Development of a Polyclonal Antibody-Based Serodiagnostic Assay for the Detection of *Xanthomonas campestris* pv. *pelargonii* in Geranium Plants

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ABSTRACT

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Polyclonal antisera were produced by immunizing New Zealand white rabbits with a phosphate-buffered saline suspension of *Xanthomonas campestris* pv. *pelargonii*. Antisera then were used to develop an immunogold silver stain dot-immunobinding assay for the detection of this pathogen in geranium plants. Immunoglobulins also were purified and used to develop a direct enzyme-linked immunosorbent assay (ELISA). This assay was used to determine the presence of *X. c. pelargonii* in symptomatic and asymptomatic plants. Relationships among strains of *X. c. pelargonii*, various pathovars of *X. campestris*, unrelated bacteria, and fungal pathogens of geranium were examined by direct ELISA. High reactivity was observed for *X. c. pelargonii*, but five of seven other tested pathovars also gave moderate reactions. Movement of *X. c. pelargonii* in root inoculated geranium cuttings also was examined.

Bacterial wilt of florists' geranium (*Pelargonium* × *hortorum* L. H. Bailey) caused by *Xanthomonas campestris* pv. *pelargonii* is recognized as the most important disease of this ornamental crop (10). Symptoms associated with this disease include a general wilting of the plant, localized water-soaked lesions, and/or V-shaped lesions on the leaves, and a stem rot. These symptoms can be confusing to the grower as well as the plant pathologist, because the V-shaped lesions and the general wilting of the plant also may be the result of improper cultural practices such as overwatering, nutrient imbalance, and inadequate light (10).

X. c. pelargonii survives in the xylem of geranium plants. There are no effective chemical controls; therefore, prevention is the best deterrent. The primary preventive measure is the use of culture-indexed stock plants. Geraniums are vegetatively propagated from these culture-indexed plants, but culture-indexing may fail to detect an infection, and the bacterium may be carried over in infected cuttings. If the pathogen is isolated from a greenhouse, the grower is advised to discard all suspect geranium plants. This action could cost growers thousands of dollars annually.

Currently, diagnosis of bacterial wilt involves culturing and/or bioassay (6). In most cases, the bioassay has proved to be inacceptable due to the time involved and the occurrence of ambiguous results. Thus, the objective of this study was to develop a rapid, reliable polyclonal antibody-based serodiagnostic assay to detect *X. c. pelargonii* in geranium plants.

MATERIALS AND METHODS

Production of polyclonal antisera. Two-hundred-fifty-milliliter Erlenmeyer flasks containing 75 ml of complete broth (4) were inoculated with a highly virulent strain of *X. c. pelargonii* (UCRDAC 0782-29) and shaken at 80 rpm at room temperature for 3 days. Cells were harvested at 8,000 *g* for 20 min and washed three times with phosphate-buffered saline (PBS) (0.01 M potassium phosphate buffer [pH 7.2] containing 0.15 M NaCl). Bacterial cells were resuspended in wash solution and their concentration adjusted turbidimetrically to approximately 10^6 cfu/ml.

Two 3-mo-old female New Zealand white rabbits were each injected with 2 ml of a bacterial emulsion made by combining 2.5 ml of bacterial suspension with 2.5 ml of Freund's incomplete adjuvant (Difco, Detroit, MI). Injection was intramuscular in the groin, and each rabbit was injected three times. The initial injection was followed by two booster injections 10 days apart.

Sera were retrieved from the rabbits by a nonlethal bleeding of the marginal vein of the ear (7). Rabbits were bled before the first injection for normal sera. The first bleeding to recover antisera was done 14 days after the first booster injection. The blood was allowed to clot, the clot was removed by centrifugation, and the sera stored at −20 C. Antisera titers were determined by indirect enzyme-linked immunosorbent assay (iELISA) (11).

