Verticillium wilt in Australian cotton: Examining the relationship between Australian *Verticillium dahliae* isolates and virulence

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Certificate of Authorship

I, Pearl Dadd-Daigle, declare that this thesis, submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science, School of Life Sciences at the University of Technology Sydney, is wholly my own work unless otherwise reference or acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Statement

This thesis is by compilation. The first research chapter and the literature review are published papers listed in the section below. The second research chapter is a completed manuscript and has been submitted to the Australasian Plant Pathology Journal. The third research chapter is a publication style manuscript that is being prepared for journal submission. Figure and Table numbers have been edited from the original publications to match the chapter numbering of the thesis.

List of publications

Paper 1; Chapter 2

The Verticillium wilt problem in Australian cotton

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Australasian Plant Pathology. 2021 50:129–135

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Paper 2; Chapter 3

Virulence not linked with vegetative compatibility groups in Australian cotton *Verticillium dahliae* isolates

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Abbreviations

VCG	Vegetative Compatibility Groups
D	Defoliating
ND	Non-Defoliating
ISSR	InterSequence Simple Repeats

Abstract

Verticillium wilt, caused by the soil-borne phytopathogen *Verticillium dahliae*, affects many agriculturally important crops around the world. In Australia, the cotton industry, worth, on average, between $2-3 billion a year, is increasingly impacted by Verticillium wilt. The fungal pathogen is characterised into Vegetative Compatibility Groups (VCG) and further into defoliating and non-defoliating pathotypes. *Verticillium dahliae* defoliating VCG1A is reported to cause severe damage to cotton internationally, while non-defoliating VCG2A is responsible for only mild to moderate disease symptoms. In Australian cotton however, the non-defoliating VCG2A is causing more severe damage to crops in the field than the defoliating VCG1A.

A selection of isolates taken from the New South Wales (NSW) Department of Primary Industries Verticillium collection were used to infect cotton plants in controlled greenhouse conditions. The plants, encompassing four varieties, were monitored over a period of seven weeks and the disease progress scored bi-weekly. Analysis of the disease scores revealed that although disease progression is slower in plants infected with non-defoliating VCG2A isolates, both Australian defoliating VCG1A and non-defoliating VCG2A are able to kill cotton plants in glasshouse trials. Cotton variety had minimal impact on disease outcomes. This was the first confirmed report of an Australian non-defoliating VCG2A causing plant mortality in cotton plants outside of the field.

Eighty-four isolates from the NSW Department of Primary Industries Verticillium historical collection were further analysed using InterSequence Simple Repeats (ISSR). The PCR-based method resulted in a molecular fingerprint for each isolate, which was then used to produce a phylogenetic tree. Within the tree the isolates clustered into three main groups, one composed of non-defoliating VCG2A isolates, another made of both non-defoliating VCG2A and non-defoliating VCG4B, and the third contained only defoliating VCG1A isolates. These groups were labelled “Defoliating-like”, “Non-defoliating”, and “Defoliating”, respectively. Further glasshouse trials to examine the virulence of isolates in each group confirmed that the virulent VCG2A isolates all fell within the “Defoliating-like” group, the virulent

VCG1A isolates within the “Defoliating” group, and all non-virulent isolates in the “Non-defoliating” group. The inclusion of American *V. dahliae* defoliating VCG1A isolates and eight Israeli isolates of varying VCG into the ISSR study revealed that the Australian isolates appear to cluster separately and suggests that Australian *V. dahliae* isolates could be unique to Australia.

To further examine the differences between Australian and international defoliating VCG1A *V. dahliae,* four isolates were DNA sequenced using both Nanopore Minion and Illumina sequencing platforms to produce whole genomes. Isolates were analysed with 13 publicly available *V. dahliae* isolates using phylosift to build a phylogenetic tree, and gene content examined using ProgressiveMauve alignments to determine where the genes differed. The comparisons found minimal differences between the four Australian isolates, but when compared with the other 13, they appear more genetically distant. Additionally, Australian VCG1A isolates lack a set of genes identified as being in involved in defoliation of cotton plants. This work highlights, for the first time, genetic differences between Australian and international defoliating VCG1A *V. dahliae*.

Chapter 1: Thesis Overview

1.1 Overview

This thesis by compilation utilised glasshouse trials, molecular typing and whole genome sequencing to further understand the relationships between Australian *Verticillium dahliae* isolates, the Vegetative Compatibility Grouping and their virulence. The literature review, chapter 2, was submitted to the Australasian Plant Pathology journal and published in January 2021. The first results chapter, chapter 3, has been published in the Australian Journal of Crop Science. The second results chapter, chapter 4, has been submitted to the Australasian Plant Pathology Journal. The final results chapter, chapter 5, has been prepared as a manuscript for publication in the future.

1.2 Aims

The three major aims of this thesis were to:

1. Use infection models in cotton to better understand isolate pathogenicity and validate observations that were observed in the field (chapter 3)

2. Identify a molecular tool to help characterise Australian *V. dahliae* isolates (chapter 4)

3. Conduct whole genome sequencing and analyse the genome of Australian *V. dahliae* VCG1A (chapter 5)

1.3 Summary and knowledge added to the field

This thesis addressed a lack of knowledge around the types of *V. dahliae* present in Australia causing infection in cotton. It confirmed, for the first time, the presence of a virulent ND VCG2A among Australian isolates and revealed that Australia *V. dahliae* isolates taken from cotton appear to be genetically distant from international *V. dahliae* isolates.

The major findings included:

- The confirmed presence of virulent ND VCG2A *V. dahliae* in Australian cotton fields

- Confirmation of a molecular tool able to separate Australian *V. dahliae* isolates based on virulence in cotton plants

- Identification of three distinct groups within the Australian *V. dahliae* population, two virulent and one non-virulent

- Evidence that Australian *V. dahliae* isolates are genetically distant from international *V. dahliae* samples

Chapter 2: Literature Review - The Verticillium wilt problem in Australian cotton

Declaration

I declare that the below publication meets the below requirements for inclusion as a chapter in this thesis.

- I have contributed more than 50 % for the below publication.
- The below publication has been peer reviewed.
- The below publication has been formally published, and is formatted to adhere to the specific formatting requirements of the Australasian Plant Pathology Journal.
- Permission is not required by the publisher for inclusion of this publication in this thesis for non-commercial purposes.

Dadd-Daigle P, Kirkby K, Chowdhury PR, Labbate M, Chapman TA (2021). The Verticillium wilt problem in Australian cotton. *Australasian Plant Pathology.* 50:129-135 doi:10.1007/s13313-020-00756-y

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2.1 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.

2.2 Introduction

In Australia, cotton is a growing billion-dollar industry, worth, on average, between $2-3 billion a year (Cotton Australia 2022). 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 remains a major concern for the Australian cotton industry.

2.3 *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 without a host (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 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).

2.4 Vegetative Compatibility Groups

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 virulent on tomato (Tsror et al. 2001), VCG2B is highly aggressive in mint (Dung et al. 2013), VCG4A is highly virulent 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.

2.5 *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.

2.6 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. 2013; de Jonge et al. 2012), 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 its 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.

2.7 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; Li et al. 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. Kirkby, 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.

2.8 Improving future understanding 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 was recently published (Young et al. 2021) however a better

understanding of the inoculum level pre-plant to disease risk for different VCGs would clarify the effectiveness of crop rotation (Wheeler et al. 2019). 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 existing and 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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Chapter 3: Virulence not linked with Vegetative Compatibility Groups in Australian cotton *Verticillium dahliae* isolates

Declaration

I declare that the below publication meets the below requirements for inclusion as a chapter in this thesis.

- I have contributed more than 50 % for the below publication.
- The below publication has been peer reviewed.
- The below publication has been formally published, and is formatted to adhere to the specific formatting requirements of the Australasian Journal of Crop Science.
- Permission is not required by the publisher for inclusion of this publication in this thesis for non-commercial purposes.

Dadd-Daigle P, Kirkby K, Collins D, Cuddy W, Lonergan P, Roser S, Chowdhury PR, Labbate M, Chapman TA (2020). Virulence not linked with vegetative compatibility groups in Australian cotton *Verticillium dahliae* isolates. *Australian Journal of Crop Science* 14:633-640

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3.1 Abstract

Verticillium dahliae, the causal agent of Verticillium wilt, is a soil-borne ascomycete that infects numerous agriculturally important crops globally, including cotton. As a billion-dollar industry, cotton is economically important to Australia and the management of disease such as Verticillium wilt is key for the success of the industry. Internationally, defoliating *V. dahliae* isolates belonging to Vegetative Compatibility Group (VCG) 1A cause severe damage to cotton, while non-defoliating VCG2A isolates result in significantly less disease. However, in Australia, VCG2A is causing more severe damage to crops in the field than the defoliating VCG1A. This study aimed to replicate field observations in controlled greenhouse conditions. We examined and compared disease symptoms on a range of Australian commercial cotton varieties when inoculated with different *V*. *dahliae* VCGs. Seedlings were root dipped in conidial suspensions and assessed over seven weeks. The final disease score, disease over time and root length were analysed. Plant mortality resulted from both *V*. *dahliae* VCG1A and VCG2A isolates across all cotton varieties used, confirming that there are virulent VCG2A isolates present in Australia. To our knowledge, although virulent on other plant hosts, *V. dahliae* VCG2A has not previously been reported to be highly virulent in cotton. We infer that virulence cannot be defined solely by VCG in Australian *V. dahliae* isolates causing disease in cotton.

3.2 Introduction

Australian farmers have been growing cotton since the 1800s (Cotton Australia, 2016). The pursuit of a plant with greater disease resistance, lower resource requirements, improved fibre quality, and higher yield has driven the development of cotton varieties. Cotton in Australia is now a billion-dollar industry, with yields increasing from approximately 9000 bales in the 1960s to around 1.5 million bales in 2005/2006 to 2009/2010 (Cotton Australia, 2018). Australia is the third largest exporter of cotton internationally with a reputation for the highest quality of fibre produced (Cotton Australia, 2018). Disease management is now more important than ever to maintain Australia's status as an exporter of high-quality cotton.

In New South Wales (NSW), the average incidence of Verticillium wilt disease ranged from 4 to 9 % between 1984/1985 and 1988/1989 peaking at 16.6 % in the 1989/1990 season (Kirkby et al. 2013). Following the release of resistant varieties of cotton in 1990, disease incidence declined to 3 % in 1996/1997. By 2016, the average incidence of Verticillium wilt had been gradually rising (Kirkby et al. 2016). Growers had reported disease symptoms becoming more severe with large sections of dead and dying plants, particularly in irrigated fields, resulting in large yield losses (Jensen and Redfern 2017). CSD (2011) reported that yield losses in the Namoi Valley in NSW varied from two to four kg lint/ha for each percent of disease depending on seasonal conditions. Verticillium wilt caused losses in NSW of $1.9 to $3.8 million per season averaged over the five seasons up to the year 2010 (CSD 2011).

As the incidence of Verticillium wilt continues to increase, production of cotton varieties tolerant or resistant to Verticillium wilt is becoming increasingly important. In Australia, Sicala V-1 was released in 1990, followed by the release of Sicala V-2 in 1993 (Cotton Australia, 2016). Sicala V-2 was considered a breakthrough variety in terms of yield and resistance to Verticillium wilt. However, the resistance mechanism in Sicala V-2 and the subsequent varieties derived from it are temperature sensitive, meaning the tolerance breaks down below 22˚C (CSD 2011; Quinn et al. 2018). New varieties developed since the 2002/2003 season are assigned a Verticillium resistance/tolerance ranking called "V-rank". The V-rank is calculated by dividing the number of test variety plants by the number of industry standard plants with symptoms in the same trial and multiplied by 100 (Salmond 2003). A higher V-rank is indicative of a more tolerant variety. Over the past decade in Australia, three commonly grown cotton varieties include Sicot 74BRF, 71BRF and 714B3F, which have V ranks of 101, 107, and 113 respectively.

In the United States of America, the cotton variety Acala SJ-2 was produced in 1973 to decrease the disease incidence of Verticillium wilt (Smith and Cothren 1999). Today, varieties such as FibreMax are more commonly grown. Acala SJ-2 is frequently used in international studies examining *V. dahliae,* although no longer

used commercially. Acala SJ-2 does not have the resistance genes found in many modern varieties that could impact the results of virulence studies (Jiménez-Díaz et al. 2006; Korolev et al. 2001).

Verticillium dahliae Kleb. is a soil-borne ascomycete that infects the vascular system of many plant species, including cotton. It is able to survive in the soil for many years in structures known as microsclerotia, which makes the management of the pathogen difficult (Davis et al. 1994). In cotton, the pathogen is classified into two pathotypes, defoliating (D) and non-defoliating (ND), and also divided into Vegetative Compatibility Groups (VCGs). The D and ND pathotypes can be identified by a simple PCR (Mercado-Blanco et al. 2003), while assigning VCG is more complex. Vegetative compatibility groups are determined by strain interaction between two nitrogen non-utilizing mutants (*nit* mutants) on the basis that two isolates of the same VCG have the ability to form prototrophic heterokaryons (the fusion of two genetically distinct cells) (Puhalla and Mayfield 1974). Recently, Papaioannou et al. (2013a) developed a method that uses sequence data from Intergenic Spacer (IGS) regions to provide a presumptive VCG. This technique allows for faster turnaround than with the laborious production of *nit* mutants.

For decades it was thought that only ND VCG4B was present on Australian cotton farms, but in 2014 the presence of ND VCG2A was reported by Smith et al. (2014). More recently, the presence of the D VCG1A was reported in Australian cotton for the first time (Chapman et al. 2016). At that time, VCG1A was considered exotic, however the study by Chapman et al. (2016) using isolates collected and stored in the NSW long-term culture collection revealed VCG1A had been present but undetected in Australia since at least 1983. Despite this discovery, VCG1A is not consistently causing the same extent of damage in the field as reported in other countries (Chapman et al. 2016). Additionally, severe defoliation and crop losses in Australian cotton varieties have been reported from isolates belonging to the ND VCG2A pathotype (Jensen and Redfern 2017).

There are currently no reports of ND VCG2A causing severe damage or plant mortality overseas to the same extent as seen on Australian farms, revealing a clear need to further investigate the disease presentation of Australian *V. dahliae* isolates. The aim of this study was to evaluate different cotton varieties for disease symptom expression when inoculated with isolates assigned as D VCG1A, ND VCG2A and ND VCG4B under controlled glasshouse conditions and to validate field observations. Cotton varieties used in this study included the American Acala SJ-2 and the Australian cotton varieties Sicot 74BRF, 71BRF and 714B3F.

3.3 Results

Characterisation of Verticillium dahliae isolates

Isolates were assigned to the groups D VCG1A, ND VCG2A, and ND VCG4B based on PCR and sequencing analysis (Table 3.1; Figures 3.1 and 3.2). The results indicated that isolates SS61 and SS499 were the D pathotype, while all other isolates were ND. Isolates SS61 and SS499 were assigned to VCG1A; SS362, SS285, and SS262 were assigned to VCG2A; and isolates SS364, SS289, and SS94 were assigned to VCG4B.

Disease severity of infected cotton plants

Disease severity based on a 0 - 4 scale varied between isolates of the same VCG (Table 3.2) (Jiménez-Díaz et al. 2016). Two D VCG1A isolates, SS61 and SS499, and one ND VCG2A isolate SS362, had significantly ($P < 0.01$) higher disease severity scores than the remaining 5 ND isolates when analysed using a generalized linear mixed model (Table 3.3). No defoliation was observed in any cotton variety. Plants inoculated with the D isolates SS61 and SS499 took on average 25 days to mortality, while the plants inoculated with the ND isolate SS362 took an average of 35 days (Figure 3.3; table 3.4). All other isolates did not cause mortality within the timeframe of the study.

Table 3.1. *Verticillium dahliae* isolates used in the glasshouse virulence assay

Herbarium number	Isolate number	Presumptive VCG	Pathotype (D/ND)	Season collected	Valley where samples were collected
DAR82592	SS61	1A*	D	2010/2011	Namoi
DAR83206	SS499	1A	D	2015/2016	Gwydir
DAR82597	SS362	2A*	ND	2013/2014	Namoi
DAR83151	SS285	2A	ND	1983/1984	Namoi
DAR83107	SS262	2A	ND	2011/2012	Macquarie
DAR82599	SS364	4B*	ND	2013/2014	Macquarie
DAR83111	SS289	4B	ND	1983/1984	Macquarie
DAR82593	SS94	4B*	ND	2010/2011	Bourke/Walgett

*VCG determined previously using *nit* mutants (Chapman et al. 2016)

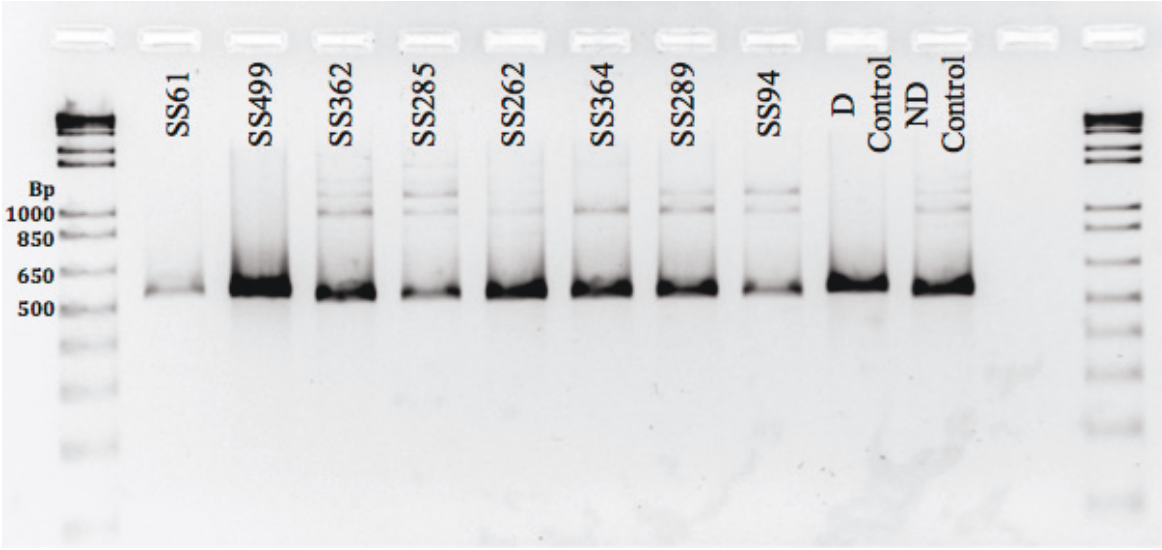

Figure 3.1. Visualisation of the defoliating and non-defoliating PCR as described by Mercado-Blanco, et al. (2003). Of the eight isolates, only SS61 and SS499 produced bands of 539 bp, consistent with the defoliating pathotype, while the other six isolates had bands of 523 bp, indicating the non-defoliating pathotype.

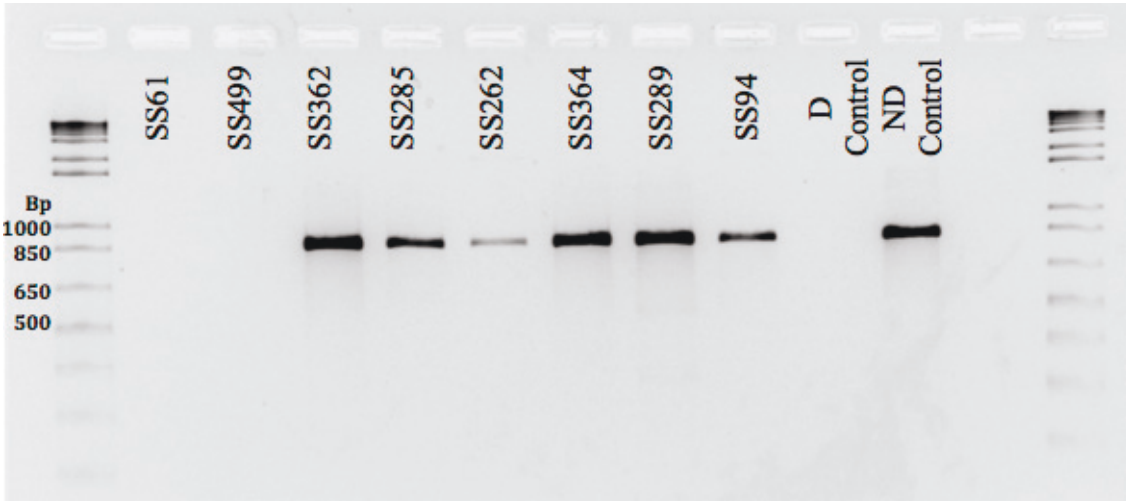

Figure 3.2. Visualisation of the confirmatory defoliating and non-defoliating PCR as described by Mercado-Blanco, et al. (2001). Isolates SS362, SS285, S262, SS364, SS289, and SS94 all produced bands of 824 bp in size, indicating that they are of the non-defoliating pathotype. Amplification of isolates SS61 and SS499 both resulted in no product being produced, consistent with the defoliating pathotype.

Disease progression over time

The area under the disease progress stairs (AUDPS) severity scores showed significant effects caused by VCG, variety and isolate (within VCG) ($P < 0.001$). The D isolates SS499 and SS61 had the highest severity means followed by the ND isolate SS362, while Acala SJ-2 and Sicot 71BRF varieties had the highest means (Figure 3.4). There were no significant interactions between isolate by variety or VCG by variety ($P > 0.05$) (Table 3.5).

Final root length of infected cotton plants

There were significant differences in root length of plants between variety ($P < 0.05$), but no significant differences in root length of plants infected by different VCG isolates (Figure 3.5). The D isolates SS61 and SS499, and the ND isolate SS362 had shorter roots than the other isolates with mean root lengths of 51.75 mm, 41.5 mm, and 75.5 mm, respectfully. The Acala SJ-2 and Sicot 71BRF cotton varieties had the shortest mean root lengths of 200 mm, and 204.6 mm, respectfully. There

were no significant interactions between isolate by variety or VCG by variety ($P > 0.05$) (Table 3.5).

Table 3.2. Disease severity scores at the end of the seven week monitoring period. Letters within the table compare varieties within each isolate. Greek letters for the mean (variety) and mean (isolate) compare variety means and isolate means respectively. For mean scores on the boundary (0 or 4), a reliable CI cannot be computed, and so is not shown.

		Mean Disease Severity (68% CI)				
Isolate	**Pathotype / VCG**	**Acala SJ-2**	**Sicot 74BRF**	**Sicot 71BRF**	**Sicot 714B3F**	**Mean (Isolate)**
SS499	D / 1A	4.00 (-)^{a}	3.39 (2.97-3.70)^{a}	4.00 (-)^{a}	4.00 (-)^{a}	3.91 (3.83-3.96)$^{\delta\varepsilon}$
SS61	D / 1A	4.00 (-)^{a}	3.79 (3.46-3.93)^{a}	3.67 (3.29-3.88)^{a}	4.00 (-)^{a}	3.92 (3.82-3.97)$^{\varepsilon}$
SS262	ND / 2A	1.03 (0.77-1.34)^{a}	0.33 (0.16-0.62)^{b}	0.82 (0.54-1.12)^{ab}	0.00 (-)^{ab}	0.52 (0.36-0.71)$^{\alpha}$
SS285	ND / 2A	1.98 (1.58-2.37)^{b}	1.53 (1.21-1.91)^{ab}	1.57 (1.24-1.97)^{ab}	1.07 (0.79-1.38)^{a}	1.46 (1.27-1.70)$^{\beta\gamma}$
SS362	ND / 2A	4.00 (-)^{a}	4.00 (-)^{a}	4.00 (-)^{a}	3.80 (3.51-3.94)^{a}	3.97 (3.91-3.99)$^{\varepsilon}$
SS289	ND / 4B	1.42 (1.12-1.83)^{b}	0.65 (0.36-0.89)^{a}	0.69 (0.42-0.95)^{a}	0.18 (0.06-0.45)^{a}	0.72 (0.54-0.88)$^{\alpha\beta}$
SS364	ND / 4B	3.33 (2.90-3.66)^{b}	1.92 (1.56-2.32)^{a}	2.86 (2.4-3.28)^{ab}	2.27 (1.79-2.76)^{ab}	2.62 (2.33-2.91)$^{\gamma\delta}$
SS94	ND / 4B	1.42 (1.10-1.85)^{a}	1.00 (0.73-1.29)^{a}	1.17 (0.92-1.53)^{a}	1.00 (0.74-1.30)^{a}	1.12 (0.97-1.33)$^{\alpha\beta}$
Mean (Variety)		3.14 (2.92-3.33)$^{\gamma}$	2.03 (1.84-2.24)$^{\alpha\beta}$	2.54 (2.32-2.76)$^{\beta}$	1.88 (1.71-2.07)$^{\alpha}$	

Table 3.3. Wald tests for fixed terms in the ordinal GLMM for disease severity

	DF	Den. DF	F-stat.	P-value
VCG	2	8.55	9.1696	0.0075
Variety	3	705	6.2602	<0.001
VCG/Isolate	5	8.99	8.0966	0.0038
VCG×Variety	6	705	0.261	0.9548
VCG/Isolate×Variety	15	705	0.4196	0.9738

Table 3.4. Analysis of deviance table for the parametric survival regression for days to mortality.

