Rapid Detection of Pectolytic *Erwinia* sp. in *Aglaonema* sp.

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SUMMARY. The purpose of this research was to develop a rapid microplate assay for detecting and presumptively identifying pathogenic pectolytic *Erwinia* sp. in ornamental propagative stock and to readily distinguish them from nonpathogenic bacteria associated with *Aglaonema*. The assay was developed by modifying an existing crystal violet–sodium polypectate (CVP) medium to visualize the depolymerization of pectate by addition of bromcresol purple (BCP), then acidifying with a dilute hydrochloric acid (HCl) solution. The assay was sufficiently sensitive to detect latent (symptomless) infections in small sections of leaf or stem tissue. Based on inoculum titration assays, 40 to 600 colony-forming units (CFU) of *Erwinia chrysanthemi* (Burkholder et al.) were required to produce symptoms in *Aglaonema* stems and leaves, respectively. The microplate assay was able to detect the pathogen at low levels (40 to 60 CFU) in tissue segments of ≈1 cm². In tests of bacterial strains isolated from 211 samples from *Aglaonema* 'Silver Queen', 'Emerald Beauty', and 'San Luis' grown in Hawaii, only the pectolytic and pathogenic *Erwinia* sp. reacted positively in the microplate assay. Other bacteria associated with *Aglaonema*, including *Pseudomonas paucimobilis* (Holmes), *P. vesicularis* (Galarneault and Leifson), and nonpectolytic *Erwinia* sp., were not detected by the assay. Pectolytic strains of *Ralstonia* (*Pseudomonas*) *solanacearum* (Smith) were differentiated from pectolytic *Erwinia* sp. by the yellow color that developed in wells of the latter strains after acidification of the medium with dilute HCl. The test is visual and can be performed with minimal equipment and cost.

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Aglaonema sp. are popular ornamental plants in the family *Araceae* and are one of the most profitable foliage plant genera in the nursery industry (MacCubbin, 1991). Several foliar and vascular diseases are reported for *Aglaonema* species and cultivars, including bacterial blights and stem rots caused by *Erwinia carotovora* Bergey, *E. chrysanthemi*, *Xanthomonas campestris* (Pammel) Dawson pv. *dieffenbachiae* (Mc Culloch and Pirone) Dye, and *Pseudomonas cichorii* (Swingle) Stapp (Wehlberg et al.,1966; McFadden, 1969; Hayward, 1972; Chase, 1983). Chemicals are generally unsatisfactory for controlling bacterial diseases; hence, the industry relies on early detection followed by sanitation for disease management (Chase, 1983).

Aglaonema cultivars are propagated by cuttings (Conover et al., 1981), and this practice facilitates the dispersal of *Erwinia* sp. Detecting latent systemic infections presents the greatest challenge to nurserymen because symptomless infections are not possible to recognize. Low levels of pathogenic bacteria may be harbored in plant tissues for long periods, resulting in visible infections only when the environment becomes conducive to disease expression. Methods have been developed to detect latent infections caused by *Xanthomonas* (Norman and Alvarez, 1994) but not for *Erwinia* sp.

In recent years, devastating outbreaks of bacterial diseases of aroids have occurred in Hawaii. Increased disease incidence may be related to the steady increase in production since 1980 (Hawaii Agricultural Statistics Service, 1989, 1994) and possible introduction of new pathogens. Current methods of detection and identification of pathogenic *Erwinia* sp. require at least 1 week, which delays appropriate response. Rapid pathogen detection techniques such as enzyme-linked immunosorbent assay (ELISA) (Ward and De Boer, 1989; Vernon-Shirley and Burns, 1992), slide agglutination tests (McLeod and Pérombelon, 1992), or more sophisticated DNA-based methods are gaining acceptance. These methods, although sensitive, require specialized laboratories and personnel. We thus investigated a simpler method based on a modification of the crystal violet polypectate medium (CVP) (Cuppels and Kelman, 1974) and the use of microplates for the detection of pectolytic *Erwinia* sp. in plant material. Using microplates permits testing of many samples in reduced space and time. Results are read visually and are easily interpreted with a minimum of equipment and training.

