FINAL REPORT: RRDP1723

This work was undertaken as part of the Digital Technologies for Dynamic Management of Disease, Stress and Yield project, through funding from the Australian Government Department of Agriculture as part of its Rural R&D for Profit program

Managing Verticillium Risk for Cotton

Contents

Managing Verticillium Risk for Cotton 1

Part 1 - Summary Details 3

Part 2 – Contact Details 3

Part 3 – Executive Summary 4

Plain English Summary 4

Executive Summary 4

Part 4 – Evaluation Report 5

Background 5

Performance summary 6

Part 5 Technical Report 7

Detailed objectives 7

Methods 8

Results 23

Outcomes 52

a) Technical advances achieved 52

b) Other information developed from research 52

Conclusion 53

Future R,D&E Opportunities and lessons learnt 53

Extension, Publication and Media 54

Part 1 - Summary Details

GRANT: Digital technologies for dynamic management of disease, stress and yield

CRDC ID: RRDP1723

Project Title: Managing Verticillium Risk for Cotton

Project Start Date: 01/07/2016 **Project Completion Date:** 31/12/2019

CRDC Goal: **Increase productivity and profitability on cotton farms**

CRDC Key Focus Area : **Protection From Biotic Threats And Environmental Stresses**

Part 2 – Contact Details

Administrator: Michelle Smith

Organisation: NSW DPI

Postal Address:	Locked Bag 21, Orange, NSW, 2800

Ph: 0263913239 Fax: E-mail: external.funding@dpi.nsw.gov.au

Principal Researcher: Dr Karen Kirkby

Organisation: NSW DPI

Postal Address:	Locked Bag 1000, Narrabri, NSW, 2390

Ph: 0267992454 Fax: E-mail: karen.kirkby@dpi.nsw.gov.au

Supervisor: Dr Will Cuddy

Organisation: NSW DPI

Postal Address:	Private Mail Bag 4008, Narellan, NSW, 2567

Ph: 0246406515 Fax: E-mail: will.cuddy@dpi.nsw.gov.au

Researcher 2: Dr Toni Chapman

Organisation: NSW DPI

Postal Address:	Private Mail Bag 4008, Narellan, NSW, 2567

Ph: 0246406219 Fax: E-mail: toni.chapman@dpi.nsw.gov.au

Signature of Research Provider Representative:

Date submitted: ________________________

Part 3 – Executive Summary

Plain English Summary

The aim of this project was to develop tool/s to inform grower decision making with regards to Verticillium wilt. Verticillium wilt is caused by the soil-borne fungal pathogen *Vertillicum dahliae*. Once introduced to a field, *V. dahliae* is considered impossible to eradicate. Failure to control the build up of disease inoculum can result in very large yield losses and fields becoming unsuitable for cotton production. Reports from growers estimate yield losses range from 10–62 per cent. As there are no control options for Verticillium, recommended best management is to rotate out of cotton to non-host crops until levels of *V. dahliae*. sufficiently reduce. Understanding the risk of verticillium disease prior to planting would mean growers could make informed decisions about whether it was safe to go back to cotton, potentially preventing significant yield losses.

Key outcomes:

- Diversity of *V.dahliae* virulence even within strains is quite high and some Australian isolates can cause more severe damage compared to international isolates.
- The development of a *V. dahliae* specific inoculum quantification assay was extremely challenging and semi-successful. The molecular soil assay can detect *V. dahliae* and the sensitivity of detection was 10-30 ppg in naturally infected soil. This is not sensitive enough when one propagule per gram of soil is enough to cause disease in more severe strains. A disease matrix was developed to industry that provides a guide to disease risk.
- A methodology was developed to determine the strain (VCG) of *V.dahliae* in soil using a combination of the dry plating soil assay and sequencing.
- A plant tissue molecular assay was developed that is faster than traditional isolating techniques and is capable of diagnosing *V. dahliae* and providing VCG in a higher proportion of infected plants. This molecular tool means industry has ability to quickly diagnose Verticillium down to VCG and growers and consultants can quickly determine which strain of *V.dahliae* they have, enabling Verticillium risk to be factored into decision to grow cotton.

Executive Summary

Using *V. dahliae* cultures in the long-term culture collection and those obtained throughout this project enabled the team to assign presumptive Vegetative Compatibility Groups (VCG's) without the use of Nit Mutants and further investigate the genetic diversity between Australian isolates. Molecular studies indicated the Australian defoliating cotton isolates are distinct from the defoliating isolates in the USA. Analysis of the USA VCG1A isolates revealed they are genetically different to the Australian VCG1A. Molecular testing has also demonstrated the presence of both pathogenic and non-pathogenic VCG1A isolates. Australian VCG1A does not always cause severe crop damage as reported overseas, while VCG2A can cause more severe damage compared to international isolates. This project has also identified that the Australian VCG2A has two distinct molecular groups which correlates with their pathogenicity (non-defoliating and defoliating) in cotton.

Isolates were also used in many laboratory and pot experiments. These were particularly useful for inoculating soil with a known inoculum to determine the minimum number of propagules required to cause disease for each VCG. Soil isolation techniques were optimised for estimating inoculum levels as well as a protocol for soil sampling in cotton fields. This work provides guidance on where to sample, how deep to sample, how many samples are required. It also outlines the methods for soil preparation and isolation techniques such as the amount of soil to be isolated per replicate and what semi-selective media to use.

Once an effective method for estimating inoculum levels was established using the dry plating technique, work was undertaken to determine the minimum inoculum level required to cause disease in fields. Extensive plots trials in commercial cotton fields were used to establish a disease risk matrix and thresholds for minimum disease incidence. Two observations of the collected data stood out. First, a large sample size of pre-plant inoculum levels showed there was an upward trend - as the ppg increased, so too did the minimum disease incidence. Second, there was considerable variation within any inoculum level. For example, in plots with 2 ppg, the disease incidence ranged from 10 - 100%.

The effect of long-term rotation following cotton with non-hosts such as durum wheat and sorghum on inoculum levels was evaluated over time. The inoculum levels significantly decreased following these crops but increased again following the planting of cotton. The value in rotation is when inoculum levels are low before the incidence levels cause yield loss. Care must also be taken when selecting alternative crops to plant in cotton fields. Biofumigant blends containing mustard and Ethiopian cabbage were confirmed hosts to *V. dahliae* as was safflower. Growers should seek advice on what crops are potential hosts for Verticillium prior to planting.

The molecular team tested the published LAMP assay using nine Australian Verticillium isolates from different VCG's, three USA VCG1A isolates and *Verticillium theobromae* as a specificity control. The LAMP assay failed to work, even after multiple optimisation efforts and consultation with the published authors and was not able to differentiate between Australian defoliating and non-defoliating pathotypes.

A plant tissue assay was developed that is faster than traditional isolating techniques and is capable of diagnosing *V. dahliae* and providing VCG in a higher proportion of infected plants. This significantly reduces the turn-around time for diagnostics. This assay was successfully validated by comparing traditional plating assays with the tissue assay on cotton and other crops. The development of a *V. dahliae* specific inoculum quantification assay was extremely challenging and semi-successful. The molecular soil assay can detect *V. dahliae* and the sensitivity of detection was 10-30 ppg in naturally infected soil. This is not sensitive enough when one propagule per gram of soil is enough to cause disease. The VCG of soil inoculum can be determined using a combination of the dry plating soil assay and sequencing.

CRDC provided separate funds to enable PhD student Pearl Dadd-Daigle to travel to the USA to present results and to participate in training at several conferences. Additional funding was made available for USA Master's student Shelby Young to travel to Australia to present the results of the collaborative studies on quantifying inoculum levels using wet and dry plating methods. Travel reports were submitted to CRDC following the funded travel from each student.

Part 4 – Evaluation Report

Background

Once introduced to a field or farm, *V. dahliae* is considered impossible to eradicate. Failure to control the buildup of inoculum can result in very large yield losses and fields becoming unsuitable for cotton production. Reports from growers estimate yield losses range from 10–62 per cent. Recently it has been discovered that there are multiple strains of *V.dahliae* affecting cotton in Australia. As there are no control options for Verticillium, recommended best management is to rotate out of cotton to non-host crops. Understanding the risk of verticillium disease prior to planting would mean growers could make informed decisions about whether it was safe to go back to cotton, potentially preventing significant losses.

With Verticillium wilt disease being a significant constraint on cotton production, this project built diagnostic capacity and increased understanding of disease epidemiology. The overall aim of this project was to improve our understanding of the strains of *Verticillium dahliae* and to develop tools that could be used to manage Verticillium risk for cotton.

The molecular diagnostic assay has been developed and tested and is robust for quantifying Verticillium in soil. Testing of the assay on soil samples has shown there is no direct relationship between inoculum quantity and disease severity. Severe disease symptoms were recorded with as little as 1 propagule per gram (ppg) of soil. The Australian VCG1A does not always cause the severe crop damage reported overseas, while VCG2A in Australia can cause more severe damage compared to international isolates. Molecular testing has also demonstrated the presence of both pathogenic and non-pathogenic VCG1A isolates. This project has also identified that the Australian VCG2A has two distinct molecular groups which correlates with their pathogenicity in cotton.

The project team collaborated with Texas A&M researchers and students. To support this project, CRDC provided separate funds to enable PhD student Pearl Dadd-Daigle to travel to the US to present results and to participate in training. Additional funding was made available for USA Master's student Shelby Young to travel to Australia to present the results of the collaborative studies on quantifying inoculum levels in soil. Following this collaboration Ms Young was successful in obtaining an International

Educational Fulbright Scholarship to research Verticillium wilt in Australia commencing July 2019 to May 2020.

Performance summary

Output 5(c) – Develop a molecular tool for Australian strains of Verticillium Dahliae.

The development of a V. dahliae specific inoculum molecular quantification assay was extremely challenging and semi-successful. The test can detect V. dahliae and the sensitivity of detection was 10-30 ppg in naturally infected soil. This is not sensitive enough when one propagule per gram of soil is enough to cause disease in more severe strains.
A plant tissue molecular assay for Australian strains of V.dahliae was developed to provide rapid diagnosis of Verticillium wilt strain. This test will mean growers and consultants can quickly determine which strain of V.dahliae they have, enabling Verticillium risk to be factored into decision to grow cotton.
ACHIEVED

	Output	Activities	Achievement Overview
Year 1	Report on potential for molecular tool to be used based on lab trials	Potential of genetic based quantitative diagnostic tool for Verticillium wilt to better manage disease risk for prior to planting. Assess efficacy and accuracy of the molecular technique vs plating technique	Performance indicator - Replicated lab experiments with naturally infected soil and positive controls with known inoculum levels used to validate the plating method and molecular technique. ACHIEVED
Year 2	Report on potential for molecular tool based on results from field samples, and recommendations for amendment to sampling/testing.	Potential of genetic based quantitative diagnostic tool for Verticillium wilt to better manage disease risk prior to planting validated with field samples. Recommended sampling strategy finalised	Sampling strategies: Performance indicator - Spatial variation of Verticillium inoculum determined to ensure sampling technique is adequate. ACHIEVED Validate Verticillium wilt tool. Performance indicator - Field samples collected and molecular based assay for identifying *V. dahliae* and determining the presence of defoliating or non-defoliating pathotypes within soil samples validated. ACHIEVED however genetic diversity of *V.dahliae* means results are not as informative for decision making as expected based on international experience.
Year 3	Decision making management tool for growers.	Decision making management tool for growers validated.	A plant tissue molecular assay for Australian strains of *V.dahliae* was developed to provide rapid diagnosis of Verticillium wilt strain. This test will mean growers and consultants can quickly determine which strain of *V.dahliae* they have, enabling Verticillium risk to be factored into decision to grow cotton. ACHIEVED

Part 5 Technical Report

Detailed objectives

Objective 1 - **Maintenance, generation and updating the NSW DPI *Verticillium dahliae* culture collection**

- **Milestone 1.0** Collection and maintenance of culture collection and generation of single spore cultures in two locations.
- Performance indicator - Representative samples have been lodged with the NSW DPI Herbarium with unique identification numbers (DAR numbers) assigned. This includes the collection of all *V. dahliae* (of cotton and other hosts) collected during this project. 100% completed.

Objective 2 - **How can we effectively measure *Verticillium dahliae* in soil?**

- **Milestone 2.1:** Establish soil sampling protocol required for soil coring at a field level.
  Performance indicator - Spatial variation of Verticillium inoculum determined to ensure sampling technique is adequate. 100% completed
- **Milestone 2.2:** Efficiency & accuracy of the molecular method vs plating method.
  Performance indicator - Replicated lab experiments with naturally infected soil and positive controls with known inoculum levels used to validate the plating method and molecular technique. 100% completed
- **Milestone 2.2.1:** Soil molecular assay developed.
  Performance indicator - Log pgDNA/g soil pre-planting (x axis) graphed against % incidence of disease (y axis). Need up to 30 samples. 100% completed
- **Milestone 2.2.2:** Plant tissue assay developed.
  Performance indicator - Plant tissue isolations compared with plant assay and VCG typed. 100% completed
- **Milestone 2.3:** Determine the minimum inoculum levels in soil of VCG1A, 2A and 4B V. dahliae needed
  to cause disease.
  Performance indicator - Replicated pot experiments in controlled conditions. 100% completed
- **Milestone 2.4:** Effect of crop rotation on inoculum levels.
  Performance indicator - Return to replicated field experiment at Farm M and take soil cores from existing six GPS points to determine propagules per gram after rotations of cotton/sorghum/wheat rotations and findings reported in progress reports. 100% completed
- **Milestone 2.5:** Validate Verticillium wilt tool.
  Performance indicator - Field samples collected and molecular based assay for identifying *V. dahliae* and determining the presence of defoliating or non-defoliating pathotypes within soil samples validated. 100% completed
- **Milestone 2.6:** Assessment of published LAMP assay for Verticillium wilt.
  Performance indicator - The existing LAMP assay will be tested on reference strains, soil samples and plant samples. 100% completed

Objective 3 - **What is the genetic diversity of *Verticillium dahliae*?**

- **Milestone 3.1:** Conduct a literature review & PhD planning.
  Performance indicator – Literature review. 100% completed
- **Milestone 3.2:** Examining the genetic diversity of *V. dahliae* in Australia and comparing it to overseas strains.
  Performance indicator – Phylogenetic trees comparing Australian isolates and overseas isolates. 100%
  completed
- **Milestone 3.3:** Genome sequencing of selected *V. dahliae* for proof of concept analysis to assess the genetic variation between virulent and avirulent strains.
  Performance indicator – Assembled genome sequence data for selected *V. dahliae* strains and assessment of genetic variation. 100% completed

Methods

Research question: Maintenance, generation and updating the NSW DPI *Verticillium dahliae* culture collection

Milestone 1.1 Collection and maintenance of culture collection and generation of single spore cultures in two locations.

Performance indicator

- Representative samples sent to NSW DPI Herbarium and unique identification DAR numbers assigned to all isolates collected during this project.
- Outcome - reference cultures kept for future research

Cotton plant tissue collected from plants showing symptoms of disease during the long-term NSW disease surveys since the 1983/1984 season. Isolates from the infected plants were maintained in the long-term culture collection and added to each season. Plant samples were collected from plants with symptoms of Verticillium wilt, Fusarium wilt and also Black root rot. Using aseptic technique, suspected Verticillium samples were surface sterilised, before being isolated onto selective media and put in an incubator in the dark at 24°C for two weeks. After microscopic examination confirmed the presence of *V. dahliae,* a 4 mm plug from the outside growing edge of the isolate was placed onto a clean plate of selective media, and once again grown in the incubator for two weeks. The number of times each sample was sub-cultured depended on contamination levels. Once a clean culture was achieved, a single spore was sampled using a hypodermic needle, and placed onto selective media. Five replicates were made for each isolate to ensure there was enough growth to send to Elizabeth Macarthur Agricultural Institute for VCG confirmation, as well as the Herbarium to be lodged and given a DAR number. Three vials were also kept at ACRI for future research.

Potential differences between isolates assigned to defoliating VCG1A as well as non-defoliating VCG2A and 4B Following on from the publication in 2016 of the new VCG1A reported in Australian cotton, several large laboratory experiments were conducted to gain a better understanding of the potential differences between Verticillium isolates assigned to defoliating VCG1A as well as non-defoliating VCG2A and 4B. Twenty one representative isolates collected from cotton grown throughout NSW were characterised for germination rate, morphology, growth rate, sporulation rate, conidia length and width as well as microsclerotia length and width.

Statistical analysis

The number of microsclerotia germinating out of the total of 20 per Petri dish at 5, 24 and 29 hours was analysed using the survival package in R version 3.5.1 (R Core Team 2018), with VCG (1A, 2A, 4B) as the explanatory factor (Therneau 2015). Differences in percent germination between VCG's were assessed using a Chi squared test. Radial growth rates were analysed by fitting a linear mixed effect model (LMEM) using the lme4 package in R (Bates et al. 2015). VCG was included as a fixed term with assay, batch, replicate and isolate as random terms. Data for length and width of both conidia and microsclerotia were assessed for each isolate. There were 100 measurements per isolate recorded for length and width for conidia, and 50 for microsclerotia. Length and width were analysed using a linear mixed effects model with VCG as a fixed term and batch and isolate as random terms. Conidial concentration was analysed using a linear mixed effects model with VCG as a fixed term with batch, replicate and isolate as random terms. A square root transformation of the data was required to improve the homogeneity of variance.

Fungal isolates and VCG typing

Cultures were obtained from single microsclerotia using a stereo dissecting microscope and sterilised hypodermic needles. A total of 21 isolates were selected from the historical culture collection belonging to VCG1A, 2A and 4B collected from various geographic locations throughout NSW, including the eight reference isolates (Chapman et al. 2016). Voucher specimens were lodged with NSW DPI Plant Pathology and Mycology Herbarium (DAR). *Nit* mutants were generated for eight isolates by our international collaborators in Spain using the method described in the first report of VCG1A in Australian cotton by Chapman et al. 2016. The VCG's of the remaining isolates were predicted using methods employed in previous work (Chapman et al. 2016). The isolate pathotype was determined using the DB19/DB22 primers (Carder et al. 1994) that distinguish between D and ND pathotypes of *V. dahliae* by amplifying different sized products. VCG was predicted by sequence analysis and comparison to reference isolates of the intergenic spacer (IGS) region, amplified using primers IGSF1 and IGSR1 (Qin et al. 2006).

Rate and number of microsclerotia germination
Sixty similarly sized individual microsclerotia of each isolate were aseptically transferred to three 90 mm plastic Petri dishes containing 20 mL of 25% PDA plus Novobiocin with lines scratched underneath to mark-up squares approximately 1 cm x 1 cm. The microsclerotia were placed in the centre of each square and incubated at 23 ±1° C in the dark. The rate of germination and number of microsclerotia were assessed using a dissecting microscope. The number of microsclerotia that had germinated after 5, 24, 29, 36 and 42 hours post transfer was recorded. Microsclerotia were considered germinated when hyphae were visible.

Morphology and microsclerotial forming types
The presence and intensity of mycelial growth was assessed on each isolate after 21 days incubation in the dark on 50% PDA. Each of the isolates was separated into four morphotypes previously described by Jabnoun-Khiareddine et al. (2010a) and Darai et al. (2014) based on colony colour and texture. Morphotype categories were defined as colony with smooth textured surface with milky white flocculose mycelium (M1), colony with grey to black surface with dense mycelium (M2), colony with white and dense mycelium in centre with black margins (strong sclerotinisation) (M3) and colony with grey to white mycelium with felt surface (M4). Each isolate was also assessed for microsclerotial development and the pattern of growth (microsclerotial forming types) after 21 days incubation in the dark on 50 % PDA using the method described in detail by Jabnoun-Khiareddine et al. (2010a). These authors defined each microsclerotial forming type as: MF type 1 - colony with few scattered microsclerotia with no clear pattern; MF type 2 - colony with more or less abundant and radially formed microsclerotia, MF type 3 - colony with abundant and radially formed microsclerotia and MF type 4 - colony with very abundant microsclerotia formed in "strand-end" pattern.

Radial growth rate and conidial concentration
The colony radial growth rate of each isolate using fungal plugs derived from single microsclerotia cultures was determined on two media: 50% PDA and 25% PDA plus Novobiocin. The pH of each medium was maintained at 5.5 for optimal growth and sporulation (El Sharawy et al. 2015). Mycelial plugs were subcultured from the advancing margins of cultures from each isolate using a 4 mm cork borer and inverted on the centre of 90 mm plastic Petri dishes containing 20 mL of each medium. Cultures were set up randomly in three batches of seven isolates. Batch one was isolated on day one, batch two isolated on day two and batch three isolated on day three, each incubated in the same incubator at 23 ±1° C in the dark for 14 days. Four radial growth measurements of each colony were recorded after 14 days for each replicate. Daily radial growth rate was calculated and expressed as mm day^{-1}. This experiment was repeated twice. Cultures were retained to assess conidial concentration, conidial size and microsclerotia size.

Conidial concentration from five replicates per isolate was assessed using a modified method described by El Sharawy et al. (2015). The cultures grown on 50% PDA plates used in the radial growth rate assessment were gently washed with 100 mL autoclaved distilled water using a sterile glass spreader to dislodge the spores. Mycelium and microsclerotia were filtered out using three layers of sterile cheesecloth. The conidial concentration was determined using an Olympus BX51 compound microscope and a haemocytometer.

Conidia and microsclerotia length and width
Conidia were retained from the filtered microsclerotia and conidial suspension from the 14 day old culture plates used for the radial growth rate assessments. Images were taken using a DP73 Olympus camera attached to the Olympus BX51 compound microscope so the length and width of 100 conidia per isolate could be determined using CellSens Standard software. Microsclerotia that were filtered from the suspension on the cheesecloth were transferred to Advantec 70 mm filter paper. The length and width (visible surface when observed under the microscope) of 50 randomly selected single microsclerotia per isolate were determined using a DP73 camera attached to an Olympus SZX9 dissecting microscope and CellSens Standard software.

