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Verticillium wilt in nursery trees: damage thresholds, spatial and temporal aspects

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Abstract Verticillium wilt can cause high losses in tree nurseries. To be able to predict disease and unravel disease dynamics over time and space, the relationship between verticillium wilt and soil inoculum densities of *Verticillium dahliae* and the nematode *Pratylenchus fallax* was studied in two 4-year field experiments with *Acer platanoides* and *Catalpa bignonioides* in the Netherlands. Best-fit regression equations showed that pre-planting inoculum densities of *V. dahliae* can be used to predict verticillium wilt over a period of at least 4 years. *Pratylenchus fallax* contributed significantly to disease severity in *A. platanoides* in some years. Disease can already occur at the detection limit of the pathogens. The 5% infection thresholds for *V. dahliae* were at 1 (*A. platanoides*) vs. 3 (*C. bignonioides*) colony-forming units (CFU) g^{-1} soil. Analysis of spatial relationships indicated that diseased plants had a higher influence on neighbouring plants at low *V. dahliae* inoculum densities (<5 CFU g^{-1} soil) than at high densities (≥5 CFU g^{-1} soil). Seventy-four percent of the diseased plants recovered during the following year. After that year, recovered plants had a significantly higher probability of becoming diseased again than plants that were healthy during the two previous years, at high inoculum densities of *V. dahliae*, indicating that inoculum density in the soil, rather than incomplete recovery, was the most important factor for disease development.

Keywords Disease incidence · Disease prediction · Isolation · Root lesion nematode · Semivariogram

Abbreviations
AUDPC Area under the disease progress curve

Introduction

Verticillium wilt, caused by the soil-borne fungus *V. dahliae* Kleb., occurs at damaging levels in numerous

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crops in many regions worldwide. As well as herbaceous plants, a large number of economically important woody species are susceptible, such as olive, avocado, cocoa, pistachio, stone fruits, and many ornamental tree species (Hiemstra and Harris 1998; Pegg and Brady 2002). In herbaceous plants like potato, verticillium wilt may result in a relatively small amount of yield loss. In tree nurseries however, wilt of trees implies a complete loss of value. Therefore, damage thresholds are low compared to those in other crops. In highly infested tree nurseries, crop losses up to 50% have been reported due to verticillium wilt (Anonymous 1990). The problem has been severe for a few decades, especially in tree nurseries located in areas where susceptible field crops were grown in the past (Hiemstra and Harris 1998). Interactions of *V. dahliae* with plant pathogenic nematodes have been observed, e.g., in potato (Martin et al. 1982; Wheeler et al. 1994), and sugar maple (*A. saccharum*) (Dwinell and Sinclair 1967). These nematodes often lower the damage threshold, and interactions have to be taken into account for reliable disease prediction at low *V. dahliae* inoculum densities.

In many countries, control of *V. dahliae* by chemicals is restricted to certain crops or application frequency (Duniway 2002) or forbidden entirely (Mus and Huygen 1992), because of potential environmental pollution and health risks. Alternative control methods, such as soil steaming in greenhouses (Corsten et al. 2002), soil solarization in areas with a Mediterranean climate (Katan et al. 1976), and biological soil disinfestation in temperate zones (Blok et al. 2000), can reduce the initial inoculum densities, but these methods are costly. To decide whether these expensive management practices are warranted, reliable disease prediction is needed. However, for nursery trees, detailed knowledge on disease prediction for verticillium wilt is currently lacking.

Disease prediction is possible by assessment of pre-planting inoculum densities of *V. dahliae*. Good relationships have been observed between soil inoculum densities and disease incidence in olive (López-Escudero and Blanco-López 2007), cauliflower (Xiao and Subbarao 1998), cotton (Paplomatas et al. 1992), potato (Nicot and Rouse 1987), and strawberry (Harris and Yang 1996). Inoculum levels of less than 1 colony-forming unit (CFU) g^{-1} soil can cause 5% (strawberry), 3–10% (cotton), or 5–15% (olive) disease incidence (Harris and Yang 1996; López-Escudero and Blanco-López 2007; Paplomatas et al. 1992). At inoculum levels of 5 CFU g^{-1} soil, disease incidence in cotton and potato can be 60–100% (Nicot and Rouse 1987; Paplomatas et al. 1992), in olive 50% (López-Escudero and Blanco-López 2007), and in cauliflower 16–20% (Xiao and Subbarao 1998). However, the harvested product (*i.e.* the quality of the harvested fruits or tubers etc.) might be relatively unaffected (Paplomatas et al. 1992; Xiao and Subbarao 1998). In ornamental trees, 10% diseased trees have been observed at soil inoculum densities of 5 CFU g^{-1} soil (Goud et al. 2004). However, large differences exist among ornamental tree species, and generally there is a lack of quantitative information about inoculum density—disease incidence relationships and damage thresholds.

Woody plant species have the ability to recover from disease over time (Hiemstra and Harris 1998). Disease is overcome by confinement of the fungus to infected xylem vessels, and the formation of new, uninfected xylem tissue (Shigo 1984). Even when severe die-back occurs, surviving plants may re-grow from the trunk base. Especially ring-porous tree species, *i.e.* hardwoods with an abrupt change in pore size between early-wood, showing large pores, and late-wood, showing small pores (Core et al. 1976), such as *C. bignonioides*, are known to be able to recover from disease without severe die-back. This is because they depend mainly on the xylem in the current annual ring (*i.e.* the part of the xylem that is replaced each year) for water and nutrient transport (Hiemstra and Harris 1998; Shigo 1984). However, recovery is irrelevant for tree nursery growers, since diseased trees (and symptomless infected or recovered trees) cannot be sold to customers. Nevertheless, recovery is an important aspect of verticillium wilt in established trees in gardens and orchards, e.g. of olive trees (López-Escudero and Blanco-López 2005) and for pathogen assessment (Markakis et al. 2009).

In practice, *V. dahliae* populations frequently occur aggregated through a field (Xiao et al. 1997) and this will affect the distribution of disease incidence. When the inoculum is aggregated in several highly infested spots, only a limited number of plants may be exposed to *V. dahliae*, depending on the number of spots and, e.g. root length of plants. Aggregation of nematode populations may contribute to the clumped distribution of verticillium wilt. In perennial crops, root infections in 1 year may cause local build-up of

inoculum and thus increase the probability of disease in the next year, also in neighbouring plants. The inoculum formed in leaves that drop and spread by wind spreads *V. dahliae* also further away from the diseased trees (Rijkers et al. 1992).

