New Insights into Dutch Elm Disease: Cell Wall Compositional, Ecophysiological, Vascular and Nanomechanical Assessments

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Abstract

Comprehensive assessments were made of the chemical profiles of woody cell wall components, and leaf growth, ecophysiological, vascular and nanomechanical traits for two Dutch elm hybrids 'Groeneveld' and 'Dodoens' which possess contrasting tolerances toward Dutch elm disease. Upon infection with *Ophiostoma novo-ulmi* ssp. *americana* × *novo-ulmi*, medium-molecular weight macromolecules of cellulose were degraded in both hybrids. A loss of crystalline and non-crystalline cellulose regions occurred in parallel. In 'Groeneveld' plants, syringyl-rich lignin provided a far greater degree of protection from cellulose degradation, but only guaiacyl-rich lignin in 'Dodoens' plants was involved in a successful defence against the fungus. Unexpectedly, we found a very high proportion of non-significant differences between the infected and non-infected plants of 'Dodoens', including similarities in leaf growth, leaf gas exchange and leaf midrib vascular traits, as well as in the nanomechanical properties of the cell walls of tracheary elements such as modulus of elasticity, adhesion and energy dissipation. Three years after initial inoculations, except for a few traits such as leaf slenderness, relative chlorophyll content, transpiration rate and sap flow density in branches, we found no evidence of a decrease in leaf trait performances among the infected plants of 'Dodoens', despite the occasional persistence of fungal hyphae in the lumens of leaf midrib tracheary elements.

Keywords: cellulose degradation, nuclear magnetic resonance, modulus of elasticity, *Ophiostoma novo-ulmi*, syringyl to guaiacyl ratio in lignin, transpiration

Introduction

Fungal metabolites and Dutch elm disease

The pathogenic *Ophiostoma novo-ulmi* isolates spread within the secondary xylem vessels of infected trees, causing the formation of vessel plugs due to tyloses and gels (Ouellette et al. 2004), which ultimately results in foliar wilting and subsequent tree death (Newbanks et al. 1983). This wilt syndrome is apparently a result of interactions between fungal metabolites and the tree (Scheffer et al. 1987). The fungus produces hydrophobin cerato-ulmin (Temple et al. 1997), a phytotoxic peptidorhamnomannan (Strobel et al. 1978, Sticklen et al. 1991), tissue-invading structures which are thought to be involved in cavitation of the water column and alteration of parenchyma cells (Ouellette et al. 2004), and cell wall degrading enzymes such as glucanases, glucosidases (Przybył et al. 2006), xylanases, laccases (Binz and Canevascini 1996a,b), exo-glycanases and glycosidases (Svaldi and Elgersma 1982). Several anatomical parameters of wood have been found to be related to Dutch elm disease (DED) resistance, and their possible use in early screening has been outlined.

Plant interactions with pathogens are characterized by a deployment of chemical signals and highly coordinated reprogramming of metabolites (Allwood et al. 2006, Lloyd et al. 2011). Biochemical profiles of biomacromolecules and their metabolic fingerprinting provide rapid classification of plant samples according to their origin and physiological state. The ability to obtain biochemical profiles and fingerprints for elms would be useful in studies to characterize the mechanisms of resistance to DED, or to identify resistant elms. Changes in the levels of cell wall components of elm wood after inoculations with *O. novo-ulmi* isolates have been investigated for the most part by the Fourier transform-infrared spectroscopy to discriminate between resistant and susceptible elms, as well as to identify metabolic profiles related to host resistance (Martín et al. 2005, 2008).

