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Phytochemistry 66 (2005) 2458–2467
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Metabolic distinction of *Ulmus minor* xylem tissues after inoculation with *Ophiostoma novo-ulmi*

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Received 28 April 2005; received in revised form 4 August 2005
Available online 19 September 2005

Abstract

Dutch elm disease (DED) is the most devastating and widespread disease of elms. The pathogen, *Ophiostoma novo-ulmi*, spreads systemically causing xylem vessels blocking and cavitation, and ultimately resulting in the development of a wilt syndrome. Twig samples from susceptible and resistant *Ulmus minor* trees were harvested at 0, 5, 15, 30, 60, and 120 days post-inoculation (dpi) with *O. novo-ulmi*. Fourier transform-infrared (FT-IR) spectroscopy, in tandem with chemometrics, was used to monitor changes in wood chemistry as consequence of infection. Principal component analysis distinguished between spectra from inoculated and control elms, and from susceptible- and resistant-inoculated elms. By 30 dpi, infected xylem showed reduced relative levels of carbohydrates and enhanced relative levels of phenolic compounds, probably due to the degradation of cell wall polysaccharides by fungal enzymes and the synthesis of host defence compounds. On 15 dpi, samples from resistant-inoculated elms showed higher levels of starch than samples from susceptible-inoculated elms, suggesting that availability of starch reserves could affect the tree's capacity for defensive responses. The results showed the power of FT-IR spectroscopy for analysing changes in the major components of elm xylem as consequence of infection by DED, and its potential for detecting metabolic profiles related to host resistance.

Keywords: *Ulmus minor*; *Ophiostoma novo-ulmi*; Dutch elm disease; Metabolite fingerprinting; FT-IR metabolite profiles; PCA; Wood degradation; Plant resistance

1. Introduction

The outbreak of the two pandemics of Dutch elm disease (DED) over the last 100 years, caused by the pathogenic fungi *Ophiostoma ulmi* (Buisman) Nannf., and the more aggressive *Ophiostoma novo-ulmi* Brasier, ravaged European and North American elm populations (Brasier et al., 2004). The pathogen spreads within xylem vessels inducing its blocking and cavitation, resulting in foliar wilting and the subsequent tree death (Newbanks et al., 1983). The wilt syndrome is apparently a result of interactions between fungal metabolites and the tree (Scheffer et al., 1987). The fungal toxin cerato-ulmin affects the host physiology by increasing respiration rates and plasma membrane permeability (Richards, 1993). Cell wall degrading enzymes, such as polygalacturonases, xylanases, pectinases or glucosidases, were reported to be involved in host colonization by the fungus (Biehn and Dimond, 1971; Binz and Canevascini, 1996; Binz et al., 1997), and in the obstruction of the sap flow by releasing partially hydrolyzed polysaccharides of host cell walls in vessel lumina (Svaldi and Elgersma, 1982). However,

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doi:10.1016/j.phytochem.2005.08.004

direct evidence for the involvement of these enzymes is still lacking.

Two of the most often reported induced defence mechanisms of elms against DED fungi are phytoalexin production and compartmentalization process. Phytoalexins are low molecular weight fungitoxic compounds accumulated by plants in response to several stress agents, notably in response to infection by microorganisms (Pearce, 1996). Elms produce several fungitoxic sesquiterpene phenolics in response to infection by *O. ulmi* and *O. novo-ulmi*, which were designated as mansonones (Duchesne et al., 1985). Rapid phytoalexin accumulation seems to be important in delaying invasion in the initial stages of DED (Duchesne et al., 1992; Ouellette and Rioux, 1992). Compartmentalization process involves the isolation of healthy xylem from infected tissues through the formation of anatomical barriers preventing spread of infection (Shigo and Tippett, 1981). Gel and tylosis formation in vessel elements hinders vertical spread of the pathogen, while barrier zones of axial parenchyma cells laid down de novo by the cambium protects the youngest wood and the cambial tissues, both of which are vital to the continuing growth and survival of the tree (Pearce, 1996). It has been suggested that lignin and suberin accumulates in reaction zones (Biggs, 1985; Rioux and Ouellette, 1991; Rioux and Baayen, 1997), but the assumption for their presence is often based on histochemistry or fluorescence microscopy. The application of complementary technologies, such as spectroscopic methods, would be desirable for a better understanding of the defence reactions of elms.