Antisera from bleeds 2–4 of Rabbit #1 and bleeds 1–4 of Rabbit #2 were pooled for Immunoglobulin G (IgG) purification by affinity chromatography. Affinity chromatography and enzyme conjugation were performed in cooperation with Agri-Diagnostics Associates (Cinnaminson, NJ). Extraction buffer, wash solution, and enzyme substrate were supplied by Agri-Diagnostics and used according to manufacturer's protocol. Cross-reactivity of the antisera was determined by direct enzyme-linked immunosorbent assay (dELISA) (2). Antisera were tested for cross-reactivity with other strains of *X. c. pelargonii*, other pathovars of *X. campestris*, other species of *Xanthomonas*, and various other bacteria and fungi. Organisms were grown on plates of nutrient agar (Difco, Detroit, MI) for 3 days at 27 C (with the exception of *Puccinia pelargonii-zonalis*). Bacteria were scraped off the plate and suspended in 1 ml of extraction buffer and adjusted to $A_{660nm} = 1.0$. Fungi were scraped off nutrient agar plates, ground in liquid nitrogen with a mortar and pestle to a fine powder, and placed in 1 ml of the extraction buffer. In the case of *P. pelargonii-zonalis*, urediospores were removed from sections of geranium leaves and treated in the same manner. Stem sections excised from a healthy (negative control) geranium plant and a geranium plant inoculated with *X. c. pelargonii* (positive control) were included in this test.

Geranium sampling method. Cross sections (approximately 1 mm thick) of stems and petioles from symptomatic and asymptomatic plants were excised with a single-edged razor blade. The blade was disinfested between incisions by dipping in 70% ethyl alcohol. Four sections were taken from each stem or petiole

as diagrammed in Figure 1. Excised sections were placed into microcentrifuge tubes containing extraction buffer and the bacteria allowed to ooze out.

Immunogold silver staining dot-immunobinding assay. A commercial IGSS kit (Auroprobe BLPlus-IntenSE II, Janssen Pharmaceutica, Piscataway, NJ) was used to develop an immunogold silver staining dot-immunobinding assay (IGSS-DIA) for detection of *X. c. pelargonii* in geranium plants (1,3). Nitrocellulose membrane (Promega Biotec, Madison, WI) was saturated in distilled water and then air dried for 15 min. Stem and petiole sections, excised from symptomatic and asymptomatic plants as described above, were placed in 100 μl of extraction buffer (50 μg/ml BSA of distilled water) in a microfuge tube. The sections were soaked for 10 min and then mixed for 10 sec. With an Eppendorf pipette, 2 μl was spotted on the nitrocellulose membrane. The samples were air dried for 15 min. Subsequent steps were as per manufacturer's instructions.

Clinical and field testing. To determine the feasibilty of using a polyclonal antibody-based serodiagnostic assay for the detection of *X. c. pelargonii*, symptomatic and asymptomatic geranium plants were collected from commercial greenhouses in Ohio as well as the Plant and Pest Diagnostic Clinic, The Ohio State University, Columbus. Geraniums were sampled as described above and analyzed for the presence of *X. c. pelargonii* by the indirect IGSS-DIA or dELISA. All serological test results (IGSS-DIA and dELISA) were confirmed with culturing and/or bioassay (6) until confidence in the serological assays was established. Approximately 35 geranium samples were tested covering a 2-yr period.

Assay verification. Eighty culture-virus-indexed (CVI) cuttings of *Pelargonium* × *hortorum* 'Yours Truly' were tagged with a number (1–80) and removed from Styrofoam growing strips. Each cutting was placed in a Styrofoam cup containing 50 ml of distilled water. The roots were allowed to soak for 10 min and then gently agitated to remove as much of the rooting media as possible. Forty cuttings (numbers 41–80) served as controls. The remaining 40 cuttings (numbers 1–40) were root inoculated with the UCRDAC 0782-29 strain of *X. c. pelargonii* suspended in distilled water. Each cutting was placed in a Styrofoam cup containing 50 ml of the bacterial suspension and allowed to soak for 10 min. The cuttings designated as controls were each placed in a Styrofoam cup containing 50 ml of distilled water for 10 min. The control and inoculated cuttings were potted in 10-cm geranium pots containing a 1:1:1 (soil:peat:perlite) sterilized potting mixture. Plants were allowed to incubate for 4 hr before placement in the greenhouse. At this time, Chapin tubes were placed in the pots for irrigation of the plants. Plants were subjected to a 16-hr photoperiod with alternating day and night temperatures of 27–31 C and 23–27 C.

