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Characterization of the wound-induced material in *Citrus paradisi* fruit peel by carbon-13 CP-MAS solid state NMR spectroscopy

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Abstract

Grapefruit, *Citrus paradisi*, were injured, inoculated with *Penicillium digitatum* and incubated under conditions favourable for the accumulation of defence related material. Histochemical examination revealed that tissues adjacent to inoculated injuries contained phloroglucinol-HCl (PG-HCl) reactive material. Solvent washed cell wall preparations of intact and injured-inoculated peel were further purified using a mixture of cell wall degrading enzymes. Samples from injured inoculated tissue contained PG-HCl reactive globular material in addition to the fragments of xylem and cuticle found in controls. The principal chemical moieties of the material that accumulates in grapefruit injuries during wound-healing were studied by solid state ^{13}C cross-polarization magic angle spinning NMR. A complete assignment of the NMR signals was made. From the analysis evidence was found that cellulose and hemicellulose are the biopolymers present in the intact peel samples, in addition, relevant quantities of cutin were found in the residues of enzyme digest. The NMR difference spectrum intact- wounded peels showed resonances which were attributed to all major functional groups of the aromatic-aliphatic suberin polyester of new material produced by the wounds. Information on the latter polyester was obtained by analyzing the $T_1\rho$ (^1H) relaxation.

Keywords: *Citrus paradisi*; Rutaceae; Wound-induced material; Plant defence; Carbon-13 solid state NMR; Relaxation times

1. Introduction

Green mold (*Penicillium digitatum* Sacc.) and blue mold (*P. italicum* Whem.) are the most frequent causes of postharvest decay of citrus fruits (Smoot et al., 1983). These fungi infect fruit through wounds made in the peel during harvesting and handling. Early on, it was observed that when fruit were held under degreening conditions, ~30 °C and high relative humidity, there was a dramatic reduction in the incidence of blue and green molds (Tindale and Fish, 1931; Hopkins and Loucks, 1948). Because of this, the wound healing process in citrus peel has been the subject of numerous histological investigations (Brown and Barmore, 1981, 1983; Baudoin and Eckert, 1985). The development of resistance to infection to green mold is associated with the deposition of a material which turns red in phloroglucinol-HCl (PG-HCl) in tissues adjacent to the injuries (Brown and Barmore, 1983; Baudoin and Eckert, 1985). Examination by electron microscopy reveals the material as amorphous, electron-dense, globules located in the lumina of the responding cells (Brown and Barmore, 1981). A similar phenomenon has also been reported in detached citrus leaves. Deposition of PG-HCl reactive material in injured leaf tissues was associated the development of resistance to infection by *Xanthamonas axonopodis* pv. *citri* (Koizumi, 1983). This PG-HCl reactive material has been described both as lignin (a phenolic polymer) and wound gum (a water insoluble carbohydrate infused with aromatic

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aldehydes) (Brown and Barmore, 1983; Baudoin and Eckert, 1985; Brown et al., 1978; Rawlins, 1933; Schneider, 1981; Stange et al., 1993a, b).

While there is doubt regarding its identity, an insoluble, PG-HCl reactive, material definitely accumulates in the tissues adjacent to injuries. Studies have shown that CP-MAS ^{13}C NMR analysis enabled the elucidation of major functional groups, cross-link sites and polymeric domain structure of insoluble plant materials such as lignin, cell wall carbohydrates and cuticular materials such as cutin and suberin (Dudley et al., 1983; Haw and Maciel, 1984; Zlotnik-Mazori and Stark, 1988; Garbow et al., 1989; Garbow and Stark, 1990; Stark and Garbow, 1992; Pacchiano et al., 1993; Ha et al., 1997; Round et al., 2000). This method works best if the abundance of the components of interest is high. Methods for purifying the induced material from solvent washed cell wall preparations of squash tissue using a commercial preparation of cell wall degrading enzymes, including cellulase, laminarinase, pectinase and xylanase, were recently reported (Stange et al., 2002). We used a similar method to purify the induced material from the peel grapefruit, and used CP-MAS ^{13}C NMR analysis to identify the main chemical moieties of the material accumulating in grapefruit peel during wound healing. Additionally, the alcohol soluble components of peel were characterized by TLC to determine if any of the induced aldehydes were analogues of lignin monomers.

