Plant Pathology (2009) **58**, 515–526 Doi: 10.1111/j.1365-3059.2008.01981.x

A PCR-based 'molecular tool box' for *in planta* differential detection of *Verticillium dahliae* vegetative compatibility groups infecting artichoke

M. Collado-Romero[a], M. Berbegal[b], R. M. Jiménez-Díaz[ac], J. Armengol[b] and J. Mercado-Blanco[a]*

[a]*Instituto de Agricultura Sostenible, CSIC, Apdo. 4084, 14080 Córdoba;* [b]*Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia; and* [c]*Escuela Técnica Superior de Ingenieros Agrónomos y Montes, Edificio C-4 'Celestino Mutis', Campus Rabanales, Universidad de Córdoba, Ctra. Madrid-Cádiz, km 396, 14071 Córdoba, Spain*

A multiplex-nested-PCR procedure was developed for *in planta* detection of *Verticillium dahliae* isolates infecting artichoke and assessment of their vegetative compatibility groups (VCGs). PCR markers were identified and assigned to *V. dahliae* VCGs, including: i) a 334 bp marker amplified from VCG1A or VCG2B^{334} isolates; ii) a 688 bp marker amplified from VCG2A or VCG4B isolates; and iii) a 688 bp and a 964 bp PCR marker amplified from VCG2B^{824} isolates. The infecting *V. dahliae* VCGs were identified in artichoke tissues according to specific patterns of amplified markers after two rounds of PCR. The PCR-based 'molecular tool box' was first optimized using DNA extracted from artichoke plants artificially inoculated with isolates representative of known VCGs. Thereafter, the efficiency of the molecular procedure was tested using DNA extracted from naturally-infected artichoke plants showing a range of symptom severity as well as from symptomless plants. The novel multiplex-nested-PCR assay was clearly superior in detecting the pathogen compared to conventional isolation procedures, and in addition was informative about the VCGs. Moreover, the PCR method allowed the detection and VCG identification of *V. dahliae* infections in symptomless but infected plants, which had yielded false negatives when checked by microbiological isolation procedures. This 'molecular tool box' has uncovered the presence of several *V. dahliae* VCGs infecting the same artichoke plants in the Comunidad Valenciana Region. In addition, it is useful for genetic and pathogenicity diversity studies of *V. dahliae* populations infecting artichoke, and may help in predicting the severity of verticillium wilt epidemics.

Keywords: *Cynara cardunculus* var. *scolymus*, molecular diagnostics, multiplex-nested-PCR, PCR markers, verticillium wilt

Introduction

Verticillium wilt of artichoke (*Cynara cardunculus* var. *scolymus*), caused by the soil borne fungus *Verticillium dahliae*, is an important biotic constraint for artichoke production. Severe outbreaks of this disease were reported in France in the 1960s (Chambonnett *et al*., 1967), Italy and Greece in the 1980s (Cirulli *et al*., 1984; Tjamos & Paplomatas, 1988), and California in the 1990s (Bhat & Subbarao, 1999). In Spain, the second largest producer in the world (FAO, 2008), the disease has been reported since the late 1990s in the Comunidad Valenciana Region in eastern-central Spain, the main artichoke production area in the country (Armengol *et al*., 2005). Since then, verticillium wilt has spread extensively in this region, causing increased concern among artichoke growers (Berbegal *et al*., 2007). Surveys carried out during 1999–2002 in infected artichoke fields in the three provinces of this region (Castellón, Valencia and Alicante) showed disease incidences ranging from 72 to 80% (Armengol *et al*., 2004). Severity of verticillium wilt of artichoke and yield losses are particularly important for cv. Blanca de Tudela, which is very susceptible to the disease, vegetatively propagated by means of stumps, and grown extensively in Spain (Armengol *et al*., 2005; Jiménez-Díaz *et al*., 2006). These authors considered that the use of infected stumps could be the main cause of the disease expansion. Indeed, the extensive use of stumps as planting material may act as an important source of primary inoculum, since plants can recover from symptoms under certain field conditions although remaining infected. Therefore, use of stumps chosen just because of the absence of disease symptoms in plants during the previous growing season, which is a common practice

*E-mail: jesus.mercado@ias.csic.es

Published online 16 December 2008

among artichoke producers in eastern-central Spain, should be avoided (Armengol *et al*., 2005). Together with the current absence of resistant varieties, the use of pathogen-free planting material in non-infested fields is the key control measure of verticillium wilt of artichoke (Armengol *et al*., 2005). So far, detection of *V. dahliae* infections in artichoke plants is performed mainly by pathogen isolation in culture media followed by identification based on morphological features. This approach is usually time-consuming and does not provide any information about genetic or pathogenicity characteristics of the isolates.

Severity of wilt disease has been found to correlate with *V. dahliae*-vegetative compatibility groups (VCGs) (Jiménez-Díaz *et al*., 2006). Vegetative compatibility is the genetically controlled ability of individual fungal isolates to undergo hyphal anastomosis and form stable heterokaryons (Leslie, 1993). Thus, vegetatively compatible isolates are placed in the same VCG. *Verticillium dahliae* isolates in different VCGs are thought to be genetically isolated populations, each sharing a common gene pool which may vary in traits such as pathogenicity (virulence and aggressiveness) (Puhalla, 1979; Rowe, 1995). After surveys of a large collection of isolates originating from infected artichoke plants, five (VCG1A, VCG2A, VCG2B, VCG2Ba and VCG4B) out of eight *V. dahliae* VCGs currently identified worldwide, have been identified in the Comunidad Valenciana Region (Collado-Romero *et al*., 2006; Jiménez-Díaz *et al*., 2006). Artificial inoculation bioassays showed that isolates belonging to these VCGs were pathogenic to artichoke. However, significant differences were identified in the severity of disease among some groups with VCG4B as the most aggressive group, followed by VCG2B, VCG2A and VCG1A. Moreover, molecular differences among isolates within the VCG2B group have been identified by polymerase chain reaction (PCR) assays using specific primers. These molecular intra groups also correlated to differences in pathogenicity. Thus, a subgroup that amplified a 334 bp PCR marker ($VCG2B^{334}$) was less aggressive than another subgroup that amplified a 824 bp PCR marker ($VCG2B^{824}$) (Jiménez-Díaz *et al*., 2006). According to the differential amplification of these two PCR markers, the population of *V. dahliae* isolates infecting artichoke plants in the Comunidad Valenciana Region can be differentiated into two groups of VCGs: VCG1A and $VCG2B^{334}$ isolates that amplify the 334 bp PCR marker; and VCG2A, $VCG2B^{824}$ and VCG4B isolates that amplify the 824 bp PCR marker (Collado-Romero *et al*., 2006; Jiménez-Díaz *et al*., 2006). However, some isolates with the same PCR marker, e.g. VCG2A and $VCG2B^{824}$, exhibited different levels of disease severity.

