Marginal Necrosis and Intercostal Leaf Spots of Cocoyam Infected by *Xanthomonas campestris* pv. *dieffenbachiae*

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ABSTRACT

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Pathogenic bacteria from intercostal and marginal lesions of cocoyam (*Xanthosoma sagittifolium* [= *X. caracu*]) leaves in Florida were both identified as *Xanthomonas campestris* pv. *dieffenbachiae* based on physiological and biochemical tests. Inoculation of bacteria into intercostal or marginal tissue produced symptoms normally observed regardless of lesion type from which the strain was originally recovered. Numbers of intercostal and marginal lesions were independent of strain source. An average of 11 marginal lesions per leaf developed when actively guttating plants were mist-inoculated, but only 2.4 marginal lesions per leaf developed on nonguttating plants. Greater numbers of intercostal lesions (13.6 per leaf) were evident when plants were inoculated during midafternoon than when bacteria were sprayed on leaves early in the morning (0.025 lesions per leaf). Addition of a surfactant to the inoculum did not affect the numbers of either intercostal or marginal lesions.

In 1985, bacterial spot of cocoyam (*Xanthosoma sagittifolium* (L.) Schott [= *X. caracu* Koch & Bouché]) was first reported in the United States (14). The causal organism was described as a strain of *Xanthomonas campestris* pv. *dieffenbachiae* (McCulloch & Pirone) Dye. In host range studies, bacterial strains from Florida cocoyam were pathogenic on a number of aroids previously reported to be hosts for *X. c.* pv. *dieffenbachiae* (13). Therefore, the proposal of a distinct pathovar name, *X. c.* pv. *aracearum* (Berniac) Dye (1,7), for the strains attacking cocoyam seems unwarranted, at least in the United States.

All original Florida strains were isolated from young, water-soaked spots in intercostal areas of the leaf blade (14). Stomates are likely portals for ingress of *X. c.* pv. *dieffenbachiae* cells that result in intercostal lesions. We also observed water-soaking and necrosis of leaf margins on many field plants. Indeed, leaf margins of plants in some fields appeared burned because of extensive damage caused by the pathogen around most of the leaf perimeter. The pattern of symptom development of these marginal lesions was similar in every respect to that seen with intercostal lesions. The marginal lesions were assumed to be caused by *X. c.* pv. *dieffenbachiae* cells entering hydathodes in receding guttation fluids.

Since these results were published, we have become aware of several reports suggesting that the marginal necrosis and the intercostal lesions may be symptoms of two distinct diseases. In Costa Rica, *X. c.* pv. *aracearum* (possibly *X. c.* pv. *dieffenbachiae*) is described as causing "bacterial spots" (8). The predominant symptom described is an abundance of water-soaked intercostal spots, with some concentration and coalesence along leaf margins. The disease is said to occur only in eastern Costa Rica. A distinct disease, bacterial marginal necrosis, also has been described (8) and has been observed in all parts of Costa Rica where cocoyam is grown. Infected leaves show only marginal lesions, with occasional systemic invasion and collapse of petioles. The causal organism is listed as *X. campestris* (Pammel) Dowson, with no specific pathovar designated.

In some islands of the French Antilles, lesions observed on diseased cocoyam are predominantly intercostal. On other islands, only marginal injury is seen (P. Prior, *personal communication*).

The main purpose of this study was to compare and identify xanthomonad strains from intercostal and marginal lesions of cocoyam grown commercially in Florida. In particular, strains were compared for development of both types of lesions to see if symptoms were dependent on the type of lesion from which the isolate was recovered. In addition, the ecology and epidemiology of hydathode invasion by the pathogen were investigated.

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MATERIALS AND METHODS

Isolation. Portions of leaves of field-grown, infected cocoyam were surface-disinfested for 30 sec in 0.5% sodium hypochlorite and rinsed in sterile, distilled water. Small (2 mm^2) sections of both marginal and intercostal lesions were cut at the junction of healthy and diseased tissues. Samples were placed in 2 ml of buffered saline (10) for 15 min. Loopfuls of buffer were then streaked on starch-methionine agar (3). Plates were incubated at 27 C for 6 days. Representative colonies were selected, and cultures were purified by repeated streaking on nutrient agar amended with 1% (w/v) glucose (GNA). Permanent cultures were maintained on silica gel beads at −4 C (16). Working cultures were kept up to 1 mo on yeast extract-glucose-calcium carbonate (YDC) slants at 4 C (6).

