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Vegetative compatibility groups in *Verticillium dahliae* isolates from the Netherlands as compared to VCG diversity in Europe and in the USA

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Abstract

In a study of vegetative compatibility in *Verticillium dahliae* in the Netherlands, a collection of 45 isolates including representatives from woody hosts, several horticultural crops and from the soil of potato fields was examined. In addition an effort was made to compare vegetative compatibility groups (VCGs) from different countries. The results of this study indicate that VCG diversity in *V. dahliae* in the Netherlands is limited. Only two VCGs were detected: VCG NL-I and VCG NL-II. The former is the predominant VCG for isolates from tree hosts. However, Verticillium wilt in trees can be caused by isolates from both VCGs. It is suggested that the predominance of VCG NL-I in tree hosts is the result of the origin of the tree and the cropping history of its growing site, rather than trees being preferential hosts for isolates from this VCG. Comparison of VCG testers from the Netherlands, from several other European countries and from the USA show that in Europe two major VCGs are present. The first one, including NL-I, is compatible with USA VCG 3 and VCG 4, whereas the second one, including NL-II, is compatible with USA VCG 1 and VCG 2. These groups are not completely separated; in some cases, testers formed heterokaryons with VCG testers from both main groups. Because of the presence of these bridge isolates and because mutants from the same isolate differ in ability to form heterokaryons, it is emphasised that careful selection of isolate testers is an essential step to get a clear picture of VCG diversity.

Introduction

Verticillium dahliae is a vascular pathogen causing wilt diseases in numerous plant species worldwide. Its wide host range (Smith et al., 1988) includes important field crops (e.g. potato and cotton), horticultural crops (e.g. tomato and strawberry), cut flowers (e.g. rose) and many tree species. Among the latter are fruit trees, such as stone fruits (*Prunus* spp), olive, pistachio, avocado and cocoa, shade trees, such as maple and Catalpa, and ornamentals such as lilac (Sinclair et al., 1987).

In the Netherlands, Verticillium wilt is a serious problem in tree nurseries growing shade trees and ornamental species. Norway maple (*Acer platanoides*), grown as selected cultivars grafted on seedling rootstocks, is often affected. There are no effective therapeutic measures to control the disease (Smith et al., 1988). Soil fumigation with steam or chemicals like methyl bromide prior to planting may be effective, but cost and environmental limitations do not allow large-scale application of these techniques. Soil solarisation, as practised in Mediterranean countries, is not feasible in the Netherlands because of climatic constraints.

The Dutch tree breeding industry is expanding. However, many soils suitable for tree growing have a complex history of agricultural use, often including potato crops. Because *V. dahliae* is common in potato fields, this means that introduction of healthy planting stock in disease-free soil is often not possible. In such

cases, the only alternative is use of resistant species or varieties, especially because of the prolonged period of exposure as a result of the host's long life. All Norway maple cultivars commonly used in the Netherlands (Hiemstra, unpublished data) as well as the rootstocks used (seedlings from the same species) are susceptible to Verticillium wilt. In 1993 a research project was initiated to select Verticillium-resistant rootstocks. Efficient selection and screening methods have been developed and a number of promising seedlings have been selected (Hiemstra, 2000). Several of these individuals have been developed into small clones which now are being tested for their value as resistant rootstocks. For a reliable test, however, the genetic variation within the population of the pathogen must be also considered.

One way to characterise genetic variation in fungi without a sexual reproductive stage, such as *Verticillium*, is to place isolates into vegetative compatibility groups (VCGs) (Rowe, 1995). Hyphae of isolates belonging to the same VCG can anastomose and form stable heterokaryons with one another, whereas hyphae from isolates belonging to different VCGs cannot. This mechanism is the only known means of genetic exchange between individuals of these fungi (Heale, 1988). As the isolates from different VCGs are genetically separate from each other, this allows fungal populations to be subdivided into distinct natural groups (Leslie, 1993).

During the last decades many workers used this approach to study genetic diversity in *V. dahliae*. Puhalla and Hummel (1983) found 16 VCGs by using microsclerotial colour mutants. Later, by using nitrate non-utilising mutants (*nit* mutants), the number of VCGs in the same collection of isolates was reduced to four: VCG 1, VCG 2, VCG 3 and VCG 4 (Joaquim and Rowe, 1990). Other workers incorporated the isolates of VCG 3 into VCG 4 and created a new group, named VCG 5 (Strausbaugh et al., 1992) or distinguished three subgroups, VCG 4A, VCG 4B and VCG 4A/B within VCG4 (Joaquim and Rowe, 1991).

Genetic subgroupings within a pathogen become of practical importance when other features such as virulence or host range can be related to them. *Verticillium dahliae* isolates from one host can infect many other hosts (e.g. Subbarao et al., 1995). However, a certain degree of host specificity has been reported in a few strains. For example, two races were distinguished on tomato, depending on interaction with cultivars containing the Ve resistance gene (Schaible et al., 1951). On cotton, defoliating and non-defoliating strains have been described (Schnathorst and Mathre, 1966) and these differences were reflected in the VCGs of the isolates (Daayf et al., 1995). The defoliating strains from cotton belonged to VCG 1 whereas non-defoliating strains belonged to VCG 2. In tomato, race-1 strains were placed in VCG 2 whereas strains of race 2 belonged to VCG 4. Also, VCG 4 seems to be a common VCG for solanaceous isolates (Jeger et al., 1996), whereas VCG 1 was predominant in woody hosts in the USA (Chen, 1994).

