# Detection of *Verticillium dahliae* Isolates Differing in Vegetative Compatibility in Infected Artichoke Plants by Multiplex, Nested PCR

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**Abstract**

**A multiplex, nested-PCR (polymerase chain reaction) protocol has been developed and optimized for the detection of *Verticillium dahliae* in infected artichoke plants, based on the amplification of specific DNA sequences of the pathogen using total DNA extracted from target tissues. Primer pairs for the procedure were designed based on DNA sequences associated with the vegetative compatibility grouping (VCG) of *V. dahliae* isolates infecting artichoke, which allowed to identify the belonging of isolates to VCGs according to the PCR marker amplicon produced in the second round of the nested-PCR assay. Thus, amplicons were associated with VCG1A and VCG2B$^{334}$ (334 bp), VCG2A, VCG2B$^{824}$ and VCG4B (688 bp), or only VCG2B$^{824}$ (964 bp). That differential detection is of significance because VCG of isolates was shown to correlate with their virulence to artichoke cultivars in previous work. Artichoke plants artificially infected with *V. dahliae* as well as naturally-infected ones sampled from commercial fields in the Comunidad Valenciana region at eastern-central Spain were used to validate the molecular detection procedure and for comparisons with conventional detection by isolating the fungus from plant tissues in culture media. The molecular detection procedure was always more efficient than the conventional detection. In all cases, results from the multiplex, nested-PCR assays using artificially-infected plants were predictive of the VCG of the infecting *V. dahliae* isolates. Also, results using plants sampled from commercial fields showed that genetic/molecular diversity exists among *V. dahliae* isolates infecting artichoke in Valencia and Castellón provinces. Moreover double infection of a plant by different *V. dahliae* isolates was demonstrated.**

## INTRODUCTION

Verticillium wilt (VW), caused by the soilborne fungus *Verticillium dahliae*, is of increasing concern for artichoke production in the Comunidad Valenciana region of eastern-central Spain since the 1990s (Armengol et al., 2005). Artichoke crops in this region account for nearly 33.5% of the total national artichoke production and contribute to Spain being the second artichoke producer worldwide, after Italy (FAO, 2004). Severity of VW attacks and yield loss are particularly important for cv. Blanca de Tudela, which is very susceptible to VW, vegetatively propagated by means of stumps, and grown extensively in Spain (Armengol et al., 2005; Jiménez-Díaz et al., 2006). Use of pathogen-free planting material in noninfested soil can control the disease. However, selection of healthy stumps based on the absence of disease symptoms in plants during the previous growing season, which is usually practiced by many artichoke producers in eastern-central Spain, should be avoided because infected plants can remain symptomless (Armengol et al., 2005). Detection of *V. dahliae* infections in artichoke is mainly by fungal isolations from plant tissues in culture media and the subsequent identification of the pathogen based on morphological features. This procedure requires time for

incubating cultures and skills in fungal identification. In this present work, we have developed and optimized a molecular-detection procedure for the detection and identification of *V. dahliae* in infected artichoke plants based on the nested-polymerase chain reaction (PCR) technique. To that aim, we used primer pairs that had been designed for the identification of cotton- and olive- defoliating and nondefoliating *V. dahliae* pathotypes (Mercado-Blanco et al., 2001, 2003). Also, because VCG of isolates was shown previously to correlate with their virulence to artichoke cultivars, we developed new primers were developed based on DNA sequences that correlate with vegetative compatibility grouping (VCG) of *V. dahliae* isolates (Collado-Romero, 2006; Collado-Romero et al., 2006; Jiménez-Díaz et al., 2006).

## MATERIALS AND METHODS

### Plant Samples

Twenty nine 'Num. 6374' and 'Num. 9444' artichoke plants infected with *V. dahliae* isolates of different VCGs were used to optimize the molecular-detection multiplex, nested-PCR protocol (Table 1). These plants were obtained from virulence assays carried out in previous work (Jiménez-Díaz et al., 2006). To validate the newly developed protocol, two leaves sampled from each of 22 plants from two fields in Valencia province (Table 2) and four leaves from each of eight plants from three fields in Castellón province (Table 3) were used for molecular detection and microbiological isolations of *V. dahliae*. Sampled plants from Castellón were selected as representatives of different levels of symptoms severity.

