

Biochemical and Morphological Responses to Abiotic Elicitor Chitin in Suspension-Cultured Sugarcane Cells

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ABSTRACT

Cells of Saccharum officinarum submitted to hydrolyzated chitin for 1 to 8h produced phenolic compounds. These alterations were observed through cytochemical methods using Toluidine Blue and Phloroglucinol/HCl. After 4 h, besides cell wall change, there was a change in nuclear pattern of chitin treated cells. There was a 96% increase in nuclear area in 6 h chitin treated material, as observed by Feulgen reaction. The treated cells showed chromatin compacted regions and a degeneration process of nucleoli. In the outer areas of cell wall, there was a polysaccharide desegregation, confirming results obtained for different plants with the use of other elicitors. Peroxidase activity was maximal after 4 h and decreased progressively. PAL activity started to increase at 4 h of incubation. These results showed that chitin hydrolyzate stimulated a defense response in sugarcane cells.

Key words: Chitin, elicitation, peroxidase, PAL, sugarcane, tissue culture

INTRODUCTION

Plant cells can respond to pathogenic attacks by activating a series of defense reactions (Ride 1992; Boller, 1995; Hawkins and Boudet, 1996). The defense system of plants includes biochemical mechanisms such as accumulation of phenolic compounds linked to cell wall, synthesis of phytoalexin, activation of enzymes involved in lignification, synthesis of hydrolytic enzymes such as chitinases and β -1,3-glucanases, and formation of cell wall structural proteins, including hydroxyproline-rich glycoproteins (Dixon et al., 2002; Verpoorte et al., 2002). Phenylpropanoids, whose metabolism may be initiated by the activation of enzymes that participate in initial

defense reactions, are important for the survival of cells under stress (Dixon and Paiva, 1995).

Besides lignification, other cellular alterations have been observed after the addition of biologically active substances to cell suspension cultures. Among these substances, some oligosaccharides obtained from xiloglucans (McDougall and Fry, 1990; Joseleau et al., 1996), pectins (Aldington et al., 1991), chitin and chitosan (Peltonen et al., 1997) have been studied. Chitin, a cell wall polysaccharide from fungi, is an active elicitor of lignification in wounded wheat leaves and wheat suspension-cultured cells (Gotthardt and Grambow, 1992; Stacey and Shibuya, 1997; Shibuya and Minami, 2001; Ramonell et al., 2002).

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Peroxidases (EC.1.11.1.7) and phenylalanine ammonia-lyase (PAL) (EC. 4.3.1.5.) are one of the enzymes activated in response to a stress caused by fungi. Peroxidases are widely distributed in the plant kingdom and are associated with several physiological processes, including growth regulation and cellular lengthening (Goldberg et al., 1986), auxin metabolism (Grambow and Langenbeck-Schwich, 1983) and lignification (Melo et al., 1995; McGhie et al., 1997). PAL catalyzes the first reaction of the phenylpropanoid biosynthetic pathway involving the conversion of L-phenylalanine into *trans*-cinnamic acid through a non-oxidative deamination reaction (Rohde et al., 2004).

Lignin is a complex heterogeneous phenolic polymer whose deposition can be induced in many cell types by diseases or wounds, indicating that the genes involved in the biosynthesis respond to developmental and environmental stimuli (Dixon et al., 2002). This process can be analysed using plant cell cultures where the cells are stimulated by the substances known to induce lignin deposition (Grima-Pettenati and Goffner, 1999).

In this work, the morphological alterations and the changes in peroxidase and PAL activities in *Saccharum officinarum* cells cultivated in suspension and exposed to a chitin hydrolyzed solution were investigated.

MATERIAL AND METHODS

Saccharum officinarum cells derived from calluses were sub-cultured every 2-3 weeks into Murashige and Skoog (1962) medium containing 3 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (MS₃). Cell suspensions were initiated by transferring the portions of callus to 20 mL of liquid medium. Cultures were maintained at 25 ± 1° C in the dark and constant agitation (120 g). The hydrolyzed chitin from the shells was acquired from Fine Chemical Kito (Palhoça, Santa Catarina State, Brazil), prepared as described by Villegas and Brodelius (1990) and 80 µL was added to the culture medium immediately following the inoculation of 2-d-old cell suspensions cultures to give a 200 µg mL⁻¹ final concentration. Flasks (125 mL) with 80 mL culture medium were used. Cells were collected after 1, 2, 3, 4, 6 and 8 h of elicitor addition. The control cultures received sterile distilled water and samples (N=3) were also