Because of the work of the first author on Verticillium resistance in trees, we were interested in the genetic variation in *V. dahliae*. To come to reliable selection and screening methods, information on genetic variation of *V. dahliae* in the Netherlands as well as information on how this relates to global variation was needed. Our objectives were: (1) to assess VCG diversity in a collection of isolates from tree hosts in the Netherlands; (2) to get an impression of total diversity in the Netherlands by comparing the diversity found in tree isolates to that in isolates collected from additional hosts; and (3) to relate the VCGs found in isolates from the Netherlands to those reported from other countries.

Materials and methods

The work was carried out in three main steps. Firstly, the VCGs in a group of isolates from woody hosts in the Netherlands were established. From each isolate a large number of *nit* mutants was generated and one or two pairs of mutants with the highest capacity to form heterokaryons were selected as isolate testers. Testers from all isolates were paired to assess the VCGs present in this group of isolates. Secondly, the variation found was compared to that in a second group of isolates from other hosts and locations in the Netherlands. For this purpose VCG testers selected from the first group were paired with isolate testers from the second group of isolates. Thirdly, the VCGs found in *V. dahliae* isolates from the Netherlands were related to those found in other countries by pairing VCG testers from the Netherlands with VCG testers from those countries.

Group 1 isolates

This group consisted of nine isolates from ash (*Fraxinus excelsior*), ten isolates from several other tree species and five isolates from potato field soils (Table 1). The ash isolates were taken from a collection of isolates established in 1988 during a nation-wide

Table 1. Data on isolates of *V. dahliae* used in this study

Isolate number	Initial designation	Host species or substrate of origin	Location[a]	Plantation type[b]	Year of isolation	Source[c]
Group 1						
1	HPSS-L	Soil of potato field	Dronten, Fl	1	1986	1
2	Tilia 1	*Tilia* sp.	Sevenum, Li	2	1993	2
3	Robinia	*Robinia pseudoacacia*	Sevenum, Li	2	1993	2
4	A.palm.	*Acer palmatum*	Horst, Li	2	1994	2
5[d]						
6	Tilia	*Tilia* sp.	Horst, Li	2	1994	2
7	F. angust.	*Fraxinus angustifolia*	Sevenum, Li	2	1993	2
8	A. capp. Azw	*Acer cappadocicum*	Sevenum, Li	2	1993	2
9	A. plat. I	*Acer platanoides*	Sevenum, Li	2	1993	2
10	A. capp. Awit	*Acer cappadocicum*	Sevenum, Li	2	1993	2
11	A. plat. II	*Acer platanoides*	Sevenum, Li	2	1993	2
12	A. plat.	*Acer platanoides*	Horst, Li	2	1994	2
13	es 136	*Fraxinus excelsior*	Amstelhoek, Ut	4	1988	2
14	es 142	*Fraxinus excelsior*	Spaarnwoude, NH	3	1988	2
15	es 107A	*Fraxinus excelsior*	Heiligerlee, Gr	3	1988	2
16	es 120	*Fraxinus excelsior*	Lelystad, Fl	3	1988	2
17	es 54A	*Fraxinus excelsior*	Zeewolde, Fl	3	1988	2
18	es 95	*Fraxinus excelsior*	Wageningen, Ge	4	1988	2
19	es Lim 1	*Fraxinus excelsior*	Rijckholt, Li	3	1988	2
20	es 24	*Fraxinus excelsior*	Zeewolde, Fl	3	1988	2
21	es 22	*Fraxinus excelsior*	Zeewolde, Fl	3	1988	2
22	57-2a	Soil of potato field	Lelystad, Fl	1	1995	1
23	58-2b	Soil of potato field	Lelystad, Fl	1	1995	1
24	60-la	Soil of potato field	Lelystad, Fl	1	1995	1
25	GS	Soil of potato field	Lelystad, Fl	1	1995	1
Group 2						
26	Afw.D10	*Fraxinus excelsior*	Wageningen, Ge	4	1988	2
27	A.plat. Hon	*Acer platanoides*	Hungary[e]	2	1996	2
28	es de Goor	*Fraxinus excelsior*	Wageningen, Ge	4	1993	2
29	S1	*Syringa vulgaris*	Aalsmeer, NH	5	1996	2
30	S12	*Syringa vulgaris*	Aalsmeer, NH	5	1996	2
31	F17	*Forsythia intermedia*	Amstelveen, NH	5	1996	2
32	F19	*Forsythia intermedia*	Amstelveen, NH	5	1996	2
33	V7	*Rosa* sp.	de Kwakel, NH	6	1996	3
34	V8	*Fragaria ananassa*	America, Li	5	1996	3
35	V9	*Fragaria ananassa*	Horst, Li	5	1997	3
36	V11	*Rosa* sp.	Horst, Li	5	1996	3
37	V12	*Rosa* sp.	Luttelgeest, Fl	6	1996	3
38	V17	*Phlox* sp.	Noordwijk, ZH	5	1996	3
39	50	*Chrysanthemum* sp.	Kwintsheul, ZH	6	1988	3
40	215	*Chrysanthemum* sp.	Nieuwe Wetering, Ut	6	1993	3
41	236	*Chrysanthemum* sp.	Unknown	6	1994	3
42	MC 1	*Rubus* sp.	Unknown	5	1996	1
43	MC-J-1	*Ribes rubrum*	Unknown	5	1996	1
44	MC-A-G-3	Soil of potato field	Wageningen, Ge	1	1994	1
45	MC-G-1	Soil of potato field	Wageningen, Ge	1	1994	1

[a]Nearest town and province.
[b]Type of plantation or culture in which host was grown: 1 = potato field; 2 = tree nursery; 3 = forest stand; 4 = shade or amenity tree; 5 = horticultural field; 6 = greenhouse crop.
[c]1 = Wageningen Agricultural University, Department of Phytopathology; 2 = first author; 3 = NAKB-Laboratory, Roelofarendsveen.
[d]Isolate No. 5 was not used because it appeared to be *V. albo-atrum* instead of *V. dahliae.*
[e]Young tree imported from tree nursery in Hungary.

