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AN ELICITOR ISOLATED FROM SMUT TELIOSPORES (*SPORISORIUM SCITAMINEUM*) ENHANCES LIGNIN DEPOSITION ON THE CELL WALL OF BOTH SCLERENCHYMA AND XYLEM IN SUGARCANE LEAVES

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Abstract

Sugarcane leaf shows the classical arrangement of cells which defines a C4 species. Vascular bundles consist of xylem, phloem and fibres, surrounded by an outer layer of sclereids and an inner ring of stone cells associated with the phloem. Some sclereids located below and above the vascular bundles act as docking cells and connect the vascular bundle to the internal surfaces of upper and lower layers of the epidermis. A compact mass of sclereids occupies the total internal volume of the leaf edge. Neither docking cells nor the internal mass of sclereids in the edge were markedly coloured by acriflavin or phloroglucinol, indicating the absence of lignin in their cell walls. However, such staining indicated that fibres of the vascular bundle and the external layer of sclereids were strongly lignified. Incubation of leaf discs with an elicitor produced by the pathogen *Sporisorium scitamineum* increased the thickness of the lignified cell walls of sclereids as well as the mid and small xylem vessels, as a possible mechanical defense response to the potential entry of the pathogen.

Introduction

Smut is a major disease of sugarcane caused by *Sporisorium scitamineum* (Syd.). Piepenbr & Oberw. 2002 (= *Ustilago scitaminea* Sydow & P. Sydow). Spore germination is achieved on the internode surface, and it is followed by the formation of appressoria, mainly on the inner scale of young buds and on the bases of emerging leaves (Waller, 1970). Entry into the bud meristem occurs between 6 and 36 h after the teliospores are deposited on the surface (Alexander & Ramakrishnan, 1980). Hyphal growth occurs throughout the infected plant, but mostly in the parenchyma cells of the lower internodes. In the upper internodes, hyphal growth concludes with the formation of the whips (sori with teliospores). Hyphae do not penetrate into the cells of the scale leaves (Singh & Budhraja, 1964) and, consequently, buds tightly enclosed within scale leaves can escape infection. Fungal hyphae have been reported to occur on the abaxial epidermis of smut-infected leaves in cv. Barbados, where penetration was obtained through open stomata. Many parenchymatous cells were invaded by smut and their cell walls were broken by actively progressing hyphae. Mucilage production accompanied the complete destruction of parenchymatous cells that harboured many fungal spores (Legaz *et al.*, 2006).

Resistance of sugarcane to smut has also been associated with the accumulation of free or conjugated polyamines in tissues (Legaz *et al*., 1998; Piñón *et al*., 1999) and the

production of several glycoproteins which can be recovered from cane juices (Martínez *et al.*, 2000). These glycoproteins affect the cytoplasmic polarity during spore germination (Fontaniella *et al.*, 2002) and impede cell polarization by inhibiting the protrusion of the germ tube. Such evidence indicates that the inhibition of teliospore germination constitutes a defence mechanism involved in the resistance of sugarcane to smut (Millanes *et al.*, 2005; Legaz *et al.*, 2005).

However, resistance of plants to disease seems to be a multifactorial process. The response phase includes the accumulation of different compounds such as phytoalexins (i.e. low molecular mass antimicrobial compounds that accumulate at sites of infection), systemic enzymes that degrade pathogens (e.g. chitinases, β-1,3-glucanases and proteases), systemic enzymes that generate antimicrobial compounds and protective biopolymers (e.g. peroxidases and phenoloxidases), biopolymers that restrict the spread of pathogens (e.g. hydroxyproline-rich glycoproteins, lignin, callose), and regulators of the induction and possible activity of defensive compounds (e.g. elicitors of plant and microbial origin, immune signals from primed plants and compounds which release immune signals) (Kuc, 1990).

Plant cells lack a circulatory immune system that is capable of quickly recognizing invaders, but are individually equipped with the ability of recognizing pathogens and turning on an effective defense system. This type of defense response is achieved through the interaction of a putative plant-derived receptor and a corresponding pathogen molecule called an 'elicitor'. Elicitor production is dependent on so-called 'avirulence' (*avr*) genes. It is still unclear for most *avr* genes whether the final elicitor is the actual Avr protein or an Avr-dependent by-product. The products of plant resistance genes (known as *R* genes) are hypothesized to be the receptor molecules that recognize specific elicitors. This *R–avr* interaction initiates what is referred to as gene-for-gene resistance (Holt *et al.*, 2000), although, in this case, smut seems to invade sugarcane cultivars with independence on their genetic and geographical origin (Chinea & Rodriguez, 1994).

Particularly, the infection of plants by fungal pathogens produces phenols and changes the role and reaction abilities of phenolic compounds (Sedlářová & Lebeda, 2001) as a primary response to elicitor signalling. The early release of preformed phenolics and subsequent intensive production after stimulation of phenylpropanoid metabolism, are a part of resistance reactions to disease in many plants (Peltonen, 1998). Within the central vacuole, the pre-existing pool of phenylpropanoids (mainly monolignols) are stored and incorporated into the cell wall following release into the cytoplasm during the initial stages of plant defense. These processes are dependent on peroxidases and other enzymes in the apoplast involved in phenolic acid esterification. Only during later pathogenesis is *de novo* synthesis of phenolic compounds switched on, following the transcriptional activation of genes for phenylpropanoid biosynthesis, closely associated with phenylalanine ammonia-lyase (Guidi *et al.*, 2005). Lignins are formed and used to increase rigidity of cell walls and phenolics are part of the hypersensitive response (Lebeda *et al.*, 2001).

