Eur J Plant Pathol (2009) 124:439–450
DOI 10.1007/s10658-009-9431-5

Changes in soluble and cell wall-bound hydroxycinnamic and hydroxybenzoic acids in sugarcane cultivars inoculated with *Sporisorium scitamineum* sporidia

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Received: 18 July 2008 / Accepted: 29 January 2009 / Published online: 28 February 2009

Abstract The accumulation of soluble and cell wall-bound phenolics in the sugarcane stems of young plants from highly resistant cv. My 5514 and susceptible cv. B 42231, inoculated or not inoculated with smut sporidia, was studied. The ratio of inoculated to uninoculated plants of some cell wall-bound phenolics, such as ferulic, caffeic, and syringic acids increased for the resistant cv. My 5514, whereas it was maintained more or less constantly for the susceptible cv. B 42231. The highest increase of this ratio in the resistant cv. My 5514 corresponded to both caffeic and syringic acids. This could result in a better capacity to cv. My 5514 for an increase in the frequency of bridges between lignin fragments through ester-ether linkages for reinforcing the cell wall and major resistance to the disease. This reinforcement of the cell wall could provide an effective barrier to pathogen entry and spread. Soluble sub-fractions of all phenolics detected showed non-stable patterns. Caffeic acid, that regulates phenylalanine ammonia-lyase activity in sugarcane, showed a significant decrease in its titre at 24 h in the resistant cultivar, principally in the free soluble fraction, whilst the susceptible cultivar enhanced it. We hypothesise that the pathway of hydroxybenzoic acids is only activated once the level of *p*-coumaric acid justifies the accumulation of hydroxycinnamic acids required for reinforcing the cell wall after inoculation.

Keywords Smut · Free phenolics · Glycoside-bound phenolic acids · Ester-bound phenolic acids · Cell wall-bound phenolic acids · *Ustilago scitaminea*

Abbreviations

CWBPA	cell wall-bound phenolic acids
EBPA	ester-bound phenolic acids
FPA	free phenolic acids
GBPA	glycoside-bound phenolic acids
HPLC	high-performance liquid chromatography
PAL	phenylalanine ammonia lyase
PDA	potato dextrose agar medium
PDB	potato dextrose broth medium

Introduction

Phenolics are widely distributed in plants and there are often large increases in the phenolic synthesis in plants after infection with plant pathogens (Matern et al. 1995). Infection by fungal pathogens produces

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changes in the role and reaction abilities of phenolic compounds. Early release of preformed phenolics and their later intensive production after stimulation of phenylpropanoid metabolism are a part of resistance reactions to disease in many plants (Peltonen 1998). Within the central vacuole, pre-existing phenylpropanoids are stored serving as a pool of compounds to be incorporated into the cell wall when released to the cytoplasm during the initial stages of plant defence. These processes are dependent on peroxidases and other enzymes in the apoplast that provide phenolic acid esterification. Only during later pathogenesis, *de novo* synthesis of phenolic compounds is switched on, following the transcriptional activation of genes for phenylpropanoid biosynthesis, which is closely associated with phenylalanine ammonia-lyase (PAL) (Saunders and O'Neill 2004).

Hydroxycinnamic acids, particularly ferulic and *p*-coumaric acids, occur widely in cell walls of graminaceous plants (Grabber et al. 1995). The content and composition of hydroxycinnamic acids and their derivatives depend on the morphological location and the differentiation stage. It has been reported that ferulic acid rapidly deposits in the cell wall at the early stage of lignification (Morrison et al. 1998).

Smut is an important disease of sugarcane caused by *Sporisorium scitamineum*. (=*Ustilago scitaminea*). It was first reported from Natal in 1877 during the early days of sugarcane culture in South Africa and now occurs or has occurred in most of the sugarcane-producing areas of the world (Singh et al. 2004). The disease affects plant growth and the juice quality (Martínez et al. 2000). Resistance to smut has been associated with the accumulation of free or conjugated polyamines in sugarcane tissues (Legaz et al. 1998; Piñón et al. 1999) and with the production of several glycoproteins in juice (Martínez et al. 2000) which affect germination of fungal spores (Fontaniella et al. 2002; Millanes et al. 2005, Legaz et al. 2005).

De Armas et al. (2007) correlated the susceptibility or resistance to smut with changes in the levels of free phenolic compounds and PAL, and peroxidase activities in discs of sugarcane leaf as a response to elicitors extracted from the smut mycelium. In this regard, susceptibility or resistance could be defined as the early inability or ability, respectively, of the plant host to produce major defence responses to the pathogen. *In vitro,* elicitors enhance the level of hydroxycinnamic and hydroxybenzoic acids and PAL activity, mainly in the resistant cultivar, compared with the susceptible cultivar. *p*-Coumaric and syringic acids are the phenolics that show major accumulation patterns.

Associated responses with an early accumulation of soluble phenolics after infection with plant pathogens, include the accumulation of cell-wall appositions such as papillae and the early accumulation and modification of phenols within host cell walls (Benhamou 2004) as well as the synthesis and deposition of the phenolic polymer, lignin. Strong evidence suggests that the esterification of phenols to cell wall materials is a common response in the expression of resistance (de Ascensao and Dubery 2000) and the presence of phenols in host cell walls is usually taken to imply an increase in resistance to fungal enzymes as well as a physical barrier against fungal penetration.

The aim of this work was to describe, for the first time, a phenolic fractionation in sugarcane and to relate susceptibility or resistance to smut with changes in the levels of soluble and wall-bound phenolic acids, produced by inoculated smut sporidia in young plants.

Materials and methods

The experiment was carried out with sugarcane plantlets obtained by tissue culture techniques. These methods allowed plants to be produced in a controlled physical and chemical environment avoiding recombination between different specimens and the interaction with other opportunistic pathogenic microorganisms, at least before the plants were transferred to the glasshouse.

