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On the nature and distribution of sclereids in sugarcane leaves

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ABSTRACT

Sugarcane leaf shows the classical arrangement of cells which defines a C4 species. The vascular bundle includes xylem, phloem and fibers. These fibers consist of outer sclereids surrounding the vascular bundle and an inner ring of stone cells involving the phloem. Some sclereids behave as docking cells since they are located below and above the vascular bundle, connecting it to the internal surface of the epidermal upper and lower layers. A compact mass of sclereids occupies the total internal volume of the leaf edge. Neither docking cells nor the more internal mass of sclereids in the edge were significantly coloured by phloroglucinol, indicating this the absence of lignin in their cell walls, whereas fibers of the vascular bundle and the most external layer of edge sclereids are strongly lignified.

KEYWORDS

Saccharum officinarum;
Leaf structure;
Sclereids.

INTRODUCTION

Sugarcane, *Saccharum officinarum* L., is a C4 plant, their leaves showing a typical Kranz anatomy.[1, 23] The blade of sugarcane leaf is a long, thin, and flat structure, which gradually tapers from the base to the tip and is supported by a midrib extending practically its full length. Leading off at acute angles from the midrib are numerous parallel veins, each of which contains a vascular bundle. On the upper and lower leaf surfaces are found a number of one- and two-celled trichomes, which develop from the epidermal cells lying between the veins. Abaxial epidermis showed well-defined stomata and many regular granules or ribbons that characterised epicuticular waxes of *n*-aldehyde nature[9]. Stomata are more numerous on the lower than on the upper surface of the leaf.

A cross section of a sugarcane leaf shows a systematic arrangement of cells. The lower and upper epidermal layers are made up of brick-shaped cells with their long axes parallel to the leaf and with their cell walls thick and lignified. Bulliform cells, located above or to one side of small vascular bundles, often extend to the upper epidermis, thus making the epidermal layer very thin at this point. The conducting tissues or vascular bundles are found within circular- to oval-shaped groups of cells. The vascular bundle includes xylem, phloem and phloem fibers, all of which surrounded by a ring of large cells known as bundle sheath cells. These, and those immediately adjoining them on the outside of the bundles (mesophyll tissue), form the photosynthetic

Regular Paper

tissues. Situated above and below each large- and medium-sized vascular bundle is a small group of long, slender but very thick-walled cells known as fiber cells. These also occur at the base of each small bundle and a few are sometimes found lying above the bundle.[16]

Many authors claim about the role of the mechanical resistance of plant tissues to pathogen invasion by producing biopolymers that restrict the spread of pathogens such as hydroxyproline-rich glycoproteins, lignin and callose,[13] mainly consisting of suberized or lignified periderm, sclereids, xylem, and phenolic phloem parenchyma cells. Transgenic tobacco plants with suppressed levels of the phenylpropanoid biosynthetic enzyme L-phenylalanine ammonia-lyase and correspondingly low levels of chlorogenic acid, the major soluble leaf phenylpropanoid product, exhibit more rapid and extensive lesion development than wild-type plants after infection by the virulent fungal pathogen *Cercospora nicotianae*.[15] An appropriate mechanical stimulation of cucumber leaves significantly improves plant resistance and alters the activity of phenylalanine ammonia-lyase leading to synthesis of lignin. The effects of the stress on these cellular fundamental events are eliminated when the adhesion between plasma membrane and cell wall is disrupted.[25] Microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) bark attacked by stem-boring weevils (*Pissodes strobi*) or through mechanical wounding demonstrated significant accumulation of transcripts resembling dirigent protein (DIR) genes, a subfamily direct stereoselective phenolic coupling reactions in the formation of lignans and lignins.[18] Manipulation of the *CAD* (cinnamyl alcohol dehydrogenase) gene causes changes in enzyme activity, lignin content and in the composition of the cell wall in the transgenic plants of *Linum usitatissimum,* consisting of a reduction in the lignin level in the CAD-deficient plants. However, the resistance of the transgenic lines to *Fusarium oxysporum* was over two-fold lower than for the non-transformed plants.[28]

Concerning sugarcane plants, de Armas *et al*.[5] found that the cultivar Mayarí 55–14, highly resistant to smut, strongly increases phenylalanine ammonia-lyase activity after incubation of leaves on a smut elicitor without accumulation of free hydroxycinnamic acids. This has been interpreted as a polarization of the use of these phenolics towards lignin synthesis in order to avoid smut penetration. Thus, a better knowledge of lignified tissues of sugarcane leaves is previously required to the elucidation of a mechanism of mechanical resistance to the pathogen.

