Forest Pathology WILEY-BLACKWELL

For. Path. 46 (2016) 215–228 doi: 10.1111/efp.12247

Physiological and biochemical differences among *Ulmus minor* genotypes showing a gradient of resistance to Dutch elm disease

By M. Li[1], R. López[1,2,3], M. Venturas[1], J. A. Martín[1], J. Domínguez[1], G. G. Gordaliza[1], L. Gil[1] and J. Rodríguez-Calcerrada[1,4]

[1]Grupo de Investigación en Genética y Fisiología Forestal, ETSI Montes, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain; [2]INRA Clermont Université, Clermont-Ferrand, France; [3]Present address: Hawkesbury Institute for the Environment, UWS, Science Road, Richmond, 2753 NSW, Australia; [4]E-mail: jesus.rcalcerrada@upm.es (for correspondence)

Summary

Dutch elm disease (DED) spread across Europe and North America in the 20th century killing most natural elm populations. Today, breeding programmes aim at identifying, propagating and studying elm clones resistant to DED. Here, we have compared the physiology and biochemistry of six genotypes of *Ulmus minor* of variable DED resistance. Leaf gas exchange, water potential, stem hydraulic conductivity and biochemical status were studied in 5-year-old trees of AB-AM2.4, M-DV2.3, M-DV2 × M-CC1.5 and M-DV1 and 6-year-old trees of VA-AP38 and BU-FL7 before and after inoculation with *Ophiostoma novo-ulmi*. Leaf water potential and net photosynthesis rates declined, while the percentage loss of hydraulic conductivity (PLC) increased after the inoculation in susceptible trees. By the 21st day, leaf predawn and midday water potential, stomatal conductance to water vapour and net photosynthesis rates were lower, and PLC was higher in trees of susceptible (S) genotypes inoculated with the pathogen than in control trees inoculated with water, whereas no significant treatment effect was observed on these variables in the resistant (R) genotypes. Fourier transform infrared spectroscopy analyses revealed a different biochemical profile for branches of R and S clones. R clones showed higher absorption peaks that could be assigned to phenolic compounds, saturated hydrocarbons, cellulose and hemicellulose than S clones. The differences were more marked at the end of the experiment than at the beginning, suggesting that R and S clones responded differently to the inevitable wounding from inoculation and repeated sampling over the experimental course. We hypothesize that a weak activation of the defence system in response to experimental wounding can contribute to the susceptibility of some genotypes to *O. novo-ulmi*. In turn, the decline in shoot hydraulic conductivity and leaf carbon uptake caused by the infection further exacerbates tree susceptibility to the fungus.

1 Introduction

In the twentieth century, two pandemics of Dutch elm disease (DED) spread across Europe and North America. The first, detected in 1920, was caused by the fungus *Ophiostoma ulmi*, whereas the second, which started in 1970 and is still ongoing, was caused by the more aggressive *Ophiostoma novo-ulmi* (Brasier 1991). Both pandemics devastated elm populations; for example, in Europe, more than 90% of mature *Ulmus minor* Mill. trees were lost to DED (Brasier and Buck 2001).

The pathogen causing DED is transmitted by several species of bark beetles, mostly *Scolytus* and *Hylurgopinus* (family Curculionidae). These insects feed on twig phloem where fungal spores are deposited and germinate. The fungal hyphae grow into the outer vessels of xylem where they can spread upwards, downwards and laterally through vessel pits, and sporulate as budding cells (yeastlike phase).

Several are the mechanisms by which DED affects its host. The fungus, once inside xylem vessels, produces cell wall-degrading enzymes such as xylanases, pectinases or glucosidases (Binz and Canevascini 1996; Przybył et al. 2006). Early research on pathogenicity of *O. ulmi* suggested that the hydrophobin *cerato-ulmin* was a wilt toxin that affected elm physiology by reducing transpiration and increasing leaf respiration (Takai 1974; Richards 1993). However, further research showed that *cerato-ulmin*-disrupted mutants of *O. novo-ulmi* were also pathogenic (Bowden et al. 1996). *Cerato-ulmin* is now considered a parasitic fitness factor which contributes to the transmission of the disease by protecting spores from desiccation and increasing their adherence to bark beetles (Temple et al. 1997; Bernier et al. 2015). Molecules secreted by the fungus also enhance xylem vulnerability to cavitation by reducing the sap surface tension and by degrading intervessel pit membranes (Newbanks et al. 1983; Solla and Gil 2002a; Cochard et al. 2009). Xylem dysfunction is caused mainly by induced embolism (Newbanks et al. 1983). Nevertheless, losses in hydraulic conductivity are also due to vessel occlusion by tyloses and gels produced by the tree to prevent fungal spreading as well as by substances secreted in the sap by the fungus (Ouellette et al. 2004). As a consequence of reduced shoot hydraulic conductivity, water supply to leaves ceases, leaf water potential declines, and the stomata close to conserve the water homoeostasis (Aldea et al. 2006; Venturas et al. 2014). Hydraulic failure has interactive impacts on tree carbon metabolism. The most immediate impact is the reduction of leaf photosynthesis because of impaired water and CO_2 availability to mesophyll cells; but the fungal infection may also limit the photosynthetic capacity because of the reduction of chlorophyll content and photochemical efficiency of PSII in chloroplasts (Oliveira et al. 2012). Less straightforward effects of fungal invasion on the tree carbon balance might arise from a potential increase of respiration related to the upregulation of the defence system (Landis and Hart 1972; Richards 1993). In the longer term, massive fungal spread can cause massive embolism of the hydraulic system and dieback (Thomas et al. 2002; Wullschleger et al. 2004; Jacobsen et al. 2012). New shoots can be produced from the roots, but eventually, sustained hydraulic and carbon limitations may compromise the ability of the tree to resprout (Goodsman et al. 2013).

Received: 13.7.2015; accepted: 27.10.2015; editor: C. G. Fossdal

Elm trees have a variable resistance to DED. Breeding programmes have devoted a continuous effort towards the identification of resistant genotypes of *U. minor* (Franke et al. 2004; Collin and Vernisson 2006; Martín et al. 2015), and several genotypes of marked resistance to DED have been propagated *ex situ* over the last decades. This plant material has provided an excellent opportunity to investigate the aspects underpinning DED resistance. For example, today we know that elms with narrower and scattered vessels tend to be more resistant to DED than those with larger xylem vessels or larger vessel groups (Solla and Gil 2002a), as these later characteristics favour hydraulic conductance, fungus spreading and xylem cavitation. Compared to anatomical studies, fewer studies have evaluated the importance of physiological and biochemical characteristics for DED resistance. Martín et al. (2005, 2008a) have compared the biochemical profiles of elm species and genotypes of contrasted DED resistance with Fourier transform infrared (FT-IR) spectroscopy analysis. Actually, the use of FT-IR fingerprinting has proved useful for detecting chemical changes after fungal infections (Dorado et al. 2001; Pandey and Pitman 2003; Martín et al. 2005, 2008a). Moreover, research on water relations and gas exchange in response to pathogen infection (e.g. Meinzer et al. 2004; Domec et al. 2013) has rarely been conducted in relation to DED (Oliveira et al. 2012; Urban and Dvořák 2014; Venturas et al. 2014).

Plants have constitutive phenological, physical and chemical barriers against fungus infection. Early flushing can be a phenological barrier against DED (Ghelardini and Santini 2009); physical barriers involve some properties of the plant surfaces that prevent fungus penetration, such as hard cuticles or small stomatal pores; whereas chemical barriers are mainly 'phytoanticipins', antimicrobial compounds that are present in plants before fungal infection, or produced after infection from pre-existed constituents (Vanetten et al. 1994; Iriti and Faoro 2009), including saponins, cyanogenic glycosides and glucosinolates (Bowyer et al. 1995; Osbourn 1996; Bidart-Bouzat and Kliebenstein 2008; Zagrobelny et al. 2008). Once constitutive barriers fail, the fungus is recognized by plant cell receptors that elicit a complex signalling cascade that eventually induces a defence response. It is worth noting that sometimes the defence response is induced not by the pathogen itself, but rather by concomitant factors such as wounding (Steele et al. 1998; Nagy et al. 2000; León et al. 2001; Garcia et al. 2015). Compartmentalization is a mechanism by which trees isolate the pathogen in a localized area (Shigo and Tippett 1981; Pearce 1996). In the case of DED, the formation of suberized tyloses in xylem vessels restricts the vertical spread of the fungus and produces the isolation of healthy vessels from infected ones (Ouellette et al. 2004); more so in resistant than susceptible clones of *Ulmus* × *hollandica* Mill. (Elgersma 1973). One of the most effective barrier against DED is probably the wall 4 of the CODIT model (Shigo and Tippett 1981), constituted by suberized or lignified parenchyma cells formed *the novo* by the cambium, which prevents the radial spread of the pathogen from earlywood vessels towards the cambial cells (Ouellette et al. 2004; Martín et al. 2005). However, compartmentalization may be insufficient for trees to prevent fungal infections; rather, this response is usually integrated within other biochemical responses involving the production of secondary metabolites such as mansonones (elm phytoalexins; Duchesne et al. 1992; Ouellette and Rioux 1992; Vanetten et al. 1994; Grayer and Kokubun 2001; Witzell and Martín 2008). Once trees are infected, there may be a negative feedback of fungus spreading on subsequent tolerance. Genotypes capable of maintaining relatively high rates of hydraulic conductance and net CO_2 assimilation after infection could have a larger pool of non-structural carbohydrates, and thus more respiratory substrates to synthesize defence compounds and new tissue after shoot dieback (Kozlowski 1992). On the contrary, a rapid closure of stomata upon infection could slow down the dispersion of pathogens through xylem sap and allow for the tree to restrict the damages by compartmentalizing or killing the pathogen.

