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Phytochemistry 63 (2003) 679–686
PHYTOCHEMISTRY
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Soluble and wall-bound phenolics and phenolic polymers in *Musa acuminata* roots exposed to elicitors from *Fusarium oxysporum* f.sp. *cubense*

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Received 6 January 2003; received in revised form 22 April 2003

Abstract

The accumulation of soluble and wall-bound phenolics and phenolic polymers in *Musa acuminata* roots exposed to cell wall-derived elicitor from the pathogen, *Fusarium oxysporum,* f.sp. *cubense*, race four, was investigated. The root tissue from the banana cultivar 'Goldfinger' was found to respond strongly and rapidly towards the elicitor through the increased synthesis of phenolic compounds. Following elicitation, the conjugated and non-conjugated phenolic metabolites in the induced root tissue were extracted and quantified. Induced phenolic synthesis was rapid and reached near maximum values after 16 h. High-performance liquid chromatography revealed both compositional and quantitative differences between induced phenolics (*p*-coumaric, ferulic, and sinapic acids) and those constitutively present (*p*-coumaric- and ferulic acid). In addition, vanillic acid was found in the ester-bound fraction and protocatechuic acid in the cell-wall bound fraction of elicited tissue. The deposition and accumulation kinetics of polymerized phenolic monomers as lignin and lignin-like polymers was quantified over a time period of 0–36 h and found to reach maximum values after 24 h. Ionization difference UV spectra of lignin indicated compositional differences in the newly synthesized lignin fraction and correlated with increased concentrations of ferulic acid and sinapic acids esters. The results show that the increased flux through the phenylpropanoid pathway resulted in the synthesis of cinnamic acid and benzoic acid derivatives that were esterified and incorporated into the cell wall fraction as part of the anti-microbial defenses activated in the root tissue.

Keywords: *Musa acuminata*; Musaceae; Banana; Phenolic compounds; Lignin; Fusarium wilt

1. Introduction

Phenolics are widely distributed in plants and there are often large increases in phenolic synthesis in plants after infection with plant pathogens (Matern et al., 1995). Some occur constitutively and function as pre-formed inhibitors (phytoanticipins) associated with nonhost resistance, while others, (phytoalexins), are formed in response to the entrance of the pathogen and their appearance is considered as part of an active defence response (Nicholson and Hammerschmidt, 1992). Phenolic based defence responses are characterised by an early accumulation of phenolic compounds at the infection site. It is thought that rapid accumulation of toxic phenols may result in the effective isolation of the pathogen at the original site of entrance (Matern and Kneusel, 1988; Fernandez and Heath, 1998). Associated responses include the accumulation of cell-wall appositions such as papillae and the early accumulation and modification of phenols within host cell walls (Shiraishi et al., 1989) as well as the synthesis and deposition of the phenolic polymer, lignin.

Evidence strongly suggests that esterification of phenols to cell-wall materials is a common theme in the expression of resistance (Fry, 1987), 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. Moreover, the accumulation of polymerized phenols occurs as a rapid response to infection (Grand et al., 1987; Hammerschmidt, 1984). The esterification of phenylpropanoic acids to the host cell wall (Matern and Kneusel, 1988) and cross-linking of such phenylpropanoid esters via ether bonding, has been suggested to lead to the formation of lignin-like polymers by supplying lignin

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doi:10.1016/S0031-9422(03)00286-3

attachment sites to the matrix polysaccharides (Lewis and Yamamoto, 1990). Accordingly, phenols in the primary cell wall were suggested to function as a template for further lignin deposition and thus esterification and lignification may be regarded as contiguous rather than separate processes that gradually integrate (Matern et al., 1995). Lignin, a polymer of aromatic phenolics, is formed as a response to mechanical damage or wounding and many plants respond to microbial attack by deposition of lignin and other wall-bound phenolic material at the point of attack (Boudet et al., 1995).

