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Genetic and molecular characterization of *Verticillium dahliae* isolates from woody ornamentals in Belgian nurseries

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Abstract

Isolates of *Verticillium dahliae* were collected from affected trees (*Acer* spp., *Tilia* spp. and *Robinia* spp.) and soils in Belgian ornamental nurseries. Nitrate non-utilizing mutants were produced and vegetative compatibility groups (VCGs) were classified based on complementation tests with reference tester strains. Of the 30 isolates analysed, 12 were classified as VCG2B and 18 as VCG4B following the American classification. In order to distinguish VCG2B from VCG4B, specific polymerase chain reaction primers were designed based on the sequence of a VCG2B-associated Direct Amplification of Minisatellite-region DNA (DAMD) band generated with the core sequence of the phage M13 minisatellite DNA. Using this test, amplification products were generated for all the VCG2B isolates characterized in this study. In contrast, no signal was seen on ethidium–bromide agarose gel for VCG4B isolates. Pathogenicity tests were carried out in a glasshouse on maple-rooted cuttings inoculated with conidial suspensions of *V. dahliae* belonging to both groups (VCG2B/VCG4B). Some strains proved to be highly aggressive, while others did not. However, these different behaviours were not correlated with the VCGs.

Introduction

Verticillium dahliae is an important soil-borne fungus causing vascular wilt on a wide host range, including woody plants. The fungus produces microsclerotia in the dying tissues of the infected plant. These structures survive in soil over a range of moisture and temperature conditions for many years (Harris, 1998). This concerns particularly tree nurseries, as infected field plots can be re-used for non-susceptible hosts only. Several ornamental tree species, notably maples (*Acer* spp.), are susceptible to the disease (Schreiber and Mayer, 1992; Smith and Neely, 1979; Townsend et al., 1990). Symptoms in affected woody plants include wilting and intercostal necrosis of leaves, discolouration of the xylem (also referred to as 'streaking') and decline (Hiemstra, 1998; Sinclair et al., 1981).

Genetic diversity in populations of *V. dahliae* has been studied using vegetative compatibility group (VCG) analysis (Joaquim and Rowe, 1990; Puhalla, 1979), whereby isolates whose hyphae anastomose and form viable heterokaryons are assigned to a VCG. As no sexual state is known for *V. dahliae*, the only source of gene flow within the species may be by parasexual interactions following the establishment of heterokaryons. Therefore, isolates from the same VCG are thought to comprise a genetically distinct population (Katan, 2000; Leslie, 1993; Puhalla, 1979). Joaquim and Rowe (1990), using nitrate non-utilizing mutants (*nit*), identified four VCGs in *V. dahliae*, two of them (VCG2 and VCG4) being further divided into two subgroups A and B. Later, this technique was used extensively to characterize *V. dahliae* populations in various countries (Chen, 1994; Daayf et al., 1995; Dobinson et al., 1998; Elena, 1999; Korolev et al., 2000; Strausbaugh, 1993; Zeise and Tiedmann, 2001). In maple, VCG analysis of 42 *V. dahliae* isolates in North America revealed that the vast majority of them

were from the VCG1 group, with a few belonging to VCGs 2 and 4 (Chen, 1994). In Europe, Hiemstra and Rataj-Guranowska (2000), using their own VCG classification system, identified two VCGs among isolates isolated from ornamental plants.

In spite of their usefulness, VCG analyses are time-consuming and cumbersome. In addition, *V. dahliae* isolates may fail to produce mutants (Bhat and Subbarao, 1999; Joaquim and Rowe, 1991) or produce only one type of mutant, thus preventing the opportunity to perform intra-strain compatibility analyses (Katan, 2000). As a consequence, improved methods are needed for the rapid classification of *V. dahliae* populations in VCG. Molecular markers have been developed to characterize VCG 4A and 4B isolates of *V. dahliae* associated with potato early dying (Dobinson, 2000). A polymerase chain reaction (PCR) test has also been developed to differentiate cotton-defoliating (D) and non-defoliating (ND) pathotypes (Pérez-Artés et al., 2000), these pathotypes being associated with different VCGs (Katan, 2000). Among the DNA-based methods available for studying population genetics, the Direct Amplification of Minisatellite DNA (DAMD, Heath et al., 1993) is a promising technique. Indeed, as for RAPD analysis, no knowledge of the organism's nucleic acid sequence is required and a single reaction can produce several amplification products. Moreover, DAMD patterns are highly reproducible (Santini and Capretti, 2000).

The objective was to assess VCG diversity within a collection of *V. dahliae* isolates obtained from ornamental woody plants and soils in various Belgian nurseries as well as to correlate the aggressiveness of isolates to maple with their VCG. Finally, PCR primers were designed based on the sequence of a VCG2B-associated DAMD band in order to discriminate between VCG2B and VCG4B isolates.