The objective of this study was to identify PCR markers useful to differentiate *V. dahliae* VCGs, and suitable for use in a multiplex-nested-PCR procedure for the *in planta* detection of the infecting VCG(s). The optimized PCR-based 'molecular tool box' should be more efficient than conventional isolation methods, and ideally provide information on the possible correlation between VCG diversity and severity of wilt, making it useful for the management of this disease.

Materials and methods

Culturing of *Verticillium dahliae* and fungal DNA extraction

A collection of 260 *V. dahliae* isolates representing all VCGs currently known (VCGs 1A, 1B, 2B, 2Ba, 3, 4A, 4B and 6) were used in this study. All isolates have been previously characterized at the genetic (VCG assignment) and molecular (AFLP profiling) levels (Collado-Romero *et al*., 2006; Jiménez-Díaz *et al*., 2006) except isolate 171 of VCG4A (Table 1) and the unique VCG3 isolate used in this study (70–21) (kindly provided by R. Rowe, OARDC Ohio State University, OH, USA). *Verticillium dahliae* isolates were stored as single-spore cultures on plum lactose yeast extract agar (PLYA) (Talboys, 1960) plates covered with liquid paraffin at 4°C in the dark (Bejarano-Alcázar *et al*., 1996). Fungal DNA was extracted from mycelia of 6- to 7-day-old cultures of isolates in Czapek-Dox broth (Difco Laboratories), or by scraping off the surface of colonies developed on a cellophane disk placed on PLYA, as described in Collado-Romero *et al*. (2006). DNA was extracted from 20 mg of finely ground, freeze-dried mycelia using the DNeasy Plant Kit (Qiagen GmbH) according to the manufacturer´s instructions. DNA purity and concentration were determined spectrophotometrically using a Biophotometer (Eppendorf AG) and by agarose gel electrophoresis according to standard procedures (Sambrook *et al*., 1989). DNA extractions were stored at –20°C until used. A modification of the HotSHOT method described by Truett *et al*. (2000) was used for rapid, small-scale DNA extraction. Briefly, small amounts of mycelia were scraped off from PLYA grown colonies with the tip of a sterile pipette, and disrupted in 20 μL of 25 mM NaOH, 0·2 disodium EDTA, pH 12, solution in 0·2-mL PCR tubes. These mixtures were then incubated for 1 h at 95°C and 5 min at 4°C in a thermocycler after which 20 μL of 40 mM Tris-HCl, pH 5, was added. Five- to eight-microlitre aliquots from these lysates were directly used in PCR assays.

Sequencing of the 1410 bp PCR marker and design of primers MCR2B and INTND3r

Diagnostic PCR markers associated with *Verticillium dahliae* VCGs used in this study are shown in Table 2. In addition, other sets of primers were designed in order to help with the differentiation of VCGs. Thus, the 1410 bp amplicons of 17 *V. dahliae* isolates obtained with primer pair NDf/NDr were sequenced in at least two isolates per VCG (Table 1). These isolates were representative of all VCGs from which this marker was amplified and included a heterokaryon self incompatible (HSI) isolate (V4I, Table 1). Five microlitres of the PCR product amplified as explained in the following section were analysed by electrophoresis. PCR products were purified using the

Table 1 *Verticillium dahliae* isolates from which the 1410 bp PCR marker has been sequenced

Isolate[a]	VCG	1410 bp sequence type[b]	Sequence Accession Number	Host plant	Geographic origin
V142I[c]	2A	NDf/r[1]	DQ266237	Cotton	Spain
V145I	2A	NDf/r[1]	DQ266236	Olive	Spain
V716I	2A	NDf/r[1]	DQ266234	Olive	Italy
V404I	2A	NDf/r[1]	DQ266238	Artichoke	Spain
V589I	2A	NDf/r [1]	DQ266235	Artichoke	Spain
V4I	HSI[d]	NDf/r [1]	DQ266239	Cotton	Spain
V160I[c]	4B	NDf/r[1]	DQ266232	Cotton	Israel
V392I	4B	NDf/r[1]	DQ266231	Olive	Spain
V803I	4B	NDf/r[1]	DQ266230	Olive	Spain
V684I	4B	NDf/r[1]	DQ266233	Artichoke	Spain
V282I[c]	$2B^{824}$	NDf/r[2]	DQ266227	Cotton	Israel
V357I	$2B^{824}$	NDf/r[2]	DQ266228	Cotton	China
V479I	$2B^{824}$	NDf/r[2]	DQ266229	Artichoke	Spain
131-M	4A	NDf/r[2]	DQ266243	Potato	USA
171[c]	4A	NDf/r[2]	DQ266242	Potato	USA
V560I[c]	6	NDf/r[3]	DQ266240	Pepper	USA
V561I	6	NDf/r[3]	DQ266241	Pepper	USA

[a]*Verticillium dahliae* isolate details can be found in Collado-Romero *et al.* (2006).
[b]Sequencing was done in both orientations using primers NDf and NDr (Mercado-Blanco *et al.*, 2001).
[c]Isolate sequences used for comparisons among different VCGs.
[d]HSI, heterokaryon self incompatible. This isolate was similar molecularly and exhibited the same pathogenicity phenotype as isolates of VCG2A (Korolev *et al.*, 2001; Collado-Romero *et al.*, 2006).

Table 2 Diagnostic PCR markers associated with *Verticillium dahliae* vegetative compatibility groups used in this study

	PCR markers (bp)				
VCG[a]	526–543[b]	548/462[c]	334[d]	1410/824/688[e]	964[f]
1A	+	+	+	–	–
1A*	+	–	+	–	–
1B	+	–	+	–	–
2A	+	–	–	+	–
$2B^{824}$	+	–	–	+	+
$2B^{334}$	+	–	+	–	–
2Ba**	+	–	+	–	–
3	+	–	+	–	–
4A	+	–	+	+	+
4B	+	–	–	+	–
6	+	–	–	+	–

[a]VCG, vegetative compatibility group. *VCG1A cotton isolates from Greece and Turkey (Collado-Romero *et al.*, 2006). **2Ba, artichoke isolates that complement with artichoke *nit* mutants but not with international reference testers (Jiménez-Díaz *et al.*, 2006).
[b]Polymorphic *V. dahliae*-specific sequence (Collins *et al.*, 2005) amplified with primer pair DB19/DB22 (Carder *et al.*, 1994).
[c]Sequences amplified with primer pairs D1/D2 (548 bp) (Pérez-Artés *et al.*, 2000) or INTD2f/INTD2r (462 bp) (Mercado-Blanco *et al.*, 2002).
[d]Sequence amplified with primer pair DB19/espdef01 (Mercado-Blanco *et al.*, 2003).
[e]Sequences amplified with primer pairs NDf/NDr (1410 bp), INTND2f/INTND2r (824 bp) (Mercado-Blanco *et al.*, 2001) and INTND2f/INTND3r (688 bp) (see text for details).
[f]Sequence amplified with primer pair INTND2f/MCR2B (see text for details).
+, amplification of the PCR marker; – , no amplification.

