FINAL REPORT 2013/101

Strategies to Manage Soil-borne Fungi and Mitigate Sugarcane Yield Decline

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ABSTRACT

Culture–dependent and culture-independent analyses of sugarcane rhizosphere fungal and oomycete communities were conducted across two cropping seasons at two sites. Agronomic treatments included replant continuous cane, cane-legume rotations and longer-term (LT) ratoon cane. A native forest soil was included as a comparison with cane cropping.

Relative abundances of fungal taxa were used to define impacts of agronomic treatments on the structure of the fungal microbiome. Fungal communities of LT ratoon cane were significantly different ($P<0.003$) from replant continuous cane and cane-legume rotations. Forest soil communities were highly differentiated ($P<0.001$) from the adjacent sugarcane cropping treatments and had the greatest ($P<0.001$) diversity and taxonomic eveness.

The majority of differences among sugarcane fungal rhizosphere soil communities were attributed to 33 taxa, their abundance differing ($0.001<P<0.05$) among cropping treatments. These included numerous soil and root-associated saprophytes, potentially pathogen antagonistic *Trichoderma, Epicoccum, Humicola*, *Bullera* and *Clonostachys* spp., plant pathogenic *Fusarium* spp. and functionally diverse Helotiales, Hypocreales, Myriangiales and Pleosporales comprising mycorrhizal, mycoparasitic and plant pathogenic species.

Culture-dependent root endophytic fungal communites were dominated by saprophytic *Chaetomium*, *Mortierella* and *Penicillum*, pathogen antagonistic *Trichoderma* and plant pathogenic *Fusarium* spp. Oomycete communities were dominated by plant pathogenic *Pythium spinosum. Pachymetra chaunorhiza* was not isolated from roots or rhizosphere soils. Saprophytic and plant pathogenic taxa were more abundant ($P<0.001$) in replant continuous cane and cane-legume rotations. *Trichoderma* and *Epicoccum* spp. were more abundant under LT ratoon cane ($P<0.001$) and produced metabolites that inhibited root pathogens *in vitro*, implying a mechanism for rhizosphere selection and disease suppression by these taxa.

EXECUTIVE SUMMARY

The broad aims of this research were to deliver fundamental knowledge of rhizosphere-fungal interactions in sugarcane cropping systems and agronomic factors impacting on the incidence and suppression of root diseases. This information forms the basis of disease prediction and management strategies focused on selection of planting sequences and targeting of inputs to suppress disease, thereby enhancing root health and maximising resource-use efficiency.

Culture–dependent microbiological and culture-independent DNA sequence analyses of sugarcane rhizosphere fungal and oomycete communities were conducted across two cropping seasons at two sites in the Herbert region of northern Queensland. Agronomic treatments included replant continuous cane, cane-legume rotations and longer-term (LT) ratoon cane. A native forest soil was included as a comparison with cane cropping.

There were no significant differences in total soil nitrogen among replant continuous cane, cane-legume rotations or LT ratoon cane 5 weeks prior to cane planting (T0), indicating no residual soil N remaining from the legume rotations. Soil organic carbon was significantly greater in rhizosphere soils of the actively growing sugarcane crops than in pre-plant soils and significantly increased with each year of cane cropping, regardless of the agronomic treatment. Soil carbon levels were however, relatively low compared with those reported in ratoon cane of a similar duration.

Relative abundances of fungal taxa were used to define impacts of agronomic treatments on soil-borne fungal communities of sugarcane. Communities under LT ratoon cane were significantly different from replant continuous cane and cane-legume rotations whereas, those under the replant cane treatments were not significantly different. Forest soil fungal communities had the highest diversity and were significantly differentiated from the adjacent sugarcane crops.

The majority of differences among sugarcane soil communities were attributed to 33 fungal taxa, their abundances significantly different among cropping treatments. These included numerous root-associated saprophytes, 5 pathogen antagonistic and potentially disease suppressive taxa, plant pathogenic *Fusarium* and 5 broad groups (orders) comprising plant-beneficial mycorrhizal and mycoparasitic fungi and taxa related to wilt, stem rot and root rot pathogens of sugarcane.

Microbiological analyses indicated that root colonising fungal communites were dominated by 3 primarily saprophytic fungi, pathogen antagonistic *Trichoderma* and plant pathogenic *Fusarium* spp. Oomycete communities were dominated by plant pathogenic *Pythium spinosum. Pachymetra chaunorhiza* was not isolated from roots or rhizosphere soils at either site, despite DNA diagnostics detecting the presence of the pathogen.

Pathogen antagonistic *Trichoderma* and *Epicoccum* spp. were significantly more abundant under LT ratoon cane and produced metabolites that significantly inhibited *Fusarium* growth and killed *Pythium*. These metabolites indicate a potential mechanism to enhance competiveness of *Trichoderma* and *Epicoccum* in rhizosphere soils, leading to increased abundance and suppression of root pathogens. These root endophytic fungi and their secondary metabolites require investigation as potential disease suppressive treatments in sugarcane.

In summary microbiological and DNA sequence analyses of sugarcane roots and rhizosphere soils indicated that communities of saprophytic *Chaetomium* and *Mortierella* spp. and plant pathogenic *Fusarium* and *Pythium* spp. were generally significantly more abundant in early ratoon replant continuous cane and cane-legume rotations compared with LT ratoon cane. In contrast abundance of endophytic fungal antagonistic *Trichoderma* and *Epicoccum* spp. increased with the time under cane cropping, being greatest in LT ratoon cane.

The results of this study contrast with the generally accepted view that LT ratoon cane increases communities of soil-borne root pathogenic fungi, resulting in higher root disease incidence. *Fusarium* (30%) and *Pythium* (64%) root infection were high in these sugarcane fields, but both were significantly lower (10%) in LT ratoon cane compared with replant continuous cane and cane-legume rotations. Significantly greater root (25%) and rhizosphere abundance (105%) of disease suppressive *Trichoderma* in LT ratoon cane may account for the suppression of these pathogens.

Broad taxonomic groups of fungi (orders) reported to contain plant beneficial and sugarcane plant pathogenic species also significantly decreased in abundance under LT ratoon cane. These fungi remain largely unidentified and further research is required to isolate and identify the plant pathogenic species and monitor how their abundance shifts in response to cropping sequences.

