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Pathogenicity and virulence of the two Dutch VCGs of *Verticillium dahliae* to woody ornamentals

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Abstract

Two experiments were performed in two consecutive years to test whether isolates of different vegetative compatibility groups (VCGs) differ in their ability to cause disease in woody ornamentals, to study the host specificity of the isolates and to get an insight into disease development in woody hosts. A range of woody ornamental plant species, including *Acer campestre*, *Acer platanoides*, *Acer pseudoplatanus*, *Catalpa bignonioides*, *Cotinus coggygria*, *Robinia pseudoacacia*, *Rosa canina*, *Syringa vulgaris* and *Tilia cordata*, were root-dip inoculated with six isolates of *Verticillium dahliae*, belonging to the two VCGs that occur in the Netherlands (VCG NL-1 and VCG NL-2). Isolates belonging to each VCG caused severe symptoms of verticillium wilt in most plant species tested. Disease progress differed between plant species, but was generally the same for the two VCGs. No overall differences in virulence were observed between the two VCGs for external wilt symptoms, number of dead plants, or shoot length. No significant VCG × plant species interactions were present for these characteristics. However, isolates of VCG NL-1 caused more vascular discolouration than did isolates of VCG NL-2. Isolates within VCGs often differed considerably in their virulence to certain hosts, as shown by highly significant isolate × plant species interactions. Isolates were more virulent on their original host. These findings imply that VCG identification does not contribute to disease prediction for a range of woody hosts.

Abbreviations: AUDPC – area-under-the-disease-progress curve; VCG – vegetative compatibility group.

Introduction

In tree nurseries all over the world considerable losses are reported from verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* (Piearce and Gibbs, 1981; Sinclair et al., 1989). In the Netherlands, 30% of the area used for tree production is estimated to be infested (Anonymous, 1990). Growing susceptible tree species on infested soil can cause up to 50% loss (Anonymous, 1990; Goud et al., 2000). Crop rotation is the only way to manage verticillium wilt, since chemicals for disinfestation of the soil become less and less available (Annis and Waterford, 1996; Anonymous, 1990), while non-chemical methods for the temperate zones are still in development (Blok et al., 2000). Most of the current methods used to quantify the soil inoculum level do not provide information about the virulence of the soil population (Termorshuizen et al., 1998), though it is known that isolates can differ significantly in virulence to woody hosts (Ashworth, 1983; Donohue and Morehart, 1978; Schnathorst and Sibbett, 1971; Tjamos, 1981).

Because the sexual cycle is absent in *Verticillium*, parasexuality is considered the single means of exchange of genetic material between individual mycelia (Heale, 1988). Vegetatively incompatible strains belong to different vegetative compatibility groups (VCGs) and therefore are genetically isolated

from each other. This means that VCGs may vary in many characteristics, including those related to pathogenicity and virulence (Rowe, 1995). Work on pathogenic isolates of *Fusarium oxysporum* has shown that VCGs are subdivisions within formae speciales, i.e., the intra-specific pathogenicity grouping. Each forma specialis of *F. oxysporum* contains one to many VCGs, and each VCG occurs within one forma specialis (Katan and di Primo, 1999).

Vegetative compatibility grouping in *V. dahliae* is done with the use of nitrate non-utilising (*nit*) mutants, which express mutant growth on a minimal medium. Isolates are judged to be vegetatively compatible when complementing *nit*-mutants are able to mate and produce wild-type growth. This is the result of successful fusion of hyphae between strains and subsequent heterokaryon formation (Joaquim and Rowe, 1990; Puhalla, 1985). Following this approach, Joaquim and Rowe (1990) grouped isolates from *V. dahliae* into VCG 1–4. Later, isolates of VCG 3 were regrouped into VCG 4 (Bell, 1995; Katan, 2000; Strausbaugh et al., 1992).

In the Netherlands, two VCGs of *V. dahliae* are present, provisionally called VCG NL-1 and VCG NL-2 (Hiemstra and Rataj-Guranowska, 2000). It is not exactly known how the isolates used in this study fit into the American vegetative compatibility grouping system, but tester strains of VCG NL-1 were compatible with both the American tester strains of VCGs 3 and 4, whereas the tester strains of VCG NL-2 were compatible with both the American tester strains of VCGs 1 and 2 (Rataj-Guranowska and Hiemstra, 2000).

Links of some American VCGs with pathogenicity have been reported, e.g., VCG 1 contains all cotton-defoliating strains and VCG 2 and VCG 4 contain all cotton non-defoliating strains (Daayf et al., 1995). Grouping of isolates from certain hosts into specific VCGs has been observed in several inventories, e.g., isolates from watermelon in Greece were VCG 2 (Elena, 2000), most isolates from potato were VCG 4 (Rowe et al., 2000), and most American isolates from woody hosts were VCG 1 (Chen, 1994). Such inventories can only be used for disease prediction when pathogenicity tests are performed. This has been done for single crops, like potato (Joaquim and Rowe, 1991; Strausbaugh, 1993; Strausbaugh et al., 1992), or several (Gennari et al., 2000) to many (Bhat and Subbarao, 1999) herbaceous crops, but never for a range of woody hosts. This is important because disease development in woody hosts can differ from that in herbaceous plants due to differences in anatomy and multi-year effects (Hiemstra, 1998).

All plants used in the present study were listed as susceptible to *V. dahliae* (Hiemstra, 1998) and often show wilt symptoms in nurseries in the Netherlands. The hypotheses were: (1) isolates of different VCGs in the Netherlands differ in virulence to woody hosts; (2) these isolates are host specific, and (3) disease development differs for the hosts studied. Preliminary results have been published in an abstract (Goud and Termorshuizen, 2000).

