FINAL REPORT

CRDC ID: DAN1703
Project Title: Innovative solutions to cotton diseases
Confidential or for public release? For Public Release

Part 1 – Contact Details & Submission Checklist

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Submission checklist.
Please ensure all documentation has been completed and included with this final report:

☒ Final report template (this document)
☒ Final Technical Report (see Part 3)
☒ Final Schedule 2: IP register
☐ Final financial report
☒ PDF of all journal articles (for CRDC's records)

Signature of Research Provider Representative:________________________

Date submitted: ____________

Part 2 - Monitoring & Evaluation

Achievement against milestones in the Full Research Proposal

Milestone	Achieved/ Partially Achieved/ Not Achieved	Explanation
Milestone 1.1 Conduct Scoping study - Review industry pathology issues and identify and priorities research opportunities	Achieved	A review manuscript on detection and management of Verticillium wilt was completed in 2017-18 and is under revision for a peer-reviewed publication in 2020-21.
Milestone 2.1 Agreement with fungicide suppliers established	Achieved	MTAs were signed in partner with Syngenta, ThinkBio and Adama
Milestone 2.2 Conduct efficacy testing of existing fungicides not currently registered in cotton identified as per 1.1.	Achieved	Six and eight non-cotton fungicides were assessed for their control efficacy against black root rot (BRR) and Alternaria leaf spot (ALS), respectively. Two fungicides were granted the emergency permit for controlling ALS on cotton seedlings.
Milestone 3.1 Develop and implement methodology for screening of novel products in cotton as identified in 1.1	Achieved	Novel chemistries and biocontrol agents were assessed for against BRR and ALS. Both in vitro and glasshouse assessments were undertaken; and these are being continued.
Milestone 3.2 Commercialisation and exploitation plan developed for any new products screened in this project.	Not achieved	Commercialisation and exploitation have not been initiated at this point as no new efficacious products or chemistries have been identified to date. A few potential novel products require further testing.
Milestone 4.1 Identify research gaps in cotton pathology	Achieved	Population biology and diversity of BRR and ALS pathogens are largely unknown, so they are key investigations in a subsequent project DAN2101.
Milestone 5.1 Adoption pathways and measurement of adoption	Achieved	MERI plan was developed.
Milestone 6.1 ACRI Pathology Unit to liaise with Cotton Crop Protection Specialist (Yanco) to oversee field evaluations of novel products to manage	Achieved	Discussion with newly recruited southern cotton pathologist was finalised and the southern team conducted field-based assessment of Fungicide 2 and Tebuconazole

cotton diseases in southern region		against BRR and ALS, respectively.
Milestone 6.1 Pathology research for Southern cotton	Achieved	BRR and ALS are the two major diseases for research in southern NSW.

Outputs produced *(Please refer to examples document to assist in completing this section).*

Output	Description
Products	• Mancozeb and Tebuconazole were granted an emergency application permit on seedlings and mature cotton if there is a future outbreak.
Publications	• Three scientific papers were published on peer-reviewed journals. The papers uncovered the etiology of ALS on cotton seedlings and pathogen diversity of Fusarium and Verticillium wilt diseases on cotton in NSW. • One industry article on a topical ALS disease was published on The Australian Cotton Grower Magazine.
Presentations	• Four oral and one poster presentations on cotton diseases, including one keynote were delivered at national and international conferences. • Nine oral presentations were delivered at national/regional workshop and industry engagements, including APPS-Fusarium workshop, FUSCOM and CCA workshops.

Outcomes from project outputs *(Refer to examples document).*

Outcome	Description
Increased knowledge about practices and products	• A number of non-cotton fungicides, novel chemistries and biocontrol were assessed in vitro and glasshouse for their efficacy against BRR. Such knowledge provides a guideline for further assessments of potential candidates; • *Alternaria alternata*, a causal pathogen of ALS on seedlings was successfully characterised and officially reported. *A. alternata* has been long considered a minor pathogen on cotton; therefore, its etiology and epidemiology on cotton remain largely unknown until the discovery of *A. alternata* as the main pathogen responsible for the ALS outbreak on cotton seedlings in 2017-18 season in southern NSW; • Efficacy and safety of Mancozeb and Tebuconazole which are currently granted an emergency permit for use on cotton seedlings were assessed in vitro and glasshouse conditions and considered suitable for application on seedlings.
Collaboration	• A strong network with Queensland pathology team and southern NSW team allowed for delivering a broad research topic, including disease management, surveillance and diagnostics; • Screenings of non-cotton and novel products against cotton diseases were conducted in vitro, glasshouse and field conditions. Collaboration with southern NSW team allowed for more field-based assessments being conducted;

	- Additionally, CottonInfo team help to deliver research outcomes through industry networks.

Part 3 – Technical Report

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EXECUTIVE SUMMARY

Diseases of cotton are of a significant constraint to cotton production and have been identified as key areas for investment by the cotton industry. Sustainability of the Australian cotton industry remains dependent on the continued development and adoption of cultivars that are highly resistant against major soilborne pathogens such as *Thielaviopsis basicola*, *Fusarium oxysporum* f. sp. *vasinfestum* and *Verticillium dahliae,* and a re-emerging Alternaria leaf spot (ALS) pathogen, *Alternaria alternata*. Incorporating high yielding traits with disease resistance is a difficult long-term process; and with limited resources, it is not always possible to develop a complete resistant cultivar to all diseases. Therefore, effective management of cotton diseases relies on an integrated approach. a key focus of this project was to identify non-cotton fungicides, novel chemistries and biocontrol agents, which can be integrated into management strategies of major diseases in cotton production systems.

This project has identified a number potential candidates/approaches. Several pathological research gaps that could be further investigated for their efficacy and insights into pathogen biology. These are as follows:

- Non-cotton fungicides A-16148-F and Fungicide 2, and a novel plant extract PlantY provided a potential black root rot (BRR) control, but there was a lack of control consistency and required further assessments;
- In response to the leaf spot outbreak on seedlings in 2017/18 season in southern NSW, *Alternaria alternata* was a predominant pathogen responsible for the outbreak;
- Mancozeb and Tebuconazole have been granted an emergency application permit on both seedling and mature cotton in the event of a future outbreak;
- Incorporation of a brassica crop could provide a potential alternative practice to suppress the *V. dahliae* population, but this requires a long-term field assessment for inclusive recommendations;
- BRR and Verticillium wilt pathogens are now widely distributed in NSW, but insights of their biology and diversity are largely unknown. Such knowledge is essential for developing an accurate and rapid detection approach, as well as management strategies;
- Assessing for disinfestation efficacy against BRR and Verticillium wilt pathogens of Farmcleanse-alternative products will be vital for farm hygiene practices;
- Studies focused etiology and epidemiology of a sporadic but important boll rot disease should also be a priority.

State-based agencies such as NSW DPI have historically played an important role in providing responses to industry needs and delivering leadership in cotton pathology. This project built and enhanced research capacity by the appointment of an additional cotton pathologist based at NSW DPI Narrabri. The aim was to retain grower confidence in the cotton industry to provide support and leadership of cotton pathology issues. The appointed team including Dr Duy Le and Aphrika Gregson has collaborated with Queensland cotton pathology team, southern NSW Cotton Crop Protection specialist based at Yanco Agricultural Institute and CottonInfo team. Such collaboration allowed us to investigate into a broad array of plant pathology research activities such as disease surveillance, management and diagnostics.

INTRODUCTION

The ongoing success of the Australian cotton industry is dependent on effective disease management tactics. With disease being a significant constraint on cotton production, this project generated knowledge of current and emerging cotton disease issues. Research efforts will focus specifically on conducting a review to quantify existing disease issues, then identifying suitable new/novel products for cotton disease management and developing strategies which incorporate all registered products.

New product research will involve identification of potential solutions through efficacy screening, including assessment of technical feasibility. Products of interest would be either commercially available in other commodities but not currently registered on cotton, or experimental control agents. Where potential disease management solutions are identified steps will be taken to progress these products toward registration or commercialization for use in cotton. As part of this process, scientifically rigorous screening procedures will be developed with a view to these methodologies contributing to a registration data package and/or supporting future commercialization processes.

New knowledge developed during the life of this project along with collaborative links to existing cotton pathology projects will be used to develop key components of decision matrices which will allow growers and managers to effectively assess disease risk in their area and implement regionally specific solutions. Development of communication strategies established in agreement with industry partners will also facilitate rapid tactical responses to emerging disease issues.

Where gaps in the knowledge are identified research would be undertaken to inform potential product use within an integrated disease management framework. Through these investigations of new products industry will have a greater capacity to respond to regional pathology issues (in both northern and southern NSW).

This project will also integrally link to the industry disease survey project by responding to emerging issues identified through surveillance activities.

MATERIALS AND METHODS

Scoping study

Comprehensive literature reviews of major cotton diseases, including Verticillium wilt, written by Klosterman et al. in 2009 from the journal *Annual Review of Phytopathology* and by El-Zik in 1985 from the journal *Phytopathology*; Fusarium wilt, written by Davis et al. in 2006 from the journal *Plant Disease*; and black root rot written by Pereg in 2013 from the journal *Crop and Pasture Science* were accessed. The literature described was thoroughly assessed in order to review industry pathology issues and identify and prioritise research opportunities. The scoping study also included keyword searches such as 'cotton diseases' 'new/emerging/exotic diseases', and 'Verticillium/Fusarium/black root rot management' from both national and international sources. Accessed sources include: The Cotton Research and Development Corporation's 'Inside Cotton Library', and academic literature retrieved from 'Google scholar' and archived databases of plant pathology journals.

Pathogen isolation and identification

Samples collected or received throughout survey seasons were processed as follows for pathogen recovery. The isolation was initiated with surface decontamination of the diseased plant tissue with 70% ethanol for around 10 seconds and blotted dry with paper towel. Under aseptic conditions, small sections of diseased tissue were excised and embedded into potato dextrose agar (PDA Difco) amended with 100 ppm streptomycin sulfate (Sigma Aldrich) (sPDA). The dishes were sealed with parafilm and incubated at 25 °C in darkness for two to three days. Colonies emerging from infected tissue were individually sub-cultured onto new sPDA dishes. Pure cultures were then incubated at 25 °C in darkness for at least 7 days before small plugs (0.5 cm2) were excised from the colony margins, submerged in sterile water and stored at room temperature.

Pathogen identification was based on both morphology and molecular analysis. For molecular identification, genomic DNA was obtained using the Wizard® Genomic DNA Purification Kit (Promega). Fungal mycelia (10-100 mg) were scraped off culture dishes and transferred into a 1.5 mL tight-lock Eppendorf tube. The extraction steps were followed as recommended by the manufacturer. Common fungal barcodes, including the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF1) regions were amplified with universal primers. The amplicons were then sequenced by Macrogen, South Korea and compared with known sequences deposited in the GenBank database.

In vitro assays

The poisoned food technique was deployed to evaluate the control efficacy of chemical candidates against Alternaria leaf spot pathogen, *A. alternata*. Briefly, designated concentrations were thoroughly mixed in a 55 °C-molten PDA medium (Difco). Under aseptic conditions, 15 mL aliquots of the fungicide-amended PDA were transferred to 9-cm-diameter Petri plates and left to set for 45 min. Control plates contained PDA without fungicide amendment. Active cultures of *A. alternata* were excised into 0.5 cm squares and transferred to the centre of each plate. The culture plates were sealed with parafilm and kept at 25 °C in darkness. Colonial diameters of the cultures were measured in mm at three days after incubation. There were three replicate plates per testing concentration; and the assay was repeated once. Growth inhibition of *A. alternata* was calculated based on the formula below:

$$\text{Growth inhibition (\%)} = (C-T)/C \times 100$$

C is growth diameter of control plates
T is growth diameter of tested plates

Dual culture assays were carried out against block root rot pathogen *Thielaviopsis basicola,* with putative biocontrol bacteria. Single-colony bacterial cultures were grown in nutrient broth (peptone 5g/L, yeast extract 3g/L, NaCl 5g/L, pH 6.5-7) for two days at 25 °C in darkness. Specialised media nutrient agar medium (NAM) was prepared according to the following: peptone 10g/L, yeast extract 3g/L, NaCl 5g/L, agar 15g/L, and adjusted to pH 6.5-7. Active cultures of BRR were excised into 0.5 cm squares, then placed onto the centre of ½ NAM + ½ PDA plates. An inoculum ring of biocontrol bacteria was made around the excised BRR square by dipping the base of a sterile 0.6 cm diameter Petri dish in the bacterial suspension and placing the inoculated surface to the area surrounding the BRR square.

Sterile water was used in lieu of bacterial suspension on the control plates. There were three replicate plates for each tested bacterium. Plates were sealed with parafilm and kept upside down at 25 °C for 5 days. Growth inhibition was calculated as above.

Pot trials

Seed treatments were deployed to assess the control efficacy of candidate products against the BRR pathogen. Black (untreated) cotton seeds (Sicot 75RRF Cv.) were manually coated with the tested products as follows: A mixture of 1kg of cotton seeds, 30mL of distilled water, designated volume of tested products and 3g of fluency agent (Bayer) were first thoroughly shaken, then the coated seeds were left to air-dry overnight in a fume hood cabinet. The treated seeds were either stored at room temperature for later pot trials or bagged (at a rate of 13 seeds/m) using a seed counter (Contador).

Additional assays were conducted to assess the efficacy of candidate products applied via soil drench against the BRR pathogen. To manipulate the in-furrow injection of fungicides, 15 black cotton seeds were planted in 14-cm-diameter pots containing pasteurised Searles® potting mix, sprayed with tested fungicides at designated concentrations and then covered with a thin layer of potting mix. The pots were watered daily, and disease assessments based on percentage of root necrosis were undertaken at four weeks after planting.

Foliar sprays of chemical candidates against the ALS pathogen were also assessed on seedlings. Two-week-old seedlings were inoculated with *Alternaria* conidial suspensions at a concentration of 10^4 conidia/mL. Inoculated cotton seedlings were sprayed until run-off with conidial suspensions. The control treatment consisted of seedlings sprayed with sterile water. Following inoculation, all plants were immediately covered and sealed tight with individual plastic bags to increase humidity. After two days, the covering bags were removed, and seedlings were misted three times per day to elevate canopy humidity. Disease assessment of inoculated seedlings were undertaken at 10 days after inoculation (DAI). Visual assessments for disease incidence and severity were scored using a rating scale of 0 to 5 as follows: 0 = no disease present; 1 = minute pinhead size spots less than 5% cotyledon surface diseased; 2 = dark brown lesions covering 5 to less than 25% of the cotyledon surface; 3 = necrotic lesions covering 25 to less than 50% of the cotyledons; 4 = necrotic lesions covering 50 to less than 75% of the cotyledons; and 5 = lesions coalescing covering 75% or greater the cotyledon surface. At 10 DAI, the Alternaria-inoculated seedlings were sprayed wet with tested chemistries at designated concentrations. The disease progress was monitored and assessed at 10 days after the chemical application.

Field trials

The efficacy of seed treatments against the BBR pathogen were also assessed under field conditions in the 2017/18 and 2018/19 seasons. All trials included cotton black seeds (non-treated) as the negative control and commercially treated seeds with Dynasty as the positive control. All selected fields were known to have a history of BRR.

Field 1, ACRI: The field experimental design was randomized with four 19m-long rows per replicate, and four replicates for each treatment. Two trials with different planting dates, including early planting in September 2017 and late planting in October 2017 were conducted at field 1.

Field 2, Merah North: The field trial was designed with one 14m-long row per replicate and four replicates per treatment. All treatments were randomly arranged. The trial was planted in October 2017.

Field 3, Warren: The field trial was first planted in early October 2017 and replanted in late October 2017 due to sand blast damage. There were three 14m-long rows per replicate and four replicates per treatment. All treatments were randomly arranged.

Field 4, Griffith: The field trial was designed with three 14m-long rows per replicate and four replicates per treatment, randomly arranged. The trial was planted in October 2017. All off-site trials were established and managed with the assistance of the CSIRO Cotton Plant Breeding Unit, Narrabri. Similar fields at the same site were selected in the 2018/19 season and planted during October 2018. Disease assessments were undertaken at 4-6 weeks after planting and during the early season disease surveys.

The efficacy of rotation with a brassica crop in reducing the *Verticillium dahliae* inoculum were assessed at the ACRI trial site from 2018 – 2020. A well-known brassica crop (cv. Caliente) was planted during winter (June to September). The crop was ploughed and incorporated into the soil at the end of September. Immediately after incorporation, the field was fully irrigated to simulate anaerobic conditions and to stimulate the decomposition process for 4 weeks. Soil cores collected at 10 cm depth were sent to SARDI, Adelaide for quantification of the soil inoculum before and after brassica incorporation.

RESULTS

Objective 1. Research question – Are there opportunities for new or existing products to manage cotton diseases?

Milestone 1.1. Conduct Scoping study - Review industry pathology issues and identify and priorities research opportunities

A literature review entitled ‘*Disease-related constraints to Australian cotton production*’ was completed in order to identify scope in future research directions (appendix 1). The project focuses on investigating the role of novel products and biocontrol agents in controlling cotton diseases. We decided to target four topical pathogens of the Australian cotton industry; *Alternaria* spp. causing Alternaria leaf spot, *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) causing Fusarium wilt, *Thielaviopsis basicola* causing black root rot (BRR) of cotton seedlings, and *Verticillium dahliae* causing Verticillium wilt.

The sustainability of the Australian cotton industry remains dependent on the continued development and adoption of cultivars that are highly resistant to major pathogens, including *T. basicola*, *F. oxysporum* f. sp. *vasinfectum* and *V. dahliae*, as well as emerging pathogens, namely *Alternaria* spp. Incorporating high yielding traits with disease resistance is a difficult long-term process; and with limited resources, it is not always possible to develop cultivars with complete resistance to all diseases. In the meantime, there are numerous approaches that could be applied to control disease outbreaks and minimize yield losses. For Alternaria disease, several fungicides are available on crops other than cotton, hence, it is necessary to evaluate their efficacy against *Alternaria* spp. on cotton as a short-term priority. For BRR of cotton, crop rotation and organic amendment have been shown to provide varying degrees of protection. However, these practices might also trigger the dominance of other pathogens, namely *Pythium* spp. and *Rhizoctonia* sp.; hence, the success of these practices will be mostly dependent on a thorough knowledge of field history. Chemical seed treatments with fungicides and plant activators have shown promising BRR control in cotton seedlings, but there remains a need to independently test the efficacy of novel compounds. Biocontrol of BRR appears as a potential and sustainable approach, so it warrants a further research. For Fusarium wilt, a popular management strategy is the adoption of high F-rank cultivars. Verticillium wilt is a key concern to the cotton industry currently, owing to the lack of commercial cultivars with more than limited resistance against the disease. Strategies to minimise yield losses currently rely on alternative approaches. Chemical fumigation is an important practice to suppress *V. dahliae*-infested soils; unfortunately, this is not practical to the cotton industry as the practice would be costly and uneconomic; and could have negative environmental impacts. Similarly, soil solarization using thin plastic film has proven its effectiveness in suppressing a wide range of soil pathogens, but this is typically impractical for broad acre cotton fields. However, these approaches can be adapted to control *V. dahliae* in field hot spots. The approach has been exploited in the project RRDP1724: Improving the management of cotton disease in Australian cotton farming. Biofumigation or bio soil disinfestation approaches have drawn great attention; however, consistency needs to be improved. While waiting for the development of *V. dahliae* resistant cultivars, induced resistance of cotton against *V. dahliae* is of great interest.

For this project, research was focused on the following approaches:

- Seed treatments with not-yet registered and novel compounds, and potential biocontrol agents for controlling cotton seedling disease, particularly BRR (pages 13-16; 23-27);
- Chemical control of Alternaria disease by efficacy evaluation and selection of suitable foliar fungicides for the industry (pages 16-21);
- Adopting and optimizing bio-disinfestation of soils infested with *V. dahliae* (pages 21-23);
- Exploring biocontrol agents to suppress *T. basicola* populations, and thus reducing BRR (pages 27-29).

Objective 2. Research question – What is the efficacy of existing commercially available fungicides on cotton diseases?

Milestone 2.1 Agreement with fungicide suppliers established

NSW DPI negotiated and established a collaborative partnership with Syngenta Aust Pty Ltd. Ken McKee, a Field Development Manager with Syngenta signed and executed a material transfer agreement (MTA) for the supply of four Syngenta products. The Syngenta products were assessed for their efficacy against BRR and ALS pathogens. Mr McKee recently contacted NSW DPI again in regard to further collaboration. A selection of new Syngenta products will be screened for their efficacy against BRR.

NSW DPI also established a collaborative partnership with Adama Australia Pty Ltd. Initiated by Susan Maas from CRDC and followed up with several phone conversations between Dror Dagan, Head of Portfolio – Marketing and Dr Duy Le. NSW DPI. An outcome of discussions between NSW DPI and Adama was the selection of several potential candidates with potential control efficacy against *Alternaria* spp. The formal MTA was signed by representatives from the two parties. The Adama products have been delivered and will be included in the screening program outlined in DAN2101.

NSW DPI also established collaborative a partnership with Thinkbio Pty Ltd. Dr Duy Le was contacted by Lisa Anderson, Managing Director from Thinkbio, with a potential biocontrol agent to be tested against BRR. A formal collaborative agreement was signed, and in vitro work was conducted in 2019.

NSW DPI initiated discussions with Bayer CropScience Pty Ltd to establish a collaborative partnership. NSW DPI met with representatives from Bayer including Matthew Westgarth – Business Development Manager with the aim of establishing a collaborative partnership to field test a selection of Bayer products in the 2018-19 cotton season. Potential products include fungicides to control *Alternaria* spp. However, according to Bayer's current investment strategy, ALS on cotton is not included in their short-term focus due to limited market potential.

NSW DPI is establishing a collaborative partnership with Northwest Chemicals and Distributors. Discussions between Northwest Chemicals and Distributors, James Wallace and DPI's Rod Jackson and Duy Le centre on the testing of disinfestation efficacy of Agricleanse against *V. dahliae.* A formalised collaborative agreement is currently being negotiated. Testing will be a key deliverable of DAN2101 - *Evaluate efficacy of novel chemistries, biocontrol agents and management practices to control Alternaria and Black Root Rot disease in cotton*

Milestone 2.2 Conduct efficacy testing of existing fungicides not currently registered in cotton

Black root rot (BRR)

Black cotton seed was treated with potential fungicides currently registered on crops rather than cotton from industry partners (Syngenta) were included in field screening trials for their control efficacy against BRR disease during 2017/18 season. These included:

- **Syn2**: this solo active ingredient is registered in Australian. This is formulated with a number of different active ingredients targeting at true fungi and oomycetes and the new products with this active ingredient will be registered in Australian in the next 12 months. To Syngenta, this Syn2 could be worth testing against *V. dahliae*.

- **Syn3**: this is registered Australia and presents in a formulation targeting Fusarium on cereals. This solo ingredient is suggested for testing against Fov on cotton.
- **Syn4**: this is registered in Australia as Tecto and used as a seed treatment in potatoes to control dry rot caused by *Fusarium* species.
- **Dynasty**: this is an industry standard seed treatment, which was included as a positive control.

Below is a brief summary of treatments (**Table 1**).

Table 1: A summary of chemistries used, application rates and number of field trials screened for seed treatment efficacy in season 2017-2018

Products	Formulation No.	Primary Rate Recommended		Field trials
		(Product) mL/kg	(Active) g a.i./kg	
Syn2	A-16148-F	0.8 mL	0.4 g a.i.	2 at ACRI, 3 off sites
Syn3	A-9142-G	16.7 mL	0.5 g a.i.	2 at ACRI, 3 off sites
Syn4	A-10466-D	4 mL	2.0 g a.i.	2 at ACRI, 3 off sites

Of the five trial sites, BRR incidence at the ACRI late planting and Griffith site was significantly higher than the BRR incidence recorded at the ACRI early planting and Warren trial sites. No BRR was detected at the Merah North trial site. These offsite trials were coordinated by the CSIRO Breeding group; therefore, selection of our trial sites was subjected to existing CSIRO collaborators trial site availability. BRR incidence was not significantly different among seed treatments at ACRI early and late planting and at Warren; the mean BRR incidence was as low as 1% at Warren, up to 44.8% at ACRI late planting (Figure 1). At the Griffith trial site, BRR incidence was recorded lowest at 9.9% in A-16148-F treated seeds; and was significantly different from A-10466-D which had the highest incidence at 53.5% (Figure 1). This promising candidate warranted further screening.

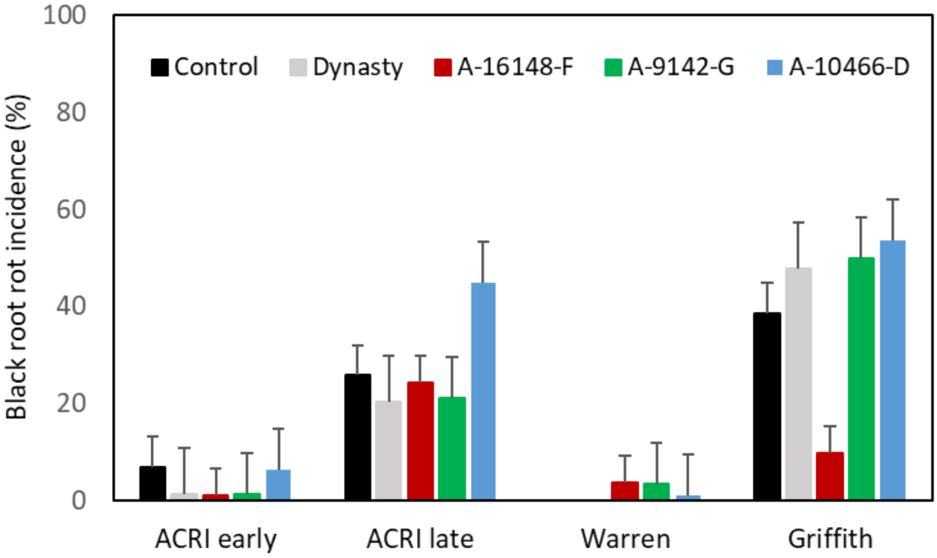


Figure 1: Black root rot incidence (%) recorded at four trial sites in 2017/18 season. Bars represent SEM (n = 10).

The efficacy of these seed treatments was also assessed against other seedling diseases. Rhizoctonia-like rot was obvious across trial sites in the 2017/28 season. The disease incidence was recorded highest at the ACRI late planting (between 80 – 95%) site, following by Warren where the incidence was between 60 – 70%. Seedlings assessed at Merah North and Griffith exhibited less Rhizoctonia-like symptoms. However, overall there was no significant difference among treatments in regard to the expression of Rhizoctonia-like rot on cotton seedlings in the 2017/18 season.

Note: Rhizoctonia-like rot (also known as collar rot) refers to cotton seedlings which exhibit superficial, irregular reddish-brown lesions commonly around the collar regions. These symptoms resemble those induced by *Rhizoctonia solani*; however, we recovered a greater number of *Fusarium* species, including *F. oxysporum*, *F. equiseti*, *F. falciforme*, than that of *R. solani*. Of the *Fusaria*, *F. oxysporum* was predominant and lacked a specific pathogenicity marker that was unique to Australian *F. oxysporum* f. sp. *vasinfectum*. However, due to biosecurity restrictions on site at ACRI we have not been able to confirm the pathogenicity of these *Fusaria* on cotton seedlings, so the name Rhizoctonia-like rot remains in use. Part of the findings involving the genetic diversity of Fusarium species recovered from cotton seedlings were published in the peer-reviewed journal Australasian Plant Pathology (appendix 2).

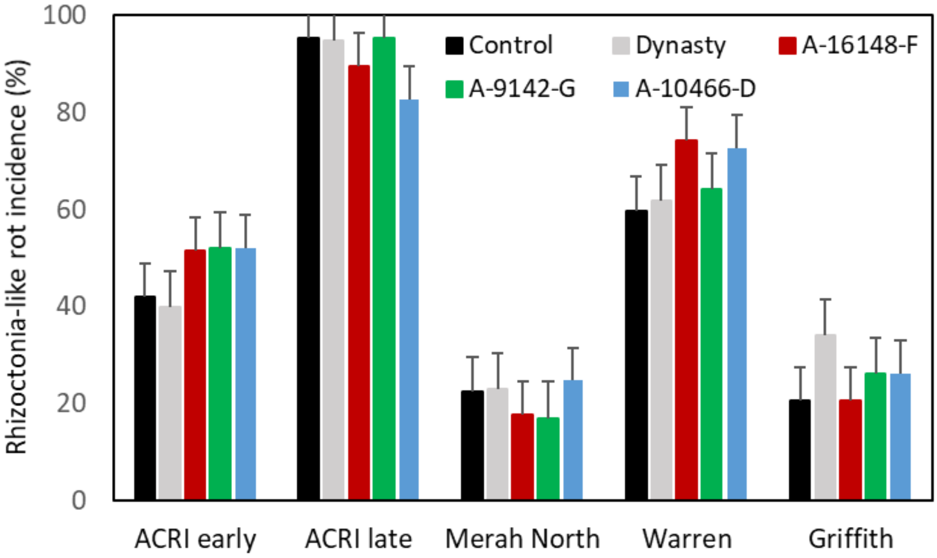


Figure 2: Rhizoctonia-like rot incidence (%) recorded at four trial sites in 2017/18 season. Bars represent SEM (n = 20).

These candidates were screened again under field conditions in the 2018/19 season. However, due to low seedling disease expression at CSIRO trial sites in the 2018/19 season, the efficacy of potential candidate A-16148-F against BRR remained inconclusive.

The two commonly used fungicides (namely Fungicide 1 and Fungicide 2) for seed treatment in cereals were assessed for their control efficacy against BRR in a pot trial. The two fungicides were supplied by Bayer and DowDuPont, respectively. Fungicide application was conducted to simulate the in-furrow injection of fungicide application under field conditions. At four weeks after planting, all seedlings across the treatments were infected with BRR, indicating a BRR incidence of 100%. The disease severity was also substantially high and was comparable across all treatments, except for the soil drench with Fungicide 2 at 3 mL/L. The BRR severity recorded in the Fungicide 2 drench treatment was reduced by approximately 16% and significantly different to other treatments (**Figure 3**). It was also noted that only the Fungicide 2 drench treatment was able to sustain BRR-infected seedling stands (**Figure 4**).

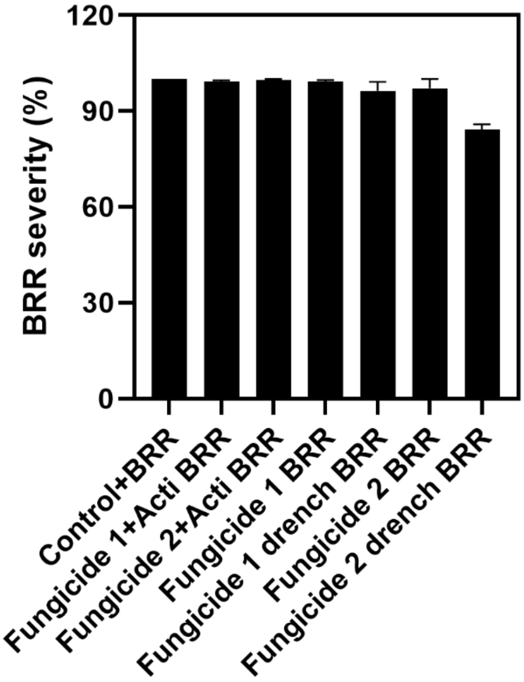


Figure 3: Variation in disease severity (%) recorded on seedlings drenched with different fungicides. Bars represent SEM (n = 10).

