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REVIEW

The Verticillium wilt problem in Australian cotton

P. Dadd-Daigle[1,2] · K. Kirkby[3] · P. Roy Chowdhury[2] · M. Labbate[2] · Toni A. Chapman[1]

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Abstract

Verticillium dahliae is a soil-borne phytopathogen and the causal agent of Verticillium wilt. It affects many agriculturally important crops around the world, including cotton. In Australia, the billion-dollar cotton industry is increasingly impacted by Verticillium wilt. Internationally it has been reported that the defoliating *V. dahliae* Vegetative Compatibility Group (VCG) 1A causes severe damage to cotton. In Australia however, the non-defoliating VCG2A is causing more severe damage to crops in fields than the defoliating VCG1A. This review examines the current research to understand the Australian *V. dahliae* situation, including current classification systems, genetic analyses and management strategies. It appears that virulence cannot be defined solely by VCG in Australian *Verticillium dahliae* isolates causing disease in cotton, and that the industry must continually adapt their practices in order to keep the disease under control.

Keywords *Verticillium* · Cotton · *Gossypium hirsutum* · *V. dahliae*

Introduction

In Australia, cotton is a growing billion-dollar industry. Cotton yields have increased from 500 kg per hectare in the 1960's to 2000 kg per hectare in 2013 (Hamilton 2016). Cotton crops are largely furrow irrigated, grown on alkaline clay soils and tend to be located near flood plains. There is often reduced or minimum tillage, tail-water recirculated and in some areas permanent bed systems (Kirkby et al. 2013). Sustainability and growth of the cotton industry is reliant on improved cotton varieties, management of soil and water resources, and control of weeds, insect and diseases (Constable 2004). Although Verticillium wilt in Australian cotton is generally well managed, other countries have seen economic losses of 50% or more (Wu and Subbarao 2014). The average incidence levels of Verticillium wilt caused by *V. dahliae* in Australian cotton are relatively low but yield losses can vary between 10 and 62% in some fields (Holman et al. 2016). However, the recent discovery of the defoliating VCG1A and the disease severity of the non-defoliating VCG2A present an additional problem for management of Verticillium wilt as incidences rise (Chapman et al. 2016; Dadd-Daigle et al. 2020; Jensen and Redfern 2017; Kirkby et al. 2013). Hence, Verticillium wilt is becoming a major concern for the Australian cotton industry.

Verticillium dahliae

Verticillium encompasses a group of soil-borne ascomycetes. As of 2011, ten *Verticillium* species have been described (Inderbitzin et al. 2011), including *V. dahliae*, the main causal agent of Verticillium wilt. *Verticillium dahliae* is responsible for disease in over 400 plant species across the world. These include many economically important crops such as olives, tomatoes, potatoes, lettuce and cotton (Bhat and Subbarao 1999; Inderbitzin et al. 2011).

The life cycle of *V. dahliae* allows it to persist on farms for many years. It survives in soil in highly melanised resistant structures, known as microsclerotia, for over 10 years (Davis et al. 1994; Klosterman et al. 2009). These microsclerotia germinate in the presence of host plants, producing hyphae that penetrate the root cortex and reach the xylem. As hyphae and conidia grow within the xylem, the plant host can express

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✉ Toni A. Chapman
toni.chapman@dpi.nsw.gov.au

[1] NSW Department of Primary Industries, Biosecurity and Food Safety, Elizabeth Macarthur Agriculture Institute, Woodbridge Road, Menangle, NSW 2568, Australia

[2] School of Life Sciences, The University of Technology Sydney, Harris Street, Ultimo, NSW 2007, Australia

[3] NSW Department of Primary Industries, Biosecurity and Food Safety, Kamilaroi Highway, Narrabri, NSW 2390, Australia

symptoms of wilting, necrosis and leaf discolouration (Klimes et al. 2015). As symptoms progress, *V. dahliae* enters a saprophytic phase where the infection expands to other tissues, such as leaves, and a mass production of microsclerotia occurs. The extent of symptoms can depend on the susceptibility of the host and the infecting strain of *V. dahliae*. While some plants suffer severe wilting and necrosis, other infections are less severe, allowing the plant to recover (Daayf 2015).

