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Genetic and Molecular Approach to *Verticillium dahliae* Infecting Sunflower

Abstract: Verticillium leaf mottle and wilt caused by the fungus *Verticillium dahliae* is a major disease of sunflower in Argentina and the USA. In the summer of 2013, an important outbreak of the disease occurred in one field in the Southwest of Spain. The determination of vegetative compatibility groups (VCGs) of *V. dahliae* is used in the genetic characterization of the fungus. Molecular markers have also been developed and applied for the analysis of *V. dahliae*, particularly for those isolates infecting artichoke, cotton and olive tree. The objective of this work was to determine the genetic and molecular features of *V. dahliae* of sunflower. Three *V. dahliae* isolates, one from Argentina collected in 2012 and two collected in Spain in 2013, were analysed. The VCGs were determined by complementation between *nit* mutants of the isolates from sunflower and the VCGs reference strains. Molecular characterization was conducted by polymerase chain reaction using primer pairs that are diagnostic of either *V. dahliae* species, defoliating or non-defoliating pathotypes, or VCGs. Complementation tests between *nit* mutants and reference strains clearly showed that the three isolates from sunflower belong to the VCG2B. The VCG2B has been identified in *V. dahliae* from crops as cotton, artichoke, eggplant, pepper and tomato among others. When molecularly analysed, the three *V. dahliae* isolates infecting sunflower had the same molecular pattern than the one found for non-defoliating isolates of *V. dahliae* pathogenic to artichoke or cotton. The results of this work show the closeness between non-defoliating isolates of *V. dahliae* infecting artichoke, cotton and sunflower and suggest that any of these three species can serve as carrier and source of inoculum for Verticillium outbreaks in them.

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Introduction

Verticillium leaf mottle and wilt of sunflower caused by the fungus *Verticillium dahliae* Kleb. was described in Spain in the 1970s. Low frequencies (1–8%) of affected fields and incidences no higher than 20% affected plants were observed, confectionary cultivars being more severely affected than oilseed cultivars (Jiménez-Díaz *et al.*, 1980). Currently, Verticillium is a major disease of sunflower in Argentina and the USA (Gulya *et al.*, 1997; Radi and Gulya, 2007; Galella *et al.*, 2012) and has secondary importance in Spain, France, Romania, Bulgaria and Turkey. *Verticillium dahliae* is a soilborne ascomycete with a wide range of host crops. It causes important yield losses in artichoke (*Cynara cardunculus* L. var. *scolymus*), cotton (*Gossypium hirsutum* L.), eggplant (*Solanum melongena* L.), lettuce (*Lactuca sativa* L.), olive tree (*Olea europaea* L.) and tomato (*Solanum lycopersicum* L.) among others (Pegg and Brady, 2002). In Spain, Verticillium constitutes an important constraint for the production of cotton, artichoke, and, particularly, of olive tree (Bejarano-Alcázar *et al.*, 1996; Korolev *et al.*, 2001; Jiménez-Díaz *et al.*, 2006; Navas-Cortés *et al.*, 2008; Berbegal *et al.*, 2010; Jiménez-Díaz *et al.*, 2011; López-Escudero and Mercado-Blanco, 2011).

Genetic resistance is the most effective method for controlling Verticillium in sunflower. Initial sources of resistance were identified in Canada in the 1950s (Putt, 1958). The inheritance of resistance in some inbred lines was found to be qualitative or of complete dominance and designated as V_1 (Putt, 1964). The same type of resistance was found 10 years later in certain inbred lines from the USDA collection, such as HA89 (Fick and Zimmer, 1974), which is widely employed to produce resistant hybrids, particularly in public sunflower breeding programs. The first race of *V. dahliae* (NA-1) was detected in the USA, and it was controlled by the resistance into HA89 (Gulya *et al.*, 1997). New races overcoming this resistance, and apparently different to each other, have been later reported: one (NA-Vd2) in the USA (Gulya, 2007) and three in Argentina (Bertero de Romano and Vázquez, 1982; Gallela *et al.*, 2004; Bazzalo, pers. comm.). Some of these new races seem to be controlled by the resistance of some entries of the USDA sunflower collection, such as PI507901 (Radi and Gulya, 2007) or by the resistance in the inbred lines HA300, HA371 and in the HAR lines (Gulya *et al.*, 1997). The

inheritance of resistance appears to be recessive or additive in some lines, and pyramiding quantitative resistance has been explored as an alternative of genetic control against *V. dahliae* in Argentina (Galella *et al.*, 2012). While races of *V. dahliae* pathogenic to sunflower, tomato and lettuce are distinguished depending on the genes of resistance they overcome, isolates of *V. dahliae* infecting other crops, such as cotton or olive tree, are not assigned to races but to pathotypes. Defoliating (D) and non-defoliating (ND) pathotypes are identified on the basis of their capacity to cause, or not, the complete fall of green leaves (Rodríguez-Jurado *et al.*, 1993; Bejarano-Alcázar *et al.*, 1996).

