

SEPARATION FROM *USTILAGO SCITAMINEA* OF DIFFERENT ELICITORS WHICH MODIFY THE PATTERN OF PHENOLIC ACCUMULATION IN SUGARCANE LEAVES

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SUMMARY

Smut caused by *Ustilago scitaminea* (Syd.) is a major disease of sugarcane. Different compounds from a crude fungal extract were separated by capillary electrophoresis. These compounds produced changes in the accumulation of free phenolics and enhanced phenylalanine ammonia-lyase and peroxidase activities in sugarcane leaves of the susceptible cv. Mayarí 5514, and the resistant cv. Barbados 42231. Smut-elicitor fractions were resolved by capillary electrophoresis as different peaks. Those inducing the highest biological activity were resolved as three main peaks corresponding to negatively charged proteins, peptides or glycopeptides of medium molecular mass. These compounds enhanced the accumulation of free phenolics, mainly hydroxycinnamic acids, by activation of phenylalanine ammonia-lyase in the resistant cultivar, and hydroxybenzoic acids in the susceptible cultivar. Another important difference was the enhancement in the resistant cultivar of peroxidase, an enzyme that uses free phenolics as substrates for the activation of important mechanisms of resistance of sugarcane leaves to the fungal pathogen.

Key words: capillary electrophoresis, peroxidase, phenolic acids, phenylalanine ammonia-lyase, sugarcane smut.

INTRODUCTION

Smut caused by *Ustilago scitaminea* (Syd.) a major disease of sugarcane (*Saccharum officinarum*), affects plant growth and juice quality (Martínez *et al.*, 2000). Spore germination occurs on the internode surface and is followed by the the formation of appressoria mainly on the inner scales of young buds and at the base of emerging leaves (Waller, 1970). Entry of mycelium into the bud meristem occurs between 6 and 36 h after teliospores are deposited on the surface (Alexander and

Ramakrishnan, 1980). Hyphae grow throughout the infected plant, but mostly in parenchyma cells of the lower internodes. In the upper internodes, hyphal growth ends with the formation of whips (sori with teliospores). Hyphae do not penetrate the cells of scale leaves (Singh and Budhreja, 1964), therefore buds tightly enclosed within scale leaves can escape infection. It has been proposed that varietal resistance of sugarcane is determined by the morphological features of the buds. However, other authors have suggested that resistance is based on chemical properties rather than on bud morphology (Lloyd and Naidoo, 1983).

Resistance to disease seems to be a multifactorial process. The response phase includes accumulation of different compounds such as phytoalexins (i.e. low molecular mass antimicrobial compounds that accumulate at the sites of infection); systemic enzymes that limit pathogen invasion (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 restricts the spread of pathogens (e.g. hydroxyproline-rich glycoproteins, lignin, callose); and regulators of the induction and/or activity of defensive compounds (e.g. elicitors of plant and microbial origin, immune signal from primed plants and compounds, which release immune signals) (Kuc, 1990).

Resistance to sugarcane smut has also been associated with the accumulation of free or conjugated polyamines in the host tissues (Legaz *et al.*, 1998; Piñón *et al.*, 1999) and the production of several glycoproteins (Martínez *et al.*, 2000) which affect polarization of the cytoplasm during spore germination (Fontaniella *et al.*, 2002), impair germ tube protrusion and, ultimately, germination of the spores. It has been proposed that the inhibition of teliospore germination constitutes a defence mechanism involved in the general pattern of the resistance of sugarcane to the smut (Millanes *et al.*, 2005; Legaz *et al.*, 2005).

Infection by fungal pathogens elicits changes in the role and reaction of phenolic compounds (Sedláková and Lebeda, 2001). Early release of pre-formed phenolics and their later intensive production after stimulation of phenylpropanoid metabolism are part of resist-

ance reactions to disease in many plants (Peltonen, 1998). Pre-existing phenylpropanoids stored in the central vacuole to be later incorporated into the cell wall in healthy plants, could be released in the cytoplasm during the initial stages of plant defence against infection. This process is dependent on peroxidases (POX) and other apoplast enzymes that provide phenolic acid esterification. Only during later stages of pathogenesis, *de novo* synthesis of phenolic compounds is switched on, which is closely associated with phenylalanine ammonia lyase (PAL) (Guidi *et al.*, 2005). Lignins are formed to strengthen cell walls, and phenolics have a bearing in the hypersensitive response (Lebeda *et al.*, 2001).

In a previous paper (de Armas *et al.*, 2007) sensitivity or resistance to sugarcane smut was correlated with changes of free phenolic compounds as well as of PAL and POX activity in host leaves induced by an elicitor from *U. scitaminea* mycelium.

The aim of this work was to partially purify the smut elicitor and to correlate its biological activity with the capillary electrophoretic pattern of different smut-elicitor fractions.

MATERIALS AND METHODS

Plant material. Field-grown 12-month-old sugarcane plants of cvs Mayarí 5514 (smut resistant) and Barbados 42231 (highly susceptible to smut), were used throughout this work. Discs 1.0 cm in diameter were cut from central part of first completely developed young leaf from different stalks. Leaves were submerged in water during disc excision. Twenty discs were used in each experiment.