FT-IR spectroscopy is a widely used technique, which is a rapid, reproducible, and stable in time. Needing only a very basic sample preparation, FT-IR spectroscopy technique has been proposed for determination of the degree of methylesterification of cell wall pectic polysaccharides (Barros et al., 2002), for distinction of wine polysaccharide extracts and identification of the wine-making processes involved (Coimbra et al., 2002), and for food additive authentication (Černá et al., 2003). The shapes of the FT-IR spectral bands and their positions on the energy scale are characteristic of particular functional groups within the molecule. However, in some cases it is not possible to clearly identify these functional groups in the spectra because of their complexity. The variation in intensity or absence of some bands in the spectra, however, reflects compositional differences between samples. Multivariate techniques such as principal component analysis (PCA) have been specifically designed to analyze complex data sets (Summer et al., 2003). A previous study revealed the potential of FT-IR spectroscopy for the evaluation of host resistance in the DED complex (Martin et al., 2005), suggesting further investigation in order to evaluate its utility. Here, we report a FT-IR spectroscopy method, coupled with multivariate analysis, for the metabolic analysis of xylem tissues in susceptible and resistant *Ulmus minor* Miller trees infected with *O. novo-ulmi*. This approach may lead to the identification of metabolic pathways connected with defence responses to DED.

2. Results and discussion

U. minor ramets inoculated with *O. novo-ulmi* showed a progressive increase of wilting symptoms over time (Table 1). The first external symptoms appeared 15 days post-inoculation (dpi). Differences between symptoms of susceptible and resistant trees were observed for the first time on 30 dpi, when the inoculated branches of susceptible trees were observed to be 100% wilted. On 60 and 120 dpi, susceptible trees showed the highest symptoms which, in the case of the ramets UPM068 and UPM171, exceeded 90% wilting, while resistant trees showed less than 25% wilting.

The resulting FT-IR spectrum of control *U. minor* trees (Fig. 1), although complex due to broad overlapping peaks, showed a well-defined pattern with several characteristic bands (Table 2). A clear separation between spectra from control and inoculated trees was detected from 30 to 120 dpi in the PCA (Fig. 2). This separation was mainly characterized by PC1 axis, which showed a positive score gradient for control trees and a

Table 1
Symptom development in *Ulmus minor* trees after inoculating with *Ophiostoma novo-ulmi* (4 May, 2004)

Clone	Origin from Spain	Susceptibility to DED[a]	Percentage of wilting leaves[b]				
			5 dpi	15 dpi	30 dpi	60 dpi	120 dpi
UPM068	Huélago, Granada	high	0	0	45 (100)	95 (100)	98 (100)
UPM158	San Nicolás, Sevilla	high[c]	0	1 (5)	20 (100)	40 (100)	60 (100)
UPM171	Puebla de Montalbán, Toledo	high	0	2 (10)	30 (100)	90 (100)	100 (100)
UPM007	Alatoz, Albacete	low	0	2 (10)	5 (25)	10 (50)	10 (50)
UPM066	Deifontes, Granada	low	0	0	5 (25)	15 (75)	20 (75)
UPM130	Pedrizas, Málaga	low	0	2 (10)	15 (70)	25 (100)	25 (100)

[a] According to a previous susceptibility test.
[b] Figures in brackets indicate the percentage of wilting leaves of the inoculated branch; dpi: days post-inoculation.
[c] Should be reclassified to medium according to the symptom development.

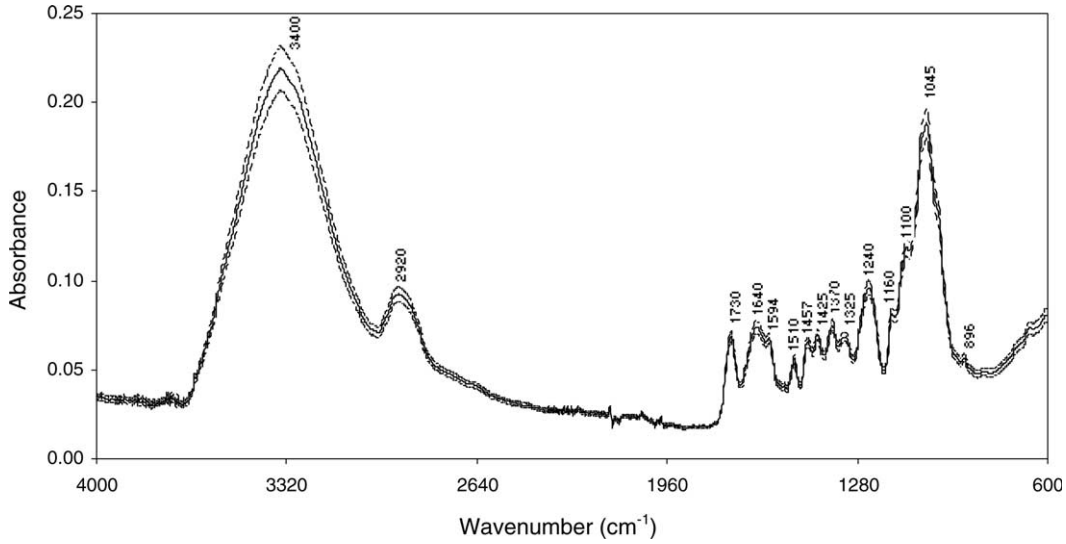

Fig. 1. FT-IR average spectrum from xylem samples harvested 0 days post-inoculation from control *Ulmus minor* trees. Dotted lines indicate 95% confidence limits.