Historically, the characterisation and classification of *V. dahliae* has been based on the symptoms exhibited by the host plant, or by the interaction of pathogen virulence and host resistance genes. Consequently, this has led to the use of host-specific terminology and classification, resulting in a number of different classification systems. *Verticillium dahliae* strains infecting tomato and cotton are divided into "races", classified by the presence or absence of the *Ave1* gene (Hu et al. 2015; Maruthachalam et al. 2010). Strains from cotton are also categorised into defoliating (D) and non-defoliating (ND) pathotypes (Daayf et al. 1995). While the D and ND pathotypes largely align to races 1 and 2, respectively, this is not true for all strains and the systems are generally not used interchangeably (Hu et al. 2015). Host-specific pathology groups also include "eggplant pathotype", "tomato pathotype", "mint pathotype" and "sweet pepper pathotype" (Dung et al. 2012; Komatsu et al. 2001; Papaioannou et al. 2013b). While these classifications are generally understood in studies that focus on strains infecting a single host type, complexity arises when investigating *Verticillium* strains independently of the plant host they infect. Currently, there is only one system that classifies all *V. dahliae* strains into groups, known as Vegetative Compatibility Groups (VCGs).

Vegetative Compatibility Groups (VCGs) in *Verticillium dahliae*

VCGs are determined by strain interaction and describe the formation of prototrophic heterokaryons, a fusion of two genetically distinct cells that occurs when two hyphal cells meet (Puhalla and Mayfield 1974). While not molecularly characterised in *V. dahliae*, related fungal models have shown that two sets of gene loci, known as *vic* (vegetative incompatibility) and *het* (heterokaryon incompatibility) govern the process. For isolates to form a heterokaryon, the alleles at the *het* or *vic* loci must be identical (Jiménez-Gasco et al. 2013). In practice, the VCG determination process requires that *V. dahliae* strains are mutated to become nitrogen non-utilizing "*nit* mutants". Mutants strains, one or two with known and the other with an unknown VCG, are placed on opposite sides of a minimal media agar plate and monitored for signs of prototrophic growth. If the mutant isolates are able to form heterokaryons, which allow growth on minimal media, the unknown isolate is assigned the same VCG as the known isolate (Joaquim and Rowe 1990). This method has led to the identification of five VCGs in *V. dahliae*, namely, VCG1 2, 3, 4 and 6, with VCG1 and VCG2 further characterised into A and B subgroups, and VCG4 into A, B and AB (Papaioannou and Typas 2015; Strausbaugh 1993).

Vegetative Compatibility Groups have been used to track the evolution and movement of *V. dahliae*. Several groups found that isolates within VCGs are phylogenetically similar (Collado-Romero et al. 2006) or fit a clonal reproductive model (Dung et al. 2013; Milgroom et al. 2014). Others argued that although isolates of the same VCG may be genetically similar, they are often phylogenetically distant, with members of different subgroups being more closely related (Jiménez-Gasco et al. 2013). In most instances VCGs are monophyletic, with some exceptions such as VCG2B (Collado-Romero et al. 2008). Following these studies, the origin of the *V. dahliae* species has been speculated to be in Europe (Short et al. 2015), while the virulent VCG1A has been traced back to North America (Milgroom et al. 2016).

Different plant hosts are often associated with different *V. dahliae* VCGs. VCG2A is known to be highly pathogenic to tomato (Tsror et al. 2001), VCG2B is highly aggressive in mint (Dung et al. 2013), VCG4A is highly pathogenic to potato (El-Bebany et al. 2013), and VCG1A is virulent in olives (Dervis et al. 2007). In cotton, it has generally been reported that VCG1A causes significant damage while VCG2A and VCG4B are less virulent, although there have been some reports of VCG2B causing damage (Dervis and Bicici 2005; Dervis et al. 2008; Elena 1999; Jiménez-Gasco et al. 2013; Korolev et al. 2001).

While VCGs are currently the most widespread method to describe *V. dahliae* populations, the genetics behind VCGs in *V. dahliae* are not well understood. In their attempt to create a high-throughput VCG screening method, Papaioannou and Typas (2015) also sought to understand the genetic relationship between the two, "strong" and "weak", heterokaryon reactions observed. These authors found that weak interactions tend to be unstable, but there is still a transfer of genetic material, suggesting that they may be vegetatively compatible. Although many other studies acknowledge that weak reactions occur, most regard only strong interactions as compatible (Strausbaugh 1993). This could impact the reliability of results examining relatedness amongst VCGs and highlights a need for a narrower classification system that does not suffer from these issues. Additionally, as the VCG determination process is labour intensive and time-consuming, several groups have attempted to develop alternative methods (Collado-Romero et al. 2009; El-Bebany et al. 2013; Papaioannou et al. 2013a). However, currently, no molecular method is as reliable as the traditional method.