The objectives of this study were: (1) to establish inoculum density—disease severity relationships and damage thresholds for young Norway maple and southern catalpa trees, taking nematode populations into account; (2) to establish the spatial pattern of verticillium wilt and its effect on the inoculum density—disease severity relationship, (3) to investigate the probability of recovered trees becoming diseased again during following years, and (4) to investigate the probability of spread of *V. dahliae* from diseased and recovered trees, and from residues of rogued trees to neighbouring trees. Therefore we followed disease development in 20 plots each at two locations for *A. platanoides* and *C. bignonioides* for a period of 4 years.

Materials and methods

Plot history

Two field experiments were performed, at Wageningen (experimental farm of Wageningen University, the Netherlands) and at Meterik (experimental farm 'Meterikse Veld', the Netherlands). In Wageningen, the soil was naturally infested and in Meterik, the soil was artificially infested with microsclerotia of *V. dahliae* obtained from dried and milled potato stems (Goud et al. 2004). Each field experiment consisted of 20 plots with different levels of *V. dahliae*. The two sets of 20 plots originated from two biological soil disinfestation experiments (Blok et al. 2000) At Wageningen, the experiment was performed in 1997 and at Meterik in 1998. Data on these experiments have been published previously (Goud et al. 2004). In short, at both locations, a 2×2 factorial field experiment in a randomized complete block design with five replications was conducted. Experimental factors were (1) the incorporation of Italian ryegrass (*Lolium multiflorum* Lam.) into the top 25–35 cm soil layer (0 or 40–54 t ha^{-1}), and (2) covering the field with nearly-airtight plastic (yes or no) (Klerks Plastic Industrie, Noordwijkerhout, the Netherlands). After the treatments the soil was left fallow till the next spring. Plots measured 8×8 m (Wageningen) or 7.5×10.5 m (Meterik). Different inoculum levels resulted from this soil disinfestation experiment, so that these plots could be used for the experiment described here to investigate the relationship between *V. dahliae* soil inoculum density and wilt in young trees during the following 4 years. The biological soil disinfestation treatments did not affect conduciveness to verticillium wilt (Goud et al. 2004). In Wageningen, the root lesion nematode *P. fallax* was present at various inoculum densities in the 20 plots (Goud et al. 2004). The nematode was identified after isolation from plant roots (Seinhorst 1977). At Meterik, plant pathogenic nematodes were not present in the soil, presumably because of two consecutive croppings of *Tagetes* sp. in the years prior to the experiment.

Layout of field experiments

At the start of the current experiment, at Wageningen in 1998 and at Meterik in 1999, each plot was planted with 60 seedlings of *A. platanoides* L. (Norway maple) and 60 seedlings of *C. bignonioides* Walt. (southern catalpa). To avoid border effects, only the inner area of each plot, measuring 5×5 m (Wageningen) or 4.5×7.5 m (Meterik) was sampled and planted with experimental trees. The outer area of each plot was planted with guard trees of the same species.

Assessment of soil inoculum levels of *V. dahliae*

To determine pre-planting inoculum levels of *V. dahliae*, 50 soil cores (0 to 25 cm deep) were collected per plot, in a V and inverse-V-pattern. Soil samples from a plot were thoroughly mixed and stored at 4°C until processed. A subsample of the soil was air-dried at room temperature, dry-sieved through a 2-mm sieve, and 12.5 g of the air-dry soil was subsequently wet-sieved through 106- and 20-μm nested sieves. The fraction retained on the 20-μm sieve was suspended in 50 ml of 0.08% water agar, and 0.8 ml of this suspension was plated on modified soil extract agar (MSEA), a semi-selective medium (Harris et al. 1993) containing 50 ppm oxytetracycline and soil extract prepared from a sandy garden soil. After incubation at 20°C for 4 weeks, colonies were counted and CFU g^{-1} dry soil calculated. Post-planting soil inoculum levels of *V. dahliae* were assessed similarly each spring, except that samples were collected within the planted rows, at approximately

15 cm distance from the base of the trees. Soil samples were taken separately for Norway maple and catalpa, except in 1999 at Wageningen, where soil samples from the two plant species were pooled per plot.

Assessment of soil inoculum levels of *P. fallax*

At Wageningen, soil inoculum densities of *P. fallax* nematodes were assessed in the soil samples that were kept at 4°C and used for assessment of soil inoculum levels of *V. dahliae*. A 200-g subsample was washed in an Oostenbrink elutriator (Oostenbrink 1960), and the nematodes and lighter soil particles were caught on four 45-µm sieves and washed onto a filter (Milac cotton sandwich, diam. 22 cm; Hygia/Hartmann, Nijmegen, the Netherlands). The filter was placed in a saucer filled with water, so that the filter was just below water level. The filter was left undisturbed for 24 h, which allowed *P. fallax* to crawl through the filter and be caught on the bottom of the saucer. In a subsample under the microscope, nematodes were identified morphologically and counted.

In Wageningen, the density of *P. fallax* nematodes was also measured in roots (Oostenbrink 1960) during 1998 and 1999, in trees that were removed (Goud et al. 2004). This was done because pre-planting soil inoculum densities of *P. fallax* were low and nematode populations are known to be able to increase rapidly to significant numbers in susceptible plant roots (Goud et al. 2004). During the first growing season, the number of *P. fallax* nematodes per gram of root was determined on a sample of roots pooled from five plants arbitrarily chosen from the row that was removed. A 50-g (fresh weight) subsample was used by cutting the clean roots into approximately 1-cm pieces, placing them in a mist incubator, collecting the nematodes in the run-off for 7 days, and counting them under the microscope (Oostenbrink 1960). During the second season, *P. fallax* was assessed in roots of four arbitrarily selected plants, which were harvested on the same day and analyzed separately using the procedure described above, but with 20 to 30 g (fresh weight) of roots per plant.

Plant origin and maintenance

Seeds of Norway maple were obtained from Boevé BV (Boskoop, the Netherlands) for the Wageningen experiment, and germinated seeds for the Meterik experiment were obtained from the municipal plant nursery of Wageningen (Wageningen, the Netherlands). Seeds of catalpa were obtained from Boevé BV for both locations. Plants were raised in potting soil in the greenhouse for 10 weeks and transplanted into the field 7 months after the soil treatments. In Wageningen, each plot was planted with three rows of 20 Norway maple seedlings (eight to ten leaves) and three rows of 20 catalpa seedlings (six to eight leaves). At planting, rows were 33 cm apart and the plants were spaced at 25-cm intervals. Each set of three experimental rows was surrounded by a guard row of the same species. After the first growing season, the middle row was harvested to provide growing space for the remaining plants and for assessment of infection. After the second growing season, a second row was removed.