MATERIAL AND METHODS

Plant material

S. officinarum (L.), cv. Barbados 42231, six months-old, field grown, was used throughout this work. 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), fertilised with nitrogen (150 kg ha^{-1}), phosphorus (75 kg ha^{-1}) and potassium (120 kg ha^{-1}) at the moment of planting. Plants were cultured in isolated greenhouses, under a mean of 250 μmol m^{-2} s^{-1} flux density of white light for 14 h per day, 35°C of mean temperature and 90% of relative humidity from April to October, and daily irrigated.[14]

Light microscopy

For structural studies, leaves 3 and 4 from the bottom of six different plants, at a middle position on the stalks, were always chosen. The central zone of the leaf blade was cut into 1.0 cm portions, which were cut in 10 μm-thick sections with a freezer microtome. Leaf cuts were stained with 0.5% (v/v) toluidin blue in 70% (v/v) ethanol[4] or alternatively in 1.5 % (w/v) saphranin in 50% (v/v) ethanol and 0.5 % (w/v) alcian blue in 90% (v/v) ethanol for 2 min.[11] Lignified structures were visualized using the phloroglucinol/HCl (PGH) test. Leaf cuts were incubated in a solution of 1% (w/v) phloroglucinol in 100% methanol overnight. Following further incubation of cleared tissues in chloral hydrate, they were subsequently mounted on slides, a few drops of concentrated hydrochloric acid were added and finally the tissues were covered with a coverslip[21]. Tissues were viewed immediately. After 10 min, lignified structures appeared cherry red-orange, but colour faded within 2–4 h.[22] The stained sections were observed using a Zeiss 60 invertoscope adapted with a CCD camera capturing images by using a Viewfinder Lite program.

Transmission electron microscopy

The microstructure of sugarcane leaves was examined by conventional TEM.[19] 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 and finally embedded in Epon. Thin sections were counter stained with uranyl acetate and lead citrate. Electronic micrographs were produced at 80 kV by TEM (Carl Zeiss EM 902). Image analysis was achieved by using the Image Tool 2.00 program.

Scanning electron microscopy

For ultrastructural studies, leaves 3 and 4 from the bottom of six different plants, at a middle position on the stalks, were always chosen. 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[20] using a JEOL JSM 6400 (Japan). Digital images were obtained by using an INCA (Oxford) program incorporated to the equipment.[14]

RESULTS

A cross section of a sugarcane leaf shows the classical arrangement of cells, which defines a C4 species (Figure 1A). On the upper and lower leaf surfaces are found a number of one- and two-celled trichomes, which develop from the epidermal cells lying between the veins . Inside the edges of leaves, having sharp marginal teethes inclined toward the apex of the leaf, a dense mass of sclereids occurs below the epidermal layer (Figure 1B). 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 (Figures 1A and B). The conducting tissues are found within the circular to oval-shaped groups of cells. The bundles are 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 is rather circular. A small, round bundle always lay next to a large vascular bundle, which usually extend from the upper to the lower epidermis of the leaf (Figure 1A). 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.[7] The large vessels were irregular in shape, having comparatively thick walls (Figure 1A) and many sides, each of which, when viewed in cross section, appear more or less as a straight line. Each vessel is formed not from a single cell but from a series of elongated cells, whose contents and end walls have disappeared.