Economic Outcomes

Reported average production losses to sugarcane root diseases in Australia are 10-15% and are therefore estimated to cost the Australian sugar industry approximately $80-100 million p.a. This research has enhanced ecological understanding of plant-microbe interactions in sugarcane cropping soils and the potential to improve root disease prediction and management.

Environmental Outcomes

Environmental benefits that may be realised through enhanced biological disease suppression include reduced and more efficient use of agrichemicals and improved weed management, via enhanced crop establishment. This will result in decreased on-farm and off-site contamination of soil and water resources.

Social Outcomes

Communication of research outcomes will further raise awareness in the grower community of the beneficial and deleterious functions of soil microbes in sugarcane cropping systems and the impacts of agronomic management practices on incidence and suppression of disease.

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1. BACKGROUND

Previous research has shown that long-term monoculture, soil compaction, excessive tillage, and depletion of soil organic matter all contribute to sugarcane yield decline (Garside *et al*., 2005). These practices have increased microbial communities deleterious to the growth and health of sugarcane roots (Magarey, 1996; Pankhurst *et al.*, 2000). Significant productivity benefits have resulted from soil fumigation and the application of chemical treatments selective for soil-borne plant pathogens (Magarey *et al.*, 1997), indicating that root diseases continue to constrain optimum sugarcane production (Egan *et al.*, 1997; Magarey, 2005).

Managing these generally intractable diseases in sugarcane monocultures represents a significant challenge due to the unavailability of effective fungicides and either the lack of disease resistant germplasm or decrease in disease tolerance, as evidenced in varietal yield decline (Garside *et al*., 2005). Incorporating legume rotations during fallow periods prior to replanting cane can improve soil N fertility and suppress soil-borne pathogens, compared with re-plant continuous cane and longer-term ratoon cane cropping systems (Garside *et al*., 2005, Pankhurst *et al*., 2000).

To date most studies have employed culture-dependent microbiological approaches to isolate and identify root pathogenic fungi in sugarcane cropping systems (Magarey, 1996; Egan *et al.*, 1997; Magarey, 2005; Viswanathan & Rao, 2011) and define the impacts of fungicides and cropping sequences on root disease incidence and crop productivity (Magarey *et al.*, 1997; Pankhurst *et al.*, 2000; Garside *et al*., 2005). However, a diverse and significant proportion of soil-borne fungi are either not amenable to direct isolation from soil or are underrepresented during isolations from root tissue (Schlatter et al., 2018). Consequently, culture-independent methods are required to describe the holistic taxonomic composition of rhizosphere soil fungal communities (Toju *et al*., 2018), identify community differentiation induced by agronomic interventions and identify previously undescribed taxa involved in expression (Schlatter *et al*., 2018) and suppression (Penton *et al*., 2018) of root disease.

The broad aims of this research were to deliver fundamental knowledge of rhizosphere-fungal interactions in sugarcane cropping systems and agronomic factors impacting on the incidence and suppression of root diseases. Culture–dependent microbiological and culture-independent DNA sequence analyses of sugarcane rhizosphere fungal and oomycete communities were conducted across two cropping seasons at two sites in the Herbert region of northern Queensland. Agronomic treatments included replant continuous cane, cane-legume rotations and longer-term (5^{th}-10^{th}) ratoon cane to investigate how cropping sequences impact on plant pathogenic and disease suppressive microbial communities.

The research addressed significant knowledge gaps associated with structural and functional attributes of soil-borne fungal communities in sugarcane production systems and their relationships to root disease incidence. The focus was to broaden our ecological understanding of these deleterious and beneficial root-microbe interactions in cane growing soils, thereby supporting the development of disease prediction frameworks and management strategies to enhance root health, resource-use and cane productivity.

2. PROJECT OBJECTIVES

The broad aims of this research are to deliver fundamental knowledge of rhizosphere-fungal interactions in sugarcane production systems and factors impacting on the incidence and suppression of root diseases. This information forms the basis of management strategies focused on selection of planting sequences and targeting of inputs to suppress root diseases, thereby enhancing root health

and maximising resource-use efficiency.

Specific Aims:

- Characterise soil-borne fungal communities in sugarcane yield decline (YD) and non-YD cropping systems via microbiological and eco-genomic analyses.
- Define the dynamics of root pathogenic fungi and disease suppressive (pathogen antagonistic) microbes via quantitative molecular ecological analyses.
- Establish linkages between rhizosphere fungal communities (sugarcane-legume), crop management and root disease incidence.

Defining the agro-ecological interactions among target YD pathogens, their antagonistic microbes and crop hosts (sugarcane-legumes) will lead to improved disease prediction and identify intervention strategies with potential to enhance root disease suppression.

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

This research has delivered fundamental knowledge of rhizosphere-fungal interactions in sugarcane production systems and factors impacting on the incidence and suppression of root diseases. This information forms the basis of management strategies focused on selection of planting sequences and efficient targeting of disease suppressive treatments to improve root health and enhance the effective use of inputs.

1. Improve understanding of the agro-ecological processes affecting root-pathogen-antagonist interactions in sugarcane production systems, via comparative eco-genomic analyses of disease suppressive and conducive soils.

Analyses of the sugarcane rhizosphere soil fungal microbiome indicated significant differentiation between fungal communities under longer-term (5^{th} – 10^{th}) ratooning cane compared with replant continuous cane and cane-legume rotations. There were however, no significant differences in fungal community structure among the replant cane and cane-legume rotations. Forest soil fungal communities had the highest diversity and were significantly differentiated from the adjacent sugarcane crops.

The majority of these differences between can cropping treatments were attributed to 33 fungal taxa. These included numerous soil and root-associated saprophytes, potentially pathogen antagonistic *Trichoderma, Epicoccum, Humicola*, *Bullera* and *Clonostachys* spp., plant pathogenic *Fusarium* spp. and functionally diverse Helotiales, Hypocreales, Myriangiales and Pleosporales comprising mycorrhizal, mycoparasitic and plant pathogenic species.

Culture-dependent and culture-independent analyses indicate that as rhizosphere abundance and root colonisation of pathogen antagonistic *Trichoderma* and *Epicoccum* significantly increased, frequencies of root pathogenic *Fusarium* and *Pythium* and root-associated saprophytic *Chaetomium* and *Mortierella* significantly decreased. Rhizosphere communities of unidentifed taxa within the orders Helotiales, Hypocreales, Myriangiales and Pleosporales (*e.g.* dematiaceous fungi) were all more abundant in rhizosphere soils of 1^{st} ratoon replant continuous cane and cane-legume rotations and signifcantly declined in longer-term ratoon cane.