Table 2
Summary of the frequencies and proposed structural assignments of the most characteristics FT-IR bands of the spectra from *Ulmus minor* xylem samples

Frequency (cm^{-1})	Assignment	References
3400	O–H stretching	Dorado et al. (2001), Pandey and Pitman (2003)
2920	C–H stretching	Dorado et al. (2001), Pandey and Pitman (2003)
1730	C═O stretching of alkyl ester	Séné et al. (1994), Dorado et al. (2001)
1640	C═O stretching and C–N stretching	Séné et al. (1994)
1594	Aromatic ring in lignin	Dorado et al. (2001), Pandey and Pitman (2003)
1510	Aromatic ring in lignin	Chen and McClure (2000), Dorado et al. (2001), Pandey and Pitman (2003)
1457	C–H bending in alkyl groups	Chen and McClure (2000), Dorado et al. (2001)
1425	C–H deformation in lignin	Chen and McClure (2000), Pandey and Pitman (2003)
1370	C–H deformation in cellulose and hemicellulose	Pandey and Pitman (2003)
1325	C–H vibration in cellulose	Pandey and Pitman (2003)
1280–1185	C–O–H deformation and C–O stretching in phenolic compounds	Séné et al. (1994)
1176	C–O stretching in starch	Aburto et al. (1999), Sun and Tomkinson (2003)
1160	C–O–C vibration in cellulose	Kacurakova et al. (2000), Pandey and Pitman (2003)
1100	C–O and C–C stretching in pectin	Kacurakova et al. (2000)
1096	C–O stretching in starch	Aburto et al. (1999), Sun and Tomkinson (2003)
1045	C–O stretching in cellulose, hemicellulose and pectin	Kacurakova et al. (2000), Pandey and Pitman (2003)
983	C–O stretching in starch	Aburto et al. (1999), Sun and Tomkinson (2003)
896	C–H deformation in cellulose, hemicellulose and pectin	Kacurakova et al. (2000), Pandey and Pitman (2003)

negative score gradient for inoculated trees. The percentage of variance explained by PC1 was about 50% on 5 and 15 dpi, and about 75% on 30, 60, and 120 dpi. When the loadings for PC1 were analyzed, a similar pattern of peaks was identified on 30, 60, and 120 dpi (Fig. 3). Two prominent positive peaks appeared at 1730 and 983 cm^{-1}, which could be assigned to pectin ester groups (Wilson et al., 2000) and to starch (Aburto et al., 1999; Černá et al., 2003; Sun and Tomkinson, 2003), respectively. Additional positive peaks were observed at 1160 (glycosidic linkage), 1100 (pectin), 1045 (xylan) and 896 cm^{-1} (β-linkage), in the carbohydrate region (1200–800 cm^{-1}). Negative peaks appeared at 1594 and 1510 cm^{-1}, characteristic of aromatic rings in lignin, at 1260 and 1215 cm^{-1}, characteristic of C–O–H deformation and C–O stretching of phenolic compounds, and at 1457 cm^{-1} for the alkyl bending band. These results showed that the xylem tissues of the inoculated elms, when compared to the control, contained lower levels of polysaccharides and higher levels of phenolic compounds. The application of the I_2/KI test for starch to the samples confirmed lower starch content in the inoculated samples with respect to the controls.

Degradation of cell wall polysaccharides caused by fungal enzymes could explain the decrease of the carbohydrate to lignin proportion in inoculated trees. Severe