Materials and methods

Experimental set-up

Two experiments were carried out in 1997 and 1998, with *Acer campestre* (field or hedge maple), *Acer platanoides* (Norway maple), *Acer pseudoplatanus* (sycamore maple), *Catalpa bignonioides* (southern Catalpa), *Cotinus coggygria* (European smoketree) and *Robinia pseudoacacia* (black locust). The plant species *Syringa vulgaris* (common lilac) and *Tilia cordata* (littleleaf linden) were tested in Experiment 1, but because they did not show clear disease symptoms (no difference between the inoculated plants and the controls) they were not included in Experiment 2. *Rosa canina* cultivar 'Laxa' (a dog rose selection which is used as rootstock) was tested in Experiment 2 only. All plants were seedlings with 6 to 8 leaves, except for plants of *T. cordata* and *A. pseudoplatanus* in the first experiment and *R. canina* in the second, which were one year old.

Experiment 1 consisted of 25 blocks in a randomised complete block design, with each block including all plant species in combination with six isolates, representing the two VCGs, and three control plants, so 25 plants were used per species × isolate combination, together with 75 control plants per species (a total of 225 plants per species). Control plants were used to distinguish between disease symptoms and plant reactions caused by environmental factors. Plants were grown in a screenhouse with a plastic cover and open sides. Temperature generally fluctuated between 10 and 35 °C and relative humidity between 20% and 100%. Experiment 2 consisted of 10 blocks in a randomised complete block design, including two control plants, so 10 plants were used per species × isolate combination, together with 20 control plants per species (a total of 80 plants

per species). Plants were grown in a ventilated greenhouse, with temperatures generally fluctuating between 15 and 25 °C and a relative humidity between 40% and 80%, but temperatures in the greenhouse exceeded 30 °C during day time 3–5 weeks after inoculation.

Plant maintenance

Pots were placed in individual saucers to prevent cross contamination. Plants were watered 2–4 times per week, depending on their needs, by filling up the saucers. Each experiment was fertilised once with 100 ml solution of 2 g l^{-1} of Kristalon fertiliser 19-6-20-3+ microelements (Hydro Agri Rotterdam BV, Vlaardingen, the Netherlands), and in Experiment 2 Osmocote Plus controlled release fertiliser 15-11-13-2+ microelements (Scotts Europe BV, Heerlen, the Netherlands) was mixed through the potting soil at a density of 4 g l^{-1} soil.

In Experiment 1, all *Acer* species, *R. pseudoacacia*, and *C. coggygria* were sprayed against powdery mildew three times during summer with Curamil (Hoechst Holland N.V. Agro Chemie, Amsterdam, the Netherlands), Rubigan Flow (Dow-Elanco BV, Antwerp, Belgium), and Baytan Flow (Bayer BV, Mijdrecht, the Netherlands) respectively, all at a concentration of 0.5 ml l^{-1}. In Experiment 2, *R. canina* was sprayed once with Funginex (Shell Nederland Chemie BV, Rotterdam, the Netherlands) and all plants were sprayed against spider mites with Masaï (Bayer BV, Mijdrecht, the Netherlands) and against woolly aphids with Pirimor (ICI Agro, Ridderkerk, the Netherlands) all at a concentration of 0.5 ml l^{-1}. After the sprays, spider mites were controlled biologically using Spidex (Koppert BV, Berkel and Rodenrijs, the Netherlands) and woolly aphids with *Cryptolaemus* spp. (Entocare BV, Wageningen, the Netherlands). Though no negative effects of the applied fungicides and pesticides to *V. dahliae* were known from the literature, pots were covered during all sprays to prevent dripping of chemicals into the soil.

Inoculation

Six *Verticillium* isolates were used from different locations in the Netherlands (Table 1). Monoconidial cultures were made of the original isolates by the first author in 1997, except for G3 and S12,2 which were received as monoconidial cultures from the researchers who isolated them. Between 1997 (Experiment 1) and 1998 (Experiment 2) monoconidial cultures were maintained on potato dextrose agar at 4 °C. Roots were washed free of soil and the plants were root-dip inoculated with a conidial suspension of *V. dahliae* at 1.0×10^6 conidia ml^{-1} for 2 min. Inoculated seedlings were planted in 0.7 litre-pots with potting soil (Nr. 4, Lentse Potgrond, Lent, the Netherlands) which had been steam-sterilised one month before. Control plants were dipped in autoclaved inoculum of the same conidial density.

Disease observations

In the first experiment, disease scores were rated 75 days after inoculation on a 0–5 scale: 0 = healthy plant, 1 = one or two leaves affected (= wilted, dead or fallen off; two top leaves showing epinasty for *Acer* species), 2 = three leaves or up to 30% of the leaves affected,

Table 1. Collection and storage information for isolates of the two VCGs of *V. dahliae* used in the experiments

