An Elicitor Isolated From *Sporisoriun scitamineum* Multiplies Xylem Bridges between Vascular Neighboring Bundles in Sugarcane Leaves

Vicente C[*], Alarcón B, Santiago R, Armas RD, Sánchez-Elordi E and Legaz ME

Team of Cell Interactions in Plant Symbioses, Faculty of Biology, Complutense University, 28040 Madrid, Spain

[*]**Corresponding author:** Vicente C, Team of Cell Interactions in Plant Symbioses, Faculty of Biology, Complutense University, 28040 Madrid, Spain, Tel: + 34 914 52 04 00; E-mail: cvicente@bio.ucm.e

Abstract

A chemical simulation of the infection of sugar cane leaves by smut (*Sporisorium scitamineum*), using an elicitor isolated from the growing fungal mycelium, provokes an increase of the pattern of lignification of both sclereids and xylem elements. The entry of the elicitor into the tissues of the plant likewise induces the production and secretion of a mucilaginous material, probably glycoproteins, that has been considered as a primary response of defense. In addition, the elicitor provokes an increase in the thickness of the lignified cell walls of sclereids surrounding the phloem bundle as well as the mid and small xylem vessels. After elicitor treatment, the appearance of transverse bundles that were born from the mid xylem elements is for the first time described. Although these transverse bundles also occurred in non-infected plants, the length reached by the new differentiated xylem tissue in transverse development, as well as their number and complexity, seem to be higher after elicitor treatment than that observed in healthy plants. The possible blockade of the xylem elements by the growing mycelium or by an excess of secreted polymers can be prevented by the increase in the number of transverse xylem by-pass between vascular neighboring bundles.

Keywords: Saccharum officinarum; Sporisorium scitamineum; Defense; Fungal elicitor; Lignification; Transverse bundles; Sclereids; Xylem

Introduction

Smut is a major disease of sugarcane caused by *Sporisorium scitamineum* (Syd.). Piepenbr and Oberw (*Ustilago scitaminea* Sydow and Sydow). The germination of spores is reached on the wet surface of the internodes, followed by the formation of appressoria, mainly in the inner scale of young outbreaks and on the bases of arising leaves [1]. The entry in the outbreak meristem happens between 6 and 36 h after the teliospores is deposited on the surface [2]. Growth of the pathogenic agent, as soon as it has penetrated in the tissues of the host, is carried out on all the parts of the infected plant, but especially in the

parenchymatous cells of the internodes, under the site of infection. In the top internodes, hyphal growth concludes with the formation of the whips (sori with teliospores). Hyphae don't penetrate into the cells of the scale leaves [3-19] and, consequently, the outbreaks loudly included inside scale leaves can avoid the infection. It has been reported that fungal hyphae develops on the abaxial epidermis of smut-infected leaves in the sensitive Barbados cv, for which the penetration was carried through the opened stomata. Nevertheless, in other cases, the fungal hyphae are able of breaking actively the layer of epidermal cells, combining the enzymatic attack with the mechanical pressure derived from the hyphal growth to progress through the intercellular spaces in the underlying mesophyll tissues [20-26]. Many parenchymatous cells are invaded by smut and their cell walls were broken by actively progressing hyphae. Mucilage production accompanied the complete destruction of parenchymatous cells that harbored many fungal spores [17].

A crude elicitor, prepared from *S. scitamineum* mycelium, reproduces some characteristic symptoms of the disease. For example, it induces high values of phenylalanine ammonia-lyase activity without accumulation of free hydroxycinnamic acids and moderately high activity values of some peroxidases, mainly in resistant cultivars [10], both enzymes related to the lignin biosynthesis. The elicitor was extracted from the fungal mycelium and autoclaved at 120°C, according to that described by Mc Ghie et al. [20] for a similar elicitor produced by *Pachymetra chaunorhiza*. The crude elicitor from smut has been separated in several active fractions composed by proteins and glycoproteins, although the most active fraction in the induction of phenylalanine ammonia lyase and peroxidase activities seems to be only composed by protein. Smut elicitor also induces the appearance of some structural changes in treated plants. Incubation of leaf discs with the elicitor produced by the pathogen *S. scitamineum* increases the thickness of the lignified cell walls of subepidermal sclereids, or docking cells, as well as those fibers surrounding the phloem, and the mid and small xylem vessels. These facts have been interpreted as a possible response of mechanical defense of the plant to impede or put back the potential entry of the pathogen [1,26].

In the present paper, we have tried to deepen in this structural defense response, giving a particular attention to the modifications of the xylem that the treatment with the fungal elicitor produce in sugarcane resistant Mayarí 55-14 CV.

