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Distribution and persistence of *Verticillium dahliae* in the xylem of Norway maple and European ash trees

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Abstract *Verticillium dahliae* colonizes the xylem vessels of susceptible host plants. Hence it can be expected that the distribution of the fungus as well as disease progress will be influenced by the anatomy of the xylem of that host. Here, we studied the spatial and temporal distribution of *V. dahliae* in relation to recovery from disease symptoms in young European ash (*Fraxinus excelsior*) and Norway maple (*Acer platanoides*) trees that differ in vascular anatomy. Quantifying the amount of *V. dahliae* DNA at different heights in the stem of inoculated trees at different time points after inoculation showed that, in the year of inoculation, the speed of colonization of these two species by *V. dahliae* was similar. Nevertheless, in the year after inoculation disease incidence and also quantities of *V. dahliae* detected in maple trees were significantly higher than in ash trees, suggesting that the xylem of ash trees is much less supportive for growth and survival of *V. dahliae* than that of maple trees. Moreover, in this second year *V. dahliae* could not be re-isolated from the wood of ash trees that had recovered from disease and was only rarely detected by PCR, only in xylem of the previous year and never in the current xylem. In contrast, *V. dahliae* easily was detected in the wood of diseased ash and maple trees. Furthermore, despite the presence of a layer of parenchyma cells between growth rings in ash trees, in symptomatic ash trees *V. dahliae* was present in the xylem of the new growth ring. We also observed that *V. dahliae* can move downward from the point of inoculation into the root collar, which possibly provides a way for infection of new growth rings by circumventing the physical barriers within the stem xylem.

Keywords *Acer platanoides* · *Fraxinus excelsior* · Recovery · Real-time PCR · Verticillium wilt

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Introduction

Verticillium wilt caused by the soil-borne fungus *Verticillium dahliae* Kleb., is a major disease worldwide that affects both herbaceous annuals and woody perennials (Pegg and Brady 2002; Smith et al. 1988). Shade tree nurseries and landscape plantings, especially in areas where field crops suffered from Verticillium wilt in the past, are agricultural settings that are often confronted with this disease (Hiemstra and Harris 1998; Riffle and Peterson 1989). Maples (*Acer* spp.) are probably the best known shade trees that are susceptible to Verticillium wilt (Gleason and Hartman 2001; Harris 1998; Townsend et al. 1990). *A. platanoides* (Norway maple) is an important shade tree species in Europe that highly susceptible to *V. dahliae* (Harris 1998; Piearce and Gibbs 1981). Ash trees (*Fraxinus* spp.) are widely cultivated

because of their high-quality timber (strong but elastic) and their ornamental value. Several species in this genus, and especially *F. excelsior*, are also highly susceptible to Verticillium wilt in nurseries as well as after being planted out in the landscape (Heffer and Regan 1996; Hiemstra 1998; Worf et al. 1994).

The disease cycle of *V. dahliae* on tree hosts was described in detail by Hiemstra (1998). Microsclerotia (resting structures) of *V. dahliae* in the soil are stimulated to germinate by exudates from nearby growing roots. Hyphae from germinating microsclerotia grow towards the roots, penetrate the root surface (Lockwood 1977; Nelson 1990; Schreiber and Green 1963) and grow inter- and intracellularly through the root cortex to reach the xylem vessels (Prieto et al. 2009). Once inside the vessels, the fungus starts to produce conidia that are spread throughout the infected tree with the flow of xylem fluid. At vessel ends or against protruding parts of vessel elements, conidia are trapped and may germinate. The new hyphae penetrate into adjacent vessel elements, produce conidia and the process continues, finally leading to systemic colonization of upper parts of infected plants (Baídez et al. 2007; Rodríguez-Jurado et al. 1993).

Fungal propagules and host defense products may block xylem vessels in infected plants. As a result, the water flow through the xylem is hampered and symptoms of water stress develop. Consequently, wilting, desiccation and defoliation are among the early symptoms of Verticillium wilt disease in trees. If the plugged vessels are not replaced rapidly, dieback of shoots, branches or even the whole tree may follow. Not all tree species show dieback to the same extent. Whereas ash is able to recover completely from Verticillium wilt (Hiemstra 1995b), serious dieback is generally observed in maple trees (Harris 1998; Piearce and Gibbs 1981).

The ability of tree species to recover from vascular infections has been related to the inherent structure of their xylem including vessel arrangement and the ability to produce new layers of healthy xylem tissue around diseased xylem (Banfield 1968; Emechebe et al. 1974; Sinclair et al. 1987; Tippett and Shigo 1981). Ash is a ring-porous tree with most of the water transport taking place in the xylem vessels of the most recent growth ring and a marginal parenchyma sheath between successive growth rings may act as a barrier zone, effectively separating the latewood vessels of one growth ring from the earlywood vessels of the next growth ring (Braun 1970; Grosser 1977; Schweingruber 1990). This implies that as long as infected ash trees are able to produce new uninfected xylem vessels every year they can substitute for the blocked vessels, which enables complete recovery, often even without dieback of the crown. In maple, which is a diffuse-porous tree, xylem vessels remain functional for several years spanning several consecutive growth rings. Therefore, loss of a major part of the water transport capacity in infected trees often cannot be sufficiently compensated by new growth rings. Consequently, these trees show much more dieback of the aerial parts and recovery, if it occurs, starts by regrowth from healthy parts of the stem base or roots (Hiemstra 1998).

De novo xylem formation as a mechanism to recover from Verticillium wilt implies the requirement to protect new xylem vessels from infection by the fungus that is already present in the tree. Compartmentalization resulting from the inherent structure of the wood, in combination with changes in anatomy and chemistry of xylem after infection, was suggested to play important roles in protecting trees against colonization by vascular pathogens (Bonsen et al. 1985; Manion 2003; Shigo 1984; Tippett and Shigo 1981; Smith 2006). However, although recovery from Verticillium wilt has been described not only for ash, but also for other tree species including almond and peach (Ciccarese et al. 1990), apricot (Taylor and Flentje 1968; Vigouroux and Castelain 1969), pistachio (Paplomatas and Elena 1998), cocoa (Emechebe et al. 1974), avocado (Latorre and Allende 1983), olive (López-Escudero and Blanco-López 2005), catalpa and sassafras (Kasson et al. 2015), there is little information about the fate of the fungus in infected trees in the years following the initial infection. Recently, Kasson et al. (2015) reported that *V. nonalfalfae* could be isolated from asymptomatic red and sugar maple several years after inoculation. Hence, studying the colonization and presence of *V. dahliae* in different tree species in the years following the initial infection would be important from the epidemiology viewpoint and for risk assessment purposes.

In this research we investigated (1) if the spread of *V. dahliae* varies between two tree species that differ in vascular anatomy, (2) if *V. dahliae* is still present in the xylem of a tree host at one year after infection, and (3) if recovery is correlated to containment of the pathogen in the xylem of the year of infection. To this end, we studied the spatial and temporal distribution of *V. dahliae* as well as recovery in stem-inoculated Norway maple and European ash trees. We monitored disease progress and quantified the amount of pathogen

that is present at different heights in the stem of inoculated trees during the year of inoculation and in the subsequent year. In the second year we also investigated the presence of the pathogen in the newly formed ring of stem xylem.

