Suppression of Bacterial Blight by a Bacterial Community Isolated from the Guttation Fluids of Anthuriums†

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Growth and survival of *Xanthomonas campestris* pv. *dieffenbachiae* in guttation fluids (xylem sap exuded from leaf margins) of anthuriums were suppressed by several bacterial strains indigenous to leaves of various anthurium cultivars. Inhibition of growth was not observed in filter-sterilized guttation fluids and was restored to original levels only by reintroducing specific mixtures of bacteria into filter-sterilized guttation fluids. The inhibitory effect was related to the species in the bacterial community rather than to the total numbers of bacteria in the guttation fluids. One very effective bacterial community consisted of five species isolated from inhibitory guttation fluids of two susceptible anthurium cultivars. The individual strains in this community had no effect on the pathogen, but the mixture was inhibitory to *X. campestris* pv. *dieffenbachiae* in guttation fluids. The populations of the individual strains remained near the initial inoculum levels for at least 14 days. The effect of the five inhibitory strains on reducing disease in susceptible anthurium plants was tested by using a bioluminescent strain of *X. campestris* pv. *dieffenbachiae* to monitor the progression of disease in leaves nondestructively. Invasion of the pathogen through hydathodes at leaf margins was reduced by applying the strain mixture to the leaves. When the strain mixture was applied directly to wounds created on the leaf margins, the pathogen failed to invade through the wounds. This bacterial community has potential for biological control of anthurium blight.

Bacterial blight of anthurium (*Anthurium andraeanum* Lind. ex André), which is caused by *Xanthomonas campestris* pv. *dieffenbachiae* (McCulloch and Pirone 1939) Dye (= *Xanthomonas axonopodis* pv. *dieffenbachiae* [27]), is an important disease in Hawaii, as well as other tropical and subtropical regions. An outbreak of bacterial blight in the 1980s had a severe impact on Hawaii's local anthurium industry (21, 22). Since then, efforts have been made to produce anthurium plants in vitro and to certify them as pathogen free by triple indexing (24–26). Changes in cultural practices, as well as strict sanitation (15), have reduced the disease problem to manageable levels. Yet, bacterial blight has not been eradicated from production fields, since the mild climate and persistent latent infections perpetuate the disease in symptomless plants (5, 17). Also, the pathogen can be introduced into clean fields by aerosols (2). A recent report that bacterial blight occurs in The Netherlands and that the pathogen was isolated from propagative materials en route from The Netherlands to India (19) indicates that the disease is not restricted to tropical and subtropical regions.

No effective pesticides currently are registered for bacterial blight in Hawaii. Several resistant (tolerant) cultivars have been developed by conventional breeding and have been grown widely in recent years. However, susceptible cultivars are also in high demand because of their desirable flower shapes and colors. Alternative methods of disease control are needed to ensure protection of the crop from future disease outbreaks.

A bioluminescent strain of *X. campestris* pv. *dieffenbachiae* has provided valuable information on the infection process in bacterial blight, especially during the latent systemic phase of

infection (4). Use of the bioluminescent strain has also allowed accurate evaluation of cultivar susceptibility in the foliar infection phase without dependence on symptom expression (5). While conducting susceptibility evaluation tests in the greenhouse, we observed that the severity of leaf infection in a certain cultivar occasionally was unusually variable in replicates. For example, no infections occurred in one or two plants (replicates) even though the rest of the plants examined were severely infected (severity of leaf infection reaching 100% toward the end of disease assessment). This phenomenon was observed more frequently with some cultivars (e.g., cultivars ARCS and UH1060) than with others. We concluded that other host-related factors or biological agents were responsible for the occasional suppression of disease in certain cultivars. It appeared that the hydathodal (guttation) fluid played a key role in the suppression, because the hydathode is the primary entry point for the pathogen. Physiological events induced by the host defense mechanisms did not explain the observations made with anthuriums, since spontaneous disease suppression occurred in highly susceptible cultivars as well as resistant cultivars and the suppression was not accompanied by rapid necrotic reactions, which are typical of hypersensitive responses.

While much is known about biochemical and physiological events in host-bacterium interactions, biotic factors in guttation fluids have been inadequately studied. Microorganisms indigenous to a guttation fluid may play a significant role in determining the fate of a pathogen before it becomes successfully established in hydathodes. Therefore, we examined the role(s) of indigenous bacterial communities on suppression of leaf infection by the anthurium bacterial blight pathogen, *X. campestris* pv. *dieffenbachiae*.

(A preliminary report of the results has been published previously [3].)

MATERIALS AND METHODS

Pathogen and culture media. Bioluminescent strain V108LRUH1 of *X. campestris* pv. *dieffenbachiae* (= *X. axonopodis* pv. *dieffenbachiae* [27]) was used

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in this study (4); this strain is referred to below as strain Xcd-lux. Before using strain Xcd-lux for experiments, we confirmed that Tn4431 encoding the *lux* genes (20) was present in the strain by growing it and observing bioluminescence emissions from colonies on 523 medium (8) containing 50 µg of rifampin per ml and 10 µg of tetracycline per ml.

Peptone-glucose medium (PGM) (1% peptone, 0.5% glucose, 1.7% agar) and yeast extract-dextrose-calcium carbonate (YDC) medium (28) were used to produce Xcd-lux inocula and inocula of all other bacterial strains, respectively. The cell density of Xcd-lux was determined by dilution plate counting on PGM supplemented with 50 µg of rifampin per ml, 10 µg of tetracycline per ml, and 100 µg of cycloheximide per ml. A modified triphenyltetrazolium chloride (TZC) medium (16) supplemented with 100 µg of cycloheximide per ml was used to determine the total bacterial population sizes.