Here, we have compared the physiology and biochemistry of clones of *U. minor* of variable DED resistance before and after an experimental inoculation with an aggressive strain of *O. novo-ulmi*. Clones of high, intermediate and low resistance to DED were selected from those clonally propagated by the Spanish Elm Breeding Program. Our objectives were to investigate (i) whether there are constitutive differences in leaf physiological and shoot biochemical properties among these clones; (ii) how fast and to what extent does the inoculation with *O. novo-ulmi* affect their physiology and biochemistry; and (iii) what are the causes and implications of any potentially different response. We hypothesized that contrasting levels of DED resistance are related to differences in physiological and chemical profiles between clones, either prior or subsequent to the infection. More specifically, we hypothesized that resistant clones maintain higher rates of hydraulic conductivity and leaf gas exchange after inoculation with *O. novo-ulmi* than more susceptible clones; and that resistant clones contain and/or respond to inoculation with *O. novo-ulmi* by producing more defence compounds than susceptible clones.

2 Materials and methods

2.1 Plant material

Twenty-four *U. minor* trees representing six genotypes (four trees/genotype) were selected for the experiment. The genotype selection was based on previous evaluations of susceptibility to inoculation with *Ophiostoma novo-ulmi* performed by the Spanish Elm Breeding Program. This way, two genotypes were of high resistance to the disease, showing under 30% leaf wilting and branch dieback after inoculation with *O. novo-ulmi* ('R' genotypes AB-AM2.4, M-DV2.3), two of medium resistance (30–75% wilting and dieback; 'M' genotypes BU-FL7, M-DV2 × M-CC1.5) and two of low resistance, showing over 75% leaf wilting soon after the inoculation ('S' genotypes VA-AP38, M-DV1).

2.2 Experimental design

Trees were planted at the forest breeding centre of Puerta de Hierro (Madrid, Spain; 3°45′N, 40°27′E; 595 m a.s.l.) in spring 2010, and maintained with supplementary watering during summers to avoid water-deficit stress. Plants were

divided into two blocks, each including the six genotypes randomly planted at 1 × 1 m spacing and the two treatments applied in 2013: inoculation with *O. novo-ulmi* (hereafter inoculated trees) and inoculation with distilled water (hereafter controls). Trees were 5 years old for AB-AM2.4, M-DV2.3, M-DV2 × M-CC1.5 and M-DV1 and 6 years old for VA-AP38 and BU-FL7 when treatments were applied, as it is recommended that *U. minor* trees older than 4 years are used when studying resistance to DED (Solla et al. 2005).

The inoculation took place in the first week of May 2013, when leaves had almost fully expanded. A 0.1 ml of spore suspension (10^6 conidia ml^{-1}) was inoculated by making a horizontal incision in the stem at approximately 10–15 cm from the ground with a sharpened, sterilized razor blade reaching the xylem. The *Ophiostoma novo-ulmi* Brasier ssp. *americana* Z – BU1 was used for inoculation. The isolate was collected from DED-infected trees in Bubierca (Zaragoza, Spain) in 2009. Control trees were also cut and inoculated with distilled water in the same way. Inoculations were made on a sunny day to make sure the solutions were effectively taken up by trees.

Sampling for leaf and branch physiology was conducted on days 0, 7, 14, 21, and 30 after the inoculations while sampling for branch biochemistry was conducted on days 0 and 21. To decide which leaves or branches to sample, we first evaluated the health status of the tree. Either dark green or yellowing leaves were selected according to the predominant status of tree foliage (healthy or yellowing); that is, a yellowing leaf was sampled when trees had >50% of leaves yellowing. Similarly, branches of similar diameter (approximately 0.8–1.0 cm) were sampled for hydraulic conductance and chemistry studies according to the predominant health status of the tree; that is, branches had some yellowing leaves when most tree leaves were yellowing. The idea behind the non-random sampling was to have a realistic picture of the physiological and chemical changes that were taking place after the inoculation at the tree level (see Domec et al. 2013). Withered leaves or branches containing withered leaves were never sampled.

2.3 Gas exchange

Leaf gas exchange was measured with a LI-6400 portable gas exchange system (Li-Cor Inc., Lincoln, NE, USA) equipped with a 6-cm^2 chamber coupled to a LED light source (LI-6400-02B) in branches at the lower tree crown. Measurements were typically conducted from 10:00 to 13:00 h under ambient air vapour pressure deficit (1–2 kPa), and artificially adjusted microenvironmental conditions of 800 μmol m^{-2} $second^{-1}$ photon flux density, 400 ppm CO_2 concentration, and 25°C air temperature. Net photosynthesis rate (P_n), stomatal conductance to water vapour (g_s), internal CO_2 concentration (C_i) and transpiration rates (E) were recorded. Dark respiration rate (R_d) was subsequently measured in the same leaf, but before, the leaf was covered with aluminium foil paper for 30 min to prevent light-enhanced respiration (Atkin et al. 1998). Area-based gas exchange rates were expressed per unit leaf dry mass after measuring the mass of the leaf area enclosed in the cuvette.

2.4 Water potential

Predawn and midday leaf water potentials were measured with a pressure chamber (PMS Instrument Company, Albany, OR, USA) on the same days as gas exchange and hydraulic conductance measurements.

2.5 Hydraulic conductance

Xylem hydraulic conductance was measured using a XYL'EM embolism-meter (Bronkhorst, Montigny les Cormeilles, France). Stem segments from 1-year-old branches were selected to avoid the cold induced embolism from previous winter. Branches from the upper tree crown (>0.75 m long) were cut under water, to avoid air entering through the cut surface. They were placed with their cut end in a bucket containing water, covered with double black plastic bags, and transported to the laboratory (<10 min) where measurements were performed immediately. Their bark was carefully removed from the end to be connected to the conductivity apparatus, and both segment ends were shaved with a new razor. Initial hydraulic conductivity (K_i) was measured with a solution of 10 mM KCl and 1 mM $CaCl_2$ in deionized degassed water at 2–3 × 10^{-3} MPa pressure. After measuring K_i, segments were flushed with the same solution at 0.18 MPa for 10 min to remove embolisms and to obtain the maximum hydraulic conductivity (K_{max}). The percentage loss of hydraulic conductivity (PLC) was then calculated as: PLC (%) = 100 × (K_{max}−K_i)/K_{max}; with this value corresponding to native embolism. Initial specific stem conductivity (K_s) and maximum specific stem conductivity (K_{smax}) were calculated by dividing K_i and K_{max}, respectively, by the xylem cross-sectional area. All the leaves on the branches above the sampled segment were collected and then scanned to know the total leaf area on the branch (A_L). Then, the specific leaf hydraulic conductivity (K_L) was obtained by dividing initial hydraulic conductivity by the total leaf area on the branch.

2.6 Biochemistry

Stem segments collected for biochemistry analyses were immediately frozen in liquid nitrogen after cutting and later stored at −80°C until required. Frozen branch samples were lyophilized and milled into a fine powder to conduct Fourier transform infrared (FT-IR) spectroscopy analysis (600–4000 cm^{-1}). An infrared spectrophotometer FT-IR Perkin–Elmer 1600 (Perkin Elmer Inc, Boston, MA, USA) was used to obtain the absorption spectra of each sample. Each pellet was prepared with 240 mg KBr and 3 mg of sample powder ground into an agate mortar uniformly, and then pressed in a vacuum microenvironment using a pulling press (Perkin Elmer Inc) specially constructed for the preparation of KBr pellets for IR

spectroscopy and pre-adjusted at 10×10^4N. Three measurements were made per sample for obtaining the final IR spectrum. Spectra were displayed at a resolution of 4 cm^{-1} at the absorbance mode from 4000 to 600 cm^{-1}. Information on the relationship between absorption peaks, chemical assignments and tentative compound groups is given in Table 1.

2.7 Statistical analyses

Physiological variables were analysed by repeated-measures ANOVA to test the main effects of time, inoculation treatment and susceptibility to DED, and their interaction effects on data means. Measurement day was considered as the repeated-measures factor, whereas genotype was nested into the DED susceptibility factor. At each day, *t*-tests – or Welch tests when variances were significantly different – were used to compare means between the two infection treatments, for each group of DED susceptibility (high, intermediate and low). Control trees of each group were compared by one-way ANOVAs before the inoculation to test for potential constitutive differences among them. Some variables were log10-transformed so that the test requirements of normality and homogeneity of variance were observed. These analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA), with 95% confidence limits ($p < 0.05$).

FT-IR spectra were processed using OPUS 2.1 Software (Opus software Inc, San Francisco, CA, USA). For each spectrum, a total of 19 peaks were measured. The baseline of each peak was first added and then the height of the peak, from the maximum absorbance value to the peak baseline, was measured. Analysis of variance was used to compare means of peak heights among tree groups of different DED susceptibility, with Tukey's post hoc tests further employed to separate significantly different groups. A principal component analysis (PCA) of the 19 peak heights per sample was also used to explore for potential differences in the broad biochemical profiles of clones with different DED susceptibility and between treatments. The scatter plot of PC1 and PC2 scores helped identifying any clustering in samples; loadings of original variables were also shown in the plots to visually inspect the influence of specific peaks on data scattering. Furthermore, the relationship between branch chemical profile (quantified through the PC1) and the susceptibility to DED of each genotype (quantified through the percentage of wilting after inoculation) was analysed via linear regression analysis. PCA analyses were made with SPSS 17.0.

3 Results

3.1 Wilting status

The results of wilting after inoculation with *O. novo-ulmi* of this experiment confirmed the contrasted susceptibility among genotypes observed in the previous susceptibility test (Table 2): wilting progressed faster in S than R clones. First symp-

Table 1. List of the most characteristic FT-IR bands and proposed structural assignments of the spectra from *Ulmus minor* samples.

