

Heritability of *Ulmus minor* resistance to Dutch elm disease and its relationship to vessel size, but not to xylem vulnerability to drought

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Dutch elm disease (DED) is a vascular wilt disease that causes the occlusion and cavitation of xylem vessels. Therefore, it is hypothesized that those elms that are less vulnerable to cavitation by drought might be more resistant to DED. To test this hypothesis, the relationship between xylem vulnerability to cavitation and susceptibility to DED was examined in progenies of crosses between susceptible and resistant individuals of *Ulmus minor*. Hydraulic conductivity and xylem vulnerability curves were evaluated and anatomical features such as vessel size, length and grouping were measured. Next, elms were inoculated with *Ophiostoma novo-ulmi*, the cause of DED, and pre-dawn and midday water potentials, stomatal conductance and wilting percentages were assessed. Progenies of R × R crosses showed significantly lower mean wilting percentages (30–50%) than the progeny of S × S crosses (75%). Fifty percent conductivity loss was reached at *c.* –1 MPa, pointing out a high vulnerability of this species to drought-induced cavitation. Crown wilting percentage as a result of inoculation and xylem vulnerability to cavitation by water stress did not show any significant correlation. Nevertheless, significant differences in theoretical hydraulic conductivity and vessel size parameters (diameter, length and size distributions) were found among the tested progenies. Susceptible trees had significantly wider and longer vessels. Xylem structure of resistant elms seems to restrict pathogen spread rather than prevent cavitation.

Keywords: Dutch elm disease, elm breeding, *Ulmus minor*, water stress, wood anatomy, xylem vulnerability curves

Introduction

Two pandemics of Dutch elm disease (DED) during the past century have killed most European and North American mature elms (Brasier, 2000). The first pandemic was caused by the fungus *Ophiostoma ulmi* and the second, and more destructive one, by *Ophiostoma novo-ulmi*. As a consequence, conservation of native elm genetic resources became a major concern in Europe and great efforts have been put into breeding disease-resistant elms (Solla *et al.*, 2005a; Santini *et al.*, 2008). In Spain, *Ulmus minor* has been the most affected species and the main focus of the Spanish Elm Breeding Programme (Martín *et al.*, 2010). Further knowledge on tree features related to DED resistance and their heritability is needed for guiding tree selection in elm breeding programmes, and to assess the effect of introducing resistant clones in natural elm populations which need to be restored.

Xylem dysfunction due to cavitation is the primary effect of abiotic stresses such as drought (e.g. Hacke *et al.*, 2000; Vilagrosa *et al.*, 2012), freeze–thaw cycles (e.g. Davis *et al.*, 1999; Sevanto *et al.*, 2012) and biotic stressors such as herbivores (e.g. Tyree *et al.*, 1994a) and pathogens (e.g. Newbanks *et al.*, 1983; Kuroda, 1991; Raimondo *et al.*, 2010). Stem-specific hydraulic conductivity and stomatal control are important factors related to the vulnerability of the xylem to cavitation by water stress (e.g. Sparks & Black, 1999; Brodribb & Cochard, 2009). Decreasing plant conductivity as a result of xylem cavitation amplifies the effect of water stress on the leaves, and could increase the sensitivity of the stomatal response to drought (Sperry, 2000; Brodribb & Cochard, 2009). Some xylem structural traits such as mechanical stem strength, xylem density, vessel and fibre wall thickness, and fibre wall area have been positively correlated with cavitation resistance to water stress (Hacke *et al.*, 2001; Jacobsen *et al.*, 2005). On the other hand, wide pit membrane pores have been linked to high xylem water transport efficiency and increased vulnerability to water stress-induced embolism (e.g. Pammenter & Vander Willigen, 1998; Christman *et al.*, 2009). In fact, inter- and intraspecific differences in xylem vulnerability to cavitation have been correlated to a differential pit membrane pore size distribution (e.g. Vander Willigen *et al.*, 2000), whereas environmentally induced differences in vessel

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sizes explain changes in plant hydraulic conductance (e.g. Pammenter & Vander Willigen, 1998). As *U. minor* is capable of withstanding moderate summer drought, understanding how all these parameters involved in drought-induced cavitation may be linked to DED resistance could be of great interest to elm breeders.

Wilting and die-back in DED-infected elms are caused by hydraulic failure, either by direct vessel blocking or by inducing embolisms as a consequence of fungal invasion (Newbanks *et al.*, 1983). When elm bark beetles (*Scolytus* and *Hylurgopinus* spp.) feed, they introduce fungal spores into xylem vessels. Once inside the xylem vessels, the fungus produces cell wall-degrading enzymes (Scheffer & Elgersma, 1982), toxic substances (Takai, 1974; Sticklen *et al.*, 1991) and the hydrophobin cerato-ulmin (Takai, 1974; Van Alfen & Turner, 1975). These substances may ultimately affect sap tensile properties and could therefore be related to xylem dysfunction by embolism (Newbanks *et al.*, 1983). The host reacts to infection by producing antifungal compounds (e.g. mansonones; Duchesne *et al.*, 1990) and lignin (Martín *et al.*, 2007), and by causing the occlusion of the vessel lumen by tyloses, gums and gels (Shigo & Tippett, 1981; Martín *et al.*, 2005b), which implies hydraulic compartmentalization. As a consequence, water transport sufficiency will rely on vessel redundancy (Ewers *et al.*, 2007).

