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Relationship between Symptom Development and Actual Sites of Infection in Leaves of Anthurium Inoculated with a Bioluminescent Strain of *Xanthomonas campestris* pv. dieffenbachiae

R. FUKUI,* H. FUKUI, R. MCELHANEY, S. C. NELSON, AND A. M. ALVAREZ

Department of Plant Pathology, University of Hawaii at Manoa, Honolulu, Hawaii 96822-2279

Received 26 June 1995/Accepted 16 November 1995

The infection process of bacterial blight of anthurium was monitored with a bioluminescent strain of *Xanthomonas campestris* pv. dieffenbachiae. The relationship between symptom expression on infected leaves (assessed visually) and the extent of bacterial movement within tissues (evaluated by bioluminescence emission) varied among anthurium cultivars. In several cultivars previously considered susceptible on the basis of symptom development alone, bacterial invasion of leaves extended far beyond the visually affected areas. In other cultivars previously considered resistant, bacterial invasion was restricted to areas with visible symptoms. In three cultivars previously considered resistant, leaves were extensively invaded by the bacterium, and yet few or no symptoms were seen on infected leaves. The pathogen was consistently recovered from leaf sections emitting bioluminescence but not from sections emitting no light. At an early stage of infection, no significant differences in the percentages of infected areas as determined by visual assessment were observed in any of the cultivars. However, differences among cultivars were detected by bioluminescence as the disease progressed, because bacterial invasion was not always accompanied by symptom expression. In susceptible cultivars, the advancing border of infection was 5 to 10 cm inward from the margins of the visible symptoms and often reached to the leaf petiole even when symptoms were visible in <10% of the total leaf area. Comparisons of anthurium cultivars in which a nondestructive method was used to quantify the severity of leaf infection by a bioluminescent pathogen have enabled us to evaluate susceptibility and resistance to bacterial blight accurately. Such evaluations will be of importance in breeding resistant cultivars for disease control.

Bacterial blight of anthurium (*Anthurium andraeanum* Lind. ex André), caused by *Xanthomonas campestris* pv. dieffenbachiae (McCulloch and Pirone 1939) Dye 1978, is a difficult disease to control in Hawaii, since the mild climate and the persistent latent infection perpetuate the disease in symptomless plants. The disease became a serious problem statewide in the later half of the 1980s and caused a major decline of wholesale production in the local anthurium industry in 1988 (31). Bacterial blight is less serious now than it was in the late 1980s, but the disease is still a persistent threat to Hawaiian anthurium growers. Strict sanitation practices in combination with other control measures have been successful in reducing the incidence of bacterial blight. In theory, the use of tissue culture to produce putative pathogen-free plants is the most effective method of managing the disease. In reality, pathogen-free plants are difficult to obtain, since healthy looking propagative materials may carry the bacterium symptomlessly for more than a year (1, 22) and the bacterium could be reintroduced into the field by aerosols (4). A previous report described the detection of the pathogen on symptomless leaves on some farms by a miniplate enrichment–enzyme-linked immunosorbent assay system (21), suggesting that some portions of plants could be infected latently and might provide inoculum for secondary spread in the field. However, the actual extent of symptomless (latent) infection has not been demonstrated clearly.

A bioluminescent strain of *X. campestris* pv. dieffenbachiae should be a valuable tool for studies of the infection process in bacterial blight, especially in latently infected plants. A strain of *X. campestris* pv. dieffenbachiae was genetically engineered to carry the *lux* genes encoding luciferase, an enzyme responsible for light (photon energy) production. Only living cells can produce light, because the production of the substrate for the enzymatic reaction requires active electron transfer (23). Bioluminescence has been used successfully to detect genetically engineered bacteria inside various plant parts (8, 28, 29), in the rhizosphere (5, 6, 11, 13), and in soil (12). Light production by a bioluminescent bacterium was easily recorded with X-ray film (30). Although bioluminescence is useful in detecting sites of bacterial growth, a method to quantify the amount of infection (i.e., infected area per leaf) by bioluminescence has not yet been developed. Such a method would be particularly useful for evaluations of varietal susceptibility and the effects of stress, different fertilization schemes, and various antibacterial chemicals on blight severity. These effects have been assessed only by visual symptoms.

The objectives of this study were to compare the development of visual symptoms with the extent of bacterial invasion as detected by bioluminescence and to develop a quantitative method for measuring the amount (severity) of leaf infection by using bioluminescence. Various cultivars of anthurium were inoculated with a bioluminescent strain of *X. campestris* pv. dieffenbachiae and evaluated as leaf infection progressed.

(Preliminary reports of this work have been published [2, 14].)

MATERIALS AND METHODS

Plant materials and growing conditions. Twelve cultivars of anthurium were tested in this study. ARCS (UH1068), Asahi, Blushing Bride (UH798), Ellison Onizuka (UH711), Kalapana (UH1016), Marian Seefurth, Pink Elf (UH1070), Rudolph (UH965), Tropic Mist (UH780), and UH1060 (unnamed) cultivars planted in 10-by-10-cm cinder pots were obtained from Hawaiian Heart, Inc. (Mt. View, Hawaii). The Mickey Mouse (previously called Madame Butterfly)

* Corresponding author. Mailing address: Department of Plant Pathology, University of Hawaii at Manoa, St. John 315, 3190 Maile Way, Honolulu, HI 96822-2279. Phone: (808) 956-8054. Fax: (808) 956-2832. Electronic mail address: ryo@hawaii.edu or RioF@eWorld.com.