The plants were sampled (10 days postinoculation) and assayed (by dELISA) for the presence of *X. c. pelargonii*. At this time plants inoculated with *X. c. pelargonii* showed no symptoms of bacterial wilt. The base stem section (section A in Figure 1) was excised and cut in half across its diameter. One half was placed in a microcentrifuge tube containing extraction buffer.

A loopful of extraction buffer from each microcentrifuge tube containing the half-stem section was streaked onto a yeast extract-dextrose-$CaCO_2$ (YDC) agar plate (9). All plates were incubated at 28 C and observed daily for characteristic growth.

Movement of the bacterium in inoculated plants. Two hundred CVI cuttings of *Pelargonium* × *hortorum* 'Yours Truly' were tagged with a number from 1 to 200 and removed from Styrofoam

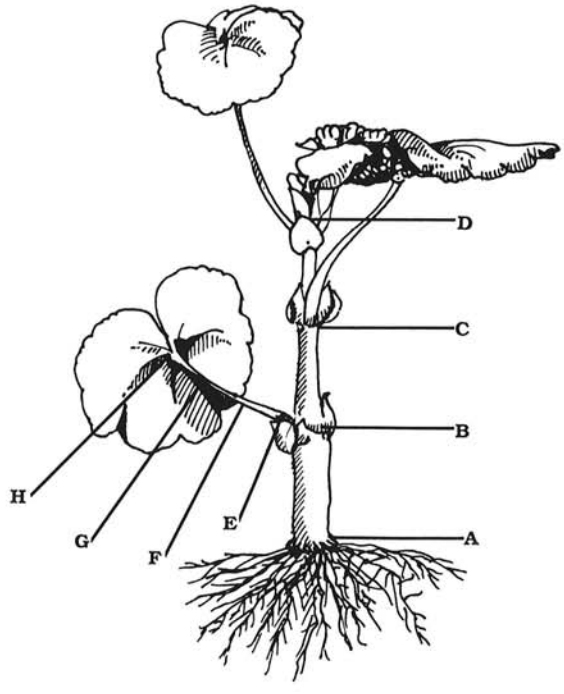

Fig. 1. Diagram of a geranium plant showing location of various sampling points (A–D, stem sections; E–H, petiole sections). Each point was sampled on all cuttings tested.

TABLE 1. Disease severity index for root inoculated geranium cuttings infected with *Xanthomonas campestris* pv. *pelargonii*

Rating	Leaf symptoms
1	No visible symptoms
2	Incipient wilt of leaves and/or marginal yellowing of leaves
3	Definite wilt of leaves and/or initiation of V-shaped lesions and/or moderate amount of yellowing
4	Severe wilt of leaves and/or V-shaped lesions and/or severe amount of yellowing
5	Complete collapse and/or necrosis of leaves

TABLE 2. Reaction of bacterial strains and fungi with antiserum prepared to *Xanthomonas campestris* pv. *pelargonii* (UCRDAC 0782-29) by dELISA

