Australian Government

Cotton Research and Development Corporation

SUMMER and HONOURS SCHOLARSHIP APPLICATION 2015-16 SEASON

1.	**Project Title** (Maximum 85 char)	:	Constructing a Model to Investigate Rhizopheric Competent Microorganism Interactions and Peptide Secretions
2.	**Proposed Start Date**	:	**29th Feb 2016**
	Proposed Cease Date	:	**18th November 2016**
	Scholarship Type	:	**Honours**
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1. Executive Summary:

Verticillium dahliae is a highly destructive fungal pathogen that causes an annual global product loss in cotton of 20%. Currently there are no effective treatments for verticillium wilt, but bioinoculants might provide an environmentally friendly solution. This project aimed to: develop methods to study the interactions between the cotton plant *Gossypium hirsutella*, the pathogen *V. dahliae* and a rhizopheric competent fungal symbiont, *Beauveria bassiana*. To quantify root colonisation by the pathogen and/or symbiont in single or multiple inoculations, and to identify patterns in peptides secreted by the plant, pathogen and symbiont during single or multiple inoculations. Soil was replaced by vermiculite and the plants were cultivated using Hoagland's nutrient solution allowing complete control over their nutrient availability and uptake. This study found that there was no significant difference in the susceptibility of two cotton cultivars Sicot F-1 and Siokra 1-4. Results indicated that pre-infection with *B. bassiana* cause no interference with *V. dahliae's* ability to colonises and infect the plants. *B, bassiana* did however supress the infection of a contaminating unidentified *Aspergillus*. Post-infection of *B. bassiana* did have a significant effect on the colony forming units of *V. dahliae* when it was introduced as a pre-infection, this interaction also occurred when the fungi's roles were reversed. A significant difference in peptide secretion profiles was observed when comparing UV chromatographs from different treatments.

2. Background:

Verticillium dahliae (Kleb), the causal agent of 'verticillium wilt' is a highly destructive fungal plant pathogen that infects over 400 plant species, including multiple highly important commercial crops, such as cabbage, tomato, olive and cotton. In the hundred years since the first reported case, *V. dahliae* has spread to across the globe and is now present in over 95% of the worlds cotton growing areas (Mehboob ur *et al.*, 2007; Xue *et al.*, 2013). Annually *V. dahliae* causes a product loss of 20% in cotton and is the second highest single cause of yield loss in cotton after *Helicoverpa amigera* (Australia, 2016; Xue *et al.*, 2013). Transmission into new areas is facilitated by farming equipment already contaminated with *V. dahliae*, through soil or through infected plant material. In 2011 it was discovered that *V. dahliae* could be transmitted through seeds harvested from infected plants (Göre *et al.*, 2011). Control of verticillium wilt is extremely difficult due to the high resistance of microsclerotia to environmental stress and antimicrobial agents, as well as their ability to exist in the soil for decades. Control is further hampered by the broad host range of *V. dahliae* and a lack of effective fungicides (Thomma & Fradin, 2006; Zhang *et al.*, 2014). Methods to control verticillium wilt have included, cultivation of resistant lines, crop rotation, chemical fumigation, soil-solarisation and biological control agents (Lüders *et al.*, 2008).

Currently the industry preferred method of controlling the damage caused by verticillium wilt is to use resistant cotton cultivars. One possible alternative is to use microorganisms to control verticillium wilt and protect plants against infection. *Beauveria bassiana* is one potential biological control agent. *B. bassiana* is an endophyte and an entomopathogenic fungus and is known to establish its self on plant roots it is also a member of the Hypocreales order. Endophytic fungi from the order Hypocreales, such as *Trichoderma spp.*, (Phylum Asocomycota, Order Hypocreales) were first identified as potential biological control agents in the early 1930's (Weindling, 1932). pest control.

Trichoderma species have been shown to parasitize other fungi by (Seidl *et al.*, 2009), who suggested that parasitism of fungi by *Trichoderma* may be facilitated by the modification of receptors in *Trichoderma* that are sensitive to nitrogen levels by peptide secretions produced from the host fungus. Low levels of environmental nitrogen induce upregulation of genes that causes a signal cascade promoting the production of extracellular enzymes that hydrolase the hosts cell walls, allowing the *Trichoderma* to penetrate the host's hyphae and access cellular nutrients (Benhamou & Chet, 1997; Seidl *et al.*, 2009; Viterbo *et al.*, 2002). Small peptides are thought to be important in this activity (Zheng *et al.*, 2011).