Growth of plants. All plants were grown in an air-conditioned greenhouse with a diurnal temperature range of 20–29 C. The house was covered with 30% black polypropylene shading (30% light attenuation). The growth medium was a commercial potting mix (Metro-Mix 300, W. R. Grace & Co., Cambridge, MA). Throughout all tests, plants were irrigated at the pot rim by means of an automatic watering system and were supplied 3 days after potting with 30 g per pot of a slow-release fertilizer (Osmocote 20-20-20, Sierra Chemical Co., Milpitas, CA). Propargite (Omite, Uniroyal Chemical Co., Middlebury, CT) was applied as needed for mite control.

Cocoyam plants were propagated from cormels in 18.9-L plastic pots filled with soil mix to within about 3 cm of the rim. Plants were inoculated at the two- to three-leaf stage, approximately 4 wk after potting. Dieffenbachia (*Dieffenbachia fournieri* Makey ex M. T. Mast) cuttings with two or three leaves were obtained from a local commercial nursery and inoculated in the five- to six-leaf stage.

Inoculum preparation. Inocula were prepared by flooding 48-hr GNA cultures with sterile buffered saline. Suspensions were adjusted to $A_{600} = 0.18$ (determined with a spectrophotometer) and diluted to appropriate concentrations as needed.

Physiological, biochemical, and pathogenicity tests. Physiological, biochemical, and pathogenicity tests on cocoyam

and dieffenbachia were carried out as described previously (14). Cabbage plants, cv. Princess, were grown from seed to the two-true-leaf stage (about 3 wk). The plants were mist-inoculated early in the morning with a 2.0×10^8 cfu/ml suspension of bacteria (two plants per isolate), then enclosed in plastic bags for 24 hr to ensure a postinoculation period of high humidity, arranged on a bench in a completely randomized design, and observed for symptom development over the next 21 days (4).

Inoculation of intercostal and hydathode sites. Strains from the two types of lesions were compared for disease development when introduced into intercostal tissue and along leaf margins of 5- to 6-wk-old greenhouse-grown plants. Eight strains were chosen for this study: MI11, MI12, MI14, and MI16, which were originally recovered from intercostal lesions, and MH19, MH21, MH22, and MH23, which were recovered from marginal lesions. To investigate development in intercostal tissue, bacterial suspensions (about 2.0×10^6 cfu/ml) were introduced into intercostal tissue midway between the midvein and the leaf margin using a disposable syringe with a 27-gauge needle. Leaves were covered with plastic bags for 24 hr after inoculation.

A system was designed to expose plants to bacterial suspensions at sites along edges of leaves where guttation indicated the likely presence of hydathodes. Household plastic sponges were cut into blocks measuring $7.6 \times 2.5 \times 2.5$ cm. A slit 0.6 cm deep was made 1.3 cm in from the edge of the long axis of the sponge block. Sponges were then moistened thoroughly in distilled water and autoclaved at 121 C for 15 min.

Cocoyam plants were observed 11 mornings (0700–0800 hours) over a 2-wk period to identify hydathode sites along leaf edges. Points where guttation droplets appeared at least nine mornings were considered likely sites for hydathodes. A small dot was made proximal to each hydathode site with a waterproof marker as a permanent means of identifying potential inoculation sites. Sponges were soaked in a 2.0×10^8 cfu/ml suspension of test bacteria, then set in 50-ml beakers of the same bacterial suspension with the long axis of the blocks perpendicular to the bottom of the beaker. The edge of the leaf at the inoculation site was eased about 5 mm into the slit in the sponge. Care was taken not to insert the leaf edge so far as to bring the sponges into contact with tissue proximal to the epithem (thin-walled parenchyma tissue at the leaf edge through which water moves to hydathodes). Wooden scaffolding on which to place the beakers was constructed all around the perimeter of the greenhouse benches. The horizontal platforms on the scaffolding were raised or lowered to align the sponges and leaves. The inoculated leaves were left positioned as naturally as possible. Sponges remained moistened by capillarity and were left in place until 1100 hours the next day.

Twelve intercostal inoculations (four per leaf) and 12 hydathode inoculations (three to five per leaf) per strain were randomized among leaves. All inoculations were done between 0700 and 0930 hours; active guttation typically was evident until 0830–0900 hours. Inoculation sites were scored for symptom development 21 days later. This experiment was carried out twice.