TABLE 1. Average monthly temperatures in the greenhouse during the experiments

Parameter	Avg monthly temp ± SD (°C)							
	Expt I[a]			Expt II[b]		Expt III[c]		
	April 1994	May 1994	June 1994	August 1994	September 1994	November 1994	December 1994	January 1995
Minimum	20.7 ± 0.8	21.8 ± 0.8	22.9 ± 0.7	24.5 ± 0.7	24.4 ± 0.8	22.2 ± 1.1	20.0 ± 1.0	18.2 ± 1.3
Maximum	28.1 ± 1.5	28.8 ± 1.3	30.8 ± 1.1	31.9 ± 1.0	32.2 ± 0.9	29.5 ± 1.1	28.0 ± 0.8	27.5 ± 0.9

[a] Inoculation on 25 April 1994.
[b] Inoculation on 11 August 1994.
[c] Inoculation on 16 November 1994.

cultivar was obtained from Greenpoint Nursery (Hilo, Hawaii). Some plants of Kalapana and Tropic Mist cultivars were propagated from tissue culture in our laboratory and grown in 10-by-10-cm cinder pots until use. Some plants of Rudolph cultivars were propagated from tissue culture and were obtained from A. R. Kuehnle, Department of Horticulture, University of Hawaii. Rooted Top shoots of Tropic Ice (UH1051) were obtained from M. Chun, Komohana Experimental Station, Hilo, Hawaii, and were grown in cinder pots until use. All the cultivars were *A. andraeanum* Hort. hybrids bearing typical heart-shape spathes, except for ARCS, Pink Elf, and UH1060, which were interspecific hybrids bearing tulip-shaped spathes. Most plants were 20 to 30 cm in height, and some of the Tropic Ice plants were >30 cm when used for inoculation studies.

In the first and second experiments (experiments I and II, respectively), the plants were taken out of their pots to remove cinders and all fertilizer pellets from the roots, repotted with new cinders in 10-by-10-cm pots, and fertilized with pellets of Nutricote (13-13-13 plus micro elements in a 70-day release formulation) (Chisso Asahi Co., Ltd., Tokyo, Japan) at a rate of about 0.5 g of fertilizer per pot (≅65 mg of nitrogen per pot [≅80 kg of nitrogen per ha]) 2 weeks before the inoculation. Some large plants (Tropic Ice) were planted in 12.5-cm-diameter pots. All plants were grown in a greenhouse shaded with two layers of Saran sheet (70% shading each) and were watered every other day. Asahi, ARCS, Ellison Onizuka, Kalapana, Marian Seefurth, Tropic Ice, and Tropic Mist plants were used for the first experiment, and Blushing Bride, Kalapana, and Rudolph plants were used for the second experiment.

In the third experiment (experiment III), plants were repotted as described above 4 weeks before the inoculation. Meanwhile, the plants were watered every other day and fertilized once a week by drenching them with Hoagland's solution (with Fe-EDTA but without nitrogen) at a rate of 150 ml per pot (20). The concentration of micronutrients in Hoagland's solution was modified for anthurium to contain 46.3 μM H_3BO_3, 9.15 μM $MnCl_2 \cdot 4H_2O$, 0.81 μM $ZnCl_2$, 0.29 μM $CuCl_2 \cdot 2H_2O$, and 0.01 μM $Na_2MoO_4 \cdot 2H_2O$ (modified Hoagland's solution). Two days prior to the inoculation, the plants were fertilized with modified Hoagland's solution containing 70 μg of nitrogen (in the form of NH_4NO_3) per ml at the same drench rate. At the drenching, the fertilizer solution was collected in a plastic saucer placed underneath the pot and reapplied to the plants during the next day. Fertilization with modified Hoagland's solution containing 70 μg of nitrogen per ml was repeated 4 and 7 days after the first treatment and weekly thereafter until the end of the experiment (for a total of seven times, resulting in 73.5 mg of nitrogen per pot in total), with collecting and redrenching each time. All plants were grown in the same greenhouse and watered every other day (except on the day of treatment). ARCS, Kalapana, Marian Seefurth, Mickey Mouse, Pink Elf, Rudolph, Tropic Ice, and UH1060 plants were used for the third experiment. The average minimum and maximum temperatures in the greenhouse during the experiments were recorded (Table 1).

Bacterial strain. The bacterial strain used was bioluminescent strain V108LRUH1 of *X. campestris* pv. dieffenbachiae (2, 14). A local Hawaiian strain of *X. campestris* pv. dieffenbachiae (V108) resistant to rifampin was transformed to bioluminescent strain V108LRUH1 by mating it with *Escherichia coli* HB101 harboring plasmid pUCD607 in which a transposon sequence (Tn*4431*) containing the *lux* genes was inserted (30). The presence of the *lux* genes in the bacterium was verified by the production of light from colonies grown on 523 medium (17) amended with 50 μg of rifampin per ml and 10 μg of tetracycline per ml.

Leaf inoculation. Strain V108LRUH1 was grown on agar plates of peptone-glucose medium (1.0% Bacto Peptone, 0.5% glucose, and 1.7% agar) at 28°C for 3 days. Actively growing cells were resuspended in sterile distilled water, and the cell concentration of this bacterial suspension was adjusted to approximately 10^8 CFU/ml with a spectrophotometer (an optical density at 600 nm of 0.1 ≅ 10^8 CFU/ml). This suspension was diluted 100-fold with distilled water (≅10^6 CFU/ml) and was used as an inoculum. Individual plants were placed inside plastic bags and inoculated with the bacterium by spraying the cell suspension uniformly onto every leaf until runoff occurred. Then, the openings of the plastic bags were closed, and the plants were incubated overnight at room temperature (23 ± 1°C). The next day, the plants were removed from the plastic bags and returned to the greenhouse, where they were arranged in a randomized complete block design of six to eight blocks.

