European Journal of Plant Pathology **109:** 351–359, 2003.

Ultrastructural changes and production of a xanthan-like polysaccharide associated with scald of sugarcane leaves caused by *Xanthomonas albilineans*

Maria Teresa Solas[1], Dolores Piñón[3], Ricardo Acevedo[3], Blanca Fontaniella[2], María Estrella Legaz[2] and Carlos Vicente[2,*]
[1]*Department of Cell Biology,* [2]*Department of Plant Biology, Complutense University, 28040 Madrid, Spain;* [3]*INICA, Avda. van Troy, Ciudad Habana, Cuba;*
[*]*Author for correspondence (Fax: +34913945034; E-mail: cvicente@bio.ucm.es)*

Accepted 10 December 2002

Key words: gum, *Saccharum officinarum*, *Xanthomonas albilineans*, xanthans

Abstract

Leaf scald is a vascular disease of sugarcane caused by *Xanthomonas albilineans*. Scalded leaves show white-yellowish streaks alternating with green zones in parallel to the main veins. These zones develop large bulliform cells, probably as a consequence of the wilting process. Moreover, a gum exudate occludes phloem and bundle vessels, and partially enters mesophyll cells. Some lysigenic cavities appear near the xylem. However, the white-yellowish streaks show both phloem and xylem completely occluded by the gum and the overall mesophyll appears to be full of this bacterial secretion, as revealed by scanning electron microscopy. The gum in conducting tissues has been purified from juice obtained from scalded stalks by precipitation with isopropyl alcohol and size-exclusion chromatography. It was identified as a xanthan-like polysaccharide and found to be composed of glucose, mannose and glucuronic acid by acidic hydrolysis and capillary electrophoresis.

Abbreviations: CE – capillary electrophoresis; i.d. – inner diameter; o.d. – outer diameter.

Introduction

Leaf scald, a vascular disease of sugarcane, is caused by the bacterium *Xanthomonas albilineans*. The initial characteristic symptom of disease is a white-yellowish streak ('pencil-line') 1–2 mm wide on the leaf along the main veins. The streaks may later become more enlarged and the affected leaf becomes wilted and necrotic. The white-yellowish pencil line may also be visible on the leaf sheaths (Lopes et al., 1998). Yellowish stripes occur at the leaf tips, and the vascular bundles exude a yellowish gum when cut (Purseglove, 1979). Symptoms of the first phase of leaf scald disease are seen after ratooning or in young shoots growing from an infected plant cane. Later, these symptoms may disappear, although plants remain infected. Alternatively, plants may be infected, but grow without showing any symptoms.

The bacterium is transmitted by infected cuttings and by implements used to cut stalks. There is also evidence for soil and water transmission. The disease is kept out of production areas through the quarantine of varieties introduced from other growing areas. In areas where the disease is endemic, resistance is used to manage the disease. Additionally, canes can undergo a cold soak/hot water treatment before planting.

Two main pathways are used by the bacterium to produce disease and death of the infected plants. First, the pathogen is confined mainly to the leaf and stalk vascular bundles which are often partially or completely occluded with a gum-like substance secreted by the bacterium, and mature stalks may suddenly wilt and die, sometimes without the prior appearance of other symptoms (Martin and Robinson, 1961). These events are related to the wilting but they do not appear to be related to leaf chlorosis which is restricted to

the appearance of white-yellowish streaks. The second mode involves a mixture of antibacterial compounds produced by chlorosis-inducing isolates of *X. albilineans*. The antibacterial mixture produced in culture is bactericidal to *Escherichia coli* and was given the trivial name albicidin. Near the minimum inhibitory concentration, albicidin causes a complete block to DNA synthesis, followed by partial inhibition of RNA and protein synthesis, as assessed by incorporation of radioactive precursors (Birch and Patil, 1985). A gene (*xabA*) required for albicidin biosynthesis encodes a peptide of 278 amino acids, including the signature sequence motifs for phosphopantetheinyl transferases that activate polyketide and non-ribosomal peptide synthetases. XabA seems to be a phosphopantetheinyl transferase required for post-translational activation of synthetases in the albicidin biosynthetic pathway (Huang et al., 2000a). Huang et al. (2000b) generated transgenic sugarcane plants that express an albicidin detoxifying gene (albD), which was cloned from a bacterium that provides biocontrol against leaf scald disease. Plants with albicidin detoxification capacity did not develop chlorotic disease symptoms in inoculated leaves, whereas all untransformed control plants developed severe symptoms.