VCG	Isolate	Host of origin	Location	Date	Isolation by	Storage
NL-1[1]	G3	Potato (*Solanum tuberosum* L.)	Wageningen	1–7–1994	A.J. Termorshuizen, Wageningen University	Agar plugs in water at 4 °C
NL-1	es120mc-1	Ash (*Fraxinus excelsior* L.)	Lelystad	10–3–1988	J.A. Hiemstra, Plant Research International	Agar plugs in water at 4 °C
NL-1	AplatImc-1	Norway maple (*Acer platanoides* L.)	Grubbenvorst	28–9–1993	J.A. Hiemstra, Plant Research International	Agar plugs in water at 4 °C
NL-2 [2]	A40mc-2	Red currant (*Ribes rubrum* L.)	Buurmalsen	26–1–1996	B. Wessels, Plant Protection Service	Potato dextrose agar plates at 4 °C
NL-2	A59mc-1	Blackberry (*Rubus fruticosus* L.)	Spijk	20–9–1996	W. Veenbaas, Plant Protection Service	Potato dextrose agar plates at 4 °C
NL-2	S12,2	Lilac (*Syringa vulgaris* L.)	Aalsmeer	30–9–1996	J.A. Hiemstra, Plant Research International	Agar plugs in water at 4 °C

[1]Compatible with American VCGs 3 and 4; [2]compatible with American VCGs 1 and 2.

3 = 30% to 60% of the leaves affected, 4 = 60% to 80% of the leaves affected, 5 = more than 80% of the leaves affected or plant dead. In *A. pseudoplatanus* only the four top leaves could be taken into account in Experiment 1, due to a hypersensitivity reaction of the older leaves to the anti-mildew spray with Baytan Flow. Shoot length was measured at the end of the growing season. Vascular discolouration was observed for all plants from 15 blocks on cross sections of the stem base and rated on a 0 (absent) to 5 (xylem tissue of the cross section completely discoloured) scale. Stem pieces of all 72 plants in one block and random samples from the other blocks were plated on ethanol agar (Nadakavukaren and Horner, 1959) to check for presence of the fungus. The 10 remaining blocks were kept in a frost-free greenhouse during winter and transferred back in spring to score for either death or regrowth. In the second experiment, disease scores were taken every 2 or 3 weeks for 28 weeks and area-under-the-disease-progress curves (AUDPC) were calculated (Campbell and Madden, 1990). Death of plants was also recorded. For *C. bignonioides* and *R. pseudoacacia* the assessment of the disease scores was stopped 18 weeks after inoculation because all diseased plants were either dead or cured after initial symptom expression. Vascular discolouration was observed as in Experiment 1 after the last disease score was taken. Stem pieces of all plants in one block and random samples from the other blocks were plated on modified soil extract agar (Harris et al., 1993) to check for presence or absence of the fungus.

Statistical analyses

Data presented in the second part of the Results section (concerning VCG main effects) were analysed using a mixed statistical model. This is a model in which fixed and random variables can be combined. In this analysis, plant species, VCG, and plant species × VCG interaction were treated as fixed variables, whereas block, isolate (within VCG), and plant species × isolate (within VCG) interaction were treated as random variables. This was done because the individual isolates were chosen randomly from each VCG, and no information was available about whether their variability represented adequately the variability within their VCG. This type of data analysis is conservative, in the sense that variation is first ascribed to the random effects and after that to the main effects (e.g., VCG). AUDPC and shoot length data were square-root transformed and analysed using the MIXED procedure of SAS version 8 (SAS Institute Inc., Cary, NC, USA). Residuals of the transformed data were normally distributed. Ordinal data (classes) of disease scores of Experiment 1 and vascular discolouration of Experiments 1 and 2 cannot be analysed adequately by parametric statistical models because class data are not normally distributed and step sizes between classes are not always similar for all classes (Oude Voshaar, 1994). Moreover, averages of ordinal scores may not always be meaningful. The threshold model (McCullagh, 1980) overcomes these problems by quantifying the ordinal data and constructing an underlying continuous scale. On this continuous scale thresholds are estimated between class 0 and 1, class 1 and 2, etc. Step sizes between these thresholds correspond with step sizes between classes. Next, data are fitted on this scale in the way that represents best the frequencies of plants scored in different classes. Data were fitted best when a normal distribution was used to create this continuous scale (i.e., the 'link function'). The analysis of the ordinal data, using a mixed statistical model, was performed with the use of the CLASS procedure addition (Candy and Wilkinson, 1997; Keen, 1998) of GENSTAT version 5.4.1 (Genstat, Rothamsted Experimental Station, Harpenden, UK). Because vascular discolouration was absent in *S. vulgaris* and *R. canina*, these species were not included in the statistical analysis of the discolouration data. Plant death is a binomial phenomenon (either alive or dead) and is therefore not normally distributed. Plant death data for each experiment were analysed by means of the MIXED procedure of SAS version 8. Because this procedure assumes normality, the analysis was checked using a Chi-square test (Snedecor and Cochran, 1989), after pooling the data for all blocks and all isolates within VCGs. Data for *R. pseudoacacia* and *R. canina* had to be omitted during calculation of the Chi-square of Experiment 2, because none of the plants of those species died.

Data presented in the last part of the Results section (concerning individual isolates) were analysed using a statistical model with fixed effects only: isolate, plant species, isolate × plant species interaction, and block. AUDPC and shoot length data were square-root transformed and analysed using the GLM procedure of SAS version 8. Residuals of the transformed data were normally distributed. Ordinal data (classes) of disease scores of Experiment 1 and vascular discolouration of Experiments 1 and 2 were analysed using the threshold model GENMOD of SAS version 8, using the normal distribution as link function (see above). Specific questions, e.g., whether there were differences between VCGs, were estimated during the analysis

using orthogonal contrasts (Snedecor and Cochran, 1989), assuming that the three isolates are correct representatives of each VCG. Analysis of the binomial plant death data by means of the GLM procedure of SAS version 8 was checked using a Chi-square test.