survey for ash wilt disease in forest plots and landscape plantations (Hiemstra, 1995). Isolates from several additional woody hosts were collected from diseased trees in tree nurseries in 1993 or 1994. In all cases, *V. dahliae* was isolated from the xylem of young branches of trees showing defoliation or loss of leaves. Isolates were stored as small discs (5–8 mm diameter) from microsclerotial colonies on malt extract agar (MEA) plates kept in sterile water in 30-ml flasks stored at about 4 °C (refrigerator) in the dark. The potato strains were isolated from soil samples of several different fields by means of the soil dilution technique (Nadakavukaren and Horner, 1959). These isolates were maintained on potato dextrose agar (PDA) at 4 °C.

Group 2 isolates

This group consisted of 15 isolates from horticultural crops, including cut flowers and flowering or fruiting shrubs, three isolates from tree hosts and two isolates from potato (Table 1). The strains from tree hosts and from lilac and *Forsythia* were isolated and stored as described above. The other isolates originated from samples of diseased plants sent to the laboratories of the Dutch Plant Health Service and the former NAKB (now Naktuinbouw) by commercial growers. These isolates were cultured on PDA.

Generation and characterisation of nit *mutants*

Monoconidial cultures on MEA or PDA were prepared from all isolates. The procedure developed by Cove (1976) and modified by Puhalla (1985) and Rataj-Guranowska et al. (1993) was adapted to recover *nit* mutants. Mycelial transfers from monoconidial cultures of group 1 isolates were placed onto PDA, cornmeal agar (CMA) and minimal medium (MM) amended with 1.6 g l^{-1} L-asparagine (Correll et al., 1987) and up to 60 g l^{-1} $KClO_3$ (two plates of each medium) and incubated at 24 °C in the dark. After 2–3 weeks of incubation, mycelial fragments from up to 60 putative mutants (faster growing sectors) per isolate were transferred to small Petri dishes (5 cm diameter) with MM and stored at 5 °C for later use. All putative mutants were transferred to 10-cm plates with MM on which they were grown for several weeks at 24 °C in the dark to test their mutant character and stability. Each plate was inoculated at 21 points with individual putative mutants (*cf.* Rataj-Guranowska et al., 1993). Appearance of complementing pairs of mutants on these plates (*in-situ* complementation) was recorded 7–30 days after inoculation. At the same time, the phenotype of all mutants was characterised by subculturing on MM and MM with hypoxanthine (H) replacing $NaNO_3$ as N source. All plates were incubated at 23–25 °C in the dark. Colony morphology was assessed after 4–7 days of growth. Mutants that formed thin, expansive colonies on both MM and H-medium were classified as *nit*M, whereas mutants that grew sparsely on MM but profusely on H-medium were called *nit*1. In preliminary experiments differentiation between *nit*1 and *nit*3 appeared problematic because many mutants did not show any growth on basal medium amended with nitrite, a phenomenon also reported by Strausbaugh et al. (1992) and Chen (1994). Therefore, no further attempts were made to distinguish between *nit*1 and *nit*3. Consequently, mutants referred to as *nit*1 may include *nit*3 mutants. Because the objective was to assess vegetative compatibility and the *nit*1 (or *nit*3) mutants were paired with *nit*M mutants this does not compromise the objective.

The procedure for generation of *nit* mutants from group 2 isolates was largely the same as for the isolates in group 1, but fewer mutants were collected (up to about 30 per isolate). All *nit* mutants were generated on MM amended with 1.6 g l^{-1} L-asparagine and 5% $KClO_3$. All putative mutants were transferred to MM to test their mutant character and stability. After 2–3 weeks the phenotype of the remaining (i.e. not reverted) mutants was characterised by subculturing them on MM and H-medium as described for group-1 isolates.

Selection of 'isolate testers'

It is possible to use different combinations of *nit* mutants for VCG characterisation of isolates. This is usually done by combination of *nit*1 and *nit*M mutants (Correll et al., 1987). Combination of *nit*M with *nit*M has also been reported to be useful for efficient formation of heterokaryons (Baayen and Kleijn, 1989; Rataj-Guranowska et al., 1993). Taking this observation into account, we aimed at selection of at least one good *nit*M/*nit*1 or *nit*M/*nit*M pair.

For group-1 isolates, the results of the *in-situ* complementation on MM plates (see above) were used for a first, preliminary selection of mutants. Only the *nit*1 mutants from complementing pairs of mutants observed on the MM plates and all *nit*M mutants were taken into consideration. With these mutants for each isolate, all possible *nit*M–*nit*1

and *nit*M–*nit*M combinations were made on new MM plates. Heterokaryon formation was recorded after 14 days and, if possible, one or two 'pairs of isolate testers' (i.e. mutants to be used in complementation tests between isolates) were chosen for each isolate from the stable mutants that produced good heterokaryons most frequently.

For group 2 isolates, all available stable mutants per isolate were used in the complementation experiments to select isolate testers. Again, at least one *nit*M–*nit*1 or *nit*M–*nit*M pair was chosen for each isolate. If this appeared not possible, two *nit*M mutants, or else one or two *nit*1 mutants, were chosen for complementation tests between isolates.