Research question: How can we effectively measure *Verticillium dahliae* in soil?
Milestone 2.1 Establish soil sampling protocol required for soil coring at a field level

Performance indicator

- Spatial variation of Verticillium inoculum determined to ensure sampling technique is adequate
- Outcome – recommended sampling strategy published in industry format (eg fact sheet)

A considerable effort has been made to devise a suitable sampling procedure for Verticillium microsclerotia to reflect more accurately the propagule density for determining appropriate management strategies. *V. dahliae* propagules have been found to be aggregated in soil with a negative binomial distribution (Wheeler et al, 2000). To overcome this and to achieve a measure of variability known as the coefficient of variation (*cv*) of less than 10-25%, the methodology recommends collection of many samples and combine these as a single composite sample.

The area where composite samples are collected from a field may affect the estimate of the mean density of propagules. The objective of this study was to assess the influence of sampling area on estimates of population density mean and variance and hence aggregation of propagules and assess potential links with NDVI maps to assist in sampling a field. The information gained from this work was used to further develop a soil sampling plan for this current Verticillium inoculum research.

A 40 ha commercial cotton field near Narrabri was sampled repeatedly to determine the effects of sampling area size on the estimated mean and variance of soil populations of *V. dahliae.* The field was divided into contiguous quadrants of 10,000m^2. Each quadrant was assigned consecutive identifying numbers with the position of every five cores being recorded using a GPS. A total of 400 soil cores were collected on the same day. Ten cores per quadrant were taken using a step point transect method. Soil was air dried for two to four weeks to kill conidia and mycelium before being sieved and weighed for isolations. Subsamples from each of the ten cores within each quadrant were combined to create a bulk sample providing a total of 40 bulk samples.

For each of the 440 samples, dilution plate assays were conducted five times. This consisted of taking 10 g soil from each sample and mixing with 90 mL deionised water and mixing thoroughly on an agitator then pipetting five 1 mL aliquots onto semi-selective media in petri plates. The plates were incubated in the dark at 22°C for 14 days and colonies of *V. dahliae* were identified and counted under a dissecting microscope on 20x magnification. Every sample was isolated and recorded including the bulk samples. The mean and variance for each sample was determined. The population density of *V. dahliae* in each of the 400 individual cores and the 40 bulk samples was determined. Following that, density determinations for single quadrants were combined mathematically, not experimentally, to estimate larger sampling areas of 1 m^2, 10,000 m^2 (1 ha), 40,000 m^2 (4 ha) and 160,000 m^2 (16 ha). Twenty replicates of each sampling size were analysed and averaged and repeated for 10 replications to determine the mean and variance (Figure 1). The sampling position to be analysed was determined by using a random number generator in Excel. A seed number is used to generate each set of random numbers without pattern.

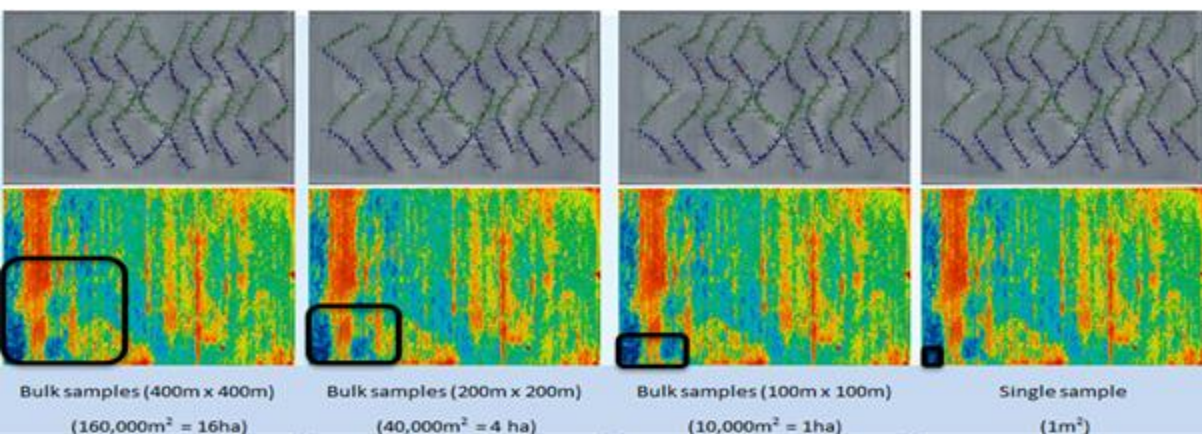


Figure 1. NDVI output for a single 40 ha field showing the representative sampling quadrat size.

Intense isolated diseased patches were indicated by the NDVI map from some individual soil samples within the 40ha field (Figure 3) suggesting potential aggregation of the pathogen. The aggregation parameter was calculated using k = [(mean2 - VAR/n)/(VAR-mean)], where n was the number of samples collected for a sampling unit size, n = 20 for each quadrant size. The data from this was used to determine the mean and variance for each quadrant size (1m^2, 1ha, 4ha and 16ha). Following that, density determinations for single quadrants were combined mathematically, not experimentally to estimate larger quadrant areas. The data from 20 replicates of each quadrant size were analysed and the mean and variance determined. The sampling position within the field to be analysed was determined by using a random number generator in Excel. A seed number was used to generate each set of random numbers without pattern. Intense isolated patches were recorded from some individual soil samples within the 40ha field indicating aggregation of the pathogen. The aggregation parameter was calculated using k = [(mean2 - VAR/n)/(VAR-mean)], where n was the number of samples collected for a sampling unit size, n=20 for each quadrant size.

Horizontal and vertical inoculum levels

Soil samples were collected three weeks after planting cotton crops from three fields. Soil was collected from depths of 0-10 cm and 11-20 cm within the plant line in the permanent bed and within the irrigation furrow. Each soil sample was stored at room temperature for four to five weeks to facilitate conidia and hyphal death. Soil samples were sieved, rolled and weighed in preparation for soil isolations. Each individual and bulk soil sample was isolated five times per sample and the average propagules per gram (ppg) of soil determined. Cotton is planted within the 1-5 cm depth below the soil surface and the roots grow within the 10 to 20 cm infested region of the soil profile. Consequently, it is important to promote effective soil sampling and the need to sample from potentially infected vertical zones where most inoculum will be situated.

Determining soil isolation technique, selective media, optimum weight of soil to isolate and depth of soil sample

The optimum soil isolation technique was determined by comparing a dilution plate technique (wet) with a direct dry plating method. Bulk soil samples were collected from 12 fields (six in NSW and six in Texas, USA) and placed into labelled bags then air dried for 14 days at 25°C to kill present viable conidia and hyphae (Butterfield & DeVay 1977). Once dry, the soil was thoroughly mixed and rolled with a metal pipe to achieve uniform small particle size. Thorough mixing of the soil was important when working with the small subsample sizes needed for the direct dry plating method (Goud & Termorshuizen 2003). Five subsamples of 0.2 g of bulk soil sample (for dry plating) and 10 subsamples of 20 g of the same soil sample (for wet plating) were weighed out from each bulk soil sample collected at each depth. Depth of soil to sample was determined by collecting soil from 2-12 cm, 13-24 cm in Assay One and 2-12 cm, 13-24 cm and 25-37 cm depths in Assay Two (following year). Within each field, 20 samples were taken at each depth from three quadrant areas and mixed thoroughly for a bulk sample. Each field had three bulk samples at each depth. The optimum selective media to be used to isolate *V. dahliae* from soil was determined by comparing the results of isolations from each sample on four media: Potato dextrose agar (PDA), acidified PDA, 25% Sorensen's NP-10 and acidified Sorensen's NP-10. Optimum media was based on recovery of *V. dahliae* colonies and ease of identifying the pathogen.

The optimum weight of soil used for direct dry plating to isolate was determined by comparing the results of four soil samples and using four subsample weights: 0.1, 0.2, 0.5 and 1g. Each soil sample had five replicate subsamples and the means were compared. Optimum soil preparation was determined by rolling four bulk soil samples and splitting each of the samples in half, then sieving one lot and leaving the other half of each sample un-sieved. The optimum time to sample was determined by collecting soil samples from three fields using the method described above at pre-plant, December, February and following harvest and incorporation. Inoculum levels for each sample were determined by the direct dry plating method.

Statistical analysis

For the optimum weight of soil, the number of microsclerotia per plate were analysed as a generalized linear model with a log link function (poisson regression) using the glm function in R (R Core Team 2019). The model had sample weight (0.1, 0.2, 0.5, 1 g), treatment (sieved, un-sieved) and their interaction as testable factors. For Assay One and Two, the inoculum level (ppg) was analysed as a linear mixed model using the lme function in the nlme R package (Pinheiro et al. 2018). For Assay One, the optimum media,

plating method and depth and the four types of media were fixed terms, plating method (wet, dry), and depth. For Assay Two, the fixed terms were plating method (wet, dry), depth (2-12, 13-24, and 25-37 cm). To make comparisons between Assay's One and Two, only the data for the one media (Sorensen's NP-10) in study was analysed. The ppg data was squared root transformed as inspection of residual plots on untransformed data showed variation increased with ppg. Predicted means from the above models were obtained using the predictmeans function from R package predictmeans (Luo et. al. 2018).

Milestone 2.2: Efficiency & accuracy of the molecular technique vs plating method

Performance indicator

- Replicated lab experiment with naturally infected soil and positive controls with known inoculum levels used to validate plating method and molecular technique
- Outcome - confidential update on potential for molecular tool to be used based on lab and soil trials reported to CRDC

Comparison of dry plating method and Ct values obtained with DNA

A comparison of the dry plating technique of soil with a known number of propagules per gram was compared with the molecular assay to determine whether an indication of Verticillium inoculum in the soil could be determined using molecular techniques. Initially, the protocol for dry plating techniques were used to establish initial inoculum levels in the soil samples and these were compared with Ct values determined using DNA.

Comparison of dry plating method and PREDICTA®B method

Twenty-four bulk soil samples were collected from a field with a history of Verticillium wilt in May 2019. Each soil sample was split in half and one half provided to Mr Rob Long. The paired samples were assessed using two methods. One set of soil was isolated using the dry plating method described earlier and the second set analysed using PREDICTA®B method. The results were compared for potential correlations in inoculum levels.

Milestone 2.2.1: Soil molecular assay developed

Performance indicator

- Log pgDNA/g soil pre-planting x axis graphed against % incidence of field y axis (need up to 30 samples)
- Outcome – Findings published in Spotlight article or Cotton Grower article

Previously, DNA from soil was extracted using a Qiagen DNeasy PowerMax Soil Kit which did not allow for high sample throughput, making the soil extraction labour intensive. Testing of the high throughput DNA extraction kit, Qiagen's MagAttract PowerSoil DNA KF, automated on a Thermofisher KingFisher 96 instrument with paired samples extracted with a Qiagen DNeasy PowerMax Soil Kit (Manual) was performed to assess how comparable the DNA obtained from the high throughput method was to our previously used manual method.

Milestone 2.2.2: Plant tissue assay

Performance indicator

- Plant tissue isolations compared with plant assay and VCG typed
- Outcome – Findings published in Spotlight article or Cotton Grower article

The development of a fast-turn-around molecular assay for *V. dahliae* has included the optimisation of a number of molecular assays to identify N and ND pathotypes as well as VCGs direct from both stem and soil samples. Traditionally, analysis is conducted by the isolation and culturing of pure cultures from stem and soil samples. These isolates then require DNA extractions and molecular work, which in total can take up to 6 weeks.

While VCG1A can be identified via the D/ND assays, VCG2A and 4B cannot currently be differentiated by PCR. To determine the VCG of *V. dahliae* isolates, a short segment of DNA known as the Intergenic Spacer (IGS) was sequenced and compared to reference isolate sequences of known VCG. Identification of VCG via IGS PCR was performed by initially extracting DNA from stem samples using a Qiagen DNeasy plant mini prep kit. This DNA extraction method can extract DNA from *V. dahliae* within the plant stem. Extracted DNA is then confirmed for *V. dahliae* DNA using a real-time PCR assay designed by Gharbi et al (2016) and then amplified using the *V. dahliae* specific IGS primers developed by Qin et al (2006) and adapted by Jiminez-Gasco et al (2013).

To further improve turnaround time, it was determined in this study that these PCR conditions (Qin and Jimineze-Gasco primers) were able to be performed as a real-time assay, thereby removing the need for

the Gharbi et al real-time assay, reducing the time and cost of the diagnostic. The DNA concentration could then still be confirmed using the NanoDrop and sent for sequencing. This process of analysis direct from stem tissue removes the culturing bottleneck and enables rapid diagnostics, reducing the time to result by weeks (Figure 2). The plant tissue assay was tested on 368 samples from several commodities.

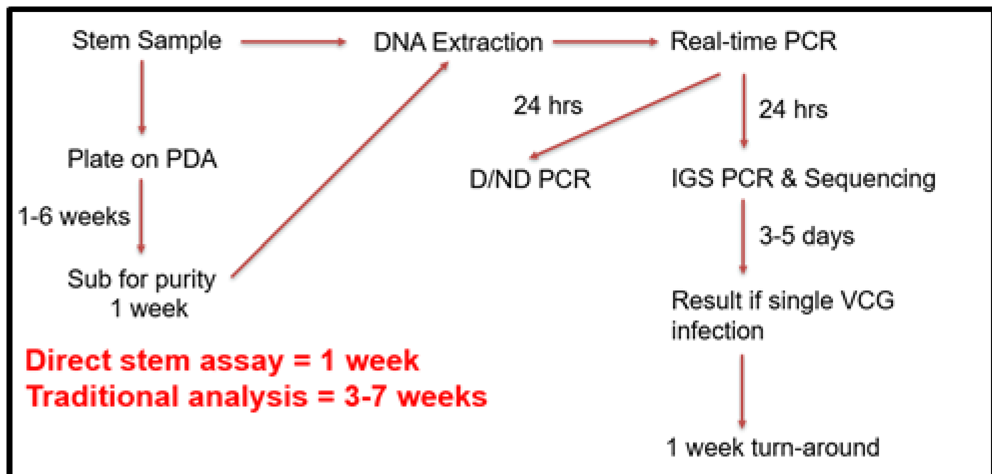


Figure 2: Flowchart comparing workflow for direct stem assay with traditional analysis methods. Utilising the direct stem assay, identification of *V. dahliae* can be achieved much quicker compared to traditional analyses.

To analyse IGS results, initially, IGS sequences were assigned a VCG by performing multiple pairwise nucleotide alignments with reference sequences and a VCG designated by a corresponding reference match using Geneious version 9.1.8 (https://www.geneious.com, Kearse et al., 2012). A publication by Papaioannou *et al.* (2013) showed that nucleotide sequences within the *V. dahliae* IGS were composed of repeat regions in which patterns of repeats could be used for VCG identification. Due to these repeats, pairwise nucleotide alignments proved to be difficult as the repeat elements comprising the IGS interfered with obtaining clean alignments.

To address this, custom annotations were designed in Geneious 9.1.8 to identify patterns of repeats in sequenced IGS which were then compared to the key in the Papaioannou paper to infer VCG based on IGS repeat patterns (Figure 3). An in-house custom python script was also written to automatically annotate and report IGS repeat patterns which can be used to classify large volumes of sequences (https://github.com/Jwebster89/IGS-checker).

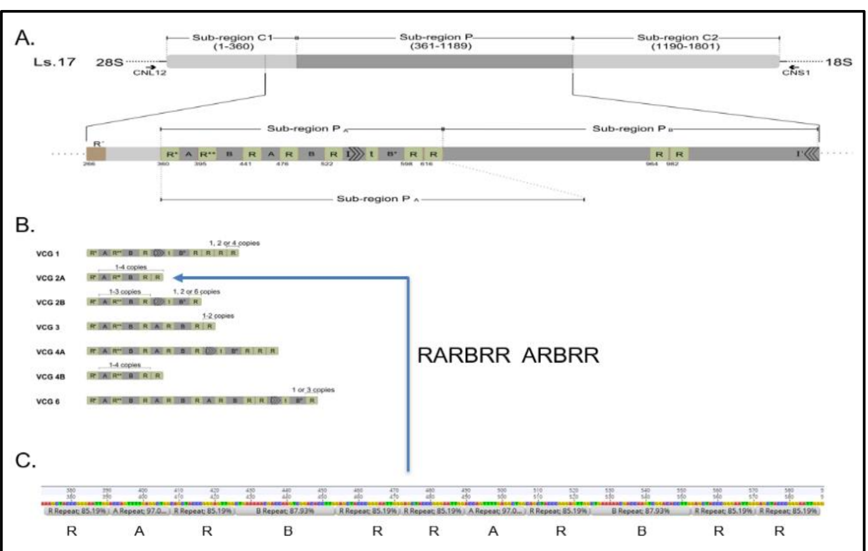


Figure 3. Using annotations of IGS repeat sequences to identify presumptive VCG's. **A)** Sequences of IGS are composed of multiple sub-regions with sub-region P_A containing strings of nucleotide repeats ranging in length from 15-28 base pairs. **B)** *Verticillium dahliae* of different VCG have been shown to have unique sets of repeats in this region allowing presumptive VCG identification based on the pattern of these repeats (Papaioannou *et al* 2013). **C)** Sequencing of IGS repeat patterns then may be matched to known patterns to identify presumptive VCG's.

Interestingly, melt-curves obtained from the IGS PCR adapted to a real-time assay, also showed potential for this assay to be able to differentiate VCG's 2A and 1A when performed on pure isolate DNA extracts as well as from several stems. Analysing VCG from melt-curves would forego the need to send PCR products away for sequencing. Melt-curve analysis of real-time PCR products gives rise to the potential of one to two day turn-around of VCG diagnostics from complex environmental samples such as stems and soil, as well as being able to identify mixed infections. To further investigate this, a high-resolution melt-curve kit was tested on samples including pure culture isolates, stem samples, hybrid samples and soil samples using our current IGS real-time assay. This assay however targets a region approximately 4x larger than the recommended PCR product size for the high-resolution melt-curve and failed to produce a higher resolution melt-curve than the initial assay.

Milestone 2.3: Determine the minimum inoculum levels in soil of VCG1A, 2A and 4B *Verticillium dahliae* needed to cause disease symptoms.

Performance indicator

- Replicated pot experiments in controlled conditions
- Outcome – Published article outlining the baseline threshold for Australian VCG

Pot experiment

A replicated, randomised pot experiment was set up in the controlled temperature growth room at ACRI to assess the minimum inoculum levels needed to cause disease symptoms. Results from a previous study in Diseases of Cotton XI recorded external symptoms at 140 ppg. Foliar symptoms had been recorded in fields with as little as 20 ppg. Inoculum levels tested were 0, 1, 10 and 20 ppg. Given all plants grown in pots in the controlled growing environment had disease, it was necessary to conduct field trials to develop thresholds for disease expression under natural field conditions.

Field trials

Intensive field trials to assess potential relationship between inoculum and disease severity

During March and April 2017, intensive field assessments were carried out on three commercial cotton fields (Farm F, Farm L and Farm AM) to assess the incidence of Verticillium wilt, severity of foliar symptoms and stem discolouration. On Farm F (Figure 4) and Farm L (Figure 5) a total of 200 soil cores were taken with every 10 being bulked to give 40 cores from 0-10 cm and 11-20 cm at each sampling point along two transects. On Farm AM (Figure 6) 120 soil cores were taken with every 10 being bulked to give 12 cores from 0-10 cm and 11-20 cm at each sampling point along six transects. For each of the fields, soil cores were taken during March/April 2017 and air dried to kill conidia and mycelium. Each soil sample was split, rolled and weighed in preparation for soil isolations. The incidence of disease was calculated as a percentage of plants with vascular discolouration present.

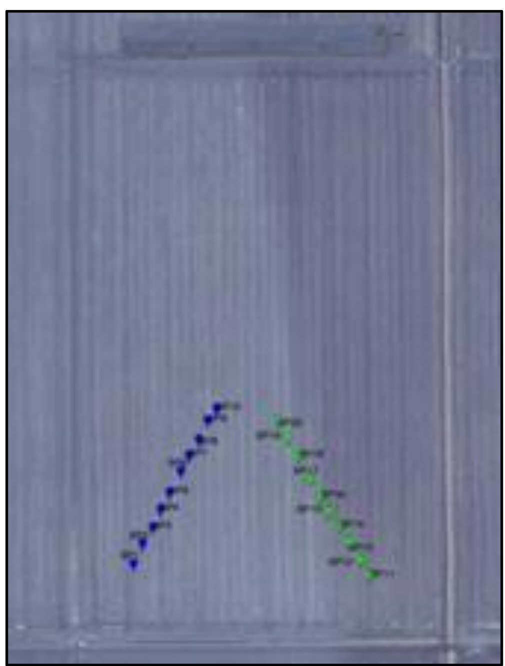


Figure 4. Farm F transects, with flags illustrating the 20 survey locations.