Recently, it has been found that the sensitivity or resistance of sugarcane to smut can be related to changes in the levels of free phenolic compounds, and phenylalanine ammonia-lyase (PAL) and peroxidase (POX) activities in the leaves. Elicitors from *S. scitamineum* enhance the activity of PAL and consequently increase the levels of hydroxycinnamic and hydroxybenzoic acids. However, a decrease in the amount of free hydroxycinnamic acids was found when the highest PAL activity was reached (De Armas *et al.*, 2007).

The purpose of the current work was to relate changes in the levels of phenolics substances, induced by a smut elicitor, previously described to the increase of lignin deposit in supporting tissues that might be indicative for biochemical and structural resistance responses of sugarcane leaves. According to the previous hypothesis, the decrease in the amount of free hydroxycinnamic acids is related to an increase in the lignin content of these leaves.

Material and Methods

Plant material: Six months- old field grown plants of *Saccharum officinarum* L., cv. Barbados 42231 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 250µmol m^{-2} s^{-1} of white light, a photoperiod of 14 h, a 90% relative humidity, and were watered daily (Legaz *et al.*, 2006).

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 & Barnett medium (Lilly & Barnett, 1951) 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 10mM 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 4h at 38°C. After centrifugation, the pellet was washed once with 5 mL methanol and dried under air flow for 2h. The dried pellet was washed with 10mM phosphate buffer, pH 6.8, then resuspended in 25 mL of the extraction buffer, autoclaved (120°C for 30 min.) and re-centrifuged. 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) were floated in Petri plates on 15 mL 10 mM phosphate buffer, pH 6.8, containing 4% isopropylic alcohol for 2 h at 37ºC 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 (De Armas *et al.*, 2007). Control experiments were performed in the absence of elicitor. Three replications using leaf samples from different stalks were made.

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 0.5 cm portions, and 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 (Clark, 1981) 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 (Johansen, 1940) 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 (Soylu, 2006). 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 colour faded within 2–4 h (Vallet *et al*., 1996).

Image analysis: To study the development and differentiation of sclerenchyma, as well as variations in thickness of the cell wall of xylem elements, an image analysis was performed by using the program Image Tool 2.0 on phloroglucinol-stained sections (the number n of replicates was done in the corresponding table).

For the study of the area occupied by lignified cells in a cross section of leaf blade, two zones of reference were arbitrarily chosen. The first zone includes the epidermal cells that contact with docking cells (subepidermal sclereids that connect with the conductive bundles), longitudinal plates or strands of hypodermal sclerenchyma associated to almost all of the longitudinal bundles of the blade (Colbert & Evert, 1982), as well as the proper docking cells and the vascular bundle, including bundle sheath cells. The second zone includes exclusively vascular tissues and associated sclereids.

To analyze the thickness of the sclereid cell wall in treated and control leaf discs, three areas were measured in each cell: the total area of the cell, the area of the cell lumen and, for difference between both, the area of the cell wall. Then, the percentage of the cell surface that represents the lignified cell wall of sclereids was calculated for control leaf discs and for those treated with the elicitor. An identical procedure was used to study variations in the thickness of the cell wall in the xylem elements of treated and control leaf discs.

Statistics: Statistical significance of the differences between means of the analyzed parameters was evaluated by Student's t-test. The F-test was used to test heterogeneity of variances. Differences were considered significant when $p \leq 0.05$.

Fluorescence microscopy: Samples were cut into 10 μm thick cross sections as above and stained for 5 minutes in 0.1% acriflavin (Christiernin, 2006). Acriflavin was used because of this fluorophore reacts with lignin even in cell walls poorly lignified. Sections were placed on a slide previously covered with a layer of polylysine (50 μg mL^{-1} MilliQ water) for 12 h and fixed with Mowiol-DABCO to avoid the decay of fluorescence. The stained cells were observed using an Olympus DP50 fluorescence microscope fitted with a CCD camera for capturing images by using a Viewfinder Lite program. Wavelengths used were 488/568 nm for excitation and 530/600 nm for imaging. The microscope was equipped with a device to avoid the red fluorescence of chlorophylls.

Results

A cross section of a sugarcane leaf shows the classical arrangement of cells, which defines a C4 species. A dense mass of sclereids occurred below the epidermal layer (Fig. 1A). Thin-walled bulliform cells were confined to the adaxial epidermis (Fig. 1B). The vascular comprised three- sized classes of cells, large, medium and small (Colbert & Evert, 1982; Legaz *et al*., 2006), and varied in shape from rhomboid and oval in the former to circular in the latter.