Monitoring and quantifying leaf infection. The severity of leaf infection was assessed by two methods, visual assessment and bioluminescence. For the visual assessment method, any visible symptoms observed on the abaxial surface of the leaf (together with the entire margin of the leaf) were traced on a transparent sheet. Visible symptoms included water-soaked spots or lesions and subsequent necrotic lesions. Chlorotic areas were excluded as a visible symptom, since the advancing margins of chlorosis on leaves were not well defined. The areas (in square centimeters) of visible symptoms and of the entire leaf then were measured with a computer digitizer program (Sigma Scan; Jandel Scientific, Corte Madera, Calif.), and the area of the leaf with symptoms was expressed as a percentage of the total leaf area.

For the bioluminescence method, leaves emitting light produced by the bacterium were exposed to X-ray film (Fuji "New RX" medical X-ray film; Fuji Photo Film Co., Kanagawa, Japan) by attaching the film firmly to the bottom side of the leaf with plastic paper clips. Upon exposure, each X-ray film was covered with a layer of lightproof paper to protect the film from bioluminescence emitted from neighboring leaves and from sudden exposure to other light sources. The entire process of exposure was done in the dark, and the films were exposed for 7 ± 1 h. After the films were developed, the margin of the leaf traced onto the transparent sheet was drawn onto each corresponding film. Then, the image of bioluminescence emission (which appears in black) on the film was compared visually with a set of standard area diagrams previously constructed to represent areas of infection amounting to 2.5, 5, 10, 15, 20, 25, 35, 45, 55, 65, 75, 85, and 95% of a leaf, and the closest percentage value (including 0 and 100%) was given to each image on the X-ray films. Values of 30, 40, 50, 60, 70, 80, and 90% were also used when an image on the film appeared to fall exactly between two standard area diagrams. The disease severity index (in 1% increments) was expressed as the average percentage of infected leaf area determined by the bioluminescence method.

To generate a positive image of bioluminescence emission, the developed negative film was scanned by a computer to produce a digitized image, which was retouched to remove background jargon and then converted to a positive image with a computer photographic program (Adobe Photoshop; Adobe Systems Inc., Mountain View, Calif.).

Assessments of leaf infection by the bioluminescence method were performed three times in each of three experiments: 25 to 28, 35 to 43, and 49 to 56 days after the inoculation (representing the early, middle, and late stages of infection, respectively). Visual assessments were done on the following days. Visual assessments were not done on the third assessment date, since leaf blight was so severe by that time that it was impractical to define visible symptoms. For experiments I and III, leaf infection was assessed with the youngest immature leaf (in the stage of either unfolding, expanding, or hardening) and the next youngest, fully hardened leaf of each plant, and the leaf that was verified to have a greater severity of infection was used for data analysis. The youngest leaf that was still in the stage of differentiating and emerging was not used for assessment. For experiment II, leaf infection was assessed with the first four youngest leaves of each plant only by the bioluminescence method, and all leaves were used for data analysis.

Statistical analysis. Prior to statistical analysis, percentage data were transformed by the arcsine transformation. The transformed data were submitted to analysis of variance to examine the effects of main factors. Assessment method and day were considered the repeated measure factors in a factorial arrangement. Means were separated by the Student-Newman-Keuls test or the protected least-significant-difference test.

Reisolation of the bacterium from leaves in various stages of infection. A subset of Kalapana plants were inoculated by the method described above, and infected leaves were exposed to X-ray film at the late stages of infection (7 to 9 weeks after inoculation). The following day, several 0.25-cm^2 (5-by-5-mm) sections of leaf tissue were cut from various areas of infected leaf (from areas within, near, and apart from bioluminescence emission, about 5 mm apart from each other) and ground in a small mortar with 2 ml of 10 mM phosphate buffer (pH 6.9). The ground leaf tissue extract (100 μl) was added to 900 μl of phosphate buffer, vortexed for 1 min, and then serially diluted (to 10^{-6}). Each dilution (100 μl) was plated onto 523 medium containing 50 μg of rifampin per ml and 10 μg of tetracycline per ml to determine the cell concentration in each leaf section.

RESULTS

Monitoring of the infection process in various anthurium cultivars by the visual assessment and bioluminescence methods. In the early stage of infection (25 days after inoculation [experiment I]), the proportion of infected leaf area, as determined by the visual assessment method, was not significantly different among cultivars (0.1 to 4.1%) and was similar to that determined by the bioluminescence method for all tested cultivars except for Asahi (Table 2). The percentage of infected area as determined by either method was a maximum of 8% for these cultivars. For Asahi, however, the percentage of infected area as determined by the bioluminescence method was considerably greater than that determined by visual assessment at this time. The images of bioluminescence emission on the exposed X-ray film and the visible symptoms on the transparent sheet revealed that the front edge of bioluminescence emission proceeded beyond the margin of water-soaked or necrotic lesions for this cultivar (Fig. 1A).

By 38 days after inoculation, the actual infection advanced markedly in Marian Seefurth, Ellison Onizuka, and Asahi (41 to 59% by the bioluminescence method), but the percentage value determined by the visual assessment method did not increase so markedly and was significantly less than that determined by the bioluminescence method for these cultivars (Table 3). In Marian Seefurth, for example, the front edge of the bacterial growth advanced far beyond the margin of visible symptoms (Fig. 1B). In Asahi, the entire leaf area was infected in some cases (Fig. 1A). On the other hand, the percentages of infected areas as determined by the two methods were still comparable at this time for Tropic Ice, Tropic Mist, and Kalapana. For Kalapana, for example, the images of bioluminescence emission and traced visible symptoms still overlapped each other almost exactly (Fig. 1C). For ARCS, symptom development in relation to the actual infection was exceptionally unusual in that few or no visible symptoms were observed at this time (Fig. 1D), although the percentage of infected area as determined by the bioluminescence method was similar to the value for Kalapana (Table 2).