Treatment of sampled tissues from artificially- and naturally-infected artichoke plants: microbiological isolation and detection, and DNA extraction.

Roots and the main vein from leaves of artificially-infected plants were carefully and individually washed under tap water and surface disinfested in 1% NaClO for 2 min. Pieces (0.5-cm-long) of disinfested tissues from 14 plants were plated onto water agar amended with 30 mg/L of chlorotetracycline. Leaf sampled from commercial fields were treated similarly, except that isolations were made from four fragments of vascular bundles per leaf onto potato-dextrose agar supplemented with 0.5 g/L of streptomycin sulphate. Cultures were incubated at 25ºC in the dark for 7 to 15 days and colonies of *V. dahliae* were identified on the basis of conidiophore morphology and the formation of dark microsclerotia. In all cases, the remaining tissues not used for microbiological detection were lyophilized and ground to a fine powder using a mill (Mixer Mill 301, Retsch, Germany) and 12-mL stainless containers with two balls using a mill Twenty 30-mg-samples of powdered tissue were used for extraction of total DNA using DNeasy Plant Kit (Qiagen GmbH, Hilden, Germany) with a final elution in 75-100 µL of the buffer supplied in the kit. DNA quantification was done in 0.7% agarose gels stained with ethidium bromide according to standard procedures.

### Multiplex, Nested-PCR Assays and VCG-PCR Markers Association

For the molecular detection of *V. dahliae*, two sequential PCR reactions (nested-PCR) and five combinations of primers pairs (multiplex) were used. Primer pairs NDf/NDr and DB19/DB22 were used jointly in the first round of amplification. In previous work for the characterization of a collection of 109 *V. dahliae* isolates from artichoke where characterized by means of specific-PCR assays and VCG belonging (Collado-Romero, 2006; Jiménez-Díaz et al., 2006), we showed that primer pair NDf/NDr amplifies a sequence of 1410 bp only from isolates of VCGs 2A, $2B^{824}$ and 4B; whereas primer pair DB19/DB22 amplifies a polymorphic (523-bp/539-bp) sequence specific of *V. dahliae* isolates (Carder et al., 1994). After the first round of amplification, a second round of PCR was carried out using 1 µL of amplification products and three combinations of primer pairs. Two of those combinations (i.e., INTND2f/INTND3r; INTND2f/MCR2B) amplify sequences that are internal to the 1410-bp amplicon produced in the first round of PCR. Thus, primers INTND2f/INTND3r amplify a

sequence of 688 bp from isolates of VCGs 2A, $2B^{824}$ and 4B; and primers INTND2f/MCR2B amplify a sequence of 964-bp only from isolates of $VCG2B^{824}$ (Collado Romero, 2006). Finally, the third primer pair used in the second round of PCR, DB19/espdef01, amplifies a 334-bp sequence internal to the polymorphic sequence amplified by primers DB19/DB22 (Mercado-Blanco et al., 2003) from isolates of VCG1A and $VCG2B^{334}$ (Jiménez-Díaz et al., 2006). Amplification conditions for the first PCR round were (25 µL total volume): 2.5 µL 10 × reaction buffer (Biotools, B&M Labs, Madrid, Spain), 400 nM of each primer DB19 and DB22, 200 nM of each of primers 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). PCR conditions were 94ºC 4 min, 35 cycles of 94ºC 1min, 54ºC 45 s and 72ºC 1 min, and a final step of 5 min at 72ºC. For the second round of PCR amplification, conditions were (25 µL total volume): 2.5 µL 10x reaction buffer, 200 nM of each of primers DB19 and espdef01, 100 nM of each of primers INTND2f, INTND3r and MCR2B, 800 nM of each dNTP, 1.5 mM $MgCl_2$, 1 µL of PCR product from the first round, and 0.75 U EcoTaq polymerase (Ecogen S.R.L., Barcelona, Spain). PCR conditions were 94ºC 4 min, 25 cycles of 94ºC 1min, 60ºC 1 min and 72ºC 1 min, and a final step of 5 min at 72ºC. In all cases, positive controls (consisting of *V. dahliae* DNA) and negative controls (no DNA) were used simultaneously with the assayed material. PCR products of the first and second rounds of PCR were electrophoresed in 1% agarose gels with ethidium bromide, and visualized under UV light.

