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ORIGINAL PAPER

Sap flow-based quantitative indication of progression of Dutch elm disease after inoculation with *Ophiostoma novo-ulmi*

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Abstract
Key message **The rate of progression of Dutch elm disease can be continuously and quantitatively estimated from sap flow measurements.**
Abstract Response of sap flow to inoculation with *Ophiostoma novo-ulmi*, a causal agent which causes vascular mycosis called Dutch elm disease, was studied in a field experiment comprised of 4-year-old wych elm trees (*Ulmus glabra*). Sap flow was measured on inoculated trees using the trunk heat balance method with external heating (EMS 62, Czech Republic) throughout the experiment. The first detectable symptoms of reduction in sap flow occurred 6 days after inoculation and all inoculated trees died within 16 days. Our experiment confirmed the ability of *O. novo-ulmi* to quickly kill young elm trees. The disease progressed faster than in previous experiments utilizing *O. ulmi*. To the best of our knowledge, this is the first experiment using sap flow measurements on trees inoculated by *O. novo-ulmi*. The trunk heat balance sap flow method is an effective non-invasive tool for continuous quantitative monitoring of the progression of vascular tree diseases, and show increased potential for field and greenhouse studies on changes in xylem hydraulic conductivity in a wide range of broadleaved and coniferous tree species.

Keywords Dutch elm disease · Sap flow occlusion · Elm dieback · Xylem dysfunction · Xylem hydraulic conductivity · Trunk heat balance method

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Introduction

Ophiostoma novo-ulmi Brasier (1991) is a causal agent which causes vascular mycosis called Dutch elm disease (DED). It is the most important disease of elm trees (*Ulmus* spp.) throughout the Northern hemisphere. DED was first observed in the early 1900s in the area of Northwestern Europe (Spierenburg 1921; Brasier 1991). DED was caused solely by *O. ulmi* (Buism.) Nannf. until the 1940s but this species lost its aggressiveness (Brasier 1988) and was replaced by a new form of pathogen. The newer, more aggressive form was divided into two races—an Eurasian (EAN), probably originating in the area of Moldavia and Ukraine, and a North American race (NAN), (Brasier 1979). The more aggressive form of *O. ulmi*, *O. novo-ulmi* was described by Brasier (1991) as a new species. Ten years later, Brasier and Kirk (2001) designated races EAN and NAN as subspecies; *Ophiostoma novo-ulmi* ssp. *novo-ulmi* (EAN) and *Ophiostoma novo-ulmi* ssp. *americana* (NAN).

The conidia of *Pesotum ulmi* (anamorph of *O. ulmi/novo-ulmi*), transmitted on the body surface and digestive tract of *Scolytidae* beetles, germinates in the trachea of the last tree ring of the twig and starts to grow within and against the direction of sap flow. Consequently, the xylem vessels are blocked up and sap flow declines. Many processes decrease the hydraulic conductivity in the xylem,

such as fungal mycelium (Fransen 1931), fungal toxins (Van Alfen and Turner 1975), xylem degrading enzymes and tissues that invade cavitation structures (Ouellette et al. 2004), and tyloses, the tree's built-in defense system. Therefore, sap flow measurements can provide real-time quantitative information on the progression of DED.

Sap flow measurements were used to continuously monitor blue stain fungi on conifers (Yamaoka et al. 1990; Kirisits and Offenthaler 2002; Rice and Langor 2008), but were never used to study DED. Field experiments are typically based on the visual symptoms like defoliation and tip die-back. The advantage of observational surveys is an opportunity to monitor large numbers of trees for a relatively low cost; however, they do not provide quantitative data. Because of the fast progression of DED, this type of study requires frequent visits to the forest. Still, observational data based on defoliation and tip die-back lag behind the actual loss of xylem hydraulic conductivity. Because of this, more studies measuring change in xylem hydraulic conductivity are required. Since direct measurements of hydraulic conductivity are destructive, transpiration or sap flow measurements are a valuable tool for indirect assessment. So far, all water flow-related studies were done on trees infested by the original species *Ophiostoma ulmi* (Roberts 1966, 1972; MacHardy and Beckman 1972; Van Alfen and Turner 1975; Roberts and Schreiber 1977) yet, to our best knowledge, no sap flow measurements were conducted on trees infested by *Ophiostoma novo-ulmi*. Furthermore, previous studies usually measured transpiration potometrically, thus mixing transpiration of the decaying crown and newly emerged base sprouts (Roberts 1966), or gasometrically under controlled conditions (MacHardy and Beckman 1972; Roberts 1972) while current studies focus on xylem hydraulic traits of resistant versus susceptible elm cultivars (Solla and Gil 2002; Solla et al. 2005; Ďurkovič et al. 2013; Venturas et al. 2013). Therefore, the goals of this study are to assess the validity of data collected from thermal-based sap flow measurements of trees with vascular diseases, such as DED, and specifically, to detect the propagation rate of DED using the sap flow measurements.