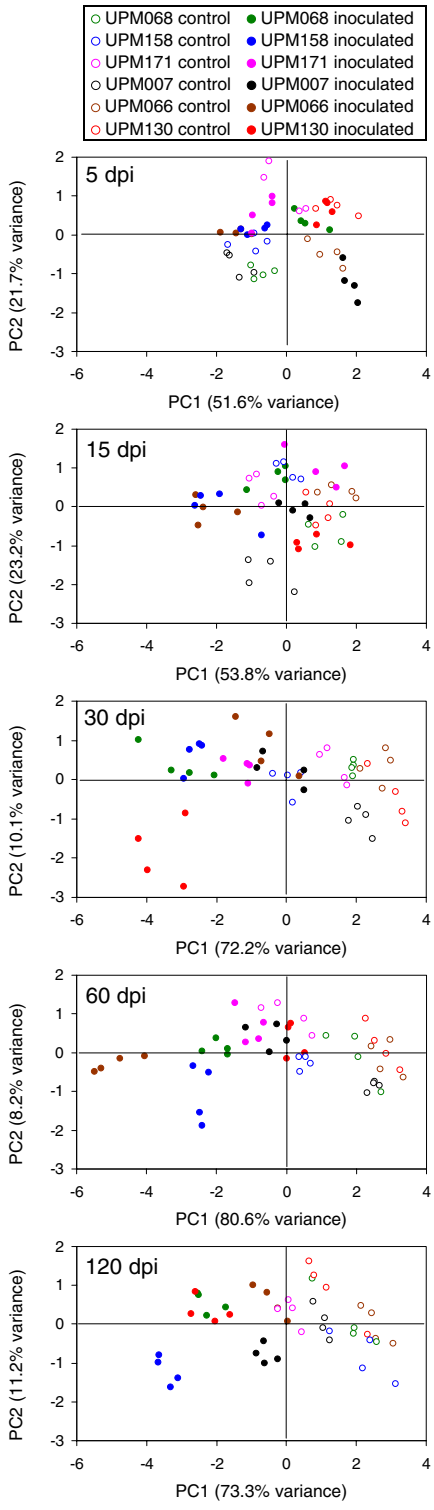

Fig. 2. PCA scores scatter plots of spectra from xylem samples harvested 5, 15, 30, 60 and 120 days post-inoculation (dpi) from control and inoculated *Ulmus minor* trees.

erosion of the vessel walls takes place during cell wall degradation caused by *O. ulmi* (Scheffer and Elgersma, 1982). The depletion of starch content in the inoculated xylem tissues as infection progressed could be related with fungal degradation of parenchyma cells or with the great amount of energy probably required for the activation of the host defence system (Shigo and Tippett, 1981). In addition, this activation probably contributed to the enhancement of lignin and other polymeric phenols, formed in plants as a response to pathogens within host cell walls (Rioux and Ouellette, 1991; Nicholson and Hammerschmidt, 1992). The enhanced level of the 1457 cm^{-1} peak in the inoculated trees probably corresponds to the accumulation of aliphatic compounds (e.g., lipids) in the tissues. In order to test this hypothesis, the temporal evolution of the absorbance at 1457 cm^{-1}, and at 2920 cm^{-1} (alkyl C–H groups) was studied (Fig. 4). From 30 to 120 dpi, both bands increased in inoculated trees with respect to the control trees. Changes in intensity of both bands were analyzed through a Pearson's correlation analysis. The correlation was significant ($r = 0.88$; $P \leqslant 0.01$), suggesting that both bands represented the same metabolite or a group of co-ordinately metabolites. The accumulation of aliphatic compounds as a response to infection could be related with the suberization of parenchyma cell walls and tyloses. Suberin is a lipidic polymer composed by long-chain aliphatic monomers associated with significant quantities of phenolic compounds (Rocha et al., 2001; Graça and Pereira, 2004). The suberized cells are resistant to fungal degradation and could also act as an effective waterproof seal, reducing drying and helping to maintain an inimical microenvironment for fungal development (Pearce, 1996). Previous research confirmed suberin and lignin production in DED-infected *U. minor* trees through FT-IR spectroscopy and histochemical tests (Martin et al., 2005), and in *Ulmus americana* L. through histochemical tests (Rioux and Ouellette, 1991).

Considering inoculated trees alone, there was a clear separation between samples from susceptible and from resistant trees on 15 dpi (Fig. 5(a)). In this case, the separation was mainly characterized by PC2, which showed positive scores for susceptible trees and negative scores for resistant trees. The loadings plot of PC2 (Fig. 5(b)) showed a prominent negative peak at 983 cm^{-1}, suggesting a higher starch proportion in samples from resistant than from susceptible trees. As the PCA did not show differences between the spectra of susceptible and resistant trees on 0 dpi, the mentioned differences on 15 dpi could be related with a higher degradation of starch directly by fungal action or by a higher expense of energy for defensive responses in the susceptible clones. A reduction in starch reserves stored in xylem parenchyma cells of stressed trees has been associated with increased susceptibility to pathogens including *Armillaria* spp. (Wargo, 1972). Depletion of energy reserves, mainly linked to the amount of starch present in the tissues, has been suggested to