Changes in the chemical profiles of cell wall components

Our recent experiments were aimed at the assessment of host responses and the metabolic profiles of wood components for two Dutch elm hybrids 'Groeneveld' (susceptible clone) and 'Dodoens' (tolerant clone) having contrasting survival strategies upon infection with the causative agent of the current DED, i.e., *O. novo-ulmi* ssp. *americana* × *novo-ulmi* (Figure 1). Upon artificial inoculations of ten-year-old plants, we found that medium-molecular weight macromolecules of cellulose were degraded by the fungal cellulolytic enzymes in both hybrids. Cellulose degradation resulted in the occurrence of secondary cell wall ruptures and cracks in the vessels but rarely in the fibres. ^{13}C magic angle spinning nuclear magnetic resonance spectra revealed that a loss of crystalline and non-crystalline cellulose regions occurred in parallel (Figure 2). Within the infected annual growth ring of 'Groeneveld' plants, a loss of the amorphous region reached 3.13% and a loss of the crystalline region reached 5.26%. Within the infected annual growth ring of 'Dodoens' plants, a loss of the amorphous region reached 5.26% and a loss of the crystalline region reached 26.09%. The rate of cellulose degradation was influenced by the syringyl to guaiacyl ratio in lignin. Syringyl-rich lignin in non-infected plants of 'Groeneveld' contained a 2.4 times higher proportion of methoxyl groups than guaiacyl-rich lignin in non-infected plants of 'Dodoens' (or a 1.4 times higher proportion of methoxyl groups for the infected plants of 'Groeneveld' than that for the infected plants of 'Dodoens'). Thus, the cellulose microfibrils of 'Groeneveld' were provided with a denser steric protection by methoxyl groups from the accessibility of the fungal cellulolytic enzymes than those of 'Dodoens'. This might be the reason why the medium-molecular weight macromolecules of cellulose of 'Groeneveld' were degraded to a lesser extent than those of 'Dodoens'. Both hybrids responded to the medium-molecular weight cellulose degradation with the biosynthesis of high-molecular weight macromolecules of cellulose, resulting in a significant increase in values for the cellulose degree

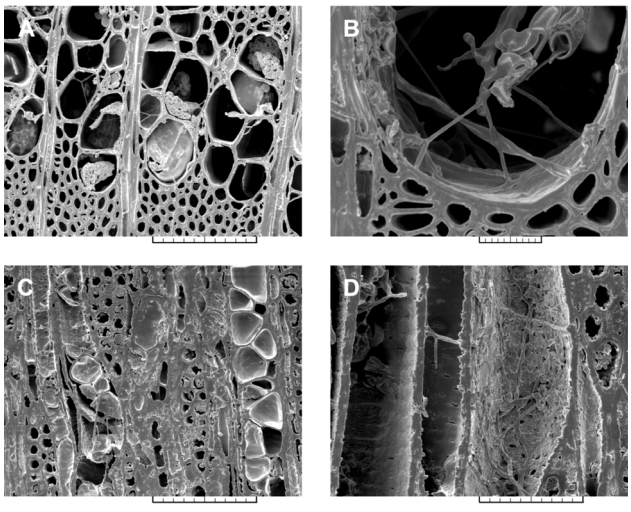

Figure 1. Scanning electron microscopy images of tylose formation **(A, C)** in response to the spread of *Ophiostoma novo-ulmi* ssp. *americana* × *novo-ulmi* hyphae within the secondary xylem vessels of 'Groeneveld' plants **(B, D)**. **A:** Cross-section, scale bar = 100 µm. **B:** Cross-section, scale bar = 20 µm. **C:** Tangential section, scale bar = 100 µm. **D:** Tangential section, scale bar = 50 µm