It has been suggested that resistance to DED shown by certain elm species, such as *Ulmus pumila* (Siberian elm), originates from the adaptation of anatomical or physiological features to xeric environments (Solla *et al.*, 2005b). *Ulmus pumila*, native to the steppe and to the Gobi and Tarim deserts, corresponds to a xeromorphic species with greater conductive safety against water stress. In early spring, when the susceptibility of elms to *O. novo-ulmi* reaches its peak, *U. pumila* forms smaller vessels than those of *U. minor* (Solla *et al.*, 2005b). This fact is supposed to restrict *O. novo-ulmi* spread through the xylem vessels and to reduce the xylem susceptibility to cavitation by the pathogen action in *U. pumila*. However, direct evidence of the relationship between drought resistance and susceptibility to DED is still lacking. It is hypothesized that (i) the xylem of DED-resistant elms is less vulnerable to drought-induced cavitation than the xylem of susceptible elms, and (ii) the xylem architecture of drought-resistant elms slows the systemic colonization by the pathogen. Therefore, the main objectives of this work were to: (i) explore the heritability of *U. minor* resistance to DED, (ii) compare the xylem vulnerability of resistant and susceptible *U. minor* progenies to water stress-induced cavitation, and (iii) evaluate the relationship between this vulnerability and resistance to DED.

Materials and methods

Plant material

In 2005, five *U. minor* clones from Spain, selected for their low level of susceptibility to DED (crown wilting after infection <40%; hereafter referred to as resistant clones, R), as well as

two *U. minor* clones from Spain, selected for their high level of susceptibility to DED (crown wilting after infection >80%; hereafter referred to as susceptible clones, S) were subjected to controlled pollinations. As a result, progenies of one S × S, seven R × R and two R × S crosses were grown for the dual purposes of: (i) obtaining breeding progenies (F₁) of resistant elms within the framework of the Spanish Elm Breeding Programme, and (ii) exploring the heritability of *U. minor* resistance to DED. Resulting seeds were sown within the same year. Seedlings were grown at Puerta de Hierro nursery (Madrid, 40°27'22"N, 3°45'5"W) under partial shading (60%) in 0.35 L Forest Pot containers (1:3 perlite:peat substrate, v/v). Plants were well watered and fertilized every 3 months. In 2007, seedlings were transplanted to an experimental plot located within the same nursery following a 0.5 × 1.0 m spacing design. Seedlings were grouped in experimental units of three plants of the same family, and these units were randomly distributed in four complete blocks. The plot was surrounded by a border line of elms which were not selected for any measurements.

In spring 2010, there were 1616 seedlings available in the plot. Their mean height was 2.8 m; each seedling had over 15 side branches. To obtain vulnerability curves and perform anatomical and physiological measurements, eight trees were randomly selected from each one of the following crosses: CR-PB1 × TO-AL1 (S × S), J-CA2 × TO-AL1 (R × S) and GR-DF3 × AB-AL1 (R × R) (24 trees in total). The R × S and R × R crossings were randomly selected within their kind. The remaining 1592 trees in the sample plot were used for the susceptibility test.

Vulnerability curves

Vulnerability curves (VC) were determined for one branch of each one of the 24 selected trees from 1 to 15 June 2010, just before DED inoculation. The branches selected for VCs were 2–3 years' old, 2–4 mm diameter, from the south-orientated top half of each tree. These branches were cut under water and immediately transported to the laboratory. Sections of these branches, 30 cm in length, were re-cut under water just before determining their VC. Hydraulic conductivity (K, kg m s⁻¹ MPa⁻¹) was measured following the methodology first described by Sperry *et al.* (1988). VCs were obtained by applying the air injection methodology, as described by Sperry & Saliendra (1994), using two 20 cm double-ended pressure chambers. Flushing and hydraulic conductivity measurements were performed using a filtered (0.2 µm) degassed 50 µM KCl solution in order to buffer any potential ionic effect on hydraulic conductivity (Zwieniecki *et al.*, 2001; Gascó *et al.*, 2006). Native embolisms were removed with high-pressure (0.16 MPa) flushing for 30 min. Maximum hydraulic conductivity (K_{max}) was then measured. Thereafter, increasing preset air pressure steps (0.1, 0.3, 0.6, 0.9, 1.2, 1.5, 2.0, 2.5, 3.0 and 4.0 MPa) were held inside the chamber for 10 min, and K was measured after releasing each pressure level. The percentage loss of conductivity (PLC) was calculated as: PLC = 100(K_{max} - K)/K_{max}. A minimum of 90 PLC was reached in every sample. Each VC was fitted to an exponential sigmoid (PLC = 100/(1 + exp[a(Ψ - b)]); Pammenter & Vander Willigen, 1998) using SIGMAPLOT v. 10.0 (Systat Software Inc.). Applied pressures (MPa) required to reach 50 PLC (P₅₀, b parameter of the fitted sigmoid) and 80 PLC (P₈₀) were obtained from the fitted model equations. Maximum xylem-specific conductivity K_{x,max} (kg m⁻¹ s⁻¹ MPa⁻¹) was calculated dividing K_{max} by its xylem cross-sectional area (m²) (e.g. Hultine *et al.*, 2006). All measurements were performed in a laboratory at 20°C.

Wood density and xylem anatomical features

Wood density (WD, g cm^{-3}) was determined with 2.5 cm-long segments cut from basal pieces of the branches used to obtain VCs. Stem segments were split longitudinally, and bark and pith were removed with a razor blade. Xylem segments were soaked in degassed water overnight. Afterwards, their fresh volume was determined, according to Archimedes' principle, by immersing each sample in a water-filled test tube placed on a balance (e.g. Hacke *et al.*, 2000). The weight of displaced water was converted to sample volume using a water density of $0.9982071 \text{ g cm}^{-3}$ at 20°C . Afterwards, samples were stored at 75°C for 48 h and the dry weight was then measured. Wood density was calculated as the ratio of dry weight to fresh volume.