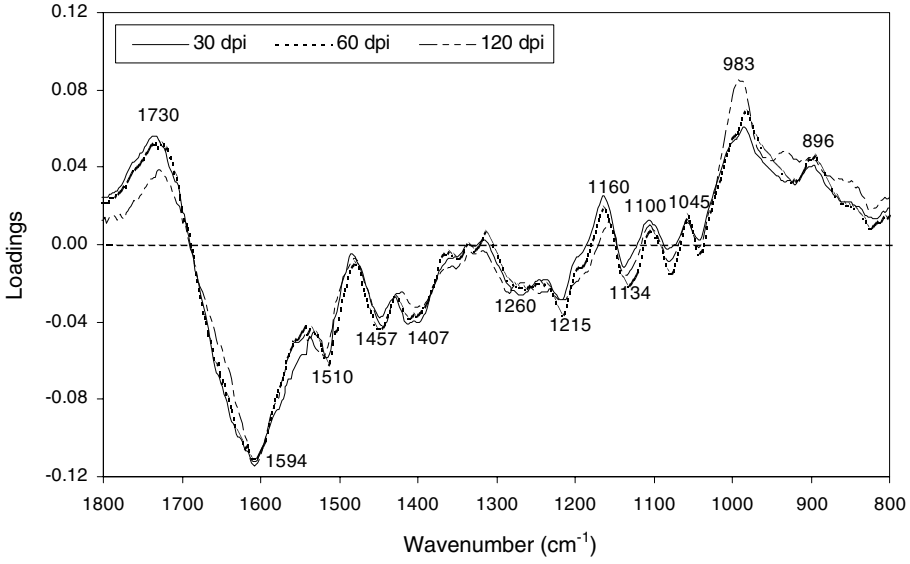

Fig. 3. Loadings plot of PC1, main factor of distinction between spectra from xylem samples harvested 30, 60 and 120 days post-inoculation (dpi) from control and inoculated *Ulmus minor* trees (see Fig. 2).

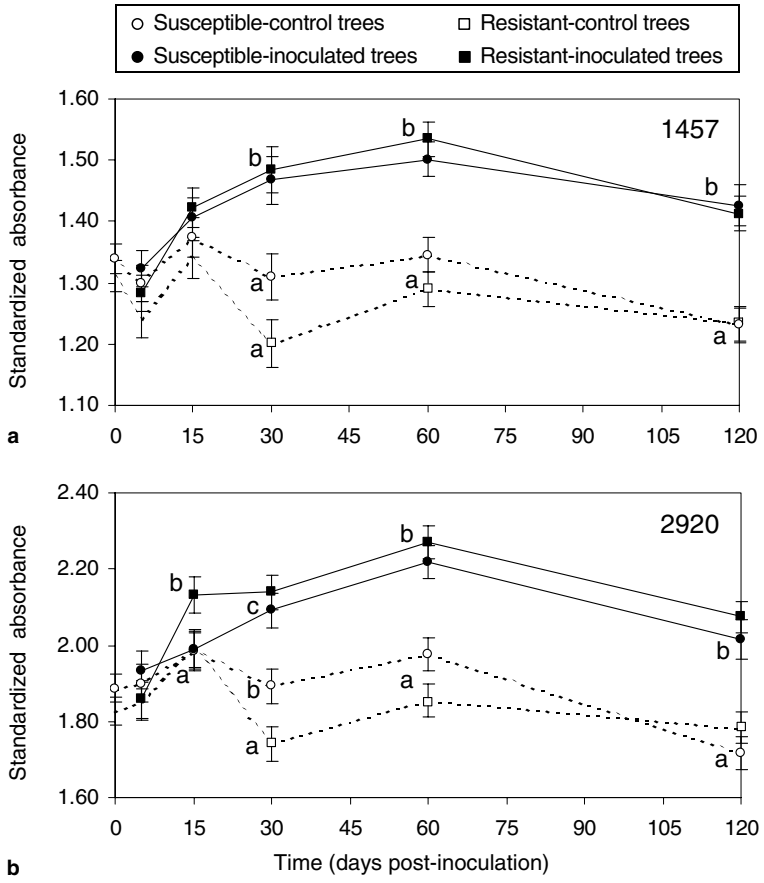

Fig. 4. Temporal evolution of the mean spectral absorbances (standardized values) at (a) 1457 cm^{-1}, and (b) 2920 cm^{-1}, from xylem samples from susceptible- and resistant-inoculated *Ulmus minor* trees. Mean spectral absorbances were calculated from 12 FT-IR spectra, obtained from each group of trees (3 clones per group, 4 replicates per clone). Different letters indicate differences among groups of trees ($P < 0.05$) and bars indicate 95% confidence limits.

explain the higher formation of barrier zones in the larger parts of elms compared with annual shoots and small branches (Shigo and Tippett, 1981; Bonsen et al., 1985), and may also be a determining factor for trees to survive recurrent infection (Shigo et al., 1986). American elms (*U. americana*) usually produce a heavy