In this work, changes in the leaf ultrastructure of healthy and scalded plants were studied in order to obtain a better knowledge of this sugarcane disease and to characterize the gum exuded in the diseased tissue.

Materials and methods

Plant material, culture conditions and ultrastructural studies

Field grown sugarcane, *Saccharum officinarum*, cv. Louisiana 55–5, was used throughout this work. Plants were developed from agamic seeds (segments of stalk internodes containing a vegetative bud), naturally infected with *X. albilineans* or uninfected, and grown in field conditions at the Real Jardín Botánico Alfonso XIII (Complutense University, Madrid) for 8 months.

The ultrastructure of sugarcane leaves obtained from healthy or scalded plants was examined by conventional scanning electron microscopy (SEM). The third pair of leaves from 20 different specimens of 8-month-old healthy (control) or scalded plants were always used. These leaves showed the characteristic morphology of white-yellowish streaks along the main veins before first wilting symptoms would appear. Samples were fixed in 2% glutaraldehyde (v/v) in 0.1 M phosphate buffer, pH 7.2, post-fixed with osmium tetroxide, washed, dehydrated in acetone, critical-point dried, sputter-coated with gold/palladium and scanned at 20 kV using a Jeol JSM 6400 (Jeol, Tokyo, Japan).

Preparation of sugarcane extracts

Twenty sugarcane stalks from healthy and diseased plants were mechanically crushed immediately after being cut and the crude juice was filtered through filter paper. Filtered juices were centrifuged at 2800 × *g* for 15 min at 2 °C (de Armas et al., 1999). The pellet was discarded and 50 ml of the supernatant used for xanthan extraction after being lyophylized. Alternatively, leaves of both healthy and scalded plants were homogeneized in a mortar with liquid nitrogen and extracted with MilliQ-grade water. Cellular debris were removed by centrifugation at 14 000 × *g* for 20 min at 2 °C and the supernatant used for xanthan extraction.

Extraction of xanthan

Extraction of xanthan was carried out according to the method described by Galindo and Albiter (1996) with some modifications. Lyophylized juice was resuspended in 50 ml Milli-Q grade water. This suspension as well as supernatant from leaf extraction was maintained at 60 °C for 30 min with shaking and then, centrifuged at 10 000 × *g* for 10 min at 2 °C. Supernatant (supernatant 1) was collected and the pellet (pellet 1) was re-extracted with 25 ml Milli-Q grade water at 60 °C, shaking for 30 min and then centrifuged at 20 000 × *g*. This process was repeated twice, obtaining supernatants 2 and 3.

All the supernatants obtained (supernatants 1, 2 and 3) (100 ml) were combined and precipitated with 100 ml of *iso*-propyl alcohol containing 3% (w/v) of KCl with shaking and then the mixture was maintained at 4 °C for 2 h, before being centrifuged at 14 000 × *g* for 20 min at 2 °C. The supernatant was discarded and the pellet, containing precipitated xanthans was dissolved in 10 ml of 10 mM sodium phosphate buffer, pH 6.8, and stored.

Samples of 5.0 ml of this crude preparation of xanthan were filtered through a 15 cm × 25 cm inner diameter (i.d.) column of Sephadex G-10 (Omnifit System, Supelco, Bellefonte PA, USA) pre-equilibrated with

10 mM sodium phosphate buffer, pH 6.8. The first 24 × 1.0 ml fractions of eluate were discarded. Fractions 25–41 were collected, and 8 ml of those were loaded onto a Sephadex G-50 column (30 cm × 2.5 cm i.d.), pre-equilibrated as above. Fractions 54–160 containing high- and mid-molecular-mass polysaccharides were assayed for carbohydrates (Dubois et al., 1956).

Acidic hydrolysis and sugar extraction

The fractions obtained from Sephadex G-50 filtration containing carbohydrates were hydrolyzed with 6 N HCl at 80 °C overnight and then were dried under reduced pressure. Each residue was ground with 3 ml of cold 80% (v/v) ethanol, stored for 2 h at 2 °C, and then, centrifuged at 19 000 × *g* for 15 min at 2 °C. After evaporation to dryness, xanthan precipitates were dissolved in 700 µl 10 mM sodium borate buffer, pH 9.2, and used for capillary electrophoresis (CE) analysis.