	Df	Deviance	Resid. Df.	-2 × LL	P-value
			183	751.22	
VCG	2	130.90	180	620.32	<0.001
Variety	3	11.61	177	608.71	0.009
VCG/Isolate	5	85.56	149	523.15	<0.001
VCG×Variety	6	20.17	140	502.97	0.003

Table 3.5. Wald F-test statistics for fixed terms in the model

AUDPS				
	DF	DenDF	F-stat	P-val
VCG	2	5.8	100.4000	<0.001
Variety	3	112.5	12.0400	<0.001
VCG × Isolate	5	6.3	20.8000	<0.001
VCG × Variety	6	112.6	1.9430	0.080
VCG × Isolate × Variety	15	112.6	0.7364	0.743
Log10 Root length				
	DF	DenDF	F-stat	P-val
VCG	3	3.1	5.100	0.105
Variety	3	77.3	5.955	0.001
VCG × Isolate	5	4.2	5.993	0.050
VCG × Variety	9	76.9	1.423	0.193
VCG × Isolate × Variety	15	77.9	1.445	0.148

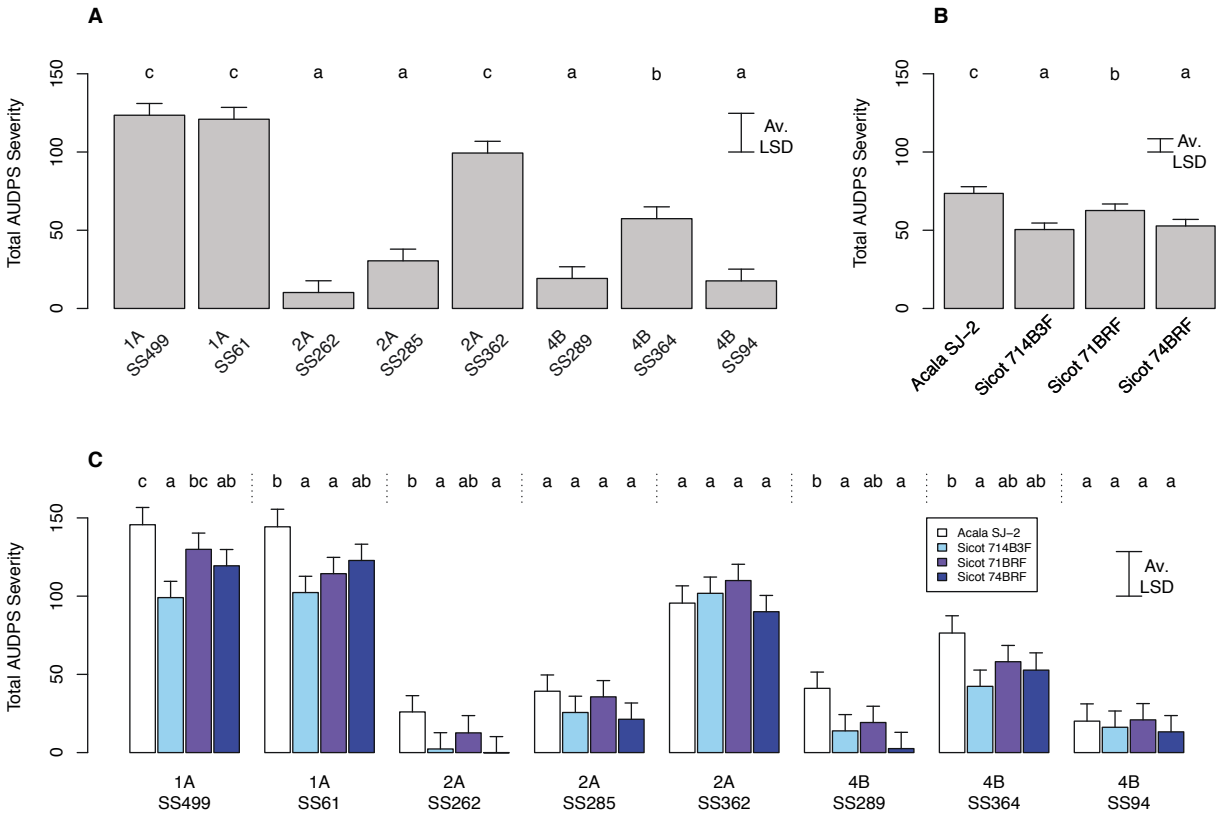

Figure 3.4. Total AUDPS severity. **A)** AUDPS means for each isolate. **B)** AUDPS means for each cotton variety. **C)** AUDPS means for each isolate by variety.
Av LSD indicates average least significant difference.
Different lower case letters indicate significant difference between bars.

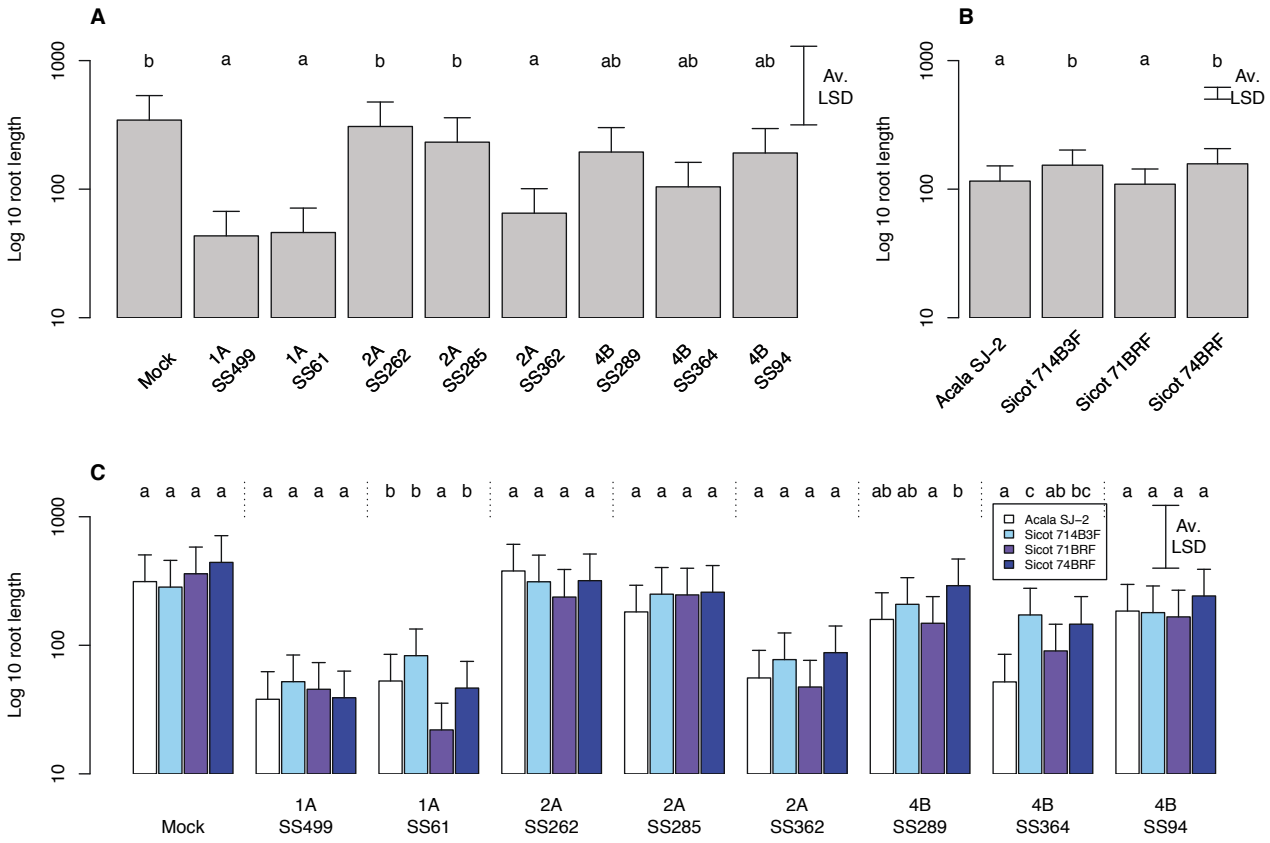

Figure 3.5. The average root length of infected plants. **A)** Average root lengths for each isolate. **B)** Average root lengths for each cotton variety. **C)** Average root lengths for each isolate by variety.

Av LSD indicates average least significant difference.

Different lower case letters indicate significant difference between bars.

3.4 Discussion

Verticillium wilt is a persistent problem on many Australian cotton farms. Average disease incidence varies between farms with some fields presenting large patches of defoliated plants, high yield losses and plant death (Jensen and Redfern 2017). The isolates recovered from these disease-affected areas were characterised and designated as ND VCG2A, which conflicts with symptoms reported internationally (Dervis et al. 2008; Korolev et al. 2008). This study found Australian isolates from ND VCG2A vary in their ability to cause symptoms from mild foliar symptoms to plant death.

Studies that look at *V. dahliae* pathogenicity in cotton often use older cotton varieties such as Acala SJ-2 which are more susceptible to infection and disease (Jiménez-Díaz et al. 2016). This study found that there was a significant effect of the disease progress over time (AUDPS scores) for the varieties, as Acala SJ-2 was more susceptible across the board. The interaction between isolate and variety was not significant as disease outcomes were fairly consistent across plants infected with the same isolate regardless of cotton variety. However, the effects of both VCG and isolate within VCG were significant ($P < 0.01$). This indicated that while there is a difference in disease progress over time between the VCGs, there is variation between isolates within each of the VCGs. For this reason, in Australia, knowing the VCG of a *V. dahliae* isolate is not an effective measure of *V. dahliae* pathogenicity or useful for predicting potential disease outcomes. This is evident by the fact that plants infected with the ND VCG2A isolate SS362 caused plant mortality at the same level of severity as plants infected with the D VCG1A isolates SS499 and SS61.

The root lengths were equally consistent with the AUDPS and disease severity scores. Plants that had higher AUDPS and higher disease severity scores had shorter roots, indicating that they had either spent fewer resources towards growing or died before the roots could get larger. The two more susceptible cotton varieties also had shorter roots lengths, consistent with expectations for susceptible varieties. The lack of a significant interaction between isolates and cotton variety also points to the isolate being a key factor in the determination of plant disease outcome. Additionally, these results show that while the recent cotton varieties used in Australia can slow disease progression, they do not make a notable impact on disease outcome regardless of the VCG type.

Observations made in fields, where the ND VCG2A isolates have been obtained from, varied from no defoliation, mild to severe foliar symptoms, to complete defoliation as well as plant death despite being a ND pathotype (Jensen and Redfern 2017). However, plants in this study did not defoliate when infected with any of the isolates, including the D VCG1A SS61 which has previously been shown to defoliate (Jiménez-Díaz et al. 2016). This defoliation did not appear significant

given mortality was observed in both previous studies. In the field VCG1A causes minimal disease symptoms (Chapman et al. 2016). In our study, both D SS61 and D SS499 were highly virulent and resulted in plant death in the least amount of time compared to the other VCGs. This could be contributed to several reasons, such as the infection method or the plant environment. Root dipping introduces conidia into the plant at concentration levels that may be higher than natural infection. Natural infection also relies on conidia and hyphae germinating and infecting a plant root. Additionally, the use of an artificial potting mix that most likely had a different microbiome to field soil may have also affected the infection process. The absence of competitors and antagonistic microbes in the potting soil may have enhanced infection by *V. dahliae*. Infection percentage is guaranteed when artificially inoculating plants and the number of conidia inside the plant are also higher.

Greenhouse trials are designed with the intent to produce pathogenicity results providing the optimum conditions for the pathogen and not necessarily the host plant. The root dipping method, with conidia as the only inoculum source, used in this study (Trapero et al. 2013) is not representative of the natural infection process. The consistent temperature of the greenhouse also differs from the hot days and cold nights that plants are subjected to on cotton farms. The difference in these conditions could account for some of the disparity.

Previous research suggests that the separate VCGs of *V. dahliae* are generally virulent on different hosts. While VCG1A is known to be virulent in cotton and olives (Dervis et al. 2007), VCG2A is generally virulent in tomatoes (Tsror et al. 2001), and VCG4B is virulent in mint and potato (Dung et al. 2013). In Israel however, the ND VCG2B is the most pathogenic *V. dahliae* in cotton rather than VCG1A (Korolev et al. 2001). *Verticillium dahliae* is an adaptable pathogen, and has been shown to have a high number of translocatable elements, allowing it to rearrange its genetic material in response to new hosts (de Jonge et al. 2013). It is likely that a subset of Australian VCG2A *V. dahliae* isolates may have adapted from a separate host, have acquired additional virulence genes from another source (Chen et al. 2017), or have simply mutated as a response to the continuous

selective pressure of growing cotton. No obvious morphological differences between virulent and non-virulent isolates have been found (data not shown). As virulence is currently determined through *in vivo* assays and field reports, genome analysis and better molecular tools for the analysis of virulence would be useful to further understand the Australian VCG2A.

3.5 Materials and Methods

Verticillium dahliae isolates and molecular characterisation

Eight *V. dahliae* isolates derived from single microsclerotia initially collected from diseased cotton plants in fields with varying disease symptoms throughout NSW were selected from the Australian Cotton Research Institute Pathology Long Term Culture Collection stored in Narrabri. These isolates were also lodged with the NSW DPI Plant Pathology and Mycology Herbarium in Orange and given unique DAR identification numbers (Table 3.1). The isolates were grown on Potato Dextrose Agar (PDA) for two weeks at 23˚C before DNA was extracted using a QIAGEN DNeasy extraction kit (cat no 69106; Venlo, The Netherlands).
Isolates were characterised as either D or ND pathotypes. Pathotypes were identified by 539 bp (D) or 523 bp (ND) bands observed on an electrophoresis gel (Figure 3.1). The PCR was run as described by Mercado-Blanco, et al. (2003). Non-defoliating strains were further distinguished by 824 bp band (Figure 3.2) using the method described by Mercado-Blanco, et al. (2001). Four isolates with VCGs determined in a previous study (Chapman et al. 2016) were included as reference isolates. Presumptive VCGs were determined for the remaining isolates using the variable IGS region which were amplified via PCR to give a 1600 bp segment of DNA, as described by Qin, et al. (2006). The resulting product was cleaned using a QIAquick PCR purification kit (Qiagen), DNA concentration confirmed using the NanoDrop, and sent for sequencing at the Westmead Millennium Institute, NSW Australia. The sequence was then analysed using Geneious version 9.1.5 (https://www.geneious.com, Kearse et al. 2012).

Pathogenicity Assay

Each of the isolates were grown in Potato Dextrose Broth at room temperature constantly shaking at 180 RPM for seven days. Conidial suspensions were subsequently filtered through four layers of sterile cheesecloth and adjusted to a concentration of $1x10^6$ conidia/ml following microscopic counts using a haemocytometer (Trapero et al. 2013). Cotton seeds encompassing four varieties (Table 3.6) were triple washed with sterile milli-Q water, and germinated on blotting paper for 72 hours. Single germinated seeds were planted in root trainers 12 cm x 4 cm containing twice pasteurised Debco native potting mix supplemented with Osmocote (N:P:K ratio of 21.2:1.9:5.7; manufactured by Scotts Australia PTY LTD, NSW, Australia) at the recommended rate of ½ a tablespoon per perennial plant. Seedlings at the two true leaf stage were gently removed from the root trainers and loose soil removed from the roots, and inoculated via root dipping in conidial suspension.

One centimeter of the root was cut from the base of each tap and lateral root before the seedlings were immersed in the conidial suspensions or sterile water for 25 minutes. Seedlings were then transplanted into 175 mm pots containing twice pasteurised Debco native potting mix supplemented with the recommended rate of Osmocote. The seedlings were arranged in a randomised complete block design with the six replicates, each forming one block, spread out over two rooms, and observed for seven weeks. Plants were maintained in a glasshouse at a temperature of 22 ± 2˚C, and watered as needed.

Table 3.6. Cotton varieties used in the glasshouse virulence assay

Cotton Variety	Description	V-rank
Acala SJ-2*	Developed in 1973 to help improve crop resistance to Verticillium wilt	
Sicot 71BRF**	Bollgard II® stacked with Roundup Ready Flex®	107
Sicot 74BRF**	Bollgard II® stacked with Roundup Ready Flex®	101
Sicot 714B3F**	Bollgard® 3 stacked with Roundup Ready Flex®	113

* o V-rank established for Acala SJ-2

** Australian varieties of cotton

Disease assessment

The disease severity of each plant was recorded bi-weekly over seven weeks using a 0 – 4 scale monitoring the external foliar symptoms for a total of 14 assessments (Jiménez-Díaz et al. 2016). A score of 0 indicated no external foliar symptoms, "1" was 1 - 33% of leaves with foliar symptoms, "2" indicated 34 - 66% of leaves affected, "3" was 67 - 99% affected, and "4" indicated 100% disease and plant death. Disease severity over time was quantified for each individual plant over the seven weeks post inoculation using the area under the disease progress stairs (AUDPS). The AUDPS was calculated using the trapezoidal method (Simko and Piepho 2011) using the formula:

$$\text{AUDPS}=\left[y_1\times\left\{\frac{t_2-t_1}{2}+\frac{D}{2(n-1)}\right\}\right]+\left[\sum_{i=2}^{n-1}\left(y_i\times\frac{t_{i+1}-t_{i-1}}{2}\right)\right]+\left[y_n\times\left\{\frac{t_n-t_{n-1}}{2}+\frac{D}{2(n-1)}\right\}\right]$$

where $\{y_1\}$ and $\{y_n\}$ = assessments at the first and last observations

$\{t_1\}$, $\{t_2\}$, $\{t_{n-1}\}$, and $\{t_n\}$ = times of the first, second, penultimate and final observations, respectively

$D = t_n - t_1$

n = the total number of observations

Root measurements

Roots from each plant were cut at ground level and soil removed by rinsing under running water. The clean roots were placed in paper envelopes and stored until analysis was undertaken. Each root sample was individually imaged by placing the roots of a single plant flat in a clear tray, the roots separated, and distilled water added until all the roots were just covered. The roots were scanned and the resulting image was analysed to estimate root length using WinRHIZO Pro V. 2009c (Regent Instruments Canada INC) (Arsenault et al. 1995). This was repeated for each collected root sample.

Statistical analysis

Linear mixed models were fitted to the AUDPS and log10 Root length and a generalized linear mixed model (GLMM) for disease severity with an ordinal response. For days to mortality, a parametric survival regression was fitted. For AUDPS, disease severity and days to mortality, data for the "mock" strain was excluded from analysis as it was all zero. Each model consisted of the fixed effects of VCG, isolate (within VCG), variety and all interactions, and, for the mixed models, random effects of room, replicate and their interactions with VCG, isolate and variety. All mixed models were fitted using ASReml-R (Butler 2018) in the R statistical environment, version 3.6 (R Core Team 2019). The parametric survival regression for days to mortality was fitted using the survreg function in the survival package (Therneau 2015). Pairwise comparisons for disease severity and days to mortality were determined using Wald and likelihood ratio tests respectively (using a dummy variable to create an appropriate reduced model). For disease severity, a mean score was computed (Hannah and Quigley 1996) with a 68% CI (corresponding to ±1SE for a normal distribution) calculated using Monte Carlo simulation with 1000 simulations.

3.6 Conclusion

On Australian cotton farms, both D VCG1A and ND VCG2A isolates are capable of causing disease and significant yield loss. It is not clear why VCG1A is not exhibiting the extensive disease symptoms on Australian farms as reported

overseas. The ND VCG2A isolates on the other hand are causing a spectrum of disease symptoms ranging from mild foliar symptoms to plant death both in the field and in this study. However, ND VCG4B isolates cause less severe disease symptoms. It appears that, at least in Australia, VCGs are not a useful pathogenicity indicator of *V. dahliae* in cotton and an alternate classification system is needed.

3.7 Acknowledgements

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Chapter 4: Identification of a *Verticillium dahliae* subpopulation affecting Australian cotton

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Declaration

I declare that the below manuscript meets the below requirements for inclusion as a chapter in this thesis.

- I have contributed more than 50% for the below manuscript.
- The below publication has been formatted to adhere to the specific formatting requirements of Australasian Plant Pathology Journal.

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Production Note:
Signature removed
prior to publication.

4.1 Abstract

Verticillium dahliae is an agriculturally important phytopathogen. While it infects many crop varieties, it is of notable importance to cotton industries around the world. The impact of the disease on the billion-dollar Australian cotton industry is increasing. Internationally, the defoliating vegetative compatibility group (VCG) VCG1A of *V. dahliae* causes significant damage to cotton plants. However, in diseased Australian cotton, the VCG classification system of *V. dahliae* does not adequately reflect the disease symptoms observed. The non-defoliating VCG2A isolates have caused significant damage, a problem not readily observed overseas, and the defoliating VCG1A isolates have not always caused widespread severe symptoms as expected. This study examined a selection of *V. dahliae* isolates derived from diseased cotton plants in NSW, Australia, America and Israel and classified them into groups using Inter-Simple Sequence Repeats (ISSR). A subset of these isolates were tested in a pathogenicity assay using cotton plants. The combined results confirmed that Australia has VCG2A isolates capable of causing significant disease ("defoliating-like") and that when analysed using ISSR, they are distinct from the non-virulent VCG2A populations. Additionally, there is a group of VCG1A isolates that are able to cause significant damage to cotton plants. The ISSR analysis provides a better understanding of the *V. dahliae* populations circulating within Australia and is a useful tool for diagnostic use, with a potential use for diagnostic confirmation of a causative strain.

4.2 Introduction

The vascular pathogen, *Verticillium dahliae,* is the causal agent of Verticillium wilt and affects numerous plant species including agriculturally important crops such as tomatoes, olives, lettuce, potatoes, and cotton (Bhat and Subbarao 1999; Inderbitzin et al. 2011). Australia is the third largest exporter of cotton in the world and produced around 1.5 million bales in 2005/2006 to 2009/2010 seasons (Cotton Australia 2016). Over the last 10 years, the incidence of Verticillium wilt has increased with large patches of dead and dying plants apparent in some fields (Jensen and Redfern 2017; Kirkby et al. 2016). Despite attempts at breeding varieties resistant to *V. dahliae,* none of the currently available varieties have true

resistance to Verticillium wilt. Rather, these varieties are temperature sensitive and are rendered susceptible when the temperatures drop to 20-22˚C (CSD, 2018). Additionally, a subpopulation of *V. dahliae* that is characterized as only moderately virulent in most of the world has increasingly caused severe damage in Australia. This suggests that virulence may vary more widely in Australian *V. dahliae* populations than has been reported internationally (Dadd-Daigle et al. 2020).

Verticillium dahliae isolates have traditionally been classified into Vegetative compatibility groups. These groups are determined by their ability to form prototrophic heterokaryons – the fusion of two genetically distinct cells (Puhalla and Mayfield 1974). Compatibility is traditionally tested using strains with mutations altering their ability to utilise nitrogen. Two nitrate non-utilizing mutant strains that are of the same VCG are able to form prototrophic heterokaryons and will survive on media containing a nitrogen source only accessible to heterokaryons, while strains that are not compatible will not form heterokaryons (Joaquim and Rowe 1990). This process is controlled by two sets of gene loci, *het* and *vic*, which must be identical between strains for them to form heterokaryons (Glass and Kaneko 2003; Jiménez-Gasco et al. 2013; Leslie 1993). The VCG can also be largely predicted by sequencing the Inter-Genomic Spacer (IGS) region (Papaioannou et al. 2013), though some difficulties remain particularly when differentiating VCG2A and VCG4B.