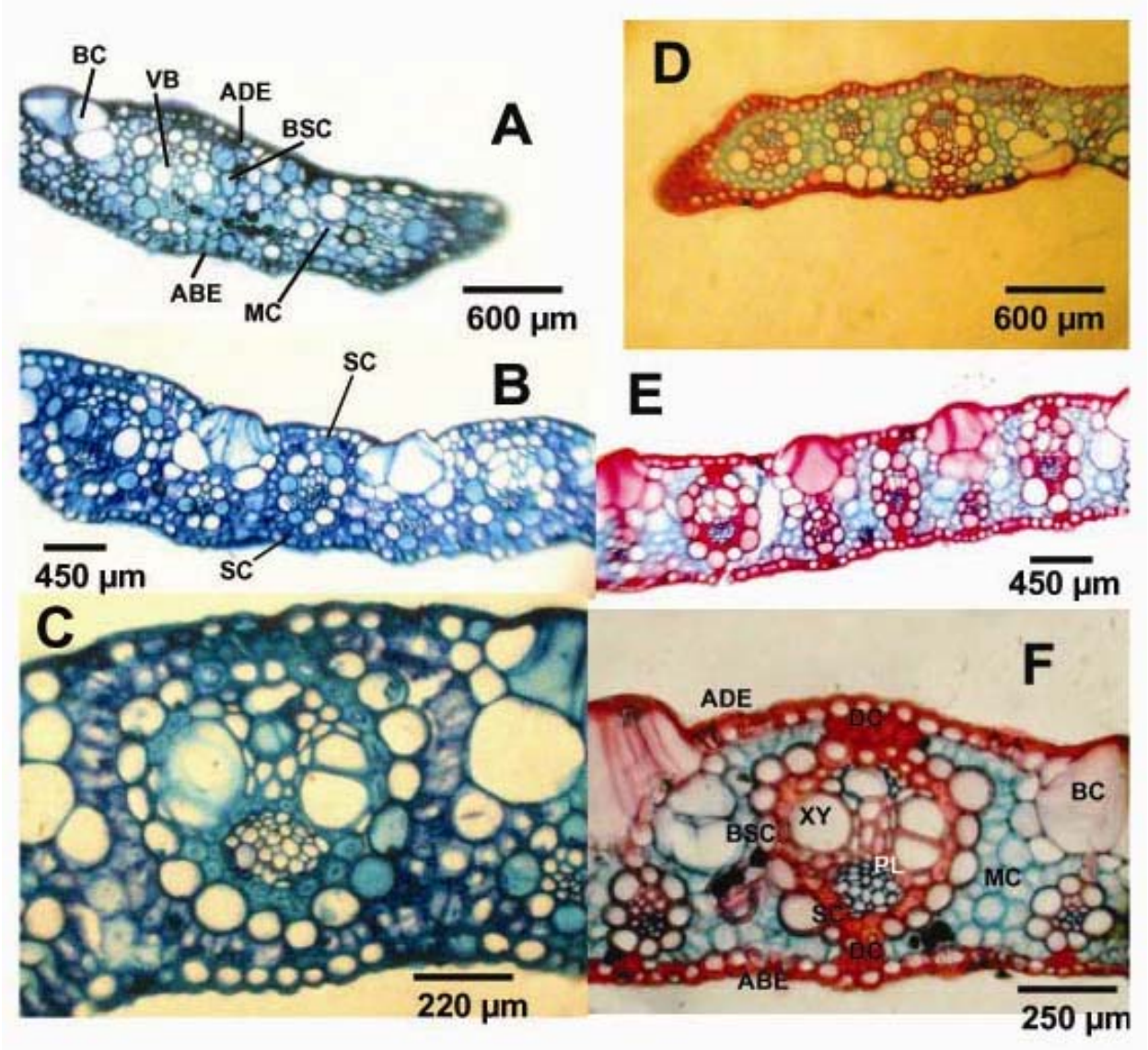

Fig. 1. A) Cross section of the edge of a leaf blade of sugarcane showing bulliform cells (BC) in the adaxial, upper (UE) and lower (LE) epidermis, bundle-sheath cells (BSC) surrounding the vascular bundle (VB) and mesophyll cells (MC). B) Cross sections of the central zone of a leaf blade of sugarcane showing sclereids (SC) below epidermal layers. C) Magnification of a cross section of a sugarcane leaf showing the vascular bundle and associated tissues. Sections in A, B, and C were stained with toluidin blue. D) Cross section of the edge of a leaf blade of sugarcane, identical to that shown in A) but stained with saphranin-alcian blue. E) Cross sections of the central zone of a leaf blade of sugarcane. F) Magnification of a cross section of a sugarcane leaf showing the vascular bundle and associated tissues, where DC= docking cells, PL= phloem, SC= stone cells (sclereids in the vascular bundle), and XY= xylem. Sections in D, E, and F were stained with saphranin-alcian blue.

A small, round bundle was always adjacent to a large vascular bundle, which extended from the upper to the lower epidermis (Fig. 1C). The xylem is made up of open tubes or vessels associated with smaller and thicker walled elements. The large bundles comprised two large vessels connected with smaller vessels. The irregular-shaped large vessels had comparatively thick walls (Fig. 1C). The function of the small cavity phloem fibres within the vascular bundle is mainly to provide strength to the leaf.

These fiber cells occurred in groups forming strands and extended longitudinally. Sclereids also are located below and above the vascular bundle, connecting it to the internal surface of the epidermal, upper and lower layers (Colbert & Evert, 1982). They are docking cells that immobilized and ensured the total vascular bundle inside the spatial structure of the leaf. The distribution and nature of these sclereids were evaluated by use of two different staining methods, viz., saphranin-alcian blue and acriflavin and phloroglucinol. The cytoplasm of parenchymatous cells was stained by alcian blue and the cell wall by saphranin, producing a red colour. Further observation by light microscopy revealed a compact mass of red- stained sclereids occupying the total internal volume of the edge (Fig. 1D). This is in agreement with that of Colbert & Evert (1982), which described that leaf margins have sclerenchyma at all level of the blade.