Materials and methods

DEVELOPMENT OF MICROPLATE ASSAY. The semiselective CVP medium was selected for the assay because depolymerization of sodium polypectate causes liquefaction of the medium and is one of the most reliable quick and presumptive methods for identifying soft rotting bacteria (Cuppels and Kelman, 1974). Nevertheless, its use in petri plates for large-scale testing is expensive and inefficient. The expense can be reduced by adapting the test to 96-well microplates, but additional reagents are required to detect liquefaction of the medium and the pectolytic activity of the test organism.

Tetrazolium violet (10 mg·L^{-1}), triphenyltetrazolium chloride (10 mg/L), and bromthymol blue (0.8 and 1.2 mg·L^{-1}) were added to separate preparations of CVP medium, and while still melted, the media were dispensed into individual wells (180 µL/well) of a 96-well Micro Test III (Falcon 3075) microplate. Known strains of *E. carotovora* subsp. *carotovora* Bergey (A2321), *E. chrysanthemi* (A1956), *Erwinia* (*Pantoea*) *herbicola* Ewing and Fife (Eh-1) and a pectolytic strain of *R. solanacearum* (A3908) were inoculated into separate wells (≈10^6 CFU/well). Plates were incubated for 48 h at 28 °C followed by addition of various amounts of different stock solutions, including ruthenium red (0.1%) (McKay, 1988), copper acetate (10%) (Hugovieux-Cotte-Pattat et al., 1992), or bromcresol purple (BCP) (1.5% in ethanol). Copper acetate forms a complex with polypectate but not with the hydrolysis products of polypectate that

are released to the liquefied medium following pectolytic activity of an organism. The last reagent, BCP, is a pH indicator (yellow in acid and purple at pH 6.8 or above). Concentrations of each reagent were adjusted to intensify color reactions. Color development was initially measured with a spectrophotometer at wavelengths of 405, 450, 490, and 650 nm to compare it to visual assessment in the microplate well system. For BCP, color changes were recorded at 15-s intervals for 15 min. Following the initial reaction with BCP, different amounts and concentrations of hydrochloric acid (0.1 or 1 N HCl) were added to the wells (5, 10, 15, 20, 30, or 50 µL/well); the change in pH was recorded every 15 s. The color reactions resulting from changes in pH were used to evaluate differences between various bacterial species, including pathogenic and nonpathogenic *Pseudomonas* and *Erwinia* species, and other epiphytes isolated from aroids.

Evaluations of assay sensitivity. A bacterial suspension of *Erwinia chrysanthemi* (strain A4682), was prepared in sterile saline solution (0.85% NaCl) and adjusted to A_{600} = 0.2. Bacteria in the inoculum were enumerated by plating on Miller and Schroth (MS) medium (Miller and Schroth, 1972). Ten-fold dilutions (10^{-1} to 10^{-10}) of the inoculum were prepared for infiltration into leaves and stems of *Aglaonema* 'Emerald Beauty'. About 150 µL of each dilution were inoculated into leaf tissue using syringes fitted with 23-gauge needles, and the area showing water congestion was delineated with permanent ink. Ten microliters of each dilution were inoculated into the stems 2 cm above ground level. Leaf and stem inoculations were made with four replications for each dilution. Four plants were used for each dilution and inoculation site.

Five days after inoculation, leaf sections (1.4 ± 0.15 cm^2) from one plant were ground in 0.5 mL phosphate buffered saline (PBS) (9.6 mM PO_4^{2-}, 0.85% NaCl). Stem pieces (≈1 cm^2) were cut from a second plant at the site of inoculation and ground in PBS. From each test sample, eight aliquots (10 µL each) were spotted onto MS medium agar in petri plates, and eight additional aliquots (10 µL each) were dispensed into each of eight wells of the CVP-microplates. The CVP-microplates were evaluated after 48 h incubation with addition of BCP, as described previously. MS plates were examined after 72 h incubation for presence of orange colonies, characteristic of enteric bacteria. The two remaining plants for each test (two replications per dilution of inoculum) were kept in the greenhouse for an additional month to observe symptom development.