Verticillium dahliae are also classified into defoliating (D) and non-defoliating (ND) pathotypes. These pathotypes are assigned by whether the leaves of the infected plant defoliate, D pathotype, or not, ND pathotype (Daayf et al. 1995). This senescence of leaves is due to the growth of hyphae and conidia within the plant's xylem, blocking water and nutrient flow (Klimes et al. 2015). Although some plants suffer wilting, necrosis and defoliation, others have only mild disease and are able to recover (Daayf 2015).

As a host adaptive pathogen, isolates from different *V. dahliae* VCGs may have varying pathogenicity on different host species. For example, while most VCGs can cause some disease in many plant species, VCG1A is known to severely impact

cotton and olives (López-Escudero and Mercado-Blanco 2011), VCG2A causes the most disease in tomatoes and VCG4B is virulent in potatoes. However, in Israel it has been reported that cotton is most impacted by VCG2B (Korolev et al. 2001), and Australian cotton appears to be impacted more by VCG2A (Dadd-Daigle et al. 2020). It was not until 2016 that VCG1A was reported in Australian cotton (Chapman et al. 2016). Contrary to observations from other countries, the Australian VCG1A *V. dahliae* does not consistently cause defoliation (Chapman et al. 2016). Additionally, the ND VCG2A has been reported to cause complete defoliation on cotton farms throughout New South Wales (NSW) despite its classification as a "non-defoliating" pathotype (Jensen and Redfern, 2017). A better understanding of the difference between *V. dahliae* isolates within and between VCGs is needed.

Molecular tools are often used in understanding pathogen populations. One such tool, Inter-Simple Sequence Repeats (ISSR) typing, is a form of genetic fingerprinting. ISSRs are sections of DNA flanked by microsatellites, repetitive regions of DNA. By designing primers that target these flanking microsatellites, the ISSRs are amplified by the polymerase chain reaction (PCR). The resolution of resulting amplicon sizes by agarose gel electrophoresis forms a fingerprint that can be used to compare isolates and assess their relatedness (Wang et al. 2005). ISSRs have been used to investigate the link between *V. dahliae* virulence and VCG in cotton. ElSharawy et al. (2015) found that the isolates separated according to virulence when analysed by ISSR typing.

Currently, Australian cotton growers and consultants request confirmation of the *V. dahliae* pathotype and VCG affecting their fields. However, as neither the pathotype nor VCG classification describes isolate virulence, a different method of classifying Australian cotton isolates is needed. This would assist growers in understanding the range of symptoms observed in their fields, tracking disease over time and monitoring the exposure of their fields to the different *V. dahliae* populations. As the only current method for determining virulence is through *in vitro* studies or relying on field observations, neither which is rapid enough for diagnostic purposes, an alternate method of characterising isolates by virulence is

needed. Therefore, this study aimed to determine whether ISSR typing is able to characterise Australian *V. dahliae* isolates by virulence rather than VCG.

4.3 Materials and methods

Verticillium dahliae cultures and molecular characterisation

The 84 Australian isolates were initially derived from single microsclerotia collected from diseased cotton plants on commercial cotton fields throughout NSW, Australia. Isolates were stored at the Australian Cotton Research Institute at Narrabri, in the Long-Term Culture Collection, and lodged with the NSW DPI Plant Pathology and Mycology Herbarium in Orange where the isolates were designated a unique identification number (DAR number). Three American isolates provided for this study by Dr Terry Wheeler from Texas A&M University were collected from diseased cotton plants on farms in America and the DNA transported to Australia for examination. Isolates were grown on Potato Dextrose Agar (PDA) at 23˚C for two to four weeks before DNA was extracted using the QIAGEN DNeasy extraction kit (cat no 69106) according to the manufacturer's instructions. DNA from six *V. dahliae* isolates taken from various agricultural crops in Israel was provided by Leah Tsror (Lahkim), from the Gilat Research Center.

Each isolate's pathotype was determined by PCR, as described by Mercado-Blanco et al. (2003), where a 539 bp band indicated a Defoliating (D) pathotype and a 523 bp band indicated Non-defoliating (ND). The method described by Mercado-Blanco et al. (2001) was used to further identify ND strains by the presence of 824 bp band. Presumptive VCGs were determined using the method described by Qin et al. (2006) in which the variable IGS regions are amplified via PCR to give a 1,600 bp segment of DNA. The QIAquick PCR purification kit (Qiagen) was used to clean the resulting product and was then sent to the Westmead Millennium Institute for sequencing. The resulting DNA sequence was analysed by alignment with sequences typed using Geneious version 9.1.5 (https://www.geneious.com, Kearse et al. 2012) (Chapman et al. 2016).

Thirteen of the 84 *V. dahliae* isolates were used in the virulence assay (Table 4.1). These were chosen to include isolates that had their VCG previously determined using *nit* mutants (Chapman et al. 2016); isolates with virulence determined in previous virulence assays (Dadd-Daigle et al. 2020); and isolates of unknown virulence.

Virulence testing in cotton plants

Cotton seeds supplied by Cotton Seed Distributors for varieties Acala SJ-2 and Sicot 714B3F were triple washed with sterile Milli-Q water and germinated on blotting paper for 72 hours. Germinated seeds were transferred to root trainers (12 cm x 4 cm) filled to the top with twice pasteurised Debco native potting mix. Seedlings that reached the two true leaf stage were removed from the root trainers and loose soil gently removed from the roots.

To challenge the cotton plants, *V. dahliae* isolates were grown at room temperature in Potato Dextrose Broth, constantly shaking at 180 RPM, for seven days. Four layers of sterile cheesecloth were used to filter the conidial suspensions. Final conidial suspension concentrations were adjusted to $1x10^6$ conidia/mL using a haemocytometer (Trapero et al. 2013). Seedlings were then inoculated with the appropriate conidial suspension *via* root dipping. Specifically, one centimetre of root was cut from the base of each tap and lateral root before the seedlings were immersed in the conidial suspensions or sterile water (controls) for 25 minutes. Seedlings were then transplanted into 175 mm pots containing twice pasteurised Debco native potting mix supplemented with the recommended rate of Osmocote (N:P:K ratio of 21.2:1.9:5.7; manufactured by Scotts Australia PTY LTD, NSW, Australia). The plants were arranged in a randomised complete block design with one plant per pot as a single replicate, totalling 6 replicates per variety and isolate combination, and observed for 7 weeks. Plants were maintained in a glasshouse at a temperature of 22 ± 2˚C and watered as needed. This assay was repeated.

Assessment and analysis of disease in cotton plants

During the 7 week observation period, plants were assigned a twice-weekly disease score to quantify external disease symptoms. The scores ranged from 0 – 4, where a score of 0 indicated no external foliar symptoms, "1" was 1 - 33% of leaves with foliar symptoms, "2" was 34 - 66% of leaves affected, "3" was 67 - 99% affected, and "4" was plant death (Jiménez-Díaz et al. 2016). The scores were then used to determine disease severity over time using the area under the disease progress stairs (AUDPS). The AUDPS was calculated using the trapezoidal method (Simko and Piepho 2011) using the formula:

$$\text{AUDPS}=\left[y_1\times\left\{\frac{t_2-t_1}{2}+\frac{D}{2(n-1)}\right\}\right]+\left[\sum_{i=2}^{n-1}\left(y_i\times\frac{t_{i+1}-t_{i-1}}{2}\right)\right]+\left[y_n\times\left\{\frac{t_n-t_{n-1}}{2}+\frac{D}{2(n-1)}\right\}\right]$$

where $\{y_1\}$ and $\{y_n\}$ = assessments at the first and last observations

$\{t_1\}$, $\{t_2\}$, $\{t_{n-1}\}$, and $\{t_n\}$ = times of the first, second, penultimate and final observations, respectively

$D = t_n - t_1$

n = the total number of observations

Measurement of Root Length

At the end of the observation period, roots were collected by severing the plant at the soil line and rinsing under running tap water to remove loose soil. Each root was placed in a clear tray, covered with distilled water, roots separated with tweezers and scanned. Analysis was performed on the scanned image to estimate root length using the WinRHIZO Pro V. 2009c software (Regent Instruments Canada INC) (Arsenault et al. 1995).

Statistical Methods

The AUDPS (removing the mock strain) and root length values were modelled using a linear mixed model with fixed effects of variety, VCG and isolate (within VCG) and their interactions. Random effects consisted of run and replicate within run and all their interactions with variety, VCG, and isolate (within VCG) and variety by VCG and isolate (within VCG) (run only). Disease severity was fitted as

an ordinal response using a generalized linear mixed model, with the same fixed and random terms. Mean scores for disease severity were estimated from the predicted score probabilities (Hannah and Quigley 1996), with an estimated 68% confidence interval determined by Monte Carlo simulation with 1000 simulations. All mixed models were fitted with the ASReml-R software (Butler 2019) in the R statistical software environment (R Core Team 2019).

Inter-Simple Sequence Repeats PCR assay

The ISSRs were determined using the PCR method described by ElSharawy et al. (2015). Thirteen primers (Supplementary Table S4.1) were used to analyse 84 *V. dahliae* isolates chosen at random from the NSW Verticillium culture collection, three American isolates, and six *V. dahliae* isolates obtained from a variety of host plants in Israel were supplied by Leah Tsror from the Gilat Research Center (Supplementary Table S4.2). The isolates were assigned a VCG or, in cases where the VCG was unable to be determined, D or ND pathotypes using the methods described previously. The bands resulting from the PCR assays, present between the 500 bp and 3000 bp mark, were compared and given a score of 0 if no band was present or 1 if a band was present. The binary scores were then analysed using hierarchical clustering (UPGMA method) on the Jaccard distances with R (R Team, 2019, using the hclust function) to produce a dendrogram.

4.4 Results

Characterisation of Australian V. dahliae isolates

The PCR and sequencing analyses were used to presumptively assign *V. dahliae* isolates to D VCG1A, ND VCG2A, and ND VCG4B (Table 4.1). Isolates SS61, SS363, SS429, SS434, and SS60 were assigned the D pathotype, while all other isolates used in the glasshouse assay aligned with the ND pathotype. The D isolates SS61, SS363, SS429, SS434, and SS60 were VCG1A; SS362, SS425, SS285, SS262, SS256, SS448, and SS414 were VCG2A; and isolate SS284 was assigned to VCG4B.

Table 4.1. *Verticillium dahliae* isolates used in the glasshouse virulence assay

Herbarium number	Isolate number	Presumptive VCG	PCR band results	Pathotype (D/ND)	Season collected	Valley of origin
DAR82592	SS61	1A*	539 bp[1]	D	2010/2011	Namoi
DAR82598	SS363	1A*	539 bp[1]	D	2013/2014	Namoi
DAR83138	SS429	1A	539 bp[1]	D	2014/2015	Namoi
DAR83143	SS434	1A	539 bp[1]	D	2014/2015	Macintyre
DAR83175	SS60	1A	539 bp[1]	D	2010/2011	Macintyre
DAR82597	SS362	2A*	523 bp[1]; 834 bp[2]	ND	2013/2014	Namoi
DAR83206	SS425	2A	523 bp[1]; 834 bp[2]	ND	2015/2016	Gwydir
DAR83151	SS285	2A	523 bp[1]; 834 bp[2]	ND	1983/1984	Namoi
DAR83107	SS262	2A	523 bp[1]; 834 bp[2]	ND	2011/2012	Macquarie
DAR82954	SS256	2A	523 bp[1]; 834 bp[2]	ND	2011/2012	Namoi
DAR83149	SS448	2A	523 bp[1]; 834 bp[2]	ND	2014/2015	Namoi

DAR83126	SS414	2A	523 bp[1]; 834 bp[2]	ND	2014/2015	Namoi
DAR83109	SS284	4B	523 bp[1]; 834 bp[2]	ND	1995/1996	Namoi

*VCG determined previously using *nit* mutants (Chapman et al. 2016)

[1] Mercado-Blanco et al. (2003)

[2] Mercado-Blanco et al. (2001)

Pathogenicity of Australian V. dahliae isolates in cotton plants

When examining disease score (Figure 4.1), AUDPS (Figure 4.2), and root length (Figure 4.3), the pathogenicity assay revealed significant differences between isolates within VCGs and amongst different VCGs ($P < 0.001$), but no significant interaction with variety ($P > 0.001$) (Table 2). In regard to disease score (Figure 4.1), the VCG1A isolates (SS434, SS61, SS60, SS429 and SS363) significantly impacted the cotton plants compared to the mock infected plants (Figure 4.4). There was also a small increase in severity on the Acala SJ-2 variety compared to the infected Sicot714B3F plants when infected with VCG1A isolates. Isolates from VCG2A (SS285, SS262, and SS425) and VCG 4B (SS284) all caused minimal to no significant disease in either cotton variety. Both varieties suffered severe damage when infected with the VCG2A isolates SS256, SS448, SS362 and SS414. The root length results revealed that the isolates with higher disease scores also had significantly shorter roots regardless of cotton variety.

The AUDPS scores revealed that plants infected with all VCG1A and VCG2A isolates SS256, SS448, SS362 and SS414 died significantly faster than the remaining isolates. There was significant difference observed between cotton varieties, with Acala SJ-2 plants having higher scores than Sicot 714B3F.

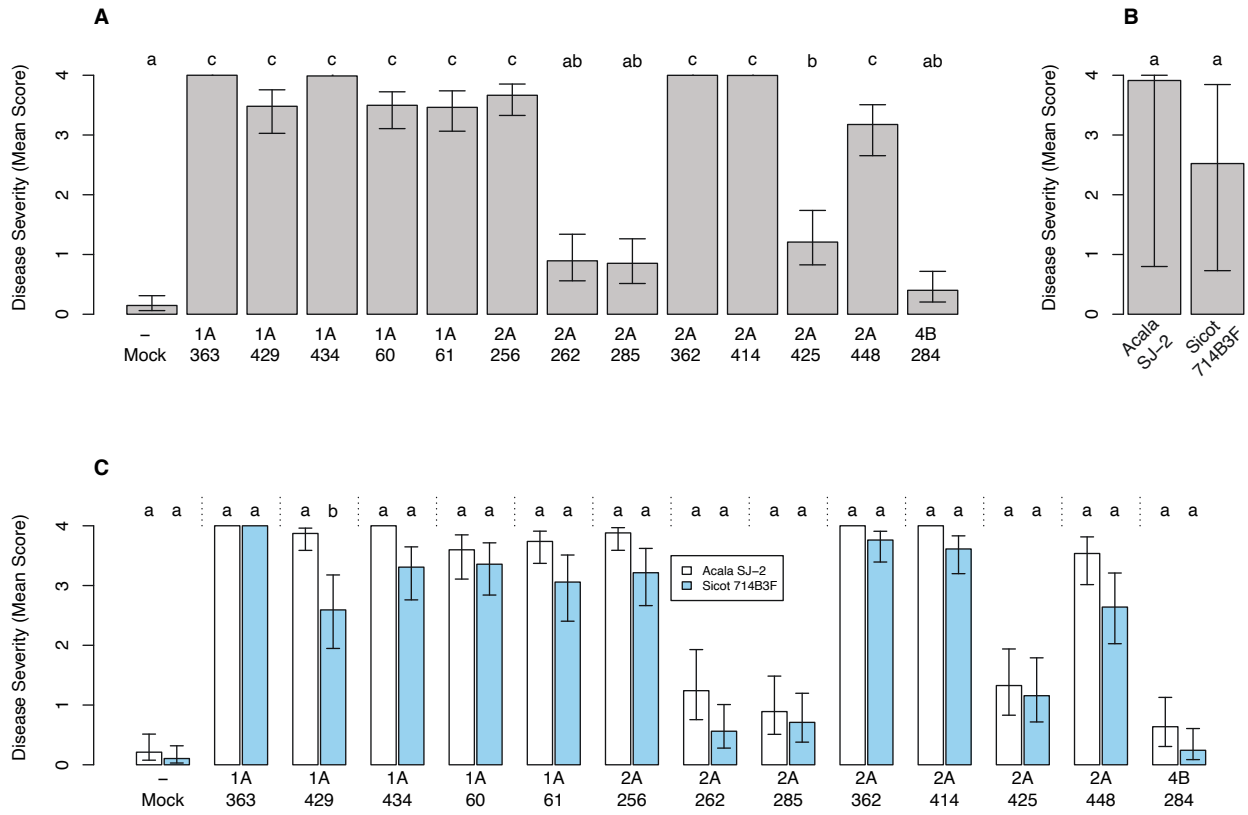

Figure 4.1. Disease severity scores. A) Mean disease severity for each isolate. B) Mean disease severity for each cotton variety. C) Mean disease severity for each isolate by variety.

Error bars indicate ±1 standard error.

Different lower-case letters indicate significant difference between bars.

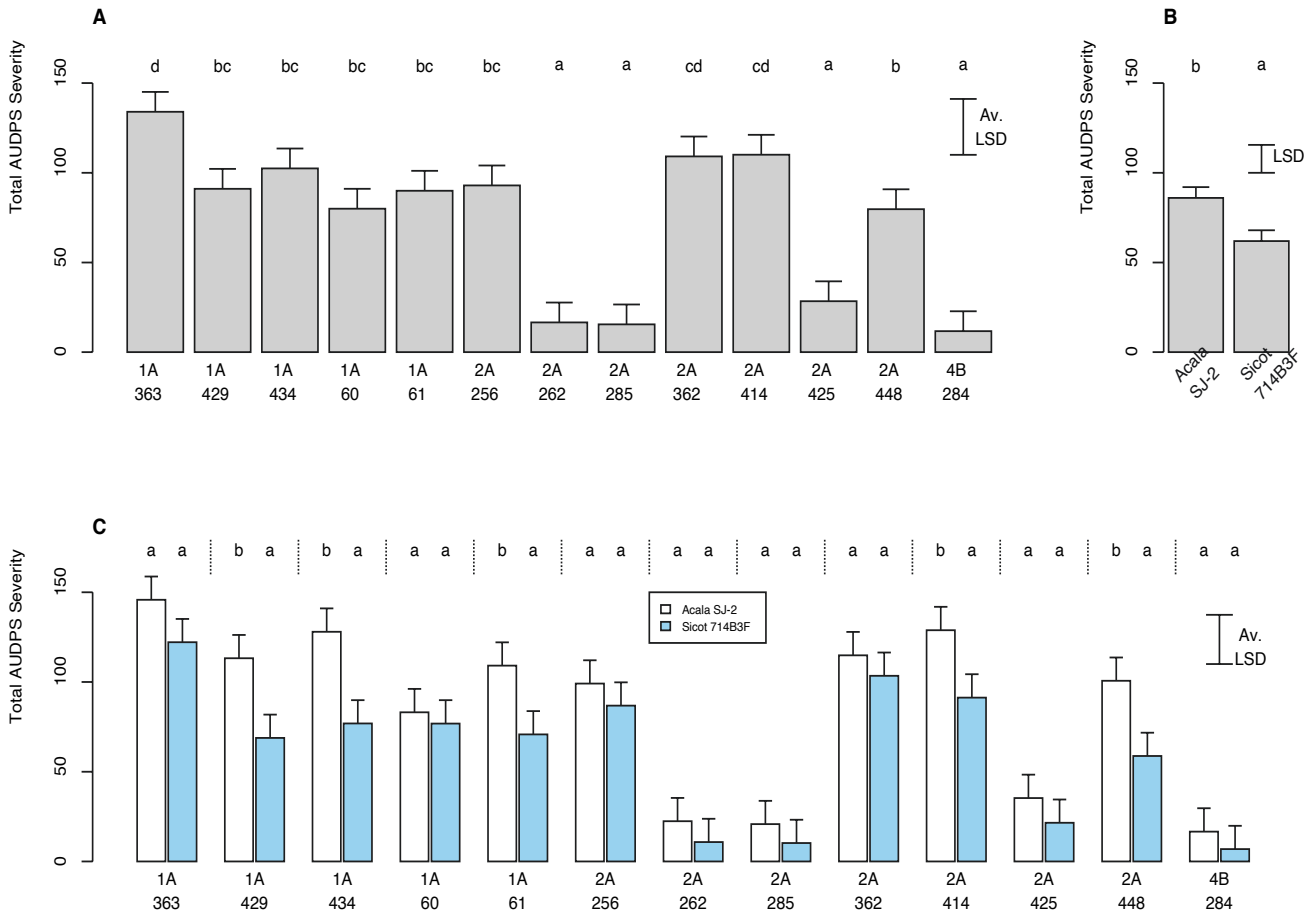

Figure 4.2. Total AUDPS severity. A) AUDPS means for each isolate. B) AUDPS means for each cotton variety. C) AUDPS means for each isolate by variety. Av LSD indicates average least significant difference.
Different lower-case letters indicate significant difference between bars.

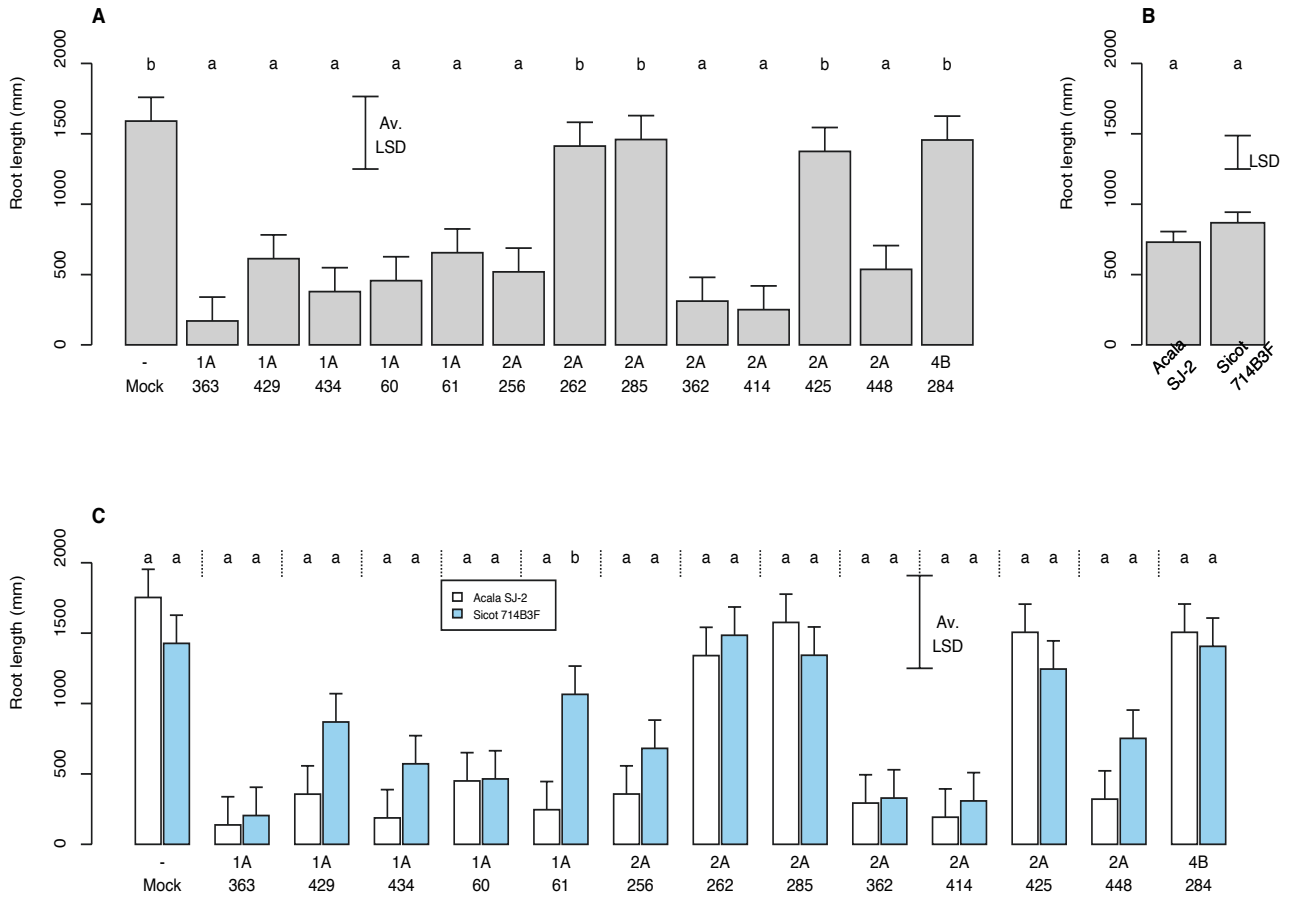

Figure 4.3. Average root length. A) Average root lengths for each isolate. B) Average root lengths for each cotton variety. C) Average root lengths for each isolate by variety.

Av LSD indicates average least significant difference.

Different lower-case letters indicate significant difference between bars.