These defense mechanisms appears to be important determinants affecting the host response, and may be possible contributing factors to the resistance mechanism in *Musa acuminata* (Valette et al., 1998; de Ascensao and Dubery, 2000). The aim of the current work was to identify changes in the levels of soluble and wall-bound phenolics metabolites that may be indicative of underlying resistance-related biochemical pathways. In this communication we report on the chemical nature and accumulation kinetics of several soluble and wall-bound phenolics as well as phenolic polymers and lignin in root tissue from the banana cultivar 'Goldfinger' in response to an elicitor fraction derived from the cell wall of *Fusarium oxysporum* f.sp. *cubense* race four, the causative agent of Fusarium wilt disease.

2. Results and discussion

Most phenolic compounds are present in plants in conjugated form, principally with a sugar residue linked through one or more of the phenolic hydroxyl groups, or as conjugated esters. The free phenols and phenolic acids are generally considered together, since they are usually identified together during plant analysis. Alkali or acid hydrolysis of extracts releases a number of ether-soluble phenolic acids that are either associated with lignin combined as ester groups or present in the alcohol-soluble fraction bound as simple glycosides (Harborne, 1991).

2.1. Changes in the content of phenolic acids following treatment with elicitor

Total soluble phenolic acids, free phenolic acids, ester-bound phenolic acids, phenolic glycosides and phenolic acids esterified to the cell wall were quantitatively determined for both control roots and roots treated with elicitor. Fig. 1 shows the effect of elicitation on the accumulation of total soluble phenolic content and the content of phenolic acids in the four different fractions for elicited roots. Increased phenolic synthesis was already measureable at 4 h and reached plateau values at 16 h post-elicitation, that stabilised at 24 and 36 h, the last point in the time series. The rapid synthesis of

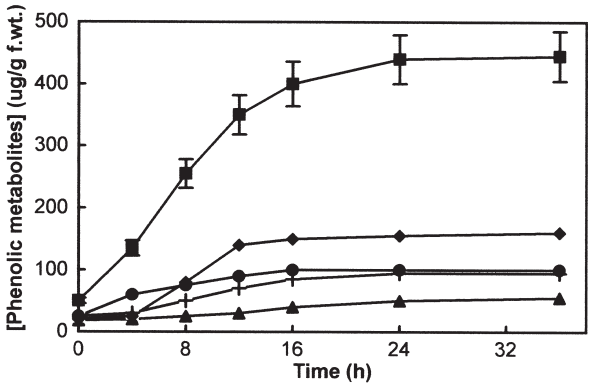

Fig. 1. Effect of elicitation on the accumulation of total soluble (■), free phenolics (▲), phenolic esters (+), phenolic glycosides (●) and cell wall-bound phenolic acids (◆) for banana root tissue. MeOH extractable material from elicitor-treated roots were quantified with the Folin–Ciocalteau reagent at the time points as indicated. Glycoside and ester-bound phenolics were released following acid and base hydrolysis, respectively. The cell wall bound fraction was released from the alcohol insoluble residue following saponification. Bars represent the standard error (mean ± S.E., $n = 9$).

low molecular weight phenols, triggered by the exposure of plant tissues to cell wall-derived elicitors of pathogen origin, is a well described phenomenon, forming part of the defense arsenal of plants (Nicholson and Hammerschmidt, 1992). However, the determination of the various sub-classes of phenolics is seldom performed or mentioned in the literature.

In the case of the banana root: *Fusarium* elicitor interaction, comparison of the phenolic levels at 36 h compared to 0 h, indicates a 4.5-fold increase in total phenolics, while the sub-fractions of cell wall-bound phenolics, ester-bound phenolics, glycoside-bound phenolics and free phenolics increased 6.3-, 4.2-, 3.0-, and 2.3-fold respectively. The comparative values for the unelicited controls exhibited only a 1.2 to 1.5-fold increase during the same period, probably due to a wound response. Little is known about lignification and cell wall reinforcement as a defense response of *Musa acuminata,* but the level of induction found in this study is similar to host responses reported in other plant: pathogen interactions (Codignola et al., 1993; Cvikrová et al., 1993; Candela et al., 1995) and the high increase in cell wall bound phenolics correlates with the deposition of lignin (section 2.3).