2. Determine host-based selection of fungal communities (beneficial and deleterious) in a sugarcane-legume rotation, enabling identification of cropping strategies to enhance disease suppression.

In this study sugarcane root infection by plant pathogenic *Fusarium* (30%) and *Pythium spinosum* (64%) was high frequency and dynamic in comparison to their low, relatively stable abundance in the rhizosphere soil microbiome. This suggests selection of plant pathogenic genotypes of these taxa by actively growing sugarcane and temporal stability of saprophytic or weakly pathogenic genotypes within rhizosphere soil communities.

Communities of saprophytic *Chaetomium* and *Mortierella* spp. and plant pathogenic *Fusarium* and *Pythium* spp. were generally more abundant ($P<0.001$) in roots and rhizosphere of early ratoon replant continuous cane and cane-legume rotations. In contrast, abundance of endophytic fungal antagonistic *Trichoderma* and *Epicoccum* spp. increased ($P<0.001$) with the time under cane cropping, being greatest in longer-term (5^{th} – 10^{th}) ratoon cane.

Secondary metabolites produced by *Trichoderma* and *Epicoccum* spp. actively inhibited fungi and oomycetes *in vitro* and act as a selective mechanism to enhance rhizosphere competition and root colonisation. Multiple years of ratoon cane is expected to apply relatively uniform rhizosphere selection over time, increasing the abundance of these fungal antagonists and suppressing root-associated saprophytes, plant pathogenic *Fusarium* and *Pythium* spp. and unidentified functionally diverse Pleosporales, Hypocreales and Myriangiales fungal taxa.

3. Improve capacity to predict the disease suppressive status of soils and manage plant pathogenic and beneficial fungal communities.

The disconnect between high frequencies of root infection by plant pathogenic *Fusarium* spp. and *Pythium spinosum* and low soil inoculum (Output 2, above) has implications when using higher taxonomic (non species–specific) DNA-based diagnostics in soil as predictors of *Pythium* and *Fusarium* root disease severity in sugarcane and rotaton crops.

In constrast, there was a positive association between root colonisation and abundance of pathogen antagonistic *T. harzianum* rhizosphere soil inoculum with a species-specific DNA diagnostic. The link between increasing *T. harzianum* abundance and decreasing *Pythium* and *Fusarium* root infection suggests that the *T. harzianum*-specific diagnostic may be a predictor of root disease suppression.

Correlating soil-borne inoculum, root colonisation and culture-independent molecular diagnostic (PredictaB, CSIRO) data sets will assist with determining relationships among plant pathogenic (*Pythium*, *Fusarium*) and beneficial (*Trichoderma*) inoculum levels, incidence of root infection and disease severity in sugarcane.

4. Provide resources (DNA extracts) for subsequent eco-genomic and quantitative diagnostic analyses of sugarcane soil microbiota (*i.e.* bacteria, invertebrates).

Existing DNA extracts provide a resource to conduct targeted taxa-specific analyses of oomycete (*Pythium*, *Pachymetra*) and unidentified potential plant beneficial and sugarcane pathogenic species within the rhizosphere microbiome of sugarcane (orders Helotiales, Hypocreales and Myriangiales). These extracts can also be analysed to investigate dynamics of bacterial and dematiaceous (Pleosporales) fungal communities, enabling identification of proposed pathogenic taxa and examining broader microbial interactions in the rhizosphere that impact on root disease expression.

The primary target audience for Outputs 1-3 includes SRA, researchers and advisors.

Target audience for Output 4 is SRA and researchers.

At this stage, there has been no adoption of the project outputs.

Recommendations for further RD&A associated with these outputs are discussed in section 8.

3.2. Outcomes and Implications

1. Contribute to the development of novel biological options and management strategies to decrease root disease incidence and improve sugarcane productivity.

Trichoderma spp are recognised antagonists of plant pathogenic fungi and oomycetes, with genotypes of *T. harzianum* and *T. gamsii* registered for the suppression of root and foliar diseases. Sugarcane root endophytic *Epicoccum* have previously reported to have antibiosis activity against sugarcane root pathogenic fungi. Metabolites of *T. harzianum* and *E. nigrum* strains isolated in this research inhibited *Fusarium* growth and were lethal to *P. spinosum*. These rhizosphere competent, pathogen-antagonistic *T. harzianum* and *E. nigrum* strains and their secondary metabolites require investigation as potential disease suppressive treatments in sugarcane.

2. Provide environmental benefits associated with decreased reliance on synthetic fungicides and increased adoption of conservation cropping practices.

During the course of this project all research activities were conducted under controlled environment conditions using sugarcane plants and cropping soils. Consequently, no new information is available on adverse or beneficial environmental impacts resulting from the research.

Economic Outcomes
Reported average production losses to sugarcane root diseases in Australia are 10-15% and are therefore estimated to cost the Australian sugar industry approximately $80-100 million p.a. This research has enhanced ecological understanding of beneficial and deleterious plant-microbe interactions in sugarcane cropping soils. This information forms the basis of disease prediction and management strategies focused on selection of planting sequences and targeting of inputs to suppress disease, thereby enhancing root health and sugarcane productivity.

Environmental Outcomes
Environmental benefits that may be realised through enhanced biological disease suppression include decreased reliance on synthetic fungicides and more efficient use of agrichemicals. This will result in decreased on-farm and off-site contamination of soil and water resources.

Social Outcomes
Communication of research outcomes will further raise awareness in the grower community of the beneficial and deleterious functions of soil microbes in sugarcane cropping systems and the impacts of agronomic management practices on incidence and suppression of disease.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

To date, no research publications have arisen from the project.

At this stage, there has been no adoption of the project outputs.

Following submission of the final report, articles summarising research outputs will be submitted to CaneConnection (Pfeffer), HCPSL (Di Bella) and Terrain Natural Resource Management (Waring) newsletters.

4.2. Industry communication messages

The aims of this research were to deliver fundamental knowledge of rhizosphere-fungal interactions in sugarcane cropping systems and agronomic factors impacting on the incidence and suppression of root diseases. This information forms the basis of disease prediction and management strategies

focused on selection of cropping sequences and targeting of inputs to suppress disease.