Elicitor purification. The elicitor was partially purified according to de Armas *et al.* (2007). Briefly, teliospores of *U. scitaminea* (20 mg) isolated from whips collected from cv. Barbados 42231 diseased plants in experimental crops of the National Institute for Sugarcane Investigation (INCA) at Matanzas, Cuba, were incubated in 200 ml of sterile Lilly and Barnett (1951) medium at 38°C for 5 days. Mycelium was harvested, washed in distilled water, dried with filter paper, weighted 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 80% (v/v) methanol were added to the pellet and the mixture shaken for 4 h at 38°C. After a further centrifugation, the pellet was washed once with 5 ml methanol and dried under air flow for 2 h. After a further wash with 10 mM phosphate buffer, pH 6.8, the pellet was resuspended in 25 ml of the same buffer and the mixture autoclaved (120°C for 30 min). Following centrifugation (10 000 g for 20 min at 4°C) the supernatant was used

as a crude elicitor.

Partial purification of the *U. scitaminea* crude elicitor (0.3 mg ml⁻¹ of protein and 0.8 mg ml⁻¹ of carbohydrates) was by gel filtration. An aliquot (5 ml) of fungal extract was chromatographed through two Sephadex columns, first a G-10 column (15×2.5 cm) then a G-50 column (30×2.5 cm) connected in series. Elution was with 10 mM phosphate buffer, pH 6.8, collecting 3 ml fractions. The void volume of the column (80 ml) was determined from the elution pattern of the tracking dye blue dextran (Sigma, St. Louis, MO, USA). Each fraction was assayed for carbohydrate content according to Dubois *et al.* (1956) and for protein according to Warburg and Christian (1941). Based on protein and carbohydrate profiles, eight different fractions of the elicitor were collected. The elicitor activity of each fraction was tested based on its effect to induce change in the accumulation of phenolic acids and in the PAL and POX activity in sugarcane leaves.

Effect of smut elicitors on sugarcane leaves. 20 leaf discs (*ca.* 0.5 g fresh weight) of both sugarcane cultivars were floated in Petri dishes in the dark on 10 mM phosphate buffer, pH 6.8, containing 4% isopropyl alcohol for 2 h at 37°C. Later, a third of the volume of each fraction was added and incubated in the dark from 24 h in the same conditions. The final volume of incubation was 15 ml. Controls were the same as above without the elicitor. For all the experiments, three replicates were made using leaf samples from different stalks.

Preparation of cell-free extracts from sugarcane leaf discs. Leaf discs incubated with or without elicitor were washed with distilled water and immediately ground to a fine powder in liquid nitrogen, which was resuspended in 5 ml 10 mM phosphate buffer, pH 6.8. Following centrifugation (10 000 g for 15 min at 2°C), the supernatant was used for enzyme assays (when needed, cell free-extracts were dialyzed against phosphate buffer) and the pellet for the extraction of phenolic acids.

Extraction and analysis of phenolic acids. The pellet was extracted with 5 ml 80% (v/v) methanol at 70°C for 1 h with continuous shaking, centrifuged at 15 000 g for 15 min at 2°C and the supernatant dried in air flow. Dry residues were dissolved in 0.2 ml acetonitrile and used for HPLC analysis.

HPLC separation was carried out using a Spectra Physics 8810 liquid chromatograph. Analytical conditions were as follows: column Tracer Excel 120 ODSB (25 cm×4.6 mm i.d.); injection, 10 µl; mobile phase, solvent A, acetonitrile (100%); solvent B acetic acid/water (2/98, v/v); gradient from 100% B (for 7 min), to 25% A + 75% B (time 35 min, then maintained for another 5 min), to 100% B (time 50 min); flow rate 0.9 ml min⁻¹; temperature, 25°C; absorbance units at full scale 0.005;

detector UV-Vis SP8490 (λ 270 nm); internal standard, 0.5 mg·ml⁻¹ salicylic acid. Standards (Sigma, St. Louis, MO, USA). were gallic acid (retention time 6.60 min), protocatechuic acid (retention time 10.46 min), *p*-hydroxybenzoic acid (retention time 16.23 min), chlorogenic acid (retention time 23.11 min), caffeic acid (retention time 23.73 min), syringic acid (retention time 24.77 min), *p*-coumaric acid (retention time 27.40 min), ferulic acid (retention time 28.49 min), benzoic acid (retention time 30.01 min) and cinnamic acid (retention time 39.48 min). Quantitative estimation of each phenol was done by using the slope of the straight line obtained by linear regression from different injected mass of phenol and their corresponding area counts.

Enzymatic assays. POX activity was assayed using reaction mixtures containing 2.7 ml 10 mM phosphate buffer, pH 6.8, 10 μ l guaiacol, 200 μ l hydrogen peroxide, and 50 μ l of supernatant from cell-free extracts. A unit of specific activity was defined as 1.0 unit of absorbance at 470 nm per mg protein per min. Controls were assayed in the absence of hydrogen peroxide (Milanes *et al.*, 2005).

