CGA FINAL REPORT

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number:

CGA: **CGA1911**

Project Title: **Additional support Fulbright Scholarship to study Verticillium wilt**

Project Commencement Date: **Project Completion Date: 31/05/20**

Part 2 – Contact Details

Administrator: Elsie Hudson

Organisation: CottonInfo

Postal Address:

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Part 3 – Final Report

Background

1. **Outline the background to the project.**
   Ms Shelby Young was awarded a Fulbright Postgraduate Research Award to study Verticillium wilt in Australia. Her program, commencing at the end of July 2019, helped build and enhance her pathology skills and knowledge while benefiting Australian cotton growers. Her project consisted of three main experimental initiatives: field-validation of an inoculum and risk assessment tool for Verticillium wilt in cotton, examining relationships between irrigation and nitrogen levels and Verticillium wilt, and examining differences in metabolomic profiles of *Verticillium dahliae* isolates.

   Dr Karen Kirkby developed a risk-based model for inoculum levels, but it needs further field validation and testing. Research findings aimed to contribute to efforts in decreasing the risk of Verticillium wilt through better understanding the inoculum levels and nutritional management and improve growth across the cotton industry.

With Dr John Baird of New South Wales Department of Primary Industry, Ms Young sampled for inoculum present at the time of planting in test plots receiving varying levels of irrigation and nitrogen to examine any effects on disease incidence late season.

Ms Young partnered with DPI and Dr Jonathan Plett of Western Sydney University to examine differences in the metabolomic profiles of four *Verticillium dahliae* isolates during the infection process in working toward better understanding their differing behaviours in the field.

The grassroots grant was be used to support Ms Young by covering consumables and travel costs associated with her Fulbright projects involving Verticillium wilt in cotton. Ms Young's Fulbright also received support from the Lower Namoi Cotton Grower Association in gaining access to regional cotton fields, and from New South Wales Department of Primary Industry and Western Sydney University's Hawkesbury Institute for the Environment through lab space, access to experimental plots, and technical assistance.

In March 2020, the Fulbright Program suspended all of their programs globally due to the COVID-19 pandemic and associated risks. Ms Young was thus restricted from completing various aspects of each project.

Objectives

2. List the project objectives (from the application) and the extent to which these have been achieved.

Objective 1 - **Grow capacity in the field of cotton pathology.**

- **Milestone 1.1:** Publish Verticillium wilt inoculum detection methods manuscript to academic journal
  Performance indicator: "Method for estimating *Verticillium dahliae* inoculum in Australian cotton soils" submitted to Crop & Pasture Science, has been reviewed and accepted for publication with minor revisions. 90% completed

Objective 2 - **Utilise the access to a USA Fulbright Scholarship student to study Verticillium wilt in Australia.**

- **Milestone 2.1:** Examine properties of genetically diverse *Verticillium dahliae* isolates through untargeted metabolomic profiling
- Performance indicator: Experiment using untargeted metabolomic profiling to examine differences in isolates at different stages of disease development in cotton plants. 80% completed
- **Milestone 2.2:** Examine possible effects of nitrogen and irrigation on Verticillium wilt incidence in cotton
  Performance indicator: Determine initial inoculum in plots designed to receive differing levels of irrigation and fertiliser; conduct end-of-season disease assessment to determine Verticillium wilt incidence

Objective 3 - **Utilise and ground truth the innovative risk-based model for inoculum levels developed by Dr. Karen Kirkby.**

- **Milestone 3.1:** Validate Verticillium wilt tool
  Performance indicator: Field samples collected and examined for inoculum levels and pH. Survey for field conditions developed to gather information from growers. Assess fields for late-season incidence of Verticillium wilt. 50% completed

Objective 4 - **Increase networking between research projects and growers, consultants, and researchers.**