Results

Disease development

Isolates of each of the VCGs were capable of causing disease symptoms in most of the woody plant species. The most prevalent symptoms were acute wilt, necrosis, and defoliation. *Acer* species and *C. bignonioides* showed all three symptoms, whereas *C. coggygria* mainly showed acute wilt, *R. canina*, a slowly developing necrosis, and *R. pseudoacacia* and *T. cordata*, defoliation. Wilting plants of the *Acer* genus, especially *A. platanoides*, often showed epinasty of the two top leaves as a first symptom. In *S. vulgaris* curling of the leaves was observed and wilt or death of leaves was absent. Inoculated plants were generally different from the controls except for *S. vulgaris* and *T. cordata*. Plating of stem pieces revealed that control plants were not infected by *Verticillium*. Leaf curling of *S. vulgaris* and defoliation of *T. cordata* could have been a result of the mock inoculation procedure or natural senescence.

Disease development over time is described in Figure 1 for Experiment 2 for all isolates on *A. campestre*, *C. bignonioides* and *C. coggygria*. Disease developed slowly in *A. campestre* (Figure 1a) but eventually disease scores reached high values for the most virulent isolates. Disease progress of *A. platanoides* and *A. pseudoplatanus* was similar to that in Figure 1a (data not shown). For *R. canina* the trend was similar, but disease scores were lower because of formation of new branches after onset of the disease (data not shown). *C. bignonioides* (Figure 1b) and *R. pseudoacacia* (data not shown) developed severe symptoms, including death of some *C. bignonioides* plants, during the first four weeks after inoculation. After that, all living diseased plants recovered. *C. coggygria* plants (Figure 1c) showed acute wilt within four weeks after inoculation. Seven weeks after inoculation most of the inoculated plants were killed and disease did not progress in the remainder of the inoculated plants. Disease progress curves within plant species were generally the same for each VCG.

Vascular discolouration was present in most inoculated plants of all plant species except *S. vulgaris*

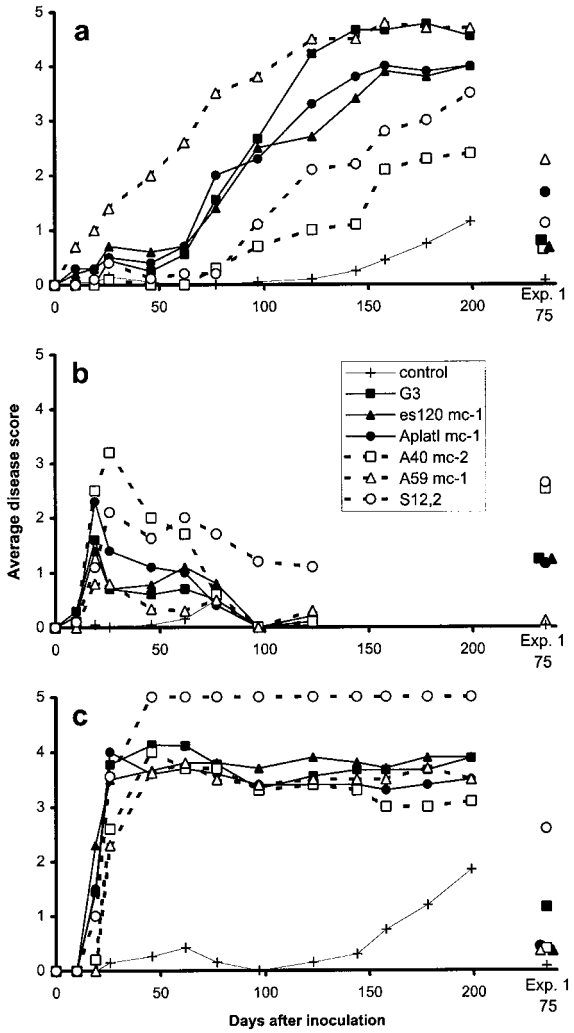

Figure 1. Disease progress curves of Experiment 2 and average scores from Experiment 1 (Exp. 1) 75 days after inoculation (data points on the right), for a: *Acer campestre*, b: *Catalpa bignonioides* and c: *C. coggygria*. Solid lines with closed markers represent isolates of VCG NL-1 of *V. dahliae*, dashed lines with open markers represent isolates of VCG NL-2, and + indicates the control.

(Experiment 1) and *R. canina* (Experiment 2), which rarely showed vascular discolouration symptoms. All other plant species showed significant differences between inoculated plants and controls, in which vascular discolouration was absent. *T. cordata* (Experiment 1) showed clear vascular discolouration even though symptom expression was unclear. Vascular discolouration was grey/green to black in *Acer* species and *T. cordata*, light brown in *C. bignonioides*, and dark brown in *C. coggygria* and *R. pseudoacacia*.

VCG effects

Disease scores were not significantly different for the two VCGs ($P = 0.75$), neither were AUDPC values ($P = 0.52$) (Figure 2). Also for individual plant species significant differences between VCGs were not present in both experiments. Furthermore plant species × VCG interactions were neither significant in Experiment 1 ($P = 0.61$) nor in Experiment 2 ($P = 0.22$). Differences between plant species were highly significant ($P < 0.0001$) in both experiments.