QIAquick PCR purification kit (Qiagen GmbH) and sequenced at the Servicio de Secuenciación de ADN del CIB (CSIC) using primers NDf and NDr. Sequences were then edited with the EditSeq program and aligned using the CLUSTALV method implemented in the MegAlign sofware (DNAStar Inc.). PCR primer MCR2B (5′-CTC-CTTGGGGCCAGCGTGTA-3′) was designed based on two single nucleotide polymorphisms (SNPs) (nucleotide positions 1192 and 1194) found in the sequence and associated to $VCG2B^{824}$ (Accession number [AN]: DQ266227) (Table 3). The MCR2B primer was used as the reverse primer in combination with primer INTND2f to amplify a sequence of 964 bp (Fig. 1).

Primer INTND3r (5′-AAATAGCCGAGGCCACG-CATAGCA-3′) was designed to amplify, in combination with primer INTND2f, a sequence of 688 bp within the 1410 bp (NDf/NDr) and 824 bp (INTND2f/INTND2r) markers (Fig. 1). INTND3r was designed using Primer-Select™ 5·0 software (DNAStar, Inc.).

PCR assays using purified fungal DNA

DNA extracted from the 17 selected isolates of *V. dahliae* (Table 1) was used as templates in single PCR assays using primer pair NDf/NDr that amplifies a sequence of 1410 bp associated with the cotton and olive non-defoliating (ND) pathotype of *V. dahliae* (Mercado-Blanco *et al.*, 2001). Primer pair INTND2f/INTND2r were used to amplify a sequence of 824 bp within the 1410 bp marker (Mercado-Blanco *et al.*, 2001) which is specifically found in *V. dahliae* isolates belonging to VCGs 2A, $2B^{824}$, 4B, 6

Table 3 Positions of the single nucleotide polymorphisms (SNPs) among vegetative compatibility groups of *Verticillium dahliae* and one indel found within sequences NDf/r[1], NDf/r[2] and NDf/r[3] (1313 nucleotides aligned)

		Polymorphic nucleotide positions											
VCG	Sequence type	34	577	622	784	850	991	1066	1150	1171	1192	1194	1256
VCG2A/VCG4B	NDf/r[1]	C	T	C	G	C	C	C	T	A	C	G	G
VCG2B^{824}/VCG4A	NDf/r[2]	C	C	T	A	A	T	T	C	G	–	C	A
VCG6	NDf/r[3]	A	T	T	G	C	C	C	T	A	C	G	G

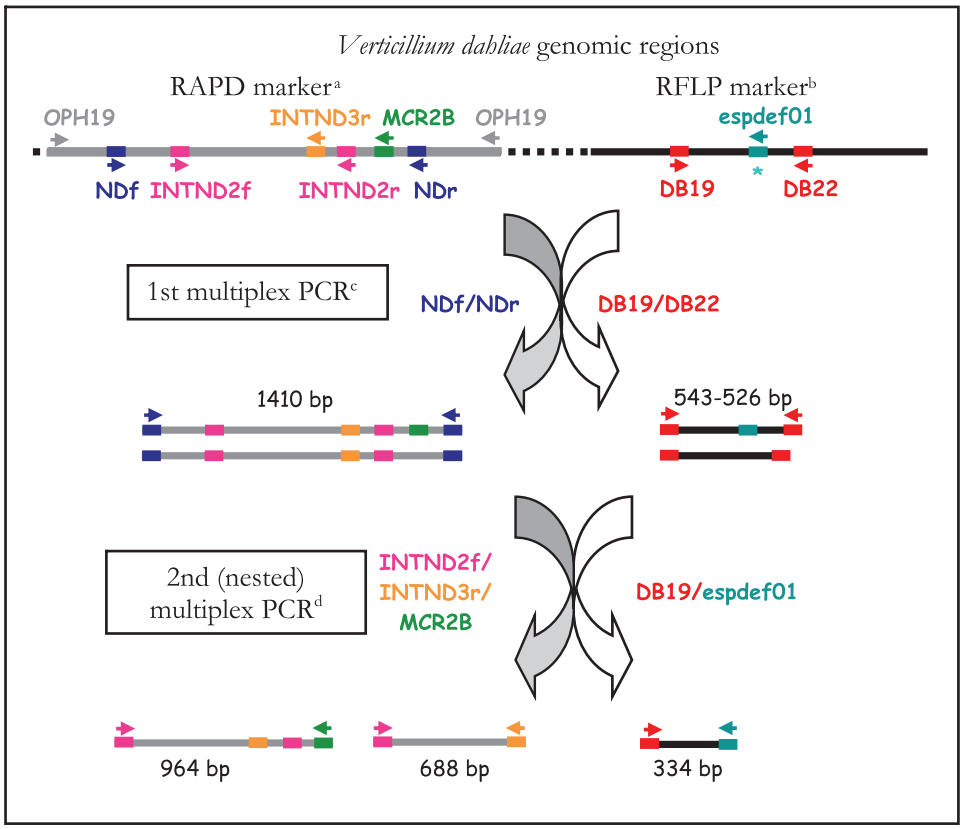

Figure 1 Overview of the multiplex, nested-PCR strategy optimized in *Verticillium dahliae* infecting artichoke. Arrowheads denote forward (➡) or reverse (⬅) primer. [a]RAPD marker from which primer pair NDf/NDr was designed (Pérez-Artés *et al.*, 2000; Mercado-Blanco *et al.*, 2001). [b]RFLP marker from which primer pair DB19/DB22 was designed (Carder *et al.*, 1994). [c]First multiplex PCR with primer pair NDf/NDr and DB19/DB22. [d]Nested multiplex PCR using both primer triplet INTND2f/INTND3r/MCR2B and primer pair DB19/espedef01. *Presence of an indel in this sequence; espdef01 primer is located in this polymorphic site (Mercado-Blanco *et al.*, 2003). See text for details on PCR conditions.

(Collado-Romero *et al.*, 2006) and 4A (Collado-Romero, 2006) (Table 2). Primers INTND3r and MCR2B (see above) were used jointly and in combination with primer INTND2f in PCR assays. Schematic location of all PCR primers used in this study is shown in Fig. 1. PCR reaction mixtures contained: 10–20 ng fungal DNA, 100 nM of each primer, 200 nM of each dNTP, 2 mM $MgCl_2$, 2·5 μL 10× reaction buffer and 0·75 U DNA polymerase (Biotools, B&M Labs) in 25 μL total volume. The PCR cycling protocols were: 94°C 5 min, 30 cycles of 94°C 1 min, 54°C 1 min (for primer pair NDf/NDr) or 60°C 1 min (for the combination INTND2f/INTND3r/MCR2B), 72°C 1 min, and a final step of 6 min at 72°C.