Materials and methods

Plant and fungal material Two-year-old seedlings of Norway maple (*Acer platanoides*) (79 trees) and ash (*Fraxinus excelsior*) (74 trees) were stem-inoculated on August (8th) 2013 with *V. dahliae* isolate Vd1 that originates from maple (collection of Applied Plant Research (PPO), Wageningen University and Research Center). The inoculum was prepared by adding small fragments from a potato dextrose agar (PDA) culture to liquid Czapek-Dox medium in Erlenmeyer flasks. The flasks were put in a shaker at 100 rpm at room temperature in the dark for about seven days to allow conidiospores to be produced. After passing through cheese cloth, the conidiospore suspension was centrifuged to remove growth medium, and the pellet resuspended in sterile water. The concentration of conidia was determined and diluted to 10^6 conidia/ml. This conidiospore suspension was used for inoculation of healthy ash and maple trees. To this end, a horizontal incision a few millimetres deep was made through the bark of the stem into the xylem with a snap-off cutter, around 30 cm above soil level. One-2 drops of 50–100 μl of conidial suspension were put on the cutter blade with a disposable transfer pipette, with the blade still inside the incision (Fig. 1). Within a few seconds the conidial suspension was drawn into the stem as a result of the low pressure potential within the xylem vessels. Additionally, 35 maple trees and 27 ash trees were not inoculated, as controls. For each species, 40 inoculated trees were kept for assessment of disease progression, and the remainder of the inoculated trees (39 maple trees and 34 ash trees) were used for quantification of the pathogen.

Fig. 1 Illustration of stem-inoculation of a maple seedling. With a snap-off cutter a horizontal incision of about 5 mm deep was made through the bark into the xylem of the stem. With the knife still inside the incision, 1–2 drops of 50–100 μl of a conidiospore suspension were put on the knife with a disposable transfer pipette. The conidiospore suspension was drawn into the stem within a few seconds as a result of the negative pressure potential within the xylem vessels

Sampling To monitor the upward and downward distribution of *V. dahliae* from the point of inoculation within the stem of inoculated trees, and also to examine changes in *V. dahliae* biomass in infected trees over time, 10 cm samples were taken at different heights from the stem of five individual trees at different time points: 0 days post inoculation (dpi; i.e. about an hour after inoculation), 10 dpi, 24 dpi and 60 dpi; as well as 8 months past inoculation (mpi), 11 mpi and 14 mpi. As the ash trees were shorter than the maple trees, we tested eight samples from ash trees (P1 = 5 cm below the soil level, P2 = 5 cm above the soil level, P3 = 5 cm below the inoculation point, and P4 to P8 = 5, 10, 20, 40 and 60 cm above the inoculation point, respectively) and ten samples from maple trees (P1-P8 as for ash trees and P9 and P10 at 80 and 100 cm above the inoculation point, respectively) (Fig. 2). These samples were used for quantification of *V. dahliae* DNA and for reisolation of the pathogen through plating.

To investigate movement of *V. dahliae* from the xylem of the growth ring of the year of inoculation into newly formed xylem vessels of the next year's growth

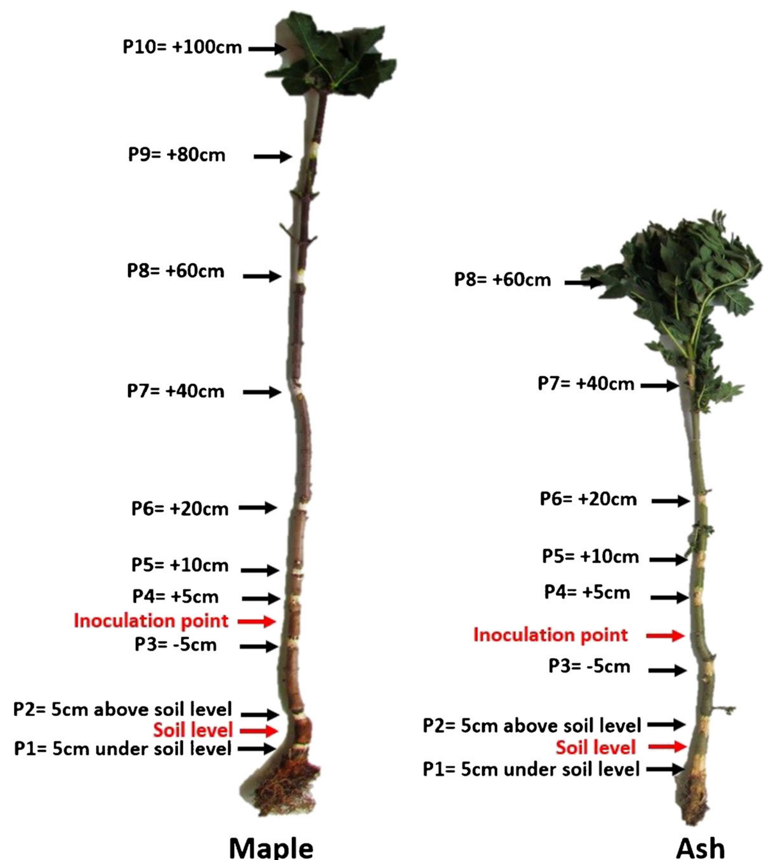

Fig. 2 Locations in the stem of inoculated trees sampled for detection and quantification of *V. dahliae* DNA shown on an uprooted maple (*left*) and ash (*right*) tree photographed with most leaves removed before taking samples. Positions of the soil level and inoculation point are indicated

ring, we analysed subsamples from the xylem of two successive years in both species by plating assays using samples collected at 11 mpi and 14 mpi. Presence of *V. dahliae* in the xylem of the growth ring of the year of inoculation as well as in that of the next year of ash (both recovered and symptomatic trees) and maple trees was further studied by real-time PCR at two time points in the year after inoculation (11 mpi and 14 mpi). To this end, xylem subsamples from both growth rings, separated by using scalpel and forceps under a binocular at three points above the inoculation point (P4, P6, P8 in maple trees, and P4, P5, P6 in ash trees) were examined.

DNA isolation Stem samples were first washed under running tap water for 1–2 min, dried with cleaning paper and left to dry for a few minutes on cleaning paper. The bark was removed under sterile conditions and small (2–5 mm) pieces of woody tissue (300–400 mg) removed by using a sterilized scalpel and transferred to a 2-ml tube containing 1 ml lysis buffer AP1 of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and 4–5 stainless steel beads (3.2 mm diameter, BioSpec, Bartlesville, US/Canada). The tubes were incubated for 15–30 min at 65 °C and then shaken in a Retsch® mixer mill (MM 400, Retsch, Haan, Germany) for 15 min at 30 Hz. After centrifugation at 10,000 rpm for 5 min, 400 μl of the suspension was used for total genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA wasquantified using a BioPhotometer (Eppendorf AG, Hamburg, Germany) and concentrations were equalized by adding elution buffer or DNase-free water.