For anatomical measurements the basal 2 cm were cut off the stem segments used to determine VCs. They were then placed in a formaldehyde–acetic acid–70% ethanol (5:5:90, v:v:v) fixative until cross sections were prepared. Fifteen-micrometre thick transverse sections were obtained using a sliding microtome (Leica SM 2400). Next, they were stained with safranin 0.1% (w/v), dehydrated through an alcohol series, mounted on microscope slides, and fixed with Canada balsam for light microscopy observation. As it has been estimated that 90% of the xylem flow of elms is restricted to the outermost (current) sapwood ring (Ellmore & Ewers, 1985), four radial 500- μm -wide sectors, spaced 90° apart, were randomly selected within the 2010 growth increment of these transverse sections. In these sectors interior vessel diameters were measured radially, ignoring those smaller than $20 \mu\text{m}$. Vessel density per mm^2 and groups of vessels (contiguous vessels; McNabb *et al.*, 1970) were also counted. An image analysis system (Image Pro Plus 4.5, Media Cybernetics) attached to a light microscope (Olympus BX50) was used to measure all these variables at $\times 100$ magnification.

Vessel transectional area (VTA, %) was obtained by dividing the area occupied by the vessels in a sector (wall excluded) by the total area of the sector, multiplied by 100 (e.g. Solla *et al.*, 2005b). The theoretical hydraulic conductance (THC, μm^2) predicted by the Hagen–Poiseuille equation (e.g. Giordano *et al.*, 1978; Solla *et al.*, 2005b) was determined by dividing the sum of the fourth power of all the internal vessel radii found within a sector by the total area of the sector (A_S) (i.e. $\text{THC} = \sum r_n^4 / A_S$). Vessels were classified in three categories of diameters, small ($<40 \mu\text{m}$), medium ($40\text{--}70 \mu\text{m}$), and large ($>70 \mu\text{m}$), because large and medium vessels are invaded more frequently by hyphae and spores than small ones (Pomerleau, 1970). The theoretical contribution to hydraulic flow of the vessels was studied in relation to their size. For example, the contribution of large vessels to flow (CLVF) was calculated as: $\text{CLVF} = \sum D_i^4 / \sum D_n^4$, where D is the vessel diameter, i are vessels larger than $70 \mu\text{m}$, and n corresponds to all the vessels within the sector (e.g. Solla *et al.*, 2005b; Pinto *et al.*, 2012).

Subsequently, the tangential lumen span (b) and the thickness of the double wall (t) between two adjacent vessels were measured for all paired vessels within a sector; and intervessel wall strength, $(t/b)^2$, was calculated following Hacke *et al.* (2001).

Finally, vessel length distributions were calculated. The same stems used to build VCs were flushed again (after having removed 2 cm from the basal end for the anatomic features measurements) at 0.16 MPa for 30 min to remove any embolism. Then a two-component silicone (Ecoflex 0030; Smooth-On, Inc.), dyed with a red pigment (Silc Pig; Smooth-On, Inc.), was injected under pressure (0.2 MPa) for 40 min

through the basal end of each stem (e.g. Sperry *et al.*, 2005; Cai *et al.*, 2010). Transversal cuts at set distances from the basal edge (5, 10, 30 mm, and every other 30 mm thereon until no silicone-filled vessels were found) were observed under an Olympus BX50 light microscope. The percentages of silicone-filled and empty vessels were calculated in four perpendicular radial sectors of the outermost growth ring, counting a minimum of 25 vessels per sector. It was evaluated in this ring because it had the longest vessels, and it has been estimated that it is responsible for 90% of conductivity (Ellmore & Ewers, 1985). The percentage of filled vessels (PFV) was fitted to the following exponential curve: $\text{PFV} = 100 \times \exp(-bx)$, where x is the distance from the stem segment base (mm) and b is a vessel-length distribution parameter (bVL) (e.g. Sperry *et al.*, 2005). Therefore, the percentage of vessels (P_V) belonging to a determined length class was calculated with the following equation: $P_V = 100 [(1 + km) \exp(-km) - (1 + kM) \exp(-kM)]$; where $k = \text{bVL}$, and m and M are the minimum and maximum lengths of the distance class, respectively. Vessel length was plotted for 10 mm classes. The maximum vessel length (VL_{max}) was established as the last length (mm) at which a silicone-filled vessel was observed. Intermediate cuts were also performed within the last 30 mm stem segment in order to estimate more accurately VL_{max} .

Susceptibility test

The highly virulent CU-HU isolate of *O. novo-ulmi* subsp. *americana* was used for the controlled inoculation of the 1592 trees in the sample plot used for the disease resistance trial. The isolate was collected from an infected *U. minor* tree in Huelves (Cuenca, Spain) in 2002. The fungus was stored *in vitro* in 2% malt extract agar at 4°C in the dark, and subcultured at 6-month intervals. Two weeks before carrying out the inoculation, the fungus was grown in Tchernoff's medium (Tchernoff, 1965). The inoculum consisted of bud cells suspended in sterile distilled water (10^6 spores mL^{-1}). Two inoculum droplets were introduced into the xylem of every tree by drawing them from a syringe while transversally cutting through the bark into the wood of the main stem 20 cm above the ground (Solla *et al.*, 2005a). The seedlings were inoculated on 13 and 14 May 2010. Disease severity was evaluated by direct observation of the wilting percentage of the crown (WP, %) 20, 60 and 120 days after inoculation (d.a.i.).

On 18 June 2010, after VCs were determined, the 24 seedlings set aside for detailed anatomical and physiological investigation were also inoculated, following the same methodology as in the previous test. Disease severity was assessed by direct observation of WP 20 d.a.i.

Water potential and gas exchange

Leaf water potential (Ψ_{leaf} , MPa) at pre-dawn (Ψ_{pd}) and midday (Ψ_{md}) and stomatal conductance (g_s , $\text{mmol s}^{-1} \text{m}^{-2}$) at midday of the 24 selected elms were measured just before inoculation, and 10, 13, 17, 25, 33 and 47 d.a.i., to monitor the effects of DED on these parameters. The measurements were always carried out on three previously marked branches, oriented south, east and west, from the lower part of the crown. One leaf per marked branch was collected each time for water potential evaluation. The leaves were kept in double black bags with a piece of wet paper at 4°C until measurements were done in the laboratory with a pressure chamber (model 1000, PMS Instrument Company) during the following 3 h. Stomatal conductance was evaluated with a porometer (model SC-1, Decagon Devices Inc.)

on one leaf per marked branch (without detaching it) before taking samples for midday water potential measurements.

