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Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*

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Abstract

Two sugarcane plots were set up in Guadeloupe with disease-free tissue cultured plants in a banana growing location distant from sugarcane fields. Thirteen weeks after planting sugarcane in the field, a *Xanthomonas albilineans* strain belonging to serotype 3 (strain XaS3) was detected in water sampled at sunrise on the leaves in the first plot. This strain randomly invaded the sugarcane canopy. Seven weeks later, a new strain belonging to serotype 1 (strain XaS1) appeared on leaves and populations of strain XaS1 progressively increased on the leaf surface, whereas populations of strain XaS3 progressively decreased. Leaf scald symptoms were first noted 26 weeks after sugarcane planting. However, only strain XaS1 was isolated from leaves and a few sugarcane stalks showing symptoms. Both strains also colonized the second field plot, which was studied at the end of the experiment to avoid human interference of aerial contamination of sugarcane. After inoculation of three sugarcane cultivars by the decapitation technique, strain XaS1 was as virulent or more virulent than five other strains of *X. albilineans* isolated from diseased sugarcane plants in Guadeloupe. Although strain XaS3 colonized a few stalks, it failed to produce any symptoms and was the least virulent strain. Leaf surface colonization by *X. albilineans* was reproduced in a greenhouse trial by spraying the pathogen on sugarcane foliage. After 8 weeks, the pathogen was isolated from disinfected leaf blades. Although the leaf scald pathogen is thought to be mainly transmitted by infected cuttings, aerial transmission of *X. albilineans* is also known to occur. These results indicate the importance of sugarcane phyllosphere colonization by virulent strains in the epidemiological cycle of leaf scald disease in Guadeloupe.

Introduction

Leaf scald, caused by *Xanthomonas albilineans*, is one of the major diseases of sugarcane (*Saccharum* spp.) and occurs in at least 66 countries of the world (Rott and Davis, 2000). It is a vascular bacterial disease that can cause severe yield losses (Martin and Robinson, 1961). Numerous haplotypes and at least three serotypes of the pathogen occur (Davis et al., 1997; Rott et al., 1994b). Leaf scald is managed by planting healthy seed-cane from plants issued from disease-free tissue culture propagation (Feldmann et al., 1994; Flynn and Anderlini, 1990) or from hot-water treated plants (Egan and Sturgess, 1980), and by use of resistant cultivars (Walker, 1971). Management of the disease is complicated by latent infection. Stalks can be infected by the pathogen for several months without showing symptoms. Improvement of diagnostic techniques has allowed detection of the pathogen in numerous symptomless plants (Comstock and Irey, 1992) revealing that *X. albilineans* seems to be randomly distributed among stalk internodes (Pan et al., 1999). Symptomless sugarcane plants can therefore constitute inoculum sources for contamination of the crop. Additionally, various epidemiological factors play a role in field contamination. Leaf scald is transmitted mechanically by

knives and harvesters and by planting infected setts (Ricaud and Ryan, 1989). However, other means of dissemination have been suspected. The pathogen was found in the rhizosphere of infected roots suggesting a possible transmission by root contact (Klett and Rott, 1994). *Xanthomonas albilineans* was also found in guttation droplets (Sordi and Tokeshi, 1986; Autrey et al., 1995), on the leaf surface of symptomatic and symptomless plants, and in aerosols above a diseased field (Klett and Rott, 1994). Leaf scald in Mauritius spread from sugarcane to maize grown between sugarcane rows (Autrey et al., 1995). These data support aerial contamination of sugarcane plants next to contaminated stalks. Leaf scald outbreaks in Florida, Louisiana and Texas were suspected to be linked to strains of *X. albilineans* that survive better epiphytically or have a greater propensity toward aerial transmission (Davis et al., 1997). In Guadeloupe, symptoms that were attributed to aerial transmission of leaf scald were first observed in sugarcane fields in 1993. These symptoms were 3–20 cm elongated necrotic stripes, generally originating from the leaf margin, from which a yellowish stripe ran down the leaf parallel to the main vein. The necrotic stripes were 0.5–1 cm wide, and the chlorotic stripes were 0.2–0.4 cm wide. *Xanthomonas albilineans* was isolated from necrotic leaves but not from the stalks. Similar symptoms were described in Mauritius in 1995 (Autrey et al., 1995) and in Florida in 2001 (Comstock, 2001). Since 1993, the presence of symptoms attributed to aerial contamination was checked each year, in Guadeloupe, in nursery plots established with disease-free material propagated by tissue culture. In this Caribbean island, 1993 was followed by 2 years of drought, and no leaf scald symptoms were observed in nurseries during that period. With the return of normal rainfall in 1996, elongated necrotic leaf symptoms attributed to leaf scald were observed in a nursery plot established with disease-free tissue cultured plants of cultivar B69566 that is susceptible to leaf scald (Rott et al., 1995). *Xanthomonas albilineans* was isolated from 18 out of 20 symptomatic leaf samples, but the pathogen was not isolated from the stalk internodes surrounded by symptomatic leaves. However, 3 months later, *X. albilineans* was isolated from 6 out of 108 (5%) stalks sampled from plants with leaf symptoms attributed to aerial transmission of leaf scald (J.H. Daugrois and L. Costet, unpublished data). These results were the first evidence of sugarcane infection in a disease-free established sugarcane field in the Carribean, by the leaf scald pathogen after aerial transmission. Similar observation was reported from Mauritius (Saumtally et al., 1996). The aim of the present study was to investigate establishment and progress of aerial transmission of *X. albilineans* on three sugarcane cultivars in disease-free fields and to determine the associated plant colonization process. Additionally, virulence of epiphytic *X. albilineans* isolates was compared with virulence of isolates previously sampled from diseased plants in Guadeloupe.

Materials and methods

Plant material

All experiments were conducted with disease-free tissue cultured plantlets. Plants were propagated *in vitro* and transferred to the greenhouse (Feldmann et al., 1994). Four-week-old greenhouse plants were used for field and greenhouse experiments.

Bacterial strains

Seven strains of *X. albilineans* isolated in Guadeloupe were used for virulence tests in greenhouse experiments (Table 1). Bacteria were stored in sterile distilled water at $-20\,^{\circ}C$ until inoculation assays were performed. They were grown on Wilbrink's medium (Dye, 1980) and propagated in susceptible cultivar CP68-1026. Cultivar CP68-1026 was inoculated with a 10^8 cfu ml^{-1} pathogen suspension by the decapitation technique (Rott et al., 1997). Strains of *X. albilineans* were isolated from symptomatic leaves, subcultured and grown for 48 h on Wilbrink's medium and used to inoculate the sugarcane plants. Material used for plant inoculation was disinfected with 95% ethanol and flamed between each strain.

