The Effects of Propagation Techniques on Leaf Vascular Anatomy, Modulus of Elasticity, and Photosynthetic Traits in Micropropagated and Grafted Plants of the Dutch Elm Hybrid ‘Dodoens’

Jaroslav Ďurkovič, Ingrid Čaňová, and Lucia Javoříková
Department of Phytology, Technical University, 96053 Zvolen, Slovakia

Monika Kardošová
Department of Forest Protection and Game Management, Technical University, 96053 Zvolen, Slovakia

Rastislav Lagaňa
Department of Wood Science, Technical University, 96053 Zvolen, Slovakia

Tibor Priwitzer
Forest Research Institute, National Forest Centre, 960 92 Zvolen, Slovakia

Roman Longauer
Department of Silviculture, Mendel University, 61300 Brno, Czech Republic

Jana Krajináková
Department of Biology, University of Oulu, 90014 Oulu, Finland

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ABSTRACT. Understanding how plants are able to change their structural, physiological, and mechanical properties in response to various propagation methods can help to improve both their performance and their survival when transferred to field conditions. To identify changes between the routinely applied vegetative propagation techniques of in vitro micropropagation and splice grafting we assessed leaf performance for any differences in midrib vascular traits, nanomechanical properties of tracheary element cell walls, and photosynthetic traits in the dutch elm hybrid cultivar Dodoens (i.e., open-pollinated Ulmus glabra ‘Exoniensis’ × Ulmus wallichiana P39). The propagation techniques appear to have had a direct effect on a large portion of the vascular traits. In the micropropagated plants, the water-conducting area within the primary xylem tissue contained a significantly greater number of tracheary elements which suggests hydraulic safety. In the grafts, the water-conducting area contained a significantly smaller number of tracheary elements, in which the lumen areas were slightly larger than those of the micropropagated plants, resulting in a significantly higher size to number ratio which may indicate a fast and more effective water transport system. Quantitative nanomechanical mapping measurements from atomic force microscopy (AFM) revealed that the tracheary elements of the micropropagated plants formed stiffer cell walls quantified by the reduced Young’s modulus of elasticity (MOE) than those of the grafts. The effect of the rootstock might contribute to the differences in vascular traits, as well as to the differences in cell wall stiffness and cell wall deformation observed between the stock types. The micropropagated plants were subjected to a more sensitive stomatal regulation of gas exchange resulting in the lower rates of net photosynthesis and transpiration. But the higher values of both instantaneous water-use efficiency (WUEinst) and chlorophyll fluorescence yields found in the micropropagated plants indicate a higher acclimation capacity to stressful environmental conditions specifically for this stock type. Both stock types formed compact homogeneous clusters clearly separated from each other in the multivariate leaf trait analysis.

The initial elm breeding programs, launched in the Netherlands, were focused on the identification of resistant elm trees to dutch elm disease (DED) mostly from the native European species, U. glabra and Ulmus minor. Asian elms, particularly U. wallichiana and Ulmus pumila, proved to be a useful additional source of DED-resistance genes (Santini et al., 2010; Smalley and Guries, 1993). A series of new dutch elm hybrid cultivars were released in the second half of the last century. Several hundred thousand grafted trees planted widely in Europe (Gerhold, 1970; Hiemstra et al., 2006) now show a varying degree of resistance to Ophiostoma novo-ulmi, the causative agent of the current DED pandemic (Brasier, 1991).

For many years, tolerant elm genotype selection and breeding has been the most successful strategy for elm recovery (Martin et al., 2015). Tissue culture techniques have been of
great help for propagating DED-tolerant trees identified by elm breeding and conservation programs. Reliable protocols have been established for routine in vitro micropropagation of several elm species and commercial cultivars (Biroščíková et al., 2004; Conde et al., 2008; Corredoira et al., 2002; Fenning et al., 1993; Mezzetti et al., 1988). Thereby, in vitro culture techniques may contribute significantly to the efforts of tree improvement through existing germplasm conservation, in vitro selection for DED tolerance under highly controlled conditions, and the rapid distribution of new and improved cultivars (Dušković et al., 2010; Eshita et al., 2000; Harvengt et al., 2004; Shukla et al., 2012). In addition, transgenic elms showing reduced DED symptoms have been regenerated (Newhouse et al., 2007) and small-scale field tests of transgenic elms have been established (Merkle et al., 2007).