Explant material and in vitro callus initiation

Callus culture was initiated from leaf roll tissue of mature sugarcane cultivars My 5514 (resistant to smut) and B 42231 (susceptible to smut) according to Linacero et al. (2001). A 10 cm-long meristematic tissue stack was surface-sterilised by rinsing the leaf roll in tap water, followed by swabbing with 70% (*v/v*) ethanol and 5% commercial sodium hypochlorite in sterile water. After removing aseptically the outermost mature leaves, the innermost ones were sliced into approximately 1 mm-thick sections. Leaf sections were then placed on a medium for callus induction

(MS+2) composed of a basal medium containing Murashige and Skoog (MS) inorganic salts and sucrose (30 g l^{-1}) and 12 g l^{-1} of agar as solidified agent supplemented with 2,4-dichlorophenoxyacetic acid (2 mg l^{-1}). The pH of the medium used to in vitro culture was adjusted to 5.7 before autoclaving at 121°C for 15 min. After one month of culture in darkness at 26°C, the induced calli were transferred to a propagation medium, MS medium supplemented with 2,4-dichlorophenoxyacetic acid (3 mg l^{-1}), indolacetic acid (1.2 mg l^{-1}) and kinetin (0.2 mg l^{-1}) and cultured under the same conditions. Subcultures were made in this last medium every 15-20 days. Callus tissue was then transferred to a regeneration, MS medium supplemented with kinetin (2 mg l^{-1}) and indolacetic acid (1.3 mg l^{-1}) and cultured under a 16 h-photoperiod of 12 W m^{-2} at 25°C until the complete development of the root system.

Regenerated plantlets were then transferred to sterile compost in plastic pots, sprayed with distilled water immediately after potting and covered with a plastic bag. Plants were maintained under a 16 h-photoperiod of 12 W m^{-2} at 25°C and hardened off over a 14 day period by gradually opening the plastic bag. Plants were then transferred to a glasshouse and grown under natural daylight. Plants with four complete expanded leaves were used for smut inoculation.

Teliospore germination and isolation of mating cell types

Teliospores of *S. scitamineum* collected from an infected field of cv. B 42231 (susceptible to smut) in Cuba, were rinsed three times in sterile distilled water containing 50 μM streptomycin sulphate (Sigma Chem. Co.) and plated onto potato dextrose agar (PDA) (Difco) media containing 50 μM streptomycin sulphate. Plates were incubated in the dark for 24 h at room temperature. Serial dilutions of the resultant cultures were made in potato dextrose broth (PDB), streaked onto PDA and incubated as before. Single sporidial colonies were then isolated, transferred to new PDA plates and incubated as before (Singh et al. 2004)

In order to determine the mating type of each isolate, random mating experiments were performed. An aliquot of each isolate was re-suspended in PDB and 10 μl of each isolate was mixed and plated onto PDA containing 1% charcoal (Sigma Co.). Mating reaction was then evidenced by the appearance of aerial mycelium (Banuett and Herskowitz 1989) and the isolates were arbitrarily designed as either plus or minus.

Inoculation procedure

Plants were inoculated using a Hamilton syringe with 50 μl of a suspension containing 2×10^6 sporidia ml^{-1} of a 1:1 mixture (plus and minus) of the isolated mating cell types (inoculated plants) or with 50 μl of sterile water (non-inoculated). Inoculation was carried out in the apical portion of the stem through the leaf sheath to ensure its contact with the meristematic region of the stem, which is the specific site of mycelium penetration and development in nature, when airborne dispersed teliospores are deposited on vegetative buds. Inoculum was infected into the stem 3 cm above the first leaf with a visible dewlap. Smut-inoculated and non-inoculated plants were sampled at 0, 24, 48, 72, 96 and 120 h post-inoculation. Three plants were used for each treatment.

Preparation of cell-free extracts from plants

Stem segments of 6 cm (3 cm above and below the point of inoculation) of the inoculated and non-inoculated plants sampled at different time intervals were ground in liquid nitrogen. Samples of 0.5 g of the fine powder were extracted with 7 ml 80% (*v/v*) aqueous MeOH during 24 h at 4°C and the suspension was centrifuged at 12,000 *g* for 15 min at 4°C. After centrifugation the supernatant was saved. The remaining precipitate was extracted and centrifuged as above. The two supernatants were combined, reduced to 7 ml under a stream of air and stored at -20°C. Aliquots were used in order to determine different soluble phenolic acid fractions according to de Ascensao and Dubery (2003) (free phenolic acids, MeOH soluble ester-bound phenolic acids and MeOH soluble glycoside-bound phenolic acids). The remaining precipitate was dried at 60°C for 24 h. The resulting alcohol-insoluble residue yielded the cell wall material which was used to extract the ester-bound cell wall phenolic acids after alkaline hydrolysis. All the analytical measurements were done in duplicate from three different biological samples.

Free phenolic acids

Aliquots of 1.5 ml of the stored supernatant were acidified with 240 μl of 1 M HCl and extracted twice with 2 ml anhydrous diethyl ether for 30 min with shaking at 35°C. The extraction was repeated twice. Organic phases were evaporated to dryness in a speedvac. Residues were redissolved in 0.1 ml acetonitrile and used for HPLC analysis.

Glycoside-bound phenolics

Aliquots (1.5 ml) of stored supernatant were hydrolysed with 1 ml concentrated HCl for 1 h at 96°C in a sealed vial, and then extracted twice with anhydrous diethyl ether The ether extracts were reduced to dryness in a speedvac and the remaining precipitate was resuspended in 0.1 ml acetonitrile for HPLC analysis.