2.2. HPLC analysis of the four different phenolic fractions

Elicitor treatment of roots resulted in a greater number of separated metabolites compared to the corresponding controls. The retention times of the phenolic acids isolated from elicitor-treated roots were compared with an external standard composed of a mixture of nine phenolic acids: the benzoic acids; gallic ($R_t = 2.21$), *p*-hydroxybenzoic ($R_t = 3.23$), protocatechuic ($R_t = 4.61$), vanillic

($R_t = 7.43$) and syringic acid ($R_t = 11.47$); as well as the cinnamic acids, caffeic ($R_t = 8.52$), *p*-coumaric ($R_t = 14.54$), ferulic ($R_t = 18.00$) and sinapic acid ($R_t = 19.71$).

HPLC separation of free phenolic acids, extracted from elicitor treated Goldfinger roots as well as the corresponding controls revealed the elution of one major peak, ferulic acid, the area of the peak due to elicitation being twice that of the corresponding control (chromatogram not shown).

Analysis of glycoside-bound phenolic acids for elicitor-treated roots revealed the elution of two peaks, *p*-coumaric acid and ferulic acid. A moderate increase in the intensity of the peaks and in the complexity of the chromatogram pattern of glycoside-bound phenolic acids, compared to the corresponding control, was observed, (chromatogram not shown). Treatment of roots with elicitor resulted in the appearance of one new peak, caffeic acid, that was not present in the control sample.

In accordance with the results found with Folin–Ciocalteau determination, treatment of roots with elicitor induced a significant number of ester-bound phenolic acids that could be separated by reverse-phase HPLC. As shown in Fig. 2A, elicitor treatment induced two new peaks, peak 4, vanillic acid and peak 1, sinapic acid, compared to the corresponding control. Two peaks, *p*-coumaric acid and ferulic acid were constitutively present in the control samples and significantly increased in the elicited tissue extracts. Thus, all the cinnamic acid precursors of lignin were present in the extract.

Elicitor treatment also induced a strong increase in the amount of phenolic material bound to the cell wall. As shown in Fig. 2B, elicitation induced four peaks: 1, sinapic acid; 2, ferulic acid; 4, vanillic acid and 5, protocatechuic acid, in contrast to the two peaks; 6, ferulic acid and 7, *p*-coumaric acid, that were constitutively present. All the lignin precursors were again found to be present in the extracts from elicited tissue. The presence of sinapic acid in the ester- and cell-wall bound fractions in the extracts from elicited tissue is interesting since it was not found to occur in the control tissue. Previously, the presence of a sinapyl alcohol dehydrogenase in elicited banana roots was reported (De Ascensao and Dubery, 2000).

Many monocotyledon plants have lignin to which various aromatic acids, predominantly *p*-hydroxybenzoic, ferulic and *p*-coumaric acids are esterified. General or non-specific esterification (Vance et al., 1980) takes place in cell walls and might in itself play a role in resistance. High levels of caffeic- and ferulic acids have also been indicated by histochemical staining to occur in banana cultivars with partial resistance to nematodes (Valette et al., 1998).

2.3. Accumulation of phenolic polymers and lignin following elicitation

Accumulation of lignin and phenolic polymers in cell walls of banana root tissue was assayed quantitatively by derivatization with thioglycolic acid from alcohol-insoluble residues (AIR) of root cell wall material. UV spectra of solutions containing lignin thioglycolate (LGTA) from 10 mg AIR in 0.5 M NaOH were obtained from control and elicited roots over time and the absorbance at the characteristic λ_{max} of 280 nm (Barber and Ride, 1988) determined. As shown in Fig. 3A, the first detectable increase in lignin occurred between 4 and 8 h following elicitor treatment. Lignin accumulated at a rapid and relatively constant rate during the 12–24 h interval, followed by a slow increase up to 36 h. Elicitor treatment induced a 6.0-fold increase in lignin content compared to the untreated controls, which responded with a 1.8-fold increase over the same time period.