Materials and methods

Site description and plant material

The field experiment was performed in South Moravia (Czech Republic), 35 km south from Brno (GPS: 48°57′12.7″N, 16°36′52.1″E), 175 m a.s.l. The mean annual temperature at the site is 9.5 °C, and the annual precipitation was between 450 and 500 mm (Urban et al. 2012a). The plot was part of a forest stand signed 236C12a and belongs to the State forest enterprise Židlochovice. Four-year-old wych elms (*Ulmus glabra*) of Slovak origin (Slánské vrchy) were used as plant material. Trees were planted within the plot in March 2009 at 2 years of age. At the time of inoculation (2011) they were 3–4 m high.

Inoculation with fungi

Inoculation was done on 1 June 2011, approximately 3 weeks after full leaf development (Solla et al. 2005). Three trees were inoculated with three different strains of *O. novo-ulmi.* One of the strains (H327, Brasier and Kirk 2001) was used as a reference strain and the other two strains came from the current collection of the author (Table 1; Dvořák et al. 2009). The fourth tree was inoculated with distilled water and the fifth was the control. The inoculum consisted of a bud-cell suspension of the fungi grown in Tchernoff's medium (Tchernoff 1965), (Fig. 1d). Conidia were centrifuged to eliminate the medium and suspended in sterile distilled water (10^6 spores ml^{-1}). Two droplets of inoculum were introduced into the xylem by drawing them into the lower third of the main branch of the crown from the tip of a syringe while cutting transversally through the bark into the wood (Fig. 1b). Given the limited number of measured trees and similarities of DED progression speed in all pathotypes, the inoculated trees are considered one group.

Sap flow measurements

Sap flow was measured on five trees from 30 May to 15 July, 2011. We used the trunk heat balance method with external heating (SF 62, EMS Brno, Czech Republic, Cienciala and Lindroth 1995; Lindroth et al. 1995; Urban et al. 2012b) and maintained a constant temperature difference of 4 °C between the heated and the reference parts of the sensor. Sensors were installed on the inoculated trees above the points of inoculation (Fig. 1a, c). Data were collected every minute while 10 min averages were stored in the memory of the datalogger. During the data processing, sap flow was first expressed per unit of crown ground projected area to enable comparison with reference evapotranspiration (ETo). Daily sums of sap flow were then standardized on variation in atmospheric evapotranspirative demand by dividing by the grass reference evapotranspiration for a particular day (Allen et al. 1998).

Meteorological measurements

Actual meteorological variables were measured by a combined sensor for global radiation, air temperature and air humidity with built-in datalogger (Minikin RTH, EMS Brno, Czech Republic) placed at a clearance ca. 200 m

Table 1 Details of inoculum introduced into the measured elms

Isolate	Sp.	Subsp.	Source	Isolated by	Date	Reference
H327	*O. novo-ulmi*	*novo-ulmi*	Brezno (SK)	J. Jamnický	1979	Brasier and Kirk (2001)
MUAF1747	*O. novo-ulmi*	*americana*	Louny (CZ)	M. Dvořák	2008	Dvořák et al. (2009)
MUAF1744	*O. novo-ulmi*	*novo-ulmi*	Brno (CZ)	M. Dvořák	2008	Dvořák et al. (2009)