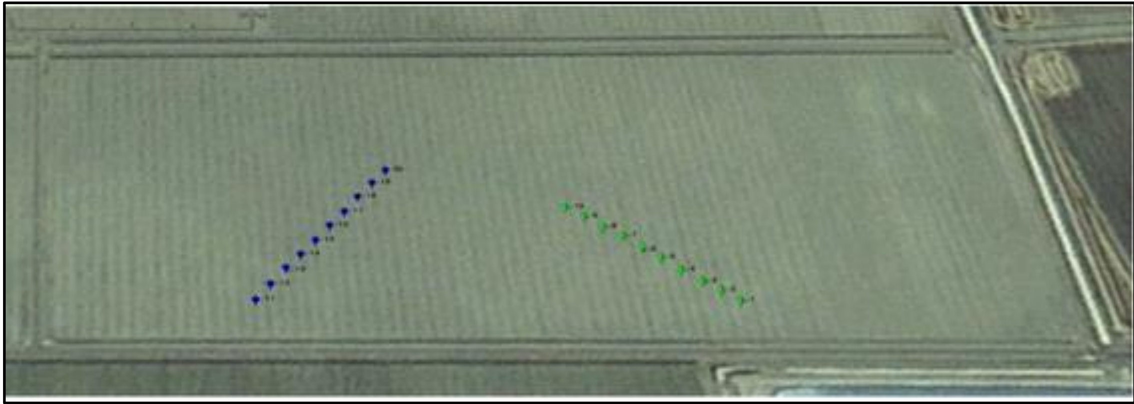


Figure 5. Farm L transects, with flags illustrating the 20 survey locations.

Figure 6. Farm AM transects in Field L2 on the bottom and L3 on the top with flags illustrating the 60 survey locations over two fields.

Disease incidence in each field was recorded as the percentage of infected plants out of 200 plants assessed at 20 sampling points. The severity of foliar symptoms (Foliar Disease Index) was assessed using the 0-4 scale where 0 = no external symptoms, 1 = 1-33% foliar affected, 2 = 34-66% foliar affected, 3 = 67-97% foliar affected and 4 = dead plant (Figure 7).

Figure 7. Foliar disease severity using the disease severity scale 0-4 where 0 = healthy with zero foliar symptoms, 1 = 1-33% foliar affected, 2 = 34-66% foliar affected, 3 = 67-97% foliar affected and 4 = dead.

The severity of stem discolouration (Stem Disease Index) was assessed using the 0-3 scale where 0 = no vascular discolouration, 1 = 1-33% vascular discolouration, 2 = 34-67% vascular discolouration, 3 = 68-100% vascular discolouration (Figure 8).

Figure 8. Stem disease severity using the disease severity index scale 0-3 where 0 = healthy with zero vascular discolouration, 1 = 1-33% vascular discolouration, 2 = 34-67% vascular discolouration, 3 = 66-100% vascular discolouration.

The potential relationship between the percentage of plants with stem disease symptoms and the inoculum levels from both farms with Verticillium wilt present were pooled and the combined data set analysed using linear regression. The confidence level of all analyses was set at 95% and <0.05 was considered as statistically significant. The inoculum at 0-20 cm was determined for each transect based on the composite samples.

Intensive field trials to assess potential relationship between inoculum pre-planting and disease incidence

140 bulk soil samples were collected pre-planting and isolated from 140 plots from three commercial cotton fields during the 2017/2018 cotton season. Five subsamples were taken from each of the bulk soil samples and isolated using the dry plating method using 0.2 g soil/plate. The plates were incubated in the dark at 23°C for 14 days. Soil was washed from the surface of the plates and left to air dry in the biological safety cabinet before being assessed under a dissecting microscope at 20x magnification. The number of colonies germinated (propagules) were counted per plate and x by the dilution factor. The average of the five plates was reported as the plot average of ppg of dry soil. In total, 700 soil isolations were made to establish plot averages for 140 plots.

In February 2018, the incidence of disease was recorded in each of the plots. Twenty plants per plot were cut at the base of the stem and vascular discolouration recorded as potential Verticillium infection. Infection was confirmed following plant tissue culture on semi selective media. In the 140 plots, there were 26 different inoculum levels ranging from 0 to 36 ppg. Each plant was examined for external symptoms as well as internal stem discolouration. The average incidence of Verticillium wilt was calculated for each plot with the same inoculum level to establish the minimum disease incidence at each inoculum level.

In 2018/2019, field validation of the newly developed thresholds was carried out on four commercial cotton fields. The pre plant inoculum levels were established and predicted incidence of disease for each field fell into the thresholds. In February 2019, the incidence of disease in these four fields was calculated using the method described above. The correlation between predicted and observed was tested.

Milestone 2.4: The effect of crop rotation on inoculum levels.

Performance indicator

- Return to replicated field experiment at Farm M and take soil cores from existing six GPS points to determine ppg after rotations of cotton/sorghum/ wheat rotations and findings reported in progress reports
- Outcome - findings published in Spotlight article or Cotton Grower article

Long term field case study

A commercial cotton field with extremely high levels of non-defoliating Verticillium inoculum was selected as a case study to follow the effects of long-term rotation of non-host crops such as sorghum and durum on inoculum levels. The field study was established in May 2013 and inoculum levels

estimated using the plating method following each crop rotation. Soil samples from six GPS marked locations within the field were collected from May 2013 to December 2018. Collections times were May 2013, December 2014, July 2016, December 2017 and December 2018. An additional 20 random soil samples were added to the field study and assessed in December 2016, 2017 and 2018. Soil preparation and isolations were done using the method described earlier.

Additional crop rotation crops assessed during 2018/2019
Inoculum levels were assessed in two fields following Industrial Hemp cultivation. Inoculum levels increased from 1 to 6 pgg in one field and remained the same in the other field. No infected plants were recorded. Tissue isolations from the same farm were made from Safflower plants showing disease symptoms. In a biofumigant trial run by a local agronomist, the inoculum levels were assessed across the plots where biofumigant was planted at different planting rates per hectare. Volunteer Ethiopian cabbage and mustard plants from this crop were taken to the laboratory and isolated and VCG determined. The inoculum levels in a field trial near Breeza were assessed pre and post planting of winter and summer rotational crops. Inoculum levels were assessed under cabbage, ryecorn, onion, mustard, canola, faba beans, field peas, wheat, durum, barley, fallow and broccoli.

Milestone 2.5: Validate Verticillium wilt tool.
Performance indicator

- Field samples collected and molecular based assay for identifying *V. dahliae* and determining the presence of defoliating or non-defoliating pathotypes within soil samples validated.
- Outcome - molecular based assay for identifying *V. dahliae* in soil and determining the presence of defoliating or non-defoliating pathotypes within soil samples validated and offered as a commercial service by NSW DPI.

The plant tissue assay was optimised for fast throughput results in order to reduce the method to one real-time PCR that can determine the VCG of *V. dahliae* infections within a host plant. Traditionally, diagnostics would require a length culturing phase to be able to identify *V. dahliae* from a host and further complicating the matter was that it was not always possible to isolate. The plant tissue assay was tested on 139 cotton samples and further validation performed by testing stems and isolates cultured from these same stems.

The soil assay was developed to test for the presence of *V. dahliae* in a field, pre-planting. The soil assay was optimised to work with high-throughput DNA extraction to enable large volume testing as well as obtaining real-time results.

Milestone 2.6: Assessment of published LAMP assay for Verticillium wilt.

Performance indicator

- The existing LAMP assay will be tested on reference strains, soil samples and plant samples.
- Outcome - recommendation if the published LAMP assay is a viable option for use under Australian conditions

The existing LAMP assay was tested on reference strains, soil samples and plant samples using the protocol as published by Moradi, A., Almasi, M. A., Jafary, H., & Mercado-Blanco, J. (2014). A novel and rapid loop-mediated isothermal amplification assay for the specific detection of *Verticillium dahliae*. *Journal of applied microbiology*, *116*(4), 942-954. Primers for a loop-mediated isothermal amplification (LAMP) were tested on nine different *V. dahliae* isolates of various VCG. Samples were tested using the published protocol.

Milestone 3.1 Conduct a literature review & PhD planning.
Performance indicator

- Literature review
- Outcome – draft review manuscript

PhD planning was completed within a few months of the PhD commencing. The literature review was formatted as a manuscript and submitted for publication in the Australasian Plant Pathology Journal on the 22/11/20

- Dadd-Daigle. P. Kirkby. K., Chowdhury. P., Labbate. M and Chapman. T. “Literature review - The Verticillium wilt problem in Australian cotton”.

An extension was granted until the 7th October 2019 due to factors outside the student’s control. Initially, a contact offered to sequence samples but then retracted the offer due to problems at their facility. Another course of action for sequencing the fungal genomes was identified further delaying the progress of Pearl’s research. Further issues procuring the correct permits for sequencing equipment resulted in more delays. Ultimately the sequencing was outsourced and has now been completed.

Milestone 3.2: Examining the genetic diversity of *Verticillium dahliae* in Australia and comparing it to overseas strains.

Performance indicator

- Phylogenetic trees comparing Australian isolates and overseas isolates
- Outcome – published paper

DNA was extracted and isolates were characterised as defoliating and non-defoliating pathotype and assigned VCG’s. A large sample of the NSW long term culture collection of *V. dahliae* was analysed via Inter-simple sequence repeats (ISSR) with a virulence assay expanding on the results of the ISSR assay. Additional genome sequencing for the selected VCG1A isolates was completed. The isolates selected for sequencing were sequenced both using Nanopore Minion and Illumina Myseq technologies. These provide long-read and short-read sequence data, respectively. Together the two technologies allow for accurate genomes to be constructed, as long-read sequences, although not offering as much coverage, allow gaps and repetitive regions to be resolved. The short-read sequences provide much higher coverage and reduce the error rate of the final sequence. These assembled genomes were analysed against each other and against other VCG1A genomes that are publicly available.

Due to copyright issues, the PhD experiment results will not be provided here, instead they will be published as follows:

- Dadd-Daigle. P. et al. “Virulence not linked with Vegetative Compatibility Groups in Australian cotton *Verticillium dahliae* isolates”.
- Dadd-Daigle. P., Kirkby, K., Roser. S.,Lonergan. P., Chowdhury. P., Labbate. M and Chapman. T. “Virulence varies in Australian cotton *Verticillium dahliae* isolates”.
- Dadd-Daigle. P. et al. “Whole genome analysis of Australian *V. dahliae* Vegetative Compatibility Group 1A”.

Copies of all manuscripts have been submitted to CRDC for permission to publish.

To confirm the diversity of pathotypes of Australian Verticillium isolates, multi-locus sequence typing (MLST) using five genes: actin (ACT), oxaloacetate transport protein (OX), Elongation factor 1a (EF-1a), glyceraldehyde-3-phosphate dehydrogenase (GPD) and tryptophan synthase (TS) (Kasson et al 2014 and Inderbitzin et al 2011) was investigated.

Milestone 3.3: Genome sequencing of selected *Verticillium dahliae* for proof of concept analysis to assess the genetic variation between virulent and avirulent strains.

Performance indicator

- Assembled genome sequence data for selected *V. dahliae* strains and assessment of genetic variation
- Outcome - recommendation on the viability for diagnostic assay development to identify the pathogenicity potential of *V. dahliae*

Genomes of select *V. dahliae* VCGs? were genome sequenced with short and long read sequencing technologies in order to assemble genomes of these isolates and achieve a greater understanding of the pathogen, identify pathotype differences and design diagnostic assays that may differentiate between pathogenicity. The isolates sequenced included:

-X003 - A Noogoora burr isolate with an unclear IGS sequence

-SS280 - A cotton isolate with a nit mutant test indicating VCG1

-SS362 - A cotton isolate with an IGS sequence indicating VCG2A that is shown to cause high levels of disease in cotton plants.

-15/607D - A Noogoora burr isolate with an IGC sequence indicating VCG2A isolate from the same location as the cotton isolate SS362.

Sequencing of these isolates using PacBio technology was planned in order to produce largely complete genomes and comparative genomics to be used to identify unique regions that could be possible diagnostic targets for VCG assessment. Multiple commercial kits were assessed for their capacity to produce high molecular weight DNA and were unable to produce the strict concentration and purity required for PacBio sequencing. Manual extractions using enzymatic and salt lysis methods were also tested for their effectiveness. These methods, while extracting DNA, also appeared to co-purify other compounds with similar chemical structure to DNA, confounding DNA quantification and quality results. To obtain long read sequence data, DNA extracts were run on an Oxford Nanopore MinION instead of the PacBio platform. Multiple extraction methods were tested for their ability to produce high quality, high molecular weight DNA for sequencing reads. Short read sequencing was also performed on all isolates using an Ion Torrent S5. All four isolates were multiplexed onto one Ion Torrent 530 sequencing chip at equimolar ratios.

Short Read Assemblies

Ion Torrent reads were assembled using SPAdes assembler (version 3.11.1) and a core SNP analysis was then performed using Snippy 4.4.5 on SPAdes assembled genomes with *V. dahliae* JR2v4.2.0 (Genbank accession GCA_000400815.2) as the reference sequence. The core SNP alignment comprised 224,934 SNPs and a phylogenetic tree was built using the Neighbour-Joining algorithm in Geneious 9.1.8.

Other genomes of *V. dahliae*, obtained from NCBI, were also analysed with Snippy and included in the SNP phylogeny. Annotation of the *V. dahliae* genomes was performed using funannotate (https://funannotate.readthedocs.io/en/latest/index.html). SPAdes assemblies were initially cleaned by aligning the shortest contigs against the rest of the SPAdes assembly with minimap2 to detect repetitive segments. This process was looped starting from the shortest contigs to the N50 of the assembly, removing any contigs that had a percentage overlap of 95% or more with a 95% or higher identity. Remaining contigs were then sorted from largest to shortest and repeat regions were softmasked using a combination of RepeatModeler/RepeatMasker. Ab-initio gene prediction was performed with Augustus, Genemark, Snap and GlimmerHMM and BUSCO was used to train Augustus and tRNAScan-SE was used to predict tRNA regions. Evidence modeller was then run to generate consensus gene models from all the data obtained.

Functional annotation was performed by initially running predicted protein regions through Interproscan 5 (which provides annotations using CATH-Gene3D, CDD, HAMAP, PANTHER, Pfam, PIRSF, PRINS, PROSITE profiles, PROSITE patterns, SFLD, SMART, SUPERFAMILY and TIGRFAMS). Additional functions were annotated using EggNog Mapper, Diamond blastp of UniProtKB and MEROPS, CAZyme and GO ontology. Annotations were then assessed for completeness using BUSCO (Benchmarking Universal Single-Copy Orthologs). These BUSCO profiles showed that annotation profiles appeared to be incomplete with a large proportion of single copy orthologs either missing or fragmented. To address this, training of Ab-initio gene predictors was performed using *V. dahliae* JR2 RNA-seq data, publicly available on NCBI's Sequence Read Archive (SRA). This RNA-seq data was assembled against the JR2 genome with Trinity and PASA to produce *V. dahliae* specific gene prediction models. Gene prediction and functional annotation was performed a second time. Functional annotation of these genomes represents a significant step forward in the understanding of these pathogens. To date, only one publicly available Verticillium genome has functional annotation associated with it, VdLs.17.

Clusters of Orthologous Groups (COGs) are widely used to classify proteins from sequenced genomes based on the orthology concept. Differences in the abundances of these COG categories is indicative of the differences in metabolic processes occurring between isolates. Peptidases (those classified by MEROPS) and carbohydrate-active enzymes (CAZymes) are also used in this way to identify differences in metabolism signified by varying functional protein profiles. This could indicate a difference in metabolism, e.g. glycoside hydrolases catalyse the hydrolysis of glycosidic bonds, such as those in cellulose, hemicellulose and starch, found commonly in the biomass of plants and may reflect a difference in SS280's pathogen-host interactions. However, with 75% of the predicted proteins not having a functional annotation, these relative abundance profiles are still subject to change.

Due to the size of SS280, genome sequence data from SS280 was mapped to *V. dahliae* JR2 reference sequence with the Geneious Mapper method in Geneious 9.1.8 while saving a list of unused contigs. Large unused contigs were checked using BLAST to determine whether they all belonged to *V. dahliae*.

The BLAST results confirmed sequencing was from *V. dahliae* and not a contaminating organism. To also help confirm whether the sequencing was a result of mixed *V. dahliae* cultures, sequences were checked for IGS that did not match known VCG2A IGS sequences. These results showed only one VCG sequence type. Cultures were also single spored to ensure purity of the culture.

While *V. dahliae* has not had an observed sexual phase and is haploid in its structure, it is closely related to the amphidiploid *Verticillium longisporum.* Phylogenetic analyses have shown that *V. longisporum* separates into three separate lineages that have formed from the hybridisation of two haploid parents (Inderbitzin 2011). These three lineages are composed of combinations of four different parents, two unknown ancestral species provisionally named species A1, and D1 as well as two *V. dahliae* lineages D2 and D3. These three lineage combinations were formed by hybridisation of A1 x D1, A1 x D2 and A1 x D3. While no diploid *V. dahliae* are known today, an unknown species has in the past hybridised with *V. dahliae* to form the amphidiploid *V. longisporum*, which may provide an answer to the large genome of SS280.

To identify regions of interest in pathogenic versus non-pathogenic VCG2A, assembled contigs of SS362 and SS425 were separately aligned to the *V. dahliae* JR2 reference sequence using minimap2 and Alvis to visualise sequence alignment to the reference. This alignment shows the presence/absence of assembled sequence in relation to the reference sequence. This was performed for both SS362 and SS425 so that comparisons could be made between these two draft genomes (as pairwise alignments of these genomes to the reference which consists of 8 chromosomes, produces eight graphs and pairwise alignment of SS362 and SS425 draft genomes would produce thousands of graphs).

Results

Milestone 1.0 Collection and maintenance of culture collection and generation of single spore cultures in two locations.

Selected *V. dahliae* isolates were used by USA Master's student Ms Shelby Young for experiments in Milestone 2.1. Selected *V. dahliae* isolates were used to evaluate the efficacy and accuracy of the plating method and the development of the molecular technique discussed in full in Milestone 2.2. Selected *V. dahliae* isolates were used by PhD student Ms Pearl Dadd-Daigle and team members for genetic studies, discussed in full in Milestones 3.1, 3.2 and 3.3. A total of 240 isolates have been lodged with the Herbarium with assigned unique DAR (Table 1).

Table 1. NSW DPI culture collection.

Pathogen	Number isolates	Storage location	VCG assigned	DAR assigned
Verticillium dahliae	168	ACRI & Herbarium	Yes	Yes
Fusarium oxysporum f. sp. *vasinfectum*	32	ACRI	N/A	Yes
Thielaviopsis basicola	40	ACRI & Herbarium	N/A	Yes

Potential differences between isolates assigned to defoliating VCG1A (eight isolates) as well as non-defoliating VCG2A (seven isolates) and 4B (six isolates) fungal isolates.

Rate and number of microsclerotia germination

The rate of germination between microsclerotia derived from different VCG's differed significantly. With germination at 5, 24, 29 and 36 hours of 16.9, 90.4 98.8 and 100%, VCG2A germinated quicker ($\chi^2_{(df=1)}$ =11, P=0.0009) than VCG4B (12.5, 83.3, 96.9 and 99.7%), which in turn was quicker ($\chi^2_{(df=1)}$ =25.3 P<0.0001) than VCG1A (5.2, 74.0, 89.5 and 98.3%) the slowest to germinate.

Morphology and microsclerotial forming types

Verticillium dahliae isolates had more or less erect, totally hyaline and verticilliate conidiophores with two or four phialides at each node. Conidia were also produced at the phialide tips. Colony colour and texture was variable between pathotypes and VCG's. Morphotypes included colony with smooth textured surface with milky white flocculose mycelium (M1), colony with grey to black surface with dense mycelium (M2), colony with white and dense mycelium in centre with black margins (strong sclerotinisation) (M3) and colony with grey to white mycelium with felt surface (M4) (Figure. 9).

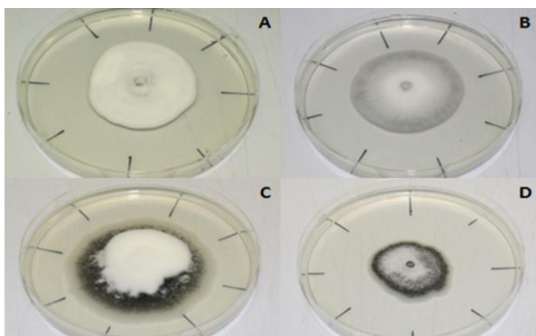


Figure 9. Colony morphotypes. (A) Morphotype 1 Smooth textured surface with milky white flocculose mycelium, (B) Morphotype 2 Grey to black surface with dense mycelium, (C) Morphotype 3 White and dense mycelium in centre with black margins (strong sclerotinisation) and (D) Morphotype 4 Grey to white colony with felt surface.

Seven isolates from VCG1A were categorised as morphotypes M2 and M3. VCG2A isolates were M1, M3 and M4. VCG4B isolates were M1, M2 and M4. All 21 isolates produced microsclerotia; however, the pattern in which the microsclerotia formed and the intensity of sclerotinisation was variable between isolates and did not differentiate by pathotype or VCG. Microsclerotial development was the same in the two media. The isolates fell into three of the four microsclerotial forming types (MFT) previously described in detail by Jabnoun-Khiareddine et al. (2010) (Figure 10). Eleven of the isolates' microsclerotial development was radial, while the remaining ten isolates formed a strand-end growth pattern. None of the isolates separated into MFT1. Isolate morphology and microsclerotia forming types were not related to each other or to pathotype or VCG assignment.

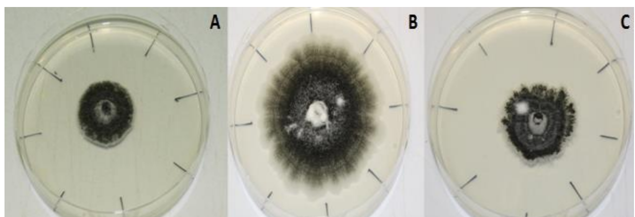


Figure 10. Microsclerotial forming types (A) MFT 2: colony with more or less abundant and radially formed microsclerotia, (B) MFT 3: colony with abundant and radially formed microsclerotia and (C) MFT 4: colony with very abundant microsclerotia formed in "strand-end" pattern.