Wavelength (cm^{-1})	Assignment	Tentative functional groups	Reference
3394	O–H stretching	Carbohydrates and glycoconjugates	Dorado et al. (2001), Pandey and Pitman (2003)
2922	C–H stretching	Saturated hydrocarbons (fatty acids, suberin)	Dorado et al. (2001), Pandey and Pitman (2003)
1738	C=O stretching	Fatty acids, aliphatic and aromatic carbonyl-containing compounds	Sene et al. (1994), Dorado et al. (2001)
1622	C=O, C–N, and C=C stretching	Lignin, proteins, peptidoglycans	Sene et al. (1994), Dorado et al. (2001), Pandey and Pitman (2003)
1514	C=C stretching	Aromatic ring in lignin	Chen and McClure (2000), Dorado et al. (2001), Pandey and Pitman (2003)
1427	C–H deformation	Lignin, phenolic compounds	Chen and McClure (2000), Pandey and Pitman (2003), Sene et al. (1994)
1374	C–H deformation	Cellulose and hemicellulose	Pandey and Pitman (2003)
1318	C–H vibration	Cellulose	Pandey and Pitman (2003)
1248	C–O–H deformation and C–O stretching	Phenolic compounds	Sene et al. (1994)
1161	C–O–C vibration	Cellulose	Kacurakova et al. (2000), Pandey and Pitman (2003)
1108	C–O and C–C stretching	Pectin	Kacurakova et al. (2000)
1054	C–O stretching	Cellulose, hemicellulose and pectin	Kacurakova et al. (2000), Pandey and Pitman (2003)
888	C–H deformation	Cellulose, hemicellulose and pectin	Kacurakova et al. (2000), Pandey and Pitman (2003)
<825	C-H and O-H bending; O-O, O-OH and P-O-C stretching, CH2 rocking; N-H wagging and twisting	Polysaccharides (glucose, maltose, starch); cell wall glycoconjugates; lipid peroxides; aromatic containing compounds; phospholipids; nucleic acids; lipids; nitrogen containing compounds	Pavlovic and Brandao (2003), Chalmers and Griffiths (2002), Stuart (2004), Coates (2000)

toms of wilting were found on day 14, and by day 21 more than 50% of tree foliage exhibited wilting in S clones. Clones M and R also showed visible symptoms of wilting, but to a lower extent (8–20%). Wilting increased to approximate 90, 50, and 20% in clones S, M and R, respectively, on day 30 (Table 2). Six months after inoculation, 37.5% of the S trees were dead, whereas all M and R clones were alive. One control tree of the susceptible genotype M-DV1 inoculated with water exhibited high percentage of wilting by day 30, possibly due to natural infection with the pathogen.

3.2 Water potential

No constitutive significant differences in Ψ_{pd} ($p = 0.43$) or Ψ_{md} ($p = 0.22$) were observed among S, M and R clones before the inoculation. Afterwards, there were clear differences in both variables among treatments and clones (Table 3). Both Ψ_{pd} and Ψ_{md} were barely affected by the inoculation treatment in R genotypes (Fig. 1). However, the response of M and S clones was similar and more intense; differences between treatments were appreciable at day 21, when, for example, control trees of M clones had 2 MPa higher Ψ_{md} than M inoculated trees. Differences between treatments persisted on day 30 although they were of lower magnitude and statistical significance (Fig. 1).

3.3 Gas exchange

No constitutive significant differences in g_s ($p = 0.97$), P_n ($p = 0.92$) or R_d ($p = 0.42$) were observed among S, M and R clones before the inoculation. Both g_s and P_n tended to decline after inoculation with *O. novo-ulmi*, although significant differences between control and inoculated trees were only found in S clones (Fig. 2; Table 3). The inoculated S trees had 3 and 10 times lower P_n than controls on days 21 and 30, respectively. In the case of R_d, the only significant difference between inoculated and control trees was found for S clones on day 30, when R_d was two times higher in the inoculated trees (Fig. 2). Both g_s and P_n in inoculated trees declined exponentially in response to increasing water stress (Fig. 3). The decline appeared to be steeper in R clones, but the fact that Ψ_{pd} did not fall to values as low as in the other clones precluded a reliable comparison of stomata sensitivity to water stress.

3.4 Hydraulic conductance

No constitutive significant differences in K_L ($p = 0.55$), K_{smax} ($p = 0.51$) or PLC ($p = 0.61$) were observed among S, M and R clones before the inoculation. However, all these variables were significantly affected by *O. novo-ulmi* in S clones. Significant differences in K_L were observed on day 21, when inoculated S trees had two times lower water supply to leaves than controls. PLC was approximately more than two times higher in S inoculated plants than in S controls on day 21. No significant differences between treatments were observed for R and M clones for these variables. In addition, on day 21 K_{smax} was significantly lower for S inoculated clones than controls (Fig. 4).

3.5 Biochemistry

Results of PCA of FT-IR spectral peaks indicated that clones separated according to their susceptibility to DED. Including only control trees in the analysis, R clones tended to occupy the positive side of the PC1 and S clones the negative, left side (Fig. 5a). The same analysis applied to data of day 21 showed a clearer separation between R and S clones along the PC1 (Fig. 5b). The absorption peaks that weighted more on PC1 (and thus on the separation of susceptibility groups) appeared at 3394 (O–H, N–H and C–H stretching), 2922 (C–H aliphatic stretching), 1738 (C=O stretching of ester groups), 1514 (aromatic C=C skeletal stretching), 1374 cm^{-1} (C–H symmetric deformation), and 1248 (C–O–C stretching) cm^{-1} (see Fig. 5c,d; Table 1). When plotting the average wilting percentage of clones (as a surrogate of tree susceptibility to DED) against the average scores of PC1 (as a surrogate of shoot chemical profile), a trend for S clones to have negative PC1 scores and R

Table 2. Evolution of wilting percentage for six genotypes of variable susceptibility to DED after inoculation with *O. novo-ulmi* (I) or water (C; controls).

		Wilting leaves (%)							
		Day 1		Day 14		Day 21		Day 30	
Genotype	Previous DED susceptibility characterization	I	C	I	C	I	C	I	C
M-DV1	Susceptible (S)	0	0	5 ± 5	0	67.5 ± 7.5	12.5 ± 12.5	90	30 ± 30
VA-AP38	Susceptible (S)	0	0	12.5 ± 7.5	0	55 ± 35	2.5 ± 2.5	87.5 ± 7.5	10 ± 10
M-DV2*M-CC1.5	Intermediate (M)	0	0	5 ± 5	0	20	2.5 ± 2.5	72.5 ± 12.5	10 ± 10
BU-FL7	Intermediate (M)	0	0	0	0	20	0	30 ± 10	0
AB-AM2.4	Resistant (R)	0	0	0	0	7.5 ± 2.5	0	20	0
M-DV2.3	Resistant (R)	0	0	0	0	15 ± 5	0	22.5 ± 2.5	0

Table 3. Repeated-measures ANOVA testing the effects of time, treatment, susceptibility to DED and the interactions of treatment × susceptibility, time × treatment, and time × treatment × susceptibility on means of leaf and shoot physiological variables: predawn water potential (Ψ_{pd}), midday water potential (Ψ_{md}), leaf net photosynthesis (P_n), stomatal conductance to water vapour (g_s), transpiration rate (E), dark respiration (R_d), internal CO_2 concentration (C_i), leaf mass per area (LMA), initial specific stem hydraulic conductivity (K_s), maximum specific stem hydraulic conductivity (K_{smax}), specific leaf hydraulic conductivity (K_L) and percentage loss of hydraulic conductivity (PLC).

	Time	Treatment	Susceptibility	Treatment × Susceptibility	Time × Treatment	Time × Treatment × Susceptibility
Ψ_{pd}	0.00	0.00	0.00	0.00	0.00	0.04
Ψ_{md}	0.00	0.00	0.43	0.00	0.00	0.02
P_n	0.00	0.01	0.05	0.66	0.00	0.56
g_s	0.00	0.03	0.00	0.47	0.09	0.87
E	0.00	0.01	0.01	0.56	0.04	0.99
R_d	0.00	0.32	0.12	0.59	0.25	0.17
C_i	0.00	0.85	0.03	0.23	0.66	0.23
LMA	0.00	0.37	0.08	0.03	0.08	0.88
K_L	0.65	0.86	0.95	0.42	0.36	0.57
K_s	0.08	0.64	0.23	0.94	0.01	0.40
K_{smax}	0.01	0.96	0.08	0.56	0.18	0.29
PLC	0.10	0.26	0.55	0.93	0.00	0.38

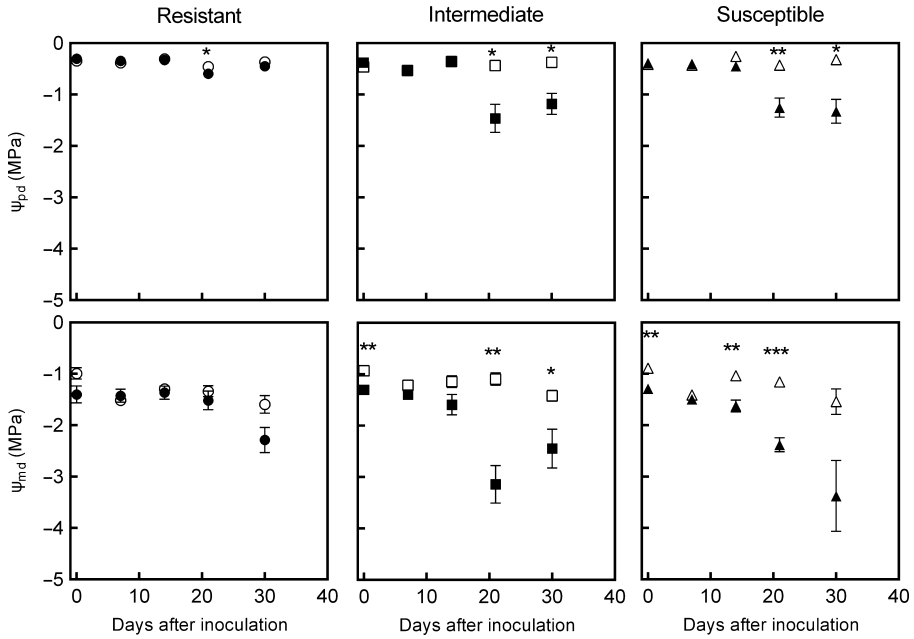

Fig. 1. Time course of predawn (Ψ_{pd}) and midday (Ψ_{md}) water potentials of resistant (circles), intermediate (squares) and susceptible (triangles) clones (each including two genotypes) subjected to inoculation with *O. novo-ulmi* (solid symbols) or water (controls; open symbols). Symbols are means ± standard error (n = 4). Differences between inoculation treatments are based on *t*-tests ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***).

clones to have positive PC1 scores was evident at day 0 and significant at day 21 (Fig. 5e,f), supporting the observation that the biochemical profile differs among clones according to their susceptibility to DED. Considering individual absorption peaks, only that at 3394 cm^{-1} at day 0 and that at 2922 cm^{-1} at day 21 were significantly different among clones (highest in R and lowest in S in both cases; Fig. 5g,h).