DNA quantification Real-time PCR assays were performed using a *V. dahliae*-specific primer pair designed based on the internal transcribed spacer (ITS) region (Van Doorn et al. 2009) (VerDITSF: 5′-CCGGTCCATCAGTCTCTCTG-3′, VerDITSRk: 5′-CACACTACATATCGCGTTTCG-3′) and a primer pair for plant cytochrome oxidase (*COX*) (Weller et al. 2000) to quantify the amount of *V. dahliae* DNA and plant

DNA, respectively. All real-time PCR reactions were performed in a STRATAGENE Max 3000P™ real-time PCR machine (Agilent Technologies, Santa Clara, United States). The real-time PCR program consisted of an initial step of denaturation for 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 40 s at 62 °C, and 40 s at 72 °C. The quantities of *V. dahliae* and plant DNA were determined using a standard curve by plotting the logarithm of a ten-fold dilution series prepared from 10 ng/µl DNA suspension of *V. dahliae* isolate V117 (supplied by F. J. Lopez-Escudero of the Laboratory of Plant Pathology, Department of Agronomy, University of Córdoba, Spain), and a ten-fold dilution series prepared from 10 ng/µl plant (maple/ash) DNA suspension, respectively, against the threshold cycle (Ct) obtained in the real-time PCR assays. The relative quantity of *V. dahliae* DNA in the tested samples was calculated based on the quantity of *V. dahliae* DNA (ng) in 100 ng total DNA (i.e. including pathogen and plant DNA as quantified by simultaneously conducting plant-specific real-time PCR and pathogen-specific real-time PCR) isolated from inoculated plant tissues.

Disease assessment To monitor disease progress, 40 inoculated trees were selected randomly from the group of inoculated trees and severity of disease symptoms on these trees recorded at the day of inoculation (0 dpi = days post inoculation) and at the end of the growing season in the year of inoculation (60 dpi) and, after the dormant period in the winter from 2013 to 2014, at 11 mpi (mid-season; mpi = months post inoculation) and 14 mpi (end of the growing season) in the year after inoculation. Disease symptoms for each tree were rated on a scale from 0 to 4 based on the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis or dieback (0 = no symptoms; 1 = slight (<30%) foliar symptoms; 2 = severe foliar symptoms (>30%) with or without slight (<10%) dieback of top or shoot tips; 3 = severe dieback of top or shoot tips (>10%); 4 = dead plant) (scale modified from Hiemstra 1995a).

Reisolations To re-isolate *V. dahliae*, stem samples of 10 cm in length were first washed under running tap water. After drying, the bark was peeled off and chips from xylem of the two most recent growing years were taken and disinfected in 10% chloramine-T hydrate 98% for 1 min. Afterwards, wood chips were washed with sterile water for 30 s and dried on Whatman filter paper. Chips then were placed onto PDA and incubated at 24 °C in dark for 7 days.

Results

Disease incidence In this work, 79 maple trees and 74 ash trees were stem-inoculated with a *V. dahliae* conidiospore suspension to investigate the disease progression and distribution of the pathogen. To monitor disease progression, the severity of disease symptoms on 40 inoculated trees of each of the two species was recorded in a time course (0 dpi, 60 dpi, 11 mpi, 14 mpi). Two months after inoculation (i.e. at the end of the growing season) disease symptoms had developed in both species, although the percentage of diseased trees varied strongly. At this time point, 75% of the inoculated ash trees showed symptoms of Verticillium wilt, with 55% of the trees showing severe symptoms (Table 1B), whereas only 17.5% of the inoculated maple trees showed disease symptoms, with 5% displaying severe symptoms (Table 1A). Early in the following growing season (11 mpi), the disease incidence in ash trees was decreased strongly, with 70% of the trees being devoid of disease symptoms, whereas disease incidence in maple was strongly increased with only 35% of the trees remaining symptomless. During that second season, incidence and severity of disease increased again in both species, with ash being notably less affected than maple. At the end of the second growing season (14 mpi) 37.5% of the ash trees remained symptomless and 40% showed only slight leaf symptoms. In contrast, the disease index for maple trees had strongly increased by that time, resulting in 80% showing symptoms, including 30% dead trees (Fig. 3).

Upward movement of V. dahliae To investigate upward movement of *V. dahliae* within the stem of maple and ash trees, different heights of the inoculated stems were analysed in a time course by real-time PCR for presence of the pathogen. The results of the real-time PCR analysis of samples collected at different heights of the inoculated stems showed that in ash trees *V. dahliae* was already present at 5, 10 and 20 cm above the inoculation point on the day of inoculation, at 40 cm above the inoculation point at 10 days, at 60 cm above the point of inoculation at 24 days, and at the top of the stem, 80 cm above the point of inoculation at 60 days after inoculation (Fig. 4a). On the day of inoculation, *V. dahliae* was detected at 5 and 10 cm

Table 1 Number (#) and percentage (%) of diseased trees of ash (A) and maple (B) at different time points after inoculation with *V. dahliae*

A. Ash (*Fraxinus excelsior*)

Disease Index (DI)[a]	2013				2014			
	0 dpi[b] (9-Aug.)		60 dpi (14-Oct.)		11 mpi[c] (14-July)		14 mpi (23-Sep.)	
	#	%	#	%	#	%	#	%
0	40	100	10	25	28	70	15	37.5
1	0	0	8	20	7	17.5	16	40
2	0	0	22	55	2	S	4	10
3	0	0	0	0	1	2.5	2	5
4	0	0	0	0	2	5	3	7.5

B. Maple (*Acer platanoides*)

Disease Index (DI)	2013				2014			
	0 dpi (9-Aug.)		60 dpi (14-Oct.)		11 mpi (14-July)		14 mpi (23-Sep.)	
	#	%	#	%	#	%	#	%
0	40	100	33	82.5	14	35	8	20
1	0	0	5	12.5	9	22.5	7	17.5
2	0	0	2	5	4	10	8	20
3	0	0	0	0	6	15	5	12.5
4	0	0	0	0	7	17.5	12	30

[a] Disease symptoms of each tree were rated on a scale from 0 to 4 based on the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis or dieback (0 = no symptoms; 1 = slight (<30%) foliar symptoms; 2 = severe foliar symptoms (>30%) with or without slight (<10%) dieback of top or shoot tips; 3 = severe dieback of top or shoot tips (>10%); 4 = dead plant)

[b] dpi = days post inoculation

[c] mpi = months post inoculation

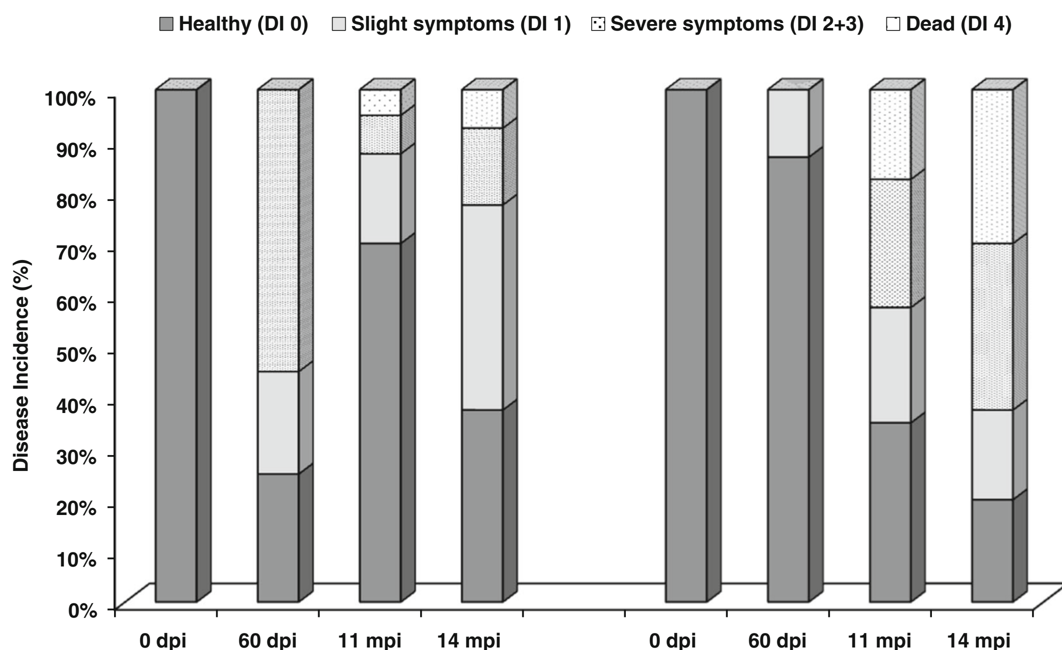

Fig. 3 Disease incidence in ash (*left*) and maple (*right*) at different time points. Disease index (DI) categories: trees without symptoms (DI 0), with slight symptoms (DI 1), severe symptoms (DI 2 + 3) and dead trees (DI 4). Dpi = days post inoculation, mpi = months post inoculation

