FINAL REPORT
TEMPLATE

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Project Title: Transformation of *Verticillium dahliae* causal agent of Verticillium wilt of cotton with the GFP gene

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 Reports (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: 30/11/2021

Part 2 - Monitoring & Evaluation

This data forms part of CRDC's M&E data collection. Please complete all fields and add additional rows into each table if required.

Achievement against milestones in the Full Research Proposal

Milestone	Achieved/ Partially Achieved/ Not Achieved	Explanation
1.1 Literature review and research plan	Achieved	Literature review completed outlining Verticillium infection pathway research studies previously conducted.
1.2 Transformation protocol	Achieved	A practical and feasible protocol for transformation of Australian *Verticillium dahliae* was adapted from existing methodologies for Fusarium species in combination with the review of published protocols.
2.1 Transformation of *Verticillium* with the green fluorescent protein (gfp) gene	Achieved	Successfully labelled *V. dahliae* with GFP gene informed by milestone 1.2.
2.2 Using confocal laser scanning microscopy, the	Partially Achieved	The stability of the GFP-tagged non-defoliating (ND)

infection, colonisation, virulence and host plant resistance of isolates from different strains/virulence quantified.		*V. dahliae* strains was confirmed prior to successful studies into the infection and colonisation of Australian cotton using confocal microscopy. From candidate *V. dahliae* strains, successful transformants were exclusively from non-defoliating strains. Therefore, differentiation in infection and colonisation between D and ND isolates could not be assessed.
3.1 Colonisation of *V. dahliae* in cotton seeds	Achieved	In collaboration with University of Queensland (Prof. Elizabeth Aitken) experimentation was completed March-September 2021. Direct stem inoculation did not facilitate a systemic infection and translocation of GFP-tagged *V. dahliae*; hence, *V. dahliae* was not visualised in the seeds
3.2 Colonisation of cotton weeds	Achieved	Eight weed species important to the cotton farming system were sourced from NSW cotton production areas was made available to University Queensland in early 2021 and experimentation was completed between March-September 2021. GFP-tagged *V. dahliae* was able to infect five weed hosts confirming through conventional isolation. Visualisation of the infection under the confocal microscope was only detected in *N. banthamiana* host.

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

Output	Description
Literature Review	Literature Review entitled *Verticillium wilt: Epidemiology and host interaction of a severe phytopathogen affecting cotton (Gossypium hirsutum)*
Thesis's	**Gregson, A (2019)** Transformation of a non-defoliating strain of *Verticillium dahliae* with the green fluorescent protein, and its colonisation on Upland cotton **Morrison, S (2021)** Fluorescent proteins as a tool for studying *Verticillium dahliae* on cotton (*Gossypium hirsutum*) and associated weed species
Presentations	**Gregson, A (2020)** Cotton, Verticillium wilt and a jellyfish: GFP-tagged *V. dahliae* as a tool in the study of plant-pathogen interactions. Online seminar run by Australian Association of Cotton Scientists, 8th October 2020 **Morrison et al. (2021)** Analysis of infection by *Verticillium dahliae* in cotton seed and weed species from cotton-producing regions of Australia. Australasian Plant Pathology Society Online Conference, 23-26th November 2021
Honours Scholarship	Ms Gregson completed Honours scholarship investigating GFP-tagging techniques in the study of Verticillium wilt pathogen on Australian cotton

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

Outcome	Description
Industry capacity building	Researcher develops greater knowledge of disease epidemiology and cotton host interaction of the Verticillium wilt pathogen by completing an Honours degree
Adoption of research methods and techniques by other researchers	The use of GFP-tagged strains generated in this project have been incorporated into RNA based pesticide research lead by Professor Neena Mitter at the ARC Research Hub for Sustainable Crop Protection
Collaboration	Collaboration between research partners; University of Queensland, Queensland Department of Agriculture and Fisheries, and NSW Department of Primary Industries on next phase of GFP-*V.dahliae* experimentation and screening of Australian cotton cultivars and host weeds species research.

Part 3 – Technical Report 1

Gregson, A (2019) Transformation of a non-defoliating strain of Verticillium dahliae with the green fluorescent protein, and its colonisation on Upland cotton

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Executive Summary

Verticillium wilt caused by the soilborne fungus *Verticillium dahliae*, is one of the most challenging and economically significant diseases of cotton in Australia and worldwide. Host resistance is regarded as the most effective control strategy, however the biological complexity of the pathogen and controversy regarding the mechanisms of resistance hinder plant breeding efforts. Previous studies have utilised GFP-tagged isolates of *V. dahliae* to investigate the host – pathogen interaction on cotton, providing insights into the host resistance response and little understood areas of the disease cycle. Here, we establish GFP-tagged isolate Vd71-3 as a tool for evaluating infection and colonization on cotton cultivars tolerant and susceptible to Verticillium wilt. Isolate Vd71-3 was obtained by transforming a GFP vector construct into highly virulent non-defoliating strain Vd71171 isolated from a diseased Upland cotton plant in NSW, Australia. Prior to study on cotton, pathogenicity of Vd71-3 was deemed consistent with that of the parent wildtype, indicating that GFP expression does not dramatically alter virulence. Confocal laser scanning microscopy observations confirmed existing descriptions of early infection on cotton, including germination of conidia by 24 hours post-inoculation, formation of an infection peg, intercellular colonisation of the root tips but not lateral root junctions, preferential colonisation of the xylem vessels, and acropetal movement of conidia in vessels. Extensive fungal occlusion of the vessels was also observed, not previously captured elsewhere on cotton.

V. dahliae was recovered from six of the eight weed species that were inoculated with the transformed VCG 1A and 2A strains. The VCG 2A transformant was recovered more frequently from weeds than the VCG 1A transformant, suggesting that *V. dahliae* VCG 2A may have higher infectivity towards weed hosts in Australian cotton fields. *V. dahliae* was not recovered from seeds from cotton plants that were subject to direct stem inoculation, although vascular tissue adjacent to the site of inoculation was colonised. Further investigation is needed to understand whether *V. dahliae* VCG 1A and 2A strains are capable of infecting Australian cotton seed using alternative inoculation techniques.

Technical report 1
Introduction

Cotton (*Gossypium* spp.) is the most widely produced natural fibre in the world, grown in over 100 temperate and subtropical countries including Australia. In Australia, cotton is grown predominantly under irrigated conditions between the Tropic of Capricorn in Queensland (Qld) and south-west New South Wales (NSW). High quality lint and cottonseed is produced for export, generating on average a gross value of production of $2 billion Australian dollars (Cotton Research and Development Corporation, 2014).

The four cultivated species of cotton belong to the genus *Gossypium*. Of these, *Gossypium hirsutum*, commonly referred to as Upland cotton, is high yielding and widely adapted to diverse growing regions and consequently predominates the total cotton-planted area globally (Zhang et al., 2014). The high prevalence of Upland cotton in the farming system results in low genetic diversity across growing regions. For this reason, pest and disease pressure poses a significant constraint on cotton production worldwide. Amongst those diseases, Verticillium wilt is one of the most challenging and economically significant (Klosterman et al., 2009).

In Australia, Evans and Paull (1967) indicated that Verticillium wilt was first reported in the Namoi Valley of NSW in 1959. Since then, Verticillium wilt has been reported in all the major cotton growing regions of NSW and Qld (Smith, 2020). Over a 5-year period, Cotton Seed Distributors Ltd. estimated yield losses of between A$1.9 and A$3.8 million per season were attributable to Verticillium wilt in NSW (Anon, 2011). New detections in previously non-infested fields, farms, or regions continue to be reported (D. P. Le, personal communication, September 1, 2019). In severe cases where field inoculum load is high and infection widespread, cotton cropping is discontinued.

The ubiquitous phytopathogen *Verticillium dahliae* is the causal agent of Verticillium wilt and is typified by its high degree of genetic diversity and ability to infect over 400 plant species (Efsa Panel on Plant Health, 2014). *V. dahliae* is a soilborne, vascular wilt fungus possessing two spore stages, conidia and microsclerotia, both of which are asexually produced and lead to infection. Conidia are hyaline, ovoid and single celled (Inderbitzin et al., 2011). Microsclerotia are thick walled, compact aggregates of individually melanised cells which serve as the long-lived primary inoculum of Verticillium wilt. The life cycle of *V. dahliae* has been described by Klimes et al. (2015) and follows three major phases: a (1) dormant, (2) parasitic and (3) saprophytic phase. In the dormant phase, plant root exudates stimulate the germination of microsclerotia, forming germ tubes, then hyphae, which extend toward host roots. Extensive hyphal networks are established on the root surface, from which fungi penetrate directly through the epidermal tissue and intercellularly in the cortex, thus initiating the parasitic phase. Hyphae breach the endodermis to reach the root vasculature, preferentially colonising the xylem. Despite being far less nutritionally-rich than the phloem, the low osmotic pressure and dead tracheary elements of the xylem are easily accessed, making the xylem a niche for vascular wilt pathogens including *V. dahliae* (Yadeta & Thomma, 2013). From here, the fungus proliferates in the above-ground vasculature. Budding produces conidia throughout the parasitic phase; these conidia are carried in the transpiration stream where they germinate and initiate more localised sites of colonisation. It is accepted in the literature that the colonisation of the vascular elements coincides with the appearance of external symptoms. Disease symptoms on Upland cotton

include vascular browning, stunting, leaf chlorosis and necrosis, flower and boll abscission and defoliation (Klosterman et al., 2009). The third and final phase of the disease cycle involves the production of microsclerotia on dead or dying tissues (Schnathorst, 1981). Inoculum is deposited into the soil as crop residues decompose, and may be further distributed by farming practices such as cultivation and overland irrigation (Baroudy et al., 2018), or disseminated through infected weed hosts (Evans, 1971; Johnson et al., 1980). Control is confounded by the pathogens longevity in the soil, expansive host range and a lack of effective disease management strategies (Klosterman et al., 2009).

Little is understood regarding the pathogen's population biology; however genetically distinct populations are divided into vegetative compatibility groups, as well as pathotype such as on cotton and olive (Jiménez-Díaz et al., 2011; Klosterman et al., 2009). Vegetative compatibility groups (VCGs) refer to isolates which can undergo genetic exchange with each other (Puhalla & Hummel, 1983). The phenomenon of vegetative compatibility in fungi was hypothesised to have evolved to curb the spread of mycoviruses between fungal populations (Caten, 1972). In doing so the organism has effectively created subspecies groupings limiting genetic exchange. Since the first report of Verticillium wilt on cotton in the United States by Carpenter in 1914, (as cited by Zhang et. al, 2014) six VCGs affecting Upland cotton have been identified internationally (Pegg & Brady, 2001).

On olive and cotton hosts, *V. dahliae* is differentiated into two pathotypes: defoliating (D) and non-defoliating (ND) based on the isolates ability to cause defoliation of the green leaves and stems (Jiménez-Díaz et al., 2011; Schnathorst & Mathre, 1966). International literature affirms defoliating isolates exhibit greater virulence; developing earlier, more rapidly and incurring more significant yield losses than the non-defoliating pathotype. In contrast, the non-defoliating pathotype is found in several VCGs, more genetically diverse and more widely occurring (Friebertshauser, 1982; Jiménez-Díaz et al., 2017; Korolev et al., 2001; Pérez-Artés et al., 2000; Zhengjun et al., 1998). Total remission has been reported on olive infected with the non-defoliating pathotype (Jiménez-Díaz et al., 2017), and late-stage infection on cotton may cause no yield loss (Pullman & DeVay, 1982). However highly virulent isolates within the non-defoliating pathotype, equally virulent to defoliating isolates, have been characterised on Israeli cotton (Korolev et al., 2001).

At present, VCGs 1A, 2A, and 4B have been detected on Australian cotton (Chapman et al., 2016). Of these three VCGs, the predominating strain in Australia is VCG 2A, belonging to the non-defoliating (ND) pathotype (D. Le, personal communication, September 1, 2019). Additionally, 2A isolates are often recovered from severely affected cotton in NSW (Dadd-Daigle et al., 2020). Preliminary pathogenicity studies conducted by Smith et al. (2014) determined that VCGs 1A and 2A are highly pathogenic and VCG4B is mildly pathogenic on Australian cotton.

Cotton breeding in Australia has developed new Upland varieties with improved tolerance to Verticillium wilt, however Upland cotton remains susceptible to Verticillium wilt infection at any stage of growth from seedling to mature plant. Due to a lack of heritable immunity within the Upland cotton germplasm, the breeding of highly tolerant or resistant cultivars is being pursued through interspecific breeding (Zhang et al., 2014). However, the genetic complexity of the pathogen and a lack of knowledge regarding the mechanisms of resistance hinder plant breeding efforts. Additionally, the colonisation process of *V. dahliae* throughout the entire disease cycle has only recently begun to be investigated in cotton (Zhang et al., 2013) and other hosts (Wang et al., 2013;

Zhang et al., 2018). To do so, researchers have utilised fluorescent reporter proteins such as the green fluorescent protein (GFP) isolated from *Aequorea Victoria* to study filamentous fungi *in planta* (Lorang et al., 2001; Shimomura et al., 1962). Staining or tagging of fungi with chemical or biological markers, such as reporter proteins, is necessary to distinguish the organism-of-interest amongst the many bacterial and fungal organisms endemic to plant tissues. In contrast to histological staining techniques, however, high resolution micrographs of GFP-expressing fungi can be visualised using confocal laser scanning microscopy (CLSM), with minimal preparation of tissues.

A growing number of studies have utilised GFP-tagged *V. dahliae* to investigate resistant versus susceptible interactions on lettuce, olive, and cotton, providing insights into the host resistance response, and little understood aspects of the disease cycle (Carrero-Carrón et al., 2018; Vallad & Subbarao, 2008; Zhang et al., 2020). In a study of resistant versus susceptible lettuce cultivars, resistance was associated with the shedding of infected roots or confinement to individual xylem vessels (Vallad et al., 2008). In susceptible versus resistant cotton cultivars, comparable levels of *V. dahliae* were detected during root invasion, however the amount of *V. dahliae* detected during the vascular phase of infection was disproportionately higher in the susceptible than the resistant variety (Zhang et al., 2013). This suggests effective resistance against *V. dahliae* lies in the ability of the host to restrict entry to the vasculature, rather than restricting root invasion.

Agrobacterium tumefaciens-mediated transformation (ATMT) has proven to be an efficient transformation technique in filamentous fungi (Mullins et al., 2001). Modified forms of GFP, including enhanced green fluorescent protein (eGFP), with increased fluorescence, stability *in vivo* and a 'red shift' which raises the excitation maxima to 488 nm, are ideally suited to fluorescence microscopy (Lorang et al., 2001). To visualise whole-fungi *in planta*, strong constitutive expression, such as that achieved under the control of *Aspergillus nidulans* promoter translation elongation factor (TEF), can express GFP cytoplasmically in all fungal morphotypes (Mullins et al., 2001). Additionally, antibiotic resistance is included as a selective marker for transformant recovery. Before using GFP-*V.dahliae* isolates, pathogenicity should be assessed against the wildtype isolate to confirm genetically modified isolates are suitable for research.

Host resistance is currently the most viable option to control *V. dahliae* on Upland cotton. GFP-tagged *V. dahliae* has been established as an effective tool in the study of host-pathogen interactions, revealing novel insights into host response and pathogen biology. Much remains uncertain regarding the mechanisms of host resistance in cotton, and the extent and nature of *V. dahliae* colonisation *in planta*. Despite high levels of pathogenic variation present in *V. dahliae* populations, most studies use isolates from within the defoliating pathotype. This research project proposes to transform Australian isolates amongst the non-defoliating pathotype of *V. dahliae*, representative of endemic strains affecting Upland cotton, with the *GFP* gene, and establish their suitability for study on cotton through laboratory and glasshouse assays related to fungal growth and pathogenicity. Furthermore, this research will investigate the interaction of GFP-tagged *V. dahliae* on a commercially available tolerant and susceptible cotton cultivar.

Materials and method

Generation of fluorescent Verticillium dahliae

Preparation of Fungal Material

Three *V. dahliae* isolates, set out in Table 1, were provided by Dr Linda Smith (Department of Agriculture and Fisheries, Queensland) and stored in the Queensland Plant Pathology Herbarium collection. Vegetative compatibility grouping of isolate Vd71171 was performed by Professor Rafael M. Jiménez Díaz (University of Cordoba) using nit-m testing (Appendix 1). Isolate Vd71172 and Vd71181 pathotype established by PCR (Mercado-Blanco et al., 2003) conducted by Dr Linda Smith.

***Table 1**. Australian Verticillium dahliae isolates from G. hirsutum provided by Dr Linda Smith for experimentation*

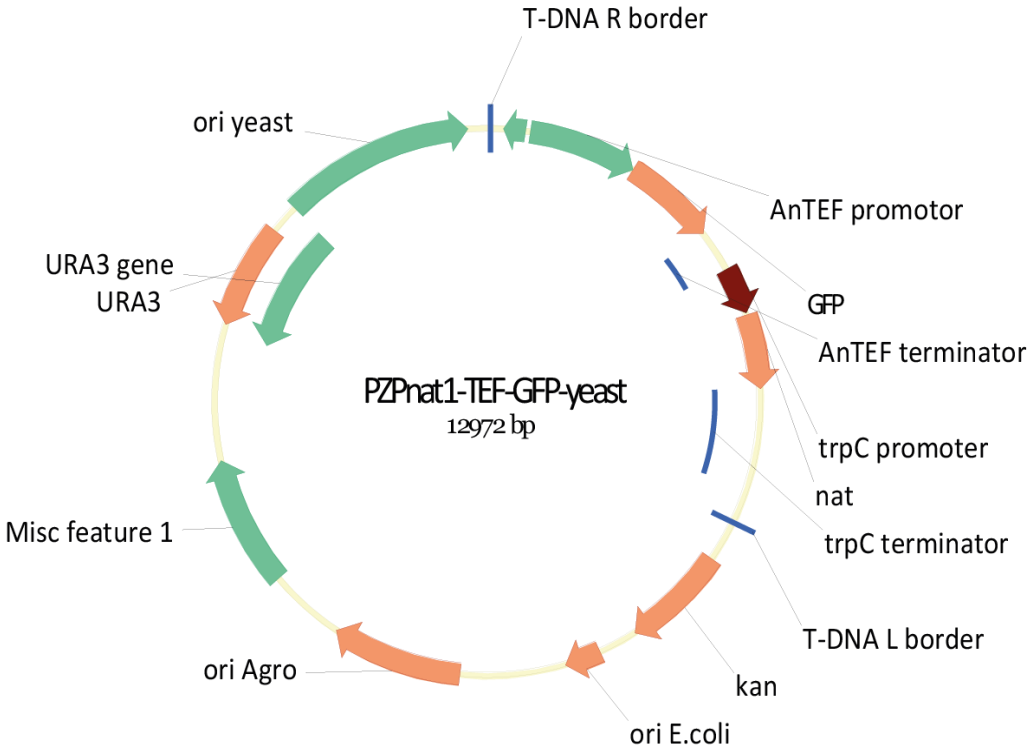
Isolate	BRIP	VCG	Pathotype	Locality	Host
V. dahliae "Vd71171"	71171	2A	Non-defoliating	Namoi Valley, NSW, Australia	*Gossypium hirsutum* (Upland cotton)
V. dahliae "Vd71172"	71172	-	Non-defoliating	Macintyre Valley, NSW, Australia	
V. dahliae "Vd71181"	71181	1A*	Defoliating	Gwydir Valley, NSW, Australia	

*Presumptive VCG based on pathotype

Single-spored isolates were recovered from water storage by plating onto potato dextrose agar (PDA Difco) and maintained at room temperature (approximately 22°C) in a 12 hour light/dark cycle for 2 weeks. Conidia were harvested by flooding plates with 10 mL sterile distilled water (SDW) and disrupting the surface mycelia with a no. 22 scalpel blade, followed by filtration through sterile Miracloth (Calbiochem, USA) and funnel. Spore concentrations were determined using a haemocytometer and recorded at concentrations of $1 \times 10^7 - 1 \times 10^8$ conidia/ mL. Aliquots of each isolate were either stored in 1.5 mL Eppendorf tubes at -80°C, or maintained at room temperature for further experimentation.

Fungal Transformation Vector

Fungal transformation vector pZPnat1-TEF-GFP was constructed by Dr Donald Gardiner (CSIRO Agriculture & Food), cloned in yeast, and maintained in *Escherichia coli* (Figure 1.). Within the T-DNA region, enhanced green fluorescent protein (eGFP) gene was placed under the control of highly expressed constitutive *Aspergillus nidulans* promotor translation elongation factor (TEF). The neourseothricin resistance gene was also included in the T-DNA region as the binary vector selectable marker.



Backbone		T-DNA Region	
ori yeast	Yeast origin of replication	**T-DNA R border**	Agrobacterium T-DNA right border repeat region
URA 3 gene	Cloning in yeast	**AnTEF promoter**	TEF promoter from *Aspergillus nidulans*
URA 3		**GFP**	GFP gene
Misc feature 1	Star region from plasmid VS1	**AnTEF promoter**	TEF terminator
ori Agro	Agrobacterium origin of replication	**trpC promoter**	trpC promoter from *Aspergillus nidulans*
Ori E.coli	E. coli origin of replication	**nat**	Neourseothricin resistance gene
Kan	Kanamycin resistance gene	**trpC terminator**	trpC terminator
		T-DNA L border	Agrobacterium T-DNA left border repeat region

Figure 1. *Fungal transformation vector pZPnat1-TEF-GFP-yeast containing Agrobacterium T-DNA region (T-DNA R border to T-DNA L border) on PZP series backbone. Annotation of features within the backbone and T-DNA region included in legend.*

Sanger sequencing of the T-DNA region of pPZPnat1-TEF-GFP to confirm the presence of the eGFP transgene was performed by the Australian Genome Research Facility (AGRF) using their purified DNA service following their guidelines with five different primers made available from the CSIRO Fungal pathogen primer collection (Table 2). Reactions were set up with 10 pmol of primer and either 2 or 4 µL of DNA from a mini-prep (equating to approximately 200 and 400 ng of DNA respectively) in a total volume of 12 µL. Sequence chromatograms were downloaded from AGRF and analysed in Contig Express (LifeTechnologies) with manual trimming of chromatograms for high quality regions prior to assembly against the expected pPZPnat1-TEF-GFP sequence.

***Table 2**. Description of primer sequences used in Sanger sequencing of TDNA region of pPZPnat1-TEF-GFP*

Primer name	Sequence	Target notes
DG1227	GACGCTGTGGATCAAGCAAC	Binds in the *A. nidulans* TEF promoter to sequence into the 5' end of eGFP
DG010	AAGCACTGCACGCCGTA	Binds in the middle of eGFP to sequence towards the 5' end of the CDS
DG720	ATGGTGAGCAAGGGCGAGGAGC	Binds across the start codon of eGFP to sequence into the coding sequence
DG898	TCTCGTTGGGGTCTTTGCTC	Binds close to the 3' end of eGFP sequence
DG134	GTAAAACGACGGCCAGT	M13f sequencing primer binds between repeat region 1 and the TEF promoter to sequencing into the TEF promoter

Triparental Mating

Two strains of *Agrobacterium tumefaciens*, AGL-0 and LBA4404 provided by Dr Donald Gardiner, were selected as candidate recipients for the target plasmid pZPnat1-TEF-GFP. *E. coli* conjugation helper plasmid RK2013 was selected to facilitate conjugation and transfer of the target plasmid into recipient Agrobacterium. Strains involved in triparental mating are summarised in Table 3.

***Table 3.** Cultured organisms utilised in triparental mating for uptake of target plasmid pZPnat1-TEF-GFP into recipient Agrobacterium, LBA4404 or AGL-0*

Organism	Strain	Resistance genes	Role
Escherichia coli	RK2013	Kanamycin	Helper plasmid
Escherichia coli harbouring pPZPnat1-TEF-GFP	pZPnat1-TEF-GFP-yeast	Neourseothricin Kanamycin	Bacterium harbouring target plasmid
Agrobacterium tumefaciens	AGL-0	Rifampicin Ampicillin	Recipient bacterium
Agrobacterium tumefaciens	LBA4404	Rifampicin Streptomycin	Recipient bacterium

Each of the Agrobacterium and *E. coli* cultures (n = 4) were grown for 24 hours on Luria broth (LB) (PD Difco) at 28°C on an orbital shaker (180 rpm), then pelleted down and resuspended in 5 mL of LB. Aliquots of 100 µL from each culture were vortexed in a sterile Eppendorf tube before plating onto solid LB without selection using a sterile L-shaped spreader, in duplicate. Control cultures and triparental mating cultures are set out in Table 4. Cultures were incubated for 24 hrs at 28°C in the dark.

***Table 4**. Combinations of bacterial strains used in triparental mating. Aliquots of each culture were combined as follows in control and triparental mating cultures to obtain cultures of A. tumefaciens with T-DNA region from pZPnat1-TEF-GFP.*

Control cultures	**Triparental mating cultures**
E. coli conjugation helper RK2013 + *E. coli* harbouring pZPnat1-TEF-GFP	*A. tumefaciens* strain AGL-0 + *E. coli* conjugation helper RK2013 + *E. coli* harbouring pZPnat1-TEF-GFP
A. tumefaciens strain LBA4404 + *E. coli* harbouring pZPnat1-TEF-GFP	*A. tumefaciens* strain LBA4404 + *E. coli* conjugation helper RK2013 + *E. coli* harbouring pZPnat1-TEF-GFP
A. tumefaciens strain LBA4404 + *E. coli* conjugation helper RK2013	
A. tumefaciens strain AGL-0 + *E. coli* harbouring pZPnat1-TEF-GFP	
A. tumefaciens strain AGL-0 + *E. coli* conjugation helper RK2013	
E. coli conjugation helper RK2013	
E. coli harbouring pZPnat1-TEF-GFP	
A. tumefaciens strain LBA4404	
A. tumefaciens strain AGL-0	

From successful triparental mating cultures, streak dilution was performed using a sterile inoculating loop onto yeast mannitol (YM) plates containing Rifampicin, Kanamycin, and either Ampicillin or Streptomycin according to the strain of Agrobacterium (see Table 3). Each antibiotic was used at a concentration of 100 mg / L. Plates were incubated for 48 hours in the dark at 28°C. Using a sterile pipette tip, a single colony was selected and added to 7.5 mL of LB plus antibiotics, as above, then incubated for 48 hours at 28°C on an orbital shaker (180 rpm) in the dark.

To confirm the presence of pZPnat1-TEF-GFP in Agrobacterium, plasmids were first purified using a QIAprep spin mini-prep-kit (QIAgen, USA) prior to undergoing plasmid digest. Reactions were set up with 1 µL of Cutsmart™ buffer (New England BioLabs) and 4 µL of DNA from the mini-prep with restriction enzymes *Nco*I-HF and *Bam*HI in a total volume of 10 µL, then analysed via electrophoresis on agarose gel in TAE buffer using 3 µL aliquots of each sample (Figure 2).

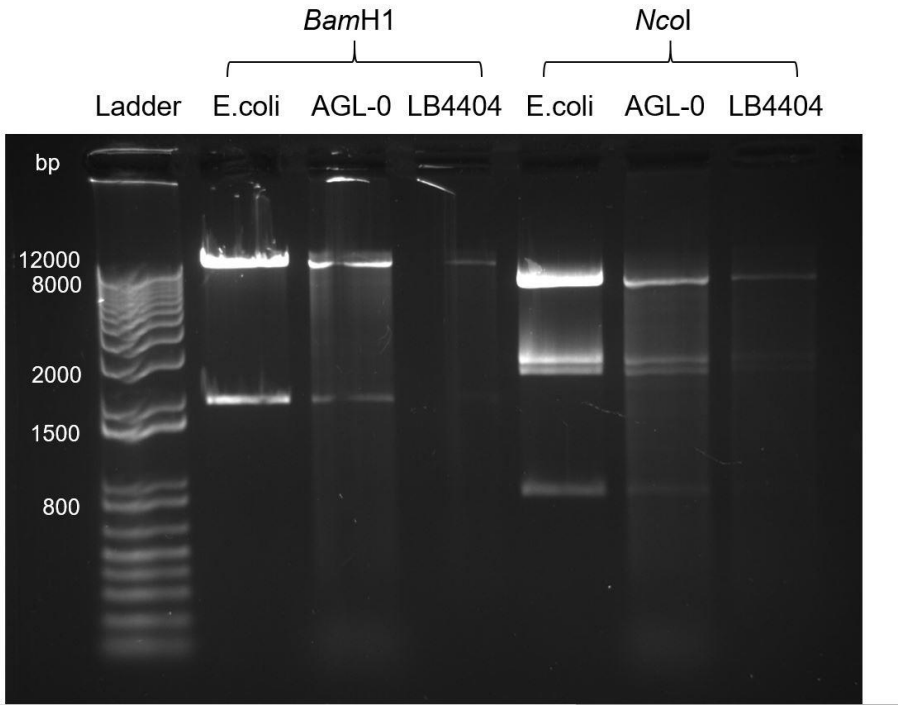


Figure 2. *Restriction digest of pPZPnat1-TEF-GFP in E. coli and Agrobacterium strains AGL-0 and LB4404, using restriction enzymes Bam*HI *and Nco*I*. Bands correspond to restriction sites (Bam*H1*: 8133, 11139; Nco*I*: 855, 2341, 2597, 7179) of pPZPnat1-TEF-GFP, confirming presence of the conserved plasmid in E. coli, as well as Agrobacterium strains AGL-0 and LB4404. 1 kb+ ladder (Invitrogen).*

Agrobacterium-mediated transformation

To confirm an optical density (OD) of 660nm after incubation, 1 mL of each Agrobacterium culture was transferred to a cuvette and the OD read using a spectrophotometer. In an Eppendorf tube, 1 mL from each culture was centrifuged for 1 minute at 13,000 rpm and resuspended in 20mL of sterile induction media (IM) prior to incubation for 6 hours at 28°C in a 100 mL conical flask. Co-cultivation of Agrobacterium and *V. dahliae* was then performed on induction media plus acetosyringone (IM + AS). Conidia were harvested from actively growing cultures of *V. dahliae*, or retrieved from storage at -80°C, as described previously. Using a haemocytometer or automated cell counter (Nexcelom Bioscience Cellometer®, ThermoFischer Scientific), spore suspensions were adjusted to a target concentration of 2 x 10^5 conidia / mL with SDW. A 1:4 ratio of *V. dahliae* to Agrobacterium spores per mL was spread across a plate using a sterile L-shaped spreader in two replicates, then sealed with Parafilm M® film and incubated for 48 hours at 22°C in the dark. After 48 hours IM + AS plates were overlaid with PDA amended with antibiotics at double concentrations to account for proliferation into the IM + AS media. Antibiotics Cefotaxime at 100 µg / mL, and Neourseothricin at 100 µg / mL were used for bacterial and fungal inhibition, respectively. Plates were then sealed with parafilm and incubated at 22°C in the dark for 10 – 14 days. After the incubation period, plates were screened using a blue-light torch and GFP filter glasses (NightSea, Lexington, MA, USA) to detect green fluorescence of putative transformants. Transformants were sub-cultured to PDA to obtain pure cultures and grown for at least 7 days in the dark. Small plugs (0.5 cm^2) were excised from the colony margin and submerged in SDW in 2.5 mL cryotubes and stored at room temperature.

For a full list of media and recipes see Appendix 1.

Colony appearance, Growth rate and Fecundity

Colony morphology was examined using a Nikon Eclipse Ci-L light microscope and recorded using a Nikon DS Fi3-L4 camera. Isolates Vd71171, Vd71-3, and Vd71-6 were inoculated onto 15 mL ½ PDA in five replicates and grown at 24°C in a 12 hour light / dark cycle to assess growth rate. Colony diameter was measured at a set time daily for 4 days post-inoculation. The mean increase in diameter per day, per isolate was calculated across five replicates and subjected to analysis of variance.

At 7 days post inoculation, plates were flooded with 15 mL of SDW and colonies disrupted with an L-shaped sterile spreader to free spores. Using a sterile syringe, 10 mL of solution was extracted from each plate and filtered through sterile Miracloth (Calbiochem) held within a funnel. The solution was then vortexed, and 10 µL extracted to determine the spore concentration using a haemocytometer. In *R*, a generalised linear model (with poisson regression) was used model the spore count data and emmeans applied for pairwise comparison.

Pathogenicity Assay

Plant material and Inoculation

Cotton varieties Siokra 1-4 and Sicot 714 B3F were selected to represent a susceptible and tolerant plant-pathogen interaction, respectively. Non-treated seed (lacking fungicide) of each variety was obtained from Dr Donald Gardiner (CSIRO), and Cotton Seed Distributors (CSD), respectively. Seed viability was tested prior to planting by germinating a random sample of 100 seeds on moistened paper towel. Cotton plants were raised from seed in seedling trays using Searles® premium potting mix in a temperature-controlled glasshouse for 10 days, until the emergence of true leaves.

Cotton seedlings of uniform height and growth stage were gently uprooted from seedling trays and the roots washed free of potting mix prior to root-dip inoculation. Inoculations were carried out by suspending 48 seedlings per treatment in 500 mL of a 1 x 10^5 conidia / mL suspension of either Vd71171 or Vd71-3 for 5 minutes, or in 500 mL of sterile distilled water as a control.

Inoculated and control plants were re-planted into 140 mm diameter pots of steam-pasteurised UQ 23 mix (see Appendix 1). Four plants per pot, 12 pots per treatment were included. Pots were placed on saucers and contained within a biohazard bag to capture run-off. Pots were randomly arranged across a single bench in a 25 ± 1°C temperature controlled PC2 OGTR-certified glasshouse. After inoculation, 6 g of MPK fertiliser (Osmocote®) was applied to the soil surface, and plants watered every 2 - 4 days.