Also, the cell wall of epidermis, bulliform cells, xylem vessels, stone cells (surrounding the phloem) and docking cells appeared bright red whereas bundle-sheath cells retained saphranin to a lesser extent (Fig. 1E and F). Acriflavin produced yellow fluorescence in the cell wall of sclereids at the leaf's edge (Fig. 2A), as well as in docking cells and sclereids surrounding xylem and phloem elements (Fig. 2B). Elicitation seemed to increase fluorescence (Fig. 2C and D). However, acriflavin-stained lignin produced a dispersion of the fluorescence emitted from the edges of the cell walls. This implied some indetermination in the quantification of areas by image analysis. For this reason, this analysis was always achieved by using cross sections stained with phloroglucinol.

Only those outer sclereids surrounding the vascular bundle, the inner ring of stone cells, and the external cells at the leaf's edge were stained by phloroglucinol, specific for lignins (Fig. 3C). Neither docking cells nor the internal mass of sclereids in the edge were significantly colored by the phenol (Fig. 3A and B). Both epidermal and bundle-sheath cells clearly showed the green color of chlorophylls (Fig. 3B).

Image analysis of the development and differentiation of sclerenchyma, was evaluated in two major leaf zones. The first zone included vascular vessels, stone cells and surrounding bundle-sheath cells as well as docking sclereids in contact with epidermal cells. The second zone only included vascular tissues and associated sclereids. Both zones were considered as the total area the first and the vascular bundle area the second one.

Cross sections of control leaves, showed that the area occupied by docking sclereids associated with the abaxial epidermis was higher than that of the adaxial epidermis, and was calculated as 3.61% and 3.14% of the total leaf area respectively. Sclereids surrounding the vascular vessels, and stone cells between xylem and phloem elements, represented about 11% of the total area and 42% of the vascular bundle area. The area occupied by docking cells was reversed following smut elicitation. The area of adaxial sclereids was about 2.75% whereas that of the abaxial sclereids was approximately 2.40% of the total area. A decrease in the area occupied by sclereids in the vascular bundle was shown after elicitor treatment, and amounted to approximately 9% of the total area, and 30.33% of the vascular bundle area (Table 1). As deduced from data in Table 1, the area of the complete vascular bundle slightly increases after the treatment. Image analysis of individual xylem vessels revealed that the area of the cell wall increased after elicitation in middle and small vessels but decreased in the bigger vessels (Table 2).