**Random Amplified Polymorphic DNA Assays to Assess PCR Quality of Extracted Plant DNA**

Multiplex, nested-PCR assays sometimes yielded negative results for *V. dahliae*. In these cases, quality of the extracted plant DNA used for PCR assays was further tested by random amplified polymorphic DNA (RAPD)-PCR assays to confirm that lack of *V. dahliae* DNA amplification was due to absence of the pathogen in the assayed plant tissues and not due to the presence of inhibitors of the PCR reaction. Amplification conditions for those assays were (25 µL total volume): 2.5 µL 10× reaction buffer, 2.5 mM $MgCl_2$, 500 nM of primer OPH19 (Operon Technologies, CA, USA), 0.75 U DNA polymerase (Biotools, B&M Labs) and 3 µL of plant DNA. PCR conditions were 94ºC 4 min, 30 cycles of 94ºC 1min, 37ºC 1 min and 72ºC 1 min, and a final step of 6 min at 72ºC.

**RESULTS AND DISCUSSION**

*V. dahliae* DNA was detected by multiplex, nested-PCR in all artificially-infected artichoke plants, either in the assayed roots or leaves (Table 1). Samples that were negative for *V. dahliae* in nested-PCR assays were also negative in RAPD-PCR assays. However, *V. dahliae* DNA amplification was possible for some of those negative detections when the total plant DNA was diluted 1:10 in water. When plant samples where tested both by microbiological and molecular detection procedures, all samples that were positive by isolation in pure culture were also positive by molecular detections. However, the molecular detection protocol yielded 92.3% positive detections compared with 50% achieved with microbiological isolations.

Since the molecular detection procedure optimized in this work aims to be applicable for the certification of pathogen-free artichoke planting material, we focused the detection in those plant parts that may harbour *V. dahliae* and can be easily sampled without need of uprooting the plant. For this purpose, basal leaves of artichoke plants were sampled from commercial fields and only vascular bundles from the main vein were used for DNA extraction. Microbiological detection using that material was positive for six leaves from five out of 22 plants from commercial fields in Valencia province (Table 2). Conversely, the multiplex, nested-PCR detection procedure was positive for 13 leaves from 10 of the 22 plants, and most of the extracted DNA samples were of PCR quality as indicated by results of RAPD-PCR assays (Table 2). Besides being of higher detection

efficiency compared with microbiological isolations, the molecular detection procedure was informative about the nature of isolates infecting the plants. Thus, most of the infected plants from Valencia were infected either by isolates of VCG1A or VCG2B$^{334}$ since seven plants yielded the 334-bp marker. However, because only one out of 109 *V. dahliae* isolates from artichoke in the Comunidad Valenciana region was of VCG1A (Jiménez-Díaz et al., 2006), it is most probable that the infecting isolates were of VCG2B$^{334}$. In addition, one plant from Valencia was infected by two different isolates, as indicated by the simultaneous amplification of both the 334-bp marker and the 688-bp marker, that never are amplified in a single *V. dahliae* isolate.

DNA extracted from leaves sampled from crops in Castellón province were of lesser PCR quality than those from Valencia, as indicated by RAPD-PCR assays. Therefore, all DNA samples from Castellón were diluted 1:10 in water to counteract the possible presence of PCR inhibitors that may give rise to false negative. Such a lesser DNA quality might be due to the fact that those samples were subjected to two rounds of freeze and defreeze processes and had been stored for about 9 moths before used for the molecular detection procedure. Nevertheless, the multiplex, nested-PCR assay yielded positive detection for 28 leaves from eight plants compared with positive detection in 19 leaves from seven plants by microbiological isolations (Table 3). All plant except one were found infected by *V. dahliae* isolates in two different VCGs, as indicated by the simultaneous amplification of the 334- and 688-bp markers, or the 334-, 688- and 964-bp markers. These results indicated the existence of molecular/genetic diversity in the population of *V. dahliae* infecting artichoke in those fields, and also identified the presence in them of VCG2B$^{824}$, the most virulent VCG to artichoke (as indicated by amplification of the 964-bp marker) (Jiménez-Díaz et al., 2006).