Understanding the anatomical, physiological, and metabolic processes that allow plants to modify their phenotype in response to environmental conditions can help to improve both field performance and plant survival (Brito et al., 2009). Field assessment of gas exchange will give information about stomatal control of net photosynthetic rate, transpiration rate, and WUE (Benowicz et al., 2002). In addition, measurements of chlorophyll a fluorescence yields can be used for assessing photochemical efficiency and photo-inhibition in response to environmental stresses (Berger et al., 2007; Desotgiu et al., 2012; Zhao et al., 2015). Chlorophyll a fluorescence competes for excitation energy with photosynthesis (i.e., photosynthesis) and with nonradiative decay processes, thereby providing information for both processes (Carvalho et al., 2001).

The improved force-distance curve-based AFM techniques are a powerful tool to combine nanoinaging with quantitative mapping of physical, chemical, and biological properties. Recent developments in PeakForce quantitative nanomechanical mapping (PeakForce QNM; Bruker Nano Surfaces, Santa Barbara, CA) optimized this new AFM technique to the point that it enables high-resolution imaging of algae (Pletikapić et al., 2012) and woody plant cell walls (Dušković et al., 2012, 2013; Ren et al., 2015), as well as fibrillar protein aggregates and native proteins (Pfeuntdschuh et al., 2014; Sweers et al., 2011). At the same time, with the imaging process, mapping measurements quantify the nanomechanical properties of material surfaces to nanowave (or even piconewton) and nanometer resolution. The probe is usually oscillated at low frequencies to enable capturing and analyzing the individual force-distance curves each time the AFM tip taps on the sample’s surface. The maps extracted from arrays of force-distance curves provide crucial information on the nanomechanical properties of biological systems. The mechanical properties of the cellular microenvironment, notably its rigidity and stiffness, possess a regulatory role for a variety of cellular responses including adhesion, migration, shape, and division (Dufrené et al., 2013; Janney et al., 2009). Determination of the nanomechanical attributes of plant cell walls is of great importance for evaluating both the effects of hybridization and the results of plant breeding. Thus for the cell walls of xylem tissue, quantifications of MOE, adhesion, and cell wall deformation play a key role in the assessment of the material stiffness, toughness, and its adhesive properties (Dušković et al., 2012, 2013).

Previous studies have revealed that the dutch elm hybrid ‘Dodoens’, a tree appreciated in cities and rural landscapes, shows a tolerance to the current prevalent strain of DED. O. novo-ulmi ssp. americana × novo-ulmi (Dušković et al., 2013, 2014). Phenotypic expressions for the leaf and branch hydraulic, anatomical and morphological traits have revealed a constitutive strategy of defense for their possible role in DED tolerance (Plichta et al., 2016). The effects of propagation techniques on cell wall chemistry and wood anatomy of ‘Dodoens’ plants were assessed in our recent study (Dušković et al., 2015). Here, we focused on changes in the leaf midrib vascular, nanomechanical, and ecophysiological traits with respect to the propagation technique. The objectives for this study were 1) to determine whether routinely applied vegetative propagation techniques that maintain true types of elm genotypes tolerant to DED (i.e., in vitro micropropagation vs. widely used grafting of past decades) would affect the indicators of leaf vascular architecture, gas exchange, and chlorophyll a fluorescence yields; 2) to identify the differences between the examined stock types with respect to the nanomechanical properties of tracheary element cell walls, to reveal any mechanical advantages for either stock type when using multi-parametric quantitative imaging of force-distance curve-based nanomechanical mapping; and 3) to find possible correlations among vascular, nanomechanical, and ecophysiological traits that could contribute to the maximum physiological leaf performance of ‘Dodoens’ plants in relation to the technique of propagation.

### Materials and Methods

**Plant material, study site, and sampling.** The experiments were conducted on clonally micropropagated and grafted plants of the dutch elm hybrid cultivar Dodoens, which has shown a tolerance to an aggressive isolate M3 of O. novo-ulmi ssp. americana × novo-ulmi (Dušković et al., 2013, 2014). The procedure of in vitro micropropagation through axillary and adventitious shoot formation has previously been described in detail (Krajňáková and Longauer, 1996). The grafted plants were obtained through splice grafting which consisted of joining a ‘Dodoens’ scion onto the stem of a DED-tolerant Ul. pumila ‘Pusztta’ rootstock to reduce the possibilities of DED transmission in the soil by root graft connections (Green et al., 1985). Grafting of scions onto rootstock was performed 2 cm upward from the root-stem junction, and the scions used for both grafting and in vitro culture establishment originated from identical donor plants. Three-year-old micropropagated and grafted plantlets of uniform size were selected and then planted in the experimental field plot at Banská Bela, Slovakia (lat. 48°28′N, long. 18°57′E, altitude 590 m). No postplanting treatments such as irrigation and fertilization were applied. The climate and soil conditions of the study site were described previously (Dušković et al., 2015). The experiments were conducted on five plants per stock type unaffected by DED and chosen randomly to remove any positional effects in the field. Measurements were performed on fully expanded leaves in the tenth growing season following planting. Scanning electron microscopy images of the plant leaf material used in this study are presented in Fig. 1A–D.