In Meterik, 60 seedlings of each species were planted into each plot in two rows (50 cm apart) of 30 plants. One row was harvested after the first growing season, and plants were thinned alternately in the second row after the second growing season. After the shoot length measurements in 2000 at Meterik and Wageningen, and in 2001 at Meterik, the tops of the plants were cut at 1.80 m (unless they were smaller) with a pruning knife to achieve a higher and more profitable width/height ratio. This is common practice by growers to prevent trees from becoming too slender. Plants were fertilized twice with 75 kg of N ha^{-1} (50% NO_3/50% NH_4) and once with 100 kg Mg ha^{-1} (MgO) during each growing season. Weeding was done mechanically or by hand at regular intervals. Norway maple plants were sprayed periodically with fungicides to control mildew (three times with Rubigan, 20 ml per 100 l of water [Dow Elanco, Wilrijk, Belgium]; twice with Meltatox, 250 ml per 100 l [BASF, Arnhem, the Netherlands]; and three times with Baycor Flow, 60 ml per 100 l [Bayer, Mijdrecht, the Netherlands]).

Wilt symptoms

Disease severity was rated monthly during summer and autumn for 4 years on each plant using a 0 to 5 scale, where 0 = healthy, 1 = up to 5% leaves showing wilt, 2 = 6 to 40% wilt or defoliation, 3 = 41 to 60% wilt or defoliation, 4 = 61 to 80% wilt or defoliation, and 5 = more than 80% wilt or defoliation or plant dead). During winter, it is not possible to observe wilt symptoms because of natural defoliation due to low temperatures. AUDPC values (Campbell and Madden

1990) were calculated for each individual plant, based on disease severity sores, and averaged per plot for each plant species for each growing season. Plants that died during 1 year were included in the disease assessment of that year, but not in disease assessments of following years. Dead trunks of trees that died back during 1 year and re-grew from the base during the following years were not included in the disease ratings of the following years.

Recovery from disease

Recovery was rated based on the presence or absence of disease during successive growing seasons. A plant was considered healthy when it received a maximum disease score of 0 or 1 during a particular growing season, and it was considered diseased when it received a maximum disease score of 2 or higher. A plant was considered as having recovered from disease when it was diseased in one growing season and healthy during the next growing season.

Infection with *V. dahliae*

After the first growing season in Wageningen, the incidence of infection with *V. dahliae* was assessed on all 20 plants of each plot of each plant species that were removed. Plants were harvested per block. Blocks 1 to 5 were harvested in 1998 on 9 September, 25 September, 19 October, 29 October, and 10 November, 1998, respectively. Infection was confirmed by surface sterilizing the base of the main trunk in 1% sodium hypochlorite for 1 min, washing in sterile water, aseptically removing the bark, cutting discs with a pruning knife, and placing five to seven discs onto MSEA (Harris et al. 1993). After incubation for 4 weeks at 20°C, presence of *V. dahliae* was determined. In Meterik, confirmation of infection by plating was sought only incidentally, when symptoms were unclear.

Inoculum density—disease relationships

Data were analysed with SAS version 8.0 (SAS Institute Inc., Cary, NC). The GLM procedure was used to calculate regression equations for AUDPC values or percentages of diseased or infected plants, in relation to *V. dahliae* inoculum densities in the soil and *P. fallax* inoculum densities in the soil or the roots. Soil inoculum densities of *V. dahliae* and *P. fallax* and numbers of *P. fallax* per plant root were $\log(x+1)$-transformed and proportions of infected, diseased and healthy plants were arcsine-root-transformed, where appropriate.

Recovery

The FREQ procedure of SAS was used to perform Chi-square tests of numbers of diseased and healthy plants with different disease histories. Numbers of plants assessed in the third and fourth experimental years with the same disease history were pooled per location and tree species (i.e. plants diseased in year 1—healthy in year 2—assessed in year 3 were pooled with plants healthy or diseased in year 1—diseased in year 2—healthy in year 3—assessed in year 4) . The REPEATED statement in the GENMOD procedure of SAS was used to perform a weighted regression of probability of disease on pre-planting inoculum density of *V. dahliae*, using the numbers of plants as weighing factors. During this analysis, the regression of numbers of diseased and healthy plants after recovery (diseased–healthy–*diseased* or diseased–healthy–*healthy*) on *V. dahliae* inoculum density was compared with the regression of numbers of diseased and healthy plants that were healthy during the two preceding years (healthy–healthy–diseased or healthy–healthy–healthy) on *V. dahliae* inoculum density.

Spatial aspects

Spatial aspects concerning diseased trees were analysed by calculating unidirectional semivariograms from the AUDPC values of individual plants, based on disease severity scores, using the data from the first experimental year from one row of 20 (Wageningen) or 30 (Meterik) plants per plant species. The semivariogram formula is: $\gamma(h) = c \times [(3h/2a) - (h^3/2a^3)]$ when $h < a$ and $\gamma(h)=a$ when $h>a$, where c is the maximum level (the horizontal part, or 'sill' of the curve), h is the distance (cm) between two plants, and a is the range of influence of a diseased plant (Clark 1979). Semivariograms were calculated by squaring the difference in AUDPC between two plants at a certain distance apart from each other; summing all differences of plant pairs at that distance; and dividing this figure by 2 × the number of pairs. The number of pairs 25 cm apart (the smallest distance) was n-1; the number of pairs 50 cm apart was n-2; etc. For each plot, individual data points

of the semivariograms were calculated and plotted in Microsoft Excel version 97 SR-1 (Microsoft, Chicago, USA). From the data points, ranges and sills were determined graphically per plot for each plant species. The sills were log-transformed. The ranges and sills were analysed in two ways using the GLM procedure in SAS: for each plot, they were related directly to *V. dahliae* inoculum densities and they were analysed for different *V. dahliae* inoculum density classes, viz. low and high inoculum densities: <5 and ≥5 CFU g^{-1} soil, respectively. These classes were chosen because 5 CFU g^{-1} soil represents a damage threshold of 10% diseased trees in tree nurseries (Goud et al. 2004).

