previous tests with mature plants, *P.* × *domesticum* was more resistant to bacterial blight than was *P.* × *hortorum* (7,9). Stephens and Tuinier (19) inoculated 63 hybrid seed geranium cultivars in the greenhouse with *X. c.* pv. *pelargonii* and found the hybrids used here (White Orbit, Appleblossom Orbit, Scarlet Orbit, and Red Orbit) to be susceptible to bacterial blight (19). We used the same methods with eight regal geranium cultivars (including Olga, Virginia, and Melissa) and found them to be resistant to bacterial blight when compared with cutting and seed-propagated cultivars of *P.* × *hortorum*. Plantlets of regal geranium inoculated in vitro were significantly more resistant to bacterial blight than those of hybrid seed geranium cultivars (Table 1). Thus, the in vitro assay can be used to detect differences in bacterial blight resistance similar to that observed in the greenhouse. The strains of *X. c.* pv. *pelargonii* used in the test can affect the development of disease. Kivilaan and Scheffer (8) showed that different strains of *X. c.* pv. *pelargonii* varied in aggressiveness to cutting geraniums. We observed similar differences with our four strains of the pathogen in our assay.

Somaclones of *P. scabrum, P. betulinum, P. grandiflorum, P. multicaule, P. hispidum*, and *P.* × *domesticum* were significantly more resistant to bacterial blight than were those of *P. denticulatum* and *P.* × *hortorum*. Previously, mature plants of *P.* × *domesticum, P. acerifolium, P. tomentosum*, and *P. scarboroviae* were reported to have resistance (9).

The use of an in vitro inoculation of plantlets or shoots grown in a controlled environment has been suggested as a way to screen large amounts of germ plasm (5,15,17). Such tests have been used to evaluate disease resistance in *Asparagus* (17), papaya (15), and peaches (5). We report here that somaclones of *Pelargonium* species can be inoculated in vitro with *X. c.* pv. *pelargonii* soon after shoot regeneration. Large numbers can be rapidly evaluated for resistance to bacterial blight. Moreover, the procedure requires little space, and survivors can be established in soil in the greenhouse.

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