PAL activity was measured according to de Armas *et al.* (2007). Cell free extracts from sugarcane leaf discs were dialyzed against 0.1 mM sodium phosphate buffer, pH 6.8, for 24 h at 4°C in the dark. Reaction mixtures contained 0.6 μ l 0.1 M Tris-HCl buffer, pH 8.8, 0.4 ml supernatant from dialyzed cell free extracts and 2 ml 10 mM L-phenylalanine (Sigma, St. Louis, MO, USA) as a substrate, in a final volume of 3.0 ml. Reaction was started by adding the substrate and carried out at 37°C from 0 to 6 h, measuring the absorbance of the mixture at 30 min intervals, using a Unicam Helios β spectrophotometer (Unicam, Cambridge, UK). The increment of the absorbance at 275 nm in the linear section of each time-course was used to calculate the amount of cinnamic acid produced and the slope of the straight line after fitting experimental data by linear regression was used as the reaction rate value. PAL activity was then expressed as μ g of cinnamic acid produced per mg protein per min. Protein was estimated according to Lowry *et al.* (1951).

Capillary electrophoresis. Fractions of smut-elicitor were frozen at -20°C, lyophilised at -80°C and 10 mbars vacuum and stored at -20°C. Lyophilised samples were resuspended in 10 mM sodium borate buffer, pH 9.2 (15 μ l m⁻¹ elicitor volume). Mesityl oxide (Fluka, Riedel-de Haën, Germany) at a concentration of 4% (v/v) in the same buffer was used as neutral marker.

Capillary zone electrophoresis was performed using a P/ACE MDQ Instrument from Beckman Coulter (Fullerton, CA, USA). Detection was monitored at 200 and 280 nm. New capillaries were conditioned with 1.0 M NaOH for 10 min at 60°C, and then 0.1 M NaOH

for 10 min at 60°C. Equilibration of the capillary was done by washing it with 25 mM sodium borate buffer, pH 9.2 for 30 min at 25°C and finally with the same buffer for 30 min at 25°C under a voltage of 17 kV. Regeneration of the capillary surface between runs was performed by rinsing it as follows: 0.1 M NaOH for 10 min, Milli-Q grade water for 10 min and 25 mM sodium borate buffer, pH 9.2 for 15 min. The buffer used as electrolyte was 25 mM sodium borate buffer, pH 9.2 (Legaz and Pedrosa, 1993). Solutions (around 40 nl) were injected under pressure (0.5 psi for 5 sec) and separated at 11 kV using 25 mM borate buffer, pH 9.2, as electrolyte. Data were acquired by using a 32 KaratTM (v 4.0) software.

Different peaks in electropherograms were established through their apparent electrophoretic solute mobility, μ'_a , which was calculated as

$$\mu'_a = (lL)/(t'_m V),$$

where *l* is the effective capillary length (to the detector), *L* the total capillary length, *V* the applied voltage across the capillary and *t'_m* (corrected migration time) the difference between migration time of the compound and that of the neutral marker (Pedrosa and Legaz, 1995).

Statistical analysis. Statistical significance of the differences between means of the analysed parameters was evaluated by Student's T-test. The F-test was used to test heterogeneity of variances. When needed, data were log transformed prior to analysis. Differences were considered significant when $P \leq 0.05$.

RESULTS

Characteristics of the partially purified fractions of the smut elicitor. Carbohydrate and protein profiles of the partial purified fungal extract (crude elicitor) isolated from *U. scitaminea* mycelium are shown in Fig. 1. According to these profiles, eight fractions were considered, fraction 1, 84-92 ml; fraction 2, 93-101 ml; frac-

Table 1. Proteins and carbohydrates concentrations from each fraction of the elicitor, separated through Sephadex G10 and G50, columns (see also Fig. 1).

Fraction number	Protein concentration (μ g ml ⁻¹)	Carbohydrate concentration (μ g ml ⁻¹)
1	25.0	37.1
2	29.3	95.0
3	13.2	29.1
4	7.6	5.8
5	4.2	0
6	2.6	12.5
7	5.0	16.8
8	1.3	31.6