Plant materials and growth conditions. The following eight cultivars of anthurium were obtained from local growers on the island of Hawaii: UH908 ('Alii'), UH1068 ('ARCS'), UH711 ('Ellison Onizuka'), UH1016 ('Kalapana'), H33 ('Marian Seefurth'), 'Nitta,' UH780 ('Tropic Mist'), and UH1060 (no common name). Anthurium plants (height, 30 to 40 cm) were transplanted into black cinder in pots (10 by 10 cm) and were fertilized with pellets of Nutricote (13-13 plus microelements in a 70-day release formulation; Chisso Asahi Co., Ltd., Tokyo, Japan) at a rate of about 0.6 to 0.7 g per pot. The plants were grown in a glasshouse with shading provided by two layers of saran (70% light transmission each). The daily minimum and maximum temperatures in the glasshouse were 18 to 22 and 26 to 30°C, respectively.

Survival of Xcd-lux in guttation fluids of anthurium plants and isolation of inhibitory bacterial strains. Guttation fluids were collected from cultivar ARCS, Marian Seefurth, and UH1060 plants (eight plants per cultivar). The youngest leaf of each plant was disinfested by spraying 70% ethanol onto the upper and lower surfaces and wiping the surfaces with Kimwipe tissue soaked with 70% ethanol. The disinfested leaves were each covered with a clean plastic bag in the evening, and the plants were watered. The bags were removed from the leaves early in the morning on the following day, and guttation fluids were collected individually. The leaves normally produced 100 to 500 µl of guttation fluid per leaf overnight. It was rare that more than 1.0 ml of guttation fluid was collected from one plant, and none of the cultivar ARCS and UH1060 plants produced more than 1.0 ml of guttation fluid overnight. Guttation fluids were collected from leaves that produced more than 500 µl overnight (six, six, and two cultivar ARCS, Marian Seefurth, and UH1060 samples, respectively), and 500 µl of each fluid was placed in a sterile test tube (100 by 13 mm) and used to determine the effect of the fluid on the growth of Xcd-lux. The remaining samples of guttation fluids were stored at 5°C for 10 days before bacterial strains were isolated from the inhibitory fluids at the end of the test.

An inoculum used for *in vitro* tests was produced by growing Xcd-lux on PGM for 2 days at 28°C and suspending the cells in sterile 10 mM phosphate buffer (pH 6.9). The density of the suspension was adjusted to $\sim 10^9$ CFU/ml, and 7.00 log CFU/ml was added initially to each sample in a test tube. The test tubes were covered with caps, sealed with Parafilm, and incubated at 28°C (without shaking) for 7 days. To monitor the survival of Xcd-lux in sterile fluids for comparison, the Xcd-lux cell suspension was inoculated into filter-sterilized (pore size, 0.2 µm; Supor Acrodisc 25; Gelman Sciences, Ann Arbor, Mich.) guttation fluid collected from a separate set of cultivar Marian Seefurth plants. After 1, 3, and 7 days of incubation, 50 µl of the guttation fluid was removed from each sample and added to 450 µl of sterile phosphate buffer. The resulting solution was serially diluted (10-fold) and plated onto PGM containing 50 µg of rifampin per ml, 10 µg of tetracycline per ml, and 100 µg of cycloheximide per ml.

Bacteria were isolated from the guttation fluids that were inhibitory to Xcd-lux by streaking subsamples (stored at 5°C) onto TZC medium containing 100 µg of cycloheximide per ml. The resulting plates were incubated at 28°C for 3 days to allow individual bacterial colonies to develop, and 10 dominant strains were isolated and transferred to YDC and TZC media. Five of the 10 strains, designated strains GUT3, GUT4, GUT5, GUT6, and GUT9, were selected for further study since they exhibited fast colony growth and had a distinctive colony morphology on YDC and TZC media. GUT3, GUT4, and GUT5 were isolated from guttation fluid from cultivar Marian Seefurth plants, and GUT6 and GUT9 were isolated from guttation fluid from UH1060 plants. Below these five bacterial strains are referred to guttation bacteria. Cells of the guttation bacteria were stored in 25% glycerol in distilled water at -80°C until they were used.

Effects of guttation bacteria on growth and survival of Xcd-lux in filter-sterilized guttation fluids. Guttation fluids were collected from plants of four different cultivars (cultivars Marian Seefurth, ARCS, Kalapana, and Nitta); the fluids collected from each cultivar were pooled and then filter sterilized. Cell suspensions of guttation bacteria and Xcd-lux were prepared in sterile 10 mM phosphate buffer and adjusted to concentrations of $\sim 2.0 \times 10^8$ CFU/ml. Fifteen microliters of the Xcd-lux cell suspension was inoculated into 1.47 ml of each filter-sterilized guttation fluid in a sterile test tube. To this preparation we added 15 µl of the GUT3, GUT4, GUT5, GUT6, and GUT9 cell suspension or 15 µl of a mixture containing equal volumes of the cell suspensions of the five strains. Four replicate samples (one for each cultivar) were used for each strain and for the mixture. As a control, a cell suspension of Xcd-lux (15 µl) was inoculated into filter-sterilized guttation fluid and 15 µl of sterile phosphate buffer was added to replace the cell suspension containing the guttation bacteria. The tubes were incubated at 28°C as described above. After 0, 3, 7, and 10 days of incubation, a

100-µl subsample was removed from each tube, and the cell densities of Xcd-lux and all guttation bacteria were determined by dilution plate counting on PGM containing 50 µg of rifampin per ml, 10 µg of tetracycline per ml, and 100 µg of cycloheximide per ml and TZC medium containing 100 µg of cycloheximide per ml, respectively. Cultivars were considered as blocks, and the results were expressed as means for four replicates.

A similar test was conducted to monitor the densities of individual guttation bacteria. Cell suspensions of Xcd-lux and a mixture containing the five guttation bacteria were inoculated into two tubes containing filter-sterilized guttation fluid from cultivar Marian Seefurth. The tubes were incubated and the cell densities of Xcd-lux and the guttation bacteria were determined 0, 1, 3, 7, and 14 days after inoculation as described above. In this test, the cell densities of the five guttation bacteria were determined individually on the basis of the different colony morphologies of the bacteria on TZC medium containing 100 µg of cycloheximide per ml.