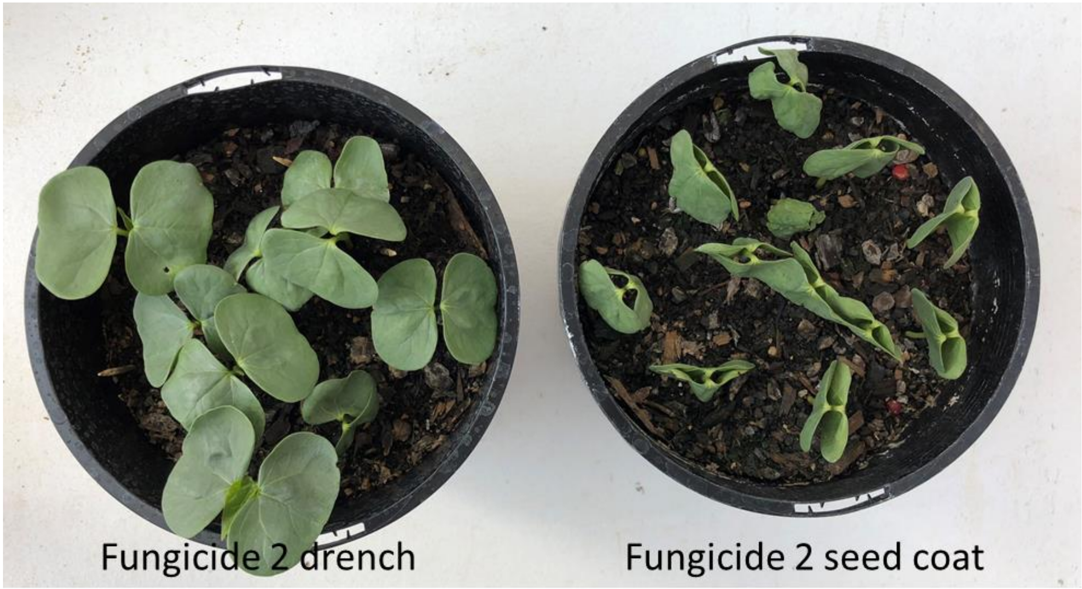


Figure 4: Simulated in-furrow application of Fungicide 2 resulted in healthier looking seedlings (left) compared to seedlings received seed treatment with Fungicide 2 (right).

The efficacy of Fungicide 2 was further evaluated under field conditions in the 2019/20 season. Tim Green, a newly appointed Plant Pathologist based in Yanco coordinated and conducted the field trial. Trial results will be analysed in consultation with Dr Duy Le and published in the November 2020 DAN1903 Progress Report.

Alternaria leaf spot (ALS)
In response to the ALS outbreaks on cotton seedlings in 2017/18 season in southern NSW, evaluation of foliar fungicides efficacy again ALS pathogens was promptly undertaken. Additionally, due to the minor concern of ALS on seedlings, the outbreaks were questioned if there was a new *Alternaria* species associated with it. Therefore, a characterisation work was conducted to confirm the *A.*

alternata as a main causal agent responsible for the outbreaks. The results were published in Australasian Plant Pathology journal (appendix 3).

A number of foliar fungicides that have been registered for control of *Alternaria* spp., including *A. alternata* on other crops rather than cotton have been undertaken the screening for their efficacy against *Alternaria* spp. causing early and late leaf spots of cotton (Table 2).

Four representative isolates recovered from cotton seedlings were selected for our fungicide efficacy screening. Two isolates RvL and CBL were identified as *A. alternata*; and two isolates WL and WYP were identified as putative *A. eureka*.

Table 2: A summary of fungicides used and application rates for in vitro screening for their efficacy against *Alternaria* spp. recovered from cotton leaf spot

Fungicides [1]	Recommended rate (mL/ha)	In vitro application (mL/L medium)
Mancozeb	3000	30
Fungicide 3	200	2
Tebuconazole	300	3
Fungicide 4	300	3
Fungicide 5	300	3
SYN545974	300	3
Fungicide 6	600	3
Fungicide 7	500	5

[1] These fungicides were supplied by Syngenta and Bayer. Mancozeb and Tebuconazole are currently granted an emergency permit for controlling of ALS.

The in vitro results indicated that efficacy of the tested fungicides was dependent on *Alternaria* species. Growth of unidentified *Alternaria* isolates WYP and WL were highly suppressed by all tested fungicides irrespective of the application rate (**Figure 5**). Fungicide 4 was less sensitive on WYP and WL isolates, but was able to suppress more than 50% growth of the two isolates. Initial characterisation work of the *Alternaria* population collected during ALS outbreaks in the 2017/18 season determined that unidentified Alternaria comprised a small proportion and appeared least virulent to cotton seedlings in pot trials. Therefore, to some extent the lesser sensitivity to Fungicide 4 is of minor concern.

Of the *Alternaria* isolates recovered in the 2017/18 outbreak, *A. alternaria* accounted for 83%. For the two representative *A. alternata* isolates RvL and CBL, mycelial growth was also dependent on application rates. All the eight tested fungicides excepting for Fungicide 4 suppressed 50% growth of RvL and CBL isolates at label recommended rates (**Figure 5**). Some fungicides worked well applied at half the strength of the recommended label rate (e.g. Fungicide 3 and Fungicide 6). However, complete growth suppression was only achieved for isolates at double rates (e.g. Mancozeb against isolate RvL and Fungicide 5 against isolate CBL). Among the tested fungicides, Fungicide 4 was the least sensitive (**Figure 6**). Growth suppression against *A. alternata* RvL and CBL isolates was recorded as low as at 15% (**Figure 5**). The lack of control efficacy of Fungicide 4, a registered fungicide for controlling *Alternaria* species on fruit and vegetable crops indicated that *A. alternata* from cotton could have developed some level of chemical resistance. Additionally, the fungus itself is prone to the development of fungicide resistance. Hence, foliar fungicide application against ALS should be closely monitored. The in vitro screening undertaken revealed that Mancozeb and Tebuconazole maintained their high control efficacy against *A. alternara* RvL and CBL; subsequently, the two candidates were granted for emergency permit extensions for seedling applications.

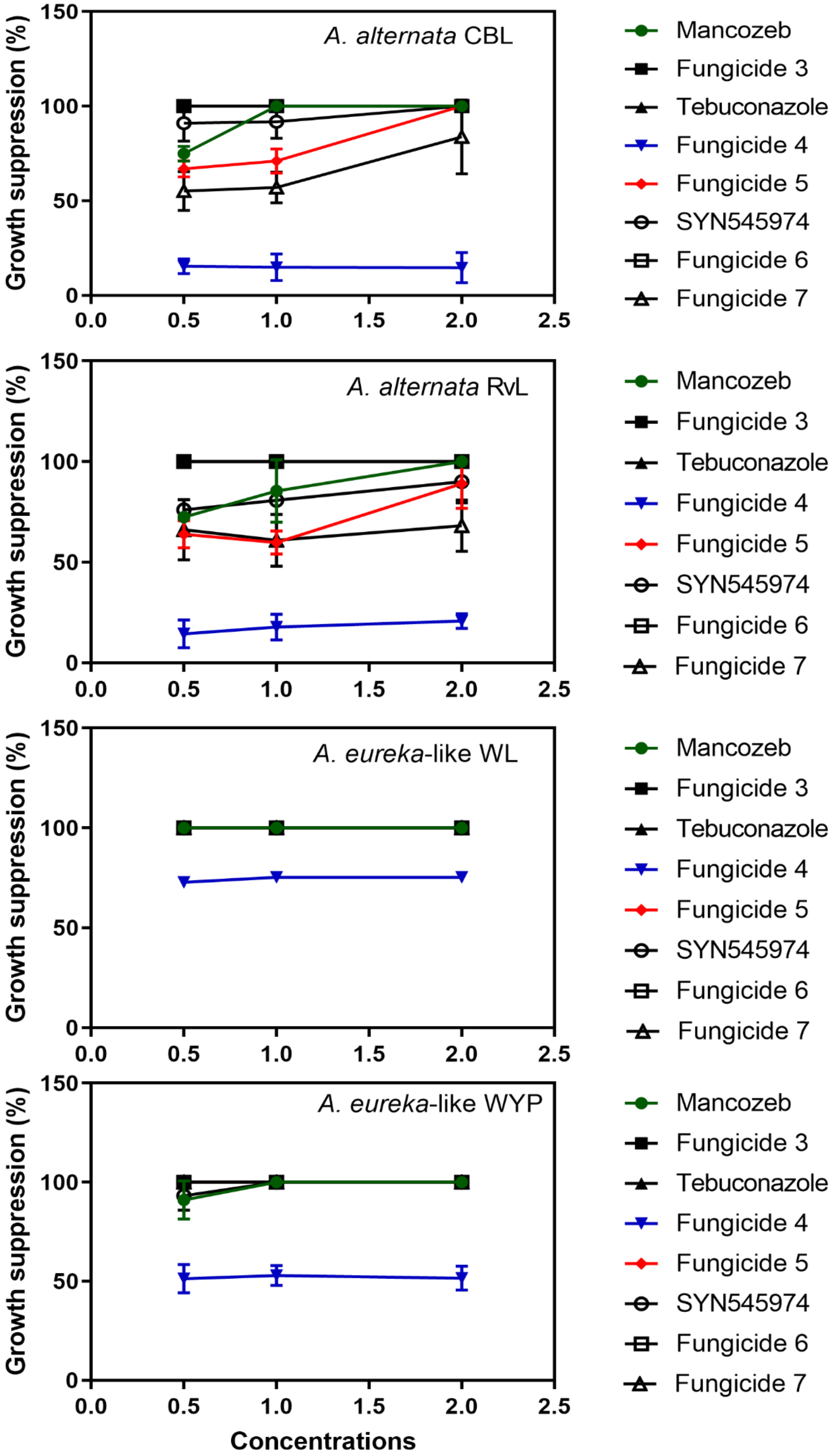


Figure 5: Growth suppression (%) of tested fungicides against *A. alternata* (isolates RvL and CBL) and putative *A. eureka*-like (isolates WL and WYP). Concentrations indicated as follows: 0.5 = ½ recommended label rate (RLR); 1 = RLR; 2 = double RLR. Bars represent SEM (n = 8).

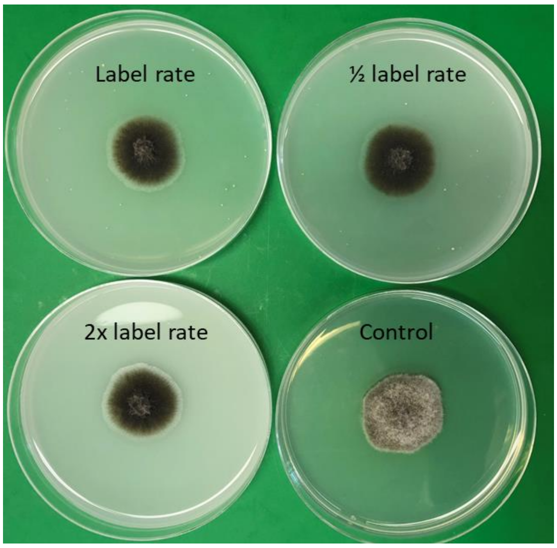


Figure 6: In vitro assay assessing growth of *A. alternaria* RvL isolate on Fungicide 4 amended medium. Cultures were at three days after incubation at 25 °C in darkness.

Due to the lack of registration for these fungicides on cotton, they were further assessed for their control efficacy and crop safety in pot trials. Fungicides were applied to *Alternaria* pre-inoculated seedlings at 7 days post infection. Disease severity was assessed 10 days after the fungicide application. Disease index progress was lower on seedlings which received Tebuconazole, Fungicide 3, Fungicide 6, and Fungicide 8 sprays than that of the *Alternaria* infected control seedlings (**Figure 7**). However, the disease index progress only decreased by 8.1 to 11.3% on the seedlings that received Fungicide 3 and Fungicide 8 sprays, respectively. Additionally, fungicide application timing (being pre- or post-infection sprays) will be assessed in further trials in order to optimise and increase their control efficacy.

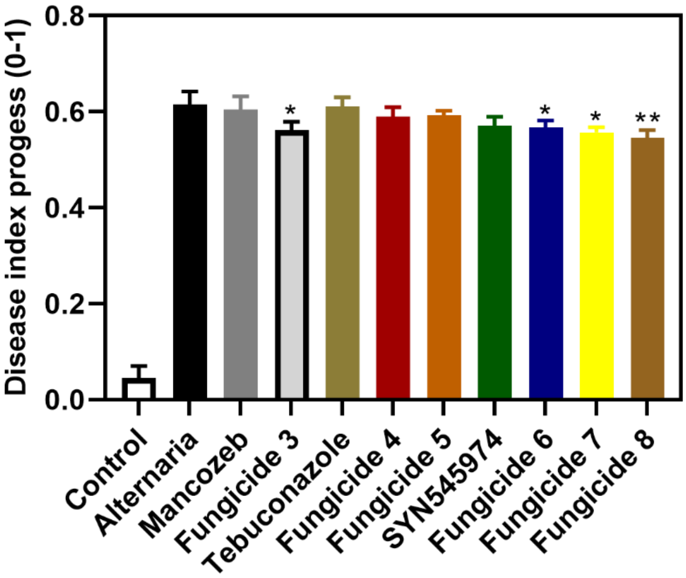


Figure 7: Disease index progress (0-1) recorded at 10 days after spray. Application rates were as per recommended on the labels. Bars represent SEM (n = 10).

Since young cotton seedlings are also prone to spray damage, an independent trial, was also conducted to assess crop safety of these fungicides. Seedlings free of Alternaria leaf spot were sourced for experimentation. At the recommended rates, minor necrosis on cotyledons and true leaves were observed on seedlings at one day after spray with Fungicide 3 and Fungicide 7 (**Figure 8A, 8B**). A greater percentage of leaf burn after Fungicide 6 application was observed on seedlings (**Figure 9**). On average around 7.5 and 20% necrosis was recorded on cotyledons and young true leaves, respectively (**Figure 8A, 8B**). Additionally, growing points and emerging leaves from seedlings that had received these fungicides were stunted and deformed, resembling hormone damage. Mancozeb and Tebuconazole appeared safe to cotton seedlings when applied at the recommended label rate in our pot trial assessment.

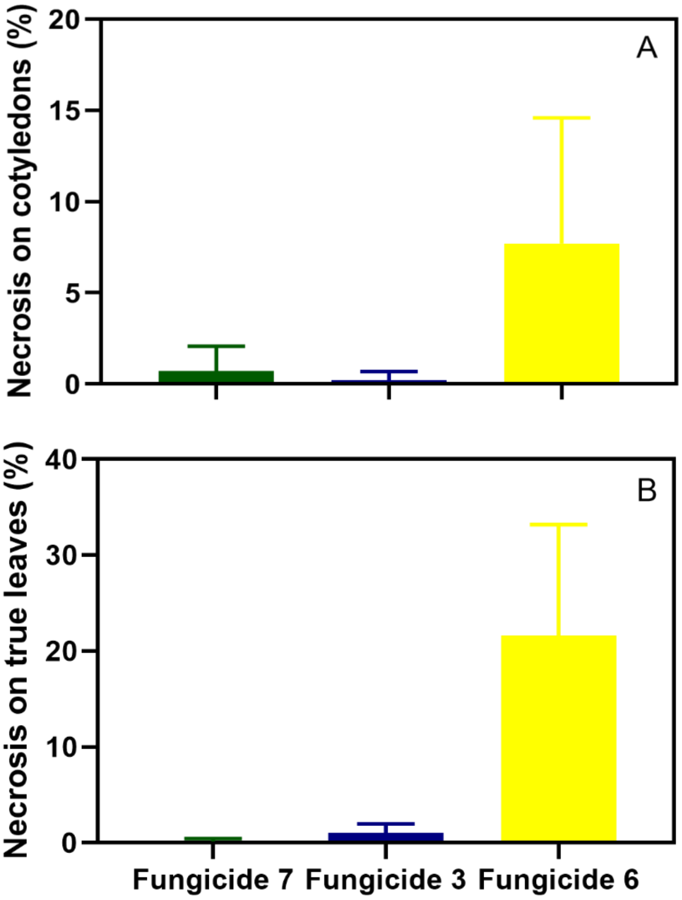


Figure 8: Spray damage on cotyledons and young true leaves were assessed based on percentage of necrosis (Bars represent standard deviations).

Figure 9: Leaf necrosis and stunted growing points were observed on young seedlings at 10 after application of Fungicide 6.

In coordination with Tim Green and his team, a field trial was conducted in the 2019/20 season to assess the control efficacy of Mancozeb and Tebuconazole against ALS. Trial results will be analysed in consultation with Dr Duy Le and published in the November 2020 DAN1903 Progress Report.

Verticillium wilt

The efficacy of incorporation of mustard biomass for disinfestation of *V. dahliae* soil inoculum under field conditions was conducted over a two-year period. The ACRI field trial (1.2 ha) was divided into multiple blocks (strip treatments) where either a brassica crop (cv. caliente) was planted after cotton during wintertime or left fallow. Caliente is a mustard line well-known for its fumigation capacity and consequently is widely adopted for suppressing soilborne pathogen inoculum. Caliente was planted in June 2018 and 2019 and the green biomass was incorporated into soil late September of 2018 and 2019. The field was flooded soon after incorporation to assist the decomposition of biomass and the release of fumigating compounds. A month after incorporation, cotton was planted and assessed for Verticillium incidence in March 2019. Additionally, soil cores pre- and post-incorporation were collected and sent to SARDI for quantification of *V. dahliae* DNA concentrations.

The mean wilt incidence recorded in the 2018 crop was around 10%. Due to hail-storm damage, the 2019 cotton crop was terminated and no disease assessment possible. The mean wilt incidence increased to 13 and 16% in the 2020 crop following the two fallows and brassica crops, respectively (**Figure 10**). However, the incidences were not significantly different among the treatments.

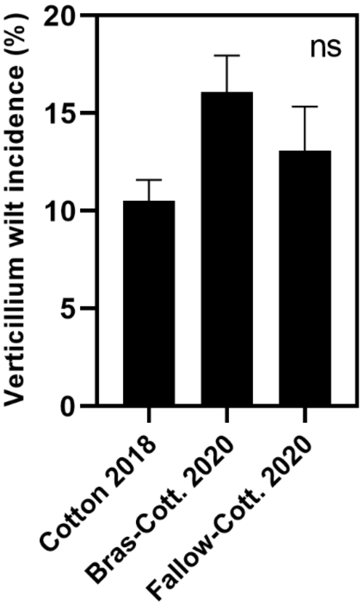


Figure 10: Verticillium wilt incidence in cotton recorded at before brassica rotation in 03/2018 and after either two fallows or two brassica rotations in 03/2020. Bars represent standard errors of means (n = 38 - 112).

Inoculum of *V. dahliae* present in field soil after each crop was indicated by the DNA concentration in the top 10 cm of soil. The DNA concentration after a cotton crop in 2018 was 20.75 pg/g soil; which dropped to 0.2 and 3.3 pg/g soil after two fallows and brassica crops, respectively in 2019 (**Figure 11**). The DNA concentration rebounded significantly after a cotton crop in 2020. The level of *V. dahliae* DNA in the fallow-cotton blocks was approximately 2.5 times higher than in the brassica-cotton blocks, indicating that growing and incorporating the biomass of a winter fumigation crop may provide a degree of soil buffer capable of slowing down the accumulation of *V. dahliae* inoculum. Experimentation is ongoing, with hopes of reporting conclusive data after a longer-term trial.

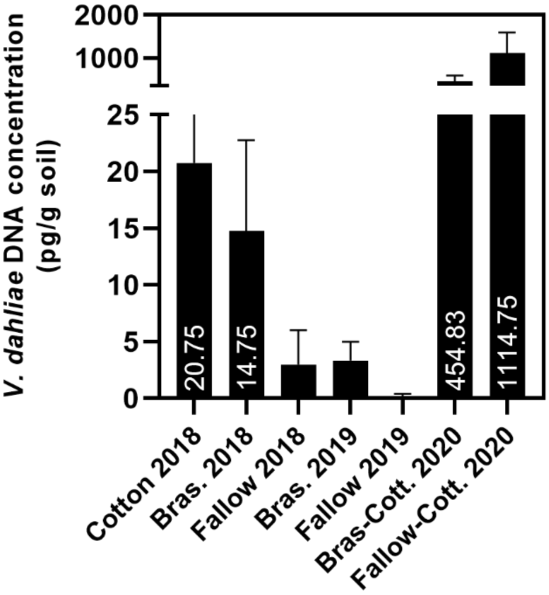


Figure 11: *V. dahliae* DNA concentration (pg/g soil) in soil sampled a month after each crop. Bars represent standard errors of means (n = 3 - 6).

Susceptibility of four brassica cultivars, including Caliente 199, Terra, Nemat and Rojo towards *V. dahliae* was assessed in two pot trials. In the two root dip trials, *V. dahliae* was able to infect and cause plant wilting and leaf necrosis of the four cultivars at four weeks after inoculation. Vascular discolouration was also visible in cross stem cut sections (**Figure 12**).

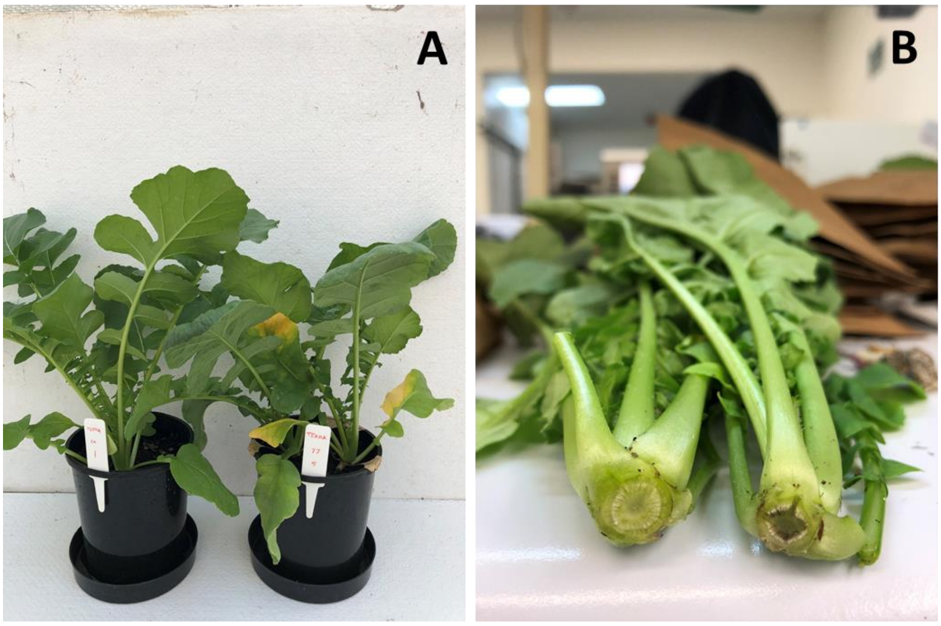


Figure 12: A brassica cv. Terra showed yellow leaf necrosis after inoculation with non-defoliating isolate Vert77 (A); correspondingly grey vascular discoloration was visual in cv. Terra inoculated with Vert 77.

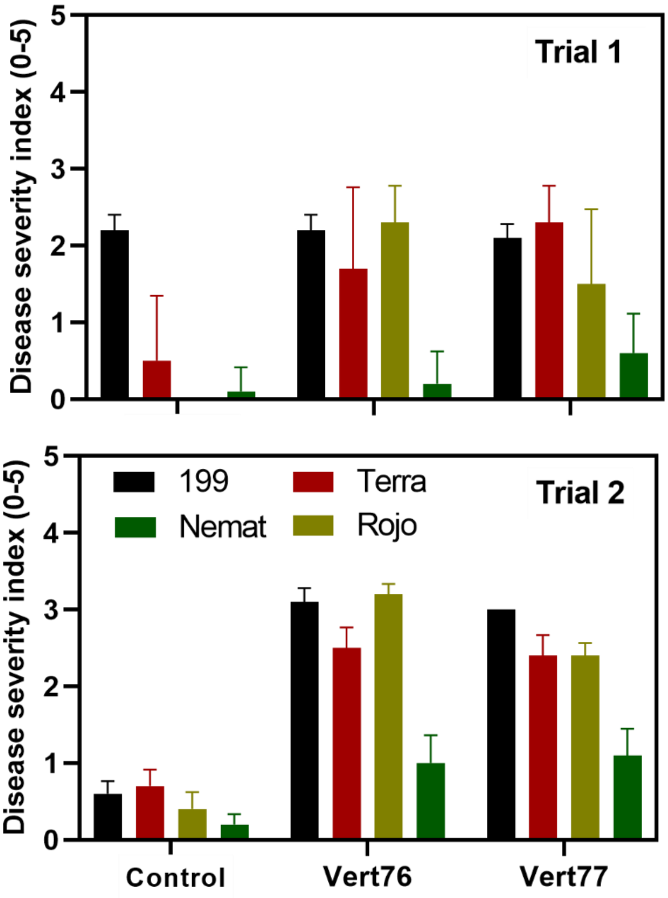


Figure 13: Susceptibility of four biofumigation brassica cultivars indicated by the disease severity index (0 - 5) towards the defoliating isolate Vert76 and non-defoliating isolate Vert77. Bars represent standard errors of means (n = 10).

In the two trials, cv. Nemat was statistically the least susceptible to both defoliating and non-defoliating isolates. The disease severity index was around one (Figure 8), indicating that there was only some degree of necrosis on cotyledons. The other three cultivars were more susceptible to *V. dahliae*. The disease severity index was between 2 to 3 on other cultivars, indicating that wilt symptoms were more pronounced on true leaves. Additionally, diseased plants showed some degree of vascular discoloration and consequently *V. dahliae* were recovered upon trial termination at four weeks after inoculation. Although these four biofumigation brassica cultivars could be infected and conlonised by *V. dahliae*, the efficacy of biomass incorporation of cv. Caliente in slowing down the *V. dahliae* inoculum in soil warrants further investigation.

Objective 3. Research question – What is the efficacy of new/novel products for management of cotton disease?

Milestone 3.1 Develop and implement methodology for screening of novel products in cotton as identified in 1.1

Field trials

The assessment of new/novel chemistries and biocontrol agents against the BRR pathogen *T. basicola* was the focus of this milestone due to the significance of the disease across NSW. A novel active ingredient under development, Syn1 from Syngenta was included in the first field screening conducted in the 2017/18 season. According to a Syngenta representative, Syn1 was evaluated for its efficacy against an array of fungal pathogens on canola and cereals. Furthermore, it was suggested that cotton seed treatment with Syn1 might protect cotton from seedling diseases. Additionally, two novel plant extracts (Rob 1 and Rob2) and two potential biocontrol agents (Rob3 and Rob4) from Dr Mensah, Biopesticides NSW DPI were also included in the first field screening (**Table 3**).

Table 3: A summary of novel chemistries and biocontrol agents used, application rates and number of field trials screened for seed treatment efficacy in season 2017-2018

Products	Formulation No.	Primary Rate Recommended		Field trials
		(Product) mL/kg	(Active) gai/kg	
Syn1	EXF10670-V	9 mL	0.4 gai	2 at ACRI, 3 off sites
Rob1	PlantW	20 mL	0.5 gai	2 at ACRI, 3 off sites
Rob2	PlantY	20 mL	0.9 gai	2 at ACRI, 3 off sites
Rob3	DAT511	20 mL	$2x10^{10}$ spores	2 at ACRI, 3 off sites
Rob4	DAR78162	20 mL	$2x10^{10}$ spores	2 at ACRI, 3 off sites

Seedling stands

Early planting at ACRI (September 2017) had the lowest number of seedling stands, where the percentage of seedling survival was less than 42% on average (**Figure 14**). The trial at the Merah North site, planted in the second week of October 2017, had the highest percentage of seedling stands, at over 80% on average (**Figure 14**). It has been established that low soil temperatures in September are a key factor affecting seedling stands. Average seedling stands at ACRI late planting (October 2018), and Warren and Griffith trial sites were approximately 64%, 60% and 54%, respectively (**Figure 14**). Although the percentage of seedling stands in some treatments were recorded at higher levels than those from the control treatments, statistical analyses did not identify treatment effects in any of the trial sites.

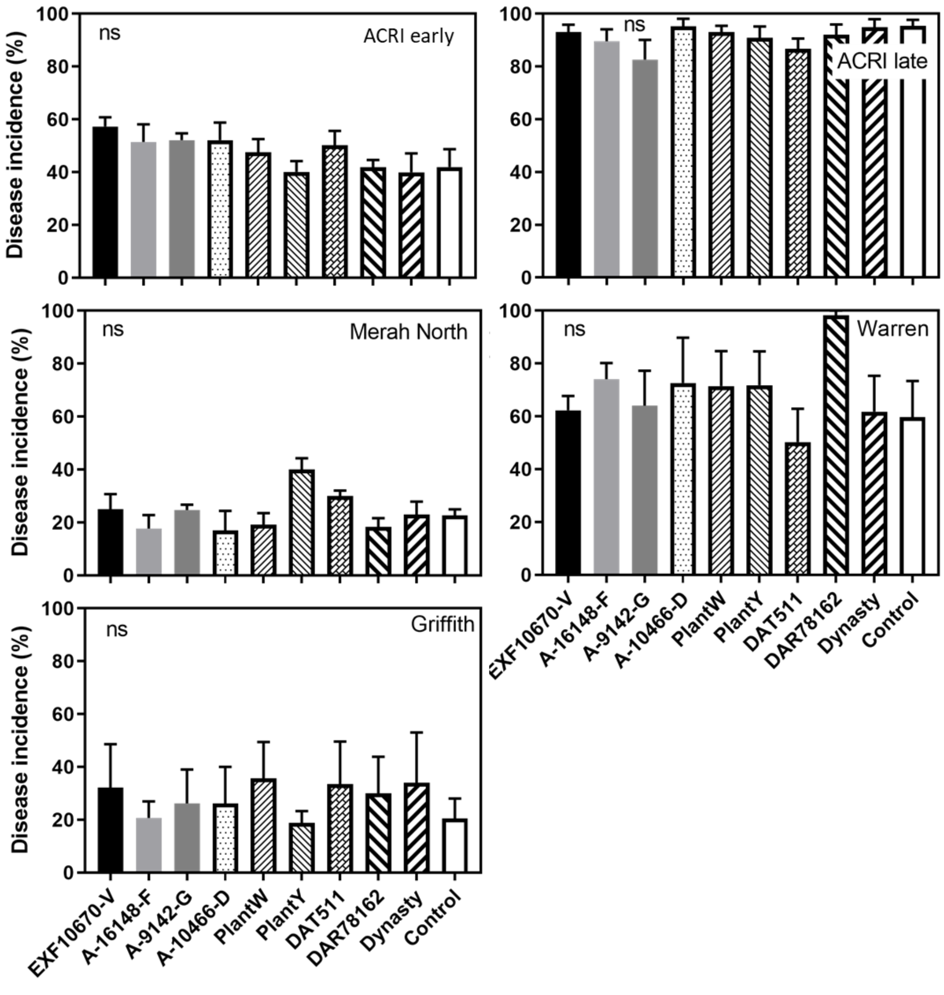


Figure 14: Percentage of seedling stands at five trial sites in Namoi, Warren and Griffith in 2017/18 season. Bars represent SEM (n = 4 - 5).