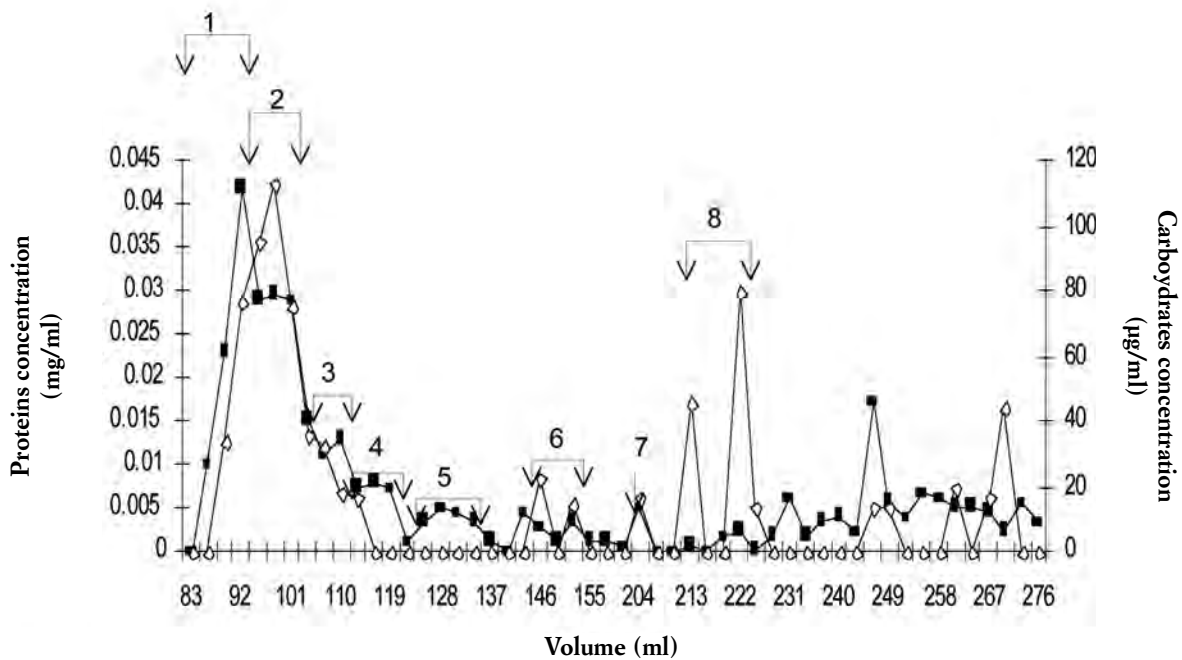


Fig. 1. Carbohydrates (empty symbols) and proteins (filled symbols) profiles of the partial purification of *U. scitaminea* crude elicitor, carried out by gel filtration through Sephadex G-10 and G-50 columns.

tion 3, 102-110 ml; fraction 4, 111-119 ml; fraction 5, 123-134 ml; fraction 6, 144-152 ml; fraction 7, 202-204 ml and fraction 8, 211-222 ml. Protein and carbohydrate concentrations of each fraction are shown in Table 1. Fraction 5 only contained protein, the others contained both protein and carbohydrates.

Effect of elicitor fractions upon phenolic content and POX and PAL activity in sugarcane leaves. According to the elution profile of partially purified elicitor and the content of both protein and carbohydrate, the effect of these eight fractions on the accumulation of phenolics in cvs Mayarí 5514 and Barbados 42231 leaves was assayed. During the incubation of leaf discs in the corresponding fraction, the following concentrations were used: 75 µg of protein and 111 µg of carbohydrates of fraction 1; 87.9 µg of protein and 285 µg of carbohydrates of fraction 2; 39.6 µg of protein and 87.3 µg of carbohydrates of fraction 3; 22.8 µg of protein and 17.4 µg of carbohydrates of fraction 4; 16.8 µg of protein of fraction 5; 7.8 µg of protein and 37.5 µg of carbohydrates of fraction 6; 5 µg of protein and 16.8 µg of carbohydrates of fraction 7 and 5.2 µg of protein and 126.4 µg of carbohydrates of fraction 8.

The content of free phenolics obtained from leaves of the susceptible cv. Barbados 42231, and from those of the resistant cv. Mayarí 5514, incubated in the absence (control treatment) or in the presence of each fraction of the partially purified elicitor, are shown in Fig. 2A. The content of free phenolics was similar in control treatments of both cultivars and no changes were ob-

tained when incubation was in fractions 1 to 4. Fractions 5 to 7 produced biological effect in both cultivars with values of phenol accumulation higher than those obtained for control treatments. The highest value of phenol accumulation was obtained with fraction 5, whereas fraction 8 produced only a slight effect on cv. Barbados 42231.

The effect of the different elicitor fractions upon PAL activity is shown in Fig. 2B. Again the highest values of enzyme activity were obtained with fractions 5, 6 and 7. Very similar pattern of POX activity was obtained for the resistant cv. Mayarí 5514, whereas only fraction 5 slightly increased POX activity in the susceptible cv. Barbados 42231. Always the resistant cultivar showed activities higher than those obtained for the susceptible cultivar for both enzymes (Fig. 2C).

Concentrations of individual hydroxycinnamic acids, caffeic, *p*-coumaric, ferulic and chlorogenic acids, and those of hydroxybenzoic acids, gallic, protocatechuic, *p*-hydroxybenzoic and syringic acids, were analyzed. Results are shown in Fig. 3. The highest values of compound concentration corresponded to *p*-coumaric (Fig. 3B) and syringic (Fig. 3H) acids, from hydroxycinnamic and hydroxybenzoic acids series, respectively.

No changes were obtained for any individual phenol when the incubation of leaf discs of both cultivars was on fractions 1 to 4. Only the concentration of protocatechuic acid (Fig. 3F) slightly increased for fractions 3 and 4 in the susceptible cultivar. Fractions 5 to 8 showed different patterns of accumulation of free phenolics for both cultivars.