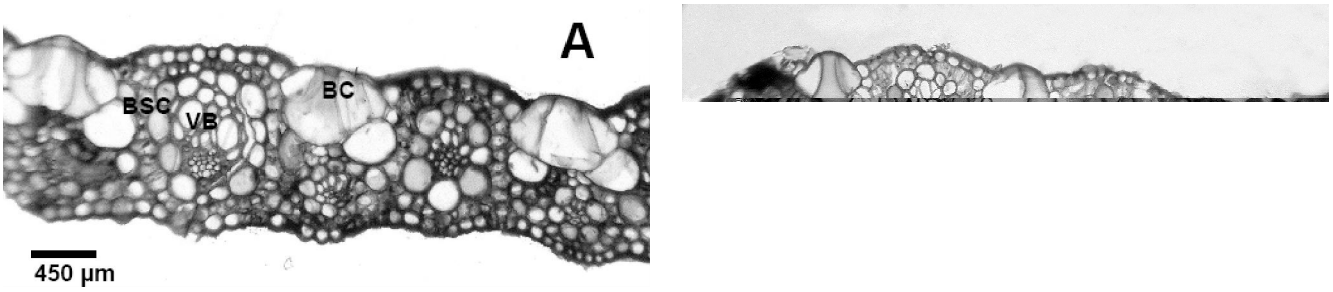

Figure 1 : A) Cross section of a leaf blade of sugarcane showing bulliform cells (BC) in the adaxial epidermis, bundle-sheath cells (BSC) surrounding the vascular bundle (VB) and the mesophyll. B) Cross section of the edge of a sugarcane leaf showing lateral trichomes (TC) and sclereids (SC) filling the blade edge. Sections were stained with toluidin blue.

According to the literature[16], the vascular bundle includes phloem fibers, the function of which, due to their structure and arrangement, is mainly to give strength to the leaf. They are long, slender, pitted cells with pointed ends and with thick walls which are lignified. Their cell cavities are extremely small. These fiber cells may occur singly but they are usually in groups forming strands, which extend longitudinally. Sclereids also are located below and above the vascular bundle, connecting it to the internal surface of the epidermal, upper and lower layers. They are docking cells that immobilized and ensured the total vascular bundle inside the spatial structure of the leaf. The distribution and nature of

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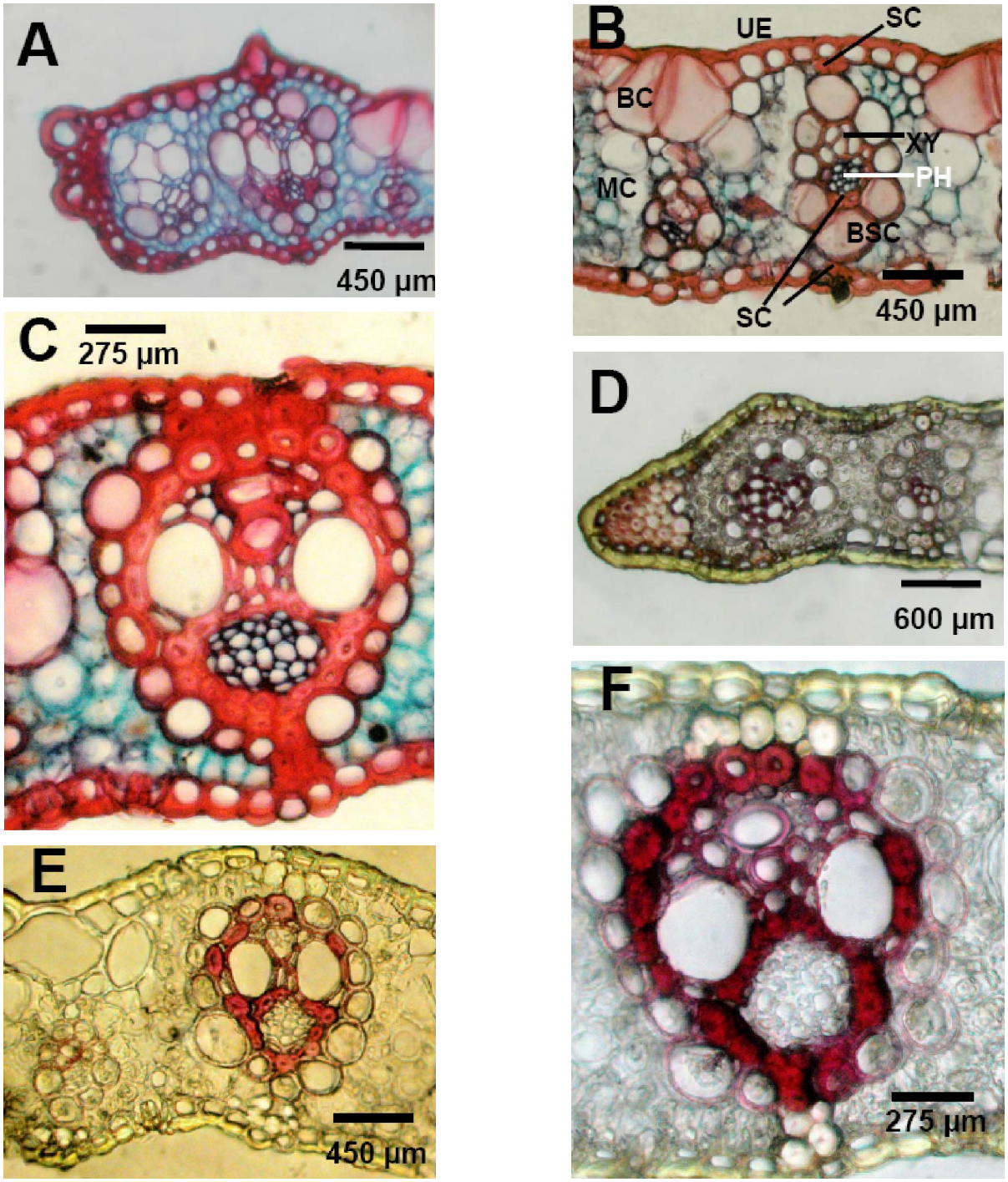