Plant samples used to optimize the multiplex-nested-PCR procedure

Twenty six 4-month-old artichoke plants of the susceptible seed hybrid cv. Nun. 9444 (Nunhems Seeds Ltd.) were artificially-inoculated with *V. dahliae* isolates representative of VCGs 1A, 2A, 2B^{824}, 2B^{334}, 4B and an HSI isolate (two plants per isolate). Three non-inoculated plants were used as controls. Artichoke plants were inoculated and cultured as previously described (Jiménez-Díaz *et al.*, 2006). Briefly, disinfested (1% NaClO, 2 min), germinated artichoke seeds were sown in trays filled with potting mixture (peat/sand, 2:1, vol/vol, autoclaved twice

Table 4 Multiplex-nested-PCR detection of *Verticillium dahliae* DNA in artichoke plants artificially inoculated with representative isolates of different VCGs and a single heterokaryon self incompatible isolate

		Roots		Leaves	
Plant[a]	VCG[b]	*V. dahliae* isolation[c]	PCR markers[d] 334 bp/688 bp/964 bp	*V. dahliae* isolation[c]	PCR markers[d] 334 bp/688 bp/964 bp
Controls	–	–	–/–/–	–	–/–/–
A-1		na	+/–/–	na	+/–/–
A-2	1A	na	+/–/–	na	–/–/–
B-1		–	–/–/–	–	–/–/–
B-2	2A	–	–/–/–	+	–/+/–
C-1		–	–/–/–	–	–/+/–[e]
C-2	2A	–	(–/+/–)	–	–/–/–
D-1		na	–/+/–[e]	na	–/+/–[e]
D-2	2A	na	(–/+/–)	na	–/+/–
E-1		na	–/+/–	na	–/+/–
E-2	HSI	na	–/+/–[e]	na	–/+/–
F-1		na	+/–/–	na	+/–/–[e]
F-2	2Ba	na	–/–/–	na	+/–/–[e]
G-1		na	+/–/–[e]	na	+/–/–[e]
G-2	$2B^{334}$	na	+/–/–[e]	na	+/–/–[e]
H-1		na	–/–/–	na	–/–/–
H-2	$2B^{824}$	na	–/+/+	na	–/+/+
I-1		–	–/+/+	+	–/+/+[e]
I-2	$2B^{824}$	–	(–/+/+)	+	–/+/+[e]
J-1		+	–/+/+	+	(–/+/+)
J-2	$2B^{824}$	–	–/+/+	–	–/+/+[e]
K-1		–	–/+/+[e]	–	–/+/+[e]
K-2	$2B^{824}$	–	(–/+/+)	–	–/+/+
L-1		–	–/+/+[e]	+	–/+/+[e]
L-2	$2B^{824}$	+	(–/+/+)	+	–/+/+[e]
M-1		+	–/+/+[e]	+	–/+/+[e]
M-2	$2B^{824}$	–	(–/+/+)	–	(–/+/+)

[a]Two plants (–1 and –2) inoculated with the same isolate. Two non-inoculated plants were used as negative controls.

[b]Vegetative compatibility groups used for inoculation. $2B^{824}$, VCG2B artichoke isolates that amplified a 824 bp PCR marker; $2B^{334}$, VCG2B artichoke isolates that amplified a 334 bp PCR marker; 2Ba, artichoke isolates that complement with artichoke *nit* mutants but not with international reference testers (Jiménez-Díaz *et al.*, 2006); HSI, heterokaryon self incompatible.

[c]*Verticillium dahliae* isolation on water agar amended with 30 mg L^{-1} chlorotetracycline (see text for details). +, *V. dahliae* successful isolation; –, no isolation; na, not assayed.

[d]PCR marker(s) amplified by multiplex-nested-PCR after the second round of multiplex-PCR. Samples from which *V. dahliae* specific PCR marker amplification was only possible after 1:10 (v/v) dilution of template DNA in UHQ water are shown in brackets. Previous RAPD-PCR tests performed on the undiluted DNA samples yielded no amplification (see text for details).

[e]DNA samples from which *V. dahliae* specific PCR markers were also visualized in agarose gels after the first round of multiplex-PCR (see text for details).

at 120°C). Plants were grown in a greenhouse at 20–24°C for 6 weeks until inoculation. *Verticillium dahliae* inocula (10^6 conidia mL^{-1}) were prepared by flooding 7- to 10-day-old cultures on potato dextrose agar (PDA) with sterile distilled water and filtering the suspension through sterile cheesecloth. Plants were uprooted, their roots thoroughly washed in tap water, slightly trimmed, and dipped for 10 min in each conidial suspension. Finally, plants were transplanted (one per pot) to plastic pots filled with the autoclaved potting mixture and maintained in the greenhouse as before. Root and leaf tissues from these plants (Table 4) were used to optimize the multiplex-nested-PCR procedure. To further validate the molecular detection procedure, naturally infected artichoke plants from commercial fields were tested. Two leaves from 22 individual plants sampled from two artichoke fields in the Valencia province and four leaves from each of eight individual plants from three fields in the Castellón province were assessed by molecular detection and microbiological isolation of *V. dahliae*. Artichoke plants from Castellón province were selected representing different levels of disease severity (Table 5). Disease severity was assessed by the symptom severity in individual plants scored on a 0 to 5 scale according to percentage of foliar tissue affected (foliar symptoms including chlorosis, stunting and wilting) in which: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = 76 to 100% of foliar tissue affected and 6 = dead plant.

Table 5 Multiplex-nested-PCR detection of *Verticillium dahliae* and VCG(s) identification in eight naturally-infected artichoke plants from three different fields in Castellón province

Field number and plant sample[a]	Severity[b]	Leaf	*V. dahliae* isolation[c]	1st PCR[d]	Marker amplified after nested-PCR (in bp) 334	688	964	Predicted infecting VCGs[e]
F2.P1	0	A	2/4	+	+	+	+	(1A, $2B^{334}$) and $2B^{824}$ and/or (2A, 4B)
		B	0/4	–	+	–	–	
		C	2/4	+	+	+	+	
		D	1/4	+	+	+	+	
F3.P1	0	A	0/4	–	–	–	–	(1A, $2B^{334}$) and $2B^{824}$ and/or (2A, 4B)
		B	0/4	+	+	+	+	
		C	0/4	–	–	–	–	
		D	4/4	–	+	+	+	
F1.P1	1	A	0/4	–	–	+	–	(1A, $2B^{334}$) and $2B^{824}$ and/or (2A, 4B)
		B	0/4	–	+	–	–	
		C	0/4	–	+	–	–	
		D	3/4	–	–	+	+	
F1.P2	2	A	3/4	–	–	–	–	(1A, $2B^{334}$) and (2A, 4B)
		B	0/4	+	+	+	–	
		C	2/4	+	+	+	–	
		D	1/4	+	+	+	–	
F1.P3	2	A	2/4	–	+	+	–	(1A, $2B^{334}$) and (2A or 4B)
		B	0/4	–	+	+	–	
		C	4/4	–	+	+	–	
		D	1/4	–	+	+	–	
F1.P4	3	A	4/4	+	–	+	+	(1A, $2B^{334}$) and $2B^{824}$ and/or (2A, 4B)
		B	4/4	+	+	+	+	
		C	4/4	++	–	+	+	
		D	3/4	++	–	+	+	
F2.P2	4	A	0/4	–	+	–	–	(1A, $2B^{334}$)
		B	0/4	–	+	–	–	
		C	0/4	–	–	–	–	
		D	0/4	–	–	–	–	
F1.P5	5	A	4/4	+	+	+	–	(1A, $2B^{334}$) and (2A, 4B)
		B	4/4	+	+	+	–	
		C	0/4	+	+	+	–	
		D	4/4	+	+	+	–	

[a]F1, F2 and F3 indicate three different commercial fields in Castellón province. P, artichoke plant sampled in the corresponding field. Plants were randomly selected according to different disease symptom severity.