2. Results

Separation of the ethanol-soluble materials by TLC revealed that the injury-inoculated extracts contained three aldehydes not present in control tissue (Fig. 1). A predominant one was found elute in 65% methanol, (R_f 0.60) and two minor ones, eluting in 50 and 35% methanol respectively (R_fs 0.31 and 0.19). Under the chromatographic conditions used R_fs for the lignals were 0.52 (*p*-coumaryl aldehyde), 0.50 (coniferyl aldehyde), and 0.39 (sinapyl adehyde).

Following curing, tissues adjacent to injuries in inoculated grapefruit turned red when mounted in PG-HCl. Microscopic examination of the purified material revealed that the control grapefruit sample consisted of xylem elements, which coloured intensely in PG-HCl, and remains of the cuticle, which gave no histochemical reaction (Fig. 2A). The purified material of injury-inoculated samples contained an abundance of amorphous globular, PG-HCl reactive, particles in addition to the fragments of xylem and cuticle observed in the control sample (Fig. 2B). Weights and yields of purified material were 1.10 g (4.6% of extractive free cell wall preparations) for control and 2.41 g (6.8%) for injury-inoculated samples.

2.1. ^{13}C CP-MAS NMR spectra

CP-MAS ^{13}C NMR spectra of the intact-cured (Fig. 3A) and injured-inoculated-cured (Fig. 4A) grapefruit peels exhibited similar spectral patterns, which were ascribed to cellulose (C) and hemicellulose (HC) polymeric components. In fact, according to previous investigations (Kolodziejski and Maciel, 1982; Dudley et al., 1983; Haw and Maciel, 1984) the resonances in the range from 60 to 110 ppm can be assigned to the following carbohydrate carbons. The two peaks at 104.5 and 101 ppm represent the C_1 (C) and C_1 (HC) respectively. The intense peaks at 71.5 and 68.5 can be considered as overlapping signals due to C_2 (C, HC), C_3 (C, HC) and C_5 (C, HC). The *downfield* shoulders at 84 and 89 ppm originate from the C_4 (C, HC) and the *upfield* shoulders at about 63 ppm are due to C_6 (C, HC). In addition, the peak at 53 ppm can be attributed to a methoxyl carbon present in some of the monomer units of HC, while the peak at 172 ppm corresponds to the acetate carboxyl groups of HC, acetate methyl signals of which are visible at 21.5 ppm, and the peak at 170 ppm can be assigned to carboxyl groups of uronic acids also present in HC.

Compared to the intact-cured tissue, CP-MAS ^{13}C NMR spectrum of the corresponding purified material (Fig. 3B) changed dramatically, exhibiting a new set of intense signals in the bulk-methylene region at 10–40