Figure 2 : A) Cross section dyed with saphranin-alcian blue of the edge of a sugarcane leaf showing two lateral teeth and some sclereids just below the epidermis. B) The arrangement of xylem vessels (XY) and phloem elements (PH) surrounded by bundle-sheath cells (BSC) in a cross section of a sugarcane leaf dyed with saphranin-alcian blue. Some sclereids (SC) can be observed below the phloem and connecting the vascular bundle to the internal surface of both upper and lower epidermis. C) Magnification of a vascular bundle dyed with saphranin-alcian blue showing large, mid and small xylem vessels, a ring of sclereids surrounding the vascular bundle and an internal ring of sclereids surrounding the phloem. D) Cross section of the edge of a sugarcane leaf dyed with phloroglucinol showing a dense mass of sclereids occupying the total internal volume of the edge. E) The arrangement of xylem and phloem surrounded by bundle-sheath cells in a cross section of a sugarcane leaf dyed with phloroglucinol. Only sclereids surrounding the vascular bundle are stained in red by the dye. C) Magnification of a vascular bundle dyed with phloroglucinol showing large, mid and small xylem vessels, a ring of sclereids surrounding the vascular bundle and an internal ring of sclereids surrounding the phloem. Only sclereids and the cell wall of xylem vessels are stained in red by the dye.

these sclereids has been herein studied by used two different staining methods: saphranin-alcian blue and phloroglucinol. The cytoplasm is stained by alcian blue whereas the cell wall was revealed by the red colour of saphranin. Observation of stained cross-section of sugarcane leaves by light microscope revealed trichomes on the external surface of the leaf edge as well as a compact mass of sclereids occupying the total internal volume of the edge, strongly stained in red colour (Figure 2A). Also the cell wall of epidermis, bulliform cells, xylem vessels, stone cells surrounding phloem, and docking cells connecting the vascular bundle to both epidermal layers appeared strongly coloured in red whereas bundle-sheath cells retained saphranin in less extent (Figure 2B and C). However, only those outer sclereids surrounding the vascular bundle, the inner ring of stone cells involving the phloem, and the more external cells filling the leaf edge were stained by phloroglucinol, specific for lignins. Neither docking cells nor the more internal mass of sclereids in the edge were significantly coloured by the phenol (Figure 2D, E and F). Both epidermal and bundle-sheath cells clearly showed the green colour of chlorophylls (Figure 2F).

The study by TEM of the cross-section of sugarcane leaves revealed that docking, subepidermical and stone cells in the vascular bundle had very thick cell wall traversed by abundant pits and a very small lumen (Figure 3A). Image analysis revealed that the most thick cell walls, and so the smallest lumen, corresponded to the docking cells below the abaxial epidermis whereas the largest internal space was exhibit by the stone cells (Figure 3B, C and D, and TABLE 1). Bundle-sheath cells had large, agranal chloroplasts with abundant starch bodies (Figure 4A) whereas mesophyll chloroplasts differentiated a complex system of grana, did not accumulate starch but a discreet number of lipidic drops could be seen (Figure 4B). Plasmodesmata connected bundle-sheath cells to the phloem (Figure 4C) and the lumen of the stone cells to the cytoplasm of phloem elements (Figure 4D). In the first case, an incipient desmotubule from the stone cell contacted to the external surface of the phloem cell wall just in the zone of abundant pits neighbouring the residual endoplasmic reticulum (Figure 4C).