DNA sequencing was used to define impacts of agronomic treatments on sugarcane rhizosphere fungal communities. Those under long-term (5^{th}-10^{th}) ratoon cane were significantly different from replant continuous cane and cane-legume rotations whereas, those under the latter two treatments were not significantly different. Forest soil fungal communities were significantly more diverse and differentiated from the adjacent cane crops.

The majority of differences among rhizosphere communities were attributed to 33 fungal taxa, their abundances significantly different among cropping treatments. These included numerous root-associated saprophytes, pathogen antagonistic and potentially disease suppressive genera (5), plant pathogenic *Fusarium* and broad groups (5 fungal orders) comprising plant-beneficial mycorrhizal and mycoparasitic fungi and taxa related to wilt, stem rot and root rot pathogens of sugarcane.

Microbiological analyses indicated that root colonising fungal communites were dominated by primarily saprophytic taxa, pathogen antagonistic *Trichoderma* and plant pathogenic *Fusarium*. Oomycete communities were dominated by plant pathogenic *Pythium spinosum. Pachymetra chaunorhiza* was not isolated from roots or rhizosphere soils at either site, despite DNA diagnostics detecting the presence of the pathogen.

Pathogen antagonistic *Trichoderma* and *Epicoccum* spp. were significantly more abundant under long-term ratoon cane and produced metabolites that significantly inhibited *Fusarium* growth and killed *Pythium*. These root endophytic fungi and their secondary metabolites require investigation as potential disease suppressive treatments in sugarcane.

Collectively microbiological and DNA sequence analyses of sugarcane roots and rhizosphere soils indicated that saprophytic communities and plant pathogenic *Fusarium* and *Pythium* spp. were significantly more abundant in early ratoon replant continuous cane and cane after legume compared with long-term ratoon cane. In contrast, abundance of disease suppressive *T. harzianum* and *Epicoccum* spp. increased with time under cane cropping.

These results contrast with the generally accepted view that long-term ratoon cane increase communities of root pathogenic fungi, resulting in higher root disease incidence. *Fusarium* (30%) and *Pythium* (64%) root infection were high in these sugarcane fields, but both were significantly lower (10%) in longer-term ratoon cane. Significantly greater root (25%) and rhizosphere abundance (105%) of disease suppressive *T. harzianum* may account for the suppression of these pathogens.

Fungal orders reported to contain plant beneficial and sugarcane plant pathogenic species also significantly decreased in abundance under long-term ratoon cane. These fungi remain largely unidentified and further research is required to isolate and identify the plant pathogenic species and monitor their dynamics in response to cropping sequences.

5. METHODOLOGY

5.1. Field Sites, Cropping Treatments and Sample Collection

Analyses of sugarcane rhizosphere fungal and oomycete communities were conducted across two cropping seasons at two sites. Intra- and inter- site comparisons were made among replant continuous cane, cane-legume rotations and longer-term (5th-10^{th}) ratoon cane cropping treatments. A native forest soil was included as a comparison with cane cropping treatments at the Ingham site (below).

Five weeks prior to planting cane (T0) soil samples were taken from bare plots (bulk soil) and within rows of ratoon cane cropping treatments. Samples were designated as pre-plant (T0). Actively growing cane plants were sampled 6-weeks post-emergence in 2016 (T1) and 2017 (T2) and processed to collect rhizosphere soils and roots.

Stone River (SR) 18° 41.924' S, 146° 0.977' E
Sugarcane-legume nitrogen management trial (SRA project 2015/074).

The trial used a complete randomised block design with 4 replicate plots of the following treatments

- Continuous Cane (C-C FT): Full tillage (FT) new-plant (T1) and 1st ratoon (T2) sugarcane after 4th ratoon cane.
- Cane-Soybean (C-S FT): Full tillage (FT) new-plant (T1) and 1st ratoon (T2) sugarcane following soybean (S) rotation.
- Cane-Soybean (C-S MT): Minimum tillage (MT) new-plant (T1) and 1st ratoon (T2) sugarcane following soybean (S) rotation.

Ratoon cane samples were taken from 4 replicate plots (20 m long) established along a 100 m transect in a cropping strip adjacent to the trial. The treatment was defined as

- Ratoon Cane (RC ZT): Zero tillage (ZT) 4th (T1) and 5th (T2) ratoon sugarcane plots.

Ingham Herbert Cane Productivity Services Limited (IHCPSL) 18° 41.924' S, 146° 0.977' E
Commercial sugarcane-legume cropping system (SRA project 2014/004)

Cane samples were taken from 4 replicate plots (20 m long) established along 100 m transects in a adjacent cropping strips. The treatments were defined as

- Cane-Lablab (C-L MT): Minimum tillage (MT) replant (T1) and 1st ratoon (T2) sugarcane following lablab bean (L) rotation.
- Ratoon Cane (RC ZT): Zero tillage (ZT) 4th (T0, T1) and 10th (T2) ratoon sugarcane plots adjacent to C-L MT.
- Forest: Undisturbed forest vegetation adjacent (~ 10 m) to sugarcane plots.

Eight soil microcores (2 cm diameter x 20 cm depth) (T0) and 8 cane plants (T1 and T2) were sampled from the 4 internal rows of each plot. There were 4 sampling points per plot, paired samples being taken every 5m along a diagonal transect. Four replicate plots of each treatment were sampled at each site. Root systems of sugarcane plants were soaked in water and agitated to isolate the adhering rhizosphere soils (T1 and T2).

In 2016 a total of 224 T0 soils (128 from SR, 32 from IHCPSL), 224 T1 soils (rhizosphere) and 160 cane plants (128 from SR, 32 from IHCPSL)) were processed.

In 2017 (T2) 224 rhizosphere soils and 192 cane plants (128 from SR, 64 from IHCPSL) were processed. In addition

5.2. Soil Physio-Chemical Properties

Site-specific composite pre-plant (T0) and rhizosphere (T1 and T2) soil samples (100 g) representing the 4 Stone River and 3 Ingham cropping treatments were subjected to full elemental, pH and EC analyses to provide an overview of the soil chemical status of each site.

Soil analyses of total non-purgeable organic carbon (TOC) and total nitrogen (TN) from each replicated plot (7 treatments x 4 reps x 3 sampling times) were conducted to investigate linkages between soil nutrient (C and N) and microbial community dynamics under the different agronomic treatments.