Radial growth rate and conidial concentration

There was no main effect of VCG or medium on radial growth rate, but there was a significant interaction effect. Each of the isolates that were assigned to VCG1A had higher growth on 25% PDA plus Novobiocin than on 50% PDA; however, the reverse was true for VCG2A where seven of eight isolates had slower growth rates on 25% PDA plus Novobiocin. For VCG4B, there was an even split with three of six growing faster on 25% PDA plus Novobiocin (Figure 11).

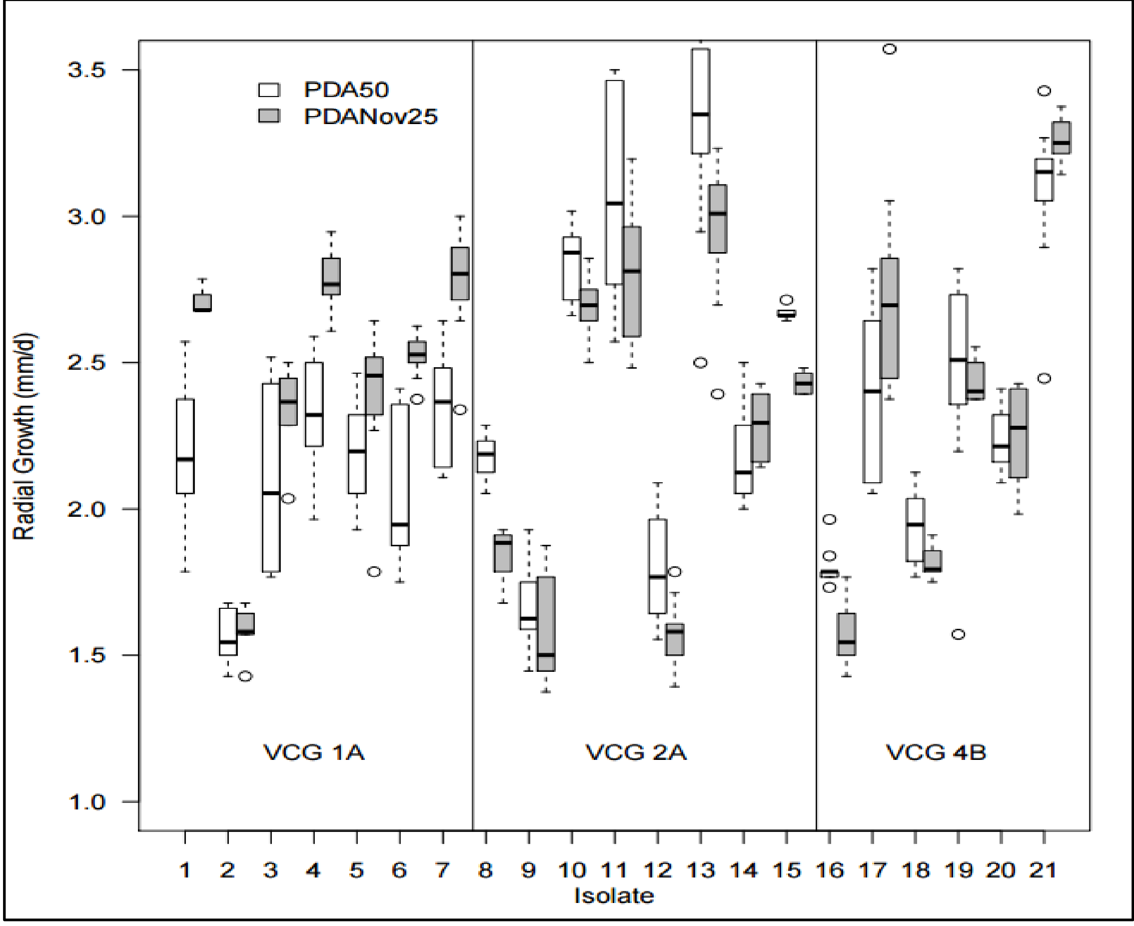


Figure 11. Average growth rate per day of isolates 1-21 as per Table 1 assigned to VCG1A, 2A and 4B on two media. o indicates outliers.

There was no significant difference (P=0.19) in conidial concentration of isolates from different VCGs. The predicted number of conidia (square root transformed) ranged from 138 (19,000) for VCG1A to 218 (48,000) per mL for VCG4A with large variation in conidial concentration of isolates within each VCG.

Conidia and microsclerotial length and width

Conidia were hyaline, ovoid to elongate in shape. Conidial length and microsclerotia width differed significantly between VCG's (Table 2). Conidial length was significantly different (P=0.006) for isolates assigned to each VCG with the average length of 5.26, 5.61 and 5.46 µm for VCG 1A, 2A and 4B respectively. Conidial width between isolates did not differ significantly between VCG's. Both D and ND isolates produce round and elongate microsclerotia on PDA. There was no significant difference (P=0.12) in microsclerotial length between VCG's, whereas there was a significant difference (P=0.007) for microsclerotial width between VCG's. Microsclerotial length ranged from 56.6, 65.3 and 62.2 µm while microsclerotial width ranged from 34.7, 42.5 and 38.0 µm for VCG1A, 2A and 4B respectively.

Table 2. Average conidial length, width and microsclerotial length and width.

VCG	Pathotype	Conidial length (µm)	Conidia width (µm)	Microsclerotial length (µm)	Microsclerotial width (µm)
1A	D	5.26^{a}	2.61^{a}	56.59^{a}	34.65^{a}
2A	ND	5.61^{b}	2.63^{b}	65.33^{b}	42.49^{b}
4B	ND	5.46^{b}	2.67^{b}	62.16^{ab}	37.98^{a}

Milestone 2.1 Develop a suitable soil sampling protocol that considers the spatial variability of the *Verticillium dahliae* pathogen in cotton soil.

The average inoculum level for the field was 4.41 ppg calculated from 40 bulked samples. Intense aggregation of the pathogen was found in both the individual and bulk soil samples (Figure 12).

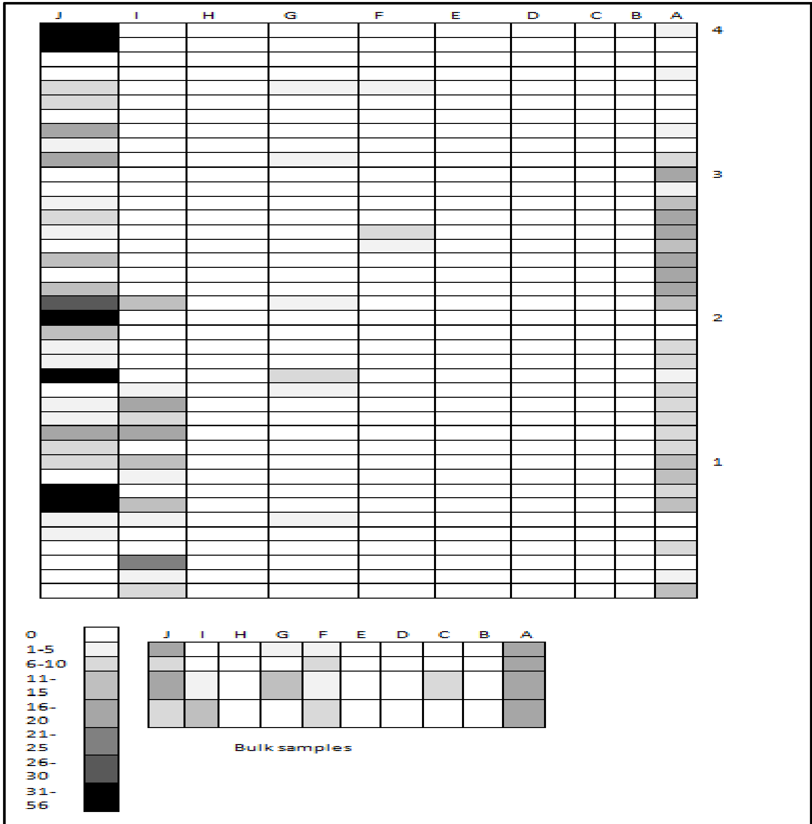


Figure 12. Pictorial view of inoculum levels in the 40ha field. Intense isolated aggregated patches were recorded within the individual sampling areas (top) and within bulked samples (bottom), indicated by dark shades.

The estimate of mean density of *V. dahliae* in this commercial cotton field was affected by quadrant size and number with heterogeneity of variance found in three of 10 replicated data sets (Table 3). The true mean estimated from the 40 bulked soil samples from the 40ha field was 4.41 ppg. Single soil samples were associated with the large variances in three of the ten replicates. Also decreasing aggregation parameters (*k*) towards zero corresponds to increasing aggregation of the pathogen. In three replicates of the sampling area of $1m^2$ a single sample was responsible for the higher than normal variance within the 20 samples assayed. The mean density of the 4ha quadrant size (4.5 ppg) was closest to the true mean of 4.41 ppg and had a lower variance.

Table 3. Estimated mean density, variance (VAR) and aggregation index (*k*) of *Verticillium dahliae* microsclerotia from sampling a range of quadrant sizes in a commercial cotton field.

	Single sample ($1m^2$)			Bulk samples (100 x 100m) ($10,000m^2$ = 1ha)			Bulk samples (200 x 200m) ($40,000m^2$ = 4 ha)			Bulk samples (400 x 400m) ($160,000m^2$ = 16ha)		
Rep	**Mean**	**VAR**	k^a	**Mean**	**VAR**	k^a	**Mean**	**VAR**	k^a	**Mean**	**VAR**	k^a
1	2.0	32.84	0.1	4.6	51.0	0.4	5.1	26.31	1.2	4.8	23.46	1.1
2	2.6	29.09	0.2	7.2	63.3	0.9	5.5	37.84	0.9	4.9	21.57	1.3
3	3.8	120.38	0.1	4.2	51.3	0.3	3.4	21.09	0.6	5.7	26.87	1.4
4	3.2	157.64	0.0	6.0	49.7	0.8	4.7	26.64	0.9	4.3	17.14	1.3
5	2.8	30.48	0.2	3.4	44.3	0.2	5.0	30.53	0.9	4.6	24.05	0.9
6	2.4	32.67	0.1	4.6	29.1	0.8	3.9	25.88	0.6	4.7	22.75	1.1
7	3.2	48.17	0.2	4.0	38.7	0.4	4.5	30.26	0.7	4.1	12.89	1.7
8	1.6	12.46	0.2	4.2	36.2	0.5	4.1	22.94	0.8	1.9	4.52	1.2
9	2.2	17.64	0.3	5.4	44.3	0.7	4.9	26.31	1.1	5.2	24.34	1.3
10	3.6	65.52	0.2	5.4	49.3	0.6	4.2	27.75	0.7	4.2	14.87	1.5
	2.7	54.7	0.1	4.9	45.7	0.6	4.5	27.6	0.8	4.8	22.6	1.2

[a] The aggregation parameter *k* was calculated as *k* = [(mean2 - VAR/*n*)/(VAR-mean)], where *n* was the number of samples collected for a sampling unit size, n=20.

Quadrant size had a significant effect (P<0.001, LSD 0.747) on predicted mean inoculum levels. Quadrant size had a significant effect (P=0.011, LSD 22.14) on variance. Quadrant size also had a significant effect (P<0.001, LSD 0.1824) on aggregation (Table 4).

Table 4. Effect of quadrant size on inoculum, variance and aggregation.

Quadrant size	Predicted inoculum	Variance	Aggregation ***(k^a)***
1 m^2	2.74^b	54.7^a	0.160^b
1 ha	4.90^a	45.6^{ab}	0.560^a
4 ha	4.53^a	27.6^{bc}	0.840^a
16 ha	4.44^a	19.2^c	1.280^a

Looking at the predicted mean for inoculum in Table 3 for each quadrant size, there was no significant difference between 4 ha and 16 ha for quadrant size, variance or aggregation. Either 4 ha or 16 ha quadrant size are the preferred sampling areas for determining inoculum levels in cotton fields. The actual sample size should also consider total field size, soil type, and crop history. Based on this, three sampling strategies were developed where one bulk sample consists of 20 individual soil samples thoroughly mixed. In the laboratory, five subsamples from each bulk soil sample should be used to determine average ppg.

- Field average - three bulk sample from 4 ha or 16 ha – more if disease is suspected to be variable
- Highest density - one bulk sample from 1 ha
- Hot spots - one bulk sample from 1 m^2 from the area affected

Horizontal and vertical inoculum levels

Inoculum levels were higher in the top 10 cm of soil profile. Estimates of the inoculum in the vertical soil profiles at Farm F showed the top 10 cm of soil contained 77% of the inoculum with 280 ppg in the top 0-10 cm compared with 85 ppg in the 11-20 cm profile (Figure 13). Similar results were found at Site two with 71% of the total estimated population in the top 10 cm with an average of 120 ppg in the 0-10 cm compared with 49 ppg in the 11-20 cm profile. There was no significant difference in the ppg horizontally across the beds or within furrows at Farm W. The average inoculum within furrows was still relatively high with 37 ppg at 0 - 10 cm and 32 ppg at 11-20 cm (Figure 14).

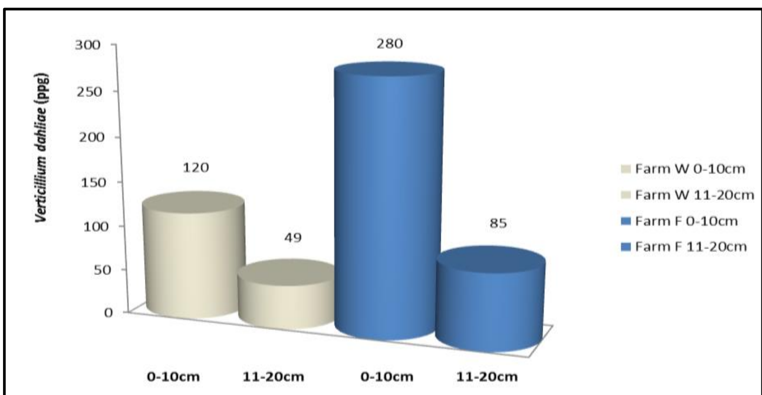


Figure 13. *Verticillium dahliae* inoculum levels in soil samples at two depths from two farms.

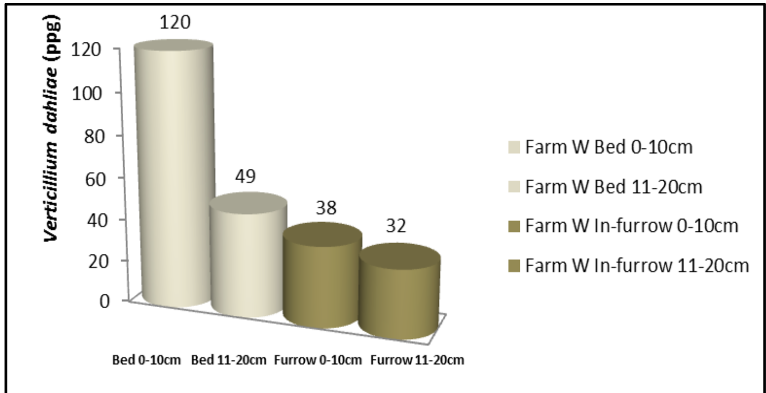


Figure 14. *Verticillium dahliae* inoculum levels in soil samples at two depths from two farms across permanent beds and in-furrows.

Determining soil isolation technique, selective media, optimum weight of soil to isolate and depth of soil sample

Soil preparation had a significant effect (P<0.001) on the recovery of ppg. The weight of soil deposited directly onto the Sorensen's NP-10 media had a significant effect (P<0.001) on the number of ppg detected in dry soil with larger amounts of soil having a negative impact on the number of colonies observed (Figure 15). Un-sieved soil recovered more ppg than sieved soil. The optimum weight of soil was 0.2 g un-sieved soil per plate. These findings agree with Goud and Termorshuizen (2003) and Kabir et al. (2004) but contradict the findings of Butterfield and DeCay (1977). Kabir et al. (2004) reported the amount of soil plated on media can influence the estimation of propagules recovered.

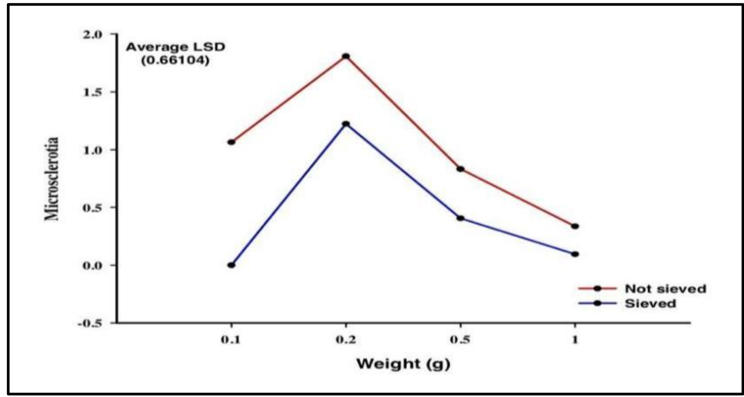


Figure 15. Number of microsclerotia (log scale) observed per plate using 0.1, 0.2, 0.5 and 1.0 g sieved and un-sieved soil per plate.

Field study results for depth to sample

The first study quantified the inoculum levels at 2-12 cm and 13-24 cm depths using four different media. The results show the highest number of ppg in both USA and Australian soils was detected using

Sorensen's NP-10 media. Isolation using dry plating consistently recovered higher ppg than the wet plating method in both studies and for all soil samples in both the USA and Australia.

In both studies, there was no significant difference in ppg detected at two sampling depths (2-12 cm and 13-24 cm) in Australian and USA soils, sampled both pre-planting and post-harvest and processed using the dry plating method on Sorenson's NP-10 media.

In the second study, higher ppg were recorded at 25-37 cm depth in post-harvest Australian soils (Table 5). The opposite was true for USA pre-planting soils where lower ppg was detected at the 25-37 cm depth (Table 6). This opposite occurrence may be attributed to the movement of infected plant tissue from the surface to lower depths during the mechanical incorporation of plant matter into the soil following harvest.

Table 5. Average number of germinated *V. dahliae* microsclerotia (MS) in propagules per gram (ppg) in Australian pre-planting and post-harvest soils using Sorenson's NP-10 media.

Plating method	Sampling depth (cm)	Australian pre-planting (ppg [z])	Australian post-harvest (ppg [z])
Dry	2-12	30.4[a]	5.4[bc]
	13-24	25.7[ab]	7.9[b]
	25-37		13.6[a]
Wet	2-12	18.5[bc]	2.3[d]
	13-24	17.4[c]	3.2[cd]
	25-37		5.1[bc]

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

Table 6. Average number of germinated *Verticillium dahliae* microsclerotia (MS) in propagules per gram (ppg) in USA soils sampled pre-planting and post-harvest using Sorenson's NP-10 media.

Plating method	Sampling depth (cm)	USA pre-planting (ppg [z])	USA post-harvest (ppg [z])
Dry	2-12	51.2[a]	10.8[a]
	13-24	43.8[a]	6.1[a]
	25-37	27.0[b]	
Wet	2-12	22.6[bc]	2.2[b]
	13-24	15.1[cd]	0.7[c]
	25-37	11.6[d]	

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($P \leq 0.05$).

A manuscript has recently been submitted by Shelby Young to Crop and Pasture Science following a copy being sent to CRDC for approval to publish and is under review.

Milestone 2.2 Efficiency & accuracy of the plating method compared with the developing molecular technique.

The comparison between dry plating and the molecular assay using Ct values obtained from DNA showed that with increasing inoculum levels observed in the dry plating method, a respective decrease in Ct was observed in the molecular assay (Figure 16). As expected, higher inputs of DNA result in a lower time until positive. The comparative results show values above a Ct of 25 representing little to no initial inoculum and values below Ct 25 representing high Verticillium soil load, with these results corroborated by the dry plating method. More samples with known ppg were tested in order to optimise the microsclerotia counting technique. Individual microsclerotia were counted and talcum powder was used as a medium with high contrast to ensure selected microsclerotia were successfully incorporated into bleached soil and sand. These samples were extracted for DNA using the high-throughput DNA

extraction method outlined below and tested with the real-time molecular assay. However, results from this were inconclusive and are likely a result of the use of talcum powder. We hypothesize that the small particle size of the talcum powder interferes with the DNA extraction method that utilises charged particles to attract DNA.

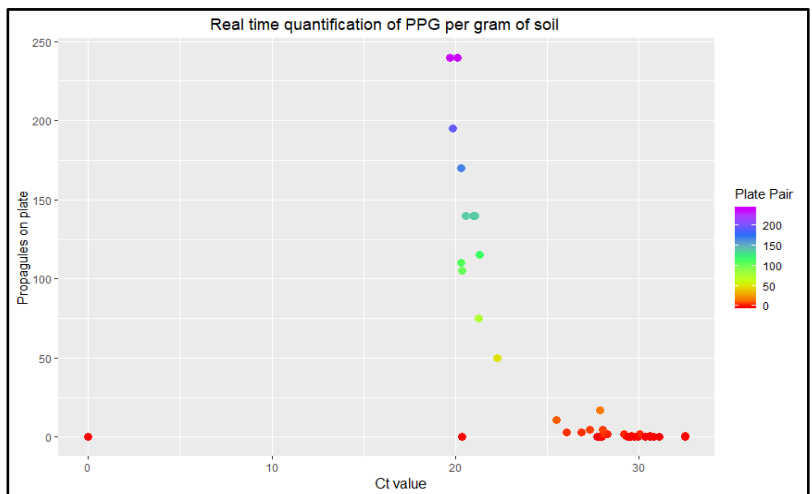


Figure 16. Comparison of ppg determined with soil plate isolations (Plate Pair) and Ct value determined using DNA.

Milestone 2.2.1 Soil molecular assay developed.