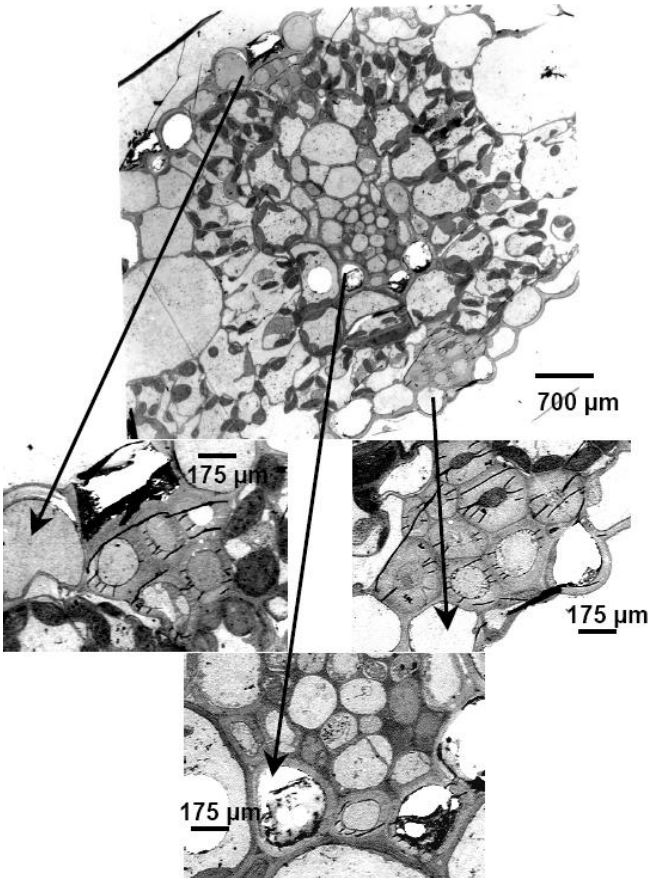

Figure 3 : A) TEM micrograph of a cross section of a sugarcane leaf where UE = upper (adaxial) epidermis, LE = lower (abaxial) epidermis, SC = sclereids, MC = mesophyll cells, occurring between two vascular bundles, BSC = bundle sheath cells surrounding the vascular bundle, XY = xylem composed by large, middle and small vessels, and PH = phloem. B) Magnification of sclereids located just below the adaxial epidermal layer. C) Magnification of sclereids surrounding the phloem. D) Magnification of sclereids located just below the abaxial epidermal layer. These, and similar views, were used for image analysis.

TABLE 1 : Quantification by image analysis applied on TEM preparations of the cell wall area of sclereids associated to the vascular bundle of sugarcane leaves. Values are the mean of 20 replicates and they are given as area units from Image Tool ± standard error. Numbers in brackets represent the per cent value of area considering as 100 per cent of total area of the cell.

Parameter	Adaxial face	Vascular bundle	Abaxial face
Cell area	3,624 ± 383 (100%)	2,111 ± 284 (100%)	4,154 ± 397 (100%)
Lumen area	1,377 ± 95 (38%)	940 ±98 (44.5%)	958 ± 96 (23.1%)
Cell wall area	2,247 ± 223 (62%)	1,171 ± 111 (55.5%)	3,196 ± 298 (76.9%)