[b]Disease severity was assessed by the symptom severity in individual plants scored on a 0 to 5 scale according to percentage of foliar tissue affected (foliar symptoms including chlorosis, stunting and wilting) in which: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = 76 to 100% of foliar tissue affected and 6 = dead plant.

[c]Number of *V. dahliae* positive isolations on PDA amended with 0·5 g L^{-1} of streptomycin sulphate from four surface-disinfested tissue pieces (vascular bundles) per leaf. Pathogen isolation assays were performed in four different leaves (A, B, C and D) per sampled artichoke plant.

[d]+, amplification of the polymorphic *V. dahliae*-specific 526/543 bp marker (DB19/DB22 primers pair (Carder *et al.*, 1994); ++, amplification of both the 526/543 bp and the 1410 bp (NDf/NDr primer pair) (Mercado-Blanco *et al.*, 2001) markers; –, no amplification.

[e]Predicted VCG groups infecting the plant according to the PCR markers amplified after multiplex-nested-PCR.

Processing of infected artichoke tissues: *V. dahliae* isolation and DNA extraction

The complete root and the main vein from two leaves of each *V. dahliae* artificially-inoculated artichoke plant were thoroughly washed under tap water and then surface-disinfested in 1% NaClO for 2 min. For *V. dahliae* isolation, four pieces (0·5 cm-long) of disinfested tissue from 14 individual plants were plated onto water agar amended with 30 mg L^{-1} of chlorotetracycline (Sigma Chemical Co.). Leaves from naturally-infected one-year-old artichoke plants sampled from commercial fields were treated similarly, except that *V. dahliae* isolations were made from four fragments of the main vein per leaf onto PDA (Biokar Diagnostics) plates supplemented with 0·5 g L^{-1} of streptomycin sulphate (Sigma-Aldrich). Plates were incubated at 25°C in the dark for 7 to 15 days and emerging mycelium from plant tissues was identified as *V. dahliae* on the basis of conidiophore morphology and the formation of dark microsclerotia. In all cases, the remaining main vein or roots not used for microbiological detection were lyophilized and ground to a fine powder in 12-mL disinfested stainless steel containers with balls using a mill (Mixer Mill 301, Retsch). Samples of powdered plant tissue

(20–30 mg) were used for extraction of total DNA using a DNeasy Plant Kit (Qiagen) following the manufacturer's instructions. DNA purity and concentration were determined as indicated above. Two non-inoculated artichoke plants were submitted to the same process and used as negative controls in both microbiological isolation and PCR tests.

Multiplex-nested-PCR assays and determination of the *V. dahliae* DNA detection limit

Two different regions of the *V. dahliae* genome known to be polymorphic in different VCGs (Mercado-Blanco *et al*., 2003; Collins *et al*., 2005; Collado-Romero *et al*., 2006) were the basis for the design of specific PCR primers. A general overview of the multiplex-nested-PCR procedure is schematically displayed in Fig. 1. Primer pairs NDf/NDr (Mercado-Blanco *et al*., 2001) and DB19/DB22 (Carder *et al*., 1994) were used jointly in the first round of amplification as previously described (Mercado-Blanco *et al*., 2003). After the first round of amplification, a second PCR (nested-PCR) was carried out using 1 μL of the amplification product from the first round as template and the five nested primers (INTND2f/INTND3r/MCR2B and DB19/espdef01) (Fig. 1). Amplification conditions for the first PCR round were: 2·5 μL 10× reaction buffer (Biotools, B&M Labs), 400 nM of each primer DB19 and DB22, 200 nM of each primer NDf and NDr, 800 nM of each dNTP, 1·5 mM $MgCl_2$, 3 to 20 ng of total DNA extracted from plants, and 0·75 U DNA polymerase (Biotools, B&M Labs) in 25 μL total volume. PCR conditions were 94°C 4 min, 35 cycles of 94°C 1 min, 54°C 45 s, 72°C 1 min, and a final step of 5 min at 72°C. For the second round of PCR amplification, conditions were: 2·5 μL 10× reaction buffer, 200 nM of each of primers DB19 and espdef01, 100 nM of each primer INTND2f, INTND3r and MCR2B, 800 nM of each dNTP, 1·5 mM $MgCl_2$, 1 μL of the first PCR product, and 0·75 U DNA polymerase (Biotools, B&M Labs) in 25 μL total volume. PCR conditions were 94°C 4 min, 25–30 cycles of 94°C 1 min, 60°C 45 s and 72°C 1 min, and a final step of 5 min at 72°C. In all assays, positive controls [pure *V. dahliae* DNA from isolates V138I (VCG1A), V176I (VCG2A) and V474I (VCG2B^{824}) (Collado-Romero *et al*., 2006)] and negative controls (no DNA and DNA from non-inoculated artichoke plants) were included. PCR products from the first and second rounds of PCR were separated by electrophoresis according to standard protocols (Sambrook *et al*., 1989).

In order to determine the minimum quantity of *V. dahliae* DNA that could be detected in infected artichoke tissues, the multiplex-nested-PCR procedure described above was performed using as templates serial dilutions (from 10 ng to 0·01 pg) of DNA extracted from three *V. dahliae* isolates representative of VCGs 1A (isolate V138I), 2A (isolate V176I), and 2B^{824} (isolate V474I). These isolates were therefore representative of all PCR marker patterns expected with primer combinations used in this study (Table 2). The PCR assays were performed both with pure *V. dahliae* DNA and *V. dahliae* DNA plus 10 ng of a DNA mixture extracted from three non-inoculated artichoke plants.

Random amplified polymorphic DNA-PCR (RAPD-PCR): assessment of PCR-quality plant DNA

Multiplex-nested-PCR assays (see above) occasionally yielded negative results for detection of *V. dahliae* DNA. To exclude the possibility of false negatives due to the presence of PCR inhibitors, quality of the extracted plant DNA was further tested by random amplified polymorphic DNA (RAPD)-PCR assays. Amplification conditions were: 2·5 μL 10 × reaction buffer, 2·5 mM $MgCl_2$, 500 nM of primer OPH19 (Operon Technologies), 0·75 U DNA polymerase (Biotools, B&M Labs) and 3 μL of plant DNA in 25 μL total volume. PCR conditions were 94°C 4 min, 30 cycles of 94°C 1 min, 37°C 1 min and 72°C 1 min, and a final step of 6 min at 72°C.