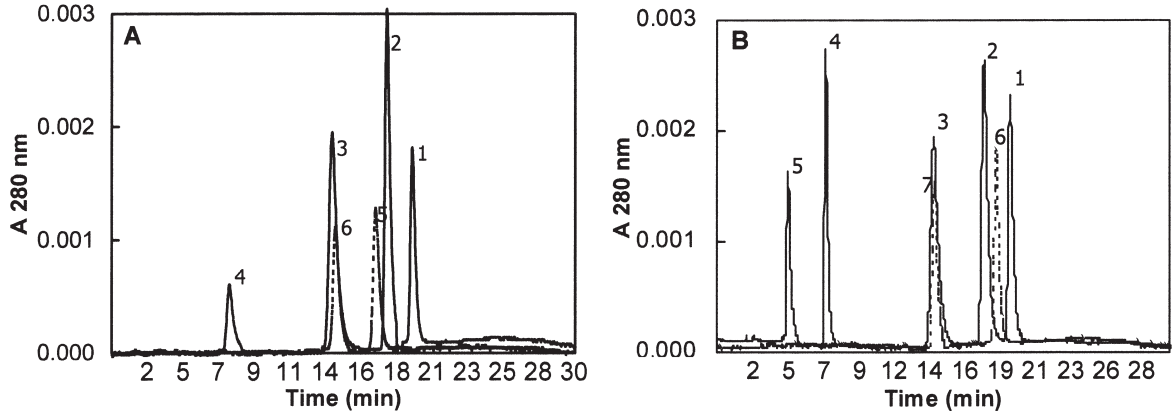

Fig. 2. HPLC chromatograms of phenolic acids present in elicitor treated (——) and control (……) root tissues: (A) ester-bound phenolics present in MeOH extracts following alkaline hydrolysis, (B) cell wall-bound phenolics following saponification of MeOH extracted, dried cell wall material. All extracts were made at 24 h post-elicitation and were separated by reverse phase HPLC on a C_{18} column using a 10–40% MeOH gradient in 5% formic acid. Separation was monitored at 280 nm for 30 min.

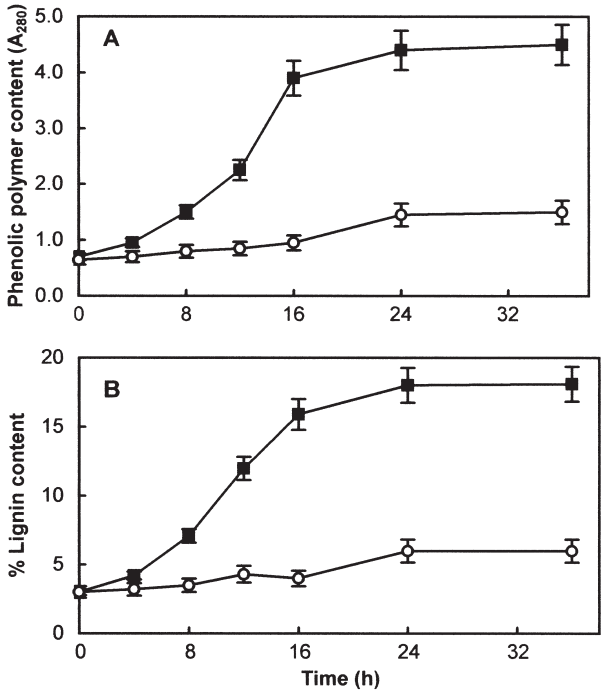

Fig. 3. The effect of elicitation on the time-dependent accumulation of (A) phenolic polymers and (B) lignin in elicitor-treated (■) and control (○) at 0, 4, 8, 12, 16, 24, and 36 h intervals. Bars represent the standard error (mean ± S.E., $n = 9$).