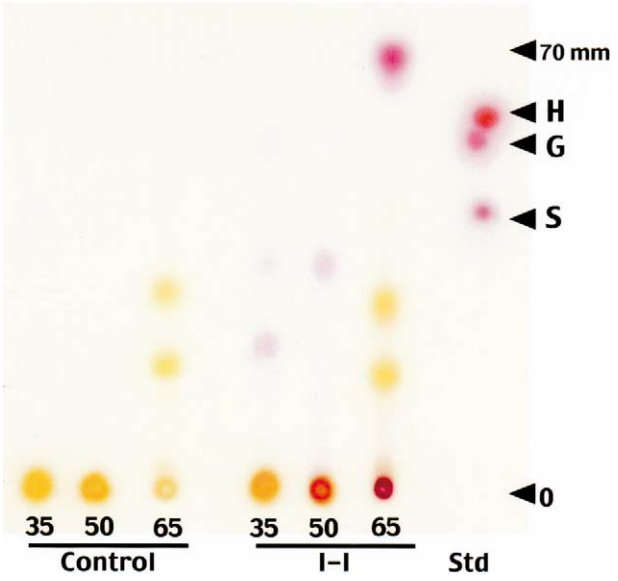

Fig. 1. Analysis of ethanol soluble aromatic aldehydes from grapefruit peel tissue by TLC. Intact (control) and injured–inoculated (I–I) peel was extracted with 80% ethanol, concentrated, and separated on a C_{18} column. Fractions were eluted in 35, 50 or 65% methanol, and amounts equivalent to 10 mg fresh weight tissue were spotted onto TLC plates. Standards (Std) spotted were 0.5 μg of *p*-coumaryl aldehyde (H), coniferyl aldehyde (G) and sinapyl aldehyde, (S). Plates were developed in hexane–EtOAc (3:5) and visualized by spraying with PG-HCl. Solvent front travelled 115 mm, only the portion of plate where spots were visualized is shown.

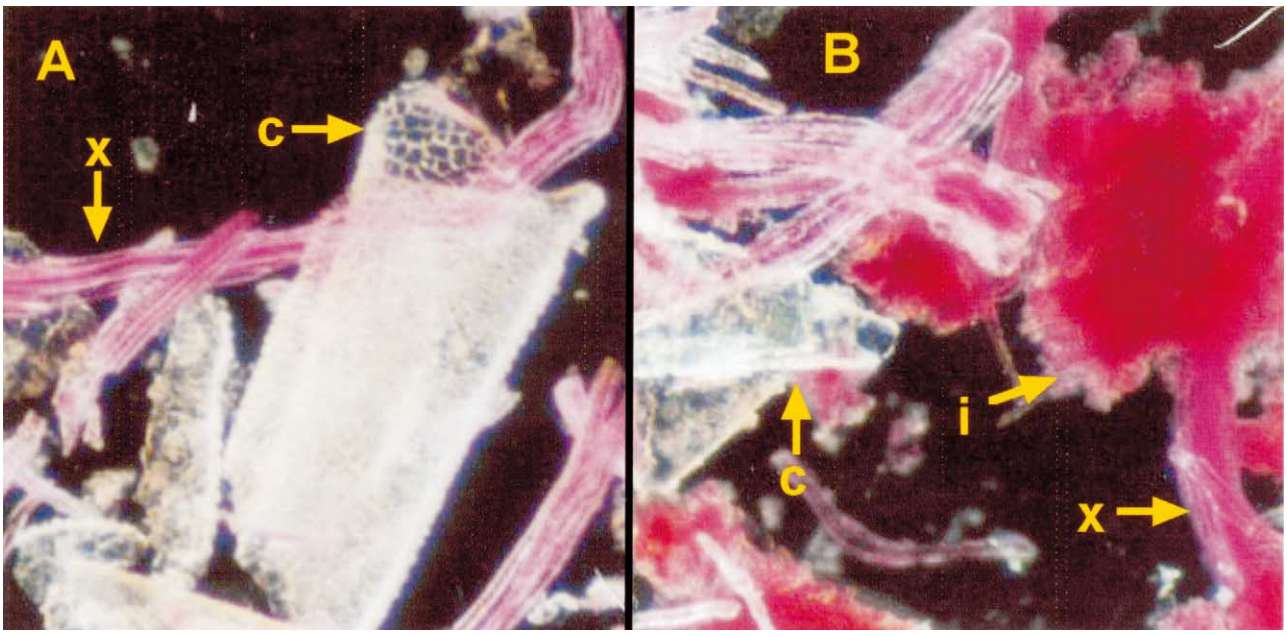

Fig. 2. Purified material of enzyme digested control (A) and injured–inoculated (I–I) (B) grapefruit peel. Samples were mounted in PG-HCl and view under dark field illumination. The control sample contained fragments of xylem (x) which coloured and cuticle (c) which gave no histochemical reaction. The I–I sample contained the induced, PG-HCl reactive, material (i), as well as fragments of xylem and cuticle present in the control sample. Images are 0.5 mm on edge.