Results

Inoculum density and disease dynamics

In Wageningen, pre-planting inoculum densities (1998) ranged from 0 to 50 CFU g^{-1} soil (average 11.4 CFU g^{-1} soil; standard error of the mean (SEM) 2.5). The average inoculum densities rose to 15 CFU g^{-1} soil in 1999 and 2000 and dropped to 10 CFU g^{-1} soil in 2001. In Meterik, pre-planting inoculum densities (1999) ranged from 0–15 CFU g^{-1} soil (average 6.5 CFU g^{-1} soil; SEM 0.9). The average inoculum densities dropped to 3 CFU g^{-1}

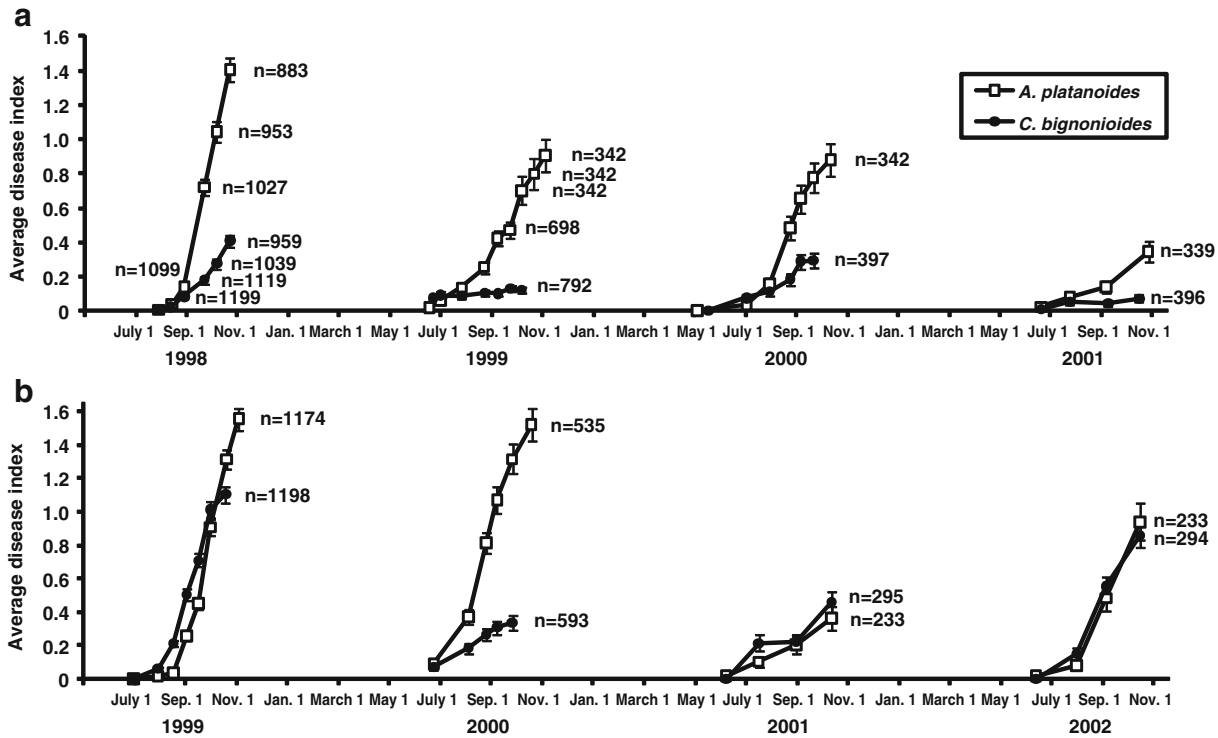

Fig. 1 Disease progress in *A. platanoides* (*open squares*) and *C. bignonioides* (*black dots*) in the 20 plots during each growing season of the four experimental years at two locations. **a**: Wageningen; **b**: Meterik. During late autumn and winter, disease can not be observed externally, because of natural leaf drop as a result of low temperatures. Average disease index scores were calculated from the individual disease index scores for all living plants in the control plots. Individual disease scores were given on a 0–5 scale (0: healthy plant—5: more than 80% of the leaves wilted or dropped). At the start of the experiment 60 plants were present in each of 20 plots (n= 1200). After thinning at the end of year 1, a maximum of 800 plants remained at Wageningen (in 1999) and 600 at Meterik (in 2000) and at the end of year 2 a maximum of 400 plants remained at Wageningen (2000–2001) and 300 at Meterik (2001–2002). Actual numbers were lower because damaged plants and dead plants of previous years were not scored. At Wageningen during 1998 and 1999 at the end of the growing season, plants were harvested per block to assess nematode colonization in the roots. This explains why numbers of plants were lower during autumn. Averages of disease scores are presented here for illustration purposes only. Data analysis was performed on AUDPC values as described in the Materials and methods section

Table 1 Best-fit regression models of annual area under the diseases progress curve (AUDPC) related to soil inoculum densities of *V. dahliae* (Wageningen and Meterik) and densities of *P. fallax* per g soil or root (Wageningen), as determined at the beginning of the experiment and annually in spring

Wageningen						Meterik		
A. platanoides								
Year [a]	R^2	Intercept	*V. dahliae* CFU g^{-1} soil (Vd)[b]	*P. fallax* g^{-1} root (Pf)[c]	Interaction (int)	R^2	Intercept	*V. dahliae* CFU g^{-1} soil (Vd)[b, d]
1	0.94	0.03	42.8×log (Vd+1)	0.19×Pf	0.19×int	0.55 [e]	−9.45	48.7×log (Vd+1)
		0.99[f]	<0.0001 [f]	0.0070	0.0016		0.51	0.0032
2	0.62	21.4	36.2×log (Vd+1)	–[g]	–	0.50	24.8	132.6×log (Vd+1)
		0.17	0.002	ns [g]	ns		0.46	0.0040
3	0.50	33.8	1.64×log (Vd+1)				–	–
		0.12	0.006	na[h]	na		ns	ns
4	0.72	15.2	1.61×Vd			0.44	−0.66	4.49×Vd
		0.077	0.0002	na	na		0.97	0.099
Year	R^2	Intercept	*V. dahliae* CFU g^{-1} soil before planting (Vd) [i]	*P. fallax* g^{-1} soil (Pf) [j]	Interaction (int)	R^2	Intercept	*V. dahliae* CFU g^{-1} soil before planting (Vd) [d, i]
1	0.95	−0.58	3.86×Vd	39.3×Pf	−2.67×int	0.55 [e]	−9.45	48.7×log (Vd+1)
		0.93	<0.0001	0.0045	0.0068		0.51	0.0032
2	0.53	11.1	1.25×Vd	42.2×Pf	–	0.76	31.2	114.5×log (Vd +1)
		0.13	0.021	0.040	ns		0.12	<0.0001
3	0.36	24.3	2.31×Vd	–	–		–	–
		0.033	0.003	ns	ns		ns	ns
4	0.52	4.07	1.52×Vd	–	–		–	–
		0.44	0.0002	ns	ns		ns	ns
C. bignonioides								
Year	R^2	Intercept	*V. dahliae* CFU g^{-1} soil (Vd)[b]	*P. fallax* g^{-1} root (Pf)[c]	Interaction (int)	R^2	Intercept	*V. dahliae* CFU g^{-1} soil (Vd)[b, d]
1	0.84	7.47	0.88×Vd	0.048×Pf	−0.0096×int	0.84 [e]	24.5	69.7×log (Vd+1)
		0.005	0.0003	0.46	0.0073		0.35	<0.0001
2	0.43	5.54	0.36×Vd	–	–		–	–
		0.006	0.0011	ns	ns		ns	ns
3	0.50	18.2	0.597×Vd				–	–
		0.07	0.0331	na	na		ns	ns
4		–	–				–	–
		ns	ns	na	na		ns	ns