Scoring and Isolation

Disease severity was scored using an ordinal rating scale of 0 - 5, adapted from Zhang et al. (2014) shown in Table 5. Plants were assessed for external symptoms at 4 weeks post-inoculation, according to the percentage of chlorosis, necrosis, and or wilt affecting true leaves. Results were

statistically analysed by two-way ordinal regression using ordinal package and ANOVA function from RVAideMemoire, which produces an analysis of deviance table with multiple comparisons of groups.

***Table 5**. Rating scale used to assess disease severity of cotton plants inoculated with V. dahliae*

Score	Description of symptoms
0	Healthy, no symptoms
1	1-20% total leaf area affected
2	21-40% total leaf area affected
3	41-60% total leaf area affected
4	61-80% total leaf area affected
5	81-100% total leaf area affected, and or plant death

At 4 weeks post-inoculation, all plants including the control were destructively sampled for *V. dahliae* re-isolation. First, a 10 - 15 cm segment from the base of each plant stem was sampled and transported to the laboratory in a sealed container. In a biosafety cabinet, plant sections were surface sterilised with 70% ethanol for 5 seconds, blotted dry and an approximately 5 mm long, 1 - 2 mm thick section from the plant vasculature excised using a sterile no. 22 scalpel blade. Where visible, darkened regions of discolouration within the vasculature were excised. Sections were embedded into PDA amended with 100 ppm streptomycin sulfate (Sigma Aldrich) sealed with Parafilm M® film and incubated in the dark at room temperature until colony growth could be observed. All soils and materials were disposed of according to OGTR requirements (NLRD IBC/1165/SAFS/2018).

Confocal Microscopy Experiment

Planting Material and Inoculation

Cotton varieties Siokra 1-4 and Sicot 714 B3F, selected to represent susceptible and tolerant varieties respectively, were raised from non-treated seed (lacking fungicide) in seedling trays using Searles® premium potting mix in a temperature-controlled growth cabinet until the emergence of true leaves (Figure 3). Seedlings of uniform height and growth stage were then gently uprooted from seedling trays and the roots washed free of potting mix prior to root-dip inoculation. Inoculations were carried out by suspending seedlings in 1 x 10^6 conidia/mL solution (as described above) of either Vd71171 or Vd71-3 for 5 minutes. Control inoculations were carried out by suspended seedlings in SDW for 5 minutes. Immediately following the inoculation procedure, two seedlings were re-planted per pot, 1 - 2 pots per time series. Plants were maintained at 24°C day / 20°C night temperatures and 16 hour light, 8 hour dark photoperiod and watered every 2 - 4 days.

Plant Sampling

Sampling of plant material for microscopy began at 4 hours post-inoculation (hpi), followed by 24 hpi, 5 days post inoculation (dpi), 7 dpi, and 10 dpi. Whole plants were gently uprooted, washed free of soil and wrapped in paper towel before being transported to the laboratory for sectioning. Using a

sterile double edged razor blade, 0.5 - 1 mm sections were excised by hand from the main root, root cap, lateral roots, lateral root junctions, basal stem, and petiole (Figure 3).

Confocal Microscopy

Transverse and longitudinal sections were mounted in distilled water on a microscope slide with cover slip for confocal microscopy. In some cases, whole root tips examined. A Zeiss 700 Laser Scanning Microscope was used to perform confocal microscopic examinations using the 488 nm laser to detect green fluorescence. Samples were examined within 12 hours of sectioning to avoid autofluoresence from plant phenolic compounds, or the decline of green fluorescent protein activity. Observations were repeated across two experiments. Images were processed and fungal structures measured using ZEN Blue 3.1 (Zeiss).

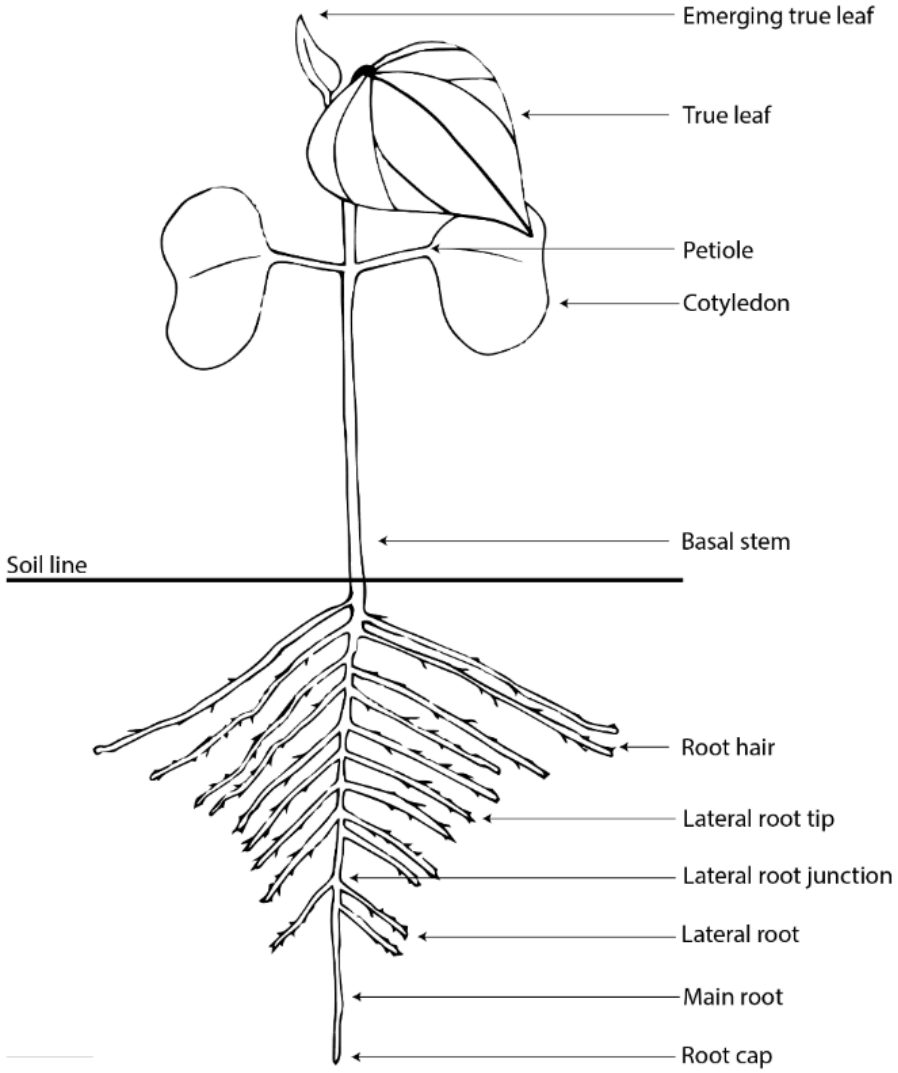


Figure 3. *Anatomy of a cotton seedling approximately 10 -14 days after sowing. Plant sections excised by hand for confocal microscopy include root cap, main root, lateral root, lateral root junction and tip, basal stem, and petiole of true leaves.*

Statistical Analysis

All analyses were conducted in *R* (R Core Development Team, 2010) using packages emmeans (Singmann et al., 2020) and RVAideMemoire (Hervé, 2020). Graphs were produced using the R package ggplot2 (Wickham, 2016), or Microsoft Excel (2010).

Results

Verticillium dahliae *can be transformed to express GFP*

Presence of the GFP transgene in recipient Agrobacterium was confirmed via plasmid digest (supplementary data referenced from Methods). A total of 21 Agrobacterium-mediated transformed *V. dahliae* with pZPnat1-TEF-GFP were obtained as determined initially by fluorescence under excitation with blue light. Of these, 20 were transformed from Vd71171 (Vd71-1 – Vd71-20), and 1 from

Vd71172. No transformants were recovered from isolate Vd71181. Of the two recipient Agrobacterium strains, 19 transformants were produced using AGL-0, and two transformants using LBA4404. Of the stable transformants generated, Vd71-3 and Vd71-6 were selected for the uniformity and strength of fluorescence under blue-light in all fungal structures.

A GFP expressing transformant was identified with normal colony appearance and growth rate but slightly altered fecundity

Colonies of wildtype parent Vd71171 after 2 weeks on PDA appeared white, with raised elevation from aerial hyphae, and circular. Upon microscopic examination no microsclerotia were observed. The colony appearance of isolate Vd71-3 was consistent with that of Vd71171 (Figure 4). Isolate Vd71-6 appeared dark and on microscopic examination an abundance of microsclerotia had formed, flat in elevation, and with irregular colony margins (Figure 4).

To assess for differences in growth rate, colony diameter of isolates Vd71171, Vd71-3, and Vd71-6 on PDA was measured daily for 4 days, then observations per isolate per day pooled and the means used in analysis of variance. The difference in growth rate of isolates was not statistically significant, with a p value of 0.8779. The mean diameter of isolate Vd71171 was 16.5 mm (n = 20, sd = 3.673*),* 15.9 mm for Vd71-3 (n = 20, sd = 2.597), and 14.65 mm for Vd71-6 (n = 20, sd = 3.673), shown in Figure 5.

To assess the fecundity of isolates on solid media, the number of conidia present in a set volume of SDW after 7 days growth on PDA was measured. *In vitro* fecundity (production of conidia / mL) of isolates Vd71171, Vd71-3 and Vd71-6 was statistically significantly different (p value <0.001). The mean fecundity of isolate Vd71171 was 2.2×10^6 and significantly higher than that of Vd71-3 (1.6×10^6), and Vd71-6 (1.0×10^6) (Figure 6). Despite the statistically significant differences in fecundity on solid media, it is not clear what impact this has on pathogenic traits at this stage.

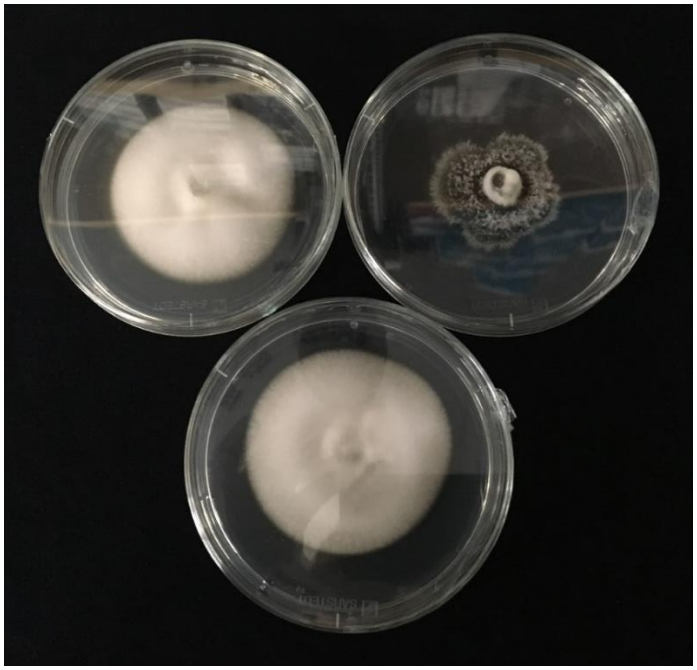


Figure 4. Colonies after 14 days on PDA, frontal view. Top left: transformant isolate Vd71-3; Top right: transformant isolate Vd71-6; Bottom, centre: Vd71171 wildtype.

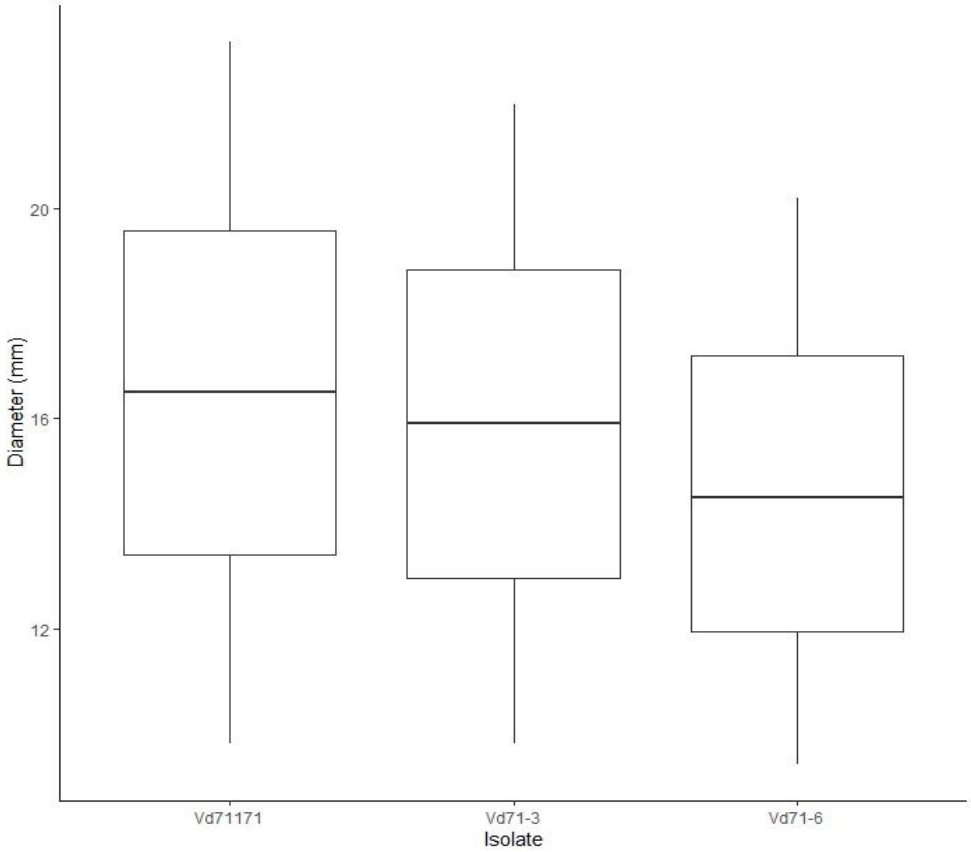


Figure 5. *Mean growth rate of GFP transformed isolates of V.dahliae (Vd71-3 & Vd71-6) and wildtype (Vd71171) measured by colony diameter (mm) and pooled from observations over 4 days on PDA. Bars represent the median value. Whiskers represent minimum and maximum diameter (mm) recorded within an isolate.*

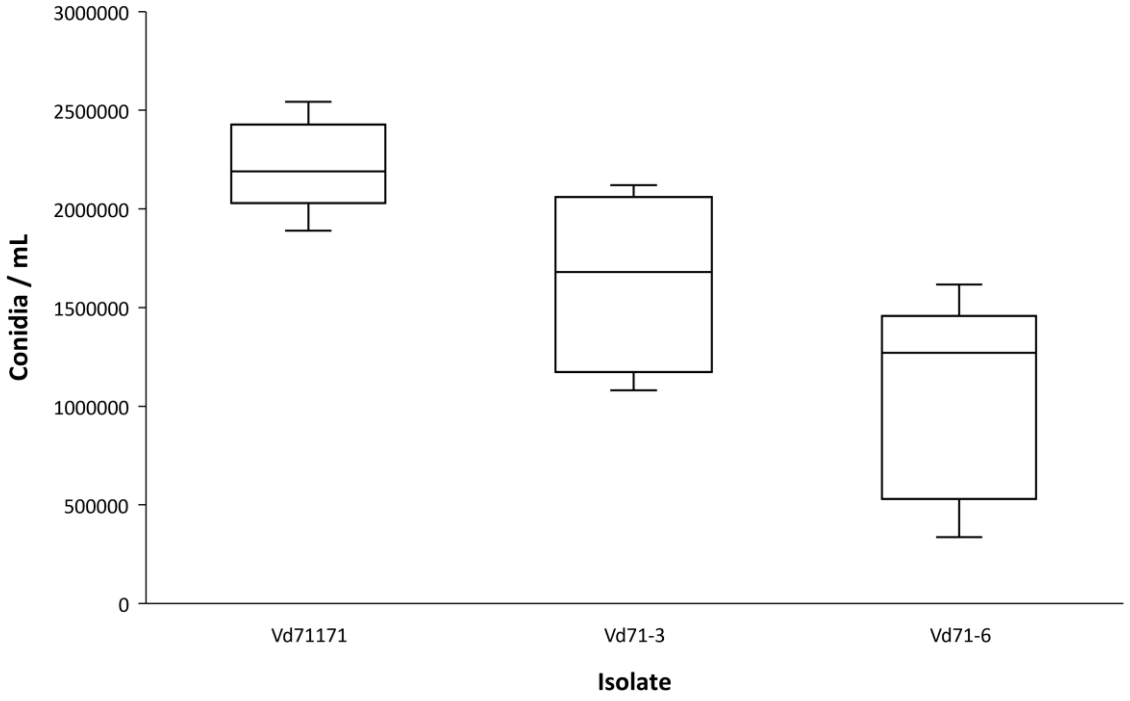


Figure 6. *Fecundity of transformant (Vd71-3 & Vd71-6) and wildtype (Vd71171) V.dahliae isolates after 7 days on PDA, as measured by conidia per mL. Bars represent the median. Whiskers represent minimum and maximum count of conidia / mL within an isolate.*

GFP transformation does not dramatically alter pathogenic traits in V. dahliae

Disease Severity Assessment

To assess if pathogenicity from insertion of the GFP gene was affected, fluorescent *V. dahliae* isolate Vd71-3 and parental wildtype Vd71171 were applied via root dip inoculation to two cotton varieties, tolerant variety Sicot 714 and susceptible variety Siokra 1-4. Plants were incubated for 4 weeks in the glasshouse and pathogenicity was assessed using a disease severity scale of 0 – 5 (Table 5). Typical Verticillium wilt disease symptoms, including chlorosis, necrosis, wilt and defoliation were observed at 4 weeks post-inoculation, and are shown in Figure 7. No external disease symptoms were observed on control plants.

There was no significant difference in disease severity between isolate Vd71171 and Vd71-3 on either cotton variety Sicot 714 or Siokra 1-4 (Table 6). However, plants inoculated with isolate Vd71171 were recorded with the highest mean disease severity; $\bar{x}$ = 3.312 (n = 48, sd = 2.344) on susceptible variety Siokra 1-4, and a mean disease severity of $\bar{x}$ = 2.479 (n = 48, sd = 2.509) on tolerant variety Sicot 714, shown in Figure 8. Disease severity ratings of 5 (*81 – 100% total leaf area affected, and or plant death)* based on symptoms (Figure 7) were recorded in all treatments. Symptomless plants (disease severity rating of 0) accounted for at least a third of the total ratings in each treatment, specifically: 31 % of Siokra 1-4 inoculated with Vd71171, 50 % of both Siokra 1-4 inoculated with Vd71-3 or Sicot 714 inoculated with Vd71171, and 63 % of ratings in Siokra 1-4 inoculated with Vd71-3. Symptomless plants were tagged to assess whether *V. dahliae* could be recovered from internal tissues. More plants showing symptoms (57 of 96) were observed amongst the susceptible variety than the tolerant variety when inoculated with *V. dahliae.* Of the diseased plants, the mean disease severity was lower on tolerant variety Sicot 714 ($\bar{x}$ = 1.865 ;n = 96, sd = 2.329), than on susceptible variety Siokra 1-4 ($\bar{x}$ = 2.896 ;n = 96, sd = 2.452).

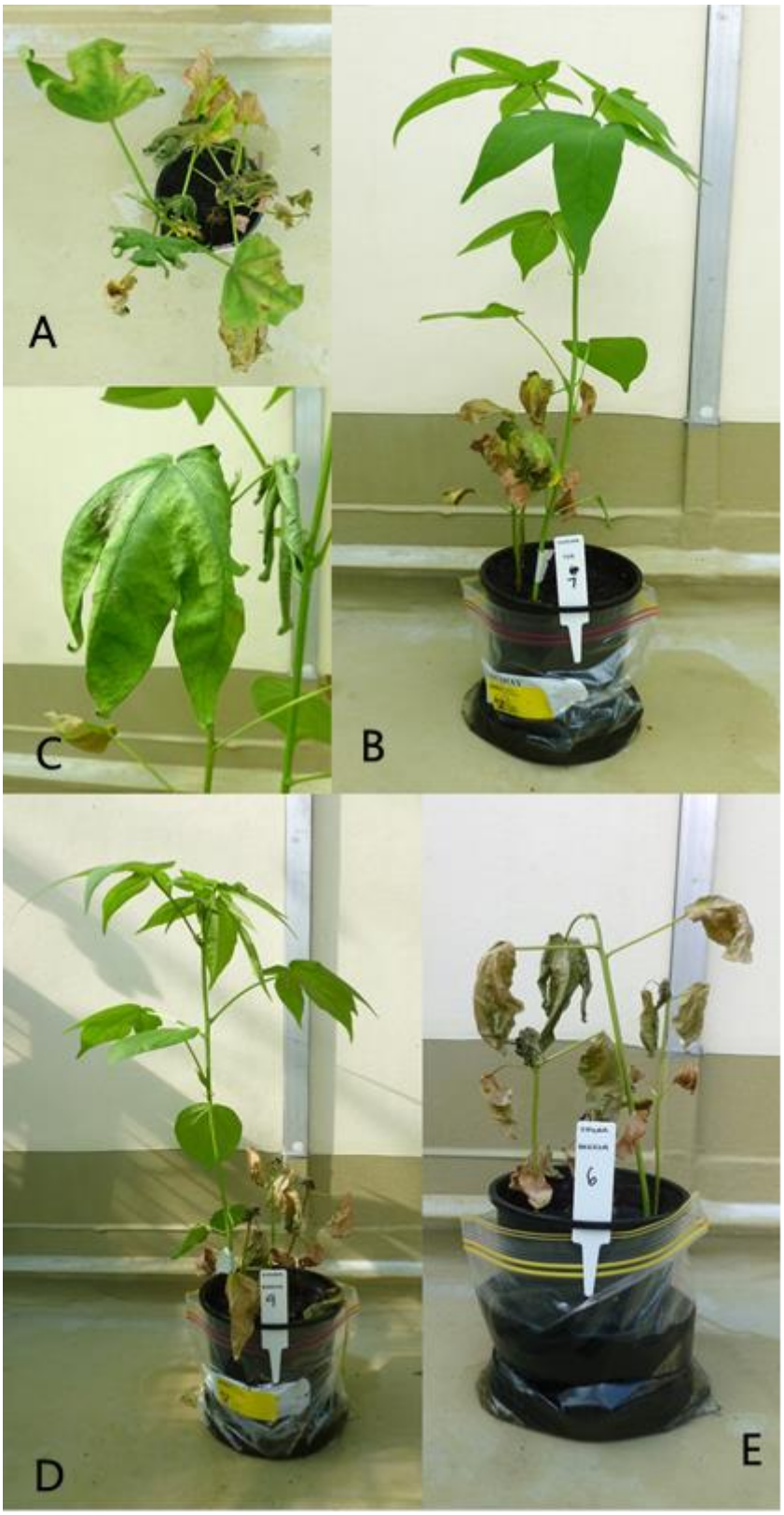


Figure 7. *A, B, C) Siokra 1-4 plants inoculated with transformant isolate Vd71-3, 4 weeks post-inoculation. A) Overhead view of leaf symptoms, including necrosis at the leaf margins and interveinal chlorosis. B) A plant exhibiting no external symptoms amongst severely affected seedlings. C) Leaf wilt and chlorosis, as well as early necrosis at the leaf margin. D, E) Siokra 1-4 plants inoculated with wildtype isolate Vd71171, 4 weeks post-inoculation. D) A plant exhibiting no external symptoms amongst severely affected seedlings. E) Severely affected seedlings exhibiting advanced necrosis of the leaf tissue.*

Table 6. *Multiple comparisons of groups analysis indicating no significant difference (p value > 0.05) in disease ratings between the two factors variety (Siokra, Sicot), and isolate (Vd71171, Vd71-3)*

Comparison of groups	Estimated marginal mean	z ratio	p value[1]
Siokra Vd71171 - Sicot Vd71171	0.414	1.811	0.2678
Siokra Vd71171 - Siokra Vd71-3	0.414	1.811	0.2678
Sicot Vd71171 - Sicot Vd71-3	0.405	1.99	0.1915
Siokra Vd71-3 - Sicot Vd71-3	0.405	1.99	0.1915

[1]α = 0.05

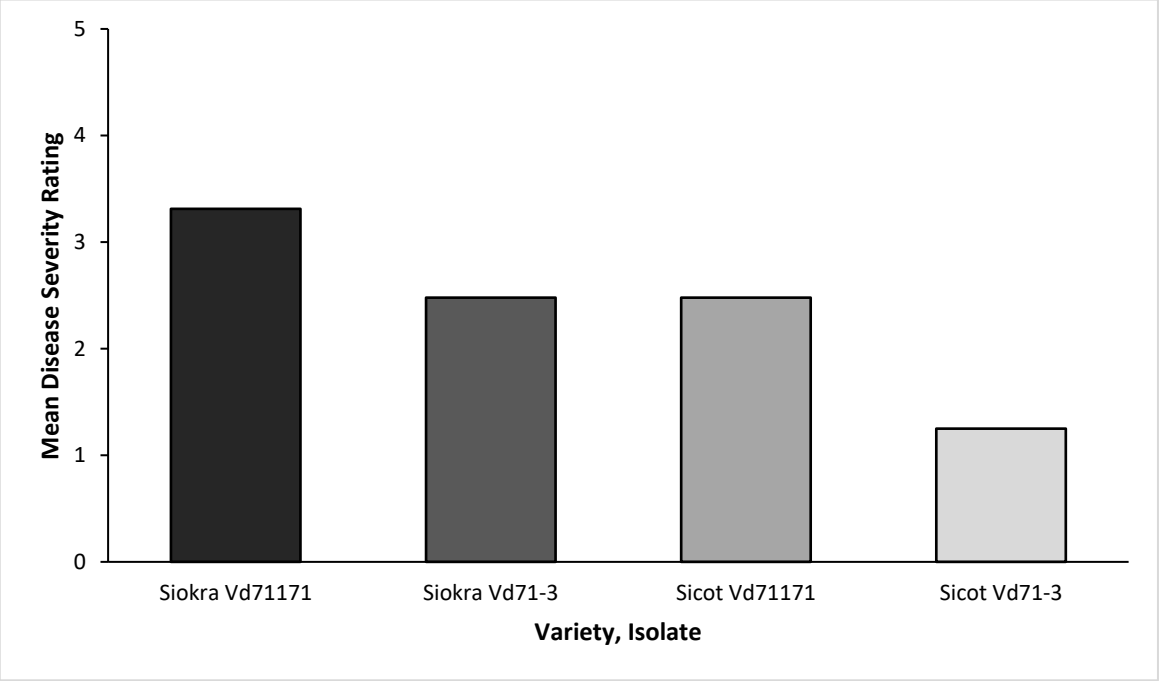


Figure 8. *Mean disease severity rating 4 weeks post inoculation with either wildtype Vd71171, or transformant Vd71-3 on susceptible cultivar Siokra 1-4, and tolerant cultivar Sicot 714. Means shown are calculated from severity ratings prior to analysis using R package emmeans. Estimated marginal means used to determine statistical significance are shown in Table 6.*

Re-isolation

At 4 weeks post-inoculation, all plants including those in control treatments, were destructively sampled. Tissue pieces were surface sterilised and placed on media for later determination of any *V. dahliae* growth. Recovery frequencies from diseased, symptomless, and non-inoculated control plants are shown in Table 7. In total, *V.dahliae* was recovered from 85% of the 99 plants showing external symptoms. On examination under blue-light isolates recovered from Vd71-3 inoculated plants in all cases was fluorescing (n = 33), indicating that the GFP transgene was stable and expressed during infection. Fluorescent *V. dahliae* was also recovered from three of 93 plants showing no external symptoms at 4 weeks post inoculation these being from two plants inoculated with Vd71-3, on both cotton varieties, and from one plant inoculated with Vd71171 on Sicot 714. *V.dahliae* was not recovered from any of the control treatments (n = 48) sampled.

Table 7. *The percentage of Vd71-3 and Vd71171 isolates recovered upon termination (4 weeks post inoculation) of the pathogenicity assay*

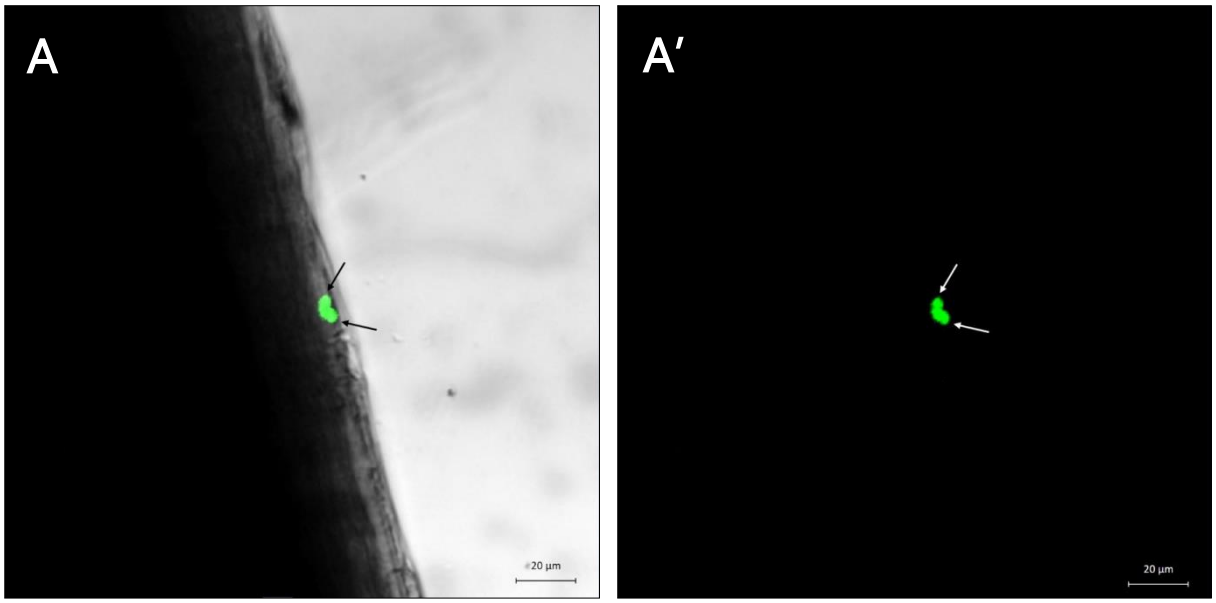
Isolated from	**Vd71-3**	**Vd71171**
Diseased[1] Siokra 1-4	100% (24)	94% (31)
Symptomless[2] Siokra 1-4	4% (1)	7% (1)
Non-inoculated Siokra 1-4	0	0
Diseased Sicot 714	39% (7)	91% (20)
Symptomless Sicot 714	3% (1)	0
Non-inoculated Sicot 714	0	0

Numbers in parentheses indicate the number of *V. dahliae* diseased plants.
[1] Diseased plants were determined based on *V. dahliae* symptoms such as wilting, leaf chlorosis and necrosis.
[2]Plants inoculated with *V.dahliae* with a disease severity score of 0 were considered symptomless.

Using confocal microscopy to follow the infection on two cotton cultivars

Root Colonisation 4 hours, 24 hours and 5 days post inoculation

Fluorescing *V.dahliae* isolate Vd71-3 was applied via root dip inoculation to two cotton varieties, tolerant variety Sicot 714, and susceptible variety Siokra 1-4, at the seedling stage. Using confocal laser scanning microscopy (CLSM), conidia on the surface of root tips was first observed 4 hours post-inoculation (hpi) with Vd71-3, on both the tolerant and susceptible cotton varieties (Figure 9). Germination of conidia was first observed at 24 hpi (Table 8). Germ tubes could be observed emerging from conidia on the surface of root tips (Figure 10, A), as well as hyphal elongation up to 18 µm on Sicot 714, and 34 µm on Siokra 1-4. On the susceptible cotton variety, at 24 hours post inoculation hyphae appeared to have penetrated the root epidermis (Figure 10, B), appearing swollen (2.7 µm) then narrowed to approximately 1.3 µm after penetration. The formation of an infection peg approximately 3 µm wide was captured on the root surface at 24 hpi (Figure 10, C). Abundant conidia were observed on the surface of root tips at 24 hpi. By 24 hours post inoculation, germ tubes were visible on 46 % (n = 13) of conidia on the susceptible variety, and 47 % of conidia (n = 19) on the tolerant variety, in single frames captured during CLSM.