ppm as well as significant spectral modifications in the carbohydrate and carboxylic regions. Furthermore, weak signals appear in the aromatic-olefinic region from 110 to 160 ppm. The chemical shift assignments were made with the aid of the difference spectrum B–A of Fig. 3C and previous NMR analyses on polymeric components such as cutin, suberin and aliphatic waxes (Zlotnik-Mazori and Stark, 1988; Garbow and Stark, 1990; Pacchiano et al., 1993). The difference spectrum B–A was obtained in such a way that the subspectrum corresponding to the carbohydrate components in the region 60–110 ppm of both B and A spectra, did not show dispersion-like or negative signals. Despite the overlapping of residual signals from C and HC polymeric components, the spectral features of the difference spectrum correspond quite closely to those reported in the literature (Zlotnik-Mazori and Stark, 1988; Garbow and Stark, 1990; Pacchiano et al., 1993; Round et al., 2000) for the aliphatic and aromatic ester linked moieties of the cutin polymer and for aliphatic waxes, in agreement with the findings from the microscopy analysis.

As to the methylene region, the presence of waxes can be recognized mainly from the intense and well resolved peak at 33 ppm ($-(CH_2)_n-$) (Garbow and Stark, 1990; Pacchiano et al., 1993) and from the highest field shoulder attributable to the terminal methyl groups, while the methylene groups of the cutin polyester exhibit the peaks at about 29 and 25 ppm. Furthermore, the peaks at 40 and 42 ppm are due to methylene groups close to oxygenated carbons ($-\underline{C}H_2-CH_2-OCOR$) of cutin and/or wax.

In the region 50–110 ppm, the $-\underline{C}H_2-COR$, $>\underline{C}H-COR$ and phenolic $-O-CH_3$ resonances of the cutin, respectively, are assigned to the peaks at 64, 72 and 55 ppm, some of which are superimposed with residual signals from carbohydrates. Finally, the peaks at 168 and 172 ppm were assigned to the $-CH_2-COR$, $>CH-COR$ groups of cutin (Zlotnik-Mazori and Stark, 1988). However, it cannot be excluded that acetate residues or acetate esters, also formed during the digests, contribute to the intense and sharp peak at 168 ppm (Garbow and Stark, 1990; Pacchiano et al., 1993). Because of the low intensity of the signals, no attribution was made to the aromatic and olefinic moieties of the cutin in the downfield region beyond 110 ppm.

We must point out that, due to the experimental CP-MAS ^{13}C NMR conditions (contact time $=700$ μs) signals from the keto group of hydroxyoxo-fatty acid constituents in the cutin aliphatic moiety are attenuated to the limit of detection because of scarce and weak dipole–dipole interactions. However, spectra recorded at longer contact times (1–5 ms) enhanced this resonance at 209 ppm, thus indicating that hydroxyoxo-fatty acid constituents are present, in agreement with literature data on the grapefruit cutin (Deas et al., 1974; Baker et al., 1975; Gérard et al., 1992) and other cutin NMR spectra (Zlotnik-Mazori and Stark, 1988; Garbow and Stark, 1990; Pacchiano et al., 1993).

Fig. 4B, where the CP-MAS ^{13}C NMR spectrum of the Driselase residue obtained from the injured-inoculated-cured tissue is displayed, shows sets of signals due to cutin, C and CH polymeric components. However,