Materials and methods

Fungal isolates

Isolates of *V. dahliae* listed in Table 1 were collected from infected trees and soils in ornamental nurseries located in regions that represent the main centres of ornamental production in Belgium (more than 70% of the production area in 1999, Economisch Raad voor Oost-Vlaanderen, EROV-Belgium). Pieces of stems were surface-disinfected in 0.5% NaOCl for 30 s, rinsed in sterile distilled water (SDW) and placed aseptically on potato dextrose agar (PDA; DIFCO Laboratories) amended with oxytetracycline (50 mg l^{-1}). Cultures were incubated at room temperature (22–26 °C) in the dark until sporulation developed. Soil samples were air-dried for 2 weeks at room temperature (~18–20 °C) and then ground in a mortar and pestle. A 25 g sub-sample was suspended in 100 ml 0.08% agar and aliquots of 1 ml soil suspension were plated onto a sodium polygalacturonate medium as described by Butterfield and Devay (1977). Plates were incubated at 21–23 °C in the dark for 21 days. Following incubation, plates were washed and agar portions containing colonies of *V. dahliae* were recovered, blended with 200 µl SDW and then transferred in 20 ml SDW containing 0.5% NaOCl and 5% ethanol. The microsclerotia were collected on sterile Gelman 1.2 filters. Liquid was removed by vacuum filtration and filters were washed with SDW before being transferred onto PDA amended with

Table 1. Origin, geographical location (in Belgium) and year of isolation of *V. dahliae* isolates used in this study

Strain number	Host origin/soil	Geographical location	Year of isolation
2040	*Acer rubrum*	Lesdain	1995
2053	*Tilia* sp.	Lesdain	1995
2072	*Tilia* sp.	Lesdain	1996
2084	*Acer negundo*	Lesdain	1996
2095	*Acer rubrum*	Aalter	1996
2103	*Acer platanoides*	Lesdain	1997
2122	*Tilia cordata*	Beernem	1998
2123	*Acer pseudoplatanus*	Maldegem	1998
2124	*Acer ruffinerve*	Eindhout	1998
2125	*Acer capillipes*	Eindhout	1998
2127	*Acer pseudoplatanus*	Waarschoot	1998
2128	*Tilia cordata*	Beernem	1998
2129	*Acer siboldanium*	Aalter	1998
2133	*Acer pseudoplatanus*	Lesdain	1998
2134	*Acer platanoides*	Lesdain	1998
2135	*Acer rubrum*	Lesdain	1998
2136	*Acer platanoides*	Lesdain	1998
2137	*Acer platanoides*	Wetteren	1998
2138	*Acer campestre*	Wetteren	1998
2139	*Tilia platyphyllos*	Oosterzele	1998
2140	*Acer platanoides*	Oosterzele	1998
2141	*Acer campestre*	Oosterzele	1998
2142	*Acer campestre*	Oosterzele	1998
2143	*Acer pseudoplatanus*	Wetteren	1998
2144	*Tilia pallida*	Wetteren	1998
2145	*Acer platanoides*	Wetteren	1998
2146	*Robinia* sp.	Oosterzele	1998
2150	Soil	Wingene	1998
2151	Soil	Maldegem	1998
2153	Soil	Lesdain	1998

oxytetracycline (50 mg l^{-1}). Colonies of *V. dahliae* developing from the microsclerotia were subcultured onto PDA. Monoconidial cultures were generated from all *V. dahliae* isolates and kept in long-term storage on PDA with paraffin oil (VWR International) at 4 °C. Isolates from other countries were kindly provided by Dr Geiger (INRA-Montfavet, France) and were isolated from tomato (strain 1 (VCG2) from France and strain 3 (VCG4) from Brazil), cotton (strain D3 (VCG1) from Russia) or soil (strain E (VCG1) from Russia). These isolates have already been characterized by Daayf et al. (1995). Other *Verticillium* species were recovered from potato (*V. nigrescens*, strain 2049), tomato (*V. tricorpus*, strain 2047) and hop (*V. albo-atrum*, strain 2101). A *V. albo-atrum* strain (NL group, strain 2081) was also provided by Dr Carder (Crop Protection Department, HRI, East Malling, UK).

