First report of the presence of *Verticillium dahliae* VCG1A in Australia

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Abstract For 32 years, cotton grown in NSW has been monitored for Verticillium wilt, with isolates of *Verticillium dahliae* Kleb. stored in the NSW Department of Primary Industries culture collection. An increase in disease severity in the 2013/14 season suggests a more virulent pathogenic strain may have been introduced. Eight *V. dahliae* isolates were selected for vegetative compatibility group (VCG) analysis using molecular assays and *nit* mutant testing. Two of the eight isolates were identified as the highly virulent defoliating VCG1A, making this the first record of VCG1A in Australia.

Keywords Verticillium wilt · Cotton · Defoliating · Non-defoliating

Verticillium dahliae Kleb. is a strictly asexually-reproducing, soil borne fungus in the phylum Ascomycota, that causes vascular disease in about 400 plant species and is responsible for major economic loss in a diverse range of crop production

systems worldwide (EFSA 2014; Inderbitzin et al. 2011; Pegg and Brady 2002). The problems caused by *V. dahliae* are exacerbated by its extensive and expanding host range (Subbarao et al. 1997) and its ability to survive in soil by means of microsclerotia, in the absence of a host, for up to 14 years (Wilhelm 1955). Cotton is an economically important crop, with exports from Australia worth more than \$2 billion annually. The ongoing revenue from this product is integral to Australian agribusiness. Verticillium wilt is a major problem in cotton worldwide and was first observed in Australia in 1967 (Evans and Paull 1967). Crop and disease monitoring has occurred since 1983 on a seasonal basis, with plants exhibiting symptoms of Verticillium wilt collected for isolation of *V. dahliae*.

Strains of *V. dahliae* infecting cotton are classified into two pathotypes: defoliating (D) and non-defoliating (ND). These strains are further categorised according to vegetative compatibility groups (VCGs) which are linked to host adaptation and often correlate with virulence on specific crops. In Australia, commercial cotton crops have been monitored and surveyed for the distribution, incidence and severity of Verticillium wilt caused by *V. dahliae* since 1983 (Kirkby et al. 2013). Verticillium wilt was described as the first major disease recorded in cotton following surveys in the 1984/85 season (Kirkby et al. 2013). The mean percentage incidence of Verticillium wilt in NSW varies between seasons but remains generally low; with the state average surpassing 10 % only on four occasions. Prior to the 2013/2014 period, it was believed there was only a single VCG type present in Australian cotton; however, this changed in 2014 with the detection of the ND VCG2A strain (Smith et al. 2014). Since then, there have been ongoing concerns expressed by some growers and consultants of an increase in the severity of disease symptoms associated with Verticillium wilt along with an observed increase in the state average incidence from 5.5 % in 2013/2014 to 7.1 % in

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2014/2015. The decision was made to further investigate the etiological agent using molecular tools to clarify the possible VCG present. A total of eight *V. dahliae* isolates, including representative historical isolates from the NSW DPI culture collection were examined.

Culture collection isolates of *V. dahliae* were originally obtained from affected cotton grown in commercial farms in NSW between 1983 and 2015. Pure cultures were grown on potato dextrose agar (PDA), single-spored, then stored in vials with distilled water at 12 °C. Mycelia (100 mg) from active cultures on PDA were used for DNA extraction as per manufacturer's instructions (Extract-N-Amp Plant PCR Kit, Sigma-Aldrich). Three molecular approaches were used for VCG and pathotype testing. Initially, eight isolates were tested using the method described in Berbegal et al. (2011), which provides a presumptive VCG type through the use of polymorphic markers. The primer pairs VdSSR1F/VdSSR1R, VdSNP1F/VdSNP1R, VdSNP2F/VdSNP2R, and VdSNP3F/VdSNP3R were used as described and sequenced in both directions to determine the multilocus genotypes for each isolate. This result prompted the use of primers DB19/DB22 (Carder et al. 1994) to amplify a PCR marker specific to *V. dahliae*. The size of the amplified product distinguished between D and ND strains; 539 and 523 bp respectively. The third method was to amplify the Intergenic spacer (IGS) region using the primers VdIGSF1 and VdIGSR1 (Qin et al. 2006). Comparison of the amplicon sequence to known *V. dahliae* VCG typed reference isolates determined the presumptive VCG of each isolate. Molecular analysis of the samples using the primer pairs derived from microsatellite sequencing (Berbegal et al. 2011) revealed two isolates (DAR82592 and DAR82598, Table 1) to be VCG1A, the cotton D pathotype,

thought to be exotic to Australia. The two Australian VCG1A isolates, when compared with the reference isolates (Papaioannou et al. 2013), aligned in the subregion P. This result prompted analysis using the Carder et al. (1994) and Qin et al. (2006) methods with both identifying the presumptive VCG1A isolates as D pathotype, thus supporting the initial VCG1A result. The eight isolates were sent to R.M. Jiménez-Díaz at the Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Córdoba, Spain for blind VCG testing using nitrate-nonutilizing (*nit*) mutants.