Previously, DNA from soil was extracted using a Qiagen DNeasy PowerMax Soil Kit which did not allow for high sample throughput, making the soil extraction labour intensive. Testing of the high throughput DNA extraction kit (Qiagen's MagAttract PowerSoil DNA KF) automated on a Thermofisher KingFisher 96 instrument with paired samples extracted with a Qiagen DNeasy PowerMax Soil Kit (Manual) was performed to assess how comparable the DNA obtained from the high throughput method was to our previously used manual method (Table 7). Twelve separate soil samples were each sub-sampled six times with three sub-samples prepped with the manual extraction method and three sub-samples prepped with the high-throughput extraction method. All samples were then run on a Qiagen Rotor Gene Q using the primers Vd-IGS F/R and protocol outlined in Gharbi *et al* (2016). An extension of the project has enabled further optimisation of this assay to continue.

Table 7: Table showing triplicate real-time PCR Ct values of soil samples tested with a manual vs high-throughput DNA extraction kit.

Sample	Replicate 1	Replicate 2	Replicate 3	Average	ppg on plate	Method
2	23.95	27.63	25	25.53	26	Manual
3	24.41	24.89	24.83	24.71	30	Manual
5		27.02	31.85	29.44	25	Manual
15			28.51	28.51	27	Manual
22	30.89		24.01	27.45	29	Manual
30		26.88	29.3	28.09	5	Manual
44	23.01	27.25	25.36	25.21	2	Manual
47	26.26	25.31	27.49	26.35	3	Manual
51	24.8	24.51	26.96	25.42	6	Manual
58	25.39	26.66	25.09	25.71	3	Manual
101	26.55	24.54	26.2	25.76	0	Manual
303	24.62	25.09	24.67	24.79	0	Manual
	Average variance between Manually extracted samples = 5.86					
2	27.7	27.68	26.83	27.40	26	High Throughput
3	25.65	24.99	26.44	25.69	30	High Throughput
5	26.48	26.7	28.71	27.30	25	High Throughput
15	25.61	24.97	27.74	26.11	27	High Throughput
22	27.4	26.82	27.25	27.16	29	High Throughput
30	26.32	23.6	28.87	26.26	5	High Throughput
44	27.03	26.86	25.05	26.31	2	High Throughput
47	26.27	26.33	26.68	26.43	3	High Throughput

51	27.84	29.43	27.29	28.19	6	High Throughput
58	27.81	24.38	26.34	26.18	3	High Throughput
101	25.76	25.08	27.15	26.00	0	High Throughput
303	24.78	25.76	25.86	25.47	0	High Throughput
	Average variance between High Throughput extracted samples = 3.06					

Variance between the triplicate samples was lower in samples extracted with the high-throughput DNA extraction kit. Five samples extracted manually resulted in no amplification. This showed the high throughput DNA extraction kit produced a more consistent DNA extract with less variability in samples. Upon successful testing of Qiagen's MagAttract PowerSoil DNA KF, soil samples from two fields, Farm 1 and Farm 2, were investigated with the soil molecular assay for soil-borne *V. dahliae* at pre-planting, mid-season and post-harvest timepoints. Each field consisted of 24 plots with triplicate samples tested per plot. The results of this molecular assay were compared to the soil plating method and are shown in Figure 17 below.

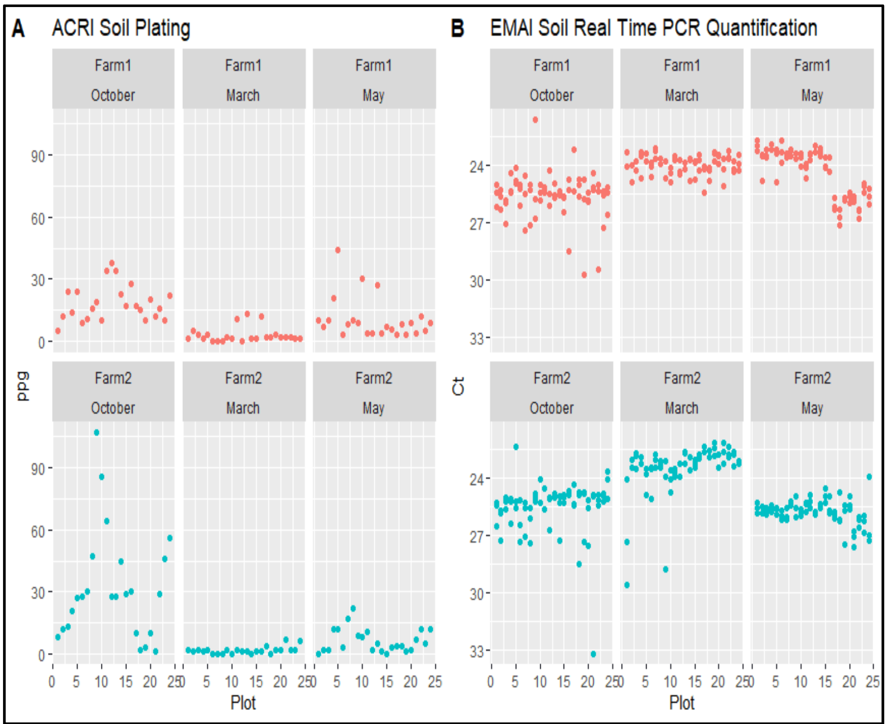


Figure 17. A) Number of ppg of *V. dahliae* in soil samples at pre-planting (October), March and post planted (May) timepoints in Farm 1 (red) and Farm 2 (green) fields. **B)** Triplicate samples of soil tested with the IGS real-time molecular assay on pre-planting, March and post-harvest timepoints in Farm 1 and Farm 2 fields.

To test the accuracy of the soil plating method, five bulk soil samples were sub-sampled three times with each sub-sample having five replicate plates. Analysis of variance found variation between individual bulk samples (as expected) and no significant difference between sub-samples or replicates. With the soil plating method, individual plots at pre-planting have a higher variability than in the mid time point, with *V. dahliae* numbers appearing to drop significantly five months later in March (Figure 18 and 19). Post-harvest inoculum levels increase after harvest as a result of infected plant material returning to the soil. This highlights the spatial variability of inoculum levels throughout a field. Generally, field average inoculum levels at Farm 1 and Farm 2 were highest pre-plant, falling significantly in March 2019 and rising again following harvest and incorporation of stubble (Figure 20).

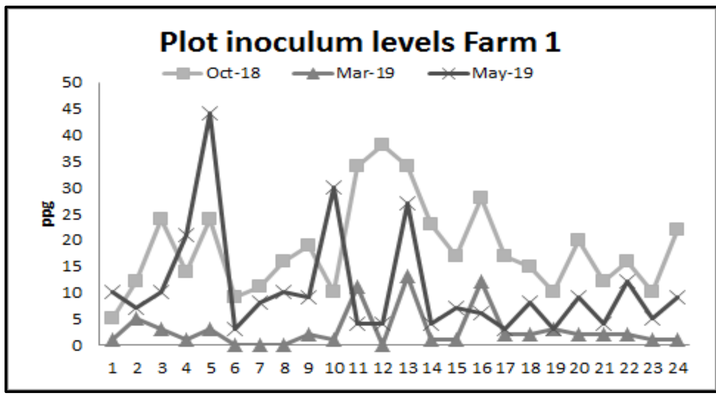


Figure 18. Inoculum levels at Farm 1 pre-plant in October 2018, March 2019 and post-harvest in May 2019.

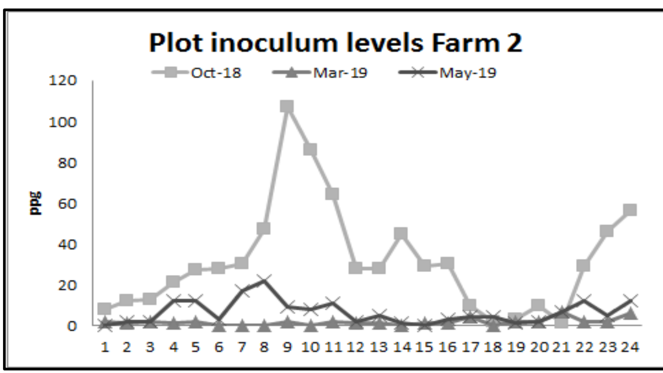


Figure 19. Inoculum levels at Farm 2 pre-plant in October 2018, mid-season in March 2019 and post-harvest in May 2019.

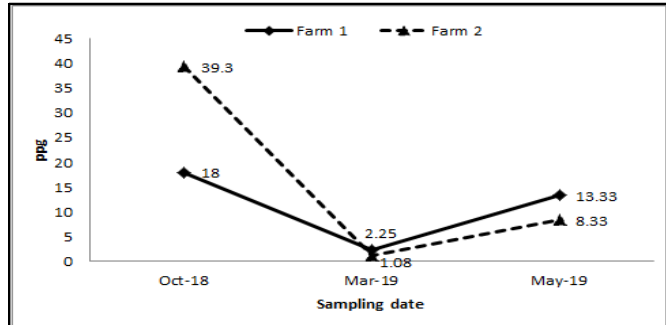


Figure 20. Average field inoculum levels at Farm 1 and Farm 2 pre-plant in October 2018, mid-season in March 2019 and post-harvest in May 2019.

Comparison of dry plating method and PREDICTA®B technique

There was no correlation between inoculum levels using the dry plating technique and the PREDICTA®B technique (Figure 21). This is not surprising given the dry plating quantifies the viable microsclerotia and PREDICTA®B quantifies total DNA (live and dead) from the Verticillium. The variability in the size of individual microsclerotia, conidia and hyphae in soil may account for some of the variation in the PREDICTA®B inoculum levels. The difference in observations between the soil isolation plating method and the molecular techniques are due to the fundamental differences in their mechanisms of analysis, where the soil plating method can identify and quantify viable and culturable *V. dahliae* regardless of the size of microsclerotia. On the other hand, the molecular assay can identify and quantify culturable and unculturable *V. dahliae* which may include live, dead, dormant microsclerotia of mixed sizes, conidia and hyphae.

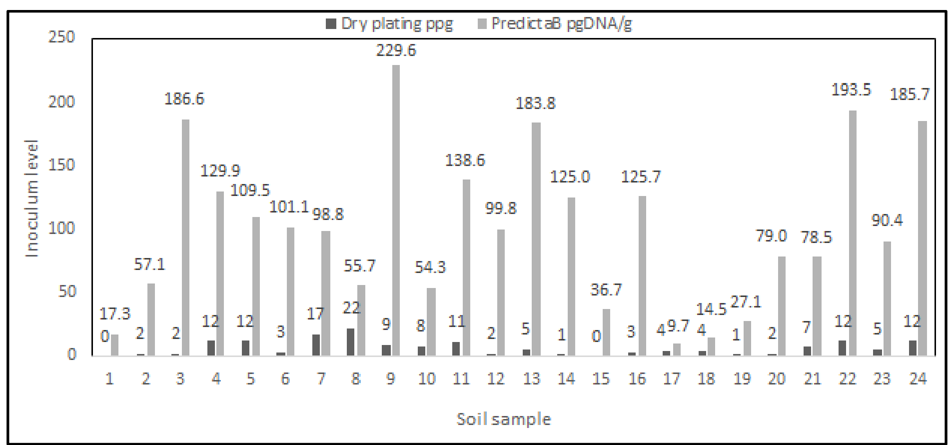


Figure 21. Comparison of inoculum levels in 24 soil samples using dry plating and PREDICTA®B methods.

Milestone 2.2.2: Plant tissue assay.

The plant tissue assay was then performed across 368 samples in various hosts of which 60% of samples were positive (Table 8). When tested on pure cultures (n=67), this assay had a 93% success rate and the stems from which these cultures were isolated matched 100% of the time with the pure culture results indicating a high success rate for this assay. Results of 60% sample positive from the overall cohort of samples is likely indicative of samples not containing *V. dahliae* as opposed to failed assay results.

Table 8. Detection of *Verticillium dahliae* using tissue assay method**.**

Sample	Positive	Negative	Percentage Positive
Cotton	111	28	80
Almond	0	2	0
Broccoli	1	0	100
Chickpea	1	5	17
Eggplant	11	0	100
Ethiopian Cabbage	3	0	100
Mustard	3	2	60
Noogoora Burr	73	101*	41
Oak	0	1	0
Olive	1	0	100
Safflower	15	1	94
Soil Plates	5	4	56

*Many of the Noogoora burr samples returned a positive PCR result, but sequencing was not clear enough to provide a presumptive VCG due to more than one VCG present.

Milestone 2.3: Determine minimum inoculum levels of *Verticillium dahliae* VCG1A, 2A and 4B needed to cause disease symptoms

Pot experiment

The growth room replicated pot experiment showed all plants infected with 1ppg from VCG1A, 2A and 4B showed external symptoms of Verticillium wilt as did plants infected with 5, 10 and 20 ppg. Similar findings were reported by Shiraishi *et al.* (2014) with the vegetable plant Udo in Japan.

Field trials

Intensive field trials to assess potential relationship between inoculum and disease severity

The average incidence of Verticillium wilt for Farm F in Field 4B was 53% (105/200). 105 plants had vascular discolouration with a stem disease index of 1.14. 103 plants showed external foliar symptoms with a foliar disease index of 1.015. The total number of plants within each of the 0-3 stem scale and 0-4 foliar scale are represented in Figure 22. These results confirm not all infected plants exhibit external foliar symptoms. Inoculum levels ranged from 0 - 21 ppg.

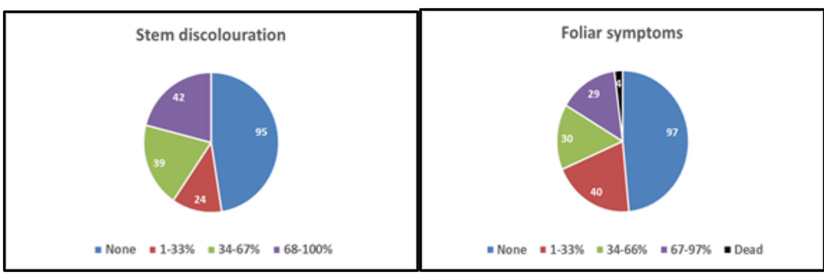


Figure 22. The number of plants from Farm F assessed for stem disease index (left) and foliar disease index (right) scales represented by each colour.

The average incidence of Verticillium wilt for Farm L in Field 13 was 90% (180/200). 180 plants had vascular discolouration with a stem disease index of 1.76. 179 plants showed external foliar symptoms with a foliar disease index of 1.65. The total number of plants within each of the 0-3 stem scale and 0-4 foliar scale are represented in Figure 23. These results confirm not all infected plants exhibit external foliar symptoms. Inoculum levels ranged from 0 - 22 ppg.

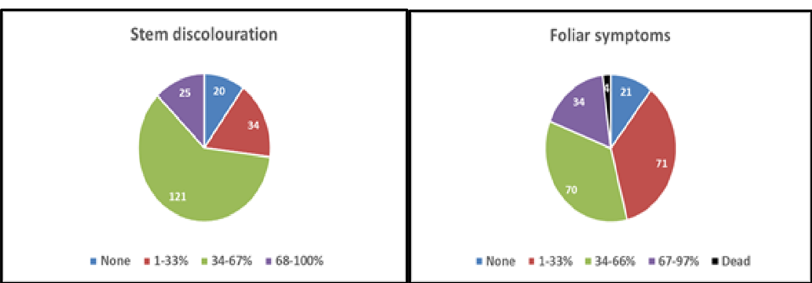


Figure 23. The number of plants from Farm L assessed for stem disease index (left) and foliar disease index (right) scales represented by each colour.

Despite the fields at Farm AM having a history of Verticillium wilt in previous years, the incidence of Verticillium wilt in Field L2, Field L3 West and Field L3 East was zero. The potential relationship between the percentage of plants with stem symptoms and the inoculum levels from both farms with Verticillium wilt present were pooled. The inoculum level (ppg) did not have a significant effect on disease index as high disease indices were recorded with as little as 1 ppg. Figure 24 shows a minimum of 1 to 11 ppg can cause stem disease index ranging between 0.8 and 2.5.

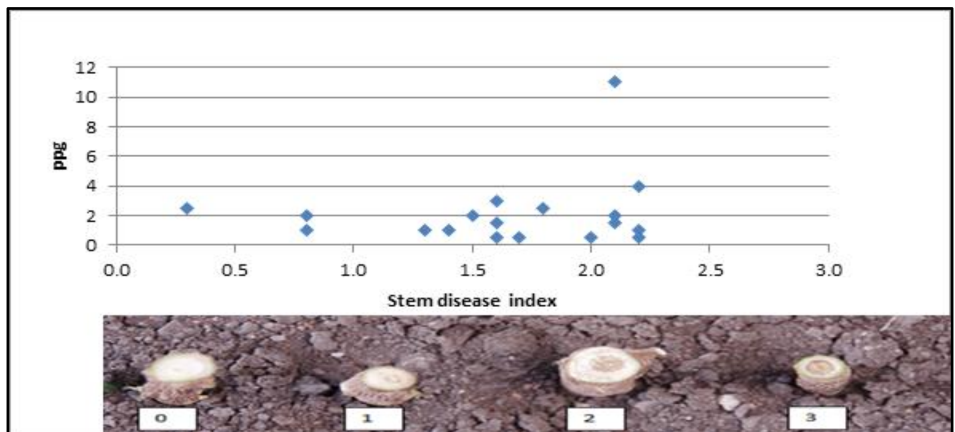


Figure 24. Inoculum levels (ppg) and stem disease index scores from two fields assessed in the 2016/2017 season shows no relationship with stem discolouration.

The information gained from these field trials using bulked soil samples led to a change in protocol to determine the potential risk associated with inoculum levels. A more comprehensive study of pre-planting inoculum levels within field plots and disease incidence was then undertaken the following season to develop thresholds for disease incidence.

Intensive field trials to assess potential relationship between inoculum pre-planting and disease incidence

Intensive field assessments were carried out to assess the symptom expression and incidence of Verticillium wilt. Not every infected plant exhibited external foliar symptoms. In fact, 41% of infected plants had no external symptoms and disease was confirmed by vascular browning and tissue isolations. The lack of significant relationship between ppg and foliar disease index carried out the previous season may be explained by the fact not all infected plants had foliar symptoms. A reasonable relationship (R^2 = 0.0622) was observed between pre-plant ppg in September/October and the minimum disease incidence record in late season (Figure 25).

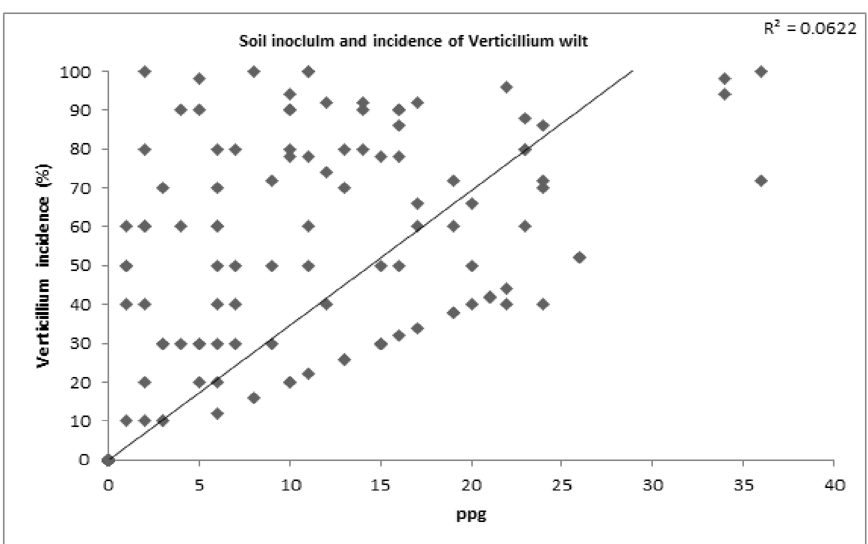


Figure 25. Relationship between pre-plant inoculum levels and incidence of Verticillium wilt.

The average disease incidence of each plot where inoculum levels were replicated had no strong correlation (R^2 = -0.998) but a reasonable grouping into three inoculum ranges of 1 - 10, 11 - 22 and 23 - 36 ppg (Figure 26).

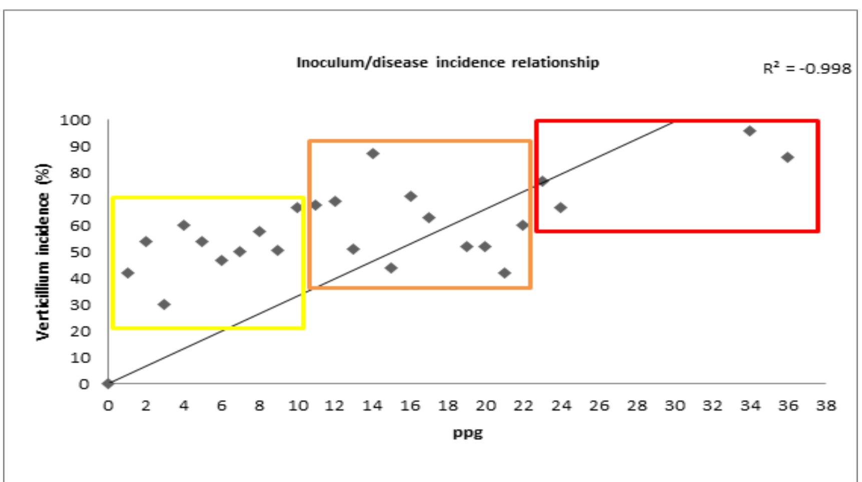


Figure 26. Disease risk matrix indicating relationship between ppg and minimum disease incidence. Mean of 26 separate inoculum levels ranging from 0 - 36 ppg.

Data from the extensive plot trials was used to develop a disease risk matrix which had limited field validation using four commercial cotton fields in 2018/2019 (Figure 27). Each of the fields had variable levels of disease incidence. The relationship between the predicted minimum disease incidence and pre-plant inoculum level was very good (R^2 = 0.9508).