Black root rot incidence and severity

Low levels of BRR were observed at sites in the ACRI early planting, Merah North and Warren trial sites. Thus, no statistical analyses were done for these sites. Conversely, BRR incidences were as high as 33% and 39% on average at the ACRI late planting and Griffith trial sites, respectively. Where observed, BRR incidences recorded on A-16148-F treated seedlings were lower than those from controls (**Figure 15**). The plant extract PlantY performed well at the Griffith site, where the BRR incidence was comparable to the A-16148-F treatment. However, the performance of PlantY was not consistent at the ACRI site (**Figure 15**). The BRR incidence recorded in other seed treatments were not significantly different from Dynasty (standard control) and the non-treated control.

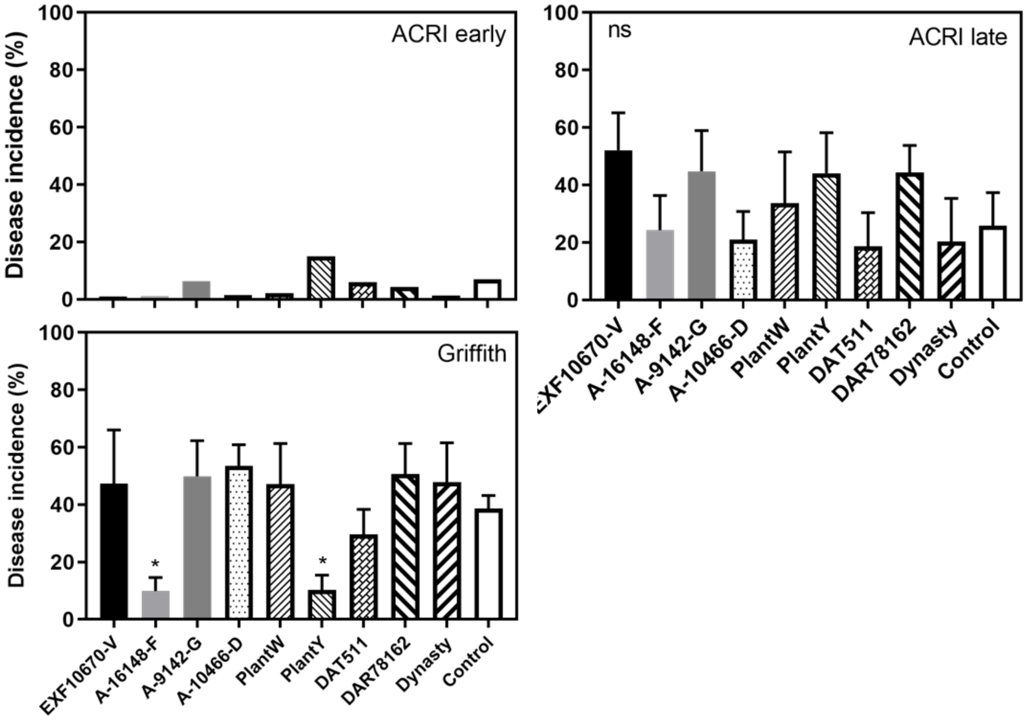


Figure 15: BRR incidence (percentage of seedlings with symptoms of BRR) at ACRI and Griffith sites in 2017/18 season. Bars represent SEM (n = 4).

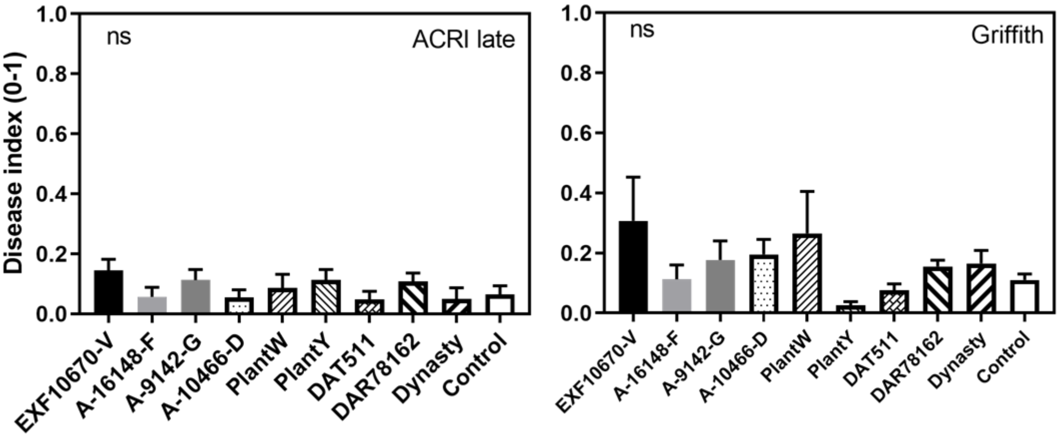


Figure 16: BRR severity index (0 = healthy seedlings; 1 = entire blacken tap root) at ACRI early planting and Griffith sites in 2017/18 season. Bars represent SEM (n = 4).

Due to low levels of BRR disease expression at the ACRI early planting trial site, only BRR severity indices were formally analysed at the ACRI late planting and Griffith trial sites. Disease indices (0-1), indicating the severity of the diseased seedlings recorded in the two trial sites were as low as 0.08 and 0.16 at ACRI late planting and Griffith, respectively (**Figure 16**). Again, PlantY appeared to perform well at the Griffith site, where the lowest BRR index was recorded. However, due to high variation among treatments, there was no statistical difference among treatments.

Rhizoctonia-like rot incidence and severity

Rhizoctonia-like rot was prevalent across all trial sites. At most sites, typical symptoms including reddish lesions and girdles around collar regions were observed on young seedlings (3-4 weeks after planting), and little damping off was observed. The disease incidence was recorded as high as 90% on average (**Figure 17**) at the ACRI late planting trial site, while Rhizoctonia-like rot incidence was lowest at the Merah North trial site. The PlantY extract appeared to perform well at the Griffith trial site, however disease incidence was not significantly different to those recorded elsewhere. Overall, seed treatments with several novel chemistries and biocontrol agents did not result in any treatment responses to Rhizoctonia-like rot across the five trial sites in the 2017/18 season.

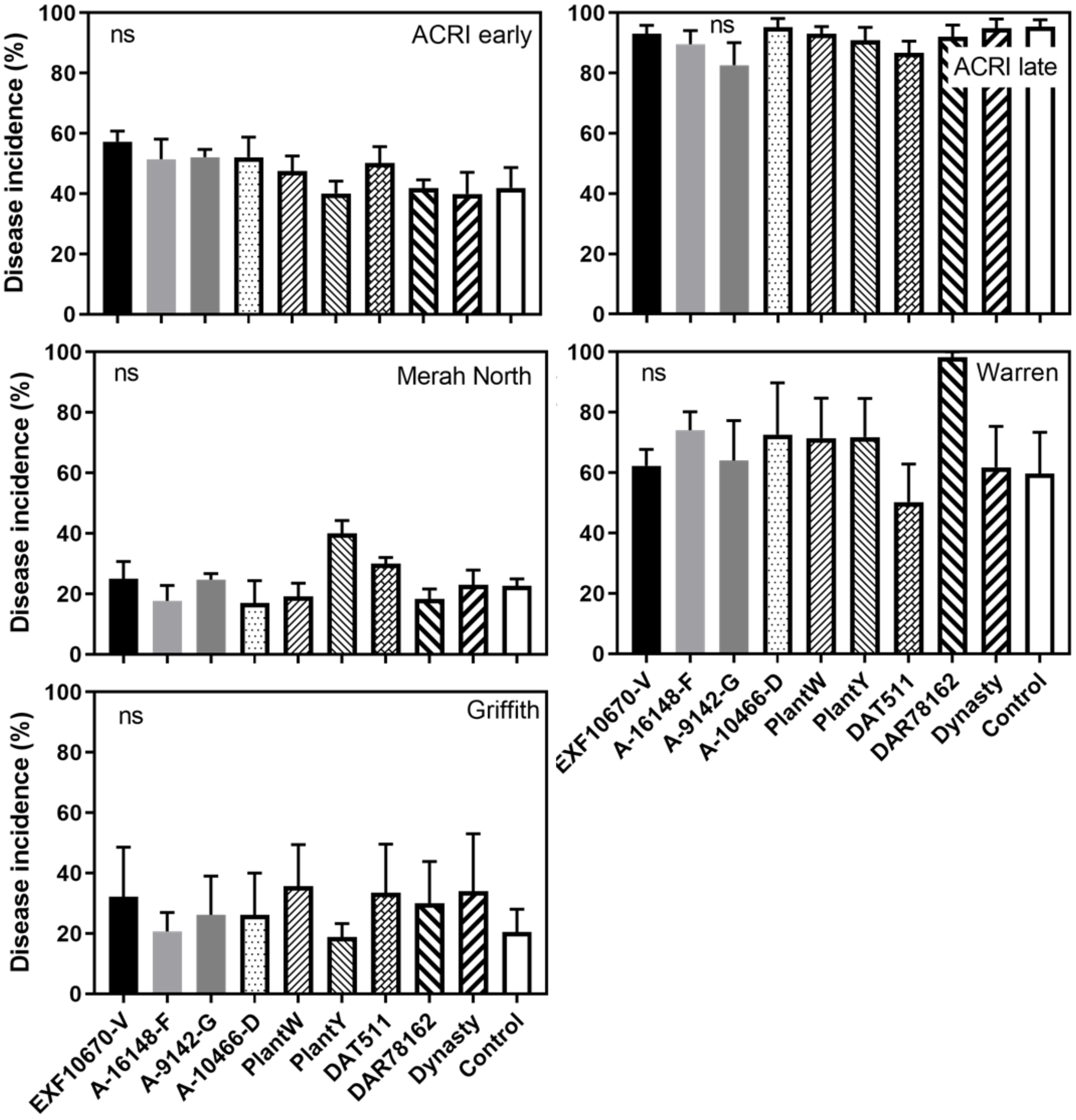


Figure 17: Rhizoctonia-like rot incidence (percentage of seedlings with symptoms of reddish lesions and girdles) at five trial sites in Namoi, Warren and Griffith in 2017/18 season. Bars represent SEM (n = 4 - 5).

Rhizoctonia-like rot symptoms were mostly superficial around the collar regions. In cases of severe infection, girdles around the collars were also observed. Disease severity indices (0 – 1) were recorded below 0.1 on average at the Merah North and Griffith sites (**Figure 18**). Disease indices were higher at the ACRI early planting and Warren trial site but remained below 0.2 on average. The Rhizoctonia-like rot index was recorded as high as 0.4 at the ACRI late planting trial site. These results indicate that disease symptoms were likely induced by a relatively mild pathogen. Irrespective of the different degrees of disease expression across five trial sites, there was no treatment effect found among treatments (**Figure 18**).

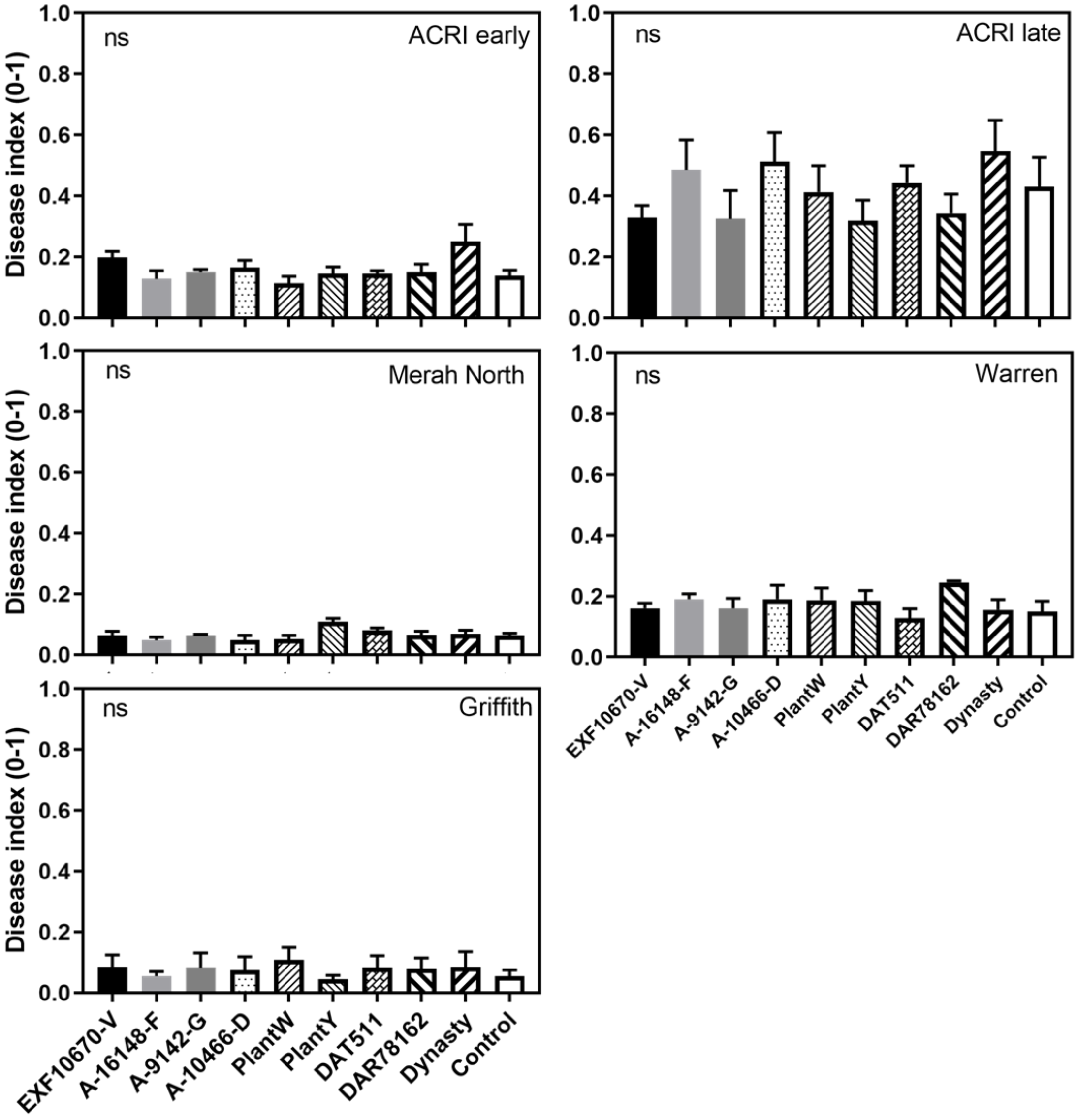


Figure 18: Rhizoctonia-like rot severity index (0 = healthy seedlings; 1 = damping off seedlings) at five trial sites in Namoi, Warren and Griffith in 2017/18 season. Bars represent SEM (n = 4 - 5).

In summary, seedling stands were more likely affected by planting dates rather than treatment effects. Despite BRR incidences and disease indices being lowest in the A-16148-F and Plant Y treatments; statistical significance was not consistent between sites. Black root rot and Rhizoctonia-like rot disease expression on un-treated control cotton seedlings were comparable to other treatments including standard treated control with Dynasty. Selected candidates, including A-16148-F and Plant Y were included in subsequent field trials in the 2018/19 season. However, BRR and Rhizoctonia-like rot disease expression was minimal at CSIRO-coordinated trial sites. The efficacy of treatments A-16148-F and Plant Y remain inconclusive.

In vitro assays

Control efficacy of a biofertiliser product (Biocontrol 1) supplied by Thinkbio Pty. Ltd. was assessed in vitro against the BRR pathogen. Isolates of *Bacillus* species were successfully reisolated and purified from the Biocontrol 1. Additionally, bacterial isolates Bac555 and Bac1568 recovered from grapevine by Dr Melanie Weckert (retired NSW DPI researcher) were included in the assessment. The two isolates provided by Dr Weckert had demonstrated a certain antagonistic effect against grey mould, *Botrytis cinerea* of grape bunch. An isolate, Bac35, recovered from cotton seedlings from our collection was also assessed for comparison. In a dual culture assay (**Figure 19**), Biocontrol 1 was able to suppress growth of the BRR pathogen by around 30% (**Figure 20**). Compared to Bac35 and Bac1568, Biocontrol 1 was a moderate antagonist. Since Biocontrol 1 is registered as a biofertilizer, it is worth assessing cotton growth promotion of Biocontrol 1 in pot trials.

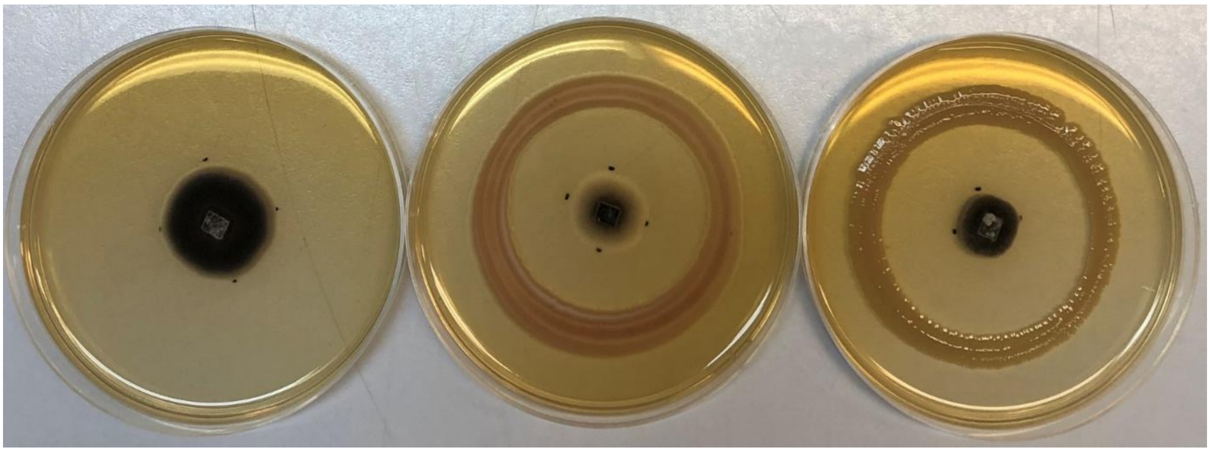


Figure 19: In vitro dual interaction assay assessing growth suppression of beneficial bacteria against black root rot pathogen. Control plate (left); Biocontrol 1 plate (middle); Bac35 (right).

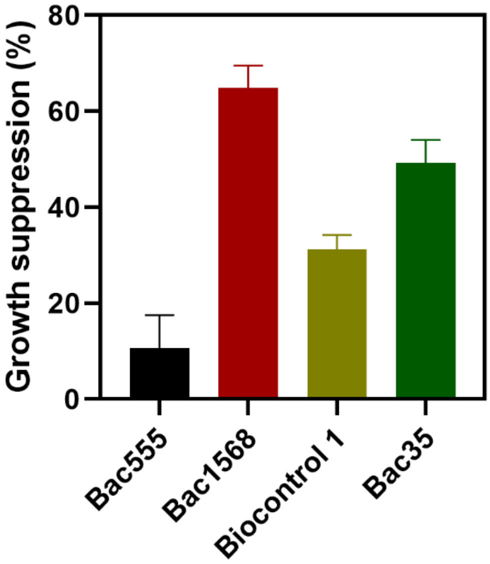


Figure 20: Growth suppression (%) of four tested bacterial strains against black root rot pathogen. Bars represent SEM (n = 8).

Glasshouse trial

A glasshouse trial was undertaken to screen for control efficacy of novel biocontrol agents against BRR. Application via soil drench to simulate the in-furrow injection of fungicides was evaluated for the biocontrol agents' efficacy against the BRR pathogen. The tested biocontrol agents included: endemic *Trichoderma gamsii* and *T. harzianum*, which were recovered from cotton soil; endemic bacterial strain Bac35 recovered from cotton seedlings; Biocontrol 1; and Biocontrol 2 from Sumitomo Pty. Ltd. containing *Streptomyces* species. Soil drenches with these agents were performed at planting and BRR disease expression was assessed at four weeks after planting.

There were no obvious necrotic symptoms on seedlings treated with biocontrol agents via soil drench, indicating that the agents did not negatively impact cotton seedlings to some extent. However, these biocontrol agents did not protect seedlings grown in artificially BRR-inoculated potting mix. The disease severity recorded on seedling co-inoculated with BRR-biocontrol agent was as high as those of the BRR-inoculated control seedlings (**Figure 21**). For example, disease severity of Biocontrol 1 BRR (co-inoculation of Biocontrol 1 and BRR) was recorded at 99% in comparison to 100% on the BRR control (**Figure 21**).

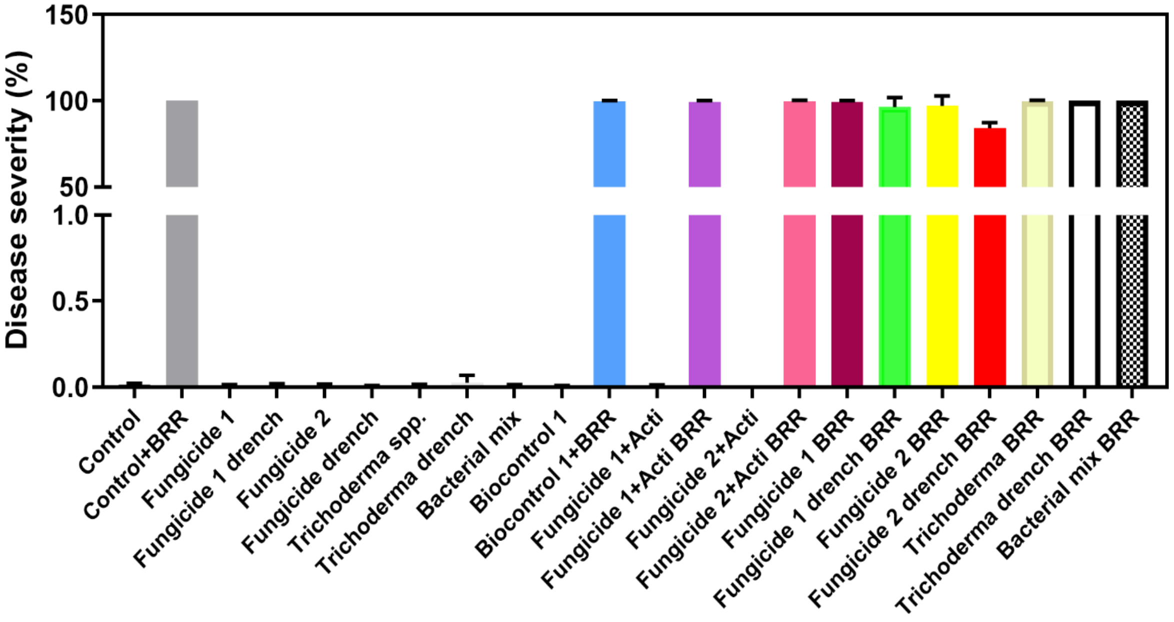


Figure 21: Variation in disease severity (%) indicated by the percentage of typical BRR necrosis recorded on seedlings treated with different biocontrol agents and fungicides. Bars represent SD.

Biocontrol agents can protect host plants from pathogens via different modes of action. These can directly antagonise pathogens or promote host plants' growth; providing indirect protection for the host. In our assay, dry biomass (g) of seedlings inoculated with the tested biocontrol agents was not significantly different from that of the non-treated control seedlings. For example, the dry biomass of Bac-inoculated seedlings was 2.5 g, comparable to 2.3 g in the non-treated control (**Figure 22**). The control efficacy of biocontrol agents is highly dependent on inoculation methods that allow for successful colonisation in soil habitats. Therefore, additional assessment toward optimisation of the inoculation method is warranted.

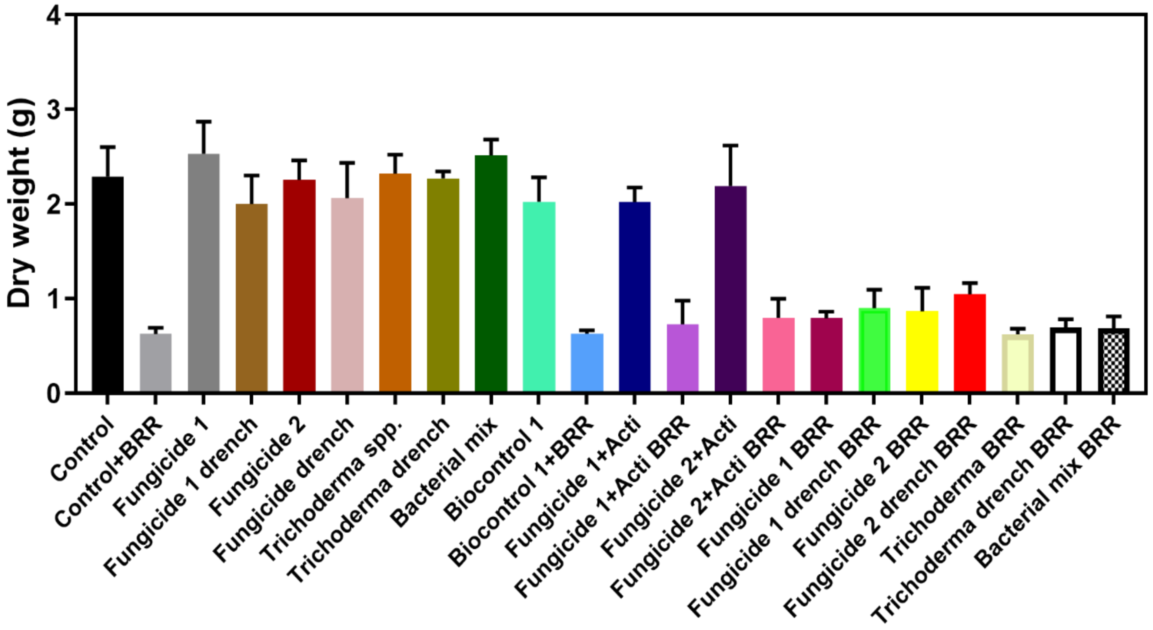


Figure 22: Recorded dry weight (g) of 10 seedlings at the end of the trial. Bars represent SD.

Milestone 3.2 Commercialisation and exploitation plan developed for any new products screened in this project

Milestone 3.3 Technology business strategy options developed

DAN1703 has not successfully identified an effective candidate for controlling either BRR or ALS pathogens. Therefore, commercialisation has not been pursued at this point in time.

Objective 4. Research question – How should innovative solutions be integrated into an integrated disease management strategy?

Milestone 4.1. Identify research gaps in cotton pathology

ALS of cotton, especially on cotyledons is generally considered a minor disease in Australia. Due to the emergence of Alternaria disease in the 2017/18 season on cotton crops in southern NSW, efficacy screening of commercially available fungicides currently registered on crops other than cotton became an immediate research focus for this project. Additionally, knowledge about the pathogen and its associated disease in Australia is limited; therefore, in addition to the undertaking of fungicidal screening work in DAN1703, further research is required to characterise the population biology of the Alternaria pathogen. The information generated by this research provides crucial insights into our understanding of the pathogen and assists with the identification of potential disease management strategies. Additionally, once fungicides are applied it is necessary to monitor fungicide resistance within pathogen populations. To manage resistance and to reduce the number of sprays, research targeted at monitoring (spore trap studies) the airborne inoculum (inoculum thresholds) of *Alternaria* should also be conducted.

Alternaria leaf spot remained problematic during the 2018/19 season and emerged again early in the 2019/20 season. Significant knowledge gaps remain regarding epidemiology and population diversity of this pathogen. Additional research into ALS and related pathogens will be crucial for the development of effective control strategies.

Preliminary data collected from this project as well as from the 'National Disease Survey' has confirmed that *Fusarium* spp. and the black root rot pathogen, *T. basicola* are major pathogens on cotton seedlings. In addition, the relatively high Fusarium wilt incidence expressed on mature cotton in 2017/18 would suggest that the occurrence of Fusarium in the industry will persist. Therefore, future research should focus on characterisation of these *Fusarium* populations to understand the evolutionary movement and host adaptation (breaking of resistance) of the pathogens. Fusarium wilt pathogens on Australian cotton are well known for their ability to evolve and adapt to cotton crops from endemic strains. Hence, it is theorised that the industry wide adoption and the continuing use of the high F-rank cultivars (not immune cultivars) could have altered the population biology of *Fusarium* spp. In conclusion more research investment in this area should be considered as the potential benefits to industry would be considerable.

Updated Fusarium research findings from Prof Liz Aitken and Dr Linda Smith have indicated that the Fusarium collection may be more diverse than first thought. A considerable number of Fusarium isolates collected from the late season survey in 2017/18 were not identified to VCG level, though most of them were compatible with Fov VCG01111. None of the isolates recovered from the early season survey in 2017/18 were positive for Fov-SIX gene profiles, which may suggest that early season collection may include either non-Fov or new Fov strains. Extra support for pathogenicity assays would help confirm or disprove this theory.

The Fusarium wilt pathogen of cotton remains a major biosecurity concern. Methodologies for monitoring both the disease and the pathogen are currently based on field symptoms and a specific molecular marker. However, there is a lack of monitoring of the pathogen population. Currently, the Fusarium wilt pathogen is collected every year during the survey seasons in both QLD and NSW.

Additionally, both states' herbaria have archived a considerable number of cotton *Fusarium* isolates since 1993 when the disease was first detected in Australia. Therefore, it is important to assess the evolution of the pathogen over time by comparing the archived collections with the prevailing collection. This research will provide insights into pathogen populations for improved disease management.

Recent research conducted by Dr Karen Kirkby indicated the potential for a new strain of *V. dahliae* in Australian cotton. The research was carried out on the historical culture collection. Therefore, it is important to continue to collect a *V. dahliae* collection representative of the prevailing populations currently in cotton systems and monitor for population diversity. This will allow the industry to keep up with the pathogen evolution of *V. dahliae* as well as the occurrence and distribution of potential emergent strains.

Data from the disease survey (RRDP1724) revealed that boll rot may contribute significantly to yield reduction. Due to high rainfall late in the 2019/20 season, boll rot was prevalent in northern NSW cotton crops. Incidence was as high as 48% in a field in the Macquarie. The mean incidence in the Macquarie, Namoi and Gwydir valleys was around 15%. Due to the sporadic occurrence of boll rot, this disease has been overlooked. There has not been comprehensive research on the etiology and epidemiology of the disease. Therefore, the actual causal agent(s) of cotton boll rot in Australia have not been characterised. Such research warrants further investigation and will offer insight into the development of management strategies and preparedness.

Come Clean Go Clean is a cornerstone farm practice to minimise soilborne pathogen movements through human activity. However, the main soil disinfectant (FarmCleanse) has been phased out. There are many other alternative products available on market, but their efficacy against major soilborne pathogens on cotton are unknown. Therefore, this is an important focus for future research.

Objective 5. Research question – What percentage of growers adopted practices that reduce the incidence of diseases on their farms at project completion?

Milestone 5.1 Adoption pathways and measurement of adoption.

NSW DPI discussed with Warwick Waters, Manager CottonInfo in regard to assisting the development of a monitoring, evaluation, reporting and improvement (MERI) plan. A draft MERI plan for all NSW DPI Ag cotton pathology research projects was completed. Key elements of the plan were to capture baseline data to document current industry practice, and both qualitative and quantitative methods will be used to assess industry adoption and project impact.