3. Aims and Objectives:

The project aimed to quantify and describe the interaction of *B. bassiana* (the 'symbiont' and *V. dahlia* (the 'pathogen') during early stages of infection in the roots of cotton plants by quantifying the infection following single or sequential inoculation, and to describe differences and similarities in patterns of peptide secretion in different combinations of plant, pathogen and symbiont. Three main objectives:

- To Develop methods to study the interaction between the cotton plant, *Gossypium hirsutella*, the pathogen *V. dahliae*, and a rhizospheric-competent fungal symbiont, *Beauveria bassiana*.
- To Quantify colonisation of the root by the pathogen and/or symbiont following single or multiple inoculations.
- To Identify patterns in peptides secreted by the plant, pathogen and symbiont during single or multiple inoculations.

4. Materials and Methods:

Two varieties of cotton, Sicot F-1 and Siokra 1-4 were obtained from CSIRO under material transfer agreement.

Vermiculite was autoclaved at 121ºC for 30 minutes to ensure material sterility. Seeds were surface sterilised by immersion in a 5% bleach solution for 2 minutes, then rinsing them with milli q water. Seed were laid out in rows of 10 x 13 in 29x35cm seed trays filled with autoclaved vermiculite, and lined with aluminium foil with glad wrap covering the top. The trays were placed in a growth room with a 14-hour photoperiod, temperature set to 24-18 ºC, and relative humidity at 60% for two weeks.

Two-week-old seedlings where inoculated by immersing the roots into a solution containing either *V. dahliae* spores provided by Queensland's Department of Agriculture and Fisheries (DAFF), or *B. bassiana* spores provided by the Invertebrate Microbiology Group (IMG) at QUT. In the one-week inoculation experiments plants were inoculated with a single fungus or water and allowed to grow for one week. In the two-week inoculation experiments plants were inoculated with water or one fungus in week one, then

inoculated with water or the other fungus in week two. Time taken for colonisation to occur was identified through light microscopy using (Vierheilig *et al.*, 1998) method of root staining for visualising root colonisation.
To quantify the level of root colonisation, roots were surface sterilised by immersion into a 10% sodium hypochlorite solution, weighed and homogenised with a mortar and pestle in 3mL of 0.05% tween80. 200uL of the homogenate was then plated onto a petri dish containing a modified version of Strasser *et al.* (1996) selective medium: containing Streptomycin (100mg/L) and tetracycline (50mg/L) in a PDA medium. Plates were incubated at 25^{o}c for one week after which colony forming units were counted. Secreted peptides were collected by placing the pants into jars containing sterile deionised water for three days one week after inoculation. Samples containing peptides were delated using C18 membrane stage tips, to purify peptide samples. Peptides were analysed at QUT in the Central Analytical Research Facility (CARF) using a Shimadzu 8050 LCMS AB Sciex instrument with UV detection set at 280 nm. CFUs were analysed using two way anova with poisson errors.