Material and Methods

Plant material

Six months-old field grown plants of *Saccharum officinarum* L., cv. Mayari 55-14 were used throughout this study. Plants were developed from agamic seeds and cultured on soil in the Real Jardín Botánico Alfonso XIII (Complutense University, Madrid). Seeds were planted in April on clay soil mixed with 25% sand (w/w), and fertilized with nitrogen (150 kg ha^{-1}), phosphorus (75 kg ha^{-1}) and potassium (120 kg ha^{-1}) at planting. Plants were grown from May to October in isolated greenhouses, under a light intensity of 250 μmol m^{-2} s^{-1} of white light, a photoperiod of 14 h, a 90% relative humidity, and were watered daily [17].

Elicitor preparation

Teliospores of *S. scitamineum* (20 mg in dry weight) were isolated from whips collected from diseased Barbados 42231 plants in experimental crops of the National Institute for Sugarcane Investigation (INCA) in Matanzas, Cuba. The collected teliospores were incubated in 200 mL of sterile Lilly and Barnett medium [18] at 38°C for 5 days. The mycelium formed was harvested, washed with distilled water, lightly dried with filter paper, weighed and ground to a fine powder in liquid nitrogen

(3.6 g wet weight). The powder was extracted with 25 mL of 10 mM Tris-HCl, pH 8.8. Following centrifugation (5000 × g for 10 min at 4°C), 20 mL of 80% (v/v) methanol was added to the pellet and the mixture was shaken for 4 h at 38°C. After centrifugation, the pellet was washed once with 5 mL methanol and dried under air flow for 2 h. The dried pellet was washed with 10 mM phosphate buffer, pH 6.8, then resuspended in 25 mL of the extraction buffer, autoclaved (120°C for 30 min.) and recentrifuged. The clear supernatant was used to elicit the sugarcane leaves.

Treatment of sugarcane leaves with the smut elicitor

Twenty discs of sugarcane leaves (0.5 g total fresh weight approximately), obtained from the central zone of leaves 3 and 4 from the bottom of six different plants, at a middle position on the stalks, were floated in Petri plates on 15 mL 10 mM phosphate buffer, pH 6.8, containing 4% (v/v) isopropylic alcohol for 2 h at 37°C in the dark. Thereafter, 0.5 mL of the elicitor solution was added to the plates and incubated in the dark for a further 6 h [10]. Control experiments were performed in the absence of elicitor. Three replicates using leaf samples from different stalks were made.

Light microscopy

Leaf discs were cut in 10 μm-thick sections using a freezer microtome. Sections were stained with 0.5% (v/v) toluidin blue in 70% (v/v) ethanol [8] or alternatively in 1.5% (w/v) saphranin 50% (v/v) ethanol and 0.5% (w/v) alcian blue in 90% (v/v) ethanol for 2 min [15] in order to observe the integrity of the leaf structure. Lignified structures (mainly sclerenchyma and xylem vessels) were visualized using the phloroglucinol/HCl (PGH) test. Sections were incubated overnight in a solution of 1% (w/v) phloroglucinol in absolute methanol. Following further incubation of cleared tissues in chloral hydrate, the sections were mounted in a few drops of concentrated hydrochloric acid and covered with a coverslip [27-33]. The stained sections were observed immediately using a Zeiss 60 invertoscope fitted with a CCD camera for capturing images using a Viewfinder Lite program. After 10 min, lignified structures appeared cherry red-orange, but color faded within 2–4 h [34].

Scanning electron microscopy

The ultrastructure of sugarcane leaves was examined by conventional scanning electron microscopy (SEM). 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 by SEM [32] using a JEOL JSM 6400 (Japan). Digital images were obtained by using an INCA (Oxford) program incorporated to the equipment [17].

Transmission electron microscopy

Ultrastructural TEM analysis was performed by softening recently prepared leaf discs in a phenol:acetic acid (1:1 v/v) mixture for 3 days and then the material was dehydrated in an alcohol series and fixed with 2.5% (v/v) glutaraldehyde in Milloning (EMS, Hatfield, PA, USA) buffer, pH 7.3 [22] for 6 h, washed in the same buffer and post-fixed with a mixture of 1% osmium tetroxide and 3 % potassium ferricyanide (1:1) for 2 h. Tissues were dehydrated in an acetone series and embedded in Epon-812 resin (EMS, Hatfield, PA, USA) for 3 days at 70°C [27]. Ultrathin sections (600 Å) obtained with an OmU-2 Reichert-Jung ultratome (Wien, Austria) were examined on a Jeol 1010 electron microscope (Tokyo, Japan).