**Leaf histology and midrib vascular traits.** Midrib sections (0.4 × 0.4 cm), excised from the leaf base, were fixed in 5% (v/v) glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.0, dehydrated in ethanol and propylene oxide, and embedded in Spur embedding medium (Dušković et al., 2013).
Cross-sections, ≈1.5 μm thick, were cut using an automated rotary microtome (RM2255; Leica Biosystems, Nussloch, Germany) with glass knives, and stained with toluidine blue and basic fuchsin. Sections were observed using a light microscope (BX50F; Olympus Europa, Hamburg, Germany). The thickness of the leaf, mesophyll, palisade, and spongy parenchyma was measured using the NIS-Elements AR 3.0 image analysis software (Laboratory Imaging, Prague, Czech Republic). Measurements were performed on five sun leaves per plant, one section per leaf, two measurements per section (on the left side and on the right side from the leaf midrib).

Vascular characteristics of the leaf midrib primary xylem; i.e., tracheary element lumen area (A) and tracheary element densities (N) per 0.1 mm² of the primary xylem area, were determined using the NIS-Elements AR 3.0 image analysis software (Laboratory Imaging). The additional indicators of leaf vascular architecture such as tracheary element lumen fraction \((F = A/N)\) and the tracheary element size:number ratio \((S = A/N)\) were calculated as described in Zanne et al. (2010).

Total relative conductivity (RC) per 0.1 mm² of the primary xylem area was calculated as the sum of individual RCs divided by the area of a cross-section of primary xylem (Đurković et al., 2012), whereas the individual RC was calculated as the fourth power of the equivalent circle diameter of the tracheary element lumen (Zimmermann, 1983). Lignin autofluorescence in cell walls of elements was detected by excitation at 360 nm using a barrier filter with a transmission cutoff at 470 nm, and photographed using a fluorescence microscope (DM4000 B; Leica Microsystems, Wetzlar, Germany) equipped with a digital color camera (DFC490; Leica Microsystems). Measurements were performed on five sun leaves per plant, one section per leaf midrib, one measurement per section.

**Multiparametric quantitative nanomechanical mapping and cell wall nanomechanics.** Midrib sections (0.4 × 0.4 cm), excised from the leaf base, were fixed in 5% (v/v) glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.0, dehydrated through...
Fig. 2. Basic principles illustrating how the different nanomechanical properties are extracted from the force-distance curve using multiparametric quantitative nanomechanical mapping measurements. The image shows a typical force-distance curve for the tracheary element cell wall in the Dutch elm hybrid ‘Dodoens’. The adhesion is the vertical distance between the base line and the lowest portion of the retraction curve. The deformation is the horizontal distance between the contact point and the turn-away point (representing maximum indentation at peak force setpoint). The reduced Young’s modulus of elasticity can be extracted by extrapolating the initial portion of the retraction curve after the contact point using the Derjaguin–Muller–Toporov model fit. The energy dissipation can be calculated by integrating the area between the two curves.

Table 1. Leaf midrib vascular traits in the Dutch elm hybrid ‘Dodoens’.

| Stock type | Micropropagated | Grafted | P value |
|------------|-----------------|---------|---------|
| Trait | (mean ± se) | (mean ± se) | |
| A (10–4 mm2) | 7.41 ± 0.42 | 7.93 ± 0.34 | 0.0709ns |
| N (no./0.1 mm2) | 597 ± 33 | 429 ± 20 | 0.0001*** |
| F (10–3 mm2) | 41.8 ± 1.3 | 33.1 ± 0.9 | 0.0001*** |
| S (10–3 mm2) | 1.42 ± 0.17 | 1.97 ± 0.16 | 0.0043** |
| RC (10–6 mm4) | 6.90 ± 0.47 | 6.66 ± 0.40 | 0.9872 |

*ns = nonsignificant or significant at P < 0.01 or 0.001, respectively, based on nested analysis of variance.