5. Results:

Internal colonisation was detected to begin at day 5, and by day 7 all plants inoculated had internal colonies. In the one-week single inoculation experiment there was a significant difference in the number of CFU of *B. bassiana* between the two cultivars. The mean CFU's/100mg of root were significantly higher in Siokra1-4 (mean= 2266) than in Sicot F1 (mean= 786), (p=0.0013). There was no significant difference in *V. dahliae* CFU's between cultivar types detected in the one week experiment. An unidentified species of *Aspergillus* was also identified on the agar plates in all treatments including controls. Analysis found a that there was a significant difference in mean CFU between cultivar type (p=0.0013) with Sicot F-1 mean= 407 having a higher amount Siokra 1-4 (mean = 83). There was no significant difference in CFU's of *Aspergillus* between treatments. There was, however, a significant difference in the proportion of plants in which *Aspergillus* was detected in variety Siokra 1-4 when comparing to control (*Aspergillus* detected in 87% of plants) with those inoculated with *B. bassiana* (*Aspergillus* detected in 40%) (p=0.028), and when comparing those infected with *V. dahliae* (*Aspergillus* detected in 93% of plants) to *B. bassiana* (*Aspergillus* detected in 40%) (p= 0.00739).
In the two-week experiment, there was no significant difference in number of CFUs between cultivar type following incubation at week 1 (p= 0.2553) or week 2 (p = 0.2115). There was no significant difference in the proportion of plants with *B. bassiana* present between cultivars (p=0.1814). Pre-treatment of plants with *V. dahliae* did not have any significant effect on number of CFU of *B. bassiana* when comparing Inoculation with Verticillium then superinoculation with Beauveria (V-B) (Sicot F-1 mean= 3760, Siokra mean= 2339) compared to treatment with water followed by inoculation with Beauveria (W-B) (Sicot mean= 3087, Siokra mean=6611) (Sicot F1 p= 0.999, Siokra 1-4 p= 0.2341). There was also no significant difference on the proportion of plants with *B. bassiana* present when pre-treated with V. dahlia (Sicot F-1 p=0.9999, Siokra 1-4 p= 0.9999). However, superinfection with Verticillium appeared to reduce the CFUs of Beauveria. There was a significant reduction in mean *B. bassiana* CFUs in Siokra 1-4 following superinfection with *Verticillium* (B-V) (mean= 2070) to a second treatment with water (B-W) (mean= 6611) (p= 0.01325). When cultivar type is removed as a factor and the treatments are compared across the experiment there is an increase in the significance of the difference when comparing B-W and B-V (p= 0.001224) (B-W mean= 5321) (B-V mean= 1737)
There was no significant difference in the number of *Verticillium* CFUs between plant types but there was a significant difference in the proportion of plants with *V. dahliae* present with Siokra 1-4 having a higher proportion (43%), compared to Sicot F-1 (32%), p=0.04. When comparing plants by treatment there was a significant difference between cotton cultivars for the two-week *V. dahliae* incubation V-W p= 0.0001105 with Sicot F-1 having a higher mean CFU count (mean= 2080) compared to Siokra 1-4 (mean= 249). There was also no significant difference in mean CFUs of *V. dahliae* when comparing pre-treatment with *B. bassiana* B-V to superinfection V-B p= 0.303. However, when comparing mean CFUs of *V. dahliae* between V-W and V-B treatment there is a significant reduction in CFUs in Sicot F-1 p= 0.001355 (V-W mean= 2080, V-B mean= 588).
This result was not replicated when comparing the same treatment in Siokra 1-4 (p= 0.9999) (V-W mean= 249, V-B mean= 439). Comparing the proportion of plants where *V. dahliae* was detected in these treatments there was a significant reduction in plants superinfected with *B. bassiana*, (V-B = 59%) compared to those superinfected with water (V-W = 100%) when cultivar was removed as a factor (p= 0.0017). Closer inspection showed that within cultivar only Sicot F-1 showed a significant reduction in proportion of plants with *V. dahliae* when superinfected with *B. bassiana* (p= 0.0456) (V-W = 100%, V-B = 50%), Siokra 1-4 (p= 0.459) (V-W= 100%, V-B= 66%).
There was no significant correlation between *V. dahliae* and *B. bassiana* CFUs in both V-B (p= 0.3075) and B-V (p=0.1585) treatments.
There was no significant difference in the mean number of Aspergillus CFUs between cotton cultivars in experiment 2 (p= 0.4102). There was also no significant difference in CFU counts between treatments. There was a significant difference in the proportion of plants with Aspergillus present when comparing treatments (table 1). In all cases, inoculation with *B. bassiana* resulted in a significant reduction in CFU's of Aspergillus sp.

Comparison	P value	% Plants with *Aspergillus* present	
V-W to V-B	P= 0.04215	V-W= 90	V-B= 52
W-V to W-B	P= 0.01104	W-V= 100	V-B= 52
W-V to W-B	P= 0.03407	W-V= 100	W-B= 50

Table 1: Significant differences in proportion of plants with *Aspergillus* detected by treatments.

UV Chromatogrpahs were obtained for 15 samples, 5 from each of the single infection treatment in experiment 1. A subset of samples from each treatment in experiment 2 are completing analysis in CARF.
Sorensen dice coefficient test in R was used to compare the on presence and absence of peaks in the UV chromatograph. A significant difference in pattern of presence and absence of peptides between all three treatments: cotton alone ('C'), cotton with *V. dahlia* ('V'), and cotton with *B. bassiana* ('B') (Anosim R significance = 0.001). Results indicate that there are three main clusters with one outlier (sample B3). Samples form clusters by treatment as follows:

Cluster 1: B8, V8, B4, B6 and B2
Cluster 2: C2, C4, C1 and C10
Cluster 3: V6, C12, V2, V13 and V14