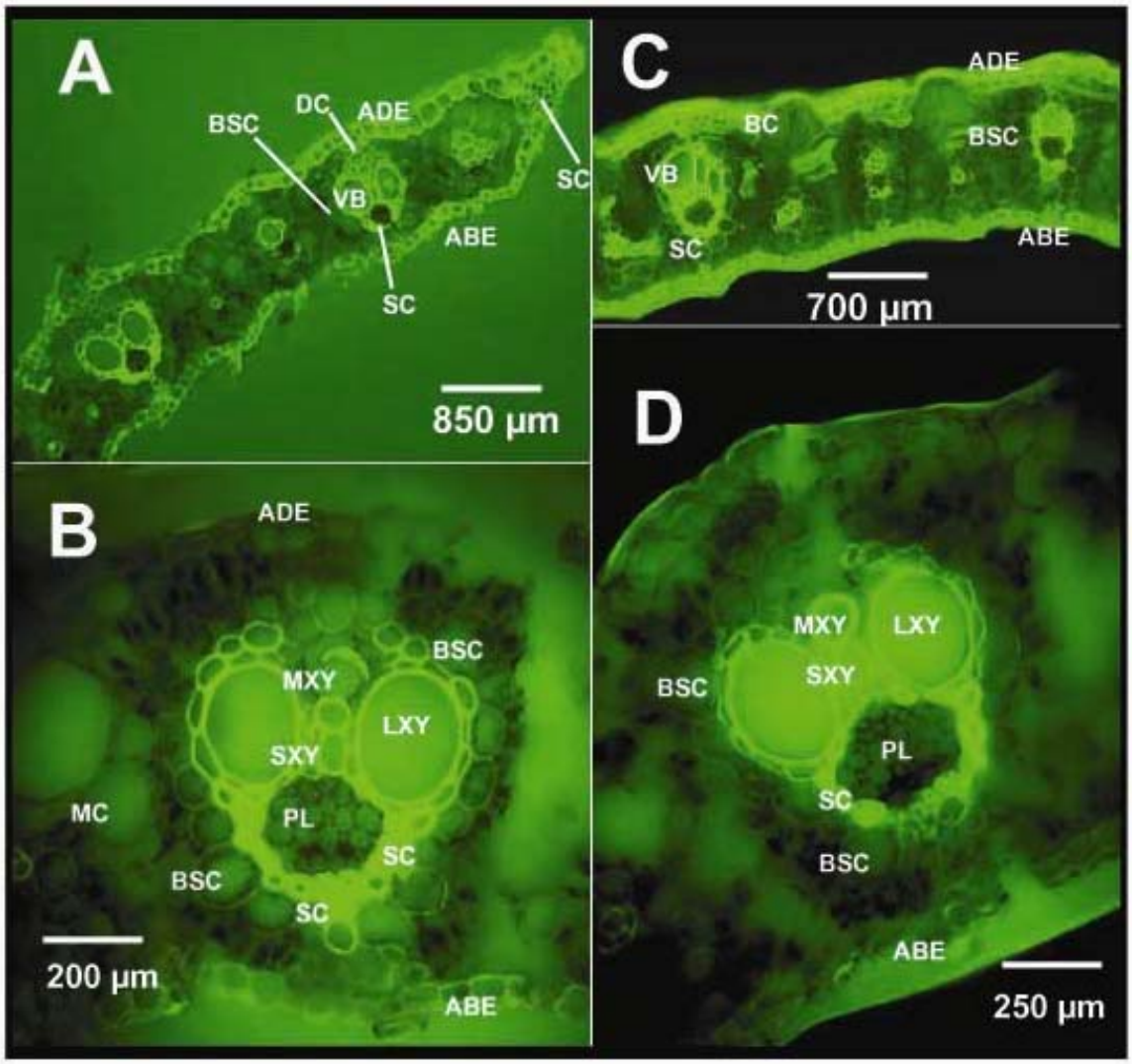

Fig. 2. Visualization of fluorescence emission from acriflavin-stained cell walls. A) Cross section of the edge of a leaf blade of sugarcane without elicitor treated showing docking cells (DC) near the adaxial, upper epidermis (UE), bundle-sheath cells (BSC) surrounding the vascular bundle (VB) and stone cells (sclereids) (SC) surrounding both phloem and xylem. B) Central zone of a cross section of leaf blade previously treated with the fungal elicitor showing some bulliform cells (BC). C) Magnification of a cross section of a sugarcane leaf showing the xylem, composed by large (LXY, mid- (MXY), and small (SXY) vessels and associated tissues, bundle-sheat cells (BSC), phloem (PL), and sclereids (SC) surrounding the vascular bundle. D) Magnification of a cross section of a sugarcane leaf previously treated with the smut elicitor, showing identical tissue organization that had been found in C).

By using image analysis, the area of the cell wall could be calculated by subtracting the cell lumen area from the total cell area. Elicitation caused a reduction in the size of the cell lumen and the ensuing expansion of the cell wall in docking cells, sclereids associated with the vascular bundle and stone cells, where the area of the cell wall represented more than 94% of the total cell area. However, the walls of identical cells in control leaves were about 88% of the total cell area. The highest increase of the cell wall area (Δ= 6.97% respect to the control) was observed in stone cells associated to phloem vessels, although docking cells also increased their cell wall area to a similar extent (Δ= 6.95% respect to the control). The highest increase of the cell wall area induced by the elicitor was observed, nevertheless, for cell of the leaf edge (Δ= 11.14%), although the area of the cell lumen represented about 11% of the total cell area.

Table 1. Area of Sclerids in cross sections of sugarcane leaves treated and untreated with the smut elicitor. Results were obtained by image analysis of phloroglucinol-stained sections, as described in material and methods. Values are the mean 20 replicates ± standard error. * indicates values for elicitor-treated leaf discs that differ at p≤ 0.05 from untreated discs.

Parameter	Control			Elicitor		
	Area counts	Per cent of the total area	Per cent of the vascular bundle area	Area counts	Per cent of the total area	Per cent of the vascular bundle area
Total area	600,095 ± 52,519			841,296 ± 67,428		
Area of subepidermal, abaxial sclereids	21,692 ± 1,984	3.61		20,226* ± 1,617	2.40	
Area of subepidermal, adaxial sclereids	18,867 ± 1,389	3.14		23,137 ± 1,548	2.75	
Bundle vascular area	158,886 ± 13,226	24.47		251,388 ± 17,487	29.88	
Area of sclereids from the vascular bundle	66,729 ± 5,341	11.12	42.00	76,265* ± 6,654	9.06	30.33
Area of the vascular bundle sclereids/area of the vascular bundle	0.42			0.30		
Total area of sclereids	107,288 ± 88,973	17.88		119,628 ± 9.841	14.22	
Total area of sclereids/total area	0.18			0,12		

Table 2. Area of the cell wall of xylem elements in sugarcane leaves untreated or treated with the smut elicitor. Values are the mean of 20 replicates ± standard error. * indicates values for elicitor treated discs that differ at p≤0.05 from untreated leaf discs.

Xylem vessel	Treatment	Total area	Lumen area	Cell wall area	Cell wall area as per cent of total cell area	Per cent of increase (+) or decrease (-) of cell wall area
Biggest	Without elicitor	8,191 ± 719	5,817 ± 515	2,374 ± 192	28.98	
	With elicitor	52,516* ± 548	43,395* ± 3,780	9,121* ± 867	17.37	-11.61
Mid	Without elicitor	4,419 ± 376	2,906 ± 213	1,513 ± 138	34.24	
	With elicitor	18,062* ± 1,654	11,196* ± 1,064	6,866* ± 592	38.01	3.77
Small	Without elicitor	2,561 ± 211	1,169 ± 118	1,392 ± 122	54.35	
	With elicitor	5,219* ± 486	1,864* ± 1,064	3,355* ± 289	64.28	9.93