Table 1. Xanthomonas albilineans strains used in the greenhouse experiments

Strain	Date of isolation	Sampling location in Guadeloupe	Serotype[1]
GPE5SR	1988	Grande Terre	1
GPE27	1991	Basse Terre	1
GPE30	1991	Marie Galante	1
GPE34	1994	Basse Terre	1
GPE42	1998	Grande Terre	1
XaS1	1997	Basse Terre	1
XaS3	1997	Basse Terre	3

[1]Serotypes according to Rott et al. (1994b).

Aerial transmission of X. albilineans *(field experiment)*

Two trials of 350 m^2, separated by 15 m, were established on 22 May 1997 at Capesterre, in a highly humid location of Guadeloupe. The trials were surrounded by banana fields and were 3 km from sugarcane fields. Three sugarcane cultivars with different resistance levels to leaf scald were used: B8008 (resistant), B69566 (susceptible) and CP68-1026 (highly susceptible) (Rott et al., 1995; 1997). Each trial consisted of 10 rows of 22 m of sugarcane. Two plants of B8008 and two plants of B69566 were alternately planted on the row, and each row consisted of 36 plants. In the first trial, 14 plant pairs (two consecutive plants on the row) of B8008 and 14 plant pairs of B69566 were randomly replaced by 28 plant pairs of CP68-1026. Both trials were divided into 45 quadrats of 3 m × 2.6 m comprising eight plants each (four plants on each of the two neighbour rows). Cultivar CP68-1026 was represented in the first trial in 28 quadrats. Plants were grown according to standard commercial practices (Rott et al., 1995).

During plant growth, the dew and/or rain water available on leaves early in the morning was used for detection of *X. albilineans*. Until the first detection of *X. albilineans*, 2 ml of pooled water droplets were sampled from the leaf surface of sugarcane plants. Droplets were taken from 10 quadrats randomly chosen in each trial, and sampling was performed every 3 weeks. As soon as *X. albilineans* was detected for the first time in the field, the sampling procedure was modified and eight droplets (total volume of 80–200 µl) were sampled from each quadrat and pooled in 2 ml of sterile, distilled water. Each pooled sample and its 100-fold dilution in sterile distilled water were plated on XAS medium, modified Wilbrink's medium supplemented with KBr 5 g l^{-1}, benomyl 2 mg l^{-1}, cycloheximide 100 mg l^{-1}, propiconazole 10 mg l^{-1}, cephalexin 25 mg l^{-1}, novobiocin 30 mg l^{-1} and kasugamycin 50 mg l^{-1} (Davis et al., 1994), using the Spiral system (Interscience, 78 860 St Non-La-Breteche, France) to determine population size of *X. albilineans*. Bacterial populations were recorded as log[(cfu/droplet) + 1] for each sample of eight droplets in 2 ml of water. Sampling was undertaken 6, 9, 10, 13, 16, 17, 18, 20, 23, 24 and 28 weeks after planting for the first trial and 6, 9, 10, 13, 16 and 26 weeks after planting for the second trial. Necrotic leaf symptoms attributed to *X. albilineans* (Autrey et al., 1995; Comstock, 2001) were recorded on 28 November 1997 (27 weeks after planting) for each plant-pair in both trials and on 9 December 1997 (29 weeks after planting) for each stalk in the first trial. Symptoms were recorded according to a 0–3 scale: 0: no symptom, 1: one short (1–10 cm) necrotic lesion per stalk, 2: several short necrotic lesions or one large (>15 cm) necrotic lesion per stalk, 3: two or more large necrotic lesions per stalk.

Isolation of the pathogen from leaf lesions and symptomless leaf tissue was attempted after disinfecting the leaf surface with 95% ethanol. Surface-disinfected and air-dried leaf tissues were cut into small pieces in 2 ml sterile, distilled water and, 40 min later, 50 µl of the suspension was streaked on XAS medium. Isolation of the pathogen from stalks was performed with 38-week-old plants. A total of 240 stalks were sampled. Stalk colonization by *X. albilineans* was assessed by the stalk blot isolation technique with XAS medium (Davis et al., 1994). Visual identification of *X. albilineans* was randomly verified by serology (Rott et al., 1994b).

Epiphytic survival of X. albilineans *(greenhouse experiment)*

Experiment setting. Ninety-six 4-week-old plants of sugarcane cultivar CP68-1026 were transplanted into 4 l pots containing an equal volume of field soil and crushed volcanic rock and divided into 12 blocks. Plants were automatically fertilized by sprinkling irrigation with water containing 16 mg l^{-1} of Mairol OR fertilizer (Mairol Gmbh & Co., 89547 Gussenstadt, Germany). After 3 months of growth, plants were sprayed with 5.5 l of a 10^8 cfu ml^{-1} suspension of *X. albilineans* strain GPE5SR resistant to streptomycin and rifampicin (Rott et al., 1994b). This inoculation was performed during late afternoon after the last irrigation. Four Petri dishes containing Wilbrink's medium amended with 50 mg l^{-1} streptomycin and 50 mg l^{-1} rifampicin (WSR medium) (Rott et al., 1994a) were placed between pots at floor level. Two days after inoculation, a bacterial film was visible on the Petri dishes.

Determination of X. albilineans *populations on plants during plant growth.* Epiphytic *X. albilineans* population densities were estimated in each block immediately and 1, 3, 7, 10, 14, 17, 24, 28 and 38 days after inoculation. Two different sampling procedures were performed. The first procedure consisted of washing two leaf parts per block with sterilized distilled

water. Six centimetre-cross sections from the middle of leaves ranked L + 2 (= second fully emerged leaf from the top) at inoculation were dipped for 1 min in a Petri dish containing 10 ml of water. During immersion of the leaves, 5 ml of water was pipetted several times and gently released along the leaf surface. The second method consisted of sampling 1 ml of water available between the stalk and the sheath of the L + 2 leaf. One water sample was randomly taken per block.