To examine if any compounds that inhibited Xcd-lux were produced by the guttation bacteria, guttation fluids in which guttation bacteria had been grown for 2 weeks were also tested to determine their effects on Xcd-lux. Two milliliters of filter-sterilized guttation fluid collected from cultivar Marian Seefurth plants was inoculated with a cell suspension of each bacterial strain or a mixture of the five strains (two replicates per strain) and incubated at 28°C as described above. After 2 weeks, all of the guttation fluid samples were individually filter sterilized, and 1.5 ml of each filtered sample was inoculated with 15 µl of a suspension of Xcd-lux cells. The effects of the filtered guttation fluids on Xcd-lux were examined by determining the number of CFU per milliliter after 0, 1, 3, and 7 days of incubation at 28°C. For comparison, two tubes containing filtered guttation fluid which had not been inoculated previously with any bacteria were incubated with Xcd-lux. This experiment was conducted twice.

Survival of Xcd-lux in filter-sterilized and nonsterile guttation fluids from various anthurium cultivars. Guttation fluids were collected from cultivar Alii, ARCS, Ellison Onizuka, Kalapana, Marian Seefurth, Nitta, Tropic Mist, and UH1060 plants (four plants per cultivar). Unlike the tests described above, guttation fluid was repeatedly collected from the same leaf for 2 to 4 consecutive days by placing a new plastic bag onto the leaf each day. Samples obtained from the same leaf were pooled in a sterile glass tube and stored at 5°C until the amount of guttation fluid exceeded 4 ml for all plants. The initial population of total bacteria in each guttation fluid was determined by dilution plate counting on TZC medium containing 100 µg of cycloheximide per ml. Then, 2 ml of each subsample was sterilized by filtration, and 1.485 ml was placed in a sterile test tube. An equivalent amount of the nonfiltered guttation fluid was placed in a second tube. The remaining portions of the samples were stored at 5°C and used for isolation of bacteria at the end of the experiment.

Survival of Xcd-lux in guttation fluids was determined by inoculating 15-µl portions of a cell suspension (adjusted to a density of $\sim 2.0 \times 10^8$ CFU/ml) into the tubes containing filter-sterilized or nonfiltered guttation fluids (four replicates each). For comparison, 15-µl portions of the suspension were inoculated into equivalent amounts of sterile distilled water and phosphate buffer (two tubes each). The tubes were incubated as described above. One hundred microliters of each filtered sample was removed from one replicate tube for each of eight cultivars within 20 min after inoculation and used to estimate the initial Xcd-lux population size by dilution plate counting. After 7 and 14 days of incubation, the cell densities of Xcd-lux were determined by dilution plate counting by using 100 µl of guttation fluid from each tube. After the inhibitory guttation fluids were identified, four or five dominant strains (identified on the basis of distinctive colony morphologies on TZC and YDC medium plates) were isolated from the corresponding original fluids that had been stored at 5°C. The pH values of the guttation fluid samples were determined after the last sample was collected by using pH indicator strips (range, pH 4.5 to 10.0, with 0.5-pH unit increments; Baxter Scientific Products, McGaw Park, Ill.).

This experiment was repeated with cultivar ARCS, Kalapana, Marian Seefurth, Nitta, and Tropic Mist plants. All of the procedures used were identical to the procedures described above, except that the survival of Xcd-lux in guttation fluids was determined 7 and 15 days after inoculation and additional strains of indigenous bacteria were not isolated.

Inhibitory effects of various bacterial mixtures on growth of Xcd-lux in filter-sterilized guttation fluid. Bacteria isolated from inhibitory guttation fluids from various cultivars in the experiment described above were mixed in different combinations and coinoculated along with Xcd-lux into filter-sterilized guttation fluids. Six different bacterial mixtures (mixtures A through F), each consisting of four or five strains, were used, and the inhibitory effects of these mixtures on Xcd-lux in filter-sterilized guttation fluid were compared. Mixture A consisted of strains GUT3, GUT4, GUT5, GUT6, and GUT9; mixtures B, C, and D each consisted of five strains that were isolated from guttation fluids obtained from cultivars Alii, Marian Seefurth, and UH1060, respectively; mixture E consisted of four strains that were isolated from a different guttation fluid obtained from cultivar Marian Seefurth; and mixture F consisted of two strains isolated from guttation fluids obtained from cultivar Ellison Onizuka and three strains isolated from guttation fluids obtained from cultivar Nitta. The bacterial strains tested were not identified. Each bacterial strain was grown for 2 days at 28°C on YDC medium plates, the cells were suspended in sterile phosphate buffer, and the concentration was adjusted to an optical density at 600 nm of 0.25 (equivalent to $\sim 3.0 \times 10^8$ to 4.0×10^8 CFU/ml). Then, the cell suspensions were mixed at

different ratios to prepare four replicates (1:2:1:2:1, 2:1:2:1:2, 1:2:2:1:2, and 2:1:1:2:1 for mixtures consisting of five strains; 1:2:1:2, 2:1:2:1, 1:2:2:1, and 2:1:1:2 for mixture E consisting of only four strains), and 15 μ l of each mixture was inoculated into 1.47 ml of filter-sterilized guttation fluid from cultivar Marian Seefurth. This procedure ensured that slight differences in the mixing ratios (expected in experiments conducted at different times) did not drastically affect the inhibitory effects of the mixtures. Guttation fluids were then inoculated with 15- μ l portions of the Xcd-lux cell suspension and incubated as described above. The size of the initial Xcd-lux population was confirmed by dilution plate counting by using a 100- μ l subsample taken from the first replicate tube of each treatment. The effects of the mixtures on the survival of Xcd-lux were examined by determining the Xcd-lux cell densities (with four replicates) in 100- μ l guttation fluid samples taken 4 and 8 days after inoculation. As a control, the growth of Xcd-lux in filter-sterilized guttation fluid containing no bacterial mixture was determined.