Complementation within group 1 and selection of VCG testers

All possible *nit*1–*nit*M and *nit*M–*nit*M combinations were made using the isolate testers of all isolates in this group. Pairings were made on MM at 23 °C and repeated at least once. The degree of complementation was recorded using five classes: (1) very strong (profuse growth of aerial mycelium); (2) strong (with substantial aerial mycelium); (3) weak (aerial mycelium much more limited but clearly visible); (4) very weak (with an often uncertain zone of sparse mycelium submerged in the agar); and (5) no prototrophic growth. Complementation classes 1, 2 and 3 were considered as positive reactions. Class 4, being very weak or uncertain, was checked once again and considered negative if the result was class 4 or 5. Results for each isolate were pooled. If one or more mutants from an isolate were compatible with one or more mutants from another isolate, isolates were considered to belong to the same VCG. Finally the results of the individual crossings between isolate testers were used to select tester mutants representing the observed vegetative complementation groups. These tester mutants were used as VCG testers during the rest of the study.

Complementation between VCG testers from group 1 and isolate testers from group 2

The VCG testers from group 1 were combined with the isolate testers of each isolate in the second group. All possible *nit*1–*nit*M and *nit*M–*nit*M combinations were made twice and heterokaryon formation was examined after 10 and 14 days using the same five classes as in group 1.

Complementation between VCG testers from the Netherlands and those from other countries

The NL testers were paired with testers received from the USA, UK, Greece and Spain (Table 2). Testers were inoculated at 15 points on MM in 10-cm Petri dishes (Rataj-Guranowska et al., 1993). All possible combinations between NL testers and testers from other countries were repeated at least once. Heterokaryon formation was scored after 7, 14 and 21 days.

Results

Generation of nit *mutants*

Initial efforts to generate *nit* mutants from group-1 isolates on media with 2.5% chlorate yielded low numbers of mutants (0–6 mutants per isolate). No substantial differences were observed in the numbers of mutants recovered on the different media used (data not shown). Increasing the chlorate concentration to 5% or 6% (most isolates) led to a much higher frequency of *nit* mutants. Collection of mutants was repeated for 17 isolates and this time from 25 up to 62 mutants per isolate were collected. A total of 766 putative mutants was collected from the isolates in group 1. Generally, the number of mutants collected from group-2 isolates was lower than with group 1. In this group no *nit* mutants could be recovered from one isolate (No. 40), fewer than 15 *nit* mutants were obtained from three isolates, and 15 up to 37 mutants were recovered per isolate from the remaining 16 isolates.

Characterisation of mutants

Mutants from group-1 isolates usually were characterised within 1 week after collecting them from the chlorate-amended medium. Even then part of the putative mutants reverted to prototrophy before their phenotype could be characterised. From the 575 true mutants, that were stable enough to be characterised, 134 (23%) were of the *nit*M type. The percentage of *nit*M mutants per isolate, however, varied from 4% to 60%. With group-2 isolates the time between collection and characterisation of mutants varied with the isolate from 2 weeks up to 3 months. As a result, the number of mutants that reverted before being characterised was higher than in group 1. One isolate (No. 44) produced only *nit*M mutants, and many isolates yielded

Table 2. Data on VCG testers of *V. dahliae* from other countries used in this study

Country	Local VCG designation	Isolate No.	Isolated from	Year of isolation	Tester strains	
					*nit*1	*nit*M
Greece	I	1V	*Gossypium* sp.	1993	$1V_{10}$	$1V_3$
Greece	Ii	7V	*Lycopersicon esculentum*	1993	$7V_4$	$7V_6$
Greece	Ii	8V	*Lycopersicon esculentum*	1993	$8V_6$	—
Greece	I	30V	*Lycopersicon esculentum*	1988	—	$30V_3$
UK	β	320[b]	*Fragaria ananassa*	1985	—	—
UK	α	327	*Fragaria ananassa*	1986	B1	C2
UK	β	F3[s]	Soil	1989	I1	H1
UK	α	F4[1]	Soil	1989	—	D1
UK	α	F6[s]	Soil	1989	5a	—
USA	1	V44	*Gossypium* sp.	—	491	492
USA	2	PH	*Pistacia vera*	—	495	496
USA	2	115	*Gossypium* sp.[a]	—	497	498
USA	3	PCW	*Capsicum* sp.	—	499	500
USA	3	70–21	—	—	501	502
USA	4A	BB	*Solanum tuberosum*	—	503	504
USA	4B	S39	Soil	—	505	506
Spain	ND[c]	50S-3	*Gossypium* sp.	—	50S-3 nit1[b]	50S-3 nitM
Spain	D[d]	58S-2	*Gossypium* sp.	—	58S-2 nit1[b]	58S-2 nitM

[a]Isolate from Syria.
[b]Tester mutant reverted before it could be used.
[c]Non-defoliating isolate, USA VCG 2A; new code No. V214 l.
[d]Defoliating isolate, USA VCG 1; new code No. V220 l.

no or only a few *nit*M mutants. However, the average percentage of *nit*M mutants in this group (28%) was about the same as in group 1.

Selection of 'isolate testers'

The extent of prototrophic growth after pairing of compatible mutants from one isolate varied with the mutants used. In group 1, from two up to 11 mutants per isolate (all *nit*M mutants and all *nit*1 mutants involved in *in-situ* complementation on the MM plates during assessment of mutant stability) were used in new pairings to select isolate testers. One isolate (No. 20) appeared to be self-incompatible. From this isolate, one *nit*1 and one *nit*M mutant were selected at random for use as isolate testers. From all other isolates, at least one pair of mutants, selected from those that were most efficient in formation of heterokaryons, were chosen as isolate testers. In some cases after some time one or two new testers had to be chosen because of reversion of the first tester pair. As a result, 65 isolate testers (2–4 per isolate) were used in the pairings between isolates. In group 2, two to four mutants per isolate (i.e. a total of 64 mutants: 44 *nit*1/3 and 20 *nit*M) were selected as isolate testers. As often as possible *nit*1–*nit*M pairs were selected. However, as a result of the scarcity of *nit*M mutants, for seven isolates only *nit*1 isolate testers were available. In these cases at least two, and usually three, *nit*1 mutants were selected as isolate testers.