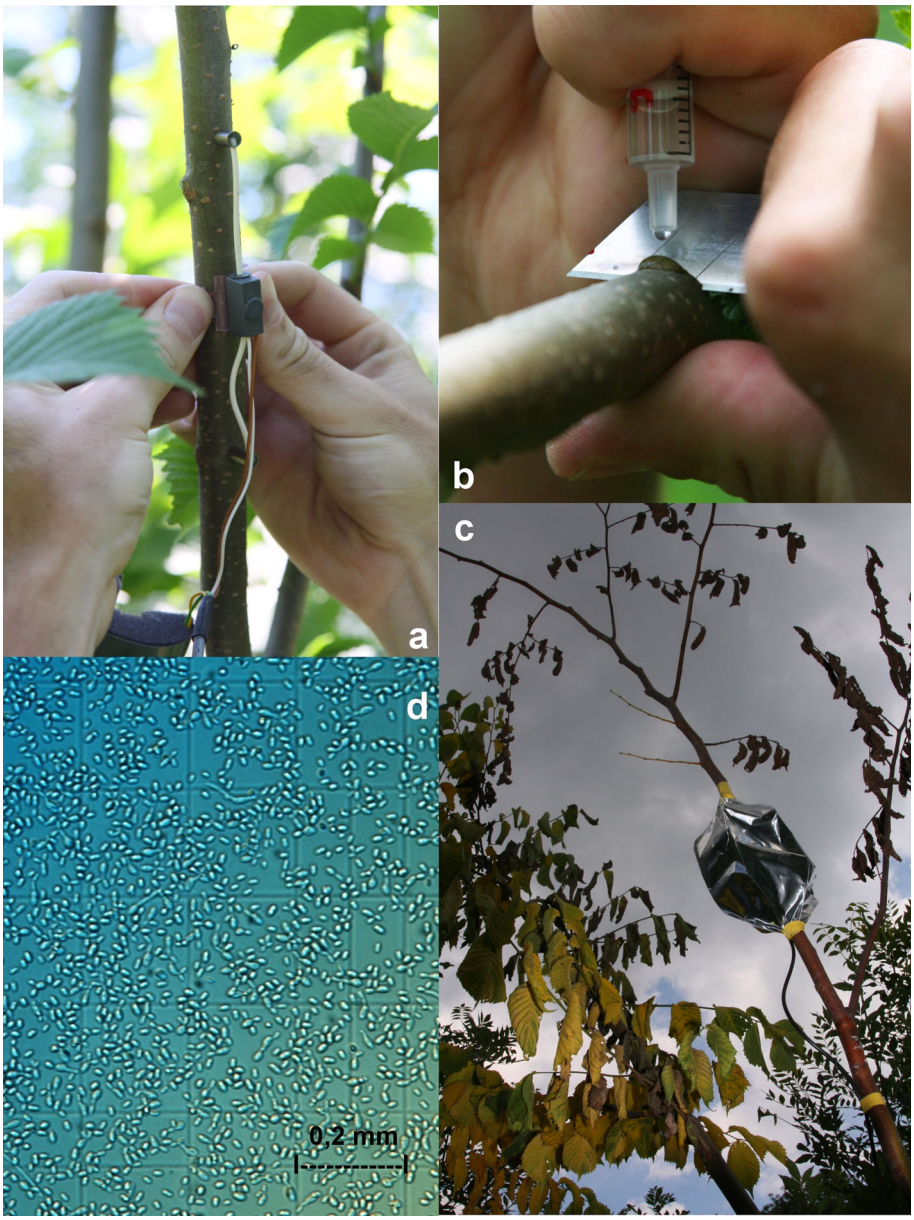

Fig. 1 **a** installation of the trunk heat balance sap flow sensors, **b** process of inoculation of mycelium to the sample tree, **c** view of the inoculated tree with installed sap flow sensor 4 weeks after inoculation, **d** suspension of spores in Tchernoffs medium

from the research plot. Reference evapotranspiration (ETo) was calculated from available meteorological data according to the Penman–Monteith FAO approach (Allen et al. 1998). Soil water potential was measured by gypsum blocks (Delmhorst Inc., Towaco, NJ, USA), depths of 20, 50 and 100 cm, and readings were recorded by SP3 datalogger (EMS Brno, Czech Republic). The sensors indicated high water availability for the entire period of the experiment (soil water potential as recorded never fell below −0.04 MPa in any depth).