Table 1 (continued)

Wageningen						Meterik		
Year	R^2	Intercept	*V. dahliae* CFU g^{-1} soil before planting (Vd)[i]	*P. fallax* g^{-1} soil (Pf)[j]	Interaction (int)	R^2	Intercept	*V. dahliae* CFU g^{-1} soil before planting (Vd)[d, i]
1	0.87	8.58	1.02×Vd	15.5×Pf	−3.50×int	0.84 [e]	24.5	69.7×log (Vd+1)
		0.002	<0.0001	0.18	0.0015		0.35	<0.0001
2	0.81	7.70	0.938×Vd	10.1×Pf	−2.46×int		–	–
		0.02	0.0011	0.51	0.053		ns	ns
3	0.53	18.5	0.807×Vd	–	–		–	–
		0.06	0.020	ns	ns		ns	ns
4	0.64	3.61	−0.344×Vd	−8.17×Pf	1.39	0.40	22.5	2.68×Vd
		0.38	0.25	0.26	0.017		0.09	0.035

[a] In Wageningen, year 1–4 was 1998–2001, and in Meterik, year 1–4 was 1999–2002, respectively

[b] *V. dahliae* soil inoculum density assessed annually in spring each year

[c] *P. fallax* density g^{-1} (fw) root assessed annually in autumn each year

[d] No nematodes present in Meterik;

[e] Year 1 assessment is identical to pre-planting assessment

[f] *P*-value for significance of F-test

[g] Not significant

[h] Not assessed

[i] Pre-planting *V. dahliae* soil inoculum density, assessed early 1998 at Wageningen and early 1999 at Meterik

[j] Pre-planting *Pratylenchus* soil inoculum density, assessed early 1998

soil in 2000, and rose again to 4 CFU g^{-1} soil in 2002. Disease usually became prevalent in mid-summer and increased until the end of October (Fig. 1). In November, leaves usually started to drop because of natural senescence and external wilt symptoms could not be determined.

Inoculum density—disease severity relationships

Significant relationships between detected inoculum densities of *V. dahliae* in the soil and AUDPC values were observed in both plant species in most years (Table 1). In *A. platanoides* in Wageningen, numbers of *P. fallax* nematodes g^{-1} root made a significant ($P<0.05$) contribution to AUDPC in 1998. Both the main effect and the interaction term were positive and significant. However, in 1999, numbers of *P. fallax* nematodes g^{-1} root were low compared to 1998, and contributions were not significant (Table 1). In *C. bignonioides* in Wageningen in each year, the number of *P. fallax* nematodes g^{-1} root did not cause a significant increase of AUDPC. In *C. bignonioides*, fits were improved when AUDPC values of the second, third and fourth experimental years were related to pre-planting soil inoculum densities of *V. dahliae* (1998 in Wageningen and 1999 in Meterik) and *P. fallax* (Wageningen only), compared with annual inoculum densities (Table 1). In 1998 and 1999, pre-planting soil inoculum densities of *P. fallax* contributed significantly to disease in *A. platanoides* (Table 1).

Isolation and damage thresholds

Percentage of isolation of *V. dahliae* from the trunk base of *A. platanoides* and *C. bignonioides* was positively correlated with pre-planting inoculum density of *V. dahliae* in the soil in each plant species ($P<0.01$) (Wageningen 1998 only) (Fig. 2). Numbers of *P. fallax* g^{-1} root of *A. platanoides* and *C. bignonioides* were positively correlated with pre-planting inoculum density of *P. fallax* in each year ($P<0.05$) (Wageningen only) (data not shown).

Spatial relations

Figure 3 shows four examples of disease spread over time and space, where the numbers of diseased trees in 1998 ranged from 2 to 19. Most of the time,

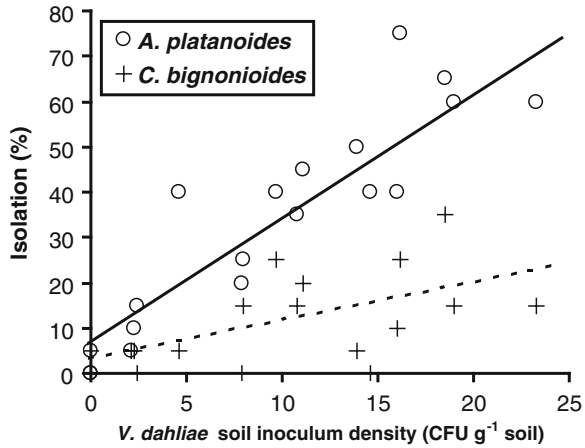

Fig. 2 Percentages of isolation of *V. dahliae* from the trunk base of *A. platanoides* and *C. bignonioides* at Wageningen, 1998. The *solid line* is the regression line for *A. platanoides* (%=5.6+2.9× *V. dahliae* inoculum density; $R^2=0.83$). The *dashed line* is the regression line for *C. bignonioides* (%=2.5+0.85×*V. dahliae* inoculum density; $R^2=0.36$)

diseased plants occurred clustered together during the first year. Plants that were diseased during later years were often close to spots with a diseased plant during the first year. This happened, for example, in plants that were rogued 1998 (middle rows) in plot 13 (plants 9 and 10), plot 14 (plants 8 and 10) and plot 18 (plant 9). Regressions of sills of semivariograms (representing variability in disease) on pre-planting soil inoculum densities of *V. dahliae* were significant in both plant species at each location ($P<0.01$). Sills were significantly higher at high than at low inoculum densities of *V. dahliae*. Regressions of ranges of influence of diseased plants on pre-planting soil inoculum densities of *V. dahliae* were significant for *A. platanoides* in Wageningen and *C. bignonioides* in Meterik ($P<0.05$), where ranges were larger at low inoculum densities of *V. dahliae*. However, they were not significant for *C. bignonioides* in Wageningen and *A. platanoides* in Meterik (data not shown).