- **Milestone 4.1:** Promote project at academic conferences
  Performance indicator: Presentations at the Association of Australian Cotton Scientists and Australasian Plant Pathology Society annual conferences. 100% completed
- **Milestone 4.2:** Network amongst growers and consultants
  Performance indicator: Attended Lower Namoi Cotton Grower meetings, communicated with multiple growers to sample fields across the region, participated in CCA regional workshop. 100% completed
- **Milestone 4.2:** Network amongst researchers
  Performance indicator: Attended AACS and APPS conferences, engaged in Fulbright alumni and awardee events, presented in seminar series at Western Sydney University Hawkesbury Institute for the Environment and the Elizabeth Macarthur Agricultural Institute. 100% completed

Objective 5 - **Improve adoption of new research (keep everyone in the loop with new disease advances).**

- **Milestone 5.1:** Presentation of findings
  Performance indicator: Present findings of the study on the relationship of inoculum and disease, findings from the examination of metabolomic profiles, and findings from examining relationship of irrigation, nitrogen, and Verticillium wilt. 0% completed

Methods

3. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related projects.

Objective: Grow capacity in the field of cotton pathology.
Milestone 1.1 Publish inoculum detection methods manuscript to academic journal
Performance indicator:

- "Method for estimating *Verticillium dahliae* inoculum in Australian cotton soils" submitted to Crop & Pasture Science, has been reviewed and accepted for publication with minor revisions. 90% completed

Ms Young earned her Master of Plant and Soil Science degree from Texas Tech University in August 2019. One of her thesis research projects was a collaboration with Dr Kirkby that developed a method for estimating *Verticillium dahliae* inoculum in cotton field soils by comparing multiple techniques from international literature using samples from both New South Wales and Texas, USA. Upon acceptance of her thesis, Ms Young and Dr Kirkby prepared this portion of the thesis into a manuscript for submission into the journal *Crop and Pasture Science.* The manuscript has been accepted for publication with minor revisions. Ms Young is addressing the reviewer comments and re-submitting the updated manuscript no later than the 30th of August 2020.

Objective: Utilise the access to a USA Fulbright Scholarship student to study Verticillium wilt in Australia.

Milestone 2.1 Examine properties of genetically diverse *Verticillium dahliae* isolates through untargeted metabolomic profiling

Performance indicator:

- Experiment using untargeted metabolomic profiling to examine differences in isolates at different stages of disease development in cotton plants.

Isolate Selection

Four isolates were selected from VCG4B (non-defoliating), VCG1A (defoliating), and VCG2A (non-defoliating). As the VCG2A group splits into two genetically different groups, two isolates were selected from the VCG2A: one "non-defoliating" and one "defoliating-like."

Plant Preparation

Cotton seeds (Acala SJ-2) were set in the glasshouse in large Petri dishes on filter paper soaked in distilled water to germinate for 96 hours. 100 seeds were planted into root trainers in twice-pasteurised Debco Native potting soil augmented with Osmocote at the recommended rate. Cotton was grown in the glasshouse until the two-true-leaf stage.

Inoculum Preparation

Inoculum was prepared in accordance with the methods outlined in an unpublished manuscript by Pearl-Dadd Daigle with the exception of preparing the potato dextrose broth in-lab. The methods of preparing the potato dextrose broth are as follows. 200g of unpeeled potatoes were sliced and boiled in 1L RO for 30minutes. The resulting mixture was filtered through cheesecloth, saving the effluent, which is the potato infusion. 20g of dextrose (Cooper's) was mixed with the potato infusion and boiled until fully dissolved. This process was repeated to result in a total of 4L potato dextrose broth, which was autoclaved broth for 15 minutes at 121°C. Plugs from the four prepared isolates were inserted into 1L Schott bottles containing potato dextrose broth and continually shaken for 7 days. After the 7 days, the fungal-broth mixtures were filtered through triple-layered cheesecloth to remove hyphae. Conidial concentrations were then augmented to $1x10^6$ conidia per mL by adding the fungal-broth mixture to 600mL of RO water, using a haemocytometer to determine the number of conidia per mL.

Plant Inoculation

Plants were inoculated using a root-dip method. Plants were gently removed from their root trainers and the bottom 1cm of their roots was removed with sterilised scissors. Plants were placed in the prepared inoculum solution for 30 minutes, then planted into 1L pots with Debco Native potting soil augmented with Osmocote at the recommended rate and left to grow until harvested at 4 days, 2 weeks, and 3 weeks post-inoculation.