Numbers of dead plants were not significantly different for the two VCGs in Experiment 1 (mixed $P = 0.47$; Chi-square $P = 0.18$) and Experiment 2

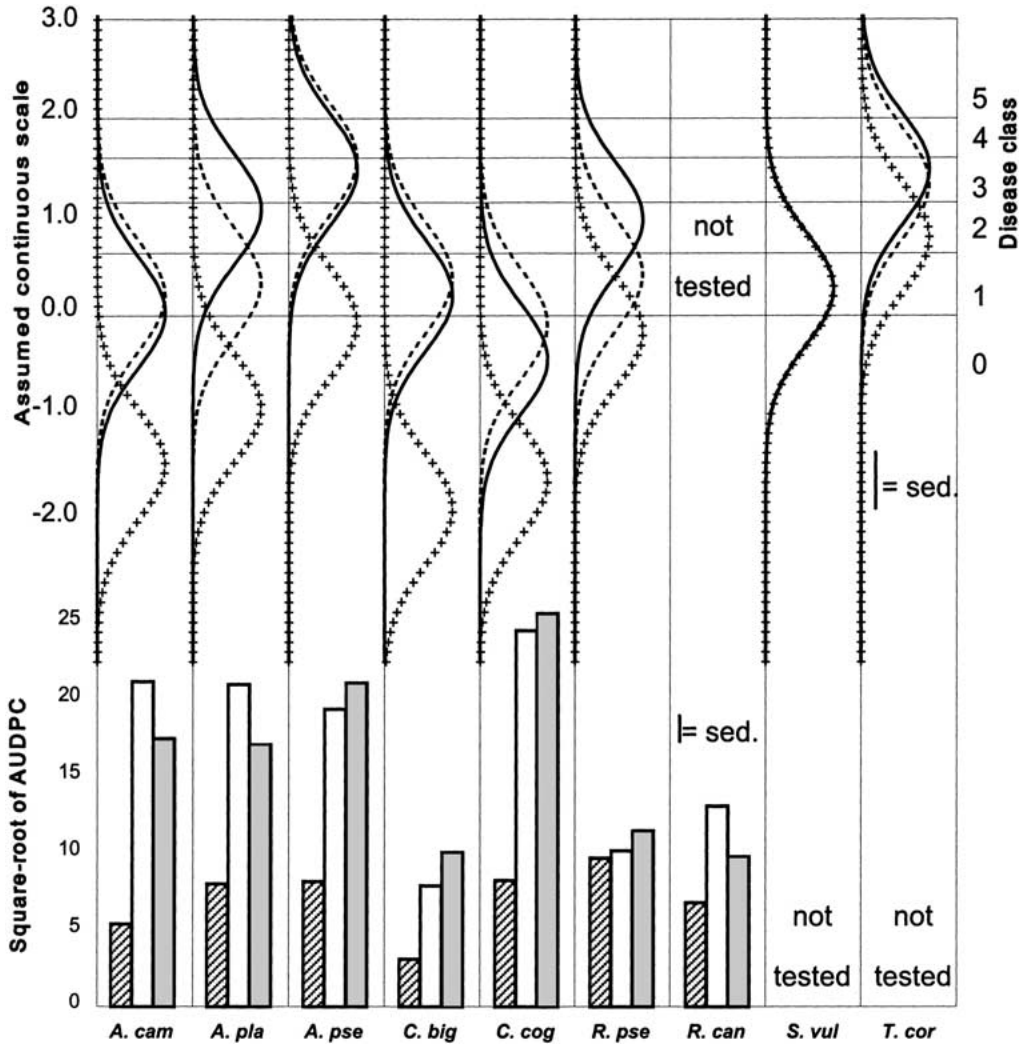

Figure 2. Estimated frequency distributions of the disease scores on an assumed underlying continuous scale (Experiment 1 – top) combined with average square-root transformed AUDPC (Experiment 2 – bottom) for the two VCGs of *V. dahliae* and the control. Experiment 1: —— = VCG NL-1, - - - - = VCG NL-2, ++++ = control. Experiment 2: White bars = VCG NL-1, grey bars = VCG-NL-2, striped bars = control. *A. cam* = *Acer campestre, A. pla* = *Acer platanoides, A. pse* = *Acer pseudoplatanus, C. big* = *Catalpa bignonioides, C. cog* = *Cotinus coggygria, R. pse* = *Robinia pseudoacacia, R. can* = *Rosa canina, S. vul* = *Syringa vulgaris,* and *T. cor* = *Tilia cordata*. Horizontal lines indicate the thresholds between disease classes of Experiment 1. Individual graphs shifted towards higher classes (top) and taller bars (bottom) imply more disease. sed. = standard error of differences within plant species between VCGs. *S. vulgaris* and *T. cordata* were tested in Experiment 1 only and *R. canina* was tested in Experiment 2 only.

(mixed $P = 0.85$; Chi-square $P = 0.79$) (Table 2). No consistent significant plant species × VCG interaction was present for number of dead plants in Experiment 1 (mixed $P = 0.62$; Chi-square $P = 0.03$) and Experiment 2 (mixed $P = 0.54$; Chi-square $P = 0.23$). Differences in numbers of dead plants between plant species were highly significant (mixed and Chi-square $P < 0.0001$) in both experiments.

For vascular discolouration no significant differences were observed between the two VCGs in Experiment 1 ($P = 0.22$) or Experiment 2 ($P = 0.37$). There were no significant plant species × VCG interactions in Experiment 1 ($P = 0.49$) or in Experiment 2 ($P = 0.17$). For *A. platanoides* and *R. pseudoacacia* there was a repeatable trend of more vascular discolouration after inoculation with isolates of VCG NL-1 compared with isolates of VCG NL-2. However, this trend was significant only for *A. platanoides* in Experiment 2 ($P < 0.05$). For other plant species there were no trends. Differences in vascular discolouration between plant species were highly significant ($P < 0.0001$) in both experiments.