After incubation for 14 days, all remaining samples of guttation fluids (~1.3 ml) were individually filter sterilized, and 1.0-ml aliquots were placed in sterile tubes. Then 10 μ l of an Xcd-lux cell suspension was inoculated into each tube, and the survival of Xcd-lux was examined after 7 days of incubation as described above.

Effects of some organic and mineral nutrients on inhibition of Xcd-lux by guttation bacteria. Sterilized 10% D-glucose, 10% peptone, and 10% yeast extract solutions were prepared by autoclaving, and 15 μ l of each solution was added to 1.455 ml of filter-sterilized guttation fluid from cultivar Marian Seefurth in a test tube (four replicates per treatment). As a control, sterile distilled water was added to the guttation fluid. Then, 15 μ l of an Xcd-lux cell suspension and 15 μ l of a mixture of cells of the five guttation bacteria were added to the guttation fluid in order to examine the effects of the three organic nutrients (final concentration of each nutrient, 0.1%) on inhibition of Xcd-lux by the guttation bacteria. For comparison (as a second control), guttation fluid inoculated with only Xcd-lux (plus 15 μ l of sterile distilled water and 15 μ l of phosphate buffer) was prepared in order to examine the survival of Xcd-lux when guttation bacteria and nutrients were not added. The tubes were incubated at 28°C as described above, and the densities of Xcd-lux and total bacterial cells were determined 3, 7, and 14 days after inoculation. The size of the initial population of Xcd-lux was determined by using four additional tubes containing guttation fluids from cultivar Marian Seefurth.

In a similar test, the effects of three mineral nutrients on inhibition of Xcd-lux by the guttation bacteria were determined. Solutions containing 10 mM CaCl_2 , 10 mM MgCl_2 , and 10 mM EDTA (ferric sodium salt) (Fe-EDTA) were filter sterilized, and 15 μ l of each solution was added to 1.455 ml of filter-sterilized guttation fluid from cultivar Marian Seefurth (four replicates per treatment). Then 15 μ l of an Xcd-lux cell suspension and 15 μ l of a cell suspension containing the guttation bacteria were inoculated into the guttation fluid in order to determine the survival of Xcd-lux in the guttation fluid in the presence of each mineral nutrient (final concentration, 100 μ M). Two controls were prepared as described above, and the densities of Xcd-lux and total bacterial cells were determined 3, 7, and 14 days after inoculation.

Effects of guttation bacteria on the ability of Xcd-lux to infect anthurium leaves. Cultivar Marian Seefurth plants were used in the experiment performed to determine the effects of guttation bacteria on the ability of Xcd-lux to infect anthurium leaves. This cultivar is known to be highly susceptible to bacterial blight (5). The experiment consisted of the following four treatments (10 plants per treatment, one leaf per plant): leaves that were treated with bacteria and wounded (by notching at four sites around the leaf margin); leaves that were not treated but were wounded; leaves that were treated with bacteria and not wounded; and leaves that were not treated and not wounded. Before inoculation, the surfaces of the leaves were disinfested with 70% ethanol, and the plants were placed inside clean plastic bags. Strains GUT3, GUT4, GUT5, GUT6, and GUT9 were grown on YDC medium plates for 2 days, and cells of each strain were suspended in sterile distilled water and adjusted to an optical density at 600 nm of 0.1 (cell densities, $\sim 1.0 \times 10^8$ CFU/ml). Equal volumes of the five cell suspensions were mixed, and the mixture was sprayed onto the foliage of 20 plants until runoff occurred. Twenty nontreated plants were sprayed with sterile distilled water. The plants were kept wet for 4 h by sealing the bags. The plants were removed from the bags at night and placed in a glasshouse to allow slow drying of the leaves. The next day, one-half of the plants in each treatment group were wounded by cutting (depth of cut, ~ 5 mm) the margin of the youngest leaf on each plant at four equidistant sites. One drop of inoculum containing the mixture of the five guttation bacteria (concentration of each strain, $\sim 2.0 \times 10^8$ to 3.0×10^8 CFU/ml) was applied directly to each wound with a pipette. Sterile distilled water was applied to nontreated plants. The remaining plants in each treatment group were neither wounded by notching nor inoculated with the bacterial mixture. After the treated leaves were dried at room temperature, all of the plants were spray inoculated with a suspension of Xcd-lux cells (concentration, $\sim 10^6$ CFU/ml) as described previously (5). The next day, the plants were arranged in a complete randomized design in the glasshouse.

The severity of leaf infection was determined by autophotography of the infected leaves in which X-ray film was used to record the bioluminescence of Xcd-lux, and the percentages of infected leaf area were used as disease severity indices as described previously (5). The severity of disease was assessed twice (27

and 41 days after inoculation with Xcd-lux) for nonwounded plants and four times (14, 21, 31, and 41 days after inoculation) for wounded plants.

The experiment was repeated by using six cultivar Marian Seefurth plants per treatment. Twelve plants were wounded by notching the two youngest leaves on each plant, and 12 plants were not wounded. One-half of the wounded plants were sprayed with the mixture of guttation bacteria, and the other half were sprayed with sterile distilled water. The nonwounded plants were treated in the same way, as described above. All of the plants were later inoculated with Xcd-lux. The severity of leaf infection was determined by assessing two leaves per plant (12 observations for each treatment). The severity of disease was assessed three times (19, 32, and 44 days after inoculation) for nonwounded plants and three times (19, 27, and 38 days after inoculation) for wounded plants.

Statistical analysis. The data from the *in vitro* tests performed to determine the inhibition of Xcd-lux growth in the guttation fluids (and the data for the total bacterial population) were analyzed by analysis of variance. Sampling day was considered the repeated measurement in factorial designs. Means were separated by the Student-Newman-Keuls (SNK) test or by Fisher's least-significant-difference (LSD) test. Mean values were expressed with one standard deviation when appropriate.