Soil Harvest and Metabolite Extraction

Plants were gently removed from each pot without disturbing the soil attached onto the root surface, this soil was considered as "rhizosphere soil" while the soil not attached to the roots was considered as the "bulk soil." The soil from unplanted pots was considered as "bare soil". Metabolites in rhizospheric soil, bulk soil and bare soil were sampled for metabolite profiling following a protocol modied from Pétriacq et al (2017). To extract metabolites from the rhizospheric soil, the whole root systems together with the rhizospheric soil, were rinsed in 10ml of cold metabolite extraction solution (50:49.9:0.1 v/v methanol:water:formic acid solution, kept at 4°C) for 30 sec in a 50ml tube by gently swirling the solution about the roots. After rinsing, the metabolite extracts were filtered through a piece of miracloth and

kept on ice in a 15 ml centrifuge tube. The roots were gently rinsed of all soil, oven-dried in a 50ml tube without lid, and their dryweight recorded. The remaining soil in the pots (i.e. bulk soil) was homogenized and approximately 100g was weighed and mixed in 15ml of aforementioned metabolite extraction solution for 30 sec. The metabolite extracts of bulk soil were then filtered as described above and kept in ice. In the same manner, metabolites from bare soil of unplanted pots were also extracted. Within 15 minutes, the metabolite extracts were centrifuged at 3500 x g for 5 min at 4°C Before placing sample tubes in the centrifuge, additional metabolite extraction solution was added to each tube using a 1000 µl pipette for so that all were of equal volume. After centrifuging, the supernatants were collected and filtered through 0.22µm PES filter (Millex-GP sterile polyethersulfone syringe filter, Millipore, United State) into a 50ml tube. Following filtration, an equal part of deionized water was added prior to freezing each sample in liquid nitrogen, and then stored at -80°C until metabolic profiling was performed.

Pétriacq, P., Williams, A., Cotton, A., McFarlane, A.E., Rolfe, S.A. and Ton, J. (2017), Metabolite profiling of non-sterile rhizosphere soil. *Plant J*, 92: 147-162. doi:10.1111/tpj.13639

At this point in the experiment, COVID-19 hindered access to lab facilities. All samples are stored at -80°C at the Hawkesbury Institute for the Environment for processing by Dr Jonathan Plett's lab at a later date. The procedure that will be followed at that time is as follows.

Metabolite Profiling

The frozen metabolite extracts will be freeze-dried at -56°C for three days. The dried extracts will then be resuspended in 400 µl of cold metabolite extraction solution and sonicated in a 4°C water bath for 15 minutes, followed by centrifugation at 14000 x g for 10min at 4°C. Afterwards, the supernatants will be collected and diluted four times with the aforementioned metabolite extraction solution and 120 µl of the final diluted extracts will then be transferred into glass vials for metabolite profiling on the UPLC-ESI-MS platform. This four-times final dilution will be determined based on a four pooled-biological quality control (PBQC) sample run previous to the actual samples. Four Blank samples (metabolite extraction solution only) will also run alongside the samples. Metabolite profiling running in both negative (ESI^-) and positive (ESI^+) electrospray ionization mode will be performed following the analytical procedure described in Pétriacq et al (2017), using the nanoACQUITY UltraPerformance Liquid Chromatography (Waters, United Kingdom) system coupled with a SYNAPT G2-S Mass Spectrometer (Waters, United Kingdom). The system will be operated in high resolution mode integrated with ion mobility to enhance separation of ions. Metabolic data pre-processing including peak alignment, peak picking and deconvolution will be performed using the Progenesis QI programme (Nonlinear Dynamics Ltd., United Kingdom). Metabolite identification will be carried out with the Progenesis MetaScope (Nonlinear Dynamics Ltd., United Kingdom) to search for matching neutral mass, m/z and fragment mass against public databases including PubChem, KEGG and ChEBI. The data matrices containing the metabolite identity and peak intensity will be exported and further analyses performed on R platform (version 3.5.1).