Statistical evaluation of data

For the purpose of statistical analyses, all infested trees were used as one group regardless of the strain of inoculated *O. novo-ulmi*. A *t* test ($\alpha = 0.05$) was used to compare day ratios of sap flow (Q) to ETo between groups of inoculated and control trees. Regression lines were fitted to each of these groups through the least square approach. Linear regression was used to fit the group of control trees

$$y = a + b\frac{Q}{\mathrm{ETo}},$$

and three-parameter sigmoidal curve for the group of inoculated trees

$$y = \frac{a}{1 + e^{\frac{\frac{Q}{\mathrm{ETo}}}{b}-x_0}},$$

where Q was the day sum of sap flow per unit of ground area, ETo was reference evapotranspiration and a, b and x_0 were empirically derived coefficients. Respective 95 % confidence intervals were computed for each of the regression lines. These confidence intervals were later used to analyze timing of the onset of statistically significant differences between two regression curves and to find a day when sap with 95 % confidence levels ceased to flow in a group of inoculated trees. We always worked on a 95 % confidence level. SigmaPlot 12.3 software (Systat, Inc. USA) was used for the evaluations.

Results

No change in sap flow was visible during the first 6 days after inoculation (Fig. 2). The first significant deviation between regression lines characterizing groups of inoculated and control trees, respectively, occurred on the seventh day of the experiment. The first statistically significant difference between the group of infested trees and control trees as estimated by *t* test appeared 10 days after inoculation ($p = 0.04$). The reason for the difference between these two approaches was low evapotranspiration atmospheric demands and inclement weather in days 7–9 of the experiment (Fig. 2b). Finally, sap ceased to flow through the xylem of all infested trees no later than 16 days after