Table 4.2. Wald F-test statistics for fixed terms in the models

Disease severity				
	DF	Den DF	F-stat	P-val
Variety	1	4.70	6.560	0.054
VCG	3	10.00	12.600	<0.001
VCG/Isolate	10	12.20	4.490	0.008
Variety × VCG	3	8.29	0.125	0.943
Variety × VCG/Isolate	10	14.30	0.455	0.893
AUDPS				
	DF	Den DF	F-stat	P-val
Variety	1	2.86	25.70	0.017
VCG	2	3.26	19.90	0.015
VCG/Isolate	10	10.30	19.10	<0.001
Variety × VCG	2	3.30	1.46	0.352
Variety × VCG/Isolate	10	10.50	1.44	0.284
Root length				
	DF	Den DF	F-stat	P-val
Variety	1	3.13	3.17	0.169
VCG	3	6.82	20.40	<0.001
VCG/Isolate	10	9.38	7.54	0.002
Variety × VCG	3	3.23	3.16	0.175
Variety × VCG/Isolate	10	6.38	1.93	0.208

Figure 4.4. Cotton variety Acala SJ-2 seven weeks after infection with various V. dahliae isolates. A) Infected with VCG1A isolates SS434, SS61, SS60 and sterile water from left to right. B) Infected with VCG4B isolate SS284 and sterile water from left to right. C) Infected with VCG2A isolates SS285, SS262, SS256, SS362, SS414, SS448, and sterile water from left to right.

Relatedness of Australian and international isolates using Inter-Simple Sequence Repeat Analysis

Inter-Simple Sequence Repeat analysis was performed on 92 isolates (Supplementary Table S4.3) and resulted in varying banding patterns for the 13 primers used. The number of amplicon bands varied with the primer options tested, with 815 having the least (4) and 834 having the highest number (18) of bands. In the dendrogram (Figure 4.5), the Australian isolates formed a distinct group while the American and Israeli isolates clustered more closely with each

other, though still separated by VCG classifications and geographic origin. In regard to the Australian isolates, although the VCG1A isolates separated into their own cluster, the VCG2A and VCG4B did not, aside from one group of seemingly highly related VCG2As. When examined in conjunction with the collected virulence data, it appears that there are three distinct groups that are varied in their ability to cause disease. These groups have been labelled “defoliating”, “non-defoliating”, and “defoliating-like”.

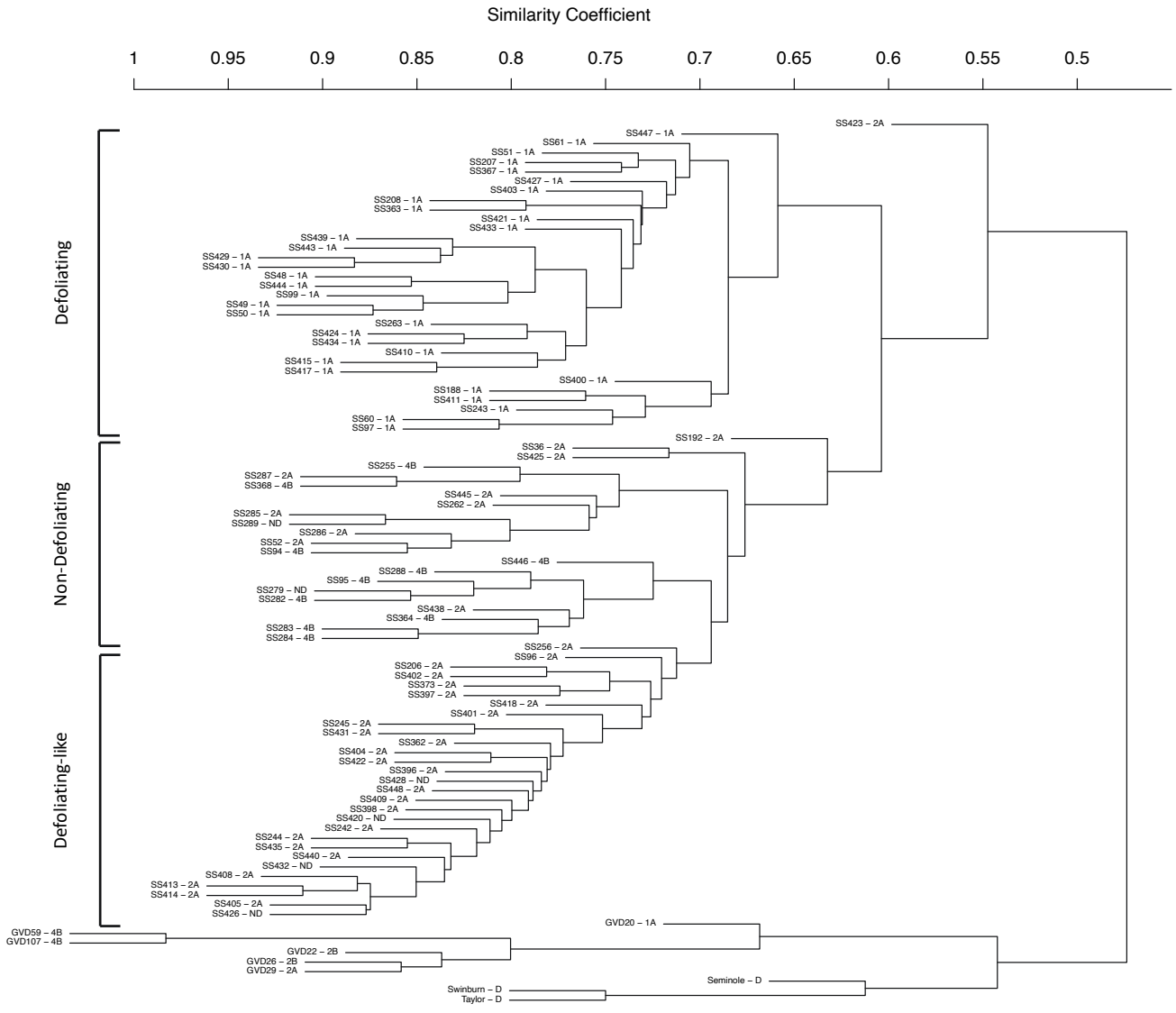

Figure 4.5. Inter-simple sequence repeats analysis reveal that Australian isolates appear to cluster into virulent “defoliating” and “defoliating-like” groups composed of exclusively VCG1A and VCG2A respectively, and a non-virulent “non-defoliating” group comprised of both VCG2A and VCG4B. American and Israeli isolates clustered together, distinct from the Australian isolates.

4.5 Discussion

Verticillium wilt is an ongoing issue for the Australian cotton industry (Kirkby et al. 2013). While VCG1A is devastating to cotton industries internationally, Australian cotton is seemingly less affected (Chapman et al. 2016). Instead it appears that the ND VCG2A is causing more severe damage to the extent of complete defoliation (Dadd-Daigle et al. 2020; Jensen and Redfern 2017).

In accordance with previous results, both VCG1A and VCG2A isolates were able to cause plant mortality (Dadd-Daigle et al. 2020). However, unlike the previous study, there appeared to be some disparity in the severity of VCG1A isolates between the two cotton varieties. Sicot 714B3F plants were not as significantly impacted by the isolates as the Acala SJ-2 counterparts, although there were no significant differences for the VCG2A or the VCG4B isolates. The disease over time scores were lower for all Sicot 714B3F, when compared to the same isolate infected in an Acala SJ-2 cotton variety, confirming that this variety is better at withstanding *Verticillium* infection. As the Sicot 714B3F is a relatively new variety with a high V-rank, it is expected that fewer plants would be impacted compared to Acala SJ-2, an old variety that is no longer grown. These results suggest that while the Sicot 714B3F variety is more likely to resist initial infection by *V. dahliae*, once the infection process has started the plant is likely to succumb to the disease.

Over the years multiple molecular methods have been used to describe *V. dahliae* populations. One of these methods, Inter-Simple Sequence Repeats, has previously been used to analyse the relationship between phylogeny and virulence among cotton isolates (ElSharawy et al. 2015). This study utilised the same method as ElSharawy et al. (2015) and found three distinct groups which varied in their ability to cause disease, the “defoliating”, “non-defoliating”, and “defoliating-like” groups. The term “defoliating-like” chosen due to field reports of infected plants defoliating despite having a ND pathotype. The ISSR also suggested that the Australian *V. dahliae* populations are unique, as neither the three American isolates nor the six Israeli isolates were closely related to the Australian *V. dahliae* and formed a separate cluster. While the VCG1A isolate taken from a cotton plant in Israel was more similar to the American cotton isolates than the other Israeli

isolates, they still tended to group by VCG and geographic origin. Although this could be due to several reasons such as geographical isolation, limited trade in cotton germplasm or Australia's tight biosecurity regulations, it is unclear why the international isolates clustered separately. It would be worthwhile to apply the ISSR methodology to a larger number of isolates from both these regions to see whether similar patterns of virulent isolates appear.

The "defoliating-like" VCG2A isolates appear to be unique to Australia. Although countries such as Israel have reported VCGs other than VCG1A causing disease in cotton (Korolev et al. 2001), VCG2A is causing significant damage in Australia (Dadd-Daigle et al. 2020). The ISSR results suggest that the defoliating-like VCG2A are closely related to each other and are more similar to the ND VCG2A than the D VCG1A. Studies into the genetics of *V. dahliae* have labelled this asexual phytopathogen as having a "plastic genome" (Amyotte et al. 2012; Faino et al. 2016). It has been suggested that adaption happens via recombination through transposons, horizontal gene transfer (Chen et al. 2017), and alternative splicing (Jin et al. 2017) of peptides leading to differential expression of genes at post translation levels. Additionally, Milgroom et al. (2014) have suggested that *V. dahliae* isolates of the same and different VCGs may have recombined into a new variant of VCG2B which differ in terms of pathogenicity and virulence from other VCG2B isolates. However, the specifics of how the Australian *V. dahliae* populations have evolved has not been examined.

There is a clear need for a better understanding of how the defoliating-like VCG2A differ from both the ND VCG2A and D VCG1A, and why the Australian D VCG1A is not causing damage of the same extent to cotton plants observed internationally. Australian conditions could be playing a role in the differential expressions of genes; however, genomes of Australian isolates could also hold meaningful insights into these differences observed. Additionally, as the ISSR method was able to differentiate between virulent isolates within Australian *V. dahliae* isolates of the same VCG it unravels options for the development of rapid molecular diagnostic tools. The next step in understanding and analysing the Australian *V. dahliae* populations clearly requires exploration into the genome sequences of these

pathogens. Along with whole genome sequencing, tools such as genotyping by sequencing could assist in understanding the relatedness between isolates. A greater genomic understanding will also assist in the tracing of exotic isolates within Australia.

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Chapter 5: Australian *Verticillium dahliae* Vegetative Compatibility Group 1A isolates lack known genes for virulence in cotton

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Declaration

I declare that the below manuscript meets the below requirements for inclusion as a chapter in this thesis.

• I have contributed more than 50% for the below manuscript.

• The below publication has been formatted to adhere to the specific formatting requirements of APS Phytopathology Journal.

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5.1 Introduction

Verticillium dahliae is a soil-borne phytopathogen that infects numerous crops globally causing the disease Verticillium wilt. To better understand the variation within *V. dahliae* populations, traditionally, *V. dahliae* was classified into 'vegetative compatibility groups' (VCG). These are based on fungus interactions *in situ* with other isolates of the same or different VCG (Joaquim and Rowe 1990; Puhalla and Mayfield 1974). Molecular tests are now also used to provide a "presumptive" VCG, however discrepancies still rely on the traditional method for verification (Chapman et al. 2016). Different VCGs tend to be more virulent in different plant hosts. For example, VCG2A is known to cause disease in tomatoes (Tsror et al. 2001), VCG4A is associated with potatoes (El-Bebany et al. 2013), and VCG1A is virulent in cotton (Jiménez-Díaz et al. 2006).

Verticillium dahliae invades plant tissues through the roots, where the hyphae proliferate in the xylem, resulting in wilting or necrosis of the plant tissue (Klimes et al. 2015). Proteins such as VTA3 and SOM1 have been identified as playing a role in root penetration (Bui et al. 2019), while VDTH14, VdRac1 and VdCla4 have been shown to be important for growth of the fungus once inside the host plant (Hoppenau et al. 2014; Tian et al. 2015). Others, including NLP1 and NLP2 play a role in tissue necrosis (Santhanam et al. 2013). While all these factors contribute to *V. dahliae* virulence, there is evidence of lineage specific (LS) regions of DNA which contain genes important for virulence in specific hosts (de Jonge et al. 2013; Klosterman et al. 2011). The LS region identified in the Chinese *V. dahliae* strain Vd991, isolated from cotton, has been shown to confer greater virulence when introduced into isolates not normally virulent in cotton (Chen et al. 2017). Additionally, the pathogenic ability of Vd991 was reduced when these LS genes were knocked out, with infected plants more closely resembling mock infected plants.

The Australian billion-dollar cotton industry has been managing *V. dahliae* induced Verticillium wilt for many years. In 2016, a re-examination of historical *V. dahliae* isolates found that *V. dahliae* VCG1A had been present in Australian cotton since at least 2011 (Chapman et al. 2016). However, these Australian VCG1A isolates did

not appear to cause the disease to the same extent as had been reported overseas, with generally mild to no defoliation observed, and hence had remained unnoticed (Chapman et al. 2016). When Australian *V. dahliae* VCG1A isolates, taken from cotton plants, were tested in glasshouse trials they rapidly induced plant death regardless of cotton variety (Dadd-Daigle et al. 2020). It is currently unclear why Australian VCG1A isolates do not appear to cause severe plant disease in the field. It is also unclear if VCG1A isolates originated from a single incursion or from multiple incursions events into Australia from international isolates. Previous studies that examined Australian VCG1A isolates phylogenetically found they formed several clusters within a larger VCG1A group (Chapter 4). This study utilised whole genome sequencing to obtain the genome sequences of four Australian *V. dahliae* VCG1A isolates taken from cotton that were identified as being from separate clusters within the VCG1A group. These four isolates were compared against each other to locate regions of difference and identify whether the isolates were genetic variants which could signal multiple incursion events. The isolates were also compared with sequences of publicly available *V. dahliae* genomes to investigate and identify regions of interest that could contribute to the differing virulence in the field.

5.2 Materials and methods

Australian Verticillium dahliae isolates

The four Australian *V. dahliae* isolates used in this study (Table 5.1) were initially grown from single microsclerotia taken from infected cotton plants on commercial cotton fields in NSW, Australia. The isolates were stored in the Long-Term Culture Collection at the Australian Cotton Research Institute in Narrabri, and also lodged with the NSW DPI Plant Pathology and Mycology Herbarium in Orange. The Herbarium assigned all isolates a unique identification number (DAR number).

Table 5.1. Australian *Verticillium dahliae* isolates genome sequenced in this study

Herbarium number	Isolate number	Presumptive VCG	Season collected	Valley of origin
DAR82592	SS61	1A	2010/2011	Namoi
DAR83138	SS429	1A	2014/2015	Namoi
DAR83143	SS434	1A	2014/2015	Macintyre
DAR83175	SS60	1A	2010/2011	Macintyre

DNA extraction of V. dahliae isolates

The method used to extract high-quality DNA from *V. dahliae* was adapted from two separate protocols (Kaur et al. 2017; Schwessinger and McDonald 2017). The four isolates were grown on potato dextrose agar for two weeks in the dark at 23 ˚C. The plates were subsequently flooded with sterile water and gently rubbed with a plastic spreader ("hockey stick") to dislodge conidia. The conidia were filtered through four layers of cheese cloth, collected in a 50 ml falcon tube and then centrifuged at 4 °C at 3620 x g for 15 min. After removing the supernatant, re-pelleting, and then resuspending in sterile water, the conidia were divided into 4 microtubes for each isolate to obtain 50 mg of conidia dry weight per tube. The conidia were frozen at -80 ˚C and lyophilized.

Sterile acid-washed sand was added to each tube containing lyophilized conidia and the conidia were ground using a micro-pestle. Liquid nitrogen was used to keep the tube cool. The ground sand and conidia were added to a new tube containing 1 ml of fresh extraction buffer (Supplementary table S5.1) with RNase A per 50 mg of conidia. The suspensions were mixed gently at 200 rpm for two minutes and then incubated at room temperature for 30 minutes, mixing by inverting every 5 minutes. Proteinase K was added as per the manufacturer's guidelines, mixed gently at 200 rpm for 20 minutes and then incubated on ice for 5 minutes. A 0.2 volume of 5 M Potassium acetate was added, the suspension was mixed by inversion before being incubated on ice for a further 5 minutes. The

supernatant was transferred to a new tube, the DNA pelleted and washed in ethanol as described by (Kaur et al. 2017).

Nanopore Minion and Illumina sequencing

The extracted DNA was then prepared for either Nanopore Minion Sequencing or Illumina sequencing. Nanopore Minion sequencing was conducted at the Garvan Institute of Medical Research. Samples were sequenced on the GridION Release 18.12.4 using minknow-core-gridion 3.1.20. The raw sequencing files were basecalled during sequencing with the guppy basecaller (ont-guppy-for-minknow v2.0.10).

The Illumina sequencing was performed at the UTS NextGeneration Sequencing facility with an IlluminaMiSeq® sequencer. All samples were sequenced with MiSeq V3 –2x300bp, and isolates SS429, SS60 and SS61 were sequenced again using MiSeq V2 –2x250 bp to improve coverage.

Assembly, sequence processing and annotation

The raw reads from Oxford nanopore sequencing were first run through porechop to remove adaptor sequences (v0.2.4, https://github.com/rrwick/Porechop). The long-read data was assembled using CANU v1.8 (Koren et al. 2017). The finished CANU assemblies were run through ntEdits (v1.2.3 https://github.com/bcgsc/ntEdit) to polish the sequences using the raw reads from the Illumina sequencing platform. Parameters for ntEdits were set for over 30 times coverage and kmer sizes used were 200, 180, 180 and 120 for SS434, SS60, SS429 and SS61, respectively. Reads produced by MiSeq V2 and V3 were merged into a single Fastq file for each isolate, and read quality was assessed with FastQC (v0.11.8 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Genomes were annotated with the Funannotate software v1.4.0 (https://github.com/nextgenusfs/funannotate). The software was run using the default settings and *V. longsporium* annotation data was used as the busco-seed-species input, and gene families corresponding to ORFs were identified using Pfam,

interpro and eggnog databases. The PANTHER classification system was then used to classify putative open reading frames into functional groups using the default settings. (Mi et al. 2018; Mi et al. 2019).

Phylogenetic and comparative genomic analysis

Phylogenetic relationships between the four Australian isolates, SS434, SS60, SS429 and SS61, and 13 *V. dahliae* genomes, publicly available on NCBI, were analysed using the Phylosift software package (Darling et al. 2014). The Phylosift package uses 40 marker genes to draw phylogenetic inferences, of which at least 33 have full-length eukaryotic homologs. The resulting phylogenetic tree was visualised and annotated using iTOL (Letunic and Bork 2019). A general overview of the gene families contained in the five Australian VCG1A and the two Chinese VCG1A isolates was produced using the online PANTHER classification system gene lists tool (Mi et al. 2018; Mi et al. 2019).

The MAUVE move contigs tool was used to tile the assembled scaffolds against the finished *V. dahliae* Vdls.17 reference genome (Genbank Accession number gb|ABJE00000000.1). Re-ordered scaffolds with the highest weight score were aligned using the ProgressiveMauve alignment tool. Regions of low sequence identity were noted for each genome, and the gene content of these regions were identified from the annotation files.

Virulence gene screening

The sequences of 29 candidate virulence genes from previously published studies were collected to form an inhouse database of *V. dahliae* virulence genes. NCBI BLASTn search was used to screen for the presence of genes in the four *V. dahliae* VCG1A genomes sequenced in this study (SS434, SS60, SS429, SS61) and three *V. dahliae* VCG1A genomes of isolates taken from cotton and shared online (Gwydir, Vd991 and CQ2).

5.3 Results

Genome assembly of the four Australian isolates

The four assembled genomes varied in number of contigs from 17 (SS429) to 42 (SS60) (Table 5.2). The total coverage for all isolates was acceptable with the lowest coverage of 81 for SS60 and 203 for SS61. Isolate SS60 had the lowest n50 and n90 values at 2095 kb and 602 kb, respectively, while SS429 had the highest n50 and n90 values at 3519 kb and 3221 kb, respectively. Predicted genome sizes were all similar and varied from 35.6 mb (SS61 and SS434) to 35.9 mb (SS429). The GC content was 51 for all isolates except SS434, which had a GC content of 52. These assembly statistics are comparable to *V. dahliae* genomes sequenced previously. Previous studies that utilised Minion sequencing and CANU assembly have achieved around 50-100x coverage, with between 18 – 69 contigs and predicted a ~35 mb genome size (Chavarro-Carrero et al. 2020).

Table 5.2. Genome statistics for the four assembled genomes.

Genome	SS60	SS61	SS434	SS429
Contigs	42	33	23	17
n50 (kb)	2095	3253	3201	3519
n90 (kb)	602	798	1201	3221
Predicted size (mb)	35.6	35.8	35.6	35.9
GC content (%)	51	51	52	51
Times coverage	81	203	147	173

PanGenome analysis for six VCG1A isolates

The five Australian *V. dahliae* VCG1A genomes, SS434, SS60, SS429, SS61 and Gwydir, and one Chinese *V. dahliae* VCG1A genome, Vd991, were run through the PANTHER classification system gene lists tool to produce pie charts for each genome (Figure 5.1). The tool was able to assign a molecular function for 68.5%, 66.6%, 66.6%, 67.5%, 66.9% and 66.7% of the predicted ORFs for SS61, SS60, SS434, SS429, Gwydir and Vd991, respectively, with the remaining genes denoted as “hypothetical”. The distribution of gene function varied very minimally between the six isolates. The largest portion of assigned genes were catalytic genes with

SS61, SS60, SS434, SS429, Gwydir and Vd991 containing 28%, 31.3%, 31.2%, 31.5%, 31.5% and 31.2%, respectively.

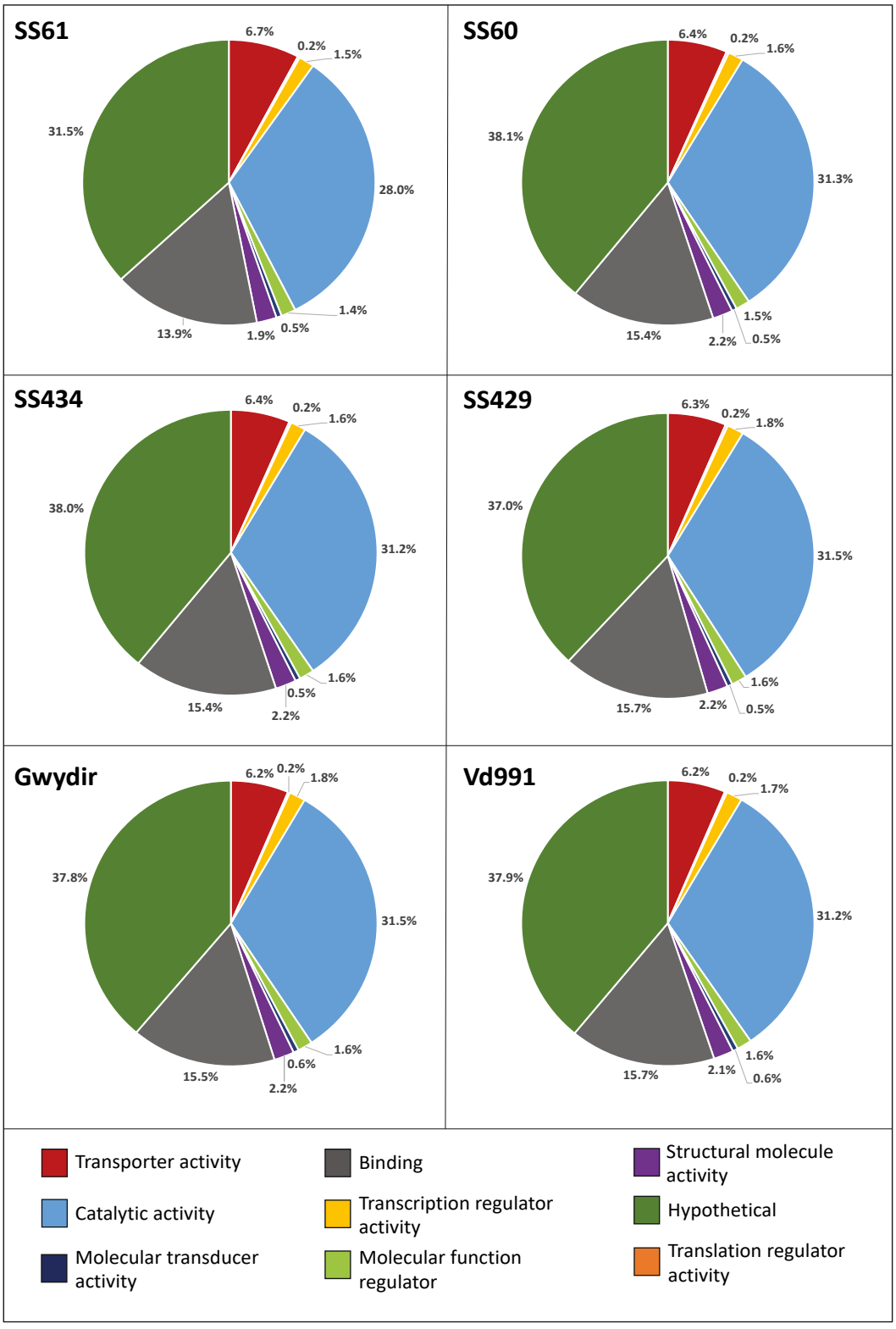

Figure 5.1. Gene content analysis of five Australian *V. dahliae* isolates and one Chinese isolate. Categories were assigned by putative molecular function according to Gene Ontology (GO) classifications.