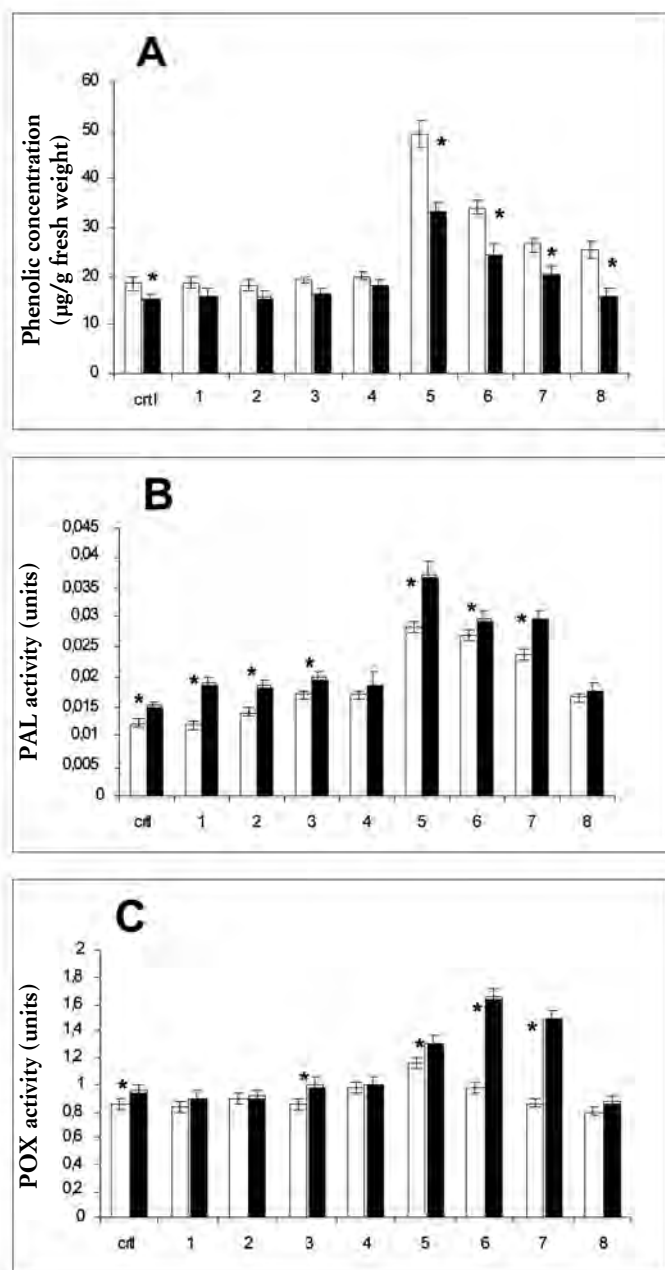


Fig. 2. Concentration of total phenolic acids (A) and assessment of PAL (B) and POX (C) activity from leaves of cvs Barbados 42231 (empty bars) and Mayarí 5514 (filled bars) incubated without (control, C) and with fractions 1 to 8 of the elicitor from *U. scitaminea*. Values are the mean of three replicates. Each replicate consists of 20 discs excised from leaves of different stalks. Vertical bars give standard error. * indicates values for treated leaves that differ at $P \leq 0.05$ from untreated (control) leaves.

In the resistant cv. Mayarí 5514, fraction 5 produced the highest increase of concentration of all phenolics analyzed. Enhanced values of phenolic concentration with respect to the control treatment were also obtained with fraction 6 for the hydroxycinnamic acids, caffeic (Fig. 3A), *p*-coumaric (Fig. 3B), ferulic (Fig. 3C) and chlorogenic acids (Fig. 3D). Fraction 7 also increased the con-

tent of ferulic acid in this cultivar (Fig. 3C). The effect was also appreciable for the content of both protocatechuic acid (Fig. 3F) and syringic (Fig. 3H) acids using fractions 6 and 7.

The phenolic accumulation pattern in the susceptible cv. Barbados 42231, was different from that of Mayarí 5514. The content of caffeic acid increased after incubation of leaf discs on fractions 5 and 6 (Fig. 3A), and that of *p*-coumaric (Fig. 3B) acid with fractions 5 to 8 with values higher than those shown the resistant by cultivar. Chlorogenic acid slightly increased after incubation on fraction 5 (Fig. 3D). For hydroxybenzoic acids, only fraction 5 enhanced the content of gallic (Fig. 3E), protocatechuic (Fig. 3F), *p*-hydroxybenzoic (Fig. 3G) and syringic (Fig. 3H) acids, with values in the susceptible cultivar higher than those found for the resistant one.

Capillary electrophoresis of different fractions of the smut elicitor. Profiles of electropherograms obtained by monitoring each fraction at 200 and 280 nm were very similar. This allowed us to establish that all compounds extracted were proteins. Results obtained at 200 nm are not shown.

According to the apparent electrophoretic mobility of solutes, μ'_a , seventeen different peaks were obtained, from 1 to 17 in correspondence with their μ'_a values. The characteristic of each peak and their relation with each fraction are shown in Table 2.

All compounds detected showed migration time values higher than that of mesityl oxide (7.70 ± 0.06 min), the neutral marker used, and for this reason, they were defined as polyanionic molecules. Their migration times varied from 11 to 33 min.