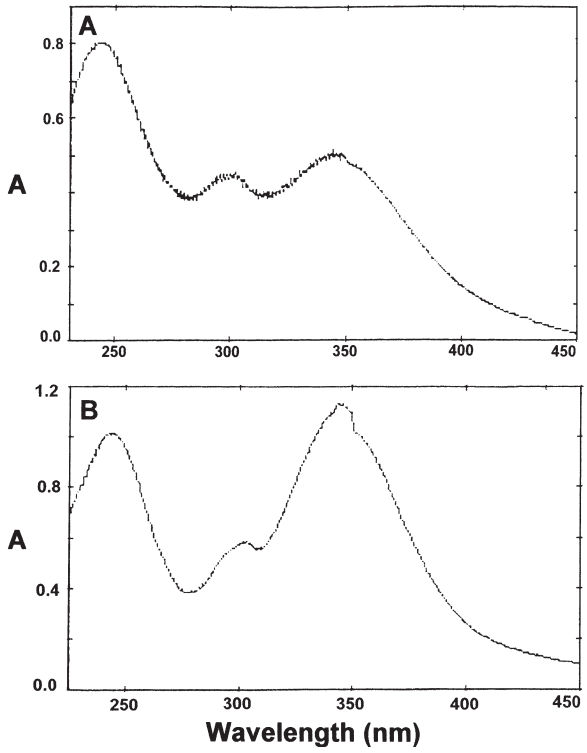

Fig. 4. Difference spectra (pH 12 vs. pH 7) of lignin from control (A) and elicitor treated (B) root tissue 36 h post-elicitation. Lignin was extracted from alcohol insoluble residues and diluted into 0.5 M NaOH or 0.5 M sodium phosphate.

While the LTGA assay is generally thought to provide a quantitative measure of lignification, it does not unambiguously demonstrate that the extracted material is lignin, as lignin-like phenolic polymers may also react in the assay (Campbell and Ellis, 1992). In order to distinguish between lignin and associated phenolic polymers, an independent assay involving digestion of the AIR root material with 25% acetyl bromide in acetic acid was used (Sasaki et al., 1996). The UV spectra of lignin-acetyl bromide complexes extracted from elicited roots taken at 0, 4, 8, 12, 16, 24, and 36 h time intervals after treatment with 25% acetyl bromide heated at 70 °C were obtained. As can be seen from Fig. 3B, the pattern of lignin accumulation is similar to that determined with thioglycolic acid. Lignin content increased 6.6-fold over time while the level of increase in the corresponding controls was only 1.9-fold.

2.4. Ionisation difference spectra

Ionisation difference spectra have been used as an indication of lignin formation. The method used in this study is based on the bathochromic shift of the characteristic 280 nm absorption maximum of lignin in alkaline solution, due to the ionisation of phenolic groups. The maxima at approximately 250 and 300 nm of difference spectra, obtained by subtracting the spectrum of the neutral (pH 7.0) from that of the alkaline (pH 12.0) solution of lignin preparations (Waldron and Selvendran, 1990), are characteristic for the absorption of the phenolate ion of simple non-conjugated aromatic hydroxyl groups. Alkaline solutions of phenolic compounds in which the hydroxyl group is conjugated through the ring with a carbonyl group have maxima greater than 300 nm. The difference spectra of pH 12 vs. pH 7 (that eliminates the contribution of non-ionisable chromophors) for roots sampled at time 0 h (Fig. 4A) and 36 h (Fig. 4B) are shown. Both curves show peaks or shoulders at 250, 300 and 350 nm, but the relative height of the peak at 350 nm for the spectrum of lignin from root samples taken at 36 h varies greatly from that taken at 0 h. On the basis of these different spectra, the composition of lignin extracts from roots samples taken at 36 h differ significantly from those taken at 0 h, not only in having a greater amount of simple phenolic groups, but also by the amount of conjugated (esterified) side chains as indicated by the major increase in the 350 nm peak. These differences in composition correlate with the increased concentrations of ferulic and sinapic acids as determined by HPLC.

Lignin is derived from the oxidative polymerization of the monomeric units, *p*-coumaryl, coniferyl and sinapyl alcohols; which give rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units in various ratios

according to their origin (Humphreys and Chapple, 2002). The relative abundance of these different monolignol residues in lignin varies and the defense-related synthesized lignin may differ in composition, adding an anti-microbial function to a structural one. Although lignin is undoubtedly a component of structural barriers that form in the banana root tissue, the primary reinforcement of the cell wall with hydroxycinnamoyl esters rather than lignin, can offer several advantages to the plant. In contrast to lignification, the synthesis of cell wall-bound esters may commence immediately on exposure to pathogen challenge (Ride, 1983). The esterification with hydroxycinnamic acids may then lead to modified cell wall polysaccharides that resist the action of lytic enzymes produced by the fungal pathogen. These esters may become cross-linked due to the action of extracellular cell wall-bound peroxidases and provide the platform for later lignification.