Vegetative compatibility group analysis

Nitrate non-utilizing (*nit*) mutants were produced from wild-type monoconidial strains on corn meal agar (CMA, DIFCO) containing 30 g l^{-1} potassium chlorate at 24 °C. Mutants were transferred to a minimal medium (MM) (Correll et al., 1987). Single conidia from cultures of *nit* mutant on PDA were subcultured on MM for confirmation of their mutant status. The *nit* mutants were identified as *nit*1, *nit*3 or *nit*M as described by Joaquim and Rowe (1990). Complementation between complementary *nit* mutants was performed on MM using VCGs [V-44 (VCG1), PH (VCG2A), 115 (VCG2B), PCW (VCG3), BB (VCG4A) and S-39 (VCG4B)] tester strains kindly provided by Dr Rowe (Ohio Agricultural Research and Development Center, OARDC Wooster). Every pairing was repeated at least twice. Plates were scored for prototrophic growth after 1–2 weeks of incubation. The VCGs were classified according to Joaquim and Rowe (1990).

Extraction and amplification of DNA

Total genomic DNA was extracted from fungal mycelium (Lee and Taylor, 1990). The protocol included cell disruption, incubation of the extract at 65 °C during 1 h in extraction buffer (50 mM Tris–HCl (pH 7.2) containing 50 mM EDTA, 3% SDS and 1% β-mercaptoethanol), and two phenol : chloroform (1 : 1) extractions. The DNA was precipitated with alcohol, dried under vacuum and resuspended in 10 mM Tris–HCl buffer (pH 8.0). DNA concentrations were estimated by electrophoresis on 2% agarose gel. For the DAMD analysis with the core sequence of M13 minisatellite DNA [5′ GAG GGT GGC GGT TCT 3′], the temperature profile was as follows: denaturation at 98 °C for 15 s, annealing at 50 °C for 60 s and extension at 72 °C for 100 s for a total of 40 cycles (Messner et al., 1996). The primers PolyF [5′ ACT GTT CAG TGG GGA CAG 3′] and PolyR [5′ CGG AAT TGG AAA TTT TAG GGG T 3′] were selected with the GCG programme (Wisconsin Package Version 10.0, Genetics Computer Group (GCG)) from the DNA sequence of the VCG2-associated DAMD marker. The PCR reaction (50 µl) consisted of 0.4 µM of each primer (PolyF and PolyR), 200 µM dNTPs, 5 µl 10× reaction buffer, 2.5 U *Taq* DNA polymerase (*Taq* Platinum, Invitrogen Life Technologies Ltd), 2.5 mM $MgCl_2$ and fungal DNA. The cycling profile consisted of 5 min at 95 °C followed by 30 cycles: 30 s at 95 °C, 1 min at 53 °C, and 1 min at 72 °C, and a final extension of 10 min at 72 °C. Amplification reactions were carried out in a Mastercycler 96 Gradient thermocycler (Eppendorf Netheler-Hinz GmbH, Germany). The amplification products were separated by electrophoresis on 2% agarose gels in TAE buffer (40 mM Tris-acetate (pH 8.0), 1 mM EDTA) stained with ethidium–bromide and visualized under UV light. The length of the amplification products was estimated by comparison with a 100 bp DNA ladder (Invitrogen Life Technologies Ltd). All reactions were repeated at least three times, on different DNA preparations.

DNA cloning and sequencing

After PCR with primer M13 on total genomic DNA from VCG2B and VCG4B isolates, two DAMD amplification products were recovered from gel and purified using the QiaEx II Gel Extraction kit (Qiagen, Germany). Those products corresponded respectively to a 608 bp (for the DAMD-VCG2B marker) and to a 587 bp (for the DAMD-VCG4B marker). Both purified DNA fragments were cloned into the plasmid PCR-Script2 using the TA Cloning kit and Invα competent cells (Invitrogen, Life Technologies Ltd). Ampicillin (100 µg ml^{-1}) resistant transformants were checked by PCR and 10 expected recombinant clones for both DAMD-markers were subcultured. Plasmid DNAs were isolated using the High Pure Plasmid Isolation Kit (Roche Diagnostic GmbH, Germany) and stored in TE (10 mM Tris–HCl (pH 8.0), 1 mM EDTA)

at −20 °C. Sequencing reactions were carried out following the manufacturer's instructions (Thermo Sequenase™ DYEnamic Direct Cycle Sequencing kit with 7-deaza-dGTP, Amersham Pharmacia Biotech Inc, USA) and sequencing was conducted on an automatic DNA sequencer (GeneReadIR™ DNA Analysis System L4200S-1, Li-Cor Inc., Lincoln NE, USA). Both strands were sequenced using as primers the M13 universal and reverse primers coupled with the IRD800 fluorescent dye (BioLegio, Malden, the Netherlands). The DNA sequence corresponding to the VCG2B-DAMD marker and those corresponding to the VCG4B-DAMD marker were aligned using a software package for sequence analysis (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison WI) in order to design the primers PolyF and PolyR specific to VCG2B isolates. The complete sequence of the VCG2B-DAMD has been deposited in the EMBL nucleotide sequence database (accession number AJ489439).