In the plant inoculation tests, the severity of disease was assessed by three examiners. The average values calculated from the data collected by the three examiners (percentage data) were transformed by the arcsine transformation and then analyzed by analysis of variance. It was confirmed in this and previous studies that treatment-examiner interactions were not significant when disease severity data were assessed by three examiners (data not shown). Assessment day was considered the repeated measurement factor in factorial arrangements, and means were separated by the protected Fisher's LSD test.

RESULTS

Survival of Xcd-lux in guttation fluids of anthurium plants.

Populations of Xcd-lux in nonsterilized guttation fluids collected from individual anthurium leaves declined at various rates during incubation for 7 days. The initial inoculum size was 7.00 log CFU/ml, and the size of the population progressively declined to 4.15 ± 1.16 log CFU/ml for cultivar Marian Seefurth (six samples), to 4.81 and 6.46 log CFU/ml for cultivar UH1060 (two samples), and to 5.94 ± 0.44 log CFU/ml for cultivar ARCS (six samples) after 7 days of incubation. The sizes of the populations of Xcd-lux inoculated into filter-sterilized guttation fluids (two samples) were 7.06 and 7.48 log CFU/ml. Individual guttation fluids typically contained five to eight predominant bacterial species, as judged by colony types and morphology observed on TZC medium.

Effects of guttation bacteria on survival of Xcd-lux in the filter-sterilized guttation fluid. The size of the Xcd-lux population in the filter-sterilized guttation fluids remained close to the initial population size in the absence of guttation bacteria (Fig. 1A). Individual guttation bacteria had no effect on the growth or survival of Xcd-lux when they were coinoculated into the filter-sterilized guttation fluids (Fig. 1B through F). When Xcd-lux was coinoculated with the mixture of five guttation bacteria, the size of the Xcd-lux population declined progressively during incubation; the sizes of the populations of Xcd-lux coinoculated with the bacterial mixture 3, 7, and 10 days after inoculation were significantly different ($P = 0.01$) from the sizes of the corresponding populations when Xcd-lux was inoculated alone (Fig. 1A and G). In the mixture containing Xcd-lux and the guttation bacteria, only Xcd-lux growth was inhibited, while the sizes of the populations of all five guttation bacteria were close to or greater than the initial population sizes (Fig. 2).

When Xcd-lux was inoculated into filter-sterilized guttation fluids from cultivar Marian Seefurth in which the mixture of five strains or individual indigenous strains had been grown for 14 days before the preparation was filter sterilized, the size of the Xcd-lux population dropped from the initial level (7.09 ± 0.05 log CFU/ml) only to 6.73 ± 0.20 log CFU/ml (for the mixture) or 7.04 ± 0.07 log CFU/ml (for GUT5) after 7 days of incubation. The density of Xcd-lux cells in the guttation fluid

FIG. 1. Growth and survival of Xcd-lux in filter-sterilized guttation fluids when it was coinoculated with guttation bacteria. Data points represent means of four replicates. (A) Xcd-lux inoculated alone. (B) Xcd-lux inoculated with GUT3. (C) Xcd-lux inoculated with GUT4. (D) Xcd-lux inoculated with GUT5. (E) Xcd-lux inoculated with GUT6. (F) Xcd-lux inoculated with GUT9. (G) Xcd-lux inoculated with strains GUT3, GUT4, GUT5, GUT6, and GUT9. Values marked by asterisks were significantly different ($P = 0.01$) from the corresponding values for Xcd-lux inoculated alone, as determined by the SNK test.

that had not been inoculated with any bacteria was 7.10 ± 0.02 log CFU/ml after 7 days of incubation.

Growth and survival of Xcd-lux in guttation fluids from various anthurium cultivars. When filter-sterilized guttation fluids from different cultivars were examined, the average sizes of the populations of Xcd-lux determined 7 and 14 days after inoculation did not vary significantly among the cultivars and were 6.0 log CFU/ml or more for all cultivars (Fig. 3). In nonfiltered guttation fluids, in contrast, the sizes of the Xcd-lux populations declined to different levels depending on the cultivar. The average sizes of the populations of Xcd-lux mea-

FIG. 2. Growth and survival of Xcd-lux and guttation bacteria in filter-sterilized guttation fluid. The sizes of the populations of individual strains were determined separately. Symbols: ●, Xcd-lux; ○, GUT3; △, GUT4; ×, GUT5; □, GUT6; ▲, GUT9. Data points represent the means of two replicates.

FIG. 3. Survival of Xcd-lux in guttation fluids from various anthurium cultivars (first trial). The bars represent the means of four replicates. Bars marked by the same letter were not significantly different ($P = 0.01$), as determined by the SNK test. The estimated size of the initial inoculum of Xcd-lux was 6.41 ± 0.09 log CFU/ml (mean of eight observations). The sizes of populations of Xcd-lux in sterile distilled water and phosphate buffer 14 days after inoculation were 6.01 and 5.70 log CFU/ml, respectively. These two values were not significantly different from the initial size of the population of Xcd-lux, as judged by the LSD value (1.25 log CFU/ml) for this experiment. The numbers in parentheses are the logarithms of the initial sizes of the populations of all bacteria (mean of four replicates) in guttation fluids from the cultivars. The differences in the initial sizes of the populations of all bacteria were not significant for cultivars, as determined by the SNK test. The data for the first measurement (3 days after inoculation) are not shown.

sured 14 days after inoculation were significantly smaller ($P = 0.01$) in the nonfiltered fluids than in the filtered fluids for all cultivars (Fig. 3). After 7 or 14 days of incubation, the average size of the population of Xcd-lux in nonfiltered guttation fluids from cultivar Marian Seefurth was significantly smaller than the average size of the population of Xcd-lux in nonfiltered guttation fluids from cultivar ARCS, Kalapana, or Tropic Mist. There was no significant difference in the average sizes of the populations of all bacteria in nonfiltered guttation fluids among the cultivars (Fig. 3). The pH values of individual guttation fluid samples after incubation ranged from 5.5 to 7.5, but the pH values were not related to the inhibitory effects of the guttation fluids.