Inoculated plants were generally 11% shorter than control plants ($P < 0.05$). However, no significant difference was observed between the two VCGs ($P = 0.63$) and there was no plant species × VCG interaction ($P = 0.36$).

Isolate effects

Data analysis with fixed factors only showed that for disease symptoms, plant species and plant species × isolate interaction were highly significant ($P < 0.0001$) in both experiments. The interaction reveals specificity of certain isolates to cause symptoms on certain plant species. The overall isolate effect was significant as well ($P < 0.01$). Similar P values are observed for data on number of dead plants, shoot length, and vascular discolouration (Table 3).

Figures 3 and 4 illustrate the isolate × plant species interaction, e.g., for *A. campestre* and *C. bignonioides* inoculated with isolates belonging to VCG NL-2: on *A. campestre* isolate A59mc-1 was most virulent and A40mc-2 and S12,2 were among the least virulent, whereas on *C. bignonioides* the effect was opposite.

Contrasts for isolates belonging to VCG NL-1 *versus* VCG NL-2 were significant only for vascular discolouration (Table 3). Isolates of VCG NL-1 caused more vascular discolouration than did isolates of VCG NL-2 in Experiment 1 ($P = 0.0031$) and Experiment 2 ($P = 0.041$). All cross sections of two plant species × isolate combinations in Experiment 2, viz., isolate G3 (VCG NL-1) on *A. campestre* and S12,2 (VCG NL-2) on *C. coggygria*, were entirely discoloured and were given the maximum score of 5. Consequently, no variation in the data for these combinations was present, which caused problems during the statistical analysis. Therefore, data for these two combinations had to be omitted during calculation of the contrast, to allow convergence of the statistical procedure.

Table 3 also shows contrasts for *S. vulgaris* inoculated with isolate S12,2, originally isolated from *S. vulgaris*, compared with other isolates. No clear symptoms (different from the control plants) were observed in *S. vulgaris* during the growing season, but during winter three out of ten plants that had been

Table 2. Percentage of dead plants per plant species combined for Experiments 1 and 2 per individual isolate ($n = 20$) of *V. dahliae* and the controls ($n = 50$)

Host	Control	Isolate					
		VCG NL-1[1]			VCG NL-2		
		G3	es120 mc-1	AplatI mc-1	A40 mc-2	A59 mc-1	S12,2
A. campestre	4	42[2]	30	30	5	75	25
A. platanoides	12	20	26[2]	15	25	15	5
A. pseudoplatanus	6	20	15	30	10	60	55
C. bignonioides	2	0	0	0	5	5	25
C. coggygria	4	63[2]	70	45	50	35	100[2]
R. pseudoacacia	2	10	15	0	0	0	0
R. canina[3]	5	0[4]	0	0	0	0	0
S. vulgaris[5]	0	0	0	0	0	0	30
T. cordata[5]	3	10	0	20	0	0[4]	40

[1]VCG = vegetative compatibility group; [2]$n = 19$; [3]tested in Experiment 2 only, so $n = 10$ and control $n = 20$; [4]$n = 9$; [5]tested in Experiment 1 only, so $n = 10$ and control $n = 30$.

Table 3. Significance levels of fixed effect factors and contrasts for all tested variables in Experiments 1 and 2. Data for control plants are excluded

Tested variable	Disease score	AUDPC[1]	Plant death	Plant death	Plant death	Plant death	Shoot length	Vascular discolouration	Vascular discolouration
Experiment	1	2	1	1	2	2	1	1	2
Statistical procedure (SAS v. 8)	genmod	glm	glm	Chi-square[2]	glm	Chi-square	glm	genmod	genmod[3]
Factors									
Plant species	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Isolate	0.0032	0.0017	0.0005	0.021	0.012	0.22	0.084	0.0012	<0.0001
Plant species × isolate interaction	<0.0001	<0.0001	<0.0001	0.018	<0.0001	0.11[4]	<0.0001	<0.0001	<0.0001
Block	0.10	0.22	0.012	—[5]	0.27	—[5]	0.72	0.0011	0.27
Contrasts									
VCG NL-1 vs. VCG NL-2[6]	0.42	0.15	0.08	0.18	0.69	0.79	0.48	0.0031	0.041
S. vulgaris isolate vs. other isolates on *S. vulgaris*	0.18	—[7]	0.014	0.0001	—[7]	—[7]	0.0005	—[8]	—[7]
A. platanoides isolate vs. other isolates on *A. platanoides*	0.0002	0.14	0.66	0.49	0.86	0.87	0.27	0.54	0.0016
A. platanoides isolate vs. other isolates on all *Acer* species	<0.0001	0.035	0.27	0.46	0.97	0.95	0.73	0.88	0.0021

[1]AUDPC = area-under-the-disease-progress curve.
[2]Chi-square test was performed to check binominal plant death data analysis.
[3]To facilitate convergence of the statistical procedure for the contrast statements, two plant species × isolate combinations were excluded, viz., isolate G3 (VCG NL-1) on *A. campestre* and S12,2 (VCG NL-2) on *C. coggygria*. All plants of these two combinations were scored in the highest vascular discolouration class.
[4]Data for *R. pseudoacacia* and *R. canina* were omitted during calculation of the Chi-square, because none of the plants of those species died.
[5]Blocks were pooled during calculation of the Chi-square.
[6]VCG = vegetative compatibility group.
[7]*S. vulgaris* was tested in Experiment 1 only.
[8]*S. vulgaris* did not show vascular discolouration.

inoculated with this isolate died (Table 2), while none of the *S. vulgaris* plants inoculated with other isolates died (glm $P = 0.014$; Chi-square $P = 0.0001$). This isolate also caused the largest reduction in shoot length (25%) on *S. vulgaris* ($P = 0.0005$). The same isolate was the most virulent on *C. coggygria* causing the severest symptoms and 100% mortality in both experiments (Table 2).