In addition, as the adhesion pull-off force is influenced by the high tip radius and surface energy (Fischer-Cripps, 2011), the raw data of adhesion were normalized to the reference tip radius of 100 nm (Amitay-Sadovsky et al., 2002). Adhesion at the reference tip radius (ADHref) was calculated according to the following equation:

\[ \text{ADH} = \text{ADH}_{\text{ref}} + \alpha (R - R_{\text{ref}}) \]

where ADH is adhesion pull-off force, \( \alpha \) is the slope of an adhesion–tip radius regression line, \( R \) is the actual tip radius, and \( R_{\text{ref}} \) is the reference tip radius of 100 nm.

GAS EXCHANGE. An open portable photosynthesis system with an infrared gas analyser (LI-6400 XTR; LI-COR, Lincoln, NE) was used for in situ gas exchange measurements. Net photosynthetic rate (\( P_n \)), transpiration rate (\( E \)), stomatal conductance (\( g_s \)), and intercellular CO2 concentration (\( C_i \)) were measured under a saturating photosynthetic photon flux density of 1200 ± 5 \( \mu \text{mol} \text{~m}^{-2} \text{~s}^{-1} \) and an ambient CO2 concentration of 370 ± 5 \( \mu \text{mol} \text{~mol}^{-1} \) using the 6400-08 standard leaf chamber with the 6400-02B light-emitting diode red/blue light source (LI-COR). WUEinst was calculated as the ratio of \( P_n \) to \( E \) (Campbell et al., 2005). During measurements, microclimatic conditions inside the assimilation chamber were kept constant (leaf temperature 21 ± 1 °C, air relative humidity 70% ± 5%). The vapor pressure deficit ranged from 0.8 to 1.3 kPa.
Measurements were performed on six sun leaves per plant, five measurements per leaf.

**Chlorophyll fluorescence and chlorophyll content.** Chlorophyll a fluorescence yields were measured on sun-exposed leaves using a portable fluorometer (Plant Efficiency Analyser; Hansatech Instruments, Kings Lynn, UK). Leaves were kept for 30 min under leaf clamps for dark adaptation. After the initial measurement of dark-adapted minimal fluorescence ($F_0$), leaves were exposed to a saturating irradiance of 2100 μmol·m$^{-2}$·s$^{-1}$ for 1 s to measure the maximal fluorescence of dark-adapted foliage ($F_m$). Variable fluorescence ($F_v$) was calculated as $F_v = F_m - F_0$, and the variables such as maximum biochemical efficiency of photosystem II ($F_v/F_m$), variable-to-initial fluorescence ratio ($F_v/F_0$) and potential electron acceptor capacity of photosystem II – “area” (i.e., area above the induction curve between $F_0$ and $F_m$) were determined. Measurements were performed on ten sun leaves per plant, two measurements per leaf (one per adaxial surface and one per abaxial surface).

Relative chlorophyll content was estimated with a portable chlorophyll meter (CL-01; Hansatech Instruments) and the results were expressed as the chlorophyll index (CHLI) (Cassol et al., 2008). Measurements were performed on seven sun leaves per plant, three measurements per adaxial leaf surface.

### Table 2. Nanomechanical traits of tracheary element cell walls of the dutch elm hybrid ‘Dodoens’.

| Trait  | Micropropagated | Grafted | $P$ value |
|--------|-----------------|---------|-----------|
| MOE (MPa) | $4.144 ± 0.47$ | $3.067 ± 0.08$ | 0.0002*** |
| ADH$_{ref}$ (nN) | $16.94 ± 0.47$ | $17.61 ± 1.13$ | 0.3219 NS |
| DEF (nm) | $2.52 ± 0.09$ | $2.29 ± 0.08$ | 0.0446* |
| DIS (eV) | $1,893 ± 132$ | $2,118 ± 157$ | 0.2716 NS |

*M OE = reduced Young’s modulus of elasticity; ADH$_{ref}$ = adhesion at the reference tip end radius of 100 nm; DEF = deformation; DIS = energy dissipation. NS, *, ***Nonsignificant or significant at $P < 0.05$ or 0.001, respectively, based on nested analysis of variance.