5.3. Quantifying Oomycete DNA in Rhizosphere Soils with PredictaB Diagnostics

Rhizosphere soils (T1 and T2) were analysed with the PredictaB *Pythium* clade F assay and the *Pachymetra chaunorhiza* diagnostic assay currently under development (http://www.pir.sa.gov.au/research/services/molecular_diagnostics/predicta_b).

5.4. Quantifying Viable Soil-borne Fungal and Oomycete Inoculum and Root Infection

5.4.1. Viable Rhizosphere Inoculum of Oomycete and *Mortierella* Species

Rhizosphere soils (T1 and T2) representing the 7 agronomic treatments at the 2 sites were analysed for oomycete (*Pythium* and *Pachymetra* spp.) and *Mortierella* communities by soil dilution plating on the oomycete semi-selective medium VP3 (Harvey *et al.* 2008). Oomycete and *Mortierella* spp. were quantified as propagules (spores) g^{-1} soil.

5.4.2. Isolation of Endophytic and Plant Pathogenic Fungi and Oomycetes from Sugarcane Roots

Fungal and oomycete isolations from sugarcane roots were conducted using established protocols (Harvey et al. 2008; Petrovic et al. 2013). Briefly, root systems were removed from each of the 8 plants sampled from each plot and soaked in water to remove the adhering rhizosphere soil. A minimum of 20 root segments per plant (each 2-3 cm) showing symptoms suggestive of fungal or oomycete infection (*i.e.* lesions, lack of lateral and fine roots, cortical pitting) were excised and washed overnight under running tap water. Root segments were surface sterilised (ethanol and hypochlorite), blotted dry and 5 segments per plant were plated onto generalist Potato Dextrose Agar (PDA), oomycete-selective (VP3) and fungal-selective (DRBC) media. Isolation frequencies of fungal and oomycete colony phenotypes were expressed as the proportion of root segments infected by the respective phenotypes.

In total, over 5,280 root segments (2,400 from T1, 2,880 from T2) were plated and colony phenotypes of the recovered endophytic fungi and oomycetes were described. Collectively, 3 oomycete (VP3), 6 fungal (DRBC) and 6 generalist (PDA) root-infecting colony phenotypes were identified, each being isolated from both trial sites. Fungal and oomycete strains representing a minimum of 2 isolates per phenotype and 5 isolates per agronomic treatment were sub-cultured for further characterisation, with 2 strains of each phenotype stored as a culture for future reference.

5.5. Identification of Rhizosphere Soil and Root Endophytic Fungi and Oomycetes

Spores (conidia) or non-sporulating mycelia of rhizosphere soil and root endophytic strains were sub-cultured to their respective original isolation media to confirm pure cultures and grown in solid (PDA) or liquid media (PDB) for DNA extraction (Harvey et al. 2008; Petrovic et al. 2013). Genomic DNA was extracted from a total of 120 (T0 and T1) and 82 (T2) soil-inhabiting and root endophytic strains and sequenced (ITS-rDNA) for molecular taxonomic identification. The ITS I -5.8S –ITS 2 rRNA gene was amplified in each isolate by PCR with the following primer pairs ITS 1: 5’ TCCgTAggTgAACCTgCgg 3’ & ITS 4: 5’ TCCTCCgCTTATTgATATgC 3’ (White *et al*. 1990). DNA sequencing of amplified regions was performed at the Australian Genome Research Facility (AGRF), Adelaide, South Australia. BLASTN results (NCBI www.ncbi.nlm.nih.gov) were used to identify fungal and oomycete species belonging to each colony phenotype and consequently, isolation frequencies of fungal and oomycete taxa from roots and rhizosphere soils.

5.6. Anti-biosis of Sugarcane Root Pathogens by Endophytic *Epicoccum* and *Trichoderma*

Sugarcane root endophytic *Trichoderma harzianum* and *Epicoccum nigrum* strains isolated from 1st ratoon replant continuous cane (Stone River) and 10th ratoon cane (Ingham) were analysed for *in vitro* antibiosis of sugarcane root pathogens *Fusarium kyushuense*, *F. oxysporum*, *F. verticillioides* and *Pythium spinosum*. Wheat root disease suppressive *T. gamsii* (Stummer *et al.* 2018) was included for comparison.

Fungal strains tested for pathogen antibiosis included *E. nigrum* 7.1 (SR C-C FT), *E. nigrum* 96.1 (IHCPSL RC ZT), *T. harzianum* 2.1 (SR C-C FT), and *T. harzianum* 88.2 (IHCPSL RC ZT). Fungal and oomycete disease suppressive inoculant *Trichoderma gamsii* A5MH (Stummer *et al.,* 2018) was isolated from wheat roots at Avon, South Australia.

Sugarcane root pathogenic strains (origins in parenthesis) were isolated in 2017 (T2) *F. kyushuense* 12.2 (SR C-C FT), *F. oxysporum* 93.2 (IHCPSL RC ZT, *F. verticillioides* 16.1 (SR C-C FT), and *P. spinosum* 46.1 (SR C-S FT)

P. spinosum and all fungal strains were grown on full strength PDA amended with 100 ppm streptomycin. Antagonists were grown for 7 days at 25 °C in 100ml PD broth. The culture medium was then filtered (0.2 µm) to ensure it was cell-free and 3ml was placed into each of 6 wells in a sterile tissue culture plate. A 5mm plug of each pathogen was inoculated into plates containing 1) filtrate of each antagonist and 2) PD broth not exposed to the antagonist as a control. Pathogen growth was monitored after 24, 72 and 168 hrs (7 days). Pathogen antibiosis was determined after 7days and quantifed as the % reduction in mycelial biomass by the equation: [1– (pathogen biomass + antagonist metabolites/control pathogen biomass) x 100], where 0 equals no inhibition and 100 equals no pathogen growth. This method was also used to assess reciprocal antibiosis between pathogen antagonistic *Trichoderma* and *E. nigrum* strains.

5.7. Fungal Microbiome of Sugarcane Soils

Eco-genomic analyses (rDNA sequencing) of the sugarcane fungal microbiome was conducted on pre-planting (T0) and rhizosphere soils (T1 - T2) isolated from roots used in plant pathological, nutrient (TOC and TN), culture-dependent viable inoculum and culture-independent (PredictaB) analyses. Root systems of sugarcane plants were soaked in water and agitated to isolate the adhering rhizosphere soil. All soils were air-dried, sieved (aggregates <2 mm retained), 20 g sub-samples snap frozen in liquid N and stored at -80 °C for subsequent analyses.