Complementation within group 1 and selection of VCG testers

After pooling the results for the different testers of one isolate, two VCGs were identified (Table 3). Twenty-two out of the 24 isolates tested, including No. 20, considered self-incompatible, were shown to belong to one VCG, provisionally called NL-I. One isolate (No. 18) was considered to belong to another VCG (NL-II) because it was self-compatible, but its testers were not compatible with selected mutants of any of the other isolates. Five tester pairs (four from NL-I, obtained from three different isolates and one from NL-II) were selected as VCG testers for pairing with group 2 isolate testers.

Complementation between VCG testers from group 1 and isolate testers from group 2

One isolate (No. 26) could not be assigned to either VCG NL-I or NL-II, because its testers did not

Table 3. Results of pairing of isolate testers within group 1 of *V. dahliae* isolates[a]

Isolate		**6**	**6**	9	9	10	10	**11**	**11**	**11**	11	13	**13**	**25**	25	**18**	18	**18**	18
testers[2,3]		**2**	**3**	1	2	2	4	**2**	**21**	**10**	20	20	**44**	**4**	21	**34**	25	**36**	20
1	1	++	++	++	−	++	−	−	−	++	−	++	++	+	+	−	−	−	−
2	8	+	±	−	−	++						−	++			−	−	−	−
3	1	+	±	++	++	+	−	−	+	−	−	++	+	−	+	−	−	−	−
4	9	+	++	−	−			++	++	−	++	−	++	++	+	−	−	−	−
4	10	−	±	+	++	++	±	++	++	±	++	++	++	+	−	−	−	−	−
6	**2**	−	+	++	++		++	++	++	−	−	++	++	++		−	−	−	−
6	**3**	+	−	±	−	+	−	+	−	−	−	−	−	++		−	−	−	±
7	20	−	−	±	+	−	+	−	++			−	++	−	±	−	−	−	−
8	4	+	−	−	++	+	+	−	−	−	±	++	−		±	−	−	−	−
8	20	−	++		+	−	++	−	+	+	++	++	−	+	+	±	−	−	±
9	1		−	−	++	++	−	++	++	±	++	++	++	+	++	−	−	−	−
9	2	±	−	++	−	±	±	+	++	±	±	++	++	+	++	−	−	−	−
10	2		+	++	±	−	++	−		++	++	±	++	±	±	±	−	−	−
10	4	++	−	++	±	++	−	−	−	±	±	±	++	±	−	±	−	−	−
11	**2**	++	+	++	+	−	−	−	++	++	++	−	++	±	±	−	−	−	−
11	**21**	++	−	++	++		−	++	−	−	±	++	++	++	++	−	−	−	−
11	**10**	−	−	±	±	++	±	++	−	−	−	?	±	±	−	−	−	−	−
11	20			++	±	++	±	++	±	−	−	−	++	++	±	−	−	−	−
12	9	++	−	−	±	++	++	−	++	−	−	−	−			−	−	−	−
12	11	++	−	±	−	+	++	±	++	−	−	−	−			−	−	−	−
13	20	++	−	++	++	++	++	++	++	±	++	−	++	++	−	−	−	−	−
13	**44**	++	−	++	++	++	++	++	++	±	++	++	−	++	++	−	−	−	−
15	2	−	−	++	++	−	−	+	++	+	−	++	++	++	±	−	−	−	−
15	6	++	−	++	++	++	−	±	++	++	−	++	++	−	±	−	−	−	−
16	4	++	−	++	−	++	−	±	±	−	±	++	−	++	−	−	−	−	−
16	5	++	±	+	±	−	±	−	++	−	++	−	++	++	−	−	−	−	−
17	6	−	−	++	+	−		±	++	±	++	++	−	+		−	−	−	−
19	26	++	−				−		++			−	++	+	+	−	−	−	−
21	9	++	+	−	−	++	++	++				++	++	+	++	−	−	−	−
23	28		+	−	−	+			++				++	±	++	−	−	−	−
24	47	−	−					−				−	+	−	−	−	−	−	−
25	**4**	++	++	+	+	±	±	±	++	±	++	++	++	−	++	−	−	−	−
25	21			++	++	±	−	±	++	−	±	−	++	++	−	−	−	−	−
18	**34**	−	−	−	−	±	±	−	−	−	−	−	−	−	−	−	++	++	++
18	25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	++	++
18	**36**	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	−	−
18	20	−	±	−	−	−	−	−	−	−	−	−	−	−	−	++	++	−	−

[a]Only part of the results are shown; that is, all combinations of testers of isolates 6, 9, 10, 11, 13, 25 and 18 (columns of the table) with all other isolate testers (rows); (++) very strong/strong complementation, (+) weak complementation, (±) very weak or uncertain complementation, (−) no complementation, (blank) complementation not tested.

[b]First number (top line and left column) refers to isolate, second number (second line and second column) is number of *nit* mutant from that isolate; that is, 1–1 means isolate 1, *nit* mutant 1, also 6–2 is isolate 6, *nit* mutant 2, etc.; bold numbers indicate mutants selected as VCG testers (see text).

[c]Isolates 14 and 20 are not included in the table because of the very limited data on these isolates; isolate tester 14–1 showed weak complementation with 25–4, whereas 20–38 showed weak complementation with 6–2 and no complementation with 6–3; data on other combinations not available.

complement any of the VCG testers used. Testers from 10 isolates formed heterokaryons with NL-II testers whereas the testers of another eight isolates clearly complemented NL-I testers. Complementation with testers from both NL-I and NL-II was never observed (Table 4).