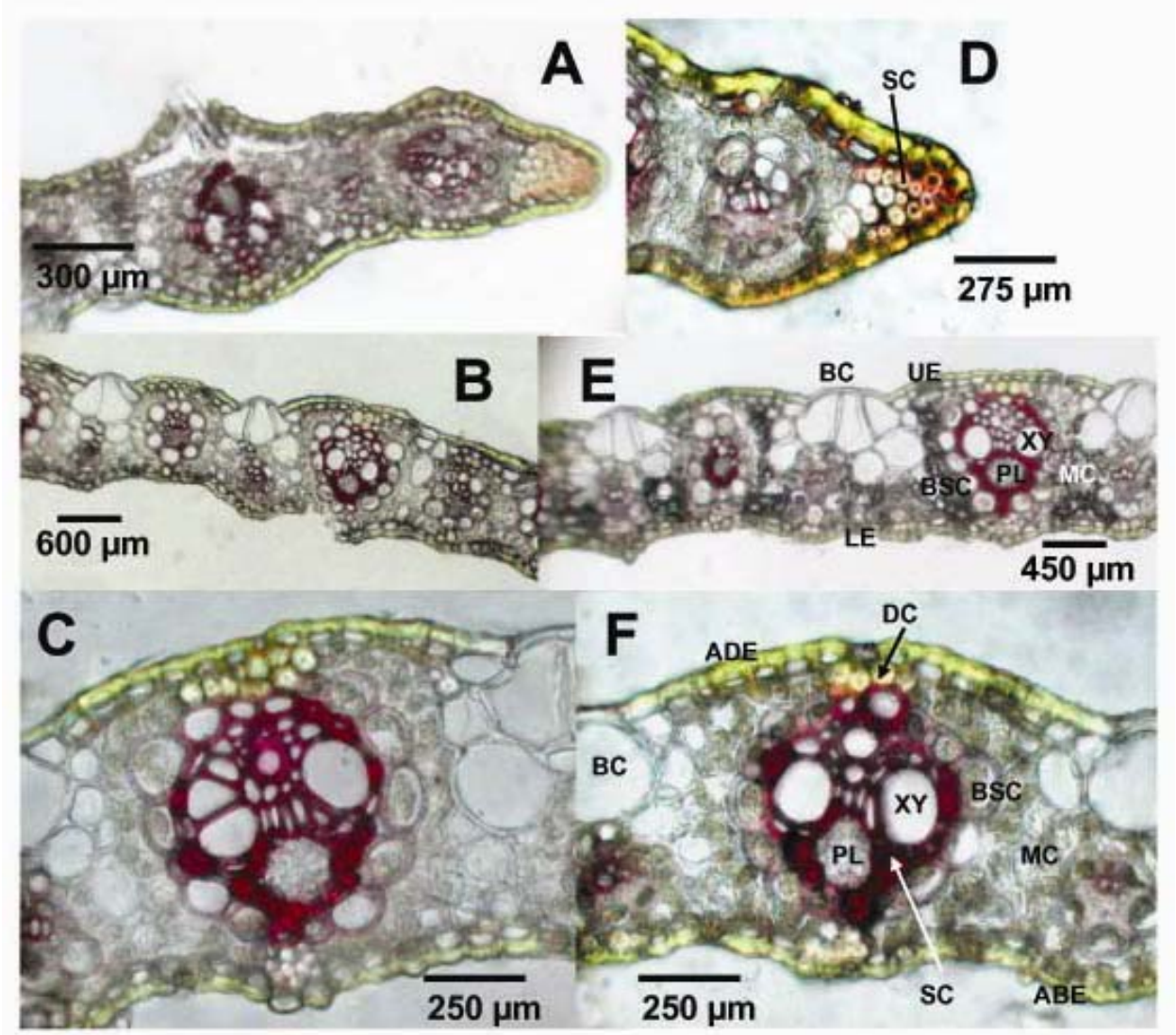

Fig. 3. A) Cross section of the edge of a leaf blade of sugarcane. B) Cross section of the central zone of a leaf blade of sugarcane. C) Magnification of a cross section of a sugarcane leaf showing the vascular bundle and associated tissues. Sections in A, B, and C were obtained from untreated, control leaf discs and stained with phloroglucinol. D) Cross section of the edge of a leaf blade of sugarcane showing sclereids (SC). E) Cross section of the central zone of a leaf blade of sugarcane, where UE = upper epidermis, LE = lower epidermis, BC = bulliform cells, BSC = bundle-sheath cells. MC = mesophyll cells, PL = phloem, and XY = xylem. F) Magnification of a cross section of a sugarcane leaf showing the vascular bundle and associated tissues, where D= docking cells (sclereids), and SC = stone cells (sclereids in the vascular bundle). Sections in D, E, and F were obtained from elicitor-treated leaf discs and stained with phloroglucinol.

Discussion

Epidermal and bundle-sheath cells of sugarcane leaves, in addition to sclereids and xylem vessels, show their cell walls strongly stained with saphranin, as it is shown in Fig. 1D-F. This does not imply that these cells have lignified cell walls, since saphranin is able to react with amino acids in a protein, or with galacturonic acid units in pectins (Warmke & Lee, 1976). In contrast, only the ring of sclereids surrounding the vascular bundle, stone cells related to phloem, and the most external sclereids in the leaf edge produce fluorescence emission after treatment with acriflavin (Fig. 2) and they are stained in red by using phloroglucinol as a chromophore. However, epidermis, the cell wall of the most internal sclereids in the leaf edge as well as that of the bundle-sheath

cells appear unstained by phloroglucinol (Fig. 3), probably because these sclereids are 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 (Walsh *et al*., 2005). Since these cells constitute the main lignified component of the leaf structure, they might be altered by pathogens or their elicitors if the mechanical properties of the leaf are involved in defense reactions against invaders.