Electropherogram of fraction 1 showed only one peak with a migration time value of 11.05 min and a μ'_a value of $1.32 \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$. This peak showed the highest value of area counts (a.c. 45374) and the number 2 was assigned to it (Table 2). In fraction 2, two different peaks were detected, the first with a migration time value of 11.04 min, identical to the peak number 2, and another at 13.06 min, to which number 4 was assigned. Fraction 3 showed four new peaks at 12.74, 13.09, 13.76 and 25.00 min which were numbered as peaks 3, 4, 6 and 14, respectively. Actually, peaks 4, 6 and 14 showed low values of area counts. Fraction 4 showed also three peaks, again peak number 6 at 13.76 min with low area counts, and another important peak (No. 9), at 14.41 min. The third peak appeared at 27.5 min and showed low area counts value.

Fraction 5, the most biological active, showed six new peaks at 10.53, 13.87, 14.18, 21.50, 28.7 and 33.3 min, that were numbered 1, 7, 8, 11, 16 and 17. Of these, peaks 7, 11, 16 and 17 showed low area counts values. Fraction 6 was resolved in two peaks, a main one at 13.45 min (number 5), and another, with a very low area counts value, at 13.76 min, corresponding to the

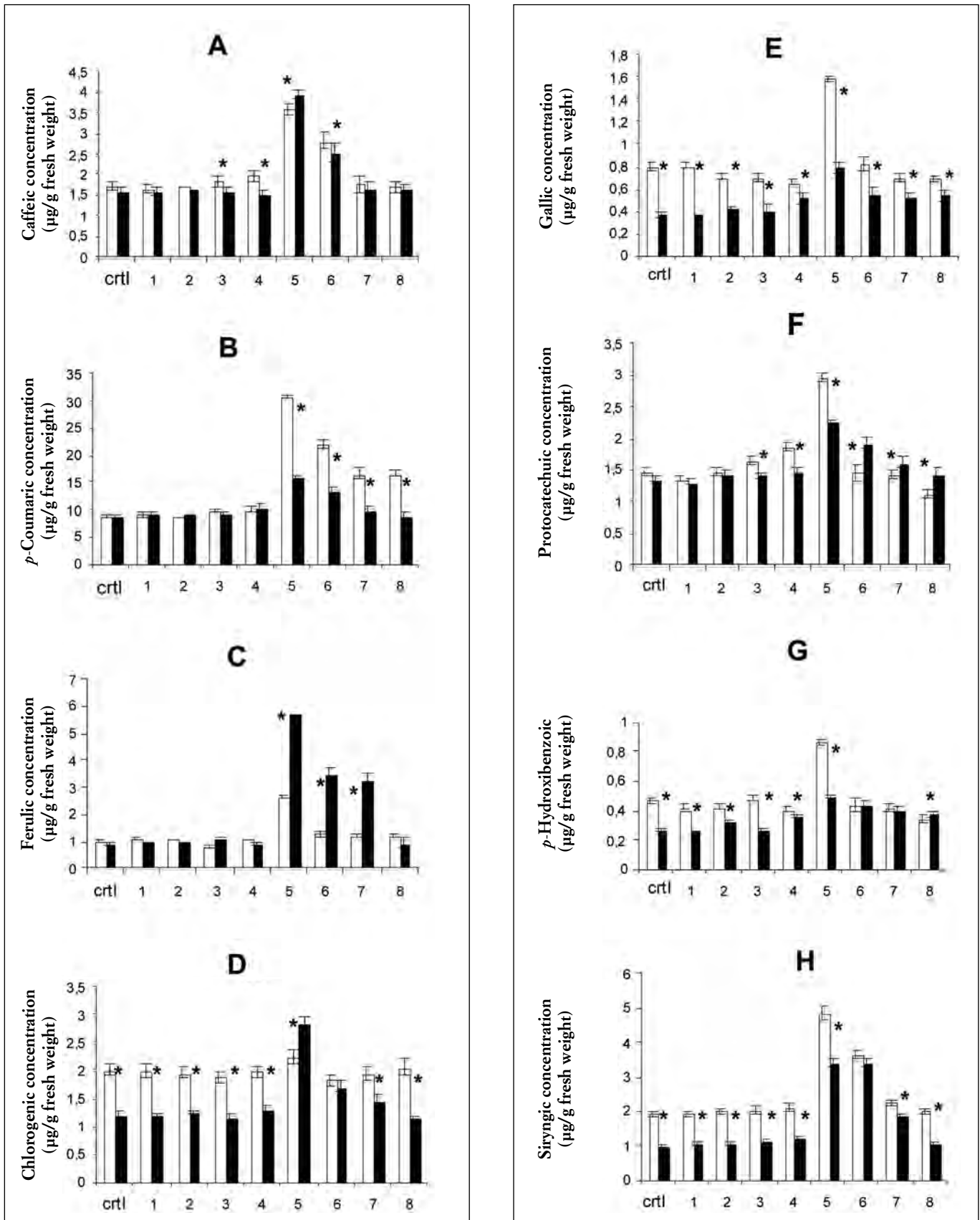


Fig. 3. Concentration of different hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA) in the leaves of cvs Barbados 42231 (empty bars) and Mayarí 5514 (filled bars) incubated without (control, C) and with fractions 1 to 8 of the elicitor from *U. scitaminea*. Values are the mean of three replicates. Each replicate consists of 20 discs excised from leaves of different stalks. Vertical bars give standard error. * indicates values for treated leaves that differ at $P \leq 0.05$ from untreated (control) leaves.

peak number 6 previously found in fraction 4. Fraction 7 showed peaks given numbers 9, 12, 13 and 16, all of which with low area counts. Finally, fraction 8 showed three peaks with a very low counts area at 14.10, 14.57 and 27.50 min, the first equivalent to the peak 8 (also in fraction 5), the second numbered as peak 10 and the third equivalent to the peak 15 from fraction 4.