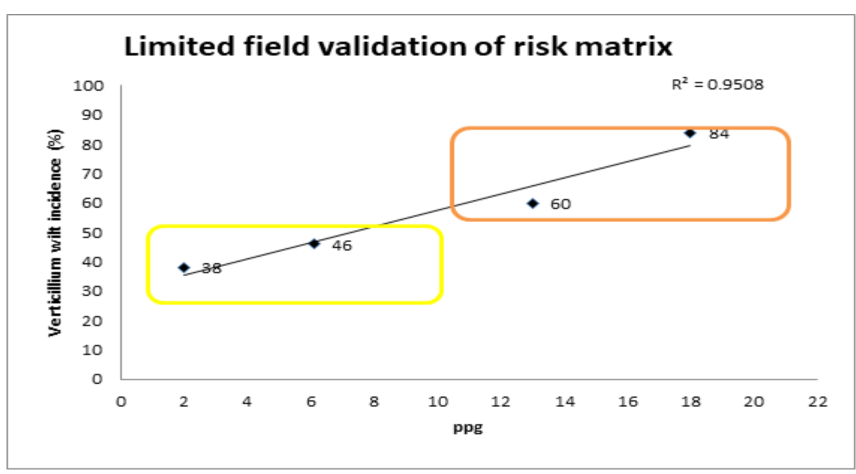


Figure 27. Limited field validation results for the disease risk matrix showing a relationship of R^2=0.9508.

The microbial community composition of soil with high and low inoculum levels was assessed by Gupta Vadakattu to look for potential differences in microbial activity. The results of the soil samples sent to Gupta showed there were differences in soil microbial profiles with low and high inoculum levels. Samples that are closer on the plot indicate greater similarity in terms of microbial catabolic diversity profiles, such as plots with low inoculum levels in Plots TD2 with 0 ppg, TD13 and TD19 with 1 ppg (Figure 28).

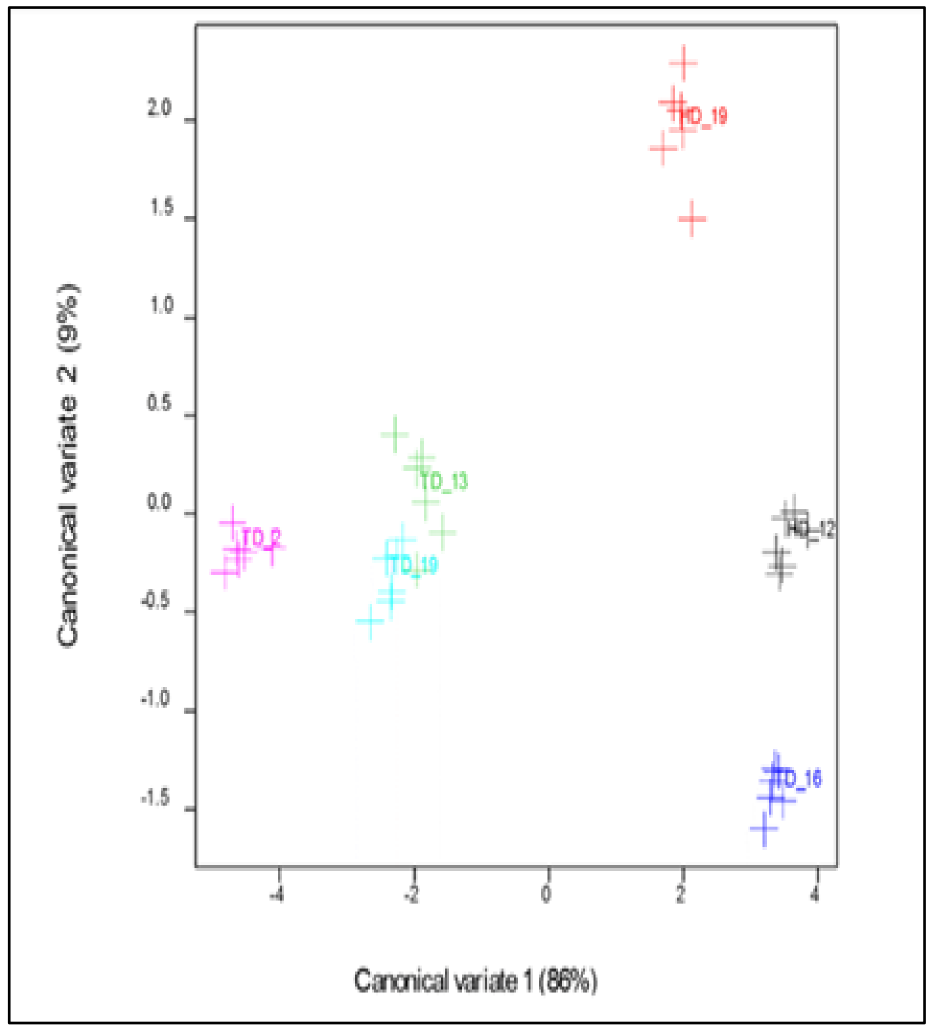


Figure 28. Multivariate analysis (Canonical variant analysis) of soil samples (indicated by colours) with low and high ppg.

A heat map generated for individual substrate-based response in microbial activity expressed as normalized values against total microbial activity was generated (Table 9). Results were obtained for average metabolic response (AMR), community metabolic diversity (CMD) and substrate induced respiration. Soil samples from Field 1 with low inoculum levels had lower AMR, CMD and substrate induced respiration compared to soil collected from Field 2 with high inoculum levels.

Table 9. Heat map showing comparisons in carbon substrate use efficiency for 31 different substrates by microbial communities in rhizosphere soil collected during October 2017 from two fields near Merah North with high and low *Verticillium dahliae* inoculum levels.

	C substrate	TD_F1_2	TD_F1_13	TD_F2_16	TD_F1_19	HD_F2_12	HD_F2_19
	Water						
Carbohydrates	Arabinose						
	Fructose						
	Galactose						
	Glucose						
	Xylose						
	Mannose						
	Maltose						
	Sucrose						
	Raffinose						
Aminoacids	Hydroxy-L-proline						
	Glycine						
	Asparagine						
	Valine						
	Serine						
	Alanine						
	Glutamine						
	Tryptophan						
	Leucine						
	Phenylalanine						
	Lysine						
	Arginine						
	Histidine						
	Aspartic						
	Methionine						
	Cysteine						
Carboxylic acids	Fumaric acid						
	Malic Acid						
	Malonic Acid						
	Oxalic Acid						
	Succinic acid						
	Tartaric Acid						
Average Metabolic Response (AMR)		2.887	2.859	3.194	2.589	3.764	4.224
Community Metabolic diversity (CMD)		10.6	11.4	14.9	9.9	16.7	17.3
Substrate Induced Respiration		3.181	4.046	5.840	3.122	6.335	7.418

- AMR is an average for the total microbial activity.
- CMD values represent the number of substrates that microbes showed significant increase in microbial activity; this is used as an index of microbial catabolic diversity i.e. diversity of active microbial community.
- Substrate induced respiration represents how responsive they are compared to background microbial activity.

Milestone 2.4: Effect of crop rotation on inoculum levels

In 2013, following a cotton crop the inoculum levels within the six sites were extremely high, averaging 706 ppg. Following the inclusion of Durum, the levels assessed in December 2014 decreased significantly to an average of 128 ppg (Figure 29). Over the years of rotation crops the inoculum levels continued to fall.

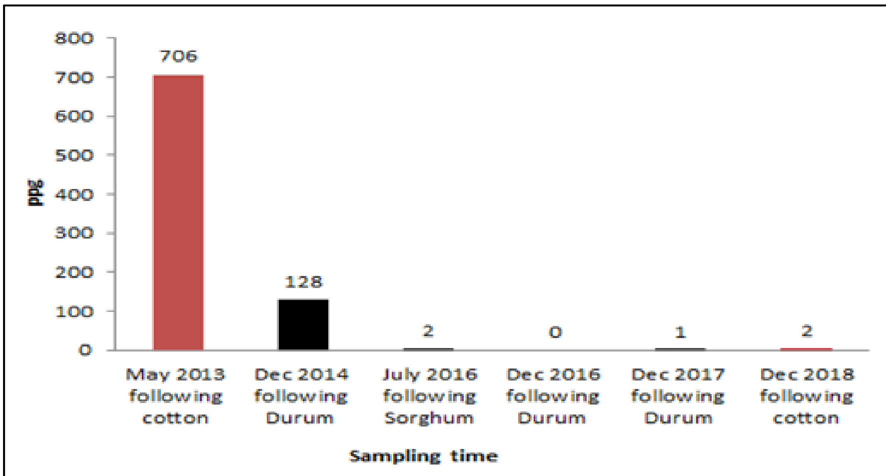


Figure 29. Reduction in inoculum levels measured as ppg in 2014 following the inclusion of Durum at six GPS sites in the field.

The average of the random sites remained low, but increased inoculum levels. In the 12 months between the Durum crop and following a cotton crop the average inoculum levels across the field increased from 0.54 to 2.25 ppg (Figure 30).

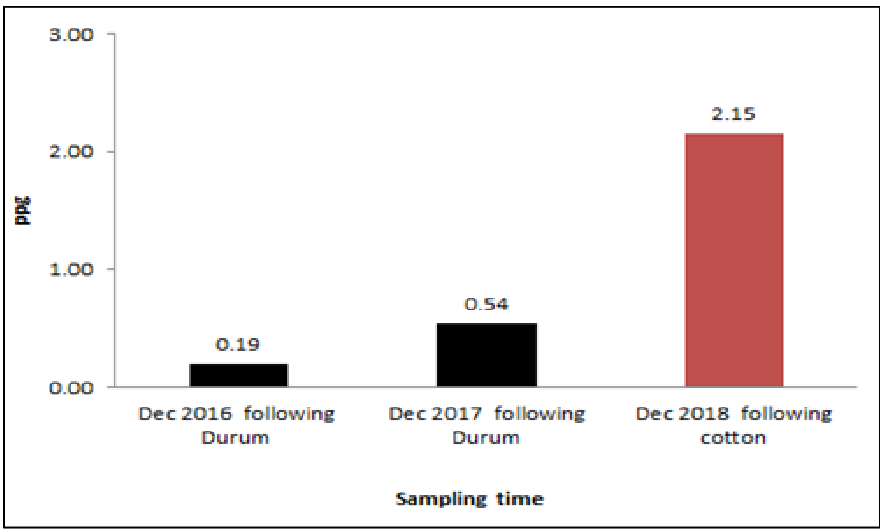


Figure 30. Increase in average inoculum level of 20 random sampling sites in December 2018 following a cotton crop.

The average incidence of Verticillium wilt during 2017/2018 cotton crop was 37.5%. Most of the infected plants retained their bolls and were not defoliated. Some completely defoliated plants retained their bolls. No large patches of dead plants were observed in the field. Since 2013, the ppg declined following the inclusion of durum wheat and sorghum in the rotation up until the planting of cotton in 2017. Inoculum levels increased following the incorporation of infected cotton material following harvest in 2018.

Similar findings have been reported by Dr Jason Woodward from field trials in 2011. Dr Woodward's presentation can be found at (http://www.plantmanagementnetwork.org/edcenter/seminars/Cotton/VerticilliumWilt/presentation_html5.html).

Additional crop rotation crops assessed during 2018/2019
Inoculum levels in two fields following Industrial Hemp increased from 1 to 6 pgg in one field and remained the same in the other field. No infected Hemp plants were observed. Tissue isolations from the same farm were made from Safflower plants showing disease symptoms and *V. dahliae* VCG1A and VCG2A were confirmed.

In the biofumigant trial run by a local agronomist, the inoculum level increased with increasing rates of biofumigant seed planted per hectare. Volunteer Ethiopian cabbage and mustard plants from this crop were taken to the laboratory and isolated. These plants were confirmed hosts for VCG1A. The inoculum levels in the Breeza trials showed both increases and decreases in inoculum levels following different rotation crops (Figure 31).

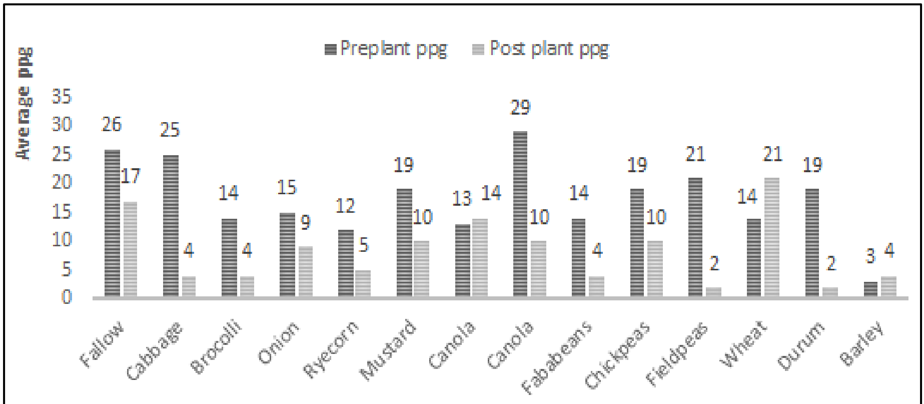


Figure 31. Pre-plant and post-harvest inoculum levels under various rotation crops.

Milestone 2.5: Validate Verticillium wilt tool.

Two molecular tools were validated for use in diagnostics of *V. dahliae* in both plant samples and soil to determine the potential impact in a range of hosts and specifically, cotton. The first of these tools is the plant tissue assay, optimised for fast throughput results. This assay has been reduced to one real-time PCR that can determine the VCG of *V. dahliae* infections within a host plant. Traditionally, diagnostics would require a lengthy culturing phase to be able to identify *V. dahliae* from a host and further complicating the matter was that it was not always possible to isolate. From our testing, this plant tissue assay has been tested on 139 cotton samples for the presence or absence of *V. dahliae*. Further validation of this assay was performed by testing on stems and the isolates obtained from these same stems.

The second of these tools is a soil assay to test for the presence of *V. dahliae* in a field, pre-planting. The soil assay has been optimised to work with high-throughput DNA extraction to enable large volume testing as well as obtaining real-time results. Unlike the stem assay, due to the nature and the complex microbial composition of soil, this DNA-based assay cannot differentiate between viable microsclerotia in the soil and residual DNA from dead microsclerotia in soil at low propagule numbers (less than 5 ppg). Testing in this project has demonstrated that as little as 1 ppg is enough to cause disease symptoms and yield loss. IGS Sequencing cannot differentiate between VCG's when there are multiple VCG's present in the system, but our method can differentiate between the defoliating and non-defoliating pathotypes. However, VCG testing may still be accomplished using this assay on isolated microsclerotia from soil plating. In addition to high-throughput, real time testing, the soil assay can give an indication of the *V. dahliae* load in the soil and therefore the risk it poses to potential crops.

These two tools provide a two-pronged approach to assess the impact *V. dahliae* may have on a crop, both in season (through the stem and soil assay) and out of season (through the soil assay) and play a role in farm management decisions.

Milestone 2.6: Assessment of published LAMP assay for Verticillium wilt.

The existing LAMP assay was tested on reference strains, soil samples and plant samples. Primers for a loop-mediated isothermal amplification (LAMP) were tested on nine different *V. dahliae* isolates of various VCG. Using the protocol as published, no positive results were obtained. Upon communicating

with the authors of the published primers, their suggestion was that their LAMP was not specific enough to differentiate between Australian pathotypes meaning it is not viable as a diagnostic for *V. dahliae* under Australian conditions.

Milestone 3.1: Conduct a literature review & PhD planning.

- PhD planning was completed within the first 6 months.
- Under review. Dadd-Daigle. P. Kirkby. K., Chowdhury. P., Labbate. M and Chapman. T. “Literature review - The Verticillium wilt problem in Australian cotton”. **Manuscript submitted** to Australian Plant Pathology

Milestone 3.2: Examining the genetic diversity of *Verticillium dahliae* in Australia and comparing it to overseas strains.

Due to copyright, the results of PhD student Pearl Dadd-Daigle’s research results are being published as manuscripts and in her thesis.

Manuscripts

- Dadd-Daigle. P. et al. “Virulence not linked with Vegetative Compatibility Groups in Australian cotton *Verticillium dahliae* isolates”. **Accepted for publication in** Australian Journal of Crop Science.
- Dadd-Daigle. P., Kirkby, K., Roser. S.,Lonergan. P., Chowdhury. P., Labbate. M and Chapman. T. “Virulence varies in Australian cotton *Verticillium dahliae* isolates”. **Draft manuscript**
- Dadd-Daigle. P. et al. “Whole genome analysis of Australian *V. dahliae* Vegetative Compatibility Group 1A”. **Preparing a draft manuscript**

Thesis

- Dadd-Daigle. P. “Verticillium wilt in Australian cotton: examining the relationship between Australian *Verticillium dahliae* isolates and virulence”.

Multi-locus sequence typing (MLST) results using five genes found three of these targets, ACT, OX and GPD, were suitable for producing an MLST for the differentiation of *V. dahliae* pathotypes (Figure 32). Individually each of these three regions were unable to differentiate between all the pathotypes. However, the final MLST represented by these three regions produced a phylogeny that was able to differentiate between VCG1A, VCG2B and VCG2A/4B. Included in this MLST scheme were five isolates of known VCG from Israel, as well as seven Australian isolates that have been nit mutant tested (Chapman et al 2016).

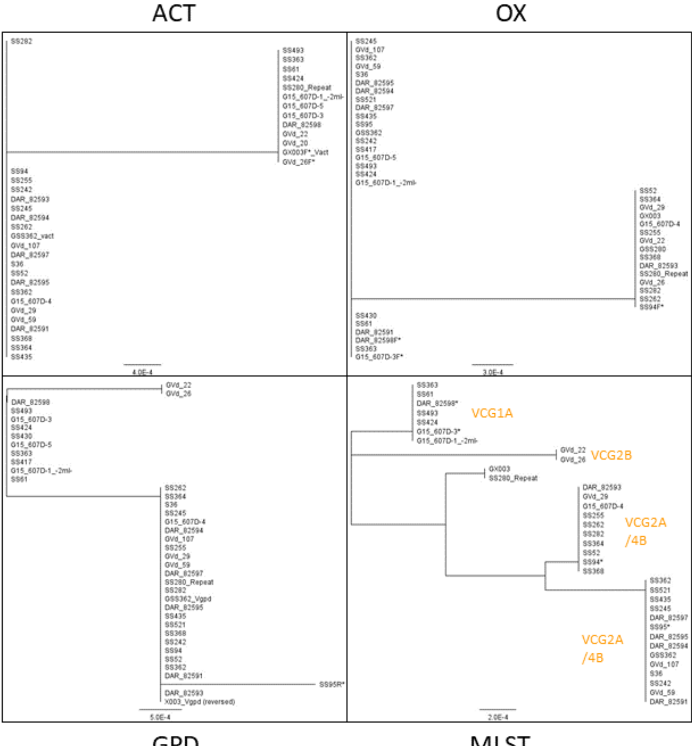


Figure 32. Phylogenetic trees of three MLST regions, ACT, VOX and GPD and a concatenated MLST of these three regions.

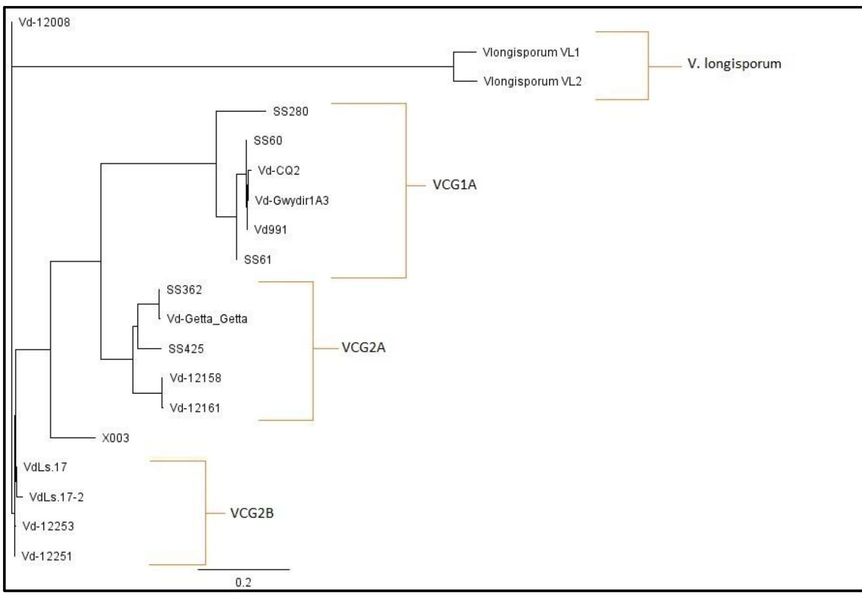
Milestone 3.3: Genome sequencing of selected *Verticillium dahliae* for proof of concept analysis to assess the genetic variation between virulent and avirulent strains.

Genomes of select *V. dahliae* were genome sequenced with short and long read sequencing technologies in order to assemble whole genomes of these isolates. Whole genome sequencing is used to achieve a greater understanding of the pathogen, identify pathotype differences between isolates and investigate methods to design diagnostic assays that may differentiate between pathogenicity.

The isolates sequenced included:
-X003 - A Noogoora burr isolate (nit mutant testing results in Spain delayed due to COVID-19)
-SS280 - A cotton isolate nit mutant tested as VCG1
-SS362 - A cotton isolate with an IGS sequence indicating VCG2A that is shown to cause high levels of disease in cotton plants.
-15/607D - A Noogoora burr isolate with an IGC sequence indicating VCG2A isolate from the same location as the cotton SS362 isolate.