Results

SEM visualization of cross section of a sugarcane health leaf shows the classical arrangement of cells, which defines a C4 species (FIG. 1A). Bulliform cells occur 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, are confined to the adaxial epidermis (FIG. 1A). The external paraclinal wall of the bulliform cells was thinner than in the common epidermis cells, according to that described by Ferreira et al. [11]. Under dry conditions, bulliform cells lose their water rapidly through their thin walls and they were collapsed. This result in an upward and inward rolling of the leaf, thus protecting the leaf from excessive evapotranspiration. Bulliforms cells acquire their maximum size after the leaves unroll from the leaf spindles [4].
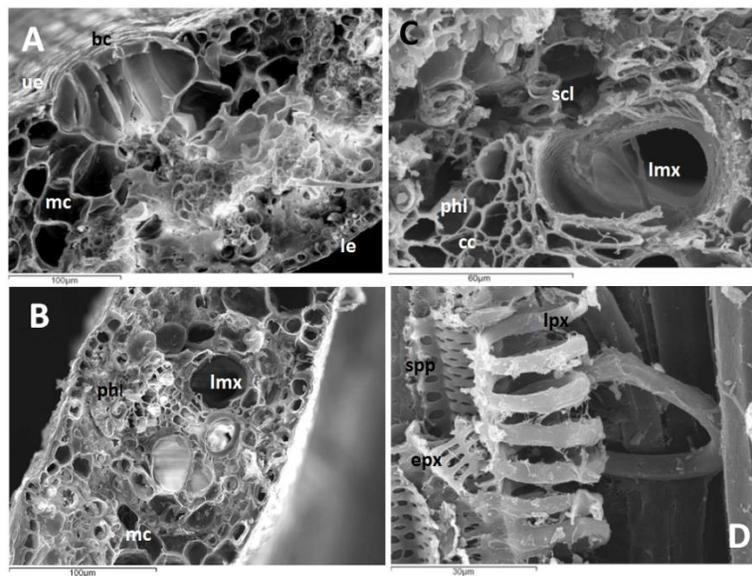

FIG. 1. SEM micrographs of the cross section of elicitor-untreated leaves from Mayarí 55-14 sugarcane cultivar. A) Ultrastructure of healthy leaf showing upper epidermis (ue), lower epidermis (le), bulliform (bc) and mesophyll cells (mc). B) Ultrastructure of healthy leaf showing mesophyll cells (mc), late metaxylem elements (lmx) and phloem (phl). C) Ultrastructure of healthy leaf showing late metaxylem elements (lmx), phloem (phl), companion cells (cc) and sclereids (fibers) surrounding phloem (scl). D) Ultrastructure of healthy leaf showing earlier protoxylem (epx), late protoxylem (lpx) and scalariform perforation plate in late metaxylem element (spp).

The conducting tissues are found within the circular to oval-shaped groups of mesophyll cells (FIG. 1B). The bundles are composed of three-sized classes of cells: large, or late metaxylem vessels, medium and small, the first two being rhomboid to oval in shape, while as a rule, the small type is rather circular. The xylem is made up of open tubes or vessels associated with smaller and thicker walled elements. The large bundles of the leaf are usually two large vessels connected with smaller vessels. The xylem of small bundles consists of only a few large pitted vessels [14]. The large vessels were irregular in shape, having comparatively thick walls (FIG. 1C). Each vessel was formed not from a single cell but from a series of elongated cells, whose contents and end walls have disappeared. Narrow metaxylem vessel elements have either scalariform (FIG.1C) or simple perforation plates. Protoxylem early-formed elements had annular thickenings whereas late formed elements had helical wall thickenings with progressively tighter coils of wall thickening (FIG. 1D).

Visualization by TEM of the vascular bundle allows observing the bundle sheath cells adhered to the more peripheral phloem and xylem elements as well as the blocks of fibers (sclereids) that reinforce the space between both types of vascular elements. The phloem elements shows an undifferentiated content as well as some sieve plates connecting two consecutive elements. The xylem elements turn out to be empty, the most peripheral showing cracks in their cell wall, which they do not manage to perforate completely, located near the fibers (FIG. 2).
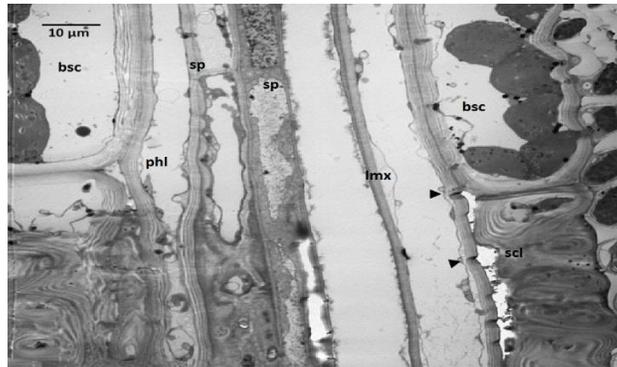