Isolates of *V. dahliae* are genetically characterized according to vegetative compatibility, which refers to the genetically controlled ability of individual fungal strains to undergo hyphal anastomosis and form stable heterokaryons. Vegetatively compatible isolates of a fungal species are placed in the same vegetative compatibility group (VCG). VCGs are identified using spontaneous nitrate non-utilizing (*nit*) auxotrophic mutants which show a thin but expansive growth on minimal medium with nitrate as a sole nitrogen source. Isolates are considered vegetatively compatible when complementing *nit* mutants anastomose and produce wild-type growth. Complementation tests are done by pairing *nit* mutants of an isolate with phenotypically distinct *nit* mutants of international testers (Jiménez-Díaz *et al.*, 2006). The efforts of several research groups from Mediterranean countries have resulted in a very interesting genetic characterization of *V. dahliae* isolates from crop species such as olive tree (Navas-Cortés *et al.*, 2009; Dervis *et al.*, 2010), artichoke (Mercado-Blanco *et al.*, 2001, 2003; Jiménez-Díaz *et al.*, 2006), eggplant (Dervis *et al.*, 2009) and cotton (Dervis *et al.*, 2008; Korolev *et al.*, 2008). These works have resulted in the location of *V. dahliae* infecting the mentioned crops into VCG1A, VCG2A, VCG2B or VCG4B. Concerning *V. dahliae* pathogenic to sunflower, only one recent work from Canada deals with genetic characterization, but no conclusive results were presented (El-Bebany *et al.*, 2013). Most of the nine isolates of *V. dahliae* showed weak reactions with testers from VCG4A and 4B, one isolate was compatible only with VCG3 testers and another one was compatible with all VCG groups except VCG2A (El-Bebany *et al.*, 2013).

Diversity of *V. dahliae* can also be characterized using molecular techniques. Among them, RAPD markers specific to D and ND pathotypes of *V. dahliae* from cotton were identified and used for the design of SCAR markers. These markers differentiate a genetically homogenous group of D isolates belonging to VCG1A. In contrast, they show a high molecular diversity of ND pathotypes belonging to 2A, 2B and 4B VCGs (Pérez-Artés *et al.*, 2000; Mercado-Blanco *et al.*, 2001, 2002, 2003). In summary, molecular markers specific to D and ND pathotypes of *V. dahliae* from cotton allow a reliable

and fast diagnosis of the pathotype of the fungal isolate, which is to some extent related to VCGs.

As mentioned, all this genetic and molecular information about *V. dahliae* was generated from the analyses of isolates infecting crops different to sunflower. The objective of this work was the determination of genetic and molecular characteristics of *V. dahliae* affecting sunflower.

Materials and methods

Fungal isolates

Two isolates of *V. dahliae* (1–13 and 2–13) were obtained from diseased plants in a field in Cadiz (Southwest Spain) where the performance of 30 hybrids was assessed in June 2013. Sunflower plants presented typical symptoms of leaf mottle and interveinal yellowing, and the fungus consistently isolated from stem tissues was morphological and molecularly identified as *V. dahliae* (García-Ruiz *et al.*, 2014). Each isolate was sampled from one different hybrid. A third isolate (1–12) was grown from a microsclerocium of *V. dahliae* from an infected sunflower plant which was collected in an experimental field at Balcarce (Argentina) in March 2012. Two monoconidial cultures per isolate were obtained from each of the three bulk isolates of *V. dahliae*, and they were used for subsequent genetic and molecular analyses.

Genetic characterization

Nit mutants of the isolates were generated on water agar chlorate (WAC) medium and identified on Czapek Dox agar (CDA) according to Korolev and Katan (1997). Colonies presenting a faint growth on CDA with no aerial mycelium were labelled as *nit* mutants and phenotyped on CDA amended with hypoxanthine as described by Correll *et al.* (1987).