Contrasts for isolate AplatI mc-1, originally isolated from *A. platanoides*, *versus* other isolates showed that this isolate caused more disease symptoms in Experiment 1 and more vascular discolouration in Experiment 2 than other isolates tested on *A. platanoides*. Tested against all other isolate × plant species combinations, AplatI mc-1 on *A. platanoides* caused above average severe wilt symptoms in Experiment 1 ($P < 0.0001$) and Experiment 2 ($P = 0.0013$). The same isolate tested on all three representatives of the *Acer* genus caused significantly more disease symptoms in Experiment 1 ($P < 0.0001$) and Experiment 2 ($P = 0.035$) than other isolates on these hosts (Table 3).

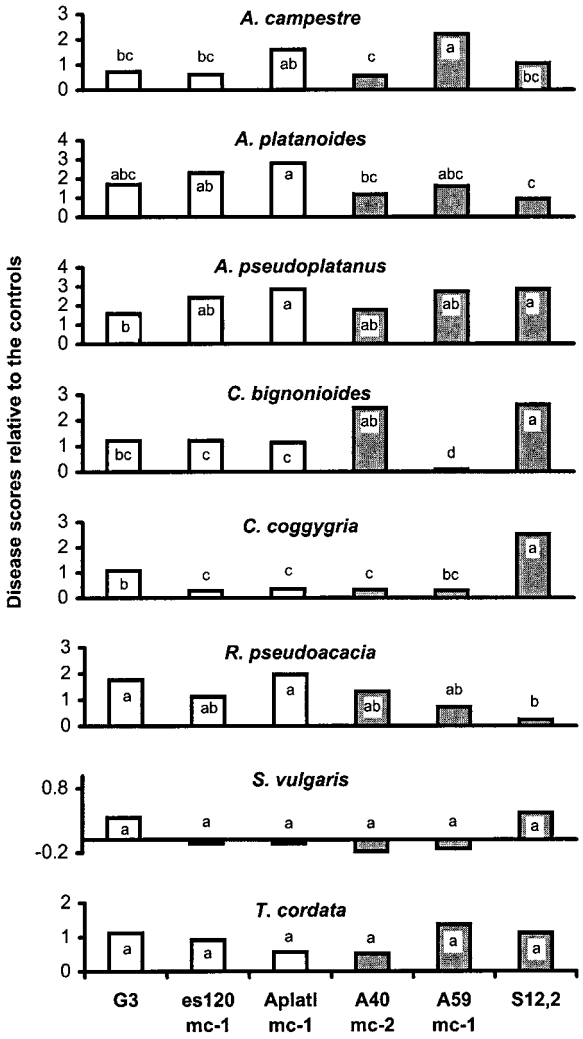

Figure 3. Disease scores of Experiment 1 for the different isolates of *V. dahliae*, presented as averages after subtraction of the average control values per plant species. The first three isolates represent VCG NL-1 (white bars), the last three isolates represent VCG NL-2 (grey bars). Different characters within plant species indicate significant differences (overall $P < 0.05$; $P < 0.0033$ per comparison). Statistics were performed on the original data as described in the Materials and methods section.

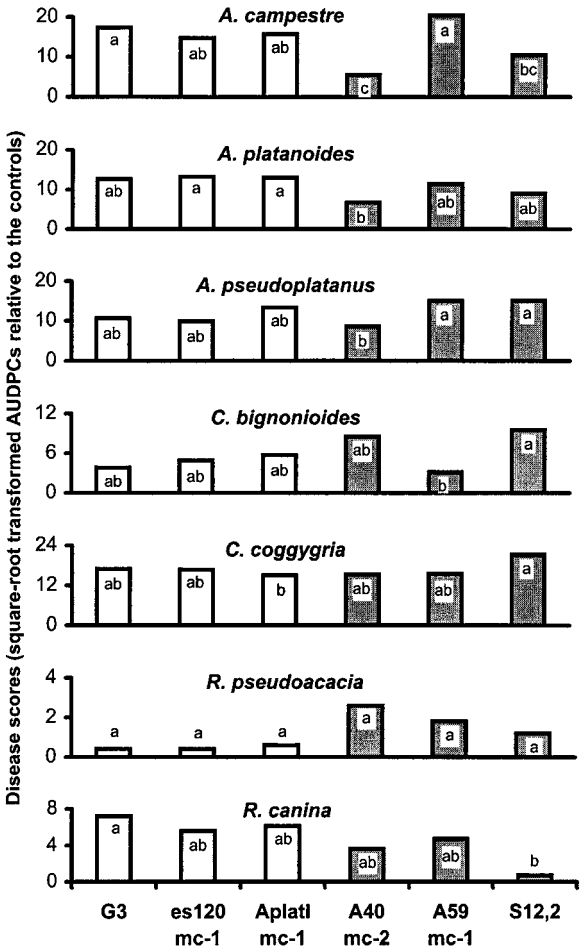

Figure 4. Square-root transformed AUDPCs of Experiment 2 for the different isolates of *V. dahliae*, presented as averages after subtraction of the average control values per plant species. The first three isolates represent VCG NL-1 (white bars), the last three isolates represent VCG NL-2 (grey bars). Different characters within plant species indicate significant differences (overall $P < 0.05$; $P < 0.0033$ per comparison). Statistics were performed as described in the Materials and methods section.