The NSW DPI pathology team played an important role in Alternaria leaf spot research and responding to the disease outbreak on seedlings in the 2017/18 season in southern NSW. Our research findings were updated and distributed by Spotlight and The Australian Cotton Grower magazines, and in the Cotton Pest Management Guide. The findings were also presented and reported to a wider science community at the AACS 2019 Australian Cotton Research Conference in Armidale, the 22nd APPS conference in Melbourne, and in a peer-reviewed journal, Australasian Plant Pathology. Consequently, the wider community is now aware of ALS on cotton. Our follow up emails after each growing season indicated that a number of growers/consultants in southern NSW harboured strong concerns about ALS infection in their cotton fields; and some also applied preventative Tebuconazole ahead of a forecasted rain event. Though this was not encouraging, ALS could be the cause of crop loss and or yield reduction paranoia for some. Several consultants in WA and the NT believed that ALS had potential to cause detrimental effects on their cotton crops, also sending their cotton leaf samples for diagnostic confirmation. A recent survey of cotton consultants conducted by Australian Crop Consultants revealed that some were less confident in consulting capacity due to the lack of knowledge and control options against ALS. Our ongoing ALS research will aim to address ALS knowledge gaps and enhance confidence and preparedness for the industry.

Our free disease diagnostic service was also widely accessed by growers and consultants across NSW as well as from WA and NT. Our fast turn-around service allowed sample senders to stay informed. The diagnostic service currently continues with support from both CRDC and NSW DPI.

New Objective 6. What are the key pathology issues that will impact productivity in cool, high yielding regions such as the southern regions of NSW?

Milestone 6.1 ACRI Pathology Unit to liaise with Cotton Crop Protection Specialist (Yanco) to oversee field evaluations of novel products to manage cotton diseases in southern region

Tim Green accepted the role of Pathologist on the project *CRDC DAN1903 Southern Crop Protection* in late 2018. He is an enthusiastic young scientist who has recently completed his honours degree whilst also working in the cereal pathology team at Wagga Wagga with Dr Andrew Milgate. Tim Green began his new fulltime role on the 14th of January 2019. *CRDC DAN1903 Southern Crop Protection* has both a research and development/extension focus and will cover both pathology and invertebrate pest management. Tim Green appointed Alison Young as a Technical Officer assisting in the disease survey, implementation and management of future CRDC DAN1703 Novel Product experiments in southern NSW and in case study development with local growers & agronomists.

Tim Green and the Yanco team visited ACRI, Narrabri and were introduced to several research teams, including both Cotton Pathology teams on the 10th Dec 2018. Discussion with the pathology teams aimed: 1) to keep Tim updated with diseases of concern in NSW and those important to southern NSW; and 2) to assist Tim in preparations for conducting a late season disease survey. Tim joined our team for the late season disease survey in the southern cotton growing valleys during the week of 18th Mar 2019. On the 28th Mar 2019, Tim returned to ACRI for laboratory training in fungal isolation and identification with our team. He has agreed to liaise with southern growers and consultants for field trials in the 2019/20 season to evaluate control efficacy of some selected candidates against BRR and Alternaria leaf spot.

Tim Green and his team joined us for the early disease survey during the first week of November 2019, as well as the late season disease survey in March 2020. His team was also undertaking three different field trials to evaluate control efficacy of selected candidate against BRR and Alternaria leaf spot. Tim also agreed to help assess the pathogenicity of selected *Fusarium* isolates on cotton seedlings and mature plants. This work will allow us to gain insights into the current status of our prevailing Fusarium wilt pathogen.

Unfortunately, Tim Green resigned in June 2020. NSW DPI successfully undertook a recruitment action in July – August and Ms Beth Shakeshaft will commence in the role at the end of October 2020. Internal NSW DPI meetings are planned during November to integrate Ms Shakeshaft into the NSW DPI pathology and plan research activities for the 2020-21 season.

Milestone 6.2 Pathology research for Southern cotton

Alternaria leaf spot and BBR are major constraints for cotton production in the southern growing regions. Anecdotally yield losses caused by BBR have been have typically been in the order of 15-20%, but in a small number of fields up to 100% yield loss has been recorded. Though yield loss has not been quantified for cotton fields which severely infected with Alternaria leaf spot, most diseased fields were sprayed resulting in a higher production input. Therefore, southern pathology research will continue to focus on management strategies for these two diseases as per the above. Additionally, Verticillium wilt has been detected in 6 southern fields (5 fields in 2017/18, 1 field in 2018/19, and 4 fields in 2019/20) over the last three seasons indicating that the disease had been introduced and is establishing in the southern region. The southern pathology team will continue to play a crucial role in monitoring, sampling and mapping the Verticillium wilt distribution, which will facilitate proper disease management options toward minimising any further spread.

Alternaria leaf spot

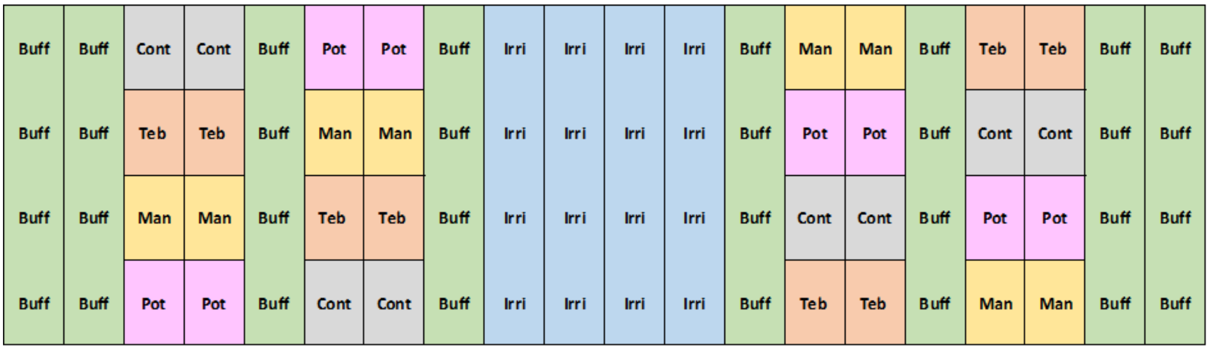
In coordination with Tim Green, a 2019-20 field trial was conducted to evaluate control efficacy of selected candidates from pot trials against Alternaria leaf spot.

Location: Leeton Farm Station C Block Bay 1

Treatments:

- Tebuconazole- 2 sprays at 440ml/ha (430g/L AI), 14 days apart, first spray when symptoms first appear (likely Jan-Feb)
- Mancozeb- 4 sprays at 2.5kg/ha (750g/kg AI), 7 days apart, first spray after flowering before irrigation (Jan-Feb)
- Potassium nitrate- 4 sprays at 10kg/ha, 14 days apart, first spray at flowering (early Jan)
- Control- No sprays

Tim Green also monitored closely the field for the disease pressure and occurrence in relation to weather changes. This will provide better understanding of the disease epidemiology. See trial design in **Figure 23** below.



Buff	Buff	Cont	Cont	Buff	Pot	Pot	Buff	Irri	Irri	Irri	Irri	Buff	Man	Man	Buff	Teb	Teb	Buff	Buff
Buff	Buff	Teb	Teb	Buff	Man	Man	Buff	Irri	Irri	Irri	Irri	Buff	Pot	Pot	Buff	Cont	Cont	Buff	Buff
Buff	Buff	Man	Man	Buff	Teb	Teb	Buff	Irri	Irri	Irri	Irri	Buff	Cont	Cont	Buff	Pot	Pot	Buff	Buff
Buff	Buff	Pot	Pot	Buff	Cont	Cont	Buff	Irri	Irri	Irri	Irri	Buff	Teb	Teb	Buff	Man	Man	Buff	Buff

Figure 23: Field trial layout for assessing the control efficacy of some tested candidates against Alternaria leaf spot.

Updated results for these trials can be found in the DAN1903 November progress report to CRDC.

DISCUSSION

DAN1703 represents an ongoing effort to identify management strategies for effective control of the major diseases of cotton. The scoping study allowed DAN1703 to focus on black root rot, a major seedling disease across NSW, and a re-emerging Alternaria leaf spot disease on seedlings. A substantial screening regime for control efficacy against the two diseases was conducted in vitro, in glasshouse and in field conditions over the time course of the project. Potential candidates, including commercially available products not yet registered on cotton, and novel chemistries/biocontrol agents were assessed. In the 2017/18 season, seed treatments with a non-cotton fungicide A-16148-F and a novel plant extract Plant Y provided a certain degree of seedling protection from BRR. Unfortunately, we failed to reassess their control efficacy in a subsequent trial conducted in the 2018/19 season due to the low level of BRR expression encountered at our trial sites. Despite the challenges encountered the two candidates retain their control potential and are worthy of further assessment. Additionally, application of a soil drench with Fungicide 2 resulted in a 16% reduction in BRR severity. Fungicide 2 drench was the only treatment that could sustain seedling health in our pot trial. Therefore, the efficacy of Fungicide 2 should be assessed further.

In response to ALS outbreaks on seedlings in southern NSW, DAN1703 screened several non-cotton fungicides for their control efficacy against the ALS pathogen. Selected candidates, including Mancozeb and Tebuconazole were nominated for an emergency permit application. The two candidates are now granted an application permit on seedlings in the event of a future outbreak. Other potential candidates are also undergoing further evaluation for their control efficacy and crop safety. CRDC and NSW DPI have supported the next stage of this research through project DAN2101 entitled “Evaluate efficacy of novel chemistries, biocontrol agents and management practices to control Alternaria and Black Root Rot disease in cotton”.

Verticillium wilt is a major disease of concern in Australia. Management of the disease is a major focus for projects RRDP1724 entitled “Improving the management of cotton diseases in Australian Cotton farming” and RRDP1723 entitled “Management Verticillium risk for cotton”. DAN1703 pursued investigations into a long term biofumigation trial by incorporation of brassica cv. Caliente biomass. Biofumigation efficacy of brassica against soilborne pathogens, including *V. dahliae* has been widely evaluated. The efficacy of this strategy is heavily dependent on the application method, particularly in minimising the loss of biofumigating chemistries such as glucosinolate and isothiocyanate. Our trial aimed to assess inoculum disinfestation efficacy of brassica incorporation under anaerobic conditions against *V. dahliae*. Though there was no significant difference in Verticillium wilt incidence recorded in brassica-amended and non-brassica blocks, the *V. dahliae* inoculum in the brassica amended block was however significantly lower than that of the non-brassica blocks. This preliminary result indicated that incorporation of a brassica crop could represent an alternative practice to suppress *V. dahliae* soil populations. Therefore, this warrants further investigation.

Project DAN1703 aimed to build and enhance NSW cotton pathology research capacity by appointment of Dr Duy Le and Aphrika Gregson. Additionally, DAN1703 set out to collaborate and support a newly appointed southern NSW cotton pathology unit in Yanco, which catered for the investigation of regionally important diseases. Personnel possessing skill sets in plant pathology such as molecular biology (which previously relied on external resources) were appointed under project DAN1703. Furthermore, DAN1703 was able to promptly respond to the ALS outbreak on seedlings in the 2017/18 season, which has been long considered minor and a late occurrence on mature crops. Accurate identification of the causal agent plays a vital role in disease management. *Alternaria alternata* was identified as a predominant pathogen responsible for the ALS outbreaks (appendix 5). By deploying molecular techniques, DAN1703 provided insights into the pathogen diversity of *Fusarium* species recovered from Rhizoctonia-like rot on seedlings (appendix 4). Additionally, our team published for the first time molecular-based support for the co-occurrence of both defoliating and non-defoliating pathotypes of *V. dahliae* in a single cotton plants under field conditions (appendix 4). DAN1703 also links with other projects; and the appointed pathology team based at Narrabri has been successfully co-ordinating a free cotton disease diagnostic service locally at NSW DPI Narrabri. Our team received and processed over 70 cotton samples, including seedlings, leaves, bolls and stem cuts over the last two years.

CONCLUSIONS

It is hoped the findings of DAN1703 will result in a greater awareness within the cotton industry that searching for an effective control approach against cotton diseases is an ongoing effort. Additionally, effective and sustainable disease management requires the incorporation of multiple approaches.

- No single chemistry nor biocontrol agent is registered against BRR disease. DAN1703 identified several potential candidates, however a lack of control consistency indicates further assessments are required;
- *Alternaria alternata* was a predominant pathogen responsible for the ALS outbreak in southern NSW in the 2017/18 season;
- Mancozeb and Tebuconazole are being granted an emergency application permit on both seedling and mature cotton in the event of a future outbreak;
- Incorporation of a brassica crop could provide an alternative practice to suppress soil *V. dahliae* population, but this requires a long-term field assessment;
- Several pathological research gaps could be further investigated for insights into pathogen biology of BRR, ALS and Verticillium wilt. Studies for etiology and epidemiology of sporadic but important boll rot disease should also be a priority.
- Assessing for disinfestation efficacy against BRR and Verticillium wilt pathogens of Farmcleanse-alternative products will be vital for cornerstone farm hygiene practices;

Key word index: Seedling diseases, black root rot, ALS, Verticillium wilt, biocontrol, fungicides

A full list of industry and scientific publications, presentations, extension activities and other outputs.

Peer reviewed publications

Le DP, Gregson A, Tran TT, Jackson R, 2020. Co-occurrence of defoliating and non-defoliating pathotypes of *Verticillium dahliae* in field-grown cotton plants in New South Wales, Australia. Plants (Basil, Switzerland) 9 (6), 750 doi:10.3390/plants9060750 (Invited article)

Le DP, Tran TT, Gregson A, Jackson R, 2020. TEF1-based sequence diversity of *Fusarium* species recovered from collar rot diseased cotton seedlings in New South Wales, Australia. Australasian Plant Pathology 49, 277-284 https://doi.org/10.1007/s13313-020-00706-8

Le D, 2020. Has Alternaria leaf spot become a major concern in your cotton fields. The Australian Cotton Grower 41 (2), 30-34 (Invited article).

Le DP and Gregson A, 2019. Alternaria leaf spot of cotton seedlings grown in New South Wales, Australia is predominantly associated with *Alternaria alternata*. Australasian Plant Pathology 48, 209-216 doi.org/10.1007/s13313-019-0617-9

Le DP, Kirkby K, Trapero C, Tran TT, Smith L, Verticillium wilt of cotton: detection and identification of the causal pathogens and their control. Frontiers in Agronomy (under revision).

Conferences and workshops

Le DP, Gregson A, 2019. The AACS 2019 Australian Cotton Research Conference, Armidale Australia October 2019 (keynote presenter)

Le DP, Gregson A, Jackson R, Smith L, 2019. Cotton diseases – the 'big four' in NSW. The 22nd APPS, Melbourne, Australia November 2019 (oral presenter)

Gregson A, **Le DP**, 2019. The AACS 2019 Australian Cotton Research Conference, Armidale Australia October 2019 (oral presentation)

Le DP, Gregson A, Ravichander P, Aitken E, Scheikowski L, Smith L, 2019. Fusarium wilt of cotton, Fusarium Workshop, the 22nd APPS, Melbourne, Australia November 2019 (invited presenter).

Le DP, 2019. Cotton diseases. Crop Consultant Australia Workshop, Griffith 22nd August 2019 (invited presenter)

Le DP, 2019. Cotton diseases. Crop Consultant Australia Workshop, Moree 29th August 2019 (invited presenter)

Le DP, 2019. Cotton diseases – updates. NSW Northwest Pathology Seminar, Tamworth 10th May 2019 (co-organiser)

Le DP, 2018. Black root rot research since 1995. FUSCOM, Griffith, Australia August 2018 (oral presenter)

Le DP, Gregson A, 2018. Innovative solutions to cotton diseases – an update. FUSCOM, Griffith, Australia August 2018 (oral presenter)

Le DP, Gregson A, 2018. Fusarium species collected from seedling and mature cotton in NSW. FUSCOM, Griffith, Australia August 2018 (oral presenter)

Le DP, Gregson A, 2018. Alternaria leaf spot, an emerging disease to Australian cotton industry. FUSCOM, Griffith, Australia August 2018 (oral presenter)

Gregson A, **Le DP**, 2018. Cotton disease diagnostics – season 2017/18 update. FUSCOM, Griffith, Australia August 2018 (oral presenter)

Le DP, Gregson A, 2018. Field screening of a novel product and biocontrol agents against seedling diseases of cotton, NSW Australia season 2017-2018. The 10th Australasian Soilborne Diseases Symposium, Adelaide, Australia September 2018 (poster presenter)

Le DP, Shafto C, Gregson A, Smith M, 2018. Genetic characterisation of black root rot pathogen of cotton in NSW Australia. The 10th Australasian Soilborne Diseases Symposium, Adelaide, Australia September 2018 (oral presenter)

Other publications

Le D, 2019. *Alternaria alternata* (Fr.) Keissl. (1912). Pathogen of the month – December 2019. APPS https://www.appsnet.org/Publications/potm/pdf/Dec19.pdf

Le D, Kirkby K, 2018. *Thielaviopsis basicola* (Berk. & Br.) Ferr. Pathogen of the month - June 2018. APPS https://www.appsnet.org/Publications/potm/pdf/Jun18.pdf

Part 4 – Summary for public release

This summary will be published on Inside Cotton, CRDC's digital repository, along with the full final report (if suitable for public release). It is designed to provide a short overview of the project for all interested parties. Please complete all fields, ensuring that this exceeds no more than two pages.

Project title:	Innovative solutions to cotton diseases	
Project details:	CRDC project ID:	*DAN1703*
	CRDC goal:	*1. Increase productivity and profitability on cotton farms*
	CRDC key focus area:	*1.3 Protection from biotic threats and environmental stresses*
	Principal researcher:	Dr Duy Le, Cotton Pathologist
	Organisation:	*NSW DPI*
	Start date:	01/07/2017
	End date:	30/06/2020
Objectives	• Scoping study for potential disease control approaches • Assessing control efficacy of non-cotton chemistries against BRR and ALS pathogens • Assessing control efficacy of novel chemistries and biocontrol agents against BRR and ALS pathogens • Identifying research gaps in cotton pathology for future investigation	
Background	The ongoing success of the Australian cotton industry is dependent on effective disease management tactics. With disease being a significant constraint on cotton production, this project will provide capacity building for both current and emerging cotton disease issues. Research efforts will focus specifically on conducting a review to quantify existing disease issues, then identifying suitable new/novel products for cotton disease management and developing strategies which incorporate all registered products. New product research will involve identification of potential solutions through efficacy screening, including assessment of technical feasibility. Products of interest would be either commercially available in other commodities but not currently registered on cotton, or experimental control agents. Where potential disease management solutions are identified steps will be taken to progress these products toward registration or commercialization for use in cotton. As part of this process, scientifically rigorous screening procedures will be developed with a view to these methodologies contributing to a registration data package and/or supporting future commercialization processes. New knowledge developed during the life of this project along with collaborative links to existing cotton pathology projects will be used to develop key components of decision matrices which will allow growers and managers to effectively assess disease risk in their area and implement regionally specific solutions. Development of communication strategies established in	

	agreement with industry partners will also facilitate rapid tactical responses to emerging disease issues. Where gaps in the knowledge are identified research would be undertaken to inform potential product use within an integrated disease management framework. Through these investigations of new products industry will have a greater capacity to respond to regional pathology issues (in both northern and southern NSW). This project will also integrally link to the industry disease survey project by responding to emerging issues identified through surveillance activities.
Research activities	• Scoping study was conducted by searching the database of 'inside cotton library', 'Google Scholar', and plant pathology journals. • Screening for the control efficacy of non-cotton fungicides, novel chemistries and biocontrol agents was conducted in vitro, in glasshouse and field conditions. • Food poisoned and dual culture assays were adopted for in vitro screening. • Seed treatments and soil drench were adopted for glasshouse and field screening. • Advanced molecular technique was deployed for identification of *Alternaria* responsible for ALS outbreak and detection of *V. dahliae*.
Outputs	• DAN1703 identified several potential candidates, but there was a lack of control consistency and required further assessments; • *Alternaria alternata* was a predominant pathogen responsible for the ALS outbreak in southern NSW in 2017/18 season; • Mancozeb and Tebuconazole are being granted an emergency application permit on both seedling and mature cotton if there is a future outbreak; • Incorporation of a brassica crop could provide an alternative practice to suppress the *V. dahliae* population, but this requires a long-term field assessment; • Several pathological research gaps could be further investigated for insights into pathogen biology of BRR, ALS and Verticillium wilt. Assessing for disinfestation efficacy against BRR and Verticillium wilt pathogens of Farmcleanse-alternative products will be vital for farm hygiene practices; • Capacity building was greatly achieved with a Narrabri based team; • A total of four peer-reviewed publications were published and one is under revision; 14 presentations at conferences and industry engagement workshops/field day; two pathogens of the month published by Australasian Plant Pathology Society. • Key communication outputs are as below.

Impacts	• DAN1703 has raised greater awareness within the cotton industry that searching for an effective control approach against cotton diseases is an ongoing effort. Additionally, effective and sustainable disease management requires incorporation of multiple approaches. DAN1703 built and enhanced cotton pathology research capacity locally by appointing a pathology team at Narrabri and supporting the appointment of the southern pathology team at Yanco. The two teams have played an important role in responding to regionally important pathological concerns.
Key publications	**Le DP**, Gregson A, Tran TT, Jackson R, 2020. Co-occurrence of defoliating and non-defoliating pathotypes of *Verticillium dahliae* in field-grown cotton plants in New South Wales, Australia. Plants (Basil, Switzerland) 9 (6), 750 doi:10.3390/plants9060750 (Invited article) **Le DP**, Tran TT, Gregson A, Jackson R, 2020. TEF1-based sequence diversity of *Fusarium* species recovered from collar rot diseased cotton seedlings in New South Wales, Australia. Australasian Plant Pathology 49, 277-284 https://doi.org/10.1007/s13313-020-00706-8 **Le D**, 2020. Has Alternaria leaf spot become a major concern in your cotton fields. The Australian Cotton Grower 41 (2), 30-34 (Invited article). **Le DP** and Gregson A, 2019. Alternaria leaf spot of cotton seedlings grown in New South Wales, Australia is predominantly associated with *Alternaria alternata*. Australasian Plant Pathology 48, 209-216 doi.org/10.1007/s13313-019-0617-9

Disease-related constraints to Australian cotton industry

Black root rot

Black root rot (BRR) is of major concern seedling disease of cotton in Australia (Pereg, 2013, Nehl et al., 2004b). The disease is caused by a wide host range hemibiotrophic *Thielaviopsis basicola* (syn. *Chalara elegans*) (Nehl et al., 2004b, Allen, 1990, HOLTZ and WEINHOLD, 1994). *T. basicola* is soilborne and characterised by production of two spore types, including thin wall endoconidia and dark thick wall chlamydospores (Paulin-Mahady et al., 2002). Though *T. basicola* isolates originated from different hosts may show a certain level of host specificity or preference, pathogenicity of a number of isolates is not limited to its original host crops (Pereg, 2013, Mondal et al., 2004, Allen, 1990). *T. basicola* causes characteristic dark brown to black necrotic lesions on the main and lateral roots of more than 230 host species (Rothrock, 1999, Gayed, 1972), which result in stunted and less vigorous seedlings, and delayed flowering and maturity (Allen, 2001). BBR is generally assumed not causing seedlings mortality directly; however, Mauk and Hine (1988) found that death of cotton seedlings were attributed solely to the severe infection of *T. basicola*. In Australian cotton, Nehl et al. (2004b) found no general relationship between BRR and seedling death though yield reduction was negatively correlated.

On cotton, *T. basicola* infection occurs as early as 12 h and 36 h after endoconida and chlamydospores, respectively come to contact the roots, and at 10 - 14 days after planting BRR lesions may be visualized (Mauk and Hine, 1988). Infection of *T. basicola* and expression of BBR on cotton is most severe when soil temperature is below 24 °C regardless of *T. basicola* optimal growth at 25 - 28 °C and water potential of soil is high (Rothrock, 1992). Several studies found that soils are also more conducive when pH is above 5.6 and clay content is high (Harrison and Shew, 2001, Meyer and Shew, 1991, Meyer et al., 1994). Additionally, the severity of BRR is also positively associated with *T. basicola* population. *T. basicola* at less than 25 cfu/g soil caused trace BRR symptoms on cotton, but at density >100 cfu/g soil typical black lesions covered almost hte entire root of cotton seedlings (HOLTZ and WEINHOLD, 1994). BRR of cotton was also observed more intense in the association with root knot nematode (*Meloidogyne incognita*) infestation, (Walker et al., 2000, Walker et al., 1998); however, no evidence has been indicated a relationship between severity of BRR and nematode damage in Australian cotton (Pereg, 2013). Collective evidence from histological studies indicated that *M. incognita* parasitism allowed *T. basicola* invasion of vascular tissues, thus probably resulting in higher root necrosis (Walker et al., 1999). On the other hand, infection of *T. basicola* is believed to open entry for infections of *Pythium* spp., *Rhizoctonia* sp. and *Fusurium* spp., which collectively form a seedling disease complex in cotton (Pereg, 2013, Roy and Bourland, 1982). Most cotton seedling diseases can be managed through seed treatment, planting time or through suppressing soil inoculum (Nehl et al., 2004b, Toksoz et al., 2009, Rothrock et al., 2012, Candole and Rothrock, 1997, Candole and Rothrock, 1998). However, the complexity of seedling diseases makes it difficult for a single approach to be successful.

Fusarium wilt

Fusarium wilt of cotton caused by *Fusarium oxysporum* f. sp. *vasinfestum* (Fov) was first reported in Australia in 1994 by (Kochman, 1995). Since then the disease was detected in many other growing regions in New South Wales and Queensland (Kirkby et al., 2013, Smith et al., 2006). Adoptions of better Fusarium resistant cultivars since 2002/2003 season onward have led to a significant reduction in disease occurrence on farms (Kirkby et al., 2013). However, Fusarium wilt still remains an economic constraint to the Australian cotton industry. Fov is both soilborne and seedborne and distributed worldwide (Davis et al., 2006, Cianchetta and Davis, 2015, Bennett et al., 2008). Losses in yield caused by Fov were estimated around 109,000 bales in the USA in 2004; complete crop losses were also noticed in single fields in California, USA (Davis et al., 2006). In Australia, Fusarium wilt was predicted to be infested on 90% of the farms in NSW by 2010 (Nehl et al., 2004a). However, with good cultivation practices, adoptions of high F-rank cultivars and Come Clean Go Clean practices, the average Fusarium wilt incidence was detected less than 10% on farms (L. Smith, unpublished data). Infection of most Fov races is often associated with nematode damage, except for California and Australian isolates which can cause severe losses to cotton industry without an association with nematode infestation (Kim et al., 2005, Davis et al., 1996, Davis et al., 2006). Based on its geographical distribution, virulence and genetic distinctions, Fov was subdivided into 6 races and 12 VCG groups (Fernandez et al., 1994, Davis et al., 2006). Australian Fov, including VCG01111 and VCG01112 was believed to be unique and evolved from its wild type origins (Wang et al., 2004, Wang et al., 2010, Davis et al., 1996). Fov can infect cotton at any growing stage. Fov-infected seedlings might result in damping off symptoms, which can be confused with symptoms caused by *Pythium* spp., *Rhizoctonia* spp., *Fusarium* spp. (Davis et al., 2006, Cianchetta and Davis, 2015). At later infection stage, Fov causes stunting, wilting, chlorosis, necrosis as well as vascular discoloration of the stems. Symptoms of Fusarium wilt is not always easily differentiated from Vericillium wilt ones though Fov symptoms from infected stems are more profound. Therefore, further confirmation from pathogen isolation is usually required for disease identification (Cianchetta and Davis, 2015, Davis et al., 2006).

Fov-infected cotton seeds can be minimized by various treatments, which subsequently resulting less seedling disease attributed to Fov (Doan and Davis, 2015). However, once Fov has colonised cotton tissue, it is of a great challenge to control. Therefore, integrated disease management approaches, including, but not limited to, using clean certified seeds, selecting high level of F-rank cultivars, managing nematode infestation, improving cultural practices for reducing soil populations of Fov are sound approaches for minimizing Fov incidence (Cianchetta and Davis, 2015, Davis et al., 2006).

Verticillium wilt

Verticillium wilt of cotton is primarily attributed to *Verticillum dahliae*, which causes vascular discolouration, leaf chlorosis, necrosis, and leaf defoliation in some severe cases (El-Zik, 1985). Occasionally, *V. albo-atrum* was also isolated from diseased cotton plants (Garber and Houston, 1966, Palmateer et al., 2004). *V. dahliae* is a soilborne pathogen in a small genus of ascomycete (Xu et al., 2012, Melero-Vara et al., 1995); however, seedborne transmission of the pathogen is also a potential source of the disease dissemination (Göre et al., 2011). *V. dahliae* has a wide host range and causes vascular wilt in more than 400 host plant species (Inderbitzin and Subbarao, 2014); and on cotton *V. dahliae* is of a pathogen of major concern worldwide (El-Zik, 1985, Allen, 1992, Göre, 2007, Xu et al., 2012, Melero-Vara et al., 1995). In the USA, cotton yield loss due to the Verticillium wilt disease was estimated up to 480 million bales over a period of 1990 - 2014 (Lawrence et al., 2016). In NSW Australia, Verticillium wilt incidence in cotton was as little as 3% to up to 16% in average over a time frame 1984 - 2012 (Kirkby et al., 2013). However, according to Allen (1992), yield loss caused by the disease in Australia can be up to 25% in years where climatic conditions favoured disease development. The disease incidence and severity were highly related to cultivars grown (Melero-Vara et al., 1995, Land et al., 2017, Göre et al., 2009, Wheeler and Woodward, 2016), seeding rates (Wheeler et al., 2010), irrigation regimes (Land et al., 2017, El-Zik, 1985, Wheeler et al., 2012), fertilization (DeVay et al., 1997, Wheeler et al., 2012), soil types (Land et al., 2017) and inoculum density (Pullman and DeVay, 1982, Wei et al., 2014, Alcázar et al., 1995).

Control of the pathogen once it reaches the vascular tissue is difficult; and no known products to date are registered against Verticillium wilt disease. Strategies for controlling Verticillium wilt rely on minimizing the risk of infection through an application of integrated packages of reducing inoculum population in soil through cultivation practices and using tolerant/resistant cultivars where applicable (Deketelaere et al., 2017, Fradin and Thomma, 2006, Klosterman et al., 2009).