Comparison of frequency of infection through stomates and hydathodes. MI and MH strains were compared for the relative frequency of intercostal and marginal lesions after mist application of bacterial suspensions.

Abundant stomates occur in both adaxial and abaxial leaf surfaces, based on examination of freehand sections of epidermis. In one series of tests, bacterial suspensions (about 2.0×10^8 cfu/ml) were gently misted with a No. 15 DeVilbiss atomizer onto the adaxial surface of leaves bearing guttation drops (0630–0800 hours). Leaves were enclosed in plastic bags immediately after inoculation until 1300 hours the next day. Five replicate leaves on greenhouse-grown plants for each strain (MI11, MI14, MH19, and MH21) were arranged in a completely randomized design. Observations on the number of marginal and intercostal lesions were made over the next 8 days. This experiment was done twice.

Because the ratio of intercostal to marginal lesions was unexpectedly high in bagged leaves, a similar series of tests was done without postinoculation bagging. In preliminary work, the diurnal opening and closing pattern of stomates of cocoyam leaves was inferred from measurements of stomatal conductance (17) using methods described by Schaffer and O'Hair (15). Measurements were

Table 1. Percentage of disease reactions from intercostal vs. marginal inoculations of cocoyam with strains of *Xanthomonas campestris* pv. *dieffenbachiae* from marginal or intercostal lesions of naturally infected plants[a]

Isolate	Lesion source	Positive disease reactions (%)	
		Intercostal[b]	Marginal[c]
MI11	Intercostal	100	100
MI12	Intercostal	92	75
MI14	Intercostal	100	92
MI16	Intercostal	100	92
MH19	Marginal	92	92
MH21	Marginal	100	75
MH22	Marginal	100	92
MH23	Marginal	100	92

[a] Based on 12 observations of each combination of strain and inoculation site.
[b] Plants were inoculated with a 2.0×10^6 cfu/ml suspension of each strain midway between midvein and margin using a syringe with a 27-gauge needle.
[c] Plants were inoculated with 2.0×10^8 cfu/ml suspension of each strain delivered by a soaked sponge attached to hydathode (guttation) sites along the leaf margin.

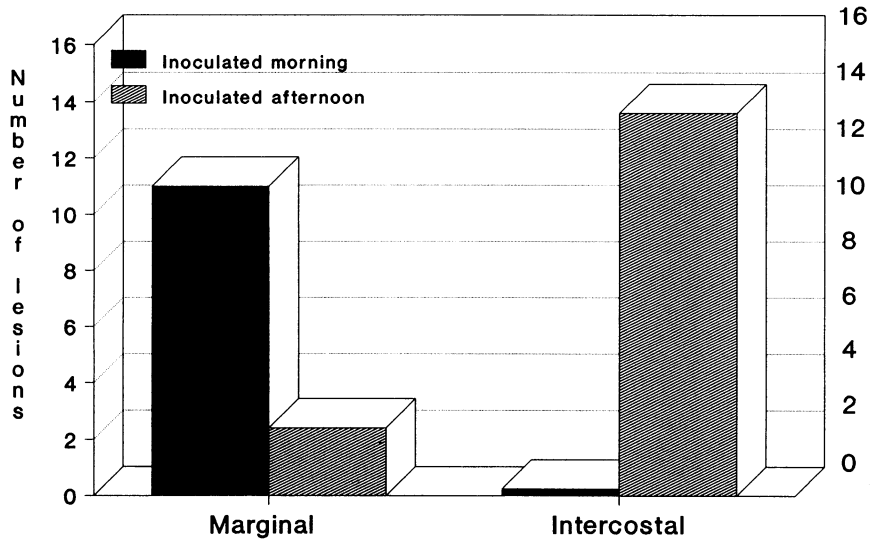

Fig. 1. The effect of time of day of inoculation on the relative numbers of marginal and intercostal lesions on leaves of cocoyam grown in the greenhouse. Leaves were mist-inoculated with a 2.0×10^8 cfu/ml suspension of *Xanthomonas campestris* pv. *dieffenbachiae*. Inoculations were done in the morning when guttation was ample or in the afternoon when guttation was not evident. Plants were not bagged after inoculation.

made every half hour from 0400 to 2200 hours using an enclosed Parkinson leaf chamber connected to a portable CO_2 analyzer (Analytical Development Co., LTD, Hoddesdom, Herts., UK). Experiments were done in May and June.