Organism tested	A_{405nm}[a]	Range
X. campestris		
pv. *pelargonii*		
OSUMJA-3	1.432	1.384–1.459
UCRDAC 0782-29	1.169	1.162–1.184
OSUMJA-5	1.608	1.525–1.678
PPDC 86-3362	1.471	1.434–1.525
pv. *begoniae*	0.467	0.415–0.543
pv. *campestris*	0.135	0.111–0.149
pv. *malvacearum*[b]	0.436	0.405–0.486
pv. *poinsetticola* 1[b]	0.439	0.435–0.443
pv. *poinsetticola* 2[b]	0.496	0.479–0.512
pv. *phaseoli*	0.024	0.014–0.035
pv. *vesicatoria* 2[b]	0.399	0.363–0.435
pv. *vesicatoria* 3[b]	0.357	0.340–0.377
pv. *hederae*[b]	0.385	0.363–0.403
Agrobacterium tumefaciens	0.003	0.000–0.010
Bacillus subtilis[b]	0.000	0.000–0.000
Erwinia chrysanthemi[b]	0.031	0.026–0.034
E. herbicola[b]	0.034	0.004–0.050
Pseudomonas aeruginosa[b]	0.034	0.000–0.059
P. cichorii[b]	0.004	0.000–0.012
P. solanacearum	0.005	0.003–0.007
Botrytis cinerea	0.000	0.000–0.000
Fusarium oxysporum	0.000	0.000–0.000
Puccinia pelargonii-zonalis	0.000	0.000–0.000
Pythium sp.	0.000	0.000–0.000
Negative geranium stem[c]	0.000	0.000–0.000
Positive geranium stem[d]	1.652	1.578–1.714

[a]Mean value of three readings.
[b]Contributed by Dr. Mike Klopmeyer.
[c]Stem from a healthy geranium plant.
[d]Geranium plant was inoculated with UCRDAC 0782-29.

growing strips. Cuttings numbered 1 to 100 served as controls. The remaining 100 cuttings were root inoculated with *X. c. pelargonii* as described above.

Random sampling of plants for detection of *X. c. pelargonii* began 24 hr after inoculation and continued for 20 days. Ten plants (five inoculated, five controls) were removed at random from the greenhouse and rated for disease severity on the basis of leaf symptoms only (Table 1). Stem symptoms were not observed. Stem and petiole sections were excised, and microcentrifuge tubes containing the excised sections in extraction buffer were placed at −20 C until testing by dELISA. Testing was completed within 24 hr. An experiment was done before this, which showed the excised stem and petiole sections could be stored in the extraction buffer at −20 C for 2 wk with no change in results.

RESULTS

Titer determination and cross-reactivity. The highest titer of the raw antisera was 1/32, 768 from rabbit #2, bleed #3. Bleeds #3 and #4 had the highest titer from Rabbit #1 at 1/4,096.

Anti-*X. c. pelargonii* (UCRDAC 0782-29) antisera reacted strongly against all strains of *X. c. pelargonii* and the inoculated plant (positive control). Other pathovars of *X. campestris* (pvs. *campestris, begoniae, hederae, malvacearum, phaseoli, poinsetticola* 1, *poinsetticola* 2, *vesicatoria* 2, *vesicatoria* 3) showed moderate or no cross-reactivity. There was no cross-reactivity with nonrelated phytopathogenic bacteria, nonphytopathogenic bacteria, selected fungi, or the healthy plant extract (Table 2).

Clinical and field testing. No false positives or false negatives were recorded on the 35 clinic and field samples tested by IGSS-DIA or dELISA. Of the 35 plants tested, 23 tested positive and 12 tested negative for *X. c. pelargonii*. Results of the serological tests were confirmed by culturing on YDC or by bioassay or both. One example of the IGSS-DIA's capability is indicated in Figure 2. Seven geranium samples were collected from a commercial greenhouse in Ohio. Five samples (Rows 1–5) were from plants produced for sale. Plants 1 and 2 were asymptomatic, while plants 3, 4, and 5 showed classic symptoms of bacterial wilt. Two of the samples (Row 6) were lateral branches from stock plants that showed symptoms of possible infection by *X. c. pelargonii*. Also included were stem and petiole sections from a healthy geranium plant (Row 7), and a symptomatic geranium plant (Row 8), which previously had tested positive for the presence of *X. c. pelargonii*. Plant 1, although asymptomatic, tested positive in both stem and petiole, while plant 2, which was also asymptomatic, tested negative in both stem and petiole (Fig. 2). Plants 3, 4, and 5 (the symptomatic plants) tested positive for *X. c. pelargonii* in both stem and petiole. The two lateral branches from stock plants tested negative for the presence of the pathogen.