2.5. Conclusion

Phenylpropanoid biosynthetic pathways are among the most frequently observed metabolic activities that are induced upon infection of plants with pathogens or treatment of plant tissue or cultured cells with pathogen-derived elicitors. Several of the 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). Previously, in a comparative study between a resistant and susceptible cv., we reported that the resistant 'Goldfinger' clone, also used in this study, has a greater capacity for phenolic metabolism with regard to the basal levels of associated enzymes and metabolites (de Ascensao and Dubery, 2000).

The results obtained in this study with regard to the partitioning of precursor carbon skeletons into the various phenolic subfractions give a detailed analysis of phenolic metabolism in elicited banana roots, indicating that the increased flux through the phenylpropanoid pathway and the lignin pathway resulted in the synthesis of phenolic monomers that were eventually esterified and incorporated into the cell wall fraction as lignin. Lignins are highly resistant to attack by microorganisms and the inducible deposition of lignin in cell walls represent an effective barrier to pathogen entrance and spread. As such, the results contribute toward understanding the biochemical basis of the genetically determined resistance of the Goldfinger banana clone towards *Fusarium oxysporum* f.sp. *cubense,* race four. This defense mechanism appears to be an important determinant affecting the outcome of the host's response (de Ascensao and Dubery, 2000), and may be a possible contributing factor to the resistance mechanism in the Goldfinger banana clones, and resistance in banana towards nematodes and fungal infection in general (Valette et al., 1998). Phenylpropanoid derivatives in *Musa acuminata* will continue to be of prime interest, considering the strong defense-related induction, adding support for a functional link between phenolic metabolism and pathogen defense.

3. Experimental

Clonally propagated, mature banana plants (cv. Goldfinger) were grown in a hydroponic system to minimize interplant variation. The heat-released, cell wall derived elicitor from the pathogen, *Fusarium oxysporum* f.sp. *cubense* race 4 was prepared as previously described (de Ascensao and Dubery, 2000). The experiments were repeated three times under the same conditions with different sets of plants and all analyses were performed in triplicate. Results of the quantitative responses at 36 h were analyzed using the Student's *t*-test for *P* values and statistical significance. Error bars on graphs of the metabolite levels in tissue samples taken at the specified time points post-elicitation show the standard error of mean ($\pm$S.E., $n=9$).

3.1. Induction of lignification in root tissues

Plants (40–50 cm) were inoculated with the solubilised elicitor through cut roots in order to simulate the natural infection process that often occur through nematode wound sites. Roots were trimmed 1 cm from the root tip to expose the vascular system before being immersed in 45 μg ml^{-1} of the elicitor dissolved in 0.25× Murashige Skoog basal salt solution. Control plants were similarly treated, but without elicitor being present. At 0, 4, 8, 12, 16, 24 and 36 h after treatment with elicitor, roots were cut 2 cm from the original cut site. Root segment samples taken from elicited and control plants at the various time intervals, were frozen in liquid N_2 and stored at -20 °C until the assays could be performed. Each assay was performed in triplicate and the individual experiments repeated three times.

3.2. Extraction of phenolics

Root segments (0.5 g) of the control and elicitor treated plants harvested at different time intervals were ground in liquid nitrogen and then extracted with 0.9 ml 80% aqueous MeOH (Torti et al., 1995). The suspension was homogenised for 1 min before being centrifuged at 12 000×*g* for 10 min. After centrifugation the supernatant was saved. The remaining precipitate was rehomogenised and centrifuged as above. The two supernatants were combined and aliquoted into four micro centrifuge tubes (0.4 ml per tube) in order to determine total soluble phenolic acids, free phenolic acids, MeOH soluble ester-bound phenolic acids and

MeOH soluble glycoside-bound phenolic acids. The remaining precipitate was dried at 70 °C for 24 h. The resulting alcohol insoluble residue (AIR) yielded the cell wall material which was used to extract the ester-bound cell wall phenolic acids after alkaline hydrolysis.

3.2.1. Total soluble phenolic acids

The aliquoted supernatant for total soluble phenolic acids determination was concentrated in a speedvac to 0.25 ml. The supernatant was then used to determine the total soluble phenolic content with the Folin–Ciocalteau reagent.