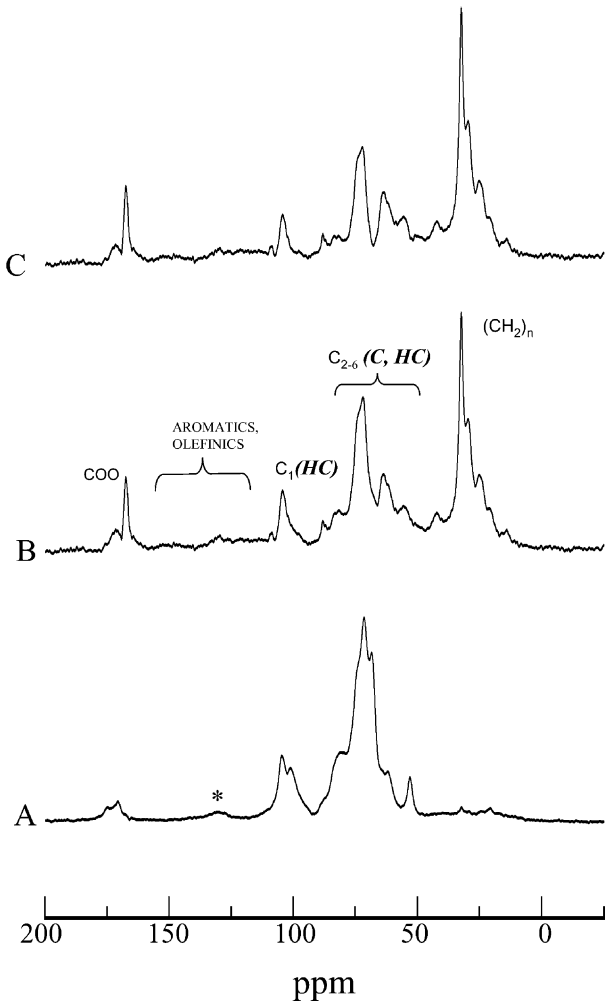

Fig. 3. ^{13}C CP-MAS NMR spectra of extractive-free cell walls from peel tissues of grapefruit, (*) Spinning sideband: (A) intact-cured peel, (B) peel Driselase residue, (C) spectrum obtained by subtraction of (B) from (A).

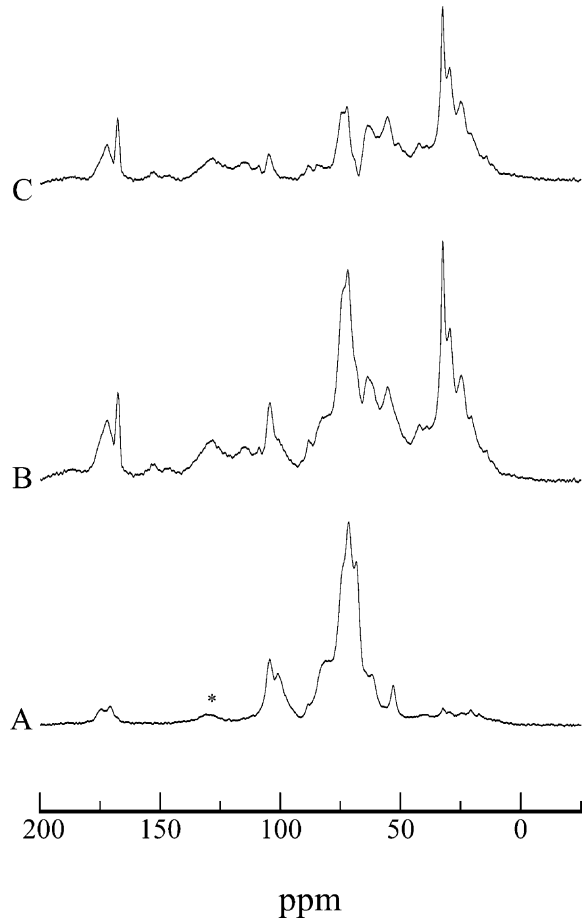

Fig. 4. ^{13}C CP-MAS NMR spectra of extractive-free cell walls from peel tissues of grapefruit, (*) Spinning sideband: (A) injured-inoculated-cured peel, (B) peel Driselase residue, (C) spectrum obtained by subtraction of (B) from (A).