Capillary electrophoresis

Zone electrophoresis was performed using a Spectraphoresis 500 system from Spectra-Physics (Fremont, CA, USA). Microbore fused-silica tubing coated with polyimide (Scientific Glass Engineering, Milton Keynes, UK) of 75 µm i.d. and 190 µm outer diameter (o.d.) with a total length of 70 cm and a separation length of 63 cm were used. The capillary was enclosed in a cassette for easy handling. On-line detection was performed with a variable-wavelength UV-VIS detector of 6 nm band width (Spectra-Physics, Fremont). Detection of saccharides was monitored at 200 nm and electrophoregrams were recorded using a SP 4290 integrator (Spectra-Physics, Fremont).

New capillaries were conditioned with 1 M NaOH for 10 min at 60 °C, 0.1 M NaOH for 10 min at 60 °C and Milli-Q grade water for 10 min at 60 °C. Equilibration of the capillary was then performed by washing with 25 mM sodium borate buffer, pH 9.2 for 30 min at 25 °C and finally with the same buffer for 30 min at 25 °C under applied voltage of 15 kV. Regeneration of the capillary surface between runs was performed by rinsing it in the following sequence: 0.1 M NaOH for 5 min, Milli-Q grade water for 5 min and 25 mM sodium borate buffer, pH 9.2 for 15 min. The buffer used as electrolyte was 25 mM sodium borate buffer, pH 9.2 (Legaz and Pedrosa, 1993).

Saccharide standards were raffinose, stachyose, D-sucrose, D-maltose, D-cellobiose, D-galactose, D-mannose, D-glucose, D-xylose, L-arabinose, D-rhamnose, D-glucuronic acid, D-galacturonic acid, mannitol, galactitol, and D-glucose-1-P (all from Sigma Chemical Co., St. Louis, MO, USA). Standard saccharides as well as sample solutions were prepared in 10 mM sodium borate buffer, pH 9.2. Voltage was applied in such manner that ions migrated from the anode to the cathode. Quantitation of monosaccharides in the hydrolysates was performed by interpolating area counts in the corresponding straight lines constructed with increasing concentrations of the corresponding standards.

Results

Leaf ultrastructure

A cross section of a healthy cane leaf showed the classical arrangement of cells which defines a C4 species. The upper and the lower epidermal layers were made up of brick-shaped cells with their long axes parallel to the leaf (Figure 1A). The bulliform cells occurred in the lamina of *S. officinarum*. These thin-walled cells of this monocotyledon, the size of which is similar to that of brick-shaped epidermal cells, were confined to the adaxial epidermis (Figure 1A). When they lose water, they contribute to the rolling of the leaf.

The conducting tissues were within the circular to oval-shaped groups of cells. The bundles were composed of three-sized classes of cells: large, medium and small, the first two being rhomboid to oval in shape, while as a rule, the small type was rather circular. A small, round bundle always lay next to a large vascular bundle which usually extended from the upper to the lower epidermis of the leaf (Figure 1A). The vascular bundle included the xylem, phloem and phloem fibers, all of which were surrounded by a ring of large cells known as the starch-bearing bundle sheath (Figure 1A,C). The xylem was made up of open tubes or vessels associated with smaller and thicker walled elements. The large bundles of the leaf were usually two large vessels connected with smaller vessels. The xylem of small bundles consisted of only a few large pitted vessels (Gunning and Steer, 1986). The large vessels were irregular in shape, having comparatively thick walls (Figure 1B) and many sides, each of which, when viewed in cross section, appeared more or less as a straight line. Each vessel was formed not from a single cell but from a series of elongated cells, whose contents and end walls had disappeared.