Results and Discussion

**Differences in vascular traits.** Significant differences were found between the stock types for a majority of leaf midrib vascular traits (Table 1). Both stock types employed different vascular anatomies. The micropropagated plants showed higher values for the trait $N$, and also for the trait $F$ which measures the relative amount of water transport space. The grafts reached higher values for the trait $S$ which measures variation in the vessel composition within the water transport space. In the micropropagated plants, the water-conducting area within the primary xylem tissue contained a significantly greater number of tracheary elements which suggests hydraulic safety (driven by embolism avoidance) (Zanne and Falster, 2010). In the grafts, the water-conducting area contained a significantly smaller number of tracheary elements, in which the lumen areas were slightly larger than those of the micropropagated plants. This difference in the leaf vascular anatomy of the grafts was also supported by a higher value for trait $S$, which may indicate a fast and more effective water transport system (Zanne and Falster, 2010) as the water flows more easily through fewer larger tracheary elements than through a large number of smaller ones. However, the performances in RC per 0.1 mm$^2$ of the grafts reached higher values for the trait $S$ which measures variation in the vessel composition within the water transport space. In the micropropagated plants, the water-conducting area within the primary xylem tissue contained a significantly greater number of tracheary elements which suggests hydraulic safety (driven by embolism avoidance) (Zanne and Falster, 2010). In the grafts, the water-conducting area contained a significantly smaller number of tracheary elements, in which the lumen areas were slightly larger than those of the micropropagated plants. This difference in the leaf vascular anatomy of the grafts was also supported by a higher value for trait $S$, which may indicate a fast and more effective water transport system (Zanne and Falster, 2010) as the water flows more easily through fewer larger tracheary elements than through a large number of smaller ones. However, the performances in RC per 0.1 mm$^2$ of the primary xylem area between the stock types were not significantly different. This suggests that a relative trade-off was achieved between a couple of vascular architecture indicators, $A$ and $S$ on one side and $N$ and $F$ on the other side. The traits $A$ and $N$, which are the primary indicators of vascular anatomy, also showed a significant negative correlation with each other ($r = -0.75, P = 0.013$). Measurements of $N$ explained
56% of the variation in $A$ [$R^2 = 0.56$ (Fig. 3A)]. Tracheary element lumen area strongly affects the capacity of xylem tissue to conduct water, whereas conduit density influences bulk xylem composition (Preston et al., 2006). The traits $A$ and $N$ are negatively correlated across angiosperms as well as gymnosperms (Sperry et al., 2008; Zanne et al., 2010).

Differences in vascular anatomy and hydraulic conductivity among the rootstocks used for grafting can often play a role in the subsequent vascular growth of a scion. Changes in vessel lumen areas over time in the graft union of various *Prunus avium* heterograft and homograft combinations were reported by Olmstead et al. (2006). The authors found that the wound-related callus formation response after grafting led to reduced vessel lumen areas in scion and graft union tissues. In this study, differences found between the stock types for vascular traits ($N$, $F$, and $S$) were phenotypically expressed in leaf midribs. This suggests that the differences in leaf vascular performances might be attributed to the rootstock. In some cases, the effect of the rootstock was found to be responsible for the physiological alterations in water transport efficiencies and hydraulic conductance; e.g., in grafted *Malus domestica* (Cohen and Naor, 2002) or *Cucumis melo* plants (Agele and Cohen, 2009). However, in this study RC per unit area of the primary xylem tissue did not seem to be affected by the propagation technique.

### Differences in MOE of Tracheary Element Cell Walls

The tracheary elements of micropropagated plants had stiffer cell walls, as indicated by the higher lignin autofluorescence (Fig. 4). The tracheary elements of micropropagated plants had stiffer cell walls, as indicated by the higher lignin autofluorescence (Fig. 4).

### Table 3. Gas exchange and chlorophyll index in the dutch elm hybrid ‘Dodoens’.

| Trait $^a$ | Micropropagated (mean ± SE) | Grafted (mean ± SE) | $P$ value |
|------------|-----------------------------|---------------------|------------|
| $P_N$ (μmol·m$^{-2}$·s$^{-1}$) | 14.2 ± 0.1 | 19.4 ± 0.3 | 0.0001*** |
| $E$ (mmol·m$^{-2}$·s$^{-1}$) | 2.38 ± 0.02 | 3.75 ± 0.09 | 0.0001*** |
| $g_S$ (mmol·m$^{-2}$·s$^{-1}$) | 273 ± 5 | 392 ± 17 | 0.0001*** |
| $C_i$ (μmol·mol$^{-1}$) | 254 ± 1 | 235 ± 2 | 0.8332 NS |
| WUE$_{inst}$ (μmol·mmol$^{-1}$) | 5.70 ± 0.05 | 5.56 ± 0.08 | 0.0489* |
| CHLI | 12.8 ± 0.3 | 11.0 ± 0.4 | 0.0001*** |