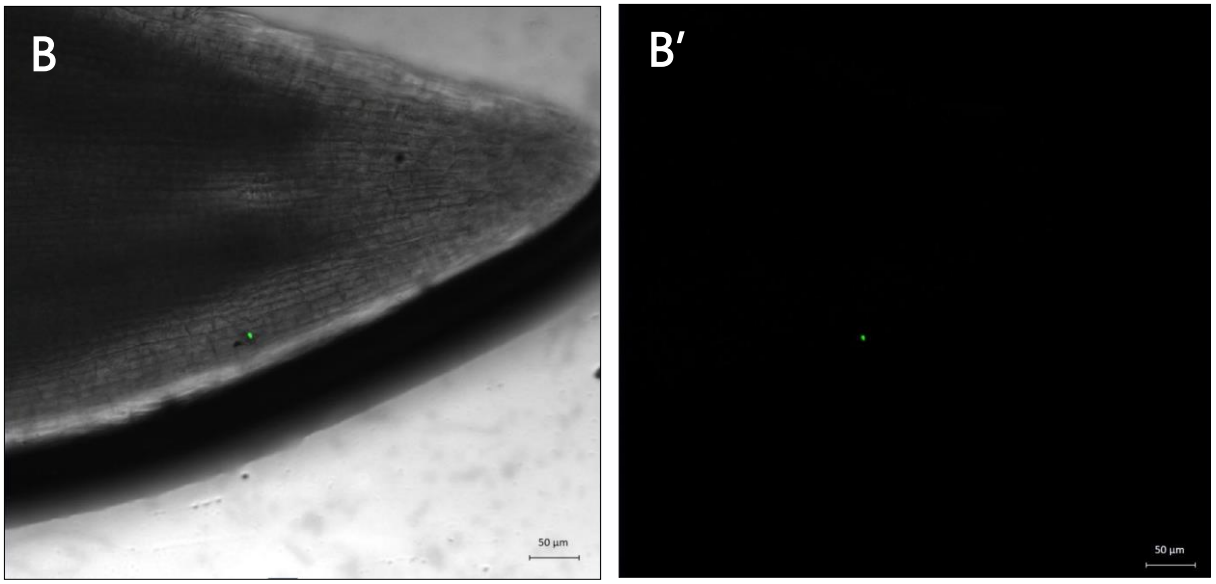
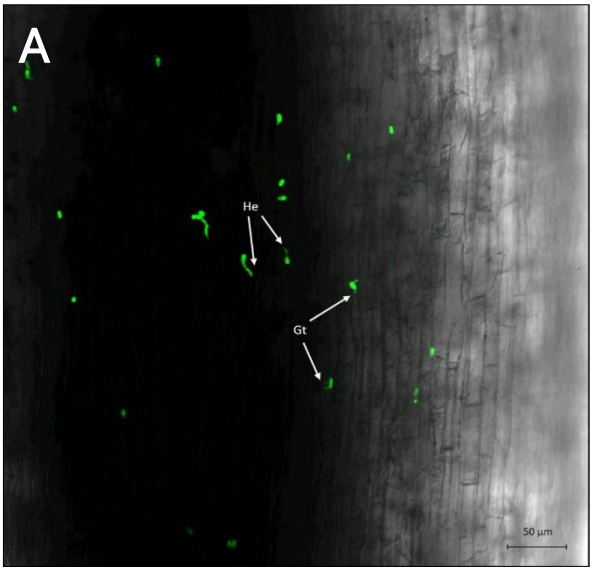


Figure 9. *Confocal laser scanning microscopy of V. dahliae transformant Vd71-3 at 4 hpi on the root tips of Upland cotton. A) Small aggregation of conidia on tolerant variety Sicot 714. A') Single-channel view. B) Single conidia on susceptible variety Siokra 1-4. B') Single-channel view.*


A
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Gt
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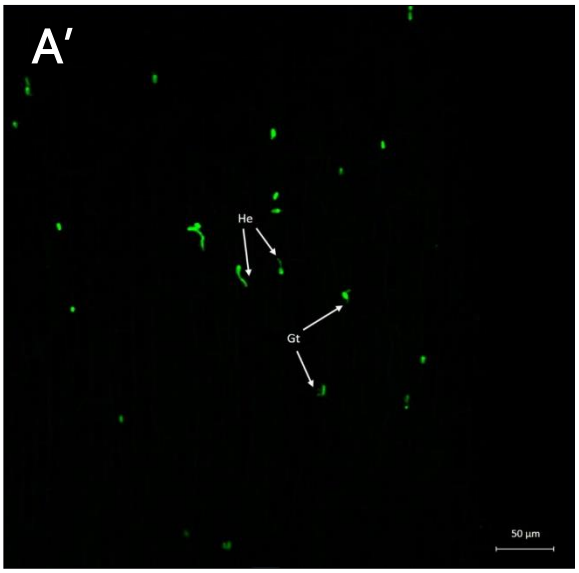
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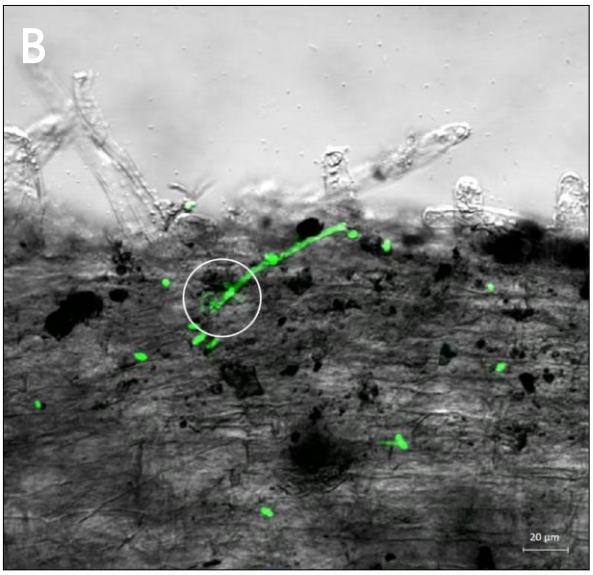
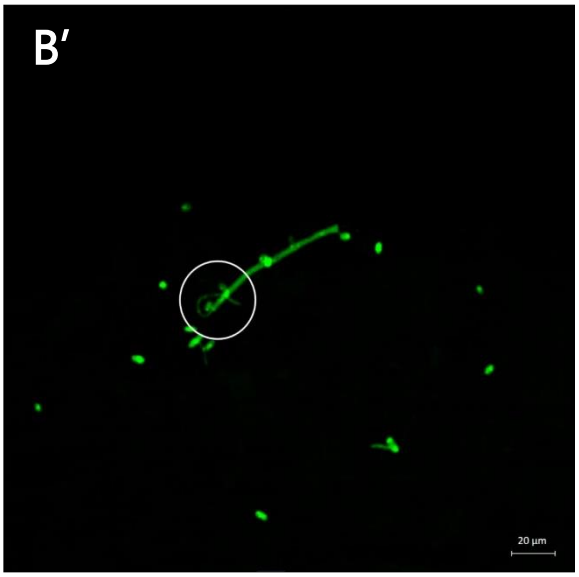
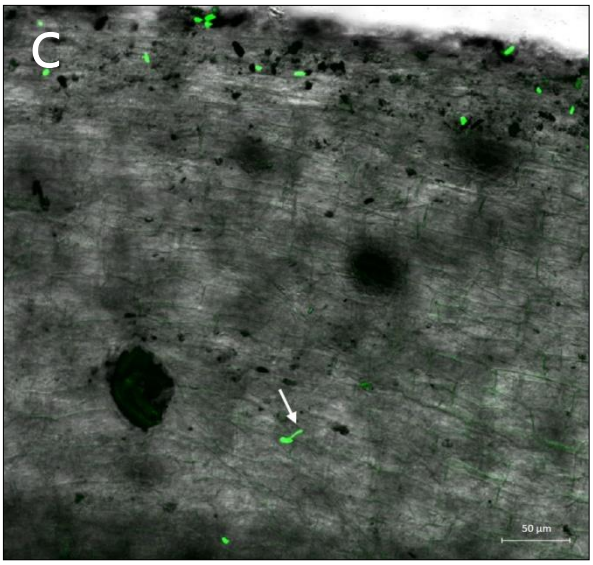

B
20 µm


B'
20 µm


C
50 µm

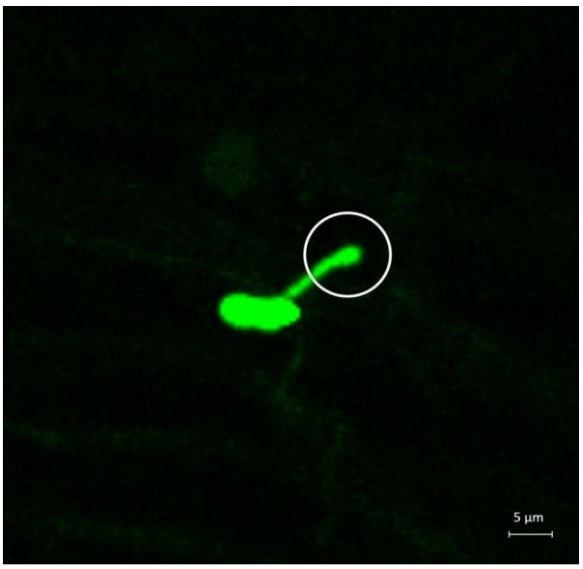

5 µm

Figure 10. *Early stage root colonisation at 24 hpi with Vd71-3 on Upland cotton. A) A single, or two, germ tubes (gt) emerging from conidia, and hyphal elongation on root tip surface of tolerant cotton variety. A') Single-channel view. B) Hyphal elongation and penetration into the root epidermis (arrow, circle) of a susceptible cotton variety. B') Single-channel view. C) Formation of an infection peg (circled) at the terminus of a germ tube, on root tip surface of a susceptible cotton variety. C') Single-channel view.*

By 5 days post inoculation (dpi) hyphal networks had established on the root tips. On the tolerant cotton variety Sicot 714, germinating conidia were observed in proximity to the root cap (Figure 11, A). Hyphal networks appeared along the surface of and within the root epidermis (Figure 12, B). On susceptible variety Siokra 1-4, root tips were colonised from the apical meristem (Figure 12, A), extending to the base of the root (Figure 12, B; C). Mycelia could be observed intercellularly within the root epidermis, appearing more densely colonised at the base of the root than at the tip. The apparent intercellular movement of hyphae from the root surface toward the root cortex was first observed at 5 dpi (Figure 13).

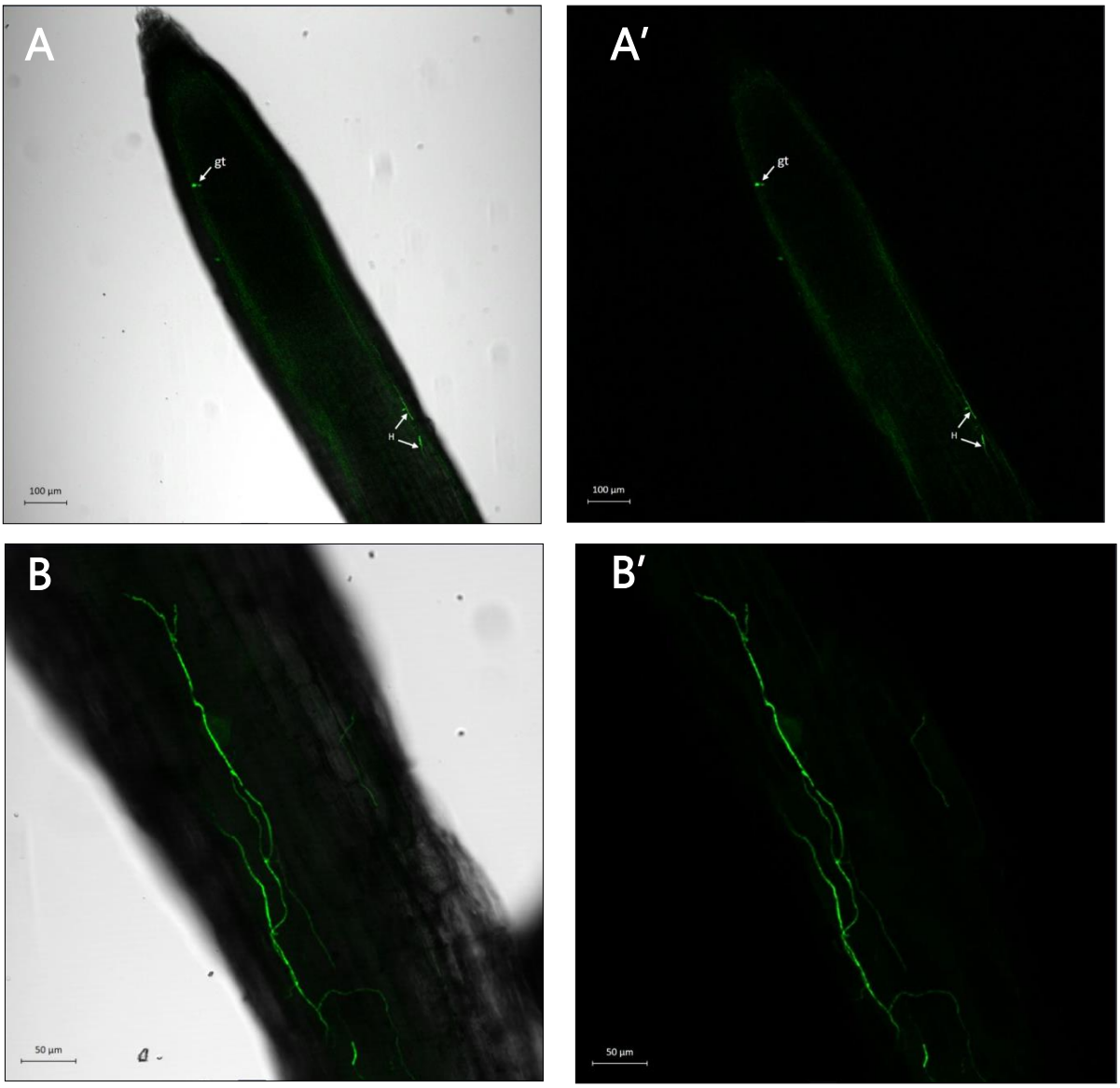


Figure 11. *Root tip colonisation 5 dpi with Vd71-3 on tolerant variety of Upland cotton. A) Germinating conidia and hyphae on root tip surface. A') Single-channel view. B) Mycelia on the root surface and within the epidermis of the root tip. B') Single-channel view.*

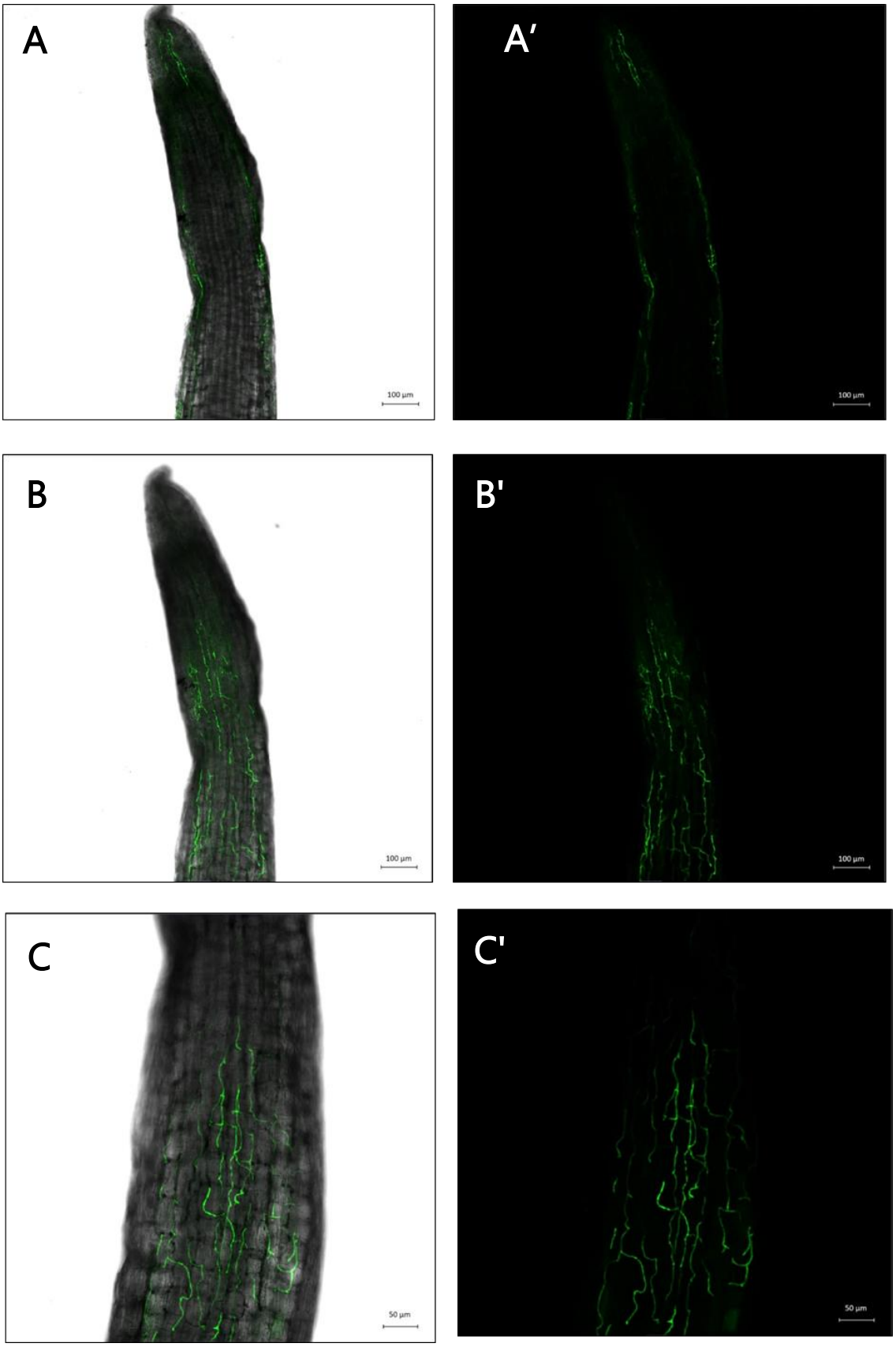


Figure 12. *Colonisation of cotton root tip of susceptible variety at 5 dpi. A) Mycelia progressing down the root cap. A') Single-channel view. B) Hyphae extending intercellularly in the root epidermis. C, C') Network of mycelia at the base of the root tip. B') Single-channel view.*

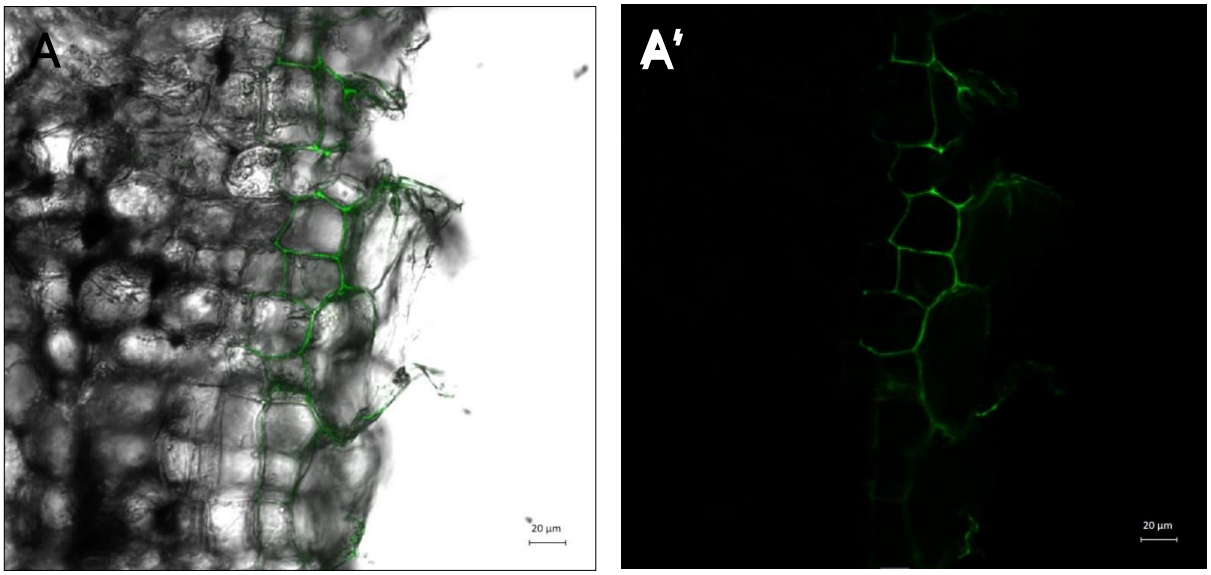


Figure 13. *A) Intercellular movement of hyphae through lateral root epidermis 5 dpi on a susceptible cotton variety. A') Single-channel view.*

Root colonisation from 7 days post inoculation

By 7 dpi, colonisation was seen at the root tip epidermis as before, as well as the vasculature of lateral and main roots in both the tolerant and susceptible cotton varieties (Figures 14 - 17). Colonisation was not observed at the junction of lateral roots with the main root at 7 dpi (Figure 14, B). However, mycelia were observed in several vessels of the vasculature of the main root adjacent to root junctions (Figure 14, A). Conidia were observed without restriction to colonised areas of the xylem, upstream of hyphal networks at a distance of several centimetres. Fungal structures observed in adjacent colonised vessels varied from mycelial network on the outermost edge of the vasculature, to sparse mycelia or single strands of hyphae closer to the stele (Figure 14, A). Within the innermost xylem vessel a single conidium was observed (Figure 14, A').

Mycelia networks and conidia were observed within the root vasculature of the tolerant cotton variety (Figure 15). In the same vessel, tightly packed, fluorescent cells appeared to occlude a section of the xylem (Figure 15). Cells were ovoid, ranging from 2.2 to 3.8 µm in length ($\bar{x}$ = 2.7 µm, n = 20) and 1.9 to 2.6 µm in width ($\bar{x}$ = 2.0 µm, n = 20). Sectioning of the root vasculature of the susceptible cotton variety did not reveal any fungal structures.

Colonisation of the above ground tissues

Basal stem sections from the susceptible cotton variety show mycelia present throughout the stem vasculature, and present in adjacent vessels by 7 dpi (Figure 16). Hyphal tips (1.7 – 2.1 µm, n = 4) are shown apparently emerging through the vessel wall into an uninfected vessel; this appears to be from adjacent colonised tissues (Figure 16, A').

Fungal structures were observed in the petiole of the susceptible cotton variety Siokra 1-4 at 7 dpi (Figure 17). Consistent with the stem vasculature, hyphae were observed in adjacent vessels of the petiole, and appeared to have penetrated the shared vessel wall (Figure 17, A). Several germinating

conidia were also observed freely within the colonised xylem vessel. The fungus was not observed in sections of the petiole from the tolerant cotton variety. The rate of colonisation, as summarised from confocal microscopy observations, is set out in table 8.

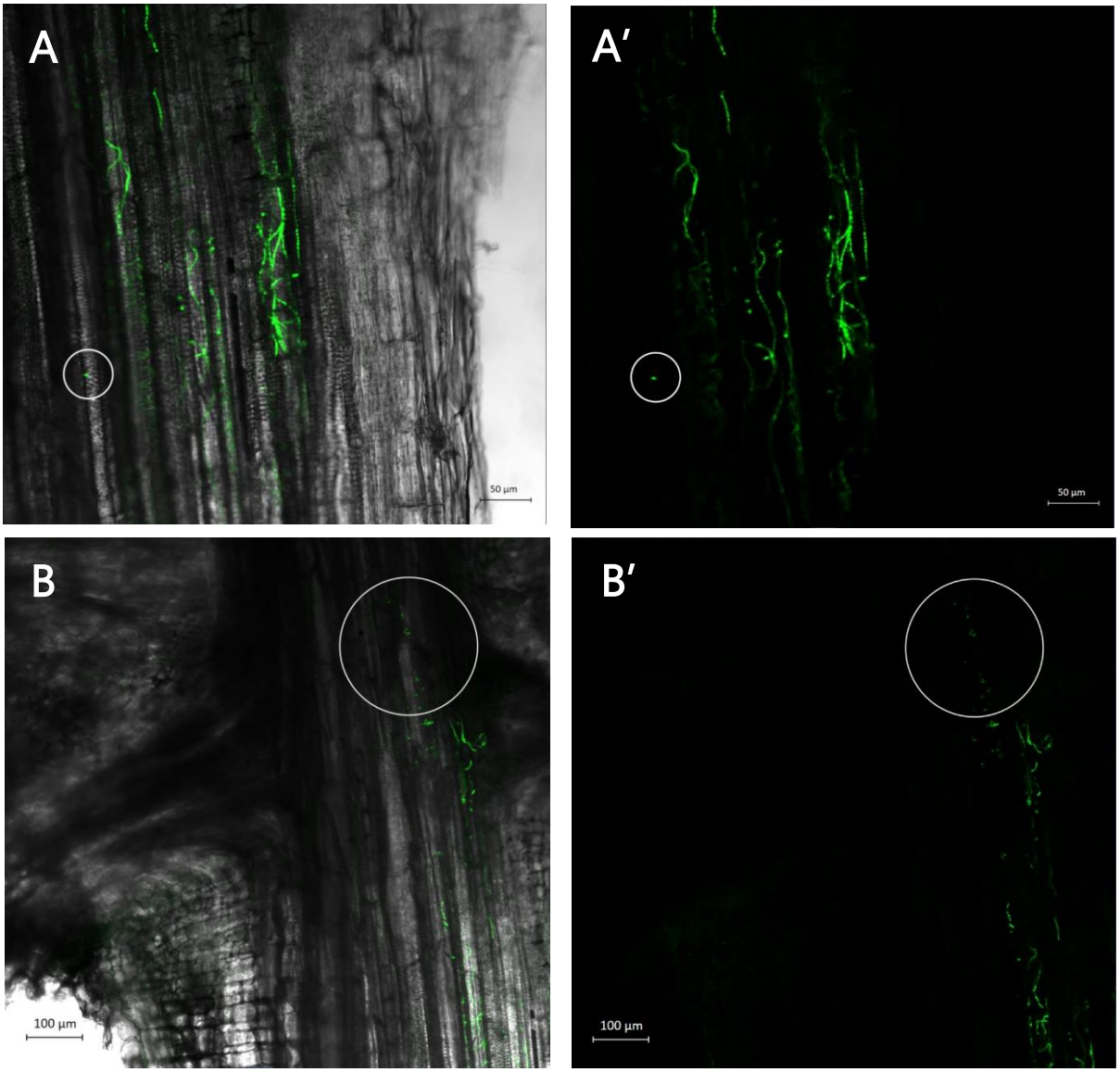


Figure 14. *Colonisation of main root vasculature in proximity to lateral root junctions at 7 dpi on tolerant cotton variety. A) Mycelia in adjacent xylem vessels. Conidia freely in the xylem (circled). A') Single-channel view. B) Conidia free within the xylem (circled), upstream of mycelia. B') Single-channel view.*

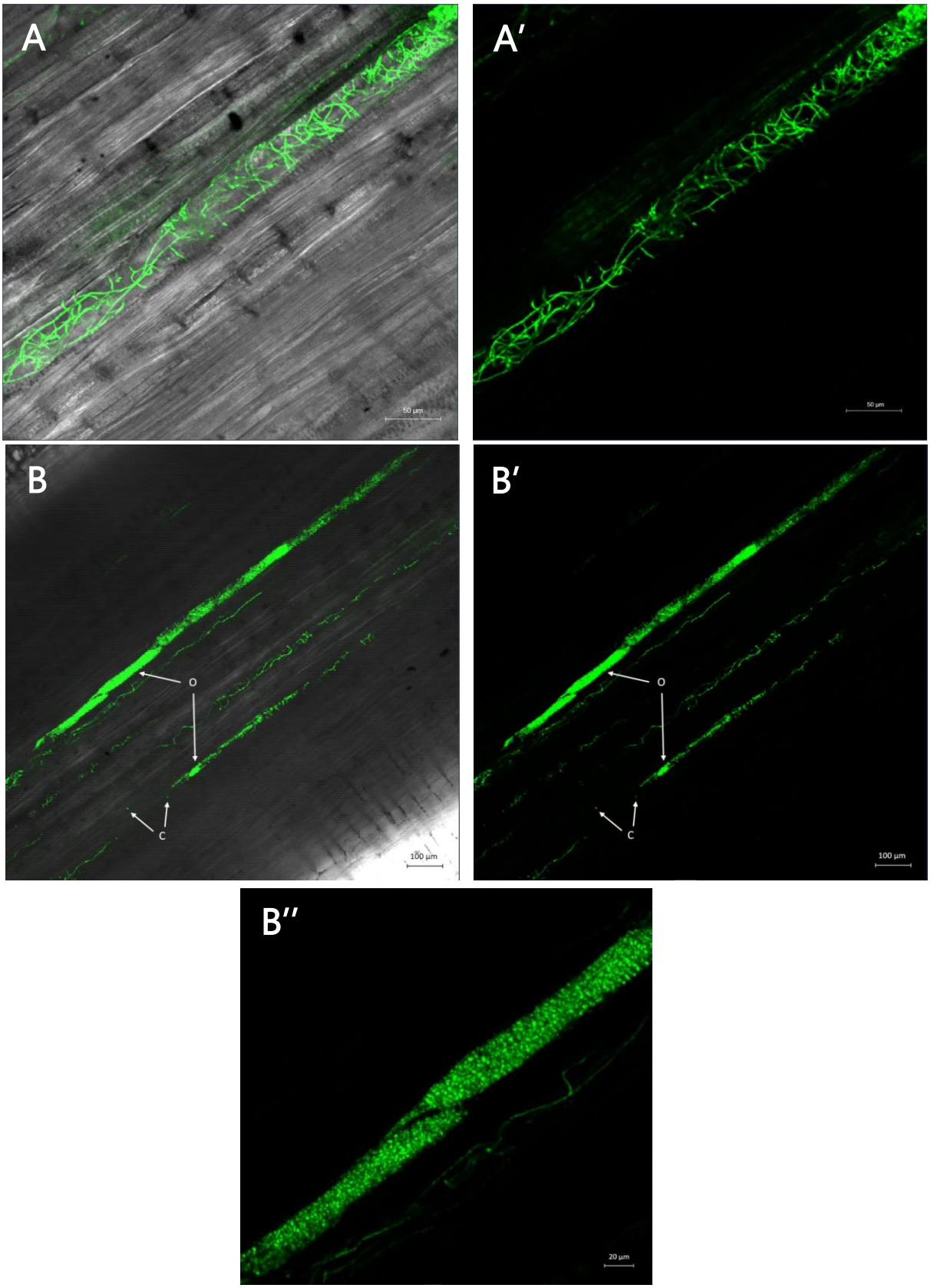


Figure 15. *Advanced stages of colonisation at 7 dpi with Vd71-3 on a tolerant cotton cultivar. A) Mycelia throughout a xylem vessel of the root vasculature. A') Single-channel view. B) Multiple xylem vessels of the root colonised by mycelia*

and conidia (c). Sites of vascular occlusion (o). B') Single-channel view. B") Single channel enlarged view of xylem vessel densely packed with conidia.

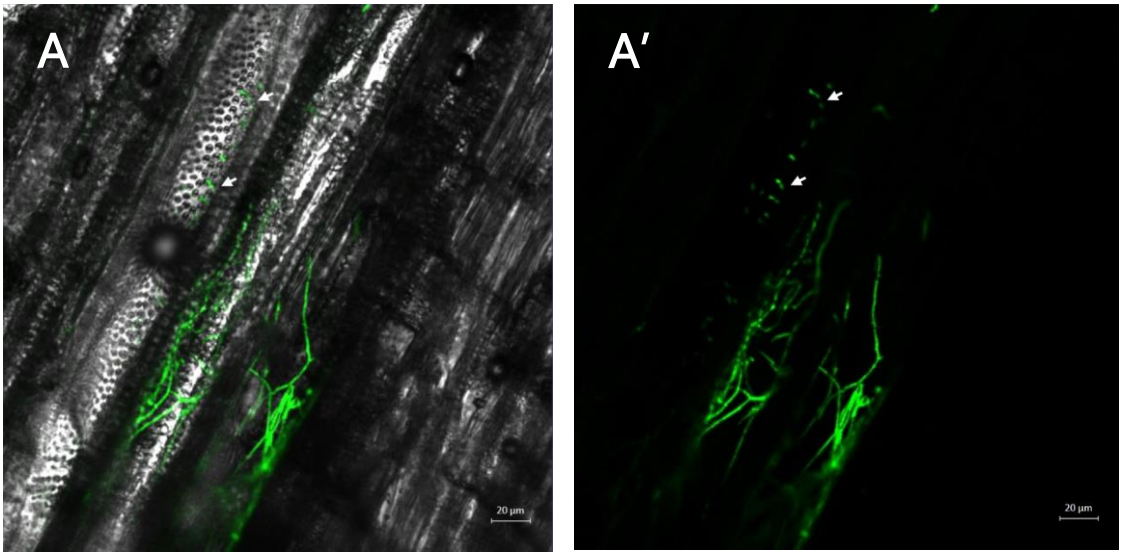


Figure 16. *A) Progression of Vd71-3 mycelia into the stem vasculature on susceptible cotton variety at 7 dpi. Arrows: Hyphae from densely colonised vessels invade uninfected adjacent vessels. A') Single-channel view.*

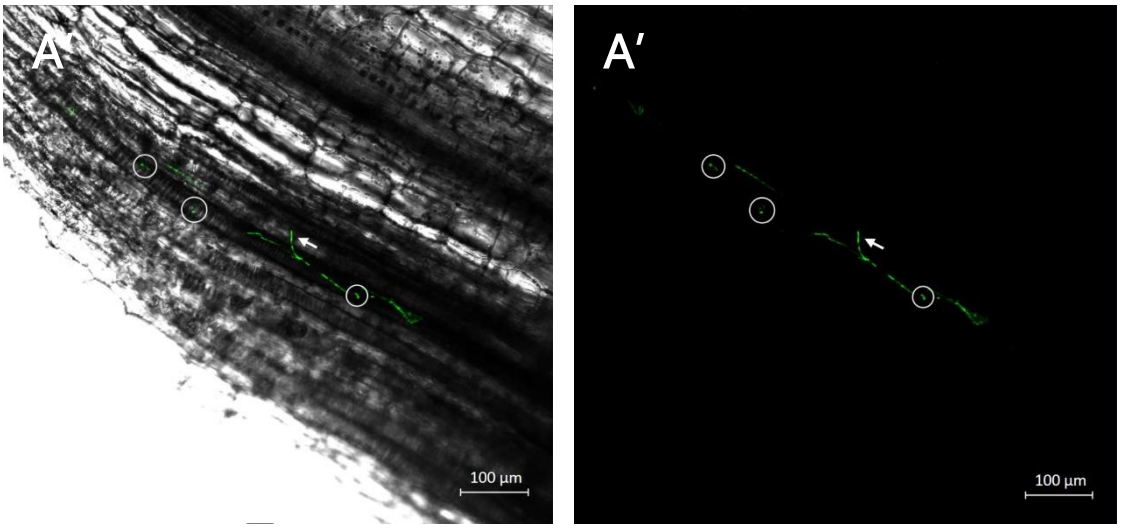


Figure 17. *A) Presence of mycelia and free conidia in the petiole of susceptible cotton variety at 7 dpi. Germinating conidia (circled) are present throughout the colonised vessel, upstream of mycelia. Arrow: Hyphae in adjacent xylem, penetrating vessel wall. A') Single-channel view.*

Table 8. *Summary of rate of colonisation based on timing of initial observation at each infection stage throughout the confocal microscopy experiment (At each observation, n = 3 samples of plant tissue section examined).*

Infection stage	**First observed[1] time**		**Fungal structures[2]**	**Number of observations[3]**
	Sicot	**Siokra 1-4**		
Germination	24 hpi	24 hpi	Conidia, germ tubes, infection peg (Siokra 1-4)	1 observation
Hyphal elongation	5 dpi	24 hpi	Conidia, hyphae	2 observations

Penetration	5 dpi	24 hpi	Conidia, hyphae	2 observations
Colonisation of the root epidermis	5 dpi	5 dpi	Mycelia	1 observation
Colonisation of the root vasculature	7 dpi	7 dpi	Mycelia, occlusion with conidia (Sicot 714)	2 observations
Colonisation of the above ground vasculature	-	7 dpi	Conidia, germ tube, hyphae	1 observation
Colonisation of the petiole	-	7 dpi	Conidia, hyphae	1 observation

[1]Time point at which infection stage was first observed.
[2]Fungal structures listed were observed on both varieties unless indicated otherwise.
[3]Independent observations.