Results

Association between PCR markers and *V. dahliae* VCGs

Correlations between PCR markers and VCG assignment of *V. dahliae* isolates were investigated as the first step for developing an effective *in planta* molecular detection procedure in infected artichoke plants (Table 2). First, all isolates used in this study amplified the *V. dahliae*-specific marker defined by primer pair DB19/DB22 (Carder *et al*., 1994) (Fig. 1, Table 2), whereas *V. dahliae* isolates of VCGs 1A, 1B, 2B^{334} and 4A amplified the 334 bp PCR marker (DB19/espdef01) (Table 2). Secondly, and as expected, all isolates that amplified the 824 bp PCR marker using primer pair INTND2f/INTND2r and the 1410 bp PCR marker (primer pair NDf/NDr), also amplified the 688 bp PCR marker (defined by primer pair INTND2f/INTND3r) (Fig. 1). Therefore, these three PCR markers (1410, 824 and 688 bp) were associated with VCGs 2A, 4A, 4B, 6, and the subgroup VCG2B^{824}. In order to differentiate among VCGs that amplified the 1410/824/688 bp markers triad, the 1410 bp amplicons from several isolates representative of the different VCGs were sequenced (Table 1). Three different sequences were identified and named as NDf/r[1], NDf/r[2] and NDf/r[3] (Tables 1 & 3). The NDf/r[1] sequence was identical in VCG4B and 2A isolates, and was also present in the HSI isolate (V4I) that is molecularly similar (Collado-Romero *et al*., 2006) and with the same pathogenicity phenotype (Korolev *et al*., 2001) as isolates in VCG2A. Sequence NDf/r[2] was found in VCG2B^{824} and VCG4A isolates. Finally, sequence NDf/r[3] was exclusively identified in the two VCG6 isolates analyzed. Based on the sequence polymorphisms found (Table 3) primer MCR2B was designed to differentiate VCG2B^{824} (sequence NDf/r[2]) from the other genetic/molecular groups that also amplified the 824/688 bp markers, that is VCGs 2A, 4B (sequence NDf/r[1]) and VCG6 (sequence NDf/r[3]).

Primer MCR2B was designed as reverse primer (nucleotide positions 1191 to 1211 of sequence NDf/r[2]), therefore having its 3′end over polymorphic nucleotide sites 1192 and 1194 (Table 3). Thus, when primer pair INTND2f/MCR2B was used in PCR assays to check the *V. dahliae* collection, the expected 964 bp marker was only amplified in VCG2B^{824} and VCG4A isolates (Table 2). Despite an identical 1410 bp sequence in VCG2B^{824} and VCG4A isolates, these two groups can be differentiated because VCG4A amplified the 334 bp PCR marker but VCG2B^{824} did not (Table 2).

In planta differential detection of *V. dahliae* VCGs by multiplex-nested-PCR

Verticillium dahliae DNA was detected by multiplex-nested-PCR in all artificially-infected artichoke plants, either in root or leaf tissues (Table 4). Samples yielding no amplification of *V. dahliae* DNA were also negative when tested by RAPD-PCR, which presumably indicated poor quality of DNA templates. However, *V. dahliae* DNA amplification was possible in 47% of these samples after diluting them (1 : 10, v/v) in UHQ water (Sigma). All tissue samples that yielded positive isolation of *V. dahliae* on culture media were also positive by molecular detection. However, the molecular detection procedure yielded 92·3% of positive detections compared to 50% of successful pathogen isolations (Table 4). DNA from non-inoculated plants did not amplify any *V. dahliae* specific markers, despite RAPD-PCR tests demonstrating that DNAs were of PCR quality (results not shown).

Once the molecular detection procedure was optimized for artificially-inoculated artichoke plants, the procedure was tested against artichoke plants sampled from commercial fields. Results obtained for root and leaf tissues (Table 4) suggested that artichoke leaves were a more appropriate target tissue to detect *V. dahliae* since they can be easily sampled without uprooting the plant. Thus, only the main vein of basal leaves of naturally-grown artichoke plants were used for DNA extraction and pathogen isolation. *Verticillium dahliae* was isolated by microbiological methods in five (six leaves) out of 22 artichoke plants sampled from commercial fields in the Valencia province (results not shown). The vast majority (89%) of DNA samples extracted from these leaves were of PCR quality, as indicated by RAPD-PCR assays (Fig. 2). The multiplex-nested-PCR procedure yielded positive detection in 10 (13 leaves) out of 22 plants. Moreover, detection was possible even after the first PCR round in five of these leaves, which suggested high levels of pathogen DNA in these samples. In all tissue samples from which *V. dahliae* isolation was achieved, the multiplex-nested-PCR procedure was successful as well, except in one leaf sample. Overall, the molecular detection procedure was more effective than the pathogen isolation procedure and, more importantly, was informative about the genetic nature (VCG) of isolates infecting the plants. Indeed, because of the amplification of the 334 bp PCR marker (Table 2), it was concluded that most of the

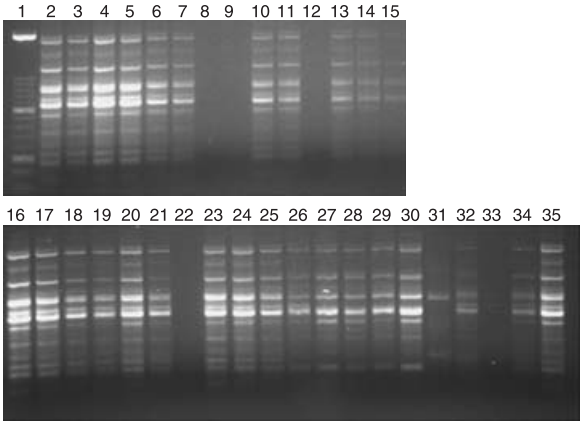

Figure 2 RAPD-PCR patterns of *Verticillium dahliae* obtained using DNA extracted from 17 out of 22 naturally-infected artichoke plants (leaves) as templates, from commercial fields in Valencia province (see text for details). Lane 1, 100 bp ladder DNA marker. Lanes 2–35, amplifications obtained from each leaf (two leaves per plant).

infected plants (seven out of 10) from the Valencia province were infected by VCG2B^{334} (either 2B^{334} or 2Ba), although not ruling out the improbable presence of a VCG1A isolate (see below). The 964 bp PCR marker was amplified from the tissue of one plant, indicating infection by a VCG2B^{824} isolate. The lack of amplification of the 334 bp marker in this plant DNA template excluded the presence of a VCG4A isolate (Table 2). Finally, one plant from the Valencia province was infected by two different isolates, as indicated by the amplification of both the 334 bp and the 688 bp markers, which were never amplified in any *V. dahliae* VCGs except VCG4A (Table 2). However, the lack of amplification of the 964 bp marker in this DNA plant sample, ruled out the infection by a VCG4A isolate. Moreover, the possibility that any of these plants could be infected by a VCG4A isolate is rather unlikely, since to the best of the authors' knowledge this group has only been found in North America (Rowe & Powelson, 2002), and previous surveys have never identified VCG4A isolates in the Comunidad Valenciana Region (Collado-Romero *et al*., 2006; Jiménez-Díaz *et al*., 2006).