Analyses were confirmed by division of data into two *V. dahliae* soil infestation classes, viz. low (<5 CFU g^{-1} soil) and high (≥5 CFU g^{-1} soil). Sills were significantly higher at high than at low *V. dahliae* inoculum densities ($P<0.01$) (Fig. 4). Ranges of influence of diseased plants were significantly higher at low than at high *V. dahliae* inoculum densities for *A. platanoides* in Wageningen and for *C. bignonioides* in Meterik ($P<0.05$). However, for *C. bignonioides* in Wageningen and

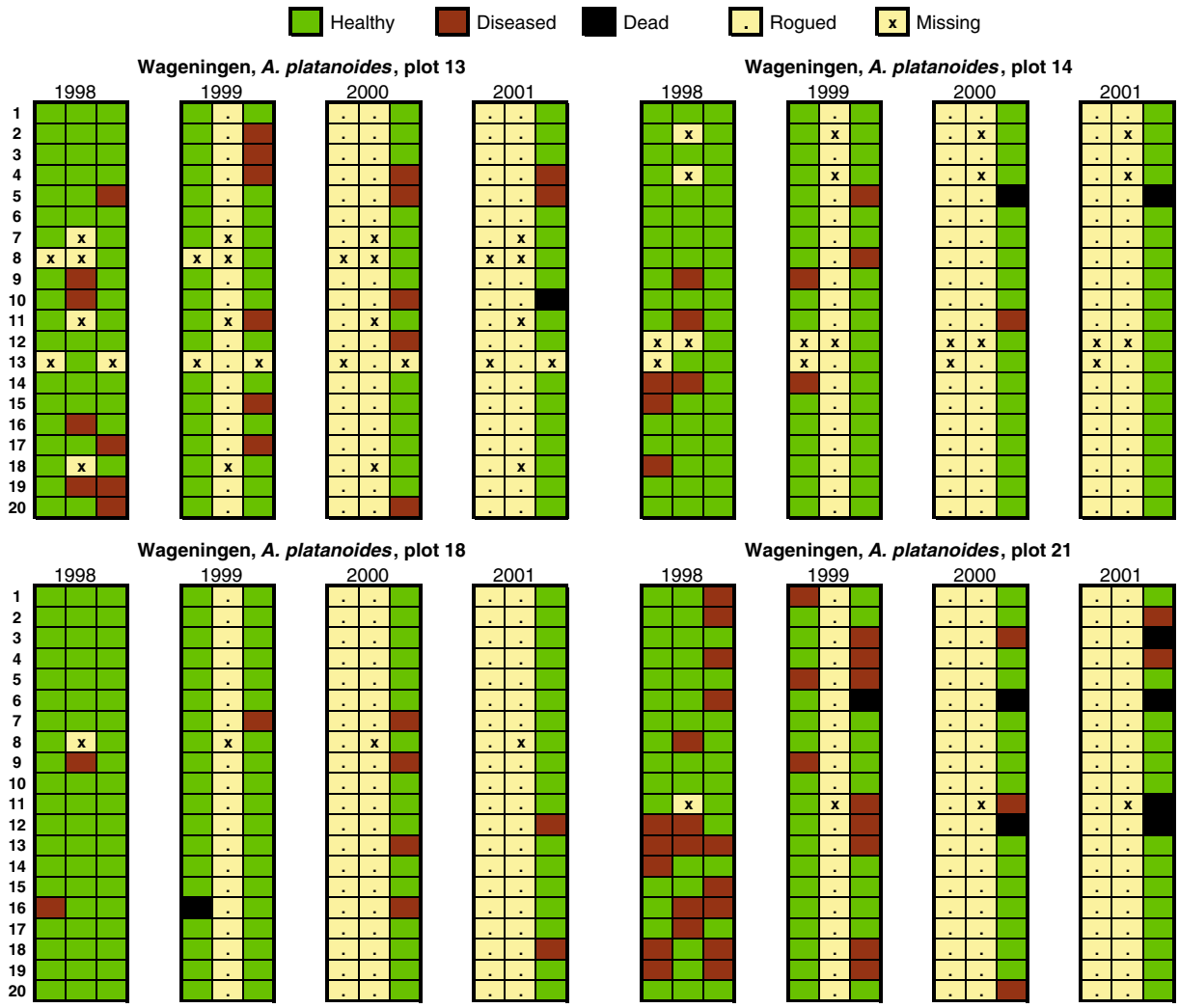

Fig. 3 Maps of four experimental plots with *A. platanoides* at Wageningen, showing the fate of the 60 trees during four consecutive years. Each cell represents one tree, planted at 33 cm row distance and 25 cm within-row distance. *Green cells* represent healthy trees, *brown cells* represent diseased trees and *black cells* represent dead trees. *Light yellow cells* indicate absent trees: 'x' means tree missing; dots indicate rogued trees

A. platanoides in Meterik ranges were not significantly different (Fig. 4). The presence of *P. fallax* at Wageningen did not increase the ranges of influence of Verticillium wilt compared to Meterik in *A. platanoides* or *C. bignonioides* (Fig. 4).

Recovery

Some 67% of the *A. platanoides* trees and 83% of the *C. bignonioides* trees recovered from disease during the following season. *Acer platanoides* mainly re-grew from the trunk base, whilst *C. bignonioides* mainly re-grew from the crown of the tree. A relatively larger number of diseased plants was observed among recovered plants (diseased–healthy) than among plants that were healthy during the two previous years, except for *A. platanoides* in Meterik (Table 2). The number of times (one or two) that a tree was diseased before recovery occurred did not have a significant effect on probability of disease in the fourth year for any of the plant species (data not shown).