Seed treatments

Seed treatments are widely adopted to protect cotton seedlings from a disease complex, including BRR (Rothrock et al., 2012, Toksoz et al., 2009, Mondal et al., 2005, Papavizas et al., 1980). Cotton seed soaked in a mixture of systemic fungicides, namely BAS 389 (N-cyclohexyl-N-methoxy-2,5-dimethyl-3- furancarboxamide), CGA-48988 (N-(2,6-dimethylphenyl)-N-(methoxyacetyl) alanine methylester) and MBC-HC1(methyl benzimidazolecarbamate HCl) had the highest rate of seedling stand that being 90% compared to under 15% in untreated control; BRR index recorded from treated seeds was as low as 1.5 compared to 3.6 (max 4) from the untreated control (Papavizas et al., 1980). Application of myclobutanil at 42 g a.i./100 kg cotton seed provided better protection of the seedlings from BRR than untreated and Dynasty CST (41.6 g a.i./100 kg seed) treated seeds (Toksoz et al., 2009). Percentage of BRR discoloration recorded from untreated and Dynasty-treated seeds was about five and two times higher, respectively than that from myclobutanil-treated seeds (Toksoz et al., 2009). In Australia, seed treatment with acibenzolar-S-methyl (known as Bion) is an option for adoption for reducing BRR of cotton (Mondal et al., 2005, Pereg, 2013). Acibenzolar-S-methyl does not have a direct fungicidal effect to the pathogen, including *T. basicola*. It induces systemic acquired resistance in cotton (Mondal et al., 2005). BRR severity was consistently decreased by 20-30% recorded from cotton seed soaked in acibenzolar-S-methyl solution (50 µg/mL) for up to 5 hours before planting, compared to untreated seeds (Mondal et al., 2005). Toksoz et al. (2009) recorded a similar efficacy of seed treatment with acibenzolar-S-methyl against BRR in trials in the USA. The efficacy of acibenzolar-S-methyl seed treatment was enhanced when it was co-treated with myclobutanil even under high population of *T. basicola* (154 CFU/g soil) (Toksoz et al., 2009). However, Australian soils sampled from cotton farms are commonly found with *T. basicola* population around 200-500 CFU/g soil (Nehl et al., 2004b), which is of a real challenge for any single application to provide complete protection of cotton seedlings from BRR.

Using Fov-free seed is a good practice to minimize the introduction of Fov to new fields (Davis et al., 2006, Cianchetta and Davis, 2015). Numerous treatments have proven their efficacies in Fov elimination from infected seeds. A treatment included pre-soaking cotton seeds in 30% potato dextrose broth on a shaker at 100 rpm for 1 hour, then immersed the pre-treated seeds in either hot fungicide slurry containing azoxystrobin (0.24 g a.i.), fludioxonil (0.5 g a.i.), thiabendazole (0.42 g a.i.) and thiophanate methyl (0.7 g a.i.) or hot thiophanate methyl (0.7 g a.i.) alone at 60 °C for 20 min reduced Fov contamination by 85% without loss of seed germination and vigor (Doan and Davis, 2015). Complete elimination of Fov was achieved when seeds were treated as above with hot thiophanate methyl (0.7 g a.i.) at 70 °C for 20 min; however, seed germination and seedling vigor were decreased by 36% and 38%, respectively (Doan and Davis, 2015). Similarly, Bennett and Colyer (2010) found dry heat treatment at temperature up to 80 °C for up to 14 days effectively eliminated Fov from infected seed, but seed germination and seedling vigor were significantly declined.

Chemical control

Chemicals registered for control of soilborne diseases are very limited, except for few that can suppress the soilborne pathogen populations. Controlling of Fusarium wilt and Verticillium wilt have traditionally relied on soil fumigation to reduce viable inoculum; and subsequently reduce disease incidence and severity. Chemical fumigation is adopted widely to disinfest *V. dahliae*-infested soils growing high value crops in the horticultural and floricultural industries (Pecchia et al., 2017, Ślusarski and Pietr, 2009, Cal et al., 2004, Duniway, 2002). In California, over 90% of strawberry farms either for fruit or for runner plant production were chemically fumigated with methyl bromide and chloropicrin before planting (Duniway, 2002). Chemical fumigation is also commonly practiced on bell pepper, chrysanthemum, artichoke and lettuce farms (Pecchia et al., 2017, Atallah et al., 2011, Cirulli et al., 2010, Ślusarski and Pietr, 2009). Verticillium incidence recorded on bell pepper (*Capsicum annuum*) fields was comparable between plots fumigated with methyl bromide 80 g/m and plots treated with dazomet 50 g/m + *T. asperellum* B35. However, a combined treatment of dazomet and *T. asperellum* gave better economic returns than those of methyl bromide treatment (Ślusarski and Pietr, 2009). Dimethyl disulfide (DMDS) was evaluated for it efficacy in controlling Verticillium wilt on chrysanthemum for two consecutive years in field trials. At the rate of 600 kg of DMDS/ha, wilt disease incidence and stem yield of chrysanthemum on DMDS treated plots were comparable to those of standard chemical fumigation (either Chloropicrin or Metam sodium). Compared to the untreated control, DMDS treated plots had disease incidence reduced by 25-30%, and stem yield increased by around 10% (Pecchia et al., 2017). Soil from artichoke farms with history of *V. dahliae* infestation was disinfested with either a mixture of 1,3-dichloropropene and chloropicrin or methyl bromide alone; subsequently Verticillium wilt of artichoke was effectively controlled and bud yield was increased in the first growing season (Cebolla et al., 2004). Three applications at 21 days intervals of Prochloraz (Sportak 45% EC, 450 g/l) and prochloraz-manganese complex (Sporgon 50 WP, 46% w/w) obtained from Aventis Crop Sciences Co., Adana, Turkey at high dosages, 506 a.i. ha^{-1} and 1250 a.i. ha^{-1}, respectively decreased the Verticillium disease severity of cotton grown on naturally infested soil (Kurt et al., 2003).

Bennett et al. (2011) demonstrated that soils fumigated with a mixture (60:40 vol/vol) of chloropicrin and 1,3-dichloropropene at 295 liters a.i./ha could suppress Fov population and wilt incidence as comparable as those recorded in soils treated with conventional fumigation with methyl bromide. Fumigated plots with 0.2 kg/m^2 ammonium bicarbonate based novel fumigant and 0.4 kg m^{-2} lime and followed by covering with plastic film for 15 days before transplanting reduced Fusarium wilt incidence on cucumber to about 15% in fumigated plots compared to 52% in non-fumigated control. Yield was significantly higher in treated plots than those in control plots (Li et al., 2016). The fumigant was believed to alter soil microbial community by reducing fungal population, including Fusarium and stimulating potentially beneficial microbiomes (Li et al., 2016, Sun et al., 2015). Density of Fov in plots receiving 180 units of anhydrous ammonia was significantly declined at 35 days after application compared to plots receiving either 40 or 180 units of urea. However, the suppression was not persistent at 115 and 175 days after application (Wang et al., 1999). DMDS was also trialed for controlling of Fusarium wilt in cucurbits. DMDS treated plots were recorded with lower density of *F. oxysporum* compared to control plots; however, disease expression was not prevented (Gómez-Tenorio et al., 2015). Everts et al. (2014) for the first time demonstrated that soil drench with prothioconazoles and acibenszolar-S-methyl could provide additional management options against Fusarium wilt of watermelon. Yet, the efficacy of the applications was dependent on soil types and number of applications.

Though chemical fumigations have proven their efficacy in controlling soilborne pathogens, the fumigation exercise with regular application is not economically viable for commercial cotton production (Bennett, 2012, Bennett et al., 2011). This should probably be considered for disinfestation hot spots.

Biological control

Successful biocontrol agents at the field scale need to satisfy all of the following criteria: (*a*) reduce pathogen population in the soil bank; (*b*) reduce disease incidence on a subsequent crop; and (*c*) improve yields of infected plants to levels comparable with healthy plants or crops grown in pathogen infested soils (Deketelaere et al., 2017, Cook, 1985, Weller, 2007).

There are several potential agents discovered to control *T. basicola*, but to date there has been limited research conducted to assess biocontrol options for BRR of cotton (Schoina et al., 2011). *Pseudomonas fluorescens* strains CHAO isolated from naturally suppressive soil to tobacco BRR was able to induce suppressiveness of conducive soil, which resulted in a BRR expression on tobacco (Stutz et al., 1986, Stutz et al., 1989). *P. aureofaciens* recovered from canola rhizosphere was also very suppressive to the growth of *T. basicola* in vitro (Paulitz et al., 2000). *Paenibacillus alvei* strain K-165 is a well-studied biocontrol agent against *V. dahliae*, but K-165 also suppressed *T. basicola* growth in vitro. Percentage of root discoloration of cotton seedlings caused by T. basicola was reduced by up to 40% in pot trials, in which co-inoculation of K-165 and *T. basicola*, compared to *T. basicola* inoculated control (Schoina et al., 2011).

As to biocontrol of BRR of cotton, biocontrol of Fusarium wilt of cotton is little recorded. Soil application with *T. harzianum* (T-35) was successful to control of Fov wilt in cotton (Sivan and Chet, 1986). Applications of 5-15 g of *T. harzianum* (T-35) inoculum (dried colonised wheat-bran/peat) per kg of soil decreased Fusarium population by 4-14 times and subsequently reduced Fov incidence by up to 87% compared to untreated control (Sivan and Chet, 1986).

Biocontrol of *V. dahliae* in cotton is well researched and documented. Fungal endophytes, including *Penicillium simplicissimum* (CEF-818), *Leptosphaaeria* sp. (CEF-714), *Acremonium* sp. (CEF-193 and *Talaromyces flavus* (CEF-624) isolated from cotton roots were able to control Verticillum wilt of cotton with control efficacy varying from 26 - 67% in glasshouse and field trials. Further studies found that these isolates also help to delay Verticillium wilt expression on cotton (Yuan et al., 2017). Cotton seedlings at the two to three emerging leaf stage drenched with 10^9 CFU/ml of an endophytic strain of *Enterobacter cancerogenus* HA02 isolated from cotton root showed up to 80% and 50% protection of cotton seedlings challenged with a defoliating strain *V. dahliae* in glasshouse and field conditions, respectively. Field applications were repeated every 15 days from 18/5 to 28/8 (Li et al., 2012). Endorhiza fungi, including *Fusarium oxysporum* By125 and *Nectria haematococca* Bx247 were also reported to have control efficacy towards Verticillium wilt of cotton. The isolates also promoted plant growth and biomass by up to 30% (Zheng et al., 2011). Pre-inoculation of *Gibellulopsis nigrescens* (previously known *V. nigrescens*) recovered from cotton plants with Verticillium wilt symptoms protected cotton seedlings in a pot trial from subsequent inoculation of pathogenic *V. dahliae*. Disease incidence and severity were reduced by up to 95% and 97%, respectively. However, co-inoculation at the same time of the two fungi reduced level of protection of *G. nigrescens* up to 60% (Zhu et al., 2013). A combined treatment of three endophytic bacteria, including *Paenibacillus xylanilyticus* YUPP-1, *Paenibacillus polymyxa* YUPP-8 and *Bacillus subtilis* YUPP-2 protected 100% seedlings from infection of *V. dahliae* in pot trials. However, the protection efficacy was reduced by at least 30% on field trials, but this still achieved effective control of Verticillium wilt of cotton (Yang et al., 2013).

Amendment of dry mycelium of *Penicillium chrysogenum*, a by-product of pharmaceutical industry at a rate of 30 g/m^2 protected cotton plants from Fusarium and Verticillium wilts. Protection levels from infection of Fov and *V. dahliae* under field conditions were at average of up to 42 and 49%, respectively. The efficacy of the amendments was rate- and application-method-dependent (Dong et al., 2006). Dry mycelium of *P. chrysogenum* contained 90% organic matters and had no antagonistic effect on growth of Fov and *V. dahliae*. Hence, protection of cotton plants from the two wilt diseases was possibly attributed to induce resistance (Dong et al., 2006, Dong et al., 2003).

Crop rotation

Rotation with non-host crops is a recommended practice to reduce inoculum density of soilborne pathogens. *T. basicola* often showed its host preference or specificity, and it does not reproduce on non-hosts or crop residues, thus rotation has been demonstrated as an effective approach in reducing *T. basicola* inoculum loads (Baard and Laubscher, 1983, Reddy and Patrick, 1989, Rothrock et al., 1995, Nehl et al., 2004b). Soil where hairy vetch was grown as a winter cover crop had a significantly lower population of *T. basicola* compared to winter fallowed soil; the population was reduced by up to 80%. Subsequently, disease index was rated at 2.5 (max 5), reducing by about 30% and recovery of *T. basicola* from cotton seedlings was as little as 2% in hairy vetch grown soil compared to 30% of those from fallowed soil (Rothrock et al., 1995). Similarly, *T. basicola* population in soils, including rhizosphere and non-rhizosphere in a bean-rye (host-nonhost) cropping system was about one third of that in back to back bean cropping system (Reddy and Patrick, 1989). However, Nehl et al. (2004b) argued that wheat-cotton rotation practice may reduce *T. basicola* inoculum, but it was not sufficient to suppress BRR of cotton. Nehl et al. (2004b) also suggested BRR severity of cotton increases in accordance to number of cotton crops irrespective to rotation practices. Interestingly, although maize was not infected by *T. basicola*, propagules of *T. basicola* in soil where maize (non-host) was grown after groundnut (host) were stimulated (Baard and Laubscher, 1983). Therefore, crop rotation practice to control *T. basicola* and BRR should be carefully evaluated.

Long term (seven years) crop rotations with non-susceptible hosts of *V. dahliae* may help to reduce Verticillium population, reduction rate 1.4 microsclerotia/year, but it is better to manage water regimes to create less conducive environment for disease development (Wheeler et al., 2014). Rotation with paddy rice may suppress *V. dahliae* microsclerotia and *T. basicola* chlamydospores, but the effect of this practice is not due to the rotation with rice itself. It was due to flooding practice, which created anaerobic conditions that were severely disadvantageous for the viability of *V. dahliae* microsclerotia and *T. basicola* chlamydospores (Pereg, 2013, Yang et al., 2013). However, this is not feasible for many cotton growing areas due to shortage of water supply (Yang et al., 2013, Pereg, 2013).

Formae speciales (f. sp.) of *F. oxysporum* are supposed to be host specific, but in many cases *F. oxysporum* f. sp. can infect a wide host range. Fov can infect and incite either obvious wilt and vascular discolouration or no obvious symptoms on other crops rather than cotton, including soybean, tobacco, yellow lupin, okra, wheat, barley (Davis et al., 1996, Cianchetta and Davis, 2015, Davis et al., 2006, Smith and Snyder, 1975). Therefore, success of crop rotation to reduce incidence of Fov wilt of cotton is often limited (Cianchetta and Davis, 2015, Smith and Snyder, 1975).

Solarization and Biofumigation

Thielaviopsis basicola population in soil amended with hairy vetch was much lower than that in fallowed non-amended soil; subsequently, cotton seedlings were more vigorous and BRR index was lower in hairy vetch amended plots compared to non-amended plots (Candole and Rothrock, 1998, Candole and Rothrock, 1997). The suppressiveness of soils with hairy vetch amendment was thought to be due to the presence of volatile ammonia. Ammonia was detected at 0.14 ppm at 7 days after soil incorporation with hairy vetch, which was sufficient to kill chlamydospores of *T. basicola* (Candole and Rothrock, 1997). Similarly, the population of *T. basicola* in groundnut field was declined by up to 80% when the field was incorporated with lucerne compared to the control; however, disease incidence on groundnut was not different (Baard and Laubscher, 1983). BRR index of tobacco was decreased by up to 50% when tobacco was subsequently grown in soil with rye residue amendment, which was decomposing for at least 30 days. Decomposition of rye residues resulted in an increase of indigenous bacteria population, which appeared to be associated with microbial antagonism and antibiotic production in rye amended soil that suppressing *T. basicola* (Reddy and Patrick, 1989). An interesting fact is that soil incorporated with plant residues that are suppressive towards *T. basicola* have frequently been attributed to increases in populations of *Pythium* spp. and *Rhizoctonia* spp., which are two major seedling pathogens and responsible for seedling mortality of cotton (Candole and Rothrock, 1997, Candole and Rothrock, 1998, ROTHROCK et al., 1995). Therefore, a thorough consideration of plant residue amendment should be based on the prevalence of specific pathogens at individual locations.

Soil solarization has been trialed in fields infested with Fov and *V. dahliae*; however, this is an expensive control method for soil disinfestation and feasibly impractical for large-scale use in cotton; however, it can be applied for disease suppression in field hotspots. Additionally, the efficacy of the treatment may be persistent for multiple years (J. Katan et al., 1983, Bennett, 2012). Viability of Fov propagules buried under 30 cm deep were reduced by up to 97.5% and 58% in solarized soils under a double layer and a single layer of transparent polyethelene (PE) films for 31 days, respectively (Ben-Yephet et al., 1987). Similarly, under solarization temperatures from 37°C and 41°C nearly all chlamydospores were dead after 34 and 6 days, respectively (Bennett, 2012). Consequently, disease incidence caused by Fov at 100 days after planting was recorded as low as 5% in solarized plots, while 90% of the cotton plants showed Fov infection in the control, non-solarized plots (J. Katan et al., 1983). Population of *F. oxysporum* in plots at the same time irrigated with drip line and covered with PE film for six weeks was reduced significantly at 10 and 15 cm deep. The CFU counts/g soil were declined by 98% to nearly eliminated at the two depths, respectively (Bennett et al., 2011). Success of soil solarization greatly depends on achieving adequate soil temperature (40° C at 10 cm depth), the amount of water applied and the duration of the solarization (Bennett et al., 2011, Ben-Yephet et al., 1987). Combined treatments of organic amendment and soil solarization could shorten the solarization duration. In carnation field, plots with soil amendment of either poultry manure or pelletized *B. carinata* or olive residue compost followed by soil solarization for 4 weeks reduced Fusarium propagules by 35 times and disease incidence by 86-99% and subsequently resulting in a yield increase of 5-9 times compared to non-treated control plots (Basallote-Ureba et al., 2016). Although organic amendment and soil solarization in combinations have direct effect in suppressing Fusarium populations, the combinations did not enhance levels of soil suppressiveness in comparison to an organic treatment alone (Klein et al., 2011). Therefore, soils after solarization are more prone to Fusarium re-infestation. Additionally, solarized soils are often insufficient mycorhizal population which can cause poor establish of cotton.

Population of *V. dahliae* in plots covered with think transparent plastic film (25 - 37 µm thick) and solarized for 6 - 10 weeks was very low or undetectable and subsequently, incidence of cotton wilt was reduced to 13% compared to 55 - 90% in un-solarized plots. Additionally, Verticillum wilt on cotton was delayed by 2 -7 weeks on treated plots, but this is not an economical and sustainable practice given the *V. dahliae* population bounced back abruptly on plots following the planting of susceptible cultivars. This practice is recommended as part of integrated approach in conjuntion with crop rotations and use of resistant cultivars (Melero-Vara et al., 1995). A synergetic effect in reducing *V. dahliae* viability was observed in a combined treatment of solarization and metham sodium. Metham sodium alone at 25 ml/m^2 reduced the viability of *V. dahlia*e by 70% compared to the untreated control after a week of treatment, while *V. dahliae* population was only reduced by 3% in solely solarized plots after a week of solarization. With the same treating duration, there was no detection of *V. dahliae* plots treated with solarization and metham sodium at 25 ml/m^2 (Ben-Yephet et al., 1988). Fov, on the other hand, appeared more resistant to soil solarization and metham sodium application. Neither solarization nor metham sodium applications alone reduced Fov viability. Only the combined treatment reduced Fov viability after 6 weeks of treatment (Ben-Yephet et al., 1988).

Similarly, Bennett et al. (2011) found plant mortality caused by Fusarium in metham sodium treated plots was as high as those in untreated control.

Soil populations of *F. oxysporum* and *V. dahliae* were significantly decreased in plots amended with either broccoli or ryegrass biomass and airtight covered with plastic films compared to untreated, organic amendment only and plastic cover only (Blok et al., 2000). This potential approach can be an alternative practice for soil disinfestation where chemical, solarizing and flooding are not feasible (Blok et al., 2000). Incorporation of 0.5% (w/w) rice straw into soil that was flooded and covered with plastic films for 15 days to induce anaerobic conditions reduced the Fusarium wilt incidence in banana up to 82% in two independent field trials over two years. The treatment was considered to suppress *F. oxysporum* population and increase bacterial densities that had the antagonistic effects towards *F. oxysporum* (Huang et al., 2015). Anaerobic soil disinfestation manipulated by applying rice bran (17-20 t/ha) and water saturation for 3-6 weeks effectively protected strawberry from *V. dahliae* up to 100% under field conditions. Net economic returns were equivalent to those from chloropirin fumigation. Strawberry farmers are adopting the practice (Shennan et al., 2017).

Potentials of Brassica crops to reduce *V. dahliae* density were evaluated. Nineteen cultivars were selected and screened for their control efficacy against *V. dahliae* when amended in soils. Mortality of *V. dahliae* in naturally infested soils varied from 9 - 90% depending on soil types and cultivars tested (Neubauer et al., 2014). Microsclerotia of *V. dahliae* in soil amended with 0.4% v/v of defatted seed meals (BioFence^{TM}) of either *B. juncea* or *B. carinata* was completely eliminated in vitro assays conducted on artificially inoculated soil. However, the efficacy was reduced by 20 - 80% when these were amended into naturally infested soil (4 t/ha). The efficacy was greatly dependent on soil types and better control effect was recorded on sandy soil with low carbon contents (Neubauer et al., 2015). Glucosinolate and isothiocyanate released from the brassica meals were believed to be toxic to *V. dahliae* (Neubauer et al., 2015). Another individual assay with BioFence^{TM} derived from *B. carinata* recorded reduction of *V. dahliae* density in natural infested soil by only 27% compared to the untreated control (Wei et al., 2016). Use of brassica crops provide alternative practices to suppress the *V. dahliae* population, but they were not sufficient to eliminate the Verticillium wilt risk for some of the sensitive crops (Wei et al., 2016).

Soils amended with organic matter could protect cotton plants from *V. dahliae* infection under field conditions. Depending on the organic matter used, disease indices recorded on cotton plants were reduced by 20 to 77%. Organic amendments were believed to enhance and alter soil microbial populations, including antagonists, which subsequently suppressed Verticillium wilt (Huang et al., 2006).

Concluding remarks

Sustainability of the Australian cotton industry remains dependent on the continued development and adoption of cultivars that are highly resistant against major pathogens, including *T. basicola*, *F. oxysporum* f. sp. *vasinfestum* and *V. dahliae*. This is a difficult long term process to incorporate high yielding traits with disease resistance; and with limited resources, it is not always possible to develop a complete resistant cultivar to all diseases. In the meantime, there are numerous approaches that could be applied to control disease outbreaks and minimize yield losses. For BRR of cotton, crop rotation and organic amendment have been shown to provide varying degrees of protection. However, these practices might also trigger the dominance of other pathogens, namely *Pythium* spp. and *Rhizoctonia* sp.; hence, the successes of these practices will be mostly dependent on field history knowledge. Chemical seed treatments with fungicides and plant activators have shown promising BRR control in cotton seedlings, but the need to independently test the efficacy of novel compounds remains. Biocontrol of BRR is still widely opened for research. For Fusarium wilt, a current adoption of high F-rank cultivars has resulted in a less stressful industry about this problematic disease. Verticillium wilt is really shaking the industry at moment since there are very limited resistant cultivars available. Minimizing yield losses currently rely on alternative approaches. Chemical fumigation is always an important practice to suppress *V. dahliae*-infested soils; unfortunately, this is not a possible option for the cotton industry in term of environmental damage, cost and economic returns. Similarly, soil solarization using thin plastic film has proven its effectiveness in suppressing wide range soil pathogens, but this is possibly not practical for large scale cotton fields. However, these approaches can be adopted to control *V. dahliae* in hot spots. Biofumigation or bio soil disinfestation has been drawn great attention; however, consistency needs to be improved. While waiting for the development of *V. dahliae* resistant cultivars, induced resistance of cotton against *V. dahliae* is of a great interest.

For this particular project, research will be focused on the following approaches:

- Seed treatments with novel compounds, biocontrol agents for controlling cotton seedling disease that being BRR
- Applications of novel plant activators for promoting seedling health and inducing resistance against BRR
- Exploring biocontrol agents to suppress BRR pathogen population
- Adopting and optimizing bio-disinfestation of soils infected with *T. basicola*, Fov and *V. dahliae*
- Exploring plant activators in inducing resistance of cotton against *V. dahliae*.

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RESEARCH NOTE

TEF1 sequence-based diversity of *Fusarium* species recovered from collar rot diseased cotton seedlings in New South Wales, Australia

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Abstract

Cotton is a billion-dollar crop in Australia, especially in the regional areas of New South Wales (NSW) and Queensland. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) is of an important disease on cotton globally and in Australia. The Australian cotton industry is currently adopting highly resistant cultivars to Fov. Therefore, it is expected that Fusarium wilt pressure will be minimal on the cotton crop. However, we recovered a total of 186 putative *Fusarium* isolates from cotton seedlings exhibiting atypical symptoms of either Fusarium damping-off or Fusarium wilt sampled across NSW during the 2017/18 and 2018/19 seasons. On a basis of sequence analysis of the translation elongation factor 1-alpha (TEF1), eight *Fusarium* species were identified that being *Fusarium oxysporum* and *F. equiseti* species complex, *F. falciforme, F. nygamai, F. brachygibbosum*, *F. acuminatum, F. chlamydosporum*, and *F. redolens*. Of these, *F. oxysporum* species complex (FOSC) accounted for over 80% of the total number of isolates recovered. The predominant number of isolates (78.5%) were clustered with non-Australian Fov races 1–8. The remaining 21.5% of the FOSC isolates in our study was clustered with the Australian Fov biotypes, a unique phylogenetic group within the FOSC. Our study is the first TEF1-based assessment of *Fusarium* diversity associated with collar rot diseased cotton seedlings sampled across NSW, Australia.

Keywords *Gossypium hirsutum* · Seedling diseases · Rhizoctonia rot · *Fusarium oxysporum*

In Australia, cotton (*Gossypium hirsutum*) is mainly grown in regional areas of New South Wales (NSW) and Queensland (QLD), where cotton is produced predominantly under irrigated conditions (CRDC 2018). In the 2017/18 cotton growing season, the industry employed up to 10,000 people across 152 communities and generated about 1.9 billion dollars in revenue (CRDC 2018). Sustainability of the industry relies on successful management of weeds, pests and diseases which could cause significant yield reductions. Diseases such as black root rot, Fusarium or Verticillium wilts alone could be attributed for 5–50% yield losses. On a rare occasion complete yield loss was reported in one field in NSW (unpublished data).

Cotton seedlings are prone to infection with an array of pathogens, including damping off pathogens such as *Pythium* spp., *Rhizoctonia* spp., *Fusarium* spp. (Rothrock et al. 2012), black root rot pathogen *Thielaviopsis basicola* (Nehl et al. 2004a; Toksoz et al. 2009), and leaf spot pathogen *Alternaria alternata* (Le and Gregson 2019). Of these, damping off pathogens could cause the majority of seedling stand reduction (Roy and Bourland 1982; Rothrock et al. 1995; Rothrock et al. 2012), which subsequently resulted in skipped rows and uneven field establishment (Anderson 2014; Rothrock et al. 2012). From 1952 to 2009, seedling diseases caused substantial yield reduction in the USA, accounting for around 23% of the total 2.8% average estimated losses driven by diseases (Blasingame and Patel 2013). Across NSW, seedling mortality was on average around 30% across the seasons from 1988 to 2009, and the seedling mortality was also recorded as high as 51% in one of the surveyed fields (Anderson et al. 2010).

On cotton seedlings, *Fusarium* species were of the most frequently recovered genus from roots and hypocotyls (Melero-Vara and Jiménez-Díaz 1990; Roy and Bourland

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1982; Zhang et al. 1996). Many were reported for their pathogenicity, including *F. oxysporum*, *F. solani*, *F. verticillioides*, *F. incarnatum* (syn. *F. semitectum*), *F. graminearum*, *F. equiseti* and *F. scirpi* (Costa et al. 2005; Hillocks 1992). Of these *F. oxysporum* and *F. solani* were of the most aggressive pathogens on cotton seedlings in Louisiana (Colyer 1988), Mississippi (Roy and Bourland 1982), southern Spain (Melero-Vara and Jiménez-Díaz 1990), and Egypt (Abd-Elsalam et al. 2006). Roy and Bourland (1982) reported that *F. oxysporum* collected from cotton seedlings could incite similar wilt symptoms to those caused by Fusarium wilt pathogen. *Fusarium verticillioides, F. incarnatum* and *F. equiseti* were reported to have a variable degree of virulence on cotton seedlings (Abd-Elsalam et al. 2006; Colyer 1988; Roy and Bourland 1982; Zhang et al. 1996). However, other reports found *Fusarium* spp. isolated from cotton seedlings and surrounding soils were of minor concern in cotton seedling disease complex (Johnson et al. 1978; Johnson and Doyle 1986). Similarly, in Australia several unidentified *Fusarium* spp. recovered from cotton soils were deemed non-pathogenic to mildly virulent on cotton seedlings (Ogle et al. 1993).

Fusarium wilt caused by *F. oxysporum* f. sp. *vasinfectum* (Fov) is of the most economically important cotton disease (Davis et al. 2006). Fov can infect and colonise the vascular system of susceptible cotton at any growing stage. Fov-incited symptoms commonly include leaf chlorosis and necrosis, and profound vascular discoloration of roots and stems. Severe infection may result in plant death (Cianchetta and Davis 2015). Fov is not considered a true damping off pathogen (Davis et al. 2006), but it can kill young established seedlings, and vascular discoloration is often associated in such cases. In Australia, Fusarium wilt of cotton caused by Fov was first reported QLD in 1994 (Kochman 1995), and subsequently detected in many other growing regions in NSW and QLD (Kirkby et al. 2013; Smith et al. 2006). Fov is both soilborne and seedborne and distributed worldwide (Bennett et al. 2008; Cianchetta and Davis 2015; Davis et al. 2006). Losses in yield caused by Fov were estimated around 109,000 bales in the USA in 2004; complete crop losses were also noted in a single field in California, USA (Davis et al. 2006). In Australia, Fusarium wilt was predicted to be infested on 90% of the farms in NSW by 2010 (Nehl et al. 2004b). However, the average Fusarium wilt incidence was detected below 10% on surveyed farms lately (unpublished data) following the good cultivation practices, the adoption of highly resistant cultivars and on-farm hygiene (Come Clean Go Clean) practices. Infection of most Fov races was often associated with nematode damage, except for Australian and Californian isolates which can cause severe losses to cotton industry without an association with nematode infestation (Davis et al. 1996; Davis et al. 2006; Kim et al. 2005). Based on its geographical distribution, virulence and genetic distinctions, Fov was subdivided into 6 races and 12 VCG groups (Davis et al. 2006; Fernandez et al. 1994). Australian Fov biotypes, including VCG01111 and VCG01112 were believed to be unique and evolved from its wild type origins (Davis et al. 1996; Wang et al. 2004; Wang et al. 2010a, 2010b).

During our early season disease surveys in the previous two seasons between 2017 and 2019, many putative *Fusarium* isolates were recovered from cotton seedlings exhibiting collar rot symptoms similar to those of Rhizoctonia rot. Symptoms typically were observed at just below ground regions on the main stems (collar regions). Initial symptoms included small, irregular reddish-brown lesions around the collar and at a later stage enlarged lesions became red to dark brown, frequently girdling the hypocotyls (Fig. 1). However, these symptoms appeared superficial since they were sloughed off when the seedlings grew bigger. Therefore, our study aimed to identify *Fusarium* species associated with cotton seedlings exhibiting collar rot symptoms sampled in the last two seasons. Currently, the internal transcribed spacer (ITS) has been proposed as the universal barcode for fungal and fungal-like oomycete species identification thanks to its comprehensive databases, simplicity for PCR amplification and DNA sequencing (Levesque and de Cock 2004; Schoch et al. 2012). However, the ITS marker is not informative enough for all *Fusarium* species identification (O'Donnell et al. 2015). Conversely, the translation elongation factor 1-alpha (TEF1) is a *Fusarium* phylogenetically informative locus for *Fusarium* species and is sufficient for species identification (O'Donnell et al. 2015; Lombard et al. 2019). Our study assessed, for the first time, species diversity of the *Fusarium* spp. associated with collar rot diseased cotton seedlings in NSW, Australia based on a sequence dataset retrieved from the TEF1 locus.