The Funannotate annotation was able to identify a number of putative genes in all four Australian isolates. It identified a total of 9097, 8644, 9268 and 9090 genes in SS60, SS61, SS434 and SS429, respectively. These results are similar to Kasson et al. (2019) who used Funannotate to examine *V. nonalfalfae* and found a total of 9,627 protein-coding genes.

Phylosift derived phylogeny of Verticillium dahliae genomes

The phylosift analysis of 13 publicly available *V. dahliae* sequences and the four isolates sequenced in this study resulted in three major clades (Figure 5.2). The isolates largely separated according to their assigned VCG. The cotton VCG1A isolates all clustered within one clade, the VCG2A isolates in another, and VCG2B isolates in a separate clade. The four Australian isolates sequenced in this study were more closely related to each other than the overseas isolates, with SS61 and SS434 sharing the most recent common ancestor. The Australian VCG1A Gwydir clustered with the Chinese cotton isolate Vd991and share a most recent common ancestor with the four Australian isolates.

The phylosift analysis relies on SNPs present in 33 marker genes, which only provides a small snapshot of the genome. However, when Fan et al. (2018) looked at the phylogenetic relationship between the five strawberry isolates, 12008, 12251, 12253, 12158 and 12161, and the tomato isolates, Vdls.17 and JR2, the isolates clustered in the same groups. As the results in this study are consistent with the results of Fan et al. (2018), it suggests that the phylosift clustering in this study is reliable.

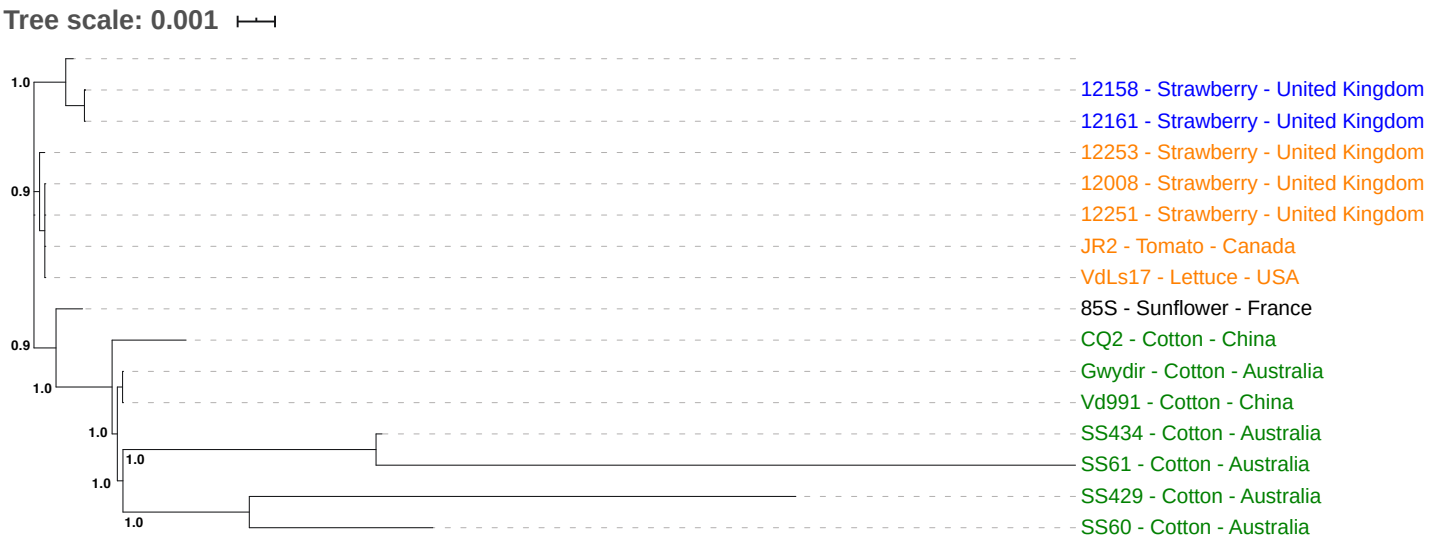

Figure 5.2. Phylogenetic tree showing the relationships between *V. dahliae* genomes. The VCG1A isolates sequenced in this study are coloured green. Blue indicates isolates identified as VCG2A. Orange indicates VCG2B isolates. Black is of unknown VCG. The scale bar represents 1 nucleotide polymorphism per 1000 nucleotides. The numbers on each node denote a confidence value between 0 and 1, with values closer to 1 indicating a higher probability of the branch at that node existing.

Comparative analysis of Australian V. dahliae VCG1A isolates

To identify regions of difference in the genomes, a comparative genomic approach was implemented using ProgressiveMauve on the 4 isolates, SS434, SS60, SS429, and SS61. The regions of low sequence identity (Figure 5.3; Supplementary table S5.2) contained predominantly house-keeping genes. In many regions low homology was found in the same gene, suggesting that across the different isolates they contained alternate copies of the same gene. 14 of the 22 regions were identified as likely containing virulence related genes (Table 5.3). Numerous regions encoded major facilitator superfamily proteins, oxidoreductases, and dehydrogenases; all proteins previously shown to impact virulence in *V. dahliae*. Of the 14 regions encoding virulence related genes, 9 were only present in isolate SS429. These included genes encoding cellulase related proteins (regions 5), necrosis inducing proteins (region 11), and NAD(P)-binding proteins (region 7),

amongst others. However, genes or similar genes could be located in different regions for the other isolates. Several regions (14, 15, 18, and 20) did not contain any identified protein coding sequence despite the low sequence identity between isolates. This suggests that non-coding regions or non-protein products such as small regulatory RNAs also differ between the isolates.

A ProgressiveMauve alignment of SS60 and Vd991 was also conducted to identify regions of difference between a foreign virulent VCG1A and an Australian VCG1A (Figure 5.4). One hundred and nine regions of low sequence identity and rearrangement of LCBs were found (Supplementary Table S5.3). Of the 109 regions with low homology, 54 contained putative genes. Of the regions containing putative genes, 50 % had gene content in the Vd991 genome that was absent in SS60. Conversely, 17 % contained putative genes present in SS60 but absent in Vd991. The other 33% consisted of regions 8, 12, 18, 48 and 83 and had the same gene content indicating alternate genes encoding similar proteins. Some of the regions identified had the same gene content but located in different regions. For example, region 24 SS60 had the same content as region 23 Vd991, and region 64 SS60 contained the same putative genes as region 78 Vd991. While the gene content was largely house-keeping genes, cellulase related proteins, major facilitator superfamily proteins, oxidoreductases, and dehydrogenases and NAD(P)-binding proteins were found in multiple regions (Table 5.4). Isolate SS60 had glycosyl hydrolase family proteins, which have cellulase activity, in two regions (24 and 83), whereas isolate Vd991 had glycosyl hydrolase family proteins in 12 regions.

Table 5.3. 14 identified low homology regions with potential virulence factors for the four Australian *V. dahliae* genomes.

Region	Isolate	Potential virulence related protein
2	SS434	-
	SS60	Dehydrogenase
	SS429	-
	SS61	-
3	SS434	Glycosyl hydrolase; Major Facilitator Superfamily
	SS60	Glycosyl hydrolase; Major Facilitator Superfamily
	SS429	Glycosyl hydrolase; Major Facilitator Superfamily

	SS61	-
4	SS434	-
	SS60	-
	SS429	Major Facilitator Superfamily; Dehydrogenase family
	SS61	-
5	SS434	-
	SS60	-
	SS429	Glycosyl hydrolase family; Cellulase
	SS61	-
6	SS434	-
	SS60	-
	SS429	Major Facilitator Superfamily
	SS61	-
7	SS434	-
	SS60	-
	SS429	Dehydrogenases; NAD(P)H-binding
	SS61	-
8	SS434	-
	SS60	-
	SS429	Major Facilitator Superfamily; Oxidoreductase; dehydrogenase
	SS61	-
10	SS434	Glycosyl hydrolase family
	SS60	-
	SS429	Dehydrogenase
	SS61	Glycosyl hydrolase family
11	SS434	-
	SS60	-
	SS429	Necrosis inducing protein; Major Facilitator Superfamily
	SS61	-
12	SS434	-
	SS60	-
	SS429	Major Facilitator Superfamily; Dehydrogenase
	SS61	-
13	SS434	-
	SS60	-
	SS429	Major Facilitator Superfamily
	SS61	-
18	SS434	Dehydrogenase; Major Facilitator Superfamily
	SS60	Dehydrogenase; Major Facilitator Superfamily
	SS429	-
	SS61	-
20	SS434	NAD(P)-binding; Major Facilitator Superfamily; Glycosyl hydrolase families
	SS60	Glycosyl hydrolase family; Major Facilitator Superfamily
	SS429	-
	SS61	-
21	SS434	-
	SS60	-
	SS429	Oxidoreductase; Dehydrogenase
	SS61	-

Table 5.4. Low homology regions with potential virulence factors for the Australian SS60 and Chinese Vd991 *V. dahliae* genomes.

Region	Isolate	Potential virulence related protein
5	SS60	-
	Vd991	Dehydrogenases; Major Facilitator Superfamily; Glycosyl hydrolase families;
10	SS60	-
	Vd991	Dehydrogenase; Oxidoreductase; NAD(P)-binding
12	SS60	Major Facilitator Superfamily
	Vd991	Major Facilitator Superfamily
21	SS60	-
	Vd991	Dehydrogenase
23	SS60	-
	Vd991	Glycosyl hydrolase family; Dehydrogenase
24	SS60	Glycosyl hydrolase family
	Vd991	-
25	SS60	Oxidoreductase
	Vd991	-
27	SS60	-
	Vd991	Glycosyl hydrolase family; NAD(P)-binding
32	SS60	-
	Vd991	Glycosyl hydrolase family
36	SS60	-
	Vd991	Glycosyl hydrolase family
45	SS60	-
	Vd991	Oxidoreductase; NAD(P)-binding; NAD(P)H-binding
46	SS60	-
	Vd991	Glycosyl transferase families; Dehydrogenases; Major Facilitator Superfamily
47	SS60	-
	Vd991	Glycosyl hydrolase family
55	SS60	-
	Vd991	Hydrolase
64	SS60	Dehydrogenase
	Vd991	Glycosyl hydrolase families
68	SS60	-
	Vd991	NAD(P)H-binding
71	SS60	-
	Vd991	Major Facilitator Superfamily
78	SS60	-
	Vd991	Dehydrogenases; Oxidoreductase
81	SS60	-
	Vd991	Dehydrogenase; Major Facilitator Superfamily
83	SS60	Major Facilitator Superfamily; Glycosyl hydrolase family
	Vd991	Major Facilitator Superfamily; Glycosyl hydrolase family
92	SS60	-
	Vd991	Dehydrogenase
99	SS60	-
	Vd991	Glycosyl hydrolase family

101	SS60	-
	Vd991	Dehydrogenase; Oxidoreductase
109	SS60	Major Facilitator Superfamily
	Vd991	Major Facilitator Superfamily

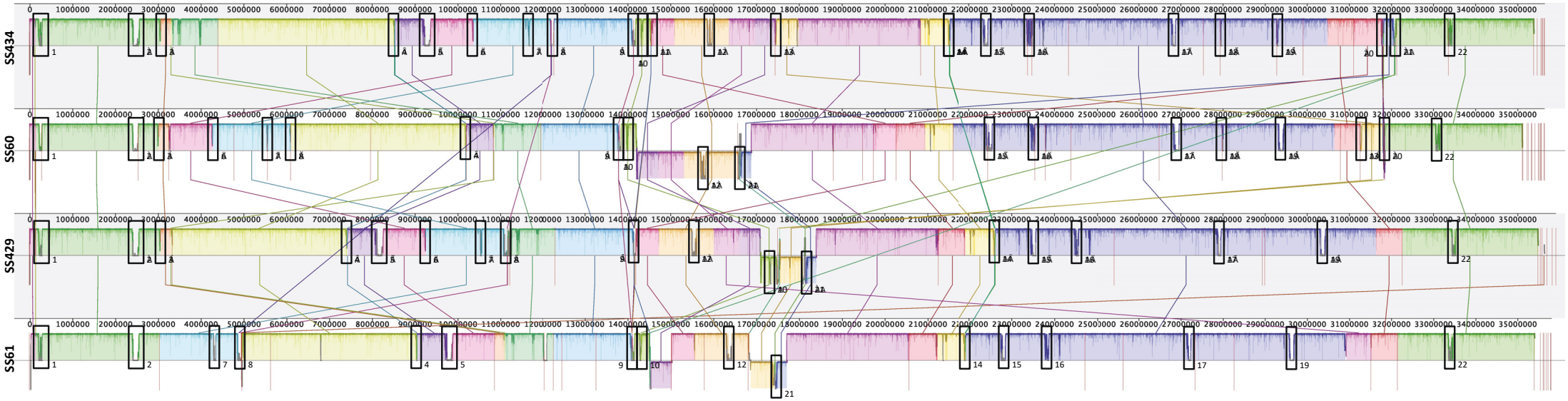

Figure 5.3. ProgressiveMauve analysis of four Australian genomes. Each coloured block represents a localised collinear block (LCB). The height of the bars signifies sequence identity, with dips towards the middle-line indicating no or low identity. Bars below the middle-line indicate areas of sequence inversion. Coloured lines indicate the movement of LCBs between the genomes. Regions of low sequence identity have been indicated by black boxes.

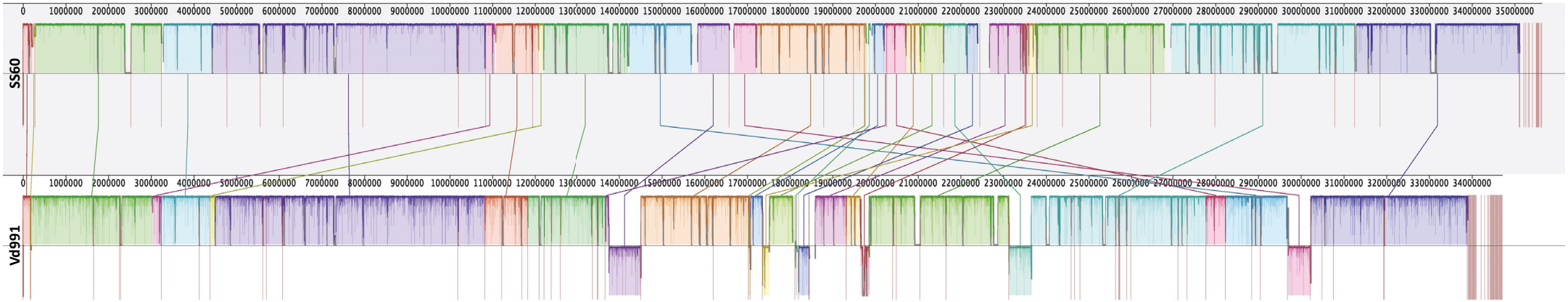

Figure 5.4. ProgressiveMauve analysis of SS60 and Vd991 reveals multiple rearrangements and regions of no or low sequence identity. Each coloured block represents a localised collinear block (LCB). The height of the bars signifies sequence identity, with dips towards the middle-line showing no or low identity. Bars below the middle-line indicate areas of sequence inversion. Coloured lines indicate the movement of LCBs between the genomes.

Blastn screen of virulence genes

Twenty-nine genes previously identified as playing a role in *V. dahliae* virulence were blasted against seven *V. dahliae* genomes known to be virulent in cotton, encompassing five Australian isolates, SS434, SS60, SS429, SS61 and Gwydir, and two Chinese isolates, Vd991 and CQ2 (Table 5.5). All genomes resulted in positive matches for 18 of the screened genes. Three of the genes, *NLP1*, *VdNUC-2*, and *VdPR1*, were not a complete match but aligned with high identity. Both the Chinese isolates, Vd991 and CQ2, contained the seven genes (*VEDA_05193* – *VEDA_05199*) previously identified as being in the lineage specific (LS) region of the Vd991 genome and important for virulence in cotton (Chen et al. 2017). These were not present in any of the Australian genomes. One gene, *VdCPC1*, was present in all genomes except for SS60.

Table 5.5. Blastn screen of virulence genes. + indicates a positive match for the gene; ~ indicates a partial match; and - indicates no match.

ID	Gene	SS434	SS60	SS429	SS61	Gwydir	Vd991	CQ2
MF946582.1	*VEDA_05193*	-	-	-	-	-	+	+
MF946582.1	*VEDA_05194*	-	-	-	-	-	+	+
MF946582.1	*VEDA_05195*	-	-	-	-	-	+	+
MF946582.1	*VEDA_05196*	-	-	-	-	-	+	+
MF946582.1	*VEDA_05197*	-	-	-	-	-	+	+
MF946582.1	*VEDA_05198*	-	-	-	-	-	+	+
MF946582.1	*VEDA_05199*	-	-	-	-	-	+	+
KF164287.1	*VdSSP1*	+	+	+	+	+	+	+
VDAG_08621	*VdSNF1*	+	+	+	+	+	+	+
VDAG_06199	*VdCP1*	+	+	+	+	+	+	+
VDAG_00190	*VdPKS1*	+	+	+	+	+	+	+
JQ665433.1	*VGB*	+	+	+	+	+	+	+
VDAG_04701	*NLP1*	~	~	~	~	~	~	~
VDAG_01995	*NLP2*	+	+	+	+	+	+	+
VDAG_06298	*VdSGE1*	+	+	+	+	+	+	+

VDAG_00329	*VdFTF1*	+	+	+	+	+	+	+
KT454782.1	*VdNUC-2*	~	~	~	~	~	~	~
VDAG_02474	*VdRac1*	+	+	+	+	+	+	+
VDAG_05856	*VdCla4*	+	+	+	+	+	+	+
VDAG_07052	*VdCYC8*	+	+	+	+	+	+	+
VDAG_00904	*VdPR1*	~	~	~	~	~	~	~
KM032761.1	*VdMsb*	+	+	+	+	+	+	+
VDAG_05890	*VdCYP1*	+	+	+	+	+	+	+
DQ026260.1	*VDH1*	+	+	+	+	+	+	+
VDAG_10113	*VdCPC1*	+	-	+	+	+	+	+
VDAG_JR2_Chr1g09120a	*SOM1*	+	+	+	+	+	+	+
VDAG_JR2_Chr1g07600a	*VTA3*	+	+	+	+	+	+	+
HE972150.1	*VTA2*	+	+	+	+	+	+	+
VDAG_01137	*VdTHI4*	+	+	+	+	+	+	+

5.4 Discussion

Internationally, *V. dahliae* VCG1A is described as defoliating and highly virulent while VCG2A is non-defoliating and causes mild to moderate symptoms in cotton (Jiménez-Díaz et al. 2006). While large sections of damage to cotton fields in Australia are caused by *V. dahliae* VCG2A isolates, rather than VCG1A (Chapman et al. 2016; Dadd-Daigle et al. 2020; Jensen and Redfern 2017; Kirkby et al. 2013), glasshouse trials have found that both Australian VCG1A and VCG2A isolates are able to cause mortality (Dadd-Daigle et al. 2020). It is unclear why Australian *V. dahliae* isolates behave differently in the field compared to international reports. Therefore, to identify differences which could contribute to virulence, four VCG1A isolates were genome sequenced. These sequences were compared both between the four Australian VCG1A isolates and between Australian and international VCG1A isolates. They were also examined to determine if Australian VCG1A isolates originated from a single or multiple incursion event.

The analysis of the four Australian isolates, SS434, SS60, SS429 and SS61 using the ProgressiveMauve tool clearly indicated multiple rearrangements had occurred. The regions of low sequence identity predominantly contained house-keeping genes, but some proteins linked to virulence such as cellulases and necrosis related proteins were also present. Rearrangements of LCBs between the chromosomes were numerous amongst the four isolates. It has been suggested that the shuffling of genes between chromosomes plays a role in the adaptive pathogenicity of *V. dahliae* (de Jonge et al. 2013), but glasshouse trials have shown that there was no significant difference between the virulence of the four isolates when examined in infected seedlings (Dadd-Daigle et al. 2020). However, there is currently no data comparing the isolates after infection in an established plant in the field. It is therefore possible that the virulence of these four isolates could vary in field conditions. While the genetic rearrangements may not impact isolate virulence, they likely provide enough variation to explain the different ISSR groupings within the VCG1A cluster (Chapter 4).

When the overall gene content was compared between the five Australian isolates and the virulent Chinese Vd991 there was very little variation apparent. However, the alignment of the SS60 with Vd991 genomes revealed multiple regions of low sequence identity and the rearrangement of LCBs. While approximately half of the identified regions had no putative gene content, 50 % of those regions had potential genes only in the Vd991 genome. Of these regions with putative genes in the Vd991 genome, quite a few contained potential virulence factors. *Verticillium dahliae* virulence has been shown to be regulated or conferred by alternate splicing (Jin et al. 2017), movement of transposons (Amyotte et al. 2012; Faino et al. 2016), epigenetic modifications (Ramírez-Tejero et al. 2020), or a genomic transfer event from another fungus or bacteria (de Jonge et al. 2012; van Kooten et al. 2019). The greater number of potential virulence factors in the Vd991 genome compared to the Australian isolates suggests that it likely gained virulence factors through a horizontal gene transfer event(s). Chen et al. (2017) suggested that several genes identified in the LS region of the Vd991 genome, shown to impact virulence, were gained during a transfer event when a *V. dahliae* was co-infecting a cotton plant with a *Fusarium sp*. Zhang et al. (2019) found that several of these

genes were responsible for *V. dahliae* causing defoliation, and knock-in studies, which introduced the target genes into previously non-virulent *V. dahliae* isolates, induced defoliation in not only cotton, but okra and olives. While all *V. dahliae* isolates examined contained 18 identified virulence genes in common, only the two Chinese VCG1A isolates contained the seven genes found in the LS region. Of the genes in the LS region, Chen et al. (2017) identified VEDA_05196 as a major facilitator superfamily protein and VEDA_05197 and VEDA_05198 were identified as being part of the NAD(P)-binding domain. These play a role in the oxidative stress response which helps to overcome the plants defence system (Chen et al. 2017). Zhang et al. (2019) showed that VEDA_05199 is likely a NAPE-PLD, which hydrolyzes N-acylphosphatidylethanolamines (NAPEs) to produce NAE. NAEs play a role in leaf senescence (Murata et al. 2015). Zhang et al. (2019) found that defoliation increased when plants infected with non-defoliating strains were supplemented with exogenous NAE 12:0, a secondary metabolite regulated by VEDA_05199, VEDA_05198, and VEDA_05197. As the Australian VCG1A isolates did not contain any of these genes, this could impact their ability to induce defoliation and explain the differences observed internationally.

