Influence of Plant Age on Symptom Development in *Ulmus minor* Following Inoculation by *Ophiostoma novo-ulmi*

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ABSTRACT

Solla, A., Martín, J. A., Ouellette, G. B., and Gil, L. 2005. Influence of plant age on symptom development in *Ulmus minor* following inoculation by *Ophiostoma novo-ulmi*. Plant Dis. 89:1035-1040.

In American and European breeding programs, numerous elm trees from many species (*Ulmus* spp.) and hybrids have been inoculated annually with the fungus *Ophiostoma novo-ulmi* (the Dutch elm disease pathogen) in screening tests for resistance. Because trees were inoculated at different ages, it appeared necessary to study the influence of host age on the symptoms shown. Four *Ulmus minor* trees and one *U. minor* × *U. pumila* tree were cloned annually from 1994 to 1999. The replicates obtained (usually $n = 6$) were inoculated on 17 May 2000 with an *O. novo-ulmi* strain. At the end of the season, 2-year-old *U. minor* trees showed average wilting of $8 \pm 3\%$ (ES), significantly less than that of 3-year-old trees ($34 \pm 5\%$) ($P \leq 0.01$). Elms that were 4, 5, 6, and 7 years old showed wilting values of around 50%. A positive relationship between the symptoms shown and the diameter of the elm xylem vessels was observed, and the implications for elm resistance and breeding are discussed. Breeders and pathologists should use trees of the same age and physiological phase when determining the relative resistance among elm clones. It is concluded that under the growing conditions of this experiment, the optimal age for *U. minor* screening was 4-year-old plants.

Additional keywords: hydraulic conductance, juvenility, plant resistance

The highly destructive vascular wilt disease of elm species (*Ulmus* spp.), Dutch elm disease (DED), is caused by *Ophiostoma ulmi* (Buisman) Nannf. and the more aggressive *O. novo-ulmi* Brasier (1). These fungi are transmitted from infected to healthy trees by elm bark beetles of the genus *Scolytus* or *Hylurgopinus* or through root grafts. In breeding programs for resistance to DED, thousands of elms have been inoculated annually with *Ophiostoma novo-ulmi* (14,15,22,25,26,28,29). Breeding cycles must be short in order to obtain resistant plants as soon as possible and control costs. However, juvenile resistance has been reported in *Ulmus* × *hollandica* Mill. (11,38), *U. americana* L. (2,16,39), and *U. minor* Mill. (14,29) that were less than 4 years old. Changing variability in symptoms with age confounds understanding of inherent resistance of the plants,

makes the selection process difficult, and impedes progress on chemotherapy and biological control studies.

Elms undergo morphological and physiological changes in developing from young seedlings into mature or flowering trees. During this interval, many properties change, including nervation, hairiness, size and form of leaves, length of petioles, tendency to form cork, leaf phenology, and wood anatomy, among others (11,18,20). It is therefore not surprising that the degree of susceptibility to DED also should change during this juvenile phase. In 1- to 3-year-old branches from *U. minor* and *U. americana*, average xylem vessel diameters and the disposition of vessels within the annual growth ring were found to differ with age (23,31). Vessels of 1- and 2-year-old branches are diffusely distributed and narrower in diameter than vessels of 3-year-old branches, which are ring porous. Because vessels of smaller diameters in elms were reported to be related to resistance to DED (4,13,23,30–32), variations in vessel diameter with age also may be related to symptom variation. The objectives of the present work were to characterize this relationship and to determine the optimal age of *U. minor* elms for early screening.

MATERIALS AND METHODS

Plant material. Four *U. minor* clones from different locations in Spain were studied (Table 1). One additional clone, an open pollinated hybrid between *U. minor* and *U. pumila* L., was also included. The five clones were selected from 32 previously tested Spanish elm clones, because they showed similar susceptibility to DED (29), favorable rooting ability, and distinct amplified fragment length polymorphism (AFLP) fingerprinting profiles (8).

The trees were propagated annually by root cuttings at the Forest Breeding Center in Puerta de Hierro, Madrid, Spain, from 1994 to 1999. About six ramets of each clone were planted the year after propagation at the same center. The plot was a two-block design, with random experimental units of three trees per clone and 0.9×0.9 m spacing. In order to avoid edge effects, an elm border was planted around the plot. The soil at the site had a fine sandy loam texture and was amended annually in March with organic matter to enhance moisture retention. The plot was irrigated by sprinklers during spring and summer as needed.