FIG. 2. TEM micrograph of a longitudinal section of elicitor-untreated leaves from Mayarí 55-14 sugarcane cultivar showing bundle sheath cells (bcs), phloem elements (phl), sieve plates (sp), late metaxylem elements (lmx) and sclereids (docking cells) connecting the vascular bundle to the inner face of epidermis (scl). The tops of arrow indicate cracks in the external face of the lignified cell wall.

The experimental treatment of discs of young sugar cane leaves with an elicitor isolated from of *S. scitamineum* effectively provokes, as it has been described before [25], an increase in the thickness of the lignified cell walls of sclereids surrounding the phloem bundle (FIG. 3A and 3C) as well as the mid and small xylem vessels (FIG. 3B).
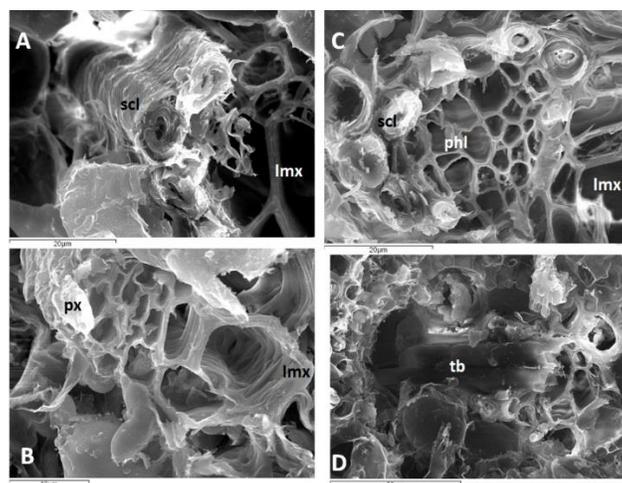

FIG. 3. SEM micrographs of the cross section of elicitor-treated leaves from Mayarí 55-14 sugarcane cultivar. A) Ultrastructure of treated leaf showing sclereids (docking cells connecting the vascular bundle to the inner face of epidermis (scl), and late metaxylem elements (lmx). B) Ultrastructure of treated leaf showing large, late metaxylem elements (lmx), mid metaxylem elements (mmx) and protoxylem (px). C) Ultrastructure of treated leaf showing

sclereids (fibers) surrounding the phloem (scl), phloem (phl) and late metaxylem elements (lmx). D) Ultrastructure of treated leaf showing transverse xylem bundle (tb).

Nevertheless, a new fact has been herein described: The appearance of transverse bundles that were born from the mid xylem elements, a fact that had not been observed before for smut infected plants (FIG. 3D). Observation by light microscope of cross sections of sugarcane leaves from healthy plants and those treated with the smut elicitor revealed that these transverse bundles also occurred in non-infected plants (FIG. 4A and 4B).
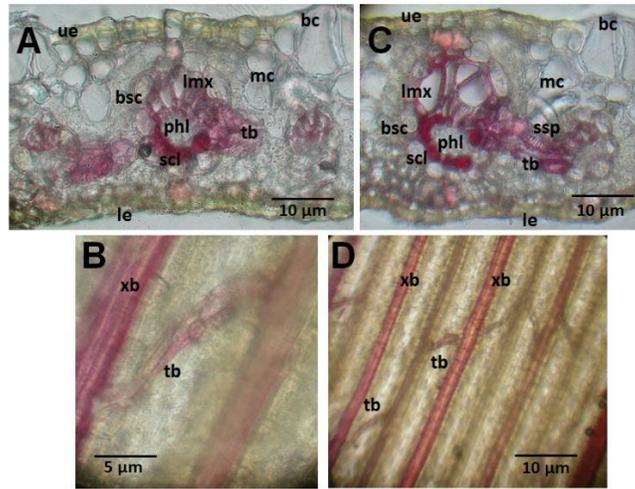