Determination of X. albilineans *populations on and in plants at the end of the experiment.* Eight weeks after inoculation, the first destructive sampling was undertaken with two stalks per block. For each stalk, the leaf blades and leaf sheaths were removed, separated and then separately washed by dipping in 300 ml of sterile, distilled water in a 0.5 l graduated cylinder and hand shaken for 1 min. The cut end of leaf blades and sheaths was kept outside the graduated cylinder sealed with cling film. All leaf blades or leaf sheaths from one plant were then homogenized for 10 min in a Warring blender in 300 ml of sterile distilled water. Sap also was extracted from a 2-cm section from each stalk internode by centrifugation at 1500*g* for 20 min. The second destructive sampling was performed 9 weeks after inoculation, and one stalk was taken in each of 5 (set 1) and 10 (set 2) randomly chosen blocks. The position (rank) of each leaf on each stalk was identified, and each leaf was treated separately. The pathogen was isolated from the leaf blade of set 1 by cutting each leaf into small pieces in 100 ml of Tris buffer saline (TBS, pH 7.5, 5 mM). Samples were shaken for 2 h on a rotary shaker before plating on WSR medium with the spiral system. The pathogen was isolated from the leaf blade of set 2 as described for those of set 1 except that leaves were previously washed and disinfected as follow. Each leaf was washed with 100 ml of 5 mM TBS by hand agitation, for 1 min, in a 250 ml graduated cylinder. It was then cut transversly into two equal parts. Both parts were then cut longitudinally and one half part of the upper part and one of the lower part were disinfected by dipping into 95% alcohol for 3 s and removing the excess alcohol by evaporation under a laminar flow cabinet. The two other half parts were disinfected by dipping into 95% alcohol for 3 s and rapid flaming. The four samples of each leaf blade of set 2 were then cut as described above. Pathogen population densities were estimated by plating 50 µl of suspension on WSR medium with the Spiral system.

Virulence tests

Two separate greenhouse experiments were conducted. In both experiments, 4-week-old plants were transplanted into 4 l pots containing an equal volume of field soil and volcanic rock. Plants were fertilized and drip-irrigated every day with 2.4 l of water containing 16 mg l^{-1} of Mairol OR fertilizer. Three-month-old plants of three sugarcane cultivars differing in resistance to leaf scald disease were used. Cultivars B69379 (susceptible), B8008 (resistant) and R570 (tolerant) were inoculated with seven strains of *X. albilineans* isolated in Guadeloupe (Table 1). Strain virulence was tested using a split plot design with three (first experiment) or four (second experiment) replications, and with the cultivars as main plots. Each treatment per block consisted of three (first experiment) or two (second experiment) plants inoculated with a strain of *X. albilineans* or distilled water (control). Plants were inoculated by the decapitation technique as previously described (Rott et al., 1997). Briefly, sugarcane stalks were cut at the third visible leaf ligule from the top with pruning shears dipped in a 10^8 cfu ml^{-1} pathogen suspension. One millilitre of the pathogen suspension was deposited with a pipette on the cut stalk.

Symptoms were recorded every 2 weeks until the end of the experiment, 12 weeks after inoculation (Rott et al., 1997). All inoculated stalks were rated individually, using a symptom severity scale ranging from 0 to 5 (0 = no symptoms to 5 = death of plant or stalk with side shoots). Ratings were used to calculate mean disease severity (DS) varying between 0 and 100 per plot. $DS = 100 \sum \text{rating}/(5T)$ where T = total number of stalks. DS recorded on newly developed, non-inoculated leaves 6, 8, 10 and 12 weeks after inoculation were used for the calculation of the area under disease progress curve (AUDPC) (Campbell and Madden, 1990) for each strain of *X. albilineans*. Three months after inoculation, two cross sections of 2 cm each were sampled from each stalk with disinfected pruning shears. The first was taken from the bottom part of the stalk from the second internode below the inoculated area that showed reduced internodes. The second was taken from the upper part of the stalk from the second internode above the inoculated area. Stalk samples were then centrifuged at 1500*g* for 20 min. The resulting sap was used to determine bacterial population densities by plating 50 µl of 10- and 1000-fold dilutions of sap extract on XAS medium with the Spiral System.

Statistical analysis

SAS computer programs 6.2 (SAS Institute Inc., Cary, NC) were used for data analysis with the exception of strength of aggregation among quadrats in field trials that was examined by spatial autocorrelation analysis using the LCOR2 software (Gottwald et al., 1992b). AUDPC of each replication of each cultivar/strain combination of the virulence tests was calculated with the data from all observation times, and compared by analysis of variance. Stalk populations of *X. albilineans* were compared by analysis of variance after rank-transformation of population density data (Rott et al., 1997).

Results

Aerial transmission to sugarcane

The first observation of *X. albilineans*-like bacteria in water droplets on leaves was made in trial one, 13 weeks after transplanting disease-free plants to the field. Priority was given to *X. albilineans* population counts in this trial and the second trial was left for sampling at the end of the experiment to observe aerial transmission of *X. albilineans* without multiple interventions during the process.

Bacteria isolated from leaf water droplets 13 weeks after transplanting, reacted positively with *X. albilineans* serotype 3 antibodies. Hereafter, this type of bacteria will be referred to as XaS3. Following first identification of XaS3 in trial one, positive quadrats were found randomly dispersed in the field during all subsequent sampling periods. No aggregated distribution was detected, and no evidence to determine entry direction of the pathogen into the field was found. The number of XaS3-positive quadrats varied from 40% to 60% until 18 weeks after planting of sugarcane in the field. Mean population densities of XaS3 rapidly increased on leaves and reached 24 cfu/water droplet 18 weeks after planting of sugarcane in the field (Figure 1). This value decreased progressively and only 2.6 cfu/water droplet were found on the leaves 28 weeks after sugarcane planting. This population density decrease was correlated with the reduction of the number of XaS3-positive quadrats. XaS3 was detected in only 14 out of 45 (31%) quadrats 24 and 28 weeks after sugarcane planting, whereas it was isolated before from 26 and 25 out

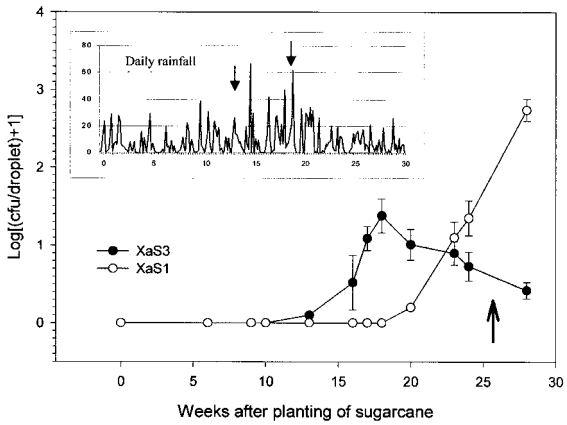

Figure 1. Xanthomonas albilineans population densities in water droplets sampled for 7 months from the leaf surface of sugarcane in the first field trial. Each vertical bar represents the standard error of the mean of 45 samples of 8 pooled droplets each. The large black arrow indicates the first observation of necrotic symptoms attributed to leaf scald. The small arrows indicate the tropical disturbances preceding strain isolation. Daily rainfall expressed in mm.