Purified DNAs from naturally-infected artichoke plants in the Castellón province were of poor PCR quality as indicated by gel electrophoresis and RAPD-PCR assays (results not shown). Therefore, all DNA samples from Castellón were diluted 1:10 in UHQ water to counteract the possible action of PCR inhibitors. Indeed, after dilution, *V. dahliae* DNA was successfully detected by multiplex-nested-PCR in all sampled plants (27 leaves) (Table 5). DNA quality of the samples that did not amplify any marker (five leaves, Table 5) were further checked by specific PCR adding 1 ng of DNA from isolate V138I and using conditions described for the first round of the multiplex-nested-PCR procedure. In all cases, there was amplification of the expected marker showing that the PCR reaction was not inhibited. Moreover, the molecular detection procedure was more effective than *V. dahliae* isolation, which was only possible in 18 leaves from seven

plants (Table 5). For this group of plants, all leaves positive in *V. dahliae* isolation also amplified pathogen DNA. It is remarkable that all plants except one were infected by *V. dahliae* isolates of at least two different VCGs, as indicated by the simultaneous amplification of the 334 and 688 bp PCR markers, or the 334, 688 and 964 bp PCR markers (Table 5). The detection of several VCGs in these plants suggested molecular/genetic diversity within the *V. dahliae* population infecting artichoke in these fields. Moreover, it is worth mentioning the consistent presence of VCG2B^{824} isolates (revealed by the amplification of the 964 bp marker) in the three surveyed fields.

Detection limit of *V. dahliae* DNA

The minimum quantity of *V. dahliae* DNA that could be detected with all primer pairs either in the presence or absence of artichoke DNA was 0·1 pg (i.e., no amplification with 0·01 pg of *V. dahliae* DNA), regardless of the isolate used. In all cases, the PCR pattern obtained was as expected from each VCG representative isolate used (Table 2).

Discussion

The use of resistant cultivars is an economically efficient control measure for the management of verticillium wilt in artichoke (Cirulli *et al*., 1994). However, no resistant cultivars of commercial interest are currently available. Due to its high quality, cv. Blanca de Tudela is the traditionally predominant variety grown in Spain. However, this genotype is very susceptible to verticillium wilt and is vegetatively propagated by means of stumps, which may contribute to the spreading of the disease (Armengol *et al*., 2005). Therefore, the use of pathogen-free planting material in noninfested or low inoculum density soils (i.e. by soil fumigation and/or soil solarization) (Ciccarese *et al*., 1985; Tjamos & Paplomatas, 1988) is, among others, a key control measure for this crop (Cirulli *et al*., 1994; Armengol *et al*., 2005).

In the present work, a PCR-based 'molecular tool box' for the identification of *V. dahliae* VCGs infecting artichoke plants is described. Currently, diagnosis of infected artichoke plants is based on assessment of symptoms followed by *V. dahliae* isolation from plant tissues and identification based on morphological features. However, pathogen isolation may suffer from inconsistency. For example, seasonal changes in *V. dahliae* isolation have been reported in artichoke and other crops (Armengol *et al*., 2005; Berbegal *et al*., 2007). Seasonality may be influenced, among other factors, by unfavourable environmental conditions for the *in planta* growth of *V. dahliae* or by anatomical and physiological changes in the plants (Schreiber & Mayer, 1992). Thus, positive isolation of *V. dahliae* from artichoke plant tissues is more frequent in autumn and late spring, associated with the appearance of severe symptoms (Armengol *et al*., 2005; Berbegal *et al*., 2007). The multiplex-nested-PCR procedure developed here could overcome these problems, providing a powerful tool for the detection of the pathogen at the subspecies level (i.e. VCGs) and in different plant growing situations. Indeed, the molecular procedure was more efficient than the microbiological isolation procedure in both artificially- and naturally-infected plants. Moreover, the procedure uncovered *V. dahliae* presence in symptomless plants, which makes it an excellent implement in certification schemes of pathogen-free planting material. The rare event of no *V. dahliae* PCR marker amplification but positive pathogen isolation (just one leaf out of 44 samples analyzed in Valencia province), may be explained because of the different leaf sections used for each procedure (pathogen isolation or molecular detection) assayed, and the possibility of an uneven *V. dahliae* colonization of the vascular tissue in this particular sample. Similarly, sample DNA degradation could explain this result. Nevertheless, this seemed to be of minor relevance in this study (only one sample), and can be overcome by increasing the number of leaf tissue samples. Moreover, results obtained from Castellón samples (where the same sections of the leaf were used for both methods) confirmed the advantages of the molecular procedure.

From the results obtained in this work, two important premises have to be considered for an appropriate implementation of the PCR-based 'molecular tool box' designed here in certification schemes of *V.dahliae*-free planting material. First, results from artificially-infected plants showed that detection was equally efficient using either root or leaf tissues. In addition, results from naturally-infected plants showed that a strategy of sampling four leaves per plant was sufficient to qualify it as an infected plant. Therefore, leaves from plants (either in a nursery or in a commercial field) are suitable targets for infection assessment using the PCR-based procedure. Secondly, results also showed that the quality of the purified plant DNA is a key factor for the success of the molecular detection procedure. Thus, an optimum PCR-quality DNA was achieved when plant tissues were frozen immediately after sampling. However, when plant tissues were submitted to two freeze/thaw cycles, as happened with artichoke plants from Castellón province, the DNA quality was considerably reduced and negatively affected molecular detection. However, no difference in detection effectiveness was found regardless of whether leaves were severely wilted or green.

The multiplex-nested-PCR procedure proved to be superior to the pathogen isolation methods because it provided information about the infecting isolate, since amplified PCR markers correlated to the VCG. According to the current available data on the *V. dahliae* population structure infecting artichoke in the Comunidad Valenciana Region (Jiménez-Díaz *et al*., 2006), three groups of VCGs can be differentially identified by the multiplex-nested-PCR procedure: i) isolates amplifying exclusively the 334 bp PCR marker (VCGs 1A and 2B^{334}); ii) isolates amplifying exclusively the 688 bp PCR marker (VCGs 2A and 4B); and iii) isolates amplifying the 688 bp and the 964 bp PCR markers (VCG2B^{824}). Thus, the use of these PCR markers provided information about the genetic