Similar results were obtained in the second trial of this experiment. For all cultivars, the sizes of the populations of Xcd-lux determined 7 and 15 days after inoculation were significantly smaller ($P = 0.01$) in the nonfiltered guttation fluids

FIG. 4. Survival of Xcd-lux in guttation fluids from various anthurium cultivars (second trial). The bars represent the means of four replicates. For each incubation time, bars marked by the same letter were not significantly different ($P = 0.01$), as determined by the SNK test. The estimated size of the initial inoculum of Xcd-lux was 6.72 ± 0.08 log CFU/ml (mean of five observations). The sizes of populations of Xcd-lux in sterile distilled water and phosphate buffer determined 15 days after inoculation were 6.41 and 5.91 log CFU/ml, respectively. These two values were not significantly different from the initial size of the population of Xcd-lux, as judged by the LSD value (0.95 log CFU/ml) for this experiment. The numbers in parentheses are the logarithms of the initial sizes of the populations of all bacteria (mean of four replicates) in guttation fluids from the cultivars. The differences in the initial sizes of the populations of all bacteria for the cultivars were not significant, as determined by the SNK test.

than in the filter-sterilized guttation fluids (Fig. 4). As observed in the experiment described above, the average size of the population of Xcd-lux determined 15 days after inoculation into the nonfiltered guttation fluids from cultivar Marian Seefurth was significantly smaller than the average size of the population of Xcd-lux in the guttation fluids from cultivar ARCS, Kalapana, or Tropic Mist. The average sizes of the populations of all bacteria in nonfiltered guttation fluids were not significantly different among the cultivars (Fig. 4).

Inhibitory effects of various bacterial mixtures on growth of Xcd-lux in filter-sterilized guttation fluid. All six bacterial mixtures that were added to filter-sterilized guttation fluids significantly ($P = 0.01$) reduced the sizes of the populations of Xcd-lux during 8 days of incubation in filter-sterilized guttation fluid. As judged by the sizes of the populations of Xcd-lux determined 8 days after inoculation, the inhibitory effect of mixture A (consisting of GUT3, GUT4, GUT5, GUT6, and GUT9) was significantly greater ($P = 0.01$) than the inhibitory effects of mixtures B, D, and E (Fig. 5). The inhibitory effects of mixture C (containing five other strains obtained from cultivar Marian Seefurth) and mixture F (containing five strains obtained from cultivars Ellison Onizuka and Nitta) were similar to the inhibitory effects of mixture A. Mixture E was the least inhibitory of the six bacterial mixtures tested, although it

FIG. 5. Survival of Xcd-lux in filter-sterilized guttation fluid inoculated with various mixtures of bacterial strains isolated from guttation fluids from several anthurium cultivars. Mixture A consisted of the five guttation bacteria (GUT3, GUT4, GUT5, GUT6, and GUT9). Mixtures B, C, and D consisted of five strains isolated from guttation fluids from cultivars Alii, Marian Seefurth, and UH1060, respectively. Mixture E consisted of four strains isolated from a different guttation fluid sample from Marian Seefurth. Mixture F consisted of two strains isolated from cultivar Ellison Onizuka and three strains isolated from cultivar Nitta. The bars represent the means of four replicates. For each day, bars marked by the same letter are not significantly different ($P = 0.01$), as determined by the SNK test. The estimated size of the initial inoculum of Xcd-lux was 6.69 ± 0.08 log CFU/ml (mean of seven observations).

consisted of four strains that were isolated from an inhibitory guttation fluid from cultivar Marian Seefurth. None of the guttation fluid samples was inhibitory to Xcd-lux when all bacteria (including Xcd-lux) were removed by filtration after 14 days of incubation and Xcd-lux was reinoculated into the filtered fluids (data not shown).

Effects of organic and mineral nutrients on inhibition of Xcd-lux by guttation bacteria. When glucose, peptone, and yeast extract (each at a concentration of 0.1%) were added to guttation fluid, they all reversed the inhibition of Xcd-lux by the guttation bacteria, and peptone was the most efficacious compound (Fig. 6). At 7 days after inoculation, the size of the population of Xcd-lux in the guttation fluid containing peptone (in the presence of guttation bacteria) was significantly greater ($P = 0.01$) than the size of the population in the absence of guttation bacteria (in the absence of additional nutrients). By 14 days after inoculation, the sizes of the populations of Xcd-lux in the guttation fluids containing glucose, peptone, and yeast extract were not significantly different than the sizes of the population of Xcd-lux in the fluid containing no guttation bacteria. Peptone and yeast extract significantly ($P = 0.01$) increased the number of total bacteria. However, glucose did not have any impact on the number of total bacteria despite the fact that it enhanced survival of Xcd-lux in the presence of guttation bacteria (Fig. 6).

None of the mineral nutrients had the same effects as the organic nutrients on the survival of Xcd-lux and the number of total bacteria (Fig. 7).

Effects of guttation bacteria on suppression of foliar infection by Xcd-lux. Pretreatment of anthurium leaves with mixtures of guttation bacteria significantly reduced infection by Xcd-lux of both intact (nonwounded) and wounded (notched) leaves (Fig. 8). Spraying guttation bacteria onto intact leaves reduced the disease severity index to approximately two-thirds the value obtained for nontreated leaves by day 41 (Fig. 8A) in

FIG. 6. Effects of organic nutrients (concentration, 0.1%) added to guttation fluid on the inhibition of Xcd-lux by guttation bacteria. The densities of Xcd-lux and total bacterial cells were determined 3 days (data not shown) and 7 and 14 days after inoculation. Bars marked by the same letter were not significantly different ($P = 0.01$), as determined by the SNK test. The initial densities of Xcd-lux and total bacteria were 6.34 ± 0.06 and 6.71 ± 0.04 log CFU/ml (means of four replicates), respectively.