Data processing and statistical analyses

A repeated measures ANOVA was run for WP data considering block, crossing type and family (nested in crossing type) as main factors, and the basal diameter of the trees was used as a covariable. To assess the narrow sense heritability (h^2) of WP a mixed effect ANOVA with family, block and interaction of block by family was applied. Variance components for family (σ_f), family-block interaction (σ_{fxb}) and residual errors (σ_e), with the respective associated standard errors (SE), were estimated by restricted maximum likelihood. h^2 was estimated as:

$$h^2 = \frac{\sigma_a}{\sigma_P}$$

where σ_a is the additive genetic variance and σ_P the total phenotypic variance. σ_a was considered as being twice the genetic variance between families, as trees from the same family were full-sibs ($2\sigma_f$), whereas total phenotypic variance was estimated as:

$$\sigma_P = \sigma_f + \sigma_{fxb} + \sigma_e$$

One-way analyses of variance (ANOVA) were performed using the type of crossing (R × R, R × S and S × S) as the independent variable for those physiological variables estimated once on every tree. GLM was used to analyse results of those variables measured in several samples per tree (e.g. anatomical measurements), considering the type of crossing and each tree (nested within the type of crossing) as fixed factors. Considering these same factors, differences in Ψ_{pd} , Ψ_{md} and g_s were checked against time after inoculation using repeated measures ANOVA. Pairwise comparisons of means were made by multiple range tests using Fisher's least significant difference (LSD) intervals at 95% confidence level. In order to understand the

relations between variables, Pearson's product-moment correlation coefficient (r) and its statistical significance (P -value) were calculated using the mean value of each variable for each tree. Analyses were performed using STATISTICA v. 7.0 (StatSoft Inc.).

Results

DED progress and disease resistance

In the susceptibility test, crown wilting as a result of DED inoculation varied from 0 to 100% within the seedlings (data not shown). All factors considered in the repeated measures ANOVA for WP ($n = 1592$) were significant except for the date of measurement (Table 1). Wilting percentages (least squares means) were 32–46% in R × R families, 48–59% in R × S families and 75% in the only S × S crossing tested (Table 2). WP of the R × S family selected for the detailed analysis (J-CA2 × TO-AL1) was not significantly different ($P = 0.07$) from the two R × R ones (GR-DF3 × AB-AL1 and MA-PD2 × AB-AL1; Table 2). Narrow sense heritability for WP was 0.54, showing that resistance to DED is genetically controlled.

Hydraulic conductivity and vulnerability to cavitation

Vulnerability to cavitation (P_{50} and P_{80}), $K_{x_{max}}$ and absolute conductivity (K_x) did not differ significantly among the types of crosses (Fig. 1; Table 3). Loss of conductivity began at -0.3 MPa and progressed at a similar rate in all crosses, i.e. there were no differences in the slope of VCs ($P = 0.87$; Table 3).

Despite P_{80} and $K_{x_{max}}$ not differing between crossing types, these variables were positively correlated with WP 20 d.a.i. for the 24 selected trees ($P < 0.05$; Table S1). Nevertheless, the coefficient of correlation was low in both cases ($R^2 < 0.20$).

Table 1 Percentage of the explained variation and significance value from the repeated measures ANOVA for explaining the evolution of wilting percentage (WP), pre-dawn water potential (Ψ_{pd}), midday water potential (Ψ_{md}) and stomatal conductance (g_s)

Factor	WP		Ψ_{pd}		Ψ_{md}		g_s	
	<i>P</i>	%	<i>P</i>	%	<i>P</i>	%	<i>P</i>	%
Block	***	2.04	–	–	–	–	–	–
Family (crossing type)	***	4.85	–	–	–	–	–	–
Plant (crossing type)	–	–	**	4.82	***	9.19	***	30.81
Crossing type	***	9.59	n.s.	0.32	**	1.51	n.s.	0.32
Basal diameter	**	0.42	–	–	–	–	–	–
Error (between subjects)		63.64		2.72		4.01		5.61
Date	n.s.	0.02	***	47.13	***	63.55	***	20.66
Date × Block	***	0.17	–	–	–	–	–	–
Date × Family (crossing type)	***	0.47	–	–	–	–	–	–
Date × Plant (crossing type)	–	–	***	24.48	***	10.29	***	20.66
Date × Crossing type	n.s.	0.05	***	3.77	***	1.75	*	1.61
Date × Basal diameter	*	0.05	–	–	–	–	–	–
Error (within subjects)		18.70		16.75		9.69		14.97

P is the significance level of the factor (n.s.: not significant; * <0.05 ; ** <0.01 ; *** <0.001). % is the percentage of variability explained by the factor. Factors that have not been considered in the model are represented with a dash (–).