FIG. 4. Light micrographs of cross or longitudinal sections of leaves of sugarcane, cv. My 55-14 without any treatment (A and B) and treated with the smut elicitor (C and D), stained with phloroglucinol/HCl. A) Structure of the cross section of sugarcane leaf showing upper epidermis (ue), lower epidermis (le), bulliform (bc), mesophyll (mc), and bundle sheath cells (bsc), late metaxylem elements (lmx), phloem (phl), sclereids (fibers) surrounding the phloem (scl), and transverse bundle (tb). B) Structure of the cross section of sugarcane leaf showing the xylem bundles (xb) and a single transverse bundle (tb). C) Structure of the cross section of sugarcane leaf showing upper epidermis (ue), lower epidermis (le), bulliform (bc), mesophyll (mc), and bundle sheath cells (bsc), late metaxylem elements (lmx), phloem (phl), sclereids (fibers) surrounding the phloem (scl), scalariform perforation plate in late metaxylem element (spp), and transverse bundle (tb). D) Structure of the cross section of sugarcane leaf showing the xylem bundles (xb) and a single transverse bundle (tb).

They appeared even in the late metaxylem elements with evident scalariform perforation plates. The same transverse bundles were observed in cross sections of sugar cane leaves treated with the elicitor though, in this case, the length reached by the xylem elements in transverse development, as well as their number and complexity; seem to be higher than that observed in healthy plants (FIG. 4C and 4D). These connections represented a real, differentiated branched of the middle zone of xylem, trying to connect two different vascular bundles, separated in the space, by means of new differentiated xylem tissue growing according to a transverse pattern. Parallel veins run the length of the pine needles are also connected at intervals by very small transverse veins [7], similar to those observed in (FIG. 4B and 4D). Large veins supply water rapidly over the whole lamina, from the basis to the tip, while small veins distribute it locally and slowly to everything broad of the leaf.
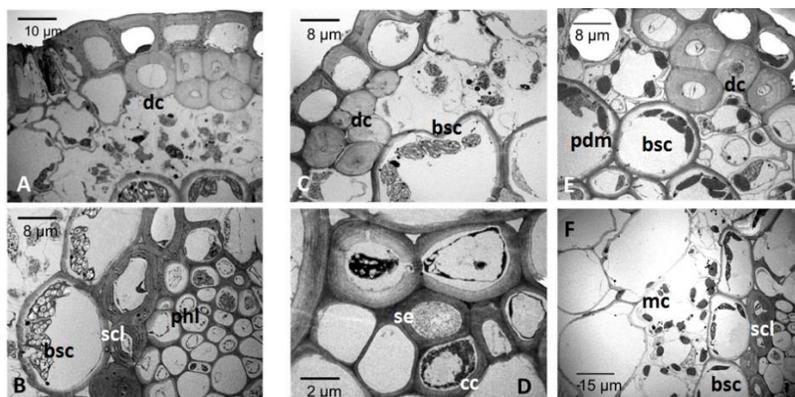
FIG. 5. TEM micrograph of a cross section of elicitor-treated leaves from Mayarí 55-14 sugarcane cultivar showing A) docking cells (dc); B) docking cells (dc), and bundle sheath cells (bcs); C) docking cells (dc), bundle sheath cells (bcs), and plasmodesmata (pdm) connecting two neighboring bundle sheath cells; D) bundle sheath cells (bcs), sclereids (fibers) surrounding the phloem (scl), and phloem elements (phl); E) sieve elements (se), and companion cells (cc); F) mespophyll cells (mc), bundle sheath cells (bcs), and sclereids (fibers) surrounding the phloem (scl).

The vascular bundles were surrounded by two bundle sheaths (FIG. 5B and 5F) showing agranal chloroplasts [3]. Plasmodesmata traversed the walls of chlorenchymatous bundle-sheath cells and mestome-sheath cells (FIG. 5E) according to that described by Robinson-Beer and Evert [23]. Many parenchymatous cells were invaded by smut in Barbados cv., sensitive to smut. Some of these cells were found with their cell wall broken by actively progressing hyphae. The production of mucilage was evident in the mesophyll, in which large and complex mycelium packages completely destroyed the parenchymatous cells. Healthy leaves did not contain the invader agent or its elicitor and ultrastructural damage was not observed. However, fungal hyphae and spores actively invaded the interior of the parenchymatous cells in infected plants. Many of them were completely destroyed. However, leaves and buds from Mayari cv., defined as resistant to smut, suffered the invasion by the fungal mycelium following a pattern of distribution similar to that described for cv. Barbados [31], but no intracellular penetration were observed.