Milestone 2.2 Examine possible effects of nitrogen and irrigation on Verticillium wilt incidence in cotton

Performance indicator:

- Soil Sampling
- Assessment of inoculum levels
- Late-season field incidence of Verticillium wilt

Soil Sampling

Bulk soil samples were collected from 9 experimental plots at the Australian Cotton Reseach Institute. Within each plot, 20 samples were taken from three quadrant areas and mixed thoroughly for a bulk sample; each field had three bulk samples. Soil was collected from a depth of 2-12cm for all samples and placed into labelled bags.

Soil Preparation and Plating
Samples were air dried for 14 days at 25°C to ensure the death of hyphae and conidia. After drying, the soil was thoroughly mixed and rolled with a metal pipe to achieve uniform small particle size. From each mixed and rolled bulk sample, five subsamples of 0.2g of soil were weighed out and plated onto five Petri dishes containing Sorenson's NP-10 media. All plates were then incubated for 14 days at 24°C.

Assessment for Inoculum Levels
After incubation, soil was gently washed from each plate. Plates were then examined under a dissecting microscope for the presence of germinated microsclerotia. Microsclerotia were counted and multiplied by the dilution factor to get number of microsclerotia per gram of soil.
At this point in the experiment, COVID-19 hindered access to lab facilities and restricted all travel. The following processes were not able to be completed but were planned as follows.

Late-Season Incidence of Verticillium Wilt
Within each plot, 50 plants are cut and assessed for the presence of Verticillium wilt. Incidence is derived for each plot and compared with the inoculum levels previously assessed.

Objective: Utilise and ground truth the innovative risk-based model for inoculum levels developed by Dr. Karen Kirkby
Milestone 3.1 Validate Verticillium wilt tool
Performance indicator:

- Soil sampling
- Assessment of inoculum levels
- pH
- Grower survey to gather information on field conditions
- Late-season field incidence of Verticillium wilt

Soil Sampling
Bulk soil samples were collected from 21 fields in the Lower Namoi cotton growing region. Within each field, 20 samples were taken from three quadrant areas and mixed thoroughly for a bulk sample; each field had three bulk samples. Soil was collected from a depth of 2-12cm for all samples and placed into labelled bags.

Soil Preparation and Plating
Samples were air dried for 14 days at 25°C to ensure the death of hyphae and conidia. After drying, the soil was thoroughly mixed and rolled with a metal pipe to achieve uniform small particle size. From each mixed and rolled bulk sample, five subsamples of 0.2g of soil were weighed out and plated onto five Petri dishes containing Sorenson's NP-10 media. All plates were then incubated for 14 days at 24°C.

Assessment for Inoculum Levels
After incubation, soil was gently washed from each plate. Plates were then examined under a dissecting microscope for the presence of germinated microsclerotia. Microsclerotia were

counted and multiplied by the dilution factor to get number of microsclerotia per gram of soil.

pH
As each field was split into three subsections, the bulk soil sample from the middle section in each field was assessed for pH.

At this point in the experiment, COVID-19 hindered access to lab facilities and restricted all travel. The following processes were not able to be completed but were planned as follows.

Grower Survey for Field Conditions
A survey was developed to for cotton growers in order to gather information on the conditions of each field sampled.

Late-Season Incidence of Verticillium Wilt
Within each of the three sections of each field, 100 plants are cut and assessed for the presence of Verticillium wilt. Incidence is derived for each section of each field and compared with the inoculum levels previously assessed.

Objective: Increase networking between research projects and growers, consultants, and researchers.
Milestone 4.1 Promote project at academic conferences
Performance indicator:

- Presented at the Association of Australian Cotton Scientists annual conference
- Presented at the Australasian Plant Pathology Society annual conference

Milestone 4.2 Network amongst growers and consultants
Performance indicator:

- Attended Lower Namoi Cotton Grower meetings
- Communicated with multiple growers to sample fields across the region
- Participated in CCA regional workshop

Milestone 4.2 Network amongst researchers
Performance indicator:

- Attended and presented at Australian Association of Cotton Scientists annual meeting
- Attended and presented at Australasian Plant Pathology Society conference
- Engaged in Fulbright alumni and awardee events
- Presented in seminar series at Western Sydney University Hawkesbury Institute for the Environment
- Presented in lecture series at the Elizabeth Macarthur Agricultural Institute

Objective 5 - **Improve adoption of new research**
Milestone 5.1: Presentation of findings
Performance indicator:

- Present findings of the study on the relationship of inoculum and disease, findings from the examination of metabolomic profiles, and findings from examining relationship of irrigation, nitrogen, and Verticillium wilt.