Discussion

Generation of GFP-*expressing* V.dahliae *isolates well suited for study on cotton*

The transformation of filamentous fungi with reporter proteins such as GFP, offers a highly controlled, simplified approach to the study of plant – pathogen interactions, especially when combined with confocal laser scanning microscopy. In this study, a GFP vector construct, pPZPnat1-TEF-GFP was utilised to produce a total of 21 *V. dahliae* transformants by *Agrobacterium tumefaciens*-mediated transformation (ATMT), adapted from Mullins et al. (2001). Of the transformants, over 90% were produced with recipient Agrobacterium strain AGL-0, suggesting LBA4404 was not as efficient in the *V. dahliae* transformation. Of the stable transformants generated, Vd71-3 and Vd71-6 were selected for the uniformity and strength of fluorescence under blue-light in all fungal structures, and subject to further characterisation.

The mean growth rate of isolates Vd71-3, Vd71-6 and Vd71171, as measured by mm diameter increase over 4 days, was not significantly different. This result indicates expression of the GFP transgene does not slow or alter typical growth in *V. dahliae*.

Fecundity is a measure of the ability of a fungus to reproduce. Vascular wilt pathogens such as *V. dahliae* succeed in rapidly colonising host tissues by production and release of conidia in the xylem; conidia are carried in the transpiration stream throughout the plant and then germinate, instigating new sites of colonisation (Fradin & Thomma, 2006). *In vitro* fecundity (conidia / mL) of isolates Vd71-3, Vd71-6 and wildtype isolate Vd71171 were significantly different from each other ($p < 0.0001$). The comparatively low ($\bar{x}$ = 1.0 x 10^6 conidia / mL) fecundity of Vd71-6 (Figure 6) interestingly is accompanied by an increase in microsclerotial production (Figure 4). The production of microsclerotia, the hardy, specialised resting structures of *V. dahliae*, occurs in the limited saprophytic phase on dead, dying tissues (Klimes et al., 2015). One hypothesis is the GFP insertion site has altered or interrupted a cellular pathway related to conidia or microsclerotia production. It is unknown at present what impact this may have on the pathogenicity of isolates.

Artificial inoculation of Upland cotton cultivars did not show a significant difference in pathogenicity between isolate Vd71-3 and wildtype isolate Vd71171. The inoculum concentration of 1 x 10^5 spores/ mL used in this experiment was less than that used in similar studies on cotton, typically 1 x 10^6 – 2 x 10^7 spores/ mL (Zhang et al., 2014). The concentration used in this study was selected to minimise the risk of the highly virulent isolate Vd71171 eliciting total plant death prior to assessment. Despite this, severe disease and plant death after 4 weeks incubation was observed in all treatments, excluding the control. The temperature of the glasshouse was set to 25°C during the experiment, the optimum temperature conducive to Verticillium wilt disease development on cotton (Xu et al., 2012). As expected, the greatest number of diseased plants and highest mean disease severity was recorded from the susceptible variety, Siokra 1-4 treatment, over the tolerant variety, Sicot 714 (Figure 8). Of the 192 cotton seedlings inoculated with *V. dahliae*, 48% appeared healthy and symptomless 4 weeks after inoculation. The root dip method utilised in this study is a popular inoculation technique for studying *V. dahliae* on cotton (Klosterman et al., 2009; Zhang et al., 2014). However, achieving an even distribution of inoculum on plant roots can be challenging, as evidenced in this experiment. To improve artificial inoculation, a second inoculation 1 week after the first was shown by Zhang et al. (2014) to achieve more uniform infections.

V. dahliae was recovered from 85% of diseased plants, and upon examination under blue-light in all cases fluoresced when the GFP strain Vd71-3 was used. This implies that the GFP transgene was stable and expressed during infection. The recovery of *V. dahliae* from symptomless plants (Table 7) supports a limited degree of asymptomatic infection amongst inoculated plants. Asymptomatic, or latent, infection of *V. dahliae* has been reported on olive (Karajeh & Masoud, 2006), as well as many monocotyledonous and weed species (Malcolm et al., 2013).

Fecundity could be a contributing factor in the lower mean disease severity observed in plants inoculated with Vd71-3 compared to the wild type isolate (Figure 8). Isolate Vd71171 produced significantly more spores than Vd71-3; additionally during subsequent re-isolation, isolate Vd71171 was recovered more frequently than isolate Vd71-3 from diseased plants (Table 7). Greater conidia production *in planta* would likely increase colonisation given the apparent spread and instigation of new infection loci from conidia in the transpiration stream (Beckman et al., 1976). This would therefore incite increased levels of disease severity, as postulated by Schnathorst (1963) (as cited by Pegg & Brady, 2001). High levels of colonisation *in planta* could influence isolation frequency from infected tissues as observed in the wildtype inoculated plant compared with the GFP-tagged strains.

Insights from Confocal Microscopy

Isolate Vd71-3 able to uniformly and stably express a GFP fluorescent signal was used to investigate the interaction of *V. dahliae* on Upland cotton. Furthermore, of the two transformant isolates tested, isolate Vd71-3 possessed comparable growth rate, morphology, pathogenicity, and slightly altered fecundity to the wildtype, Vd71171. Two cultivars, Sicot 714 B3F and Siokra 1-4, representing a host with partial resistance (tolerance) and a susceptible host, respectively were selected.

Conidia were observed on the root tip 4 hours post inoculation; however germination was not observed at that stage. By 24 hours post inoculation, germ tubes were visible on 46 % (n = 13) of conidia observed on the susceptible variety, and 47 % of conidia (n = 19) on the tolerant variety, in

single frames each, captured during CLSM. Similarly, on lettuce cultivars, germination of conidia was first observed 12 to 48 hours following inoculation with a GFP-tagged *V. dahliae* isolate (Vallad et al., 2008). Zhang et al. (2013) reported germination of conidia earlier, at 2 hours post inoculation, on cotton inoculated with a defoliating, 1A isolate of GFP-tagged *V. dahliae*. Neither group reported any significant difference in the rate of germination between a susceptible and tolerant cultivar, which differed to the cultivars used in this study.

The formation of a conspicuous infection structure, such as appressoria, has not been reported in studies on cotton with *V. dahliae* (Bishop & Cooper, 1983; Zhao et al., 2014), and no appressoria were observed in the present study. At 24 hours post inoculation, a narrow infection peg (3 µm) was observed on the surface of a cotton root tip (Figure 10, C, C'), as well as hyphal swelling to approximately 2.7 µm, which appeared to narrow after penetration (Figure 10, B'). Hyphal swelling has previously been observed using CLSM in infection studies on Arabidopsis, oilseed rape, sunflower and cotton, where it appeared to assist penetration of the root epidermis through formation of a narrow infection peg (Eynck et al., 2007; Zhang et al., 2013; Zhang et al., 2018; Zhao et al., 2014). Eynck et al. (2007) suggested hyphal swelling was a consequence of cytoplasm accumulating in the hyphal tip in response to the mechanical resistance of host tissue. Once the epidermis is breached, hyphae returned to a normal diameter (Bishop et al., 1983; Zhang et al., 2013).

Colonisation of the root surface, observed on whole-mounted cotton root-tips, was observed at 5 days post inoculation (Figure 11). An ultrastructural study of root invasion by vascular wilt diseases conducted by Bishop et al. (1983), revealed undifferentiated tissues of the root tip allowed intercellular growth of invading *V. albo-atrum* hyphae to progress longitudinally into immature xylem elements, thus establishing successful infection of the vascular tissues. Zhang et al. (2013) reported the same mechanism in *V.* dahliae using a GFP-tagged strain. Our results also support this description (Figures 10 – 12).

Pegg et al. (2001) described root tips as sites of penetration of *V. dahliae* on cotton hosts. Zhang et al. (2013) postulated that lateral root junctions (Figure 3) were also a penetration site. To investigate this, sections of the lateral root junctions on both cultivars were prepared for confocal microscopy 5 and 7 days post inoculation, however penetration, or colonisation, as described by Zhang et al. (2013) was not observed at either time point. Fungal structures within the xylem, but not at the junction, of a lateral root can be seen in Figure 14. Sections were examined in 2 independent observations, on at least 2 – 3 samples per cultivar.

Exclusively intercellular colonisation was observed in the root tissues at 5 dpi (Figure 12), and is clearly shown in the root epidermis at 5 dpi (Figure 13). Unlike other hosts, intracellular colonisation was observed rarely in *V. dahliae* CLSM studies on cotton and Arabidopsis (Zhang et al., 2013; Zhao et al., 2014). Garber and Houston (1966) suggested both intra- and intercellular movement of hyphae through the endoderm, however Zhao et al. (2014) reported movement from cortical cells into xylem vessels proceeded intercellularly. Entry from the root cortex, through the endoderm and into the xylem vessels was not captured by CLSM in this experiment.

In observations of the root and stem tissues, mycelia of *V. dahliae* were restricted to individual xylem vessels of the vasculature (Figures 14 - 17). Most descriptions describe the movement of *V. dahliae*

mycelia longitudinally in the xylem, perforating tracheary elements, also observed in this study (Fradin et al., 2006; Zhang et al., 2013). Additionally, evidence of transverse colonisation was seen in multiple observations (n =2), where hyphal tips emerging from a vessel wall appeared continuous with hyphae in an adjacent vessel (Figure 16). Zhao et al. (2014) captured hyphae extending transversely in the xylem of Arabidopsis roots inoculated with fluorescent strain V592-GFP1, with swelling of the hyphal tip, as seen during mechanical invasion of the root tip. Colonisation similar to that seen in Arabidopsis was also observed in the vessel elements of the petiole (Figure 3) by 7 days post inoculation on the susceptible cotton cultivar (Figure 17). This observation is well in advance of descriptions by Zhang et al. (2013), who observed conidia and mycelia at the petiole base 30 days post inoculation, using a virulent defoliating GFP isolate of *V.dahliae.* Not only was their *V. dahliae* strain, TV7 (VCG 1A, D), used in the study by Zhang et al. (2013) genetically distinct to that used in the current study, infection was studied on Chinese cotton cultivars with differential resistance to Australian cultivars. A higher spore concentration (1×10^7 spores/ mL) than applied here (1×10^5 spores/ mL) was used to inoculate plants. The faster rate of colonisation when inoculated at a lower concentration suggests either (1) isolate Vd71-3 used in the current study is more virulent on the tested cotton cultivars, or (2) the rate of colonisation observed in the Zhang et al. (2013) study was influenced by the cultivar used. Care should be taken with either interpretation, reserving judgment until isolates can be studied under the same experimental conditions.

At 7 days post inoculation, an intense fluorescence signal was observed in the xylem of the tolerant cultivar Sicot 714, within a vessel densely colonised by mycelia (Figure 15). The size, shape and fluorescence of the structures suggest a massive occlusion by conidia. Eynck et al. (2007) using CLSM observed conidia of GFP-tagged *Verticillium longisporum* densely clustered at a tracheid end on *Brassica napus* (oilseed rape)*.* Vascular occlusion by fungal pathogens and the secretion of plant compounds such as tyloses to restrict their passage, are proposed to instigate classic wilt (Pegg & Brady, 2001). Restricting xylem vessels colonised by *V.dahliae* in the lateral roots was identified as an important response in resistant lettuce cultivars (Vallad et al., 2008). Similarly, the cotton interaction observed here could point toward the restriction of the fungus at the border pit membranes of the xylem, described by Fradin et al. (2006). Though interesting and worthy of further investigation, the interaction captured in Figure 15 was only observed once, and was not observed in any tissue sections sampled from the susceptible variety. Unlike studies in lettuce, the putative restriction observed here did not successfully prevent longitudinal colonisation within the vessel, as evidenced by the presence of mycelia upstream of the sites of occlusion (Figure 15). Furthermore, isolated conidia in the xylem were observed in multiple occasions, tissue sections and time points, which point toward systemic movement within the plant vasculature (Figures 14, 15 and 17).

Conclusions and Future Work

Overall, the rate of colonisation between a tolerant and susceptible cotton cultivar during early infection of the root tissues did not dramatically differ (Table 8). Hyphal elongation and penetration of the root surface of the susceptible variety Siokra 1-4 was observed earlier than on the tolerant variety Sicot 714, however repeating observations would be prudent considering the qualitative nature of microscopy data. Furthermore, quantitative methodologies such as real-time PCR, effectively demonstrated by Zhang et al. (2013) and Eynck et al. (2007), would complement this and

future microscopy-centred studies. For example, the presence of fungal structures was not observed in the petiole of tolerant cotton tissues, nor lateral root junctions, as previously described by other authors when examined using confocal microscopy (Zhang et al., 2013).

Global production regions are afflicted by different VCGs and pathotypes of *V. dahliae*, for example, VCG 1A in Spanish cotton and olive, and VCG 4 on tomato and potato in the USA (Bhat & Subbarao, 1999). Mounting evidence points toward evolution of the pathogen within regions including the development of moderate host-specificity (Klimes et al., 2015). The 2A, non-defoliating strain of *V. dahliae* is an important pathogen on Australian cotton (Dadd-Daigle et al., 2020), predominantly isolated from severely diseased Upland cotton in NSW.

We propose GFP-tagged *V. dahliae*, Vd71-3, generated in this study, would be a valuable tool in the study of Australian Upland cotton cultivars response to local strains of *V. dahliae*. Additionally, we suggest the use of complementary quantitative methodologies such as real-time PCR to elucidate mechanisms of host resistance and enabling a deeper understanding of this complex and important phytopathogen.

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Appendix

Supplementary Materials and Methods

Preparation of Medias used in triparental mating

Yeast Mannitol (YM) media prepared as follows: Agar (BD Difco), yeast extract, mannitol, NaCl, MgSO47H2O, and K2HPO4 were transferred to a clean Schott bottle, distilled water (DW) added and adjusted to pH 7.0 using a pH meter prior to autoclaving at 121°C for 15 minutes.

10mM Acetosyringone (AS) was prepared by dissolving 0.0196 g acetosyringone (3', 5'-dimethoxy-4-hydroxyacetophenone, MW 196.2) in 10 mL DW on a shaker, then adjusted to pH 8.0 with 5 M potassium hydroxide (KOH). In a laminar flow cabinet, the solution was filter sterilised using a 0.45 µM filter (Corning®) and transferred to Eppendorf tubes and stored at room temperature.

1 M 2-N morphoethane sulfonic acid (MES) was prepared by dissolving 19.25 g of MES in 80 mL of DW , adjusted to pH 5.3 with 5 M KOH, then additional DW added to a final volume of 100 mL. In a laminar flow cabinet, solution was filter sterilised using a 0.45 µM filter (Corning®) in 9mL aliquots and stored at -18°C. Prior to use, aliquots were thawed in a room temperature water bath.

1 M glucose was prepared by dissolving 9.01 g of 1 M glucose in 50 mL DW, then filter sterilised using a 0.45 µM filter (Corning®) in a laminar flow cabinet and stored in aliquots at room temperature.

Induction media (IM) was prepared as set out in Mullins et al. Briefly, MM salt stock, 10mM glucose, and 0.5 % glycerol were transferred to a clean Schott bottle, SDW added then autoclaved at 121°C for 15 minutes. To make IM agar, agar (BD Difco) powder was added prior to autoclaving. After autoclaving, the solution was cooled to 50°C in a water bath and 1 M MES stock added to a final concentration of 40mM, and 1 M glucose added to a concentration of 10mM.

IM + AS media prepared as described for IM, with the addition of 10 mM AS (final concentration 200 µM) after cooling.

UQ 23 Potting Media

This Media was designed by K. Hayes, The University of Queensland, and prepared according to the following recipe. The pH is adjusted to 5.5 - 6.5, with either $FeSO_4$ if pH is high or Dolomite if pH is low, to suit the needs of a wide range of plants grown in the research glasshouses.

Base Media	**Fertilizers and other augments (m^{-3})**
70% Composted Pine Bark 0-5mm	1Kg Yates Flowtrace
30% Coco Peat	1Kg Iron Sulphate Heptahydrate
	0.4Kg Superphosphate
	0.03Kg Copper Sulphate
	1Kg Gypsum
	1Kg Dolomite

Key word index:
Verticillium wilt, Verticillium dahliae, GFP, transformation, agrobacterium, non-defoliating, confocal microscopy

TECHNICAL REPORT 2

Morrison, S (2021) Fluorescent proteins as a tool for studying Verticillium dahlia on cotton (Gossypium hirsutum) and associated weed species

Abstract

Verticillium dahliae Kleb. is a soil-borne fungal pathogen that causes Verticillium wilt, a systemic vascular disease affecting a range of economically important crop species, including upland cotton (*Gossypium hirsutum* L.). Populations within *V. dahliae* can be categorised into vegetative compatibility groupings (VCGs), which differ in host range, pathogenicity, and distribution. Recently, two pathogenic *V. dahliae* strains, VCG 1A and VCG 2A, have emerged from Australian regions of cotton production and are associated with recent increases in Verticillium wilt incidence in Australian cotton. To assist disease management strategies, a more comprehensive understanding of how these strains have disseminated and predominated in Australian cotton regions is needed. The study at hand used fluorescently-tagged transformants of *V. dahliae* VCG 1A and 2A to study pathogen infection in Australian cotton varieties and in associated weed species. In this study, a VCG 1A strain recovered from the Namoi Valley cotton region was transformed with the *mCherry* fluorescent gene from *Dicosoma* sp. using *Agrobacterium* transformation methods. This fluorescent transformant, alongside a previously transformed GFP-tagged VCG 2A *V. dahliae* isolate, was used to investigate infection patterns *in planta*. Australian weed species and cotton varieties were inoculated with the transformant strains and visualised *in planta* for detection of fluorescent fungal structures using confocal laser-scanning microscopy.

V. dahliae was recovered from six of the eight weed species that were inoculated with the transformed VCG 1A and 2A strains. The VCG 2A transformant was recovered more frequently from weeds than the VCG 1A transformant, suggesting that *V. dahliae* VCG 2A may have higher infectivity towards weed hosts in Australian cotton fields. *V. dahliae* was not recovered from seeds from cotton plants that were subject to direct stem inoculation, although vascular tissue adjacent to the site of inoculation was colonised. Further investigation is needed to understand whether *V. dahliae* VCG 1A and 2A strains are capable of infecting Australian cotton seed using alternative inoculation techniques. This study contributes to a better understanding of *V. dahliae* and its interaction with both cotton and weeds. In the long term these findings may assist future management approaches for Verticillium wiltin the Australian cotton industry.

Keywords: *Verticillium dahliae*, VCG, cotton, GFP, mCherry, Australia, weed species, Cotton seed

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List of Abbreviations

ANOVA	Analysis of variance
BRIP	Queensland plant pathology herbarium code
CSIRO	Commonwealth Scientific and Industrial Research Organisation
D	Defoliating
DAF	Department of Agriculture and Fisheries
DNA	Deoxyribonucleic acid
DPI	Department of Primary Industries
eGFP	Enhanced Green Fluorescent Protein
GFP	Green Fluorescent Protein
GLM	Generalised linear model
ITS	Internal transcribed spacer
LB	Luria broth
NPK	Nitrogen (15%) / Phosphorus (9%) / Potassium (11%) fertiliser
ND	Non-defoliating
NSW	New South Wales
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
QAAFI	Queensland Alliance for Agriculture and Food Innovation
QLD	Queensland
RFU	Relative fluorescence units
SDW	Sterile distilled water
SEM	Standard Error of the Mean
TEF	Translational elongation factor
T-DNA	Transfer DNA
T-PMT	Photomultiplier tube for transmitted light
UQ	University of Queensland
VCG	Vegetative compatibility grouping

1. Introduction

Cotton (*Gossypium* L.) is a dicotyledonous, perennial shrub cultivated for its soft fibres that develop around the seeds of the cotton plant (Oosterhuis, 1990). Grown in over 80 countries and in a range of ecological niches, cotton is the most economically important source of natural fibre worldwide (Rathore et al., 2006). The genus *Gossypium* contains over 50 species, of which four are cultivated globally as annual crops (Cope, 2018). *Gossypium hirsutum* L., known as upland cotton, is the most widely grown of these species, representing 90% of cotton production globally (Applequist et al., 2001, Cope, 2018, Hu et al., 2019). In Australia, upland cotton production comprises an expanding multi-billion-dollar industry that employs over 12,000 people nationwide (Cotton Australia, 2021).

Verticillium dahliae Kleb. is an asexual, soil-borne member of the Ascomycota which acts as a monocyclic phytopathogen to over 400 plant species (Dadd-Daigle et al., 2021, Fradin and Thomma, 2006, Wheeler and Johnson, 2016). It is speculated that *V. dahliae* originated in Europe (Dadd-Daigle et al., 2021, Short et al., 2015), and has since spread to major temperate cropping regions worldwide (Requena-Mullor et al., 2021). *V. dahliae* is the primary causal agent of Verticillium wilt in many economically important crop species, including upland cotton (Martín-Sanz et al., 2018). Verticillium wilt is a systemic disease that arises from the colonisation and subsequent occlusion of the host vasculature by the pathogen (Berlanger and Powelson, 2000). Resulting symptoms include wilting and stunting of the host, dropping of foliage, discolouration of vascular tissue, development of necrotic lesions, and plant death (Klimes et al., 2015). In Australia, Verticillium wilt is reported to cause reduction in cotton yields in the range of 10% to 62% under pathogen conducive conditions (Holman et al., 2016).

Eradication of *V. dahliae* is challenging once it becomes established in the field. The fungus can persist in soils for up to 14 years in the absence of a susceptible host as melanised resting structures called microsclerotia (Klosterman et al., 2009). The longevity of these microsclerotia makes it difficult to eradicate the fungus through conventional disease management strategies, such as crop rotation or field fallowing (Dadd-Daigle et al., 2021). Microsclerotia remain dormant in soils until they are triggered to germinate by the presence of plant root exudates, which activate hyphae that infect the roots of susceptible hosts (Dadd-Daigle et al., 2021, Mol and Termorshuizen, 1995). Upon entering the host xylem tissues, hyaline, asexual conidiospores are produced, which disseminate systemically and colonise the above-ground host vasculature (Berlanger and Powelson, 2000). The accumulation of fungal material and the resulting host defence responses lead to occlusion of the xylem vessels, inducing the Verticillium wilt disease symptoms (Berlanger and Powelson, 2000). Upon senescence of

the plant, the pathogen permeates the surface of the decaying tissues, producing microsclerotia, after which the fungus returns to dormancy in the soil (Powelson, 1970).

Pathogenic strains of *V. dahliae* are generally categorised based on pathotype (Bellahcene et al., 2005) or race (Baroudy et al., 2019). In cotton, *V. dahliae* pathotypes are described as either defoliating (D) or non-defoliating (ND), depending on the symptoms induced in the host. The D pathotype is generally considered more severe, whilst ND isolates may vary in disease aggression (Jimenez-Diaz et al., 2006, Korolev et al., 2000, Pérez-Artés et al., 2000). Populations within *V. dahliae* can be further classified on a sub-species level based on vegetative compatibility. This refers to the ability of isolates within a fungal species to exchange genetic information through the successful formation of vegetative heterokaryons (Leslie, 1993). Due to the strictly asexual nature of the fungus, hyphal anastomosis provides the only mechanism of genetic exchange between strains of *V. dahliae* (Collado-Romero et al., 2008). Consequently, vegetative compatibility groups (VCGs) within *V. dahliae* have classically been considered as genetically isolated populations that may differ from each other in physiology, virulence, environmental response, and host range (Collado-Romero et al., 2008, Milgroom et al., 2014).

Until recently, the only *V. dahliae* isolate reported in Australian cotton fields was the mildly virulent VCG 4B strain. However, in 2014, Smith et al. (2014) isolated a second strain, VCG 2A, from cotton-growing regions in the Namoi Valley, NSW. Subsequent investigations by Chapman et al. (2016) led to the recovery of a second novel strain, VCG 1A, from the NSW Department of Primary Industries (DPI) historical collections. Of these three VCGs of *V. dahliae* reported to be in Australian cotton, VCG 2A and 4B strains are internationally considered to be ND pathotypes of *V. dahliae*, whilst VCG 1A generally corresponds to the highly virulent D pathotype (Chapman et al., 2016, Holman et al., 2016, Smith et al., 2014). Overseas it is VCG 1A that has been associated with severe yield losses in cotton, as well as in olive production (Jimenez-Diaz et al., 2006). However, typical disease presentation and similar crop losses caused by VCG 1A internationally have not been widely observed in Australia (Dadd-Daigle et al., 2021). On the other hand, VCG 2A has been associated with widespread disease and yield losses in Australian cotton fields, despite there being no reports of VCG 2A causing the same damage overseas (Dadd-Daigle et al., 2021, Dervis et al., 2008). The reasons behind the disparities occuring between these novel isolates are currently unknown.

It has been suggested that the dominating presence of *V. dahliae* VCG 2A observed in Australian cotton fields may be attributed to its ability to colonise and maintain inoculum capacity on weedy hosts common to these regions (Dadd-Daigle et al., 2021, Yildiz et al., 2009). Alternate hosts have the potential to act as intermittent reservoirs for *V. dahliae*, increasing inoculum levels in the field

(Keykhasaber et al., 2018). Pathogenic *V. dahliae* VCG 2A strains have been reported infecting economically important weed species from cotton fields overseas (Yildiz et al., 2009). In Australia, susceptibility to *V. dahliae* has been described previously in a range of native and non-native weedy hosts by Evans (1971); although these reports do not characterise the VCGs associated with these infections. The study by Evans also suggests the potential for *V. dahliae* to endophytically colonise weed species localised to Australian cotton-growing regions. Endophytic infection of hosts within the cotton field would pose an additional challenge to *V. dahliae* management by potentially enhancing carry over of inoculum. It is possible that *V. dahliae* VCG 2A has become the dominant strain in Australian cotton fields by endophytically adapting to a broad range of local hosts (Dadd-Daigle et al., 2021).

A better understanding of how *V. dahliae* moves between geographic areas will guide strategies to limit the spread of VCG 1A and 2A in Australian cotton-growing regions. Established modes of transmission for *V. dahliae* include movement of infected plant material or soil, irrigation, and use of contaminated equipment (Fradin and Thomma, 2006). In cotton, there is evidence suggesting that seed-borne transmission of the pathogen is also possible. Recently, a study on commercially available seed lots reported widespread distribution of *V. dahliae* in cotton seed from Turkish fields (Göre et al., 2014). The study identified *V. dahliae* isolates belonging to the VCGs 1A, 2A, 2B, and 4B from infected seed. At present, *V. dahliae* has not been reported to infect seed from Australian cotton varieties. The potential for the emerging Australian VCGs to be transmitted by Australian cotton seed therefore remains an ongoing concern for the industry.

The implementation of fluorescent reporter technologies can provide novel insights into the complex interactions between *V. dahliae* and its associated hosts. Integration of fluorescent proteins into plant pathogens has facilitated understanding of infection and colonisation patterns in studies on other filamentous fungi (Lorang et al., 2001, Spellig et al., 1996, Eshel et al., 2002). The Green Fluorescent Protein (GFP), isolated from *Aequorea victoria* (Shimomura et al., 1962), and the mCherry fluorescent protein, optimised from *Dicosoma* sp. (Shaner et al., 2004), are two widely used fluorescent proteins in *V. dahliae* transformation (Gregson, 2020, Li et al., 2016, Su et al., 2018). *V. dahliae* isolates tagged with GFP or mCherry have been used to understand pathogen intercellular dissemination in cotton (Li et al., 2016), infection of seed in sunflower (Zhang et al., 2018), and pathogenic processes in the model species *Nicotiana benthamiana* (Su et al., 2018). In Australia, a GFP-tagged isolate of the novel VCG 2A strain was recently used to investigate infection patterns of *V. dahliae* in susceptible and resilient Australian cotton varieties (Gregson, 2020).

The overarching aim of this study is to use fluorescent proteins to observe infection by the emerging *V. dahliae* VCGs 1A and 2A *in planta,* to determine what has allowed these strains to persist in Australian cotton-growing regions. For this purpose, a fluorescing transformant of the VCG 1A isolate with the mCherry protein will be developed and used alongside a GFP-tagged VCG 2A isolate transformed previously (Gregson, 2020). These two fluorescently transformed variants can be used to understand VCG-based distinctions in host range and infectivity in Australian species. Specifically, this study aims to:

1) Generate mCherry-expressing *V. dahliae* VCG 1A transformants,
2) Investigate the potential for *V. dahliae* VCG 1A and 2A strains to infect weed species common to regions of cotton production in Australia and
3) Assess the capacity for these strains to infect the seeds of susceptible and tolerant Australian cotton varieties.

This will lead to insights into *V. dahliae* spread and persistence in Australian cotton fields. It is hypothesised that *V. dahliae* VCG 2A will be the more prevalent isolate in both weed species and seed studies, given its affiliated disease severity in Australian cotton.

2. Methods

2.1 Development of *V. dahliae* VCG 1A mCherry transformant

2.1.1 Origin and preparation of fungal strains and plant material

Fungal isolate BrT0003, a GFP-expressing derivative of *V. dahliae* VCG 2A strain Vd71171 (BRIP 71171) (Sabburg et al., 2021) was provided by Professor Elizabeth Aitken (School of Agricultural & Food Sciences, University of Queensland (UQ)). *V. dahliae* VCG 1A strain Vd71181 (BRIP 71181) originating from Gwydir Valley, NSW, was provided by Dr Linda Smith (Department of Agriculture & Fisheries (DAF), QLD). Vegetative compatibility classifications of *V. dahliae* isolates have been described previously by Gregson (2020). BrT0003 and Vd71181 fungal colonies were incubated for 14 days in the dark at 25°C on half-strength potato dextrose agar (1/2 PDA) (PDA Difco) media. Spores were harvested from plates by flooding plates with 10 mL of sterile distilled water (SDW), disrupting surface mycelia with a no. 22 scalpel, and filtering suspension through sterile Miracloth (Calbiochem, USA). Spore concentrations were determined using a haemocytometer (Neubauer Improved Haemocytometer, LaborOptik, UK).

Untreated, de-linted cotton seed from Australian upland cotton varieties Siokra 1-4 and Sicot 714 B3F were obtained from the Elizabeth Aitken laboratory group. The Siokra 1-4 variety is more susceptible

to Verticillium wilt, whilst Sicot 714 B3F is generally considered to be more tolerant (Gaspar et al., 2014, Gregson, 2020). Seven weed species common to the cotton-growing region of Narrabri, NSW, Australia, were provided by Dr Duy Le (NSW DPI, Narrabri). The species used were: flaxleaf fleabane (*Conyza bonariensis*), common sowthistle (*Sonchus oleraceus*), wild oats (*Avena fatua*), windmill grass (*Chloris truncata*), feathertop Rhodes grass (*Chloris virgata*), liverseed grass (*Urochloa panicoides*), and awnless barnyard grass (*Echinochloa colona*). Seeds from a native tobacco-related species with known susceptibility to *V. dahliae*, *Nicotiana benthamiana*, were provided by Dr Donald Gardiner (Queensland Alliance for Agriculture and Food Innovation (QAAFI), UQ).

2.1.2 Preparation and construction of mCherry plasmid

The fungal transformation vector pPZPnat1-TEF-GFP-yeast (Sabburg et al., 2021) containing the *enhanced GFP* (*eGFP*) gene was digested with the *Bam*HI restriction enzyme (Figure 1) and the plasmid backbone was gel purified using the MinElute Gel Extraction Kit (QIAGEN, Germany) as per the manufacturer's instructions. The following components were used to create the target pPZPnat1-TEF-mCherry-yeast (Figure 1) plasmid: the backbone excised from the pPZPnat1-TEF-GFP-yeast vector, the promoter of the highly expressed constitutive *translation elongation factor 1 alpha* (*TEF*) gene from *Aspergillus nidulans*, and a fungal codon-optimised fragment of the *mCherry* gene which was synthesised by Integrated DNA Technologies and provided by Dr Donald Gardiner. Fragment primers were designed with overlaps to allow for yeast recombinant-based cloning (Table 1). The plasmid pPZPnat1-TEF-mCherry-yeast was constructed in *Saccharomyces cerevisiae* (strain BY4743) using yeast transformation methods described by Gietz and Schiestl (2007). Plasmid transformation into yeast was confirmed by plating colonies onto yeast synthetic drop-out (lacking uracil and tryptophan) media (Sigma, USA) amended with 2% glucose.