$^aP_N$ = net photosynthetic rate; $E$ = transpiration; $g_S$ = stomatal conductance; $C_i$ = intercellular CO$_2$ concentration; WUE$_{inst}$ = instantaneous water-use efficiency; CHLI = chlorophyll index. NS, * Nonsignificant or significant at $P < 0.05$ or 0.001, respectively, based on nested analysis of variance.
cell walls quantified by MOE (Table 2). In addition, MOE was positively related to the number of tracheary elements per unit area ($r = 0.66$, $P = 0.036$). Measurements of $N$ explained 44% of the variation in MOE [$R^2 = 0.44$ (Fig. 3B)]. From a mechanical point of view, a significant increase in $N$ in the micropropagated plants was accompanied by a significant increase in MOE of cell walls to render a greater stiffness for the primary xylem tissue (Durković et al., 2013). Thus, the micropropagated plants may benefit directly from a higher MOE over the grafts by having a reduced risk of tracheary element implosion when an embolism spreads and cavitation of the water column occurs as a result of the stressful conditions caused by $O. novo-ulmi$, the causative agent of DED (Martin et al., 2009, 2010; Ouellette et al., 2004). However, the reasons why the grafts had less stiff cell walls than the micropropagated plants are not completely clear. In our previous study (Durković et al., 2015), we found that rootstock significantly affected the relative amounts of lignin and holocellulose as well as neutral saccharides (mainly $\alpha$-glucose and $\alpha$-xylose) in the stems of ‘Dodoens’ grafts. In addition, do Nascimento et al. (2011) reported different relative amounts of sucrose in latex samples of mature Hevea brasiliensis trees which were grafted onto various rootstocks. Gonçalves et al. (2006) found that the concentration of several leaf metabolites such as starch, total phenolics, total chlorophyll, and total carotenoids, was influenced by the rootstock genotype in grafted $P. avium$ trees. The above studies indicate the direct effect of the rootstock on the relative amounts of cell wall biopolymers and metabolites in both the stem and the leaves of a scion. Thus, alterations in the content and distribution of cell wall biopolymers might be one of the major reasons for the differences in both MOE and the cell wall deformation discovered between the grafts and the micropropagated plants in this study.

The other nanomechanical traits that are related to the adhesive properties of the cell wall surface; i.e., ADH ref and energy dissipation, did not differ significantly according to the propagation technique (Table 2). The quantitative AFM imaging of tracheary element cell walls is shown in Fig. 4. These images show the structure of the cell wall fragment surface in the peak force error and the height AFM channels. The peak force error channel produces a map of the peak force for each pixel in the image during the scan, and reveals the difference between the setpoint and the actual imaging force value. This error-signal provides a sensitive detection technique for the visualization of fine cell wall surface details. The height channel shows a height profile (i.e., cell wall topography).

**DIFFERENCES IN ECOPHYSIOLOGICAL TRAITS AND THICKNESS OF LEAF TISSUES.** Most of the ecophysiological traits assessed varied according to the applied propagation technique (Table 3). The micropropagated plants reached higher values for both WUE$_{inst}$ and CHLI, whereas the grafts had higher values for $P_N$, $E$, and $g_S$. No difference between the stock types was found for $C_t$. The measurements of $g_S$ revealed that the grafts were subjected to a less sensitive stomatal regulation of photosynthesis and transpiration than were their micropropagated counterparts. A higher $g_S$ increased carbon uptake during photosynthesis ($r = 0.73$, $P = 0.016$ (Fig. 5A)). Measurements of $g_S$ explained 53% of the variation in $P_N$ ($R^2 = 0.53$) indicating that a smaller proportion in variation was left for the nonstomatal component of photosynthesis. A higher $g_S$ also

### Table 4. Chlorophyll $a$ fluorescence in the dutch elm hybrid ‘Dodoens’ determined on adaxial and abaxial leaf surfaces.

| Trait               | Micropropagated (mean ± se) | Grafted (mean ± se) | $P$ value |
|---------------------|-----------------------------|---------------------|-----------|
| **Adaxial surface** |                             |                     |           |
| $F_v/F_m$           | 0.786 ± 0.003               | 0.749 ± 0.004       | 0.0001*** |
| $F_m/F_0$           | 3.63 ± 0.07                 | 3.02 ± 0.07         | 0.0001*** |
| “Area” (Mb$^-1$)    | 32.80 ± 0.78                | 24.38 ± 0.69        | 0.0001*** |
| **Abaxial surface** |                             |                     |           |
| $F_v/F_m$           | 0.794 ± 0.003               | 0.768 ± 0.003       | 0.0001*** |
| $F_m/F_0$           | 3.88 ± 0.06                 | 3.34 ± 0.05         | 0.0001*** |
| “Area” (Mb$^-1$)    | 24.36 ± 0.74                | 20.98 ± 1.17        | 0.0098*** |

$F_v$ = variable fluorescence yield in the dark-adapted state; $F_m$ = maximal fluorescence yield; $F_v/F_m$ = maximum photochemical efficiency of photosystem II; $F_0$ = minimal fluorescence yield; $F_v/F_0$ = variable-to-initial fluorescence ratio; “Area” = potential electron acceptor capacity of photosystem II.