In response to the inoculation with *O. novo-ulmi*, no clear distinction in the biochemical profile was observed between inoculated and controls on any date; only a light separation was visible for S clones at day 21 (Fig. 6). Pooling data of infected and control trees to analyse differences in absorption peaks among clones, we observed significantly higher peaks at 2922, 1374, 1248 and 888 cm^{-1} in R than S clones, with M clones showing intermediate values (Fig. 7).

4 Discussion

Thousands of *U. minor* genotypes have been screened for DED resistance in the last decades within the Spanish Elm Breeding Program. Thanks to this continuous effort, today, seven *U. minor* genotypes – including the M-DV2.3 genotype studied here – have been registered as resistant clones by the Spanish Administration and are planned to be used in reforestations

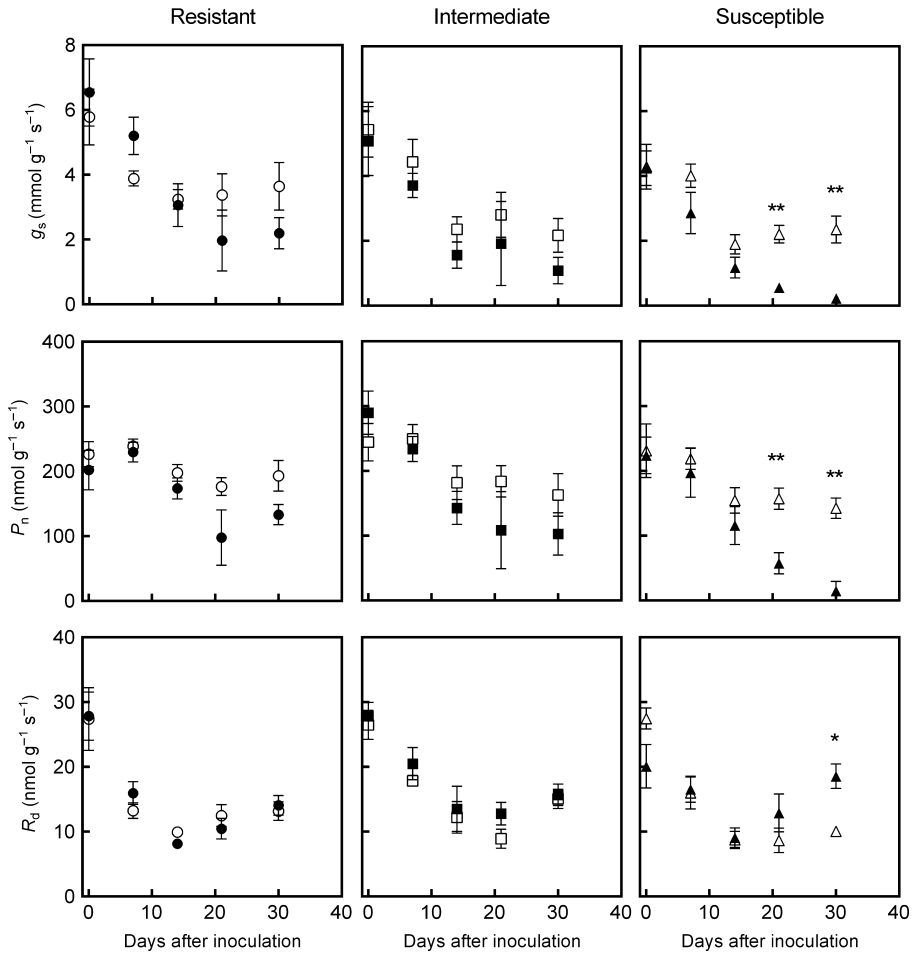

Fig. 2. Time course of leaf mass-based stomatal conductance to water vapour (g_s), net photosynthesis (P_n) and dark respiration (R_d) of resistant (circles), intermediate (squares) and susceptible (triangles) clones (each including two genotypes) subjected to inoculation with *O. novo-ulmi* (solid symbols) or water (controls; open symbols). Symbols are means ± standard error (n = 4). Differences between inoculation treatments are based on *t*-tests ($p < 0.05$, *; $p < 0.01$, **).

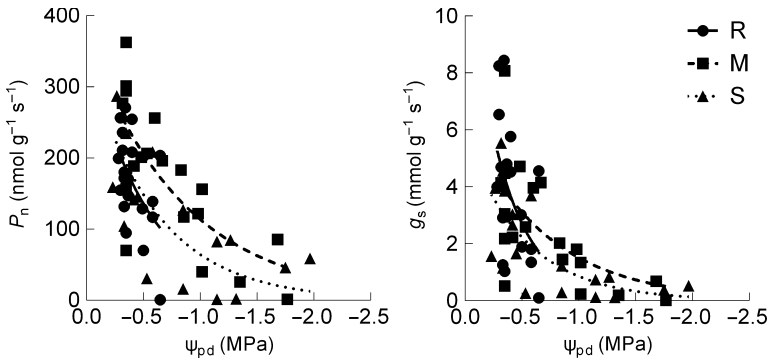

Fig. 3. Relationship between leaf predawn water potential (Ψ_{pd}) and leaf mass-based net photosynthesis (P_n) and stomatal conductance to water vapour (g_s) in resistant (R; circles), intermediate (M; squares) and susceptible (S; triangles) clones (each including two genotypes) with inoculation.

in their natural habitats (Martín et al. 2015). Indeed, here, clones M-DV2.3 and AB-AM2.4 showed a remarkable resistance to DED. These clones exhibited a different biochemical profile than more susceptible ones and their basic physiological functions – tree water status, shoot hydraulic conductivity and leaf gas exchange – were not affected for 30 days since the inoculation with the pathogen *O. novo-ulmi*.

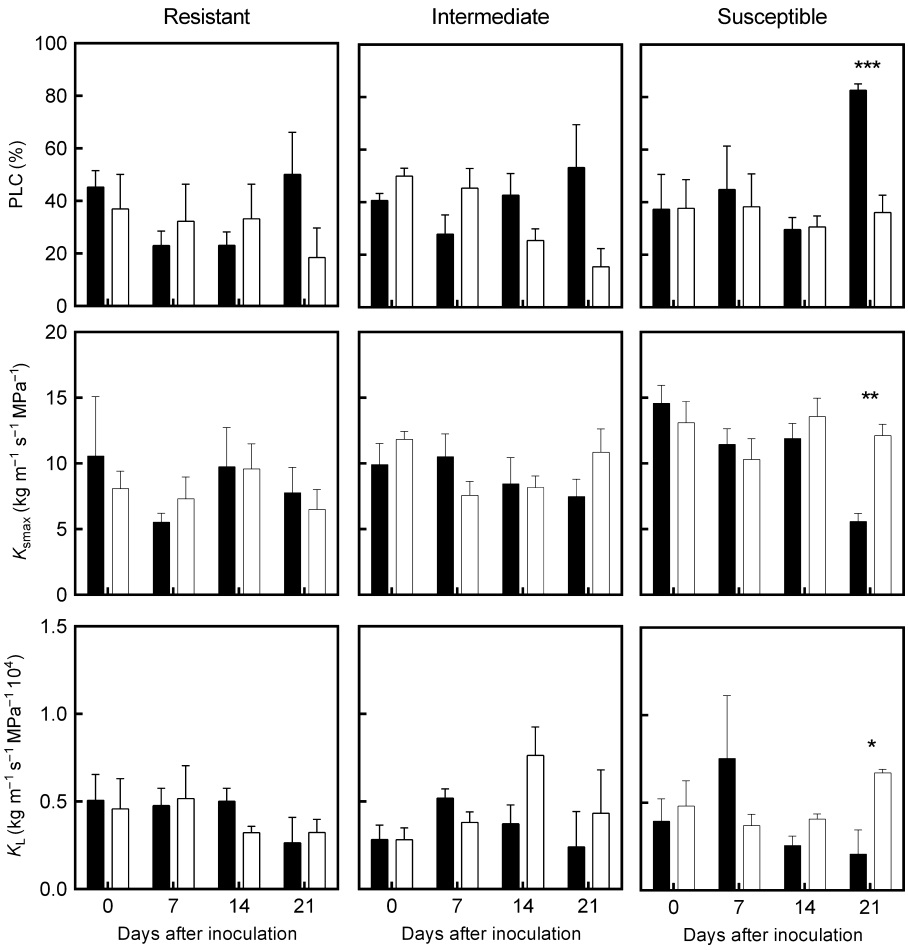

Fig. 4. Time course of per cent loss of hydraulic conductivity (PLC), maximum xylem specific conductivity (K_{smax}) and specific leaf hydraulic conductivity (K_L) of resistant, intermediate and susceptible clones (each including two genotypes) subjected to inoculation with *O. novo-ulmi* (solid bars) or water (controls; open bars). Symbols are means ± standard error (n = 4). Differences between inoculation treatments are based on *t*-tests ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***).