The plasmid was then extracted from yeast cells using the Zymoprep™ Yeast Plasmid MiniPrep II kit (Zymo Research), transferred to *Escherichia coli* TOP10 via heat-shock transformation methods (Green and Sambrook, 2020) and grown at 37 °C on Luria broth (LB) media (10g NaCl/L) amended with kanamycin 50 μg/mL. Plasmids were extracted from *E. coli* cultures using the QIAprep® Spin Miniprep kit (QIAGEN, Germany). Extracted plasmids were verified by Sanger sequencing prior to *Agrobacterium* transformation (data not shown).

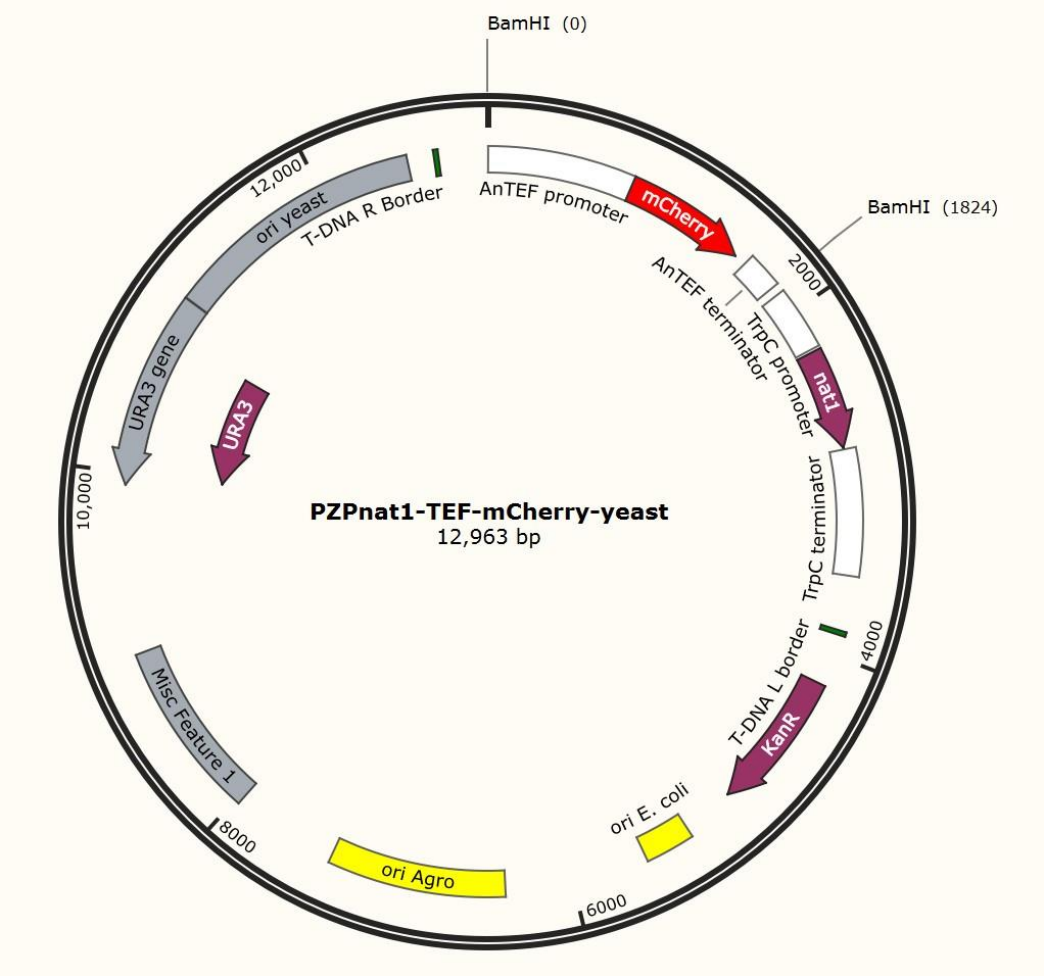

Figure 1. The pPZPnat1-TEF-mCherry-yeast plasmid showing the mCherry fragment and TEF promotor and terminator regions. Locations of the antibiotic resistance genes nourseothricin (*nat1*), kanamycin (*KanR*), and the uracil selective marker for *S. cerevisiae* (URA3) are presented in maroon. TrpC promoter/terminator from *A. nidulans.*

Table 1. Primers for yeast recombinant-based cloning of TEF promoter/terminator and mCherry fragments into backbone

Primer name	Description	Sequence* (5'-3')
DG1346	**Forward primer for TEF promoter**	cctcaccgcggcccatggtctagaactagt**ggatcc**AACGGGC
DG1347	**Reverse primer for TEF promoter**	gcccttggagaccatGGTGAAGGTTGTGTTATGTTTTGTGGA
DG1348	**Forward primer for mCherry**	aacacaaccttcaccATGGTCTCCAAGGGCGAGGAGGA
DG1349	**Reverse primer for mCherry**	aaatcgaatgtccgcTCATTTGTACAGCTCGTCCATACCG
DG1350	**Forward primer for TEF terminator**	gagctgtacaaatgaGCGGACATTCGATTTATGCCG
DG1351	**Reverse primer for TEF terminator**	CTCGAGGTCGACAAGCTTGT

* lowercase = homology arms of primer, uppercase = sequence with homology to PCR template, bold/highlighted = *Bam*HI restriction site

2.1.3 Plasmid transformation into *Agrobacterium tumefaciens* by electroporation

Agrobacterium tumefacien strains AGL1 and EHA105, provided by Dr Donald Gardiner and Dr Andrew Urquhart (CSIRO, Agriculture & Food), were used to transfer the pPZPnat1-TEF-mCherry-yeast plasmid into *V. dahliae*. *Agrobacterium* AGL1 and EHA105 strains were grown on LB media for 24 hrs at 30°C. For AGL1 the selective antibiotics rifampicin and ampicillin were added to the LB media to a concentration of 50 µg/mL; no antibiotics were used for EHA105. *Agrobacterium* colonies were then transformed with the pPZPnat1-TEF-mCherry-yeast plasmid by electroporation as described by Urquhart and Idnurm (2017).

2.1.4 Transformation of *V. dahliae* VCG 1A strain

The *V. dahliae* isolate Vd71181 was transformed with the pPZPnat1-TEF-mCherry-yeast plasmid using methods from Urquhart et al. (2018) with the following adaptations. *V. dahliae* spores were harvested from ½ PDA media as described above in section 2.1.1, and spore concentration was adjusted to 10^8 spores per mL. A volume of 50 µL of *Agrobacterium* suspension and 500 µL of *V. dahliae* spore suspension were pipetted onto 145 mm diameter Petri plates containing 25 mL of induction media (Gardiner and Howlett, 2004) (Appendix 7.1) and incubated in the dark at 22°C for 3 days. Plates were overlaid with molten ½ PDA amended with nourseothricin at a concentration of 50 µg/mL and cefotaxime at 100 µg/mL. Emerging transformant colonies were assessed for spore and mycelial mCherry fluorescence under a Zeiss 700 Laser Scanning Microscope at a laser emission wavelength of 555 nm. Five brightly fluorescing colonies were selected for subsequent analyses.

2.2 Comparison of transformant isolates with *V. dahliae* VCG 1A parent

2.2.1 Comparison of morphology of transformant strains against parent Vd71181 isolate

Macroscopic morphology was assessed by plating fluorescent colonies (putative transformants) and the parent isolate on ½ PDA media and incubating plates in the dark for 10 days at 25°C, after which colonies were assessed for any abnormal appearance compared with the Vd71181 isolate. Transformant spore morphology was assessed using the Cytation 1 Cell Imaging Multi-Mode Reader (BioTek) and Gen5 imaging software. Transformants exhibiting obvious variations from the parent or inconsistencies in fluorescence along hyphae were omitted from subsequent comparison analyses.

2.2.2 Comparison of growth rate of transformant strains against parent Vd71181 isolate

Growth rates of fluorescent transformant strains were measured using the Cytation 1 imager (BioTek). Spores were harvested from transformant and parent isolate plates by flooding with SDW (as described in section 2.1.1.); the spore concentration of the resultant suspension was measured using

a haemocytometer and adjusted to 2×10^6 spores per mL. The suspensions were then diluted with potato dextrose broth (PDB) to a concentration of 2×10^5 spores per mL. A volume of 100 µL of the final spore suspension in PDB was aliquoted into each well of a 96-well MicroWell™ flat plate (ThermoFisher Scientific, USA). Bright field and mCherry fluorescence images of spores were then taken over 30 hrs at 1-hr imaging intervals. The Object Sum Area (μm^2), fluorescence total intensity (RFU), and cell count were calculated for each image. Maximal growth rate (μm^2/hr) was determined as the maximal slope along the Object Sum Area growth curve and was calculated using the BioTek Gen5 software. For each isolate five replicate wells were assessed by analysis of variance (ANOVA).

2.2.3 Quantification of fluorescence in transformant strains

Fluorescence total intensity was measured using the Cytation 1 Cell Imaging Multi-Mode Reader (BioTek) as described above. Transformant fluorescence was calculated as the ratio of final fluorescence total intensity measured at 30 hrs to the initial spore count in each well. For each isolate five replicate wells were assessed by ANOVA statistical analyses.

2.2.4 Comparison of fecundity of transformant strains against parent Vd71181 isolate

Fecundity of transformants was determined by *in vitro* spore production of plated colonies. A 5 mm diameter mycelial plug was taken from the leading edge of transformant and parent Vd71181 colonies and placed in the centre of 90 mm plates set with 25 mL of ½ PDA. Plates were incubated in the dark at 25°C for 10 days. Spores were then harvested with 10 mL of SDW and 10 µL of the resulting spore suspension was used for calculating spore concentration (spores per mL) using a haemocytometer. The mean number of spores for each isolate was calculated from five replicates. A negative binomial generalised linear model (GLM) and post hoc Tukey comparison were performed to compare transformant fecundity with the parent strain. Dispersion of fecundity data was assessed with the *AER* package in *RStudio*.

2.2.5 Pathogenicity assay of transformant strains

Pathogenicity of fluorescent putative transformants were compared with the parent Vd71181 strain to confirm that virulence was maintained following transformation. Sicot 714 B3F seeds were germinated in trays of pasteurised UQ23 potting mix (Appendix 7.2) in a temperature-controlled growth facility. Conditions were maintained at 28°C/24°C (day/night) with a 16hr photoperiod and 64%/80% humidity until 8 days post germination. Spores were harvested from transformants and the Vd71181 parent, and adjusted to a concentration of 10^5 spores/mL in 500 mL of SDW. Cotton seedlings of similar size were gently uprooted and roots washed with SDW. Inoculation of seedlings was conducted by immersing roots in spore suspensions for 5 minutes and repotting into 0.64 L pots using UQ23 mix amended with NPK fertiliser (Osmocote®, Everris, The Netherlands) (4 g/L). A negative

control was included where seedlings were root-dipped in SDW only. Pots were randomised and seedlings were grown at 24°C/20°C (day/night) post inoculation. Soil was top watered every second day. After 4 weeks, leaf chlorosis, necrosis, and defoliation, and plant height of seedlings were scored using a Likert rating scale of 0 to 5 (Figure 2) adapted from Cirulli et al. (1990) for *V. dahliae* induced symptoms (Table 2). Cross-sections of seedling stems approximately 5 mm in height were sampled, surface sterilised with 70% ethanol for five seconds, and plated onto ½ PDA media amended with streptomycin 100 µg/mL and nourseothricin 50 µg/mL (for transformants only) using methods described previously (Le et al., 2020). After 10 days of incubation at 25°C, plated stem tissue was checked for apparently *V. dahliae* growth. Colonies emerging from transformant-inoculated plates were assessed for fluorescence under a Zeiss 700 Laser Scanning Microscope.

Single spore isolates were obtained for culture from stem sections plated onto ½ PDA from plants inoculated with all treatments (putative transformants, parent Vd71181 isolate, and SDW controls). DNA was extracted from each isolate using a rapid extraction buffer as described by Liu et al. (2011). DNA was then amplified by PCR (Appendix 7.3) and assessed for presence of *V. dahliae* genomic material by gel electrophoresis. The *Verticillium*-specific primers used for PCR (Lievens et al., 2006) amplify a product size of 200 bp in the internal transcribed spacer 1 (ITS 1) region (Table 3).Comparison of *V. dahliae* isolation frequency from seedlings between parent and transformant isolates was conducted with Fisher's Exact test for count data.

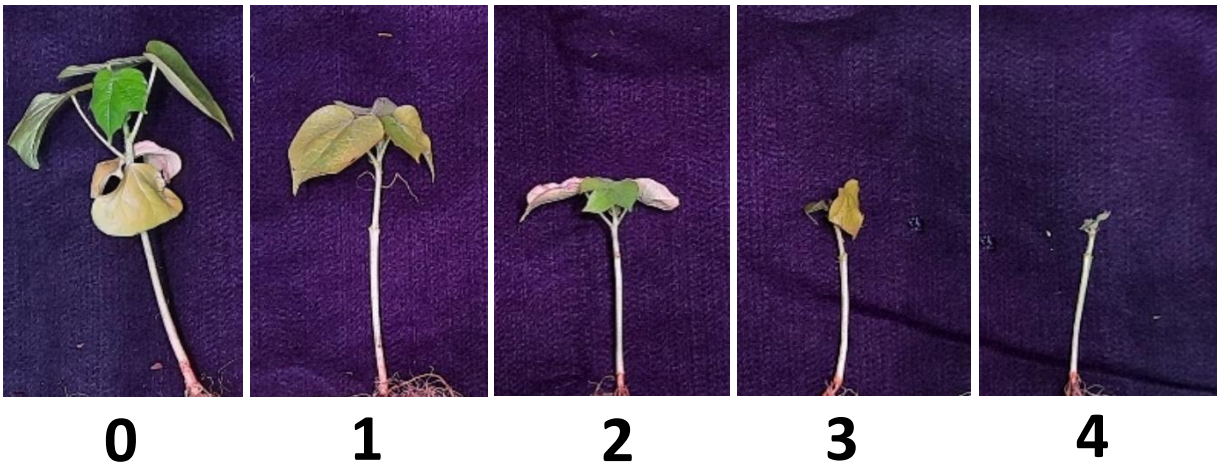

Figure 2. Disease scores for pathogenicity assay comparing the pathogenicity of mCherry-fluorescing transformants against the *V. dahliae* parent isolate Vd71181 in Sicot 714 B3F cotton seedlings. Scale of 0 to 4 depicted above indicates severity of necrosis, chlorosis, and wilting of foliage, and stunting of seedling stems when compared to the uninoculated control plants. Disease scores are based on disease scoring guide adapted from Cirulli et al. (1990) (Table 2). Images show plants assigned to disease scores of 0 to 4. A disease score of 5 indicates a dead plant.

Table 2. Disease scoring guide for above-ground plant tissues used in cotton pathogenicity studies and weed studies. Adapted from Cirulli et al. (1990).

Symptoms	Affected leaves (%)	Degree of stunting compared to control[a]		
		None or very slight (<10%)	Moderate (11%-50%)	Severe (>50%)
None	0	0	-	-
Slight leaf chlorosis, flaccidity, necrosis	1-9	1	2	3
Moderate leaf chlorosis, flaccidity, necrosis, slight defoliation	10-24	2	3	4
Severe leaf chlorosis, flaccidity, necrosis, moderate defoliation	25-50	3	4	4
Plants with severe or complete defoliation	>50	4	4	4
Dead plants	–	5	5	5

[a]scored based on the percentage in height reduced when compared to the uninoculated control plants

Table 3. *V. dahliae*-specific primers from Lievens et al. (2006) amplifying a 200 bp ITS product

Name	Primer type	Organism	Sequence (5'-3')	Target
ITS1-F	Forward	Fungi	CTTGGTCATTTAGAGGAAGTAA	18S rDNA
ST-VE1	Reverse	*Verticillium* spp.	AAAGTTTTAATGGTTCGCTAAGA	ITS 1

2.3 Weed studies experiment

2.3.1 Preparation and inoculation of plant material

Seeds from weed species described in section 2.1.1 were germinated in pasteurised UQ23 potting mix and kept in growth cabinets at ambient laboratory humidity with a 12-hr photoperiod. Wild oat (*Avena fatua*) seeds were grown at 15°C/10°C (day/night) due to the low germination temperature required for the species (Whittington et al., 1970). All other weeds were grown at 25°C/15°C (day/night). Upon emergence of the second true leaf, weed seedlings were root-dipped in fungal spore suspensions of either the GFP-tagged VCG 2A *V. dahliae* transformant, BrT0003, or an mCherry-fluorescing VCG 1A transformant developed from the experiments above (section 2.1). Plant roots were dipped for 10 mins using the same methods described for the pathogenicity assay above (section 2.2.5). A negative control inoculation with SDW only was included. Plants were then individually potted into 0.26 L pots of UQ23 soil amended with NPK fertiliser (Osmocote®, Everris, The Netherlands) (1 g/L) and left for 1-

hour before watering. Pots were randomised and seedlings were kept at 24°C/20°C (day/night) and top-watered every second day.

2.3.2 Assessing for disease and fungal growth in plant material

After 4 weeks post inoculation, plants were scored for disease symptoms according to Table 2 and stem cross sections were taken as before (section 2.2.5), sterilised (Le et al., 2020) and plated onto ½ PDA media amended with streptomycin (100 µg/mL), cefotaxime (100 µg/ml), chloramphenicol (25 µg/mL) and nourseothricin (50 µg/mL). After 10 days of incubation at 25°C plates were assessed for *V. dahliae* growth. Comparison of *V. dahliae* recovery rate between VCG 1A and 2A transformants across all weeds was conducted using Pearson's chi-squared test with Yates' continuity correction. Further analysis of recovery rate of *V. dahliae* transformant strains within weed species was conducted with Fisher's Exact test for count data.

2.4 Cotton seed studies experiment

2.4.1 Preparation and inoculation of plant material

Cotton seeds from the varieties Sicot 714 B3F and Siokra 1-4 were sown into 2 L pots of pasteurised UQ23 potting mix amended with NPK fertiliser (Osmocote®, Everris, The Netherlands) (4 g/L). Initially, two seeds were sown in each pot and placed in a growth cabinet at 28°C/24°C (day/night) with 62%/80% humidity and a 16-hr photoperiod to allow for germination and growth. After 10 days, seedlings were thinned to one plant per pot. Upon maturation to flowering, approximately 8 weeks after seed germination, the temperature in the growth cabinet was reduced to 24°C/20°C (day/night) and the following day cotton flowers were wound-inoculated. Inoculations were carried at a stage just prior to anthesis when flower buds had developed but petals had not yet unfurled (Figure 3). Inoculations were made by making a 2 mm-wide, 5 mm-deep incision immediately below the node at the base of the flowering stem. A 5 mm mycelial plug growing on ½ PDA was taken from either the GFP-tagged or mCherry-tagged *V. dahliae* transformants described in section 2.3.1 and pushed into the wound site. A negative control of inoculation with ½ PDA media only was included. The inoculated stem was then wrapped in tape. Between 3-5 flowers were inoculated per plant. The plants were placed back into a growth cabinet at the temperature regime of 24°C/20°C (day/night). Pots were randomised and plants were bottom-watered at the base of the pot every second day starting from the day prior to inoculation.

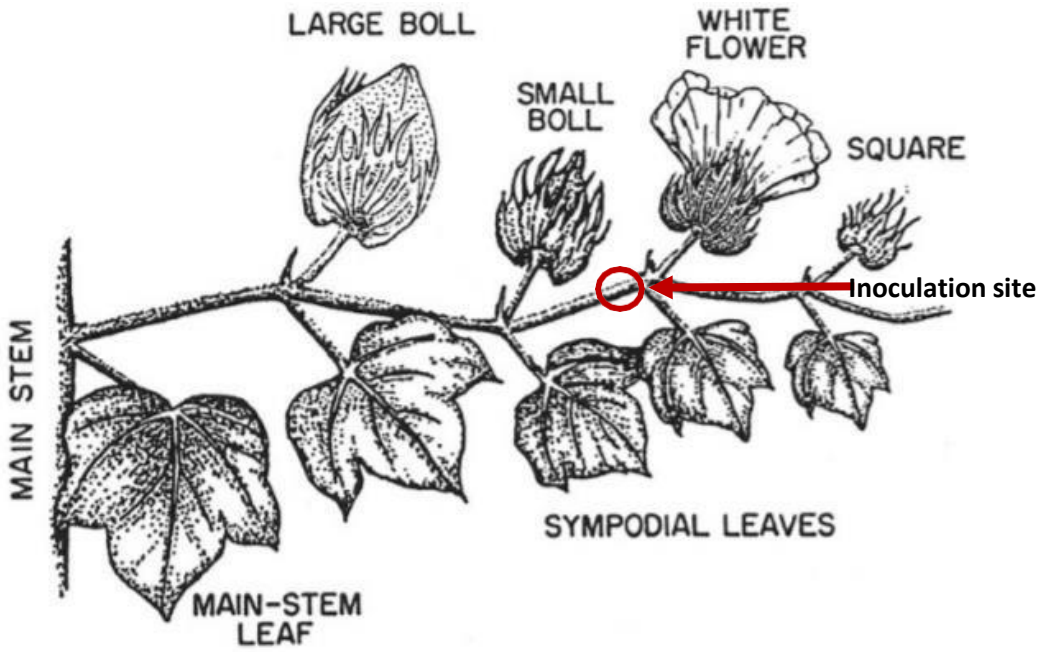

Figure 3. Depiction of flowering branch of cotton plant adapted from Oosterhuis, 1990. Red circle indicates site of wound inoculation relative to flowering bud. Inoculations conducted during anthesis of cotton flower.

2.4.2 Assessing for fungal growth in plant material

Bolls from inoculated stems were harvested upon maturation, approximately 50 days after inoculation. Cotton seeds were delinted by mixing bolls with 98% sulphuric acid (Emsure®, Merck, USA) (~10 mL per 100 g of bolls) until lint was dissolved. Seeds were then doused in a 0.1 M solution of sodium carbonate anhydrous (Univar Solutions, USA), washed twice in SDW, and dried on blotting paper. Ten seeds from each plant were randomly selected, surface sterilised by washing with 1.2% sodium hypochlorite solution (ChemSupply, Australia) for 5 minutes, and then rinsed with SDW for 2 minutes. Seeds were halved longitudinally and plated cut-side-down onto ¼ PDA media amended with cefotaxime (100 µg/mL) and streptomycin (100 µg/mL). After 10 days of incubation at 25°C plates were assessed for *V. dahliae* growth.

2.5 *In planta* and *in vitro* analyses with confocal microscopy

Confocal microscopy was conducted using a Zeiss 700 Laser Scanning Microscope. The previously available GFP *V. dahliae* transformant (Gregson, 2020, Sabburg et al., 2021), BrT0003, was excited with a 488 nm cyan laser, at a master gain of 640. The mCherry transformants obtained in this study were observed under a 555 nm yellow-green laser at a master gain of 809.

2.5.1 Confocal microscopy in pathogenicity assay

Cotton seedlings inoculated with the mCherry-transformed *V. dahliae* were assessed *in planta* for mycelial growth. Longitudinal and transverse sections taken from the base of the stem and at the stem-root junctions were hand dissected and imaged using a 555 nm yellow-green excitation laser.

2.5.2 Confocal microscopy in weed studies

After 10 days of incubation, fungal colonies emerging from plated stem tissue were viewed under fluorescence microscopy. Both transverse and longitudinal sections from plant stems and roots were analysed and observations of fluorescent fungal structures *in planta* were recorded. Above-ground sections were taken from the root-stem junction, base of the stem, leaf nodes, and leaf surfaces. Below-ground sections were taken from the primary root tips, secondary root tips, and sites of lateral root formation.

2.5.3 Confocal microscopy in cotton seed studies

Developing cotton bolls, including seeds, locules containing seeds, calyx (sepal), and nectaries were hand sectioned longitudinally and observed under confocal microscopy. Samples were taken ranging between 2 and 6 weeks post-inoculation. Transverse and longitudinal stem sections were also taken at 5 mm, 10 mm, and 20 mm above and below the wound inoculation site. A total of three bolls and corresponding stems were dissected and studied for each treatment group.

2.6 Statistical analyses

All statistical analyses were conducted in *RStudio* using the packages *rcompanion, AER, FSA, stats, emmeans,* and *MASS*.

Disease score comparisons for the pathogenicity assay and weed experiments were conducted using the non-parametric, rank-based Kruskal Wallis H test. Multiple comparison p-values of disease scores were generated using the Dunn test and adjusted with the Benjamini-Hochberg method. All datasets were tested for normality with the Shapiro-Wilk normality test, and homogeneity of variance using Levene's test. The threshold for statistical significance was a p-value of $p < 0.05$. Graphs were created using Microsoft Excel and the *ggplot2* package in *RStudio*.

3. Results

3.1 *V. dahliae* transformation with the fluorescent protein mCherry

Following *Agrobacterium*-mediated transformation with the fluorescent protein mCherry, a total of 53 transformant colonies of *V. dahliae* Vd71181 strain were obtained, as determined by confocal microscopy (Appendix 7.4). Thirty-six of these transformants grew from plates that were transformed using the AGL1 *Agrobacterium* recipient strain. Twenty transformants were obtained from plates with the EHA105 *Agrobacterium* strain. Of the total 53 transformants, five isolates were selected based on the brightness and uniformity of fluorescence in spores and hyphae (Table 4). These isolates were used for comparative analyses against the parent strain Vd71181.

Table 4. Transformant (T) isolates selected for comparison against *V. dahliae* VCG 1A parent, Vd71181, that originated from Gwydir (Gw) Valley, NSW. Isolates selected for brightness and uniformity of fluorescence. Table includes corresponding *Agrobacterium* strain used for transformation of mCherry protein into *V. dahliae.*

Isolate	Origin strain	VCG	*Agrobacterium* strain
GwT0028	Vd71181	1A	AGL1
GwT0029	Vd71181	1A	AGL1
GwT0030	Vd71181	1A	AGL1
GwT0069	Vd71181	1A	EHA105
GwT0073	Vd71181	1A	EHA105

3.2 Morphology of transformants compared to the parent Vd71181 strain

After 14 days of incubation on ½ PDA, colony growth of the parent Vd71181 strain appeared white and circular, with abundant, hyaline hyphae protruding from the substrate surface. No microsclerotia were visible in parent colonies. Macroscopic morphology of all transformants was consistent with the parent strain (Figure 4). Spores were harvested from isolates and spore morphology was observed under the Cytation 1 plate imager (BioTek). Spores from parent colonies were hyaline, cylindrical to ovate, and approximately 5 µm in length. Spores from transformants GwT0069, GwT0030, GwT0073, and GwT0028 were morphologically similar to the parent strain (Figure 4). Isolate GwT0029 produced spores that visually appeared longer than that of the parent strain (data not shown).

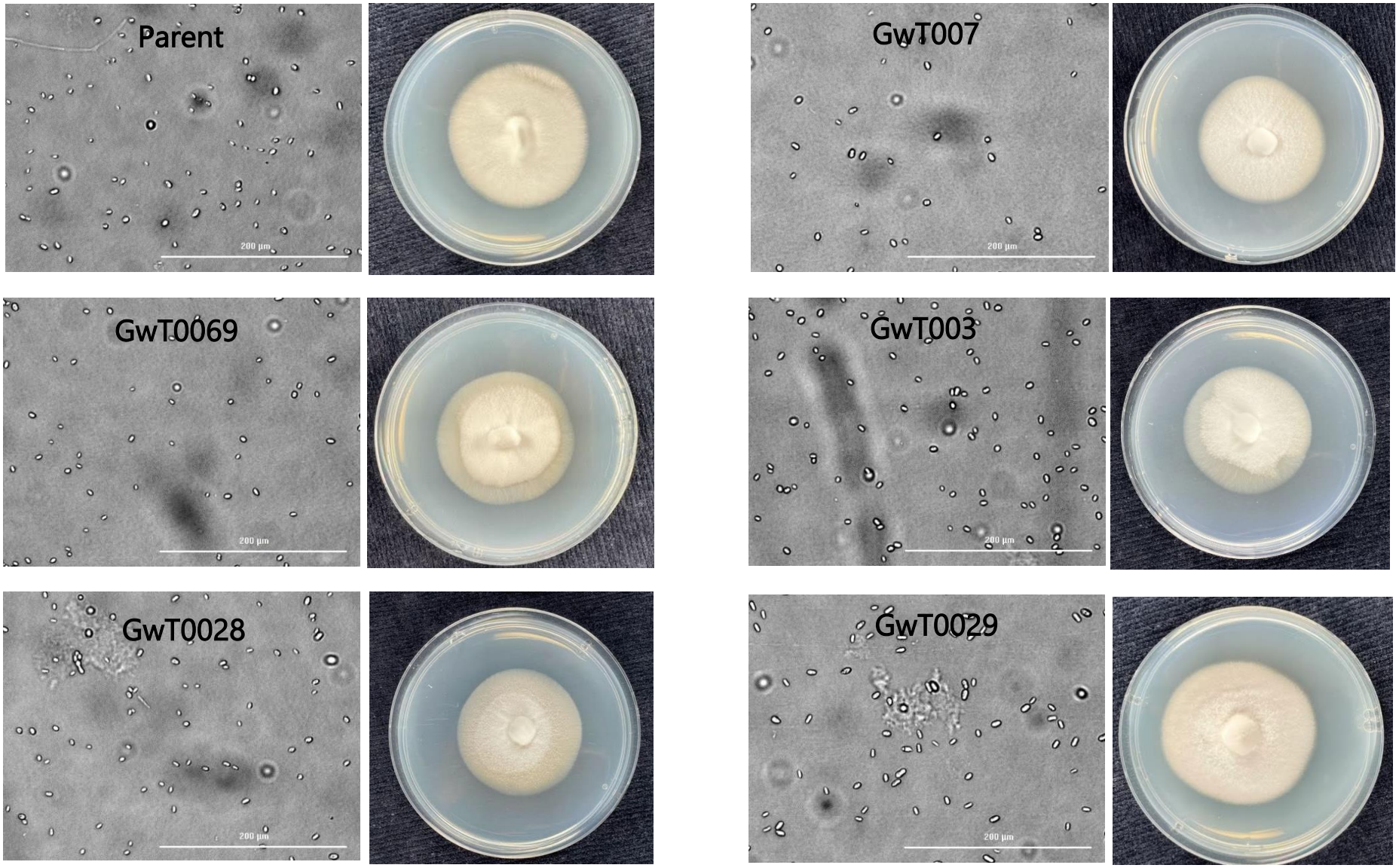

Figure 4. Microscopic and macroscopic features of mCherry transformants compared to the parent Vd71181 *V. dahliae* strain. Microscopic features imaged using the Cytation 1 Biotek imager. Colonies grown for 10 days on ½ PDA. Scale bar = 200 µm.

3.3 Growth rates of transformants compared to the parent Vd71181 strain

The maximal growth rates of parent and transformant isolates were determined using the BioTek Gen5 software and the growth rate means of replicates were compared by analysis of variance. The mean growth rate of the parent Vd71181 strain was 6.17×10^5 µm^2/hr ± 2.26×10^4 (SEM) (n = 5). There was no significant difference between parent growth and the growth rate of transformant isolates (p = 0.101) (Figure 5).

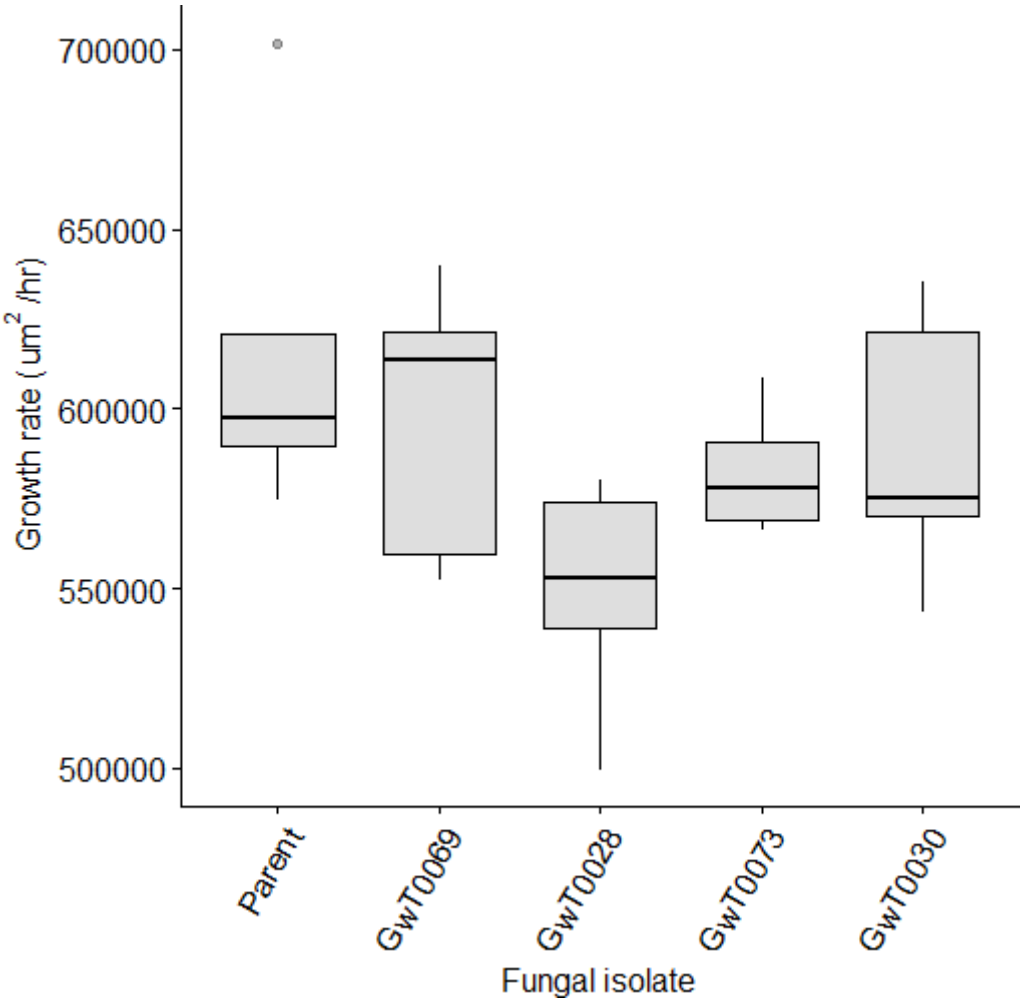

Figure 5. Growth rates (µm^2/hr) of parent Vd71181 *V. dahliae* strain and mCherry-tagged transformants in ½ PDB media over a 30 hr period. Medians and IQRs of growth rates represented by boxes and taken from five replicates (n = 5). Maximum and minimum values shown as whiskers. Statistical analyses performed with one-way ANOVA (p = 0.101), indicating no significant difference between growth rates.