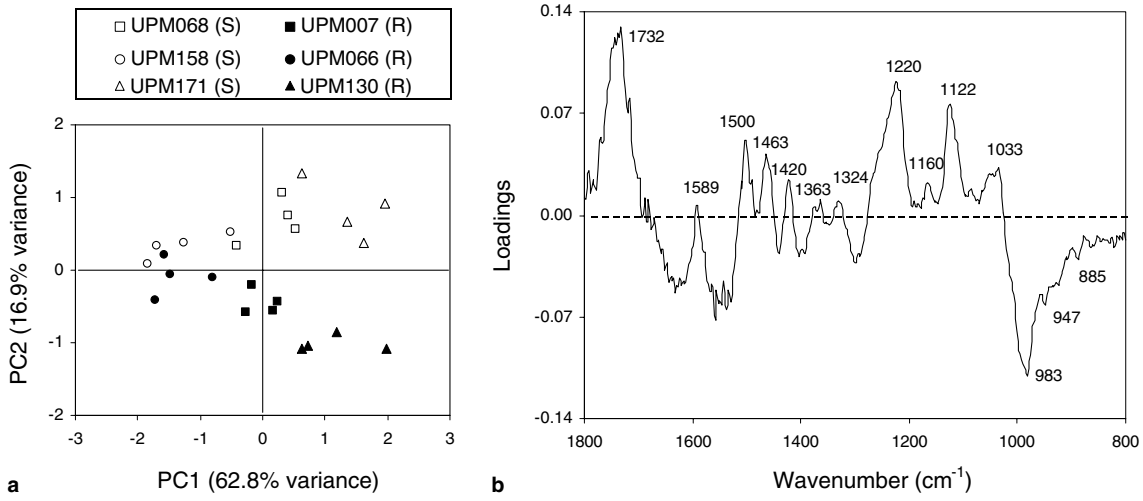

Fig. 5. PCA of spectra from xylem samples harvested 15 days post-inoculation from susceptible (S) and resistant (R) inoculated *Ulmus minor* trees. (a) PC1–PC2 scores scatter plot, (b) loadings plot of PC2.

seed crop before the new growth rings begin to develop, with the consequent drain on energy reserves. This fact has been suggested as a factor favouring the high susceptibility of this elm species (Shigo and Tippett, 1981).

Absolute and relative absorbance changes for the diagnostic spectral wavenumbers, 983 cm^{-1} (starch), 1160 cm^{-1} (carbohydrates), 1457 cm^{-1} (aliphatic chains), 1510 cm^{-1} (aromatic rings), and 1730 cm^{-1} (esters), are shown in Fig. 6. The analysis of the relative absorbances confirms, in most cases, the results obtained with the PCA. However, this analysis detected several differences between susceptible- and resistant-control trees that were not appreciable with PCA. The A_{1160}/A_{1510} ratio (Fig. 6(g)) was proposed as a good reference to calibrate carbohydrate to lignin content (Pandey and Pitman, 2003). The lower values of this ratio for inoculated compared to control trees on 30, 60 and 120 dpi would indicate a reduction of the carbohydrate to lignin proportion as consequence of infection. Differences between susceptible- and resistant-inoculated trees were observed on 15 dpi at A_{983}/A_{1730} and A_{1457}/A_{1510} ratios. In the case of A_{983}/A_{1730} ratio (Fig. 6(f)), the higher values for resistant trees could be related with different starch content, as discussed above. For the A_{1457}/A_{1510} ratio (Fig. 6(h)), the lower values for resistant trees could be related to a higher proportion of aromatic structures in these trees due to the production of host defence compounds (e.g., phytoalexins or lignin-like material), but more research is needed. Significant differences between susceptible- and resistant-control trees were found on 15 dpi for 983/1730 cm^{-1} ratio, on 30 dpi for the three studied ratios, on 60 dpi for 983/1730 cm^{-1} and 1160/1510 cm^{-1} ratios, and on 120 dpi for 983/1730 cm^{-1} and 1457/1510 cm^{-1} ratios. The interpretation of such differences is complex, but the potential to use these ratios as markers of resistance is evident. A more thorough investigation of this promising application is in progress.

3. Concluding remarks

This study shows the power of FT-IR for metabolic profiling. Although minor compounds are not covered by this approach, one single analysis allows a number of quite different cell constituents to be covered. For *U. minor*, the FT-IR spectra showed a decrease of the carbohydrate to lignin proportion in infected xylem, as revealed by the changes in the bands at 1594, 1510, 1160, and 1100 cm^{-1}. Furthermore, the starch level in the xylem tissues after inoculation, according to the band at 983 cm^{-1}, seems to be related with tree susceptibility, confirming previous literature. It may be concluded that FT-IR together with PCA is a valid tool for analysing plant-pathogen interaction of DED, and could be used in the future for monitoring resistant elm genotypes.

4. Experimental

4.1. Plant material

Six *U. minor* clones from different locations in Spain were used in this work (Table 1). Clones were selected from previously tested 38 Spanish elm clones, since they showed different AFLP fingerprinting profiles (Gil et al., 2004) and markedly different responses to DED in the susceptibility tests described below.