Single-spore cultures of each isolate were grown on PDA plates at 25±1 °C in the dark for 7 days for complementation tests of *nit* mutants. *Nit* mutants were generated on water agar-chlorate (WAC) (Korolev and Katan 1997) according to the methods of Collado-Romero et al. (2006) and Jiménez-Díaz et al. (2006), and phenotyped according to the methods of Correll et al. (1987). Complementation tests were performed as described in Collado-Romero et al. (2006) and Jiménez-Díaz et al. (2006). Phenotypically distinct *nit* mutants of an isolate were paired with complementing *nit* mutants of the Ohio Agricultural Research and Development Center (OARDC, The Ohio State University, Wooster, OH), reference strains of *V. dahliae* VCGs (VCG1, strain T9M [Code used by the OARDC to label the NitM mutant]; VCG3, strain 70–21; and VCG4A, strain 131 M) and Israeli *nit* testers (VCG2A, strain Cot200; VCG2B, strain Ep8M, and VCG4B, strain Pt15M) (Korolev et al. 2000) in all possible combinations.

Israeli *nit* testers of VCG2A, VCG2B, and VCG4B were previously shown to correlate with the

Table 1 VCG typing of eight historical collection isolates of *Verticillium dahliae* using the three described molecular assays and *nit* mutant testing for final VCG confirmation

Australian Culture Collection Accession Numbers	Year Isolated	SSR1 ^a	SNP1 ^a	SNP2 ^a	SNP3 ^a	DB19/DB22 ^b	IGS Sequence ^c	GenBank Accession Numbers	Presumptive Molecular VCG ^d	<i>Nit</i> Mutant Result
DAR82591	2009/2010	3	4	1	1	ND	2A	KU743353	2A	2A
DAR82592	2010/2011	4	2	2	2	D	1A	KU743354	1A	1A
DAR82593	2010/2011	3	4	1	1	ND	No Result	KU743355	2A	4B
DAR82594	2011/2012	3	4	1	1	ND	2A	KU743356	2A	2A
DAR82595	2011/2012	3	4	1	1	ND	2A	KU743357	2A	2A
DAR82597	2013/2014	3	4	1	1	ND	2A	KU743358	2A	2A
DAR82598	2013/2014	4	2	2	2	D	1A	KU743359	1A	1A
DAR82599	2013/2014	1	3	4	1	ND	4B	KU743360	4B	4B

^a Primer names from Berbegal et al. 2011 to amplify the polymorphic markers for multilocus genotyping and result from the alignment, the collective result allowed for assignment of the presumptive molecular VCG (4)

^b Primer names from Carder et al. 1994 to amplify the product to distinguish between the ND and D pathotype

^c IGS sequence result using the protocol from Qin et al. 2006 and alignment with the isolates from Papaioannou et al. 2013

^d Confirmed presumptive VCG

Fig. 1 **a** Cotton leaf mottle characteristic of *Verticillium* Wilt; **b** Vascular discoloration in a cotton stem infected with *Verticillium dahliae*; **c** Cotton plants attacked by non-defoliating (left) and defoliating VCG1A (right) strains of *V. dahliae*

international OARDC reference strains (Korolev et al. 2000; Korolev et al. 2008; Rowe 1995). The cotton defoliating strain T9 (the original defoliating pathotype from cotton in California) belongs to subgroup VCG1A (Bell 1994). Only isolates of this subgroup caused severe defoliation of cotton in previous studies (Daayf et al. 1995; Korolev et al. 2008). Thus, isolates strongly compatible with T9 (or strongly compatible with other testers) may be assigned to VCG1A (Korolev et al. 2008).

Plates for complementation tests were scored for prototrophic growth after 14 and 28 days of incubation. Complementation was indicated by the formation of a dense, aerial growth where mycelia from an unknown and a tester strain had met and formed a prototrophic heterokaryon. In such a case, the unknown mutant was considered vegetatively compatible with the tester strain and was assigned to the VCG of the tester strain. All tested isolates produced *nit1* mutants, with DAR82594 forming NitM mutants. Complementary heterokaryons formed between *nit* mutants of the tested isolates and the international OARDC reference strains and Israeli tester strains. The morphology of heterokaryons in pairings of compatible *nit* mutants included prototrophic growth and formation of dense to scattered aerial mycelium and development of microsclerotia. Based on their positive complementation reactions with specific testers, the eight tested isolates were assigned to VCGs as shown in Table 1. Complementation tests were repeated once with the same results.

Single spore cultures of all eight *V. dahliae* have been deposited in the NSW DPI Herbarium culture collection with assigned accession numbers as indicated in Table 1. These results confirm that *V. dahliae* VCG1A is present in Australia and that one of the isolates collected and stored from the 2010/2011 season, was also VCG1A. This work confirmed that *V. dahliae* VCG1A defoliating pathotype is present in Australian cotton, with symptoms illustrated in Fig. 1.

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