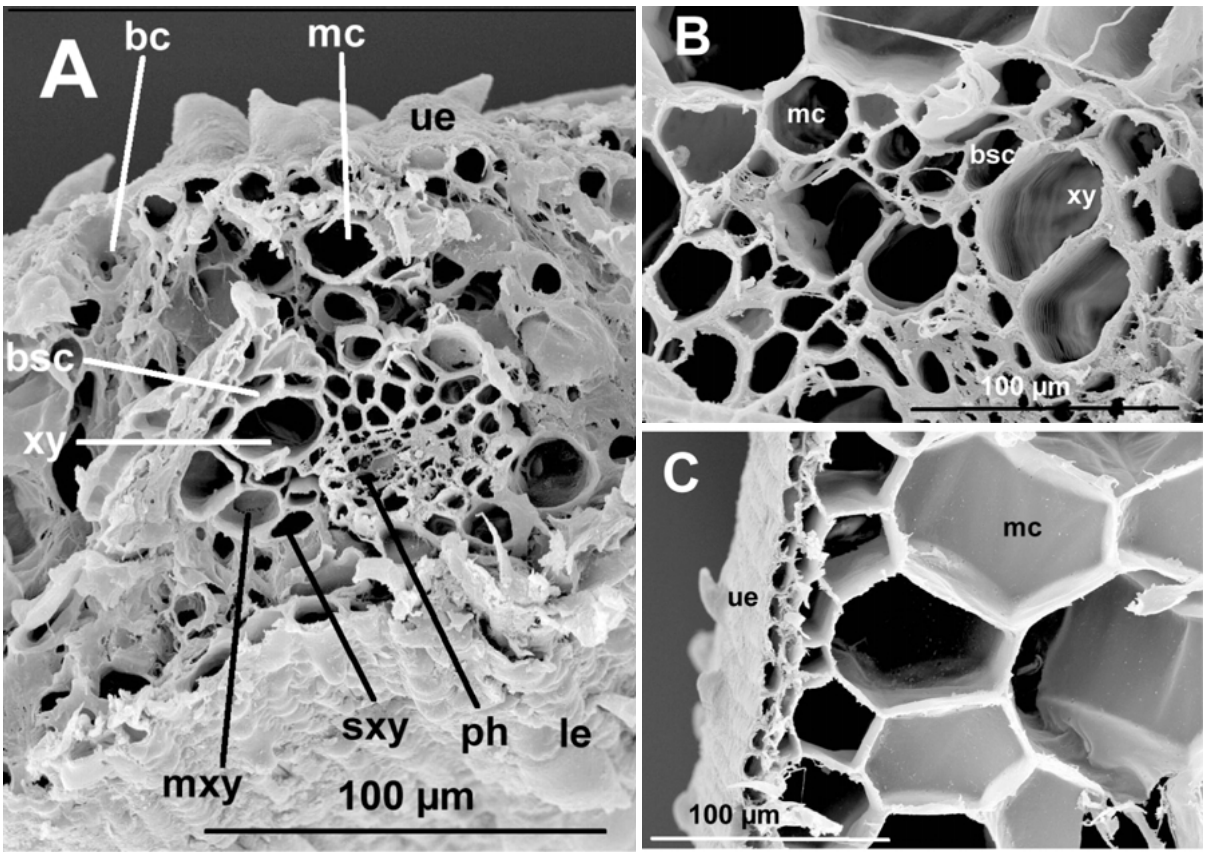

Figure 1. Ultrastructure of leaves from healthy sugarcane plants. (A) Scanning electron micrograph of a cross section of a sugarcane leaf from a healthy plant of Louisiana 55-5 cultivar. Above upper epidermis (up) a layer of bulliform cells (bc) and photosynthetic mesophyll cells (mc) can be seen. The bundle vessels consist of three kinds of xylem elements, large (xy), medium (mxy) and small vessels (sxy) as well as phloem elements (ph), all of them surrounded by bundle sheath cells (bsc). Lower epidermis (le) shows a very regular relief. (B) Magnification of the large xylem vessels (xy) showing helical lignification and surrounded by bundle sheath cells (bsc). Mesophyll cells (mc) and phloem elements (ph) are also visible. (C) Scanning electron micrograph of a cross section of sugarcane leaf showing lower epidermis (le) and mesophyll cells (mc).

Primary xylem elements showed helical lignification (Figure 1B).

With leaf infection, the cells of the starch-bearing sheath and those of chlorophyll-bearing parenchyma immediately lost their green color. This was limited to a few bundles and explains the sharply defined margin of the lesions (Martin and Robinson, 1961). From these scalded leaves, two different zones were chosen to analyze ultrastructural paths, those from the green or from the bleached, yellowish zones (Figure 2).

SEM observations of the green zones revealed that the upper epidermis, as well as the immediately subjacent mesophyll, were not significantly altered, but bulliform cells showed a dramatic enlargement (Figure 3A). The transversal diameter of bulliform cells from scalded plants was about four times longer than that of the same cells from healthy plants. The pathogen was confined mainly to the leaf and stalk vascular bundles, particularly the phloem bundles and neighboring mesophyllic cells (Figure 3B), which were often partially or completely occluded, with a gum-like substance. The gum may be a built-up mass of individual cells. In some instances, cell walls of the occluded xylem had disintegrated. This disintegration occurred in the leaf bundles, but was more commonly found in the tissues adjacent to the air spaces or lacunae of the stalks, thus forming the lysigenic cavities (Figure 3A,C) full of gum and cellular debris.