of 45 (57% and 55%) quadrats, 17 and 23 weeks after sugarcane planting (Figure 2). When XaS3 densities started to decrease, a second type of *X. albilineans* strain was identified in the sampled water droplets. This new type of *X. albilineans* grew slower than XaS3 on XAS medium. After 6 days of growth, its average colony size was 1 mm compared to 3 mm for XaS3. This bacterium was isolated 20 weeks after setting up the two trials and reacted positively with *X. albilineans* serotype 1 antibodies. This type of bacteria will be referred to as XaS1 in the rest of this study. Population densities of XaS1 on the leaves increased until the end of the experiment when 5.5×10^2 cfu/droplet of water were determined (Figure 1). The mean XaS1 population at the end of the study, 28 weeks after planting of sugarcane in the field, was 200 times higher than that of XaS3. The increase of the population size of XaS1 was correlated with the increase of the number of XaS1 contaminated quadrats: 47% of the quadrats were contaminated 23 weeks after planting, whereas 98% were contaminated 5 weeks later (Table 2). The increase of XaS1 population density occurred as the XaS3 population density decreased (Figure 1). In addition, aggregated distribution was detected for XaS1 at week 23, and quadrat contamination by this strain clearly began from the west and then progressively covered the entire field (Figure 3). At the end of the experiment (28 weeks after planting), population

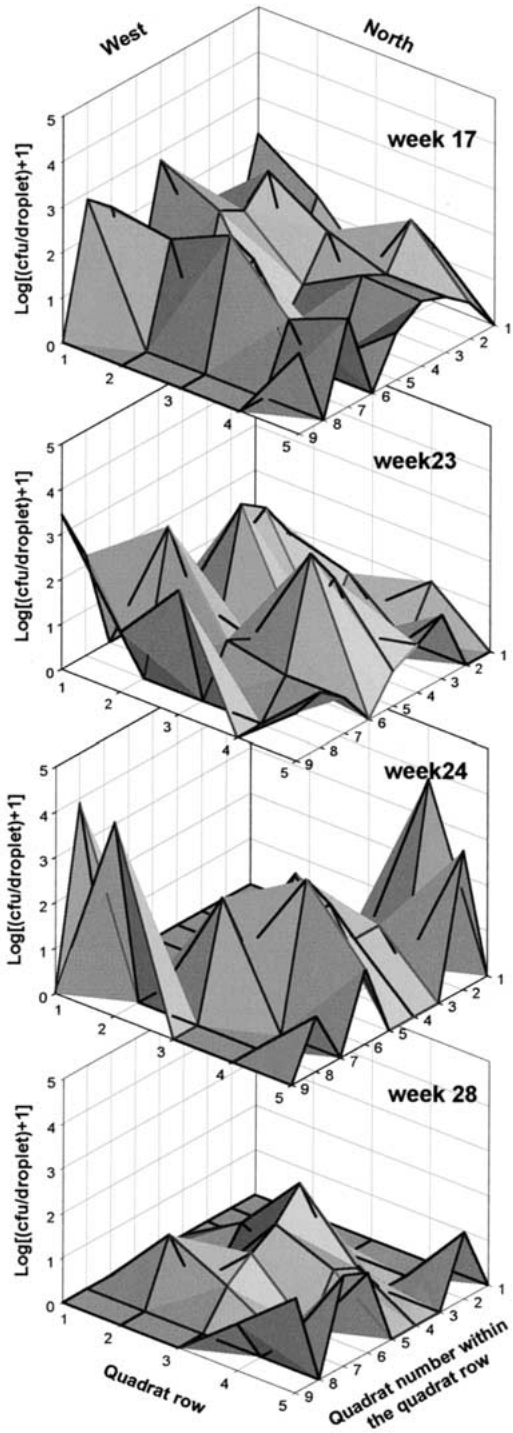

Figure 2. Distribution of *X. albilineans* serotype 3 (XaS3) in field trial one at four different dates of sampling. Pathogen population densities are expressed as the means of log 10[(cfu/droplet) + 1] per quadrat.

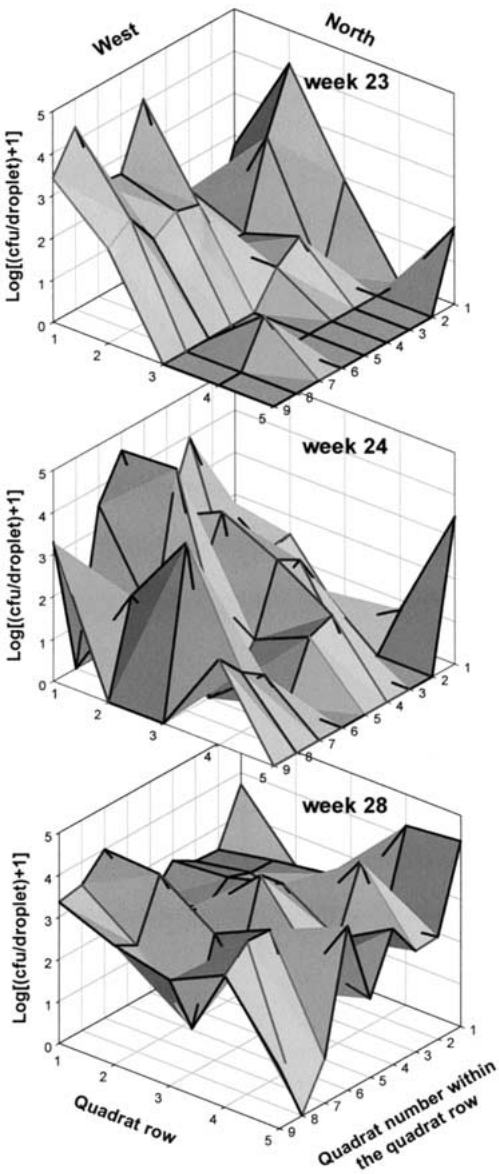

Figure 3. Distribution of *X. albilineans* serotype 1 (XaS1) in field trial one at three different dates of sampling. Pathogen population densities are expressed as the means of log 10[(cfu/droplet) + 1] per quadrat.

densities of the pathogen varied between 10 and 2.4×10^4 cfu/water droplet in each of the 44 contaminated quadrats (Figure 3). However, for 34 (77%) of the 44 contaminated quadrats, population densities varied only between 10^2 and 8×10^3 cfu/water droplet.