Hybridization assays

The VCG2B-DAMD marker cloned in plasmid PCR-Script2 was excised by digestion with EcoRI, resolved by electrophoresis in a 2% agarose gel and purified using the QiaEx II Gel Extraction kit (Qiagen, Germany). DNA was labelled and used as a probe in hybridization experiments using the ECL direct nucleic acid labelling and detection systems based on enhanced chemiluminescence (Amersham Pharmacia Biotech Europe GmbH, Germany) according to the manufacturer's instructions. For Southern blots, agarose gels were treated and transferred onto nylon membrane (Hybond N+, Amersham Pharmacia Biotech Europe GmbH, Germany) as described by Sambrook et al. (1989).

Pathogenicity tests on maple rooted cuttings

Maple (*Acer campestre*) stems were collected in August 2001. Stem pieces about 5 cm long with one bud were dipped in cutting powder (0.4% 1-naphtyl acetamide, Rootone®, Scotts Belgium), treated with a mixture of two fungicides (Rovral, Aventis CropScience/Pomarsol, UCB s.a.) and placed in peat pellets (AS Jiffy Products Ltd, Norway) in a glasshouse under natural temperature and photoperiod conditions. Two months after planting, the stem pieces were transferred to a soil and compost (1 : 1, v/v) mixture, grown for 1 month, and then uprooted. Their roots were washed in tap water and immersed in 300 ml of a conidial suspension (10^7 conidia ml^{-1}) for 3 min. The inoculated plants were placed in plastic pots filled with the soil and compost (1 : 1, v/v) mixture and kept in a glasshouse at 20–25 °C under a 16 h photoperiod and a relative humidity of 70–90% for 80 days. Controls were dipped in water. There were seven replicates in a randomized complete block design, each block comprising one inoculated plant per isolate and two control plants. Ten *V. dahliae* strains were evaluated in the assay, five belonging to VCG2B (2040, 2123, 2133, 2135 and 2138) and five to VCG4B (2125, 2127, 2129, 2134 and 2141). Foliage symptom severity was scored on a scale of 1–7, based on the percentage of leaves showing necrosis (1 = plant without any symptoms, 2 = 1–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–99%, 6 = 100% and 7 = dead plant) 60 and 80 days after inoculation. Analysis of variance, Dunnett's two-tailed-*t*-test and contrast method were performed using the statistical SAS software (SAS Institute, Cary, NC Version 8.2) for each date of measurement. Colonization of plants by *V. dahliae* was first evaluated by visual observation of the vascular discolouration in stem tissues. For plants without any vascular symptoms, surface-disinfected stem segments were placed onto PDA and incubated at room temperature for 10 days.

Results

Vegetative complementation group analysis

Isolates of *Verticillium* collected from infected trees and soils in different ornamental nurseries were classified as *V. dahliae* on the basis of the presence of microsclerotia and verticilliate conidiophores (Hawksworth and Talboys, 1970) and the absence of chlamydospores in their cultures. The different species of maple and lime included in this study, as well as robinia, were infected by *V. dahliae* (Table 1).

All 30 *V. dahliae* isolates produced *nit* mutants. In total, 357 *nit* mutants were classified based on their phenotype. Ten isolates produced *nit*1 and *nit*M mutants, 14 produced *nit*1 only and 6 produced *nit*M mutants only (Table 2). The number of *nit*1 mutants was approximately twice as high as that of *nit*M mutants; no

Table 2. Vegetative complementation group analysis of 30 isolates recovered from ornamental nurseries using the OARDC tester strains

Strain	*nit*1	*nit*M	Reference tester strains[2]						VCG[1]
			V44 1	PH 2A	115 2B	PCW 3	BB 4A	S-39 4B	
2040	8	54	−	+	++	−	−	−	2B
2053	0	9	−	+/−	++	−	−	−	2B
2072	0	4	−	+	++	−	−	−	2B
2084	0	4	−	+/−	++	−	−	−	2B
2095	22	1	−	+/−	++	−	−	−	2B
2103	70	22	−	+/−	++	−	−	−	2B
2122	5	0	−	+/−	++	−	−	−	2B
2123	3	0	−	+/−	+	−	−	−	2B
2124	1	0	−	−	−	−	−	++	4B
2125	0	2	−	−	−	−	−	++	4B
2127	14	2	−	−	−	−	−	+	4B
2128	4	2	−	−	−	−	−	++	4B
2129	5	2	−	−	−	−	−	+	4B
2133	10	2	−	+	++	−	−	−	2B
2134	2	2	−	−	−	−	−	++	4B
2135	18	0	−	+	++	−	−	−	2B
2136	5	2	−	−	−	−	−	+	4B
2137	7	0	−	−	−	−	−	+	4B
2138	2	0	−	+/−	++	−	−	−	2B
2139	2	0	−	−	−	−	−	++	4B
2140	11	1	−	−	−	−	−	++	4B
2141	10	0	−	−	−	−	−	++	4B
2142	2	0	−	−	−	−	−	++	4B
2143	17	0	−	+/−	++	−	−	−	2B
2144	4	0	−	−	−	−	−	+	4B
2145	7	0	−	−	−	−	−	+	4B
2146	0	1	−	−	−	−	−	++	4B
2150	0	2	−	−	−	−	−	++	4B
2151	8	0	−	−	−	−	−	+	4B
2153	8	0	−	−	−	−	−	++	4B