Outcomes

4. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

5. Please report on any:

a) Feedback forms used and what the results were
b) The highlights for participants or key learnings achieved
c) The number of people participating and any comments on level of participation

b) Highlights and Key Learnings Achieved

Ms Young was able to strengthen her understanding of and skills relating to plant pathology. Collaborative relationships between multiple entities were strengthened and formed. NSW DPI, WSU HIE, CRDC, and the Lower Namoi CGA were represented in research presentations alongside representation of the Fulbright Program, the largest international exchange program in the world.

c) Participation (by location)

Lower Namoi Cotton Growers Association:

Elsie Hudson provided assistance in connecting with growers to locate fields for sampling. Mandy Gilmour provided assistance in handling financials.

New South Wales Department of Primary Industry Australian Cotton Research Institute:

Dr Karen Kirkby provided guidance on experimental design for the inoculum threshold validation project and irrigation and nitrogen project. Dr John Baird provided the test plots and treatments for the irrigation and nitrogen project. Dr Kirkby and Sharlene Roser provided technical assistance and guidance on lab practices for all projects. Peter Lonergan provided expertise and assistance on graphics for presentations.

New South Wales Department of Primary Industry Elizabeth Macarthur Agricultural Institute:

Dr Will Cuddy and Dr Toni Chapman made arrangements to accommodate Ms Young in the Plant Pathology labs at EMAI. Dr Toni Chapman provided guidance on the experimental design of the metabolomics project. Peal Dadd-Daigle provided guidance for inoculum preparation. Dr John Webster and Leena Koop provided technical assistance and guidance on lab practices.

Western Sydney University Hawkesbury Institute for the Environment:

Dr Jonathan Plett and Dr Krista Plett provided guidance on experimental design and other aspects of the metabolomic analysis project. Dr Jonathan Plett's lab will process and analyse samples and data.

Conclusion

6. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

Ms Young further developed and increased her knowledge and skills in plant pathology, particularly in determining *V. dahliae* inoculum levels present in field soils. Growers were informed of their inoculum levels. Due to COVID-19, work was unable to be completed to determine Verticillium wilt incidence and therefore the examination of the correlation between inoculum and disease did not occur. Future work is warranted in this and especially in field-validating the threshold matrix developed by Dr Kirkby for use by growers and consultants. As with the inoculum threshold work, late-season sampling to determine disease incidence was not completed in the field trial for irrigation and nitrogen at ACRI. Further work is warranted here as well as better understanding the potential interaction of

nitrogen soil amendments with Verticillium wilt inoculum would directly benefit the Australian cotton industry.

The metabolomic profiling is also currently unfinished due to COVID-19. Fortunately, however, the samples are currently in frozen storage at the Hawkesbury Institute for the Environment and will be processed as COVID-19 restrictions ease. The results will be forwarded to CRDC upon completion.

Ms Young garnered great value in the networking opportunities provided through these projects; she developed new and strengthened existing connections between NSW DPI, WSU, the cotton industry, and the Fulbright program. These relationships will continue to provide value to the Australian cotton industry through future projects and collaborations. One of Ms Young's goals is to return to Australia and continue working closely with these connections in pursuit of a PhD.

Extension Opportunities

7. Detail a plan for the activities or other steps that may be taken:

(a) To tell other CGAs/growers/regions about your project.
(b) To keep in touch with participants.
(c) For future projects.

b and c) Keeping in touch with participants.

Ms Young is in the process of compiling an application for the Fulbright Future Award to return to Australia and pursue a PhD. Most participants involved in this set of projects are in some way involved with the project to be proposed in the application. If successful, the collaborative relationships formed and strengthened through this Verticillium wilt work will continue to provide contributions to the cotton industry via PhD research outcomes.