By combining carbon-related and hydraulic measurements we delineated the sequence of functional changes occurring in *U. minor* trees when inoculated with *O. novo-ulmi*. Susceptible clones started to show physiological alterations as soon as 14 days after the inoculation; shoot hydraulic conductivity decreased relative to control trees, resulting in lower supply of water to individual leaves, lower water potentials, and lower rates of stomatal conductance and net CO_2 assimilation. The reduction in hydraulic conductivity and water potential is consequence of pathogen infection, something observed in some vascular tree diseases (Duniway 1973), and that has previously been reported for DED (Newbanks et al. 1983; Urban and Dvořák 2014). This can be due to induced embolism or to direct vessel blockage by the fungus and compounds it secretes. The accumulation of gels and tyloses can also block xylem vessels of *U. minor* trees in response to *O. novo-ulmi* (Ouellette et al. 2004) and explain the associated decline in water transport capacity. Actually, the significant differences observed in susceptible clone's hydraulic parameters 21 days after inoculation indicate that both induced embolism and vessel blockage contribute to losses in conductivity. If increased PLC was only due to induced embolism, K_{smax} for inoculated and control plants would have been equal after flushing. However, K_{smax} was reduced to half that of control plants. On the other hand, the high reduction in native K_s, which lead to 85% PLC, cannot be explained only by vessel blockage, supporting Newbanks et al. (1983) induced embolism hypothesis. It is to be noted that *U. minor* is highly susceptible to drought-induced cavitation (Venturas et al. 2013, 2014) and any conductivity losses may lead to runaway cavitation. These restrictions in leaf water potential caused a stomatal limitation to photosynthesis, as suggested by the similar leaf internal concentration of CO_2 in infected and control susceptible trees (data not shown). However, other studies have reported that fungal infections can limit leaf photochemical efficiency and photosynthetic capacity (Santos et al. 2005; Oliveira et al. 2012). Contrarily, an increase in respiration can take place soon upon pathogen inoculation in response to an increased demand of respiratory products to set up defence mechanisms (Landis and Hart 1972). Leaf dark respiration increased in inoculated susceptible trees relative to controls, but only in senescing leaves late after inoculation, which does not point to a defence response but to a senescence-related peak (Tetley and Thimann 1974). It is possible that leaf water stress, which typically causes a reduction in mitochondrial respiration rates (e.g. Rodríguez-Calcerrada et al. 2011), offset any defence-induced increase in respiration.

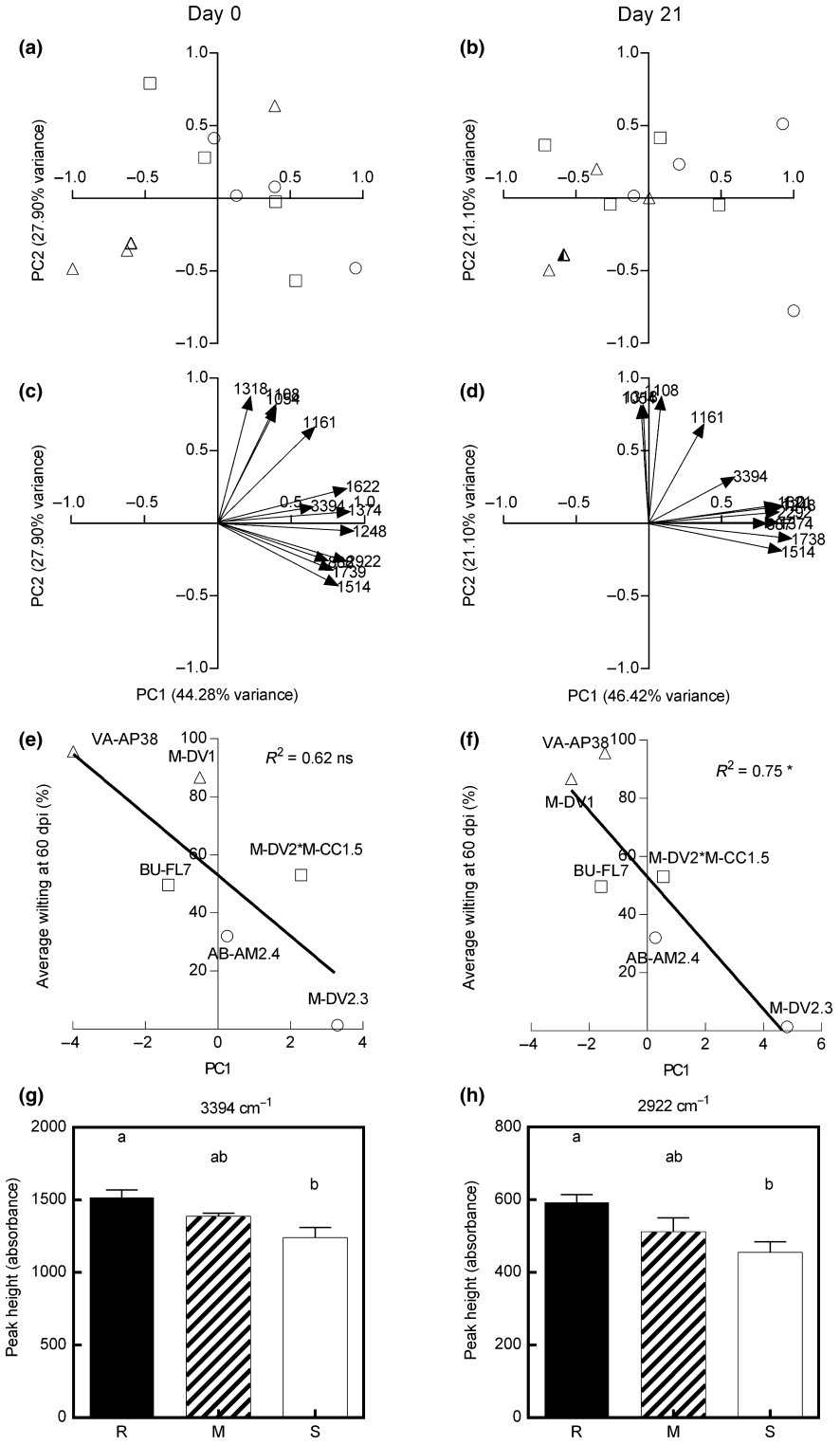

Fig. 5. (a,b) PCA scatter plots of FT-IR spectroscopy data from branches of resistant (circles; AB-AM2.4 and M-DV2.3 pooled), intermediate (squares; BU-FL7 and M-DV2 × M-CC1.5 pooled) and susceptible (triangles; VA-AP38 and M-DV1 pooled) clones on days 0 and 21 since inoculation; (c,d) weight of absorption peaks (see Table 1 for details on related chemical compounds) in PCA on days 0 and 21 since inoculation; (e,f) relationship between average wilting percentage of clones after inoculation (as a surrogate of tree susceptibility to DED) and the average PC1 scores (as a surrogate of shoot chemical profile) on days 0 and 21 since inoculation; (g,h) comparison of peak heights at 3394 cm^{-1} (related to absorption of carbohydrates and glycoconjugates; day 0) and 2922 cm^{-1} (related to saturated hydrocarbons; day 21) among resistant (R), intermediate (M) and susceptible (S) clones (each including two genotypes). Different letters indicate significant differences at $p < 0.05$ after Tukey's HSD test in (g) and (h). A half-black and half-white symbol in (b) refers to one susceptible tree with wilting leaves. All data are from control (c; open symbols) trees inoculated with water at day 0.

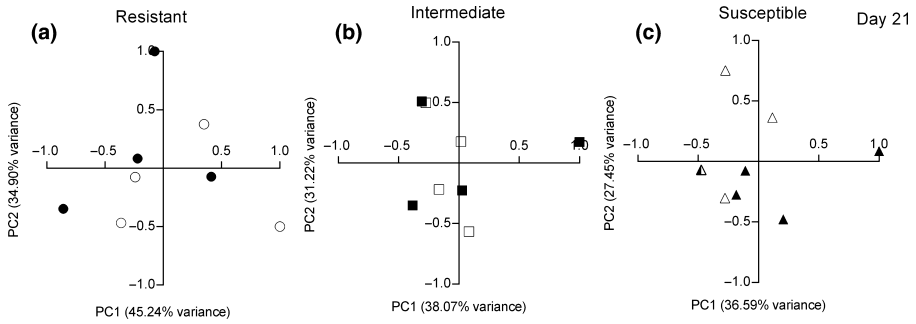

Fig. 6. (a–c) PCA scatter plots of FT-IR spectroscopy data from branches of resistant (circles; AB-AM2.4 and M-DV2.3 pooled), intermediate (squares; BU-FL7 and M-DV2 × M-CC1.5 pooled) and susceptible (triangles; VA-AP38 and M-DV1 pooled) clones on day 21 since inoculation with *O. novo-ulmi* (solid symbols) or water (open symbols).