The effects of strain source, guttation, and addition of surfactant to the inoculum suspension were studied in a $2 \times 2 \times 2$ factorial experiment. Strains MI11 and MH21 were used. Leaves were mist-inoculated as described previously. Leaves were inoculated while hydathodes dripped freely (0700–0800 hours) or when no guttation was observed (1400–1500 hours). Half the leaves in both the morning and the afternoon were treated with bacterial suspensions amended with two drops of polyoxyethylene sorbitan monolaurate (Tween 20). Each treatment combination was replicated four times in a completely randomized design. Numbers of intercostal and marginal lesions were counted 14 days later. Data were subjected to analysis of variance (ANOVA) after appropriate transformation. The experiment was done twice in the winter months.

RESULTS

Physiological, biochemical, and pathogenicity tests. Strains from intercostal and marginal lesions reacted similarly in physiological and biochemical tests. All strains were gram-negative rods. No fluorescent pigment was produced on King's medium B. Strains were catalase-positive and oxidase-negative; hydrolyzed gelatin, aesculin, and starch; and were negative for nitrate reduction. All strains elicited hypersensitivity in tobacco and produced xanthomonadin pigment. Asparagine was not utilized as a sole source of carbon and nitrogen. Proteolysis occurred in litmus milk. Acid was produced from D(+)-galactose, D-glucose, maltose, D-mannose, and sucrose. Acid was not produced from dulcitol, erythritol, inositol, and mannitol. Variable reactions between strains were recorded for glycerol, lactose, and sorbitol. Pathogenic reactions in cocoyam were similar for all strains. All strains except MI18 were pathogenic in dieffenbachia, and none produced symptoms in cabbage.

Inoculation of intercostal and marginal sites. Overall, 98% of inoculations of intercostal tissue with strains from either intercostal or marginal lesions resulted in symptoms 21 days later (Table 1). When soaked sponges were used to deliver inoculum at hydathode sites, the percentage of successful marginal infections was similar for strains from both lesion types (90% for intercostal strains, 87% for marginal strains).

Comparison of stomates and hydathodes as portals for ingress of *X. c.* pv. *dieffenbachiae*. In experiments where leaves were bagged immediately after inoculation, intercostal lesions predominated. Under these conditions, the number of intercostal lesions averaged 60 per leaf, compared with 19 marginal lesions per leaf.

When plants were not bagged after inoculation, the relative number of marginal and intercostal lesions was highly dependent on the time of day of inoculation (Fig. 1, Table 2). An average of 11 marginal lesions was recorded on leaves inoculated early in the morning when leaves were guttating, with only 2.4 marginal lesions on nonguttating plants inoculated in the afternoon. The associated damage of the epithem tissue also was significantly greater on leaves inoculated in the morning (Table 2). In contrast, the number of intercostal lesions was higher in plants inoculated in the afternoon (13.6 lesions per leaf) than in those inoculated in the early morning (0.25 lesions per leaf) (Fig. 1, Table 2). There was also a significantly greater ($P \leq 0.01$) percentage of intercostal tissue damage after inoculation in the afternoon.

Stomatal conductance increased rapidly at and shortly after sunrise (Fig. 2). The stomatal conductance decreased slowly from midmorning until sundown; a fairly stable proportion of stomates remained open during daylight hours.

Strains from intercostal and marginal lesions produced similar responses in test leaves, as indicated by the lack of significant F tests for strain in the ANOVA (Table 2). Furthermore, addition of surfactant did not result in increased numbers of intercostal or marginal lesions in either the morning or the after-

Table 2. F values in analyses of variance for number of marginal and number of intercostal lesions and the associated tissue damage on greenhouse-grown cocoyam leaves as a function of strain, time of day of inoculation, and surfactant addition[a]

		Marginal lesions		Intercostal lesions	
Source of variation	df	Number of lesions[b]	Epithem damaged[c] (%)	Number of lesions[b]	Intercostal tissue damaged[c] (%)
Strain[d]	1	1.599	0.026	2.214	0.004
Time[e]	1	16.001**[f]	0.843**	23.106**	0.103**
Surfactant[g]	1	0.319	0.046	0.001	0.001
Strain × time	1	0.018	0.003	2.60	0.006
Time × surfactant	1	0.076	0.060	0.025	0.003
Strain × surfactant	1	0.011	0.019	0.470	0.010
Strain × time × surfactant	1	0.058	0.013	0.030	0.002
Error	24	1.29	0.041	2.047	0.009

[a] Based on observations 8 days after inoculation of four replicate leaves spray-inoculated with a 2.0×10^6 cfu/ml suspension of test bacteria.
[b] Count data were converted to square root equivalents before analysis.
[c] Percentage data were converted to arcsin-square root equivalents before analysis.
[d] MI11, originally from an intercostal lesion, and MH21, originally from a marginal lesion.
[e] Inoculated at 0730–0830 hours, when plants were actively guttating, or at 1400–1500 hours, when no guttation was observed.
[f] ** = Significant at $P = 0.01$.
[g] Two drops of polyoxyethylene sorbitan monolaurate per 250 ml of inoculum preparation.