The isolates were genetically characterized in complementation tests that were done by pairing *nit* mutants of the isolates with the complementary mutants of the international OARDC (The Ohio State University, Wooster, Ohio, USA) reference testers and Israeli *nit* testers: T9 isolate (VCG1A), Ep8M and Ep52 isolates (VCG2A), Cot200 and Cot254 isolates (VCG2B), 131M isolate (VCG4A) and Pt15M isolate (VCG4B). All testers were provided by Professor R. Jiménez-Díaz (University of Córdoba, Spain).

Pairings were done following the methodology by Collado-Romero *et al.* (2006) and Jiménez-Díaz *et al.* (2011). Mycelial plugs of *nit* mutants of test and tester isolates were placed 1–1.5 cm apart on CDA in petri plates at 24°C in the dark. Plates were scored for prototrophic growth after 14 and 28 days of incubation. Positive complementation was indicated by the formation of a dense, aerial growth where mycelia from the test (unknown) *nit* mutant and the tester strains had met and formed a prototrophic heterokaryon. In such a case, the test *nit* mutant was considered vegetatively compatible with the tester strain and was assigned to its VCG.

Molecular characterization

Molecular characterization of the isolates was performed using the diagnostic markers previously described and used for isolates of *V. dahliae* infecting olive tree and artichoke (Carder *et al.*, 1994; Mercado-Blanco *et al.*, 2001, 2002, 2003; Collado-Romero *et al.*, 2009).

Total genomic DNA from each isolate was purified using the DNeasy Plant Mini extraction kit (Qiagen Iberia SL, Madrid, Spain) according to the manufacturer's instructions. Quality and concentration of DNA samples were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Finally, DNA samples were adjusted to a final concentration of 10 ng/μL and stored at −20°C until required.

The primer pairs used for PCR reactions were DB19/DB22 (Carder *et al.*, 1994), DB19/espdef01 (Mercado-Blanco *et al.*, 2003), INTD2f/INTD2r and INTND2f/INTND2r (Mercado-Blanco *et al.*, 2002), INTNDf/INTNDr (Mercado-Blanco *et al.*, 2001), and INTND2f/INTND3r (Collado-Romero *et al.*, 2009). Optimised PCR assays were carried out in a final volume of 25 μL containing 0.4 μM each primer, 800 μm dNTPs, 2.5 μL 10 × PCR buffer (800 mM tris–HCl, pH 8.3–8.4 at 25°C, 0.2% Tween 20 wt/V), 0.75 U Taq-DNA Polymerase (Dominion MBL, Córdoba, Spain), 1.5 mM (DB19/DB22 primers) or 2 mM (rest of primers) $MgCl_2$.

Amplification conditions were as follows: 4 min denaturation at 94°C; followed by 35 cycles of 1 min denaturation at 94°C, 1 min of annealing at 54°C (DB19/DB22), 62°C (DB19/espdef01), 64°C (INTD2f/INTD2r, INTND2f/INTND2r, INTNDf/INTNDr), 60°C (INTND2f/INTND3r), and 1 min of extension at 72°C; and a final extension step of 6 min at 72°C. All reactions were done in a T1 Thermocycler (Whatman Biometra, Goettingen, Germany). Amplification products were separated by horizontal electrophoresis in 2% agarose gels containing 0.05 μl/ml SafeView Nucleic Acid Stain (NBS Biologicals,

Huntingdon, UK) and visualised over a UV light source. A 100- to 2,000-bp or 100- to 1,000-bp ladder (Dominion MBL, Cordoba, Spain) was included in the electrophoresis.

Results and discussion

The isolates of *V. dahliae* resulted in six (1–12 and 2–13) or seven (1–13) *nit* mutants. All the mutants were phenotyped as *nit1* mutants. Pairings between complementary mutants of the isolates and OARDC and Israeli *nit* testers resulted in the identification of the three isolates of *V. dahliae* from sunflower as belonging to VCG2B (Table 1). Isolates of *V. dahliae* from cotton, artichoke, eggplant, pepper and tomato, among other crops, have also been assigned to VCG2B (Collins *et al.*, 2005; Jiménez-Díaz *et al.*, 2006; Dervis *et al.*, 2009; Korolev *et al.*, 2009; Berbegal *et al.*, 2010; Papaioannou *et al.*, 2013). Recent research on *V. dahliae* from sunflower in Canada suggests the isolates belong to 3, 4A or 4B VCGs (El-Bebany *et al.*, 2013), although genetic characterization of the isolates is not clearly determined nor the pathotype of the isolates (D or ND) is mentioned. In any case, our work provides consistent results about the adscription of the isolates of *V. dahliae* affecting sunflower in Argentina and Spain included in the study, to VCG2B and, together with the results by El-Bebany *et al.* (2013), suggests that isolates of the fungus infecting sunflower in Canada are genetically distant from those from Argentina and Spain. Our work constitutes the first approach to an international genetic study of *V. dahliae* pathogenic to sunflower.