above the inoculation point in maple, while at 10 days after inoculation the fungus was also detected at 20, 40 and 60 cm. At 24 days after inoculation *V. dahliae* DNA was detected at 80 cm and at 60 dpi the fungus was detected at 100 cm (i.e. in the top of the stem) (Fig. 4b). These results suggested that the speed of *V. dahliae* colonization in the inoculated ash and maple trees does not differ between the two species.

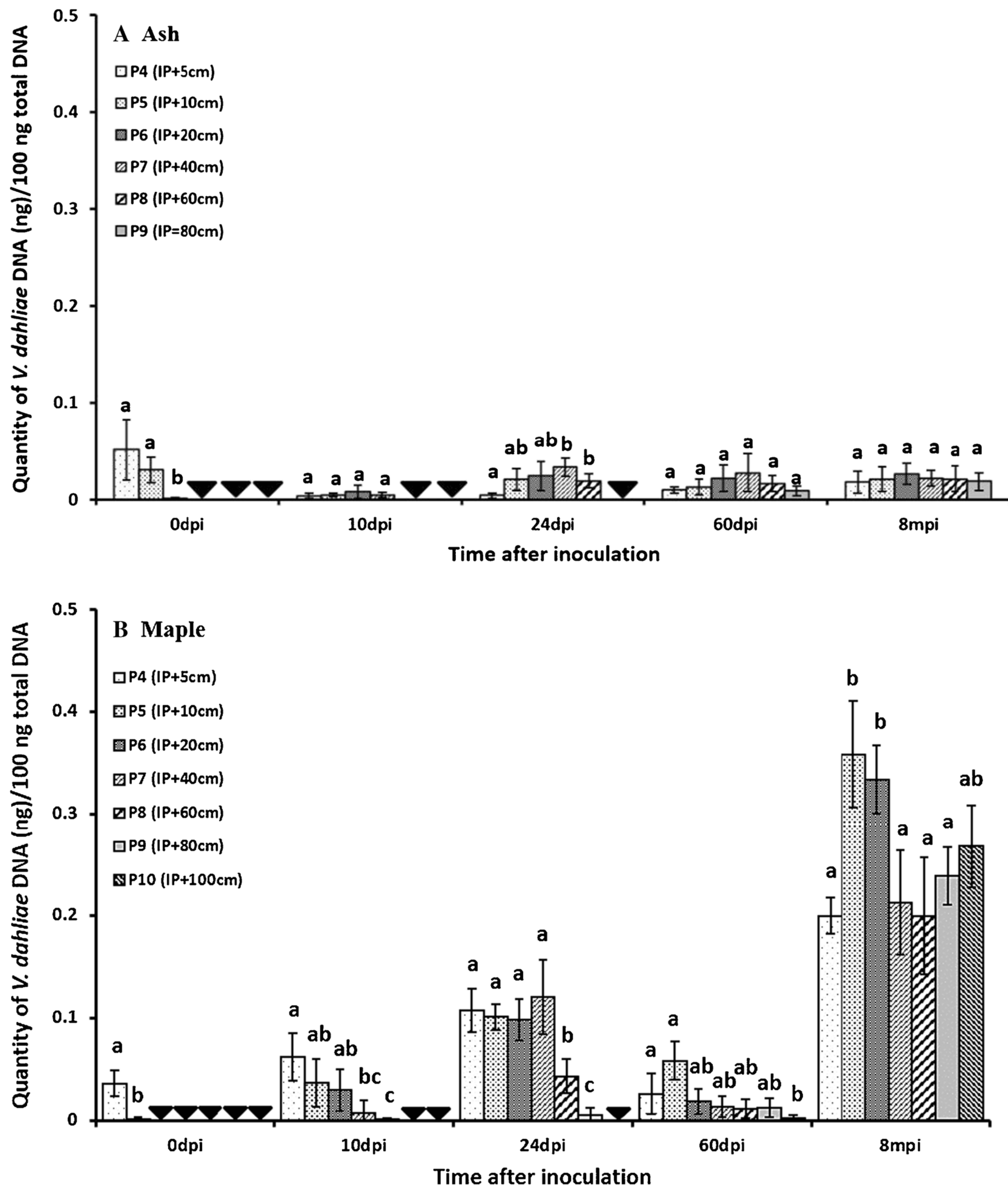

Fig. 4 Quantities of *V. dahliae* DNA detected at different heights above the inoculation point (IP) in the stem of inoculated ash (**a**) and maple trees (**b**). Assessments were conducted at 0, 10, 24, 60 dpi and 8 mpi. Each bar is the mean value of *V. dahliae* DNA quantities detected at corresponding stem positions in 5 trees. Error bars show standard errors. An inverted solid triangle (▼) indicates that *V. dahliae* DNA was not detected (threshold value 0.001 ng of DNA according to the standard curve). Significant differences in quantities of *V. dahliae* DNA detected in different stem positions at each time point are indicated by *different letters* above the bars (P = 0.05)

Downward movement of V. dahliae The potential for downward movement of *V. dahliae* after stem inoculation was studied by analysis of stem samples at three points below the inoculation point (Fig. 5). Directly after inoculation, high amounts of *V. dahliae* DNA were detected at 5 cm below the inoculation point in both species. At 10 dpi, *V. dahliae* DNA was detected also at 5 cm above the soil level in both species, while at 24 days after inoculation *V. dahliae* DNA was detected also at 5 cm below the soil level. Analysis of P1, P2, and P3 samples taken at 8 mpi (i.e. in the next growing year) showed that *V. dahliae* was still present at these three sites in both species.