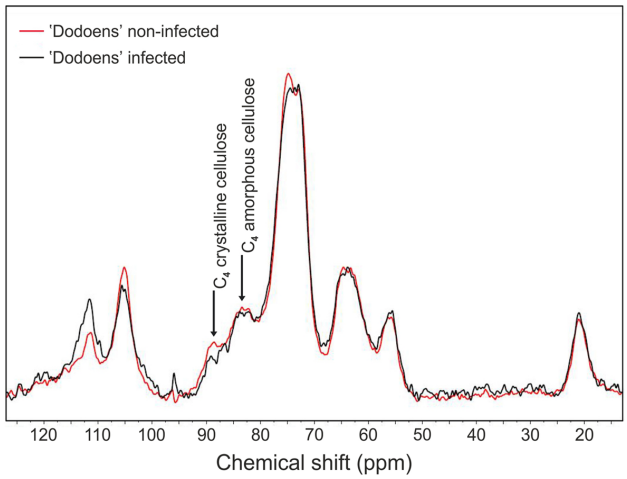

Figure 2. Solid-state ^{13}C magic angle spinning nuclear magnetic resonance spectra of extractives-free samples from the non-infected and infected plants of the Dutch elm hybrid 'Dodoens'. Signals at 83 ppm and 89 ppm correspond to C_4 carbon atoms of amorphous and crystalline cellulose, respectively. The decrease in signal intensities at both resonances reveals that losses in crystalline and non-crystalline cellulose regions have occurred in parallel. Figure is adopted from Ďurkovič et al. (2015)

of polymerization and polydispersity index. Other common responses included an increase in lignin content, a decrease in relative proportions of D-glucose, and an increase in proportions of D-xylose. Different responses between the Dutch elm hybrids were found in the syringyl to guaiacyl ratio in lignin. In 'Groeneveld' plants, syringyl-rich lignin provided a far greater degree of protection from cellulose degradation, but only guaiacyl-rich lignin in 'Dodoens' plants was involved in a successful defence against the fungus (Ďurkovič et al. 2014).

Leaf trait dissimilarities between 'Groeneveld' and 'Dodoens' plants

Strong dissimilarities in leaf trait performances were observed between non-infected plants of the examined Dutch elm hybrids 'Groeneveld' and 'Dodoens'. 'Dodoens' plants, tolerant to DED, had significantly higher values for leaf mass per area, leaf tissue thickness, tracheary element lumen area, relative hydraulic conductivity per unit area, gas exchange parameters such as net photosynthetic rate, stomatal conductance, transpiration and intercellular CO_2 concentration, as well as chlorophyll index and chlorophyll *a* fluorescence yields. 'Groeneveld' plants, susceptible to DED, had stiffer cell walls of primary xylem tracheary elements quantified by the nanomechanical modulus of elasticity, also a higher value for water-use efficiency and a lower leaf water potential. Taken together for both hybrids, the leaves with a large carbon and nutrient investment in leaf mass per area tended to have a greater leaf thickness and a higher net photosynthetic rate, but leaf mass per area (related to carbon economy) was independent of relative hydraulic conductivity per unit area (related to leaf water flux) (Ďurkovič et al. 2013).

Leaf trait similarities between infected and non-infected plants of 'Dodoens'

Three years following initial inoculations, strong similarities between infected and non-infected plants of 'Dodoens' were found among vascular traits, where all the examined traits (i.e., tracheary element lumen area, number of tracheary elements per unit area, tracheary element lumen fraction, tracheary element size to number ratio and relative hydraulic conductivity per unit area) showed non-significant differences. The proportion of trait similarities was also very high among leaf growth traits such as leaf dimensions, leaf area, leaf mass per area and leaf thickness, with the only exception being the infected plants of 'Dodoens' having more slender leaves. Although some differences were found among ecophysiological traits, the similarities were proportionally greater (60%). Previous studies have shown that DED fungus is able to colonize remote areas in the plant, such as the leaf midrib and secondary veins (Pomerleau and Mehran 1966, Nasmith et al. 2008). Therefore, we hypothesized that leaf trait performances of infected plants of 'Dodoens' related to gas exchange rates, chlorophyll *a* fluorescence yields, leaf growth traits, and nanomechanical properties of tracheary element cell walls would be significantly decreased. Unexpectedly, the infected plants had significantly higher values for chlorophyll *a* fluorescence parameters; however, these higher chlorophyll fluorescence yields did not have a direct impact on the more effective biological functioning of photosystem II in comparison with the non-infected plants. The reaction centres of photosystem II were intact functionally in both types of 'Dodoens', moreover, their F_v/F_m ratios were far higher than the threshold value of 0.725 that indicates the onset of reversible changes in reaction centres of photosystem II (Čaňová et al. 2012). The infected plants showed a significantly lower chlorophyll index, but again, no impact on the net photosynthetic rate was observed in comparison with the non-infected plants. In addition, tracheary element cell walls in the leaf midrib primary xylem also shared similarities between infected and non-infected plants of 'Dodoens' for the nanomechanical properties which were derived from the atomic force microscopy (AFM) PeakForce mapping measurements. Non-significant differences were found for modulus of elasticity, adhesion and energy dissipation of the cell walls. AFM imaging of the cell wall surfaces of tracheary elements in the leaf midrib primary xylem is presented in Figure 3. After the termination of leaf expansion, except for two traits (leaf slenderness and relative chlorophyll content), we found no evidence of a decrease in leaf trait per-