collected at each time. Each experiment was repeated three times. Suspensions were filtered on sinterized glass funnels (40-90 µm porosity) and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde solution in 50 mM phosphate buffer, pH 7.0 for 4 h at 4°C. Dehydration was made in ethanol solutions and clearing in xilol for 30 min, followed by immersion in paraffin and serial cuts with 7 µm thickness. After removal of paraffin, the material was stained with: Toluidine Blue (TB) 0.025% (λ_{\max} = 620-630 nm) in McIlvaine's buffer (pH 4.0) for detection of anionic groups and metachromasy (λ_{\max} <580-590 nm) (Vidal, 1977); Periodic Acid-Schiff (PAS) reaction for the detection of neutral polysaccharides, according to Cortelazzo (1992). The reaction with phloroglucinol/HCl (Vallet et al., 1996) was employed for lignin detection. Feulgen's reaction (Mello and Vidal, 1978) for DNA detection was employed and nuclei diameter measurement was performed on ten cells per slide (N= 40) from the treated and control materials. For Transmission Electron Microscopy (TEM), cells were fixed for 18 h in a solution containing 2.5% glutaraldehyde, 3% sucrose and 5 mM CaCl₂ in phosphate buffer (pH 7.2), followed by several washes and post-fixation with 1% osmium tetroxide. Dehydration was made in ketone, followed by sample inclusion in resin Spurr (1969). Ultra-thin cuts were stained with uranyl acetate and lead citrate. Observations were made using a TEM Zeiss (Leo 906) at 80 kv. Peroxidase activity of culture medium was assayed spectrophotometrically through the oxidation of guaiacol (George, 1953). Phenylalanine ammonia-lyase (PAL) activity was assayed in lyophilized material (Dörnenburg and Knorr, 1997). Enzyme activity was analyzed by ANOVA-*one way* and means were compared by Tukey's test or *t* test to compare two averages of treatments ($P \leq 0.05$).

RESULTS

The results obtained by staining methods are presented in Table 1. PAS reaction, which identified neutral sugars in the cell wall, revealed control cells with a more regular form (Fig. 1A), while in 6 h heterogeneity, many elongated cells were observed (Fig. 1B). Control cells presented metachromasy when stained with Toluidine Blue.

Table 1- Structures stained in sugarcane cells elicited by a chitin hydrolyzate.

Structure ^a	Control	Chitin elicited					
		1h	2h	3h	4h	6h	8h
<i>Cell wall</i>							
Acidic sugars (TB)	+++ ^b	+++	++	+	++	+++	+++
Neutral sugars (PAS)	+++	+++	+++	+++	+++	+++	+++
Metacromasy (TB)	+++	++	+	-	+	++	+++
Lignin (Phloroglucinol)	-	+	++	+++	++	+	-
<i>Cytoplasm</i>							
Protein (XP)	++	+	+	+	+	++	++
Starch grain (PAS)	+++	+++	+++	+++	+++	+++	+++

^a TB Toluidine Blue, PAS Periodic Acid Schiff, XP Xylidine Ponceau

^b (-) no stain, (+) fairly stained, (++) stained, (+++) strongly stained

This staining was reduced in chitin treated cells. The staining intensity reduction was continuous until 3h and after 4h, the staining intensity again increased (Table 1).

A positive reaction for phloroglucinol/HCL was not observed in control material (Fig. 1C), but treated material presented a progressive positive staining up to 3 h of incubation (Table 1), followed by a decrease at 8 h (Fig. 1D).

Feulgen reaction in control cells revealed the presence of smaller nuclei ($9.18 \pm 1.5 \mu\text{m}$, Fig. 1E), in relation to treated cells, which presented diameters of 11.2 ± 2.5 and $11.3 \pm 2.2 \mu\text{m}$ after 4 and 6 h, respectively (Fig. 1F). After 8 h of incubation, the nuclei diameters showed similar value ($8.69 \pm 1.9 \mu\text{m}$) as that of control material. Electron micrograph of control material showed less compacted chromatin in nuclei (Fig. 2A), while in material submitted to chitin hydrolyzate, cells with greater chromatin compactation and degenerating nucleoli were observed (Figs. 2B-C).