The gum exudate produced by *X. albilineans* completely filled the leaf tissues corresponding to the

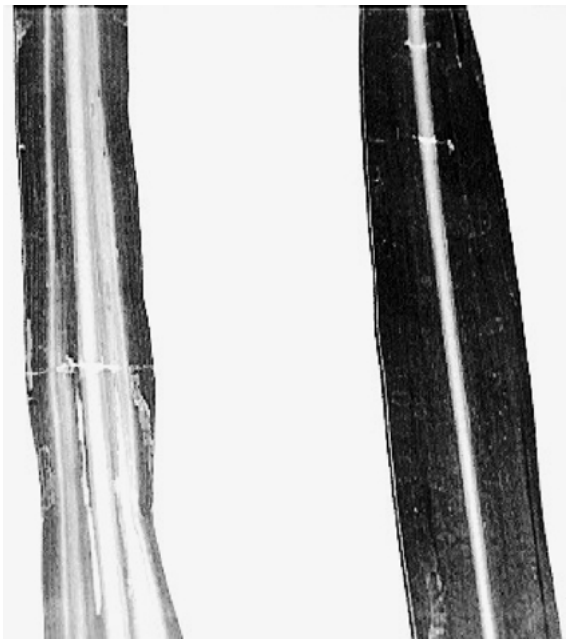

Figure 2. Visual symptoms of a scalded leaf of sugarcane (left) compared with a leaf from a healthy plant (right).

white-yellowish strip of scalded leaves (Figure 4A). Large gum deposits surrounded mesophyll cells and exerted mechanical pressure on some bulliform cells leading to wall deformations. Sometimes, there were small spaces between the bulliform cells and gum deposits and, in this case, wall deformation was not observed. Both xylem vessels and phloem, but mainly large xylem elements (Figure 4B,C) were completely occluded by the plugging material, forming visible lysigenic cavities (Figure 4A,C). Filamentous prolongations of deposited gum produced from infected tissues, occupied the intercellular space (Figure 4D) and completely altered the leaf structure. Differences between healthy and scalded tissues are summarized in Table 1.

The chemical nature of the exudated gum

Analysis of the fractions collected after filtration through Sephadex columns of the *iso*-propanol-precipitated fraction from aqueous extracts of diseased leaves revealed peaks identified as mannose (11.79 min), glucose (13.8 min), glucose-1-P (19.47 min) and glucuronic acid (19.90 min). Sometimes, a small peak corresponding to cellobiose (10.33 min) appeared. A peak at 22.45 min was not identified. The occurrence of both mannose and glucuronic acid (mannose/glucuronic acid ratio = 0.78) and glucose could be considered as indicative of the existence of a xanthan-like polysaccharide in extracts obtained from diseased sugarcane leaves, but the amount of glucose was too high in the hydrolysate to consider this a true xanthan. This large amount of glucose could have derived from starch obtained from bundle sheath cells and was partially extracted with *iso*-propanol. To test this hypothesis, the gum obtained from sugarcane stalks, which mainly accumulated sucrose instead of starch, was analyzed. The hydrolysate obtained from *iso*-propanol-precipitated juice always contained large amounts of cellobiose, glucose, mannose, glucose-1-P and glucuronic acid. In this case, the mannose to glucuronic acid ratio was 0.82. Moreover, cellobiose (β-D-glucosyl-[1 $\rightarrow$ 4]-D-glucose), which appeared in the electrophoregram, was considered a potential source of glucose. As described in the literature, incomplete acidic hydrolysis of xanthan produced a large amount of cellobiose (Christensen and Smidsrod, 1996). The ratio of both free glucose and that occurring as cellobiose to mannose or glucuronic acid (Table 2) was calculated as 2.5 and 2.05, respectively, for fractions obtained from stalks extracts. As a conclusion, the gum produced and exudated by *X. albilineans* could be defined as a xanthan-like polysaccharide (glucose/mannose and glucose/glucuronic acid ratios for a true xanthan are 1.0 and 2.0, respectively) on the basis of its monosaccharide composition.

Discussion

The epidermis of sugarcane leaves did not suffer significant modifications after infection with *X. albilineans*, perhaps because the walls of these cells are thick and lignified. After infection, the volume of bulliform cells increased, reaching near the maximum size described for this class of cells, about 100 μm (Esau, 1977). The observed increase of the size of bulliform cells seems to be related to the progressive loss of water caused by the occlusion of conducting vessels. During dry conditions, they lose their water rapidly through their thin walls and collapse which results in an upward and inward rolling of the leaf, thus protecting the leaf from excesive evaporation. The leaves of a wilted cane plant assume an erect position since it is necessary for the curving blades to straighten before it begins to roll. The bulliform cells acquire their maximum size after the leaves unroll from the leaf spindles (Bowes, 1996).