Total metagenomic DNA was extracted from 0.25 – 1.0 g of soil using PowerSoil® DNA isolation kit following the manufacturer's protocol (MO BIO Laboratories Inc., Carlsbad, CA, USA). DNA was purified, quantified and diluted to 2 ng/µl for subsequent analyses. In total (336) DNA samples were extracted, 112 per sampling time (T0, T1 and T2). Preliminary testing showed that optimal sequence reads were achieved by pooling 2 of the 4 sub-replicates from within each replicated plot. In total 96 samples were sequenced sequencing from the SR site (4 treatments x 4 replicates x 2 sub-replicates x 3 times) and 72 samples from the IHCPSL site (3 treatments x 4 replicates x 2 sub-replicates x 3 times).

High throughput sequencing (Illumina MiSeq) and bioinformatic diversity profiling of fungal community structure was performed at AGRF (www.agrf.org.au). The internal transcribed space region (ITS1) of the nuclear ribosomal RNA gene was amplified with primers ITS 1F-2. Fungal operational taxonomic units (OTUs) were clustered at minimum identity of 97% and fungal taxa identified using Unite databases (Unite version 7.2).

5.8. Quantification of *Trichoderma harzianum* in Rhizosphere Soil by qPCR

Abundance of pathogen antagonistic *T. harzianum* (Zhang *et al*., 2015) and *T. gamsii* (Stummer *et al*., 2017) were quantified in all pre-plant (T0) and rhizosphere soils (T1 and T2) by species-specific qPCR (Stummer *et al.* 2013; Stummer *et al.* 2018). *T. harzianum* and *T. gamsii* inoculum was quantified in all 168 soil DNA extracts used for soil fungal microbiome analyses and expressed as *Trichoderma* conidial equivalents g^{-1} soil.

DNA of all *Trichoderma* pure cultures were also assayed with these *T. harzianum* and *T. gamsii* qPCR diagnostics and the results compared with identifications based on ITS-rDNA sequencing (5.5 above).

5.9. Data Analysis

Statistical analysis of culture-dependent (root and soil isolation frequencies), culture-independent (qPCR, microbiome relative abundance, diversity indices) and soil nutrient (TOC, TN) data used Genstat v17, (VSN International Ltd) to test for treatment effects (one-way ANOVA) and treatment x

time interactions (two-way ANOVA). Fisher's least significant difference (LSD) comparison (P=0.05) was performed to determine significant differences among means.

Relative abundance (OTUs) of fungal taxa in the sugarcane soil fungal microbiome were analysed using Primer6 (Primer-E Ltd, Plymouth, UK) (Clarke & Warwick, 2001). Data were square-root transformed and dissimilarity matrices constructed using the Bray Curtis algorithm. Taxonomic diversity among fungal communities in sugarcane was determined based species richness (S), eveness (J') and the Simpson Index (λ), the latter two indices ranging from a minimum of 0 to a maximum of 1. Fungal OTUs with relative abundance >0.05% were included in cluster analysis and ANOSIM to determine differentiation among soil fungal communities in response to sugarcane agronomic treatment and sampling time. The ANOSIM statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. An R value close to 1.0 suggests dissimilarity between groups while an R value close to 0 suggests an even distribution of high and low ranks within and between groups. The significance of taxonomic similarities among rhizosphere fungal communities were assessed using the similarity profile test SIMPROF (Clarke *et al*. 2008).

6. RESULTS

6.1. Total Nitrogen and Organic Carbon Contents in Sugarcane Cropping Soils

Analyses of total nitrogen (TN) and non-purgeable organic carbon (TOC) in pre-plant (T0) and 6-week post-emergent rhizosphere (T1 – T2) soils were conducted to investigate linkages between nutrient and microbial community dynamics in sugarcane cropping treatments.

There were no significant differences in pre-plant (T0) soil TN among replant continuous cane, ratoon cane or cane-legume rotations at either site (data not shown). Consequently, there was no residual soil TN remaining from soybean or lablab bean rotations 5 weeks prior to cane planting. Similarly, there were no significant differences in rhizosphere soil TN among sugarcane cropping treatments following fertiliser application (20 – 30 Kg N Ha^{-1}) at planting (data not shown).

Soil TOC significantly (P<0.001) increased each year of cane cropping, regardless of the agronomic treatment (Table 1). TOC was significant greater in T2 compared with T1 rhizosphere soils, both being significantly higher than in pre-plant (T0) soils. Lower levels of TOC in pre-plant (T0) and rhizosphere soils of replant cane (T1) is likely due to recent tillage increasing microbial mineralisation of the labile organic fraction, thereby reducing TOC relative to the less disturbed ratoon cane treatments (T2).

At Ingham there were no significant differences in soil TOC within or between pre-plant (T0) or rhizosphere (T1) cane-legume rotation or ratoon cane treatments (Table 1). There was however, a significant difference (P<0.001) in T2 rhizosphere TOC of long-term (10^{th}) ratoon cane and the 1^{st} ratoon of the cane-lablab rotation (Table 1). Forest soil TOC showed a similar trend to that of the long-term ratoon cane treatment, significantly (P<0.001) increasing from T1 to T2 (Table 1)

At Stone River, soil TOC was not significantly different prior to planting cane (T0) or among rhizosphere soils (T1) of continuous (replant) cane and cane-soybean rotations. Rhizosphere (T1) soil TOC of 4^{th} ratoon cane was significantly (P<0.001) greater than replant continuous cane and the cane-soybean rotation (Table 1). The significantly higher rhizosphere TOC in the T2 cane-soybean rotation full tillage treatment was unexpected, as there were no differences between replant cane tillage treatments the previous year and no increase in TOC under longer-term 4^{th} and 5^{th} ratoon cane (Table 1).

Table 1 Total non-purgable organic carbon (TOC) in sugarcane soils at 5 weeks pre-planting (T0) and 6 weeks post-emergence in 2016 (T1) and 2017 (T2), in response to crop rotation and tillage treatments at Ingham and Stone River Qld.