***, ****Significant at $P < 0.01$ or 0.001, respectively, based on nested analysis of variance.
supported a higher rate of \( E [r = 0.90, P < 0.001 \text{ (Fig. 5B)}] \), and measurements of \( g_s \) explained 81\% of the variation in \( E (R^2 = 0.81) \). In addition, \( g_s \) was negatively linked with WUE\text{inst} (Renninger et al., 2015) when individuals conserving water in leaves through a lower \( g_s \) had an increased value of WUE\text{inst} \([r = -0.72, P = 0.019 \text{ (Fig. 5C)}]\). Previous studies have shown that \( g_s \) plays a pivotal role in predicting carbon and water exchange between the atmosphere and terrestrial plants (Cernusak et al., 2009; Lin et al., 2015; Orchard et al., 2010). When interpreting the differences in the gas exchange results, one should note that the type of propagation technique also affects the degree of rejuvenation in the planting stock (Bonga and von Aderkas, 1992; Tetsunura et al., 2004). In U. glabra, Đurković et al. (2010) reported a lesser degree of rejuvenation, accompanied with an earlier onset of flowering and seed production, for the grafts than for the micropropagated plants. The authors observed a similar pattern to that described in this study; i.e., with higher rates of gas exchange (especially \( P_N \)) for the U. glabra grafts than for the micropropagated plants. It seems that in vitro micropropagated plants are subjected to a greater degree of rejuvenation, which in turn affects the stomatal regulation of photosynthesis in a more sensitive way than in the grafts. It may, thus, be a reason why the micropropagated plants obtained lower photosynthesis than the grafts. Our results confirmed that stomatal behavior was an important regulator of gas exchange and water use for both the micropropagated and grafted plants of ‘Dodoens’. It could be assumed that due to a higher photosynthetic carbon gain per unit transpirational water vapor loss found in the micropropagated plants, this stock type could have a higher acclimation capacity to stressful environmental conditions than the grafts. This assumption was supported by additional data coming from chlorophyll \( a \) fluorescence yields (see below).

The micropropagated plants reached significantly higher values for \( F_m/F_{m0} \), \( F_v/F_{v0} \), and potential electron acceptor capacity than the grafts for both leaf surfaces (Table 4). For both stock types, higher values of \( F_m/F_{m0} \) and \( F_v/F_{v0} \) ratios were recorded for abaxial than for adaxial surfaces, whereas the contrary was true for the variable “area.” Typically, for nonstressed plants a characteristic \( F_m/F_{m0} \) ratio of open photosystem II is in the range of 0.75–0.85. A rapid decline in \( F_m/F_{m0} \) is a sensitive and early indicator of a change in photosynthesis and in the physiological status of the plant in general (Bolhár-Nordenkampf et al., 1989). Measurements in this study revealed that \( F_m/F_{m0} \) ratio for the grafts reached an average value of 0.759 as opposed to 0.790 for the micropropagated plants. Thus, the reaction centers of photosystem II were functionally intact irrespective of the propagation technique. Moreover, their \( F_m/F_{m0} \) ratios were far higher than the threshold value of 0.725 which indicates the onset of reversible changes in the reaction centers of photosystem II (Čaňová et al., 2012). The variable \( F_v/F_{v0} \) estimates the maximum primary yield of photochemistry of photosystem II. A decline in \( F_v/F_{v0} \) ratio has been used as an indicator of drought stress (Li et al., 2006; Percival and Sheriffs, 2002). The variable “area” determines the potential acceptor capacity for electron transport during the primary processes of photosynthesis and has been shown to be a sensitive indicator of salinity (Panda et al., 2006). Despite lower values found in the grafts, chlorophyll \( a \) fluorescence yields were found within an optimum range for both nonstressed stock types.