Isolation and characterization of bacteria associated with Aglaonema plants. In total, 211 bacterial strains were isolated from July 1993 to March 1994 in Hawaii from *Aglaonema* hybrids 'Silver Queen', 'Emerald Beauty', and 'San Luis' that exhibited lesions, spots, or water-soaked areas on leaf blades, stems, or petioles. Samples were cut into 1-cm^2 sections and ground in 0.25 mL of PBS. Fifteen microliters of the suspension was streaked onto a nonselective but differential medium containing triphenyl-tetrazolium chloride (TTC) (Kelman, 1954). The TTC was reduced from 0.005 to 0.001% to lower its toxicity. Plates were incubated for 72 h at 28 °C. Colonies were then streaked with a single pass onto three diagnostic media, MS, yeast dextrose calcium carbonate (YDC) (Wilson et al., 1967), and CVP for further identification.

Colonies that formed orange colonies on MS medium and showed pectolytic activity on CVP medium were clearly enteric bacteria and possibly *Erwinia* sp. Further tests to identify pectolytic *Erwinia* sp. were the following: acid production from trehalose, growth at 36 to 37 °C, use of methyl-α-D-glycoside and palatinose (Dickey and Kelman, 1988), and oxidation–fermentation of glucose and lactose, sensitivity to erythromycin, indol production, and hypersensitivity on tobacco (Chase, 1987). Known strains of *Pseudomonas* and *Erwinia* sp. and the saprophytes isolated from *Aglaonema* in Hawaii were evaluated for reactivity by the CVP tests in microplates.

Pathogenicity tests. Bacterial strains were tested for pathogenicity in intact plants and in detached leaves of *Aglaonema* 'Silver Queen' and 'Emerald Beauty'. Bacterial suspensions containing ≈10^8 CFU/mL in distilled water were prepared by adjusting the optical density to A_{600} = 0.1 with a spectrophotometer (Spectronic 20). Inoculations were performed by injecting 10 µL into the stems of *Aglaonema* plants (≈2 months after transplant) and by infiltrating ≈150 µL of the same suspension into detached leaves. Plants were incubated in plastic bags for 24 h. One plant per strain was used for stem inoculations, and two leaves with three injections each were used for the detached leaf assay. Symptoms were evaluated after 72 h incubation for detached leaves and 5 d for potted plants. Incubation conditions were 23 °C 16 h light (100 lux, fluorescent), 23 °C 8 h dark. Known strains of *Erwinia carotovora* subsp. *carotovora* (A2321, originally E33 A. Chase, Univ. of Fl) and *Erwinia chrysanthemi* (A1956, originally D6 A. Chatterjee, Univ. of Missouri) were used as controls.

Results and discussion

Microplate assay. Some of the reagents, such as ruthenium red and copper acetate, were good indicators of pectolytic activity in petri plates. However, they require a large agar surface for a distinctive reaction and were not appropriate for the microplate system. The addition of triphenyl-tetrazolium chloride, tetrazolium violet or bromthymol blue to the CVP medium did not reveal pectolytic activity in the microplates. CVP medium containing tetrazolium violet (10 $mg \cdot L^{-1}$) resulted in the best detection of pectolytic activity in the microplate system, but only after addition of 20 µL of the BCP stock solution. If the sodium polypectate was depolymerized (hence, liquefied) the BCP diffused very rapidly, causing the entire well to turn a red-wine color in <15 s. BCP also formed a red-wine color when reacted with pure galacturonic acid. The BCP solution is yellow-orange, and this color was initially imparted to the microplate wells until it diffused into the neutral-pH medium and, in the presence of pectolytic *Erwinia* or *Ralstonia* species, changed to a red-wine color in <15 s. After 10 min, the BCP conferred a red-wine color or purple color to all the wells, regardless of pectolytic activity. To distinguish pectolytic facultative anaerobes (*Erwinia* sp.) from pectolytic aerobes (*Ralstonia*), 50 µL of hydrochloric acid (0.1 N HCl) were added to each well to detect the extent of liquefaction. The HCl produced an instantaneous pH change (turning the medium yellow) if the medium was completely liquefied by an *Erwinia*,

Table 1. Detection of different bacterial species grown in microplates containing modified crystal violet polypectate (CVP) medium.