Similar results were obtained for trial 2 in which both serotypes were also identified (Table 2) and, as

Table 2. Leaf surface population of the leaf scald pathogen in two field trials, 5 and 6 months after planting of sugarcane in the field

Trial	Weeks after planting	Number of quadrats[1] contaminated by *X. albilineans*[2]			*X. albilineans* population densities[3]	
		XaS3	XaS1	XaS3 and XaS1	XaS3	XaS1
1	23	25 (55)	21 (47)	10 (22)	0.90	1.08
1	28	14 (31)	44 (98)	14 (31)	0.42	2.74
2	26	22 (49)	24 (53)	10 (22)	0.70	0.93

[1] Positive quadrats out of 45, in parentheses the percent corresponding value.
[2] XaS3 and XaS1 refer to serotype 3 and 1 of *X. albilineans*, respectively.
[3] Densities expressed as log[(cfu/droplet) + 1] (mean of 45 quadrats).

Figure 4. Necrotic symptoms of leaf scald on sugarcane leaves of cultivar B69566 after aerial contamination by *X. albilineans*.

in trial 1, the distribution patterns of strains XaS1 and XaS3 were different (data not shown).

Necrotic symptoms were observed on sugarcane leaves in trial one 6 weeks after the first detection of XaS1 in water droplets (Figures 1 and 4). Symptoms appeared as elongated necrotic lesions which extended along leaf veins with yellowish stripes. Thirty out of 180 plant-pairs (17%) showed these lesions 27 weeks after planting sugarcane in the field. A second observation made on each stalk showed that all three sugarcane cultivars had some necrotic leaf symptoms, but the percentage of symptomatic stalks and symptom severity varied according to cultivar (Table 3). Lesions were more frequent and more severe on cultivar B69566 than on the two other cultivars, including cultivar CP68-1026 which is highly susceptible to leaf scald. Most symptoms were recorded in the west part of the field (Figure 5) where high populations of XaS1 were found 6 weeks earlier (Figure 3). The coefficient of determination (r^2) between symptom severity observed at

week 29 and XaS1 bacterial populations in the water droplets collected on the leaves at week 23, 24 and 28 was 0.71, 0.52 and 0.24, respectively. Necrotic symptoms were also observed on few sugarcane leaves in trial two 27 weeks after planting. Samples were taken in trial one from symptomatic and asymptomatic leaves to isolate the pathogen from internal leaf tissues. XaS1 was recovered from all the 20 samples taken from the area surrounding lesions. It was also isolated from one sample out of 20 asymptomatic leaves. XaS3 was never recovered from the 40 leaf samples. Stalk infection by *X. albilineans* was investigated at the end of trial one, and XaS1 was found by stalk blot isolation with XAS medium in the lower part of two stalks out of 80 of cultivar B69566 (susceptible) and two stalks out of 72 of cultivar CP68-1026 (highly susceptible). It was also isolated from the upper part of one stalk out of 80 of cultivar B8008 (resistant).

Table 3. Necrotic leaf lesions observed on three sugarcane cultivars 29 weeks after planting (trial one, 9 weeks after first detection of *X. albilineans* XaS1 in water droplets)

Cultivar	Number of stalks observed	Percentage of stalks with score[1]			
		0	1	2	3
B69566 (S)[2]	1463	82.9	8.8	5	3.3
B8008 (R)	1290	96.1	2.6	0.9	0.4
CP68-1026 (HS)	212	94.8	3.3	1.9	0

[1] Score: 0 = no symptom, 1 = one short (1–10 cm) necrotic lesion per stalk, 2 = several short necrotic lesions or one large (>15 cm) necrotic lesion per stalk, 3 = two or more large necrotic lesions per stalk.
[2] S = susceptible to leaf scald, R = resistant, HS = highly susceptible.

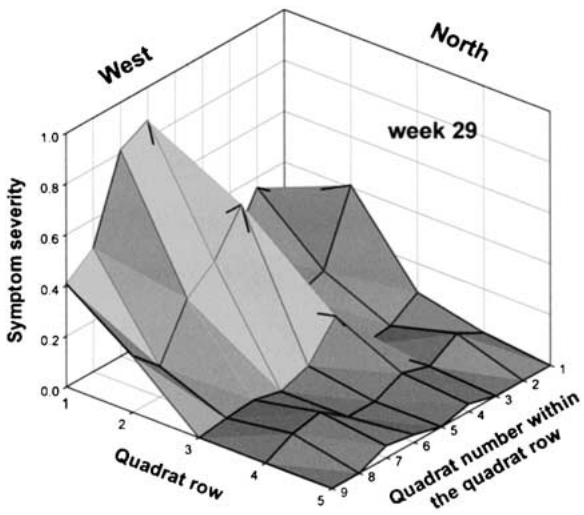

Figure 5. Symptom severity per quadrat in field trial one. Symptoms were recorded on week 29 on each stalk according to a 0–3 scale. Score: 0 = no symptom, 1 = one short (1–10 cm) necrotic lesion per stalk, 2 = several short necrotic lesions or one large (>15 cm) necrotic lesion per stalk, 3 = two or more large necrotic lesions per stalk. Data represent the mean of rating per quadrat.

Epiphytic survival of X. albilineans *(Greenhouse experiment)*

When sugarcane foliage was sprayed with *X. albilineans* strain GPE5SR in the greenhouse, mean pathogen populations isolated by leaf washing rapidly decreased on the second fully emerged leaf from the top (Figure 6). From then on, only a few bacteria (20–80 cfu ml^{-1} water) were sporadically found in some blocks until 28 days after inoculation. GPE5SR also was found in water available between the stalk and the sheath of the same leaf during 3 weeks after foliage inoculation (Figure 6). Eight weeks after inoculation, *X. albilineans* was isolated by leaf washing and leaf blade homogenization following leaf blade washing (Table 4). The pathogen was also isolated from leaf sheaths by washing and by homogenization but occurrence of positive samples was lower (Table 4).