Discussion

The observation that isolates from each of the VCGs can cause verticillium wilt in woody hosts, and that no difference in virulence between VCGs was found has major consequences for disease prediction in woody hosts. Absence of a link between VCG and virulence implies that VCG determination of the population of *V. dahliae* present in infested soil does not contribute to disease forecasting in woody hosts in general. This was expressed clearly in all external disease symptoms and was repeated in both experiments. Other unknown characteristics at the individual isolate level were found to be of higher importance than VCGs.

No reports exist on virulence testing of VCGs on a range of woody hosts. Bhat and Subbarao (1999) tested 14 herbaceous crops and observed no correlation among VCG and reduction of plant length and root and shoot dry weight. Unfortunately, data for external

wilt symptoms are not presented in their paper. Gennari et al. (2000) found no differences in virulence between three Italian VCGs tested on tomato and melon. On the other hand, virulence differences between VCGs were reported frequently for individual host species, e.g., for potato (Joaquim and Rowe, 1991; Strausbaugh, 1993; Strausbaugh et al., 1992) and cotton (Daayf et al., 1995; Korolev et al., 2000), though sometimes different results were obtained when isolates from different countries were used.

We repeatedly observed more severe vascular discolouration caused by isolates of VCG NL-1 than that caused by isolates of VCG NL-2. This is an indication that different VCGs can cause differential reactions in plants. Apparently, these reactions do not affect wilt symptoms and plant length. Our findings are not in agreement with those of Bhat and Subbarao (1999), who observed no difference in vascular discolouration between VCGs. Vascular discolouration differs from other symptoms because it can extend beyond infected tissue (Mace, 1989) and it is permanent: if the plant recovers from the disease by formation of new vascular tissue, external symptoms can disappear, but vascular discolouration of the older xylem tissue remains visible. Vascular discolouration is generally absent in *S. vulgaris* (Van der Meer, 1925) and can be present (Nienhaus et al., 1992) or absent (McCain, 1976) in *R. canina*.

Isolates within VCGs can vary considerably with respect to virulence (Bao et al., 1998; Elena, 1999; Gennari et al., 2000). All isolates used in the present study were not absolutely host specific, despite the highly significant plant species × isolate interaction. Some degree of specificity was visible in most of the isolates, e.g., in the two isolates that were more virulent on their original host. This latter phenomenon has been reported before (Douhan and Johnson, 2001; Mol, 1995; Resende et al., 1994; Sinclair et al., 1989), and also in *Acer* (Adams and Tattar, 1976; Hiemstra, 1995), though host specificity appears not to be a rigid character (Jeger et al., 1996). Fordyce and Green (1963) have shown, through repeated inoculation and isolation, that isolates from peppermint that were not pathogenic to tomato became pathogenic after one or more passages through tomato plants. Quantitatively, potato is the most important host in the Netherlands, because *V. dahliae* can increase well on this host. Van der Meer (1925) already observed that woody and herbaceous plants on former potato fields were often infected. The potato isolate used in this study had an average virulence, compared with the other isolates. Adams and Tattar (1976) observed that isolates from potato, tomato, and chrysanthemum were less pathogenic on *A. saccharum* than isolates from woody hosts. More potato isolates should be tested to discover trends.

Recovery of diseased *C. bignonioides* and *R. pseudoacacia* plants in Experiment 2 is most probably caused by the high air temperatures in the greenhouse. *V. dahliae* hardly grows at temperatures above 30 °C (Schnathorst, 1981), whereas *C. bignonioides* and *R. pseudoacacia*, the two most thermophilic plant species tested, reacted with rapid growth resulting in recovery from the disease. Other plant species tested did not grow faster at high temperatures. Isolation of *V. dahliae* from recovered *C. bignonioides* and *R. pseudoacacia* plants at the end of the experiment showed that the fungus was still present. Recovery of infected trees during hot summer periods has been reported for apricot (Taylor and Flentje, 1968), avocado (Latorre and Allende, 1983; Zentmyer, 1949), and olive (Wilhelm and Taylor, 1965). Recovery of diseased trees should be further examined, focusing on unravelling the mechanisms involved and gathering quantitative information to get insight about the relative importance of recovery.

The highly significant differences between plant species indicate the importance to test a range of species. VCG determination may be useful only for disease prediction in specific crops. More isolates need to be tested, because it is unclear whether three isolates is a sufficiently large number to represent an entire VCG. Future research also needs to investigate whether most isolates of VCG NL-2, like the tester isolates, are compatible with American VCG-1 and VCG-2 tester isolates, thus being an entire bridging population. A situation like that exists in *Armillaria* where three North American intersterility groups are partially (two groups) or fully (one group) interfertile with one European intersterility group (Anderson *et al*., 1980; Guillaumin *et al*., 1991). Research on disease prediction for a range of woody hosts should focus on molecular characterisation of isolates to discover virulence genes. For example, differentiation between the defoliating and non-defoliating strains of *V. dahliae* has been shown for cotton (Pérez-Artéz et al., 2000) using random amplified polymorphic DNA analysis. This type of approach could help to discover genes that contribute to virulence. Because of significant effects of both isolate and isolate × plant species observed in our study, one should search for genes involved with virulence

to woody hosts in general, and virulence to specific (woody) plant species.

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