6. Discussion and Conclusions:

There was no significant difference between plant varieties in mean number of CFUs of *V. dahliae* or proportion of plants with *V. dahliae* present in both experiments 1 and 2. This was surprising as Sicot F-1 is listed as more resistant to verticillium wilt then Siokra 1-4 (Reid *et al.*, 2004) and we expected to find a reduced CFU count for *V. dahliae* in Sicot F-1. There was again no significant difference of cotton variety on mean count of CFUs of *V. dahliae* after 1 week of incubation in experiment 2, but a significant difference between cultivars in the mean number of CFUs of after two-weeks, with a higher mean number of CFUs observed in Sicot F-1. This is directly opposite to the expected result as Sicot F-1 listed as more resistant to verticillium wilt then Siokra 1-4. However, the proportion of plants in which CFUs of *V. dahliae* were detected was significantly higher in Siokra 1-4 plants compared to Sicot F-1. Combining this with similar observations in experiment 1, we speculate that the reported effect of increased resistance in Sicot F-1 has its main effect later in the infection cycle, and does not reduce the initial growth of *V. dahliae* during root colonisation. There was a surprising difference in cultivar response to colonisation by *B. bassiana* in experiment 1, with a significantly higher mean CFU count in Siokra 1-4. However, there was no significant difference in the number of *B. bassiana* CFUs between cotton cultivars in experiment 2. Furthermore, incubation for one or two-weeks had no significant impact on the mean number of *B. bassiana* CFUs in the single fungal inoculation treatments. Looking at total CFUs there was no significant difference between the cultivars.
Contamination by an unidentified Aspergillus species occurred in all treatments including controls, although it was not detected in all control plants (28%). In experiment 1 Aspergillus had a significantly higher mean CFU count in Sicot F-1. These differences were not detected in experiment 2.

Pre-treatment with *B. bassiana* had no impact on CFUs of *V. dahliae*, or on the proportion of plants infected with *V. dahliae*. Similarly, pre-treatment with *V. dahliae* had no significant impact on the number of CFUs of the *B. bassiana* superinfection or the proportion of plants in which *B. bassiana* was found. There appears to be a significant effect of superinfection by a second fungus on CFUs of the first fungus inoculated. There was a significant impact on *B. bassiana* CFUs when *V. dahliae* was introduced as a superinfection in the Siokra 1-4 cultivar, and a similar trend in Sicot F-1, though in the small number of plants used in this experiment a significant difference was not detected. Similarly, was a significant reduction in mean CFUs of *V. dahliae* when plants were superinfected with *B. bassiana* (V-B) when compared to controls treated with water (V-W) but this time it was significant in variety Sicot F-1, but not in Siokra 1-4. Within cultivars there was also a significant reduction in proportion of plants with *V. dahliae* when superinfected with *B. bassiana* only in variety Sicot F-1 but not in Siokra 1-4.

One possible explanation for these observations and for the limited effects of incubation for 2 weeks on CFU count is that colonisation of the root only occurs in root tips, and that colonisation declines in older sections of roots. It is possible that the CFU method only estimates the amount of fungus found in the root tips, which are re-colonised from the older root area as the root extends. CFU count thus remains constant between week 1 and week 2 post inoculation in single inoculations. Where a fungus is inoculated by dipping a second time, the direct inoculation may lead to a greater establishment in the root tip by the superinfecting fungus, which then reduces infection of the root tip by the more gradual spread of the fungus down the root from the first infection.

There was a significant difference between treatments, with samples to clustered within treatments, and a significant difference between all three treatments: cotton alone, cotton with *V. dahliae*, and cotton with *B. bassiana*. Closer inspection indicates that there are some peaks which appear to be unique to the different treatments. This indicates that there is a difference in secretions between treatments that supports further investigation into peptide profiles and identity (to be completed January 2017).

There are limitations associated with isolation dependent studies in microbiology, and it is possible that CFU counts underestimated the presence of fungus, one of the greatest limitations we had was with our CFU data. Only 1/15th of the root grindate was used for plating, and it is possible that in the samples where no fungal growth was detected that fungus may have been present at low levels in the remainder of the samples. This could be addressed through PCR of markers such as ITS and next generation sequencing techniques that quantify relative abundance of reads within amplicons (Deagle *et al.*, 2013)
Overall, all three aims were achieved despite limitations on resources. A model with which to investigate the interactions between rhizospheric-competent fungi in planta was developed and tested and its use demonstrated in practice.

7. Highlights:

This project identified that a superinfection of *B, bassiana* in plants infected with verticillium could significantly reduce the number of CFUs. We also identified that there was a significant difference in the peptide secretion profiles between cotton plants inoculated with water, *B. bassiana* or *V. dahliae*.

8. Future Research:

Future studies should be encouraged to further purse the peptide interactions between plants and fungi with the aim to identify secreted peptides, and quantify the amount secreted.

9. Presentations and Public Relations:

Not applicable

10. Reference List:

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