Table 2 Crown wilting percentages (WP) at 20, 60 and 120 days after inoculation (d.a.i.) and least squares means WP from repeated measures ANOVA

Family (♀ × ♂)	Crossing type ^a	n ^b	WP ^c 20 d.a.i.	WP 60 d.a.i.	WP 120 d.a.i.	Least squares means WP ^d
MA-PD2 × GR-DF3	R × R	220	29.6 ± 1.0	32.0 ± 1.2	34.1 ± 1.3	32.5 ± 1.2 a
J-CA2 × GR-DF3	R × R	162	33.9 ± 1.3	33.3 ± 1.4	34.6 ± 1.5	33.9 ± 1.4 ab
M-DV4/5 × GR-DF3	R × R	71	34.6 ± 1.5	34.4 ± 1.9	38.9 ± 2.6	35.7 ± 2.2 abc
M-DV4/5 × J-CA2	R × R	199	37.9 ± 1.2	36.2 ± 1.2	36.9 ± 1.2	36.8 ± 1.3 bc
J-CA2 × M-DV4/5	R × R	194	44.0 ± 1.4	37.6 ± 1.4	37.5 ± 1.3	40.0 ± 1.3 ce
MA-PD2 × AB-AL1	R × R	22	50.1 ± 4.8	46.1 ± 5.2	41.4 ± 5.3	43.6 ± 3.9 de
GR-DF3 × AB-AL1	R × R	193	46.7 ± 1.7	48.2 ± 1.7	47.5 ± 1.9	46.3 ± 1.4 d
J-CA2 × TO-AL1	R × S	240	48.9 ± 1.5	46.9 ± 1.6	45.8 ± 1.6	47.5 ± 1.2 d
M-DV4/5 × TO-AL1	R × S	266	57.8 ± 1.3	59.9 ± 1.5	59.2 ± 1.6	59.2 ± 1.1 f
CR-PB1 × TO-PB1	S × S	25	69.6 ± 5.3	73.8 ± 5.2	76.2 ± 5.7	74.8 ± 3.7 g

^aR: resistant; S: susceptible.

^bNumber of inoculated seedlings.

^cMean wilting percentage ± SE.

^dLetters label homogeneous groups by Fisher's LSD test ($P = 0.05$).

Anatomical features

Maximum vessel length (VL_{max}) ranged from 69 to 118 mm. $S \times S$ trees had 30–40% significantly longer conduits and a higher percentage of longer vessels (Fig. 2a; Table 3). There was a negative correlation between Kx_{max} (log-transformed) and bVL ($R^2 = 34.5$, $P = 0.0026$; Table S1): plants with shorter vessels had lower conductivity.

$S \times S$ progeny showed the widest vessels (VD; Table 3), and were unique in having vessel diameters greater than 90 μm (Fig. 2b). The progeny of the $S \times S$ cross also had larger VTA, and a THC twice as high as the other two groups (Table 3). CLVF, CMVF and CSVF did not differ among crossing types ($P > 0.05$, Table 3). As expected, Kx_{max} (log-transformed) was positively correlated with THC ($R^2 = 32.6$, $P = 0.0035$; Table S1) and VD ($R^2 = 28.8$, $P = 0.0068$; Table S1). In addition, $R \times R$ individuals showed a significantly higher VF (c. 20%) and a greater $(t/b)^2$ ($P < 0.05$; Table 3). Meanwhile, $S \times S$ saplings had significantly higher PGV (Table 3). There were no differences in WD, VPG or VGA between the groups ($P > 0.05$; Table 3).

Water potential and gas exchange after DED inoculation

Both Ψ_{pd} and Ψ_{md} progressively decreased after DED inoculation (Fig. 3a). Seventeen d.a.i., Ψ_{pd} had dropped more than 0.25 MPa and 47 d.a.i. c. 1 MPa, independent of the type of crossing (Fig. 3a). Ψ_{md} dropped from -1 MPa to almost -3 MPa in $S \times S$ progeny at the end of the experiment. From the thirteenth d.a.i., Ψ_{md} of $R \times R$ cross progeny was significantly different from $S \times S$ cross progeny.

Stomatal conductance (g_s) decreased significantly following inoculation (Fig. 3b). Ten d.a.i. $S \times S$ showed significantly higher g_s than the other two types of crossings

(Fig. 3b). From then onwards, gas exchange rates were strongly reduced to $<40 \text{ mmol s}^{-1} \text{ m}^{-2}$ in all plants, and they slowly recovered to 50–70 $\text{mmol s}^{-1} \text{ m}^{-2}$ at the end

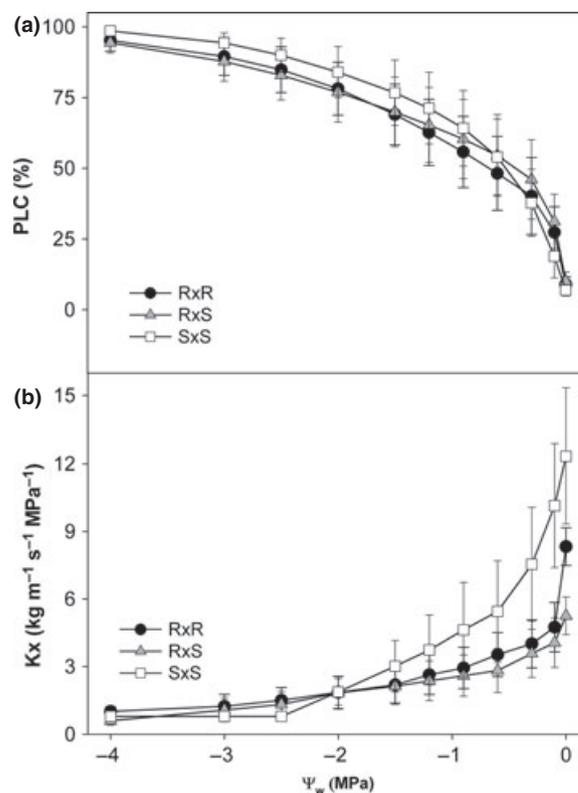


Figure 1 *Ulmus minor* vulnerability curves constructed for trees belonging to progeny of Dutch elm disease resistant (R) and susceptible (S) crosses (GR-DF3 × AB-AL1 (R × R), J-CA2 × TO-AL1 (R × S) and CR-PB1 × TO-AL1 (S × S)). (a) Percentage loss in conductivity; (b) actual hydraulic conductivity ($n = 8$ per group; means ± SE).