Complementation between VCG testers from the Netherlands and those from other countries

Testers from three isolates, the UK isolate No. 320 and the two isolates from Spain (see Table 2), reverted before the pairings could be completed, resulting in

Table 4. Complementation between *V. dahliae* testers for VCG NL-I and NL-II and isolate testers of group 2 isolates (numbering of testers as in Table 3; nitM mutants are indicated in bold; ++ very strong complementation, + strong complementation, ± weak or uncertain complementation, − no complementation)

Isolate testers		VCG-testers										Isolate testers		VCG-testers									
		NL-I								NL-II				NL-I								NL-II	
		6 **2**	6 3	11 2	11 10	11 **21**	13 **44**	25 3	25 4	18 **34**	18 36			6 **2**	6 3	11 2	11 10	11 **21**	13 **44**	25 3	25 **4**	18 **34**	18 36
26	1	−	−	−	−	−	−	−	−	−	−	27	10	−	−	−	−	−	−	−	−	+	−
26	4	−	−	−	−	−	−	−	−	−	−	27	**11**	−	−	−	−	−	−	−	−	−	+
26	5	−	−	−	−	−	−	−	−	−	−	27	16	−	−	−	−	−	−	−	−	+	−
28	2	++	−	−	−	++	+	−	+	−	−	29	**9**	−	−	−	−	−	−	−	−	+	+
28	6	+	±	−	−	++	++	−	+	−	−	29	**14**	−	−	−	−	−	−	−	−	+	+
28	13	+	±	−	−	+	+	−	+	−	−	29	21	−	−	−	−	−	−	−	−	+	−
34	6	+	±	−	−	++	++	−	+	−	−	29	3g	−	−	−	−	−	−	−	−	+	−
34	14	+	+	−	−	++	++	−	+	−	−	30	6	−	−	−	−	−	−	−	−	+	−
34	24	++	±	−	−	+	+	−	+	−	−	30	**8**	−	−	−	−	−	−	−	−	+	+
35	**1**	−	++	++	++	++	++	+	+	−	−	30	**20**	−	−	−	−	−	−	−	−	+	+
35	**3**	−	++	++	++	++	++	+	+	−	−	30	6g	−	−	−	−	−	−	−	−	+	−
35	5	++	±	−	−	++	++	−	+	−	−	31	8	−	−	−	−	−	−	−	−	+	−
35	12	++	+	−	−	+	+	−	+	−	−	31	**9**	−	−	−	−	−	−	−	−	+	+
37	2g	+	+	−	−	+	++	−	+	−	−	31	**13**	−	−	−	−	−	−	−	−	+	+
37	6g	++	−	−	−	++	+	−	+	−	−	31	3g	−	−	−	−	−	−	−	−	+	+
38	10	++	+	−	−	++	++	−	+	−	−	32	**3**	−	−	−	−	−	−	−	−	+	+
38	18	++	+	−	−	++	+	−	+	−	−	32	12	−	−	−	−	−	−	−	−	+	+
38	20	+	+	−	−	++	++	−	++	−	−	32	1g	−	−	−	−	−	−	−	−	+	−
41	2	+	±	−	−	+	+	−	+	−	−	32	11g	−	−	−	−	−	−	−	−	+	−
41	6	+	±	−	−	+	+	−	+	−	−	33	2	−	−	−	−	−	−	−	−	++	−
41	9	+	±	−	−	+	++	−	+	−	−	33	5	−	−	−	−	−	−	−	−	+	+
44	**2**	++	++	+	+	+	+	++	++	−	−	33	9	−	−	−	−	−	−	−	−	+	+
44	**9**	++	++	+	+	+	+	++	++	−	−	36	4	−	−	−	−	−	−	−	−	+	−
44	**11**	++	++	++	+	+	+	++	++	−	−	36	**9**	−	−	−	−	−	−	−	−	+	−
45	1	++	±	−	−	+	++	−	++	−	−	36	5g	−	−	−	−	−	−	−	−	+	+
45	3	++	±	−	−	+	++	−	++	−	−	39	**2**	−	−	−	−	−	−	−	−	++	++
45	9	++	±	−	−	+	++	−	++	−	−	39	4	−	−	−	−	−	−	−	−	++	−
45	**12**	++	+	++	++	−	++	+	++	−	−	39	10	−	−	−	−	−	−	−	−	++	−
												42	**3**	−	−	−	−	−	−	−	−	−	+
												42	**5**	−	−	−	−	−	−	−	−	+	+
												42	11	−	−	−	−	−	−	−	−	+	−
												42	13	−	−	−	−	−	−	−	−	−	+
												43	**1**	−	−	−	−	−	−	−	−	++	++
												43	**6**	−	−	−	−	−	−	−	−	++	++
												43	7	−	−	−	−	−	−	−	−	++	−
												43	8	−	−	−	−	−	−	−	−	++	−

(very) incomplete data for these testers. The results for the remaining testers are shown in Table 5. NL-I testers were compatible with testers of both VCG 3 and VCG 4 (A + B) from USA, VCG 1 from Greece and VCG β from UK. The testers of the NL-II group reacted strongly with VCG 1 and VCG 2 from USA, VCG 2 from Greece and VCG α from UK. In addition the testers from NL-II reacted weakly with testers VCG 3 and VCG 4 from USA and one of the tester pairs of NL-I combined very strongly with the testers of VCG 2 from USA.