compared to the spectrum of the Driselase residue from intact-cured tissue (Fig. 3B), a reduction of the intensity of the peak at 33 ppm occurs, indicating a decrease of the wax content in the sample, accompanied by a marked increase of the peak at 174 ppm and of the resonances in the aromatic-olefinic region from 110 to 160 ppm, that was ascribed to the formation of newly induced material. Subtraction of the spectrum in Fig. 4B from that in Fig. 3B gives the difference spectrum displayed in Fig. 5. This spectrum allows for a better analysis of the signals originating from the induced material in the Driselase residue of the injured-inoculated-cured tissue. This spectrum exhibits ^{13}C NMR signals typical of the suberin aliphatic-aromatic polyester, the aromatic signals due to carbons from ferulic and coumaric acids predominating over the aliphatic residues, as found for suberized tissues of wound-healing potatoes (Garbow et al., 1989; Stark and Garbow, 1992). These observations oppose what was observed in the spectra of cutin polyester (Fig. 3B) and of other cutin samples from different sources (Zlotnik-Mazori and Stark, 1988; Garbow and Stark, 1990; Pacchiano et al., 1993). The spectral assignments for the related bulk methylenes, oxygenated methylenes, methines, aromatics or olefinics and carboxyl groups of suberin are collected in Table 1.

2.2. ^{1}H NMR relaxation in the rotating-frame

To obtain further information on the induced material in the grapefruit peel after wounding and fungal inoculation we investigated the dynamics of the polymeric domains by means of the spin relaxation-times in the rotating-frame, $T_{1\rho}$ (^{1}H). This parameter is sensitive to the motions in the kHz range, and since it is mainly affected by ^{1}H spin diffusion it depends on the neighbouring spin reservoirs (Garbow and Stark, 1990; Stark and Garbow, 1992).

Table 2 collects $T_{1\rho}$ (^{1}H)'s measured in the sample of purified material of the injured-inoculated-cured peel of grapefruit and, for a comparison purpose, the corresponding parameters of cutin-wax and suberized potato epiderm. As shown, the analysis was limited to a

Table 1
^{13}C NMR assignments for suberized grapefruit peels

Suberin, carbon type	Chemical shift (ppm)	Peak No.
–(CH$_2$)–$_n$	26, 30, 33	1, 2, 3, 4
CH$_3$O–	56	5
–CH$_2$O–	64	6
>CHO–	72	7
Ar–CH–	104	8
Ar–CH–	115	9
–CH=CH–, Ar–CHC	130	10
Quat.Ar–C	150	11
COO	172	12

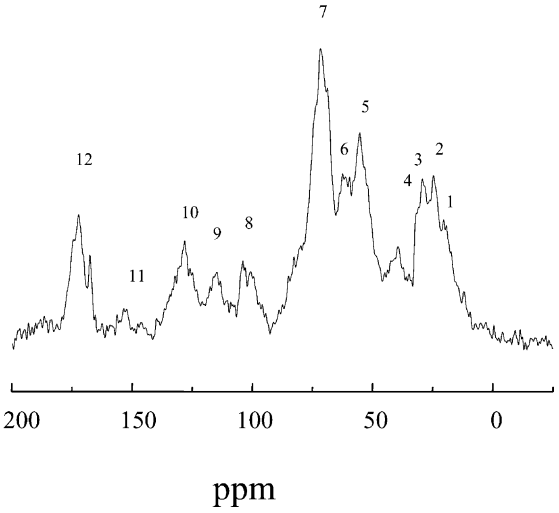

Fig. 5. ^{13}C CP-MAS NMR spectrum of the induced material in Driselase residue of injured–inoculated-cured peel tissues of grapefruit, obtained by subtraction of the spectrum in Fig. 4B from that in Fig. 3B. For the assignments see Table 1.

restricted number of peaks because of the low signal-to-noise ratio and spectral resolution limits. Furthermore, single proton $T_{1\rho}$ values were observed for all carbon peaks, so that the estimated relaxation times must be considered as average parameters depending on carbons of different polymeric components present in the sample.