Percentages of disease in recovered plants were significantly correlated with pre-planting soil inoculum densities of *V. dahliae* for each plant species at Wageningen ($P<0.05$) (Fig. 5). Disease percentages

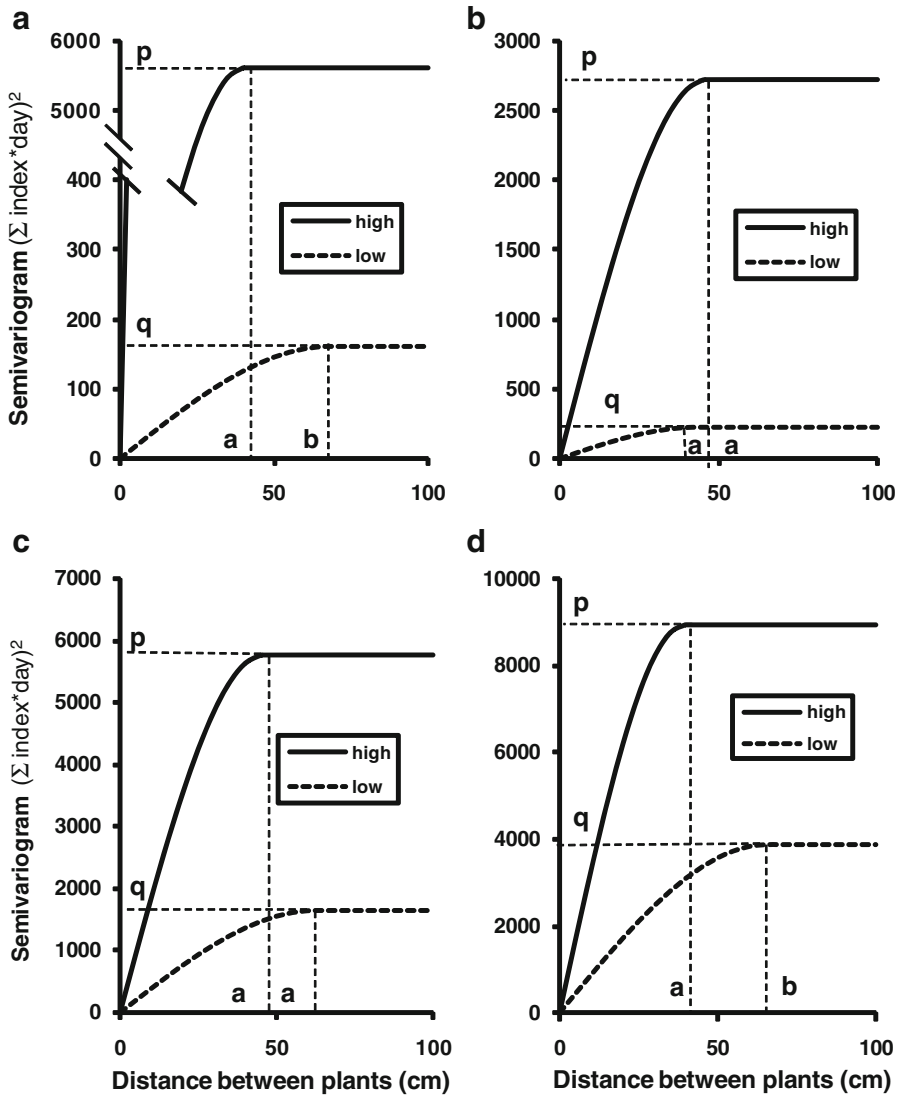

Fig. 4 Pooled unidirectional semivariograms for high (≥5 CFU g^{-1} soil) and low (<5 CFU g^{-1} soil) inoculum densities of *V. dahliae*. **a**: *A. platanoides* at Wageningen; **b**: *C. bignonioides* at Wageningen; **c**: *A. platanoides* at Meterik; **d**: *C. bignonioides* at Meterik. Different letters indicate significant differences ($P<0.05$) for ranges of influence (a or b) and significant differences ($P<0.01$) for sills (p or q)

of plants that were healthy during the two previous years were used for comparison (Fig. 5). The slopes of the regression lines were significantly different ($P<0.05$), indicating that at higher inoculum densities, a higher percentage of recovered plants became diseased again (Fig. 5). At Meterik, there were no significant relationships between probability of disease and *V. dahliae* inoculum density (data not shown).

Discussion

Significant relationships were observed between disease severity (AUDPC) and soil inoculum densities of *V. dahliae* during most years. In years with low disease levels, relationships were sometimes not significant. *V. dahliae* inoculum density usually was the most significant term in the best-fit regression equation. Most of the times, the terms for *V. dahliae* and *P. fallax* in the equations were positive, while the interaction term was negative. This indicates that *P. fallax* contributed to disease at low *V. dahliae* inoculum densities, but not at high *V. dahliae* inoculum densities. For example, at a *V. dahliae* inoculum density of 1 CFU g^{-1} soil, a *P. fallax* inoculum density of 1 g^{-1} soil can cause an increase in AUDPC of 35–65 (*A. platanoides* Table 1), which may represent 1–2% diseased plants. A lower damage threshold was also observed for other *Pratylenchus*

Table 2 Numbers of plants that were healthy and diseased after being healthy during two consecutive years and after having recovered (diseased in 1 year and healthy in the following year)

2 years before 1 year before Assessment year [a]	Healthy Healthy Healthy	Healthy Healthy Diseased	Diseased Healthy [b] Healthy	Diseased Healthy [b] Diseased	Chi-square probability level[c]
Wageningen					
A. platanoides	361	38	70	39	<0.0001
C. bignonioides	623	39	49	12	<0.0001
Meterik					
A. platanoides	259	48	62	11	0.90
C. bignonioides	333	72	70	27	0.025

[a] Numbers of plants with different disease history were different. Numbers of plants from years 3 and 4 (Wageningen: 2000 and 2001; Meterik: 2001 and 2002) and with the same disease history were pooled

[b] Plants that successfully recovered from disease

[c] Significance of difference between the ratio of healthy–healthy–diseased/healthy–healthy–healthy and the ratio of diseased–healthy–diseased/diseased–healthy–healthy

species by Martin et al. (1982) in potato and Dwinell and Sinclair (1967) in sugar maple. Our results are in contradiction to those of McKinley and Talboys (1979), who found a high effect of *P. penetrans* on verticillium wilt severity in strawberry at high *V. dahliae* densities, but not at low inoculum densities. This could be caused by the use of fumigated and partly dried soil in their experiment, resulting in disturbed relationships between soil biota.

Disease symptoms already occurred around the *V. dahliae* detection limit of approx. 0.55 CFU g^{-1} soil. AUDPC does not directly relate to financial loss, because commercially, the entire tree is lost when disease symptoms are clear (disease score 2 or higher). In some years, at 5 CFU g^{-1} soil, 1–2% diseased *A. platanoides* and *C. bignonioides* were present. In those years, the observed 5% infection thresholds were <0.55 CFU g^{-1} soil (*i.e.* the detection limit) for *A. platanoides* and around 3 CFU g^{-1} soil for *C. bignonioides*. This is low compared to cauliflower, cotton and potato, where disease incidence can be significant at such levels, however, without causing severe financial losses (Nicot and Rouse 1987; Paplomatas et al. 1992; Xiao and Subbarao 1998). It is comparable with the damage threshold for strawberry and olive where 5% and 15% infection and wilt can occur, respectively, below 2 CFU g^{-1} soil, causing significant yield losses (Harris and Yang 1996; López-Escudero and Blanco-López 2007).