It was possible to observe the integrity of cell walls in control material, with their characteristic fibrillar aspect (Fig. 2D), while a cell wall polysaccharide disaggregation was observed in treated material. This phenomenon consisted in partial release of a narrow fringe of microfibrils, which became oriented perpendicular to the surface and progressively individualized (Fig. 2E) and an enhanced reactivity to uranyl acetate staining was clearly visible.

After 3h, peroxidase isozymes activity in culture medium increased for the control cells. This increase was continuous until 8h. For chitin treated cells, peroxidase isozymes activity started to increase at 2h and reached a peak at 4h, followed by a decreased at 6 and 8h (Table 2). PAL activity was only detected at 4 h after incubation (Table 2). There was a difference between the control, with treated cells and higher values for chitin treated cells.

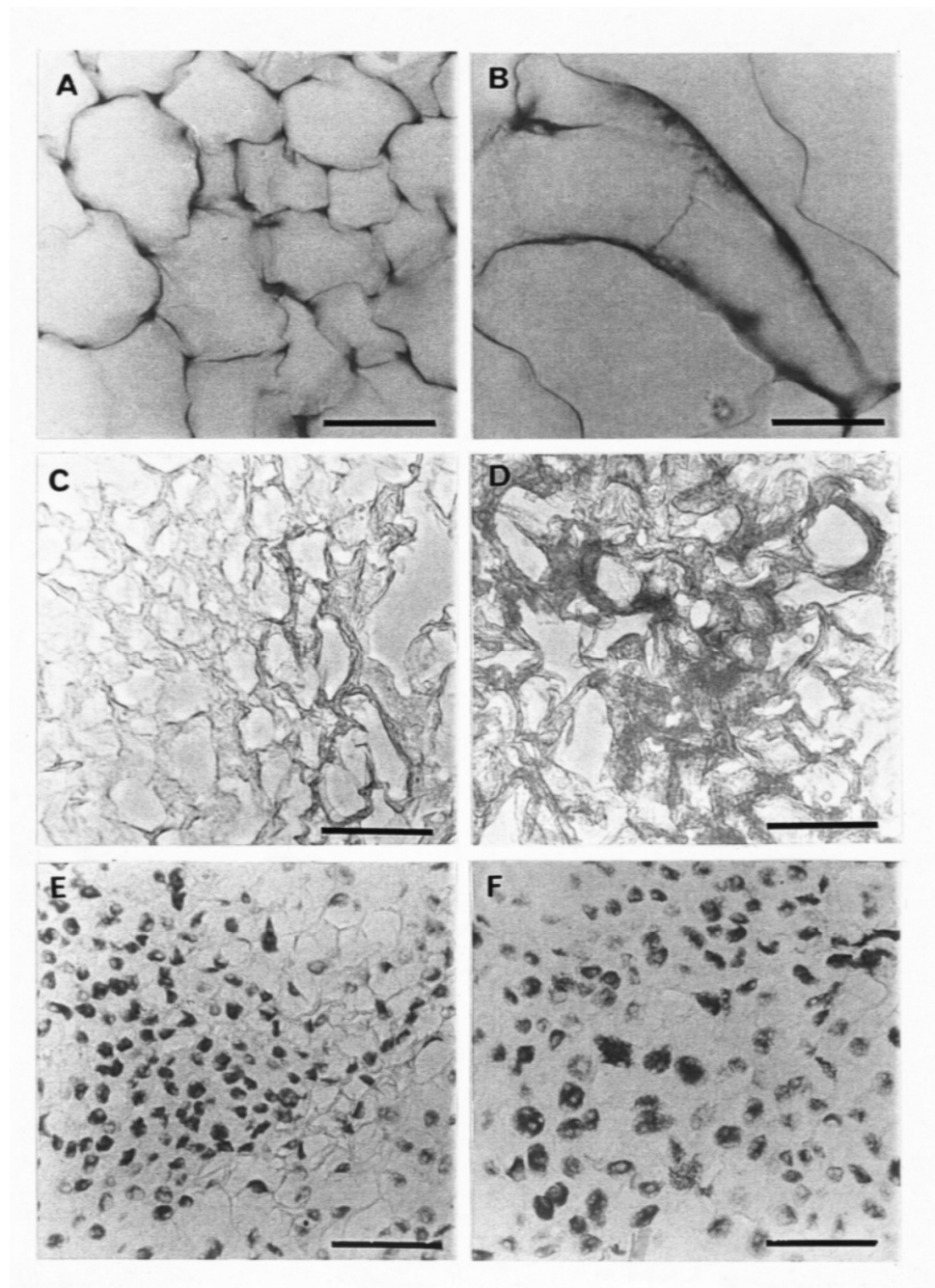


Figure 1 - Suspension cultured cells of *Saccharum officinarum*. A, C and E: Control cells; B, D and F: 6h after treatment with oligosaccharides. A-B: PAS reaction. Note the stained polysaccharides of cell walls and the elongated cells of treated material. C-D: Phloroglucinol/HCl reaction, with a positive response after 6h of incubation. E-F: Feulgen reaction. Note the nuclei smaller in control cells. Bars: A e B: 11 μm ; C, D, E, e F: 36 μm .