Discussion

Individual fungal isolates are considered to belong to the same VCG when pairing of mutants from different isolates results in good heterokaryons. If this is impossible, the isolates are considered to belong to different VCGs (Joaquim and Rowe, 1990; Puhalla, 1985). Vegetative compatibility of isolates, however, does not imply that all mutants from one isolate will form heterokaryons with mutants from the other isolate. Also, several authors have reported that the

capacity of mutants for prototrophic growth is a characteristic of individual mutants and does not necessarily reflect that of their parent strains (Joaquim and Rowe, 1990; Chen, 1994). As a consequence, it is of utmost importance to use good tester mutants when assessing vegetative compatibility between isolates. Therefore, in this study we decided to generate many mutants per isolate (preferably more than 30) and select the best combining pairs of mutants before combining mutants from individual isolates.

The results of this study indicate that VCG diversity in *V. dahliae* in the Netherlands is limited. Only two VCGs were detected: VCG NL-I and VCG NL-II, with the former being the predominant VCG for isolates from tree hosts. The number of isolates tested was limited. However, they originated from different geographic areas and various woody as well as non-woody hosts grown in very different agricultural systems. Among the hosts were an arable crop (potato), horticultural crops grown in greenhouses (rose, chrysanthemum) or outside (rose, lilac, phlox, strawberry), tree nursery crops and forest trees. Because of the small sample size, it cannot be excluded that isolates belonging to other VCGs may be present in limited areas or specialised on certain hosts, but from the present results it can be concluded that in general VCG diversity in the Netherlands is limited.

Chen (1994) reported that in the USA VCG 1 is the predominant VCG for isolates from woody hosts. Our results show that in the Netherlands there is also a predominant VCG for isolates from woody hosts: VCG NL-I. Some isolates from trees and shrubs (isolates 18, 27, 29, 30, 31 and 32; see Table 1) belonged to a second VCG: VCG NL-II. These isolates, however, are from very specific situations. All lilac isolates come from one area, where this flowering shrub has been cultivated for over a century in a system that has some peculiar traits, including systematic wounding of the roots, moving plants to the greenhouse to force them into flowering and bringing them back to the same field after cutting the flowers, which strongly favour the build up of local *V. dahliae* populations. The *Forsythia* isolates are from the same region and therefore possibly from the same population. Of the two VCG NL-II isolates from shade trees, one (the maple isolate) was obtained from a tree imported directly from Hungary. This is probably an isolate from a population in another European area. The ash isolate is from a tree growing next to an experimental garden where many disease experiments including experiments with *V. dahliae* have been done, which makes its real origin uncertain.

In our study, VCG NL-I is the VCG of all *V. dahliae* isolates from forest trees as well as those from young nursery trees. This does not necessarily mean that woody hosts are preferential hosts (Daayf et al., 1995) for strains from VCG NL-I. It may indicate that *V. dahliae* is distributed from established populations in nurseries along with transplanted trees. When nurseries have been established on former agricultural land, which is a common situation in the Netherlands, this is likely because all potato isolates tested were in the same VCG as the tree isolates. The same mechanism has been suggested to be the cause of Verticillium in tree hosts in the USA (Sinclair et al., 1981). A similar situation was reported to cause serious infections in olive orchards in Greece (Thanassoulopoulos, 1993). In the Netherlands, Van der Meer (1925) reported that woody hosts as well as herbaceous plants on former potato fields were often infected. Apparently, for isolates from tree hosts, the origin of the tree and the cropping history of its growing site are of more importance for the VCG of specific isolates than the host species.

The presence of some VCG NL-II isolates in the group of isolates from woody hosts shows that isolates from both VCGs can be pathogenic to trees. Also the predominant VCG in trees in the Netherlands (VCG NL-I) was not compatible with VCG1, the group that was reported by Chen (1994) to be predominant in trees in the USA. Therefore, it can be concluded that Verticillium wilt in trees can be caused by isolates from different VCGs. Nevertheless the results provide information of use in breeding and selection for Verticillium resistance in trees. Apparently isolates from VCG NL-I are the only group found in practice in woody hosts in the Netherlands. Therefore, as long as detailed information on virulence and host specificity characteristics of isolates in different VCGs is lacking, the emphasis should be on VCG NL-I when selecting isolates to be used in selection and screening for resistance.

The limited VCG diversity observed in the Netherlands corresponds well with reports from other European countries. In the UK (Harris and Yang, 1995), Greece (Elena and Paplomatas, 1998; Elena, 1999), Germany (Zeise and von Tiedemann, 2001), Spain (Korolev et al., 2001) and Israel (Bao et al., 1998; Korolev et al., 2000) also two main VCGs were detected with in some cases a very limited number of isolates from a third VCG. Comparison of VCG testers from the Netherlands with those from other European countries (Table 5) showed that there are two major VCGs present in Europe. This suggests that

Table 5. Complementation between VCG testers of *V. dahliae* from different countries; results are summarised per isolate. (++ very strong complementation, + strong complementation, ± weak or uncertain complementation, − no complementation)