3.4 Transformant GwT0069 is more fluorescent than other transformants

Total mCherry fluorescence of transformants was measured by weighting fluorescence total intensity at 30 hrs post-inoculation against initial spore number. Fluorescence intensity was significantly different between isolates as determined by analysis of variance between replicate means (p < 0.001)

(Figure 6). Transformant GwT0069 (2.35×10^7 RFU ± 2.27×10^6 (SEM), n = 5) was markedly brighter than transformants GwT0028 (8.05×10^6 RFU ± 6.07×10^5), GwT0073 (1.13×10^7 RFU ± 1.53×10^6), and GwT0030 (9.51×10^6 RFU ± 4.46×10^5) ($p < 0.001$) (Figure 7). Although isolate GwT0073 appeared marginally brighter than GwT0028 ($p = 0.409$) and GwT0030 ($p = 0.819$), these differences were not statistically significant. Transformants GwT0069 and GwT0073 were selected for subsequent comparative analyses against the parent Vd71181 strain.

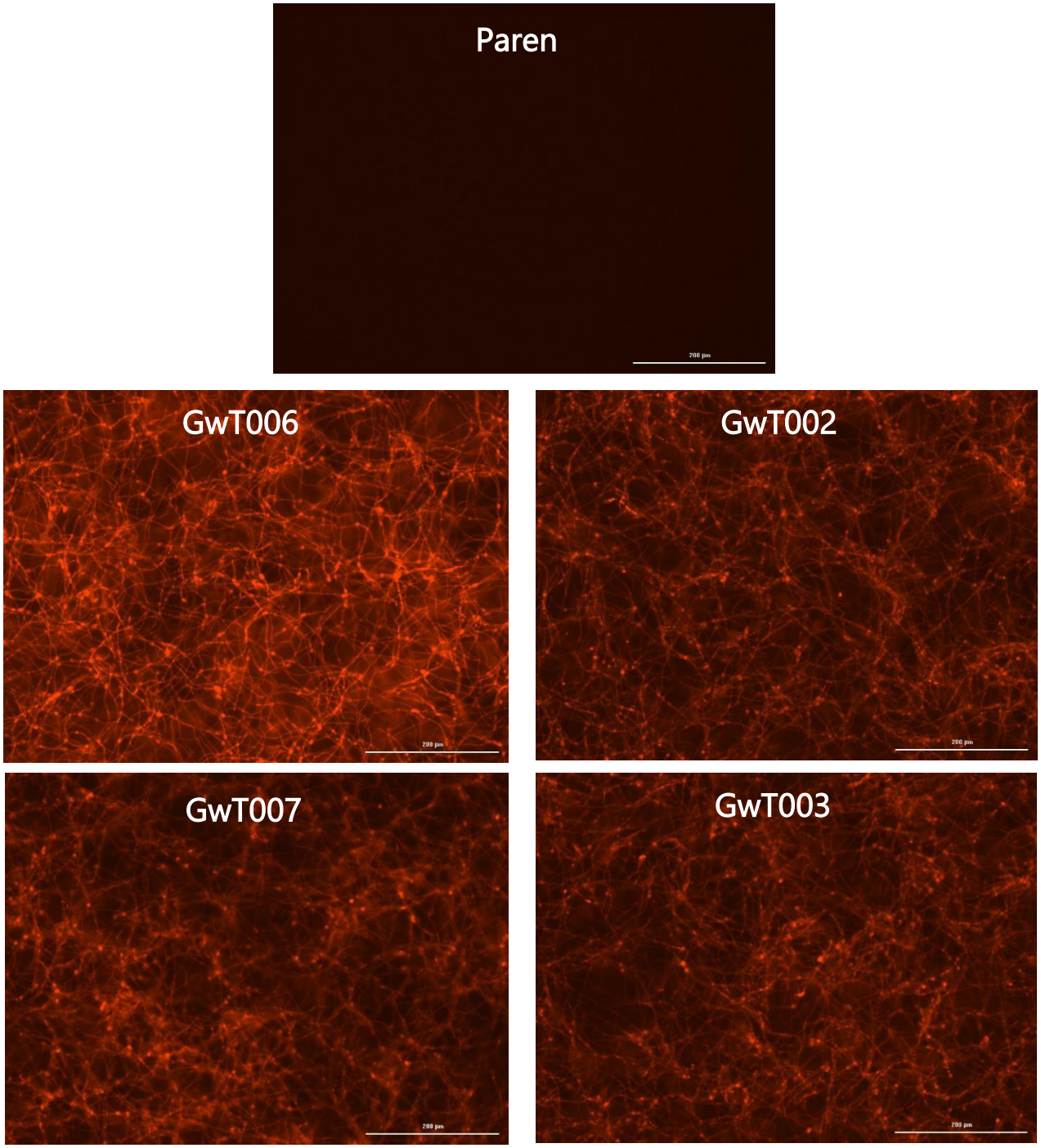

Figure 6. Fluorescence of spores and hyphae from *V. dahliae* transformant strains taken at 15 hrs post-inoculation. Imaged using BioTek Cytation 1 Multi-Reader and Gen5 software. Scale bar = 200 µm.

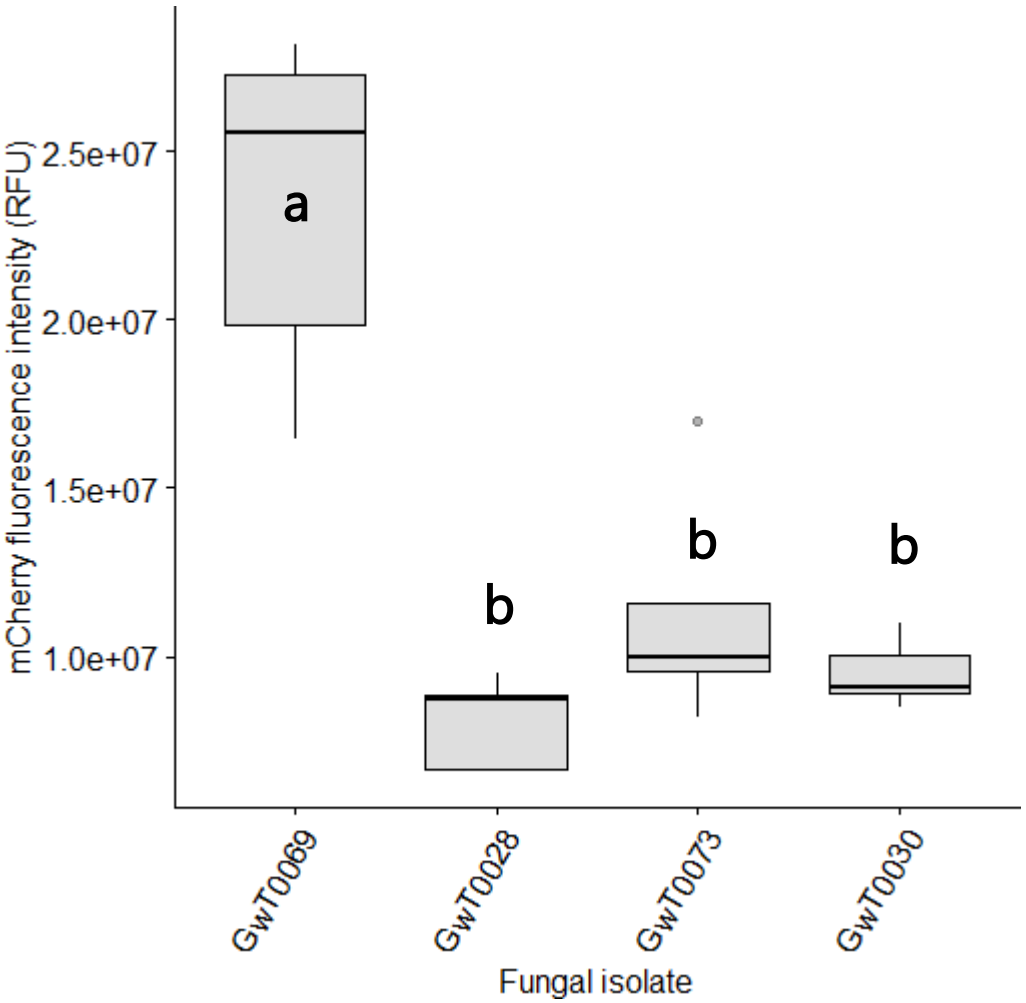

Figure 7. mCherry total fluorescence intensity (RFU) of *V. dahliae* transformant isolates GwT0069, GwT0028, GwT0073, and GwT0030. Medians and IQRs of growth rates represented by boxes and taken from five replicates (n = 5). Maximum and minimum values shown as whiskers. Statistical analyses performed with one-way ANOVA. Letter variations indicate significant difference between isolates ($p < 0.001$) as determined by post-hoc Tukey test.

3.5 Fecundity of transformants compared to the parent Vd71181 strain

V. dahliae fecundity was measured by spore concentration after growing on ½ PDA plates for 10 days using a haemocytometer. The mean fecundity for the parent Vd71181 isolate was 6.33×10^7 spores/mL ± 5.00×10^6 (SEM) (n=5) (Figure 8). There was no significant difference in fecundity between the parent isolate and the transformant GwT0069 (5.83×10^7 spores/mL ± 2.95×10^6 (SEM), n=5) ($p = 0.821$). Transformant GwT0073 (3.05×10^7 spores/mL ± 4.64×10^6 (SEM), n=5) had a lower fecundity than both the parent and GwT0069 isolates ($p < 0.001$).

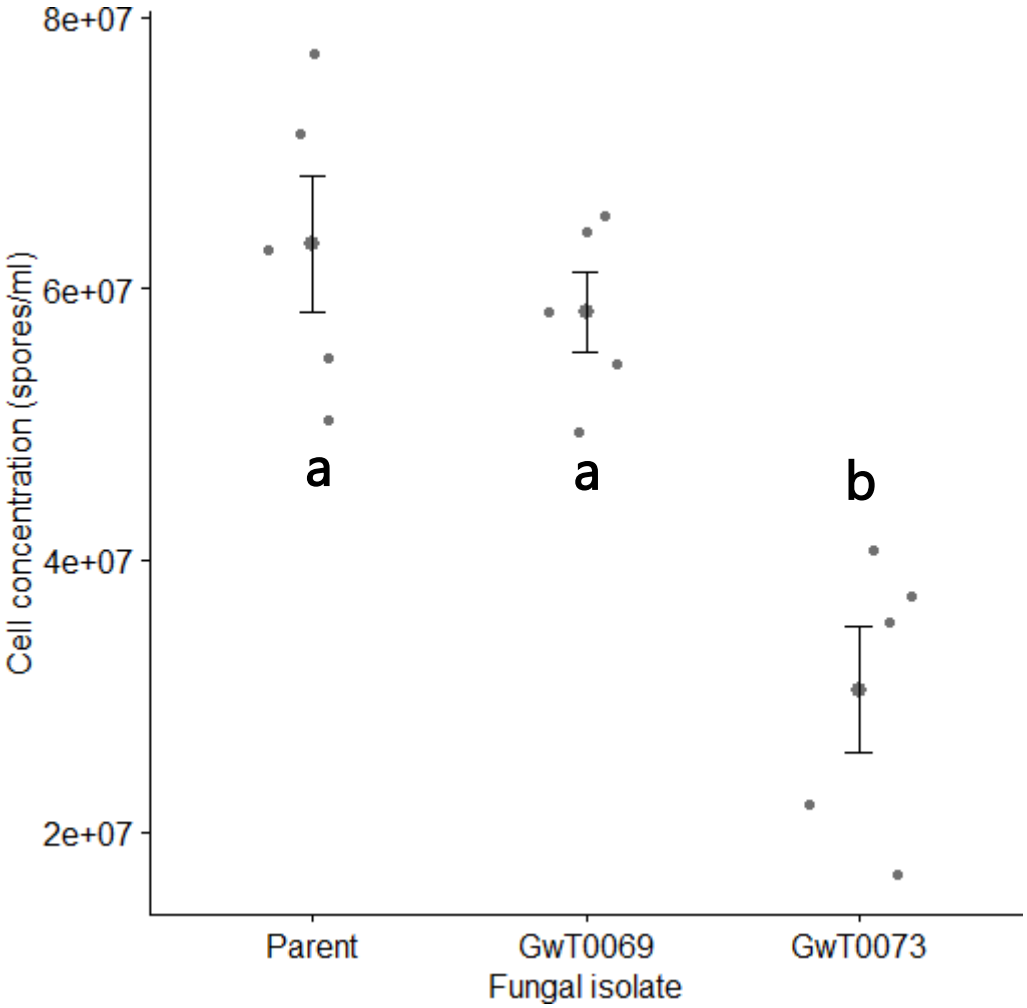

Figure 8. Fecundity of *V. dahliae* parent Vd71181 and transformant GwT0069 and GwT0073 isolates as measured by spore concentration (spores/mL) after 10 days on ½ PDA media. Means taken from five replicates (n = 5). Error bars show standard error of the mean (SEM). Statistical analyses performed with a negative binomial GLM. Letter variations indicate significant difference between isolates ($p < 0.001$) as determined by post-hoc Tukey test.

3.6 Pathogenicity of transformants compared to the parent Vd71181 strain

Sicot 714 B3F cotton seedlings were root-inoculated with the parent Vd71181 isolate and transformants GwT0069 and GwT0073. After 4 weeks of incubation external symptoms were scored and plants were assessed for internal discolouration (Figure 9). All *V. dahliae*-inoculated plants had a significantly higher mean disease rating compared to the water only inoculated negative control ($p < 0.001$) (Figure 10). There was no significant difference in the disease rating between seedlings inoculated with the Vd71181 parent isolate ($\bar{x}$ score = 3.2 ± 0.12 (SEM), n=24) and plants inoculated with either of the transformants GwT0069 (3.3 ± 0.23 (SEM), n=20) ($p = 0.887$) or GwT0073 (3.9 ± 0.19 (SEM), n=21) ($p = 0.076$). Confocal imaging showed that mCherry fluorescent isolates could be visualised *in planta* under a 555 nm yellow green laser, with limited autofluorescence from plant tissue (Figure 11).

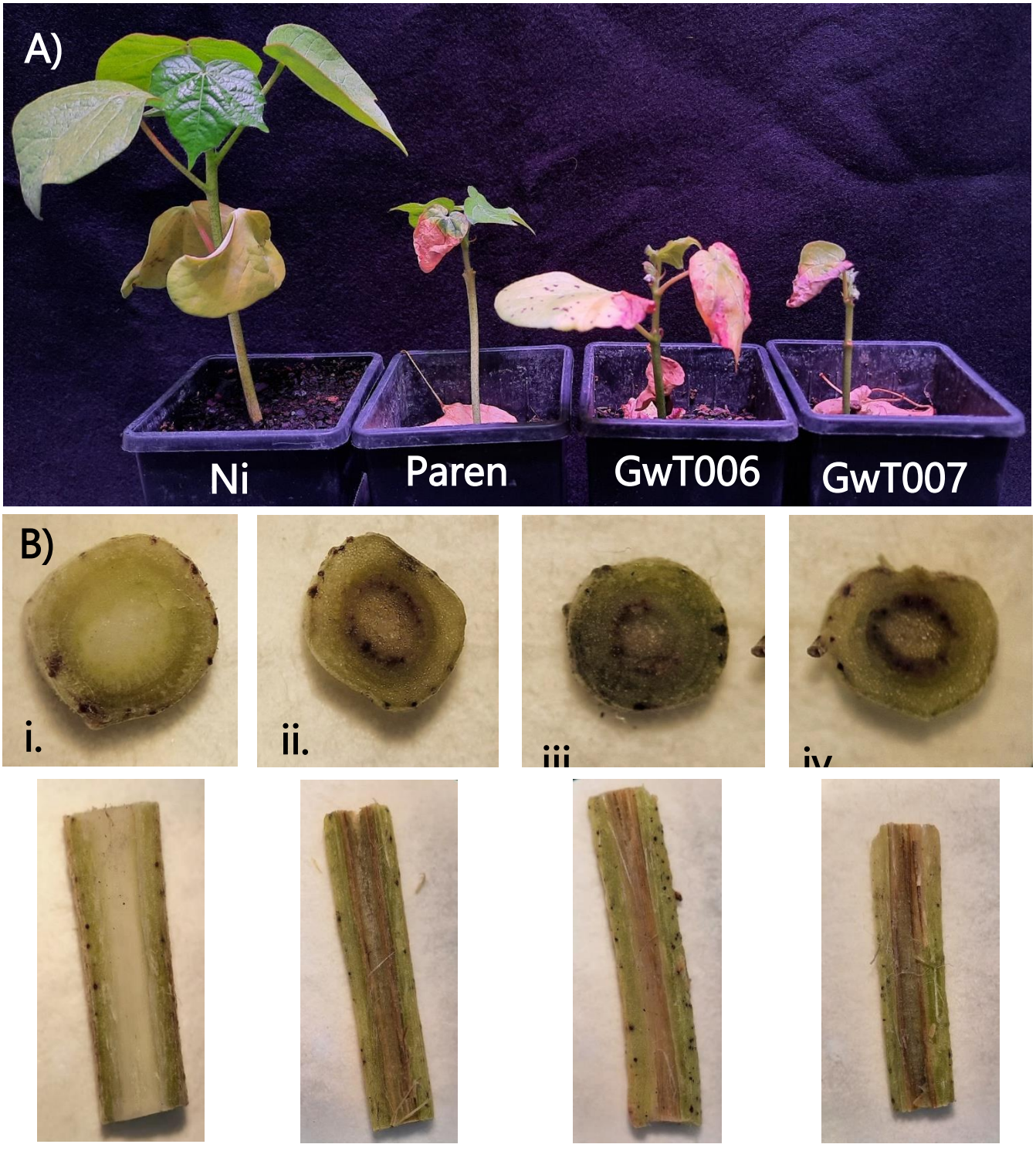

Figure 9. A) Assessing pathogenicity of GwT0069 and GwT0073 isolates against parent Vd71181 strain with Sicot 714 B3F cotton seedlings. Image taken 4-weeks post-inoculation. Disease ratings, based on Cirulli et al. (1990), were as follows: Nil = 1, Parent = 3, GwT0069 = 3, GwT0073 = 4. B) Transverse and longitudinal stem sections of i. Nil-inoculated seedling, ii. Parent-inoculated seedling, iii. GwT0069-inoculated seedling, iv. GwT0073-inoculated seedling.

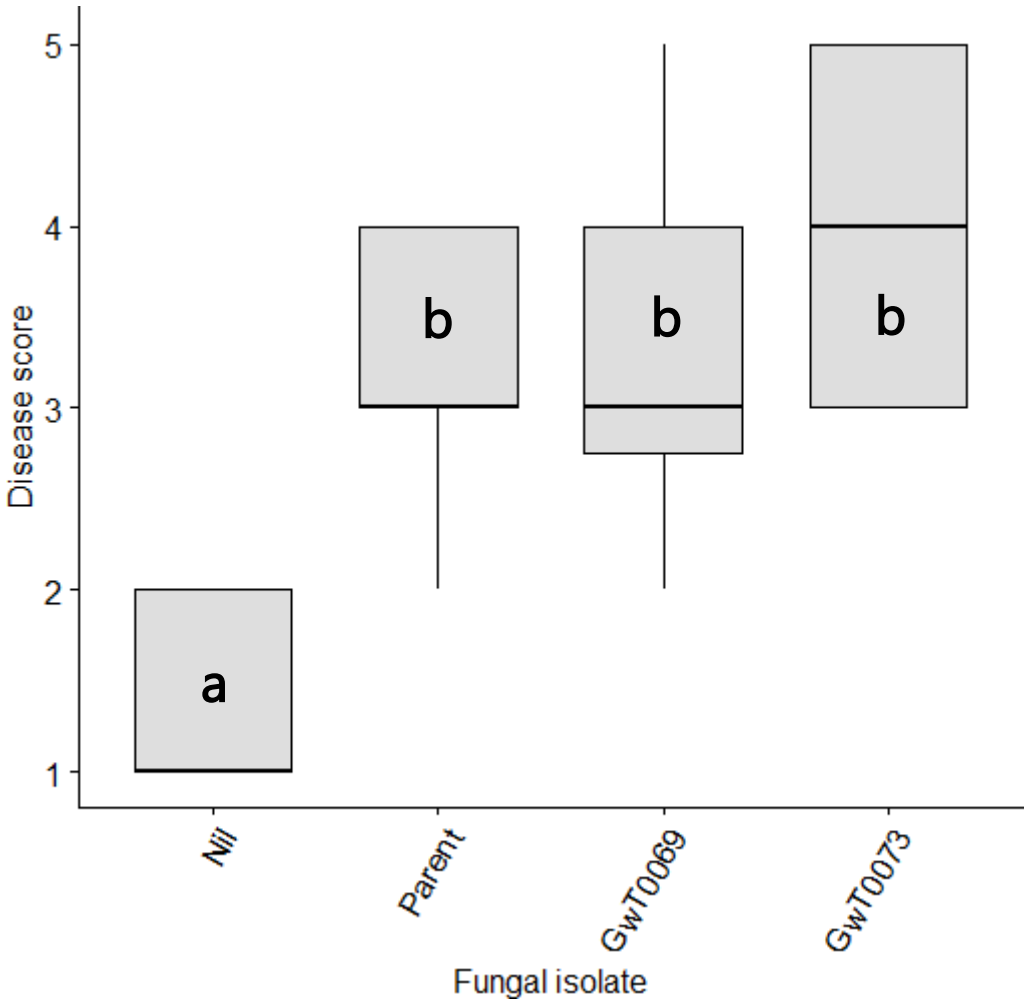

Figure 10. Comparison of mean disease scores in parent Vd71181-, GwT0069-, and GwT0073-inoculated Sicot 714 B3F upland cotton seedlings. Nil = negative control with SDW. Medians and IQRs of growth rates represented by boxes and taken from five replicates (n = 5). Maximum and minimum values shown as whiskers. Statistical analyses performed with non-parametric Kruskal-Wallis H test and multiple comparison post-host Dunn test adjusted with Benjamini-Hochberg method. Letter variations indicate significant difference between isolates ($p < 0.001$).

Seedlings were destructively sampled, and stem cross-sections were plated onto ½ PDA selective media (Figure 12). Colonies emerging from plant tissues that were white and resembled the *V. dahliae* parent Vd71181 colonies (Figure 4) were DNA extracted and amplified by PCR using *V. dahliae* ITS-specific primers (Table 3) to confirm presence of *V. dahliae* fungal DNA (Appendix 7.5). Colonies growing from transformant-inoculated plates were assessed for fluorescence using confocal microscopy. The parent Vd71181 strain was isolated from 62.5% (n=24) of inoculated seedlings. The GwT0069 and GwT0073 transformants were isolated from 45% (n=20) and 66.7% (n=21) of inoculated

seedlings, respectively. There was no significant difference in *V. dahliae* recovery between transformants and parent strain as determined by Fisher's Exact test (p = 0.761). *V. dahliae* was not recovered from any of the negative control plants inoculated with water only.

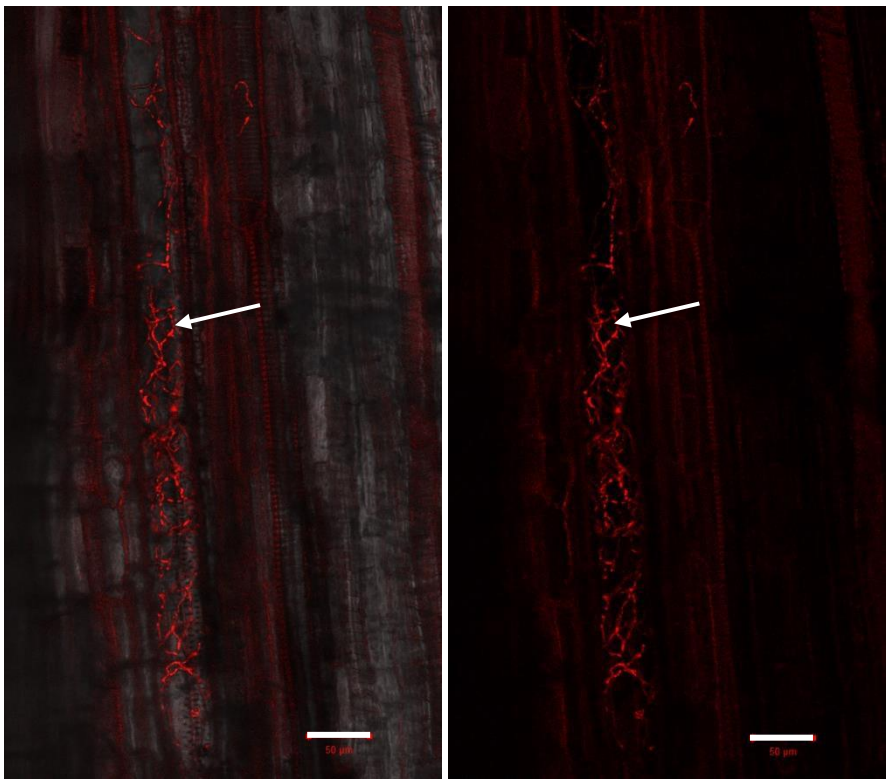

Figure 11. Confocal microscopy image of longitudinal stem section of Sicot 714 B3F cotton seedling with GwT0069 transformant mycelia colonising host xylem tissue. Left: mCherry fluorescence with plant tissue using T-PMT. Right: mCherry fluorescence channel only. Viewed at magnification 10x using EC Plan-Neofluar objective. Laser emission = 555 nm, Master Gain = 809. Arrows show fungal hyphae. Scale bar = 50 µm

Figure 12. Emerging *V. dahliae* colonies from Sicot 714 B3F seedling stem cross-section tissue after 10 days incubation on ½ PDA. Individual plates show 4 samples taken from one seedling.

3.7 *V. dahliae* re-isolation from Australian weed species

Upon emergence of the second true leaf, weed species common to cotton growing regions in Australia were root-inoculated with fluorescing transformant strains of *V. dahliae* VCGs 1A and 2A. Isolate GwT0069, transformed with the mCherry fluorescent protein, was selected for its high fluorescence intensity and similarity to the parent Vd71181 isolate, which belongs to VCG 1A. Isolate BrT0003, transformed with GFP previously by Gregson (2020), belongs to VCG 2A. GwT0069 and BrT0003 were used for all subsequent analyses. Plant stem tissue was plated onto ½ PDA at 4 weeks post-inoculation. *V. dahliae* was isolated from six of the eight inoculated weed species (Table 5). Isolate BrT0003 (VCG 2A) was isolated from all six species, whilst isolate GwT0069 (VCG 1A) was isolated from four of the weed species. *V. dahliae* was not recovered from any of the negative control plants that were inoculated with water only. Across all transformant-inoculated weed species, the recovery rate of transformant BrT0003 was 17.4% (n=190). This was significantly higher than the recovery rate of transformant GwT0069, which had a recovery of 8.9% (n=190) across all weeds ($p = 0.023$). Recovery rates of *V. dahliae* transformants were also compared within individual weed species. The frequency of *V. dahliae* recovery was higher for isolate BrT0003 than GwT0069 in *C. bonariensis, S. oleraceus, C. virgata, U. panicoides,* and *N. benthamiana*, although these differences were not significant as determined by Fisher's Exact analysis (Table 5). The highest recovery percentage within weed species of *V. dahliae* BrT0003 was 61.5% (n=26) in *N. benthamiana*, followed by 24% (n=25) in *U. panicoides*. For transformant GwT0069, the highest recovery percentage was 50% (n=26), also in *N. benthamiana*, followed by 7.7% (n=26) in *E. colona*.

Comparison of disease scores between BrT0003-inoculated, GwT0069-inoculated, and uninoculated plants were assessed by Kruskal-Wallis H analysis and post-hoc comparisons were conducted with the Dunn test. In *N. benthamiana* there was a significant difference in disease symptoms between BrT0003 and GwT0069 treatment groups ($p = 0.006$), as well as between inoculated and uninoculatedplants ($p < 0.001$) (Figure 13). GwT0069-inoculated *N. benthamiana* plants were markedly more diseased than those that were inoculated with BrT0003 (Appendix 7.6). In *C. bonariensis,* plants inoculated with BrT0003 had lower disease scores than both the GwT0069-inoculated ($p = 0.030$) anduninoculated groups ($p = 0.031$). Uninoculated *C. bonariensis* plants appeared to experience some form of stress throughout the duration of the experiment ($\bar{x}$ score $= 1.9 \pm 0.21$ (SEM), n=25) (Appendix7.6).

Table 5. Recovery into culture of *V. dahliae* transformant isolates BrT0003 and GwT0069 from stem tissue of weed species 4 weeks post-inoculation.

Family	Weed species	Isolate	VCG	Frequency[a]	Fisher's p- value[b]
Asteraceae	*Conyza bonariensis* L.	BrT0003	2A	4/26	0.349
		GwT0069	1A	1/26	
	Sonchus oleraceus L.	BrT0003	2A	2/25	0.489
		GwT0069	1A	0/25	
Poaceae	*Avena fatua* L.	BrT0003	2A	0/10	-
		GwT0069	1A	0/10	
	Chloris truncata R.Br.	BrT0003	2A	0/26	-
		GwT0069	1A	0/26	
	Chloris virgata Sw.	BrT0003	2A	3/26	0.235
		GwT0069	1A	0/26	
	Echinochloa colona L.	BrT0003	2A	2/26	1
		GwT0069	1A	2/26	
	Urochloa panicoides P.Beauv.	BrT0003	2A	6/25	0.098
		GwT0069	1A	1/25	
Solanaceae	*Nicotiana benthamiana* Domin	BrT0003	2A	16/26	0.577
		GwT0069	1A	13/26	

V. dahliae was not isolated from any of the water only control plants. [a] Number of plants from which *V. dahliae* was isolated / total plants inoculated. [b] Fisher's Exact test p-value comparing proportion of plants within species infected with BrT0003 versus GwT0069.

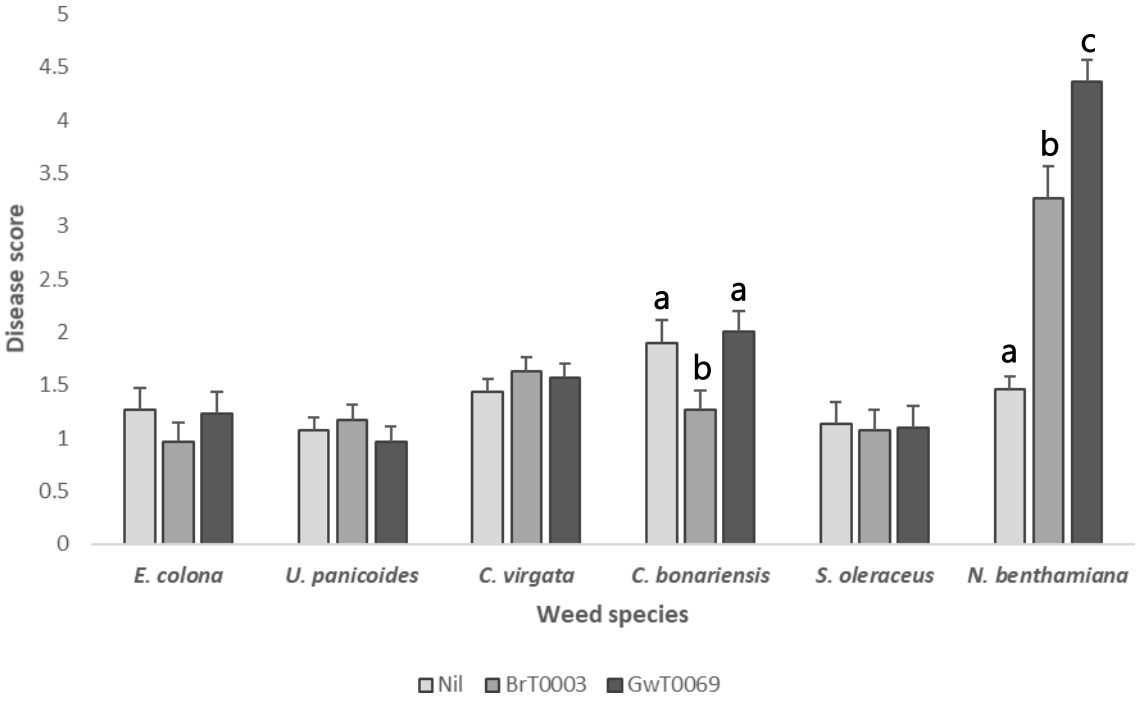

Figure 13. Comparison of mean disease scores in weed species 4 weeks after inoculation with BrT0003 and GwT0069 *V. dahliae* transformants. Scoring method described in section 2.2.5, adapted from Cirulli et al. (1990). Nil = negative control with SDW. Statistical analyses performed with non-parametric Kruskal-Wallis H test and multiple comparison post-host Dunn test adjusted with Benjamini-Hochberg method. Letter variations indicate significant difference within weed species ($p < 0.05$). Error bars represent the standard error of mean (SEM). Light grey bar = disease score of Nil-inoculated plants, middle grey bar = disease score of BrT0003-inoculated plants, dark grey bar = disease score of GeT00069-inoculated plants

Where fungal colonies were recovered from *C. bonariensis, S. oleraceus, C. virgata, E. colona,* and *U. panicoides,* all were confirmed to be either BrT0003 or GwT0069 by fluorescence under confocal microscopy. Stems and roots were sectioned and assessed for presence of *V. dahliae*. Isolates BrT0003 and GwT0069 were observed *in planta* in *N. benthamiana* roots and leaf petioles (Figure 14). Fluorescence associated with the transformed isolates could not be detected *in planta* in any other weed species assessed.