Table 3 Wilting, hydraulic and anatomical properties of 24 trees selected from progeny of one of three crosses between elms that are resistant or susceptible to Dutch elm disease (eight per crossing type)

Variable ^a	Units	Crossing type ^b			P-value ^c
		GR-DF3 × AB-AL1 (R × R)	J-CA2 × TO-AL1 (R × S)	CR-PB1 × TO-AL1 (S × S)	
WP 20 d.a.i.	%	24.0 ± 6.9 a	25.9 ± 6.7 a	49.6 ± 8.7 b	0.0444
VC slope	MPa ⁻¹	36.0 ± 22.3 a	36.8 ± 22.4 a	22.6 ± 17.7 a	0.8661
P ₅₀	MPa	-1.10 ± 0.36 a	-1.09 ± 0.40 a	-0.90 ± 0.29 a	0.9088
P ₈₀	MPa	-1.77 ± 0.52 a	-1.72 ± 0.59 a	-1.35 ± 0.41 a	0.8280
K _{xmax}	kg m ⁻¹ s ⁻¹ MPa ⁻¹	8.33 ± 3.34 a	5.25 ± 0.84 a	12.33 ± 3.00 a	0.1441*
VL _{max}	mm	69.4 ± 10.6 a	80.6 ± 10.2 a	118.1 ± 12.2 b	0.0131
bVL		0.063 ± 0.014 ab	0.063 ± 0.013 b	0.036 ± 0.006 a	0.0581**
WD	g cm ⁻³	0.54 ± 0.02 a	0.53 ± 0.03 ab	0.45 ± 0.3 b	0.0863
VD	μm	38.6 ± 2.1 a	40.3 ± 2.2 a	46.6 ± 1.8 b	0.0000
VTA	%	33.9 ± 1.9 b	30.2 ± 1.9 a	38.0 ± 1.9 c	0.0001
THC	μm ²	62.8 ± 12.4 a	60.1 ± 12.4 a	117.1 ± 12.4 b	0.0000
VF	Vessels mm ⁻²	272.4 ± 23.9 b	231.4 ± 23.9 b	224.5 ± 23.9 a	0.0268
(t/b) ²		0.021 ± 0.005 b	0.013 ± 0.005 a	0.017 ± 0.005 ab	0.0203
PGV	%	46.0 ± 8.8 ab	34.8 ± 8.6 a	56.3 ± 8.6 b	0.0170
VPG	Vessels	2.3 ± 0.4 a	1.7 ± 0.4 a	2.5 ± 0.4 a	0.1915
VGA	Groups mm ⁻²	152.5 ± 39.3 a	100.8 ± 39.3 a	85.6 ± 39.3 a	0.4842
CLVF	%	20.6 ± 9.3 a	23.7 ± 9.3 ab	49.6 ± 9.3 b	0.0735
CMVF	%	64.1 ± 8.7 b	63.0 ± 8.7 b	39.0 ± 8.7 a	0.0911
CSVF	%	15.9 ± 4.0 a	13.4 ± 4.0 a	13.7 ± 4.0 a	0.8917

^aWP 20 d.a.i., wilting percentage 20 days after inoculation; VC slope, 'a' parameter of the exponential sigmoid: $PLC = 100/(1 + \exp[a(\Psi - b)])$; P₅₀, applied pressure at which the sample loses 50% hydraulic conductance; P₈₀, applied pressure at which the sample loses 80% hydraulic conductance; K_{xmax}, maximum xylem specific conductivity; VL_{max}, maximum vessel length; bVL, vessel length distribution parameter; WD, wood density; VD, vessel diameter; VTA, vessel transectional area; THC, relative theoretical hydraulic conductance; VF, vessel frequency; (t/b)², resistance to implosion; PGV, percentage of grouped vessels; VPG, vessels per group; VGA, vessel groups per area; CLVF, contribution of large vessels (VD >70 μm) to flow; CMVF, contribution of medium vessels (40 < VD < 70 μm) to flow; CSVF, contribution of small vessels (VD <40 μm) to flow.

^bR: resistant, S: susceptible. Mean value ± SE. Letters label homogeneous groups within a variable (P = 0.05, Fisher's LSD method).

^cP-value from the ANOVA. *log-transformed to meet ANOVA requirements; **inverse-transformed to meet ANOVA requirements.

of the experiment (Fig. 3b). R × R saplings showed significantly higher g_s values than S × S saplings 47 d.a.i.

Discussion

Wilting assessment showed that DED resistance is a heritable trait. Previous works in the Netherlands, Spain and Italy have obtained DED resistant elm genotypes by crossing *U. minor* with Asian elms (Solla *et al.*, 2005a; Santini *et al.*, 2008). The current results demonstrated the heritability of DED resistance within *U. minor*. The heritability for the resistance to wilting diseases in other species is supported by several quantitative genetic studies, e.g. in the *Fusarium xylarioides*–*Coffea canephora* or *Ceratocystis fimbriata*–*Eucalyptus* systems (Rosado *et al.*, 2010; Musoli *et al.*, 2013). As tree breeding programmes expect that genetic gains will be cumulative over generations, an additive genetic effect guarantees the efficiency of selection, and strengthens the ability to generate a bank of genetic resources of *U. minor* resistant to DED conserving the genetic integrity of the native species. Furthermore, resistant elms introduced in natural populations can transfer their resistance to DED to the local gene pool through sexual reproduction.

The WP of the R × R (GR-DF3 × AB-AL1) and R × S (J-CA2 × TO-AL1) crossings that were randomly selected for detailed anatomical and physiological observations were similar (Table 2). However, their significantly lower WP compared to the tested S × S progeny (CR-PB1 × TO-PB1) allows for a proper discussion about candidate traits involved in DED resistance mechanisms.