Ester-bound phenolics

Soluble ester-bound phenolic acids were extracted after alkaline hydrolysis under mild conditions. To 1.5 ml of the aliquoted supernatant, 1 ml 2 M NaOH was added, and the tubes allowed to stand for 3 h at room temperature. After hydrolysis, 1.5 ml 3 M HCl was added and the phenolics extracted twice with 2 ml anhydrous diethyl ether for 30 min at 35°C with shaking. Organic phases were evaporated to dryness in a speedvac and the remaining precipitate resuspended in 0.1 ml acetonitrile for HPLC analysis.

Cell wall-bound phenolic acids

Ester-bound phenolics incorporated in the cell wall were extracted after alkaline hydrolysis (Campbell and Ellis 1992). Samples of 10 mg of dry cell wall material were resuspended in 1 ml 0.5 M NaOH for 1 h at 96°C. Under these mild saponification conditions, wall-esterified phenolic acid derivatives were selectively released. The supernatant was acidified to pH 2 with HCl, centrifuged at 12,000 *g* for 10 min at 4°C and then extracted twice with 3 ml anhydrous diethyl ether. The mixed organic phases were reduced to dryness in a speedvac and the precipitate resuspended in 0.1 ml acetonitrile for HPLC analysis.

HPLC analysis of phenolics

HPLC separation was carried out using a Spectra Physics 8810 liquid chromatograph according to de Armas et al. (2007). 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%) and solvent B: acetic acid/water (2/98, *v/v*); gradient, from 100% B (for 7 min), to 25% A+75% B (time=35 min and 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, salicylic acid, 0.5 mg ml^{-1}. 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) from Sigma Chem. Co. were used as standards. Quantitative estimation of each phenol was done by using the slope of the straight line obtained by linear regression from different injected masses of phenol and their corresponding area counts.

Statistical analyses

Statistical analyses of the differences between the mean values measured from control and treated plants were performed using the Student`s t-test. Differences were considered to be significant at $P<0.05$.

Results

Changes in the content of soluble and cell wall-bound phenolics after inoculation

Total soluble phenolic acids, free, ester-bound and phenolic glycosides as well as phenolic acids esterified to the cell wall were quantitatively determined for both control stem segments and those inoculated with sporidia from both resistant and susceptible cultivars. Figure 1 shows the effect of inoculation on the accumulation of total soluble phenolic acids and those esterified to the cell wall. The ratio of cell wall-bound

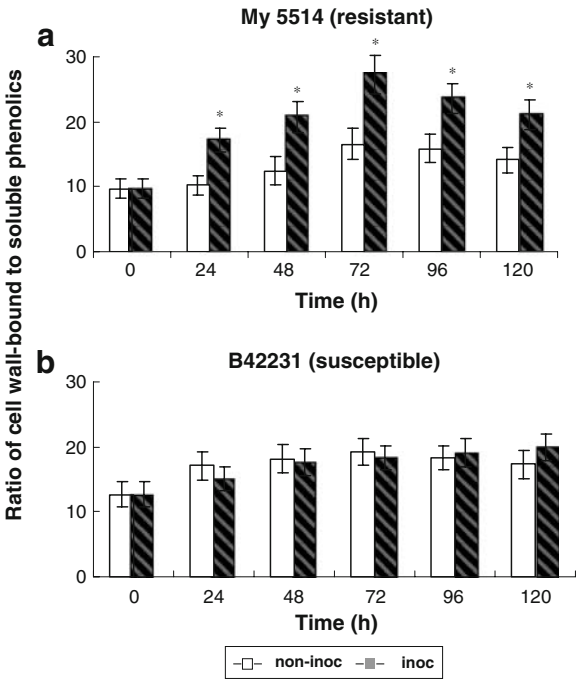

Fig. 1 Time-course of the ratio of cell wall-bound phenolics to total soluble phenolics of non-inoculated plants (empty bars) and inoculated plants (dashed bars) in cv. My 5514 (**a**) and cv. B42231 (**b**). Values represent the mean of three biological and two analytical replicates. Vertical bars represent the standard error of the means. * indicates values significantly different from the control

to total soluble phenolics increased with time up until 72 h and then decreased in the resistant cultivar (Fig. 1a). Inoculation caused a significant increase in this ratio from 24 h compared with the control. No significant difference occurred in the ratio of insoluble to soluble phenolic acids in the susceptible cultivar between control and inoculated plants (Fig. 1b).

Analysis of free hydroxycinnamic acids following inoculation

HPLC separation of the three soluble fractions of phenolic acids, free phenolic acids (FPA), glycoside-bound phenolic acids (GBPA) and ester-bound phenolic acids (EBPA) as well as the cell wall-bound phenolic acid fraction (CWBPA) was performed in both control and inoculated plants of both the resistant and the susceptible cultivars.

p-Coumaric, ferulic and caffeic acids were the main hydroxycinnamic acids detected. Table 1 shows the concentration range of those phenolic fractions that did not show any significant difference between inoculated and control plants. Concentration of free *p*-coumaric acid was very similar in both cultivars and ranged close to 2 μg g^{-1} FW (Table 1). The amount of glycoside-bound *p*-coumaric acid was higher than that observed in the free fraction. The susceptible cultivar showed a lower range, (about 50%) than the resistant cultivar (Table 1). Titer of ester-bound *p*-coumaric acid decreased with time up until 48 h in the resistant cultivar; after this time, a small increase was observed. Inoculation did not affect this time-course (Fig. 2a). In the resistant cultivar the highest accumulation of soluble *p*-coumaric acid was as an ester-bound fraction (Fig. 2b). Inoculation produced an increase of this titer that became significant from 24 h to 72 h compared with the control. CWB *p*-coumaric acid concentration was very high and was near 1 mg g^{-1} DW in both cultivars (Table 1).