Eight ramets of each clone were obtained in 1995 by root cutting and planted in an inoculation plot at

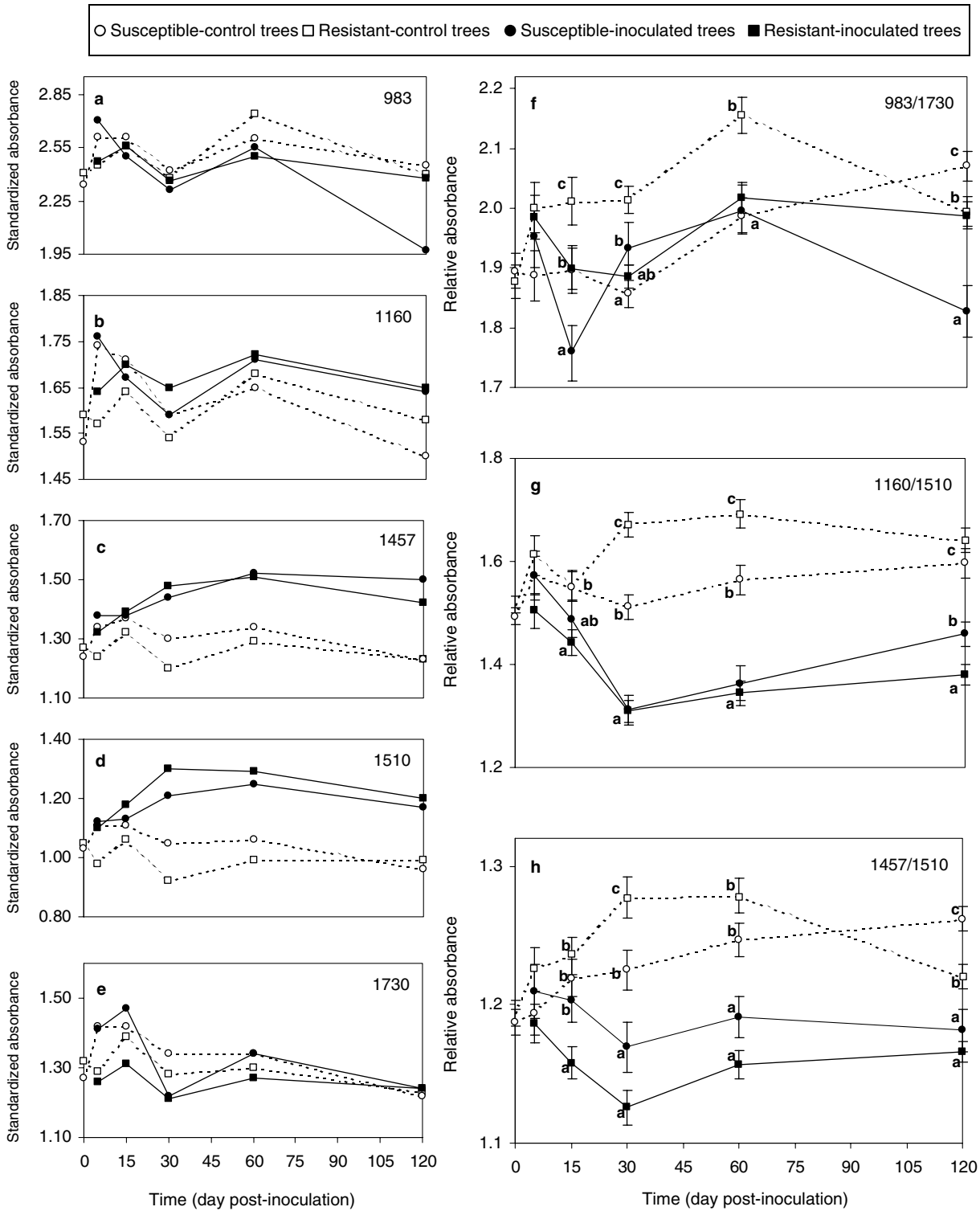

Fig. 6. Temporal evolution of absolute (a–e) and relative (f–h) mean absorbances for several diagnostic wavenumbers from xylem samples from susceptible- and resistant-inoculated *Ulmus minor* trees. (a) 983 cm^{-1}, (b) 1160 cm^{-1}, (c) 1457 cm^{-1}, (d) 1510 cm^{-1}, (e) 1730 cm^{-1}, (f) A_{983}/A_{1730}, (g) A_{1160}/A_{1510}, (h) A_{1457}/A_{1510}. Mean absorbances were calculated from 12 FT-IR spectra, obtained from each group of trees (3 clones per group, 4 replicates per clone). Different letters indicate differences among groups of trees ($P < 0.05$) and bars indicate 95% confidence limits.

the Forest Breeding Centre in Puerta de Hierro (Madrid, Spain). The plot was a two-block design, with random experimental units of 4 plants and 0.9×0.9 m spacing. For the FT-IR study, two additional ramets per clone were planted in an adjacent plot (spacing of 4×4 m). The soil at the site had a fine sandy loam texture and was amended annually (March) by organic matter to enhance moisture

retention. The plots were irrigated by sprinklers during spring and summer as required.