In the later stage of infection (56 days after inoculation), cultivars were classified into three groups in terms of susceptibility as evaluated by the bioluminescence method: susceptible, Marian Seefurth, Ellison Onizuka, and Asahi (93 to 99% by the bioluminescence method); intermediately to moderately resistant, Tropic Mist, Kalapana, and ARCS (59%); and highly resistant, Tropic Ice (5%) (Table 2). For such susceptible cultivars as Asahi and Marian Seefurth, all eight leaves used for disease assessment were defoliated because of infection, and three to five plants (of eight) were dead by 71 days after inoculation (Table 3). For Tropic Mist and Kalapana, all assessed leaves also were defoliated, but none of the plants were dead. All Tropic Ice and ARCS plants were still surviving and had lost no assessed leaves at this time.

In the similar test with some different cultivars (experiment III), some cultivars also had percentages of infected area as determined by the bioluminescence method that were significantly greater than those determined by visual assessment. In both trials, the actual extent of infection was significantly greater than the extent of symptom development for ARCS and Marian Seefurth, but they were similar for Tropic Ice and Kalapana (Table 2). In the early stage of infection (28 days after inoculation), the percentages of infected area as determined by visual assessment also did not differ considerably among cultivars (0.2 to 3.2%). The percentages of infected area, as determined by the bioluminescence method, were significantly greater than those determined by visual assessment for only Pink Elf and Rudolph.

By 43 days after inoculation, the actual infection advanced markedly in Pink Elf, Marian Seefurth, and Rudolph (43 to 55% as determined by the bioluminescence method), but the percentages of infected area as determined by the visual assessment method did not increase so markedly, resulting in considerably different values for the two assessment methods (Table 2). The difference was most pronounced for Pink Elf and Rudolph. For ARCS, the infection again progressed almost symptomlessly. The same was also observed for UH1060. For these two cultivars, the percentages of infected area as determined by the bioluminescence method were 14 to 19%, whereas visible symptoms developed in only 1.2 to 1.3% of the area of a leaf. For the rest of the cultivars (Mickey Mouse, Tropic Mist, and Kalapana), the percentage values determined by the two methods were still comparable.

In the late stage of infection (56 days after inoculation), the cultivars were classified into two groups on the basis of the percentage values determined by the bioluminescence method: susceptible, UH1060, Pink Elf, Marian Seefurth, and Rudolph (69 to 91%); and resistant to intermediate, Mickey Mouse, Tropic Mist, Kalapana, and ARCS (10 to 32%) (Table 2). By 73 days after inoculation, some Pink Elf and Rudolph plants were dead, while no resistant to intermediate cultivar plants died (Table 3).

Relationship between leaf age and the severity of leaf infection. The severity of leaf infection was greatest in the youngest immature leaves and was progressively less in older leaves (Table 4). Statistically, the percentage values for the youngest leaf and the second youngest, fully hardened leaf were not significantly different for the three cultivars tested.

Reisolation of the bacterium from infected leaves. All of the leaf sections taken from the areas with detectable bioluminescence emission contained the bacterium, and the bacterial population in such leaf sections was generally above 10^6 CFU/cm^2 (Fig. 2). The bacterium was also recovered at a high density from a leaf section taken from necrotic leaf tissue (an area with visible symptoms that had ceased to bioluminesce) (Fig. 2D). The bacterium was not found in leaf sections without bioluminescence emission, even when tissue samples only 5 mm away from the front edge of emission were taken. Exceptionally, some tissue samples without detectable bioluminescence emission contained the bacterium at low densities (3.9 and 5.1 log CFU/cm^2) (Fig. 2B and C, respectively). These leaf sections contained a part of the main leaf vein vessel.

DISCUSSION

The relationship between the extent of actual infection (evaluated with a bioluminescent strain of *X. campestris* pv. dieffenbachiae) and symptom expression on infected leaves varied among cultivars of anthurium. Thus, our measure of susceptibility was similar but not identical to the susceptibility ratings commonly accepted among growers and researchers. Of the 11 cultivars tested in this study, Asahi, Ellison Onizuka, Marian Seefurth, and Rudolph have been considered susceptible. Kalapana and Tropic Mist have been generally considered moderately resistant, but they are sometimes considered intermediate, depending on the grower. ARCS, Pink Elf, and UH1060 have been believed to be tolerant by many people. Mickey Mouse and Tropic Ice were known to be highly resistant. This study revealed that four susceptible cultivars were also susceptible as judged by the bioluminescence method, and the severity of infection as determined by the bioluminescence method was far greater than the severity determined by visual

TABLE 2. Severity of leaf infection, as measured by the visual assessment and bioluminescence methods, in various anthurium cultivars inoculated with bioluminescent *X. campestris* pv. dieffenbachiae V108LRUH1[a]