Concentration of FPA ferulic acid was not detectable in the resistant cultivar and showed values <1 μg g^{-1} FW in the susceptible plants (Table 1). In both cultivars, the highest level of the soluble fractions

Table 1 Concentration range of individual phenolics obtained from cvs My 5514 (resistant) and B 42231 (susceptible) in time-course experiments

Phenolic acid	Concentration range					
	Free (μg g^{-1} fresh weight)		Glycoside-bound (μg g^{-1} fresh weight)		Cell wall-bound (μg g^{-1} fresh weight)	
	My 5514	B 42231	My 5514	B 42231	My 5514	B 42231
p-Coumaric acid	2.0-2.8	1.7-2.6	10.6-29.4	4.5-7.4	843.6-1038.6	929.6-1062.1
Ferulic acid	n.d.	0.0-1.0	0.8-2.0	0.4-2.5	794.5-1000.4	770.2-980.3
Caffeic acid	See Fig. 4a	See Fig. 4b	5.0-15.8	7.0-19.3	57.9-150.3	152.3-229.2
p-Hydroxybenzoic acid	See Fig. 5a	See Fig. 5b	3.8-5.3	3.0-3.9	11.2-46.5	11.8-20.0
Syringic acid	See Fig. 6a	See Fig. 6b	6.3-18.3	1.7-5.7	8.9-58.8	42.2-74.0

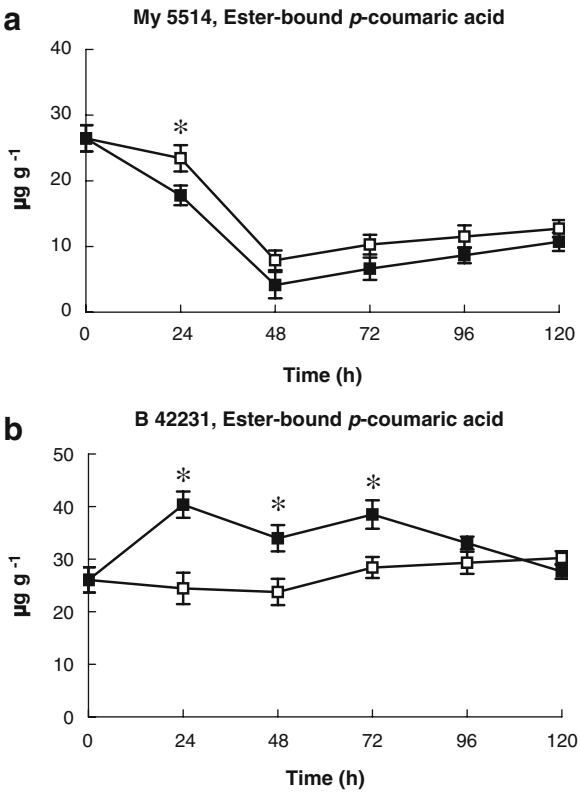

Fig. 2 The effect of inoculation on the time-dependent accumulation of soluble ester-bound *p*-coumaric acid in cv. My 5514 (**a**) and cv. B 42231 (**b**). Unfilled symbols represent the non-inoculated plants and filled symbols represent the inoculated plants. All values are the mean of three biological and two analytical replicates. Vertical bars represent the standard error of the means. * indicates values significantly different from the control

was observed for EBPA (Fig. 3a and b). The resistant cultivar maintained the concentration of ester-bound ferulic acid up to 24 h in control plants whereas inoculation produced a significant decrease of this phenolic acid after inoculation (Fig. 3a). However, the initial decrease of ester-bound ferulic acid concentration was constant with time but maintained in the susceptible cultivar. Inoculation produced significant differences from 48 h (Fig. 3b). The concentration of glycoside-bound ferulic acid was the lowest when compared with the other phenolics from the GBPA fraction (Table 1). As occurred with *p*-coumaric acid, the highest concentration of ferulic acid was detected in the CWBPA fraction of both cultivars and ranged from 0.7 to 1.0 mg g^{-1}DW (Table 1).

Concentration of caffeic acid was very similar in both the resistant (Fig. 4a) and the susceptible cultivar (Fig. 4b). There were no significant differences with time of inoculation either in the control, or in inoculated plants from the susceptible cultivar (Fig. 4b). However, concentration of caffeic acid decreased with time after 48 h, in control plants from the resistant cultivar (Fig. 4a) while inoculation produced this decrease during the first 24 h, which was significant from the beginning compared with the control. The amount of caffeic acid from the GBPA fraction did not show any significant difference with time of inoculation. Titer of caffeic acid was between 5 and 20 µg g^{-1} FW in both cultivars (Table 1) The concentration of soluble ester-bound caffeic acid was similar before inoculation in the two cultivars (Figs. 4c and d). Inoculation produced significant differences, compared with the control, only in the susceptible cultivar (Fig. 4d). In this cultivar, the highest concentration was produced at 24 h and then