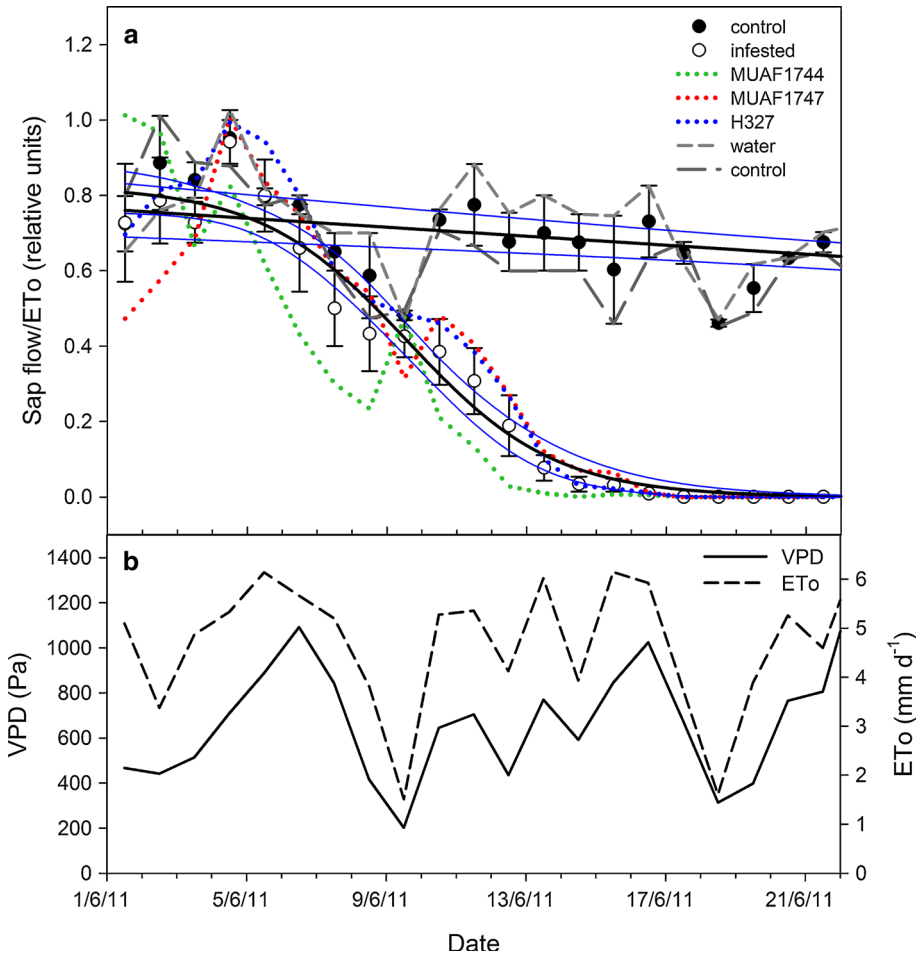

Fig. 2 **a** Sap flow relative to the reference evapotranspiration (ETo) in a group of inoculated (*open circles*) and reference (*full circles*) trees. Symbols are mean values ± standard error. Regression lines (*black solid lines*) with respective confidence intervals (*thin blue lines*) are fitted to the data from each group of the trees. Dotted lines show progress of the xylem occlusion in three trees inoculated with different strains of *Ophiostoma novo-ulmi* ssp. *novo-ulmi* (*green* and *blue line*) and ssp. *americana* (*green line*). Dashed lines show two control trees, one of them inoculated with water (*short-dashed line*) and the second one without any inoculation (*long-dashed line*). Trees were inoculated on June 1. **b** Vapor pressure deficit (VPD, *solid line*) and reference evapotranspiration (ETo, *dashed line*)

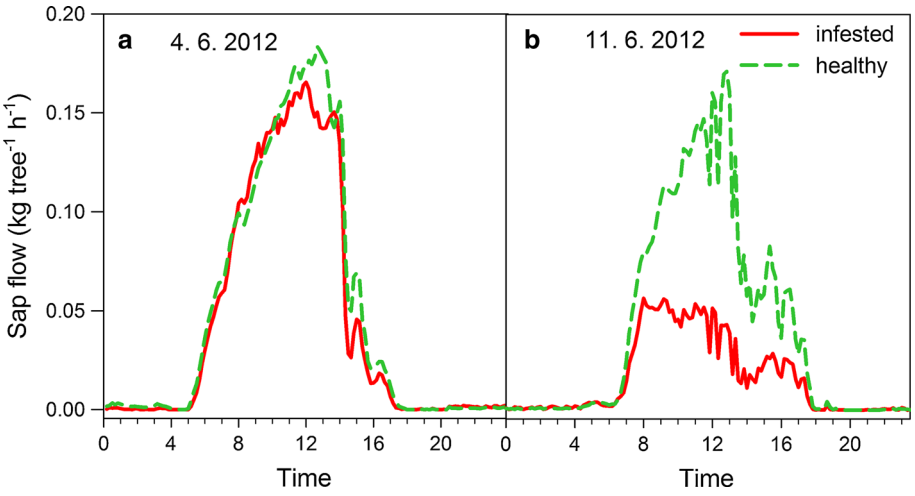

Fig. 3 Typical diurnal curves of sap flow in infested (*red*, *full line*) and control (*green*, *dashed line*) trees, 4 days (panel A) and 11 days (panel B) after inoculation

inoculation. Inoculation with water appeared to have no effect on the sap flow rates since no visible deviation was found between the control and the water-treated trees (Fig. 2).

The general progress of DED was very similar in all inoculated trees. All trees that were inoculated died within 14–16 days. No apparent difference in the speed of occlusion of sap flow occurred between trees inoculated with strains MUAF1747 and H327. The general progress of DED was about 2 days faster in a tree inoculated with strain MUAF1744, however, as only one tree was used for the treatment this result is preliminary (Fig. 2).

Each day sap began flowing at 5 am. Diurnal curves of sap flow were similar in groups of control and infested trees until the fourth day after inoculation (Fig. 3a). Later (i.e. 11 days after inoculation, Fig. 3b), the sap flow of infested trees increased until 8 am and then leveled off. No time lag in sap flow occurred between the groups of infested and control trees in any stage of experiment.

Discussion

The general progress of the disease in our experiment (16 days from inoculation to the total occlusion of sap flow) was much faster than what was reported in previous studies, i.e., 2 weeks to the start of decline and 6 weeks to eventual death (Roberts 1966) or 8–17 days to the reduction of transpiration (MacHardy and Beckman 1972). Time to total sap flow occlusion was also shorter than 1 month in Roberts and Schreibers' (1977) experiment, although their trees significantly reduced water uptake already on the fifth day after inoculation, which corresponded with our data. All previous studies were done with *O. ulmi* (although some studies suggest the possible spread of *O. novo-ulmi* already in late 70s) on *Ulmus americana*. Therefore, we may speculate on the faster progression of *O. novo-ulmi* in our experiment coming either from higher aggressiveness of *O. novo-ulmi* or increased susceptibility of *Ulmus glabra*. However, this hypothesis cannot be directly tested, since none of the trees investigated in the present study were inoculated by the original strain of *O. ulmi*. Another reason for the faster progression of the disease in the present study may be the higher susceptibility of saplings growing in the diverse environment of a field experiment than in controlled laboratory conditions of the previous experiments.