As in other plants of the Gramineae family [29] by Singh, the sieve elements of sugarcane are easily recognized by their characteristic protoplast which contain light electron-dense proteinaceous inclusions (FIG. 5D), whereas companion cell protoplasts contained more dense, differentiated organelles. Only docking cells (FIG. 5A-5C) and fibers surrounding the phloem (FIG. 5D-5F) dramatically increased the thickness of their lignified cell walls, as previously described, after enhancing both cynnamoyl alcohol and syringyl alcohol dehydrogenase activities [26]. Docking cells are subepidermal sclereids that connect with the conductive bundles [9].

Nevertheless, the conductive cells of the vascular bundles as well as the surrounding bundle sheath cells, when leaf discs were incubated on the smut elicitor, turn out to be partially stuffed of a fibrous material (FIG. 6C and 6D), possibly of mucilaginous nature, similar to that described for infected sensitive cultivars [17]. This material did not appear in control tissues without any previous treatment (FIG. 6A and 6B). However, leaves suffered a chemical-simulated infection after incubation with the smut elicitor in absence of an effective inoculation with teliospores or infective sporidia. Thus, this loose network found in (FIG. 6)might be produced by the plant tissue itself as a response to the uptake of the elicitor.
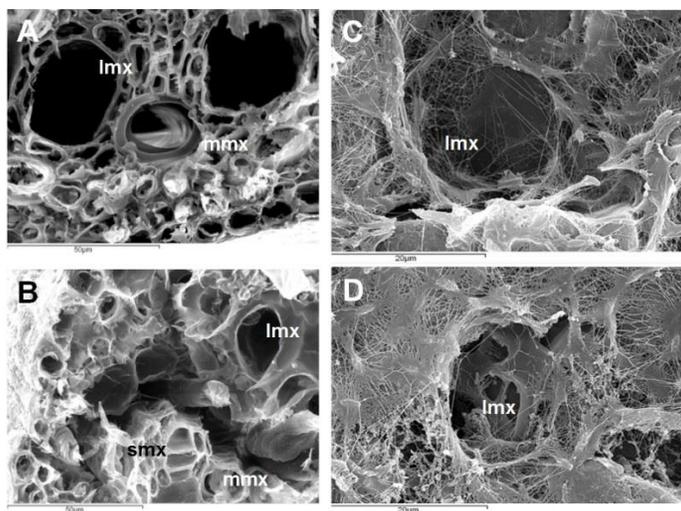
FIG. 6. SEM micrographs of cross sections of leaves of sugarcane, cv. My 55-14, without any treatment (A and B) and treated with the smut elicitor (C and D). The uptake of the smut elicitor by leaf discs (C and D) is followed by the production of a dense network of a mucilaginous material which invades the surrounding of the xylem vessels and also the late metaxylem elements (lmx) whereas this material is absent from late, mid (mmx) and small (smx) vessels from elicitor-untreated leaves (A and B).

The connection of bundle xylem vessels by means of transverse bridges [5] can be observed by SEM, using longitudinal sections of leaves. In (FIG. 7A) a sagittal section of a late metaxylem vessel surrounded by bundle sheath cells can be observed, in which three holes on line would correspond to the beginning or entry of the transverse bundles. A magnification of which can be observed in the (FIG. 7B). Similar transverse bundles can be observed in longitudinal sections of leaves treated with the fungal elicitor (FIG. 7C) whereas the take-off of such transverse bundles from some mid elements of the metaxylem can be seen in the magnification of (FIG. 7D) as well as some more boarders some phloem vessels, tangentially broken during the cut.

Discussion

Morphological and physiological alterations of sugar cane plants after smut infection have been extensively studied [26,31,35]. They mainly consisted in an increase of the lignification pattern of sclereids and docking cells [1,26] (FIG. 2 and 5) and the appearance of a dense mucilage (FIG. 6) that invaded leaf mesophyll and some xylem elements so that the plant can undergo a progressive drying. Probably, this material consisted in sugarcane glycoproteins, produced by the parenchimatous cells of the plant [13], containing a complex, branched polysaccharide moiety, described as a primary chemical response to the disease [21,24]. This observation could corroborate that supposed by Koeck et al. [16] relative to plant immunity. They hypothesize that, during plant infection, pathogens could synthesize and secrete effector proteins (elicitors), some of which were translocated into the plant cytosol where they could alter the host response to the invading pathogen. In an unsuccessful infection, such as those achieved on resistant cultivars, pathogen effectors enhanced the immune system of the plant and orchestrate the reprogramming of the infected tissue to impede growth and development of the pathogen.