3.2.2. Non-conjugated phenolic acids

The aliquoted supernatant for total soluble phenolic acids determination was concentrated to 0.25 ml and 25 μl of 1 M HCl was added to acidify the solution before extraction with 1 ml anhydrous diethyl ether (Cvikrová et al., 1993). The ether extract was reduced to dryness in a speedvac and the resulting precipitate was resuspended in 0.25 ml 50% aqueous MeOH. This solution was used to determine the free phenolic content with the Folin–Ciocalteau reagent. For HPLC analysis the same procedure was followed as above but the final precipitate was resuspended in 50 μl 80% aqueous MeOH.

3.2.3. Glycoside-bound phenolics

The aliquoted supernatant for MeOH soluble glycoside-bound phenolic content determination was hydrolysed in 40 μl concentrated HCI for 1 h at 96 °C, and then extracted with 1 ml anhydrous diethyl ether. The ether extract was reduced to dryness in a speedvac and the remaining precipitate was resuspended in 0.25 ml 50% aqueous MeOH. This solution was used to determine the phenolic glycoside content with the Folin–Ciocalteau reagent. For HPLC analysis the same procedure was followed as above but the final precipitate was resuspended in 50 μl 80% aqueous MeOH.

3.2.4. Ester-bound phenolics

Soluble ester-bound phenolic acids were extracted after alkaline hydrolysis under mild conditions (Cvikrová et al., 1993). To the aliquoted supernatant for phenolic ester content determination, 0.1 ml 2 M NaOH was added and the tubes were allowed to stand for 3 h at room temperature. After hydrolysis, 40 μl 1 M HCI was added and the phenolics extracted with 1 ml anhydrous diethyl ether. The ether extract was reduced to dryness in a speedvac and the precipitate was resuspended in 0.25 ml 50% aqueous MeOH. This solution was used to determine the phenolic ester content using the Folin–Ciocalteau reagent. For HPLC analysis the same procedure was followed as above but the final precipitate was resuspended in 50 μl 80% aqueous MeOH.

3.2.5. Cell wall-bound phenolic acids

Ester-bound phenols incorporated in the cell wall were extracted after alkaline hydrolysis (Campbell and Ellis, 1992). Dry cell wall material (AIR) was weighed and resuspended in 0.5 M NaOH (1 ml for 10 mg) for 1 h at 96 °C. Under these mild saponification conditions, wall-esterified hydroxycinnamic acid derivatives were selectively released. The supernatant was acidified to pH 2 with HCI, centrifuged at $12\,000\times g$ for 10 min and then extracted with 1 ml anhydrous diethyl ether. The diethyl ether extract was reduced to dryness in a speedvac and the precipitate was resuspended in 0.25 ml 50% aqueous MeOH. This solution was used to determine the wall-esterified phenolic acids content with the Folin–Ciocalteau reagent. For HPLC analysis the same procedure was followed as above but the final precipitate was resuspended in 50 μl 80% aqueous MeOH.

3.3. Folin–Ciocalteau assay

The method used to determine the total soluble phenolic content is based on the reduction of the phospho-molybdene/phospho-tungstate present in the Folin–Ciocalteau reagent (Swain and Hillis, 1959). The concentrated supernatant (50 μl; Section 3.2.1) was diluted to 0.25 ml with 50% MeOH and was added to 0.25 ml of 50% (v/v) Folin–Ciocalteau reagent and mixed. After 3 min, 0.5 ml of saturated aqueous sodium carbonate was added, mixed and incubated in a waterbath at 25 °C for 1 h. A blank of 50% MeOH was used instead of the sample. Ferulic acid was used as a phenolic standard to construct a standard curve ranging from 0 to 32 μg. The concentration of phenols in the various extracts was calculated from the standard curve and expressed as μg ferulic acid g^{-1} fresh weight.