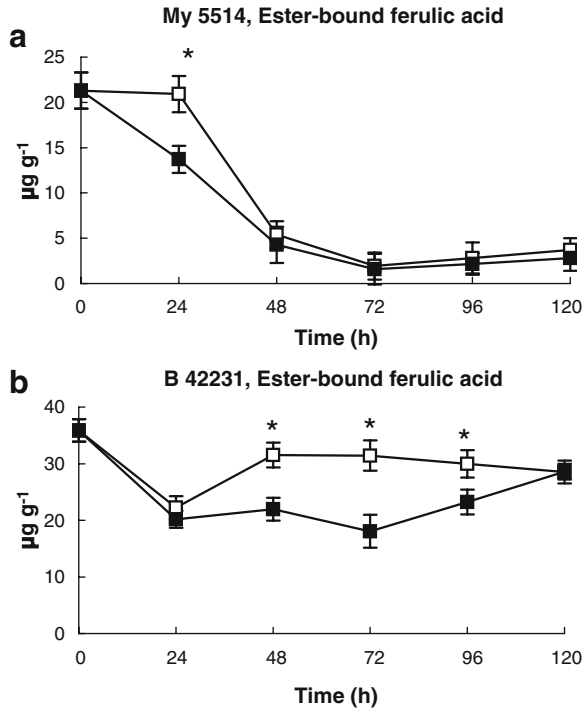

Fig. 3 The effect of inoculation on the time-dependent accumulation of soluble ester-bound ferulic acid in cv. My 5514 (**a**) and cv. B 42231 (**b**). Unfilled symbols represent the non-inoculated plants and filled symbols represent the inoculated plants. All values are the mean of three biological and two analytical replicates. Vertical bars represent the standard error of the means. * indicates values significantly different from the control

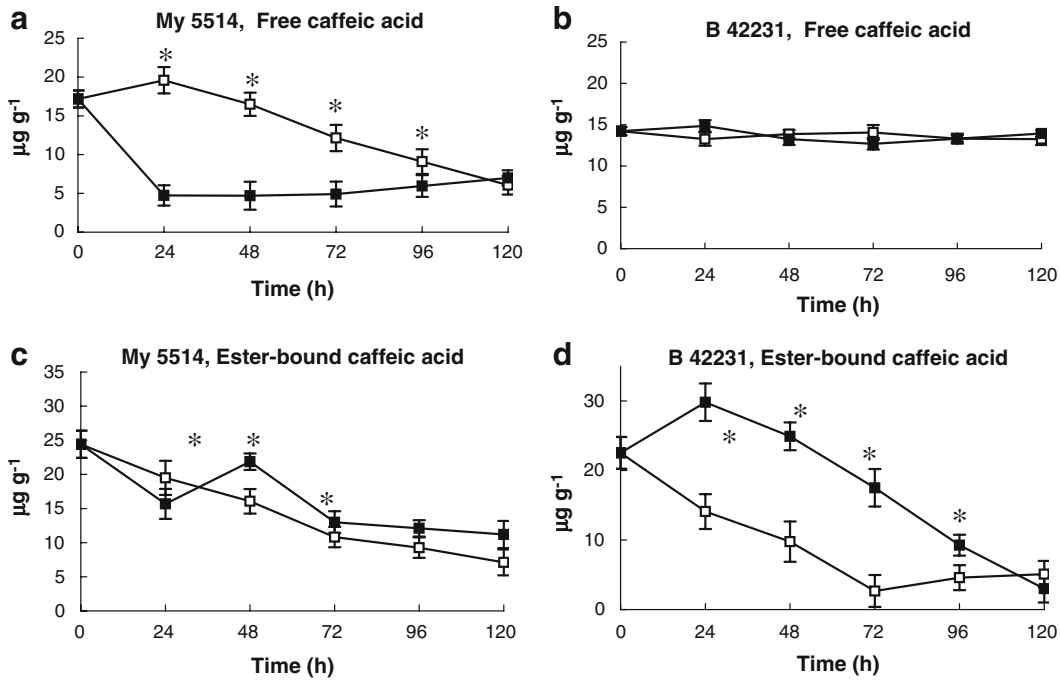

Fig. 4 The effect of inoculation on the time-dependent accumulation of caffeic acid in free (**a**), and ester-bound (**c**) soluble fractions in cv. My 5514 and cv. B 42231 (**b** and **d**, respectively). Unfilled symbols represent the non-inoculated plants and filled symbols represent the inoculated plants. All values are the mean of three biological and two analytical replicates. Vertical bars represent the standard error of the means. * indicates values significantly different from the control

diminished to the end of the treatment. In the resistant cultivar, no significant differences were observed in the concentration of soluble ester-bound caffeic acid between inoculated and uninoculated plants and between time intervals (Fig. 4c). The content of caffeic acid in the CWBPA fraction (Table 1) was much lower than the content of *p*-coumaric or ferulic acids; its concentration ranged from 58 to 230 µg g^{-1} DW.

Analysis of hydroxybenzoic acids following inoculation

The sugarcane plants contained *p*-hydroxybenzoic and syringic acids from the hydroxybenzoic acid series. Time-course of free *p*-hydroxybenzoic acid is shown in Fig. 5a and b. Titer of this phenolic was higher in the resistant than in the susceptible cultivar and its concentration decreased with time up until 72 h then increased later. The decrease in concentration was higher as a consequence of inoculation (Fig. 5a). No appreciable differences were observed either with time or as a consequence of inoculation in susceptible plants (Fig. 5b). Time-course of soluble ester-bound p-hydroxybenzoic acid is shown in Fig. 5c and d. While the amount of this phenolic was maintained in the susceptible cultivar (Fig. 5d), a gradual decrease that became significant from 24 to 72 h compared with the control, was observed in the inoculated plants of the resistant cultivar (Fig. 5c). The concentration of *p*-hydroxybenzoic acid from the GBPA fraction was low and very similar in both cultivars (Table 1). Titer of this phenolic in the CWBPA fraction was almost two times higher in the resistant than in the susceptible cultivar (Table 1).