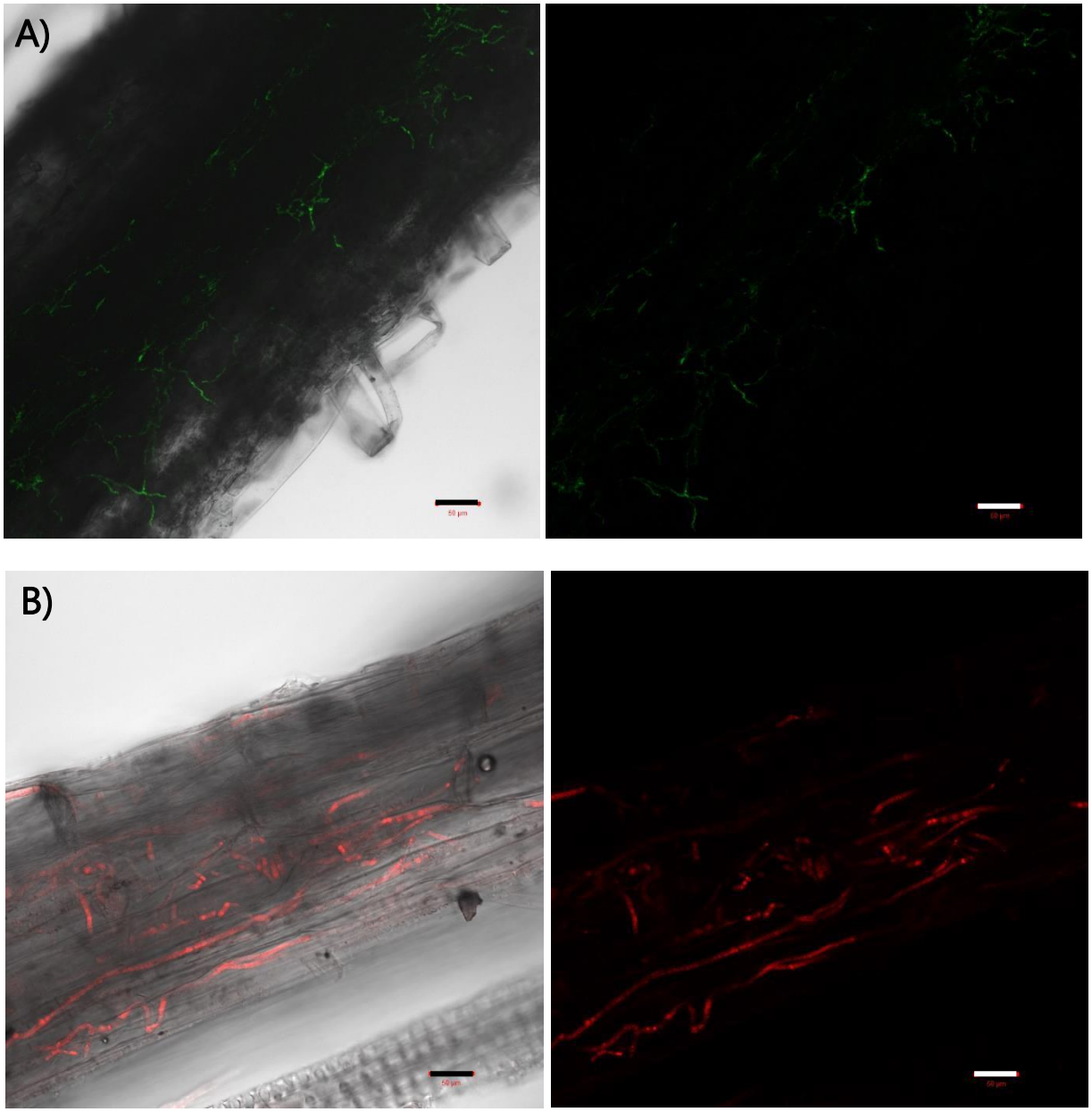

Figure 14. A) Confocal image of longitudinal section of *N. benthamiana* leaf petiole with BrT0003 transformant mycelia colonising tissue. B) Confocal image of longitudinal section of *N. benthamiana* root with GwT0069 transformant mycelia colonising tissue. Left: fluorescence with plant tissue using T-PMT. Right: fluorescence channel only. Viewed at magnification 10x using EC Plan-Neofluar objective. Laser emission = 555 nm (mCherry), 488 nm (GFP). Scale bar = 50 µm.

3.8 *V. dahliae* re-isolation from seed from Australian cotton varieties

Sicot 714 B3F and Siokra 1-4 cotton plants were wound-inoculated with the BrT0003 and GwT0069 isolates below the node of the flowering stem during anthesis. Seeds were harvested upon boll maturation and plated onto ¼ PDA for 10 days. A total of 80 seeds from eight plants were plated for each treatment group. *V. dahliae* was not recovered from any seeds after 14 days incubation. Confocal microscopy of the developing boll, locules, nectaries, and seeds did not indicate the presence of fluorescing fungal structures.

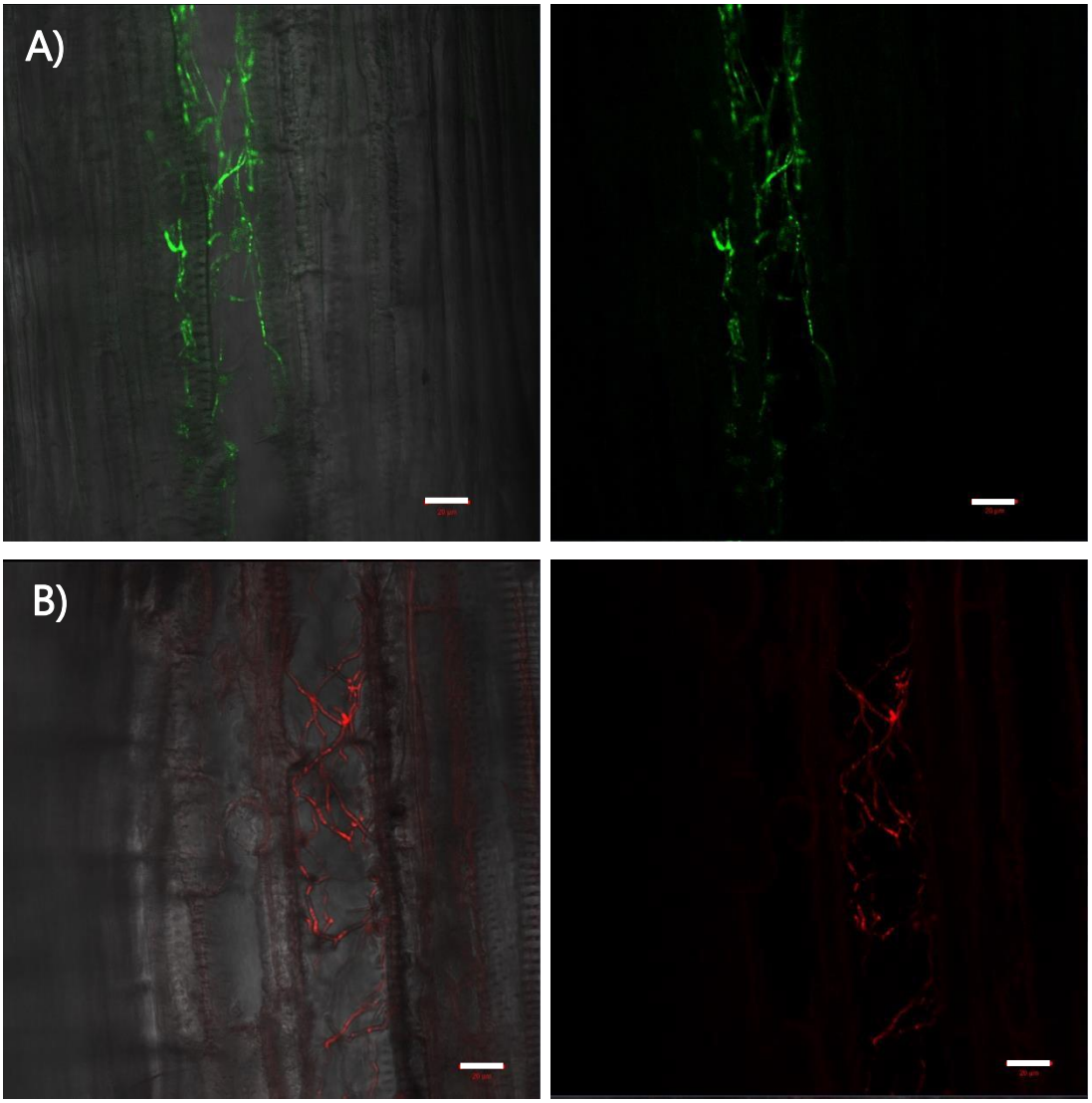

Figure 15. A) Confocal image of longitudinal section of Siokra 1-4 cotton stem with BrT0003 transformant mycelia colonising xylem tissue. B) Confocal image of longitudinal section of Siokra 1-4 cotton stem with GwT0069 transformant mycelia colonising xylem tissue. Left: fluorescence with plant tissue using T-PMT. Right: fluorescence channel only. Viewed at magnification 25x using EC Plan-Neofluar objective. Laser emission = 555 nm (mCherry), 488 nm (GFP). Sections taken 5 mm away from wound inoculation site, towards boll. Scale bar = 20 µm.

Transverse and longitudinal sections of the plant stem tissue adjacent to the wound inoculation site were observed under confocal microscopy. Mycelia of GwT0069 and BrT0003 were observed within Siokra stem vascular tissue, at the point of inoculation and in tissue both in directions towards and away from the developing boll (Figure 15). For Sicot 714 B3F, the isolate BrT0003 was imaged colonising the vasculature of stem tissue, however isolate GwT0069 was not observed in this variety (n=3) (Figure 16). Hyphal structures from both transformant isolates were observed within ~10 mm from the wound sites in both Siokra 1-4 and Sicot 714 B3F varieties and were not seen further down the stem.

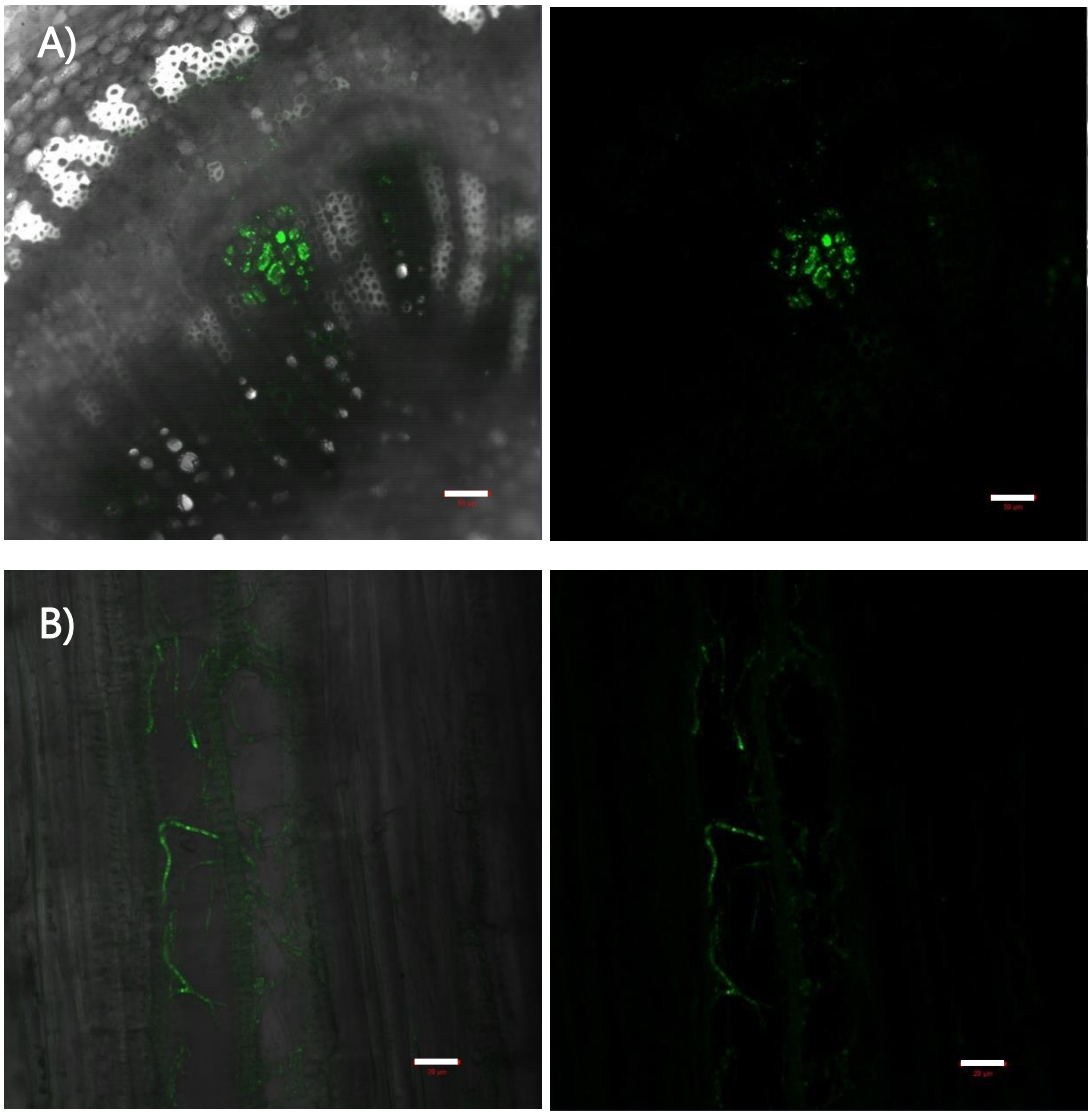

Figure 16. A) Confocal image of transverse section of Sicot 714 B3F cotton stem with BrT0003 transformant mycelia colonising xylem tissue (viewed at magnification = 10x). Scale bar = 50 µm. B) Confocal image of longitudinal section of Sicot 714 B3F cotton stem with BrT0003 transformant mycelia colonising xylem tissue (viewed at magnification = 25x). Scale bar = 20 µm. Left: fluorescence with plant tissue using T-PMT. Right: fluorescence channel only. Laser emission = 488 nm (GFP). Sections taken 5 mm away from wound inoculation site, towards boll.

4. Discussion

The emerging Verticillium wilt problem in Australian cotton has prompted investigation into the newly reported strains of *Verticillium dahliae,* namely VCGs 1A and 2A (Dadd-Daigle et al., 2021). The host range, types of inoculum reservoirs, and modes of transmission of these novel VCGs are some of the areas of particular interest to the Australian cotton industry. The study at hand aimed to develop a fluorescing transformant of a VCG 1A isolate recovered from Australian cotton fields. Using this alongside a previously transformed GFP-tagged VCG 2A isolate (Gregson, 2020), we aimed to assess *V. dahliae* pathogenicity in local potential hosts. Here, we successfully created a stable mCherry-fluorescing VCG 1A transformant and demonstrated the application of these fluorescing isolates as useful tools for research on *V. dahliae*-host interactions.

The mCherry red fluorescent protein is a well-established and reliable candidate for transformation into filamentous fungi (Ivanov and Harrison, 2014, Xiao et al., 2018). As such, mCherry was chosen in the current study for its photostability and fluorescence intensity characteristics that make it suitable for short-term observation by confocal laser scanning microscopy (Schuster et al., 2015, Shaner et al., 2004). Consistent with the established literature, we observed stable expression of mCherry in *V. dahliae*, with minimal bleaching and minimal autofluorescence from host plants. Fluorescing mycelia were clearly differentiated from host cells *in planta* when excited with a 555 nm wavelength laser. This suggests that mCherry is well suited for confocal experiments on *V. dahliae* host infection.

Transformation of the VCG 1A isolate Vd71181 with the mCherry fluorescent protein yielded 53 fluorescing isolates, of which five were selected for subsequent analyses. Isolate GwT0069 showed high similarity in morphology, growth rate, *in vitro* fecundity, and pathogenicity in cotton seedlings compared to the original parent strain Vd71181. GwT0069 was also markedly stronger in fluorescence intensity compared to other transformed isolates. Other studies have similarly found mCherry-transformed *V. dahliae* isolates to be reliable for *in planta* experiments (Su et al., 2018). These support our conclusions that uniform expression of mCherry can be generated in *V. dahliae* without detriment to fungal growth or pathogenicity.

The emergence and widespread prevalence of the Australian VCG 2A strain has prompted investigation into the capacity for Australian *V. dahliae* isolates to colonise common Australian hosts. Prior reports propose that VCG 2A may predominate in Australian cotton fields through its ability to infect and multiply in associated weed species (Dadd-Daigle et al., 2021). The study at hand, which compared the infectivity of transformant VCG 1A and 2A isolates in eight different local species, supports this notion. Here, the VCG 2A transformant, BrT0003, was re-isolated from a greater number of weed species than the VCG 1A transformant, GwT0069. Furthermore, transformant BrT0003 was

recovered significantly more frequently across all species than transformant GwT0069. This suggests that *V. dahliae* VCG 2A strains may have a broader range of weedy hosts associated with Australian cotton fields and may also have the capacity to infect these hosts with higher frequency than VCG 1A strains. Further comparisons of transformant recovery within weed species found that BrT0003 was generally isolated more frequently from plants than GwT0069. These differences within species were non-significant; however, they do support the notion that VCG 2A isolates may be more effective at colonising weeds than VCG 1A. This highlights the need to further investigate local species as potential reservoirs for *V. dahliae* VCG 2A in Australian environments.

V. dahliae was isolated from six of the eight species investigated, including the known host, *Nicotiana benthamiana* (Su et al., 2018). *N. benthamiana*, a native Australian relative of tobacco, has been used as a model species to understand *V. dahliae* infection in previous studies (Faino et al., 2012, Su et al., 2018, Yin et al., 2021). However, there is limited literature describing *N. benthamiana* susceptibilities to different *V. dahliae* VCGs. The study at hand re-isolated transformants GwT0069 and BrT0003 from *N. benthamiana* plants, suggesting that this species is a host for both *V. dahliae* VCGs 1A and 2A. It was also found that the VCG 1A transformant, GwT0069, induced higher severity of disease symptoms and frequency plant death than VCG 2A transformant, BrT0003. These findings therefore provide a deeper insight into *V. dahliae* interactions with the model species *N. benthamiana.*

To our knowledge, this is the first report of *Chloris virgata* and *Urochloa panicoides* as hosts of *V. dahliae*. This is also the first description of *V. dahliae* infection of *Conyza bariensis* and *Echinochloa colona,* although closely related members within these genera have been reported as hosts of the pathogen elsewhere (Evans, 1971, Frederick et al., 2017). Reports on the status of *Sonchus oleraceus* as a host for *V. dahliae* are conflicting (Ligoxigakis et al., 2002, Thanassoulopoulos et al., 1981, Vallad et al., 2005, Woolliams, 1966, Yildiz et al., 2009). In the present study, *S. oleraceus* was found to be infected with *V. dahliae* BrT0003 at low frequencies (8%). This coincides with previous reports of VCG 2A infecting *S. oleraceus* internationally (Frederick et al., 2017), as well as *V. dahliae* infecting this species in Australia (Evans, 1971).

V. dahliae could not be isolated from the native species *Chloris truncata* or from the globally-distributed species *Avena fatua*. Conflictingly, *A. fatua* has previously been reported as a host of *V. dahliae* at low frequencies in olive orchards in Greece (Thanassoulopoulos et al., 1981). Furthermore, *V. dahliae* VCG 2A specifically has been associated with *A. fatua* in North American regions of potato production, and has been observed to produce microsclerotia on host tissue (Frederick et al., 2017). However, given the low frequency of *V. dahliae* observed in these studies, it is unsurprising that *V.*

dahliae was not isolated from *A. fatua* in the study at hand. Further investigation is needed to understand if *A. fatua* is a susceptible host to *V. dahliae* in an Australian context.

Monocotyledonous species are generally considered to be resistant to Verticillium wilt disease, however they may remain susceptible to endophytic colonisation by the fungus, which is symptomless (Dung, 2020, Malcolm and Jimnez-Gasco, 2011). Consistent with the literature, the study at hand found that infection by *V. dahliae* was asymptomatic for all weeds belonging to the Poaceae family. Similar results have been described previously in other grass species (Krikun and Bernier, 1990). In *S. oleraceus,* a dicotyledonous weed, there was no difference in disease scores between inoculated and uninoculated groups*,* which also indicates asymptomatic colonisation of this species. This finding was consistent with other studies on *V. dahliae* infection of *S. oleraceus* in fields overseas (Frederick et al., 2017).

V. dahliae BrT0003 and GwT0069 were both re-isolated from the dicotyledonous species *C. bonariensis*. Interestingly, plants inoculated with the BrT0003 isolate had a lower overall disease score than the uninoculated control group. This difference was significant at a p-value of $p < 0.05$, but did not satisfy a more stringent threshold of $p < 001$. *C. bonariensis* seedlings were exhibiting signs of stress or disease throughout the duration of the experiment, which is evident from the disease scores of the uninoculated control plants. Given the low isolation frequency of BrT0003 from the host, it is likely that these differences in symptoms were caused by some unknown confounding factor. It is also possible that the disease rating method used (Cirulli et al., 1990), which was developed originally for assessing Verticillium wilt in eggplant, was not suitable for use in *C. bonariensis.* This warrants further investigation into whether *C. bonariensis* is susceptible to Verticillium wilt.

In the weed experiments, all *V. dahliae* colonies recovered from host stem tissue and cultured *in vitro* could be confirmed by assessment of fluorescence under confocal microscopy. Consequently, the use of fluorescently-tagged *V. dahliae* transformants provided a rapid and reliable method of isolate identification. Observation of fluorescing transformants occurred less frequently for *in planta* analyses. BrT0003 and GwT0069 were only observed *in planta* in *N. benthamiana,* which had a higher isolation frequency (~50%) of *V. dahliae* compared to other weed species. Fluorescing transformants were observed both in roots and leaf petioles of *N. benthamiana*. These observations coincide with similar studies investigating *N. benthamiana* infection with fluorescently-tagged *V. dahliae*, which observed *in planta* dissemination of fungal mycelia in plant roots, stems, and leaves (Su et al., 2018, Zhang et al., 2017). Overall, the fluorescing transformants were useful for *in vitro* diagnosis but the low infection frequency in weedy hosts limited the potential for *in planta* observation.

It is important to note that whilst this study identifies species that are susceptible to infection by *V. dahliae* VCGs 1A and 2A, it does not investigate the capacity of these hosts to increase pathogen inoculum levels in the field. *V. dahliae* microsclerotia are the primary fungal propagules that persist in soils and act as carry-over inoculum into subsequent cropping seasons (Busch et al., 1978). Therefore, quantifying microsclerotia production that occurs in plant hosts is important for Verticillium wilt disease prediction and management (Goud and Termorshuizen, 2003). Prior studies have demonstrated the potential for weed species to be infected by *V. dahliae* without increasing the microsclerotia inoculum load (Busch et al., 1978, Evans, 1971). Evans (1971) isolated *V. dahliae* from the weed *S. oleraceus* in Australian cotton fields, however concluded the species as a non-host to *V. dahliae* because infection was confined to the plant roots and did not result in the production of microsclerotia. On the other hand, a study on the monocotyledonous weed *A. fatua* found that the species was host to a tomato-pathogenic *V. dahliae* strain from North America, and that production of microsclerotia was higher in this species compared to other local weed hosts (Frederick et al., 2017). Consequently, further investigations into the capacity of local species to increase inoculum load of *V. dahliae* will help to shape future Verticillium wilt management approaches.

This study investigated the capacity for the novel Australian VCGs to infect, and thereby be transmitted by, cotton seed. Support for *V. dahliae* infection of cotton seed varies across the literature,however there is evidence suggesting that seed-based dissemination of the pathogen is possible (Göre et al., 2014, Allen, 1953). In the study at hand, *V. dahliae* was not recovered from cotton seeds following artificial wound inoculation of stems adjacent to cotton flowers. There was limited migration of fungal structures within plants when stem tissues were analysed under confocal microscopy. Furthermore, there were no obvious differences in *V. dahliae* infection between the susceptible Siokra 1-4 and the more tolerant Sicot 714 B3F variety. This suggests that the *V. dahliae* GwT0069 and BrT0003 isolates were able to infect cotton plants at the wound site but did not disseminate systemically. In previous studies on *V. dahliae* in Australian cotton, both D and ND *V. dahliae* pathotypes were isolated from the stems of mature cotton plants infected with the pathogen naturally in the field (Le et al., 2020). Therefore, timing of inoculation may be relevant to dissemination of *V. dahliae* in mature cotton plants, since stage of plant development plays a key role in susceptibility to
V. dahliae infection and mature plants are generally considered more resilient to the pathogen (Bugbee and Sappenfield, 1970). Late-stage wound inoculation was chosen as the method for inoculation to reduce seedling mortality and because it offered controlled timing during the flowering stage of the plant. As seen in the cotton seedling pathogenicity trials conducted in this study, early inoculation of seedlings resulted in a high frequency of plant death. However, it was also observed in these trials that some seedlings exhibited Verticillium wilt disease symptoms without completely

senescing. Future studies may benefit from investigating whether these *V. dahliae*-infected seedlings have the potential to recover from early onset disease and whether *V. dahliae* infection is maintained in the plant upon maturation to seed development.

In artificial stem inoculation studies, Allen (1953) found that inoculation of *V. dahliae* at the base of the cotton plant primary stem resulted in vascular discolouration extending into lower branches. *V. dahliae* was re-isolated from these discoloured vascular tissues, suggesting that the pathogen was capable of migrating from the inoculation site. Interestingly, when similar inoculations were made higher on the primary stem, near the stem tip, the study found that vascular symptoms were restricted to the immediate vicinity of the inoculation wound. This suggests that site of inoculation may also play a role in *V. dahliae* dissemination in mature cotton plants. Therefore, further studies involving alternate inoculation techniques are needed to clarify whether the *V. dahliae* VCGs 1A and 2A are capable of seed infection in Australian cotton varieties.

4.1 Future directions

The GwT0069 and BrT0003 fluorescent transformants provide a novel avenue for investigating how and why the *V. dahliae* VCGs 2A and 1A have only now emerged as threats to Australian cotton production. Utilising these transformant strains may aid future studies in revealing complex interactions, such as those between *V. dahliae* and the soil microbiome. They can also feed into co-inoculation studies to understand the relationships occurring when both VCG 1A and 2A are co-present in the field. These transformants therefore have broad application in future *V. dahliae* research.

This study provides an insight into weed and cotton interactions with *V. dahliae* under artificial inoculation but does not reflect conditions that occur in the field. External factors such as timing of infection, inoculum load, temperature, and irrigation are likely to play roles in *V. dahliae* infectivity in weedy hosts and dissemination in cotton plants (Bugbee and Sappenfield, 1970, Ondieki, 1969, Palanga et al., 2021, Tani et al., 2018). Consequently, investigation into the natural infection of these hosts under field conditions is needed. Furthermore, whilst this study identifies potential hosts of *V. dahliae*, it does not assess the capacity for these hosts to increase inoculum levels of the pathogen. Therefore, quantifying the proliferation of *V. dahliae* and production of microsclerotia propagules in the presence of these species is needed to understand whether they contribute to Verticillium wilt epidemics in the field.

4.2 Conclusions

This study demonstrates that the mCherry protein is effective at inducing stable fluorescence in *V. dahliae* VCG 1A, allowing for rapid identification of transformed isolates through confocal microscopy.

Fluorescent proteins are powerful tools for visualising the extent of *V. dahliae* infection in cotton and other hosts and can therefore be used to better understand *Verticillium* wilt in an Australian setting.

In cotton seed studies, confocal microscopy was used to assess *V. dahliae* migration in mature cotton plants, however the pathogen was not isolated from seed. Further investigation is needed to understand whether *V. dahliae* is seed-borne in Australian cotton varieties.

This is one of the few studies that assesses weed susceptibility to specific *V. dahliae* VCGs. We report five weed species from local cotton fields that have the capacity to harbour *V. dahliae*. To our knowledge, four of these have not been described as hosts of this pathogen previously. The majority of infected plants were asymptomatic, supporting the notion that *V. dahliae* VCG 2A may be colonising weeds undetected. Importantly, these hosts are economically important weed species that are widespread in cotton growing regions in Australia and resistant to conventional herbicides, such as glyphosphate (Preston, 2020). The insights provided by this study will therefore aid in developing a more coherent approach to Verticillium wilt management in the Australian cotton industry.

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Appendices

6.1 Preparation of induction media

Preparation of induction media (IM) agar used for *Agrobacterium* transformation of mCherry fragmentinto *V. dahliae* Vd71181 was taken from Gardiner & Howlett (2004) and consisted of the following:

For 1 L:

- 1 x MM salts (salts described below) (400 mL 2.5x stock)
- 5 mM glucose (0.9 g)
- 0.5 % glycerol (5 mL)
- dH20 (540 mL)
- 2 % agar (20g)

Preparation of IM agar:

- Dissolve 7.7 g MES in 50 mL water, pH to 5.3 with KOH. Filter sterilise and add directly to the IM medium after autoclaving.
- Dissolve 19 mg (or 38 mg for 200 μM) acetosyringone in 250 μL DMSO. Add directly to the IM medium after autoclaving.

MM salts for IM media (1 L of 2.5x stock)

- 3.625 g KH_2PO_4
- 5.125 g K_2HPO_4
- 0.375 g NaCl
- 1.250 g $MgSO_4.7H_2O$
- 0.165 g $CaCl_2.2H_2O$
- 6.2 mg $FeSO_4.7H_2O$
- 1.250 g $(NH_4)_2SO_4$

6.2 UQ23 potting media

This media was designed by K. Hayes (2014). Made to a recipe and have a pH of 5.5-6.5, to enable awide range of plants to be grown in the research Glasshouses.

Note: The pH is balanced with either $FeSO_4$ if pH is high or Dolomite if pH is low.The

UQ23 potting media consists of:

- 70% Composted Pine Bark 0-5mm.
- 30% Coco Peat.

Fertilizers and other augments/ M^3:

- 1 kg Yates Flowtrace
- 1 kg Iron Sulphate Heptahydrate.
- 0.4 kg Superphosphate.
- 0.03 kg Copper Sulphate
- 1 kg Gypsum

6.3 PCR details

Appendix table 1. Components for PCR reaction Mastermix for amplification of *V. dahliae*-specific ITS productof 200 bp. Primer sequence obtained from Lievens et al. (2006).

Component	**Per PCR reaction (µL)**
H_2O	16
MyTaq Reaction Buffer 5x (Bioline)	5
Taq polymerase	1
Primers (Forward+Reverse)	1 + 1
DNA	1

Appendix table 2. PCR cycling details for amplification of *V. dahliae*-specific ITS product of 200 bp.

Temperature (°C)	**Duration of temperature hold**	**Number of cycles**
94	1 min	1
94	30 s	
58	30 s	30
72	30 s	
72	5 min	1

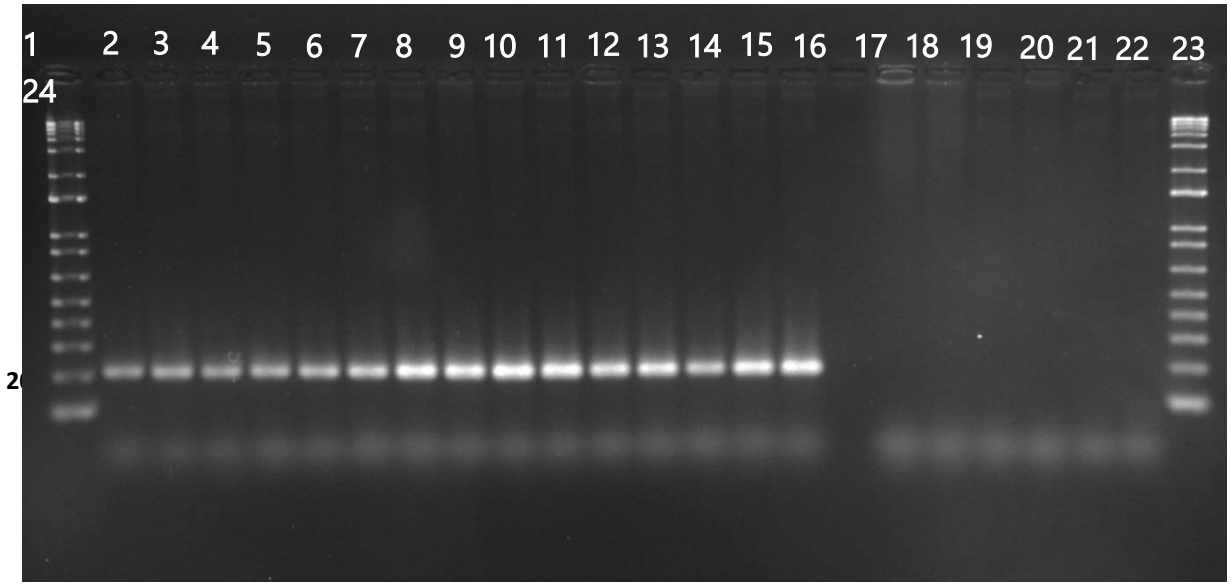
6.4 Table of putative fluorescing transformants of *V. dahliae* Vd71181 strain

Appendix table 3. Table of putative mCherry fluorescing transformant of *V. dahliae* VCG 1A Vd71181 parent isolate from Gwydir Valley, NSW. Table includes *Agrobacterium* strain used for transformation.