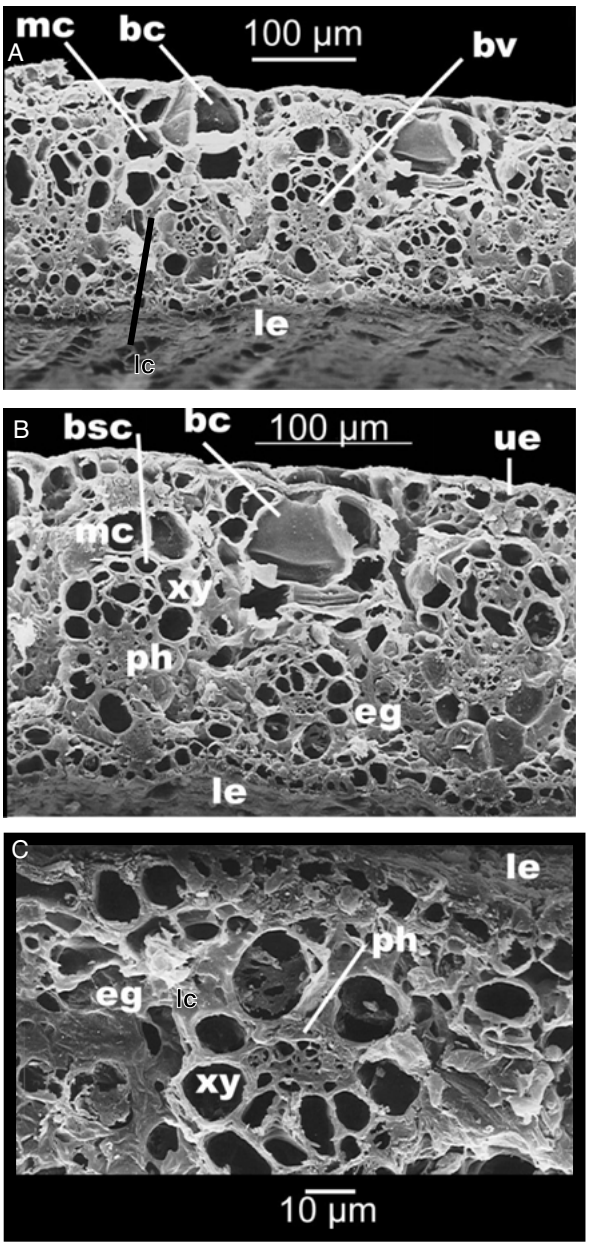

Figure 3. Ultrastructure of green streaks of scalded sugarcane leaves. (A) Scanning electron micrograph of a cross section of the green zone of a sugarcane leaf from a scalded plant of Louisiana 55-5 cultivar. Some bulliform cells (bc) appear greatly developed and unmodified photosynthetic mesophyll cells (mc) can be seen. The bundle vessels (bv) appear as occluded by gum, mainly phloem elements, sometimes forming lysigenic cavities (lc). Lower epidermis (le) shows a very regular relief. (B) Magnification of the cross section of a scalded leaf showing deposits of exuded gum (eg) occluding phloem elements and some mesophyll zones, whereas mesophyll cells (mc) occupying the layer immediately below upper epidermis (up) as well as bundle sheath cells (bsc) and large xylem elements does not apparently contain the plugging material. (C) Magnification of the large xylem vessels (xy) showing lateral lysigenic cavities (lc), surrounded by bundle sheath cells (bsc). Phloem (ph) is completely occluded by exudate gum (eg).

Vessel occlusion observed in diseased sugarcane plants is a symptom similar to that described for the black rot of Cruciferae, produced by *X. campestris* pv. *campestris*, in which bacteria enter and move through the xylem vessels of the host plants and interfere with the translocation of water and nutrients by producing plugging material which completely or partially occlude vessels (Wallis et al., 1973). Entry *of X. campestris* pv. *campestris* into plant leaves is achieved via hydathodes, as it has been found by Hugouvieux et al. (1998) for *Arabidopsis thaliana*, whereas *X. albilineans* only enters the sugarcane plants through mechanical injuries or the cutting surfaces (Rott et al., 1997). Later, *X. albilineans* may invade the parenchyma between the bundles, or the bundles just below the growing point, and cause reddish pockets of gum.