[1]Vegetative compatibility group; [2]The extent of prototrophic growth between mutants is indicated as strong (++), normal (+), weak (+/−) or absent (−). All data were confirmed by at least two different pairings.

*nit*3 mutants were recovered. After complementation with the tester strains of known compatibility groups, 12 isolates were classified as VCG2 and 18 as VCG4 (Table 2). All the isolates failed to anastomose with a VCG3 or a VCG1 tester. VCG2 showed strong complementation with tester strain of subgroup B, but all were also weakly compatible with the tester strain VCG2A. Indeed, the complementation with VCG2A tester appeared later than the complementation with VCG2B tester and resulted in a heterokaryon with very little aerial mycelium. All VCG4 isolates showed complementation with the tester strain of subgroup B only. There was no apparent correlation between isolate VCG and geographic distribution.

Discrimination between VCG2B and VCG4B isolates by PCR

PCR reactions were carried out using *V. dahliae* total genomic DNA and the core sequence of M13 minisatellite DNA as primer (DAMD, Messner et al., 1996; Stenlid et al., 1994). DNA fragments were amplified for each of the 30 *V. dahliae* isolates. Moreover, the electrophoretic profiles generated from the total DNA of other *Verticillium* species differed, thus providing an additional mean to differentiate *V. dahliae* from other *Verticillium* species (Figure 1).

Electrophoretic profiles generated from *V. dahliae* total DNA exhibited three majors bands at

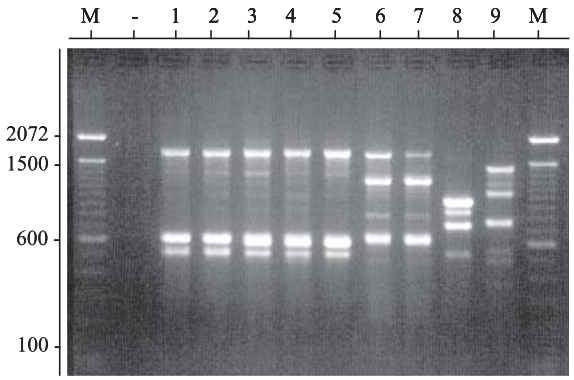

Figure 1. Banding patterns of *Verticillium* isolates obtained with the M13 primer. The isolates are *V. dahliae* VCG2 strains 2040 (1), 2143 (2), *V. dahliae* VCG4 strains 2125 (3), 2128 (4), 2141 (5), *V. albo-atrum* strain 2101 (6) and 2081 (7), *V. tricorpus* strain 2047 (8), *V. nigrescens* strain 2049 (9). M = molecular weight markers (100 bp DNA ladder, Invitrogen Life Technologies). − = negative control (no template DNA).

approximately 500, 600 and 1800 bp, as well as several faint signals. The size of the amplification fragment located at approximately 600 bp was systematically slightly higher for isolates assigned to VCG2 group compared to those assigned to VCG4 (Figure 1). This polymorphic fragment was cloned and sequenced for VCG2B and VCG4B isolates. A comparison between both sequences revealed a 21 bp sequence occurring twice in strains from VCG2B (Figure 2). This polymorphism was used to design VCG2B-specific primers and to develop a PCR assay capable of discriminating between both groups of strains. The results presented in Figure 3A show that all VCG2B strains gave a 327 bp amplification fragment, but the VCG4 strains did not, therefore confirming the VCG predictions. Hybridization with the VCG2B-DAMD marker used as a non-radioactive probe revealed huge signals for all VCG2B isolates. Among the VCG4B isolates, a faint signal was observed for two isolates (2150 and 2151) only (Figure 3B).