The phylosift analysis revealed that the Australian isolates clustered closest to the virulent Chinese VCG1A isolate, Vd991. The Australian isolates appeared to have the largest distance between them in terms of relatedness, with the exception of Gwydir, an Australian VCG1A sequenced separately (Genbank GCA_003320035.1), which clustered with the Chinese Vd991. The Australian isolates and the Chinese Vd991 appear to share a common ancestor. However, as the Australian *V. dahliae* VCG1A isolates lack the LS region, it is likely that they were introduced to Australia before the genes were acquired from *Fusarium sp*. It is unclear if there were multiple incursion events prior to this. Interestingly, SS61, originally collected in Namoi, clustered more closely with SS434, which was collected in Macintyre. Similarly, SS60 (Macintyre) clustered more closely with SS429 (Namoi). This indicates that there is movement of different VCG1A varieties across cotton growing locations. However, it is unlikely that a new VCG1A has been introduced since Vd991 acquired the LS region, as despite clustering with Vd991, the Gwydir isolate does not contain any of the LS associated genes.

5.5 Conclusion

Although genetic shuffling has occurred within the Australian *V. dahliae* VCG1A population, these changes are unlikely to have impacted isolate virulence. The lack of defoliation induced by Australian VCG1A is likely due to not producing a high concentration of the metabolite NAE 12:0 (Zhang et al. 2019). This suggests that *V. dahliae* VCG1A was present in Australia before Chinese *V. dahliae* VCG1A isolates acquired the LS genes from a *Fusarium sp.* (Chen et al. 2017). It is unclear if there were multiple incursion events prior to this. However, the phylosift analysis suggests that there was movement of isolates across cotton growing regions, which could be significant if a virulent VCG1A was introduced into Australia.

To better understand *V. dahliae* in Australian cotton, future studies that use a lower conidia concentration or that examine infection in established plants combined with transcription and metabolite studies could assist in understanding Australian VCG1A virulence. Additionally, as some Australian VCG2A isolates are able to induce defoliation it would be worthwhile to sequence their genomes and determine whether these have a high NAE 12:0 concentration or whether an alternate factor could be responsible for defoliation.

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Chapter 6: General discussion and future directions

6.1 Vegetative Compatibility Groups are not good indicators of disease potential

Although *Verticillium dahliae* causing Verticillium wilt impacts cotton fields internationally, the ability for different variants to cause disease is still not well understood. Despite this, there is a demand for a method that can characterise *V. dahliae* isolates and provide data to easily understand the disease potential for different isolates affecting crops and fields. Vegetative Compatibility Group (VCG) typing offers a simple way to categorise *V. dahliae* isolates and has been used as the main method for characterisation over the past few decades. However, there are some distinct problems with the use of VCGs for determining disease potential on Australian farms.

Firstly, the method for determining VCG requires comparison of an isolate with unknown VCG with an isolate of known VCG. Not all *V. dahliae* VCGs are present within Australia and, due to Australia's strict biosecurity laws, the isolates are not able to be brought into the country. Therefore, to undergo the traditional VCG typing method for Australian samples the unknown isolates must be sent overseas for testing. This is costly and time-consuming. Chapman et al (2016) explored typing a subset of isolates via the traditional method and then using the IGS region (Papaioannou et al. 2013) to identify VCG by comparison with isolates of known and unknown VCG. While this method provides a faster and cost-effective method for characterising *V. dahliae* isolates into VCGs, it does not always effectively distinguish between VCG2A and VCG4B. Due to this, and the reliance on user interpretation, the use of IGS regions is unlikely to have widespread adoption as a tool for characterisation. However, as no other genetic region has been identified that can distinguish VCGs molecularly, the IGS region still currently offers the best solution when the traditional method is inaccessible.

The second problem with the use of VCGs for predicting disease potential is that they do not allow for variation of virulence within the VCG. As demonstrated in

chapters 3 and 4, in Australia there appears to be a subset of *V. dahliae* VCG2A isolates that are able to cause severe disease in cotton. Ideally this virulent subset could be separated out of the VCG2A population by the use of an additional marker(s), such as defoliating and non-defoliating markers (Mercado-Blanco et al. 2003). However, Australian VCG2A isolates test positive for the non-defoliating marker (Chapters 3 and 4), suggesting that these markers are more useful for separating VCG1A from VCG2A and VCG4B, but are not able to distinguish isolates by virulence.

There has been some movement away from grouping isolates by VCGs to clonal lineages. Milgroom et al. (2014) showed that *V. dahliae* groups into 9 different clonal lineages. They found that different VCG subgroups such as VCG2A and VCG4B were phylogenetically closer than subgroups VCG2B and VCG4A (Milgroom et al. 2014) which could help explain the similarity in IGS sequences between VCG2A and VCG4B isolates. However, as molecular markers that confer lineages largely align with VCGs, clonal lineages can be inferred from VCG (Jiménez-Díaz et al. 2016; Milgroom et al. 2014). When looking at the differences in clonal lineages, Jiménez-Díaz et al. (2016) found that all 2A isolates grouped together by race, non-defoliating pathotype, and PCR genotype. They also found that 2A isolates were significantly less pathogenic than isolates of the defoliating pathotype (lineage 1A) (Jiménez-Díaz et al. 2016). This suggests that, ultimately, while lineages can be used to characterise *V. dahliae* isolates similarly to VCGs, they don't differentiate virulence within a lineage and so present the same problem as VCGs.

6.2 New molecular tools are needed to better characterise *V. dahliae*

As VCGs do not reflect disease potential and current markers are unable to further distinguish virulence, there is a clear need for new tools that are able to separate virulent from non-virulent isolates. Ideally such a tool would be simple to use and able to rapidly type a large number of isolates. Although there has been research focused on tracing isolate origins, or better understanding mechanisms of disease, few studies have attempted to find a method of distinguishing virulence either

within or outside VCG characterisations. This is likely because isolates in other large cotton growing countries, such as the USA or China, appear to be consistent with VCG1A isolates causing severe disease and VCG2A isolates causing only mild or moderate disease.

The studies that have examined virulence have largely had mixed results. ElSharawy et al. (2015) was able to use the PCR-based Inter-Simple Sequence Repeat (ISSR) to characterise *V. dahliae* cotton isolates from Turkey, finding that the ISSR assay separated the isolates according to virulence. The method applied to the Australian isolates was also able to separate isolates based on virulence (Chapter 4), providing the first tool able to separate out the VCG2A virulent and non-virulent isolates.

The ISSR assay does present some challenges. While the method is sensitive, it is time consuming and relies heavily on the user to correctly interpret the results. This leaves it open to error. It could be improved by the use of primers attached to fluorescent probes which emit fluorescence and can be detected digitally. This would both help enhance sensitivity of band detection and limit human error in correctly identifying all bands present for each sample in each of the reactions and, would greatly improve the turn-around time from sample collection to identification.

Ultimately, the ISSR assay provides a steppingstone for the development of better diagnostic tools. The application of the assay to the Australian *V. dahliae* population has facilitated the identification of isolates that differ in virulence despite being classified as the same VCG. This allows ideal candidate isolates for genome sequencing to be chosen, and these genomes can then be scanned for unique regions. Following this, cheaper and more rapid diagnostic tools can be designed, which would benefit the cotton farmers and the international research community and ideally help develop a new characterisation system for *V. dahliae*.

6.3 Whole genome sequencing helps demystify Australian *V. dahliae* virulence

Since the first report of *V. dahliae* VCG1A in Australia, it has been unclear why the Australian isolates appear to cause less severe symptoms in cotton than what has been described internationally. Although glasshouse trials using Australian VCG1A *V. dahliae* isolates have shown that the isolates are capable of rapidly killing cotton seedlings (Chapters 3 and 4), field studies have indicated that VCG1A isolates cause only mild to moderate disease symptoms with no defoliation (Jensen and Redfern 2017). This disparity in disease symptoms could be caused by a range of factors including Australian VCG1A isolates not being competitive compared to VCG2A isolates in the Australian environmental context; the microbiome of the plants or soil being different in Australia and thus preventing VCG1A disease; or the Australian *V. dahliae* population could lack the virulence genes found in international isolates.

Analysis of the Australian *V. dahliae* isolates so far indicates that Australia may have distinct *V. dahliae* populations, at least for VCG1A and VCG2A isolates. The ISSR assay indicated that Australian VCG1A *V. dahliae* isolates cluster separately to international VCG1A isolates (Chapter 4). Additionally, the genome sequencing of Australian VCG1A isolates indicated that they are distantly related to international isolates, and that the Australian *V. dahliae* isolates lacked a set of genes that have been associated with defoliation and virulence in Chinese VCG1A *V. dahliae* isolates (Chapter 5; Chen et al. 2017; Zhang et al. 2019). It is likely that the Australian *V. dahliae* populations were introduced to Australia before the international VCG1A isolates acquired the virulence genes, and are hence unable to cause the same damage. A large-scale genomic study would be needed to confirm this.

However, it is unclear whether these virulence genes are present in Australian VCG2A *V. dahliae* isolates. Given the VCG2A isolates cluster closer to Australian non-virulent VCG2A, VCG4B, and Australian VCG1A isolates than international isolates (Chapter 4), it is unlikely that Australian VCG2A *V. dahliae* have the genes found in the LS region of Chinese VCG1A. Zhang et al. (2019) suggest that there are many homologs to the *VEDA_05199*, *VEDA_05198*, and *VEDA_05197* genes

responsible for the regulation of metabolite NAE 12:0, believed to induce defoliation in *V. dahliae* isolates. It is therefore possible that Australian VCG2A isolates have separately acquired a similar set of genes, or other genes that carry out similar functions.

There are many possible ways that pathogenicity can evolve within *V. dahliae*. Alternate splicing (Jin et al. 2017), movement of transposons (Amyotte et al. 2012; Faino et al. 2016), epigenetic modifications (Ramírez-Tejero et al. 2020), or a gene transfer event from another fungus or bacteria (de Jonge et al. 2012; van Kooten et al. 2019). Co-infection of cotton plants with both *Fusarium sp.* and *V. dahliae* has been observed with both fungi recovered from a single plant (Wagner et al. 2020). As *Fusarium sp.* are common on Australian cotton farms, this is a plausible way that VCG2A isolates could have acquired greater virulence. Additionally, current cotton harvesting methods allow *V. dahliae* microsclerotia to enter the soil regardless of their ability to cause senescence. This allows non-defoliating populations to continue on farms, increasing their likelihood of acquiring genes or a mutation that confer greater pathogenicity. As Wagner et al. (2020) also recovered multiple *V. dahliae* isolates of different VCGs from single plants, the continued presence of pathogenic VCG2A isolates on farms could facilitate further spread of virulence factors into non-pathogenic isolates.

In order to better understand what is causing Australian VCG2A *V. dahliae* virulence, there are several directions that should be explored. Using the ISSR results to select isolates with high and low disease potential, the genomes of the selected isolates can be compared to look for genes known to be responsible for virulence. Additionally, comparing to virulent VCG1A genomes might identify common virulence genes. As it is possible that NAE regulating genes or unknown factors could be contributing to VCG2A virulence, metabolic and gene expression assays could assist in identifying genes linked to virulence. These analyses would also help facilitate the development of a molecular screening tool for virulent and non virulent isolates.

6.4 Conclusion

Through the work in this thesis it has been shown that Australian *V. dahliae* appears to be different to *V. dahliae* that has been reported in the literature internationally. Although Australian VCG1A *V. dahliae* isolates are able to cause significant disease in seedlings, for the first time it was demonstrated *in vitro* that some Australian VCG2A isolates are also able to cause severe disease. This was further highlighted through the application of ISSR as a molecular tool to analyse virulence in *V. dahliae* populations. This analysis revealed for the first time the presence of a defoliating-like group of VCG2A isolates amongst the Australian *V. dahliae* population and indicated an area for future genomic analysis. A look at the genomes of several Australian VCG1A isolates showed that the Australian isolates are lacking several genes that have been shown to be important for *V. dahliae* virulence in cotton. This study has laid the groundwork for future research to better target regions of interest in VCG1A isolates and other Australian *V. dahliae* VCGs for improving understanding of the mechanisms of virulence.

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Supplementary data

Table S4.1. Primer sequences used for Inter-Simple Sequence Repeat analysis.

Primer Name	Sequence
834	AGA GAG AGA GAG AGA GYT
815	CTC TCT CTC TCT CTC TG
848	CAC ACA CAC ACA CAC ARG
4	ACACACACACACACACAG
24	ACACACACACACACACTC
25	ACACACACACACACACCA
60	AGAGAGAGAGAGAGAGGG
885	GAGGAGAGAGAGAGAGA
818	CACACACACACACACAG
888	CACCACACACACACACA
866	GGTGGGGTGGGGTG
864	ATG ATGATGATGATGATG
836	AGA GAG AGA GAG AGA GYA

Table S4.2. Isolates used in the Inter-Simple Sequence Repeat analysis.

Isolate	VCG*	Host plant	Origin	Year Isolated
SS36	2A	*Gossypium hirsutum*		
SS48	1A	*Gossypium hirsutum*		
SS49	1A	*Gossypium hirsutum*		
SS50	1A	*Gossypium hirsutum*		
SS51	1A	*Gossypium hirsutum*		
SS52	2A	*Gossypium hirsutum*		
SS60	1A	*Gossypium hirsutum*		
SS61	1A	*Gossypium hirsutum*		
SS94	4B	*Gossypium hirsutum*		
SS95	4B	*Gossypium hirsutum*		
SS96	2A	*Gossypium hirsutum*		
SS97	1A	*Gossypium hirsutum*		
SS99	1A	*Gossypium hirsutum*		
SS188	1A	*Gossypium hirsutum*		
SS192	2A	*Gossypium hirsutum*		
SS206	2A	*Gossypium hirsutum*		
SS207	1A	*Gossypium hirsutum*		
SS208	1A	*Gossypium hirsutum*		

SS242	2A	*Gossypium hirsutum*
SS243	1A	*Gossypium hirsutum*
SS244	2A	*Gossypium hirsutum*
SS245	2A	*Gossypium hirsutum*
SS255	4B	*Gossypium hirsutum*
SS256	2A	*Gossypium hirsutum*
SS262	2A	*Gossypium hirsutum*
SS263	1A	*Gossypium hirsutum*
SS279	ND	*Gossypium hirsutum*
SS282	4B	*Gossypium hirsutum*
SS283	4B	*Gossypium hirsutum*
SS284	4B	*Gossypium hirsutum*
SS285	2A	*Gossypium hirsutum*
SS286	2A	*Gossypium hirsutum*
SS287	2A	*Gossypium hirsutum*
SS288	4B	*Gossypium hirsutum*
SS289	ND	*Gossypium hirsutum*
SS362	2A	*Gossypium hirsutum*
SS363	1A	*Gossypium hirsutum*
SS364	4B	*Gossypium hirsutum*
SS367	1A	*Gossypium hirsutum*
SS368	4B	*Gossypium hirsutum*
SS373	2A	*Gossypium hirsutum*
SS396	2A	*Gossypium hirsutum*
SS397	2A	*Gossypium hirsutum*
SS398	2A	*Gossypium hirsutum*
SS400	1A	*Gossypium hirsutum*
SS401	2A	*Gossypium hirsutum*
SS402	2A	*Gossypium hirsutum*
SS403	1A	*Gossypium hirsutum*
SS404	2A	*Gossypium hirsutum*
SS405	2A	*Gossypium hirsutum*
SS408	2A	*Gossypium hirsutum*
SS409	2A	*Gossypium hirsutum*
SS410	1A	*Gossypium hirsutum*
SS411	1A	*Gossypium hirsutum*
SS413	2A	*Gossypium hirsutum*
SS414	2A	*Gossypium hirsutum*
SS415	1A	*Gossypium hirsutum*

SS417	1A	*Gossypium hirsutum*		
SS418	2A	*Gossypium hirsutum*		
SS420	ND	*Gossypium hirsutum*		
SS421	1A	*Gossypium hirsutum*		
SS422	2A	*Gossypium hirsutum*		
SS423	2A	*Gossypium hirsutum*		
SS424	1A	*Gossypium hirsutum*		
SS425	2A	*Gossypium hirsutum*		
SS426	ND	*Gossypium hirsutum*		
SS427	1A	*Gossypium hirsutum*		
SS428	ND	*Gossypium hirsutum*		
SS429	1A	*Gossypium hirsutum*		
SS430	1A	*Gossypium hirsutum*		
SS431	2A	*Gossypium hirsutum*		
SS432	ND	*Gossypium hirsutum*		
SS433	1A	*Gossypium hirsutum*		
SS434	1A	*Gossypium hirsutum*		
SS435	2A	*Gossypium hirsutum*		
SS438	2A	*Gossypium hirsutum*		
SS439	1A	*Gossypium hirsutum*		
SS440	2A	*Gossypium hirsutum*		
SS443	1A	*Gossypium hirsutum*		
SS444	1A	*Gossypium hirsutum*		
SS445	2A	*Gossypium hirsutum*		
SS446	4B	*Gossypium hirsutum*		
SS447	1A	*Gossypium hirsutum*		
SS448	2A	*Gossypium hirsutum*		
Swinburn	D	*Gossypium hirsutum*	Southern high plains; Texas USA	-
Taylor	D	*Gossypium hirsutum*	Southern high plains; Texas USA	-
Seminole	D	*Gossypium hirsutum*	Southern high plains; Texas USA	-
GVD20	1A	*Gossypium hirsutum*	North Israel	1997
GVD22	2B	*Capsicum annuum*	South Israel; Lahahv	1995
GVD26	2B	*Capsicum annuum*	South Israel; Lahahv	1996
GVD29	2A	*Solanum melongena*	South Israel; Sde Uzyia	2015
GVD59	4B	*Solanum tuberosum*	South Israel; Gilat	1997
GVD107	4B	*Solanum lycopersicum*	South Israel; Ohad	2005

*Isolates unable to be assigned a VCG have their D or ND pathotype indicated instead

Table S4.3. PCR band scores for ISSR assay

Attached excel file

Table S5.1. Extraction Buffer

2.5 volume of Buffer A	0.35 M sorbitol 0.1 M Tris-HCl 5mM EDTA,pH8
2.5 volume of Buffer B	0.2 M Tris-HCl 50mM EDTA,pH8 2M NaCl 2% CTAB
1.0 volume of Buffer C	5% Sarkosyl N-lauroylsarcosine sodium salt (SIGMA L5125)
0.5 volume Polyvinylpyrrolidone (40000 MW) 10 % [w/v] (Sigma PVP40)	
0.5 volume Polyvinylpyrrolidone (10000 MW) 10% [w/v] (Sigma PVP10)	
1.4µl of RNase A (10mg/ml stock) per ml	

Table S5.2. ProgressiveMauve analysis of four Australian genomes revealed regions of low sequence identity. Region numbers refer to their position on figure 3. Regions with putative genes have been indicated with blue.

Region	Isolate	Location (bp)	Contig	PFAM	Gene content
1	SS434	201828 - 290320	11340	PF00005; PF00664	ABC transporter; ABC transporter transmembrane region
	SS60	216993 - 284247	374	PF00005; PF00664	ABC transporter; ABC transporter transmembrane region
	SS429	213254 - 287588	22	PF00005; PF00664	ABC transporter; ABC transporter transmembrane region
	SS61	204858 - 286271	5019	-	
2	SS434	2407045 - 2569869	11340	PF07716; PF00400; PF12894	Basic region leucine zipper; WD40 repeat; Anaphase-promoting complex subunit 4 WD40 domain
	SS60	2113716 - 2241996, 0 - 6009	46851, 259	PF00742; PF03447	Homoserine dehydrogenase; Homoserine dehydrogenase, NAD binding domain
	SS429	2404312 - 2563597	22	-	-
	SS61	2402995 - 2555201	5019	-	-

3	SS434	3040646 - 3061884	11340	PF04734; PF17048; PF01055; PF13802; PF16863; PF07690	Neutral/alkaline non-lysosomal ceramidase, N-terminal; Neutral/alkaline non-lysosomal ceramidase, C-terminal; Glycosyl hydrolases family 31; Galactose mutarotase-like; maltase-glucoamylase; Major Facilitator Superfamily
	SS60	459088 - 476786	259	PF00010; PF04734; PF17048; PF01055; PF13802; PF16863; PF07690	Helix-loop-helix DNA-binding domain; Neutral/alkaline non-lysosomal ceramidase, N-terminal; Neutral/alkaline non-lysosomal ceramidase, C-terminal; Glycosyl hydrolases family 31; Galactose mutarotase-like; maltase-glucoamylase; Major Facilitator Superfamily
	SS429	3034374 - 3059151	22	PF00010; PF09427; PF04734; PF01055; PF13802; PF16863; PF07690	Helix-loop-helix DNA-binding domain; Domain of unknown function (DUF2014); Neutral/alkaline non-lysosomal ceramidase; Glycosyl hydrolases family 31; Galactose mutarotase-like; maltase-glucoamylase; Major Facilitator Superfamily
	SS61	-	-	-	-
4	SS434	4104997 - 4129774	1	-	-
	SS60	2218796 - 2234590	65	-	-
	SS429	4068987 - 4090225	2	PF07690; PF00171; PF12709; PF07991; PF00179; PF01644; PF08407; PF13632; PF09463	Major Facilitator Superfamily; Aldehyde dehydrogenase family; Fungal Transforming acidic coiled-coil (TACC) proteins; Acetohydroxy acid isomeroreductase; Ubiquitin-conjugating enzyme; Chitin synthase; Chitin synthase N-terminal; Glycosyl transferase family group 2; Opy2 protein
	SS61	3404654 - 3404654	1	-	-
5	SS434	4812931 - 4958058	1	-	-
	SS60	-	-	-	-
	SS429	4779397 - 4924523	2	PF00566; PF10516; PF00076; PF00156; PF13522; PF13537; PF02102; PF14521; PF01590; PF13185; PF01564; PF17284; PF00025; PF00503; PF00933; PF01915; PF14310; PF03074; PF02036; PF00638; PF13634; PF06331; PF00378; PF16113;	Rab-GTPase-TBC domain; SHNi-TPR; Phosphoribosyl transferase domain; Glutamine amidotransferase domain; Deuterolysin metalloprotease (M35) family; Lysine-specific; metallo-endopeptidase; GAF domain; Spermine/spermidine synthase domain; ADP-ribosylation factor family; G-protein alpha subunit; Glycosyl hydrolase family; Fibronectin type III-like domain; Glutamate-cysteine ligase; SCP-2 sterol transfer family; RanBP1 domain; Nucleoporin FG repeat region; Transcription factor TFIIH complex; Enoyl-CoA hydratase/isomerase; Cellulase; Glucanosyltransferase; SH3 domain; Phorbol esters/diacylglycerol binding domain; Fes/CIP4, and EFC/F-BAR homology domain; Variant SH3

				PF00150; PF03198; PF00018; PF00130; PF00611; PF14604; PF00270; PF00271; PF04851; PF15456; PF01433; PF09127; PF17900; PF00225; PF12423; PF12473; PF16183; PF16796; PF00173; PF01435; PF08241; PF13489; PF13649; PF01237; PF15409; PF00176; PF00271; PF01412; PF14773; PF01284	domain; DEAD/DEAH box helicase; Helicase conserved C-terminal domain; Type III restriction enzyme, res subunit; Up-regulated During Septation; Peptidase family M1 domain; Leukotriene A4 hydrolase; Peptidase M1; Kinesin motor domain; Kinesin protein 1B; Kinesin protein; Kinesin-associated; Microtubule binding; Cytochrome b5-like Heme/Steroid binding domain; Peptidase family M48; Methyltransferase domain; Oxysterol-binding protein; Pleckstrin homology domain; SNF2 family; Putative GTPase activating protein for Arf; Helicase-associated putative binding domain; Membrane-associating domain
	SS61	4105509 - 4247096	1	-	-
6	SS434	5938902 - 5960140	1	PF12146; PF12697; PF00134; PF00153; PF13202; PF13499; PF13833; PF05739; PF01239	Serine aminopeptidase; Alpha/beta hydrolase family; Cyclin; Mitochondrial carrier protein; EF hand; EF-hand domain pair; SNARE domain; Protein prenyltransferase alpha subunit
	SS60	1014978 - 1036216	46854	-	-
	SS429	5901828 - 5923066	2	PF00046; PF03221; PF05920; PF07690; PF03061; PF00270; PF00271; PF00636; PF03368; PF04851; PF14622; PF04177	Homeodomain; Tc5 transposase DNA-binding domain; Homeobox KN domain; Major Facilitator Superfamily; Thioesterase superfamily; DEAD/DEAH box helicase; Helicase; Ribonuclease III domain; Dicer dimerisation domain; Type III restriction enzyme, res subunit; Ribonuclease-III-like; TAP42-like family
	SS61	-	-	-	-
7	SS434	7240792 - 7269109	1	-	-
	SS60	764748 - 777275, 0 - 7218	206, 1342	-	-
	SS429	7200178 - 7235575	2	PF04410; PF00270; PF00271; PF04851; PF13959; PF07738; PF00106; PF01073; PF01370; PF02719; PF04321; PF08659; PF13460; PF16363; PF00107; PF13602; PF00023; PF13857; PF17132; PF06807; PF16573; PF16575	Gar1/Naf1 RNA binding region; DEAD/DEAH box helicase; Helicase; Type III restriction enzyme, res subunit; Domain of unknown function (DUF4217); Sad1 / UNC-like C-terminal; short chain dehydrogenase; 3-beta hydroxysteroid dehydrogenase/isomerase family; NAD dependent epimerase/dehydratase family; Polysaccharide biosynthesis protein; RmlD substrate binding domain; KR domain; NAD(P)H-binding; GDP-mannose 4,6 dehydratase; Zinc-binding dehydrogenase; Ankyrin repeat; alpha-L-rhamnosidase; Pre-mRNA cleavage complex II protein Clp1; N-terminal beta-sandwich domain of polyadenylation factor; mRNA cleavage and polyadenylation factor CLP1 P-loop