Additional testing on geranium plants that tested negative for *X. c. pelargonii* revealed that symptoms were caused by pathogens of geranium other than *X. c. pelargonii* or were the result of improper cultural practices such as low light or lack of proper fertilization.

Assay verification. *X. c. pelargonii* was detected in 40 of the 40 root-inoculated cuttings and 0 of the 40 uninoculated control plants by dELISA. In all cases, stem sections reported as positive by dELISA (A_{405nm} = 1.5 or greater) were verified by growth typical of *X. c. pelargonii* on YDC. Stem sections that tested negative (A_{405nm} = 0.005 or less) for *X. c. pelargonii* by dELISA were verified by the lack of typical growth on YDC.

Movement of the bacteria in inoculated plants. *X. c. pelargonii* was first detected 4 days postinoculation in the base sections of the stems (Section A, refer to Fig. 1) of the inoculated plants (Figs. 3A and 4). As time progressed, the bacterium was detected simultaneously in Sections B and C (Figs. 3B and 4), and then the section from the stem apex, Section D (Figs. 3C and 4). The lowest petiole on each stem also was sampled. At 9 days postinoculation, the majority of the sections excised from the

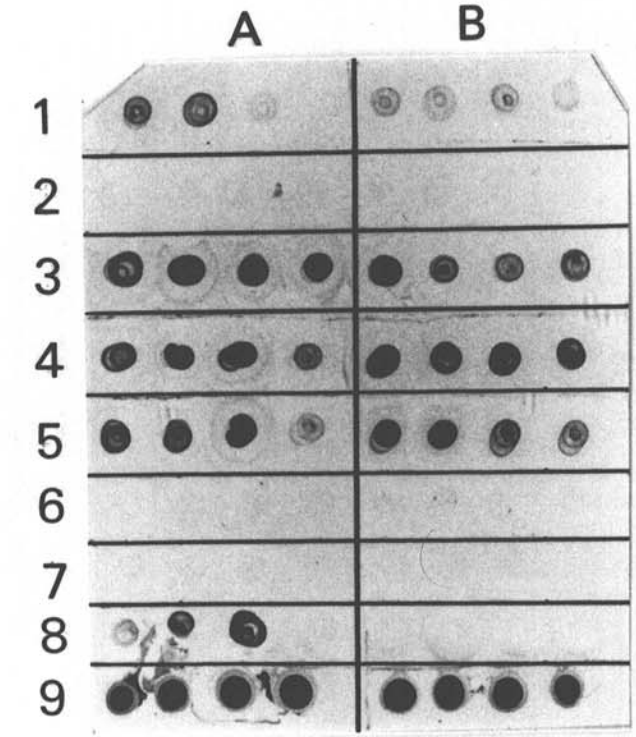

Fig. 2. Immunogold silver stained dot-immunoblot (IGSS-DIA) of sap samples taken from commercially produced geranium plants submitted to the diagnostic clinic. Column A, rows 1–5 are stem samples; column B, rows 1–5 are petiole samples. Rows 1 and 2 were asymptomatic plants; rows 3, 4, and 5 were symptomatic plants. Row 6 contained samples taken from lateral branches of stock plants. Row 7 contained samples taken from a healthy geranium; rows 8 and 9 were positive controls. Row 8 contained samples from a symptomatic geranium. Row 9 contained a suspension of *Xanthomonas campestris* pv. *pelargonii* in extraction buffer.