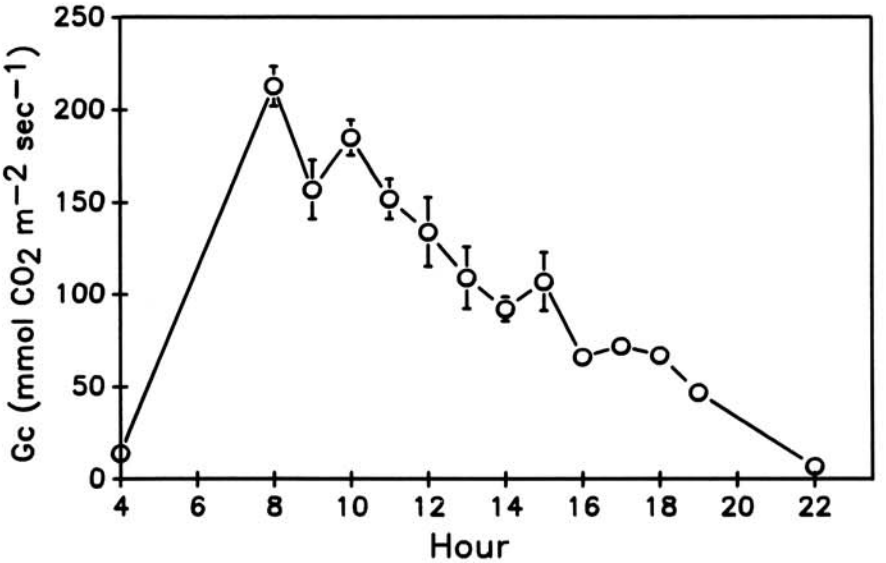

Fig. 2. Diurnal measurement of stomatal conductance for CO_2 (Gc) of cocoyam. Each point represents the mean value of measurements from single leaves of four plants. Error bars are ± 1 SE.

noon inoculations.

The first indication of infection through a hydathode was the appearance of water-soaking on the abaxial surface of the leaf approximately 5 days after inoculation. This was usually observed at premarked guttation sites. At first, the water-soaked area expanded along the leaf margin in the epithem tissue, with less development toward the interior of the leaf blade (Fig. 3). Subsequently, infections characterized by water-soaking, chlorosis, necrosis, and occasional bacterial exudate proceeded rapidly along major veins, resulting in large streaks of diseased tissue across the blade (Fig. 4).

DISCUSSION

Intercostal and marginal lesions on cocoyam leaves in Florida are both caused by *X. c.* pv. *dieffenbachiae*. When the pathogen was inoculated to cocoyam and dieffenbachia, reactions were similar regardless of strain source and were consistent with those reported previously (14). In other areas of the Caribbean basin, cocoyam plants have been observed with either intercostal or marginal lesions predominating, depending on specific location (8; P. Prior, *personal communication*). Adaptation of strains or even pathovars in the Caribbean for invasion through specific natural openings cannot be ruled out. However, environmental factors can have a profound effect on the ultimate symptom pattern observed, as evidenced by the studies reported herein.

The intercostal and marginal lesions recorded in our experiments are probably the result of pathogen invasion of stomates and hydathodes, respectively. However, the invasion of minute wounds cannot be precluded. Mew et al (11) reported that there is inherent specificity for penetration of *X. c.* pv. *oryzae* into rice leaves. Hydathodes are the primary infection courts with little colonization of stomates. In cocoyam, we found that numbers of intercostal and marginal lesions were similar for strains of *X. c.* pv. *dieffenbachiae* recovered from either type of naturally occurring lesion. However, the relative frequency of infection through either portal was heavily dependent on the time of day when plants were inoculated. This diurnal infectivity pattern, in turn, is dependent on the physiology of leaves, particularly their water status. When guttating plants were inoculated in the morning, stomates were in a period of rapid opening after being closed during the night (Fig. 2). Water congestion was concentrated in epithem-hydathode tissues. Because of the active guttation, there was a continuous path of water for pathogen entry. Cells of *X. c.* pv. *dieffenbachiae* likely move into plants with the retreating guttation fluid drawn back into leaves later in the morning (5).