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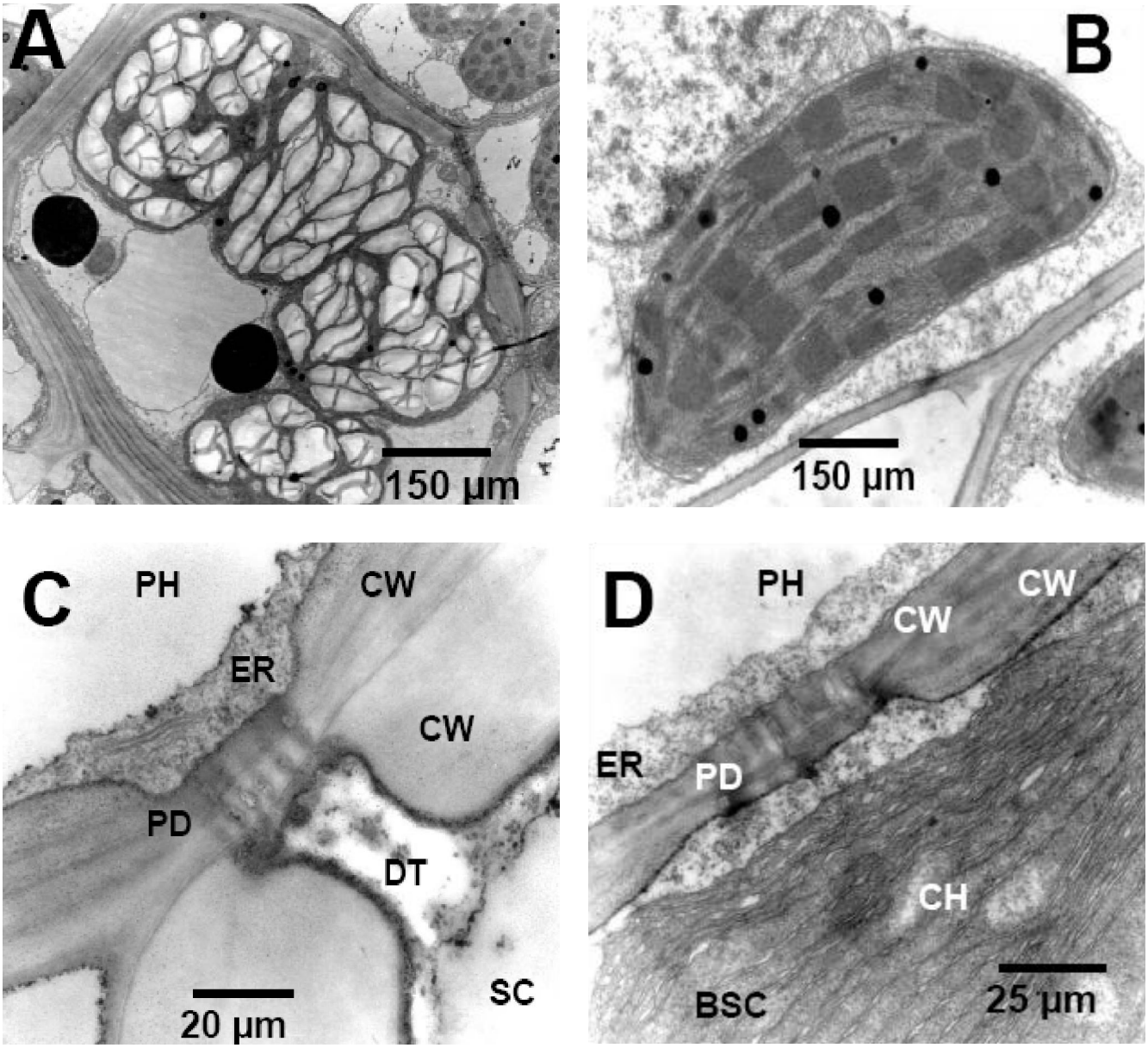

Figure 4 : A) A bundle-sheath cell showing large chloroplasts without grana, containing numerous starch inclusions. B) The chloroplast of mesophyll cells showing numerous granal membranes. C) Magnification of plasmodesmata (PD) in the cell wall (CW) of a sclereid connecting to a sieve plate (PH). DT = desmotubule, and ER = endoplasmic reticulum. D) Magnification of plasmodesmata (PD) between a bundle-sheath cell (BSC) and a sieve plate (PH). CW = cell wall, CH = chloroplast, and ER = endoplasmic reticulum.