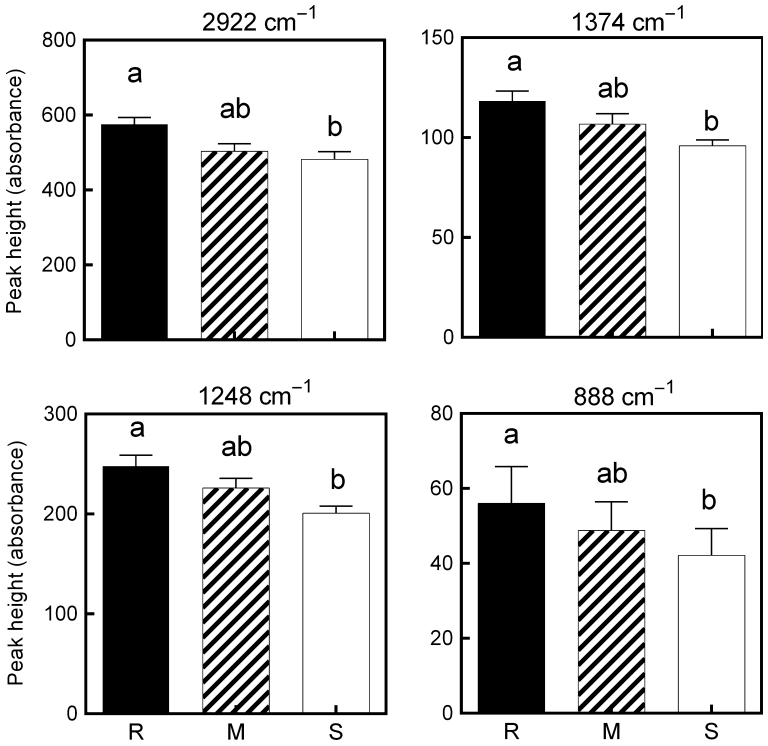

Fig. 7. Comparison of absorption peak heights at 2922, 1374, 1248 and 888 cm^{-1} wavelengths among resistant (R), intermediate (M) and susceptible (S) clones (each including two genotypes) on day 21 after inoculation. Different letters indicate significant differences at $p < 0.05$. Trees inoculated with *O. novo-ulmi* or water (controls) were pooled based on lack of chemical differences between them (see Fig. 6).

Physiological traits measured here did not show any difference indicative of constitutive resistance among clones. However, the fact that resistant clones maintained near-optimal tree water status, shoot hydraulic conductivity, foliage area, and leaf gas exchange for 1 month upon inoculation with *O. novo-ulmi* feeds forward on the capacity to overcome the fungal infection. Maintaining foliage area and net CO_2 assimilation ensures that non-structural carbohydrate reserves follow normal seasonal dynamics. On the contrary, susceptible clones suffering a progressive loss of water transport capacity and CO_2 assimilation after inoculation will likely suffer a progressive depletion of non-structural carbohydrate reserves, more so if respiratory carbon losses remain unchanged or increase, as it seems to occur (see above). In support of this, (i) higher levels of starch were found in the resistant *U. pumila* than the susceptible *U. minor* and also in resistant *U. minor* genotypes relative to susceptible ones after inoculation with *O. novo-ulmi* (Martín et al. 2008a); (ii) photosynthesis, and the concentrations of soluble sugar and starch decreased after *O. novo-ulmi* inoculation in *in vitro* cultures of *U. minor* (Oliveira et al. 2012); (iii) whereas leaf gas exchange and carbon and nutrient accumulation were not affected by *O. novo-ulmi* in mature resistant '*Dodoens*' elm (*U. glabra* 'Exoniensis' × *U. wallichiana*) hybrids (Ďurkovič et al. 2012). The shortage of carbon reserves, in turn, could limit the synthesis of defence compounds and compromise the capacity to recover from shoot die-

back (McDowell et al. 2008). While maintaining near-optimal water transport and carbon balance may help resistant trees to surmount the fungal infection, it remains unclear why the water transport is barely affected by the pathogen in these trees, while it is fast and severely restricted in others. One possibility that has been previously discussed is that clones with bigger vessels (Solla and Gil 2002b; Venturas et al. 2014) or bordered pits (Martín et al. 2009) are more prone to cavitate upon fungal inoculation or to facilitate the spread of fungal toxins and propagules. However, only the R clone M-DV2.3 showed certain anatomical properties (low proportion of large vessels) that could be related to resistance (J. Martin, Unpublished data). Another possibility is that resistant clones are able to restrict fungal spreading to areas close to the inoculation without producing gels and tyloses that block xylem vessels. The possession or rapid induction of certain defence compounds plays an important role in tree resistance to fungal infection (Duchesne et al. 1992; Grayer and Kokubun 2001; Witzell and Martín 2008). Through FT-IR spectroscopy analysis, here we explored the possibility that resistant clones displayed a broad chemical profile (before or after the inoculation) that might have conducted to a capacity to defend against fungal infection without compromising water transport capacity.

Before the inoculation, resistant clones tended to separate chemically from susceptible ones. Resistant clones scored higher in the first component of the PCA positively related to carbohydrates, suberin, fatty acids, cellulose and hemicellulose, and phenolic compounds (Fig. 7). An analysis of individual absorption peaks indicated that the peak at 3394 cm^{-1} was higher in resistant than susceptible clones. This intense peak, although rather unspecific, could be mainly assigned to carbohydrate and glycoconjugate biochemical compounds (Table 1). As mentioned above, a higher pool of non-structural carbohydrates could lead to a better capacity to stop fungal infections, due to the need of carbon substrates for the secondary metabolism (Iriti and Faoro 2009; Goodsman et al. 2013). The separation among clones using control trees was verified on day 21. Actually, the trend for more resistant clones to score higher in the PC1 was more evident and significant at $p < 0.05$, which has led us to hypothesize that repeated wounding – at stem base for water inoculation and tree top for hydraulic and biochemical sampling – enhanced the capacity to chemically defence against fungal infection. There are numerous examples of wound-induced defence activation (Steele et al. 1998; Nagy et al. 2000; León et al. 2001; Vek et al. 2013; Smith 2015), but this possibility has never been suggested in the case of DED. The resistant clones had a higher peak at 2922 cm^{-1} than susceptible clones, band characteristic of fatty acids and, in particular, of suberin-like substances (Martín et al. 2008b). Suberin is typically induced by wounding, at least locally, to isolate the injured parts of the tree (Biggs 1987; Pollard et al. 2008) and was reported to be induced in elms by chemical stress (Martín et al. 2008b, 2010). Suberin is an important cell wall phenolic polymer involved in preventing pathogen infection through the 'compartmentalization of decay in trees' (CODIT; Shigo 1984). As a hydrophobic waxy substance, suberin could also maintain xylem functionality by reducing the occurrence of cavitation (Pearce 1996; Franke and Schreiber 2007). Furthermore, both fatty acids and their derivatives jasmonic acid and oxylipins have been well reported in wound responses (Weber 2002; Tumlinson and Engelberth 2008; Upchurch 2008) and can induce systemic resistance against fungal infections (Kachroo and Kachroo 2009). In elms, soil application of carvacrol induced the accumulation of 16C and 18C fatty acids in xylem tissues and increased the plant tolerance to *O. novo-ulmi* (Martín et al. 2012).

Constitutive (phytoanticipins) or induced (phytoalexins) phenolic compounds are common and widespread secondary metabolites that play a role in defence. After pooling branches of control and infected trees, which surprisingly barely varied throughout the experimental course, it was observed that resistant clones had higher absorption peaks at 1248 cm^{-1}, band that could be associated to C–O–H deformation and C–O stretching in phenolic compounds (Sene et al. 1994), and at 1374 and 888 cm^{-1}, bands which could be mainly assigned to cellulose and hemicellulose (Kacurakova et al. 2000; Pandey and Pitman 2003). Phenolic compounds play a double role as antimicrobial and signalling compounds (Hammerschmidt 2005; Cvikrova et al. 2006; Lattanzio et al. 2006; Witzell and Martín 2008). For example, the accumulation of phenolic compounds is necessary for suberin biosynthesis (Kolattukudy 1981; Pollard et al. 2008) and inhibits growth and sporulation of *O. novo-ulmi* (Rioux and Ouellette 1991; Martín et al. 2007, 2010). Cellulose is a component of the cell wall and was found to be in higher amounts in the resistant *U. pumila* than the susceptible *U. minor*, and in more resistant genotypes of *U. minor* inoculated with *O. novo-ulmi* (Martín et al. 2008a). Again, the fact that differences among clones in height of peaks at 1374, 1248 and 888 cm^{-1} were not observed at day 0, but at day 21 after repeated sampling injury to trees, suggests that a chemical response favourable to cope with *O. novo-ulmi* was induced by wounding, and that this response was of higher extent in resistant than susceptible clones. In line with this, it has been observed that wounding promotes the accumulation of phenolic compounds (Kemp and Burden 1986; Klepzig et al. 1995), whereas insect attacks can prompt the reinforcement of the cell walls with more cellulose and reduce cell susceptibility to subsequent fungal attacks (Mohebby 2005; Furstenberg-Hagg et al. 2013). This idea is consistent with the transmission of DED by bark beetles, which cause hundreds of small wounds in branches when feeding or laying eggs (Peacock et al. 1981; Webber 2004).

In conclusion, trees of clones M-DV2.3 and AB-AM2.4 not inoculated with *O. novo-ulmi* had a different biochemical profile than those of more DED-susceptible clones VA-AP38 and M-DV1. In particular, FT-IR spectroscopy data suggest that resistant clones M-DV2.3 and AB-AM2.4 had higher pools of saturated hydrocarbons (suberin and fatty acids), phenolic compounds, cellulose, and hemicellulose. All these compounds are well known for playing direct or indirect roles in defence, which suggests that part of the resistance of M-DV2.3 and AB-AM2.4 to DED is related to a better capacity to restrict the fungal spreading. Because differences among clones were observed after 21 days of repeated destructive sampling, we hypothesize that wounding induced the activation of chemical defence mechanisms more in resistant than susceptible clones. Future experiments must be specifically planned to corroborate this hypothesis. In any case, the higher capacity of resistant clones to restrict pathogen infection is reflected in the maintenance of shoot hydraulic conductivity, and leaf

water status and gas exchange, which feed forward on the capacity of these clones to cope with the fungus and/or recover from eventual damages.

Acknowledgements

We thank Paz Andrés for assistance and guidance in the use of the infrared spectrophotometer FT-IR Perkin–Elmer 1600. We also thank Pedro Perdiguero and Carmen Collada for helpful discussions, and one anonymous reviewer and Jean-Christophe Domec for reviewing the manuscript. Funding was provided by the project OLMOS (AGL2012-35580).