(VCGs) diversity of the *V. dahliae* population infesting a specific artichoke field, which may help in establishing the population structure of this pathogen without the need for isolation procedures from plant tissues. This has been shown for the two sets of naturally-infected plants analyzed in this present study. Similarly, differential detection of VCGs led to verification of the coinfection of the same naturally-infected plant by isolates belonging to at least two or three different VCGs (one plant from Valencia and seven plants from Castellón). This finding may have important epidemiological consequences. It may also provide critical information necessary for implementing control strategies. Indeed, the amplified PCR markers are informative about the aggressiveness of the infecting *V. dahliae* isolates in artichoke, which correlates to VCG (Jiménez-Díaz *et al*., 2006). For example, the consistent presence of VCG2B^{824} isolates in samples from Castellón province was also relevant from an epidemiological perspective, since this group is one of the most aggressive VCGs in artichoke (Jiménez-Díaz *et al*., 2006). In some instances however, VCG2A which is less aggressive in artichoke than VCG4B (Jiménez-Díaz *et al*., 2006), still remained molecularly undistinguishable with the PCR markers used and between isolates of VCG2B^{334} and VCG1A, the latter group being less aggressive on artichoke. Nevertheless, available data based on broad surveys have so far shown that both groups are seldom represented (< 1% for VCG1A and < 3% for VCG4B) within the artichoke *V. dahliae* population in the Comunidad Valenciana Region (Jiménez-Díaz *et al*., 2006). However, genetic diversity was previously found within VCG2B^{334} (Jiménez-Díaz *et al*., 2006). In this group, some isolates form stable heterokaryons with international reference testers (subgroup 2Br^{334}) whereas others only do it with artichoke isolates assigned to VCG2B (subgroup 2Ba). However, this differential behaviour correlated neither with molecular differences, as revealed by AFLP fingerprinting (Collado-Romero *et al*., 2006), nor with pathogenicity in artichoke or cotton (Jiménez-Díaz *et al*., 2006). Therefore, detection in an infected artichoke plant of the 334 bp PCR marker, which may indicate the presence of a VCG2B^{334} isolate (either 2Br^{334} or 2Ba), would not compromise any epidemiological inference or disease severity prediction related to this VCG group. Finally, amplification of the 334 bp PCR marker may also indicate the rare event (see above) of a VCG1A infection.

The 688 bp PCR marker (INTND2f/INTND3r) provided the same diagnostic information yielded by the 824 bp amplicon (INTND2f/INTND2r) (Mercado-Blanco *et al*., 2003; Collado-Romero *et al*., 2006). During preliminary assays with diverse artichoke tissues, the latter primer pair occasionally produced smears after the second round of PCR. Moreover, since amplification of the 964 bp marker (INTND2f/MCR2B) allowed the differentiation between VCG2B^{824} isolates and those from VCGs 4B and 2A, and in order to obtain well-differentiated markers after nested PCR, primer pair INTND2f/INTND3r was used instead of INTND2f/INTND2r (Fig. 1). Certainly, the use of INTND2f/INTND3r primer pair (jointly with primer MCR2B) in the nested-PCR round abolished the smearing, yielded consistent results, and produced clearly distinguishable amplicons in agarose gels (688 bp marker and 964 bp marker). An amplicon of approximately 850 bp was occasionally observed in samples where the 964 bp marker was amplified (Fig. 3). This band has not been characterized in this study, but did not interfere with the effectiveness of the procedure.

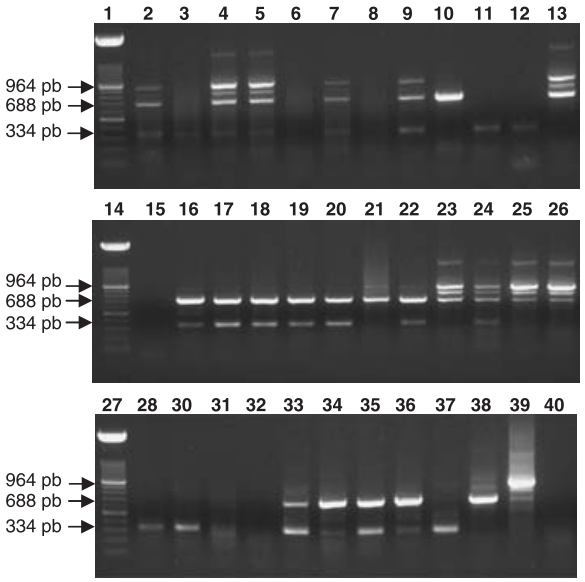

Figure 3 PCR marker patterns of *Verticillium dahliae* obtained after conducting the multiplex, nested-PCR assay using DNA extracted from naturally-infected artichoke leaves from commercial fields in Castellón province as templates. DNA templates were from four different basal leaves and corresponded to plant F2.P1 (lanes 2–5); plant F3.P1 (lanes 6–9); plant F1.P1 (lanes 10–13); plant F1.P2 (lanes 15–18); plant F1.P3 (lanes 19–22); plant F1.P4 (lanes 23–26); plant F2.P2 (lanes 28–32); and plant F1.P5 (lanes 33–36). *Verticillium dahliae* DNA from isolate V138I (VCG1A) (lane 37); *V. dahliae* DNA from isolate V176I (VCG2A) (lane 38); *V. dahliae* DNA from isolate V400I (VCG2B) (lane 39); negative control (no DNA) (lane 40). Lanes 1, 14 and 27, 100 bp ladder DNA marker. See also Table 5 for details of sampled plants.

In addition to the advantages as a diagnostic procedure for certification of pathogen-free artichoke planting material, the designed 'molecular tool box' can be useful for epidemiological studies focused in the identification and prevalence of isolates (or groups of isolates, VCGs), not only in artichoke but in other susceptible crops. The information available on the association of *V. dahliae* VCGs with specific crops and geographic areas, has so far shown that limited VCG diversity generally occurs and that correlation may exist between aggressiveness towards a given host and the VCG (Joaquim & Rowe, 1991; Strausbaugh *et al*., 1992; Korolev *et al*., 2000, 2001; Bhat *et al*., 2003; Jiménez-Díaz *et al*., 2006). Therefore, the molecular procedure developed here, and the information on pathogenicity and fungal genotypes could be applicable to other *V. dahliae* susceptible hosts such as cotton, olive, potato or tomato. An additional outcome is the potential interest of the 964 bp PCR marker to differentiate

between VCG4A and VCG4B isolates, frequently associated with potato in North America (Rowe & Powelson, 2002).

In conclusion, this work presents a powerful molecular tool which provides: i) higher efficiency in *V. dahliae* detection in artichoke-infected plants than traditional microbiological isolation procedures; ii) ability to identify genetic diversity (VCGs) within the *V. dahliae* population infecting a commercial field; and iii) relevant epidemiological information, since the diverse VCGs infecting artichoke display differential pathogenicity on this host. In addition, it may have interest in breeding programs for verticillium wilt of artichoke resistance, helping to assess whether new artichoke varieties may be differentially affected by *V. dahliae* VCGs.

Acknowledgements

This research was supported by grants AGL2003-00503 and AGL2000-1444 from Comisión Interministerial de Ciencia y Tecnología (CICYT) of Spain, and by grant GV–CAPA00-12 from Consellería de Agricultura, Pesca y Alimentación de Generalitat Valenciana. Melania Collado Romero was a recipient of a FPI fellowship from the Spanish Ministry of Education and Science (MEC). Mónica Berbegal is a recipient of a FPU fellowship from the MEC. We are indebted to José García-Jiménez for helpful advice and guidance in artichoke fields. Thanks are due to the editor and anonymous reviewers for editorial improvement and thorough revision.

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