Changes in biomass of V. dahliae in infected maple and ash trees over time In ash trees, from 10 dpi onward, the quantities of *V. dahliae* DNA detected at different heights in the stem were more or less at the same level with the differences between the quantities detected at different levels in the stem generally not being significant ($P = 0.05$) (Table 2A). In contrast, *V. dahliae* DNA quantities detected in maple varied much more in the year of inoculation the quantities detected at higher points (P8 at 10 dpi, P8 and P9 at 24 dpi, and P10 at 60 dpi) being significantly ($P = 0.05$) lower than the quantities detected at points closer to the inoculation site. However, in the year after inoculation (8 mpi), the amount of *V. dahliae* DNA in the top part (P10) did not differ significantly from the lower parts ($P = 0.05$) (Table 2B). Comparison of the mean *V. dahliae* DNA quantities in ash and maple trees at different time points, as determined by averaging the amounts detected at different heights in the stem of the examined trees, revealed that in the year of inoculation there was no significant difference between maple and ash trees at each of the time points tested, except at 24 dpi (Fig. 6; see also Table 2A and B, last lines). At this time point, the mean quantity of *V. dahliae* DNA in maple trees was significantly higher than that in ash trees ($P = 0.05$). However, from 8 mpi (start of the growing season in the year after inoculation) onward, the amounts of *V. dahliae* DNA in the stem of maple trees showed a significant increase when compared with the quantities detected at 0, 10, 24 and 60 dpi (in the year of inoculation), while quantities of *V. dahliae* DNA in the stem of ash trees did not increase ($P = 0.05$) (Table 2). Notably, from 8 mpi onward, quantities of *V. dahliae* DNA in ash trees were significantly lower than in maple trees ($P = 0.05$) (Fig. 6).

Presence of V. dahliae upon de novo xylem formation Four maple trees at each time point, as well as four ash trees at 11 mpi and six ash trees at 14 mpi, were examined. Two of the tested ash trees at each of the time points had shown clear disease symptoms in the year of inoculation but became symptomless in the year after inoculation (11 mpi/14 mpi). At each time point, one non-inoculated tree from each species was used as a control. *V. dahliae* was not found in symptomless ash trees based on plating assays, while it was recovered from most samples from old as well as new growth rings of symptomatic ash trees at both time points (11 mpi and 14 mpi) (Table 3A). In maple, *V. dahliae* was recovered from old and new growth rings of most of the tested trees at both time points, whereas the pathogen could not be recovered from two tested trees (tree 3 at 11 mpi and tree 1 at 14 mpi) (Table 3B).

In this assessment, 75% (27 out of 36) of the subsamples tested from six symptomatic ash trees at 11 mpi and 14 mpi contained *V. dahliae* DNA in the vessels of both years (Table 3A). In contrast, *V. dahliae* DNA was detected only in 25% (3 out of 12 samples) from four symptomless ash trees at 11 mpi and 14 mpi of the tested subsamples, and always in the xylem from the year of inoculation, never in the xylem of the new growth ring (Table 4A). In maple trees, *V. dahliae* DNA was detected in over 80% of all tested subsamples from new and old vessels of tested trees (Table 4B). Notably, *V. dahliae* was not detected in negative control samples from ash and maple trees when tested by real-time PCR or plating assays.

Discussion

Little is known about differences in the pattern of *V. dahliae* distribution in the stems of infected tree species that differ in anatomy of the xylem. As vascular pathogens like *V. dahliae* colonize their hosts through the xylem vessels, it can be expected that the speed and extent of colonization after a localized infection will be influenced not only by the direct interactions between the pathogen and the host (Yadeta and Thomma 2013) but also by the

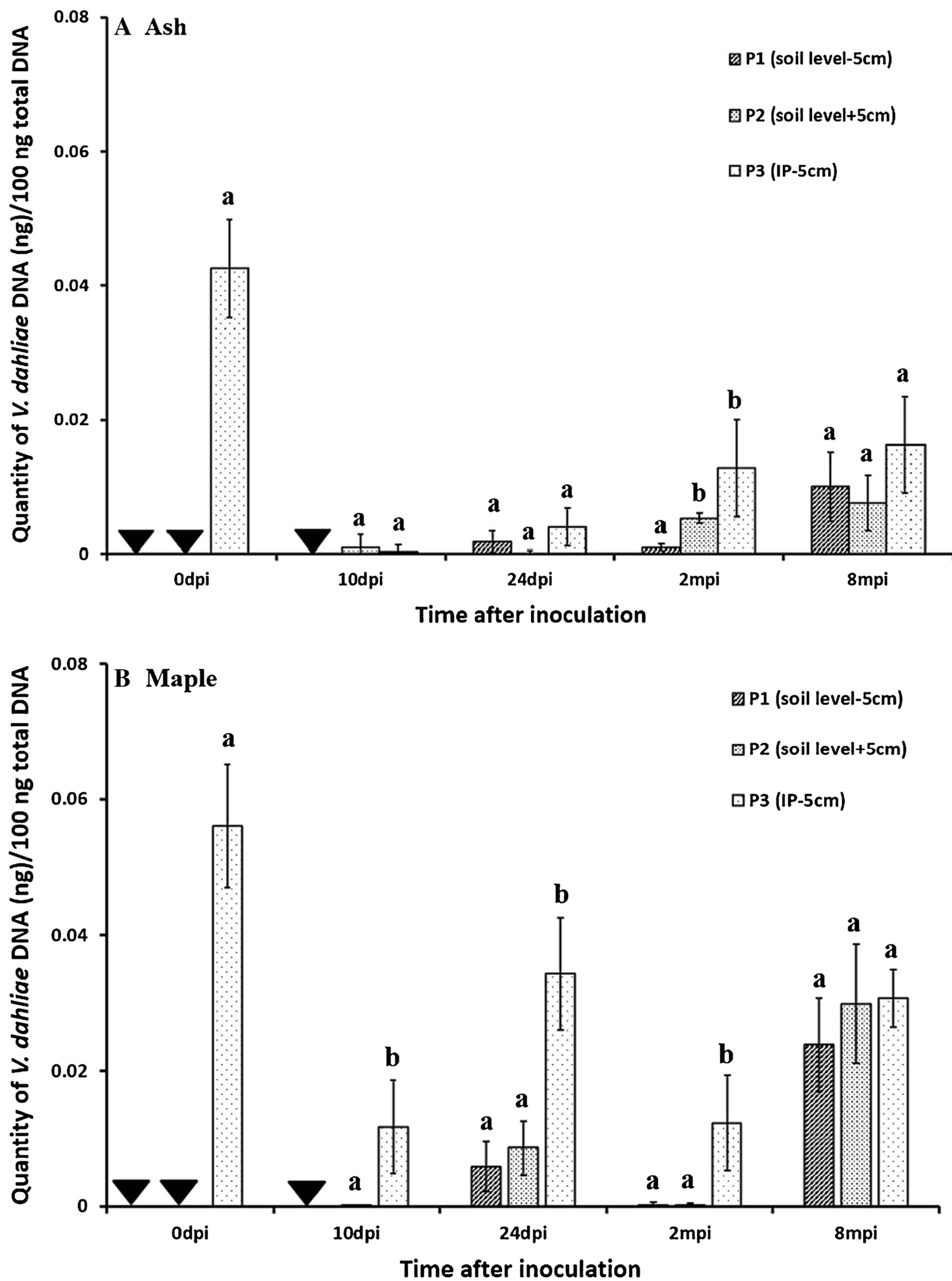

Fig. 5 Quantities of *V. dahliae* DNA detected at three points below the inoculation point (IP) in the stem of inoculated ash (**a**) and maple trees (**b**) at different time points (dpi = days post inoculation, mpi = months post inoculation). Each *bar* represent the mean of samples from 5 individual trees. *Error bars* show standard deviations. An inverted solid triangle (▼) indicates that *V. dahliae* DNA was not detected (threshold value 0.001 ng of DNA according to the standard curve). Significant differences in quantities of *V. dahliae* DNA detected in different stems positions at each time point are indicated by *different letters* added above the bars ($P = 0.05$)