Inoculation. If available, 2-, 3-, 4-, 5-, 6-, and 7-year-old ramets from each clone were inoculated on 17 May 2000. Only ramets of similar sizes within each age and clone were used (Table 1). Inoculations were carried out using a highly virulent strain of *Ophiostoma novo-ulmi* subsp. *americana*, labeled as V-NG 1. The isolate was collected in 1995 from a *U. minor* tree in Valencia (Spain) and had an in vitro growth of 4.5 mm per day on 2% malt extract agar (MEA) (20°C). A conidial suspension of the isolate was prepared by adding 2×2 mm plugs from the edge of 7-day-old cultures on MEA to 50 ml of Tchernoff's liquid medium (34) in sterile Erlenmeyer flasks, followed by shaking in darkness for 4 days at room temperature. Conidial suspensions were centrifuged at $50 \times g$ for 20 min to eliminate the medium, then suspended in sterile distilled water. At midday, a transverse cut was made with a flame-sterilized scalpel blade into the sapwood of the trees, 8 to 10 cm above the soil surface, on the north side of each tree. Two 0.1-ml drops of the conidial suspen-

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Accepted for publication 7 June 2005.

sion (1×10^6 conidia per ml) were applied by hypodermic syringe; this inoculum was immediately drawn into the xylem.

Wilting and dieback are the main parameters used for assessing elms for resistance to *O. novo-ulmi* (26,28,29). Each tree was assessed for percent leaf wilting in the crown 15, 22, 30, 45, 60, 75, 105, and 120 days after inoculation. From 1 to 15 days after inoculation, foliar wilting was recorded the day it first appeared. At 120 days after inoculation, crown dieback was also evaluated. Readings were made by three independent assessors, and the mean of the three assessments was then calculated for each tree (1).

On 18 May (1 day after inoculation), all trees were wounded slightly in order to mark the stage of xylem formation at the time when the inoculations were made. Using a flame-sterilized scalpel, the trees were wounded by making a 20×2 mm slit longitudinally on the east side of the trunk, to the depth of the vascular cambium and 2 cm above the inoculation point.

Microscopy. In January 2001, histological observations were made of xylem tissue development of all plants. The main stems were bisected at wound height, and one 10-mm-thick cylindrical wood slice per tree was sampled. Slices were immediately placed in formalin-acetic alcohol (FAA; formaldehyde:acetic acid:70% ethanol, 5:5:9 vol/vol/vol) fixative. Subsamples of approximately 15 μ m in thickness were cut using a sledge microtome, placed on glass slides, and stained with safranin 0.1% (wt/vol) before being dehydrated through an alcohol series and fixed with Canada balsam. The sections were examined under an Olympus BX50 light microscope, which was connected to a PC with appropriate software for xylem measurements (Biometry, AITIM, Madrid, Spain).

Measurements were taken from the 2000 growth increment formed before inoculation (earlywood). Four sectors, separated by 90° of radius, were randomly selected from the earlywood increment. Each sector was defined as the surface contained between two or three consecutive xylem rays (23). Measurements of interior vessel diameter were taken radially and used to calculate the vessel transectional area (VTA) and the relative theoretical hydraulic conductance (THC; μm^2). The VTA was obtained by dividing the area occupied by the vessels in a sector

(wall excluded) by the total area of the sector, then multiplying by 100 (12). The THC, predicted by the Hagen-Poiseuille equation (9), was determined by dividing the sum of the fourth power of internal vessel radii ($\sum r_n^4$) by the sector area. Vessels less than 20 μ m in diameter were ignored.

Statistics. Percentages of wilting, dieback, and VTA were normalized using the arcsine transformation $(x/100)^{1/2}$. Normality of transformed data was confirmed using the Shapiro-Wilks statistic (21). Parameters for each clone were analyzed using a multifactorial ANOVA, considering block and age as factors. Tukey tests were applied to compare averages. Regression analyses were made between average transformed values, 60 days after inoculation, of wilting, vessel diameters, VTA, THC, and vigor of the ramets (expressed in relation to growth during the year before inoculation). Statistics were carried out under Statgraphics Plus v5.1 software (Statgraphics Centurion, Herndon, VA).