Expt	Days after inoculation	Variety	Leaf area with visible symptoms (%)[b]	Disease severity index (%)[c]
I	25	Tropic Ice	0.1 a	0 A
		Tropic Mist	0.9 a	1 A
		Kalapana	1.6 a	3 AB
		ARCS	0.4 a	2 A
		Marian Seefurth	2.7 a	5 AB
		Ellison Onizuka	4.1 a	8 AB
		Asahi	3.9 a*	21 B
	38	Tropic Ice	0.3 a	1 A
		Tropic Mist	5.6 abc	7 AB
		Kalapana	7.2 bc	16 B
		ARCS	1.1 ab*	23 B
		Marian Seefurth	10.2 c*	41 C
		Ellison Onizuka	15.4 c*	55 C
		Asahi	14.5 c*	59 C
	56	Tropic Ice	ND[d]	5 A
		Tropic Mist	ND	59 B
		Kalapana	ND	59 B
		ARCS	ND	59 B
		Marian Seefurth	ND	93 C
		Ellison Onizuka	ND	99 C
		Asahi	ND	96 C
III	28	Mickey Mouse	0.7 a	1 A
		Tropic Ice[e]	0.2 a	2 A
		Kalapana	1.7 a	2 A
		ARCS	0.2 a	4 A
		UH1060	0.2 a	0 A
		Marian Seefurth	3.2 a	9 A
		Pink Elf	1.4 a*	12 A
		Rudolph	2.7 a*	10 A
	43	Mickey Mouse	2.1 a	4 A
		Tropic Ice[e]	1.5 a	5 A
		Kalapana	5.2 ab	7 A
		ARCS	1.3 a*	14 A
		UH1060	1.2 a*	19 A
		Marian Seefurth	25.2 c*	43 B
		Pink Elf	8.5 ab*	55 B
		Rudolph	13.2 b*	54 B
	56	Mickey Mouse	ND	10 A
		Tropic Ice[e]	ND	14 A
		Kalapana	ND	16 A
		ARCS	ND	32 A
		UH1060	ND	69 B
		Marian Seefurth	ND	84 BC
		Pink Elf	ND	79 BC
		Rudolph	ND	91 C

[a] Plants were grown in cinders in 10-by-10-cm pots and were fertilized with pelleted fertilizer (Nutricote) at a rate of about 0.5 g per pot for experiment I and with modified Hoagland's solution at a rate of 150 ml per pot (by drenching) for experiment III. The experiments were arranged in the randomized complete block design. The severity of leaf infection (percentage of total leaf area infected) was determined with eight and seven replicates in experiments I and III, respectively (one leaf per plant).

[b] Determined by the visual assessment method. Arcsine-transformed data were analyzed by three-way (seven or eight cultivars, two assessment methods, and two assessment dates) analysis of variance, with the assessment method and date being considered the repeated measure factors in a factorial arrangement. For both experiments, all main effects were significant at $P = 0.05$, and there were significant interactions for all combinations of the effects. According to the Student-Newman-Keuls test, values followed by the same lowercase letters are not significantly different at $P = 0.05$ for each assessment date. According to pairwise comparison by the protected least-significant-difference test, values with asterisks are significantly smaller than the corresponding values determined by the bioluminescence method.

[c] Determined by the bioluminescence method. Arcsine-transformed data were analyzed by two-way (seven or eight cultivars and two assessment dates) analysis of variance, with the assessment date being considered the repeated measure factor in a factorial arrangement. For both experiments, the main effects were significant at $P = 0.05$, and there were significant interactions for all combinations of the effects. Values followed by the same uppercase letters for each assessment date are not significantly different at $P = 0.05$ according to the Student-Newman-Keuls test.

[d] ND, not done.

[e] The values for this variety are the means of six replicates.

assessment. Asahi was supersusceptible in this regard. Kalapana and Tropic Mist were moderately resistant, and in most of the cases, the actual sites of infection were restricted to the areas with visible symptoms for these cultivars. Mickey Mouse and Tropic Ice were highly resistant as judged by both methods. However, ARCS, Pink Elf, and UH1060 were actually infected as severely as intermediate or susceptible cultivars, and yet few or no symptoms were seen with these cultivars. In addition, infected ARCS plants neither lost infected leaves (in the first trial) nor were killed because of infection, which creates an illusion that this cultivar is highly resistant.

These results confirm the need for careful vigilance against bacterial blight. Certain cultivars of anthurium can be infected symptomlessly, and hence it is dangerous to assume that the field is safe even if plants have few or no visible symptoms. The study showed that at an early stage of infection, there were no significant differences in the percentages of infected areas as determined by visual assessment among 11 tested cultivars, regardless of their actual susceptibility. Differences were seen by the bioluminescence method, but the subsequent infection process was not always accompanied by symptom development. In susceptible cultivars, the advancing boundary of bioluminescent emission was 5 to 10 cm inward from the margins of visible symptoms and often reached to the leaf petiole, while visible symptoms were seen for $<10\%$ of the total leaf area. This observation suggests that the inoculum potential of minute symptoms on leaves should not be underestimated. We excluded chlorosis as a visible symptom for this study. Although chlorosis is a characteristic symptom of bacterial blight, it usually occurs in the middle to late stage of infection, by which time the bacterium has infected the plant far more extensively than one would assume from appearance. Hence, any measures for disease management should be started as soon as visible symptoms are observed on leaves. If a few symptoms are observed on such susceptible cultivars as Asahi, Ellison Onizuka, Marian Seefurth, Pink Elf, and Rudolph, it is advisable to destroy the entire plant since one cannot estimate the extent to which the bacterium has infected the plant by that time. If minute symptoms are seen with Kalapana or Tropic Mist, removal of the infected leaf may save the plant. However, this is still a risky practice, since other symptomless leaves may carry the bacterium. Infection of ARCS or UH1060 is an example of the most dangerous situation, since infection advances almost symptomlessly. Moreover, with ARCS as well as Mickey Mouse and Tropic Ice, infected leaves are less likely to become detached and infected plants are not killed quickly. These cultivars may serve as carriers for the secondary spread of the bacterium. More anthurium cultivars, including newly released ones, should be evaluated by such methods as bioluminescence in conjunction with visual assessment to provide growers with more reliable information regarding the relationship between the extent of internal colonization and symptom expression.