References

Aldea, M.; Hamilton, J. G.; Resti, J. P.; Zangerl, A. R.; Berenbaum, M. R.; Frank, T. D.; Delucia, E. H., 2006: Comparison of photosynthetic damage from arthropod herbivory and pathogen infection in understory hardwood saplings. Oecologia **149**, 221–232.

Atkin, O. K.; Evans, J. R.; Siebke, K., 1998: Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. Funct. Plant Biol. **25**, 437–443.

Bernier, L.; Aoun, M.; Bouvet, G. F.; Comeau, A.; Dufour, J.; Naruzawa, E. S.; Nigg, M.; Plourde, K. V., 2015: Genomics of the Dutch elm disease pathosystem: are we there yet? iForest **8**, 149–157.

Bidart-Bouzat, M. G.; Kliebenstein, D. J., 2008: Differential levels of insect herbivory in the field associated with genotypic variation in glucosinolates in *Arabidopsis thaliana*. J. Chem. Ecol. **34**, 1026–1037.

Biggs, A. R., 1987: Occurrence and location of suberin in wound reaction zones in xylem of 17 tree species. Phytopathology **77**, 718–725.

Binz, T.; Canevascini, G., 1996: Xylanases from the Dutch elm disease pathogens *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*. Physiol. Mol. Plant Pathol. **49**, 159–175.

Bowden, C. G.; Smalley, E.; Guries, R. P.; Hubbes, M.; Temple, B.; Horgan, P. A., 1996: Lack of association between cerato-ulmin production and virulence in *Ophiostoma novo-ulmi*. Mol. Plant Microbe Interact. **9**, 556–564.

Bowyer, P.; Clarke, B.; Lunness, P.; Daniels, M.; Osbourn, A., 1995: Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science **267**, 371–374.

Brasier, C. M., 1991: *Ophiostoma novo-ulmi sp*. nov., causative agent of current Dutch elm disease pandemics. Mycopathologia **115**, 151–161.

Brasier, C. M.; Buck, K. W., 2001: Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). Biol. Invasions **3**, 223–233.

Chalmers, J. M.; Griffiths, P. R., 2002: Handbook of Vibrational Spectroscopy. Chichester: Wiley.

Chen, M.; McClure, J. W., 2000: Altered lignin composition in phenylalanine ammonia-lyase-inhibited radish seedlings: implications for seed-derived sinapoyl esters as lignin precursors. Phytochemistry **53**, 365–370.

Coates, J., 2000: Interpretation of infrared spectra, a practical approach. Encyclopedia of analytical chemistry.

Cochard, H.; Hölttä, T.; Herbette, S.; Delzon, S.; Mencuccini, M., 2009: New insights into the mechanisms of water-stress-induced cavitation in conifers. Plant Physiol. **151**, 949–954.

Collin, E.; Vernisson, F., 2006: Conservation and utilisation of genetic resources of European elm species. Workshop on Genetics, 18–22.

Cvikrova, M.; Mala, J.; Hrubcova, M.; Eder, J., 2006: Soluble and cell wall-bound phenolics and lignin in *Ascocalyx abietina* infected Norway spruces. Plant Sci. **170**, 563–570.

Domec, J. C.; Rivera, L. N.; King, J. S.; Peszlen, I.; Hain, F.; Smith, B.; Frampton, J., 2013: Hemlock woolly adelgid (*Adelges tsugae*) infestation affects water and carbon relations of eastern hemlock (*Tsuga canadensis*) and Carolina hemlock (*Tsuga caroliniana*). New Phytol. **199**, 452–463.

Dorado, J.; Almendros, G.; Field, J. A.; Sierra-Alvarez, R., 2001: Infrared spectroscopy analysis of hemp (*Cannabis sativa*) after selective delignification by *Bjerkandera* sp at different nitrogen levels. Enzyme Microb. Technol. **28**, 550–559.

Duchesne, L.; Hubbes, M.; Jeng, R., 1992: Biochemistry and molecular biology of defense reactions in the xylem of angiosperm trees. In: Defense mechanisms of woody plants against fungi. Ed. by Blanchette, A.; Biggs, R. Berlin: Springer, pp. 133–146.

Duniway, J. M., 1973: Pathogen-induced changes in host water relations. Phytopathology **63**, 458–466.

Ďurkovič, J.; Čaňová, I.; Lagaňa, R.; Kučerová, V.; Moravčík, M.; Priwitzer, T.; Urban, J.; Dvořák, M.; Krajňáková, J., 2012: Leaf trait dissimilarities between Dutch elm hybrids with a contrasting tolerance to Dutch elm disease. Ann. Bot. **111**, 215–227.

Elgersma, D. M., 1973: Tylose formation in elms after inoculation with *Ceratocystis ulmi*, a possible resistance mechanism. Neth. J. Plant Pathol. **79**, 218–220.

Franke, R.; Schreiber, L., 2007: Suberin-a biopolyester forming apoplastic plant interfaces. Curr. Opin. Plant Biol. **10**, 252–259.

Franke, A.; Rusanen, M.; De Aguiar, A.; Collin, E.; Harvengt, L.; Ackzell, L.; Bohnens, J.; Diamandis, S., 2004: Methods and progress in the conservation of elm genetic resources in Europe. Invest. Agrar. Sist. Recur. For. **13**, 261–272.

Furstenberg-Hagg, J.; Zagrobelny, M.; Bak, S., 2013: Plant defense against insect herbivores. Int. J. Mol. Sci. **14**, 10242–10297.

Garcia, T.; Gutierrez, J.; Veloso, J.; Gago-Fuentes, R.; Diaz, J., 2015: Wounding induces local resistance but systemic susceptibility to *Botrytis cinerea* in pepper plants. J. Plant Physiol. **176**, 202–209.

Ghelardini, L.; Santini, A., 2009: Avoidance by early flushing: a new perspective on Dutch elm disease research. iForest **2**, 143–153.

Goodsman, D. W.; Lusebrink, I.; Landhausser, S. M.; Erbilgin, N.; Lieffers, V. J., 2013: Variation in carbon availability, defense chemistry and susceptibility to fungal invasion along the stems of mature trees. New Phytol. **197**, 586–594.

Grayer, R. J.; Kokubun, T., 2001: Plant-fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. Phytochemistry **56**, 253–263.

Hammerschmidt, R., 2005: Phenols and plant-pathogen interactions: the saga continues. Physiol. Mol. Plant Pathol. **66**, 77–78.

Iriti, M.; Faoro, F., 2009: Chemical diversity and defence metabolism: how plants cope with pathogens and ozone pollution. Int. J. Mol. Sci. **10**, 3371–3399.

Jacobsen, A. L.; Roets, F.; Jacobs, S. M.; Esler, K. J.; Pratt, R. B., 2012: Dieback and mortality of South African fynbos shrubs is likely driven by a novel pathogen and pathogen-induced hydraulic failure. Austral Ecol. **37**, 227–235.

Kachroo, A.; Kachroo, P., 2009: Fatty acid-derived signals in plant defense. Annu. Rev. Phytopathol. **47**, 153–176.

Kacurakova, M.; Capek, P.; Sasinkova, V.; Wellner, N.; Ebringerova, A., 2000: FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. Carbohydr. Polym. **43**, 195–203.

Kemp, M. S.; Burden, R. S., 1986: Phytoalexins and stress metabolites in the sapwood of trees. Phytochemistry **25**, 1261–1269.