The current results argue against the hypothesis of a direct link between resistance to drought-induced cavitation and resistance to DED in *U. minor*. The shape and slope of the VCs, as well as P₅₀ and P₈₀, did not differ significantly among DED resistance groups (Table 3; Fig. 1). Therefore, although cavitation is involved in the DED syndrome (Newbanks *et al.*, 1983), the current results suggest that xylem resistance to water-stress cavitation is not related to *U. minor* resistance to DED. The low values of P₅₀ found for the species (*c.* -1 MPa) should be noted, which is in agreement with the behaviour of other riparian trees (Tyree *et al.*, 1994b; Cai & Tyree, 2010).

Previous works have shown direct relationships between certain xylem anatomical features and P₅₀. For instance, among-species P₅₀ variation has already been explained by differences in mean vessel diameter (VD) or

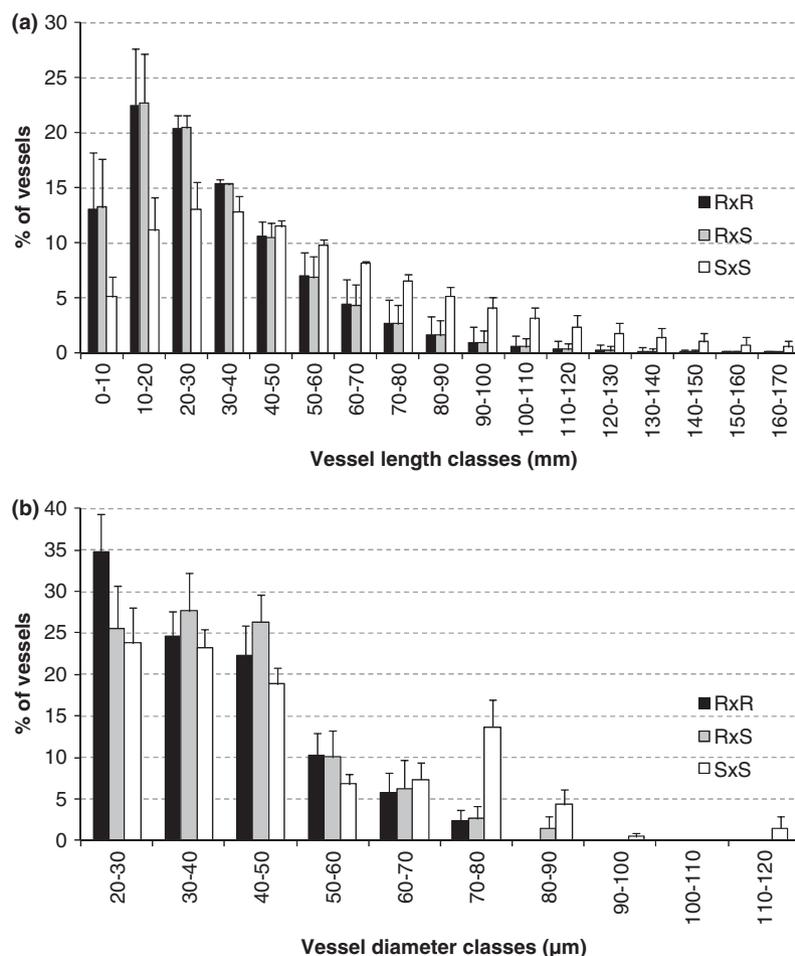


Figure 2 Vessel length (a) and diameter (b) distributions of progeny of crosses between elms that are resistant (R) or susceptible (S) to Dutch elm disease (GR-DF3 × AB-AL1 (R × R), J-CA2 × TO-AL1 (R × S) and CR-PB1 × TO-AL1 (S × S)). Measurements were carried out on the stems used to obtain vulnerability curves ($n = 8$ per group; means + SE).

pit membrane surface area (A_{pit}) (Wheeler *et al.*, 2005). Cai & Tyree (2010) further discussed this P_{50} -VD relationship and found an exponential relationship within *Populus tremuloides* when considering vessel diameter size classes. The current results did not show any relationship between VD and P_{50} in *U. minor*, although vessel size classes as described in Cai & Tyree (2010) have not been investigated. Vessel size differences between groups (Table 3; Fig. 2) indicate that vessel size is a key factor in determining xylem vulnerability to DED infection in *U. minor*, in agreement with previous research (Solla *et al.*, 2005b), but there is no evidence of its involvement in resistance to water-stress-induced cavitation.

Trees that were more susceptible to DED (i.e. progeny of the S × S cross) had longer and wider vessels (VD, VL_{max} and bVL ; Table 3, Fig. 2). Previous studies have reported that larger conduit size contributes to a faster upward movement of the pathogen and pathogen-produced toxins as a result of greater sap flow (Solla & Gil, 2002; Solla *et al.*, 2005b; Martín *et al.*, 2009). In the current study, the theoretical hydraulic conductance (THC; Table 3) was higher for progeny of the S × S cross, but there were no significant differences in $K_{x_{\text{max}}}$,

CLVF, CMVF and CSVF between groups (Table 3). Therefore, although vessel length and diameter are involved in DED resistance, it is still not clear if it is due to their effect on conductivity.

Susceptibility to cavitation caused by bubbles formed during freeze-thaw cycles is strongly correlated to vessel diameter (Davis *et al.*, 1999). Considering the reported differences in vessel size distribution parameters and wilting results (Table 3), cavitation progress in infected elms might be comparable to freeze-thaw cavitation progress. In particular, an enzymatic degradation of vessel walls may cause its blockage by vapour formation (Newbanks *et al.*, 1983), and chemical changes promoted in sap by hydrophobic substances that the fungus secretes in the vessels (e.g. cerato-ulmin) might force air bubbles out of the solution that may nucleate cavitation. A similar process has been described in pine wilt disease, where an excessive production of hydrophobic volatiles with a surface tension lower than water has been proposed to promote tracheid cavitation (Kuroda, 1991). These substances could also directly degrade pit membranes, thus lowering the threshold of cavitation, a phenomenon that is neither related to water stress. The conductivity reduction produced by these cavitation mechanisms would be