Sequencing of these isolates using PacBio technology was planned in order to produce largely complete genomes and comparative genomics to be used to identify unique regions that could be possible diagnostic targets for VCG assessment. Multiple commercial kits were assessed for their capacity to produce high molecular weight DNA and were unable to produce the concentrations required for PacBio sequencing. Manual extractions using enzymatic and salt lysis methods were also tested for their effectiveness. These methods, while extracting DNA, also appeared to co-purify other compounds with similar chemical structure to DNA, confounding DNA quantification and quality results. Assessment of DNA extracts by the Ramaciotti Centre for Genomics using Nanodrop, Qubit, Gel Electrophoresis and a Lunatic chip, were not able to quantify consistently the concentration of DNA in the samples and PacBio sequencing was recommended not to proceed due to these co-purified contaminants.

To still obtain long read sequencing data of the target genomes, DNA extracts were run on an Oxford Nanopore MinION. Sequencing reads from the MinION were not long enough and sequencing was not deep enough to be able to produce a genome that could be assembled from these complex isolates. Assembly was attempted with Canu, Unicycler and Flye long read assemblers, but all failed to produce a genome of correct length. This data likely needs to be supplemented with additional long read MinION reads in order to complete the genome assembly. Short read sequencing on the four isolates using the Ion Torrent S5 were multiplexed onto one Ion Torrent 530 sequencing chip at equimolar ratios. One sample, 15/607D, was unable to produce any reads at all on both Minion and Ion Torrent, despite DNA being quantified in the library preparations. This is believed to be due to compounds that were co-purified with the DNA, inhibiting the sequencing reaction. This sample was omitted from analysis and replaced with a non-pathogenic 2A isolate sequenced using Illumina Miseq, isolate SS425. Ion Torrent reads were assembled, and a core SNP analysis performed using Snippy 4.4.5 on SPAdes assembled genomes with *V. dahliae* JR2v4.2.0 (Genbank accession GCA_000400815.2) as the reference sequence. The core SNP alignment comprised 224,934 SNPS and a tree was built using the Neighbour-Joining algorithm in Geneious 9.1.8 (Figure 33).



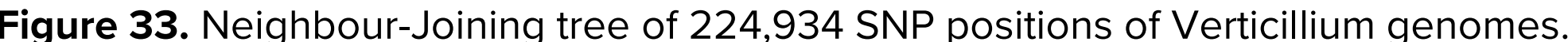


Figure 33. Neighbour-Joining tree of 224,934 SNP positions of Verticillium genomes.

Other genomes of *V. dahliae*, obtained from NCBI, were also analysed with Snippy and included in the SNP tree. This tree shows the phylogenetic relationship of all references and all genomes sequenced in this study. Isolate SS362, while demonstrated to be pathogenic, has been identified as a VCG2A and clusters with other VCG2A pathotypes from NCBI as well as SS425, a non-pathogenic VCG2A. Isolates of VCG1A also are shown to cluster based on their SNP alignments. However, SS280, while nit mutant testing showed was a VCG1A, is more distantly related and is shown to not cluster with other known 1As. This isolate also was demonstrated to not have an IGS sequence identifying it as a VCG 1A. Likewise, X003 which was also shown to have an IGS sequence the same as SS280 does not cluster with sequences of either VCG2A or VCG1A.

Annotation of the *V. dahliae* genomes was performed using funannotate (https://funannotate.readthedocs.io/en/latest/index.html). SPAdes assemblies were initially cleaned by aligning the shortest contigs against the rest of the SPAdes assembly with minimap2 to detect repetitive segments. This process was looped starting from the shortest contigs to the N50 of the assembly, removing any contigs that had a percentage overlap of 95% or more with a 95% or higher identity. Remaining contigs were then sorted from largest to shortest and repeat regions were softmasked using a combination of RepeatModeler/RepeatMasker.

Ab-initio gene prediction was performed with Augustus, Genemark, Snap and GlimmerHMM, BUSCO was used to train Augustus and tRNAScan-SE was used to predict tRNA regions. Evidence modeller was then run to generate consensus gene models from all the data obtained. Functional annotation was performed by initially running predicted protein regions through Interproscan 5 (which provides annotations using CATH-Gene3D, CDD, HAMAP, PANTHER, Pfam, PIRSF, PRINS, PROSITE profiles, PROSITE patterns, SFLD, SMART, SUPERFAMILY and TIGRFAMS). Additional functions were annotated using EggNog Mapper, Diamond blastp of UniProtKB and MEROPS, CAZyme and GO ontology.

Annotations were then assessed for completeness using BUSCO (Benchmarking Universal Single-Copy Orthologs). These BUSCO profiles showed that annotation profiles appeared to be incomplete with a large proportion of single copy orthologs either missing or fragmented. To address this, training of Ab-initio gene predictors was performed using *V. dahliae* JR2 RNA-seq data, publicly available on NCBI's Sequence Read Archive (SRA). This RNA-seq data was assembled against the JR2 genome with Trinity and PASA to produce *V. dahliae* specific gene prediction models. Gene prediction and functional annotation was performed a second time and a summary of the BUSCO profiles showing an increase in the quality of the annotations is seen in Figure 34 and Table 10.

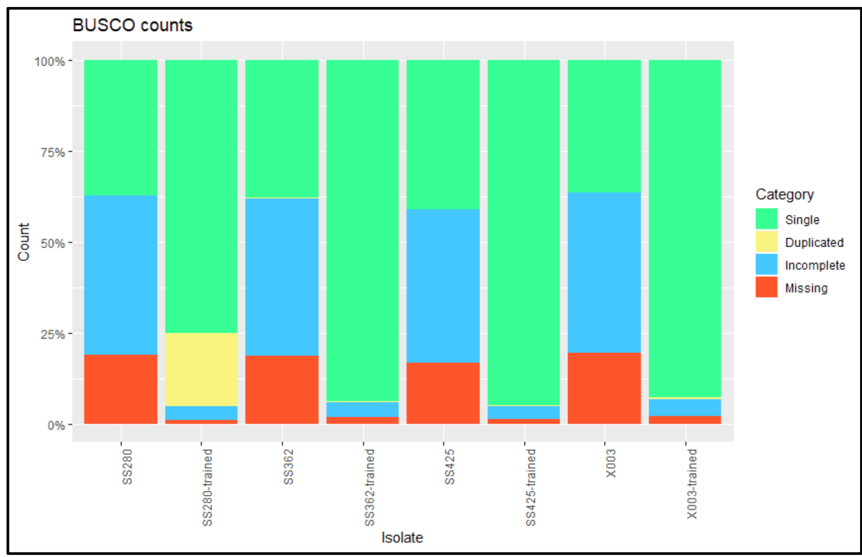


Figure 34. BUSCO profiles of annotated *Verticillium dahliae* genomes SS280, SS362, SS425 and X003 before training of ab-initio gene predictors (SS280-trained, SS362-trained, SS425-trained and X003-trained).

Table 10. *Verticillium dahliae* genome sequencing and annotation statistics.

Isolate	Total Size (bp)	No. Contigs	GC Content (%)	No. Proteins	No. tRNA	Functional Annotation
X003	31,982,796	1916	56.11	9494	227	2406 (25.3%)
SS280	64,225,913	8921	54.2	17214	379	4064 (23.6%)
SS362	32,564,961	1577	55.99	9472	228	2262 (23.8%)
SS425	34,484,710	2440	53.99	9488	234	2097 (22.1%)

Functional annotation of these genomes represents a significant step forward in the understanding of these pathogens. To date, only one publicly available Verticillium genome has functional annotation associated with it, VdLs.17. An analysis of these functional annotations gives insight into the metabolisms of the individuals sequenced and a comparison of these metabolisms is shown below in Figures 35, 36 and 37. Clusters of Orthologous Groups (COGs) are widely used to classify proteins from sequenced genomes based on the orthology concept. Differences in the abundances of these COG categories is indicative of the differences in metabolic processes occurring between isolates. Peptidases (those classified by MEROPS) and carbohydrate-active enzymes (CAZymes) are also used in this way to identify differences in metabolism signified by varying functional protein profiles.

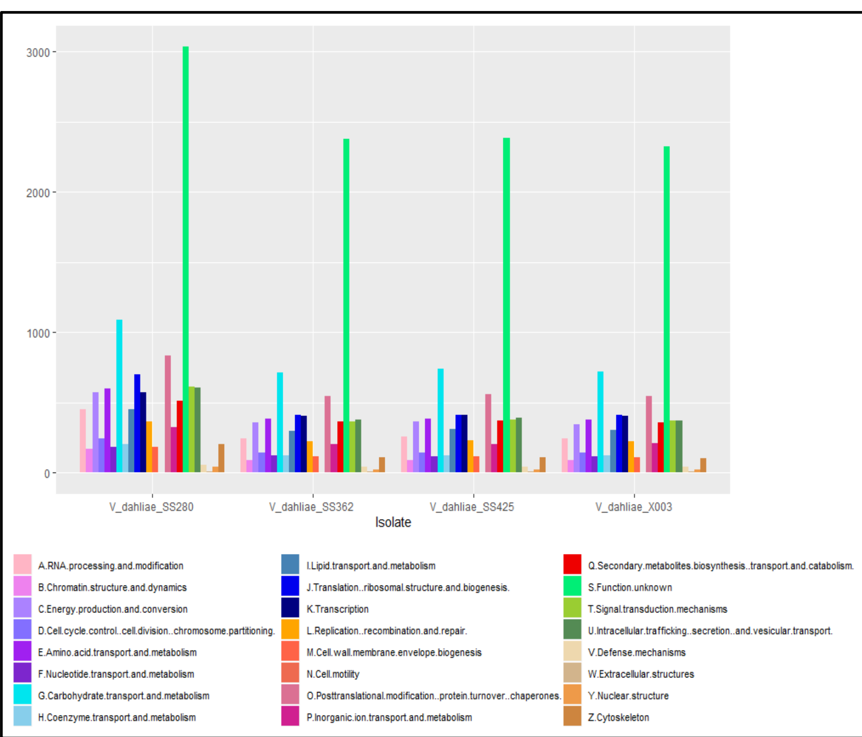


Figure 35. Cluster of Orthologous Groups (COGs) graph of *Verticillium dahliae* sequenced isolates.

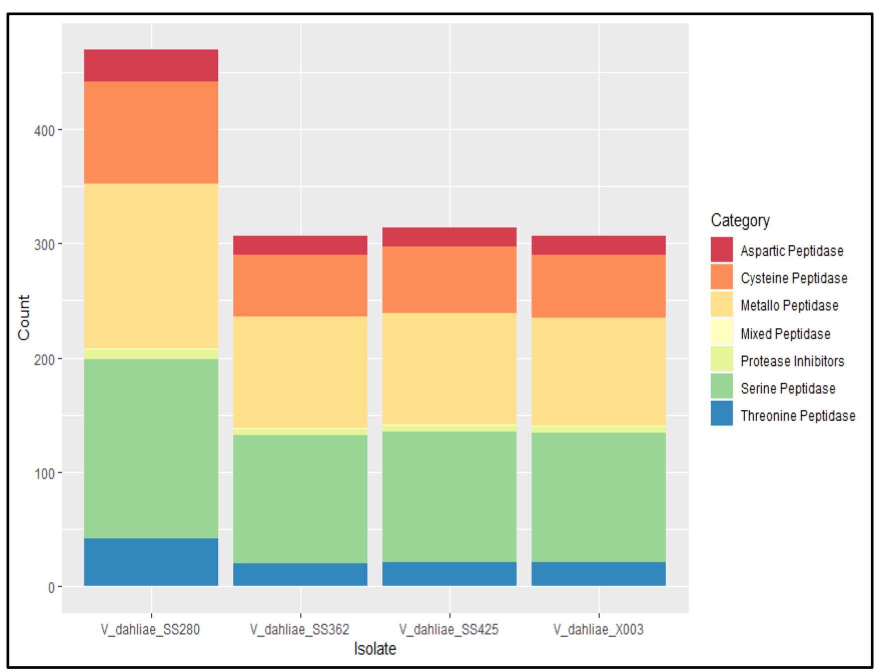


Figure 36. Counts of peptidases from MEROPS Database for genome sequenced *Verticillium dahliae* isolates.

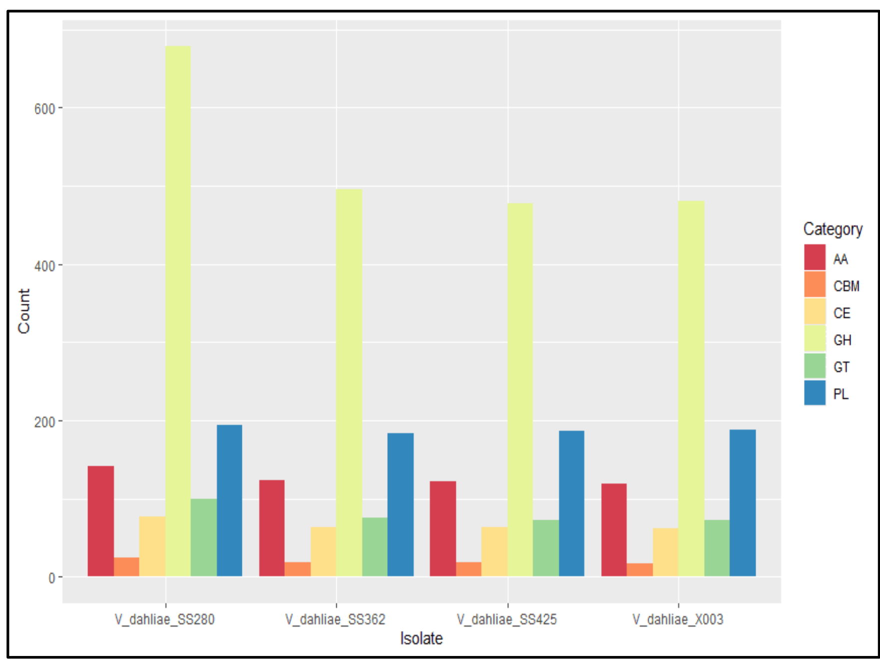


Figure 37. Counts of Carbohydrate-active enzymes (CAZymes) for genome sequenced *Verticillium dahliae* isolates. AA: Auxiliary activities, CBM: Carbohydrate-Binding Module, CE: Carbohydrate Esterase, GH: Glycoside Hydrolase, GT: Glycosyltransferase, PL: Polysaccharide lyase.

Genomes of SS362, SS425 and X003 all have similar sizes (~32-34Mb) and number of annotated proteins, as seen in Table 9. These three genomes also have almost identical COG, CAZyme and MEROP profiles. Isolate SS280 however has an assembled genome size ~2 x that of the other three isolates. As such, the total abundance of COGs, MEROPS and CAZymes are higher. The relative abundance of these categories however remains similar to the proportions of each of these categories in SS362, SS425 and X003 (Figure 38), with perhaps an increase in threonine peptidases and cysteine peptidases as well as an increase in relative abundance of glycoside hydrolases and drop in polysaccharide lyases. This could indicate a difference in metabolism, e.g. glycoside hydrolases catalyse the hydrolysis of glycosidic bonds, such as those in cellulose, hemicellulose and starch, found commonly in the biomass of plants and may reflect a difference in SS280's pathogen-host interactions. However, with 75% of the predicted proteins not having a functional annotation, these relative abundance profiles are still subject to change.

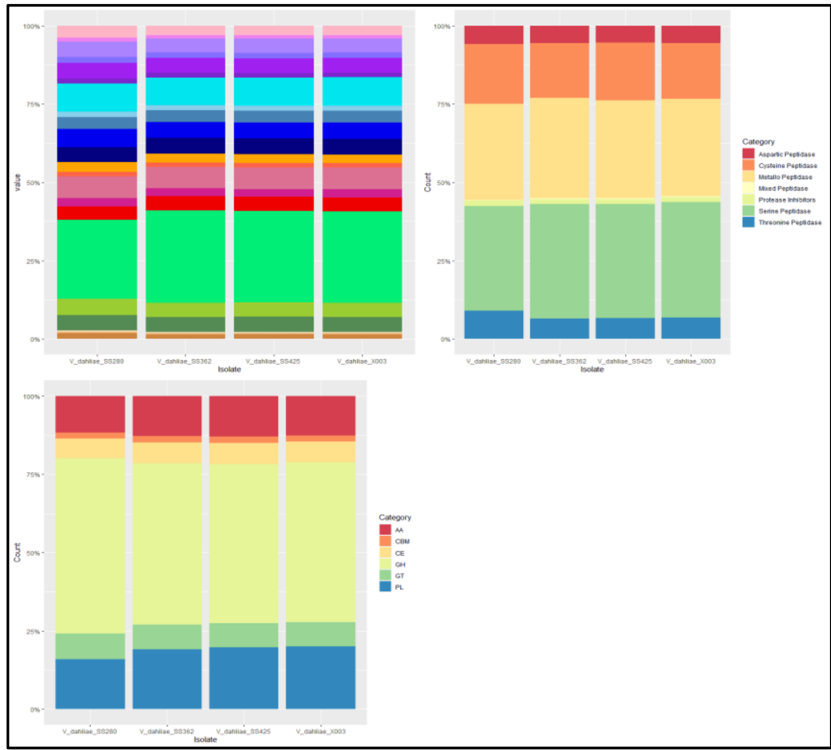


Figure 38. Relative abundance comparisons of COGs, MEROPS and CAZymes.

The genomic size difference of SS280 may be due to multiple factors such as: 1) Contamination in the genome sequencing of an unrelated organism, 2) Mixed culture of *V. dahliae* has been genome sequenced, 3) This isolate has a higher ploidy level, in this instance, diploid.

To address the first two possibilities, genome sequence data from SS280 was mapped to *V. dahliae* JR2 reference sequence with the Geneious Mapper method in Geneious 9.1.8 while saving a list of unused contigs. Large unused contigs were checked using BLAST to determine whether they belonged to *V. dahliae*. The BLAST results confirmed sequencing was from *V. dahliae* and not a contaminating organism. To also help confirm whether the sequencing was a result of mixed *V. dahliae* cultures, sequences were checked for IGS that did not match known VCG2A IGS sequences. Cultures were also single spored to ensure purity of the culture. To investigate possibility 3) would require further experimentation using techniques such as Flow Cytometry and Fluorescence-activated cell sorting (FACS) in order to accurately determine ploidy level.

While *V. dahliae* has not had an observed sexual phase and is haploid in its structure, it is closely related to the amphidiploid organism, *V. longisporum.* Phylogenetic analyses have shown that *V. longisporum* separates into three separate lineages that have formed from the hybridisation of two haploid parents. These three lineages are composed of combinations of four different parents, two unknown ancestral species provisionally named species A1, and D1 as well as two *V. dahliae* lineages D2 and D3. These three lineage combinations were formed by hybridisation of A1 x D1, A1 x D2 and A1 x D3. An unknown species has in the past hybridised with *V. dahliae* to form a diploid organism Figure 13 shows that SS280 is distantly related to *V. longisporum* and more closely related to VCG1A isolates so is likely not the diploid organism, *V. longisporum* and still a *V. dahliae*. Further experimentation is required to determine whether these observations are a result of biological processes or an artefact of sequencing.

To identify regions of interest in pathogenic versus non-pathogenic VCG2A, assembled contigs of SS362 and SS425 were separately aligned to the *V. dahliae* JR2 reference sequence using minimap2 and Alvis to visualise sequence alignment to the reference. This alignment (Figure 39) shows the presence/absence of assembled sequence in relation to the reference sequence. This was performed for both SS362 and SS425 so that comparisons could be made between these two draft genomes (as pairwise alignments of these genomes to the reference which consists of eight chromosomes, produces eight graphs. Pairwise alignments of SS362 vs SS425 would produce 1577 and 2440 graphs).

Several regions are visible as absent from the assembled genomes, though are present in the reference sequence. As the reference isolate is a VCG2B from strawberry, it is likely that these absent regions are a mixture of draft genome incompleteness as well as true biological differences between the VCG's and

host specificity. However, what is visible is that there are many regions that are present in either SS362 or SS425 and not the other. An example is the four regions in SS362 on chromosome CP0090821 that are missing while SS425 is only missing one of these regions at the end of the chromosome. A second, more striking example is the large missing section on chromosome CP0090781 for SS425 that is mostly present in SS362. Regions like these may represent areas of interest for diagnostic assay targets and regions that determine differences in pathogenicity. Further work is required to analyse these sequences in depth as well as determine regions of diagnostic interest. Additional long read sequencing such as Pacbio, ONT or 10x is likely required in order to complete these genomes to the chromosomal level.