RESULTS

Significant differences were observed in the amount of wilting depending on tree age at all intervals after the incubation period ($P < 0.05$). Wilting was first observed at 6 to 7 days after inoculation on elms of 4, 5, 6, and 7 years of age. Three-year-old ramets showed the first wilt symptoms at 11 days after inoculation, and 2-year-old ramets at 15 days after inoculation (clone UPM211) or later (UPM083, UPM089, UPM179, and UPM111). The wilting increase was greatest immediately after the day it first appeared, and wilting values remained constant after 30 days after inoculation (Fig. 1). Dieback values for 120 days after inoculation were proportional to 120 days after inoculation wilting values ($r^2 = 0.78$; $P = 0.008$), and about 20% lower on average ($P = 0.04$; data not shown).

Since leaf fall and flushing at 120 days after inoculation made wilting difficult to assess, wilting values 60 days after inoculation were used for statistical analyses. Two-year-old plants wilted less than older plants ($P < 0.05$), except for UPM083 and UPM089, whose 2- and 3-year-old ramets showed similar wilting values (Fig. 2A). Average wilting was maximum in plants that were 3 (UPM179 and UPM111) and 4 years old (UPM083, UPM089, and

UPM211). In older ramets, wilting did not differ, except for UPM211, for which wilting values decreased on 6-year-old ramets ($P = 0.03$). Comparing the *U. minor* clones, no differences between the wilting of same-age plants were found, except in UPM179, which was more susceptible than UPM083 at 3 years old, and in UPM179 and UPM211, which were more susceptible than UPM083 and UPM089 at 4 years old. Three-year-old ramets of the clone *U. minor* $\times$ *U. pumila* wilted less than 3-year-old UPM179. Also, 2- and 4-year-old hybrid ramets wilted less than UPM179 and UPM211 ramets of the same age. At 4 years of age, average height of ramets increased about 100% from the previous year (Table 1).

A diffuse xylem vessel distribution was characteristic of 2-year-old ramets (Fig. 3A), in comparison with a ring distribution in the older ramets (Fig. 3B). Discolored vessel walls and brown adjacent parenchyma cell walls were observed in inoculated trees within the current growth increments that were present at the time of inoculation. Gels, tyloses, and other extraneous material were abundant in lumina of vessels with discolored walls (Fig. 3C). Barrier zones formed outside and tangentially to the larger vessels, coinciding with the beginning of growth increments laid down after inoculation (Fig. 3D). These zones consisted of abnormal density of axial parenchyma and swollen ray parenchyma. Measurements of the xylem growth increments formed before inoculation showed similar vessel diameter distribution for clones of the same age (Fig. 4). Two-year-old ramets had more than 95% of vessels within the lowest diameter classes (20 to 39 and 40 to 59 μ m). In 4- to 7-year-old ramets, however, xylem growth increments had about 25% of vessels with diameters above 59 μ m.

Two-year-old ramets had the lowest average xylem diameters (Fig. 2B), and the oldest ramets had the highest vessel diameters, with the exception of UPM111. This hybrid had the highest average diameter value at 4 years old. Few significant differences were found for VTA between tree ages and clones (Fig. 2C). On the contrary, average TCH values (Fig. 2D) corresponded with those of average vessel diameters; these variables were significantly correlated with wilting 60 days after inoculation and ramet vigor (Table 2).

Table 1. Plant material specifications

Species	Clone	Average height (cm) of ramets $\pm$ SD (N) ^a					
		2 years	3 years	4 years	5 years	6 years	7 years
<i>U. minor</i>	UPM083	34 $\pm$ 20 (6)	53 $\pm$ 31 (6)	131 $\pm$ 29 (6)	...	194 $\pm$ 29 (6)	232 $\pm$ 38 (6)
	UPM089	25 $\pm$ 27 (6)	59 $\pm$ 33 (5)	123 $\pm$ 38 (6)	...	206 $\pm$ 43 (6)	...
	UPM179	28 $\pm$ 19 (6)	74 $\pm$ 23 (4)	109 $\pm$ 27 (6)	...	...	230 $\pm$ 47 (5)
	UPM211	34 $\pm$ 26 (4)	...	126 $\pm$ 25 (6)	...	239 $\pm$ 31 (6)	...
<i>U. minor</i> $\times$ <i>U. pumila</i>	UPM111	39 $\pm$ 18 (6)	80 $\pm$ 24 (4)	161 $\pm$ 28 (6)	239 $\pm$ 31 (6)	296 $\pm$ 39 (6)	...

^a Measured on May 2000, before inoculation.