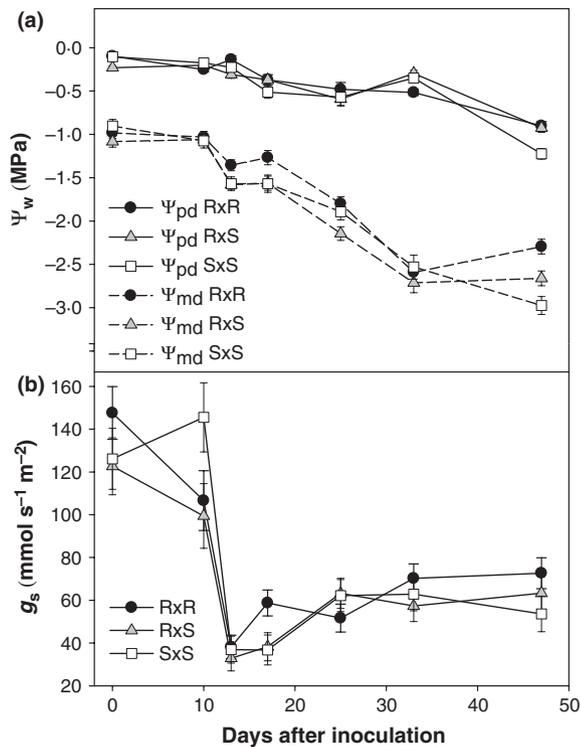


Figure 3 (a) Pre-dawn (Ψ_{pd}) and midday (Ψ_{md}) water potentials, and (b) midday stomatal conductance (g_s) for trees belonging to resistant (R) and susceptible (S) crosses following inoculation with *Ophiostoma novo-ulmi* (GR-DF3 \times AB-AL1 (R \times R), J-CA2 \times TO-AL1 (R \times S) and CR-PB1 \times TO-AL1 (S \times S); $n = 8$ per group; means \pm SE).

reinforced by the hydraulic resistance increase produced by DED toxins (Van Alfen & Turner, 1975).

Despite growing under the same environmental conditions, R \times R and R \times S elm trees showed significantly higher vessel frequency (VF), lower percentage of grouped vessels (PGV) and lower vessel diameters (VD) than S \times S saplings (Table 3). This fact may confer R \times R and R \times S plants with an intrinsically higher hydraulic safety level by building up alternative redundant water pathways (Tyree *et al.*, 1994a). These features may also hinder the colonization of new vessels by *O. novo-ulmi*, as well as providing the host with a greater ability to isolate infected vessels. The most susceptible group of elms (S \times S) showed a significantly greater VTA mean value (Table 3). Consequently, there is less room for parenchyma cells and fibres, which are responsible for chemical and compartmentalization responses to infection (Martín *et al.*, 2005a,b).

Wood density, xylem vessel implosion resistance and drought-cavitation resistance have been related within and across species (Hacke *et al.*, 2001; Jacobsen *et al.*, 2005; Awad *et al.*, 2012). However, no correlations between P_{50} , WD or $(t/b)^2$ were found when considering all 24 sampled trees (Table S1). As there were no differences in cavitation resistance (P_{50} and P_{80}) among groups, it may be that higher $(t/b)^2$ in R \times R elms results

in slower hyphal progress. Although differences in WD between resistant and susceptible elms have been reported previously (Martín *et al.*, 2009), no such differences were found in the current study (Table 3). This discrepancy could be as a result of the different methods used for evaluating WD.

Although the progression of pre-dawn (Ψ_{pd}) and midday (Ψ_{md}) water potentials were generally similar for the three types of crossing, the leaves from R \times R trees showed a higher Ψ_{md} than the leaves from S \times S plants by the end of the experiment (Fig. 3a), probably as a result of the greater wilting that S \times S trees were suffering with progression of DED. Furthermore, S \times S saplings showed significantly higher g_s during the first days after inoculation (Fig. 3b), which has previously been proposed to favour the transport of the pathogen and the generated toxic substances, ultimately increasing WP (Solla *et al.*, 2001). In addition, the earlier stomatal closure found in R \times R and R \times S crossings (Fig. 3b) would help to avoid higher water stress levels (Sparks & Black, 1999; Sperry, 2000) and, therefore, to prevent cavitation at the beginning of the infection.

In summary, despite great variability within each tested family and type of crossing, least squares means wilting percentages were lowest in R \times R elms (32–46%), intermediate in R \times S ones (48–59%) and highest for S \times S trees (75%). The results in this study show that resistance to DED is heritable. Planting resistant elm genotypes within natural populations may enhance their defence against this disease after genetic mixing. Meanwhile, xylem vulnerability to water-stress cavitation was independent of the type of crossing. Nevertheless, significant differences in theoretical hydraulic conductivity and vessel size parameters (diameter, length and size distributions) were found between progenies derived from R \times R, R \times S and S \times S crosses. Susceptible trees had significantly wider and longer vessels. These features could contribute to more rapid upward movement in susceptible trees. Finally, in agreement with a faster DED progress, favoured by a higher stomatal conductance, S \times S trees showed overall significantly lower water potential values than R \times R trees, particularly at midday, at the end of the experiment.

Acknowledgements

The authors would like to thank I. Aranda and S. Iglesias for their support to accomplish this study, and are also grateful to P. Fuentes-Utrilla, E. Miranda and J. Domínguez for their technical assistance. They would also like to thank three anonymous referees that have helped to improve this article with their comments and suggestions. This research was developed in the framework of a project funded by the Ministry of Agriculture, Food, and Environment of Spain. M.V. is sponsored by a PIF grant from the Technical University of Madrid. A.G. was sponsored by a Juan de la Cierva Postdoctoral Contract of the Ministry of Science and Innovation of Spain. The authors appreciate G. Seket's linguistic revision.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1 Correlation analysis table of the variables measured in the 24 elms selected for the detailed analysis.