Verticillium dahliae in Australian cotton

Since 1983, *Verticillium*-infected plant samples have been collected and *V. dahliae* isolates maintained and stored in the culture collection of the NSW Department of Primary Industries (Kirkby et al. 2013). The average incidence of Verticillium wilt has generally been low throughout NSW. The incidence rose from 5.5% in 2013/2014 to 7.1% in 2014/2015 and 6.3% in the 2015/2016 season (Chapman et al. 2016). Disease symptoms are becoming more severe in some patches of Verticillium wilt, with yield reductions reported to be greater than 6 bales/ha. There are concerns that this increase in severity is related to the ND VCG2A strain reported in 2014 (Dadd-Daigle et al. 2020; Smith et al. 2014).

It was previously thought that only one VCG type, ND VCG4B, was present in Australia, but in 2014, ND VCG2A was identified (Smith et al. 2014). Following the discovery of ND VCG2A, analysis of *V. dahliae* historical samples taken from the NSW Department of Primary Industries culture collection revealed the presence of the D VCG1A (Chapman et al. 2016). The D VCG1A has been the cause of severe disease and crop loss overseas (Jiménez-Díaz et al. 2006). However, despite the presence of VCG1A in the historical samples, typical VCG1A disease presentation, including the typical crop losses and complete defoliation of infected plants, has not been a widespread observation in Australia. It is not clear what is causing the disparity between the severity of D VCG1A and ND VCG2A disease in Australia and overseas. It is possible, given that VCG2A has been shown to infect weeds commonly found on cotton fields (Yildiz et al. 2009), that VCG2A *V. dahliae* has simply become the most prevalent strain on Australian cotton fields, amplified by the polyetic nature of the pathogen, and has acquired the ability to defoliate cotton plants. However, further analysis of the relationship of genetics to pathogenicity and disease severity in Australian *V. dahliae* VCGs is required.

Insights from *Verticillium dahliae* genome sequencing

In 2011 the *V. dahliae* VdLs.17 and *V. albo-atrum* genomes were sequenced using the whole genome shotgun approach via Sanger sequencing (Klosterman et al. 2011). Although the two ~ 33 Mb genomes were highly similar, there were four 300 kb regions in *V. dahliae* which had no synteny with *V. albo-atrum*. These regions were denoted "Lineage Specific" (LS) regions. The LS regions were found to be highly repetitive and represented over 50% of all identifiable transposable elements contained in *V. dahliae.* Faino et al. (2015) used PacBio long read sequences to create a "gapless" genome and have since suggested that there are problems with the initial *V. dahliae* VdLs.17 sequence. These authors argue that their method of genome assembly helps to prevent problems associated with repetitive regions that cause issues when assembling shorter contigs. Using PacBio sequencing, the VdLs.17 genome was re-assembled. The newly constructed genome indicates that 12% is composed of repetitive regions, four times higher than was previously thought.

With the availability of a *V. dahliae* reference genome, there is an increasing understanding of what makes *V. dahliae* such an adaptable pathogen with a broad host range. There are suggestions that transposons could be a major reason for the genomic diversity observed and that they contribute to the *V. dahliae* "plastic genome" driving adaption to new plant hosts (Amyotte et al. 2012; Faino et al. 2016). This is supported by de Jonge et al. (2013) who compared the VdLs.17 reference strain with 10 *V. dahliae* genomes taken from geographically separate regions and hosts. The study revealed that despite the genomes being highly similar, chromosome rearrangements had occurred between all strains. Using RNA-seq data and deletion studies, they showed that effector genes present in the LS regions were important to the development of disease (de Jonge et al. 2012, 2013), suggesting that chromosome rearrangements and these LS regions could contribute to *V. dahliae*'s adaptation to new hosts. Jin et al. (2017) explored the organism's use of alternative splicing and developed their own algorithms, alongside previously available software, to analyse *V. dahliae* cDNA sequences for common splicing events. They found that *V. dahliae* has one of the most sophisticated splicing systems in eukaryotes, outside of animals, and believe that this alternative splicing could explain some of *V. dahliae's* plasticity.