In this paper the infection of plants of the resistant variety My 55-14 has simulated by treatment with an isolated and purified elicitor from a *S. scitamineum* culture [25]. The main symptom of this simulated infection was the increase in the number of lignified transverse bundles connecting parallel xylem elements (FIG. 3 and 4). These transverse bundles occurred in non-infected plants as well as in other graminaceous plants, such as maize [28], or some palms [12], but the elicitor multiplied its presence in the tissues of treated plants leaves. This is very different to the conventional connection between xylem vessel established by way of scalariform pit fields formed by ladder-like perforations in the secondary walls of adjacent vessels, which are separated by pit membranes, which are the remnants of the primary cell walls and middle lamellae [29-35].

Conclusion

The biological signification of these transverse extensions could easily be explained. Long, wide vessels decrease resistance and increase water transport efficiency [12]. On the other hand, the extension of the connections between bundle vessels might be a fail-safe system to avoid detentions of the water flow inside the xylem (FIG. 7), produced by the accumulation of pathogenic agents, their exocellular products or embolisms, as Sing and Budhraja [30] claim for the xylem of maize plants. It would, therefore, be a new attempt to defend the varieties resistant against smut, trying to avoid the desiccation of their leaves caused by the obstruction of the xylem vessels, as a consequence of the disease. This would contribute to the maintenance of the leaf water status, altered by the synthesis of soluble glycoproteins as a defense response of the plant [13,19].
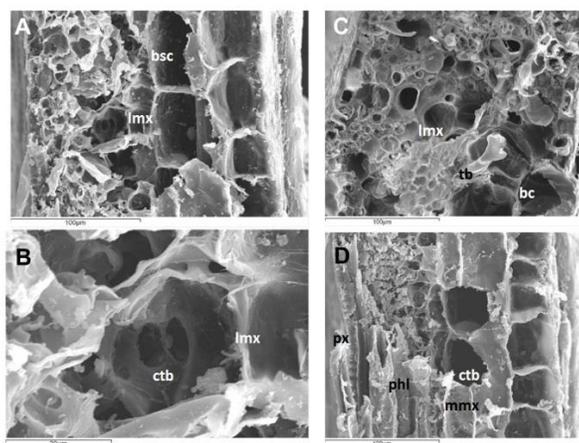

FIG. 7. TEM micrograph of a longitudinal sections of both elicitor-untreated (A and B) and elicitor-treated (C and D) leaves from Mayarí 55-14 sugarcane cultivar showing some transverse xylem bundles (tb) as well as the connecting points of these transverse elements (ctb) that begin from large (B) and mid (D) metaxylem vessels.

Acknowledgement

REFERENCES

1. Alarcón B, Santiago R, Vicente C, et al. Structural changes of lignified tissues from sugarcane leaves induced by a smut (*Sporisorium scitamineum*) elicitor. J Life Sci. 2012;6:287-300.

2.   Alexander KC, Ramakrishnan K. Infection of the bud, establishment in the host and production of whips in sugarcane smut (*Ustilago scitaminea*) of sugarcane. Proc Intern Soc Sugarcane Technol. 1980;17:1452-55.

3.   Birch RG, Patil SS. The relation of blocked chloroplast differentiation to sugarcane leaf scald disease. Phytopathology. 1983;73:1368-74.

4.   Bowes BG. A color atlas of plant structure. Manson Publishers, London. 1996.

5.   Brodersen CR, Choat B, Chatelet DS, et al. Xylem vessel relays contribute to radial connectivity in grapevine stems (Vitis vinifera and V. arizonica; Vitaceae). Am. J. Bot. 2013;100:314–321.

6.   Carlquist S. Comparative wood anatomy: Systematic, ecological and evolutionary aspects of dicotyledon wood. Springer-Verlag, Berlin .2001.

7.   Choat B, Munns R, Mc Cully M, et al. Water movement in plants. Leaf vein architecture and anatomy. In: Plants in Action, eds. by B.J. Atwell, P.K. Kriedemann, C.G.N. Turnbull. Macmillan Education Australia Pty Ltd, Melbourne, electronic edition.

8.   Clark G. Staining procedures, 4th ed. Williams and Wilkins, Baltimore, 1981.

9.   Colbert JT, Evert RF. Leaf vasculature in sugarcane (*Saccharum officinarum* L.). Planta.1982;156:136-51.

10. Armas RD, Santiago R, Legaz ME, et al. (2007) Levels of phenolics compounds and enzyme activity can be used to screen for resistance of sugarcane to smut (*Ustilago scitaminea*). Austral Plant Pathol. 2007; 36:32-38.

11. Ferreira EA, Ventrella MC, Santos JB, et al. Leaf blade quantitative anatomy of sugarcane cultivars and clones. Planta Daninha. 2007; 25:25-34.