Table 1: Genetic characterization of three isolates of *Verticillium dahliae* infecting sunflower in Argentina and Spain by means of positive or negative complementation between the test (unknown) *nit* mutant and the reference strains used as VCG testers

Test *nit* mutant	Country, year of collection	Complementation[a]				
		VCG1A	VCG2A	VCG2B	VCG4A	VCG4B
1–12	Argentina, 2012	–	–	+	–	–
1–13	Spain, 2013	–	–	+	–	–
2–13	Spain, 2013	–	–	+	–	–

Notes: [a]Pairings were done between test *nit* mutants of two monoconidial isolates from each *V. dahliae* strain and: T9 isolate (VCG1A), Ep8M and Ep52 isolates (VCG2A), Cot200 and Cot254 isolates (VCG2B), 131M isolate (VCG4A) and Pt15M isolate (VCG4B). All testers were provided by Professor R. Jiménez-Díaz (University of Córdoba, Spain).

Table 2: Molecular characterization of three isolates of *Verticillium dahliae* infecting sunflower in Argentina and Spain

Reference	Country, year of collection	Molecular markers (bp)[a]					
		526/543	1,163	824	688	334	462
1–12	Argentina, 2012	+	+	+	+	–	–
1–13	Spain, 2013	+	+	+	+	–	–
2–13	Spain, 2013	+	+	+	+	–	–

Notes: [a]Molecular markers were amplified using primer pairs and PCR conditions: DB19/DB22 (Carder *et al.*, 1994), DB19/espdef01 (Mercado-Blanco *et al.*, 2003), INTD2f/INTD2r and INTND2f/INTND2r (Mercado-Blanco *et al.*, 2002), INTNDf/INTNDr (Mercado-Blanco *et al.*, 2001), and INTND2f/INTND3r (Collado-Romero *et al.*, 2009).

Concerning molecular characterization, the three isolates amplified, as expected, the 543- or 526-bp marker specific to *V. dahliae* (DB19/DB22 primers) (Table 2). Additionally, our isolates presented the C molecular pattern that was previously described by Collado-Romero *et al.* (2006) for isolates infecting artichoke, cotton or olive tree: they amplified 1,163-, 824- and 688-bp bands. Because 1,163-, 824- and 688-bp markers are diagnostic of the ND pathotype (Mercado-Blanco *et al.*, 2001; Collado-Romero *et al.*, 2009), our molecular results place *V. dahliae* from sunflower as molecularly close to ND isolates from cotton and artichoke. Moreover, two molecular subgroups (VCG2B334 and VCG2B824) have been determined within VCG2B because they produce amplicons of either 462 or 824 bp that are associated with the D and ND pathotypes of *V. dahliae*, respectively (Mercado-Blanco *et al.*, 2001, 2003; Collado-Romero *et al.*, 2006). Because the isolates of *V. dahliae* from sunflower presented the 824- but not the 462-bp amplicons, molecular results placed them into the VCG2B824 subgroup. The results of this work locate our isolates of *V. dahliae* from sunflower as molecular and genetically close to isolates of the fungus that are pathogenic to cotton and artichoke, those of the ND pathotype and belonging to VCG2B, and distant from ND *V. dahliae* infecting olive trees (Mercado-Blanco *et al.*, 2001; Collins *et al.*, 2005; Collado-Romero *et al.*, 2006). From the phytopathological point of view, and due to the closeness of *V. dahliae* strains infecting artichoke, cotton and sunflower, root tissues of any of these three species can serve as carriers and sources of inoculum. Cross pathogenicity tests are needed to better understand the effect that the consideration of these crops into farming alternatives can have on Verticillium attacks. In the meantime, the risk of severe outbreaks or increased severities of Verticillium in any of sunflower, cotton or artichoke must be considered when they are selected for crop rotations in farming. After the

recent identification of *V. dahliae* overcoming the V1 gene in Spain (García-Ruiz *et al.*, 2014) these results also suggest the need for a pathogenic approach to the fungus focusing on the identification and characterization of the races of *V. dahliae* affecting sunflower in Europe and the Americas. In any case, a close and continuous monitoring of Verticillium outbreaks in sunflower crops worldwide is recommended.

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