## CONCLUSIONS

The multiplex, nested-PCR procedure developed and optimized in this work was in all cases (i.e., artificially- and naturally-infected plants) more efficient than microbiological isolations in pure culture for the detection of *V. dahliae* in infected plants. Moreover, the positive detection of *V. dahliae* in the two symptomsless plants assayed indicates its suitability for the certification of pathogen-free planting stumps.

The use of a set of five primers pairs adequately combined to produce three PCR markers informative of *V. dahliae* VCGs infecting the plants confers information of additional importance to the pathogen detection. Thus, the use of those PCR markers provides information about genetic diversity in the pathogen population infesting a field (diversity of VCGs) which can be of use for population studies without need of isolating the fungus from the plant. Moreover, it provides information about virulence of isolates since *V. dahliae* VCGs differ in virulence to artichoke (Jiménez-Díaz et al., 2006).

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## Tables

Table 1. *Verticillium dahliae* detection in artificially-infected artichoke plants.

| Plant[1] | VCG[2] | Roots | | Leaves | |
|---|---|---|---|---|---|
| | | RAPD[3] | Molecular detection[4] | RAPD[3] | Molecular detection[4] |
| **Control** | | + | - | + | - |
| 17-1 | 1A | Na | + | Na | + |
| 17-2 | | Na | + | - | - |
| **18-1** | 2A | - | - | - | - |
| **18-2** | | - | - | Na | +* |
| 21-1 | $2Ba^{334}$ | Na | + | Na | ++ |
| 21-2 | | - | - | Na | ++ |
| 36-1 | $2B^{824}$ | - | - | - | - |
| 36-2 | | Na | + | Na | + |
| **41-1** | $2B^{824}$ | Na | + | Na | ++* |
| **41-2** | | - | (+) | + | ++* |
| **43-1** | 2A | - | - | Na | ++ |
| **43-2** | | - | (+) | - | - |
| **48-1** | $2B^{824}$ | Na | +* | - | (+)* |
| **48-2** | | Na | + | Na | ++ |
| **50-1** | $2B^{824}$ | Na | ++ | Na | ++ |
| **50-2** | | - | (+) | Na | + |
| **52-1** | $2B^{824}$ | Na | ++ | Na | ++* |
| **52-2** | | - | (+)* | Na | ++* |
| 60-1 | 2A | Na | ++ | Na | ++ |
| 60-2 | | - | (+) | Na | + |
| **64-1** | $2B^{824}$ | Na | ++* | Na | ++* |
| **64-2** | | - | (+) | - | (+) |
| 65-1 | HSI | Na | + | Na | + |
| 65-2 | | Na | ++ | Na | + |
| 69-1 | $2Br^{334}$ | Na | ++ | Na | ++ |
| 69-3 | | Na | ++ | Na | ++ |

[1]Noninoculated, control plants. Two plants (-1 and -2) inoculated with the same isolate. In bold those plants that were assayed by microbiological detection in water agar amended with 30 mg/L chlorotetracycline.
[2]Vegetative compatibility groups of isolates used for inoculation. VCG2B334 and VCG2B824 are subgroups within VCG2B that are compatible with international reference strains but amplified different PCR marker (Jiménez-Díaz et al., 2006). VCG2Ba are isolates that are not compatible with international reference strains but show positive complementation with artichoke isolates assigned to VCG2B. HSI, heterokaryon self incompatible.
[3]RAPD-PCR assays were developed in non-diluted samples. Na = not assayed.
[4]Molecular detection by multiplex, nested-PCR. +, positive detection after 2nd round of PCR; ++, positive detection after 1st round of PCR; * positive microbiological detection; (+) positive molecular detection after 1:10 (v/v) dilution in water.