DISCUSSION

According to chromatographic profiles (Fig. 1), fractions 1, 2, 3 and 4 contained proteins of high molecular mass (higher than 870 kDa). Fractions 5, 6 and 7 contained proteins of medium molecular mass (between 9 kDa and 870 kDa), and fraction 8 seemed to be mainly composed of carbohydrates and, perhaps, small peptides (fractions of low molecular mass).

Phenolic acids are precursors of a variety of antimicrobial compounds that play an important role in plant defence responses. The synthesis of these compounds has been associated with resistance of plants to fungi and viruses (Ralph *et al.*, 2006). In this study, a non-specific response of plant leaves was shown for a susceptible and a resistant sugarcane cultivar. Both cultivars increased the phenolic content when leaf discs were incubated with fractions 5, 6 and 7 of the soluble elicitor, extracted from *U. scitaminea* mycelium, and also with fraction 8 in the susceptible cultivar (Fig. 2A). Fraction 1 to 4 did not apparently show biological activity. This seems to indicate that proteins and glycoprotein of high molecular mass in the elicitor (Fig. 1) do not participate in the elicitation response of phenolic accumulation in sugarcane leaves.

In these fractions, seven different peaks were detected by capillary electrophoresis (number 2, 3, 4, 6, 9, 14 and 15), all corresponding to proteins negatively charged, according to Pedrosa and Legaz (1995), since they migrate at times higher than that of the neutral marker. Peak 2 corresponds to a glycoprotein of high molecular mass according to Fig. 1. The beginning of its chromatographic elution overlapped with that of with fraction 1 and finished in fraction 2. This fraction 2 contains another protein, or glycoprotein, of high molecular mass (peak 4) with an apparent electrophoretic mobility higher than that of peak number 3, which completes elution in the next fraction (No. 3). Fraction 3 showed peak 3 as the most representative component; it probably corresponded to another glycoprotein of high molecular mass, the elution of which was completed in this fraction. Peak number 6, with low area counts in both 3 and 4 fractions, peak No. 14 in fraction 3, and peaks 9 and 15 in fraction 4, were proteins and glycoproteins of high molecular mass composing the raw smut-elicitor extract that did not elicit a defence response in the leaves of both sugarcane cultivars.

Fraction 5 was the most biologically active. It enhanced significantly phenolic content as well as PAL and POX activity in leaf discs of both cultivars (Fig. 2). It separated as six peaks, four of them (number 1, 7, 11 and 17) unique in this fraction plus another very important peak (number 8) showing the highest value of area counts, and number 16, with a low protein content. According to data of Fig. 1, these six peaks are proteins or peptides of mid-molecular mass because no carbohydrates were detected in this fraction. Peak 1 corresponded to a negatively charged protein, with a migration time value of 10.53 min, which had a relative electrophoretic mobility, lower than that of other peaks.

Fraction 6, with high biological activity (Fig. 2), was the fraction that more strongly enhanced POX activity in the resistant cultivar (Fig. 2C). In this case, a main unique peak (number 5) was obtained in capillary electrophoresis together with a small peak at 13.76 min with an electrophoretic pattern similar to peak 6 from fractions 3 and 4. Analyzing elution profiles (Fig. 1) corresponding to fractions 3, 4 and 6, is possible that peak 6 in fraction 3 and 4 (without biological activity) corresponds to a different protein with similar capillary electrophoretic behaviour (charge/mass ratio) of fraction 7. Similar is the case of peak 9 from fractions 4 and 7, and peak 15 in fractions 4 and 8.

Fraction 7 showed biological activity (Fig. 2), and four peaks, (9, 12, 13 and 16), that correspond to peptides or glycopeptides according to Fig. 1 are responsible for this behaviour. Fraction 8, with low biological activity, was resolved in three peaks with very low values of area counts (numbers 8, 10 and 15).