The low number of inoculated trees per *Ophiostoma* strain resulted in a limited possibility of statistical evaluation between differences in the speed of propagation among the strains. Therefore, we must regard the results of this experiment as preliminary. Regarding this limitation, reference clone H327 together with MUAF1747 may be the least aggressive (total occlusion of sap flow came on the 16th day after inoculation), while inoculation of strain MUAF1744 resulted in the total blockage of sap flow on the 12th day. Progression of the infestation was faster in this strain from the very beginning. Variability in the length of time to full sap flow occlusion was higher between individual strains of ssp. *novo-ulmi* than between the subspecies (i.e., ssp. *novo-ulmi* vs. *americana*). However, factors other than aggressiveness, like individual tree susceptibility or variability in cutting process of inoculation, may affect the speed of fungi development. Resolving this problem would require standardized (i.e., clonal) tree material and unified environmental conditions. Conversely trees in natural or managed forests are genotypically different and grow in highly variable environments. Therefore, this study may provide insight into the general behavior of trees and their pathogens under natural conditions.

Diurnal curves of sap flow in infested trees resemble trees subjected to water stress (Fig. 3b), which confirms the

hypothesis regarding occurrence of water stress in infected trees (Roberts 1966; MacHardy and Beckman 1972). The rapid increase in flow each morning was typical for control and experimental trees. However, all water available to experimental trees was depleted by 8 am, and xylem water flow reached the limit given by stem hydraulic conductance. Therefore, sap flow of infected trees stagnated or slightly decreased for the rest of the day. With stable environmental conditions (i.e., cloudless days), the time of deviation between diurnal curves of sap flow of infested and healthy trees may be an option to assess the degree of pathogen development.

Various methods are used for the monitoring of hydraulic conductance in the xylem of trees (Sack et al. 2002). While they are able to directly measure xylem conductance, their primary disadvantage is that they only provide spatially limited information at a specific time. Additionally, their use is laborious and time consuming. Methods used for sap flow measurements are not able to directly quantify changes in hydraulic conductance. However, since sap flow is a function of hydraulic conductance and differences in water potentials, changes in hydraulic conductivity can be estimated indirectly. Regarding the multitude of methods for sap flow measurements (i.e., dying, tracing of isotopes), thermal-based methods can provide continuous information about changes in the magnitude of xylem water flow. They also integrate the state of xylem hydraulic conductivity below and above the point of measurement, therefore the xylem does not need to be plugged exactly at the point of measurement. Previous assessments (Yamaoka et al. 1990; Kirisits and Offenthaler 2002) were generally positive about sap flow-based studies of fungi in conifers. But until now, no sap flow measurements were linked to the pathogens in broadleaved trees. This study proves that monitoring sap flow can also be used in ring-porous broadleaves and provide reliable information on the plugging of vessels of the wych elm tree by the *Ophiostoma novo-ulmi* and tyloses. Given the high accuracy in the wide range of flows (Urban et al. 2012b), trunk heat balance method enables continuous and accurate quantitative monitoring of the DED progression. Therefore, sap flow measurements can be an additional tool for the detailed continuous monitoring of growth of various ophiostomatoid fungi in trees with different xylem structure.

Conclusions

Sap flow measurements are a reliable indicator of the progression of Dutch elm disease in the xylem conduits. Measurements of water flow recognize the growth of *Ophiostoma* before the appearance of visual symptoms and provide quantitative information on the progression of Dutch elm disease. This method has potential to be used for indirect studies of changes in xylem hydraulic conductivity in various tree species.

Sap flow was totally occluded 16 days after inoculation of the fungi in this study. Results from this initial work suggest that progression of *Ophiostoma novo-ulmi* on *Ulmus glabra*, and consequent occlusion of sap flow, may be faster than previous research suggests for *Ophiostoma ulmi* on *Ulmus americana*.

Author contribution JU contributed to the sap flow measurements, evaluation of the sap flow data and wrote the greatest part of the manuscript. MD designed and performed the inoculation experiment, contributed to the field measurements of sap flow and completed general parts of the manuscript on DED.

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Conflict of interest The authors declare that they have no conflict of interest.

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