	SS61	1257291 - 1317465	54	-	-
8	SS434	7796520 - 7867314	1	-	-
	SS60	515214 - 538386	1342	-	-
	SS429	7744578 - 7839387	2	PF00248; PF09295; PF05160; PF00162; PF04117; PF04676; PF04677; PF03029; PF03604; PF01230; PF04677; PF11969; PF00248; PF00179; PF14461; PF00561; PF00566; PF10300; PF00382; PF07741; PF07081; PF00717; PF00296; PF00474; PF01083; PF02668; PF07690; PF00248; PF07428; PF10021; PF00732; PF05199; PF00106; PF13561; PF05721	Aldo/keto reductase family; ChAPs (Chs5p-Arf1p-binding proteins); DSS1/SEM1 family; Phosphoglycerate kinase; Mpv17 / PMP22 family; CwfJ; Conserved hypothetical ATP binding protein; DNA directed RNA polymerase, 7 kDa subunit; HIT domain; Scavenger mRNA decapping enzyme C-term binding; Aldo/keto reductase family; Ubiquitin-conjugating enzyme; Prokaryotic E2 family B; alpha/beta hydrolase fold; Rab-GTPase-TBC domain; Protein of unknown function (DUF3808); Transcription factor TFIIB repeat; Brf1-like TBP-binding domain; Peptidase S24-like; Luciferase-like monooxygenase; Sodium:solute symporter family; Cutinase; Taurine catabolism dioxygenase TauD, TfdA family; Major Facilitator Superfamily; Aldo/keto reductase family; 15-O-acetyltransferase Tri3; GMC oxidoreductase; short chain dehydrogenase; Enoyl-(Acyl carrier protein) reductase; Phytanoyl-CoA dioxygenase (PhyH)
	SS61	1816205 - 1922395	54	-	-
9	SS434	1810077 - 1909188	90	-	-
	SS60	1816494 - 1901446	1	-	-
	SS429	3278722 - 3308555	4728	PF00550; PF06985; PF01231	Phosphopantetheine attachment site; Heterokaryon incompatibility protein (HET); Indoleamine 2,3-dioxygenase
	SS61	805339 - 897370	104	-	-
10	SS434	2033076 - 2075552	90	PF04616	Glycosyl hydrolases family 43
	SS60	2025335 - 2067811	1	-	-
	SS429	3108523 - 3151701	4728	EOG09263RF8*; PF00248; PF00022; PF16010; PF00190; PF07883	Aldo/keto reductase family; Actin; Cytochrome domain of cellobiose dehydrogenase; Cupin
	SS61	1017719 - 1056656	104	PF04616	Glycosyl hydrolases family 43
11	SS434	2247577 - 2275895	90	PF00432; PF13243	Prenyltransferase and squalene oxidase repeat; Squalene-hopene cyclase
	SS60	-	-	-	-
	SS429	2909290 - 2934244	4728	PF05630; PF07690; PF00199; PF06628;	Necrosis inducing protein (NPP1); Major Facilitator Superfamily; Catalase; PAN domain; Domain of unknown

				PF00024; PF09118; PF13418; PF00205; PF02775; PF02776; PF04082	function (DUF1929); Galactose oxidase; Thiamine pyrophosphate enzyme; Thiamine pyrophosphate enzyme, C-terminal TPP binding domain; Fungal specific transcription factor domain
	SS61	-	-	-	-
12	SS434	1302256 - 1422605	63	-	-
	SS60	3758355 - 3878704	1	-	-
	SS429	1300045 - 1423933	4728	PF00387; PF00388; PF04479; PF00561; PF04083; PF10203; PF00063; PF02736; PF00191; PF06432; PF05730; PF00149; PF12850; PF05669; PF01096; PF02150; PF00314; PF05631; PF07690; PF00349; PF03727; PF00160; PF04258; PF01648; PF01039; PF00441; PF02770; PF02771; PF08028; PF10383; PF16761; PF07574; PF00153; PF00717; PF00575; PF07541; PF00400; PF00248	Phosphatidylinositol-specific phospholipase C; RTA1 like protein; alpha/beta hydrolase fold; Partial alpha/beta-hydrolase lipase region; Cytochrome c oxidase assembly protein PET191; Myosin head; Myosin N-terminal SH3-like domain; Annexin; Phosphatidylinositol N-acetylglucosaminyltransferase; CFEM domain; Calcineurin-like phosphoesterase; Calcineurin-like phosphoesterase superfamily domain; SOH1; Transcription factor S-II; RNA polymerases M/15 Kd subunit; Thaumatin family; Sugar-tranasporters, 12 TM; Major Facilitator Superfamily; Hexokinase; Cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD; Signal peptide peptidase; 4'-phosphopantetheinyl transferase superfamily; Carboxyl transferase domain; Acyl-CoA dehydrogenase, C; Transcription-silencing protein Clr2; Transcription-silencing protein, cryptic loci regulator Clr2; Nse1 non-SMC component of SMC5-6 complex; Mitochondrial carrier protein; Peptidase S24-like; S1 RNA binding domain; Eukaryotic translation initiation factor 2 alpha subunit; WD domain, G-beta repeat; Aldo/keto reductase family
	SS61	512264 - 650311	99	-	-
13	SS434	4469 - 29247	11342	-	-
	SS60	0 - 36103	302	-	-
	SS429	554449 - 579404	4725	PF00083; PF07690; PF13483; PF00856; PF00005; PF01061; PF06422; PF00385	Sugar (and other) transporter; Major Facilitator Superfamily; Beta-lactamase superfamily domain; SET domain; ABC transporter; ABC-2 type transporter; CDR ABC transporter; Chromo (CHRromatin Organisation MOdifier) domain
	SS61	556271 - 574095	5016	-	-
14	SS434	657299 - 698235	421	-	-
	SS60	-	-	-	-
	SS429	1337617 - 1383632	12	-	-
	SS61	1340642 - 1362287	8	-	-
15	SS434	1506821 - 1623630	421	-	-

	SS60	821131 - 857697, 0 - 97914	226, 190	-	-
	SS429	2197048 - 2320936	12	-	-
	SS61	2189454 - 2299184	8	-	-
16	SS434	2527224 - 2547779, 0 – 97981, 0 - 12575	421, 423, 57	PF00319; PF00533; PF12738; PF16589; PF00646; PF05426; PF13229	SRF-type transcription factor (DNA-binding and dimerisation domain); BRCA1 C Terminus (BRCT) domain; twin BRCT domain; BRCT domain, a BRCA1 C-terminus domain; F-box domain; Alginate lyase; Right handed beta helix region
	SS60	998623 - 1089645, 0 - 12062	190, 255	PF00319; PF00533; PF12738; PF05426; PF13229	SRF-type transcription factor (DNA-binding and dimerisation domain); BRCA1 C Terminus (BRCT) domain; twin BRCT domain; Alginate lyase; Right handed beta helix region
	SS429	3215352 - 3322298	12	PF00319; PF01794; PF00533; PF12738; PF13229	SRF-type transcription factor (DNA-binding and dimerisation domain); Ferric reductase like transmembrane component; BRCA1 C Terminus (BRCT) domain; twin BRCT domain; Right handed beta helix region
	SS61	3213489 - 3316871	8	PF00319; PF00533; PF12738; PF05426; PF13229	SRF-type transcription factor (DNA-binding and dimerisation domain); BRCA1 C Terminus (BRCT) domain; twin BRCT domain; Alginate lyase; Right handed beta helix region
17	SS434	2448547 - 2544798	67	-	-
	SS60	334781 - 477376	106	-	-
	SS429	2441027 - 2540843	24	-	-
	SS61	1513178 - 1609430	107	-	-
18	SS434	204 - 21593	124	PF01761; PF13685; PF01596; PF13578; PF00083; PF07690; PF00324; PF13520; PF13508; PF13673; PF08450	3-dehydroquinate synthase; Iron-containing alcohol dehydrogenase; O-methyltransferase; Methyltransferase domain; Sugar (and other) transporter; Major Facilitator Superfamily; Amino acid permease; Acetyltransferase (GNAT) domain; SMP-30/Gluconolactonase/LRE-like region
	SS60	1280 - 26234	21	PF01761; PF13685; PF01596; PF13578; PF00083; PF07690; PF13520; PF13508; PF13673; PF08450	3-dehydroquinate synthase; Iron-containing alcohol dehydrogenase; O-methyltransferase; Methyltransferase domain; Sugar (and other) transporter; Major Facilitator Superfamily; Amino acid permease; Acetyltransferase (GNAT) domain; SMP-30/Gluconolactonase/LRE-like region
	SS429	-	-		-
	SS61	-	-		-
19	SS434	4959 - 44173	180	-	-
	SS60	1327411 - 1462876	21	-	-

	SS429	1308995 - 1440896	31	-	-
	SS61	1311302 - 1436072	25	-	-
20	SS434	0 - 59835	190	PF13472; PF17996; PF13229; PF00326; PF07859; PF13450; PF00083; PF07690; PF06964; PF01532; PF02133; PF12697; PF01135; PF10176; PF00153; PF00704; PF00982; PF02358; PF00651	GDSL-like Lipase/Acylhydrolase family; Carbohydrate esterase; Right handed beta helix region; Prolyl oligopeptidase family; alpha/beta hydrolase fold; NAD(P)-binding Rossmann-like domain; Sugar (and other) transporter; Major Facilitator Superfamily; Alpha-L-arabinofuranosidase C-terminal domain; Glycosyl hydrolase family 47; Permease for cytosine/purines, uracil, thiamine, allantoin; Alpha/beta hydrolase family; Protein-L-isoaspartate(D-aspartate) O-methyltransferase (PCMT); Protein of unknown function (DUF2370); Mitochondrial carrier protein; Glycosyl hydrolases family 18; Glycosyltransferase family 20; Trehalose-phosphatase; BTB/POZ domain
	SS60	542873 - 585652	302	PF00153; PF10176; PF01135; PF12697; PF02133; PF01532; PF06964; PF00083; PF07690; PF07110; PF00326; PF07859; PF13229; PF13472; PF17996	Mitochondrial carrier protein; Protein of unknown function (DUF2370); Protein-L-isoaspartate(D-aspartate) O-methyltransferase (PCMT); Alpha/beta hydrolase family; Permease for cytosine/purines, uracil, thiamine, allantoin; Glycosyl hydrolase family 47; Alpha-L-arabinofuranosidase C-terminal domain; Sugar (and other) transporter; Major Facilitator Superfamily; EthD domain; Prolyl oligopeptidase family; alpha/beta hydrolase fold; Right handed beta helix region; GDSL-like Lipase/Acylhydrolase family; Carbohydrate esterase 2 N-terminal
	SS429	-	-	-	-
	SS61	-	-	-	-
21	SS434	276381 - 354808	190	-	-
	SS60	65750 - 87139	1337	-	-
	SS429	649988 - 689202	4725	PF00732; PF05199; PF00300; PF00026; PF06985; PF00106; PF08659; PF00083; PF00067; PF00750; PF01794; PF05746; PF08022	GMC oxidoreductase; Histidine phosphatase superfamily (branch 1); Eukaryotic aspartyl protease; Heterokaryon incompatibility protein (HET); short chain dehydrogenase; KR domain; Sugar (and other) transporter; Cytochrome P450; tRNA synthetases class I (R); Ferric reductase like transmembrane component; DALR anticodon binding domain; FAD-binding domain
	SS61	619143 - 676181	5016	-	-
22	SS434	1213330 - 1233687, 0 - 83887	149, 97	PF00117; PF06418; PF07722	Glutamine amidotransferase class-I; CTP synthase N-terminus; Peptidase C26
	SS60	1207533 - 1314480	16	PF00117; PF06418; PF07722	Glutamine amidotransferase class-I; CTP synthase N-terminus; Peptidase C26
	SS429	1179766 - 1276018	35	PF00117; PF06418	Glutamine amidotransferase class-I; CTP synthase N-terminus

	SS61	1208138 - 1325779	31	PF00117; PF06418	Glutamine amidotransferase class-I; CTP synthase N-terminus

Table S5.3. ProgressiveMauve analysis of one Australian and one Chinese *V. dahliae* genome reveals regions of low sequence identity. Regions with putative genes have been indicated with blue.

Region	Isolate	Location (bp)	Contig	PFAM	Gene content
1	SS60	127336-160171	tig00000374	-	-
	Vd991	-	-	-	-
2	SS60	203952-240435	tig00000374	-	-
	Vd991	-	-	-	-
3	SS60	248677-287141; 0-11554	tig00000374; tig00046851	PF00005; PF00664	ABC transporter; ABC transporter transmembrane region
	Vd991	-	-	-	-
4	SS60	1455396-1479110	tig00046851	-	-
	Vd991	1456713-end	NVYA01000124.1	-	-
5	SS60	2090211-2244244	tig00046851	PF00198; PF00364	2-oxoacid dehydrogenases acyltransferase (catalytic domain); Biotin-requiring enzyme
	Vd991	616267-616451; 0-9675; 0-27397	NVYA01000083.1; NVYA01000128.1; NVYA01000013.1	PF01734; PF11815; PF00106; PF13561; PF00198; PF00364; PF05705; PF03291; PF01399; PF10602; PF00155; PF00471; PF08159; PF06333; PF00135; PF01048; PF09759; PF00071; PF08355; PF08356; PF08477;	Patatin-like phospholipase; Domain of unknown function (DUF3336); short chain dehydrogenase; Enoyl-(Acyl carrier protein) reductase; 2-oxoacid dehydrogenases acyltransferase (catalytic domain); Biotin-requiring enzyme; Eukaryotic protein of unknown function (DUF829); mRNA capping enzyme; PCI domain; 26S proteasome subunit RPN7; Aminotransferase class I and II; Ribosomal protein L33; NUC153 domain; Mediator complex subunit 13 C-terminal domain; Carboxylesterase family; Phosphorylase superfamily; Spinocerebellar ataxia type 10 protein domain; Ras family; EF hand associated; Ras of Complex, Roc, domain of DAPkinase; WD40 repeat; Sugar (and other) transporter; Glycosyl hydrolases family 32; Arrestin; Major Facilitator Superfamily; Mannosidase Ig/CBM-like domain; FMN-dependent dehydrogenase; Serine carboxypeptidase; Formamidopyrimidine-DNA glycosylase H2TH domain; PAS domain; Zinc-finger of the MIZ type in Nse subunit; RING-type zinc-finger; Hexokinase; Glucosamine-6-phosphate isomerases; NDT80 / PhoG like DNA-binding family; Amidohydrolase

				PF00400; PF00083; PF07690; PF00251; PF08244; PF02752; PF07690; PF17786; PF01070; PF00450; PF06831; PF13426; PF11789; PF13445; PF00349; PF03727; PF01182; PF05224; PF01979; PF00583; PF00933	family; Acetyltransferase (GNAT) family; Glycosyl hydrolase family 3
6	SS60	304878-310351	tig00000259	PF10294	Lysine methyltransferase
	Vd991	334586-340059	NVYA01000013.1	PF00432	Prenyltransferase and squalene oxidase repeat
7	SS60	0-51168	tig00046854	-	-
	Vd991	-	-	-	-
8	SS60	370945-380066	tig00046854	PF00141; PF01822	Peroxidase; WSC domain
	Vd991	323387-330683	NVYA01000024.1	PF00141; PF01822	Peroxidase; WSC domain
9	SS60	929145-941914	tig00046854	-	-
	Vd991	885235-end; 0-6450	NVYA01000024.1; NVYA01000098.1	-	-
10	SS60	1194530-1204596	tig00046854	PF17100	NWD NACHT-NTPase
	Vd991	111752-133642	NVYA01000135.1	PF05024; PF05254; PF00570; PF01612; PF08066; PF00389; PF02826; PF00137; PF01853; PF01593; PF13450; PF13516	N-acetylglucosaminyl transferase component (Gpi1); Uncharacterised protein family (UPF0203); HRDC domain; 3'-5' exonuclease; PMC2NT (NUC016) domain; D-isomer specific 2-hydroxyacid dehydrogenase; ATP synthase subunit C; MOZ/SAS family; Flavin-containing amine oxidoreductase; NAD(P)-binding Rossmann-like domain; Leucine Rich repeat
11	SS60	740976-744396	tig00000206	-	-

	Vd991	1225566-1228303	NVYA01000135.1	PF00176; PF00271; PF04851	SNF2 family N-terminal domain; Helicase conserved C-terminal domain; Type III restriction enzyme, res subunit
12	SS60	753131-778127; 0-67195	tig00000206; tig00001342	PF00083; PF07690	Sugar (and other) transporter; Major Facilitator Superfamily
	Vd991	0-17629	NVYA01000118.1	PF00083; PF07690	Sugar (and other) transporter; Major Facilitator Superfamily
13	SS60	129217-149283	tig00001342	-	-
	Vd991	-	-	-	-
14	SS60	521780-534549	tig00001342	-	-
	Vd991	375522-388291	NVYA01000091.1	-	-
15	SS60	19689-43403	tig00000092	-	-
	Vd991	19677-43391	NVYA01000001.1	-	-
16	SS60	492152-523163	tig00000092	-	-
	Vd991	495789-524976	NVYA01000001.1	-	-
17	SS60	1021165-1039407	tig00000092	-	-
	Vd991	1026626-1044868	NVYA01000001.1	-	-
18	SS60	1181694-1241892	tig00000092	PF00025; PF00071; PF08477; PF00994; PF00241; PF10609; PF03130; PF13646; PF01096; PF12765; PF12830; PF14636; PF00610; PF12257; PF01191; PF03871	ADP-ribosylation factor family; Ras family; Ras of Complex, Roc, domain of DAPkinase ; Probable molybdopterin binding domain; Cofilin/tropomyosin-type actin-binding protein; NUBPL iron-transfer P-loop NTPase; PBS lyase HEAT-like repeat; HEAT repeats; Transcription factor S-II; HEAT repeat associated with sister chromatid cohesion; Sister chromatid cohesion C-terminus; Folliculin-interacting protein N-terminus; Domain found in Dishevelled, Egl-10, and Pleckstrin (DEP); Vacuolar membrane-associated protein Iml1; RNA polymerase Rpb5
	Vd991	1187154-1247352	NVYA01000001.1	PF00025; PF00071; PF08477; PF00994; PF00241; PF01656; PF10609; PF03130; PF13646;	ADP-ribosylation factor family; Ras family; Ras of Complex, Roc, domain of DAPkinase ; Probable molybdopterin binding domain; Cofilin/tropomyosin-type actin-binding protein; CobQ/CobB/MinD/ParA nucleotide binding domain; NUBPL iron-transfer P-loop NTPase; PBS lyase HEAT-like repeat; HEAT repeats; Transcription factor S-II; HEAT repeat associated with sister chromatid cohesion; Sister chromatid cohesion C-terminus; Folliculin-interacting protein N-terminus;

				PF01096; PF12765; PF12830; PF14636; PF00610; PF12257; PF01191; PF03871	Domain found in Dishevelled, Egl-10, and Pleckstrin (DEP); Vacuolar membrane-associated protein Iml1; RNA polymerase Rpb5
19	SS60	2201910-2236584	tig00000065	-	-
	Vd991	0-16740	NVYA01000080.1	-	-
20	SS60	617064-635140	tig00000285	-	-
	Vd991	-	-	-	-
21	SS60	161755-170876	tig00046855	-	-
	Vd991	905058-915318	NVYA01000013.1	PF00076; PF05648; PF00316; PF06966; PF00742; PF03447	RNA recognition motif; Peroxisomal biogenesis factor 11; Fructose-1-6-bisphosphatase, N-terminal domain; Protein of unknown function (DUF1295); Homoserine dehydrogenase
22	SS60	201887-242020	tig00046855	-	-
	Vd991	-	-	-	-
23	SS60	632395-681648	tig00046855	-	-
	Vd991	0-35838	NVYA01000088.1	PF11790; PF00005; PF01061; PF06422; PF14510; PF00457; PF00106; PF04082; PF08659; PF13561; PF00082; PF04113	Glycosyl hydrolase catalytic core; ATP-binding domain of ABC transporters; ABC-2 type transporter; CDR ABC transporter; ABC-transporter; Glycosyl hydrolases family 11; short chain dehydrogenase; Fungal specific transcription factor domain; KR domain; Enoyl-(Acyl carrier protein) reductase; Subtilase family; Gpi16 subunit, GPI transamidase component
24	SS60	1085700-1108232	tig00046855	PF00457; PF00005; PF01061; PF06422; PF14510; PF11790	Glycosyl hydrolases family 11; ABC transporter; ABC-2 type transporter; CDR ABC transporter; Glycosyl hydrolase catalytic core
	Vd991	441715-462161	NVYA01000088.1	-	-
25	SS60	128377-142970	tig00000001	PF02417; PF07992; PF13738	Chromate transporter; Pyridine nucleotide-disulphide oxidoreductase
	Vd991	-	-	-	-
26	SS60	252421-265190	tig00000001	PF00501; PF00378; PF16113	AMP-binding enzyme; Enoyl-CoA hydratase/isomerase
	Vd991	-	-	-	-

27	SS60	515103-547939	tig00000001	PF08241; PF13489; PF13649	Methyltransferase domain
	Vd991	253568-269552; 0-34384	NVYA01000095.1; NVYA01000112.1	PF03443; PF16499; PF17801; PF09056; PF00378; PF16113; PF00590; PF13241; PF14823; PF14824; PF09243; PF00085; PF02598; PF12708	Glycosyl hydrolase family 61; Alpha galactosidase A; Alpha galactosidase C-terminal beta sandwich domain; Prokaryotic phospholipase A2; Enoyl-CoA hydratase/isomerase; Tetrapyrrole (Corrin/Porphyrin) Methylases; Putative NAD(P)-binding; Sirohaem biosynthesis protein C-terminal; Sirohaem biosynthesis protein central; Mitochondrial small ribosomal subunit Rsm22; Thioredoxin; Putative RNA methyltransferase; Pectate lyase superfamily protein
28	SS60	776507-814814	tig00000001	-	-
	Vd991	6533-12129	NVYA01000140.1	-	-
29	SS60	1024596-1037365	tig00000001	-	-
	Vd991	0-7948	NVYA01000079.1	-	-
30	SS60	1776704-1787649	tig00000001	PF08241; PF13489	Methyltransferase domain
	Vd991	747287-758232	NVYA01000079.1	-	-
31	SS60	1798594-1906221	tig00000001	-	-
	Vd991	-	-	-	-
32	SS60	2021145-2083167	tig00000001	-	-
	Vd991	118733-end; 0-end; 0-12947	NVYA01000109.1; NVYA01000012.1; NVYA01000104.1	PF04616; PF00067; PF01494	Glycosyl hydrolases family 43; Cytochrome P450; FAD binding domain
33	SS60	2174376-2181673	tig00000001	-	-
	Vd991	105980-113277	NVYA01000104.1	PF10382; PF00018; PF03114; PF07653; PF14604	Protein of unknown function (DUF2439); SH3 domain; BAR domain; Variant SH3 domain
34	SS60	2243695-2285651	tig00000001	PF00432; PF13243	Prenyltransferase and squalene oxidase repeat; Squalene-hopene cyclase C-terminal domain
	Vd991	-	-	-	-
35	SS60	2887260-2918272	tig00000001	-	-
	Vd991	0-14625	NVYA01000102.1	PF00645; PF00454; PF02259; PF02260; PF08064	Poly(ADP-ribose) polymerase and DNA-Ligase Zn-finger region; Phosphatidylinositol 3- and 4-kinase; FAT domain; FATC domain; UME (NUC010) domain