Early season disease surveys were conducted between 3 to 6 weeks after planting in the previous two seasons 2017/18 and 2018/19 across five main cotton growing valleys in NSW. Cotton seedlings exhibiting symptoms of collar rot were

Fig. 1 Collar rot diseased cotton seedlings exhibiting irregular red brown lesions which frequently girdled the collars (red arrows)

sampled for confirmation of the causal pathogens. In total, 30 and 33 cotton seedling samples were collected during the 2017/18 and 2018/19 survey seasons, respectively (Table 1). Pathogen isolation was initiated with surface decontamination of the diseased collars with 70% ethanol for 10 s, then blotted dry with paper towel. Under aseptic conditions, small sections of diseased collars were excised and embedded into potato dextrose agar (PDA Difco) amended with 100 ppm streptomycin sulfate (Sigma Aldrich) (sPDA). The dishes were sealed with parafilm and incubated at 25 °C in darkness for 2 days. Colonies emerging from infected tissues were individually sub-cultured onto new sPDA dishes and single spore cultures established. Pure cultures were then transferred onto 1.5% water agar and incubated at 25 °C in darkness for at least 7 days before small plugs (0.5 cm^2) were excised from the colony margins, submerged in sterile water and stored at room temperature.

We obtained DNA extracts through a simple microwave protocol developed by Goodwin and Lee (1993). Briefly, mycelia (10–100 mg) were scraped off culture dishes and transferred into 1.5 mL tight-lock Eppendorf tubes. Alternatively, for some isolates, mycelia were harvested from cultures grown in potato dextrose broth (Difco). The tubes were closed tightly and microwaved on high for 5 min. Then, 100 μL TE buffer was added along with two steel beads (3.3 mm dia.) to each tube and shaken to macerate the mycelia on a tissue lyser (Retsch® MM300) for 1 min at a frequency of 28 times per second. The tubes were then centrifuged for 5 min at 13,000 rpm, the supernatants were then diluted 1:10 with DNase free water to new tubes. The diluted supernatants were used as templates for PCR.

Amplifications of the TEF1 regions were undertaken as per descriptions by Le and Gregson (2019). The primer pair EF1-728F 5’-CATCGAGAAGTTCGAGAAGG-3′ and EF2 5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′ were used in all PCR amplifications using GoTaq® G2 Green Master Mix (Promega). Each PCR mix contained: 12 μL of Green Master Mix, 6 μL of DNase free water, 1 μL of 10 mM primer mix and 1 μL of DNA template. DNase free water was included as a negative (no-template) control. PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 90 s at 72 °C, with a final elongation step of 7 min at 72 °C. Integrity of amplified PCR products was confirmed by electrophoresis. The products were run at 100 V in a 1.5% agarose gel pre-stained with GelRed (GeneTargetSolutions) for 45 min and visualised under UV light using a UVIDOC HD6 (UVITEC Cambridge). All PCR products were purified and sequenced by Macrogen Inc., South Korea. All sequences retrieved from both forward and reverse primers were manually adjusted for their consensus sequences. Multi-sequence alignments of the TEF1 and maximum likelihood (ML) phylogenetic analyses using Tamura-Nei model with 1000 bootstrap replicates were performed using MEGA 7 (Kumar et al. 2016). Sequences of related ex-type and reference specimens were downloaded from GenBank and included in phylogenetic analyses. The alignment data and the corresponding phylogenetic trees were deposited in TreeBase (submission 24,550).

A total of 186 putative *Fusarium* isolates, including 101 and 85 collected in 2017/18 and 2018/19 seasons, respectively were recovered from the diseased collars (Table 1). Those putative *Fusarium* cultures were initially identified based on colonial morphology, which was submerged to aerial on

Table 1 Summary of geographic origins, number of samples and *Fusarium* isolates recovered from cotton seedlings exhibiting collar rot sampled across main growing regions in NSW, Australia over the two seasons

Valleys[a]	Geographic origins	No. samples	No. *Fusarium oxysporum*	No. other *Fusarium* spp.
Season 2017/18				
Murrumbidgee	Southern NSW	7	17	4
Lachlan	Southern NSW	3	6	1
Macquarie	Central NSW	6	15	3
Namoi	Northern NSW	10	36	6
Gwydir	Northern NSW	4	9	4
Total		30	83	18
Season 2018/19				
Murrumbidgee	Southern NSW	5	7	0
Lachlan	Southern NSW	5	12	0
Macquarie	Central NSW	4	8	1
Namoi	Northern NSW	14	32	6
Gwydir	Northern NSW	5	16	3
Total		33	75	10

[a] These are five main growing valleys across NSW

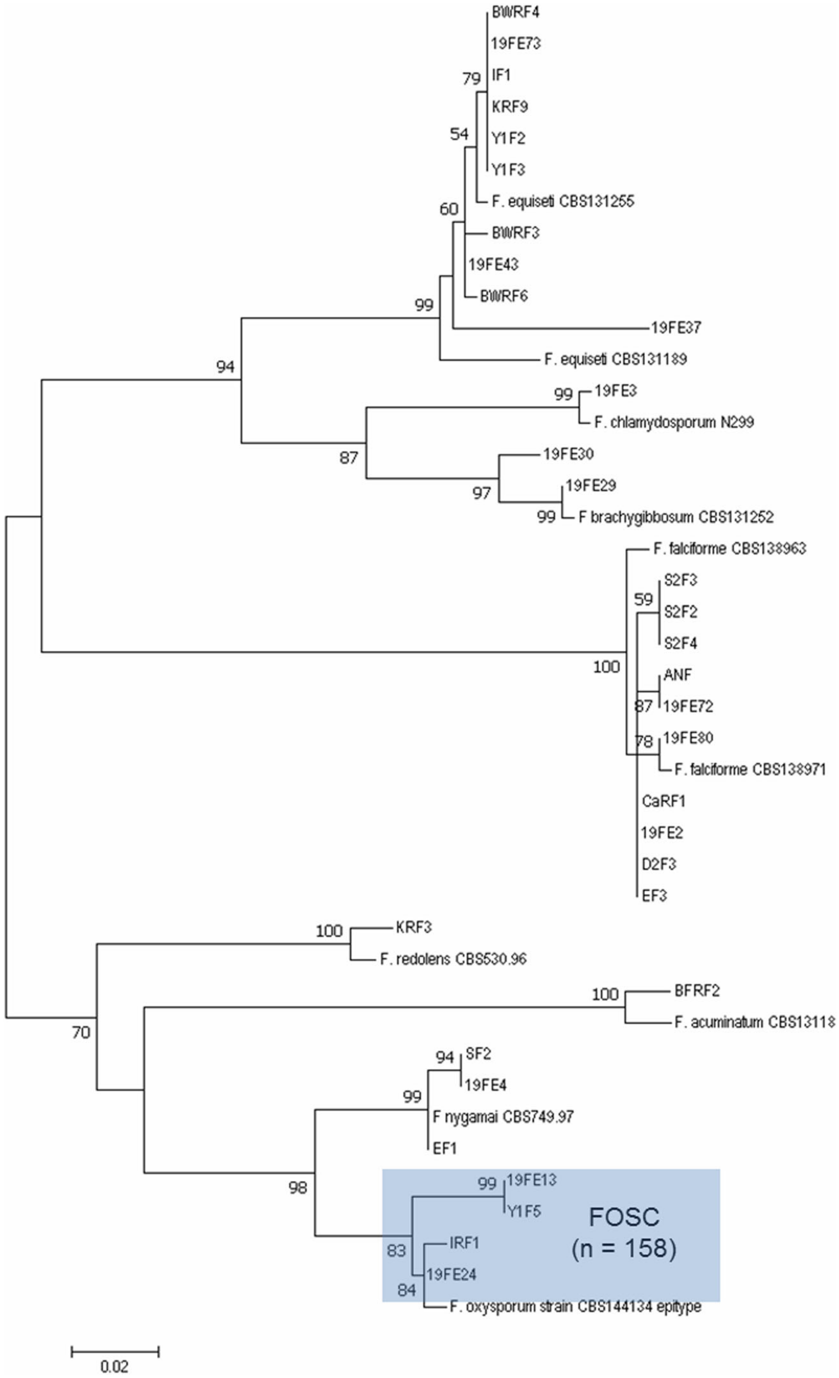
sPDA and varied greatly in colour from white to pigmented. Of these, the morphological resembling *F. oxypsorum,* which was further confirmed by TEF1 sequences, accounted for more than 80% of the collection in both seasons (Table 1). Therefore, we proposed that those *F. oxysporum* might induce collar rot symptoms. *Fusarium* spp. can induce various symptoms, including seed rot, root necrosis and post-emergence damping off on cotton seedlings (Melero-Vara and Jiménez-Díaz 1990; Ogle et al. 1993; Roy and Bourland 1982). Additionally, Costa et al. (2005) demonstrated that *F. incarnatum* incited similar symptoms to those caused by *R. solani* on cotton seedlings. However, due to strict biosecurity practices on-site at the Australian Cotton Research Institute, a Fov-free breeding research site, bioassays associated with *Fusarium* are restricted on site. Our study aimed only to provide insight into the genetic diversity of the current collection. External collaborations will warrant future pathogenicity assays to assess the virulence of these *Fusarium* spp. on cotton seedlings.

Approximately 620-bp-sequences of the above 186 *Fusarium* isolates were successfully amplified and retrieved from the TEF1 locus. Initial sequence searches at GenBank and *Fusarium*-MLST databases revealed that all 186 isolates belong to *Fusarium* genus. Sequences of representatives were deposited in GenBank and publicly available as MN101573 to MN101583. A maximum likelihood (ML) analysis revealed a majority of the collection (84.9%) clustered to *F. oxysporum* species complex (FOSC), of which they are more closely related to a *formae speciales* of *F. oxysporum* f. sp. *vasinfectum*. Isolates belonging to *F. equiseti* species complex and *F. falciforme* were of the second most abundant, accounting for 5.3% each of the total collection. Other minor species included: three isolates closely related to *F. nygamai,* two to *F. brachygibbosum,* one to *F. chlamydosporum,* one to *F. redolens,* and one to *F. acuminatum* (Fig. 2). Of these, *F. brachygibbosum* is for the first time recovered from collar rot diseased cotton seedlings in Australia.

Further ML analysis of the FOSC phylogeny consisted of 263 ingroup isolates, including 158 FOSC isolates recovered in our study and 44 well-characterised reference Fov isolates from public databases. Other 61 FOSC isolates of which many were described as new species in Maryani et al. (2019) and Lombard et al. (2019), and an outgroup isolate *F. fujikuroi* CBS 221.76 were also included in the ML analysis. This revealed that our cotton FOSC isolates were divided into two major clusters that being non-Australian Fov and the Australian Fov biotypes. Cluster 1 contained 124 FOSC isolates, accounting for 78.5% of the collection and all reference non-Australian Fov races, including races 1–8 (Fig. 3). Cluster 1 also contained FOSC isolates belonging to clades 2 and 3 as defined by O'Donnell et al. (2004). Within these two clades, a number of new species were erected, including *F. cugenagense* and *F. gossypinum* which were designated as Fov races 1–8 (Lombard et al. 2019). Historically, Fov races had been geographically distributed. Race 1 and 2 were from USA, race 3 from Egypt and race 4 from India (Armstrong and Armstrong 1960). Race 5 was from Sudan but was retracted due to its genetic and pathogenicity similarity to those of race 3 (Nirenberg et al. 1994). Race 6 came from Brazil (Armstrong and Armstrong 1978) and races 7 and 8 from China (Chen et al. 1985). However, race distributions have changed due to international trading and transportation, as well as advances in research. Race 4 was discovered in California, USA which was more virulent than Indian race 4 (Kim et al. 2005). Race 8 was also detected across many states in the USA (Cianchetta et al. 2015; Holmes et al. 2009). The Australian Fov biotypes were recovered from exported seeds to the USA (Liu et al. 2011). Fov isolates closely related to the Australian Fov biotypes have recently been reported in China (Guo et al. 2015). In Australia, Wang et al. (2010a) found approximately 15% of their FOSC recovered from *Gossypium* spp., refuge and field soils was designated to a lineage E, which was genetically closely related to the overseas Fov races 1–8. Of these, about 3% of the collection showed a certain degree of virulence to cotton in their pathogenicity assays. Additionally, Wang et al. (2010a) found none of their pathogenic isolates recovered from wilt diseased plants belonged to the lineage E. This is the first recovery of FOSC isolates from collar rot diseased cotton seedlings in NSW, which are closely related to overseas Fov races.

Cluster 2 contained the remaining 21.5% (34 FOSC isolates), all reference isolates of the Australian Fov biotypes and FOSC references of clade 1 in O'Donnell et al. (2004) (Fig. 3). Cluster 2 was divided into two subgroups. Subgroup 1 contained 4 of our FOSC isolates and all clade 1 isolates, accommodating many new species such as *F. odoratissimum*, *F. phialophorum* and *F. purpurascens*. Subgroup 2 contained 30 of our FOSC isolates and all Australian Fov biotype isolates. This confirmed the unique genetic position of the Australian Fov which was believed to be locally evolved from non-pathogenic *F. oxysporum* (Wang et al. 2010a). Genetic diversity of FOSC recovered directly from both Australian cotton soil and vascular disease cotton was well studied (Wang et al. 2010a); however, this is the first study to assess TEF1 sequence-based diversity of FOSC recovered from cotton seedling exhibiting atypical symptoms possibly incited by *Fusarium* infection on cotton. Lombard et al. (2019) have recently attempted to clear chaos in FOSC taxonomy using multi-gene phylogenetic analyses, which subsequently revealed 15 new *Fusarium* spp. Therefore, it is worth revisiting Australian FOSC population recovered from Australian cotton in order to resolve its unique taxonomic position. The Australian Fov biotypes were also unique in

Fig. 2 An unrooted maximum likelihood tree constructed from TEF1 sequences showing relationships among *Fusarium* spp. recovered from collar rot diseased cotton seedlings sampled across NSW and reference CBS isolates. Only representative isolates of FOSC were included in the tree construction. Numbers at nodes are bootstrap values of 1000 replicates



term of their pathogenicity, until the discovery of the California Fov race 4 (Kim et al. 2005), that they do not require nematodes to induce infection on cotton plants (Davis et al. 1996; Kochman 1995). The recovery of 30 FOSC isolates highly related to Australian Fov from collar rot seedlings exhibiting atypical Fusarium wilt symptoms will warrant further pathogenicity assays to assess the virulence of the prevailing FOSC collection toward current commercial cultivars. We postulate that the association of FOSC with collar rot on cotton seedlings observed over the last two seasons in NSW may be due to the adoption of highly resistant cultivars; however, more evidence is required for the confirmation of this hypothesis.

In conclusion, FOSC is prevalent on cotton seedlings growing in NSW. They are more genetically diverse than previously reported, which contains both genetic groups of the overseas Fov races and the Australian Fov biotypes based on the current TEF1 analysis. The unique TEF1-phylogenetic position of the Australian Fov biotypes could represent an independent taxon; hence, further analyses of multiple loci is

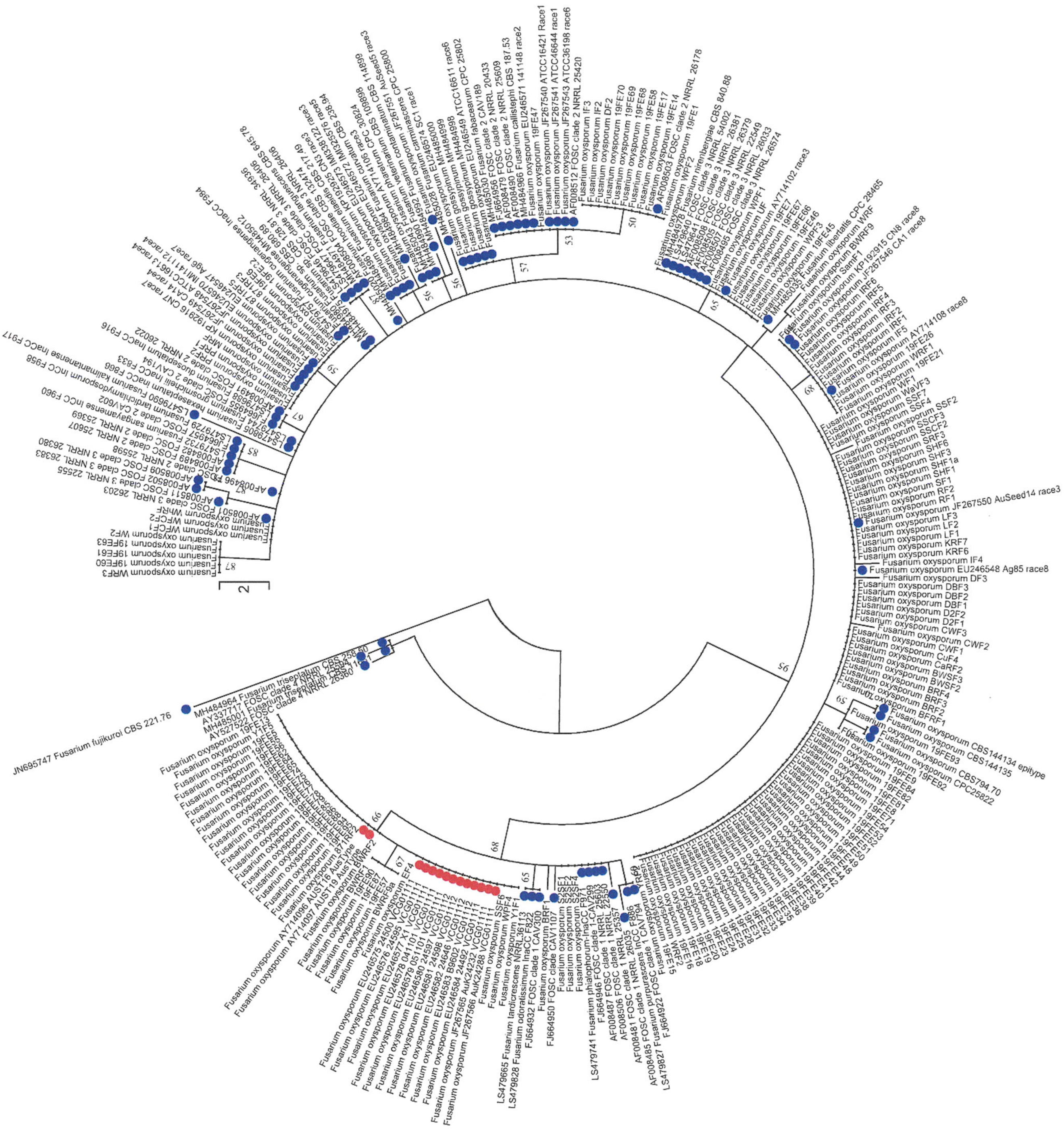


Fig. 3 A maximum likelihood tree constructed from TEF1 sequences showing relationships among FOSC recovered from collar rot diseased cotton seedlings sampled across NSW and reference isolates retrieved from GenBank. Solid red and blue dots are to indicate the Australian Fov biotypes and reference FOSC isolates, respectively. Numbers at nodes are bootstrap values of 1000 replicates (only values higher 50 are shown). The tree is rooted to *F. fujikuroi* CBS 221.76

worth pursuing to redesignate taxonomic position of the Australian Fov biotypes. Additionally, high recovery frequency of FOSC and other *Fusarium* spp. from collar rot diseased cotton seedlings will warrant future investigations into etiology and epidemiology of this disease. Wang et al. (2010b) found a significant genetic shift within the Fov population recovered from wilt cotton over three consecutive crops and suggested that Australian Fov could evolve rapidly. Therefore, disease surveillance will continue to play an important role in monitoring the occurrence and distribution of FOSC, especially those closely related to the Australian Fov biotypes on collar rot diseased cotton seedlings.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. All authors fully agree for submission of the manuscript.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Alternaria leaf spot of cotton seedlings grown in New South Wales, Australia is predominantly associated with Alternaria alternata

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ORIGINAL PAPER

Alternaria leaf spot of cotton seedlings grown in New South Wales, Australia is predominantly associated with *Alternaria alternata*

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Abstract

Alternaria leaf spot (ALS) of cotton, especially on cotyledons is generally considered a minor disease in Australia. However, severe disease outbreaks were recorded in multiple cotton crops in southern New South Wales (NSW) in the 2017/18 season. Due to ALS being considered a minor disease, causal species have not been formally characterised in Australia. In this study, putative small-spored *Alternaria alternata* was predominantly recovered from diseased cotyledons sampled from ALS hot spot fields in southern NSW as well as other locations across NSW. Species status of recovered isolates as *A. alternata* was confirmed through morphology and sequence analyses of the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF1) regions. Phylogenetic analyses of the two regions both individually or in combination resulted in a well-supported group of 69 isolates (83%) clustering with the ex-type specimen of *A. alternata* CBS 916.96, thus confirming the preliminary morphological identifications. In artificially inoculated assays on 15-day-old cotton seedlings, a subset of selected *A. alternata* isolates produced similar ALS symptoms at 7 days after inoculation (DAI) as seen in the field. Koch's postulate was fulfilled by re-isolating *A. alternata* from infected ALS cotyledons at 21 DAI. This is the first main stream published report of *A. alternata* causing ALS on cotton cotyledons in NSW, Australia.

Keywords *Gossypium hirsutum* · Minor disease · Susceptibility · Pathogenicity · Phylogenetic analyses

Introduction

Cotton is a major agricultural crop in rural areas of Australia, including New South Wales (NSW) and Queensland (QLD). In these areas, cotton is produced predominantly under irrigated conditions but smaller areas of dryland production do occur in some seasons. Cotton production contributes significantly to the economic growth and wealth of these regions. Nearly all Australian cotton is exported and generates an average value of around $1.9 AUD billion annually (CRDC 2017). Most commercial Australian cotton varieties are derived from upland cotton (*Gossypium hirsutum*) and pima cotton (*G. barbadense)* and by the 2008/09 season, more than 80% of the Australian cotton crop was planted with transgenic varieties (Naranjo 2011). Like many other crops, intensive cultivation of cotton in some growing regions has led to some major diseases of concern. Fusarium and Verticillium wilts are of the most significant diseases in both NSW and QLD (Nehl 2007), while black root rot and reniform nematodes have drawn more attention in southern NSW and in Central QLD, respectively (unpublished data).

Alternaria leaf spot (ALS) has generally not been considered an issue in cotton production across Australia (Nehl 2007). ALS incidence was reported at 100% when grown near Katherine in Northern Australia (Kochman and Nehl 2000); nonetheless, yield losses were insignificant even in the absence of fungicide application (Bhuiyan et al. 2007). However, yield losses from ALS have been recorded in other countries such as up to 40% in Israel (Bashi et al. 1983b; Rotem et al. 1988; Ephrath et al. 1989; Shtienberg and Dreishpoun 1991; Shtienberg 1993); 25–37% in India (Chattannavar et al. 2006; Hosagoudar et al. 2014); and 22–33% in Zimbabwe (Hillocks and Chinodya 1989). Most studies have found that Pima cotton is more susceptible to ALS than upland

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cotton (Bashan et al. 1991; Nehl 2007; Shtienberg 1993; Shtienberg and Dreishpoun 1991); however, currently no Pima cotton cultivars are commercially available in Australia (Eveleigh 2018). Therefore, it is assumed that ALS pressure on upland cotton in Australia will be minimal. In the 2017/18 season, cotton seedlings grown in southern NSW were severely damaged by ALS and in some fields, ALS incidence on cotyledons was observed up to 100% (unpublished data).

ALS symptoms start with pinhead necrotic lesions surrounded by a purple halo. Under favourable conditions, lesions continue to enlarge and coalesce to form irregular shapes. Severe infection may result in desiccated cotyledons (Fig. 1a), which may subsequently delay maturity. ALS of cotton in Australia is assumed to be caused by *Alternaria macrospora* and *A. alternata*; however, there has only been one study in Northern Australia that confirms the characterisation solely based on the morphology (Bhuiyan et al. 2007).

Brock et al. (1994) found that upland cotton cotyledons were more susceptible to *A. macrospora* than true leaves. Similarly, Bhuiyan et al. (2007) found that the population distribution of *A. macrospora* was more prevalent early in the season and declined significantly towards the end of the season. However, in Israel, severity of *A. macrospora* infection on cotton was observed during wet summer months when temperatures were around 20 to 33 °C (Bashi et al. 1983a), which does not align with usual growing conditions early in the season in southern NSW where average temperatures below 20 °C were recorded in the 2017/18 season (K. O'Keeffe per. Comm.). Therefore, it is proposed that the pathogen(s) causing ALS of cotton cotyledons in southern NSW might involve species other than *A. macrospora*.

This study was conducted to characterise the causal pathogens associated with ALS of cotton cotyledons across NSW based on morphological and molecular characterisation of isolates from symptomatic tissue, and pathogenicity of the recovered pathogens.

Materials and methods

Pathogen isolation and culture storage

Diseased cotton cotyledons were sampled from a number of severe ALS infested fields in the Murrumbidgee and Lachlan valleys, southern NSW as well as from other valleys in northern NSW (Table 1), where occurrence of ALS was only minor. The diseased cotyledons were surface decontaminated with 70% ethanol for 10 s and blotted dry with paper towel. Under aseptic conditions, small sections of cotyledons, which contained both healthy and necrotic tissues were excised and imbedded into potato dextrose agar (PDA Difco) amended with 100 ppm streptomycin sulfate (Sigma Aldrich). The dishes were sealed with parafilm and incubated at 25 °C in darkness for two days. Colonies emerging from infected cotyledons were individually sub-cultured onto new PDA dishes and single spore cultures established. Pure cultures were then transferred onto half strength PDA and incubated at 25 °C in darkness for at least 7 days before small plugs (0.5 cm^2) were excised from the colony margins, submerged in sterile water and stored at room temperature for subsequent experiments.

Morphological identification

Pure cultures grown on PDA were exposed to sunlight by placing culture dishes behind a glass window for two days to induce sporulation. A cellotape technique was used to prepare microscopic slides for morphological examination (Woudenberg et al. 2013). Thirty arbitrarily selected spores from representative isolates (Table 2) were assessed for conidial width and length. Preliminary species identification was based on the identification key of Simmons (2007) and confirmed with DNA sequences.

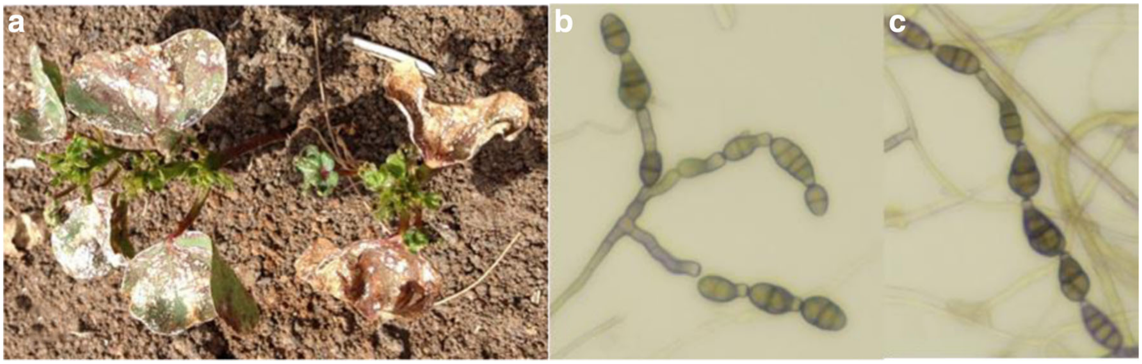

Fig. 1 Cotton cotyledons severely infected with ALS in the Murrumbidgee Valley, southern NSW in the 2017/18 season (**a**) and typical conidia in chains of *A. alternata* recovered from diseased cotyledons (**b** and **c**)

Table 1 Brief descriptions of locations and identity of fungal isolates recovered from diseased seedlings in this study

Valleys	Geographic locations	No. of samples[a]	Identity		
			A. alternata	*Alternaria* sp.	Other fungi
Murrumbidgee	Southern NSW	10	24	0	0
Lachlan	Southern NSW	5	15	1	1
Macquarie	Central NSW	4	9	3	1
Namoi	Northern NSW	8	16	3	2
Gwydir	Northern NSW	3	5	3	0
Total		29	69	10	4

[a] Samples were either collected during the early season survey or received from consultants/growers

DNA extraction

A quick and simple microwave protocol developed by Goodwin and Lee (1993) with some modifications was employed to obtain genomic DNA for PCR amplification. A small amount of mycelia (10–100 mg) was scraped off culture dishes and transferred into a 1.5 mL tight-lock Eppendorf tube and microwaved on high for 5 min. Then, 100 μL TE buffer was added along with two steel beads (3.3 mm dia.) to each tube and shaken to macerate the mycelia on a tissue lyser (Retsch® MM300) for 1 min at a frequency of 28 times per second. Tubes were then centrifuged for 5 min at 13,000 rpm, the supernatants were diluted 1:10 with DNase free water to new tubes. The diluted supernatants were used as templates for PCR.

PCR amplification

Two highly conserved gene loci, including internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF1) were selected for PCR amplifications and DNA sequencing for molecular species identification. The PCR amplifications of the ITS and TEF1 regions were carried out using primer pairs of ITS4 5‘-TCCTCCGCTTATTGATATGC-3’ and ITS5 5‘-GGAAGTAAAAGTCGTAACAAGG-3’ (White et al. 1990), EF1-728F 5’-CATCGAGAAGTTCGAGAAGG-3′ (Cortinas et al. 2006) and EF2 5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′ (Woudenberg et al. 2013). All PCR amplifications were carried out using GoTaq® G2 Green Master Mix (Promega). Each PCR mix contained: 10 μL of Green Master Mix, 8 μL of DNase free water, 1 μL of 10 mM primer mix and 1 μL of DNA template. DNase free water was included as a negative (no-template) control. PCR cycling conditions were slightly modified from Woudenberg et al. (2013) to suit amplification of both loci as follows: initial denaturation for 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 90 s at 72 °C, with a final elongation step of 7 min at 72 °C. Integrity of amplified PCR products was confirmed by electrophoresis. The products were run at 100 V in a 1.5% agarose gel for 45 min, post-stained with Diamond™ Nucleic Acid Dye (Promega) and visualised under UV light using a UVIDOC HD6 (UVITEC Cambridge). All PCR products were purified and sequenced by Macrogen Inc., South Korea.

Phylogenetic analyses

Consensus sequences for each of the isolates were obtained through manual adjustments of sequences retrieved from both forward and reverse primers. Multi-sequence alignments of individual loci and maximum likelihood (ML) phylogenetic analyses using Tamura-Nei model with 1000 bootstrap replicates were performed using MEGA 7 (Kumar et al. 2016). Sequences of related ex-type specimens were downloaded from GenBank and included in phylogenetic analyses. The sequences of the single genes were also concatenated and analysed to determine final species identification. The phylogenetic tree and the alignment retrieved from concatenated data were deposited in TreeBase (https://www.treebase.org).