ID	Parent strain abbreviation	Vector	*Agrobacterium* strain	Date transformation	Date plated	Pathotype (D/ND)	VCG	Used for analyses
GwT0024	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0025	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0026	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0027	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0028	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	Yes
GwT0029	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	Yes
GwT0030	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	Yes
GwT0031	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0032	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0033	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0034	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0035	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0036	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0037	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0038	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0039	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0040	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0041	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0042	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0043	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0044	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0045	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0046	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0047	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0048	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	

GwT0049	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0050	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0051	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0052	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0053	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0054	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0055	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0056	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0057	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0058	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0059	Gw	pPZPnat-TEF-mCherry	AGL-1	8/04/2021	22/04/2021	D	1A	
GwT0060	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0061	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0062	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0063	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0064	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0065	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0066	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0067	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0068	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0069	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	Yes
GwT0070	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0071	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0072	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0073	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	Yes
GwT0074	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0075	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0076	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0077	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0078	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	
GwT0079	Gw	pPZPnat-TEF-mCherry	EHA105	8/04/2021	22/04/2021	D	1A	

6.5 Gel electrophoresis of mCherry *V. dahliae* transformant pathogenicity assay



Appendix figure 1. Gel electrophoresis image of amplified PCR products from fungal colonies emerging from cultured stem tissue of Sicot 714 B3F cotton seedlings after 10 days incubation on ½ PDA. Gel image shows colonies taken from plants inoculated with the parent Vd71181 isolate and from plants inoculated with water only (negative control). Bands in lanes indicate presence of amplified product of 200 bp from *V. dahliae*-specificITS region. Below describes lanes in gel image and corresponding fungal isolate.

Lane 1: 1 kb plus DNA ladder (Invitrogen, Thermo Fisher Scientific)

Lanes 2 – 16: Colonies from plants inoculated with *V. dahliae* parent Vd71181Lane 17:

Negative control lane with SDW used in PCR instead of DNA

Lanes 18 – 23: Colonies from plants inoculated with water only Lane 24: 1 kb

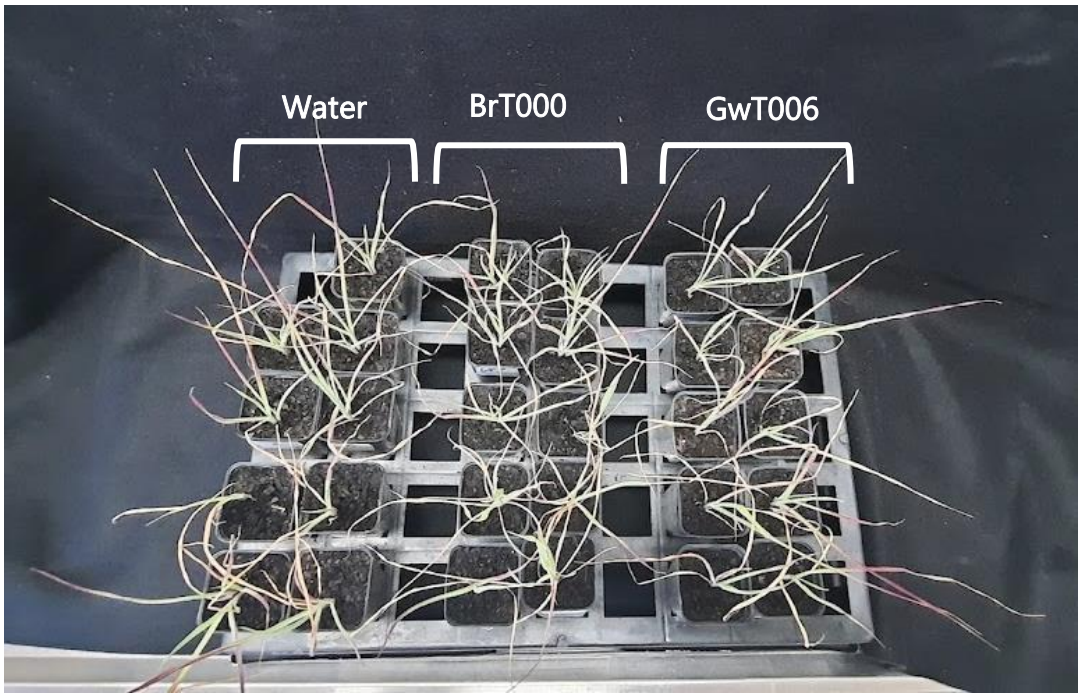
plus DNA ladder (Invitrogen, Thermo Fisher Scientific)

6.6 Images of weeds after fungal inoculation

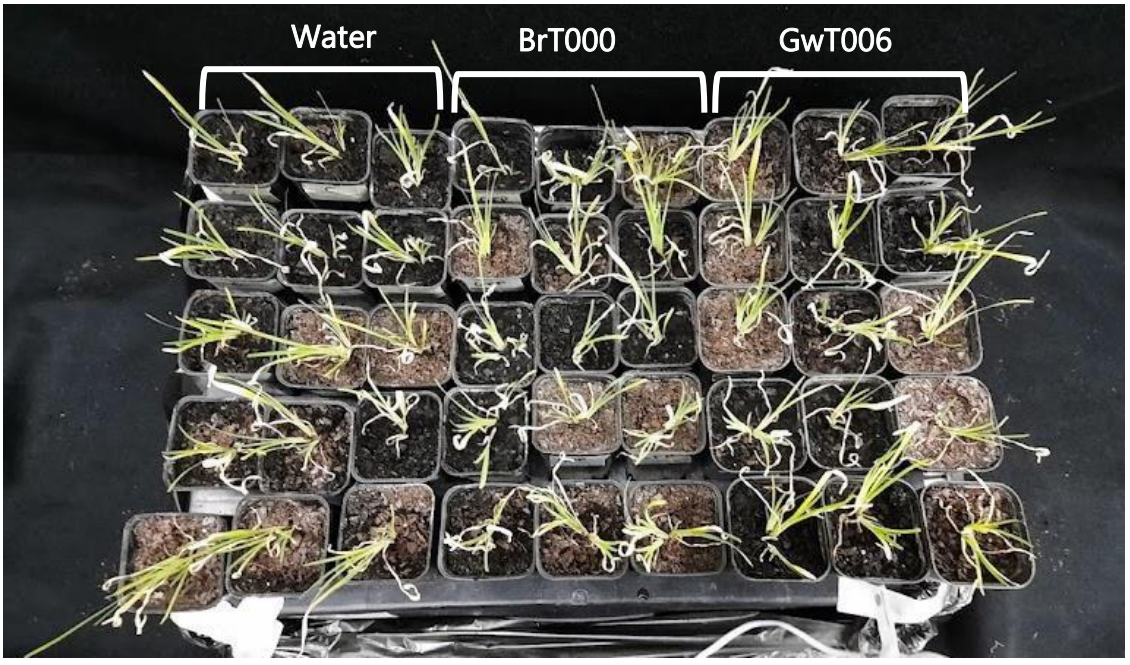
Images of weeds were taken 4 weeks after inoculation by root dipping. Replicates have been sorted by treatment group.

Appendix figure 2. *Conyza bonariensis* (flaxleaf fleabane) plants at 4 weeks post-inoculation with either water (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.

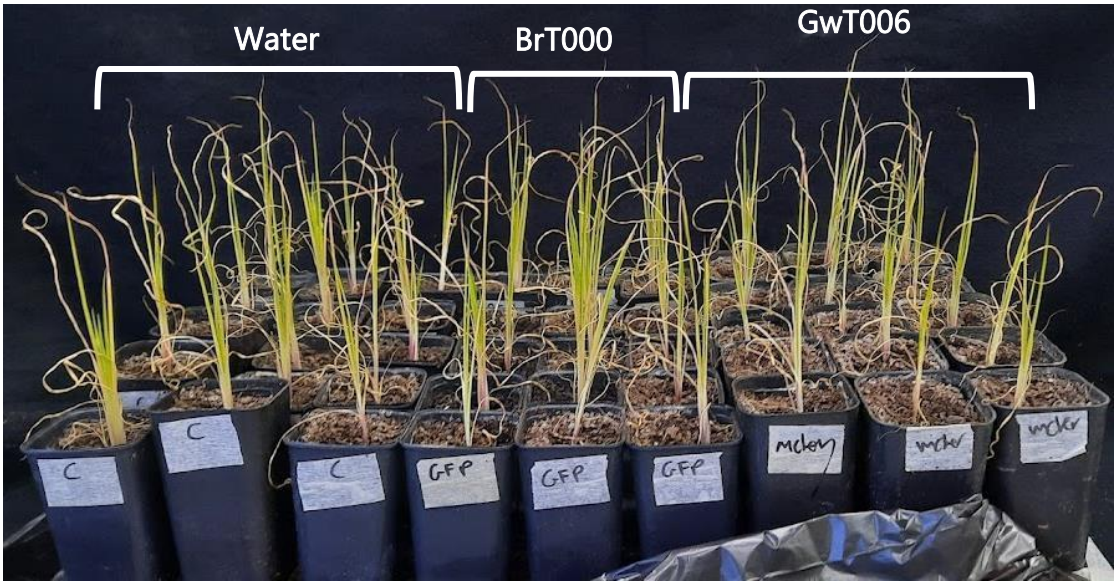
Appendix figure 3. *Sonchus oleraceus* (common sowthistle) plants at 4 weeks post-inoculation with eitherwater (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.



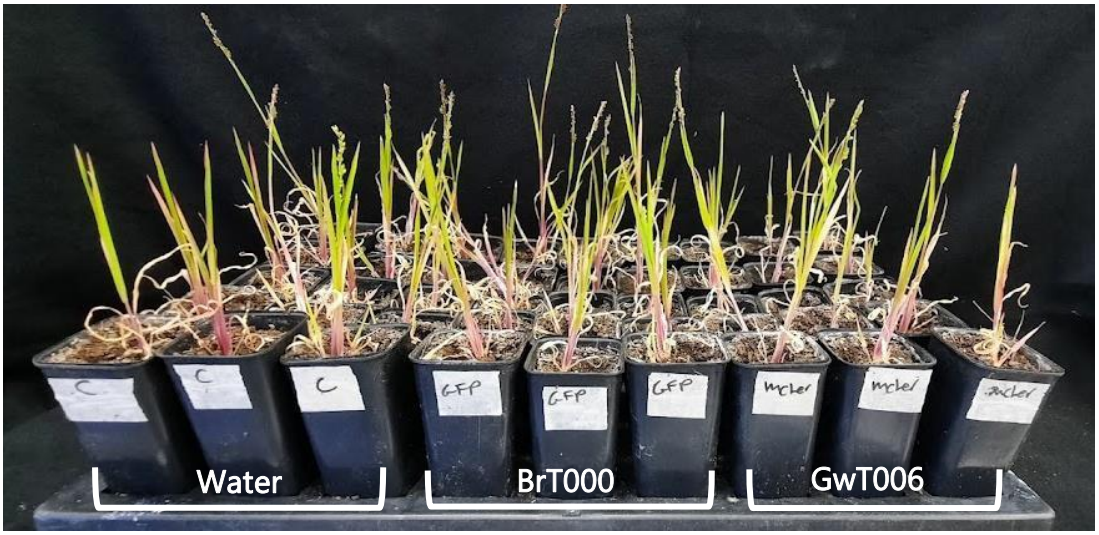
Appendix figure 4. *Avena fatua* (wild oats) plants at 4 weeks post-inoculation with either water (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.



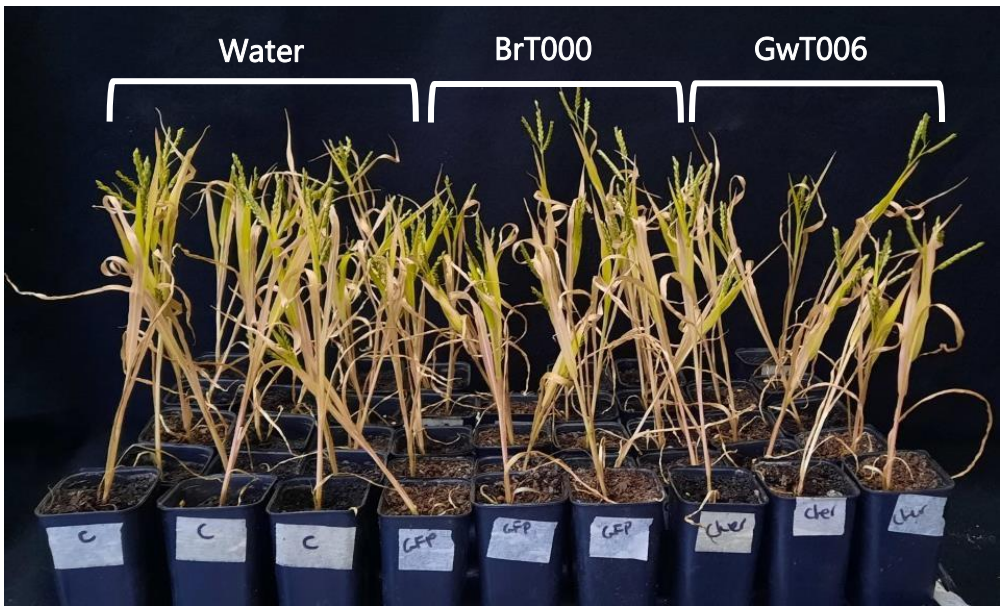
Appendix figure 5. *Chloris truncata* (windmill grass) plants at 4 weeks post-inoculation with either water (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.



Appendix figure 6. *Chloris virgata* (feathertop Rhodes grass) plants at 4 weeks post-inoculation with either water (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformantGwT0069.



Appendix figure 7. *Echinochloa colona* (awnless barnyard grass) plants at 4 weeks post-inoculation with eitherwater (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.



Appendix figure 8. *Urochloa panicoides* (liverseed grass) plants at 4 weeks post-inoculation with either water (negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.

Appendix figure 9. *Nicotiana benthamiana* (native tobacco) plants at 4 weeks post-inoculation with either water(negative control), GFP-fluorescing transformant BrT0003, or mCherry-fluorescing transformant GwT0069.

6.7 R code used in *RStudio*

Statistical analyses of data were conducted with the following code:

7.7.1. Growth rates of mCherry transformants and Vd71181 strain

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of growth rates of mCherry V. dahliae transformants and parent Vd71181 strain
#ANOVA with Tukey HSD test

my_data <- read.csv(file.choose())
library(dplyr)
dplyr::sample_n(my_data, 10)
levels(my_data$Name)
my_data$Name <- ordered(my_data$Name,
                        levels = c("Parent", "GwT0069", "GwT0028", "GwT0073", "GwT0030", "GwT0029"))

group_by(my_data, Name) %>%
  summarise(
    count = n(),
    mean = mean(Vmax, na.rm = TRUE),
    sd = sd(Vmax, na.rm = TRUE)
  )
if(!require(devtools)) install.packages("devtools")
devtools::install_github("kassambara/ggpubr")
install.packages("ggpubr")
library("ggpubr")
#Creating figure for data
q<- ggboxplot(my_data, x = "Name", y = "Vmax",
          color = "black", fill = "grey57", alpha = 0.3,
          order = c("Parent", "GwT0069", "GwT0028", "GwT0073", "GwT0030", "GwT0029"),
          ylab = expression("Growth rate ("~um^2~"/hr)"), xlab = "Fungal isolate")
q+ theme(axis.text.x = element_text(angle = 60, hjust = 1))


# Compute the analysis of variance
res.aov <- aov(Vmax ~ Name, data = my_data)
# Summary of the analysis
summary(res.aov)
TukeyHSD(res.aov)
# 1. Homogeneity of variances
plot(res.aov, 1)
library(car)
leveneTest(Vmax ~ Name, data = my_data)

# 2. Normality
plot(res.aov, 2)
# Extract the residuals
aov_residuals <- residuals(object = res.aov )
# Run Shapiro-Wilk test
```

```
shapiro.test(x = aov_residuals )
```

7.7.2. Fluorescence intensity of mCherry *V. dahliae* transformants

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of fluorescence intensity of mCherry V. dahliae transformants
#ANOVA with Tukey HSD test
my_data <- read.csv(file.choose())
library(dplyr)
dplyr::sample_n(my_data, 10)
levels(my_data$Name)
my_data$Name <- ordered(my_data$Name,
                  levels = c("GwT0069", "GwT0028", "GwT0073", "GwT0030"))

group_by(my_data, Name) %>%
  summarise(
    count = n(),
    mean = mean(Intens, na.rm = TRUE),
    sd = sd(Intens, na.rm = TRUE)
  )
if(!require(devtools)) install.packages("devtools")
devtools::install_github("kassambara/ggpubr")
install.packages("ggpubr")
library("ggpubr")
#Creating figure
q<- ggboxplot(my_data, x = "Name", y = "Intens",
          color = "black", fill = "grey57", alpha = 0.3,
          order = c("GwT0069", "GwT0028", "GwT0073", "GwT0030"),
          ylab = "mCherry fluorescence intensity (RFU)", xlab = "Fungal isolate")
q+ theme(axis.text.x = element_text(angle = 60, hjust = 1))


# Compute the analysis of variance
res.aov <- aov(Intens ~ Name, data = my_data)
# Summary of the analysis
summary(res.aov)
TukeyHSD(res.aov)
# 1. Homogeneity of variances
plot(res.aov, 1)
library(car)
leveneTest(Intens ~ Name, data = my_data)

# 2. Normality
plot(res.aov, 2)
# Extract the residuals
aov_residuals <- residuals(object = res.aov )
# Run Shapiro-Wilk test
shapiro.test(x = aov_residuals )
```

7.7.3. Fecundity of mCherry transformants and Vd71181 strain

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of fecundity of mCherry V. dahliae transformants and parent Vd71181 strain
#Negative bionomial regression test for overdispersed data with Tukey adjustment for multiple
comparisons
if(!require(psych)){install.packages("psych")}
if(!require(hermite)){install.packages("hermite")}
if(!require(lattice)){install.packages("lattice")}
if(!require(plyr)){install.packages("plyr")}
if(!require(boot)){install.packages("boot")}
if(!require(DescTools)){install.packages("DescTools")}
if(!require(ggplot2)){install.packages("ggplot2")}
if(!require(car)){install.packages("car")}
if(!require(multcompView)){install.packages("multcompView")}
if(!require(emmeans)){install.packages("emmeans")}
if(!require(MASS)){install.packages("MASS")}
if(!require(pscl)){install.packages("pscl")}
if(!require(rcompanion)){install.packages("rcompanion")}
if(!require(robust)){install.packages("robust")}
Data <- read.csv(file.choose())

Data$Treatment <- ordered(Data$Treatment,
                          levels = c("Parent", "GwT0069", "GwT0073"))


install.packages('AER')

#Dispersion of data test
library(AER)
rd <- glm(Count ~ Treatment, data = Data, family = poisson)
dispersiontest(rd,trafo=1)
dispersiontest(rd)

#Negative bionomial regression test - for overdispersed data
library(MASS)

model.nb = glm.nb(Count ~ Treatment,
                  data=Data,
                  control = glm.control(maxit=10000))

library(car)

Anova(model.nb,
    type="II",
    test="LR")

library(rcompanion)

nagelkerke(model.nb)
```

```
library(multcompView)

library(emmeans)

marginal = emmeans(model.nb,
              ~ Treatment)

pairs(marginal,
     adjust="tukey")
library(multcomp)
cld(marginal,
   alpha  = 0.05,
   Letters = letters, ### Use lower-case letters for .group
   type  = "response", ### Report emmeans in orginal scale
   adjust =  "tukey")   ### Tukey adjustment for multiple comparisons

if(!require(devtools)) install.packages("devtools")
devtools::install_github("kassambara/ggpubr")
install.packages("ggpubr")
library("ggpubr")
#Plots
ggerrorplot(Data, x = "Treatment", y = "Count",
         desc_stat = "mean_se", color = "black",
         add = c("mean","jitter"), add.params = list(color = "grey44"),
         error.plot = "errorbar" , ylab = "Cell concentration (spores/ml)", xlab = "Fungal isolate")
```

7.7.4. *V. dahliae* isolation from plant tissue in pathogenicity assay

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of isolation frequency of mCherry V. dahliae transformants and parent Vd71181
strain from cotton seedlings
#Fisher's exact test
Input =("
Isolate     Yes      No
 'Parent'    22       24
 '0069'      15       20
 '0073'      21       21
")

Matriz = as.matrix(read.table(textConnection(Input),
                     header=TRUE,
                     row.names=1))

Matriz

fisher.test(Matriz,
         alternative="two.sided")
library(rcompanion)

PT = pairwiseNominalIndependence(Matriz,
```

```
                fisher = TRUE,

                gtest  = FALSE,

                chisq = FALSE,
                digits = 3)
PT
```

7.7.5. Pathogenicity assay for mCherry transformants and parent Vd71181 strain

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of pathogenicity of mCherry V. dahliae transformants and parent Vd71181 strain on
cotton seedlings
#Kruskal-Wallis test with Dunn test
if(!require(psych)){install.packages("psych")}

if(!require(FSA)){install.packages("FSA")}

if(!require(lattice)){install.packages("lattice")}

if(!require(ordinal)){install.packages("ordinal")}

if(!require(car)){install.packages("car")}

if(!require(RVAideMemoire)){install.packages("RVAideMemoire")}

if(!require(multcompView)){install.packages("multcompView")}

if(!require(lsmeans)){install.packages("lsmeans")}

if(!require(rcompanion)){install.packages("rcompanion")}

install.packages("dplyr")


CS_exp1 <- read.csv(file.choose())
attach(CS_exp1)

Data <- CS_exp1

str(Data)

Data$Treatment <- as.factor(Data$Treatment)

str(Data)

Data$Likert.f = factor(Data$Disease,

                       ordered = TRUE)

Data$Treatment = factor(Data$Treatment,
```

```
                    levels=unique(Data$Treatment))


str(Data)


library(psych)

headTail(Data)

str(Data)

summary(Data)


xtabs( ~ Treatment + Likert.f,

      data = Data)

XT = xtabs( ~ Treatment + Likert.f,

           data = Data)


prop.table(XT,

          margin = 1)

library(FSA)

Summarize(Disease ~ Treatment,

         data=Data,

         digits=11)


kruskal.test(Disease~Treatment, data=Data)


DT = dunnTest(Disease ~ Treatment,

             data=Data,
```

```
        method="bh")


DT

#Plot graph for box and whiskers
if(!require(devtools)) install.packages("devtools")
devtools::install_github("kassambara/ggpubr")
install.packages("ggpubr")
library("ggpubr")

q<- ggboxplot(Data, x = "Treatment", y = "Disease",
        color = "black", fill = "grey57", alpha = 0.3,
        order = c("Nil", "Parent", "GwT0069", "GwT0073" ),
        ylab = "Disease score", xlab = "Fungal isolate")
q+ theme(axis.text.x = element_text(angle = 60, hjust = 1))
```

7.7.6. Frequency of isolation of transformant strains across all weed species

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of overall isolation frequency of mCherry and GFP V. dahliae transformants across weed species
#Chi-squared test
Input =("
Isolate     Yes      No
 'BrT0003'   33       157
 'GwT0069'   17       173
")

Matriz = as.matrix(read.table(textConnection(Input),
                    header=TRUE,
                    row.names=1))

Matriz


chisq.test(Matriz,
       correct=FALSE)
library(rcompanion)

pairwiseNominalIndependence(Matriz,
                    fisher = FALSE,
                    gtest = FALSE,
                    chisq = TRUE,
                    method = "fdr")
```

7.7.7. Frequency of isolation of transformant strains within weed species

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of isolation frequency of mCherry and GFP V. dahliae transformants within weed species
#Fisher's exact test
```

```
#The following inputs were changed for each weed species
Input =("
Isolate      Yes       No
 'GFP'      0         26
 'mCherry'  0         26
")

Matriz = as.matrix(read.table(textConnection(Input),
                       header=TRUE,
                       row.names=1))

Matriz

fisher.test(Matriz,
         alternative="two.sided")
library(rcompanion)

PT = pairwiseNominalIndependence(Matriz,

                         fisher = TRUE,

                         gtest  = FALSE,

                         chisq = FALSE,
                         digits = 3)
PT
```

7.7.8. Disease score comparisons of transformant-inoculated weed species

```
#Author: Sabrina Morrison
#Date: 20/10/2021
#Title: Analysis of isolation frequency of mCherry and GFP V. dahliae transformants within weed species
#Kruskal-Wallis analysis with Dunn test
if(!require(psych)){install.packages("psych")}

if(!require(FSA)){install.packages("FSA")}

if(!require(lattice)){install.packages("lattice")}

if(!require(ordinal)){install.packages("ordinal")}

if(!require(car)){install.packages("car")}

if(!require(RVAideMemoire)){install.packages("RVAideMemoire")}

if(!require(multcompView)){install.packages("multcompView")}

if(!require(lsmeans)){install.packages("lsmeans")}
```

```
if(!require(rcompanion)){install.packages("rcompanion")}

install.packages("dplyr")



CS_exp1 <- read.csv(file.choose())
attach(CS_exp1)

Data <- CS_exp1

str(Data)

Data$Treatment <- as.factor(Data$Treatment)

str(Data)

Data$Likert.f = factor(Data$Disease,

                       ordered = TRUE)

Data$Treatment = factor(Data$Treatment,

                        levels=unique(Data$Treatment))

str(Data)

library(psych)

headTail(Data)

str(Data)

summary(Data)

xtabs( ~ Treatment + Likert.f,

       data = Data)

XT = xtabs( ~ Treatment + Likert.f,

            data = Data)


prop.table(XT,

           margin = 1)


library(FSA)
```

```
Summarize(Disease ~
        Treatment,
        data=Data,
        digits=11)


kruskal.test(Disease~Treatment, data=Data)


DT = dunnTest(Disease ~
            Treatment,
            data=Data,
            method="bh")


DT
```

Communication and extension

Output	Description
Spotlight magazine article	'A new look at disease' (5 March 2019), Issue Autumn 2019
Spotlight magazine article	'Going fluoro for research' (March 2018), Issue Autumn 2018
Australian Association of Cotton Scientists (AACS) Seminar series	'Verticillium wilt, cotton, and a jellyfish' (8 October 2020), available at http://www.australiancottonscientists.org/aacs-seminar-series/
University of Queensland School of Agriculture and Food Science Honours thesis seminar	'Verticillium wilt, cotton, and a jellyfish: gaining novel insights into an established pathogen' (29 May 2020)
Virtual FUSCOM 2020	'Verticillium wilt, cotton and a jellyfish' (30 September 2020), published on the *CottonInfo: Connecting growers with research* YouTube channel (https://www.youtube.com/user/CottonInfoAust)
FUSCOM 2018, Griffith NSW	'Transformation of *Verticillium dahliae* causal agent of Verticillium wilt of cotton with the GFP gene' (28 August 2018)
Online seminar of Australian Association of Cotton Scientists, 8th October 2020	Cotton, Verticillium wilt and jellyfish: GFP-tagged *V. dahliae* as a toll in the study of plant pathogen interactions
Australasian Plant Pathology Society Online Conference, 23-26th November 2021	Analysis of infection by *Verticillium dahliae* in cotton seed and weed species from cotton-producing regions of Australia
Twitter Post	@NSWDPI_AGRONOMY (27 August 2020) "Timelapse video over 20 hours of the Verticillium pathogen in cotton fluorescing and germinating; from spores to a dense network of hyphae. Imagine this in the soil in response to plant roots?? Amazing footage by @nswdpi's @aphrika_gregson and D.Gardiner. @CottonResearch" 11 Retweets 24 Likes

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:	**Transformation of *Verticillium dahliae* causal agent of Verticillium wilt of cotton with the GFP gene**	
Project details:	CRDC project ID:	*DAN 1809*
	CRDC goal:	*1. Increase productivity and profitability on cotton farms*
	CRDC key focus area:	*2.1 Sustainablity of cotton farming*
	Principal researcher:	*Aphrika Gregson* *Research Officer Plant Pathology*
	Organisation:	NSW DPI
	Start date:	*December 2017*
	End date:	*December 2020*
Objectives	• Identify and confirm *Verticillium dahliae* disease entry and infection pathways in cotton • Apply fungal transformation techniques to genetically modify the pathogen with the green fluorescent protein (gfp) gene and carry out observations using confocal laser scanning microscopy (CLSM) • Evaluate and assess GFP-tagging technologies for potential applications in evaluating the severity of *Verticillium dahliae* infection • Understand infection processes and colonization, including the potential for colonisation of cotton seeds • Assessment *V. dahliae* colonisation in cotton weeds	
Background	Verticillium wilt caused by the soilborne fungus *Verticillium dahliae*, is one of the most challenging and economically significant diseases of cotton in Australia and worldwide. Host resistance is regarded as the most effective control strategy, however the biological complexity of the pathogen and controversy regarding the mechanisms of resistance hinder plant breeding efforts. Previous studies have utilised GFP-tagged isolates of *V. dahliae* to investigate the host – pathogen interaction on cotton, providing insights into the host resistance response and little understood areas of the disease cycle. Despite high levels of pathogenic variation present in *V. dahliae* populations, most studies use isolates from within the defoliating pathotype. This research project proposes to transform Australian isolates amongst the non-defoliating pathotype of *V. dahliae*, representative of endemic strains affecting Upland cotton.	

Research activities	This work establishes GFP-tagged isolate Vd71-3 as a tool for evaluating infection and colonization on cotton cultivars tolerant and susceptible to Verticillium wilt. Isolate Vd71-3 was obtained by transforming a GFP vector construct into highly virulent non-defoliating strain Vd71171 isolated from a diseased Upland cotton plant in NSW, Australia. Prior to study on cotton, pathogenicity of Vd71-3 was deemed consistent with that of the parent wildtype, indicating that GFP expression does not dramatically alter virulence. Confocal laser scanning microscopy observations confirmed existing descriptions of early infection on cotton, including germination of conidia by 24 hours post-inoculation, formation of an infection peg, intercellular colonisation of the root tips but not lateral root junctions, preferential colonisation of the xylem vessels, and acropetal movement of conidia in vessels. Extensive fungal occlusion of the vessels was also observed, not previously captured elsewhere on cotton. All research was undertaken at the University of Queensland and the Queensland Biosciences Precinct located in St Lucia, Brisbane.
Outputs	Principal Researcher Ms Aphrika Gregson completed a literature review entitled ***'Verticillium wilt: Epidemiology and host interaction of a severe phytopathogen affecting cotton (Gossypium hirsutum)*** Ultimately a thesis entitled ***'Transformation of a non-defoliating strain of Verticillium dahliae with the green fluorescent protein, and its colonisation on Upland cotton'*** were produced during this project, as part of the successful completion of a Ms Gregson's Bachelor of Science (Honours) degree (First Class) with the University of Queensland. In addition, Ms Sabrina Morrison (University Queensland honours student supervised by Professor Elizabeth Aitken) completed milestones 3.1 and 3.2. Experimentation entailed fluorescently-tagged transformants of *V. dahliae* VCG 1A and 2A to study pathogen infection in Australian cotton varieties and in associated weed species. In this study, a VCG 1A strain recovered from the Namoi Valley cotton region was transformed with the mCherry fluorescent gene from *Dicosoma* sp. using Agrobacterium transformation methods. This fluorescent transformant, alongside a previously transformed GFP-tagged VCG 2A *V. dahliae* isolate, was used to investigate infection patterns in planta. Australian weed species and cotton varieties were inoculated with the transformant strains and visualised in planta for detection of fluorescent fungal structures using confocal laser-scanning microscopy. Similarly, the details of the science performed are contained within Ms Morrison's thesis.

	Fluorescent proteins as a tool for studying Verticillium dahlia on cotton (Gossypium hirsutum) and associated weed species.
Impacts	This honours project will build on existing knowledge of *V. dahliae* infection pathways in cotton, and successfully adopted novel technologies such as fungal transformation and confocal microscopy to investigate endemic Australian *V. dahliae* strains under-represented in the literature. The establishment of a practical and feasible transformation protocol for *V. dahliae*, and the generation of GFP-tagged strains of Australian *V. dahliae* offers a complementary methodology in Verticillium wilt research, including: host-resistance, rotation cropping (I.e. host range studies), seedborne transmission, and inoculum production and control. The weed species and cotton reproductive structures studies contributes to a better understanding of *V. dahliae* and its interaction with both cotton and weeds. In the long term these findings may assist future management approaches for Verticillium wilt in the Australian cotton industry.
Key publications (Thesis)	Gregson, A (2019) *Transformation of a non-defoliating strain of Verticillium dahliae with the green fluorescent protein, and its colonisation on Upland cotton* Morrison, S (2021) *Fluorescent proteins as a tool for studying Verticilium dahlia on cotton (Gossypium hirsutum) and associated weeds species*