Origin	VCG	Tester isolate	Tester isolate*																				
			F3–5	30V	1V	11	13	25	6	BB	S39	PCW	70–21	PH	115	V44	50S-3	18	7V	8V	327	F4–1	F6–5	58S-2
UK	*β*	F3–5		++	++	++	+	−	++	+	++	−	−	−	−	−	−	−	−	−	−	−	−	−
Gr	I	30V	++		++	−	−	++	+	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−
Gr	I	1V	++	++		++	++	+	++	++	++	−	−	−	−	−	−	−	−	−	−	−	−	−
NL	I	11	++	−	++		++	++	++	++	++	++	++	−	−	−	−	−	−	−	−	−	−	−
NL	I	13	+	−	++	++		++	++	++	++	+	−	−	−	−	−	−	−	−	−	−	−	−
NL	I	25	−	++	+	++	++		++	++	++	−	−	−	−	−	+	−	−	−	−	−	−	−
NL	I	6	++	+	++	++	++	++		++	++	++	−	++	++	+	−	−	−	−	−	−	−	−
USA	4A	BB	+	−	++	++	++	++	++		++	++	−	−	−	−	−	+	−	−	−	−	−	−
USA	4B	S39	++	++	++	++	++	++	++	++		++	−	+	+	−	−	+	−	−	−	−	−	−
USA	3	PCW	−	−	−	++	+	−	++	++	++		++	−	−	−	−	−	−	−	−	−	−	−
USA	3	70–21	−	−	−	++	−	−	−	−	−	++		−	+	−	−	+	−	−	−	−	−	−
USA	2	PH	−	−	−	−	−	−	++	−	+	−	−		++	−	++	++	++	−	−	++	++	−
USA	2	115	−	−	−	−	−	−	++	−	+	−	+	++		−	++	++	++	−	++	++	++	−
USA	1	V44	−	−	−	−	−	−	+	−	−	−	−	−	−		−	++	−	−	−	−	−	++
ES		50S-3	−	−	−	−	−	+	−	−	−	−	−	++	++	−		++	++	++	++	++	+	−
NL	II	18	−	−	−	−	−	−	−	+	+	−	+	++	++	++	++		++	++	++	++	++	−
Gr	II	7V	−	−	−	−	−	−	−	−	−	−	−	++	++	−	++	++		−	−	++	++	−
Gr	II	8V	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++	−		−	−	−	−
UK	*α*	327	−	−	−	−	−	−	−	−	−	−	−	−	++	−	++	++	−	−		++	++	−
UK	*α*	F4–1	−	−	−	−	−	−	−	−	−	−	−	++	++	−	++	++	++	−	++		++	++
UK	*α*	F6–5	−	−	−	−	−	−	−	−	−	−	−	++	++	−	+	++	++	−	++	++		−
ES		58S-2	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−	−	++	−	

*See Table 2 for more details.

VCG diversity in Europe, including the Mediterranean area, is more limited than in the USA where up to five VCGs with several subgroups have been reported (summarised in Jeger et al., 1996).

Pairing of testers from the Netherlands with testers from USA produced some ambiguous results. Testers of VCG NL-I were strongly compatible with testers of both VCG 3 and VCG 4 (4A + 4B) from USA. This is not surprising because Strausbaugh et al. (1992) suggested that VCG 3 and VCG 4 from USA in fact are one VCG. However, some of the testers of these VCGs from USA also formed heterokaryons with VCG NL-II testers (Table 5), although more weakly than with VCG NL-I testers. The same was true for the VCG 1 and VCG 2 testers from the USA, which were strongly compatible with VCG NL-II testers but also weakly complemented some of the VCG NL-I testers.

The general picture that emerges is that of two main groups of vegetatively compatible isolates with some isolates being compatible with testers from both groups. Apparently, these two main groups, VCG 4 and VCG 2, are the two major VCGs worldwide, in several European countries, in the USA (Joaquim and Rowe, 1990; Strausbaugh et al., 1992; Strausbaugh, 1993; Subbarao et al., 1995) and Canada (Dobinson et al., 1998), and in Japan (Wakatabe et al., 1997; Ebihara et al., 1999). These groups are genetically not completely separated. In our experiments, the VCG 1 and VCG 3 testers from USA acted as bridge isolates between the two main VCGs, as did one of the testers from Spain. Similar observations of isolates being compatible with one VCG and also at least weakly with another VCG have been reported by Daayf et al. (1995) and Bao et al. (1998). Similarly, Wakatabe et al. (1997) examined a collection of Japanese isolates and reported three VCGs of which VCG J1 was compatible with both VCG J2 and VCG J3.

The presence of bridge isolates suggests that there is a large continuum of genetic variation that, depending on the testers chosen, can be subdivided into several genetically more or less separated groups. As a consequence, and because the capacity of mutants for prototrophic growth is a characteristic of individual mutants and does not necessarily reflect that of their parent strains (Joaquim and Rowe, 1990; Chen, 1994), it is of utmost importance to use good tester mutants when assessing vegetative compatibility between isolates. Consequently, the best way to get a clear understanding of how VCGs from different countries relate to each other would be to examine a fresh collection of deliberately collected isolates from all relevant geographic areas, including careful selection of isolate testers for all isolates included in the comparison. In addition, for a good comparison between VCGs from Europe and USA, it might be helpful to prepare fresh testers in the same way for the USA groups too.

Identifying VCGs in *V. dahliae* becomes much more relevant when important biological characteristics such as virulence levels or host specificity can be linked to specific VCGs. The information on these aspects, however, is far from clear. The results of the present study suggest that for tree hosts in the Netherlands the diagnostic value of VCGs may be limited because only two main VCGs were identified and isolates from both groups apparently affect tree hosts. However, some host specificity related to VCGs has been reported (Daayf et al., 1995; Jeger, 1996). Also, it was reported that the host range of individual isolates was more dependant on the VCG of the isolate than on its original host plant provenance (Zeise and von Tiedeman, 2002). On the other hand, it was reported that differences in virulence between individual isolates from one VCG may be larger than those between isolates from different VCGs (Goud and Termorshuizen, 2002). Therefore, more comprehensive information is needed on the geographical spread of VCGs in *V. dahliae*, as well as on the relation between VCG and other biological traits such as host specificity and virulence of individual isolates before the diagnostic value of VCGs in *V. dahliae* can be determined.

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