Table 2 Brief descriptions of representative isolates used in morphological and molecular identification, and pathogenicity assay

Species	Isolates	Valley of origins	GenBank accession numbers	
			ITS	TEF1
Alternaria alternata	CBL	Murrumbidgee	MK386644	MK386651
Alternaria alternata	RvL	Lachlan	MK386645	MK386652
Alternaria sp.	WL	Macquarie	MK386649	MK386656
Alternaria sp.	WYP	Gwydir	MK386650	MK386657

Pathogenicity

Cotton black seeds (cv. Sicot 75RRF) were sown in 140 mL plastic pots containing Searles® potting mix and grown in a glasshouse at 10–25 °C for 15 days prior to inoculation. There were 10 seedlings per pot and eight replicate pots per treatment. Four representative *Alternaria* isolates were selected for pathogenicity assays (Table 2). Conidial suspensions were prepared from conidia produced on PDA as described previously which were harvested through the addition of 20 mL of sterile water to cultures and lightly scraping the agar surface with a disposable L-shaped spreader. The conidial suspension was collected and adjusted to a concentration of 10^4 conidia/mL. Alternatively, the mycelial suspension was prepared by growing tested isolates, which did not readily produce conidia under the described conditions, in potato broth (Difco) for 14 days before harvesting mycelia and blending at high speed for 4 min and adjusting to a concentration of 10^5 mycelial fragments/mL. Inoculated cotton seedlings were sprayed until run-off with conidial or mycelial suspensions with the control treatment being seedlings sprayed with sterile water. Following inoculation, all plants were immediately covered and sealed tight with individual plastic bags to increase humidity. After two days, the covering bags were removed and seedlings were misted three times per day to elevate canopy humidity. All plants were grown in a temperature controlled glasshouse at 10–25 °C with humidity maintained between 15 and 65%. Disease assessments of 20 individual cotyledons on the 10 cotton seedlings per replicate pot per isolate were undertaken at 7, 14 and 21 days after inoculation (DAI). Visual assessments for disease incidence and severity were slightly modified from Mehta (1998) using a rating scale of 0 to 5 as follows: 0 = no disease present; 1 = minute pinhead size spots less than 5% cotyledon surface diseased; 2 = dark brown lesions covering 5 to less than 25% of the cotyledon surface; 3 = necrotic lesions covering 25 to less than 50% of the cotyledons; 4 = necrotic lesions covering 50 to less than 75% of the cotyledons; and 5 = lesions coalescing covering 75% or greater the cotyledon surface.

Disease incidence and index were calculated as:

$$\text{Incidence }(\%) = \frac{\text{Number of infected cotyledons}}{\text{Total number of cotyledons assessed}} \times 100$$

$$\text{Disease index} = \sum_{i=1}^{20} Xi/100$$

Disease index is calculated from 0 (clean healthy cotyledons) to 1 (defoliated cotyledons) which is a sum of the *Xi* disease severity rating (0–5 scale above) of the ith individual cotyledons (1 to 20) assessed divided by 100 which was the number cotyledons assessed (i.e. 20) multiplying with the highest disease severity rating scale (i.e. 5).

A subset of infected cotyledons was selected for re-isolation of the inoculated *Alternaria* isolates at 21 DAI. The isolation procedure was as described previously.

The effect of ALS damage caused by different *Alternaria* isolates on fresh and dry biomass production of the 10 seedlings in each replicate pot was also assessed at 21 DAI. Seedlings were uprooted, washed in running water to remove all adhering potting mix, patted dry with paper towel and then weighed to determine fresh biomass. The cotton seedlings were then placed in paper bags and dried at 70 °C for three days before weighing to determine their dry biomass.

Data collected from the pathogenicity assay were subjected to analysis of variance (ANOVA) and the separations of means were determined by Tukey's least significant difference (LSD) test ($P \leq 0.05$). The ANOVA and graphing were performed in Graphpad Prism 7.04.

Results

Isolation and identification

A total of 83 fungal isolates were recovered from ALS diseased cotyledons (Table 1). On the basis of morphology, 69 isolates were identified as putative small-spored *A. alternata*, 10 isolates were assigned to as putative *Alternaria* spp., and four isolates were non-*Alternaria*. Putative *A. alternata* isolates produced ovate to obclavate conidia with 1–5 transverse septa in single or in chains (Fig. 1b and c). Conidia with conical or cylindrical beaks ranged from 10 to 25 μm (av. 16.6 μm) in length and from 4 to 10 (av. 7.7 μm) in width. Typical characters of 10 putative *Alternaria* spp. were not readily formed in cultures. Although putative *A. alternata* were the predominantly recovered from cotyledons with ALS symptoms, multiple *Alternaria* isolates exhibiting various colony appearances on media being different to *A. alternata* were also recovered from diseased cotyledons sampled from the same fields (data not presented).

Species identification of the recovered *Alternaria* spp. were further confirmed with sequences of ITS and TEF1 regions. Sequence alignments of the ITS (570 bp) and the TEF1 (470 bp) showed a very high degree of genetic uniformity within the putative *A. alternata* population (data not presented). Sequences of the ITS region were 100% identical, while four single nucleotide polymorphisms (SNPs) were found along the TEF1 region. A total of 75 *Alternaria* spp. sequences were successfully retrieved from both of the ITS and TEF1 regions, which were concatenated for a phylogenetic analysis. The concatenated sequences containing 536 bp and 248 bp of the ITS and TEF1 regions, respectively revealed well-supported phylogenetic relationships between our *Alternaria* isolates and reference isolates (Fig. 2). The concatenated sequences of our putative *A. alternata* isolates

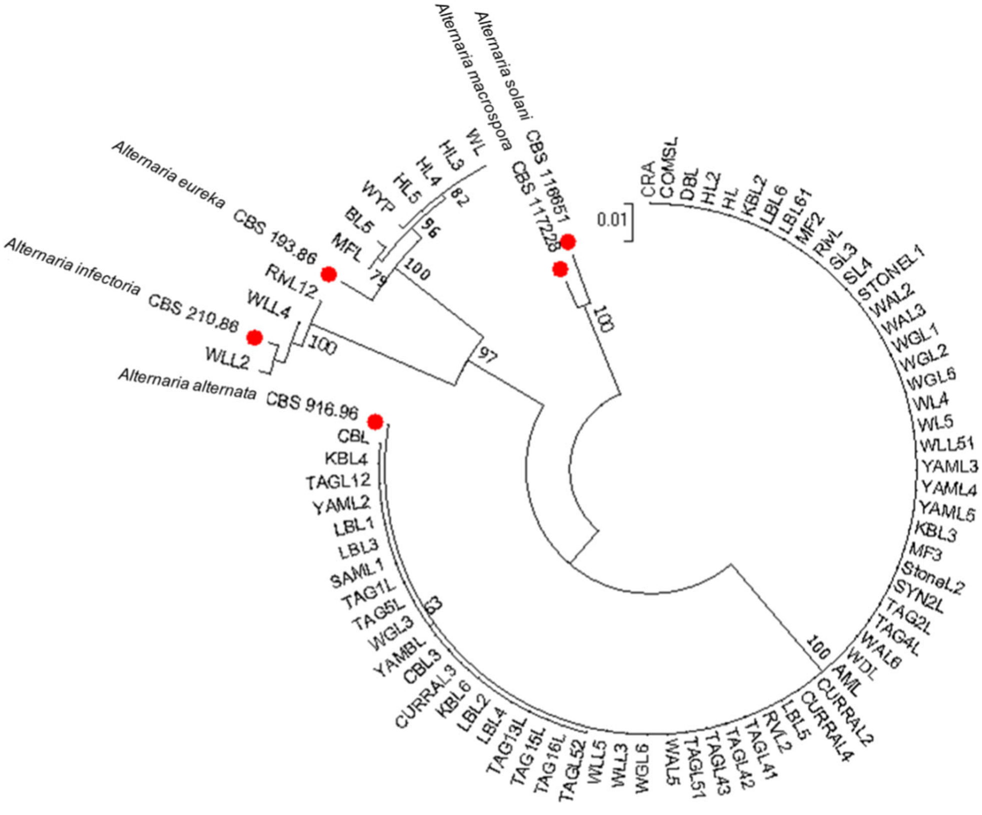

Fig. 2 Maximum likelihood tree obtained from concatenated datasets of the ITS and TEF1 regions showing the relative relationship of *Alternaria* isolates recovered from ALS cotyledons in NSW, Australia with the ex-type reference isolates (solid red circles). Numbers at nodes are bootstrap values of 1000 replicates. Only values greater than 50% are shown

were 99–100% homogeneous to the ex-type reference isolate, *A. alternata* CBS 916.96. The remaining 10 *Alternaria* isolates were either closely related to ex-type isolates of *A. infectoria* CBS 210.86 or *A. eukeka* CBS 193.86 (Fig. 2). No isolates similar to *A. macrospora* were isolated from infected cotton cotyledons in this study (Fig. 2). The concatenated phylogenetic tree was deposited to TreeBase at http://purl.org/phylo/treebase/phylows/study/TB2:S23344?x-access-code=48c049534ce3088e2a417d1e6e31d4fd&format=html

Pathogenicity

Initial symptoms of minute pinhead to small pale green lesions were observed on *A. alternata*-inoculated cotyledons as early as at 3 DAI. Disease continued to progress; and at 7 DAI necrotic lesions were more obvious and similar to recorded field symptoms (Fig. 3). Disease incidences as high as 98% and an average disease severity index of around 0.3 were recorded by 7 DAI with the two *A. alternata* isolates (Fig. 4). The control and *Alternaria* sp.-inoculated cotyledons remained healthy and green though there were a few minute pinhead lesions observed on *Alternaria* sp.-inoculated seedlings of both isolates by 7 DAI. Disease incidence was as little as 8.5 to 20% on *Alternaria* sp.-inoculated seedlings 7 DAI, and the disease indices were all below 0.04 (Fig. 4).

There was no significant difference in disease incidence and severity between cotton seedlings inoculated with *A. alternata* isolates at 14 and 21 DAI. Almost all cotyledons inoculated with *A. alternata* isolates expressed a certain degree of ALS, and disease severity indices increased by 25% between 7 and 14 DAI (Fig. 4). Minute pinhead to small lesions became more visually obvious on cotyledons inoculated with *Alternaria* sp. isolates at 14 and 21 DAI. Both disease incidence and severity induced by *Alternaria* sp. isolates increased by around 50 to 75% between 7 and 21 DAI, and were significantly different from the control (Fig. 4). However, the disease incidences and indices were still significantly lower (approx. 75%) than those recorded on seedlings inoculated with *A. alternata* isolates. Fungal colonies with similar morphology to the original tested *Alternaria* isolates were recovered from surface sterilised diseased cotyledons 21 DAI fulfilling the Koch's postulate.

Fresh biomass recorded for *A. alternata*-inoculated seedlings were significantly lower (approx. 20%) than that of the uninoculated control (Fig. 5). Conversely, seedlings inoculated with *Alternaria* sp. isolates produced fresh biomass comparable to the control treatment. Similarly, *A. alternata*-inoculated seedlings produced up to 25% lower dry biomass compared with the control and *Alternaria* sp.-inoculated seedlings (Fig. 5).

Fig. 3 Healthy cotton seedlings (left) and seedlings with symptoms of ALS on the cotyledons inoculated with *A. alternata* RvL at 7 DAI (right)

Discussion

This is the first report of *A. alternata* as the main causal agent of ALS of cotton seedlings in NSW, Australia. Additionally, this is the first publication characterising ALS pathogens on Australian cotton using both morphological and molecular techniques. Of the pathogens recovered, *A. alternata* was the most prevalent pathogen isolated from typical ALS lesions on cotton seedlings sampled across NSW. This finding is contrary to a previous study of pathogens associated with ALS in Australia, which found that *A. alternata* only became dominant later in the season when grown in northern Australia (Bhuiyan et al. 2007). However, their study was limited at Katherine in the far north of the Northern Territory of Australia where significant differences in climatic conditions compared with southern NSW are likely to influence ALS pathogen populations. In our study, species other than *A. alternate* were often recovered from one field. Likewise, studies in cotton (Bhuiyan et al. 2007) and other plant species have commonly found more than one species of *Alternaria* associated with various leaf or fruit spot diseases in crops such as apple (Harteveld et al. 2013), potato (Tymon et al. 2015) and sesame (Nayyar et al. 2017). However, none of this research has addressed the potential interaction of *Alternaria* species as disease complexes. Therefore, further research to enhance insights into the potential interaction between *Alternaria* spp. in causing severe ALS of cotton seedlings as experienced commercially in southern NSW cotton crops in 2017/18 season appears warranted.

Though *A. alternata* was predominantly recovered from ALS cotyledons and was the most virulent *Alternaria* sp. in this study, ALS of cotton is commonly associated with

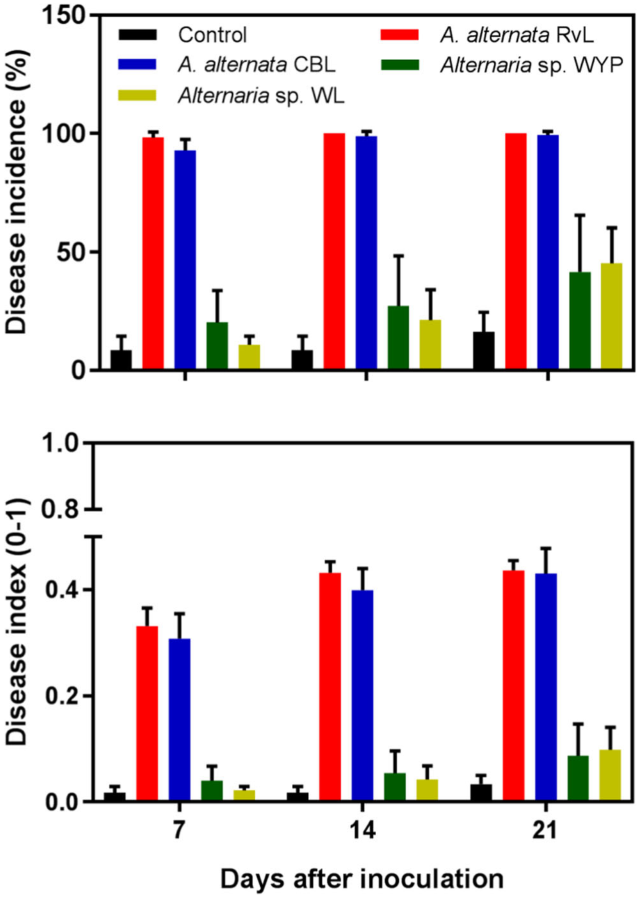

Fig. 4 Disease incidence (%) and disease index (0–1) recorded on cotton cotyledons inoculated with *Alternaria* spp. at 7, 14 and 21 days after inoculation. Bars represent standard deviation of eight replicates

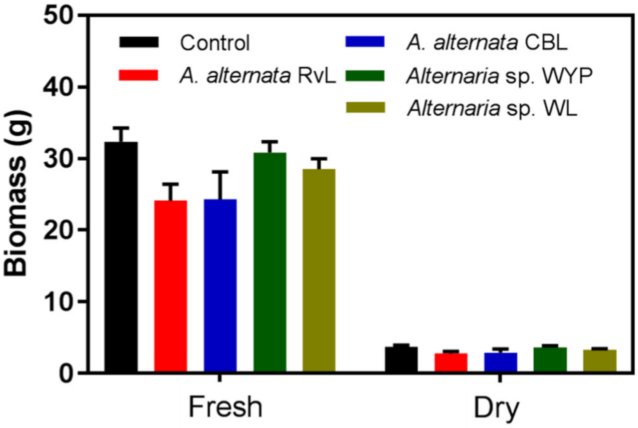

Fig. 5 Fresh and dry biomass (g) at 21 DAI of ten cotton seedlings inoculated with *Alternaria* spp. Bars represent standard deviation of eight replicates

multiple *Alternaria* spp. (Bashan et al. 1991; Bhuiyan et al. 2007; Shtienberg 1993; Rotem et al. 1988). Ten *Alternaria* spp. isolates, that did not produce characteristic morphology for identification, were also recovered in this study. Phylogenetic analyses of the ITS and TEF1 regions revealed that the 10 isolates were either related to *A. infectoria* or *A. eureka*. However, their phylogenetic positions may stand independently. Additionally, selected isolates from this group incited relatively mild ALS symptoms on artificially inoculated cotyledons with mycelial fragments, which may not be a main inoculum source under natural conditions. Therefore, further characterisation of these isolates is required for a definitive species identification, to confirm their level of pathogenicity and any potential relationship of this minor group with *A. alternata* in an ALS disease complex. None of our isolates were morphologically and phylogenetically related to *A. macrospora*, which was of the primary *Alternaria* spp. associated with ALS on cotton seedlings in northern Australia (Bhuiyan et al. 2007). *A. macrospora* has also been reported as the main ALS pathogen of cotton in Israel and India (Shtienberg 1993; Prasad et al. 2017). Previous studies have found *A. macrospora* to be more virulent and prevalent on Pima cotton (Bashi et al. 1983b; Rotem et al. 1988), which is currently not grown commercially in Australia (Eveleigh 2018). It is generally theorised that the wide spread adoption of upland cotton varieties across commercial cotton fields in Australia has possibly altered *Alternaria* populations; however, further evidence is required to support or disprove this theory.

ALS has long been considered as a minor disease by the Australian cotton industry, especially during early growth stages. Historically, ALS has been more prevalent late in the season (Mehta 1998; Bhuiyan et al. 2007), and damage to Australian cotton in Northern Australia was not significant (Bhuiyan et al. 2007). However, our study indicates that losses caused by *A. alternata* could be significant. Seedlings infected with *A. alternata* produced lower fresh and dry biomass in the glasshouse study. Although cotton plants can regrow to compensate for leaf damages during seedling stages, slow accumulation of biomass can translate into delayed maturity (Lei 2000). Further studies to determine the relationship between ALS of cotyledons and cotton yield under Australian conditions are required. Currently, no fungicide is registered in cotton for the control of ALS in Australia. Hence, cotton crops in southern NSW in 2017/18 season which were severely infected with ALS as seedlings were left uncontrolled. Studies to evaluate fungicide efficacies against *A. alternata* on cotton are required to provide the industry with an effective option to manage ALS.

In conclusion, morphological, phylogenetic and pathogenicity studies allowed confirmation of *A. alternata* as the primary causal species of severe ALS on cotton cotyledons in southern NSW production regions of Australia in the 2017/18 season. This emerging disease and pathogen need to be considered in cotton disease management programs in NSW, especially in the southern production regions where severe levels of ALS were evident in 2017/18 season and was poorly managed due to a lack of knowledge on ALS epidemiology and effective available control options. Therefore, future research to understand the epidemiology of ALS, the pathogens involved, potential disease complexes between *Alternaria* spp. along with the development and validation of effective management strategies (e.g. fungicide efficacy) is required to support continued cotton production in southern NSW.

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Article

Co-Occurrence of Defoliating and Non-Defoliating Pathotypes of *Verticillium Dahliae* in Field-Grown Cotton Plants in New South Wales, Australia

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Abstract: Verticillium wilt (VW) is a major constraint to cotton production in Australia and worldwide. The disease is caused by a soilborne fungus, *Verticillium dahliae*, a highly virulent pathogen on cotton. Commonly, *V. dahliae* is designated into two pathotypes: defoliating (D) and non-defoliating (ND), based on induced symptoms. In the previous two survey seasons between 2017 and 2019, stems with suspected VW were sampled for the confirmation of presence and distribution of D and ND pathotypes across New South Wales (NSW), Australia. A total of 151 and 84 VW-suspected stems sampled from the 2017/18 and 2018/19 seasons, respectively, were subjected to pathogen isolation. Of these, 94 and 57 stems were positive for *V. dahliae*; and 18 and 20 stems sampled respectively from the two seasons yielded the D pathotype isolates. Two stems from the 2017/18 season and one stem from 2018/19 season yielded both D and ND pathotype isolates. We also successfully demonstrated the co-infection of both pathotypes in pot trials, which was driven predominantly by either of the pathotypes, and appeared independent on vegetative growth, fecundity and spore germination traits. Our study is the first report of the natural co-occurrence of both D and ND pathotypes in same field-grown cotton plants in NSW, to which a challenge to the disease management will be discussed.

Keywords: *Gossypium hirsutum*; Verticillium wilt; pathogenecity; pathotyping; duplex PCR

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1. Introduction

Cotton (*Gossypium* spp.) is an economically important fibre crop contributing to approximately 40% of the world's natural fibre [1]. Cultivated cotton can be found in a range of ecological niches from arid to semi-arid areas of the tropical and subtropical zones and is mainly derived from Upland cotton (*G. hirsutum* L.) and Pima cotton (*G. barbadense* L.) due to superior fibre quality and quantity traits [2]. In Australia, Upland cotton is a major agricultural crop and mainly grown in regional areas of New South Wales (NSW) and Queensland and produced predominantly under irrigated conditions, but smaller areas of dryland production do occur in some seasons [3]. In the 2017/18 cotton growing season, the industry employed up to 10,000 people across 152 communities and contributed significantly to the economic growth and wealth of these regions. The majority of Australian cotton is exported, which generates an average value of around AUD 1.9 billion annually [3].

In Australia, cotton is prone to infection with an array of pathogens, including black root rot pathogen *Thielaviopsis basicola* (Berk. and Br.) Ferraris [4], leaf spot pathogen *Alternaria alternata* (Fr.) Keissl. [5], Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *vasinfectum* Snyder and Hansen, [6,7], and Verticillium wilt (VW) pathogen *Verticillium dahliae* Kleb. [8]. Of these, VW of cotton is of a major constraint to the sustainability of the cotton industry due to the lack of complete resistant resources and highly effective management strategies. The disease is associated with vascular discolouration, leaf chlorosis, necrosis, defoliation and plant death in some severe cases [9]. In NSW, VW incidence in cotton was as little as 3% up to 16% on average between 1984 and 2012 [8]. During 2016–2019, the highest average incidence of VW in NSW and Queensland was 30% and 4%, respectively [10]. According to Allen [11], yield loss caused by the disease in Australia can be up to 25% in years where climatic conditions favoured disease development. On a rare occasion, yield loss was estimated up to 50% in a severely infested field in NSW (unpublished data).

Verticillium dahliae is a soilborne phytopathogen and commonly associated with vascular wilt diseases of up to 400 host plant species; many of these are of economic importance in agriculture, horticulture and forestry [12]. *V. dahliae* was reported across many cotton growing regions such as Australia, China, Spain and the USA [8,13–15]. Virulence of *V. dahliae* was commonly found associated with its pathotypes, that being defoliating (D) and non-defoliating (ND), based on symptoms induced on host plants [16,17]. Unless otherwise stated, the D and ND pathotypes should only be referred to as pathotypes of *V. dahliae* in this study. The D pathotype was deemed to be highly aggressive, inciting defoliation, and was lethal to cotton; alternatively, the ND pathotype was considered less aggressive and did not attribute to defoliation [13]. However, *V. dahliae* isolates recovered from Australian cotton and designated as D and ND pathotypes were found to be equally lethal to its host (unpublished data). In the past two survey seasons, the ND pathotype was detected across NSW, while the D pathotype was more prevalent in the northern valleys of NSW [10]. Additionally, our initial data also indicated the presence of both pathotypes in some fields in NSW (unpublished data). Co-occurrence of both pathotypes in a cotton field were previously reported in Spain [16]. Similarly, Jiménez-Díaz et al. [18] found that co-occurrence of both pathotypes was relatively common in olive orchards in Spain. Interestingly, Mercado-Blanco et al. [19] for the first time demonstrated that co-infection of D and ND pathotypes also occurred naturally in olive trees. This reflects the complexity of the patho-system in *V. dahliae*. However, many used single isolates of *V. dahliae* for biological and pathogenicity assessments [20,21].

Interactions between *V. dahliae* with either other *Verticillium* spp. or different isolates were assessed in artificially co-inoculated assays. The observed effects varied from none to cross-protection, depending on the inoculation methods, orders and time intervals between inoculations [22,23]. For example, VW expressions on lettuce were lower in co-inoculated plants with isolates of *V. tricorpus* and *V. dahliae* compared with those inoculated with *V. dahliae* alone. Additionally, the co-inoculation relatively promoted the growth of lettuce [23]. Qin et al. [23] also reported that a soil drench with *V. tricorpus* in advance of the inoculation with *V. dahliae* appeared to provide better protection compared with simultaneous inoculation using a root-dip method. On the other hand, simultaneous inoculation of the avirulent isolate P6 and the highly virulent isolate VM of *V. dahliae* did not result in any difference in disease expression on sunflower; however, sequential inoculation of the isolate VM two days following the challenge with the isolate P6 resulted in a significantly lower disease severity in sunflower [24]. Shittu et al. [25] found that disease scores in tomato were significantly lower in plants either preceding inoculation with the non-host isolate Dvd-E6 followed by the virulent isolate Vd1 of *V. dahliae* or in simultaneous inoculation. Wheeler and Johnson [22] reported that co-inoculation with two or three different host-selective *V. dahliae* isolates did not alter potato yields, or mustard and barley biomass, but disease severity in potato increased in co-inoculated plants. On cotton, cross-protection was observed in plants grown both under artificially co-inoculated and naturally infested soils with the virulent D pathotype isolate T-1 and mildly virulent ND pathotype isolate SS-4 [26]. However, at the time, there was no evidence that supported the co-existence of both pathotypes in cotton plants [26]. To date, only Mercado-Blanco et al. [19] showed the natural co-infection and co-existence of the D and ND pathotypes in olive. Therefore, our study sought to document for the first time the natural co-occurrence of D and ND pathotypes in field-grown cotton sampled in NSW, Australia, which were also demonstrated again in our co-inoculated pot trials.

2. Results

2.1. Isolation and Pathotype

A total of 151 and 84 VW-suspected stems selected from samples collected during the 2017/18 and 2018/19 seasons, respectively, were subjected to recovery of *V. dahliae* (Table 1). Of these, 94 and 57 stems from the two seasons, respectively, yielded putative *V. dahliae* cultures on an isolating medium (potato dextrose agar amended with 100 ppm of streptomycin, sPDA). Subsequently, a total of 195 and 120 putative *V. dahliae* isolates from the 2017/18 and 2018/19 seasons, respectively, were subcultured and purified for pathotyping using duplex PCR developed by Mercado-Blanco et al. [19]. Of these putative *V. dahliae* isolates, 34 and 41 isolates accounting for approximately 17% and 34% accordingly were designated to the D pathotype (Table 1), thus confirming the identification of the pathogen.

Table 1. Number of *V. dahliae* isolates including pathotype designations recovered from Verticillium wilt (VW) disease-suspected stems sampled during the two survey seasons between 2017 and 2019.

Numbers of	2017/18 Season	2018/19 Season
VW-suspected stems [1]	151	84
V. dahliae-positive stems [2]	94	57
ND-positive stems [3]	76	37
D-positive stems	18	20
Both-pathotypes co-occurred stems	2	1
Putative *V. dahliae* isolates	195	120
D isolates [4]	34	41
ND isolates	161	79

[1] VW-suspected stems sampled during the late season disease surveys in the previous two seasons were subjected to isolation for confirmation of the associated pathogen. A minimum of three stems exhibiting typical VW peppery vascular discoloration were selected per field for pathogen isolation. [2] *V. dahliae*-positive stems were only confirmed once the corresponding pathogen was recovered. [3] D- and ND-positive stems were determined based on pathotyping results of the correspondingly recovered *V. dahliae* isolates. [4] D and ND isolates were designated using duplex PCR [19].

Of the *V. dahliae*-positive stems, 18 and 76 stems were determined to yield the D and ND pathotypes, respectively, in the 2017/18 season, and two stems yielded both pathotypes. In the 2018/19 season, 20 out of the 57 *V. dahliae*-positive stems yielded the D pathotype, and one yielded both the D and ND pathotypes. Therefore, this confirms the first report of the natural co-occurrence of both the D and ND pathotypes of *V. dahliae* in cotton in NSW, Australia.

2.2. Pathogenicity

2.2.1. Trial 1

Control plants remained healthy (disease incidence and severity = 0) during the time course of the experiment; hence, unless otherwise stated, the control data were excluded in our ANOVA analyses and graphing.

Plants inoculated with the tested *V. dahliae* isolates showed the first VW symptoms, including chlorosis and wilting on cotyledons seven days after inoculation. Disease symptoms progressed upwards, and defoliation was observed on plants inoculated with isolates 19V76 and L42, and those with co-inoculation (Table 2). We recorded various degrees of disease severity from 0 = no symptoms to 5 = dead plants four weeks after inoculation. The mean disease severity varied from 3 to 4.2 and was not significantly different among the *V. dahliae*-inoculated treatments, irrespective of either single- or co-inoculation (Figure 1).

Table 2. Number of diseased, D and ND plants recorded in the two pot trials inoculated with the tested *V. dahliae* isolates, and the number of D and ND isolates recovered upon termination (four weeks after inoculation) of the pathogenicity assays.

Numbers of	Control	19V76	19V77	19V76 + V77	L41	L42	L41 + L42
Trial 1 [1]							
Diseased plants [2]	0	6 (60)	10 (100)	8 (80)	10 (100)	9 (90)	8 (80)
D plants [3]	0	5	0	4	0	2	4
ND plants	0	1	10	4	10	7	4
D and ND positive plants [4]	0	0	0	2	0	0	2
V. dahliae isolates [5]	0	42 (100)	57 (71)	51 (91)	68 (97)	54 (86)	50 (89)
D isolates [6]	0	42	0	31	0	54	18
ND isolates	0	0	57	20	68	0	32
Trial 2							
Diseased plants	0	7 (88)	3 (38)	4 (50)	8 (100)	7 (88)	5 (63)
D plants	0	7	0	1	0	5	4
ND plants	0	0	3	3	8	2	1
D and ND positive plants	0	0	0	0	0	0	1
V. dahliae isolates	0	49 (100)	21 (100)	17 (61)	56 (100)	45 (92)	25 (71)
D isolates	0	49	0	11	0	45	21
ND isolates	0	0	21	6	56	0	4

[1] The two trials were carried out independently. A total of 10 and 8 seedlings per treatment were inoculated in trial 1 and 2, respectively. The D pathotype included isolates 19V76 and L42; the ND isolates were 19V77 and L41. [2] Diseased plants were determined based on VW symptoms such as wilting, leaf chlorosis and necrosis. Numbers in the parentheses indicate the percentage of disease incidence. [3] D plants were determined based on defoliation observed during the four weeks of the experiment. [4] D- and ND-positive plants were determined based on the recovery of both D and ND isolates from an inoculated plant. [5] All plants including the control were subjected to *V. dahliae* re-isolation individually. Numbers in parentheses indicate the frequency recovery percentage of *V. dahliae* from diseased plants. [6] The recovered *V. dahliae* isolates were subjected to pathotyping by duplex PCR [19].