FIG. 7. Effects of mineral nutrients (concentration, 100 μ M) added to guttation fluid on the inhibition of Xcd-lux by guttation bacteria. The densities of Xcd-lux and total bacterial cells were determined 3 days (data not shown) and 7 and 14 days after inoculation. Bars marked by the same letter were not significantly different ($P = 0.01$), as determined by the SNK test. The initial densities of Xcd-lux and total bacteria were 6.35 ± 0.04 and 6.72 ± 0.05 log CFU/ml (means of four replicates), respectively.

the first trial. In the second trial, however, spraying with guttation bacteria did not significantly reduce foliar infection (Fig. 8B). The effect of guttation bacteria on disease suppression was more evident in notched leaves than in intact leaves. In both trials, the disease severity index was reduced by more than 50% after guttation bacteria were applied to the wound site, and the difference was significant at all assessment times (Fig. 8C and D). Images of bioluminescence emission from the leaves recorded on X-ray film revealed that infection was initiated at the wound sites and advanced rapidly into the vascular tissues in nontreated leaves (Fig. 9). In bacterium-treated leaves, in contrast, there was no evidence that infections advanced from the wound sites, but infection through hydathodes at the leaf margins was evident (Fig. 9). In the first trial, infection occurred at 39 of 40 notched sites in the nontreated leaves but at only three sites in the treated leaves. In the second trial, infection occurred at all 48 notched sites in nontreated leaves and at seven sites in leaves treated with the mixture of guttation bacteria.

DISCUSSION

Resident bacterial communities in the guttation fluids of various anthurium cultivars were highly inhibitory to the anthurium blight pathogen, *X. campestris* pv. *dieffenbachiae*, depending on the bacterial strains in the fluids. This fact helps explain why infections occasionally do not occur in some susceptible plants even after a large inoculum of the pathogen is applied to the leaves. When guttation fluids were filter steril-

ized, the sizes of the populations of the pathogen were not significantly reduced for at least 14 days. This indicates that guttation fluid itself does not inhibit the pathogen; instead, biotic factors are involved in the inhibition. Filtration also removes other microorganisms, such as fungi, algae, and protozoans, from guttation fluids. However, the roles of such microorganisms in the inhibition of the pathogen are probably limited, since repopulating filter-sterilized guttation fluids with specific mixtures of resident bacteria (members of the bacterial community) restored the inhibitory effects of the guttation fluids.

In this study, guttation fluids were collected from leaves that had not previously been infected by the pathogen. Thus, possible toxic compounds (e.g., phytoalexins) or factors induced by the host defense mechanisms (e.g., reactive oxygen species) were not expected to be involved. Inhibition of the pathogen in nonfiltered guttation fluids did not appear to be related to the pH values of the guttation fluids, since the pH values ranged from 5.5 to 7.5 during the 2-week incubation period. This pH range is not harmful to the pathogen (1). A sudden decrease in the pH during incubation is unlikely since anthurium guttation fluid is highly buffered, possibly as a result of ions in the xylem sap that form carbonates (7).

The indigenous bacterial community may be a cofactor in the host-pathogen interaction. Thus, cultivar susceptibility could be altered indirectly (or masked) by establishing specific bacterial communities on anthurium leaves. It is not known whether inhibitory bacterial communities are formed coinci-

FIG. 8. Effects of inoculation of five guttation bacteria onto leaves on the progression of foliar infection by Xcd-lux. A mixture containing five guttation bacteria was inoculated onto wounded (notched) and nonwounded leaves of cultivar Marian Seefurth plants. The leaves were subsequently inoculated with Xcd-lux. The bars represent the means of 10 or 12 observations. Values (bars) marked by asterisks are significantly different ($P = 0.01$) from the corresponding average values for nontreated leaves. (A) First test with nonwounded leaves. (B) Second test with nonwounded leaves. One datum point for nontreated leaves was lost due to breakage of the leaf petiole before disease assessment was completed. The missing datum point was estimated by using a general linear model. (C and D) First and second tests with wounded leaves, respectively. BCAs, biocontrol agents (five guttation bacteria).

dentally or are associated with certain cultivars. The results of two repeated experiments indicated that nonfiltered guttation fluids from cultivar Marian Seefurth were more inhibitory than nonfiltered guttation fluids from cultivar ARCS, Kalapana, or Tropic Mist. Cultivar Marian Seefurth is highly susceptible to foliar infection, and the other three cultivars are resistant (5). These results suggest that certain susceptible cultivars may occasionally harbor a bacterial community that is inhibitory to the pathogen. The relationship between cultivar susceptibility and bacterial communities should be studied further with more cultivars from many sources.

The five guttation bacteria found in this study appear to be common bacterial species indigenous to anthurium leaves. Three of the five strains were tentatively identified as members of *Sphingomonas paucimobilis*, *Brevundimonas vesicularis*, and a gram-positive pleomorphic bacterium (*Microbacterium* sp.) based on standard bacteriological tests (9, 23), a fatty acid analysis, an API-NFT system (bioMérieux Vitek, Inc., Hazelwood, Mo.) analysis, and a Biolog MicroPlate system (Biolog, Inc., Hayward, Calif.) analysis. The two other strains were identified as nonfluorescent pseudomonads. In our initial attempts to isolate various bacterial strains from guttation fluids, strains that were identified as members of the same taxa as these guttation bacteria were repeatedly isolated. Notably, only the mixture containing the five guttation bacteria was inhibitory to *X. campestris* pv. *dieffenbachiae*; individual strains were not inhibitory when they were coinoculated into the guttation fluid. The mixture containing the five guttation bacteria was also better than the individual strains in suppressing leaf infection by Xcd-lux when it was spray inoculated onto the foliage of anthurium plants (4a). Moreover, only the pathogen was

eliminated from a mixture containing the pathogen and the five guttation bacteria, and the populations of the five guttation bacteria were sustained for 14 days in the guttation fluid. Such a balanced and self-sustaining bacterial community is ideal for biological control if the same phenomenon can be reproduced in planta.