Figure 6a and b show the accumulation pattern of free syringic acid. Concentration of this phenolic was always higher in the resistant cultivar than in the susceptible one. In the resistant cultivar inoculation produced a significant decrease of free syringic acid while non-inoculated plants maintained the level of this phenolic at least until 48 h (Fig. 6a). Control and inoculated plants maintained an almost stable concentration of syringic acid with time at low values in the susceptible cultivar (Fig. 6b). Titer of soluble ester-bound syringic acid showed a slight increment

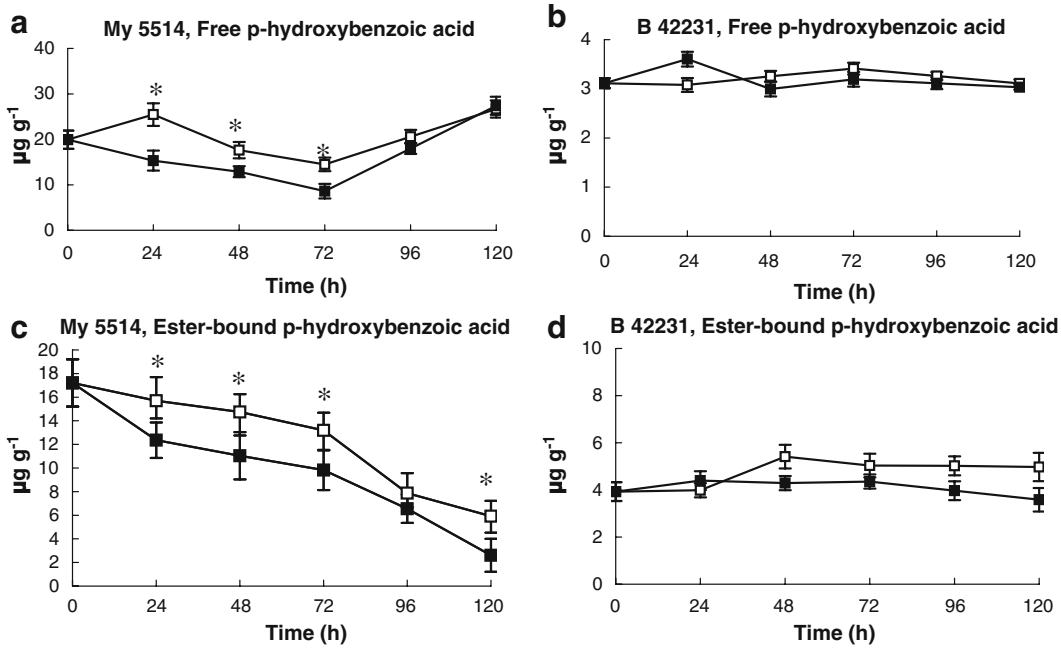

Fig. 5 The effect of inoculation on the time-dependent accumulation of *p*- hydroxybenzoic acid in free (**a**), and ester-bound (**c**) soluble fractions in cv. My 5514 and cv. B 42231 (**b** and **d**, respectively). Unfilled symbols represent the non-inoculated plants and filled symbols represent the inoculated plants. All values are the mean of three biological and two analytical replicates. Vertical bars represent the standard error of the means. * indicates values significantly different from the control

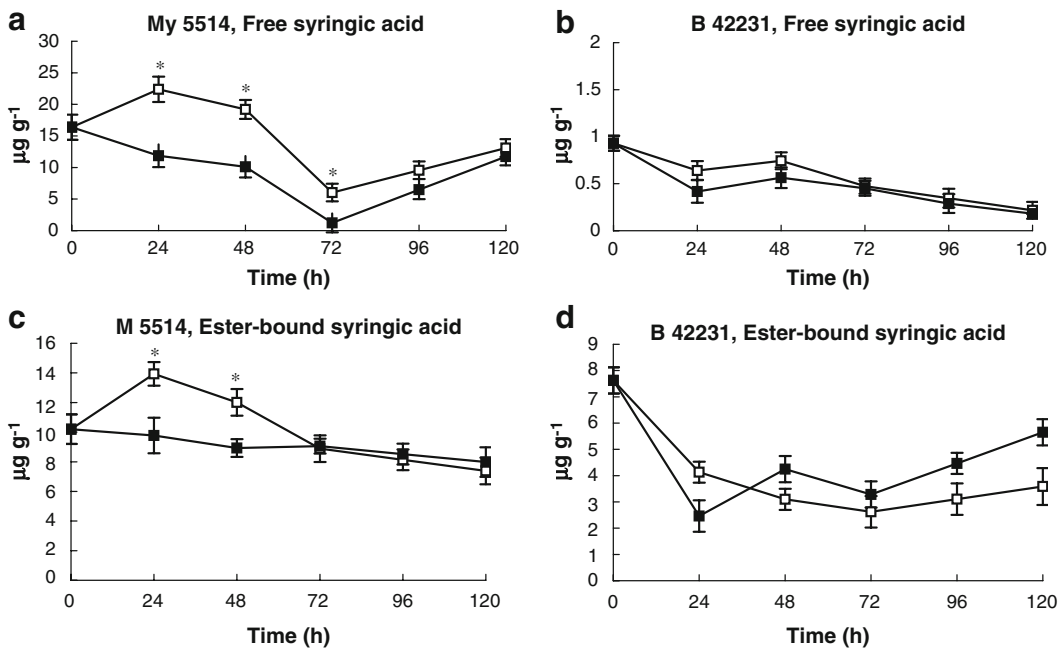

Fig. 6 The effect of inoculation on the time-dependent accumulation of soluble ester-bound syringic acid in cv. My 5514 (**a**) and cv. B 42231 (**b**). Unfilled symbols represent the non-inoculated plants and filled symbols represent the inoculated plants. All values are the mean of three biological and two analytical replicates. Vertical bars represent the standard error of the means. * indicates values significantly different from the control

after 24 h in the resistant cultivar and inoculation significantly reversed this increment (Fig. 6c). In the susceptible cultivar, the concentration of this phenolic decreased to a similar value in both inoculated and control plants during the first 24 h and was then maintained. Inoculation produced a slight and significant increase at the end of the treatment (Fig. 6d). The level of glycoside-bound syringic acid was higher in the resistant than in the susceptible cultivar (Table 1). Inoculation produced a significant decrease in this concentration only in the resistant cultivar (data not shown). Syringic acid was the main phenolic from the hydroxybenzoic acid series and its concentration was higher than that of *p*-hydroxybenzoic acid. Inoculation produced a significant increase in this titer in the resistant cultivar (data not shown).