DISCUSSION

The impact of host age on resistance of *U. minor* and *U. minor* × *U. pumila* clonal plants to *O. novo-ulmi* was studied for the first time. A significant correlation between plant age and wilting levels was found, in accordance with observations of differences in resistance noted between plants of different ages in other elm species (2,19,27,33). The low wilting values recorded for juvenile 2-year-old plants was

remarkable, and was maintained by some clones during the third year. In contrast to our results, other authors have reported obtaining typical wilting of young elm seedlings grown under greenhouse conditions (10,22,24,34). Comparisons with previous research on DED on this aspect should be interpreted carefully, however, since different elm species, the environment, inoculation method, the *Ophiostoma* species, the inoculum composition, and inoculation date

may all influence wilting expression. For instance, root-wound-inoculated elms that were grown under greenhouse conditions provide extremely favorable conditions for disease development (10).

Three-year-old seedlings will show symptoms only if they are grown under optimal conditions (11). Tested when they were 4 years old, our elms reached maximum wilting values, coincident with maximum growth rates. No differences in

Fig. 1. Changes in leaf wilting percentage in four *Ulmus minor* clones (UPM083, UPM089, UPM179, and UPM211) and one *U. minor* × *U. pumila* clone (UPM111) inoculated with *Ophiostoma novo-ulmi*. Values are averages from ramets of different ages ($n \geq 4$), and bars indicate 95% confidence limits.

Fig. 2. Average values of parameters measured in four *Ulmus minor* clones (UPM083, UPM089, UPM179, and UPM211) and one *U. minor* × *U. pumila* clone (UPM111) of different ages ($n \geq 4$): **A**, wilting percentage 60 days after inoculation with *Ophiostoma novo-ulmi*; **B**, vessel diameters from measurements taken within the external ring (earlywood) in the main stem of the trees; **C**, vessel trans-actional area, or surface area occupied by vessels relative to area of whole ring; and **D**, relative theoretical conductance, or sum of the four powers of the vascular radii contained in a sector, divide by the area of that sector. Different letters indicate differences within the same clone ($P < 0.05$), and bars indicate 95% confidence limits.

susceptibility were observed in elms older than 4 years, at which time susceptibility stabilized or even decreased, confirming previous observations on *U. americana* (19,27,39), *U. laevis* (35), and *U. procera* (33). We agree with Smalley and Kais (27), who reported that a difference of 1 or 2 years in tree age significantly affects the level of wilting, making the ranking of the clones in terms of resistance inconsistent over all ages. It is therefore desirable to use trees of similar age in studies aimed at comparing genetic resistance.

Although variation in resistance with tree age is not fully understood, we suggest two possible explanations. First, physiological changes with age might result in formation or absence of certain host products such as carbohydrates, mansonone phytoalexins (3), or other compounds that may affect pathogen growth, toxin produc-

tion, and disease development. This hypothesis needs to be tested in elms, since no previous studies have been conducted. Second, differences in xylem anatomy may be of pivotal importance. Indeed, resistance to DED is strongly associated with compartmentalization of the invaded xylem (3), evident microscopically as tyloses which prevent the movement of the fungus to other vascular elements (17). DED-resistant trees are able to quickly and efficiently induce more tyloses than susceptible elms (5,6), preventing the spread of the pathogen. It has been suggested that the smaller the size of vessels, the more slowly the pathogen disperses, and the more easily it is compartmentalized (4,13,23,31). Our results substantiate this hypothesis. New earlywood xylem vessels are considered to be the main conduits of pathogen spread in elm xylem in the infection proc-

ess of DED (32), since vessel elements from the preceding growth account for less than 10% of xylem transport (7). The relationship between wilting and ramet vigor, and between earlywood vessel diameter and ramet vigor, should be pointed out. Disease symptoms tend to be greater on more vigorous ramets of the same clone (11,30,34). In the present work, cuttings grew slowly at first, but subsequently increased in vigor. Present results indicate for the first time a clear relation between ramet vigor and diameter of earlywood xylem vessel elements in *U. minor* and *U. minor* × *U. pumila*.

Besides the effect of xylem vessel diameters on disease development, other hydraulic xylem aspects might be considered when discussing DED pathogenesis. Size of vessels is determined not only by diameter of vessel elements but also by their length. Additionally, the proportion of vessels with large diameters can favor xylem invasion by the fungus along the sap flow and deter the tree's ability to compartmentalize the disease (30–32). A high proportion of vessels with large diameters, present in elms older than 3 years that we have tested, provided high THC values, which might favor passive dispersal of the fungus during the incubation period. Once the pathogen has colonized a tree, vessels with great diameters are more vulnerable to cavitation by DED, as for water stress or by thawing (36,37). During spring, the loss or blockage of these large vessels will considerably reduce sap flow, leading to pronounced wilting levels that exceed those in elms containing narrow vessels.