Klepzig, K.; Kruger, E.; Smalley, E.; Raffa, K., 1995: Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. J. Chem. Ecol. **21**, 601–626.
Kolattukudy, P., 1981: Structure, biosynthesis, and biodegradation of cutin and suberin. Annu. Rev. Plant Physiol. **32**, 539–567.
Kozlowski, T. T., 1992: Carbohydrate sources and sinks in woody plants. Bot. Rev. **58**, 107–222.
Landis, W. R.; Hart, J. H., 1972: Physiological changes in pathogen-free tissue of *Ulmus americana* induced by *Ceratocystis Ulmi*. Phytopathology **62**, 909–913.
Lattanzio, V.; Lattanzio, V. M.; Cardinali, A., 2006: Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochemistry **661**, 23–67.
León, J.; Rojo, E.; Sánchez-Serrano, J. J., 2001: Wound signalling in plants. J. Exp. Bot. **52**, 1–9.
Martín, J. A.; Solla, A.; Woodward, S.; Gil, L., 2005: Fourier transform-infrared spectroscopy as a new method for evaluating host resistance in the Dutch elm disease complex. Tree Physiol. **25**, 1331–1338.
Martín, J. A.; Solla, A.; Woodward, S.; Gil, L., 2007: Detection of differential changes in lignin composition of elm xylem tissues inoculated with *Ophiostoma novo-ulmi* using Fourier transform-infrared spectroscopy. For. Pathol. **37**, 187–191.
Martín, J. A.; Solla, A.; Coimbra, M. A.; Gil, L., 2008a: Metabolic fingerprinting allows discrimination between *Ulmus pumila* and *U. minor*, and between *U. minor* clones of different susceptibility to Dutch elm disease. For. Pathol. **38**, 244–256.
Martín, J. A.; Solla, A.; Coimbra, M. A.; Domingues, M. R.; Gil, L., 2008b: Exogenous phenol increase resistance of *Ulmus minor* to Dutch elm disease through formation of suberin-like compounds on xylem tissues. Environ. Exp. Bot. **64**, 97–104.
Martín, J. A.; Solla, A.; Esteban, L. G.; De Palacios, P.; Gil, L., 2009: Bordered pit and ray morphology involvement in elm resistance to *Ophiostoma novo-ulmi*. Can. J. For. Res. **39**, 420–429.
Martín, J. A.; Solla, A.; Gil, L.; García-Vallejo, M. C., 2010: Phenological and histochemical changes in *Ulmus minor* due to root absorption of phenol: implications for resistance to DED. Environ. Exp. Bot. **69**, 175–182.
Martín, J. A.; Solla, A.; Garcia-Vallejo, M. C.; Gil, L., 2012: Chemical changes in *Ulmus minor* xylem tissue after salicylic acid or carvacrol treatments are associated with enhanced resistance to *Ophiostoma novo-ulmi*. Phytochemistry **83**, 104–109.
Martín, J. A.; Solla, A.; Venturas, M.; Collada, C.; Domínguez, J.; Miranda, E.; Fuentes-Utrilla, P.; Burón, M.; Iglesias, S.; Gil, L., 2015: Seven *Ulmus minor* clones tolerant to *Ophiostoma novo-ulmi* registered as forest reproductive material in Spain. iForest **8**, 172–180.
McDowell, N.; Pockman, W. T.; Allen, C. D.; Breshears, D. D.; Cobb, N.; Kolb, T.; Plaut, J.; Sperry, J.; West, A.; Williams, D. G., 2008: Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? New Phytol. **178**, 719–739.
Meinzer, F. C.; Woodruff, D. R.; Shaw, D. C., 2004: Integrated responses of hydraulic architecture, water and carbon relations of western hemlock to dwarf mistletoe infection. Plant Cell Environ. **27**, 937–946.
Mohebby, B., 2005: Attenuated total reflection infrared spectroscopy of white-rot decayed beech wood. Int. Biodeterior. Biodegrad. **55**, 247–251.
Nagy, N. E.; Franceschi, V. R.; Solheim, H.; Krekling, T.; Christiansen, E., 2000: Wound-induced traumatic resin duct development in stems of Norway spruce (*Pinaceae*): anatomy and cytochemical traits. Am. J. Bot. **87**, 302–313.
Newbanks, D.; Bosch, A.; Zimmermann, M. H., 1983: Evidence for xylem dysfunction by embolization in Dutch elm disease. Phytopathology **73**, 1060–1063.
Oliveira, H.; Sousa, A.; Alves, A.; Nogueira, A. J. A.; Santos, C., 2012: Inoculation with *Ophiostoma novo-ulmi* subsp. *americana* affects photosynthesis, nutrition and oxidative stress in *in vitro Ulmus minor* plants. Environ. Exp. Bot. **77**, 146–155.
Osbourn, A., 1996: Saponins and plant defence-a soap story. Trends Plant Sci. **1**, 4–9.
Ouellette, G.; Rioux, D., 1992: Anatomical and physiological aspects of resistance to Dutch elm disease. In: Defense mechanisms of woody plants against fungi. Ed. by Blanchette, A.; Biggs, R. Berlin: Springer, pp. 257–307.
Ouellette, G.; Rioux, D.; Simard, M.; Cherif, M., 2004: Ultrastructural and cytochemical studies of host and pathogens in some fungal wilt diseases: retro-and introspection towards a better understanding of DED. For. Syst. **13**, 119–145.
Pandey, K. K.; Pitman, A. J., 2003: FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. Int. Biodeterior. Biodegrad. **52**, 151–160.
Pavlovic, S.; Brandao, P. R. G., 2003: Adsorption of starch, amylose, amylopectin and glucose monomer and their effect on the flotation of hematite and quartz. Miner. Eng. **16**, 1117–1122.
Peacock, J. W.; Cuthbert, R. A.; Lanier, G. N., 1981: Deployment of traps in a barrier strategy to reduce populations of the European elm bark beetle, and the incidence of Dutch elm disease. In: Management of insect pests with semiochemicals: concepts and practice. Ed. by Mitchell, E. R. New York: Plenum Press, pp. 155–174.
Pearce, R. B., 1996: Antimicrobial defences in the wood of living trees. New Phytol. **132**, 203–233.
Pollard, M.; Beisson, F.; Li, Y.; Ohlrogge, J. B., 2008: Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci. **13**, 236–246.
Przybył, K.; Dahm, H.; Ciesielska, A.; Moliński, K., 2006: Cellulolytic activity and virulence of *Ophiostoma ulmi* and *O. novo-ulmi* isolates. For. Pathol. **36**, 58–67.
Richards, W., 1993: Cerato-ulmin: a unique wilt toxin of instrumental significance in the development of Dutch elm disease. In: Dutch elm disease research. Ed. by Sticklen, M.; Sherald, J. New York: Springer, pp. 89–151.
Rioux, D.; Ouellette, G. B., 1991: Barrier zone formation in host and nonhost trees inoculated with *Ophiostoma ulmi*. I. Anatomy and histochemistry. Can. J. Bot. **69**, 2055–2073.
Rodríguez-Calcerrada, J.; Shahin, O.; Del Carmen Del Rey, M.; Rambal, S., 2011: Opposite changes in leaf dark respiration and soluble sugars with drought in two Mediterranean oaks. Funct. Plant Biol. **38**, 1004–1015.
Santos, C.; Fragoeiro, S.; Phillips, A., 2005: Physiological response of grapevine cultivars and a rootstock to infection with *Phaeoacremonium* and *Phaeomoniella* isolates: an in vitro approach using plants and calluses. Sci. Hort. **103**, 187–198.
Sene, C. F.; Mccann, M. C.; Wilson, R. H.; Grinter, R., 1994: Fourier-transform Raman and Fourier-transform infrared spectroscopy (an investigation of five higher plant cell walls and their components). Plant Physiol. **106**, 1623–1631.
Shigo, A. L., 1984: Compartmentalization: a conceptual framework for understanding how trees grow and defend themselves. Annu. Rev. Phytopathol. **22**, 189–214.
Shigo, A.; Tippett, J. T., 1981: Compartmentalization of American elm tissues infected by *Ceratocystis-Ulmi*. Plant Dis. **65**, 715–718.
Smith, K. T., 2015: Compartmentalization, resource allocation, and wood quality. Curr. For. Rep. **1**, 8–15.
Solla, A.; Gil, L., 2002a: Xylem vessel diameter as a factor in resistance of *Ulmus minor* to *Ophiostoma novo-ulmi*. For. Pathol. **32**, 123–134.
Solla, A.; Gil, L., 2002b: Influence of water stress on Dutch elm disease symptoms in *Ulmus minor*. Can. J. Bot. **80**, 810–817.
Solla, A.; Martín, J.; Ouellette, G.; Gil, L., 2005: Influence of plant age on symptom development in *Ulmus minor* following inoculation by *Ophiostoma novo-ulmi*. Plant Dis. **89**, 1035–1040.

Steele, C. L.; Crock, J.; Bohlmann, J.; Croteau, R., 1998: Sesquiterpene synthases from grand fir (*Abies grandis*). Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of delta-selinene synthase and gamma-humulene synthase. J. Biol. Chem. **273**, 2078–2089.

Stuart, B. H., 2004: Infrared Spectroscopy: Fundamentals and Applications. Chichester: Wiley.

Takai, S., 1974: Pathogenicity and cerato-ulmin production in *Ceratocystis ulmi*. Nature **252**, 124–126.

Temple, B.; Horgen, P. A.; Bernier, L.; Hintz, W. E., 1997: Cerato-ulmin, a hydrophobin secreted by the causal agents of Dutch elm disease, is a parasitic fitness factor. Fungal Genet. Biol. **22**, 39–53.

Tetley, R. M.; Thimann, K. V., 1974: The metabolism of oat leaves during senescence I. Respiration, carbohydrate metabolism, and the action of cytokinins. Plant Physiol. **54**, 294–303.

Thomas, F. M.; Blank, R.; Hartmann, G., 2002: Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. For. Pathol. **32**, 277–307.

Tumlinson, J.; Engelberth, J., 2008: Fatty acid-derived signals that induce or regulate plant defenses against herbivory. In: Induced plant resistance to herbivory. Ed. by Schaller, A. Amsterdam, Netherlands: Springer, pp. 389–407.

Upchurch, R. G., 2008: Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnol. Lett. **30**, 967–977.

Urban, J.; Dvořák, M., 2014: Sap flow-based quantitative indication of progression of Dutch elm disease after inoculation with *Ophiostoma novo-ulmi*. Trees **28**, 1599–1605.

Vanetten, H. D.; Mansfield, J. W.; Bailey, J. A.; Farmer, E. E., 1994: Two classes of plant antibiotics: phytoalexins versus "Phytoanticipins". Plant Cell **6**, 1191.

Vek, V.; Oven, P.; Humar, M., 2013: Phenolic extractives of wound-associated wood of beech and their fungicidal effect. Int. Biodeterior. Biodegrad. **77**, 91–97.

Venturas, M.; López, R.; Gascó, A.; Gil, L., 2013: Hydraulic properties of European elms: xylem safety-efficiency tradeoff and species distribution in the Iberian Peninsula. Trees **27**, 1691–1701.

Venturas, M.; López, R.; Martín, J.; Gascó, A.; Gil, L., 2014: Heritability of *Ulmus minor* resistance to Dutch elm disease and its relationship to vessel size, but not to xylem vulnerability to drought. Plant. Pathol. **63**, 500–509.

Webber, J., 2004: Experimental studies on factors influencing the transmission of Dutch elm disease. For. Syst. **13**, 197–205.

Weber, H., 2002: Fatty acid-derived signals in plants. Trends Plant Sci. **7**, 217–224.

Witzell, J.; Martín, J. A., 2008: Phenolic metabolites in the resistance of northern forest trees to pathogens – past experiences and future prospects. Can. J. For. Res. **38**, 2711–2727.

Wullschleger, S. D.; Mclaughlin, S. B.; Ayres, M. P., 2004: High-resolution analysis of stem increment and sap flow for loblolly pine trees attacked by southern pine beetle. Can. J. For. Res. **34**, 2387–2393.

Zagrobelny, M.; Bak, S.; Møller, B. L., 2008: Cyanogenesis in plants and arthropods. Phytochemistry **69**, 1457–1468.