4.2. Susceptibility test

On 29 April 1998, when the trees were 4-year-old and about 2.0–2.5 m in height, the ramets for the susceptibility test were inoculated. *O. novo-ulmi* ssp. *americana* isolate OR-VR was used for inoculations because of its rapid in vitro growth rate (4.5 mm per day on 2% malt extract agar at 20 °C) and high virulence (Solla et al., 2005). The pathogen was collected in 1996 from an infected *U. minor* tree in Verín (Orense, Spain). Spore suspensions were prepared in Tchernoff's liquid medium (Tchernoff, 1965) and density adjusted in water to 10^6 ml^{-1}. Inoculations were performed by direct introduction of 0.1 ml spore suspension into the sap stream through a knife wound made at the base of the trunk (Solla et al., 2005). After 60 days the percentage of crown affected by foliar wilt was recorded. Susceptibility ratings of high, medium and low were assigned to genotypes according to their wilting percentages: 100–67%, 66–34% and 33–0%, respectively. Genotypes of high and low susceptibility were selected for the FT-IR study (Table 1).

4.3. Inoculations for FT-IR study and sample removal

On 4 May 2004, when the trees were 10-year-old and about 4–6 m in height, one ramet per each selected clone was inoculated. Inoculations were performed at the base of a 5-year-old branch (north side, and upper half of the crown), using the same methodology and inoculum as for the susceptibility test. The second ramet per clone was identically wounded and received distilled water (control). On the inoculation day, two samples were harvested from each control tree. On 5, 15, 30, 60 and 120 dpi, an additional number of samples was harvested from control and inoculated trees and the percentage of wilting leaves of each inoculated tree was recorded. Samples consisted on 3-year-old twig segments (3.0 cm length) removed from the upper third of each branch. No additional samples were harvested from non-wounded branches in order to account for the wounding effect. Adjacent branches were used for sample removal if the xylem was dry. The samples were immediately frozen in liquid N_2 and stored at −80 °C until required.

4.4. Sample preparation and FT-IR spectroscopy

Sample preparation for FT-IR analysis was optimized until good spectra with high signal to noise ratio were obtained. Spectra were collected from immediately defrosted wood material in order to prevent enzymatic degradation of the samples. After bark removal, the xylem of the outermost growth ring of the samples was extracted using a blade. The wood powder obtained was placed on a GoldenGate single-reflectance ATR accessory of a Brucker IFS-55 FT-IR spectrometer. The spectra were acquired by accumulating 64 interferograms at a resolution of 4 cm^{-1} at the absorbance mode from 4000 to 600 cm^{-1}, using OPUS 2.1 software. Four replicate spectra were obtained from each sample.

4.5. Data processing and statistical analysis

The spectra were transferred through JCAMP.DX format into the data analysis software package developed by Barros and Rutledge (Barros, 1999). All spectra were baseline corrected, normalized and centred. A window between 1800 and 800 cm^{-1}, which contains information of all major components of the xylem tissues (Martin et al., 2005), was selected in order to monitor metabolic modifications. PCA was applied in order to reduce the dimensionality of the spectral data, enabling clustering within the data set to be observed. The principal components were displayed graphically as scores scatter plots for observing any groupings in the data set. In PCA, coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Massart et al., 1998). Thus, loading plots were used to detect the wavenumbers responsible for the separation in the data. Each spectrum was auto-scaled or divided by the standard deviation, in order to obtain standardized absorbances, which were used to compare peak intensities among groups of samples. Relative absorbances were calculated by dividing the height of several diagnostic spectral wavenumbers. These ratios were analyzed using one-way ANOVA, considering the susceptibility groups as a factor. Fisher's least significant difference (LSD) procedure was applied to compare averages ($P < 0.05$). The ANOVA analysis was undertaken using the Statgraphics Plus 5.1 software.

Acknowledgements

The authors thank M. Burón and D. López for plant propagation, Dr. I. Delgadillo and Dr. S. Rocha for their help with FT-IR analysis, Dr. A. Barros for his suggestions with multivariate analyses and corrections on the text, and J.A. Ferreira for his technical support. The work was developed within the framework of an agreement established between Dirección General para la Biodiversidad (Ministerio de Medio Ambiente) and ETSI de Montes in Madrid (UPM). J.A. Martín was supported by a FPI grant from Comunidad Autónoma de Madrid.

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