The data collected in Table 2 show that for the –(CH$_2$)–$_n$ the $T_{1\rho}$ (^{1}H) values are similar to those of the cutin-wax and significantly shorter than suberin from suberized potato, indicating that cutin-wax gives a major contribution in determining the intensity of the methylene signals at 33 and 30 ppm, as was expected also from the above analysis of the CP-MAS ^{13}C NMR spectra. The remaining peaks and in particular the signal at 130 ppm, due to aromatic and olefinic carbons, exhibit values comparable to those of the suberized potato epiderm, thus substantiating the hypothesis that wounding and fungal inoculation induce the formation of suberin in the peel of grapefruit. Furthermore, these

Table 2
^{1}H Rotating-frame relaxation parameters for carbons of Driselase residue from injured-inoculate-cured tissue of grapefruit peels

Carbon type[b]	ppm	$T_{1\rho}$ (^{1}H)[a], ms		
		Wounded and fungal inoculated grapefruit peel	Suberized potato[c]	Cutin-wax[c]
–(CH$_2$)–n	30	3.6	5.0	3.8
–(CH$_2$)–n	33	3.7	4.5	3.9
–CH$_2$O–	64	7.3	6.6	3.8
>CHO–	72	7.9	6.6	4.0
Ar–CH–, *CH* (C$_1$)	104	6.7	6.6	
–CH=CH–, Ar–CHC	130	5.5	5.2	
–COO–	172	4.7	4.9	

[a] The data have an estimated uncertainty of 15%.
[b] Carbon groups in polymeric components from carbohydrate, wax, cutin and suberin.
[c] Data taken from Stark and Garbow (1992).

$T_{1\rho}$ (^{1}H)'s are quite a lot shorter than those reported for lignin (Stark and Garbow, 1992), this excludes lignin as a possibile component in the newly formed material.

3. Discussion

In the solvent-washed cell wall preparations pectin and cellulose predominate, as citrus peel contains an abundance of these components. The amount of cuticular material is low, and therefore its presence was not revealed by solid state NMR. The situation changed after digestion, which removes pectin, cellulose and some other carbohydrates, but leaves cutin. The digestion increases the relative abundance of cutin, making its presence detectable by solid state NMR spectrum. The formation of the induced material requires wounding, but its abundance is increased if a pathogen or other elicitor is present (Baudoin and Eckert, 1985). Since the quantity of the induced material is relatively low in solvent washed cell wall preparations of injured-inoculated-cured tissues, no differences were detected by solid state NMR. However, as in the case of cutin in the control samples, the relative quantity of the induced material greatly increased following enzymatic digestion and thus it was easily recognized as suberin in the NMR difference spectrum (Fig. 5). These results, together with those from the data of the dynamics in the rotating-frame, provide further evidence for the spectral characterization of the induced material as suberin.

This conclusion is quite startling. Because of its the intense reaction with PG-HCl, which is a characteristic property of these defense-induced materials in citrus and other plants, the material has been thought to be either lignin or wound gum. The data of the dynamics in the rotating-frame show that the material is not lignin. The absence of any detectable signal in the ~190 ppm

region indicates that abundance of aldehydes is negligible. While the presence of aromatic aldehydes is diagnostic for this material, they do not seem to be a primary component.

Because of its intense reaction with PG-HCl, histological studies have concluded that the induced material was lignin-like, and did not evaluate suberin as a potential component (Brown and Barmore, 1981, 1983; Baudoin and Eckert, 1985). However, the induced material is closely associated to suberin developmentally. In attached citrus leaves, phellogen forms immediately interior to the PG-HCl reactive cells, and gives rise to a boundary layer of heavily suberized cells (Koizumi, 1983; Wylie, 1928). Suberin is commonly thought of as a cell wall associated material deposited in the terminal development of specialized cells, such as wound cork (Esau, 1965). While the defensive material, appearing as amorphous intercellular globules in injured citrus fruit tissue (Brown and Barmore, 1981), does not resemble a suberized barrier in the classic sense, similar materials have been histochemically identified as suberin (Rittinger et al., 1987). Additionly, tissue containing PG-HCl reactive material from injured sweetpotatoes stained very weakly with Sudan IV, but did release 1,18-octadecenediol upon degradation (Walter and Schadel, 1983). Our findings provide focus for future histological, biochemical and molecular work needed to determine the precise composition of the phenolic and aliphatic components of induced material.