Table 2 Quantities of *V. dahliae* DNA (ng) in 100 ng total DNA (including pathogen and plant DNA) detected in samples collected at different positions in the stem of inoculated ash (A) and maple trees (B) at different time points. Values represent means ± standard error for five trees at each time point. Significant differences in quantities of *V. dahliae* DNA detected in different stems positions at each time point have been indicated by different letters added to the values ($P = 0.05$)

A. Ash	Ash							
Position in stem	(cm)[1]	0 dpi[5]	10 dpi	24 dpi	60 dpi	8 mpi[6]	11 mpi	14 mpi
P12	+140	X[2]	x	x	x	x	x	x
P11	+120	x	x	x	x	x	x	x
P10	+100	x	x	x	x	x	x	x
P9	+80	x	x	x	0.0090 ± 0.0050^{a}	0.0185 ± 0.0041^{a}	x	x
P8	+60	ND[3]	ND	0.0190 ± 0.0075^{b}	0.0161 ± 0.0081^{a}	0.0211 ± 0.0070^{a}	x	x
P7	+40	ND	0.0048 ± 0.0017^{a}	0.0333 ± 0.0093^{b}	0.0274 ± 0.0135^{a}	0.0215 ± 0.0081^{a}	0.0658 ± 0.0122^{c}	0.0131 ± 0.0021^{b}
P6	+20	0.002 ± 0.0001^{b}	0.0077 ± 0.0047^{a}	0.0242 ± 0.0149^{ab}	0.0217 ± 0.0094^{a}	0.0204 ± 0.0109^{a}	0.0074 ± 0.0024^{b}	0.0077 ± 0.0010^{ab}
P5	+10	0.0304 ± 0.0134^{a}	0.0045 ± 0.0020^{a}	0.0205 ± 0.0112^{ab}	0.0126 ± 0.0044^{a}	0.0209 ± 0.0125^{a}	0.0061 ± 0.0007^{b}	0.0090 ± 0.0040^{ab}
P4	+5	0.0516 ± 0.0211^{a}	0.0039 ± 0.0018^{a}	0.0040 ± 0.0027^{a}	0.0099 ± 0.0031^{a}	0.0177 ± 0.0102^{a}	0.0002 ± 0.0001^{a}	$0.00240.0002^{a}$
IP								
P3	−5	0.0425 ± 0.0073^{a}	0.0004 ± 0.0001^{b}	0.0039 ± 0.0018^{a}	0.0128 ± 0.0072^{a}	0.0163 ± 0.0071^{a}	x	x
P2	+5	ND	0.0009 ± 0.0002^{b}	0.0002 ± 0.0001^{a}	0.0053 ± 0.0007^{b}	0.0075 ± 0.0041^{a}	0.0002 ± 0.0001^{a}	0.0018 ± 0.0003^{a}
SL								
P1	−5	ND	ND	0.0018 ± 0.0006^{a}	0.0010 ± 0.0004^{b}	0.0099 ± 0.0051^{a}	x	x
		0.0312 ± 0.0120^{Avr4}	$0.0037 \pm 0.0017^{Avr*}$	0.0133 ± 0.0081^{Avr}	0.0134 ± 0.0085^{Avr}	0.0171 ± 0.0051^{Avr}	0.0159 ± 0.0101^{Avr}	$0.0068 \pm 0.0040^{Avr*}$
B. Maple	Maple							
Position in stem	(cm)	0 dpi	10 dpi	24 dpi	60 dpi	8 mpi[7]	11 mpi	14 mpi
P12	+140	X	x	x	x	0.2355 ± 0.0266^{a}	x	x
P11	+120	x	x	x	x	0.03473 ± 0.0316^{b}	x	x
P10	+100	ND	ND	ND	0.0026 ± 0.0022^{b}	0.2676 ± 0.0404^{ab}	x	x
P9	+80	ND	ND	0.0055 ± 0.0007^{c}	0.0123 ± 0.0092^{ab}	0.2386 ± 0.0286^{a}	x	x
P8	+60	ND	0.0010 ± 0.0001^{c}	0.0431 ± 0.0167^{b}	0.0109 ± 0.0091^{ab}	0.1995 ± 0.0568^{a}	x	x
P7	+40	ND	0.0073 ± 0.0011^{bc}	0.1205 ± 0.0362^{a}	0.0134 ± 0.0101^{ab}	0.2124 ± 0.0509^{a}	0.1521 ± 0.0214^{b}	0.2508 ± 0.0395^{a}
P6	+20	ND	0.0296 ± 0.0203^{ab}	0.0983 ± 0.0204^{a}	0.0187 ± 0.0012^{ab}	0.3334 ± 0.0330^{b}	0.2152 ± 0.0239^{b}	0.2233 ± 0.0257^{a}
P5	+10	0.0009 ± 0.0002^{b}	0.0367 ± 0.0235^{ab}	0.1010 ± 0.0125^{a}	0.0579 ± 0.0186^{ab}	0.3579 ± 0.0520^{b}	0.1971 ± 0.0230^{b}	0.2676 ± 0.0231^{a}
P4	+5	0.0360 ± 0.0130^{a}	0.0616 ± 0.0231^{a}	0.1073 ± 0.0216^{a}	0.0259 ± 0.0193^{a}	0.1995 ± 0.0179^{a}	0.1403 ± 0.0024^{a}	0.2068 ± 0.0229^{a}
IP								
P3	−5	0.0560 ± 0.0090^{a}	0.0118 ± 0.0069^{ab}	0.0343 ± 0.0082^{b}	0.0123 ± 0.0071^{ab}	0.0307 ± 0.0041^{d}	x	x

Table 2 (continued)

P2	+5	ND	ND	0.0086 ± 0.0041[c]	0.0002 ± 0.0001[c]	0.0299 ± 0.0087[d]	0.0144 ± 0.0001[a]	0.1589 ± 0.0199[a]
SL								
P1	−5	ND	ND	0.0059 ± 0.0037[c]	0.0003 ± 0.0001[c]	0.0238 ± 0.0067[d]	x	x
		0.0310 ± 0.0128[Avr4]	0.0247 ± 0.0123[Avr*]	0.0583 ± 0.0281[Avr]	0.0193 ± 0.0107[Avr]	0.2353 ± 0.0736[Avr*]	0.1438 ± 0.048[Avr*]	0.2215 ± 0.0422[Avr*]

[1] Distances (cm) from the inoculation point (IP) (P3-P12) and from the soil level (SL) (P1 and P2)

[2] X = points that did not exist in tested trees

[3] ND = indicates positions in which *V. dahliae* was not detected

[4] Avr = averages of the *V. dahliae* DNA quantities as detected at *V. dahliae*-positive positions at each time point. Mean values of *V. dahliae* DNA quantities that are indicated with asterisks in Table A are significantly different ($P = 0.05$) from the mean values of *V. dahliae* DNA quantities as calculated for other time points in ash trees. Mean values of *V. dahliae* DNA quantities that are indicated with asterisks in Table B are significantly different ($P = 0.05$) from the mean values of *V. dahliae* DNA quantities as calculated for other time points in maple trees. In each table, mean values of *V. dahliae* DNA quantities that are indicated with asterisks are not significantly differing from each other