There are an increasing number of studies suggesting that horizontal gene transfer plays an important role in *V. dahliae's* success as a pathogen. An analysis of *V. dahliae* isolated from cotton in China, revealed the presence of a virulence gene believed to have originated in *Fusarium oxysporum*, a related fungal pathogen often found infecting cotton on the same farm (Chen et al. 2017). Their deletion experiments found that removal of this gene affected the ability of the *V. dahliae* strain to infect cotton, but not lettuce or tomato, highlighting it's ability to acquire new virulence genes as it expands to different hosts. There has also been evidence of *V. dahliae* acquiring genes from the host plant and from bacteria (de Jonge et al. 2012; van Kooten et al. 2019). These studies used phylogenetic analysis to look for candidate genes that are found outside the *Verticillium* spp. They found numerous candidate genes of bacterial and plant origin, many of which could potentially aid *V. dahliae* in getting past the host plant's defences.

Management strategies for the control of Verticillium wilt

The nature of *V. dahliae* infection makes elimination of the pathogen difficult, however, multiple management strategies have been applied over the years. As the *V. dahliae* life cycle is dependent on microsclerotia present in crop soil, currently the two main strategies target either the soil itself, for example by soil fumigation, or the plants through development of resistant varieties (Short et al. 2015). Soil fumigation aims to eliminate microsclerotia in crop soil. Traditionally, methyl bromide was used to control pathogen populations, but was classified as a Class 1 stratospheric, ozone-depleting substance and international regulations dictated by the Montreal Protocol now restrict the use of this chemical (Martin 2003). Multiple studies have explored alternatives, including green manures, anaerobic soil disinfection and anaerobic digestion. Green manure is a method utilising volatile components from plant waste to reduce the number of microsclerotia (Yohalem and Passey 2011). Anaerobic soil disinfection uses microbial activity from agricultural or horticultural waste products, combined with mulched plastics, to deplete available oxygen in soil, creating anaerobic conditions to prevent fungal growth (Goud et al. 2004). Anaerobic digestion uses liquid digestate, a by-product from biogas production, as a bio-fertiliser to control microsclerotia levels (Wei et al. 2016). However, the suitability of these methods in commercial processes is still questionable. While, green manures and anaerobic digestion are still relatively new and understudied, the well-studied variants, such as *Brassica sp.*, are deemed insufficient (Neubauer et al. 2014) and anaerobic soil disinfection is not currently economically viable (Wei et al. 2016).

Production of resistant cotton varieties is a key strategy in the prevention of Verticillium wilt. The development of resistant varieties in Australia has been ongoing for more than 30 years, with the release of Sicala V-1 in 1990, and Sicala V-2 in 1994 (Liu et al. 2013). Despite successes with Sicala V-2 and subsequent varieties derived from it, the incidence of Verticillium wilt has continued to rise in recent years (Kirkby et al. 2013). This could be linked to the temperature tolerance, as currently the *V. dahliae* resistance in available cotton varieties breaks down when temperatures drop below 22˚C (Quinn et al. 2018). Although there is ongoing research into Verticillium resistance (Li et al. 2018, 2019; Zhang et al. 2018), the development of new cotton varieties that provide adequate yield is slow, and the current varieties do not provide a substantial increase in resistance (Dadd-Daigle et al. 2020). Also, without a rapid diagnostic system that classifies *V. dahliae* into groups meaningful for Australian cotton, it is difficult to develop targeted and effective strategies.

Currently, crop rotation is one of the methods used to help manage Verticillium wilt on cotton farms in Australia. Crop rotation is the practice of varying the successive crops in a particular field to assist in the control of disease and weed management. Each crop varies in its susceptibility to certain pathogens. The success of crop rotation relies on initial inoculum levels in the soil, the number of rotations with non-host crops and the wetting and drying cycles that assist in the breakdown of inoculum in the soil (Wheeler et al. 2019). For example, most cotton farmers rotate with barley or sorghum as they are not listed as host crops for *V. dahliae*. While commodity prices are the short-term driving force, farms with high disease levels are looking at rotation to ensure cotton remains sustainable in the long term (K. Kirby, personal communication, September 2016). The current recommendations to growers are long rotations with moderate irrigation to reduce overall pathogen levels and prevent widespread movement of the microsclerotia (Holman et al. 2016; Scheikowski et al. 2019).