This gum has been defined here as a xanthan-like polysaccharide. Xanthan is an industrially important exopolysaccharide produced by the phytopathogenic, Gram-negative bacterium *X. campestris* pv. *campestris* (Sánchez et al., 1997). It is composed of polymerized pentasaccharide repeating units which are assembled by the sequential addition of glucose-1-phosphate, glucose, mannose, glucuronic acid and mannose on a polyprenol phosphate carrier. This sequential transfer provides the values of 1.0 for the glucose/mannose ratio and 2.0 for the glucose/glucuronic acid ratio. Katzen et al. (1998) provide evidence that the C-terminal domain of the gumD gene product is sufficient for its glucosyl-1-phosphate transferase activity. Finally, they found that alterations in the later stages of xanthan biosynthesis reduce the aggressiveness of *X. campestris* pv. *campestris* against the plant.

Xanthan seems to be the main pathogenic factor for *X. campestris* pv. *campestris*. In fact, three extracellular polysaccharide-deficient mutants of this bacterium, gumB(−), gumD(−) and gumE(−) were constructed by Tn5 gusA5 mutagenesis (Li et al., 2001). The results of pathogenicity bioassays showed that the three mutants had reduced pathogenicity on radish leaves. In addition, the gene *udgH* in *X. campestris* pv. *campestris* coding for UDP-glucose dehydrogenase,