This infection-symptom expression relationship appears to be characteristic for each cultivar. However, the progression of leaf infection by a related pathogen of cabbage was affected by

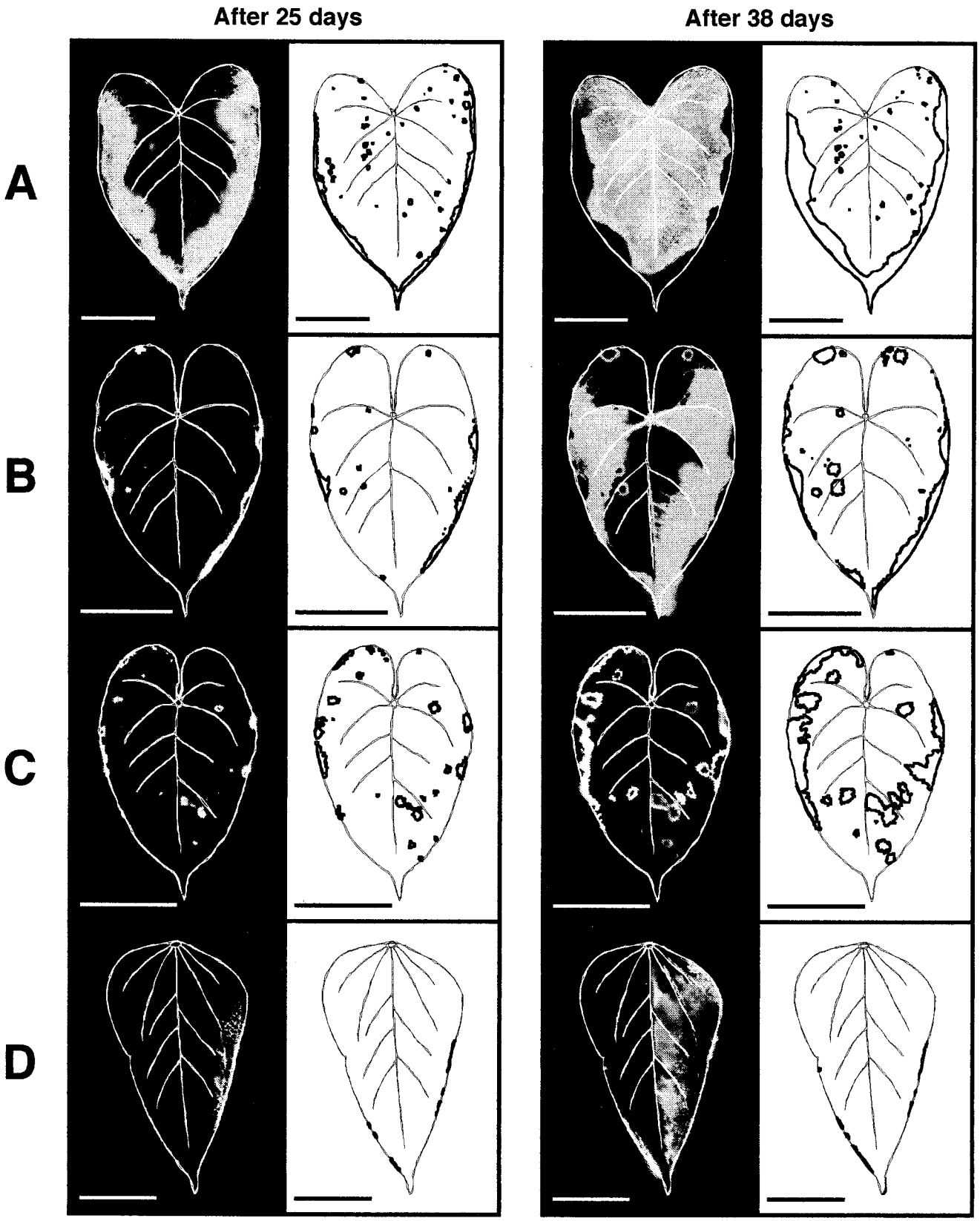

FIG. 1. Process of leaf infection by the bioluminescent strain of *X. campestris* pv. dieffenbachiae in various cultivars of anthurium, as assessed by visual symptoms (images on right) and bioluminescent emission (images on left). Relatively large leaf areas with symptoms but with no detectable bioluminescent emission were usually necrotic by the assessment day. The computer-generated images, produced with Adobe Photoshop (Adobe Systems Inc.), were made with leaves assessed at 25 and 38 days after inoculation. The images are of leaves from Asahi (A), Marian Seefurth (B), Kalapana (C), and ARCS (D) plants. Bars, 5 cm.

TABLE 3. Impact of leaf infection with bioluminescent *X. campestris* pv. dieffenbachiae V108LRUH1 on the survival of various anthurium cultivars

Expt	Variety	No. of assessed leaves defoliated	No. of dead plants
I[a]	Tropic Ice	0	0
	Tropic Mist	8	1
	Kalapana	8	0
	ARCS	0	0
	Marian Seefurth	8	4
	Ellison Onizuka	8	6
	Asahi	8	7
III[b]	Mickey Mouse	1	0
	Tropic Ice[c]	2	0
	Kalapana	1	0
	ARCS	4	0
	UH1060	5	0
	Pink Tulip	6	1
	Marian Seefurth	7	0
	Rudolph	7	4

[a] Assessed 71 days after inoculation. Values for experiment I were derived with eight plants (one leaf per plant).
[b] Assessed 73 days after inoculation. Values for experiment III were derived with seven plants (one leaf per plant).
[c] Values for this variety were derived with six plants (one leaf per plant).