36	SS60	2989415-3011305	tig00000001	PF00072; PF00512; PF02518; PF08447; PF13426; PF04117	Response regulator receiver domain; His Kinase A (phospho-acceptor) domain; Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase; PAS fold; PAS domain; Mpv17 / PMP22 family
	Vd991	80295-109482	NVYA01000102.1	PF04616; PF00448; PF02881; PF04086; PF03878	Glycosyl hydrolases family 43; SRP54-type protein; Signal recognition particle, alpha subunit, N-terminal; YIF1
37	SS60	3113459-3140822	tig00000001	-	-
	Vd991	0-24458	NVYA01000082.1	PF00365	Phosphofructokinase
38	SS60	3731857-3894210	tig00000001	PF01205	Uncharacterized protein family UPF0029
	Vd991	-	-	-	-
39	SS60	0-113993	tig00001337	-	-
	Vd991	539153-547416	NVYA01000087.1	-	-
40	SS60	622029-649392	tig00001337	-	-
	Vd991	0-39327	NVYA01000087.1	PF04516	CP2 transcription factor
41	SS60	744249-753370	tig00001337	-	-
	Vd991	97758-105055	NVYA01000092.1	PF02204; PF05764; PF08265	Vacuolar sorting protein 9 (VPS9) domain; YL1 nuclear protein
42	SS60	1163811-1185701	tig00001337	-	-
	Vd991	515496-539210	NVYA01000092.1	PF00067; PF00856	Cytochrome P450; SET domain
43	SS60	1413724-1421021	tig00001337	PF12271; PF04056; PF13519; PF00903; PF13669	Chitin synthase export chaperone; Ssl1-like; von Willebrand factor type A domain; Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily
	Vd991	769057-772706	NVYA01000092.1	-	-
44	SS60	1822886-1837480	tig00001337	-	-
	Vd991	1177675-1192268	NVYA01000092.1	PF00564; PF00571; PF00096; PF00005; PF01061; PF06422; PF12698; PF14510	PB1 domain; CBS domain; Zinc finger, C2H2 type; ABC transporter; ABC-2 type transporter; CDR ABC transporter; ABC-2 family transporter protein
45	SS60	2005668-2040327	tig00001337	-	-
	Vd991	1358269-1394752	NVYA01000092.1	PF01042; PF01593; PF13450;	Endoribonuclease L-PSP; Flavin containing amine oxidoreductase; NAD(P)-binding Rossmann-like domain; NmrA-like family; NAD(P)H-binding; Amidase; Peptidase

				PF05368; PF13460; PF01425; PF04389; PF00324; PF13520; PF05870	family M28; Amino acid permease; Phenolic acid decarboxylase (PAD)
46	SS60	2177141-2212354; 0-16941	tig00001337; tig00001339	PF03647; PF05859	Transmembrane proteins 14C; Mis12 protein
	Vd991	1529742-1571698	NVYA01000092.1	PF07287; PF00535; PF13506; PF13632; PF13641; PF01670; PF00984; PF03720; PF04616; PF17851; PF00107; PF08240; PF07690; PF07470	Acyclic terpene utilisation family protein AtuA; Glycosyl transferase family 2; Glycosyl transferase family 21; Glycosyl transferase family group 2; Glycosyltransferase like family 2; Glycosyl hydrolase family 12; UDP-glucose/GDP-mannose dehydrogenase family; Glycosyl hydrolases family 43; Beta xylosidase C-terminal Concanavalin A-like domain; Zinc-binding dehydrogenase; Alcohol dehydrogenase GroES-like domain; Major Facilitator Superfamily; Glycosyl Hydrolase Family 88
47	SS60	117272-128217	tig00001339	-	-
	Vd991	1672028-1691359	NVYA01000092.1	PF00083; PF18120; PF04616; PF17851; PF02330; PF01237	Sugar (and other) transporter; Domain of unknown function (DUF5597); Glycosyl hydrolases family 43; Beta xylosidase C-terminal Concanavalin A-like domain; Mitochondrial glycoprotein; Oxysterol-binding protein
48	SS60	514027-541390	tig00001339	PF02823; PF01613; PF00069; PF07714	ATP synthase, Delta/Epsilon chain, beta-sandwich domain; Flavin reductase like domain; Protein kinase domain; Protein tyrosine and serine/threonine kinase
	Vd991	389937-417299	NVYA01000057.1	PF02823; PF01613; PF00069; PF07714	ATP synthase, Delta/Epsilon chain, beta-sandwich domain; Flavin reductase like domain; Protein kinase domain; Protein tyrosine and serine/threonine kinase
49	SS60	32622-96468	tig00000291	-	-
	Vd991	-	-	-	-
50	SS60	127479-184029	tig00000291	PF03152	Ubiquitin fusion degradation protein UFD1
	Vd991	-	-	-	-
51	SS60	233982-248119	tig00000291	-	-
	Vd991	62566-75791	NVYA01000094.1	-	-
52	SS60	452609-466746	tig00000291	-	-
	Vd991	-	-	-	-
53	SS60	184854-197623	tig00000137	-	-

	Vd991	185042-196671	NVYA01000089.1	PF00206; PF14698; PF00501; PF00550	Lyase; Argininosuccinate lyase C-terminal; AMP-binding enzyme; Phosphopantetheine attachment site
54	SS60	443907-462605	tig00000137	-	-
	Vd991	-	-	-	-
55	SS60	568864-581405	tig00000137	PF00023; PF12796; PF13606; PF13637; PF13857	Ankyrin repeats
	Vd991	113193-125507	NVYA01000002.1	PF00122; PF00689; PF00690; PF00702; PF13246	E1-E2 ATPase; Cation transporting ATPase; haloacid dehalogenase-like hydrolase; Cation transport ATPase (P-type)
56	SS60	647231-666157	tig00000137	-	-
	Vd991	191177-210331	NVYA01000002.1	PF00732; PF05199; PF05577; PF00457; PF00457; PF01406; PF08704	GMC oxidoreductase; Serine carboxypeptidase S28; Glycosyl hydrolases family 11; tRNA synthetases class I (C) catalytic domain; tRNA methyltransferase complex GCD14 subunit
57	SS60	780715-785504	tig00000137	-	-
	Vd991	324343-330271	NVYA01000002.1	PF04082; PF01715; PF12171; PF12874	Fungal specific transcription factor domain; IPP transferase; Zinc-finger double-stranded RNA-binding; Zinc-finger of C2H2 type
58	SS60	789836-791204	tig00000137	-	-
	Vd991	-	-	-	-
59	SS60	795765-799869	tig00000137	-	-
	Vd991	173049-175101	NVYA01000078.1	-	-
60	SS60	803290-806482	tig00000137	-	-
	Vd991	178977-182170	NVYA01000078.1	-	-
61	SS60	882256-885676	tig00000137	-	-
	Vd991	258101-261293	NVYA01000078.1	-	-
62	SS60	0-13090	tig00000226	-	-
	Vd991	528661-532309	NVYA01000114.1	-	-
63	SS60	529278-559377	tig00000226	-	-
	Vd991	-	-	-	-

64	SS60	797015-856742; 0-225297	tig00000226; tig00000190	PF00275; PF01202; PF01487; PF01761; PF08501; PF13685; PF02969; PF15511; PF00125; PF06544; PF08572; PF00623; PF04983; PF04997; PF04998; PF05000; PF00096; PF06733; PF06777; PF13307; PF01494; PF07976; PF02902; PF03707; PF00004; PF00400; PF17862; PF03909; PF08567; PF00676; PF02779; PF16078; PF16870; PF09462; PF00004; PF07724; PF07728; PF10431; PF02806; PF02922; PF02936; PF00006; PF02874; PF00172; PF13191; PF03151; PF04037; PF04046; PF09733	EPSP synthase (3-phosphoshikimate 1-carboxyvinyltransferase); Shikimate kinase; Type I 3-dehydroquinase; 3-dehydroquinate synthase; Shikimate dehydrogenase substrate binding domain; Iron-containing alcohol dehydrogenase; TATA box binding protein associated factor (TAF); Centromere kinetochore component CENP-T histone fold; Core histone H2A/H2B/H3/H4; Protein of unknown function (DUF1115); pre-mRNA processing factor 3 (PRP3); RNA polymerase Rpb1; Zinc finger, C2H2 type; DEAD_2; Helical and beta-bridge domain; Helicase C-terminal domain; FAD binding domain; Phenol hydroxylase, C-terminal dimerisation domain; Ulp1 protease family, C-terminal catalytic domain; Bacterial signalling protein N terminal repeat; ATPase family associated with various cellular activities (AAA); WD domain, G-beta repeat; AAA+ lid domain; BSD domain; TFIIH p62 subunit, N-terminal domain; Dehydrogenase E1 component; Transketolase, pyrimidine binding domain; 2-oxoglutarate dehydrogenase; Mus7/MMS22 family; ATPase family associated with various cellular activities (AAA); AAA domain (Cdc48 subfamily); AAA domain (dynein-related subfamily); C-terminal, D2-small domain, of ClpB protein; Alpha amylase, C-terminal all-beta domain; Carbohydrate-binding module 48 (Isoamylase N-terminal domain); Cytochrome c oxidase subunit IV; ATP synthase alpha/beta family; Fungal Zn(2)-Cys(6) binuclear cluster domain; AAA ATPase domain; Triose-phosphate Transporter family; Domain of unknown function (DUF382); PSP; VEFS-Box of polycomb protein

	Vd991	0-6538; 0-2267; 0-132052	NVYA01000154.1; NVYA01000027.1; NVYA01000035.1	PF00319; PF01794; PF00533; PF12738; PF16589; PF01979; PF09362; PF00069; PF00179; PF01423; PF03517; PF12756; PF00326; PF00930; PF00933; PF12697; PF01699; PF10306; PF00334; PF01055; PF13802; PF17137; PF00924; PF02798; PF13409; PF13417; PF12585	SRF-type transcription factor (DNA-binding and dimerisation domain); Ferric reductase like transmembrane component; BRCA1 C Terminus (BRCT) domain; twin BRCT domain; BRCT domain, a BRCA1 C-terminus domain; Amidohydrolase family; Domain of unknown function (DUF1996); Protein kinase domain; Ubiquitin-conjugating enzyme; LSM domain; Regulator of volume decrease after cellular swelling; C2H2 type zinc-finger (2 copies); Prolyl oligopeptidase family; Dipeptidyl peptidase IV (DPP IV) N-terminal region; Glycoside hydrolase family 3; Alpha/beta hydrolase family; Sodium/calcium exchanger protein; Hypothetical protein FLILHELTA; Nucleoside diphosphate kinase; Glycosyl hydrolases family 31; Galactose mutarotase-like; Domain of unknown function (DUF5110); Mechanosensitive ion channel; Glutathione S-transferase, N-terminal domain; Protein of unknown function (DUF3759)
65	SS60	424105-432542	tig00000190	-	-
	Vd991	331110-339319	NVYA01000035.1	PF01541; PF00628; PF01388; PF02373; PF02375; PF02928; PF08429	GIY-YIG catalytic domain; PHD-finger; ARID/BRIGHT DNA binding domain; JmjC domain, hydroxylase; jmjN domain; C5HC2 zinc finger; PLU-1-like protein
66	SS60	653785-665414	tig00000190	-	-
	Vd991	560929-574383	NVYA01000035.1	-	-
67	SS60	948841-958646	tig00000190	-	-
	Vd991	-	-	-	-
68	SS60	998550-1023860	tig00000190	PF00319; PF00533; PF12738	SRF-type transcription factor (DNA-binding and dimerisation domain); BRCA1 C Terminus (BRCT) domain; twin BRCT domain
	Vd991	464626-489253	NVYA01000002.1	PF03403; PF00533; PF05368; PF13460; PF00719;	Platelet-activating factor acetylhydrolase, isoform II; BRCT domain; NmrA-like family; NAD(P)H-binding; Inorganic pyrophosphatase; Serine carboxypeptidase; alpha/beta hydrolase fold; RTA1 like protein

				PF00450; PF07859; PF04479	
69	SS60	1052363-1056696	tig00000190	-	-
	Vd991	431563-435895	NVYA01000002.1	-	-
70	SS60	1062168-1065360	tig00000190	-	-
	Vd991	423126-426318	NVYA01000002.1	PF00246	Zinc carboxypeptidase
71	SS60	1070377-1089172; 0-19394	tig00000190; tig00000255	PF05426; PF13229	Alginate lyase; Right handed beta helix region
	Vd991	378890-417882	NVYA01000002.1	PF00857; PF12253; PF03105; PF03124; PF02127; PF03171; PF14226; PF01546; PF07687; PF00083; PF07690	Isochorismatase family; Chromatin assembly factor 1 subunit A; SPX domain; EXS family; Aminopeptidase I zinc metalloprotease (M18); 2OG-Fe(II) oxygenase superfamily; non-haem dioxygenase in morphine synthesis N-terminal; Peptidase family M20/M25/M40; Peptidase dimerisation domain; Sugar (and other) transporter; Major Facilitator Superfamily
72	SS60	53606-60903	tig00000255	-	-
	Vd991	-	-	-	-
73	SS60	153708-165794	tig00000255	-	-
	Vd991	53936-66021	NVYA01000078.1	-	-
74	SS60	205242-206610	tig00000255	-	-
	Vd991	-	-	-	-
75	SS60	231709-249104; 0-10606	tig00000255; tig00000295	PF00319; PF00533	SRF-type transcription factor (DNA-binding and dimerisation domain); BRCT domain
	Vd991	555869-584144	NVYA01000002.1	PF00069	Protein kinase domain
76	SS60	516032-545903	tig00000295	-	-
	Vd991	1091166-1095814; 0-18638	NVYA01000002.1; NVYA01000119.1	-	-
77	SS60	111152-120273	tig00000076	-	-
	Vd991	111372-119808	NVYA01000086.1	-	-
78	SS60	423183-435040	tig00000076	-	-

	Vd991	423824-555202; 0-12657	NVYA01000086.1; NVYA01000085.1	PF09733; PF04037; PF04046; PF03151; PF13191; PF14630; PF00172; PF00006; PF02874; PF02936; PF02806; PF02922; PF00004; PF07724; PF07728; PF10431; PF00676; PF02779; PF16078; PF16870; PF03909; PF08567; PF00004; PF00400; PF17862; PF03707; PF00615; PF02902; PF01494; PF07976; PF06777; PF13307; PF00623; PF04983; PF04997; PF04998; PF05000; PF06544; PF08572; PF00125; PF02969; PF15511; PF00275; PF01202; PF01487; PF01761; PF08501; PF13685;	VEFS-Box of polycomb protein; Domain of unknown function (DUF382); PSP; Triose-phosphate Transporter family; AAA ATPase domain; Origin recognition complex (ORC) subunit 5 C-terminus; Fungal Zn(2)-Cys(6) binuclear cluster domain; ATP synthase alpha/beta family; Cytochrome c oxidase subunit IV; Alpha amylase, C-terminal all-beta domain; Carbohydrate-binding module 48 (Isoamylase N-terminal domain); ATPase family associated with various cellular activities (AAA); AAA domain (Cdc48 subfamily); AAA domain (dynein-related subfamily); C-terminal, D2-small domain, of ClpB protein; Dehydrogenase E1 component; Transketolase, pyrimidine binding domain; 2-oxoglutarate dehydrogenase; BSD domain; TFIIH p62 subunit, N-terminal domain; ATPase family associated with various cellular activities (AAA); WD domain, G-beta repeat; AAA+ lid domain; Bacterial signalling protein N terminal repeat; Regulator of G protein signaling domain; Ulp1 protease family, C-terminal catalytic domain; FAD binding domain; Phenol hydroxylase, C-terminal dimerisation domain; Helical and beta-bridge domain; Helicase C-terminal domain; RNA polymerase Rpb1; Protein of unknown function (DUF1115); pre-mRNA processing factor 3 (PRP3); Core histone H2A/H2B/H3/H4; TATA box binding protein associated factor (TAF); Centromere kinetochore component CENP-T histone fold; EPSP synthase (3-phosphoshikimate 1-carboxyvinyltransferase); Shikimate kinase; Type I 3-dehydroquinase; 3-dehydroquinate synthase; Shikimate dehydrogenase substrate binding domain; Iron-containing alcohol dehydrogenase; Common central domain of tyrosinase; Pyridine nucleotide-disulphide oxidoreductase

				PF00264; PF07992; PF13738	
79	SS60	443249-445073	tig00000076	-	-
	Vd991	20638-22462	NVYA01000085.1	-	-
80	SS60	1026130-1061245	tig00000076	-	-
	Vd991	604722-606520; 0-11436	NVYA01000085.1; NVYA01000103.1	-	-
81	SS60	1437154-1459589	tig00000076	PF00215	Orotidine 5'-phosphate decarboxylase / HUMPS family
	Vd991	388191-409625	NVYA01000103.1	PF03061; PF04757; PF01822; PF00149; PF04547; PF14604; PF00173; PF01070; PF07690	Thioesterase superfamily; Pex2 / Pex12 amino terminal region; WSC domain; Calcineurin-like phosphoesterase; Calcium-activated chloride channel; SH3 domain; Cytochrome b5; FMN-dependent dehydrogenase; Major Facilitator Superfamily
82	SS60	307205-473088	tig00000106	-	-
	Vd991	-	-	-	-
83	SS60	813359-899552	tig00000106	PF01728; PF07690; PF07690; PF01055; PF03134; PF06420; PF03099; PF09825; PF03656; PF13229	FtsJ-like methyltransferase; Major Facilitator Superfamily; Glycosyl hydrolases family 31; TB2/DP1, HVA22 family; Mitochondrial genome maintenance MGM101; Biotin/lipoate A/B protein ligase family; Biotin-protein ligase, N terminal; Pam16; Right handed beta helix region
	Vd991	872818-956502	NVYA01000114.1	PF01728; PF07690; PF07690; PF01055; PF03134; PF06420; PF03099; PF09825; PF03656; PF13229	FtsJ-like methyltransferase; Major Facilitator Superfamily; Glycosyl hydrolases family 31; TB2/DP1, HVA22 family; Mitochondrial genome maintenance MGM101; Biotin/lipoate A/B protein ligase family; Biotin-protein ligase, N terminal; Pam16; Right handed beta helix region
84	SS60	1100245-1104350	tig00000106	-	-
	Vd991	1159160-1163264	NVYA01000114.1	-	-
85	SS60	1123959-1150866	tig00000106	-	-
	Vd991	1183786-1209325	NVYA01000114.1	-	-

86	SS60	1406091-1421141	tig00000106	-	-
	Vd991	0-4845	NVYA01000115.1	-	-
87	SS60	117149-134706	tig00000021	-	-
	Vd991	0-6899	NVYA01000046.1	-	-
88	SS60	403112-406760	tig00000021	-	-
	Vd991	275977-279511	NVYA01000046.1	-	-
89	SS60	650900-668686	tig00000021	-	-
	Vd991	524661-596162	NVYA01000046.1	PF11917; PF01048; PF00069; PF00400; PF07714	Protein of unknown function (DUF3435); Phosphorylase superfamily; Protein kinase domain; WD domain, G-beta repeat; Protein tyrosine and serine/threonine kinase
90	SS60	723411-728428	tig00000021	-	-
	Vd991	651008-655569	NVYA01000046.1	-	-
91	SS60	908986-931788	tig00000021	-	-
	Vd991	836534-end; 0-3750	NVYA01000046.1; NVYA01000120.1	-	-
92	SS60	984462-1013421	tig00000021	-	-
	Vd991	56822-end; 0-end; 0-6155	NVYA01000120.1; NVYA01000011.1; NVYA01000129.1	PF12739; PF00107; PF13602; PF00282	ER-Golgi trafficking TRAPP I complex 85 kDa subunit; Zinc-binding dehydrogenase; Pyridoxal-dependent decarboxylase conserved domain
93	SS60	1025506-1053097	tig00000021	-	-
	Vd991	-	-	-	-
94	SS60	1201814-1215951	tig00000021	-	-
	Vd991	150464-end; 0-2528	NVYA01000107.1; NVYA01000113.1	-	-
95	SS60	1221196-1227581	tig00000021	-	-
	Vd991	7773-14841	NVYA01000113.1	-	-
96	SS60	1321298-1467550	tig00000021	-	-
	Vd991	-	-	-	-
97	SS60	2431613-2451907	tig00000021	-	-
	Vd991	967565-986491	NVYA01000157.1	PF01636; PF00069; PF08171; PF08311; PF01261;	Phosphotransferase enzyme family; Protein kinase domain; Mad3/BUB1 homology region 2; Mad3/BUB1 homology region 1; Xylose isomerase-like TIM barrel; Diacylglycerol acyltransferase; Galactose oxidase, central

				PF03982; PF13415; PF13418; PF13422; PF13854; PF08325; PF04438	domain; Domain of unknown function (DUF4110); Kelch motif; WLM domain; HIT zinc finger
98	SS60	2595105-2601718	tig00000021	-	-
	Vd991	1130373-end; 0-7059	NVYA01000157.1; NVYA01000100.1	-	-
99	SS60	2679607-2691920	tig00000021	-	-
	Vd991	85132-96761	NVYA01000100.1	PF04616; PF06738	Glycosyl hydrolases family 43; Putative threonine/serine exporter
100	SS60	2806069-end; 0-30781	tig000000211; tig00046857	-	-
	Vd991	212074-end; 0-4583	NVYA01000100.1; NVYA01000090.1	PF00067	Cytochrome P450
101	SS60	191715-194223	tig00046857	-	-
	Vd991	166134-168186	NVYA01000090.1	PF01619; PF03807; PF14748; PF05199	Proline dehydrogenase; NADP oxidoreductase coenzyme F420-dependent; Pyrroline-5-carboxylate reductase dimerisation; GMC oxidoreductase
102	SS60	463034-end; 0-37638	tig00046857; tig00000302	-	-
	Vd991	438137-440645	NVYA01000090.1	-	-
103	SS60	302579-321277	tig00000302	-	-
	Vd991	-	-	-	-
104	SS60	376915-383755	tig00000302	-	-
	Vd991	56077-64513	NVYA01000096.1	-	-
105	SS60	404277-407014	tig00000302	-	-
	Vd991	84579-87544	NVYA01000096.1	-	-
106	SS60	628111-632215	tig00000016	-	-
	Vd991	630537-633729	NVYA01000146.1	PF00535; PF13632	Glycosyl transferase family 2; Glycosyl transferase family group 2
107	SS60	1174008-1293720	tig00000016	-	-
	Vd991	0-7635	NVYA01000134.1	-	-
108	SS60	1301472-1329063	tig00000016	-	-
	Vd991	0-6261	NVYA01000081.1	PF04059	RNA recognition motif 2
109	SS60	3227764-3232097	tig00000016	PF00390; PF03949; PF07690	Malic enzyme, N-terminal domain; Malic enzyme, NAD binding domain; Major Facilitator Superfamily

	Vd991	1910633-1914966	NVYA01000081.1	PF07690	Major Facilitator Superfamily

Appendix

Attached PDF

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REVIEW

The Verticillium wilt problem in Australian cotton

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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*

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Virulence not linked with vegetative compatibility groups in Australian cotton *Verticillium dahliae* isolates

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Abstract

Verticillium dahliae, the causal agent of Verticillium wilt, is a soil-borne ascomycete that infects numerous agriculturally important crops globally, including cotton. As a billion-dollar industry, cotton is economically important to Australia and the management of disease such as Verticillium wilt is key for the success of the industry. Internationally, defoliating *V. dahliae* isolates belonging to Vegetative Compatibility Group (VCG) 1A cause severe damage to cotton, while non-defoliating VCG2A isolates result in significantly less disease. However, in Australia, VCG2A is causing more severe damage to crops in the field than the defoliating VCG1A. This study aimed to replicate field observations in controlled greenhouse conditions. We examined and compared disease symptoms on a range of Australian commercial cotton varieties when inoculated with different *V. dahliae* VCGs. Seedlings were root dipped in conidial suspensions and assessed over seven weeks. The final disease score, disease over time and root length were analysed. Plant mortality resulted from both *V. dahliae* VCG1A and VCG2A isolates across all cotton varieties used, confirming that there are virulent VCG2A isolates present in Australia. To our knowledge, although virulent on other plant hosts, *V. dahliae* VCG2A has not previously been reported to be highly virulent in cotton. We infer that virulence cannot be defined solely by VCG in Australian *V. dahliae* isolates causing disease in cotton.

Keywords: Defoliating; *Gossypium hirsutum*; Non-defoliating; Verticillium; Wilt.