We also observed several differences in the internal organization of leaf tissues between the stock types. The leaf, mesophyll, palisade parenchyma, and spongy parenchyma of the grafts had a greater thickness than those of the micropropagated plants (Table 5). The relationship between

Table 5. Thickness of leaf tissues in the dutch elm hybrid ‘Dodoens’.

| Trait                      | Micropropagated (mean ± se) | Grafted (mean ± se) | \( P \) value |
|----------------------------|-----------------------------|---------------------|--------------|
| Leaf thickness (\( \mu \)m) | 153.67 ± 4.62               | 168.50 ± 3.44       | 0.0001***    |
| Mesophyll thickness (\( \mu \)m) | 116.67 ± 3.76               | 134.41 ± 2.95       | 0.0001***    |
| Palisade parenchyma thickness (\( \mu \)m) | 56.92 ± 1.79               | 65.35 ± 2.24       | 0.0010***    |
| Spongy parenchyma thickness (\( \mu \)m) | 59.75 ± 2.63               | 69.05 ± 2.14       | 0.0015**     |

**,** ***Significant at \( P < 0.01 \) or 0.001, respectively, based on nested analysis of variance.

![Graph A](image1.png) \[ y = 85.624 + 12.867x \]
\[ R^2 = 0.376 \]

![Graph B](image2.png) \[ y = 73.561 + 3.047x \]
\[ R^2 = 0.288 \]

Fig. 6. Correlations of transpiration rate (\( E \)) with mesophyll thickness and net photosynthetic rate (\( P_N \)) with mesophyll thickness identified in the dutch elm hybrid ‘Dodoens’. (A) Relationship of \( E \) to mesophyll thickness. (B) Relationship of \( P_N \) to mesophyll thickness. Open squares show the micropropagated plants, filled squares show the grafts (\( n = 10 \)).

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Mesophyll thickness and $E$ was marginally significant [$r = 0.61$, $P = 0.059$ (Fig. 6A)], whereas the linkage between mesophyll thickness and carbon uptake ($P_N$) was nonsignificant [$r = 0.54$, $P = 0.110$ (Fig. 6B)]. Thus, the increased mesophyll thickness apparent in the grafts might have benefited from a higher transpiration rate; i.e., a higher mass flow of mineral nutrients and water from roots to the internal tissues in the leaf. In addition, the larger intercellular air spaces due to the increased thickness of the spongy parenchyma could reduce resistance in the mesophyll part of the CO$_2$ diffusion pathway (Pons et al., 2009), and thereby indirectly support a higher rate of $P_N$, especially in the grafts.
**Associations among leaf traits.** A PCA was done to evaluate how the examined leaf traits were associated (Fig. 7). The first axis explained 41% of the variation and showed strong positive loadings for $E$, $P_N$, $BS$, and the thickness of palisade parenchyma, spongy parenchyma, and mesophyll. The negative side of the axis indicated strong loadings for $N$, and chlorophyll $a$ fluorescence yields $F_0/F_m$, $F_v/F_o$, and “area.” The second axis explained 22% of the variation and showed strong positive loadings for $RC$, “area,” $A$, $F$, and the thickness of the leaf. The negative side of the axis indicated loadings for $P_N$, $E$, $N$, and $ADH_{ref}$. In addition, PCA showed that both stock types formed compact homogeneous clusters clearly separated from each other in the multivariate leaf trait analysis, except for a single specimen from the micropropagated group of plants. This specimen was positioned in very close proximity to the cluster belonging to the grafts. Distributions of the micropropagated plants showed a lesser variation in the first PCA axis (which explained a major portion of the overall variation) and a greater variation in the second PCA axis. The grafts responded conversely, with a greater variation in the first PCA axis and a lesser variation in the second PCA axis.

**Summary**

The results presented here show that several leaf midrib vascular traits ($N, F, S$), MOE, cell wall deformation, as well as ecophysiological and anatomical traits (gas exchange, chlorophyll $a$ fluorescence, and thickness of leaf tissues) varied according to the technique used for clonal propagation of the dutch elm hybrid ‘Doodoens’. The effect of the rootstock might contribute to the differences in vascular traits, as well as to the differences in cell wall stiffness and cell wall deformation observed between the stock types. The higher values of both $WUE_{ins}$ and chlorophyll $a$ fluorescence found in the micropropagated plants indicate a higher acclimation capacity to stressful environmental conditions specifically for this stock type. Taken together, the micropropagated plants reached significantly higher values for nine traits (40.9%), including chlorophyll $a$ fluorescence yields and indicators of leaf vascular architecture such as $N$ and $F$. The grafts reached higher values for eight traits (36.4%), primarily associated with gas exchange, the thickness of the leaf, mesophyll, palisade and spongy parenchyma, as well as for vascular trait $S$. Similarities between the stock types were found for five traits (22.7%).

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