Table 2. *Verticillium dahliae* detection in 22 artichoke plants sampled from two fields in Valencia province.

| Plant[1] | Leaves[2] | RAPD[3] | 1st PCR | 2nd PCR[4] | | |
|---|---|---|---|---|---|---|
| | | | | 334 bp | 688 bp | 964 bp |
| A-1-2 | 1-2 | + | - | - | - | - |
| A-33-15 | 1-2 | + | - | - | - | - |
| A-14-1 | 1-2 | + | - | - | - | - |
| B-2-7 | 1 | - | - | (+) | - | - |
| | 2 | + | - | - | - | - |
| B-2-16 | 1-2 | + | - | - | - | - |
| K-11-16 | 1 | + | - | - | - | - |
| | 2 | + | + | - | + | - |
| K-26-9 | 1-2 | + | - | - | - | - |
| K-16-4 | 1* | + | - | - | - | - |
| | 2 | + | - | - | - | - |
| C-21-8 | 1 | +/- | - | - | - | - |
| | 2 | + | + | - | + | + |
| C-25-16 | 1 | +/- | - | - | - | - |
| | 2 | - | - | (+) | - | - |
| C-22-1 | 1 | +/- | - | - | - | - |
| | 2 | + | - | - | - | - |
| H-13-5 | 1 | + | - | - | - | - |
| | 2 | + | - | - | - | - |
| P1 | 1* | + | + | + | - | - |
| | 2 | + | - | + | - | - |
| P2 | 1-2 | + | - | - | - | - |
| P3 | 1-2 | + | - | - | - | - |
| P4 | 1 | - | - | (+) | - | - |
| | 2 | - | - | - | - | - |
| P5 | 1 | + | - | - | - | - |
| | 2 | + | - | + | - | - |
| P6 | 1* | + | - | + | - | - |
| | 2 | + | - | - | - | - |
| P7 | 1 | + | - | + | - | - |
| | 2* | - | - | (+) | (+) | - |
| P8 | 1-2 | + | - | - | - | - |
| P9 | 1*-2* | + | + | + | - | - |
| P10 | 1-2 | + | - | - | - | - |

[1]Plants A-1-2 to H-13-5 are from one field; plants P1 to P10 are from the other field.
[2]Appearance of leaves 1 and 2 in the same line (1-2) indicates that results were identical for both. *, means positive microbiological detection in potato-dextrose agar amended with 0,5 g/L of streptomycin sulphate.
[3]+/-, weak amplification of Random Amplified Polymorphic DNA bands.
[4](+), positive molecular detection after 1:10 dilution in water.

Table 3. *Verticillium dahliae* detection in eight artichoke plants from three fields in Catellón province.

| Plant[1] | Severity[2] | Microbiological detection[3] | Molecular detection[4] | PCR markers (bp)[5] | Groups of VCGs[6] |
|---|---|---|---|---|---|
| C2.1.2 | 0 | 3 | 4 | 334/688/964 | (1A, $2B^{334}$)(2A, 4B)$2B^{824}$ |
| C3.5.4 | 0 | 1 | 2 | 334/688/964 | (1A, $2B^{334}$)(2A, 4B)$2B^{824}$ |
| C1.9.3 | 1 | 1 | 4 | 334/688/964 | (1A, $2B^{334}$)(2A, 4B)$2B^{824}$ |
| C1.5.2 | 2 | 3 | 4 | 334/688 | (1A, $2B^{334}$)(2A, 4B) |
| C1.7.3 | 2 | 3 | 4 | 334/688 | (1A, $2B^{334}$)(2A, 4B) |
| C1.3.1 | 3 | 4 | 4 | 334/688/964 | (1A, $2B^{334}$)(2A, 4B)$2B^{824}$ |
| C2.20.2 | 4 | 0 | 2 | 334 | (1A, $2B^{334}$) |
| C1.8.3 | 5 | 3 | 4 | 334/688 | (1A, $2B^{334}$)(2A, 4B) |
| Totals | | 19/32 | 28/32 | | |

[1]C1, C2 and C3 are different fields in Bernicaló, Castellón province.
[2]Severity of symptoms was assessed according to Cirulli et al., 1994.
[3]Number of leaves per plant for which microbiological detection was positive for *V. dahliae* after incubation of fragments of vascular bundles from leaf main nerves on potato-dextrose agar amended with 0,5 g/L of streptomycin sulphate.
[4]Number of leaves per plant for which multiplex, nested-PCR detection was positive for *V. dahliae.*
[5]PCR marker amplicons produced after the second round of amplification.
[6]In brackets, each of the predicted VCG groups infecting the plant according to the PCR markers amplicons produced in the assays.