Generalizing, seven different proteins or glycoproteins of high molecular mass, occurring in fractions 1 to 4 of the smut-elicitor, were separated by capillary electrophoresis. High molecular mass elicitors (proteins or glycoproteins) were previously detected in *Rhizoctonia solani* (Köhm) (Velazhahan and Vidhyasekaran, 2000) and *Colletotrichum falcatum* (Went) (Ramesh Sundar *et al.*, 2002) but these types of compounds from smut mycelium did not show biological activity. Other elicitors have been reported to be carbohydrates (Tepper and Anderson, 1986) or fatty acids (Bostock *et al.*, 1982). In our case, the fraction that elicits a resistant response in sugarcane leaves showed at least three important peaks, (number 1 and 8 in fraction 5) corresponding to proteins or peptides of molecular mass higher than 9 kDa and peak 5 in fraction 6 that must be a peptide or a glycopeptide. Eight more peaks (number 7, 11 and 16) in fraction 5, number 6 in fraction 6 and number 9, 12, 13 and 16 in fraction 7 with low value of area count were also detected. Nevertheless, there is some evidence (Ozretskovskaya *et al.*, 2004; Ramdas *et al.*, 2004) that very low concentration of some specific molecules may elicit a biological response of high intensity in particular cell mechanisms. These proteins, all of them negatively

charged in our experimental conditions, develop a differential defence response in both the resistant and susceptible cultivar, singly or in association with others,

In the resistant cv. Mayarí 5514, active fractions of the elicitor increased the content of caffeic, *p*-coumaric, ferulic and chlorogenic acids, from hydroxycinnamic acid series and gallic, protocatechuic, *p*-hydroxybenzoic and syringic acids, from hydroxybenzoic acid series. Nevertheless, the susceptible cv. Barbados 42231, increased the content of *p*-coumaric, and all hydroxybenzoic acids studied, with values higher than that in the resistant cultivar (Fig. 3).

According to the biosynthetic routes of the different phenolic acids analysed, caffeic acid is derived from *p*-coumaric acid, and ferulic acid is derived from the latter which is a precursor of the synthesis of syringic acid. Gallic acid is derived from *p*-coumaric acid through the *p*-hydroxybenzoic acid route. PAL activity is required to maintain or increase the synthesis of all these phenolics. Another important fact is the participation of *p*-coumaric and ferulic acids in the synthesis of lignin and in the formation of phenolic bridges that reinforce cell walls, requiring the action of the peroxidase enzymes.

According to our results (Table 2 and Fig. 3), it is possible to hypothesize that at least three important peaks, electrophoretically identified as number 1, 5, and 8 and eight more peaks with low values of area counts, from fractions 5, 6 and 7, enhance PAL activity (Fig. 2B) as well as the amount of some free phenolics (Fig. 3).

In the susceptible cv. Barbados 42231, the concentration of *p*-coumaric, caffeic and all hydroxybenzoic acids derived from *p*-coumaric acid, increased significantly. Nevertheless, in the resistant cv. Mayarí 5514 the production of *p*-coumaric acid derived from the accumulation of other hydroxycinnamic acids (caffeic, ferulic and chlorogenic acids) and reinforced cell walls through POX activation (Fig. 2C). This last enzyme is not activated by any elicitor fractions in the susceptible cultivar which justifies the high accumulation of free *p*-coumaric, caffeic, syringic and hydroxybenzoic acids. Plant peroxidases can be directly involved in defence mechanisms acting as a catalyst for the polymerisation of phenolic compounds to form lignin and suberin in the cell wall, which can act as mechanical barriers to block the spread of the pathogen in the plant (Lebeda *et al.*, 2001). The importance of peroxidases during plant resistance against pathogens has been demonstrated for the interaction between rice and *Xanthomonas oryzae* pv. *oryzae* (Chittor *et al.*, 1997), cotton and *X. campestris* pv. *malvacearum* (Dai *et al.*, 1996) and sugarcane and *U. scitaminea*. (Santiago *et al.*, unpublished information).

The synthesis of hydroxycinnamic conjugates with tyramine, in particular feruloyl-tyramine and coumaroyl-tyramine, has been associated with resistance of plants to fungi and viruses (Negrel and Jeandet, 1987; Keller *et al.*, 1996). In sugarcane, the formation of phenols conjugated

to polyamines was reported (de Armas *et al.*, 1999) and these compounds were shown to participate in the defence response of this plant to smut (Legaz *et al.*, 1998). In addition, de Armas *et al.* (2007) reported the participation of hydroxycinnamic acids in the defence-response of sugarcane to smut.

In conclusion, *U. scitaminea* produces proteins, peptides and glycopeptides of medium molecular mass that have been separated by capillary electrophoresis and elicit a defence response in sugarcane leaves to smut by activating phenylpropanoid metabolism. In the resistant cv. Mayarí 5514, PAL activity increases to enhance the production of hydroxycinnamic acids and, at the same time, POX activity increases to enhance the reinforcement of cell walls, using these phenolics as substrates. Synthesis of some conjugated hydroxycinnamic acids with antifungal properties are probably activated in the susceptible cv. Barbados 42231. Eliciting substances produced by fungi increase the concentration of hydroxycinnamic acid which accumulate in part as free phenolics whereas the rest serves for the *de novo* synthesis of some hydroxybenzoic acids that are also accumulated. However, they do not produce any antifungal substance with smut resistance properties.

Another possibility is that different proteins electrophoretically detected have different eliciting properties, some of them related to PAL activity and the activation of phenylpropanoid metabolism, whereas others must be related to the enhancement of POX activity, cell wall reinforcement, or synthesis of some phytoalexins. In the susceptible sugarcane cultivar, only the phenylpropanoid metabolism is activated together with accumulation of free phenolics, but not antifungal resistance ensues.

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