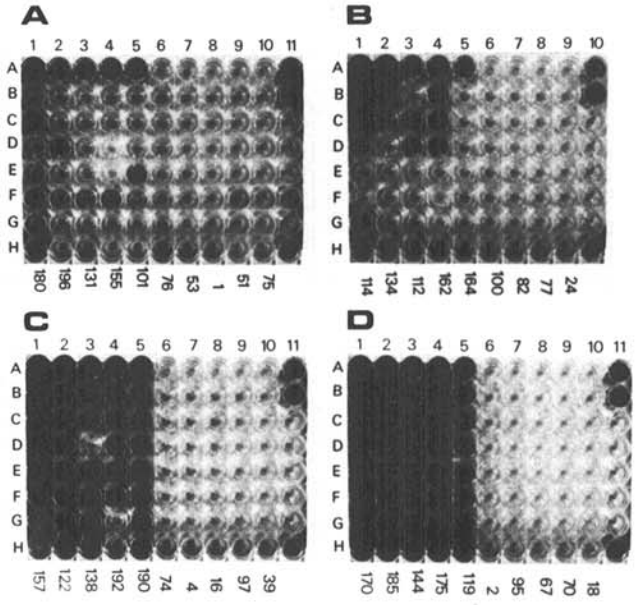

Fig. 3. Enzyme-linked immunosorbent assay plates representing days 4 (A), 7 (B), 9 (C), and 20 (D) from the bacterial movement within the plant experiment testing. Columns 1–10 (1–9 of B) represent individual plants and rows A–H represent sample locations on plant (refer to Fig 1). Columns 1–5 represent inoculated plants. Columns 6–10 (6–9 of B) represent uninoculated (control) plants. Column 11 (10 of B) contains both positive (A, B, and H) and negative (C, D, E, F, and G) controls. Plants expressed symptoms on day 9.

petioles of the five plants tested that day were positive for *X. c. pelargonii*. It was also on day 9 that symptoms of bacterial wilt were first expressed. All inoculated plants continued to test positive and express symptoms of increasing disease severity (Figs. 3D and 4). All control plants remained healthy and tested negative for the presence of *X. c. pelargonii* for the duration of the 20-day experiment (Figs. 3D and 4).

DISCUSSION

The main objective of this work was to produce a rapid and reliable polyclonal antibody-based serodiagnostic assay for the detection of *X. c. pelargonii* in geranium plants. The purified polyclonal antisera produced against *X. c. pelargonii* (UCRDAC 0782-29) were reactive with all isolates of *X. c. pelargonii*, but displayed a varying degree of cross-reactivity against other pathovars of *Xanthomonas campestris*, and nonrelated genera of phytopathogenic bacteria and fungi (Table 2).

These results are similar to those obtained with polyclonal antisera developed against other phytopathogenic bacteria (8). Although the antisera was not highly specific to *X. c. pelargonii*, the cross-reactivity observed with other pathovars of *Xanthomonas campestris* should be of minimal concern due to the host specificity of *X. c. pelargonii* in geranium. It is unlikely that other pathovars of *Xanthomonas campestris* would be associated with a diseased geranium. Cross reaction with nonrelated bacteria and fungi was insignificant.

The IGSS-DIA gave the first indication that detection of *X. c. pelargonii* in asymptomatic geranium plants was possible. Of the seven clinical samples tested using the IGSS-DIA (Fig. 2), two were asymptomatic single-stem plants. One plant tested positive for the presence of *X. c. pelargonii*. This test showed good sensitivity, but was more time consuming than desirable.

The use of gold-labeled antibody vs. an enzyme-conjugated antibody eliminated any possibility of a nonspecific reaction between the substrate and enzymes produced by the bacteria. Earlier experiments (unpublished) with enzyme-conjugated antibodies indicated the possibility of a nonspecific reaction between the substrate and the purified bacteria or plant sap extracted from *X. c. pelargonii*-infected geraniums.