Despite this possible relationship between vessel size and tree resistance, xylem vessel measurements cannot fully explain differences in susceptibility between *U. minor* and *U. minor* × *U. pumila*. Such observation is consistent with previous research (31), but results presented here for *U. minor* × *U. pumila* can be considered indicative only, because the unique clone used probably represents only a small sample of the diversity present in this hybrid. The genetic variation within the *U. minor* clones used (8) would make our results applicable to other trees of the same species, at least to those trees showing susceptibility to *O. novo-ulmi* similar to that described here. Further research is required to clarify the influence of plant age on symptom development in more susceptible or resistant elm varieties.

In this experiment, involving rooted cuttings, the shoots originated from callus buds, so they were in their juvenile phase. These shoots were anatomically similar to shoots from seedlings (data not shown), so our results might also be applicable to seedlings. We doubt if the present results will likewise apply to ramets derived from adult propagating materials, such as summer cuttings or grafted plants, as these maintain their adult phase (secondary

Fig. 3. Cross sections of the main stems of *Ulmus minor* trees (clone UPM211) of different ages inoculated with *Ophiostoma novo-ulmi* and immediately wounded: **A**, diffuse-porous wood in a 2-year-old tree (bar = 200 µm); **B**, ring-porous wood in a 6-year-old tree (bar = 200 µm); **C**, earlywood vessels occluded by gels (triangles) or tyloses and alveolar networks (arrow) in a 4-year-old tree (bar = 100 µm); and **D**, barrier zone (between triangles) surrounding the invading xylem, and earlywood vessel containing tyloses (arrows) in a 6-year-old tree (bar = 200 µm). Stained with safranin. a, growth increment after inoculation; b, growth increment before inoculation; W, wound.

Table 2. Correlation matrix and level of significance among variables

Variable	Vessel diameter ^a	VTAb	TCH ^c	Vigor of ramets ^d
Wilting ^e	0.68***f	0.28	0.61**	0.60**
Vessel diameter		0.47*	0.88***	0.81***
VTA			0.56**	0.39
TCH				0.77***

^a From measurements taken within the external ring (earlywood) at the main stem of the trees.
^b Surface area occupied by vessels relative to area of the whole ring.
^c Sum of the fourth power of the vascular radii contained in a sector, divided by the area of that sector.
^d Expressed as growth in the year before inoculation.
^e Percentage 60 days after inoculation with *Ophiostoma novo-ulmi*.
^f Asterisks denote significance at *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***).

growth, ability to flower, production of larger leaves, etc.). Additional evidence for juvenile resistance could be obtained by comparing symptoms of inoculated summer cuttings (from adult shoots) with inoculated root cuttings of comparable size

and vigor. Comparability would be more easily obtained by inoculating grafted adult plants next to juvenile ones of the same clone.

Since elms live more than 200 years, it is difficult to generalize about variation of

U. minor resistance with age. However, the present research may lead to a more reliable selection of elms resistant to DED. Screening for resistance has frequently involved the use of 2- to 3-year-old seedlings (10,39). Concerning *U. minor* and *U.*

Fig. 4. Distribution of vessels in diameter intervals of 20 μ m in four *Ulmus minor* clones (UPM83, UPM089, UPM179, and UPM211) and one *U. minor* $\times$ *U. pumila* clone (UPM111) of different ages. Values are averages from measurements taken within the external ring (earlywood) at the main stem of the trees ($n \geq 4$). Bars indicate 95% confidence limits.

minor × *U. pumila*, selection should be made at 4 years of age, when the external symptoms of the plants are maximum or coincident with those shown when they are older.

ACKNOWLEDGMENTS

We thank M^a Ruiz-Villar and Margarita Burón for their technical support, and Prof. Luis García for providing the xylem measurement software. The work has been developed within the framework of an agreement established between DGB (Ministerio de Medio Ambiente) and ETSI de Montes in Madrid (UPM). A. Solla was supported by Junta de Extremadura (Consejería de Educación y Tecnología) and Fondo Social Europeo during a visit to G. B. Ouellette in Québec.

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