PCR with the primers PolyF and PolyR and hybridization assays were also carried out using as a template either total genomic DNA from reference tester strains (mutant) or total genomic DNA from *V. dahliae* isolates from other countries belonging to different VCGs. After electrophoresis, an amplification signal was observed on ethidium–bromide agarose gel for the tester strains 115 (VCG2B) and BB (VCG4A). No signal was visible under UV light for the other reference tester strains or for the wild strains from Russia (VCG1), France (VCG2) or Brazil (VCG4) (Figure 4A). In order to lower the detection threshold, DNA was immobilized on a membrane and hybridization assays were carried out using the VCG2B-DAMD marker as a probe. This experiment revealed faint additional signals for strains D3 (VCG1), E (VCG1), 1 (VCG2) and 3 (VCG4) but also for the reference tester strain PH (VCG2A). The tester strain S-39 (VCG4B) produced a very low signal on film (Figure 4B).

Sequence analysis using the FASTA programme of the GCG package did not reveal any significant homology of the specific VCG2B-DAMD marker with known sequence.

Pathogenicity tests on maple rooted cuttings

The aggressiveness of *V. dahliae* isolates (VCG2B and VCG4B) prevailing on woody ornamentals in Belgium was evaluated on maple rooted cuttings. Analysis of variance of disease severity data carried out 60 and 80 days after inoculation revealed a significant effect of strains ($P \leq 0.001$). Only strains 2138, 2123, 2141 and 2129 caused symptoms significantly more severe compared with control plants according to Dunnett's two-tailed-*t*-test (Table 3). These strains were originally isolated from *A. campestre* (2138 and 2141), *A. pseudoplatanus* (2123) and *A. siboldanium* (2129). Moreover, comparison between VCG2B and 4B carried out by the contrast method did not reveal any significant difference ($P = 0.8915$).

All 10 isolates induced vascular discolouration in plants. Incidence of vascular symptoms ranged from 100% (isolates 2138 and 2123) to 14% (isolate 2133) 80 days after inoculation while no vascular symptom was found in the control plants. In addition, seven out of 29 inoculated plants showing no vascular symptoms gave rise to *V. dahliae* growth from stem pieces plated onto PDA. On the contrary, *V. dahliae* could not be isolated from the control plants. The cumulative number of plants found infected according to both methods (vascular symptoms and plating), was closely related to the disease severity (Table 3).

Discussion

The genetic diversity among *V. dahliae* isolates in Belgian nurseries was investigated. The genetic characterization of *V. dahliae* strains is important for a number of reasons. First, the variability of this pathogen in Europe from woody ornamentals has not been extensively studied. Second, epidemiological studies are

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1   GCGAGAATGG AAACGAGAAG GGGCGGTTGA CGAGGTTGTG ACTTGGAGGT

51  TCGGCCATGA GGGGACGCAT GTGACGCGAG CTGTGGCCCG AGCTGACTGA

101  CATCACCGTC GCCGACATCT TTGTCAGCGT CCTCTCTGCT CCTACCTACC

151  TTAGCTTTTA GGTACCTTAG GCAGGTACCT AGGTTTAGGT ACCTTAGGCA

201  GGTACCTAGG TACCCAGGTC CTTCAGGCCT TCGGCGCTCA CAGGCAAGAC

251  AGGGCACTGT TCAGTGGGGA CAGGGCACTG TTCAGTGGGG AGCCTTGGCC
                  PolyF

301  AGTGCACTGA TAGCGCAAGA AGGGAGAAGG GCCACCGTCC TGCCATTTTC

351  AGGAGATGCC CATGCCAACT TGCAAAGAAG CCCATGAGCT CCATTCTCTC

401  ACGCAGTACC TTACATGTAC AGGCCTCCTC CGATGAGGGT GGCTCGTCAG

451  CCTCGCTCGG CTTCGCATGA TCCGGGAGCT CGTTGTTGAT GTCAATCTCT

501  CATAGCTAGG TGCCTCGGTG AGATGAACCT TGGCCGCCCG CCTACAAGCT

551  TGGCCCCAGG ACCCCTAAAA TTTCCAATTC CGCGAGAAGA AACAATTGCG
                  PolyR

601  GCTGCAGG
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Figure 2. Nucleotide sequence of the 608 bp VCG2B-DAMD marker generated with the primer M13 (order N13 sequence not included). PolyF/PolyR = primers selected for the amplification of *V. dahliae* strains belonging to the VCG2B. In boxes: repeated sequence. Accession number AJ489439.