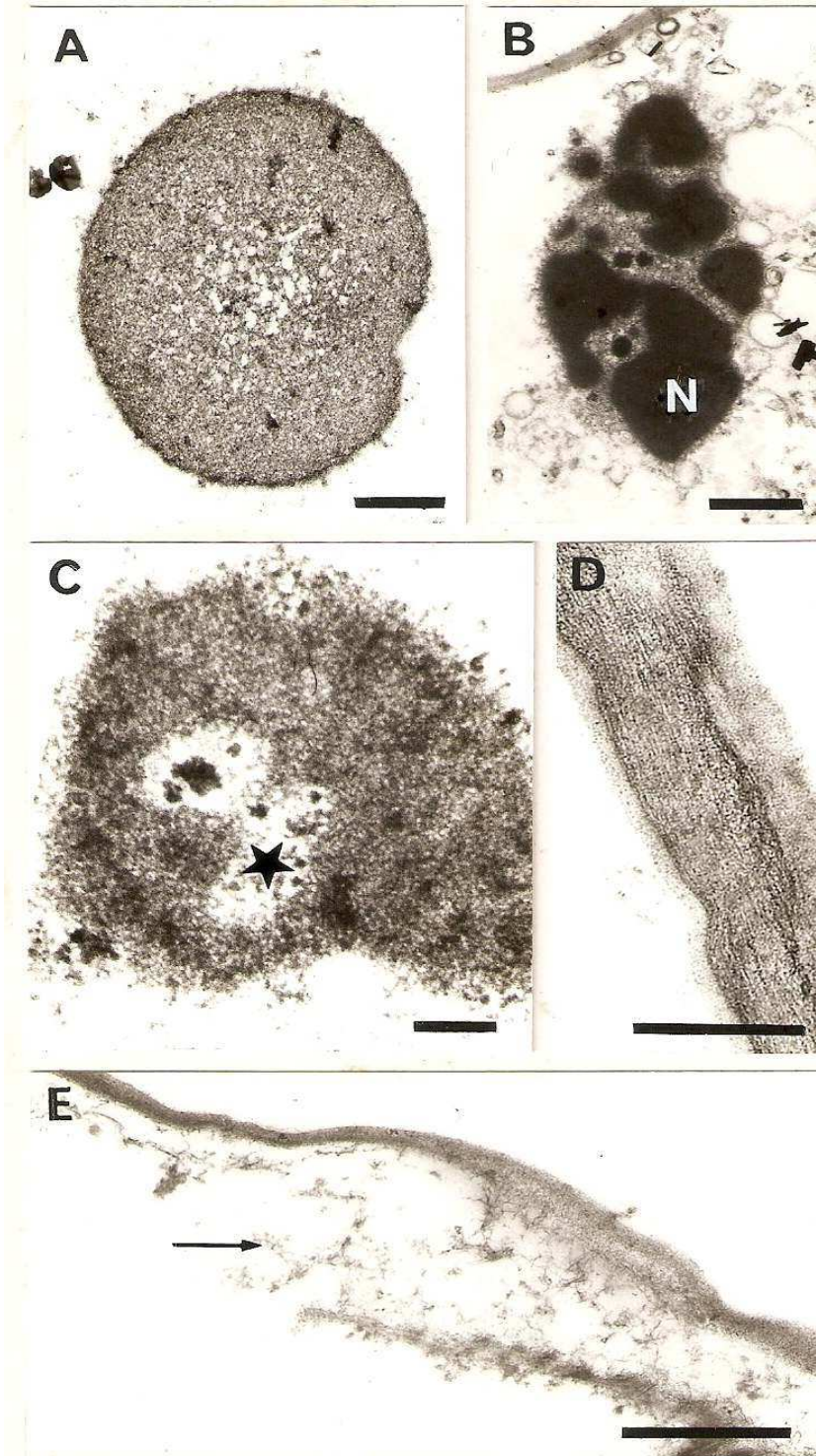


Figure 2 - Electron micrographies of control (A and D) and treated (B, C and E) material. A – Aspect of nuclei (N) with less compacted chromatin; B-C – Aspect of nuclei (N) with greater chromatin compactation and degenerating nucleoli (★) were observed. D - Cell wall of control material, with their fibrillar aspect. E: Cell wall of treated material, showing polysaccharide disaggregation with a characteristic narrow fringe of microfibrils (arrows). Bars: A e B: 1µm; C: 0,2 µm, D: 0,6 µm; E: 1µm.

Table 2 - Activities of peroxidases and phenylalanine ammonia-lyase of control and chitin-treated sugar cane cells.

Time (h)	Enzyme			
	POD (U L ⁻¹ of medium)		PAL (U Kg ⁻¹ of cells)	
	Control	Chitin	Control	Chitin
1	379 ^{d,e} ± 33	193 ^{d*} ± 80	ND	ND
2	302 ^e ± 22	264 ^{c,d} ± 48	ND	ND
3	517 ^d ± 15	377 ^{c*} ± 55	ND	ND
4	797 ^c ± 32	1220 ^{a*} ± 61	48 ^c ± 4	108 ^{c*} ± 8
6	1009 ^b ± 69	1018 ^b ± 68	117 ^b ± 8	148 ^{b*} ± 12
8	1257 ^a ± 114	969 ^{b*} ± 112	160 ^a ± 8	187 ^{a*} ± 3

Values are the mean ± SD; *n* = 3. ND = Not detected. Within a line, means with different letters are significantly different (Tukey's test, *P* < 0.05). Within each time, means for chitin-treated cells marked with an asterisk differ from the controls (*t* test, *P* < 0.05).

DISCUSSION

In the present work, the material treated with chitin hydrolyzate was less stained with TB than the control, indicating that sugarcane cells isoenzyme as a consequence of various types of stress or variations in physiological conditions are well documented, and sequential relationships between the cationic and anionic isoenzymes have been demonstrated (Gaspar et al., 1991).