[5] dpi = days post inoculation

[6] mpi = months post inoculation

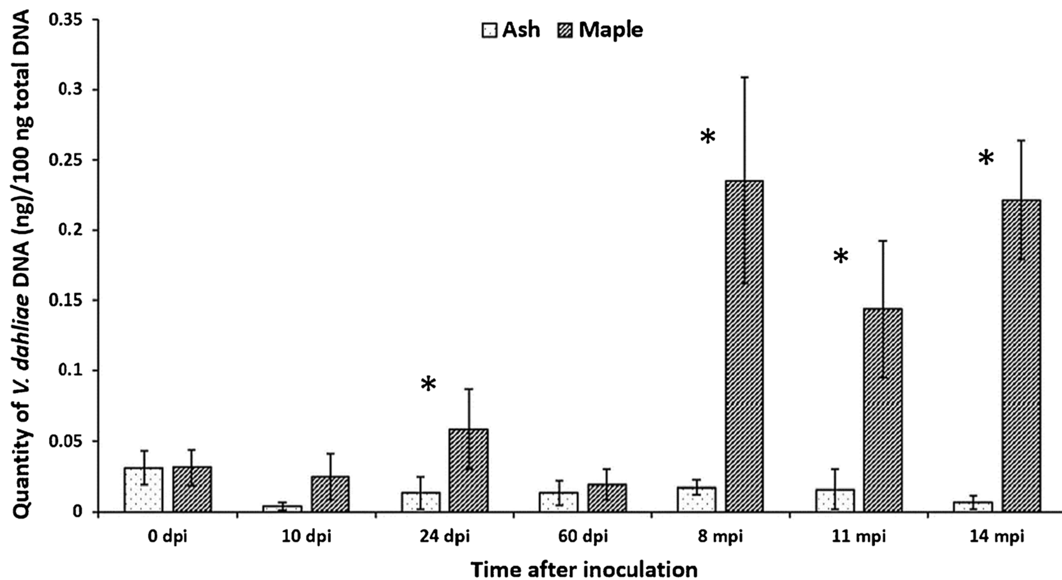

Fig. 6 Comparison between mean relative quantities of *V. dahliae* DNA detected in maple and ash trees at different time points after inoculation. Each *bar* is the mean value of *V. dahliae* DNA quantities as detected at different heights in the stem of five examined trees (see Table 2). *Asterisks* indicate significant differences in quantities of *V. dahliae* DNA detected in inoculated maple and ash trees at that time point ($P = 0.05$). Dpi = days post inoculation, mpi = months post inoculation

xylem anatomy of that host. Also, some tree species such as olive, cherry, apricot, peach, cacao, catalpa, sassafras, and ash are able to recover from Verticillium wilt; a capability in which the anatomy of the xylem is reported to play an important role (Banfield 1968; Emechebe et al. 1974; Hiemstra and Harris 1998; Sinclair et al. 1987; Tippett and Shigo 1981; Kasson et al. 2015). However, although Kasson et al. (2015) recently reported reisolation of *V. nonalfalfae* from the stem of red and sugar maple up to 4 years after inoculation, the fate of *V. dahliae* in recovered trees in the years following the initial infection is unknown. Notably, in naturally infected trees this aspect is difficult to investigate because every year new upward surges of the pathogen from infected roots are possible, as well as new infections from the soil. However, in stem-inoculated trees the infection essentially is a one-time event which makes it possible to investigate differences between tree species in their capacity to limit spread of the pathogen in the year of infection as well as in their capacity to contain the pathogen effectively and prevent it from spreading into newly formed tissues in the next year.

In this work, the spatial and temporal distribution of *V. dahliae* was investigated in relation to disease progression and recovery in stem-inoculated maple and ash trees, two species that differ strongly in vascular anatomy, with maple having a diffuse porous xylem anatomy, whereas ash has a ring porous xylem anatomy (Schweingruber et al. 2013). The main difference between these two types of xylem anatomy is that in ring porous species the xylem vessels that are formed early in the growth season have a much larger diameter (~ 2.5 to 3.5 times) than the vessels formed later in the season, whereas in diffuse porous species the diameter of the xylem vessels is more or less the same regardless of the position in the ring (Cochard and Tyree 1990; Core et al. 1979). Despite these innate differences in the anatomy of their xylem, the speed of *V. dahliae* colonization in the inoculated ash and maple trees did not really differ between the two species. This may be due to the inoculations being carried out relatively late in the season and the cut into the stem being only few millimeters deep into the xylem. As a result, the conidia were likely introduced mainly in the vessels of the outer part of the growth ring; in the case of ash in the smaller sized latewood vessels. Additionally, the plants of both species were rather young, when shoot vessel dimensions are usually smaller than in mature stems (Zimmermann 1983). The latter aspect may also explain the decrease in speed of colonization towards the top of the maple plants.

Directly after inoculation the pathogen was detected up to 10 cm both upward and downward from the inoculation site in inoculated stems of both species. As there was no time for hyphal growth, this must result from the conidial suspension being drawn into the severed vessels as a result of the low pressure potential within those vessels which is

Table 3 Re-isolation of *V. dahliae* from new and old xylem of both recovered and symptomatic ash trees (A) and from symptomatic maple trees (B) in the year after inoculation (11 mpi, 14 mpi)

A. Ash	11 mpi[e]										14 mpi													
	Recovered trees				Symptomatic trees				Control		Recovered trees				Symptomatic trees								Control	
Tested trees	Tree 1 (49)[a]		Tree 2 (26)		Tree 3 (94)		Tree 4 (16)		Tree 5 (3)		Tree 1 (19)		Tree 2 (67)		Tree 3 (95)		Tree 4 (100)		Tree 5 (17)		Tree 6 (46)		Tree 7 (81)	
Growth ring	Old[b]	New[c]	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New
Number of chips placed on agar	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15
Number of Vd-Positive chips	-[d]	-	-	-	6	2	1	1	-	-	-	-	-	-	1	-	-	-	7	2	2	2	-	-
B. Maple	11 mpi										14 mpi													
Tested trees	Tree 1 (193)		Tree 2 (131)		Tree 3 (190)		Tree 4 (154)		Tree 5 (168) Control		Tree 1 (102)		Tree 2 (148)		Tree 3 (145)		Tree 4 (132)		Tree 5 (187) Control					
Growth ring	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New	Old	New				
Number of chips placed on agar	10	20	10	20	10	15	10	15	10	15	10	15	10	15	10	15	10	15	10	15				
Number of Vd-Positive chips	1	10	4	14	-	-	-	15	-	-	-	-	1	14	-	13	10	6	-	-				

[a] Between brackets are the identification numbers of the trees from which the chips originated

[b] Old = growth ring of the year of inoculation

[c] New = growth ring of the year after inoculation

[d] - = *V. dahliae* was not recovered

[e] mpi = months post inoculation

a normal feature of functioning xylem vessels (Zimmermann 1983). In the first ten days after inoculation, the fungus moved at least 30 cm upward in ash and even 50 cm in maple, corresponding to 3–5 cm per day. From day 10 on the speed of colonization in maple decreased (Table 2), but was still well over the maximum growth rate of *V. dahliae* hyphae of about 8 mm/day (ElSharawy et al. 2015; Rampersad 2010). These results confirm the important role of conidiospore transport in the sap stream in the xylem of infected trees.