Crop Management Treatment	Total Organic Carbon (mg kg^{-1} soil) [3]		
	T0	T1	T2
Ingham (IHCPSL) [1]			
Cane-Lablab (C-L MT)	133 ^{efghi}	155 ^{defg}	179 ^{bcde}
Forest (undisturbed)	112 ^{hij}	113 ^{hij}	212 ^{b}
Ratoon Cane (RC ZT)	118 ^{ghij}	120 ^{fghij}	264 ^{a}
Stone River (SR) [2]			
Contin. Cane (C-C FT)	121 ^{fghij}	161 ^{cdef}	178 ^{bcde}
Cane-Soybean (C-S FT)	109 ^{ij}	143 ^{efghi}	261 ^{a}
Cane-Soybean (C-S MT)	108 ^{ij}	153 ^{efgh}	196 ^{bcd}
Ratoon Cane (RC ZT)	96 ^{j}	212 ^{b}	198 ^{bc}
Grand Mean		*159*	
	P	LSD	
$F_{Treatment \times Time}$ (5%)	<0.001	41.6	
$F_{Treatment}$ (5%)	0.266	24.0	
F_{Time} (5%)	<0.001	15.7	

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
   Cane-Lablab (C-L MT): Minimum tillage (MT) replant (T1) and 1st ratoon (T2) sugarcane (*Saccharum officinarum*) following summer break crop of lablab bean (*Lablab purpureus*) *i.e.* (L).
   Forest: Undisturbed forest vegetation adjacent (~ 10 m) to sugarcane plots.
   Ratoon Cane (RC ZT): Zero tillage (ZT) 4th (T0, T1) and 10th (T2) ratoon sugarcane plots adjacent to C-L MT.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
   Continuous Cane (C-C FT): Full tillage (FT) new-plant (T1) and 1st ratoon (T2) sugarcane after 4th ratoon cane.
   Cane-Soybean (C-C-S FT): Full tillage (FT) new-plant (T1) and 1st ratoon (T2) sugarcane following summer break crop of soybean (*Glycine max*) *i.e* (S).
   Cane-Soybean (C-S MT): Minimum tillage (MT) new-plant (T1) and 1st ratoon (T2) sugarcane following summer break crop of soybean (*Glycine max*) *i.e* (S).
   Ratoon Cane (RC ZT): Zero tillage (ZT) 4th (T1) and 5th (T2) ratoon sugarcane plots adjacent to C-C and C-S.
3. Rhizosphere soil total organic carbon (TOC)
   Letters in superscript indicate significant differences in pre-planting (T0) and rhizosphere (T1, T2) soil TOC.
   $F_{Treatment}$: Not significant
   F_{Time}: T2 (213 ^{a}) > T1 (151 ^{b}) > T0 (115 ^{c})

6.2. Inoculum of *Pachymetra chaunorhiza* and *Pythium* spp. in Sugarcane Rhizosphere Soils

Inoculum of oomycete root pathogens (*Pythium* and *Pachymetra* spp.) in sugarcane rhizosphere (T1, T2) and adjacent forest soils (Ingham) were quantified by culture-independent DNA diagnostics (PredictaB) and culture-dependent isolation of viable inoculum on oomycete-selective medium.

Collectively 3 broad oomycete colony phenotypes and representative pure cultures (37 in total) were identified (morphology, ITS-rDNA sequencing) as plant pathogenic *Pythium spinosum* (Clade-F), *P. glomeratum* (Clade_I) and *P. heterothallicum* (Clade_I) (Lévesque & De Cock, 2004). Whilst each *Pythium* spp. was isolated from rhizosphere soils of every sugarcane treatment and the forest soil, oomycete communities were dominated by *P. spinosum.*

Across both sites, the overall mean level of *P. spinosum* rhizosphere soil inoculum was 65 viable propagules g^{-1} soil. There were no significant differences in rhizosphere inoculum among the sugarcane tillage and rotation treatments or the adjacent forest soil (Table 2). There was however, a significant (P<0.001) increase in *P. spinosum* inoculum with time (Table 2), resulting from another year of cane growth (ratoon).

Cultue-independent quantification with diagnostics specific for *Pythium* Clade_F (PredictaB) resolved no significant differences in rhizosphere inoculum among T1 or T2 cane cropping treatments at Stone River (Table 2). At Ingham, T1 Clade_F rhizosphere inoculum was significantly (P<0.001) higher in the cane-lablab rotation compared with 4th ratoon cane and was below detection in forest soil (Table 2). Overall there was a significant decrease in *Pythium* Clade_F inoculum with time (Table 2). This result that contrasts with the culture-dependent (microbiological) assessment of *Pythium* inoculum dynamics, especially given the community was dominated *by P. spinosum* a taxon within Clade_F.

No *Pachymetra chaunorhiza* strains were isolated from rhizosphere soils at either trial site, despite the species-specific diagnostic assay (SRA project 2016/047) detecting the pathogen in all sugarcane and forest soils (Table 2). There were no significant differences in *Pachymetra* rhizosphere inoculum among the sugarcane tillage and rotation treatments at Stone River (Table 2). At Ingham, significantly (P=0.007) greater levels of *Pachymetra* inoculum were detected in replant cane following lablab bean (T1) compared with 4th ratoon cane, the latter not significant to levels detected in forest soil (Table 2). Overall there was a significant (P=0.016) increase in *Pachymetra* rhizosphere inoculum with time (Table 2), primarily due to the high levels detected in long-term (10th) ratoon cane (Table 2).

Assuming a minimum copy number of 1 target sequence per *Pa. chaunorhiza* genome and given the oospores are uninucleate, the minimum soil-borne *Pachymetra* inoculum detected equates to 100,000 oospores per kg^{-1} soil in T2 forest and cane-soybean treatments. This would place the all cane treatments at a medium to high risk of *Pachymetra* root rot (SRA Information Sheet IS1 3005). Despite this, no *Pa. chaunorhiza* strains were isolated from sugarcane rhizosphere soils or from cane roots (6.3.5, below) on oomycete-selective medium.

Table 2 Inoculum of *Pachymetra chaunorhiza* and *Pythium spinosum* in sugarcane rhizosphere soils at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld. Pathogen inoculum was quantified

by culture-independent DNA diagnostics (PredictaB) and culture-dependent isolation of viable propagules on oomycete-selective medium.