Reaction in wells	Color: Pectolytic: *Erwinia carotovora* subsp. *carotovora* *Erwinia chrysanthemi*	Color: Pectolytic: *Ralstonia* (*Pseudomonas*) *solanacearum*	Color: Nonpectolytic: *Erwinia* (*Pantoea*) *herbicola* *Pseudomonas paucimobilis* *Pseudomonas vesicularis*
After incubation for 48 h and before addition of bromcresol purple	Greyish-blue	Greyish-blue	Greyish-blue
After bromcresol purple (BCP) addition			
Instantaneous reaction (<15 s)	Red-wine	Red-wine	Yellow-orange[z]
Reaction after 10 min	Red-wine	Purple	Purple
After HCl addition			
Instantaneous reaction (<15 s)	Yellow	Purple	Purple
Reaction after 10 minutes	Yellow	Purple	Yellow with a purple ring at bottom of well

[z]The solution of bromcresol purple is yellow-orange in color; this reagent slowly diffuses into the agar turning purple at neutral or alkaline pH (after 10 min). Weakly pectolytic organisms such as *Xanthomonas campestris* pv. *campestris* give negative reactions (similar to nonpectolytic organisms).

and the medium remained yellow for at least 1 h after addition of HCl. The aerobic pectolytic *R. solanacearum* strains primarily produce pectate lyase (active at high pH). Addition of HCl to a well containing *Ralstonia* did not acidify the medium sufficiently to effect a color change to yellow even after 10 min (Table 1). Nonpectolytic aerobic or anaerobic bacteria gave delayed reactions since the indicator dye and the acid diffused slowly, resulting in a yellow band at the surface and a large red-wine-colored ring at the bottom of the well that persisted for at least 10 min. This procedure readily detected the presence of pectolytic *Erwinia* species in microplates without having to first isolate the organism, and it clearly distinguished between pectolytic *Erwinia* species and pectolytic pseudomonads, e.g., *Ralstonia*.

EVALUATION OF ASSAY SENSITIVITY. *Erwinia chrysanthemi* was detected in symptomless stem and leaf tissue both by isolation onto petri plates containing MS medium and by assay on CVP-microplates followed by color development with bromcresol purple (Table 2). Based on colony counts on MS medium, the number of CFU in the undiluted inoculum was 4×10^9 CFU/mL. Thus, 150 µL of the 10^{-6} dilution used to inoculate leaves contained ≈600 CFU. At this level, 50% of the inoculated leaves developed symptoms within 1 month (Table 2). The pathogen was detected in leaves inoculated with lower numbers of bacteria, but no symptoms developed. In contrast, only 40 CFU (10 µL of the 10^{-6} dilution) were required for symptom production in stems, and all of the stems inoculated with this suspension developed symptoms within one month. Again, the pathogen was detected in latent (symptomless) infections (Table 2).

ISOLATION AND CHARACTERIZATION OF BACTERIA ASSOCIATED WITH *Aglaonema* IN HAWAII. In total, 51 strains of enteric bacteria were recovered from 211 samples of plant tissue and were later identified as *Erwinia* sp. Of the strains, 21 showed pectolytic activity on CVP medium and were pathogenic on *Aglaonema* 'Silver Queen' and 'Emerald Beauty'. Additional biochemical

Table 2. Detection of *Erwinia chrysanthemi* in *Aglaonema* plant tissues using Miller-Schroth (MS) medium in petri plates and crystal violet polypectate (CVP)-microplates 5 d after inoculation.

Bacterial suspension dilution	Replication	Stem: No. positives/8 tested: CVP	Stem: No. positives/8 tested: MS	Stem: Symptoms[z]: 5 d	Stem: Symptoms[z]: 1 month	Leaves: No. positives/8 tested: CVP	Leaves: No. positives/8 tested: MS	Leaves: Symptoms: 5 d	Leaves: Symptoms: 1 month
10^{-3}	1	8	8	WS	WS	8	8	Chlorosis	WS
	2	8	8	WS	WS	8	8	None	WS
10^{-4}	1	8	8	None	WS,BV	8	8	None	WS
	2	8	8	None	WS,BV	8	8	None	WS
10^{-5}	1	8	8	None	WS,BV	8	8	None	WS
	2	8	8	None	BV,BV	8	8	None	WS
10^{-6}	1	8	5[y]	None	BV	8	8	None	WS
	2	8	3	None	BV	8	8	None	None
10^{-7}	1	0	0	None	None	8	8	None	None
	2	1	0	None	None	8	8	None	None
10^{-8}	1	0	0	None	None	0	0	None	None
	2	0	0	None	None	1	2	None	None
10^{-9}	1	0	0	None	None	0	0	None	None
	2	0	0	None	None	0	0	None	None