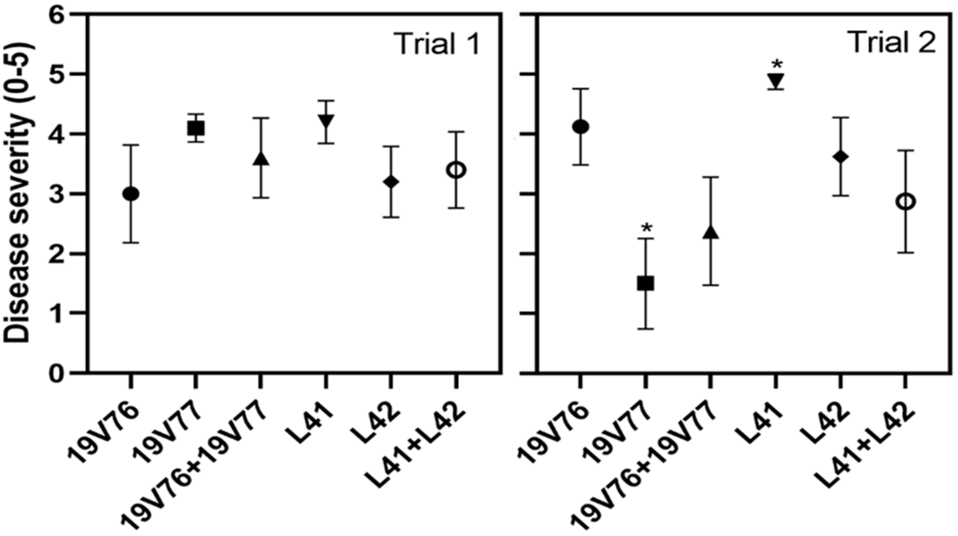

Figure 1. Mean disease severity (0: no symptoms–5: dead plants) recorded at four weeks after root dip inoculation of 2-week-old seedlings either singly or in combination with 10^4 spores/mL suspensions of the tested *V. dahliae* isolates. Bars represent standard errors of means (n = 10 and 8 in trial 1 and trial 2, respectively). Asterisks indicate significant differences among treatments (p = 0.05).

Of the diseased plants, the *V. dahliae* recovery frequencies of the inoculated isolates were from 71 to 100% (Table 2). We failed to recover any *V. dahliae* from asymptomatic inoculated plants and the control plants. A single pathotype, either D or ND, was recovered from the single inoculated plants. Both D and ND pathotypes were recovered only from the co-inoculated plants. Two plants from each of the co-inoculated treatments were co-infected with both pathotypes. The co-infections were driven by either of the pathotypes, indicated by the predominant recovery of one to the other (Table 2).

2.2.2. Trial 2

No VW disease symptoms were observed on the control plants. The first VW symptoms were noted on cotyledons of the *V. dahliae*-inoculated plants at nine days after inoculation. Defoliation was observed on plants inoculated with isolates 19V76 and L42 and their co-inoculation at four weeks after inoculation (Table 2). Disease incidence was lowest (38%) in the 19V77 treatment and highest (100%) in the L41 treatment. Means of disease severity were significantly different ($p = 0.05$) between the 19V77 and L41 treatments, but these were not different to the others (Figure 1).

Of the diseased plants, the *V. dahliae* recovery frequencies of the inoculated isolates ranged from 61 to 100% (Table 2). As in trial 1, we did not recover any *V. dahliae* from asymptomatic inoculated plants and the control plants. Diseased plants from treatments singly inoculated with either D or ND yielded a single pathotype upon isolation and pathotyping using duplex PCR. Both D and ND pathotypes were recovered only from the co-inoculated plants. A single plant from the co-inoculated L41 + L42 treatment yielded both D and ND pathotypes. The co-infection was driven by the ND pathotype; six out of seven recovered isolates were designated to the ND pathotype (Table S1).

2.3. Growth Competition

At 25 °C, the D isolates 19V76 and L42 grew respectively faster than the ND isolates 19V77 and L41 after seven days incubation in darkness, regardless of being grown in single or dual cultures (Figure 2). Colonies of 19V76 single and 19V76 dual (against 19V77) reached approximately 30 mm in diameter and were significantly higher than those of 19V77 single and 19V77 dual (against 19V76), which were around 25 mm in diameter. Similarly, growth of L42 single and L42 dual (against L41) were about 10% faster than those of L41 single and L41 dual (against L42).

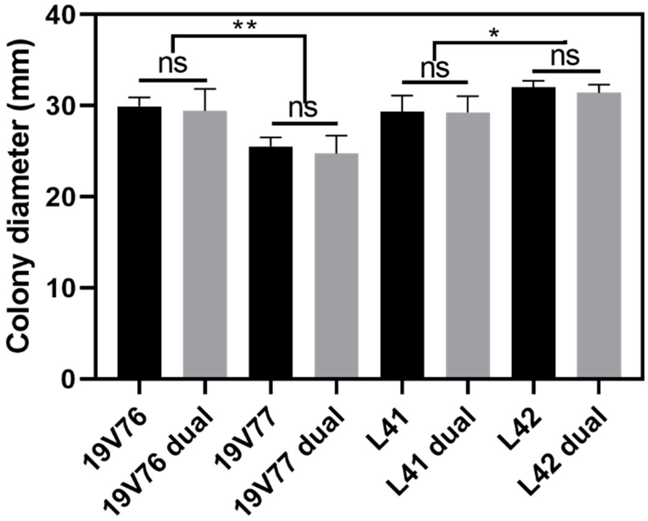

Figure 2. Colony diameter (mm) of the tested *V. dahliae* isolates recorded after seven days growing in darkness at 25 °C. Growth competition of 19V76 vice versa 19V77, and L41 vice versa L42 were assessed in dual culture assays (labelled "dual"). Data from the two assays were pooled due to the insignificant difference found between the two repeated assays. Bars represent standard errors of means ($n = 12$). Asterisks indicate significant differences among treatments ($p = 0.05$ for * and $p = 0.01$ for **).

There were no negative effects on growth of the tested *V. dahliae* isolates recorded in the dual culture assays (Figure 2). Growth of 19V76 in single culture (29.9 mm diameter) was comparable to that of the dual culture against 19V77 (29.4 mm diameter). A similar pattern was recorded on the growth of 19V77, L41 and L42 in single and dual cultures. This indicates growth competition between the D and ND isolates was not observed in our assays.

2.4. Fecundity and Germination

The in vitro fecundity (spores/mL) and germination rate (%) of the four tested *V. dahliae* isolates were isolate-dependent, but independent from pathotypes (Figure 3). Fecundity of the D isolate 19V76 was around 1.1 × 10^7 and significantly higher than that of the ND isolate 19V77 (5.3 × 10^6). On the other hand, the ND isolate L41 produced a significantly higher number of spores compared with the D isolate L42 under the same conditions.

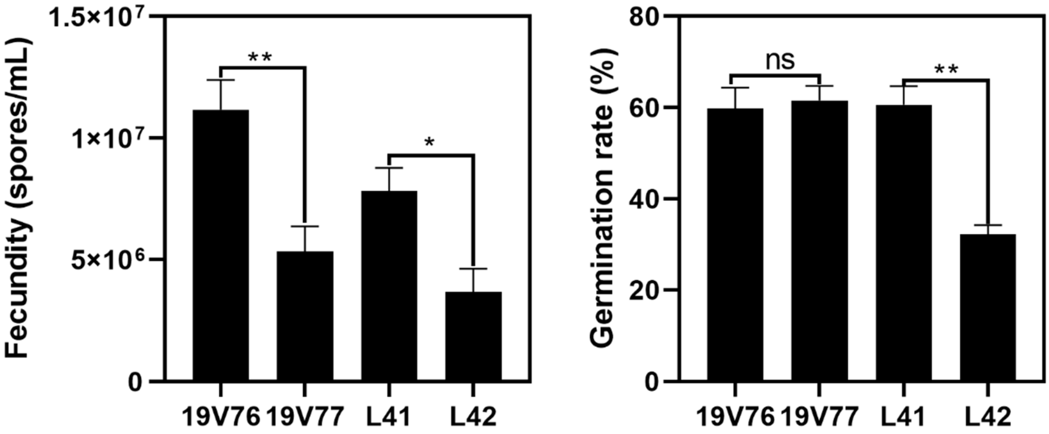

Figure 3. Fecundity (spores/mL) and germination rate (%) for the four tested *V. dahliae* isolates on potato dextrose agar amended with 100 ppm of streptomycin (sPDA) at 25 °C in darkness. Data from the two assays were pooled due to the insignificant difference found between the two repeated assays. Bars represent standard errors of means (n = 6 and 18 for fecundity and germination assessments, respectively). Asterisks indicate significant differences among treatments (p = 0.05 for * and p = 0.01 for **).

There was no significant difference in the spore germination rates of the 19V76 and 19V77 isolates on sPDA, which were recorded around 60%. Conversely, the germination rate of L42 was recorded at 32.2%, which was about half of L41 (60.6%) (Figure 3).

3. Discussion

Verticillium wilt of cotton was first reported in NSW in 1959 [27]. Over 35 years of continuous disease survey since 1984 [8], we for the first time documented the natural co-occurrence of both D and ND pathotypes of *V. dahliae* in cotton in NSW, Australia, sampled from two survey seasons between 2017 and 2019. Additionally, we were able to demonstrate the co-infection in two independently repeated pot trials using a root dip method. Therefore, we propose that co-infections of multiple *V. dahliae* isolates in a cotton plant are probably not a rare event under natural field conditions. Previously, Mercado-Blanco et al. [19] reported the co-infection of D and ND pathotypes occurred naturally in olive trees. Schnathorst and Mathre [26] artificially co-inoculated cotton with the virulent D isolate T-1 and mildly virulent ND isolate SS-4; however, there was no evidence of co-infection recorded in their study.

Our pot trials indicated that the D and ND isolates recovered from the same stem were relatively comparable in virulence on cotton, and the VW disease expressions were not significantly different between the single- and co-inoculated plants. Irrespective of treatments, initial VW symptoms appeared first on cotyledons between 7 and 9 days after inoculation and dead plants were observed across the treatments four weeks after inoculation. Similarly, Wheeler and Johnson [22] reported VW disease in potato plants co-inoculated with three isolates from potato, mint and tomato was highly comparable to plants who received a single inoculation with the potato isolate. Interestingly, Schnathorst and Mathre [26] found cross-protection on cotton when co-inoculated with virulent D and mildly virulent ND isolates. Cross-protection was more often associated with co-inoculations of avirulent and virulent isolates. Shittu et al. [25] reported the VW disease scores were reduced by half on tomato plants co-inoculated with the highly virulent Vd1 and endophyte Dvd-E6 isolates compared with those recorded from single-inoculated tomato with Vd1. Sunflower challenged prior with the avirulent isolate P6 was protected from sequential inoculation with a virulent isolate of VM, *V. dahliae*; however, simultaneous inoculation of the two isolates did not result in different disease expressions [24].

We proposed that the D and ND isolates were able to colonise cotton stems equally due to the even number of isolates recovered from the same field-sampled stems. However, in our pot trials, the recovery frequency of D and ND isolates from the confirmed co-infected stems were predominated by one to the other. Mercado-Blanco [19] directly detected both D and ND isolates from co-inoculated olive roots at 0, 7 and 21 days after inoculation; however, DNA directly obtained from stems of the same plants were only positive with the D marker. Similarly, Shittu et al. [25] suggested that tomato plants colonised by the endophyte isolate Dvd-E6 ameliorated the effectiveness of colonisation of the pathogenic isolate Vd1. We postulated that vegetative growth competition may play a minor role in co-infection and co-colonisation of the tested *V. dahliae* isolates in co-inoculated plants since there were no negative effects of one to the other detected in the dual culture assays. Fecundity and the spore germination rate of the tested isolates could partly play a role in driving this predominant colonisation. For example, the isolate 19V76 produced significantly more spores than 19V77; subsequently, in the two pot trials, the isolate 19V76 was recovered more than the isolate 19V77 in co-inoculated plants. However, we did not see a similar pattern in plants co-inoculated with the L41 and L42 isolates, though L41 produced a higher number of spores which had double the germination rate compared with L42. It is not possible to offer any conclusive recommendations from the current data set since host plant responses also play an important role in a successful colonisation of the pathogen. It will be worth using green fluorescent protein (GFP)-tagged isolates to better understand the interactions between *V. dahliae* isolates as well as with the host in planta. GFP-tagged *V. dahliae* was studied to understand its capacity to colonise cotton cultivars with different degrees of susceptibility [28].

The occurrence of both D and ND pathotypes within a single cotton plant under field conditions has also raised concerns regarding the development of disease management strategies. First, commercial Australian cotton germplasms exhibited varietal responses to D and ND pathotypes differently, and to date, there has not been a highly resistant cultivar against both pathotypes, especially to the D pathotype (C. R. Trapero per. comm.). Therefore, there will be limited cultivars available for *V. dahliae*-infested fields where the co-occurrence of both D and ND pathotypes in cotton plants were detected. However, before this raises an alarm, a thorough assessment of the damage that co-infection may cause should be pursued. The co-infection incidence under natural field conditions was detected at a low level in the previous two seasons. We continue to carry out disease surveillance, which has been ongoing for over 30 years in NSW, to enable the monitoring of the occurrence and distribution of the co-infection of both D and ND pathotypes on cotton. Second, genetic combination was also questioned. Wheeler and Johnson [22] recently reported the putative anastomosis in planta when mustard plants were co-inoculated with three isolates: potato 653, mint 111 and tomato 461. Therefore, understanding the diversity of the isolate collection recovered from co-infected cotton in this study will warrant future research.

4. Materials and Methods

4.1. Sampling and Isolating

During the 2017/18 and 2018/19 survey seasons, cotton stems from VW-suspected plants were sampled for further confirmation through isolation and identification of the actual causal agent. Where possible, at least three VW-suspected stem cuts, approximately 10–15 cm long, were sampled from each of the surveyed fields. These stem cuts were double-bagged inside a paper bag and another outside zip-lock plastic bag. The stem cuts were stored in an esky (a portable cooler) away from direct sunlight during the survey trips and immediately transferred to a 4 °C fridge/cold room after each trip until further processed.

Isolation of the putative pathogen was initiated by excising each of the stem cuts into smaller sections, 1–2 cm long, and peeling off the outer bark. Under aseptic conditions, each of the peeled sections was sprayed and left for 30 s with 70% ethanol for surface decontamination. The sprayed section was then plotted dry with paper towel and split in half using a sterile scalpel. Inner vascular discoloured tissues (wood chips) were thinly sliced with the scalpel and embedded into sPDA. sPDA was made of potato dextrose agar (PDA Difco) amended with 100 ppm streptomycin sulfate (Sigma Aldrich) and contained in Petri plates. The plates with embedded vascular tissues were sealed with parafilm and incubated at 25 °C in the dark for 3–5 days. Putative fungal colonies emerging from vascular tissues were individually sub-cultured onto new sPDA plates and single spore cultures were established. Pure cultures were then transferred onto half strength sPDA and incubated at 25 °C in darkness for at least a week before small plugs (0.5 cm^2) were excised from the colony margins, submerged in sterile water and stored at room temperature for subsequent experimentation.

4.2. Pathotyping by Duplex PCR

4.2.1. DNA Extraction

Genomic DNA was obtained using a Wizard® Genomic DNA Purification Kit (Promega, Sydney Australia) following the manufacture's protocol. However, slight modifications were deployed to suit our laboratory conditions. A small amount of mycelia (10–100 mg) was scraped off culture plates and transferred into a 1.5 mL tight-lock Eppendorf tube. Then, 50 µL Nuclei Lysis Solution was added along with two steel beads (3.3 mm dia.) to each tube and shaken to macerate the mycelia on a tissue lyser (Retsch® MM300) for 1 min at a frequency of 28 times per second. Another 450 µL Nuclei Lysis Solution was then added to each of the tubes, vortexed to homogenicity and followed by incubation in a water bath at 65 °C for 30 min. After incubation, 3 µL RNase A Solution was added to each of the tubes and followed by another incubation at 37 °C for 15 min. After cooling down at room temperature for 5 min, 200 µL Protein Precipitation Solution was added and vortexed vigorously to homogenicity and followed by a centrifugation at 13,000 rpm for 5 min. The supernatants were carefully transferred to new 1.5 mL Eppendorf tubes containing 600 µL room temperature isopropanol. The tubes were gently inverted and centrifuged at 13,000 rpm for 1 min. The supernatants were carefully decanted and the visible DNA pellets were washed twice with 70% room temperature ethanol. The DNA pellets were then air-dried under a fume hood for 30–45 min, rehydrated with 50–200 µL DNA Rehydration Solution depending on the size of the pellets and followed with an incubation at 65 °C for 1 h. The DNA solutions were then stored at −20 °C until use.

4.2.2. Duplex PCR Amplification

A duplex PCR assay developed by Mercado-Blanco et al. [19] was deployed to simultaneously characterise the two defoliating (D) and non-defoliating (ND) pathotypes. All PCR amplifications were carried out using GoTaq® G2 Green Master Mix (Promega). Each PCR mix contained: 10 µL of Green Master Mix, 8 µL of DNase free water, 1 µL of 10 mM primer mix and 1 µL of DNA template. DNase-free water was included as a negative (no-template) control. The primer mix included 3 portions of DB19 (5′-CGGTGACATAATACTGAGAG-3′), 2 portions of DB22 (5′-GACGATGCGGATTGAACGAA3′) and 1 portion of espdef01 (5′-TGAGACTCGGCTGCCACAC-3′). PCR cycling conditions were slightly modified from Mercado-Blanco et al. [19] as follows: initial denaturation for 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 90 s at 72 °C, with a final elongation step of 7 min at 72 °C. The PCR products were run at 100 V in a GelRed (GeneTargetSolutions) pre-stained 1.5% agarose gel for 45 min and visualised under UV light using a UVIDOC HD6 (UVITEC Cambridge). D pathotype isolates were predicted to contain two visible PCR amplicons at sizes of 539 and 334 bp, whereas ND pathotype isolates contained a single amplicon at a size of 523 bp [19].

4.3. Pathogenicity

Root-dip assays were conducted twice in glasshouse conditions to assess the virulence of D and ND isolates solely and in combination. Two isolates from each of the pathotypes were selected for the pathogenicity assays (Table 3). Both D and ND isolates recovered from the corresponding year were isolated from a single stem.

Table 3. A brief description including pathotypes, single-spored status, location and year of recovery of *V. dahliae* isolates from cotton used in pathogenicity assays.

Isolates	Pathotypes [1]	Single Spored	Location [2]	Year of Recovery
L41	ND	Yes	Merah North, Namoi, NSW	2018
L42	D	Yes	Merah North, Namoi, NSW	2018
19V76	D	Yes	Baan baa, Namoi, NSW	2019
19V77	ND	Yes	Baan baa, Namoi, NSW	2019
L41 + L42	Mixed	Mixed	Merah North, Namoi, NSW	2018
19V76 + 19V77	Mixed	Mixed	Baan baa, Namoi, NSW	2019

[1] Pathotypes including D and ND were designated using the duplex PCR developed by Mercado-Blanco et al. [19]
[2] There are five main cotton growing valleys in NSW, including Gwydir, Namoi, Macquarie, Lachlan and Murrumbidgee valleys.

Cotton black seeds (cv. Sicot 75RRF, a VW susceptible cultivar) were sown individually in a 100-cell plastic seedling tray containing Searles® potting mix and grown in a glasshouse at 10–25 °C for 15 days prior to inoculation. Upon inoculation, seedlings were gently removed from the seedling trays and washed free of the potting mix. The washed seedlings were root-dipped in conidial suspensions of the four tested isolates at the concentration of 10^4 conidia/mL for five minutes and then transferred into 140 mL pots containing Searles® potting mix. There were two additional co-inoculum mixtures of L41 and L42, and 19V76 and 19V77. Conidial suspensions were mixed and adjusted to 10^4 conidia each/mL. There were two seedlings per pot and five and four replicate pots for each of treatment, respectively, in trial 1 and 2. Control seedlings were treated in the same manner; however, the spore suspension was replaced with sterile water. Inoculated seedlings were maintained in the same glasshouse conditions and monitored for disease occurrence and severity. Disease ratings were as follows: 0 = no symptoms, 1 = chlorosis and wilting of cotyledons, 2 = chlorosis and wilting of first true leaf, 3 = symptoms on lower 50% of the foliage, 4 = symptoms on 51–100% of the foliage and 5 = dead plant.

Upon termination of the pathogenicity assays, all plants were subjected to re-isolation of the inoculated pathogens. The isolation of the inoculated *V. dahliae* from the collar sections was as described previously. All recovered isolates were also subjected to the duplex PCR again for determination of the pathotypes.

4.4. Growth Competition Assays

Relative growth of the four tested isolates was assessed singly at 25 °C. Briefly, actively growing *V. dahliae* cultures on sPDA were excised into 0.5 cm^2 plugs and transferred into the centre of new sPDA plates. Additionally, a dual cultures technique was undertaken to assess the growth competition of the *V. dahliae* isolates under the influence of one to another. As previously, active cultures of L41 and L42, and 19V76 and 19V77 were excised, transferred in pairs together and placed 5 cm apart onto new sPDA plates. All newly transferred plates were sealed with parafilm and incubated in darkness at 25 °C for seven days before the colony dia. was recorded in perpendicular directions. There were three replicate plates per each isolate/pair and the whole assay was repeated once.

4.5. Fecundity and Germination Assessments

Ten-day-old cultures growing at 25 °C in darkness were subjected to spore collection for fecundity and germination assessments. There were three plates per isolate for the fecundity examination. Each plate was flooded with 10 mL of sterile water and gently interrupted with a disposable L-shaped spreader. The spore concentration per mL of the collected suspension was determined using a haemocytometer. The spore suspension was then adjusted to 10^4 per mL for the germination assessment. Three 10 µL aliquots from each of the spore suspensions were individually spread onto clean sPDA plates (n = 9 per isolate). The plates were then incubated at 25 °C in darkness for two days, after which the colonies of *V. dahliae* were recorded. The experiment was repeated once.

Data collected from the pathogenicity, growth rate, fecundity and germination assays were subjected to an analysis of variance (ANOVA) and the separations of means were determined by Tukey's least significant difference (LSD) test ($p \leq 0.05$). The ANOVA and graphing were performed with Graphpad Prism 8.2.0.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/6/750/s1, Table S1: Relative number of D and ND pathotypes[1] recovered from the confirmed co-infected cotton plants in pot trial 1 and 2.

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Has Alternaria leaf spot become a major concern in your cotton fields?

By Dr Duy Le – NSW DPI, Australian Cotton Research Institute

ALTERNARIA leaf spot (ALS) on cotton has become a major concern in recent years. ALS outbreaks were reported on seedlings in southern New South Wales (NSW) in the 2017–18 season (Figure 1), and thereafter a couple of mature cotton fields in Forbes were heavily infested with ALS in the 2018–19 season (Figure 2). The disease was observed again on seedlings across NSW during our early season survey in the 2019–20 season.

ALS symptoms start with pinhead necrotic lesions surrounded by a purple halo. Under favourable conditions, lesions continue to enlarge and coalesce to form irregular shapes. Severe infection may result in blight and desiccated cotyledons/leaves (Figure 3).

Historically, ALS was considered a minor issue in cotton production across Australia. But the disease has increased in prevelance in some cotton growing regions following a run of favourable season and conditions. Whether this increased prevalence correlates to yield is not yet known. In the early 2000s, ALS was prevalent (100% incidence) in trials in Northern Australia. Pima cotton is much more susceptible, and yield losses were insignificant, even in the absence of fungicide, as infection occured after crop cut out. Pima cotton is much more susceptible, and yield losses from ALS have been recorded on Pima cotton in other countries such as up to 40 per cent in Israel, 25–37 per cent in India, and 22–33 per cent in Zimbabwe.

Our pot trials revealed that cotton seedlings infected with ALS accumulated around 25 per cent less biomass compared to healthy control seedlings. Cotton is highly effective in compensating for leaf damages, particularly during seedling stages, but slow accumulation of biomass can translate into delayed maturity.

Further studies to determine the relationship between ALS of cotyledons and cotton yield under Australian conditions are required. In Australia, though yield loss caused by ALS have been considered economically insignificant, this needs reevaluation in the current climate, with new varieties and across various crop rotations.

It is warranted to continue to monitor ALS over multiple years to ascertain the impact of ALS on cotton yield and the cotton farming systems. Most studies have found that Pima cotton is more susceptible to ALS than upland cotton, but currently no Pima cotton cultivars are commercially available in Australia. So it is assumed that ALS pressure on upland cotton in Australia will be minimal. After recent outbreak events on upland cotton, it was questioned if these were associated with a different pathogen.

Alternaria leaf spot pathogens

We collected enough morphological, molecular and pathogenicity evidence to confirm that *Alternaria alternata* was the main causal pathogen responsible for recent ALS outbreaks on seedlings in southern NSW. *A. alternata* only became dominant later in the season when grown in northern Australia.

On the other hand, *A. macrospora* has been reported as a main causal agent of ALS on Pima cotton in Australia. Cotton cotyledons were also found more susceptible to *A. macrospora* infection. But these research findings were limited to Katherine in the Northern Territory where significant differences in climatic conditions compared with southern NSW are likely to influence ALS pathogen populations. *A. macrospora* has also been reported as the main ALS pathogen of cotton in Israel and India.

FIGURE 1: ALS on cotton seedlings in southern NSW – cotyledons were severely infected

FIGURE 2: A mature cotton field in Forbes was severely infested with ALS after a chilling event in the 2018–19 season

FIGURE 3: Severe infection of ALS resulted in desiccated cotyledons (A), and mature leaf (B)

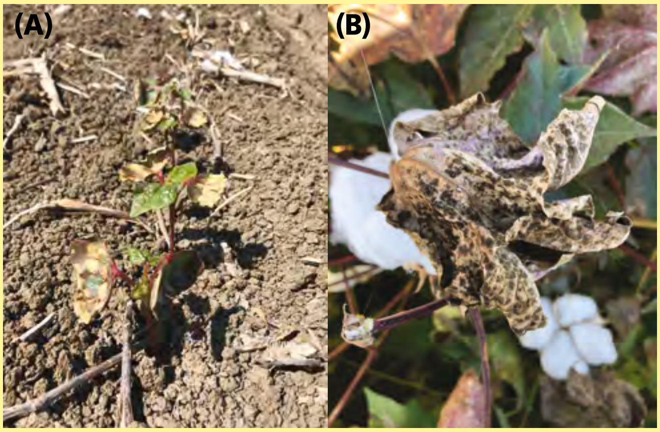
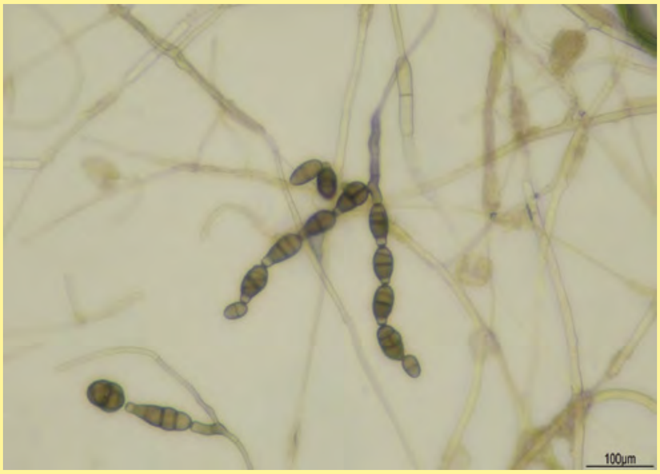


FIGURE 4: Typical chains of conidia of *A. alternata* produced on synthetic media



In Israel, severity of *A. macrospora* infection on Pima cotton was observed during wet summer months when temperatures were around 20 to 33°C, which did not align with usual growing conditions early in the season in southern NSW where average temperatures below 20°C were recorded in the 2017–18 season. But occurrence of ALS on upland cotton cotyledons in southern NSW were possibly due to the susceptibility to *A. macrospora*.

It has historically been considered that *A. macrospora* was more prevalent early in the season and declined significantly towards the end of the season. But in the past two seasons we have not recovered any isolates similar to *A. macrospora* morphologically and genetically. Instead, *A. alternata* was the main fungal pathogen recovered from ALS diseased cotyledons, leaves and bolls in the past two seasons. Additional data over multiple seasons are required for better understanding about the roles of *A. macrospora* and *A. alternata* on ALS of cotton in Australia.

Alternaria alternata belongs to section Alternata, which produces small spores (conidia) frequently in chains (Figure 4). Conidia are asexual reproductive structures and known as primary infection sources. Conidia are ovate to obclavate in shape, divided by transverse and vertical walls. Conidia with conical or cylindrical beaks range from 10 to 25 µm in length and 4 to 10 µm in width. *A. alternata* is a cosmopolitan saprophyte and pathogen.

Currently, seven pathotypes have been identified which differ in their production of host-selective toxins during conidia germination prior to penetration of the host plant. But pathotypes of the *A. alternata* which we recovered from cotton have not been identified and it is not known if it produces any toxins similar to the known ones. Such knowledge will provide insight into the pathogenicity of cotton *A. alternata* as well as towards selection of resistant lines.

Alternaria alternata is an airborne pathogen, but it can survive saprophytically in soil and plant debris. The pathogen has been frequently reported causing pre and post-harvest rot(s)/blight(s) of more than 100 host species. But strains with host specificity have also been recorded. Our in vitro study revealed that *A. alternata* had the optimal growth temperature at 25°C, but it can grow in a wide range of temperature from 5–35°C. This growth pattern was highly similar to *A. alternata* isolates recovered from cotton in New Mexico, USA. Interestingly, in our bioassays conducted on detached cotton leaves, the *A. alternata* appeared more virulent at 20°C than it did at 25°C.

Other researchers also found that cotton plants which experienced short-term pre-chilling stress at the temperature between 8–20°C were more prone to infection and damage of *A. alternata* compared with those grown at 20–28°C. In Australia, we also noticed that ALS on cotton often occurred more prevalent after chilling events such as heavy dew or rainfall. But the infection and severity of ALS on cotton are also highly dependent on inoculum loads and susceptibility of the cotton host. Such knowledge is essential for ALS management, but little is known.

Management

Like many other diseases, successful management of ALS on cotton requires an integrated approach pre, during and post growing season. Good field preparation by removing trashes, alternative hosts and volunteer cotton will help to reduce primary inoculum sources. Adoption of good tolerant/resistant cultivars is also common practice to manage ALS, but there are relatively sparse data on the susceptibility of current commercial upland Australian cotton germplasms to ALS caused by *A. alternata*. A number of fungicides are registered against *A. alternata* on horticultural crops. But ALS has been long considered a minor disease of cotton in Australia, so there are no registered fungicides for use in cotton.

An emergency permit was granted for use of mancozeb and tebuconazole in response to the outbreak of ALS in southern NSW in 2017–18. We recommend that care should be taken with fungicide applications since *A. alternata* is prone to develop fungicide resistance and late season yield impact has still not been proven. Our initial in vitro assessments found that *A. alternata* was less sensitive to a couple of tested fungicides than we expected. CRDC and NSW DPI are currently funding a project (DAN1703-Innovative Solutions to Cotton Diseases) to search for alternative control options against some major cotton diseases. Given the uncertainty about yield, we are also planning research to address whether control is warranted.

The ALS disease is a high priority. We are working with chemical suppliers to assess a number of fungicides as well as novel compounds for their in vitro control efficacy against *A. alternata*. In collaboration with Tim Green, a southern based cotton pathologist, we will further assess a number of promising candidates under field conditions at Yanco research station. *A. alternata* can survive saprophytically on crop residues, therefore burying ALS infected residue could accelerate decomposition and subsequently reduce inoculum loads.