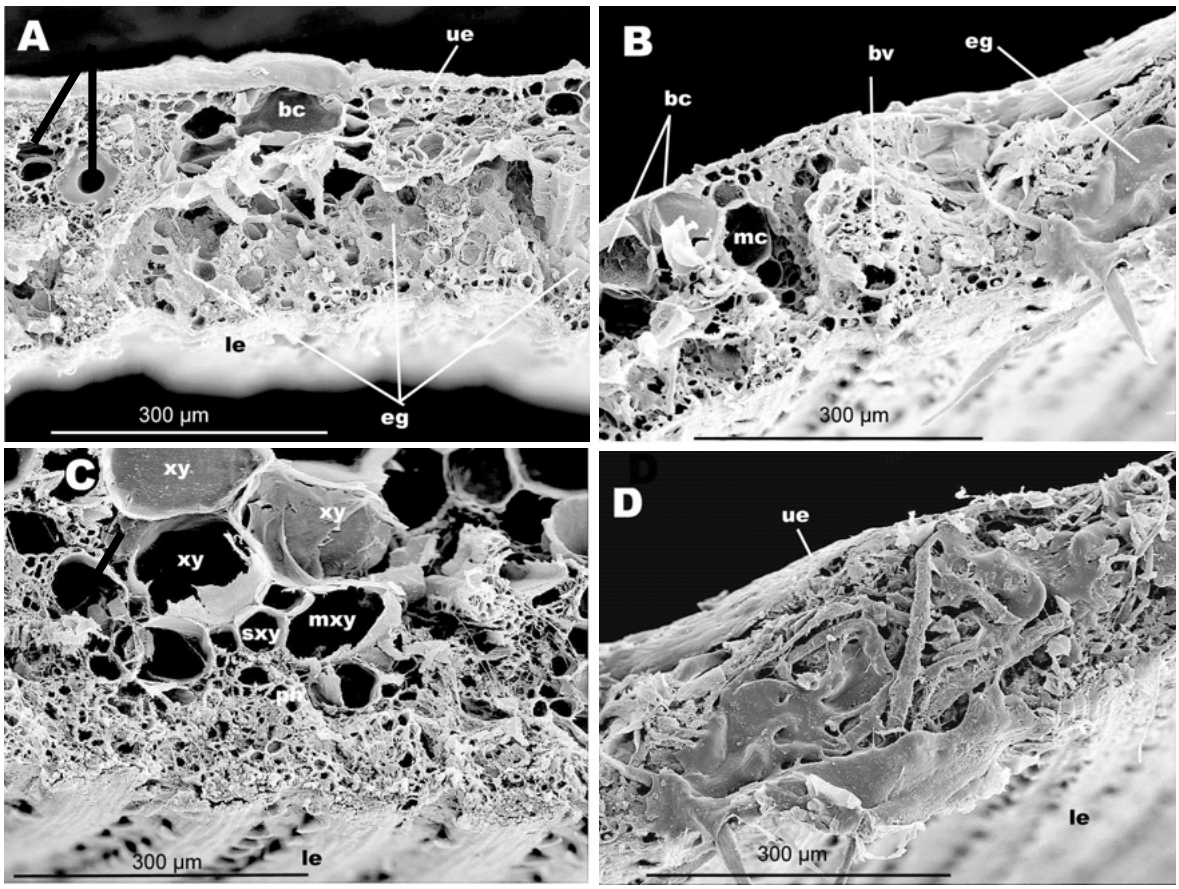

Figure 4. Ultrastructure of white-yellowish streaks of scalded sugarcane leaves. (A) Scanning electron micrograph of a cross section of the white-yellowish zone of a sugarcane leaf from a scalded plant of Louisiana 55-5 cultivar. Bulliform cells (bc) appear mechanically flattened and enlarged through their transverse axis and some photosynthetic mesophyll cells (mc) surrounded by gum (eg) can be seen. Bundle vessels (bv) and phloem appear as occluded by this gum. The bacterial plugging material can also affect both upper (ue) and lower (le) epidermis. (B) Cross section of a scalded leaf showing deposists of exudated gum (eg) which protrudes from bundle vessels (bv) although some xylem elements are not occluded by the gum. Superficial mesophyll cells (mc) are regular in shape whereas some bulliform cells (bc) seem to be enlarged through their transversal diameter. (C) Magnification of the large xylem vessels (xy) filled by plugging material and small (sxy) and mid xylem vessels (mxy) showing lateral lysigenic cavities (lc). Surface of the lower epidermis (le) has not been affected. (D) A general view of a cross section of a scalded leaf of sugarcane completely occupied by large masses of exuded gum which mask all leaf structure, including zones of the upper (ue) and lower (le) epidermis.

Table 1. A summary of ultrastructural differences observed between healthy and scalded leaves of sugarcane

Tissue (or cells)	Healthy leaves	Green streak of scalded leaves	White-yellowish streak of scalded leaves
Bulliform cells	Normal in size and shape	Enlarged	Enlarged
Mesophyll cells	Normal in size and shape	Invaded by exuded gum	Completely surrounded by exuded gum
Xylem vessels	Empty	Sometimes occluded by gum	Occluded by exuded gum
Cell walls of xylem vessels	Intact	Sometimes disintegrated	Largely disintegrated
Lysigenic cavities	Absent	Present	Present
Phloem elements	Intact	Occluded by exuded gum	Occluded by exuded gum

Table 2. Monosaccharide composition of gums isolated from leaves and stalks of scalded sugarcane plants

Homogenates obtained from	Concentration of monosaccharide released by acidic hydrolysis		
	Glucose	Mannose	Glucuronic acid
Stalks*	4.5 ± 0.37***	1.8 ± 0.2	2.2 ± 0.2
Leaves**	132.7 ± 14.3	2.8 ± 0.3	3.6 ± 0.3

*nmol per 36 μl of injected volume.
**pmol per 36 μl of injected volume.
***Including that obtained after enzymatic hydrolysis of cellobiose.

an enzyme catalyzing the conversion of UDP-glucose to UDP-glucuronic acid, was shown to be required for the biosynthesis of xanthan gum. Mutation of the *udgH* gene in *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*, the casual agent of leaf spot in pepper and tomato, was found to cause a loss of virulence (Chang et al., 2001). However, the gum isolated from diseased sugarcane leaves infected with *X. albilineans* differs from the xanthan previously described for *X. campestris* pv. *campestris*, since the ratio of glucose to mannose is about 2.0 instead of 1.0. But recently, it has been reported that the Gram-negative bacterium *Xylella fastidiosa* produces an exocellular polysaccharide similar, but not identical, to xanthan from *X. campestris* pv. *campestris*. The presence of all genes involved in the synthesis of sugar precursors, the existence of exopolysaccharide production regulators in the genome and the absence of three of the *X. campestris* pv. *campestris* gum genes suggests that *X. fastidiosa* is able to synthesize an exocellular polysaccharide probably consisting of polymerized tetrasaccharide repeating units assembled by the sequential addition of glucose-1-phosphate, glucose, mannose and glucuronic acid on a polyprenol phosphate carrier (da Silva et al., 2001). This indicates that the gum produced by *X. fastidiosa* is a xanthan-like polysaccharide identical to that produced by *X. albilineans*.

Acknowledgements

This work was supported by a grant from DGICYT (Ministerio de Educación y Ciencia, España), BFI 2000-0610 and a grant from SGCI (Ministerio de Educación y Ciencia, España) PR77-009027. We wish to thank Miss Raquel Alonso for her technical assistance and the Vicerrectorado de Investigación and Dirección del Real Jardín Botánico Alfonso XIII from the Complutense University for their financial support and help.

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