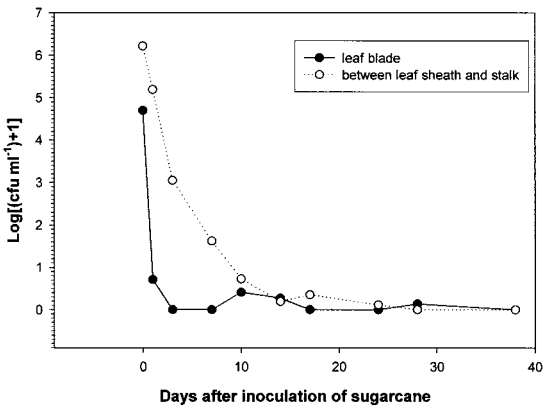

Figure 6. *Xanthomonas albilineans* populations on sugarcane leaf L+2 (= second fully emerged leaf from the top) and between stalk and L + 2 leaf sheath; counts were made for 38 days after inoculation of cultivar CP68-1026 by spraying the foliage with a 10^8 cfu ml^{-1} suspension of strain GPE5SR (approx. 55 ml per plant) (greenhouse trial).

Table 4. Isolation of *X. albilineans* (Xa) strain GPE5SR from CP68-1026 plants 8 weeks after spray inoculation of foliage in a greenhouse experiment

	Isolation methods				
	Leaf blade[1] wash	Leaf blade[1] homogenization	Leaf sheath[1] wash	Leaf sheath[1] homogenization	Stalk tissue[2] centrifugation
Number of positive samples/total samples	15/24	20/24	6/24	4/24	0/144
Mean Xa population log[(cfu/plant) + 1]	3.1	6.4	1.1	0.7	0

[1] Pooled leaf tissue of each plant.
[2] Six internodes per stalk taken from 24 stalks.

Table 5. Isolation of *X. albilineans* strain GPE5SR (Xa) on and in leaves of sugarcane cultivar CP68-1026, 9 weeks after spray inoculation of the foliage in a greenhouse experiment

Foliar ranks[3]	Set 1[1]		Set 2[2]					
	Positive leaf blades/total	Xa log[(cfu/leaf)+1]	Washing		Alcohol		Alcohol and flaming	
			Positive[4] leaf blades/total	Entire leaf blade log[(cfu/leaf) + 1][5]	Upper part log[(cfu/leaf) + 1][5]	Lower part log[(cfu/leaf) + 1][5]	Upper part log[(cfu/leaf) + 1][5]	Lower part log[(cfu/leaf) + 1][5]
L0	0/5	0						
L + 1	0/5	0						
L + 2	0/5	0						
L + 3	0/5	0						
L + 4	1/5	1.1	0/10	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
L + 5	2/5	2.8	3/9	0.6 (1)	3.0 (3)	0.0 (0)	0.8 (1)	0.0 (0)
L + 6	2/5	3.1	5/8	1.7 (2)	5.8 (5)	2.3 (2)	1.1 (1)	0.0 (0)
L + 7	2/5	3.7	2/4	0.0 (0)	2.3 (1)	0.0 (0)	2.1 (1)	0.0 (0)
L + 8	4/4	8.3	4/4	7.4 (4)	9.4 (4)	4.3 (2)	2.1 (1)	1.5 (1)

[1] 5 stalks were sampled, *X. albilineans* was isolated by leaf blade cutting without washing or disinfection.
[2] 10 stalks were sampled, *X. albilineans* was isolated by leaf blade washing and by leaf blade cutting after leaf washing and disinfection with alcohol with and without flaming.
[3] Foliar ranks L + 4 to L + 8 refer to leaves inoculated with the pathogen and foliar ranks L0 to L + 3 refer to leaves that appeared subsequently to inoculation. L + 1 = top fully emerged leaf.
[4] By at least one isolation method.
[5] Mean of all leaves tested. Number of positive leaves are given in parentheses.

Strain GPE5SR was not detected in any of the 24 stalks whose leaves were contaminated or not by the pathogen. *Xanthomonas albilineans* GPE5SR was present only on or in older leaves, which were sprayed by the pathogen (Table 5). The oldest leaves supported the highest pathogen population densities whatever the method used for sampling. Strain GPE5SR was also recovered from leaves after leaf washing and disinfection with alcohol. However, the pathogen was recovered from fewer leaves after alcohol disinfection and flaming than from leaves after disinfection without flaming (Table 5). The pathogen was more frequently recovered from the upper part of the leaves than from the lower part.

Virulence of seven strains of X. albilineans *from Guadeloupe, including XaS1 and XaS3 strains*

Virulence of XaS1, XaS3 and five other *X. albilineans* strains isolated in Guadeloupe was tested in two greenhouse trials after inoculation of sugarcane plants by the stalk decapitation method. Strains were compared by disease severity and bacterial populations in sap

Table 6. Pathogen populations in stalks of 3 sugarcane cultivars (R570, B8008 and B69379) and disease severity after plant inoculation by the decapitation method with seven strains of *X. albilineans*

Xa strain	Experiment 1			Experiment 2		
	Disease severity (AUDPC)[1]	Populations in stalks[2]		Disease severity (AUDPC)[1]	Populations in stalks[2]	
		Upper part	Lower part		Upper part	Lower part
GPE5SR	9.7 b	0.8 c	4.4 a	3.1 bc	1.7 b	4.9 ab
GPE27	12.5 ab	0.2 c	4.6 a	3.1 bc	1.6 b	5.1 ab
GPE30	21.0 a	5.7 a	6.7 a	10.1 b	5.6 a	5.9 a
GPE34	13.8 ab	2.4 b	6.2 a	0.5 c	0.4 b	3.7 b
GPE42	12.5 ab	1.8 bc	6.0 a	4.7 bc	3.9 a	5.8 ab
XaS3	0 c	0.3 c	0.9 b	0 c	0.3 b	0.5 c
XaS1	3.7 bc	0.9 c	4.4 a	17.0 a	5.2 a	6.3 a
Variance analysis						
Source	df	Pr > *F*	Pr > *F*	df	Pr > *F*	Pr > *F*
Cultivar	2	0.332	0.003	2	0.010	0.0002
Strain	6	0.0001	0.0005	6	0.0001	0.0001
Cultivar × strain	12	0.959	0.524	12	0.0045	0.214
Block	2	0.739	0.067	3	0.289	0.215
Cultivar × block	4	0.023	0.910	6	0.470	0.442

[1] AUDPC was calculated with the data from four observation times (6, 8, 10 and 12 weeks after inoculation).
[2] Pathogen populations isolated from sap extracted from the upper and lower part of stalks are expressed as the mean of log[(cfu ml^{-1} of sap) + 1] for all three cultivars tested. Variance analysis and Newman–Keuls grouping were performed after rank transformation of population density data.
Values followed by the same letter are not significantly different at $P = 0.05$.

extracted from stalks. Strain XaS3 failed to induce leaf scald symptoms, even on inoculated leaves, in any of the three cultivars (B8008, B69379 and R570) and in either experiment. Strain XaS1 induced only a few pencil line symptoms on leaves in the first experiment, and disease severity (AUDPC) was similar or lower than those of the other strains of *X. albilineans*. In the second experiment, however, XaS1 was the most virulent strain and showed the highest AUDPC value (Table 6).