In the afternoon inoculations, intercostal lesions predominated. A large portion of stomates have been open for several hours at this time of day (Fig. 2) (17), allowing the pathogen to enter substomatal cavities where they might survive and multiply. Some of the bacteria that are deposited near hydathodes may survive until the next diurnal guttation period. However, the number of marginal lesions for the afternoon inoculations was relatively low. The presence of guttation fluid that can be reabsorbed shortly after arrival of the *X. c.* pv. *dieffenbachiae* appears important in pathogen survival and ultimate infection. Cocoyam leaves are smooth and waxy. *X. c.* pv. *dieffenachiae* cells may not adhere well to leaf surfaces and may not find adequate refuge in "protected positions," sensu Leben (9).

Hydathode invasion and subsequent marginal lesions are typical of cabbage infected with *X. c.* pv. *campestris* in nature. Stomatal infection can be demonstrated under greenhouse conditions only if plants are subjected to artificial water congestion (4) or if a surfactant is used in the inoculum preparation (2). Neither hydathode nor stomatal infection of cocoyam is enhanced by use of a surfactant. When plants were bagged for 24 hr after inoculation, however, abundant stomatal infection was observed. The atmosphere inside the bags became saturated with water during the postinoculation period, and a continuous path of water was probably created from the leaf surface into the substomatal chamber. Under these conditions, *X. c.* pv. *dieffenbachiae* would be expected to penetrate stomates (12).

In nature, hydathode infection appears to occur more frequently than stomatal infection. Bagging of leaves subsequent to inoculation is a common practice in phytobacteriology. In the case of bacterial leaf spot of cocoyam, however, it resulted in an artificially high proportion of intercostal to marginal infection. This obscured initial investigations of the ecology of this disease. Very high humidity conditions may alter symptom patterns in other bacterial pathogen/host combinations. In the case of bacterial leaf spot of cocoyam, use of plastic bags after inoculation could have led one to underestimate the relative importance of hydathodes in host plant invasion by *X. c.* pv. *dieffenbachiae*.

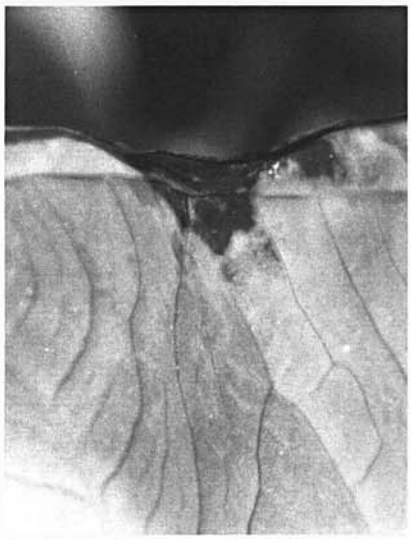

Fig. 3. The development of a bacterial leaf spot lesion at the margin of a cocoyam leaf mist-inoculated with *Xanthomonas campestris* pv. *dieffenbachiae*. Characteristic water-soaking of the abaxial surface is shown. Symptoms have spread from the hydathode entry site along the margin of the leaf as well as to the interior of the blade.

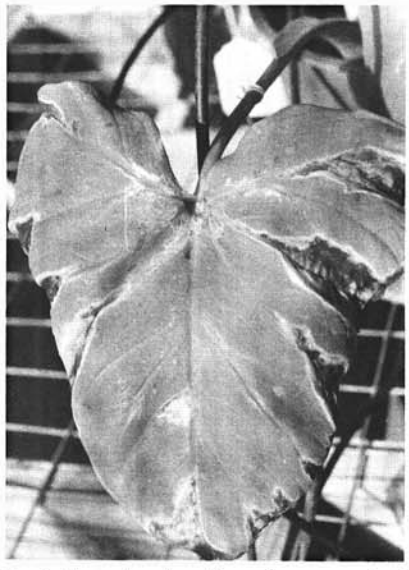

Fig. 4. Extensive invasion of cocoyam leaf blades 10 days after mist-inoculation of greenhouse-grown plants. After initial symptom development at hydathodes, pathogen invasion tends to follow major veins.

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