CWBPA in which inoculation produced significant differences

Figure 7 shows the time-course of ferulic, caffeic and syringic acids with time of inoculation. In order to assess the differences in the concentration between resistant and susceptible cultivars, data were presented as the ratio of inoculated to control plants; values >1.0 indicate that the concentration of each phenolic was higher in inoculated plants than in the controls. Values <1.0 were indicative that the concentration was higher in the control plants than in the inoculated plants.

The ferulic acid ratio was always >1.0 in the resistant cultivar at the different time periods after inoculation (Fig. 7a). Significant differences were obtained at 96 and 120 h after inoculation. In the susceptible cultivar, the ferulic acid ratio was >1.0 until 48 h and then decreased. The caffeic acid ratio between inoculated and non-inoculated plants showed significant differences in the resistant cultivar from 48 h, which was always >1.0. The ratio was almost constant and close to 1.0 in the susceptible cultivar (Fig. 7b).

Differences in cell wall-bound syringic acid concentrations between control and inoculated plants from the two cultivars are shown in Fig. 7c. The syringic acid ratio was maintained up until 24 h in both cultivars. However, after this period of time, this ratio significantly increased to 2.1 at 48 h and then was maintained in the resistant cultivar. On the contrary, a significant decrease as compared with the

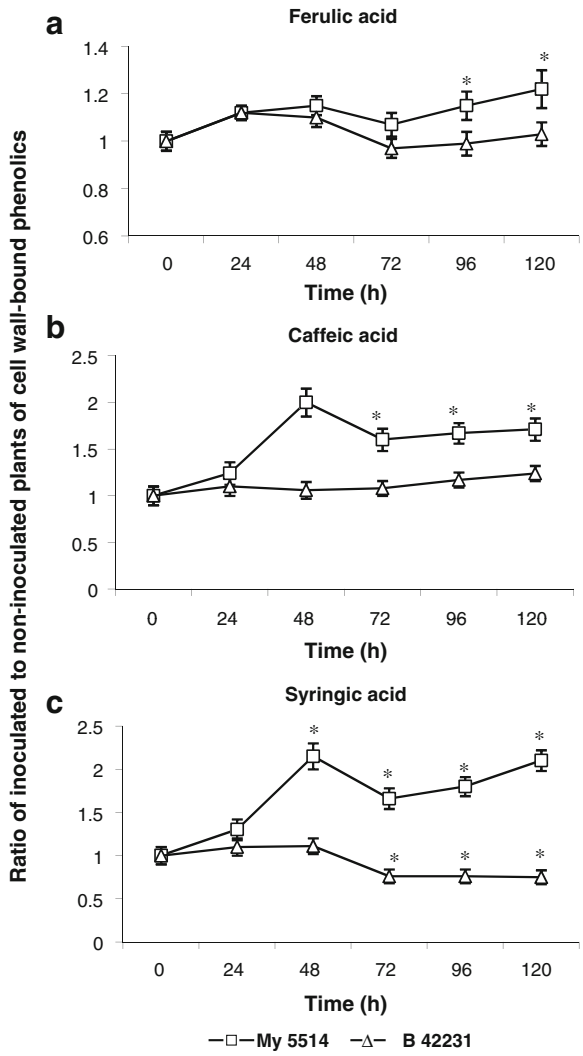

Fig. 7 Time-course of the ratio of cell wall-bound ferulic (**a**), caffeic (**b**) and syringic (**c**) acids from inoculated to non-inoculated plants in cvs My 5514 (square) and B 42231 (triangle). Vertical bars represent the standard error of the means. * indicates values significantly different from the control

control was observed in the susceptible cultivar (Fig. 7c). The increment in the content of lignin in the resistant cultivar was 27.1% 24 h after inoculation while this increment was only 4.4% in the susceptible cultivar (data not shown).

Discussion

Smut sporidia inoculated onto sugarcane young plants caused differential changes in the titer of phenolic

acids in both cultivars, the resistant and the susceptible. In general, both cultivars showed enhanced levels of phenolic acids, a typical plant defence reaction (Sedlářová and Lebeda 2001), but in this case, the resistant cultivar showed increased amounts of phenolic acids in the CWBPA fraction, more than the susceptible cultivar. This result brought about an important change in the ratio between cell wall-bound and soluble phenolic acids where higher values were observed in the resistant cultivar than in the susceptible (Fig. 1a and b). Cell wall-bound phenolic compounds constitute the factor which intervenes in host cell wall resistance to the action of cell wall-degrading enzymes (CWDE) of some pathogens (El Modofar and El Boustani 2001). The insolubilisation of phenolic compounds in the cell wall can modify its mechanical properties and decrease its extensibility (Ikegawa et al. 1996), and consequently the cell wall is less biodegradable (Matern et al. 1995). Therefore, the abundance of phenols in the cell wall makes polysaccharides less sensitive to the CWDE of pathogens (Matern et al. 1995; Ikegawa et al. 1996). In addition to this mechanical role, cell wall-bound phenolics inhibit the activity and the production of CWDE by pathogens (El Modofar and El Boustani 2001).

The main phenolic acids detected in soluble as well as in cell wall-bound fractions in decreasing order of their quantities were, *p*-coumaric acid> ferulic acid> caffeic acid> syringic acid> *p*-hydroxybenzoic acid. The amounts of these phenolics in the tissue used in this experiment (stems of young plants), were always higher than those observed in leaves of the same cultivars reported by de Armas et al. (2007). The content and composition of hydroxycinnamic acids and their derivatives are dependent on the morphological location and the differentiation stage (Morrison et al. 1998).