Pathogen population densities of XaS3 in stalk sap were very low in both experiments regardless of stalk location. Additionally, only 12 stalks out of 46 were infected by this strain. Population densities of XaS1 in the lower stalk location were 2.6×10^4 and 2.0×10^6 cfu ml^{-1} of sap in trials one and two, respectively. These densities were similar or higher than those of the five other strains (GPE5SR, GPE27, GPE30, GPE34 and GPE42) (Table 6). In the upper part of the stalk, XaS1 populations were low in the first experiment (8.5 cfu ml^{-1} of sap) but similar to those of other strains (GPE5SR, GPE27 and GPE42). In the second experiment, these values reached 1.6×10^5 cfu ml^{-1} of sap when disease severity was much higher. The bacterial population density in the cultivars varied, with high levels in the tolerant cultivar R570 and low levels in the resistant cultivar B8008 (data not shown). Significant cultivar × strain interaction was only observed once for *X. albilineans* population densities in the upper part of the stalk and was not repeatable. It was, therefore, not taken into consideration to show evidence for presence of *X. albilineans* races.

Discussion

Leaf scald has previously been demonstrated to be transmitted in the field by infected setts and cutting implements. Based on symptoms, sugarcane infection by aerial contamination of disease-free plants was reported in Mauritius (Saumtally et al., 1996). Sampling water droplets from the leaf surface of sugarcane allowed us to show evidence for epiphytic existence and/or aerial dissemination of *X. albilineans* prior to plant infection, even if the field is not located in a sugarcane growing area. Water sampling on leaves is not usual for epiphytic bacterial counts (Hirano and Upper, 1983), but it was chosen because it was non-destructive. The epiphytic

population densities of *X. albilineans* are certainly higher than those present in water droplet due to bacterial adherence on leaves and/or to the part of the population that reached endophyte sites. External and internal leaf associated phytopathogenic bacteria are supposed to form a continuum due to ingression and egression process (Beattie and Lindow, 1999). Bacterial population of *X. albilineans* in water droplets may, therefore, only represent a part of the epiphytic population resulting or not from the egression process, and may have a major role in plant to plant contamination. In addition, droplet sampling did not damage or wound the leaves and avoided plant infection through the sampling procedure. Populations of *X. albilineans* appeared on the leaf surface of disease-free plants in sugarcane plots that were distant from other sugarcane fields in Guadeloupe. Two populations of *X. albilineans*, differentiated by their serological characteristics (Rott et al., 1994b) and colony size on Wilbrink's medium, were identified on the sugarcane foliage in two field trials: a non-aggressive strain belonging to serotype 3 (referred to as XaS3) and an aggressive one belonging to serotype 1 (referred to as XaS1). Identity of both strains was also confirmed with biochemical characteristics (data not shown). Serotype 3 antibodies were prepared against a strain isolated in Guadeloupe in 1970, but strains belonging to the serotype 3 group had not been found in Guadeloupe since 1985 (Rott et al., 1994b). Serotype 1 is the most widespread serotype around the world and the major one among strains of the Caribbean Islands (Rott et al., 1994b). In trial one, strain XaS3 was first detected during a 2-day weather tropical disturbance. XaS1, a serotype 1 strain, was first detected 7 weeks later and 1 week after a tropical storm (named Fabian) with heavy rains (Figure 1). These meteorological events might be responsible for long distance transport of the pathogen that invaded the sugarcane leaf canopy. Cyclones and rainstorms are known to be responsible for long distance bacterial dissemination (Gagnevin and Pruvost, 2001) and spread of bacteria in the field (Bernal and Berger, 1996). *Xanthomonas albilineans* outbreak in Louisiana was also suspected to be favoured by hurricane (Hoy and Grisham, 1994). However, initial distribution and development of XaS3 and XaS1 populations were different. When first detected, XaS3 was randomly distributed in the field, and it colonized at least half of the field canopy. In contrast, XaS1 invaded both trials from the edge of each plot, and then progressively replaced the less aggressive XaS3 on the leaves. Decline of the XaS3 population was correlated with the increase of the aggressive XaS1 population of *X. albilineans*. XaS3 showed a relative good epiphytic survival but was less competitive than the virulent strain XaS1. The two *X. albilineans* strains appeared in both trials, but the pathogen population dynamics of each differed. In the second trial, colonization of the leaves by XaS1 seemed to be delayed in comparison to trial one. Bacterial populations of XaS1 and XaS3, in the second trial, 26 weeks after planting were similar to those observed at week 23 after planting in the first trial. This observation is also supported by the lower number of necrotic symptoms that appeared in the second trial. In addition, strain XaS3 appeared to be restricted to the leaf surface of sugarcane but it could be virulent on other plants from which the strain migrated in the sugarcane plots. In contrast, XaS1 was able to invade the inner space of leaves and to induce appearance of symptoms revealing ingression possibility. Increasing population densities of *X. albilineans* serotype 1 on the leaf surface may have been due to the ability of pathogenic bacteria to multiply inside the leaf in protected areas, such as substomatal cavities or hydathodes, and then move to the surface of the leaves by exudation or when water becomes available on the leaves, or by any other egression process (Rudolph, 1993; Beattie and Lindow, 1995). The good epiphytic survival of the virulent XaS1 strain seems to be favoured by this ingression–egression process.