Hiemstra and Harris (1998) reported that ash trees may recover from Verticillium wilt, whereas maple trees usually show progressive dieback of the aerial parts. There was a strong tendency to recovery of the stem-inoculated ash trees, despite the rapid occurrence of disease symptoms in the first year. At the end of the year after inoculation, the percentage of seriously affected trees was much lower than at the end of the year of inoculation. In contrast, disease in maple trees developed much more slowly in the year of inoculation, but showed a strong increase in the second year. Notably, the difference in disease incidence in maple and ash trees correlated with a difference in quantities of *V. dahliae* DNA detected in these two species in the year after inoculation (Fig. 6). Moreover, in the year after inoculation we were not able to detect or recover the pathogen in new xylem and only rarely in old xylem of recovered ash trees (Tables 3A and 4A). Similar results were observed for olive trees infected with *V. dahliae*, where reduction in symptoms was associated with a decrease in *V. dahliae* DNA in newly developed asymptomatic shoots (Markakis et al. 2009; Mercado-Blanco et al. 2003). Therefore, it appears that recovery correlated with the inactivation of the fungus in the xylem and impeding new infections (Hiemstra 1995a, 1995b; Rodríguez-Jurado et al. 1993; Sinclair et al. 1981; Talboys 1968; Wilhelm and Taylor 1965). It has been reported that *V. dahliae* can be inactivated by high air temperature or other non-favourable environmental conditions in the field (Wilhelm and Taylor Bruce 1965; Taylor and Flentje 1968) or by antimicrobial phenolic components produced by the host (Baídez et al. 2007; Markakis et al. 2010). Based on our data it can be ruled out that the remission of symptoms in ash is caused by unfavorable environmental factors for the pathogen because the maple trees in the same field showed a steady increase in symptoms over the same two year period in which a number of the diseased ash trees recovered. Thus, recovery of the ash trees must be the result of inherent characteristics of (the xylem of) this species including anatomical characteristics as well as

Table 4 Quantities of *V. dahliae* DNA (ng) in 100 ng of total DNA (including pathogen and plant DNA) as detected in successive growth rings of ash (A) and maple (B) trees in the year after inoculation (11 mpi, 14 mpi) by real-time PCR. In each species samples from three different positions in the stem (P4, P5 and P6 in ash and P4, P6 and P8 in maple trees) were examined

A. Ash		11 mpi[e]					14 mpi						
		Recovered		Symptomatic		Control	Recovered		Symptomatic				Control
Position in stem	Growth ring	Tree 1 (49)[c]	Tree 2 (26)	Tree 3 (94)	Tree 4 (16)	Tree 5 (3)	Tree 1 (19)	Tree 2 (67)	Tree 3 (95)	Tree 4 (100)	Tree 5 (17)	Tree 6 (46)	Tree 7 (81)
P4	New[a]	ND[d]	ND	0.0243	0.0106	ND	ND	ND	ND	0.0013	ND	ND	ND
	Old[b]	ND	ND	0.0285	0.0224	ND	0.0029	ND	0.0099	0.0091	0.0136	0.0081	ND
P5	New	ND	ND	ND	0.0019	ND	ND	ND	ND	ND	0.0020	0.0026	ND
	Old	ND	0.0015	0.0199	0.0216	ND	ND	ND	0.0372	0.0019	0.0068	0.0098	ND
P6	New	ND	ND	ND	0.0035	ND	ND	ND	0.0018	ND	ND	ND	ND
	Old	0.0050	ND	ND	0.0235	ND	ND	ND	0.0006	0.0058	0.0177	0.0232	ND
B. Maple		11 mpi					14 mpi						
Position in stem	Growth ring	Tree 1 (193)	Tree 2 (131)	Tree 3 (190)	Tree 4 (154)	Tree 5 (168) Control	Tree 1 (102)	Tree 2 (148)	Tree 3 (145)	Tree 4 (132)	Tree 5 (187) Control		
P4	New	ND	ND	ND	0.2555	ND	ND	0.5568	0.0213	0.5605	ND		
	Old	ND	ND	0.0086	0.3316	ND	0.0093	0.0168	0.1929	0.2969	ND		
P5	New	ND	0.3641	0.0005	0.0514	ND	ND	0.7419	0.2209	0.5661	ND		
	Old	0.2935	0.5135	0.0113	0.1876	ND	0.0059	0.0916	0.2875	0.2273	ND		
P6	New	0.1148	0.5956	ND	0.0624	ND	0.0068	0.7965	0.2433	0.2428	ND		
	Old	0.3225	0.2415	0.0168	0.0094	ND	ND	0.0147	0.3845	0.0981	ND		

[a] Old = growth ring of the year of inoculation

[b] New = growth ring of the year after inoculation

[c] Between brackets are the numbers of the tree from which the chips originated

[d] ND = indicates positions in which *V. dahliae* was not detected

[e] mpi = months past inoculation

physiological aspects and responses to the presence of the pathogen.

In the present study, *V. dahliae* DNA was detected in both successive xylem sheaths of maple and symptomatic ash trees. Moreover, the pathogen could also be re-isolated from both xylem sheaths. This demonstrates that the fungus can still be present and alive in the xylem of a tree one year after infection. For ash it also shows that, despite the presence of a layer of parenchyma cells between two growth rings which is supposed to restrict penetration of pathogens from the xylem of one growth ring to the xylem of the growth ring of next year (Braun 1970), infection of the new xylem layer did occur. One explanation is that *V. dahliae* is able to penetrate through this layer of parenchyma. Another explanation, however, could be that infection of xylem of the new growth year in infected ash trees occurs from the root area where the percentage of vessels per unit area is much higher than in the stem and branch wood (Banfield 1968). To this end, downward movement of the pathogen within infected xylem vessels toward roots would be required. Indeed, in this work we did observe that *V. dahliae* can spread downwards in the stem of both species and with considerable speed (Table 2), as pathogen DNA was detected at 5 cm under the soil level of the main stem of stem-inoculated ash trees at 24 days after inoculation. Consequently, it is possible that infection of new xylem vessels of ash trees occurred from the root area. The fact that *V. dahliae* can move downward in infected trees implies that spread through root grafts to neighbouring trees as reported in *Ailanthus altissima* stands infected by *V. nonalfalfae* (O'Neal and Davis 2015) may be a significant aspect in Verticillium wilts of other trees as well.

Summarizing, it can be concluded that differences in the xylem anatomy of ash and maple did not significantly affect the speed and extent of the upward spread of the pathogen in stem-inoculated trees. Furthermore, despite the presence of a layer of marginal parenchyma cells between the growth rings in ash trees, infection of the new xylem layer did occur in the year after inoculation. Nevertheless, this transition to the new growth ring was not observed in recovered ash trees, while in recovered ash trees proliferation of the pathogen was also impeded suggesting that ash xylem is much less supportive for the growth of *V. dahliae* than maple xylem. Further studies are necessary to uncover the mechanisms responsible for the reduction of the presence of the pathogen in recovered trees. In this it should be kept in mind that in the present study the trees were stem-inoculated whereas natural infections take place through the root and consequently additional defence mechanisms will be involved. We also observed a rapid downward movement of the pathogen from the point of inoculation into the root collar. This may provide a way for infection of the xylem of the new growth ring by circumventing the mechanical barriers in the stem xylem. Moreover, in addition to the inoculum from infected leaves falling from diseased ash trees (Rijkers et al. 1992), it may provide new inoculum (from infected roots) for contamination of the soil or neighbouring trees through root grafts.

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Compliance with ethical standards

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

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