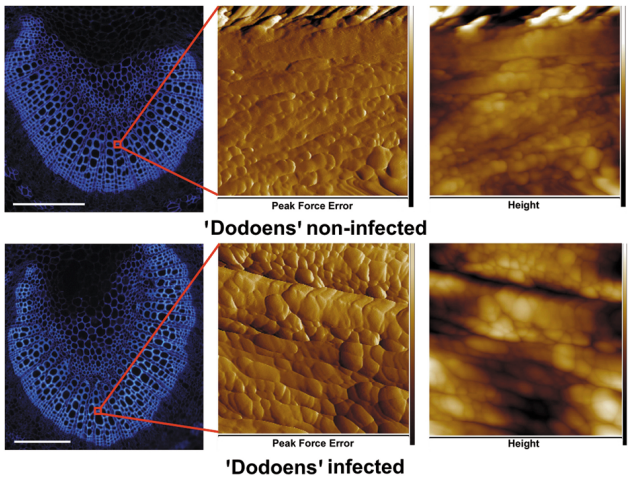

Figure 3. Lignin autofluorescence images of the primary xylem in the leaf midrib (left photos), AFM peak force error images (middle photos), and AFM flatten height images (right photos) of the cell wall surfaces of tracheary elements in the Dutch elm hybrid 'Dodoens'. Scale bars: 100 µm for fluorescence microscopy images, 1.7 µm for non-infected plant AFM images, and 1.6 µm for infected plant AFM images. Figure is adopted from Ďurkovič et al. (2013)

formances among infected plants of 'Dodoens', despite the occasional persistence of fungal hyphae in the lumens of leaf midrib tracheary elements (Ďurkovič et al. 2013).

Differences in water status of branches between infected and non-infected plants of 'Dodoens'

In the following experiment, carried out three years after the initial inoculations in the late summer, we found that infected plants of 'Dodoens' showed both a lower transpiration rate of leaves and a lower sap flow density in branches (Plichta et al. 2016). A lower transpiration rate was probably caused by the presence of fungal hyphae inside the vessels or tracheary elements, which lowered xylem hydraulic conductance. In order to maintain a physiologically normal transpiration rate, the water potential of infected plants would need to decrease below that of healthy individuals.The higher water potential gradients would lead to a higher water tension in xylem conduits and, therefore, to a higher likelihood of a cavitation event. However, leaf water potential was similar in both groups of plants, suggesting that the regulation of stomatal conductance was not impaired by the disease. Regardless of the fungal infection, 'Dodoens' leaves with a higher leaf mass per area ratio tended to have a higher leaf area-specific conductivity. Smaller leaves had an increased number of conduits with smaller diameters and thicker cell walls. Such a pattern could increase tolerance toward hydraulic dysfunction. Measurements of leaf water potential and theoretical xylem conductivity revealed that petiole anatomy could predict the maximal transpiration rate. Three years following fungal inoculation, phenotypic expressions for the majority of the examined leaf and branch traits revealed a constitutive nature for their possible role in DED tolerance of 'Dodoens' plants.

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