This hypothesis is supported by results of the greenhouse experiment in which sugarcane plants of cultivar CP68-1026 were sprayed with *X. albilineans* strain GPE5SR. The bacteria were detected on the foliage by washing a 6-cm leaf area, until 4 weeks after inoculation. However, they also were detected at the end of the experiment by washing entire leaves, when washing only 6 cm leaf areas was negative. Even though no symptoms appeared on leaves 8 weeks after inoculation, *X. albilineans* was isolated from washed leaves with or without disinfection (alcohol and flaming), indicating that some cells of *X. albilineans* reached protected areas within the leaf, confirming possibility of occurrence of ingression process in the infection cycle of *X. albilineans* virulent strains. *Xanthomonas albilineans* strain GPE5SR was not able to move to non inoculated leaves as bacterial population was only recovered from basal leaves that were present when plant surface was inoculated. This may be due to strain specificity. Strain GPE5SR, an antibiotic resistant mutant, might not be well adapted

for epiphytic competition, in contrast to wild strains (Davis et al., 1997; Klett and Rott, 1994). Additionally, the wild strain pool of *X. albilineans* may contain genotypes more fit for epiphytic conditions than the selected antibiotic resistant strain (Hirano and Upper, 1993). Absence of wind and rain splash in the greenhouse condition may also be one of the reasons for non-dispersal of strain GPE5SR. Nonetheless, as in the field, *X. albilineans* was able to enter sugarcane, at least protected sites, after leaf inoculation without wounding of the leaves. The absence of symptom development was probably due to insufficient growth time in the greenhouse (plants reached the roof of the greenhouse at the end of the experiment) or the necessity to have leaf lesions to access xylem vessels, because strain GPE5SR caused symptoms in the virulence test when sugarcane plants were inoculated by the decapitation method.

Results described herein also showed that *X. albilineans* serotype 1 moved from plant to plant in the field, eventually covering the whole leaf canopy of the sugarcane plot. This short distance movement was likely due to rain splashing during the warm, wet season (July to mid December). Xanthomonads are able to move from plant to plant or leaf to leaf (Stall et al., 1993). Klett and Rott (1994) showed that *X. albilineans* is exuded from symptomatic leaves and can be isolated from aerosols. In our study, colonization of the leaf canopy upon arrival of *X. albilineans* in the field trials was probably favoured by high humid periods during the day (90–100% relative humidity), periodic rainfalls and mild to warm temperatures (day and night temperatures ranged between 23 and 29 °C). These conditions are known to favour epiphytic life of xanthomonads (Stall et al., 1993).

Necrotic leaf lesions due to *X. albilineans* serotype 1 appeared 5–6 weeks after observation of high bacterial populations in the water available on leaves at sunrise. Symptoms were more severe in field locations where the pathogenic strain XaS1 was predominant 6 weeks earlier, indicating that epiphytic growth preceded appearance of leaf symptoms. A high correlation was also observed between symptoms and pathogen population in leaf water droplets detected 6 weeks earlier ($r = 0.86$). Similar phenomena were observed on citrus and pepper with other bacterial pathogens: *Xanthomonas campestris* pv. *citrumelo* and *X. campestris* pv. *vesicatoria* populations recoverable from the leaf surface were correlated with disease incidence that occurred several weeks later (Bernal and Berger, 1996; Gottwald et al., 1992a), but this characteristic had not been described for *X. albilineans* so far. Population size of *X. translucens* pv. *translucens* on wheat seedlings 2 days after inoculation also were predictive of bacterial leaf streak severity 5 days later (Stromberg et al., 1999). Cultivar identity was not taken into consideration during our sampling and no information is available regarding cultivar response to leaf surface contamination. Necrotic symptom severity following leaf infection varied, however, between cultivars, and response to leaf infection may therefore vary according to the cultivar. Effects of plant cultivar on foliar lesion intensity were also found in Florida after natural contamination of sugarcane by *X. albilineans* (Comstock, 2001). Nevertheless, rating cultivar resistance by foliar lesion intensity after foliar infection could differ from rating resistance after artificial plant inoculation as observed herein when the highly susceptible cultivar CP68-1026 (Rott et al., 1997) showed less foliar lesion intensity than the susceptible cultivar B69566.

The leaf scald pathogen is mainly disseminated by planting stalks taken from asymptomatic infected plants, and this material is considered to be the primary source of sugarcane infection (Ricaud and Ryan, 1989). Presence of *X. albilineans* was reported on leaf surface of symptomless plants nearby infected plants (Klett and Rott, 1994; Davis et al., 1997). However, herein is demonstrated for the first time that sugarcane leaf canopy may support high densities of epiphytic populations of *X. albilineans* prior to plant infection and symptom development in the field. Regarding the possibility of leaf canopy colonization by different strains of *X. albilineans*, it will be therefore unreasonable to predict disease incidence based on external population size without knowing strain identity and virulence. Stalk infection of sugarcane following leaf contamination is most likely the next step in the leaf scald cycle, as well as a source for a new epidemic in a newly established crop. Our results raise, therefore, the question regarding the source of the inoculum that invaded the two sugarcane trials. Several tropical weeds are known to be alternative hosts of *X. albilineans* and may constitute inoculum reservoirs when diseased sugarcane is absent (Birch, 2001; Rott et al., 1988). The majority of these weeds are non-persistant alternative hosts and some of them, as *Imperata cylindrica*, may be infected for a long-term period (Ricaud and Ryan, 1989). The planting material used in our study was disease-free, and the water droplets sampled from other plants

surrounding the sugarcane plots (various weeds, banana trees) were all negative (data not shown). Therefore, it is not possible to determine herein the distance of transport of the pathogen by air, or if other vectors or alternative hosts took part in *X. albilineans* dissemination.

Another important aspect is the variability of the pathogen present on the canopy. Results showed that at least two serotypes were able to colonize the leaf surface. Based on bacterial population densities measured in sugarcane stalks after plant inoculation, XaS1 strain was at least as virulent as other *X. albilineans* serotype 1 strains previously isolated from diseased sugarcane in Guadeloupe, whatever the cultivar inoculated. Symptoms induced by XaS1 after artificial plant inoculation were erratic between the two experiments. However, leaf scald symptoms are known to be unreliable for evaluation of sugarcane resistance to the disease, and bacterial populations are more accurate (Rott et al., 1997). Strain XaS3 was the least virulent strain in both greenhouse experiments and failed to induce symptoms. Additionally, only strain XaS1 was able to penetrate the leaves and to colonize a few sugarcane stalks in the field. More than 50 haplotypes have been identified within *X. albilineans*, and several were suspected to be associated with aerial dissemination of the leaf scald pathogen (Davis et al., 1997). Our study showed that variation in epiphytic survival exists among strains of *X. albilineans*. Additional investigations are needed to determine the genetic background of this characteristic of the pathogen and to determine the factors involved in aerial transmission of sugarcane leaf scald such as inoculum source, strain adaptability and leaf surface characteristics.

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