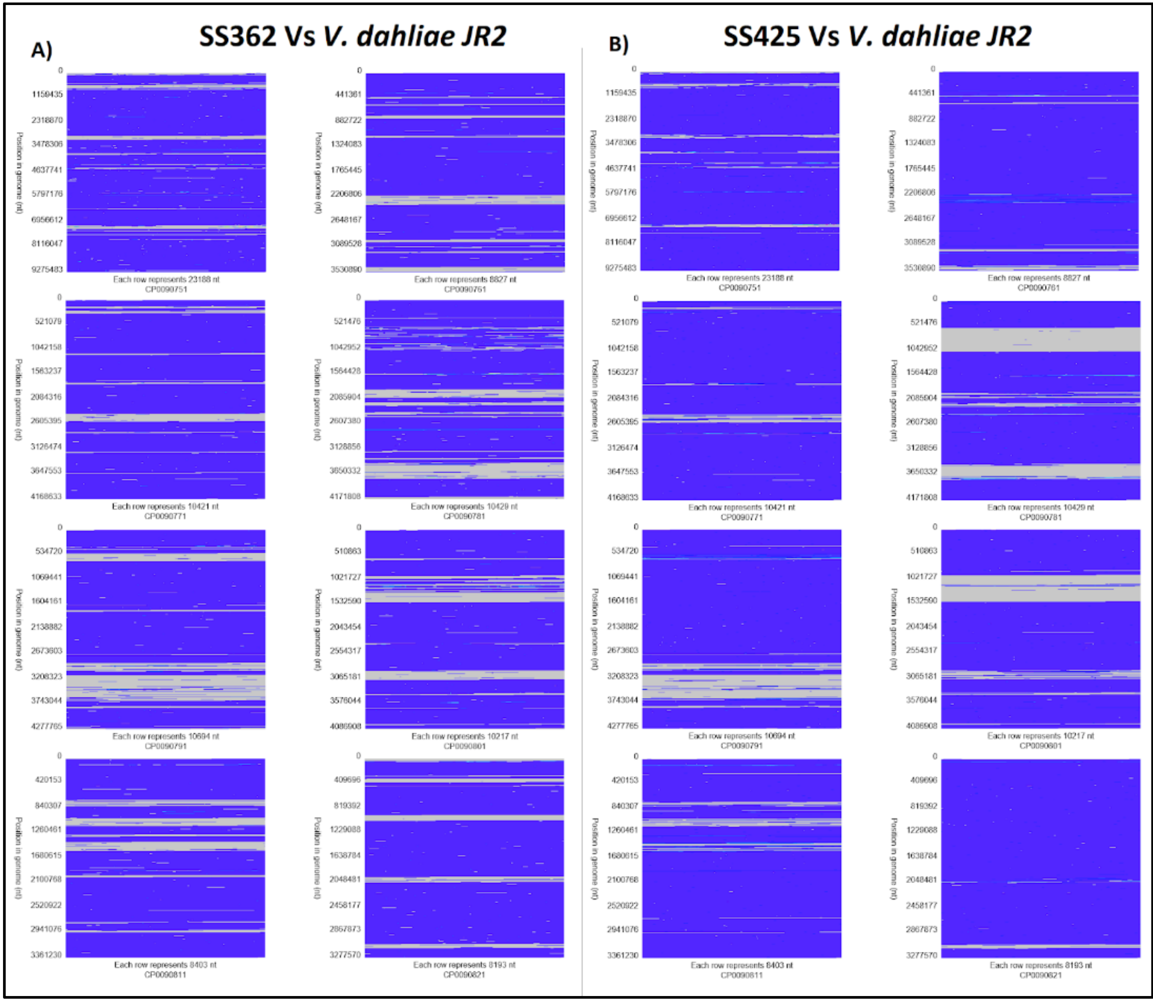


Figure 39. Presence/absence alignment of SS362 (A) Vs reference JR2 and SS425 (B) Vs reference JR2 where blue represents presence of sequencing that aligns with the reference and grey represents the absence of sequencing aligning to the reference. The differences between the two sets of data indicate regions that are present and absent in the pathogenic (SS362) and non-pathogenic (SS425) 2A VCG's. Each block represents chromosomes one through eight of *Verticillium dahliae* JR2.

Outcomes

a) Technical advances achieved

- Plant tissue assay and soil assay

These two tools provide a two-prong approach at being able to assess the impact *V. dahliae* may have on a crop, both in season (through the stem and soil assay) and out of season (through the soil assay) and play a role in farm management decisions.

b) Other information developed from research

- Melt-curve analysis of real-time PCR assay targets a region ~4x larger than what is recommended with the high-resolution melt-curve and as such failed to produce a melt-curve higher resolution than the initial assay. Future opportunities exist here to identify smaller regions of the *V. dahliae* genome that may lend themselves to diagnostic development using high resolution melt curves. This would require an in-depth analysis of genomic regions in the range of 100-300bp that are conserved in *V. dahliae*, though do not share identity across different VCG's.

- Understanding of natural fluctuations of inoculum levels during a cotton growing season. This information helped determine the optimum time to soil sample, quadrant area, depth to sample for estimating inoculum levels.

- Inoculum thresholds developed for minimum disease incidence were established in this project however field validation was not completed due to travel restrictions imposed from the Covid-19. Consequently, the cancellation of the Fulbright project and the ability for Shelby Young to assess disease incidence meant no correlations could be established. Follow up work on field validation of pre-plant inoculum thresholds and the incidence of Verticillium wilt would be beneficial.

c) Required changes to Intellectual Property register

- No changes required to the Intellectual Property register.

Conclusion

Take home messages

Two molecular tools have been developed to be used in the diagnostics of *V. dahliae* in plant and soil samples. The first of these molecular assay's targets *V. dahliae* in plant tissue. This assay was optimised to detect *V. dahliae* directly from plant tissue and foregoes the need to isolate and culture the pathogen. Traditionally, diagnostics would require a lengthy culturing phase to be able to identify *V. dahliae,* something which is not always possible as the pathogen cannot be isolated 100% of the time from infected plant tissue. Another benefit of this assay is that sequencing of the targeted amplicon allows a presumptive VCG to be determined due to specific patterns of repeat sequences inherent in targeted DNA regions.

The second of these assays has been designed to be able to give an indication of *V. dahliae* load within a soil sample. This assay has been optimised to work with a commercial high-throughput DNA extraction kit to allow large volumes of samples to be analysed concurrently. Due to the complex microbial nature of soil, this assay is currently unable to identify *V. dahliae* to VCG level. However, identification of VCG may still be accomplished using this assay on isolated microsclerotia from soil plating using direct PCR.

The significance of VCG classification for Australian cotton isolates appears to be less relevant than overseas. Internationally, isolates belonging to VCG1A are described as defoliating and can cause severe defoliation and even death of plants. Additionally, isolates belonging to VCG2A are reported to cause mild to moderate symptoms with some defoliation. There is huge variation in symptoms expressed by isolates within and between VCG1A and VCG2A. Plants infected with VCG2A have been reported to completely defoliate and even die.

Determining inoculum levels remains important for growers to assess the risk of potential disease regardless based on inoculum levels regardless of the VCG present. For this reason, we believe these two molecular tools provide a two-prong approach to assess the impact *V. dahliae* may have on a crop, both in season (through the stem and soil assay) and out of season (through the soil assay) assisting growers and consultants with farm management decisions.

Future R,D&E Opportunities and lessons learnt

(a) Future development project technology

- The industry may benefit from field validate the PREDICTA®B technology and other molecular DNA based assays to establish a better understanding of the quantification of DNA which includes the viable and non-viable components of *V. dahliae* in the soil.

(b) Future presentation and dissemination of project outcomes

- PhD candidate Pearl Dadd-Daigle thesis will be published upon submission and acceptance.
- PhD candidate Pearl Dadd-Daigle and co-authors have several draft manuscripts in various stages of development. Once finalised, permission will be sought to publish in peer reviewed journals.
- USA master's graduate Shelby Young and co-authors have submitted a manuscript to Crop and Pasture Science in March 2020 and is under review. Permission for publication was granted prior to submission.

(c) For future research

- The number of germinating cells within individual microsclerotia from each pathotype and isolates with varying pathogenicity should be investigated. This may help explain the pathogen's varying capacity to infect and cause disease with as little as 1 ppg.
- The effect of high rates of nitrogen and irrigation should be investigated to determine effect on inoculum levels. The source, time of application and source of application of nitrogen may play a role in assisting dormant microsclerotia to germinate.
- Further molecular work is warranted on the genetic differences between isolates. The ability to identify pathogenicity using molecular markers would benefit the cotton industry.
- The search for novel and biological products to control this pathogen should remain a high priority for the industry. This pathogen can survive in the soil for more than 14 years without a host and there is no chemical control option that is effective for this disease.
- One isolate that was genome sequenced, SS280, showed conflicting results between the IGS versus nit mutant testing. The MLST and SNP phylogenies also showed conflicting results. Further research is required to determine the genetic mechanisms underlying isolates that show features from multiple VCGs.

(d) Lessons learnt

- This project highlights the importance of validating molecular approaches against Australian isolates.
- This project demonstrated benefits in collaborating internationally to improve methodology approaches.

Extension, Publication and Media

Publications to date from 2016 to 2019:

Chapman, T. A., C. G. A, K. A. Kirkby and R. M. Jiménez-Díaz (2016). "First report of the presence of *Verticillium dahliae* VCG1A in Australia." Australasian Plant Disease Notes **11**(13): 1-4.

Industry publications

Holman, S., K. Kirkby, L. Smith and H. Hatnett (2016) "Fact Sheet - Vert Update: The latest in vert research."
Spotlight Magazine – Winter 2017 "Breaking the Verticillium Cycle", page 11
Spotlight Magazine – Autumn 2018 "Diagnosis for Decision Making", page 30
Spotlight Magazine – Spring 2019 "All the dirt on Vert", page 22
Spotlight Magazine – Spring 2019 "Predicting Disease Severity", page 22-23

Video

AG Cap, Women in Cotton

Publication plan

Manuscript:

- Young. S., Kirkby. K., Roser. s and Harden. S. "Method for estimating *Verticillium dahliae* inoculum in Australian cotton soils". **Submitted** to Crop and Pasture Science 9/3/20.
- Dadd-Daigle. P. Kirkby. K., Chowdhury. P., Labbate. M and Chapman. T.. "Literature review - The Verticillium wilt problem in Australian cotton". **Submitted** to Australasian Plant Pathology 22/11/19.
- Dadd-Daigle. P. et al. "Virulence not linked with Vegetative Compatibility Groups in Australian cotton *Verticillium dahliae* isolates". **Accepted for publication in Australian Journal Crop Science**
- Dadd-Daigle. P., Kirkby, K., Roser. S.,Lonergan. P., Chowdhury. P., Labbate. M and Chapman. T. "Virulence varies in Australian cotton *Verticillium dahliae* isolates". **Draft manuscript**
- Dadd-Daigle. P. et al. "Whole genome analysis of Australian *V. dahliae* Vegetative Compatibility Group 1A". **Draft manuscript being prepared**

Thesis

- Dadd-Daigle. P. "Verticillium wilt in Australian cotton: examining the relationship between Australian *Verticillium dahliae* isolates and virulence".

B. Have you developed any online resources and what is the website address?

NSW DPI Primefact sheets have just recently developed with internal reference numbers assigned. Copies of these are in Appendices A, B, C and D pending approval to publish by CRDC.

- Primefact sheet 1708, November 2019 – Soil sampling protocol for quantifying Verticillium inoculum in cotton fields
- Primefact sheet 1709, November 2019 – Quantifying Verticillium inoculum in soil using the dry plating method
- Primefact sheet 1711, November 2019 - Plant tissue assay for the identification of *Verticillium dahliae*
- Primefact sheet 1713, November 2019 - Soil Assay for the identification of *Verticillium dahliae*

Appendix A - Primefact sheet 1708, March 2020 – Soil sampling protocol for quantifying Verticillium inoculum in cotton fields.

primefact

Soil sampling protocol for quantifying Verticillium inoculum in cotton fields

March, 2020, Primefact 1708, First edition

Kirkby K, Pathologist, Biosecurity and Food Safety, Narrabri

Why sample

- Predictive – how much inoculum is in the soil at the time of sampling. What is the risk to preceding crops?
- Monitoring – have inoculum levels changed over time.
- Management – document the effect of crop rotation on inoculum levels.

Representative soil sample

Collecting a representative soil sample is essential for meaningful soil analysis. The amount of a single soil sample analysed from a field represents around 0.002%.

(40ha field 24cm deep, 1 transect bulk sample 500g, 1 gram soil analysed = %)

Variability

Inoculum levels in fields vary as a result of crop history, climate, weeds, topography, nutrition, stubble management, cultivation, biological conditions and time of sampling. Field with high variability require more soil cores for the same error than fields with low variability.

When to sample

Inoculum levels naturally fluctuate throughout the growing season. Pre-plant levels may be high if previous crop had infection. Inoculum decreases throughout the season then increases post-harvest following incorporation of infected plant material (Figure 1). Soil samples should be taken in the winter prior to planting. Soil preparation and analysis can take up to six weeks.

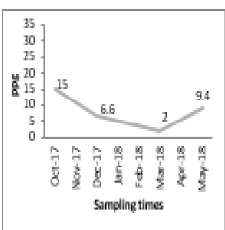


Figure 1. Natural fluctuation of *Verticillium dahliae* inoculum levels throughout the cotton growing season.

Soil sampling protocol for quantifying Verticillium inoculum in cotton fields

Where to sample

Choose fields that will potentially be planted to cotton. Consider yield results from previous seasons and problem fields. Consider soil types and topography. Take samples from within the plant line to a depth of 24 cm. Do not sample within 50 m of tail drain or head ditch.

How to sample

Collect 20 soil samples from a specified area (up to 4 ha transect) and mix thoroughly in a bucket. Each of the 20 samples should contribute equally to the representative bulk soil sample. Take a subsample from this bulk sample of around 500 g and place in a labelled bag. Double bag soil sample and place out of the sun in an esky. Take three bulk samples per field. This number of samples and transects is based on statistical precision of 95% confidence interval.

Equipment

The equipment used to take the individual soil samples needs to be fit for purpose and may vary with soil conditions or the amount of soil samples collected. It is important that with each soil sample, all depths intended in the sample are equally represented. Note – some augers don't handle very dry or wet soil.

- Corer or shovel without tapered edges, hand auger or power-driven auger, tube samplers or vehicle mounted auger
- GPS – to return to site at a later date
- Buckets, bags, labelling pens, esky
- Small spade to mix soil

Hygiene

Pests, diseases and weeds can be spread on sampling equipment. Ensure equipment is clean between sampling locations.

Sample dispatch

Do not expose samples to extreme heat.

Post samples early in the week.

If samples are taken late in the week, store in cool dry area until posting.

Respect biosecurity and movement of soil across state borders.

Acknowledgements

RRDP1723 project was supported by the Cotton Research and Development Corporation, through funding from the Australian Government Department of Agriculture as part of its Rural R&D for Profit program, and NSW DPI.

© State of New South Wales through Department of Planning, Industry & Environment 2020. The information contained in this publication is based on knowledge and understanding at the time of writing (March 2020). However, because of advances in knowledge, users are reminded of the need to ensure that the information upon which they rely is up to date and to check the currency of the information with the appropriate officer of the Department of Planning, Industry & Environment or the user's independent adviser.

Appendix B - Primefact sheet 1709, March 2020 – Quantifying Verticillium inoculum in soil using the dry plating method.

Quantifying Verticillium inoculum in soil using the direct dry plating technique

March 2020, Primefact 1709, First edition

Dr Karen Kirkby, Pathologist, Biosecurity and Food Safety, Narrabri

Soil preparation

Soil samples need to be air dried for 2 to 4 weeks to kill conidia and hyphae.

Following drying, soil needs to be mixed and rolled to a fine texture.

A 50 g subsample of the mixed soil is collected and taken to the laboratory.

Five replicates of 0.2 g samples of soil per sample are weighed into pipette tubes.

Media

20 ml of Sorensen's NP X media is poured into plastic petri dishes.

Once media is dry a cross is drawn on the base of each petri dish.

Petri dishes are labelled accordingly.

A piece of white paper is placed under the petri dish.

The soil from the pipette tubes are gently hand spread across the surface of the media. Any soil spilt is collected on the paper and poured into the petri dish.

The 5 petri dishes from the soil sample are sealed with cling wrap.

Petri dishes are incubated in the dark at 23°C for 14 days.

Microscopic examination

The petri dishes are observed under a dissecting microscope at x 20 magnification.

The number of germinating colonies is counted in each of the four segments.

The number of colonies per dish is multiplied by the dilution factor (5) and reported as propagules per gram (ppg) of dry soil.

The inoculum levels in the soil represent the viable microsclerotia in the soil at the time of sampling.

Quantifying Verticillium inoculum in soil using the direct dry plating technique

Acknowledgements

RRDP1723 project was supported by the Cotton Research and Development Corporation, through funding from the Australian Government Department of Agriculture as part of its Rural R&D for Profit program, and NSW DPI.

© State of New South Wales through Department of Planning, Industry & Environment 2020. The information contained in this publication is based on knowledge and understanding at the time of writing (March 2020). However, because of advances in knowledge, users are reminded of the need to ensure that the information upon which they rely is up to date and to check the currency of the information with the appropriate officer of the Department of Planning, Industry & Environment or the user's independent adviser.

Appendix C - Primefact sheet 1711, March 2020 - Plant tissue assay for the identification of *Verticillium dahliae.*

Plant-tissue assay for the identification of Verticillium dahliae.

March 2020, Primefact **1711**, First edition

Dr John Webster, Research Officer, Biosecurity and Food Safety, Elizabeth Macarthur Agricultural Institute.

Verticillium wilt

Verticillium wilt is a soil-borne disease caused by the fungal pathogen *Verticillium dahliae* and is known to affect over 400 host plants. Disease symptoms are varied from leaf mottling and wilting, to more severe symptoms such as necrosis, defoliation and even the death of the host. Vascular discolouration is also observed when sections of the plant stem are taken.

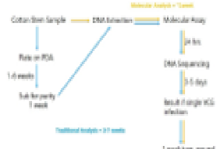
Identification of *V. dahliae*

Identification of *V. dahliae* to pathotype is routinely determined by the pathogen's vegetative compatibility group (VCG), in general, VCG 1A causes defoliation in cotton and VCG 2A being a non-defoliating pathotype.

Using this plant-tissue assay, the VCG, and therefore the pathogen's ability to defoliate a cotton host or not, can be determined directly from a host plant.

Increased Turn-around time

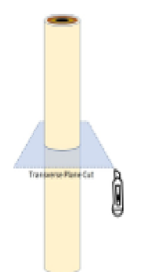
This plant-tissue assay eliminates the need to culture the *V. dahliae* pathogen as the plant-tissue assay uses a molecular method that can turn around a diagnostic much faster than traditional microbiological methods. Results from this plant tissue assay can turn around results in ~1 week. On rare occasions however, if a dual infection with two separate VCGs occurs, culturing will be required.



How to sample

Stems can be sampled from plants that are suspected of having Verticillium wilt by observing for symptoms and taking a sample of the main stem of the plant using a pair of sharp secateurs or a blade and

cutting the stem transversely into a piece ~10cm long. Observe at this stage for vascular discolouration.



Postage of samples

Samples at this stage can be placed into individual paper bags, labelled with identifying information (date, field, GPS coordinates) and placed in a postage envelope.

Samples should be treated carefully to ensure maximum viability for analysis.

- Post early in week to avoid samples sitting for extended periods of time in post.
- Avoid high temperatures
  - E.g. Placed in an Esky and not left in a hot car
- Avoid excessive moisture
  - E.g. use a cool pack or ice brick for storage and not wet ice.

Farm Hygiene

Pests, diseases and weeds can be spread on sampling equipment. Ensure equipment is clean between sampling locations to prevent the spread of pests and disease.

Acknowledgements

RRDP1723 project was supported by the Cotton Research and Development Corporation, through funding from the Australian Government Department of Agriculture as part of its Rural R&D for Profit program, and NSW DPI.

© State of New South Wales through Department of Planning, Industry & Environment 2020. The information contained in this publication is based on knowledge and understanding at the time of writing (March 2020). However, because of advances in knowledge, users are reminded of the need to ensure that the information upon which they rely is up to date and to check the currency of the information with the appropriate officer of the Department of Planning, Industry & Environment or the user's independent adviser.

Appendix D - Primefact sheet 1713 March 2020 - Soil Assay for the identification of *Verticillium dahliae.*

Soil assay for the identification of Verticillium dahliae

March 2020, Primefact **1713**, First edition

Dr John Webster, Research Officer, Biosecurity and Food Safety, Elizabeth Macarthur Agricultural Institute

Verticillium wilt

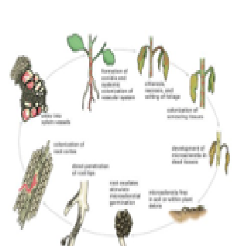
Verticillium wilt is a soil-borne disease caused by the fungal pathogen *Verticillium dahliae* and is known to affect over 400 host plants. Disease symptoms are varied from leaf mottling and wilting, to more severe symptoms such as necrosis, defoliation and even the death of the host. Vascular discolouration is also observed when sections of the plant stem are taken.

Identification of *V. dahliae*

Identification of *V. dahliae* to pathotype is routinely determined by the pathogen's vegetative compatibility group (VCG), in general, VCG 1A causes defoliation in cotton and VCG 2A being the non-defoliating pathotype.

It's in the soil

As *Verticillium dahliae* is a soil-borne pathogen, planting into soil containing populations of *V. dahliae* is the primary mechanism of infection. Infection of cotton occurs through the root tips of susceptible plants and invades the xylem. The vascular system eventually becomes clogged, preventing the flow of water and nutrients, resulting in wilting, chlorosis and plant death. Microsclerotia that form in the dying plant tissue are then released back into the soil for the process to begin again. Persistence of these microsclerotia is high and can persist in soil for greater than 14 years without the presence of a host plant.



Soil Assay

This soil assay is capable of determining the presence of *V. dahliae* in a soil sample and can be used pre-planting to indicate the risk of Verticillium wilt to potential crops. This assay can assess the load of *V. dahliae* in a soil sample rapidly, without the need for a lengthy culturing process. Testing of VCG can also be provided, though this process, but does involve a lengthier culturing process soil contains many fungi which may confound results.

How to sample

Guidelines for collecting soil samples can be obtained from Primefact 1708, "Soil sampling protocol for quantifying Verticillium inoculum in cotton fields".

Acknowledgements

RRDP1723 project was supported by the Cotton Research and Development Corporation, through funding from the Australian Government Department of Agriculture as part of its Rural R&D for Profit program, and NSW DPI.

© State of New South Wales through Department of Planning, Industry & Environment 2020. The information contained in this publication is based on knowledge and understanding at the time of writing (March 2020). However, because of advances in knowledge, users are reminded of the need to ensure that the information upon which they rely is up to date and to check the currency of the information with the appropriate officer of the Department of Planning, Industry & Environment or the user's independent adviser.