Many authors claim about the role of the mechanical resistance of plant tissues against pathogen invasion by producing biopolymers that restrict the spread of pathogens such as hydroxyproline-rich glycoproteins, lignin and callose (Kuc, 1990), mainly consisting of suberized or lignified periderm, sclereids, xylem and phenolic phloem parenchyma cells. Many examples can be invoked on this subject. Transgenic tobacco plants with suppressed levels of the phenylpropanoid biosynthetic enzyme phenylalanine ammonia-lyase and correspondingly low levels of chlorogenic acid, the major soluble leaf phenylpropanoid product, exhibit more rapid and extensive lesion development than wildtype plants after infection by the virulent fungal pathogen *Cercospora nicotianae* (Maher *et al.*, 1994). 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 (Wang *et al*., 2006). 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 (Ralph *et al*., 2006). Manipulation of the *CAD* (Cinnamyl Alcohol Dehydrogenase) gene caused 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 nontransformed plants (Wróbel-Kwiatkowska *et al.*, 2006). In the cell wall, microfibrils of cellulose are continuous along the length of the cell. Hemicelluloses and pectins have a clear horizontal orientation with little or no interconnectivity. These polysaccharide-based walls are strengthened by impregnation with the polyphenolic polymer lignin on water-conducting cells and sclereids (Boyce *et al.*, 2004). In these last cells, lignin is ramdomly oriented and fills in some of the gaps in the structure. Because of the non-crystalline nature of lignin and its low elastic modulus, it behaves more like a packing fraction being most effective at resisting compressive forces, as required by a supporting tissue. These tensile forces are supported within the matrix mostly by entanglement, hydrophobic interactions or hydrogen bonding. If microfibrils are continuous along a cell and helically wound, then the helix will open out and cell surface area will be reduced when the material is stretched, deriving from cell extension. This would explain why the area of sclereid populations in elicitor-treated leaf discs of sugarcane is lower than in the control (Fig. 3). The reduction of cell area will lead to an increase in wall thickness (Table 3) as the lignin is squashed radially (Fig. 4), according to that proposed by Hepworth & Vincent (1998).

Table 3. Area of the cell wall of different sclereids in sections sugarcane leaves untreated or treated with the smut elicitor. Values are the mean of 20 replicates ± standard error. * indicates values for elicitor treated discs that differ at p≤0.05 from untreated leaf discs.

Treatment	Without elicitor				With elicitor			
Group of sclereids	Cell area	Lumen area	Cell wall area	% of the total cell area	Cell area	Lumen area	Cell wall area	% of the total cell area
Sclereids from leaf edge	2,554 ± 232	569 ± 61	1,985 ± 178	77.7	9,616±819	1,073 ± 97	8,543*±719	88.3
Docking cells	4,157 ± 381	509 ± 46	3,648 ± 312	87.7	10,378±954	555 ± 47	9,823*±878	94.6
Sclereids surrounding bundle vessels	9,461 ± 875	1,108 ± 719	8,353 ± 846	88.3	13,110±1.137	802 ± 78	12,308*±1,111	93.9
Stone cells	5,923 ± 526	750 ± 71	5,173 ± 478	87.3	8,539±810	485 ± 48	8,054*± 782	94.3