12. Fisher JB, Tan HTW, Toh LPL. Xylem of rattans: Vessel dimensions in climbing palms. Am J Bot. 2002; 89:196-202.

13. Fontaniella B, Márquez A, Rodríguez CW, et al. A role for sugarcane glycoproteins in the resistance of sugarcane to Ustilago scitaminea. Plant Physiol. Biochem. 2002; 40:881-889.

14. Gunning BES, Steer MW. Plant cell biology: An ultra-structural approach. Edward Arnold, London. 1986.

15. Johansen DA. Plant Microtechnique. McGraw-Hill, New York. 1940.

16. Koeck M, Hardham AR, Dodds PN. The role of effectors of biotrophic and hemibiotrophic fungi in infection. Cell Microbiol. 2011;13:1849–57.

17. Legaz ME, Millanes AM, Fontaniella B, et al. Ultrastructural alterations of sugarcane leaves caused by common sugarcane pathogens. Belgian J. Bot. 2006;139:14-26.

18. Lilly VG, Barnett HL. Physiology of the fungi. McGraw-Hill, New York. 1951.

19. Martínez M, Legaz ME, Paneque M, et al. Glycosidase activities and polysaccharide accumulation in sugar cane stalks during post-collection impairment. Plant Sci. 1990; 72:193-8.

20. McGhie TH, Masel NP, Maclean D, et al. Biochemical responses of suspension-cultured sugarcane cells to a virulence factor derived from the root pathogen *Pachymetra chaunorhiza*. Austral. J. Plant Physiol. 1997;24:143-9.

21. Millanes AM, Fontaniella B, Legaz ME, et al. Glycoproteins from sugarcane plants regulate cell polarity of *Ustilago scitaminea* teliospores. J. Plant Physiol. 2005;162:253-65.

22. Milloning G. Advantages of a phosphate buffer for OsO4 solutions in fixation. J. Appl. Phys. 1961;32:1637–50.

23. Robinson-Beer K, Evert RF. Fine structure of plasmodesmata in mature leaves of sugarcane. Planta. 1991;184:307-18.

24. Sánchez-Elordi E, Manzanares VM, Díaz EM, et al. Plant–pathogen interactions: Sugarcane glycoproteins induce chemotaxis of smut teliospores by cyclic contraction and relaxation of the cytoskeleton. South African J. Bot. 2016;105:66–78.

25. Santiago R, Armas RD, Legaz ME, et al. Separation from smut (*Ustilago scitaminea*) of different elicitors which modify the pattern of phenolic accumulation in sugarcane leaves. J. Plant Pathol. 2008;90:87-96.

26. Santiago R, Quintana J, Rodríguez S, et al. An elicitor isolated from smut teliospores (*Sporisorium scitamineum*) enhances lignin deposition on the cell wall of both sclerenchyma and xylem in sugarcane leaves. Pak. J. Bot. 2010;42:2867-81.

27. Sanders WB. Observing microscopic phases of lichen life cycles on transparent substrata placed *in situ.* Lichenologist. 2005; 37:373–82.

28. Shane MW, McCully ME, Canny MJ. The vascular system of maize stems revisited: Implications for water transport and xylem safety. Ann. Bot. 2000; 86:245-58.

29. Singh AP. On the ultrastructure and differentiation of the phloem in sugarcane leaves. Cytologia. 1980;45:1-31.

30. Sing K, Budhraja TR. The role of bud scales as barriers against smut infection. Proc Bienn Conf Sugarcane Res. Devel. 1964;5:687-90.

31. Solas MT, Piñón D, Vicente C, et al. Ultrastructural aspects of sugarcane bud infection by *Ustilago scitaminea* teliospores. Sugar Cane. 1999:14-18.

32. Solas MT, Piñón D, Acevedo R, et al. Ultrastructural changes and production of a xanthan-like polysaccharide associated with scald of sugarcane leaves caused by *Xanthomonas albilineans*. Eur. J. Plant Pathol. 2003;109:351-59.

33. Soylu S. Accumulation of cell-wall bound phenolic compounds and phytoalexin in *Arabidopsis thaliana* leaves following inoculation with pathovars of Pseudomonas syringae. Plant Sci. 2006;170:942-52.

34. Vallet C, Chabbert B, Czaninski Y, et al. Histochemistry of lignin deposition during sclerenchyma differentiation in alfalfa stems. Ann. Bot. 1996;78:625-32.

35. Waller JM. Sugarcane smut (*Ustilago scitaminea*) in Kenya. II. Infection and resistance. Trans British Mycol Soc. 1970;54:405-14.