The phenylpropanoid precursors biosynthesis is controlled partly by PAL. This enzyme activity increased dramatically in sugarcane cell cultures treated with chitin oligomers. An increase in PAL activity was related to lignification in elicited pine cell cultures (Campbell and Ellis, 1992), potato cell cultures (Dörnenburg and Knorr, 1997), wheat (Vander et al., 1998) and *Citrus aurantium* (Gallão et al., 2007). The increase in PAL activity detected here reinforced these observations, since this enzyme was important in lignin biosynthesis.

Thus, it could be concluded that sugarcane cells cultivated in suspension responded to chitin hydrolyzate producing lignin in the first 6 h of elicitation, but the culture had the capacity to restore their initial values and normal conditions of cultivation after 8 h of treatment. Elicitators such as chitin have a potential to be used in tests to evaluate the defence mechanisms to biotic stress such as fungal attack.

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RESUMO

Células de *Saccharum officinarum* quando submetidas a quitina hidrolisada por 1 a 8h produziram material fenólico. Essas alterações foram observadas por meio de métodos citoquímicos como o Azul de Toluidina e Floroglucinol/HCl. Após 4 h, além das mudanças nas paredes celulares houve uma mudança no padrão nuclear das células tratadas com quitina. Por observação da reação de Feulgen, houve um aumento de 96% na área nuclear no material em 6h. Para as células tratadas foram observadas regiões de cromatina compactada e um processo de degeneração do nucléolo. Nas áreas externas da parede celular existia uma desagregação dos polisacarídeos confirmando os resultados obtidos para diferentes plantas com o uso de outros elicitores. A atividade da peroxidase foi máxima após 4 h e então decresceu progressivamente. A atividade da PAL aumentou a partir de 4 h de incubação. Estes resultados mostram que o hidrolisado de quitina estimula as respostas de defesa em células de cana.

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