[z]WS = water-soaking; BV = black veins. Observations were recorded 5 d and 1 month after inoculation.
[y]Counts of ≈28 colony forming units per each 10-µL subsample tested.

tests indicated that 20 strains were *E. carotovora* subsp. *carotovora* and one strain was *E. chrysanthemi*. *Erwinia chrysanthemi* often produces systemic infection on foliage plants and is considered the most important disease of foliage in Florida (McFadden, 1969; Chase, 1983). This pathogen has not been isolated frequently from foliage plants in Hawaii, and none of the isolated strains developed systemic infections following inoculation of intact plants. Leaf samples from aroids surveyed in Hawaii also yielded epiphytic bacteria, some of which were identified as *P. paucimobilis*, *P. vesicularis* and *E. (Pantoea) herbicola*.

Assay specificity. Only the pectolytic and pathogenic *Erwina* strains produced a positive reaction in the microplate method. Neither fungi nor saprophytic bacteria grew well on CVP medium, and none of the other common leaf inhabiting bacteria produced positive reactions after addition of BCP to the microplates. Although several *Erwinia* sp. were recovered from leaves or stems of *Aglaonema* on the MS medium in petri plates, this medium only indicates the presence of bacteria in the family *Enterobacteriaceae* and does not distinguish pectolytic from nonpectolytic bacteria (Miller and Schroth, 1972). In contrast, the CVP-microplate test distinguishes pectolytic *Erwinia* sp. from other nonpectolytic, nonpathogenic *Erwinia* sp. and other species of bacteria.

Rapid diagnostic miniplate tests have been developed for xanthomonads (Norman and Alvarez, 1994), and diagnostic tests for these and other bacterial pathogens are available commercially (Agdia, Inc. Elkhart, Indiana; BioMerieux, St. Louis, Mo.). Several tests distinguish between plant pathogenic *Xanthomonas* and *Pseudomonas* strains. The advantage of using modified CVP medium in microplates followed by addition of BCP is its reliability in the detection of facultative anaerobic pectolytic *Erwinia* sp.

Assay protocol. Regular CVP medium is prepared and tetrazolium violet ($10 mg \cdot L^{-1}$) added after autoclaving. While still melted, the medium is dispensed (180 µL/well) into microplates (Falcon 3075). After the medium has solidified, samples (10 to 25 µL in PBS) are inoculated into the wells and incubated for 48 h at 28 °C. A stock solution of BCP (1.5% in ethanol) is diluted 1:5 (v/v) with distilled water and 25 µL added to each well. Red-wine or purple color developing within 15 s indicates pectolytic activity has taken place in the well. The plates are left for 10 min at room temperature (all the wells turn either red-wine or purple color). Fifty microliters of an aqueous solution of 0.1 N HCl is added to each well, and results are interpreted according to Table 1. The entirety of the medium in the well will liquefy only if anaerobic degradation of the polypectate has occurred, allowing the acid to diffuse and give a yellow color within 15 s.

The sample collection methods for this test are flexible and depend on the purpose of the survey. Pectolytic *Erwinia* were detected directly from leaves of *Aglaonema* that had been inoculated to evaluate their recovery. Samples were collected either by cutting small leaf segments from leaves and suspending them in water, saline or PBS, or by collecting samples of rinse-water from the infected area. The collected water was plated directly into the CVP microplates, and the pectolytic strains of *Erwinia* were detected in all cases. This assay could be used to identify those stocks which are infected by pectolytic *Erwinia* sp., and would be likely to develop soft rot under favorable conditions. The identification and elimination of these stocks would improve the ability to maintain clean propagative material.

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