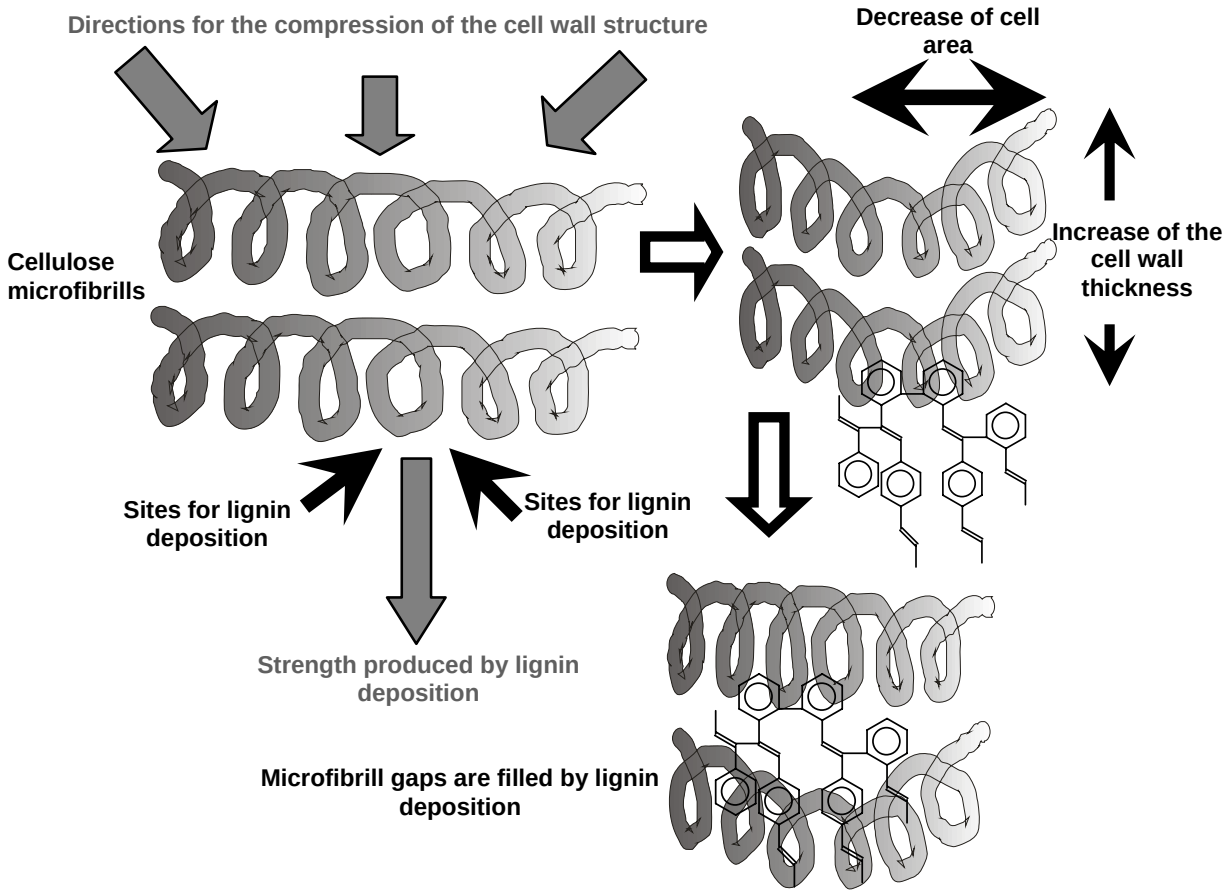

Fig. 4. A mechanistic model proposed by Hepworth and Vincent (1998) to explain the increase of the cell wall thickness and the decrease of the cell area during lignification.

However, the total area of the vascular bundle increases after treatment with the elicitor (Fig. 3C and F, and Table 1). This is in agreement with that described by Niklas (1991, 1996) for petioles of *Spathiphyllum* and *Acer saccharum*. The increase of the elastic modulus attending petiole dehydration, for example, can be linearly correlated with an increase in the relative volume fraction of tissues with lignified, thick cell walls whereas decreases in tissues with non-lignified thin walls. This seems to be true for areas occupied by docking cells and sclereids in the leaf edge (Fig. 3A and D). Although docking cells seem to be equivalent to strands of collenchymatous cells in dicotyledoneous, that cause a slight ridge of adaxial surface of the leaf, some degree of cell wall lignification, which produces slight reaction with acriflavin (Fig. 2C and D) and phloroglucinol (Fig. 3C and F), indicates that they are differentiated as sclereids in sugarcane leaf.

No cambial-like layers have been observed between xylem and phloem vessels in mature cane leaves (Fig. 3C and F). This fact is in agreement with that previously described for monocotyledoneous plants in general, and for sugarcane in particular, in which vestigial cambial activity in the bundles of the leaf seems to be restricted to the youngest, immature phase of the organ development (Moreland & Flint, 1942). Apparently, cambium is substituted by sclereids (stone cells) in mature leaf blade as well as in sugarcane stalks, as found by Oworu & McDavid (1977).

Crude elicitor prepared from *S. scitamineum* mycelium induces high phenylalanine ammonia-lyase activity without accumulation of free hydroxycinnamic acids and moderately high peroxidases activity, mainly in resistant cultivars (De Armas *et al*.,

2007). The elicitor was extracted from the fungal mycelium and autoclaved at 120°C, according to that described by McGhie *et al.*, (1977) 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 PAL and POX activities seems to be only composed by protein (in preparation).

An active elicitor preparation was obtained by autoclaving a cell wall fraction of mycelia of *P. chaunorhiza* to release soluble products (McGhie *et al.*, 1977). Fractionation of the crude elicitor preparation showed that the active component was approximately 50 kDa in size. In suspension-cultured sugarcane cells treated with the elicitor, PAL activity increased greatly after elicitor treatment of cells of a susceptible cultivar, but not with a resistant cultivar, despite accumulation of new phenolics compounds. In a similar way to that found for other thermostable proteins, both electrostatic and hydrophobic interactions, involved in the increase in the thermal stability of proteins (Spassov *et al.*, 1995), as well as the presence of additional aromatic clusters or enlarged aromatic networks on the protein surface, must be involved in the thermal stability of fungal elicitors. The aromatic clusters are found to be relatively rigid regions on the surface of the protein and often located close to the active site of the thermophilic, biologically active proteins (Kannan & Vishveshwara, 2000). This fact is probably related to the conservation of the biological activity after heating.

Peroxidases have been induced by fungal pathogens or their elicitor in many plant species. *Trichoderma harzianum*, a fungal antagonist of *Phytophthora capsici* infection on pepper plants, produces an elicitor of plant peroxidases, the induction of which can be related to the increased resistance of the plant (Ezziyyani *et al*., 2005). Two defense-related genes encoding the anionic peroxidase and acidic chitinase were also induced in transgenic *Solanum tuberosum* by the action of a broad range of fungal pathogens (Wul *et al*., 1997). Normally, increased PAL and POX activities are directly related to lignin biosynthesis. A putative promoter fragment of a *Pinus radiata* gene encoding a multi-functional O-methyltransferase (AEOMT) acting of hydroxycinamic acids and hydroxycinnamyl-CoA species, was activated by challenge of the tissue with a fungal pathogen (Moyle *et al*., 2002). Histochemical analysis in transgenic tobacco plants reveals that the promoterinduced *GUS* expression in cell types associates with lignification, such as developing vessels, phloem and wood fibers and xylem parenchyma as well as in non-lignifying phloem parenchyma. In addition, Norway spruce trees naturally infected with *Ascocalyx abietina* show differences in accumulation of soluble and cell wall-bound phenolics, contents of lignin and total peroxidase activities in the bark collected from three distinct regions of infected branches (Cvikrová *et al*., 2006).

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