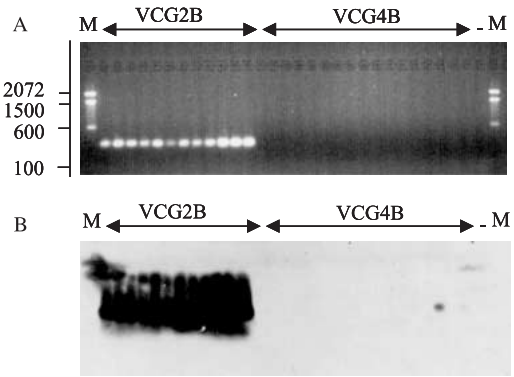

Figure 3. PCR with the primers PolyF and PolyR on VCG2B or VCG4B isolates; (A) ethidium–bromide agarose gel, (B) corresponding DNA blot hybridized with the VCG2B-DAMD marker used as probe. M = molecular weight marker (Invitrogen Life Technologies Ltd, 100 bp DNA ladder). − = negative control (no template DNA).

necessary to identify variant strains that are particularly aggressive in order to control their dissemination to countries where they are not yet present. The defoliating strains isolated from cotton and olive trees in various Mediterranean countries are a good example of such a threat (Bejarano-Alcázar et al., 1996). Third, the clear genotypic differentiation of strains showing variable aggressiveness is a prerequisite for breeding programmes aiming to produce plant cultivars resistant to the disease.

Without any sexual reproductive stage, the sole source of gene flow within a species is through parasexual interactions. Vegetative complementation tests have been developed to classify *V. dahliae* strains into different VCGs (Joaquim and Rowe, 1990; Puhalla and Hummel, 1983). *Nit* mutants were produced from 30 strains obtained from ornamental plants or soil. Some strains, however, produced only one type of mutant (either *nit*M or *nit*1), thus making it impossible to carry out all the combinations. Moreover, by

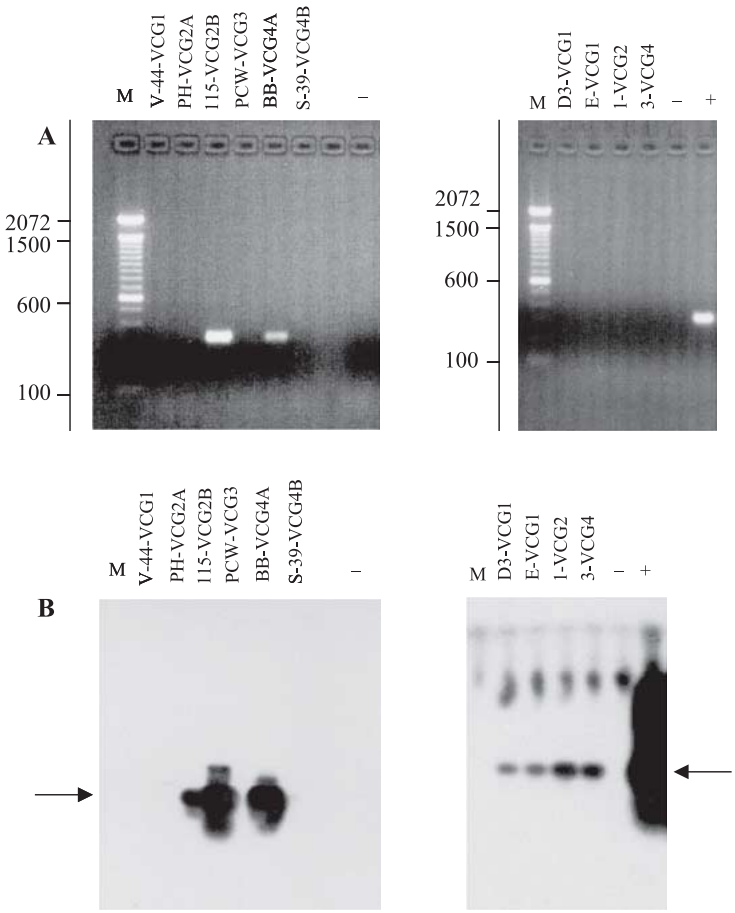

Figure 4. PCR with the primers polyF and polyR on total DNA from the reference tester strains (Dr Rowe, OARDC) and from *V. dahliae* isolated in other countries. (A) Ethidium–bromide agarose gel, (B) corresponding DNA blot hybridized with the VCG2B-DAMD marker used as a probe. − = negative control (no template DNA); + = positive control (isolate 2138, VCG2B). M = molecular weight marker (Invitrogen Life Technologies Ltd, 100 bp DNA ladder). Arrow: position of the PolyF/PolyR amplification signal.

pairing the mutants available with the complementary reference tester strains, some strains classified in one subgroup on the basis of strong complementation between mutants were also weakly compatible with the other subgroup. Similar data were reported by Elena (1999) working on *V. dahliae* strains recovered from cotton in Greece and also by Joaquim and Rowe (1991) with strains isolated from potato. Nevertheless, the data suggest that populations of *V. dahliae* in ornamental nurseries in Belgium have limited VCG diversity. This result is in accordance with that observed in the Netherlands (Hiemstra and Rataj-Guranowska, 2000).