The dELISA developed in cooperation with Agri-Diagnostic Associates proved much quicker and easier to use than the IGSS-DIA when detecting *X. c. pelargonii* in both symptomatic and asymptomatic geraniums. The IGSS-DIA took 6–7 hr, while the dELISA could be completed within 1 hr. Differences in sensitivity were observed, although no experiments on the differences in sensitivity between these two types of tests were conducted. Previous reports indicate the IGSS-DIA, in some cases is more sensitive than ELISA (5). The dELISA was also used to verify the presence of *X. c. pelargonii* as well as to detect the movement of *X. c. pelargonii* in root-inoculated geranium plants. The verification experiment using dELISA resulted in no false positive or false negatives. Although other bacteria were present on the YDC plates, only the YDC plates streaked with extraction buffer taken from microcentrifuge tubes containing inoculated stem sections showed the yellow mucoid growth typical of *X. c. pelargonii* (9). These results along with the clinical tests verify the reliability of this assay.

Reliability and a relatively short 1-hr running time give the polyclonal antibody-based dELISA a distinct advantage over traditional methods of bacterial wilt diagnosis. With regards to movement of the bacteria in root-inoculated cuttings, 4 days after inoculation, the five inoculated plants tested positive for *X. c. pelargonii* in the base sections only. At day 4 through day 9, successive stem sections excised from the inoculated plants tested positive for *X. c. pelargonii* (Figs. 3 and 4). Visible symptoms of bacterial wilt were not expressed until day 9, when all four stem sections (Sections A through D) of the five inoculated plants were positive for *X. c. pelargonii*. After day 9, all inoculated plants continued to test positive throughout the stem and showed an increased degree of bacterial wilt symptoms. These results indicate a logical progression of the bacteria from the roots to the base of the stem and eventually to the stem apex.

The lowest petiole on each stem also was tested to determine if a relationship could be made between a positive result in the petiole and the presence of *X. c. pelargonii* in the stem. A positive result in the base section of a stem did not necessarily indicate the lowest petiole would be positive. The four stem sections (A–D) could be positive and the lowest petiole would still be negative. No consistent movement of *X. c. pelargonii* into the lowest petiole was noted. No consistent relationship between the detection of *X. c. pelargonii* in the stem and the petiole was observed, indicating petioles are not reliable if used to indicate a possible stem infection. Further research needs to be conducted with larger plants to determine the movement and possible localization of *X. c. pelargonii* in infected geraniums.

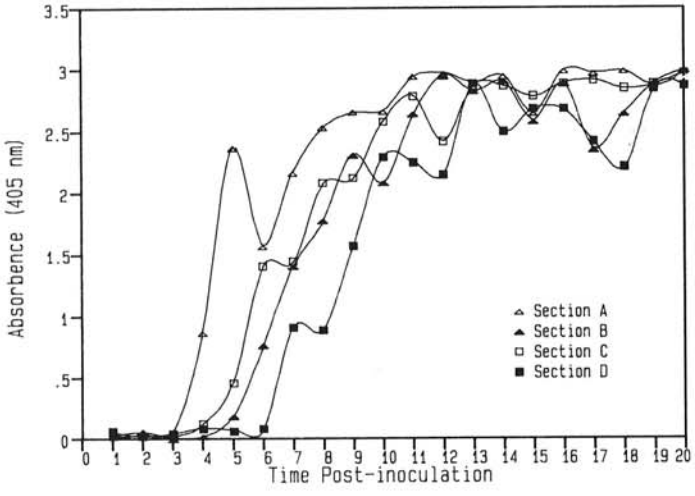

Fig. 4. Absorbance values (405 nm) versus days postinoculation of geranium plants root inoculated with *Xanthomonas campestris* pv. *pelargonii*. Inoculated plants did not show symptoms of bacterial wilt until day 9. Absorbance values (405 nm) of all uninoculated control plants were 0.003 or less.

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