TABLE 4. Relationship between the age of the leaf at the time of inoculation and the severity of leaf infection in three cultivars of anthurium inoculated with bioluminescent *X. campestris* pv. dieffenbachiae V108LRUH1[a]

Days after inoculation	Age of leaf[b]	Disease severity index (%)[c]			
		Kalapana	Blushing Bride	Rudolph	Avg[d]
35	0	5	47	34	29
	1	10	16	23	16
	2	2	5	7	5
	3	NT[e]	4	1	
Avg[f]		6 a	23 a	21 a	
49	0	15	76	67	53
	1	24	53	45	41
	2	3	35	19	19
	3	NT	21	5	
Avg[f]		14 a	55 b	44 b	
Avg	0	10	61[g]	50[h]	41 B
	1	17	35	34	28 B
	2	2	20	13	12 A
	3	NT	13	3	
Avg		10	39	32	

[a] Plants were grown in cinders in 10-by-10-cm pots and were fertilized with pelleted fertilizer (Nutricote) at a rate of about 0.5 g per pot. The experiment (experiment II) was arranged in the randomized complete block design.
[b] The youngest immature leaf of each plant at the time of inoculation was designated leaf 0, the next youngest was designated leaf 1, and so on. Data for leaf 3 were excluded from the data analysis, since some Kalapana plants did not have leaf 3.
[c] Determined by the bioluminescence method. The severity of leaf infection (proportion of infected area to the total leaf area) was determined with six replicates (three or four leaves per plant). Arcsine-transformed data were analyzed by three-way (three cultivars, three ages of leaf, and two assessment dates) analysis of variance, with the assessment date being considered the repeated measure factor in a factorial arrangement. All main effects were significant at $P = 0.05$, and there was a significant interaction only between the effects of cultivar and assessment date.
[d] Average values followed by the same uppercase letters are not significantly different at $P = 0.05$ according to the protected least-significant-difference test.
[e] NT, not tested.
[f] Average values followed by the same lowercase letters for each assessment day are not significantly different at $P = 0.05$ according to the protected least-significant-difference test.
[g] One replicate had no infection.
[h] Two replicates had no infection.

various environmental and growth factors, including temperature and fertilization (19), and these factors are probably important for anthurium, too. Previous reports indicated that disease incidence and severity as evaluated by visual assessment were lower when anthurium plants were grown at a lower temperature (≤24°C) (3) and when a fertilizer solution with a lower concentration of nitrogen was applied (25, 26). In this study, the infection-symptom expression relationships for ARCS, Kalapana, Marian Seefurth, and Tropic Ice were the same for two trials performed at different times of the year and using different fertilization schemes. On the other hand, the extents of infection in relation to the postinoculation period were somewhat different for the trials with some of the four cultivars. In experiment I, the disease severity index values for Kalapana (59%) and ARCS (59%) in the late stage of infection (56 days after inoculation) were considerably different from the value for Tropic Ice (5%). In experiment III, in contrast, the values for Kalapana (16%) and ARCS (32%) determined after the same postinoculation period were not significantly different from the value for Tropic Ice (10%). These differences are attributed to the temperature effect. Fukui et al. (15) indicated that there was a positive linear relationship between degree-days and the severity of leaf infection as determined by the bioluminescence method, and the rate of increase in leaf infection was greater for susceptible cultivars. In this study, the average temperature in the greenhouse increased in the first trial but decreased in the second trial as infection progressed. These observations suggest that differences in varietal susceptibility were much more enhanced in the first trial because of the greater cumulative temperature effect toward the late period of infection. Fertilization (specifically nitrogen) also affects the progression of leaf infection in anthurium and cabbage (15, 19). However, the influence of fertilization was probably negligible, since the total amounts of nitrogen applied were similar for two trials. These findings suggest that the infection-symptom expression relationship may be the most valuable and reliable criterion in evaluating varietal susceptibility, and such evaluations should be done under different environmental conditions. More detailed information on the effects of temperature and fertilization on symptom expression in relation to the extent of actual infection for each cultivar would be particularly useful in developing a guideline for effective blight control under field conditions.

The critical event for resistant cultivars during the infection process was the localization of the bacterium within or near the entry points (hydathodes and stomata) (8, 9). The importance of this event in disease resistance was confirmed by the results from the reisolation of the bacterium from sections of infected Kalapana leaves. This localization was not caused by the typical hypersensitive reaction, since the leaf tissues surrounding infected areas did not die (hypersensitivity-like reaction). A study of the anatomy of infected anthurium leaves showed that the bacterium was found exclusively within intercellular spaces in the epithelium, from the hydathodes to the xylem vessel members (27). Some bacterial cells were also found in the xylem vessel members. In this study, isolation of the bacterium from infected Kalapana leaves indicated that some leaf sections containing a portion of the leaf vein also carried low densities of the bacterium, probably representing the front