Inhibition of the pathogen in guttation fluids occurred in the presence of specific bacterial strains but not in the presence of bacterial strains found in different ecological niches. No mixture or pair of other leaf-inhabiting xanthomonads (*X. campestris* pv. *campestris* and *X. campestris* pv. *phaseoli*), pseudomonads (*Pseudomonas fluorescens* and *Pseudomonas syringae* pv. *syringae*), and *Erwinia herbicola* inhibited Xcd-lux in anthurium guttation fluid (4a). It is known that there is competition between bacterial species that inhabit the same ecological niche (29, 30) and between two nearly isogenic species (6, 11, 12). Various epiphytic bacteria have been used for biological control of fire blight or frost injury (10, 13, 14, 29, 30). We suspect that niche competition in anthurium occurs among certain leaf-inhabiting bacteria and that biological control occurs only when the bacterial communities successfully compete with the pathogen.

Two other bacterial mixtures (mixtures C and F) were as inhibitory to Xcd-lux as mixture A (GUT3, GUT4, GUT5, GUT6, and GUT9), implying that the same bacterial species may be found in different inhibitory mixtures or that inhibitory bacterial mixtures may be exchangeable. It is noteworthy that mixtures C and E had different inhibitory effects on Xcd-lux, despite the fact that the bacterial strains in both mixtures were isolated from inhibitory guttation fluids from cultivar Marian Seefurth. This suggests that there are key component strains (species) in a bacterial community that are responsible for inhibition and that a lack of the key organisms in bacterial mixtures eliminates the inhibitory effects on the pathogen. Thus, it may be possible to improve the efficacy of a mixture by identifying the trivial strains in the mixture and replacing them with beneficial species. More studies are needed to determine which of the five guttation bacteria which we identified play the key roles in inhibition of Xcd-lux.

When the five guttation bacteria were applied as a mixture to the leaves, they significantly reduced foliar infection and were especially effective in preventing invasion of the pathogen through wounds. The pathogen, *X. campestris* pv. *dieffenbachiae*, enters anthurium leaves through the water pores located on the upper epidermis and occupies the intercellular spaces in the epithem of a hydathode before it enters the xylem vessel members (18). Thus, notching created a readily accessible entrance for the pathogen because it exposed the vascular tissues. Guttation bacteria were directly delivered to the xylem by the notching procedure, and the inhibition of the pathogen observed in guttation fluids was reproduced in planta. However, when the guttation bacteria were applied to intact (non-notched) leaves, they were less effective in disease suppression than the guttation bacteria that were applied to notched leaves. These results may indicate that the guttation bacteria did not interfere with the pathogen efficiently on the leaf surface. Various biological factors may have affected bacterial strains on the leaf surface; these factors include survival, mobility, and subsequent colonization of the hydathodes. However, the guttation bacteria were applied at a total inoculum density of $\sim 10^8$ CFU/ml, and we expect that greater disease suppression could be achieved by using higher inoculum densities. More studies are needed to determine how guttation bacteria can be used for biological control of anthurium blight.

The mechanism of disease suppression by guttation bacteria is not known. The fact that the individual strains did not exhibit

FIG. 9. Progression of foliar infection by Xcd-lux in bacterium-treated and nontreated anthurium leaves, as monitored by bioluminescence. A mixture containing the five guttation bacteria was sprayed onto foliage of cultivar Marian Seefurth plants. On the next day, leaves were wounded by notching them (arrowheads), and the same bacterial mixture was placed on the wounds. The pathogen was spray inoculated onto the leaves about 6 h later. The white background illumination is bioluminescence from Xcd-lux recorded on X-ray film. Negative images of bioluminescence emission from infected leaves were scanned with a computer and converted to positive images by using Adobe Photoshop (Adobe Systems Inc., Mountain View, Calif.). The images represent the leaves analyzed in the first trial, which had the disease severity indices closest to the average values. Images for nonwounded leaves are not shown. Bars = 5 cm. BCAs, biocontrol agents (five guttation bacteria).

inhibitory effects on Xcd-lux in guttation fluids also suggests that the inhibition was not caused by a single, dominant factor provided by one of the strains. Addition of glucose, peptone, or yeast extract (each at a concentration of 0.1%) to the guttation fluids reversed the inhibition, suggesting that competition for organic nutrients is involved in the inhibition observed in the guttation fluids. More importantly, the observation that a complex nutrient source (peptone) was more efficacious than a single carbon source (glucose) in reversing the inhibition indicated that survival of the pathogen in the presence of guttation bacteria may be affected by the number and kinds of nutrient sources present in the guttation fluid. Siderophores are not involved in the inhibition of Xcd-lux, because addition of 100 μ M Fe-EDTA to the guttation fluid did not reverse the inhi-

bition. In addition, neither CaCl_2 nor MgCl_2 reversed the inhibition.

It is unlikely that the inhibition of Xcd-lux was caused by production of antibiotics or other toxic agents by resident bacteria, because none of the filter-sterilized guttation fluid samples was as inhibitory as nonfiltered guttation fluids containing bacterial communities were. However, antibiotics cannot be ruled out completely as the cause of inhibition because they may have bound to the filter or may have been inactivated during sterilization. It was reported previously that the inhibition of *Erwinia amylovora* by antibiotics produced by strains of *E. herbicola* was reduced in the presence of various amino acids (31). The same principle may apply for the enhanced survival of Xcd-lux in guttation fluid containing peptone. More studies

on the effects of carbon and nitrogen sources on disease suppression by guttation bacteria should provide key information which can be used for biological control of anthurium blight with mixtures of bacterial species.

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