3.4. HPLC analysis of phenolics

Only root samples collected after 24 h of elicitation and controls samples collected at the same time interval were analysed on a Beckman HPLC equipped with a 20-μl loop injection valve and connected with a UV detector at 280 nm. A Bondclone C_{18} column (Phenomenex, 300×3.9 mm, 10 μm) was used. Data were analysed by System Gold Chromatography software (Beckman). MeOH and 5% formic acid (HCOOH) were used as eluents with a gradient program from CH_3OH/5% HCOOH in the ratio 1:9 (9 min), increasing to CH_3OH/5% HCOOH 4:6 in 11 min, decreasing to CH_3OH/5% HCOOH 0:1 for 2 min and ending with CH_3OH/5% HCOOH 1:9 for 30 s. The flow rate was 2 ml min^{-1}. Before injection, the samples were filtered on Millex-SR 0.45 μm filters (Millipore). Phenols were identified by co-elution with the following standards: benzoic acid, cinnamic acid, *p*-hydroxybenzoic acid, syringic acid, gallic acid, *p*-coumaric acid, protocatechuic

acid, sinapic acid, vanillic acid, ferulic acid and caffeic acid (Codignola et al., 1989) as well as Rf values of thin layer chromatography separations (Harborne, 1991).

3.5. Thioglycolic acid derivatization of phenolic polymers

Lignin and lignin-like phenolic polymers were assayed quantitatively by derivatisation with thioglycolic acid (Barber and Ride, 1988) from alcohol-insoluble residues from banana roots. The AIR was air dried and used for lignin determination, either directly or following saponification. One millilitre of 0.5 M NaOH was added to 10 mg of dried AIR, and the tubes incubated at 96 °C for 1 h to hydrolyse cell wall bound phenolics. The mixture was neutralised with 0.25 ml of 2 M HCl and the residue collected by centrifugation at 12 000×*g* for 10 min. The residue was treated with 1 ml of 2 M HCl and 0.1 ml of thioglycolic acid. The sealed tubes were placed in a boiling water bath for 4 h at 96 °C and shaken initially to hydrate the AIR. The tubes were cooled and centrifuged at 12 000×*g* for 10 min at room temperature. Following centrifugation the supernatant was discarded and the precipitate washed once with 1 ml of H_2O. The precipitate was resuspended in 1 ml of 0.5 M NaOH and agitated gently at 25 °C for 18 h to extract the lignin thioglycolate. The samples were centrifuged at 12 000×*g* for 10 min. The supernatant was transferred to new micro centrifuge tubes and 0.2 ml concentrated HCl was added to each tube and the lignin thioglycolic acid allowed to precipitate at 4 °C for 4 h. Following centrifugation at 12 000×*g* for 10 min, the orange-brown precipitates were dissolved in 2 ml of 0.5 M NaOH, and the absorbance at 280 nm was measured.

3.6. Acetyl bromide based quantification of lignin

The lignin content of root sections was determined by the acetyl bromide method (Morrison and Stewart, 1995). Dried alcohol insoluble residues (10 mg) containing the cell wall material were washed with 25% (v/v) acetyl bromide in acetic acid and incubated at 70 °C for 30 min in 1 ml of 25% acetyl bromide in acetic acid. The mixture was cooled to room temperature, and 0.9 ml of 2 M NaOH and 0.1 ml of 7.5 M hydroxylamine hydrochloride were added and the volume made up to 10 ml with acetic acid. Following centrifugation at 12 000×g for 5 min, the absorbance of the supernatant was measured at 280 nm. A reagent blank was run with every set of estimations. The lignin content of the samples was calculated by using the specific absorption coefficient (SAC) of 20 g^{-1} l cm^{-1} for lignin and the following equation: % lignin content = {absorbance×100}÷SAC×sample conc. (g l^{-1}); Sasaki et al., 1996).

3.7. UV difference spectroscopy of lignin

Spectrophotometric determination of lignin, based on extraction of lignin in NaOH, followed by an estimation of the free phenolic groups and determination of the ultraviolet difference spectra of the lignin extracts were performed (Waldron and Selvendran, 1990). AIR (10 mg) was extracted with 1 ml 0.5 M NaOH at 96 °C for 1 h. The material was centrifuged at 12 000×*g* for 5 min, and the precipitates washed twice with 1 ml distilled water. The combined supernatant and washings were diluted to 5 ml with either 0.5 M NaOH to give a pH = 12.0, or with 0.5 M sodium phosphate to give a pH = 7.0. UV–visible absoption spectra were recorded over the 200–450 nm range. Difference spectra were plotted (absorbance at pH 12.0 vs. absorbance at pH 7.0) to indicate the content of non-conjugated phenolics (absorbance peaks around 250 and 300 nm) as well as conjugated phenolics which exhibit maxima at 300 nm and above.

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