4. Experimental

4.1. Plant material

4.1.1. Preparation of grapefruit peel samples

Fruits of grapefruit, cultivar 'Marsh', were harvested from a production grove in St. Lucie County, Florida. Fruits were washed with tap water using dish liquid and gentle brushing, wetted three times with 95% ethanol and air dried. A wounding tool, constructed from 0.64 mm thick utility blades, was used to make two parallel cuts, 1 mm deep and 1.9 mm apart in the grapefruit peel. Three pairs of cuts, from stem to blossom end were made, and fruits were rinsed in tap water until free of peel oils. Fruits were immersed for 15 s in water containing 2×10^4 *P. digitatum* spores per ml, placed in a polyethylene bag and incubated for 18 h at 20 °C. Next, fruits were incubated at 31–33 °C and saturated relative humidity for 48 h. The injured-inoculated (I–I) portion of the peel was removed, trimmed to about 2 mm thick, and with 1 mm of tissue on either side of the wounds. Control tissue was obtained by removing peel from uninjured portions of the same fruit. Tissue was homogenized in 80% ethanol (5 ml/g initial fresh weight) and filtered [Büchner; Whatman N° 1]. Ethanolic filtrates were stored at −20 °C. Ethanol-insoluble residues were suspended in 2 ml 80% ethanol/g, filtered and washed 3× with acetone by filtration. Extractive-free cell wall preparations (EFCWPs) were stored at −20 °C.

The EFCWPs, 24 g for control and 36 g for injury-inoculated, were digested in 50 mM sodium acetate (15 ml/g sample), pH 5, containing 2 U pectinase/ml and 0.02% sodium azide for 20 h at 30 °C with gentle shaking. Residues were washed 3× with water by centrifugation, 3× with acetone by filtration and air dried. Then, they were digested in 100 mM sodium acetate (20 ml/g), pH 5, containing 1% Driselase (Sigma, St Louis, Mo) and one unit pectinase/ml and shaken at 90 rpm for 90 h at 30 °C. To retard microbial activity, 4% toluene was added and digestion was done in a nitrogen purged vessel. Digests were heated under vacuum to remove toluene. The purified material was washed 3× in water, once in 80% ethanol and 3× with acetone.

4.2. Histochemical analyses

Free hand sections through injury sites and samples of the purified material were mounted in PG-HCl and examined under dark field illumination using a Nikon Optishot micoscope (Rawlins, 1933).

4.3. Isolation and separation of ethanol soluble compounds

An aliquot of the first ethanolic extract of grapefruit equivalent to 4 g fresh tissue weight was reduced to dryness and loaded onto a 1.5 cm dia column, packed with 2 cm of C_{18} sorbent (40–63 μm). The column was eluted in steps of increasing methanol concentration (20, 35, 50, 65, 80 and 100% methanol). Fractions were reduced to dryness and transferred in a small volume of 80% methanol. Samples were spotted on a silica gel TLC plate and developed in hexane–ethyl acetate (3:5 v/v). Aromatic aldehydes were visualized by spraying plates with PG-HCl. Monolignols (0.5 μg) were spotted as standards (Stange et al., 2002).

4.4. ^{13}C CP-MAS NMR analysis

The CP-MAS ^{13}C NMR spectra were recorded at room temperature on a spectrometer Varian 400 Unity Inova operating at 100.57 MHz, equipped with 7 mm probehead. Experiments were conducted on 65–80 mg freeze dried peel samples packed into a ZrO_2 rotor. A matched Hartmann-Hann condition was established at the spin-lock field of 38 kHz. The rotor was spun at the spinning rate of 4 kHz, a contact time of 700 μs was applied to obtain polarization transfer. The 90 degree pulse was 6.6 μs and a recycle delay of 5 s was used. Chemical shifts of CP-MAS spectra were obtained with respect to the methylene carbon resonance of solid

adamantine, 38.3 ppm down field from Me_4Si, determined before each measurement.

The proton relaxation times in the rotating frame $T_{1\rho}$ (^1H) were determined from an exponential fit to the decay of the carbon signal as a function of the time before cross polarization in the range 0.5–80 ms.

Acknowledgements

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