DISCUSSION

In the microscopic study of plants, saphranin is used to stain the cell wall.[26] Even in some cases, these staining properties have been attributed to the ability of saphranin to combine with polyphenolic lignins.[2, 10] In this way, epidermal and bundle-sheath cells of sugarcane leaves, in addition to sclereids and xylem vessels, would have lignified cell walls, as it is shown in Figure 2A, B and C. However, Musil[17] claims about the capability of saphranin to stain acidic macromolecules, such as collagen, whereas pectic material of the plant cell wall would also stained with the same chromophore.[26] Probably, this property of saphranin to react with amino acids in a protein, or with galacturonic units in pectins, could be explained by the presence of two amino groups in the

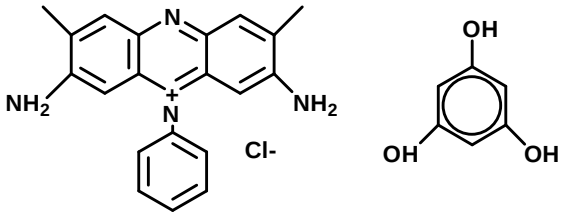

Figure 5 : Structural formulae of saphranin and phloroglucinol, dyes used to reveal cell wall and lignins, respectively.

molecule (Figure 5), able to form a pseudopeptic bond with free carboxyl groups in these macromolecules. Nevertheless, carboxyl functions of the cinnamic acids, precursors of lignols, are reduced to hydroxy groups before lignin polymerization.[27] This reduction might impede the binding of saphranin to polyphenols but the production of an oxygen bond between hydroxy groups of phloroglucinol and lignin does not. According to this, phloroglucinol is considered as a specific reagent for lignin staining.[3] This is in agreement with that observed herein, where cross-sections of sugarcane leaf are stained in red by using phloroglucinol as a chromophore only at the level of the ring of sclereids surrounding the vascular bundle, stone cells related to phloem, and the most external sclereids in the leaf edge. In contrast, epidermis, the cell wall of the bundle-sheath cells and the most internal sclereids in the leaf edge appear unstained by phloroglucinol (Figure 2D, E and F), probably because these cells alive at maturity. Then, two classes of sclereids can be defined for sugarcane leaves, each one with a precise localization: one of them, with very strongly lignified cell walls, is mainly located in the vascular bundle and in the most external zone of the leaf edge, below the epidermis, and the second one, composed by living cells poorly or not lignified, located below both adaxial and abaxial epidermis, and connecting these to the vascular bundle. Probably, these cells act as absorbers to attenuate possible contractions of the vascular bundle when leaves double by action of the wind. Living sclereids have also been found in bundles associated with long distance in the central region of sugarcane stalks.[24] The cell wall was lignified or not, this always appears as a very thick structure. On the contrary, bulliforms cells as well as bundle-sheath cells have non-lignified cell walls, since they are not stained by phloroglucinol.

Many phloem vessels directly contact to bundle-sheath cells connecting through plasmodesmata. This fact justifies the direct transport of sugars from the photosynthetic towards conducting cells. In other cases, sclereids intervene among bundle-sheath cells and phloem but abundant pits in sclereid cell wall (Figure 3) can be used to transport sugars towards the vascular elements. In sugarcane stalks, a sheath of fibres isolates the phloem apoplast from that of the storage parenchyma. This fibre sheath is narrowest to either side of the phloem fibre cap, and consists of living cells with plasmodesmata within pits in the secondary wall (Figure 4). Plasmodesmata were also arranged into pit fields between cells of the storage parenchyma. Since the vascular apoplast is isolated from the apoplast of the storage parenchyma, sucrose must move through the symplast of the fibre sheath.[24]

Moreover, sclereids of xeromorphic leaves of many plant species, such as *Hakea suaveolens*, are involved in the conduction of water to the epidermis and directly to palisade layers.[8] The role of sclereids in water movement inside the leaf has also been suggested by Gratani and Bombelli[6] for several evergreen shrubs of the Mediterranean maquis. On the other hand, the situation of sugarcane sclereids above and below the conducting vascular bundle surrounded by bundle-sheath cells suggest that fibers behave as docking cells to mechanically reinforce the central position of conducting tissues and, in addition, they are suitable for a light-guiding function, as it has been found for sclereids of *Phillyrea latifolia*. In leaves of this plant species, the amount of light transmitted through sclereids was to be up to 30-fold higher than that transmitted through the neighbouring mesophyll cells.[12] However, a similar role in sugarcane leaves deserves greater experiment and testing.

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Regular Paper

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