The abundance of *p*-coumaric and ferulic acids, mainly in the CWBPA fraction (Table 1), represents a characteristic of monocotyledons (Shibuya 1984). Jung and Deetz (1993) proposed that ferulic acid is esterified to polysaccharide components in the developing primary cell wall only, not the secondary cell wall, and provides ether bond sites for the initial monolignol attachment to the primary cell wall. However, esters of *p*-coumaric acid cannot serve as initiation sites for lignification. Though some *p*-coumaric acid is esterified to arabinoxylan in the same manner as ferulic acid, most of this is esterified to the lignin. Similarly, based on the cell wall composition of maize internodes of varying maturity, Morrison et al. (1998) concluded that the role of ferulic acid in lignification occurs early in the primary cell wall and is essential to its initiation, whereas the role of *p*-coumaric acid occurs later, in the secondary wall, and serves to bind together the growing, predominantly syringyl, lignin polymer. It is also well known that ferulic acid is associated with lignins through acid-labile bonds at the β-position of the lignin side chain, and polysaccharides through an alkali-labile ester bond with C-5 of arabinose substituents of arabinoxylans (Lam and Iiyama 2000). Sun et al. (2002) suggested that ferulic acid may also form intra- and/or inter-molecular ester-ether bridges between lignin fragments, but *p*-coumaric acid was not involved in these bridges.

Caffeic, *p*-hydroxybenzoic and syringic acids were also found in the CWBPA fraction of both cultivars, with higher values for inoculated plants of the resistant cultivar than for uninoculated plants (Table 1 and Fig. 7). *p*-Hydroxybenzoic acid occurs in the lignin fraction of walls as ester-linked forms and these are esterified mainly to lignin, not to wall polysaccharides (Kim et al. 1995); it may be involved in bridges through ester-ether linkages between lignin fragments (Sun et al. 2002). Syringic acid is not involved in ester-ether bridges between lignin molecules; it is etherified in lignin fractions (Sun et al. 2002). Major levels of all these phenolics in the resistant cultivar as well as their enhancement with time of inoculation, could indicate that the resistant cultivar has a better capacity than the susceptible cultivar for reinforcing the cell wall after inoculation with smut, and as a consequence, could have major resistance to the disease.

It is important to observe the caffeic acid pattern from soluble phenolic acids fractions. It showed a significant decrease in its titer at 24 h in the resistant cultivar, principally in FPA (Fig. 4a) and EBPA (Fig. 4b) fractions, whilst an enhancement occurred in the susceptible cultivar (Fig. 4b and d). Accumulation of caffeic acid produces a feedback inhibition of PAL activity in sugarcane, according to Millanes et al. (unpublished data) and decreased the total hyphal growth of mycorrhizal fungi (Douds et al. 1996).

According to the biosynthetic routes of the different phenolic acids analysed here, *p*-coumaric

acid is synthesised with PAL participation. Caffeic acid is derived from *p*-coumaric acid, and from this derives ferulic acid which is a precursor in the synthesis of syringic acid. *p*-Hydroxybenzoic acid is also derived from *p*-coumaric acid. PAL activity is fundamental to maintain or increase the synthesis of all these phenolics and according to de Armas et al. (2007), resistance to smut in sugarcane is associated with the possibility of maintaining high levels of PAL activity. Only the resistant cultivar was able to maintain high levels of PAL activity for longer periods of time compared to susceptible cultivars.

Soluble sub-fractions of all phenolics detected showed non-stable patterns. For example, the free fraction of the hydroxybenzoic acids detected (*p*-hydroxybenzoic and syringic acids) enhanced their levels from 72 to 120 h after inoculation for the resistant cultivar (Figs. 5a and 6b). A similar pattern was observed in *p*-coumaric acid (data not shown). This contrasts with the results of de Ascensao and Dubery (2003) that showed a constant increase of these fractions in a very short time with stability up to 24 h after *Musa acuminate* roots were exposed to elicitors from *Fusarium oxysporum*. It would be possible to hypothesise that in the present work, the route of hydroxybenzoic acids, was only activated once its precursor, *p*-coumaric acid, began its accumulation after satisfying the level of hydroxycinnamic acids necessary for reinforcing the cell wall after inoculation. This hypothesis needs to be confirmed.

Phenylpropanoid biosynthetic pathways are among the most frequently observed metabolic activities that are induced after infection of plants with pathogens. Several intermediates of the general phenylpropanoid pathway have been reported to possess antimicrobial activity and some of the lignin-specific pathway intermediates are potential phytoalexins (Barber et al. 2000). In a previous study, working with discs of sugarcane leaves from the same cultivars incubated with smut elicitors, our group demonstrated that resistance to smut was associated with high PAL activity and free phenolics accumulation (de Armas et al. 2007). The results obtained in the present study, with regard to the partitioning of phenolics into the various phenolic sub-fractions, give a detailed analysis of phenolic metabolism in sugarcane inoculated with smut sporidia, indicating that the increased flux through the phenylpropanoid pathway resulted in the synthesis of phenolic monomers that were eventually esterified and incorporated into the cell wall. This could be related to an increase of 27.1% in the content of lignin in the resistant cultivar after 24 h inoculation and only 4.4% in the susceptible cultivar. This reinforcement of the cell wall represents an effective barrier to pathogen entry and spread and could be one of the strategies developed by the resistant cultivar.

Acknowledgements This work has been supported by grants from the Agencia Española de Cooperación con Iberoamérica A/010305/07 (AECI) and Ministerio de Educación y Ciencia (Spain) BFU2006-14263 (MEC). We gratefully acknowledge the excellent technical assistance of Mrs. Raquel Alonso.

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