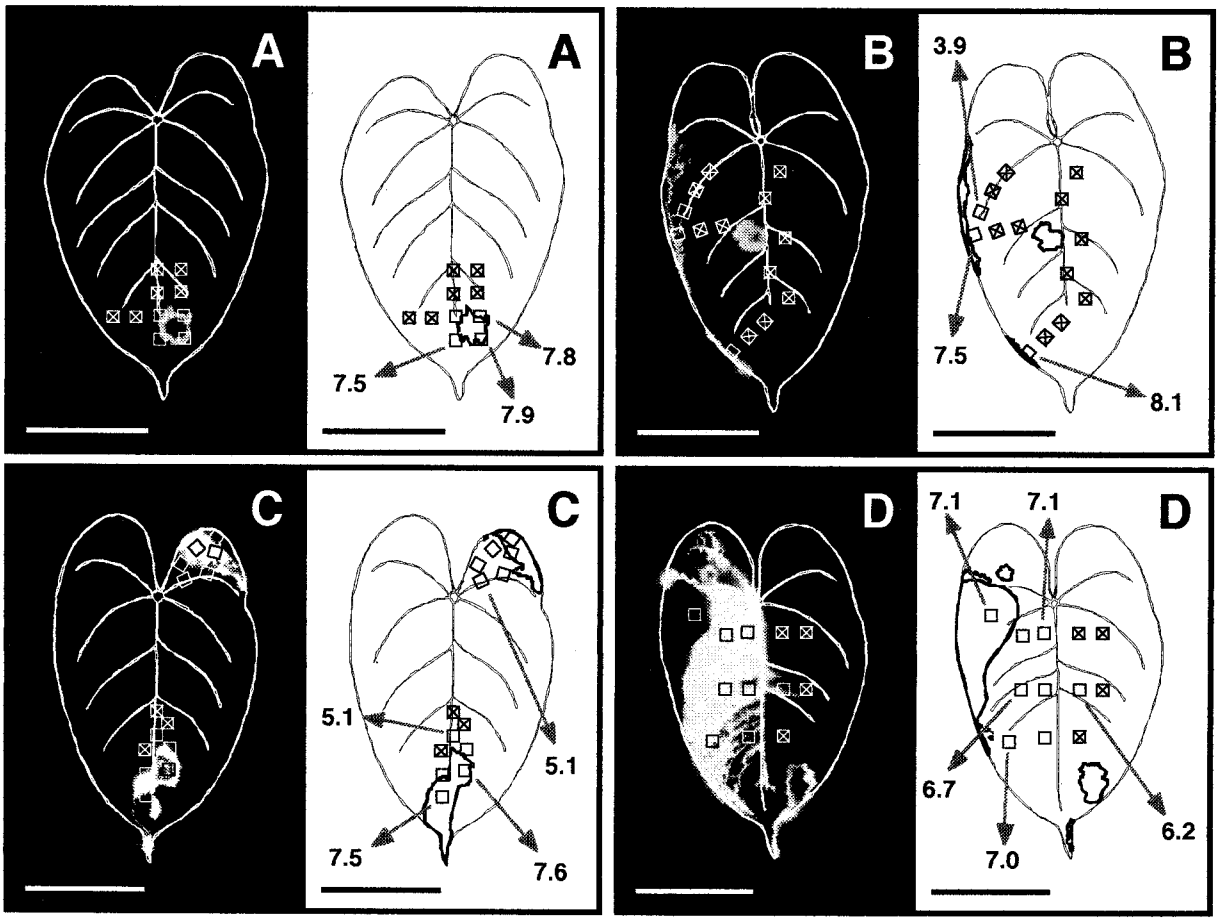

FIG. 2. Locations of the bioluminescent strain of *X. campestris* pv. dieffenbachiae in infected leaves from Kalapana plants (A to D). Visible symptoms are indicated in the images on the right, and bioluminescent emission is indicated in the images on the left. Leaf sections (0.25 cm^2) taken from the sites indicated with squares (□) contained the bacterium at a density of $>10^6$ CFU/cm^2 in most of the cases. The numbers indicate the bacterial densities in log CFU per square centimeter. Some leaf sections contained the bacterium at densities of $<10^6$ CFU/cm^2 (3.9 and 5.1 log CFU/cm^2 in panels B and C, respectively). A leaf section from a necrotic area with no detectable bioluminescent emission also contained a high density of the bacterium (D). The bacterium was not isolated from the leaf sections taken from the sites indicated with crossed squares (⊠). Isolation of the bacterium was done 7 to 9 weeks after inoculation. Bars, 5 cm.

boundary of bacterial movement. Obviously, the key event in determining varietal susceptibility is the entry of the bacterium into the xylem vessel members, and this entry probably occurs very readily in susceptible cultivars. This conclusion was further supported by the observation that infection in the leaves of Kalapana plants progressed very rapidly when the leaves were clipped at four sites around the leaf margin. The severity of infection for clipped Kalapana leaves, as determined by the bioluminescence method, was similar to that for intact Marian Seefurth leaves (data not shown).

Although it is not known whether cell wall breakdown is required for the bacterium to enter the xylem vessel members of anthurium leaves, lignification of leaf tissues along the path for bacterial movement in the hydathode appears to be important for localization of the bacterium. Bacteria entering through stomata cannot reach the xylem vessel members, since the sclerified band of cells completely surround the leaf vein (27). In this study, this outcome was seen with Kalapana or even Marian Seefurth leaves (Fig. 1B and C). The role of lignification in disease resistance was further supported by the fact that the severity of leaf infection was greatest in the youngest, unhardened leaves of three cultivars. Interestingly, no infection was found in some of the youngest leaves of Rudolph plants. Probably, such young developing leaves had undifferentiated hydathodes (and stomata) at the time of inoculation and hence escaped infection. Lignification is also known to be a part of the mechanism involved in systemically (or localized) acquired resistance induced by salicylic acid or its functional analogs in some dicotyledon plants (7, 10, 18) and in the resistance of cabbage and rice to bacterial blight caused by *Xanthomonas* spp. (16, 24). A study of the effect of salicylic acid on the infection process in bacterial blight may provide further information on varietal susceptibility.

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