In spite of the relative convenience of working with *nit* mutants, it was interesting to develop a rapid tool suitable for the study of large populations of *V. dahliae*. Only a limited number of DNA-based techniques have been linked with VCG determination. The method used is based on the amplification of DNA fragments using as primer minisatellite DNA (DAMD, Heath et al., 1993). By applying the DAMD technique to the characterization of *V. dahliae*, amplification profiles were reproductively obtained with different DNA preparations as long as the DNA was of high quality. Furthermore, although taxonomy was not the purpose of this study, species-specific patterns were observed even between *V. albo-atrum* and *V. dahliae*, two closely-related *Verticillium* species which cause wilt on many plants. If the results, however, were highly reproducible compared to RAPD, probably because

Table 3. Pathogenicity of *V. dahliae* strains from VCG2B and VCG4B on maple rooted cuttings 80 days after inoculation

(VCG)	Disease severity[a]		Plant colonization		
	Mean[b]	St dev	Test 1[d]	Test 2[e]	Total (%)
2138 (2B)	6.7*[c]	0.76	7/7	nd	100
2123 (2B)	6.6*[c]	0.79	7/7	nd	100
2141 (4B)	5.3*[c]	2.14	6/7	0/1	86
2129 (4B)	4.6*[c]	2.44	5/7	1/2	86
2127 (4B)	3.9	2.19	4/7	0/3	57
2135 (2B)	3.7	2.36	3/7	3/4	86
2040 (2B)	3.3	0.95	4/7	1/3	71
2134 (4B)	3.4	1.51	2/7	1/5	43
2125 (4B)	3.3	1.25	2/7	0/5	29
2133 (2B)	2.6	1.62	1/7	1/6	29
Control	1.9	0.86	0/14	0/14	0

[a]Disease severity determined on a scale of 1–7 based on the percentage of leaves with necrosis (1 = no symptoms, 2 = 1–25% foliage affected, 3 = 26–50%, 4 = 51–75%, 5 = 76–99%, 6 = 100%, 7 = dead plant); [b]The mean of seven replications for inoculated plants and of 14 replications for control; *[c]Difference between mean disease severity for control and inoculated plants significant at the 0.05 level (Dunnett's two-tailed-*t*-test), experimental error mean square = 2.437; error degrees of freedom = 60; [d]Visual observation of a vascular symptom carried out on all plants; [e]Plating of stem pieces onto PDA for plants lacking vascular symptoms, nd: not done.

of the length of primer (15 bp), the test interpretation was complex and it was difficult to use these molecular markers in order to discriminate between both VCGs. We therefore developed a VCG2B-specific PCR assay whereby primers were selected from the sequence of a VCG2B-DAMD polymorphic fragment. When the detection was carried out on ethidium–bromide agarose gel, an amplification fragment was observed for the VCG2B isolates only. However, after hybridization with a probe containing the sequence of the amplification product, additional signals, though weak compared to those obtained for VCG2B isolates, were observed for two isolates assigned to VCG4B. Furthermore, the PCR/hybridization assay applied to the six reference tester strains (V-44, PH, 115, PCW, BB and S-39) revealed that VCG4A and, to a lesser extent VCG2A strains, were also detected. This result was confirmed with *V. dahliae* isolates from other countries whereby strains showing strong complementation with VCG2A and VCG4A tester strains (Daayf et al., 1995) produced a signal of expected size after the PCR/hybridization assay. These variations in the intensity of the signal between VCGs were reproducible in repeated experiments and could arise as a result of difference in copy number of the target sequence, or greater homology between the primers and their annealing sites. In contrast, for VCG1, and to a lesser extent for VCG4Bs, some hybridization results were different between isolates. In this case, minor differences in the quality of the DNA preparation or heterogeneity of temperature between wells of the thermocycler, which might modify the PCR efficiency, cannot be ruled out. Nevertheless, the findings suggest that PCR with the selected primers combined with a detection in agarose gel could be used as a rapid method to differentiate between VCG2B and VCG4B isolates. These results should obviously be confirmed on a large number of isolates from different geographical origins.

Several reports have indicated variable aggressiveness of *V. dahliae* isolates from different VCGs in particular plant species (Joaquim and Rowe, 1991; Tsor et al., 2001). In order to compare strains from both subgroups isolated in Belgium in terms of aggressiveness in maple, rooted cuttings (genetically similar) constituted a better alternative to seedlings. Only 25% of maple explants, however, gave rise to rooted cuttings (data not shown). The pathogenicity test carried out in this study was therefore conducted on a limited number of strains. Nevertheless, the experiment revealed that differences in levels of aggressivity on maple do exist among isolates. Some isolates were found to be highly aggressive, while others were not. These differences were not correlated with VCG. Further studies should, however, be carried out on a greater number of strains in order to confirm these results.

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