In general, disease prediction was better at high *V. dahliae* inoculum densities (Wageningen) than at low inoculum densities (Meterik). At high inoculum densities, usually high disease levels were observed. The better fits that were sometimes obtained using pre-planting inoculum densities for disease in later years, compared with the densities of the yearly assessments are probably caused by a more random distribution of the inoculum, as a result of soil cultivation, compared to the CFUs formed in a standing crop. This indicates that disease prediction in trees remains valid for several years.

The range of influence of a tree suffering from verticillium wilt was small (0–100 cm), which can be ascribed to the low mobility of *V. dahliae* in soil (Huisman 1988). Relatively high ranges of influence of a diseased plant were observed at low *V. dahliae* soil inoculum densities several times. This suggests that verticillium wilt occurred in a clustered pattern when inoculum densities were low. Apparently, trees that were diseased during the first year(s) locally raised the inoculum density of *V. dahliae*. In plots with low inoculum densities of *V. dahliae*, a local higher density will have a relatively larger effect than in plots with high inoculum densities. After infection, the fungus mainly resides in the vascular tissue. Only when the infected plant tissue becomes moribund, microsclerotia are formed in the dying plant tissue, such as roots and petioles and can be spread through the soil to some extent by earthworms (Rijkers et al.

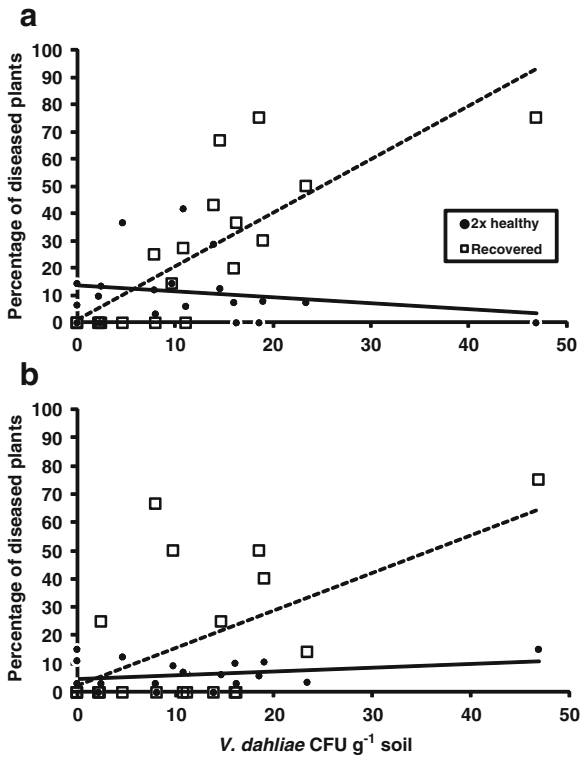

Fig. 5 Percentages of diseased plants in the year after recovery (diseased–healthy–diseased) or after two consecutive years without disease (healthy–healthy–diseased), plotted against pre-planting *V. dahliae* inoculum densities, at Wageningen. **a**: *A. platanoides*; **b**: *C. bignonioides*. *White square*, percentage of disease in recovered plants; *black circle*, percentage of disease after two consecutive years without disease; striped line, weighted regression line of disease in recovered plants on *V. dahliae* inoculum densities; solid line, weighted regression line of disease in plants that were healthy during the two previous years on *V. dahliae* inoculum densities. Regression lines were significantly different ($P<0.05$) for *A. platanoides* and *C. bignonioides*

1992) and superficially by air-blown leaves and dust. It might be sensible for growers to remove also surrounding trees or to closely monitor them for wilt symptoms. Moreover, in nurseries, root growth of surrounding trees into the empty, infested spot should be prevented by digging in a root barrier cloth and/or planting a non-host tree species on the infested spot.

The predominant observation was that recovered trees had a higher probability of becoming diseased again. This could be caused by the presence of original inoculum close to the tree (causing initial disease). The initial inoculum density seems to be the most important factor, since there was no difference between disease occurring once or twice before recovery (and thus a possible once or twice inoculum build-up). Apparently, recovered trees have not acquired induced resistance lasting until the next year. Recovery from disease is known to occur in trees, as a result of the formation of a new, uninfected ring of xylem vessels (Goud and Termorshuizen 2002; Hiemstra and Harris 1998; López-Escudero and Blanco-López 2005; Markakis et al. 2009; Shigo 1984). However, the relationship with soil inoculum density has not been investigated before. In Wageningen, high percentages of recovered plants became diseased during the next year, especially at high inoculum densities (above 15 CFU g^{-1} soil). In Meterik, disease in recovered plants was not significantly related to inoculum density, presumably because inoculum densities above 15 CFU g^{-1} soil did not occur. This is an indication that disease after recovery is mainly caused by new infections of *V. dahliae* from the soil, and not by incomplete recovery.

In summary, our results clearly demonstrate that verticillium wilt damage in tree nurseries can already occur at *V. dahliae* inoculum densities around the detection limit. Pre-planting soil inoculum densities of *V. dahliae* are highly indicative for disease during the following 1 to 4 years. Plant-pathogenic nematodes, such as *P. fallax*, may contribute to disease severity at low *V. dahliae* inoculum densities, although the effects observed were inconsistent. In tree nurseries, at low *V. dahliae* inoculum densities, diseased trees should be removed, and growth of the surrounding trees into the infested spot should be avoided. Recovered trees have a higher probability of becoming diseased again, which is caused by build-up of the inoculum in the soil.

For disease prediction in tree nurseries in practice, improvement of detection methods to detection limits of 0.1 CFU g^{-1} soil is urgently needed. Although molecular methods to quantify *V. dahliae* microsclerotia in soil have been developed by several laboratories (e.g. Bilodeau et al. 2010; Mahuku and Platt 2002), practical applications are hampered by the occurrence of inhibitory soil factors and/or the difficulty of concentrating the microsclerotia present in a field soil sample into a small reaction volume. Optimally, disease prediction should take into account the viability and the aggressiveness of the *V. dahliae* population (Chandelier et al. 2003; Goud and Termorshuizen 2002), and should also be based on the presence of other biota present in the soil sample. For this, the molecular basis of *V. dahliae*

aggressiveness to different crops and the molecular basis of soil factors that cause fungistasis or conduciveness to verticillium wilt need to be unravelled. This may eventually lead to a rapid routine testing method with a low detection limit, valid for different types of soils, which is able to not only quantify *V. dahliae*, but also provide a reliable disease prediction.

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