The development of real-time PCR protocols to determine microsclerotial load from soil samples should assist with managing crop rotation practices (Banno et al. 2011; Gharbi et al. 2016). Removal of the rotational crop plant debris has also been shown to reduce the number of microsclerotia in the soil, but does sacrifice soil health (Chawla et al. 2012). However, the known host range of *V. dahliae*, both symptomatic and asymptomatic, is expanding as the pathogen comes into contact with new plant species. There have been instances where a symptomless host has exhibited extensive vascular colonization and so contributes to the microsclerotial load despite the lack of symptoms (Wheeler and Johnson 2016). This makes selection of a suitable rotation crop more complex and highlights the need for a better understanding of the genomics of *V. dahliae*. In some instances, after multiple years of crop rotation followed by a cotton crop, the incidence of Verticillium wilt rises to match those found on farms that have had continuous cotton growth (Wheeler et al. 2019).

Given that the current attempts to mitigate Verticillium wilt on cotton farms is becoming increasingly ineffective, new strategies need to be explored for use in Australia. One area that hasn't been well examined in Australian cotton is the use of endophytes as a biological control. The idea behind this strategy is to pre-infect the plants with a microbe that will inhabit the same niche as *V. dahliae*, preventing infection by the pathogen. This has been explored with both bacterial and fungal endophytes (Li et al. 2012; Vagelas and Leontopoulos 2015) used the less virulent *V. nigrescens* to take up the niche usually filled by *V. dahliae*, preventing the infiltration of conidia by the more virulent species, while Yuan et al. (2017) looked at using unrelated fungal species as seed treatments. Although both studies saw a reduction in *V. dahliae* caused Verticillium wilt, the use of *Penicillium simplicissimum* and *Leptosphaeria* sp. also saw an increase in cotton seed production as the number of cotton bolls increased (Yuan et al. 2017). As endophytes have been shown to be beneficial in other areas of crop sustainability, such as protection from insect pests and abiotic stress (Lugtenberg et al. 2016), this area could be hugely beneficial to the Australian cotton industry which is often heavily impacted by water availability.

Improving future understanding of the Verticillium wilt problem in Australia

The nature of Verticillium wilt in Australian cotton is an interesting problem. Large patches of severe Verticillium wilt have been found to be caused by the ND VCG2A (Dadd-Daigle et al. 2020; Jensen and Redfern 2017), which is contrary to reporting on other cotton farms around the world. This could be dependent on factors other than the isolate, such as the Australian environment, or the farming conditions, and is an area that warrants further exploration. While studies to further examine the Australian *V. dahliae* population are currently being conducted, no study to date has indicated what causes the difference in disease potential between Australian and international cotton crops. In addition, the genetic analyses are revealing an increasing number of methods by which *V. dahliae* can adapt. It is no wonder that strategies that work some of the time, such as crop rotation or the use of resistant varieties, are becoming less effective (Kirkby et al. 2013; Wheeler et al. 2019).

There is an increasing need for new mitigation strategies or the development of new cotton varieties resistant to Verticillium wilt. However, in order to create and implement these strategies, the current classification system needs to be improved to better represent the *V. dahliae* present on Australian cotton farms. Characterisation of the genetics controlling virulence has improved the classification of VCGs within related *Fusarium* sp. by increasing molecular clarity between isolates and developing new classification systems (Carvalhais et al. 2019). Although there is still some debate surrounding the best tools to diagnostically identify virulent *Fusarium oxysporum* strains (Magdama et al. 2019), a similar molecular understanding could improve the VCG classification system within *V. dahliae* by establishing narrower classifications or by implementing a new system based on virulence genes unrelated to VCGs.

Future research to improve Verticillium wilt on Australian cotton farms needs to largely build on current research efforts. An improved system for quantification of inoculum in soils and a better understanding of the inoculum to disease thresholds for different VCGs can clarify the effectiveness of crop rotation (Wheeler et al. 2019). While an improved understanding of the environmental conditions and how current farming methods impact Verticillium wilt on Australian farms can help inform best farming practices (Kirkby et al. 2013). It is only through continued development of new tools and a better understanding of *V. dahliae* genetics to rapidly analyse Verticillium wilt samples that growers may be able to stay ahead of the pathogen, preventing a situation where yield loss due to disease outweighs potential yield.

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