Crop Management Treatment	*Pachymetra chaunorhiza* (K copies DNA g^{-1} soil) [3]		*Pythium* Clade_F spp. (pg DNA g^{-1} soil) [4]		*Pythium spinosum* (propagules g^{-1} soil) [5]	
	T1	T2	T1	T2	T1	T2
Ingham (IHCPSL) [1]						
Cane-Lablab (C-L MT)	15.5 ^{b}	20.8 ^{ab}	130 ^{a}	35 ^{b}	60 ^{a}	140 ^{a}
Forest (undisturbed)	0.0 ^{c}	0.1 ^{c}	0 ^{e}	0 ^{e}	5 ^{a}	105 ^{a}
Ratoon Cane (RC ZT)	2.8 ^{c}	31.6 ^{a}	6 ^{bdec}	33 ^{bc}	45 ^{a}	110 ^{a}
Stone River (SR) [2]						
Contin. Cane (C-C FT)	0.2 ^{c}	0.8 ^{c}	11 ^{bcde}	1 ^{e}	20 ^{a}	105 ^{a}
Cane-Soybean (C-S FT)	0.2 ^{c}	0.1 ^{c}	5 ^{bcde}	4 ^{cde}	20 ^{a}	95 ^{a}
Cane-Soybean (C-S MT)	0.8 ^{c}	0.2 ^{c}	4 ^{cde}	1 ^{e}	25 ^{a}	65 ^{a}
Ratoon Cane (RC ZT)	0.2 ^{c}	3.2 ^{c}	32 ^{bc}	2 ^{de}	35 ^{a}	80 ^{a}
Grand Mean	*5.5*		*19*		*65*	
	P	LSD	P	LSD	P	LSD
$F_{Treatment \times Time}$ (5%)	0.007	11.3	<0.001	31.4	0.725	54.6
$F_{Treatment}$ (5%)	<0.001	8.0	<0.001	22.2	0.113	38.6
F_{Time} (5%)	0.016	4.3	0.010	11.9	<0.001	20.6

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. *Pachymetra chaunorhiza* DNA in sugarcane rhizosphere soil.
   $F_{Treatment \times Time}$: Letters in superscript indicate significant differences in DNA of *Pachymetra chaunorhiza*.
   $F_{Treatment}$ Ingham: C-L MT (18.2 ^{a}) = RC ZT (17.2 ^{a}) > Forest (0.0 ^{b})
   $F_{Treatment}$ Stone River: RC ZT (1.7 ^{b}) = C-C FT (0.5 ^{b}) = C-S FT (0.5 ^{b}) = C-S MT (0.1 ^{b})
   F_{Time}: T2 (8.1 ^{a}) > T1 (2.8 ^{b})
4. *Pythium* Clade_F DNA in sugarcane rhizosphere soil (*i.e. P. spinosum*).
   $F_{Treatment \times Time}$: Letters in superscript indicate significant differences in soil DNA of *Pythium* spp.
   $F_{Treatment}$ Ingham: C-L MT (83 ^{a}) > RC ZT (20 ^{b}) = Forest (0 ^{b})
   $F_{Treatment}$ Stone River: RC ZT (17 ^{b}) = C-C FT (6 ^{b}) = C-S FT (4 ^{b}) = C-S MT (3 ^{b})
   F_{Time}: T1 (27 ^{a}) > T2 (11 ^{b})
5. Viable *Pythium spinosum* inoculum in sugarcane rhizosphere soils.
   $F_{Treatment \times Time}$: Letters in superscript indicate significant differences in viable *Pythium* propagules.
   $F_{Treatment}$: Not significant.
   F_{Time}: T2 (100 ^{a}) > T1 (30 ^{b})

6.3. Endophytic Fungal Colonists of Sugarcane Roots and their Relative Abundance in Rhizosphere Microbiomes

Culture-dependent microbiological (soil-borne inoculum, root isolation) and culture-independent molecular biological (rDNA sequencing, qPCR) techniques were used to determine the taxonomic structure of the sugarcane rhizosphere fungal microbiome. The taxonomic composition of fungal communities were inferred from rDNA sequencing, taxa being identified as operational taxonomic units (OTUs). Analyses focused on the dominant endophytic colonists of sugarcane roots and their relative abundances (OTUs) in response to crop rotation and tillage treatments.

In total, 202 fungal and oomycete strains consisting of 37 soil (6.2, above) and 165 sugarcane root isolates representative of each colony phenotype were identified by morphology and ITS-rDNA sequencing. Endophytic root isolates comprised 3 oomycete and 22 fungal species including recognised plant pathogenic *Pythium* and *Fusarium*, potentially plant disease suppressive *Trichoderma* and *Epicoccum* and primarily saprophytic *Chaetomium*, *Mortierella*, *Penicillium* and *Gongronella* spp.

6.3.1. Saprophytic and Root Endophytic *Chaetomium* species

Low to high frequencies (0.09 – 0.78) of endophytic *Chaetomium* spp. were isolated from surface sterilised sugarcane roots (Table 3). Among all cropping treatments, the overall mean *Chaetomium* isolation frequency was 0.41 (41%) (Table 3). *Chaetomium* strains (6) were identified as the recognised plant-associated saprophytic taxa *Chaetomium globosum* and an unidentified *Chaetomium* spp. Each *Chaetomium* taxon was isolated from sugarcane roots at both sites. There were no significant differences in *Chaetomium* root colonisation between cropping seasons at either site (Table 3).

The overall mean relative abundance of *Chaetomium spp.* (OTUs) in the sugarcane rhizosphere soil fungal microbiome was 3% (Table 3). In 2016 (T1) the relative abundance of *Chaetomium* taxa (OTUs) was significantly (P<0.001) higher under full tillage replant continuous cane (Stone River) compared with all other cropping treatments at Stone River and Ingham (Table 3). Another year of cane growth (T2) resulted in significant (P=0.004) increases in *Chaetomium* rhizosphere soil abundance in Stone River 1st ratoon cane after soybean (Table 3). There were no significant differences in the relative abundance of *Chaetomium* spp. among cropping treatments at Ingham in either 2016 (T1) or 2017 (T2) (Table 3).

Table 3 Endophytic colonisation of sugarcane roots by *Chaetomium* spp. and their relative abundance in the rhizosphere microbiome at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld.

Crop Management Treatment	*Chaetomium* spp.			
	Root Isolation Frequency [3]		OTU Relative Abundance [4]	
	T1	T2	T1	T2
Ingham (IHCPSL) [1]				
Cane-Lablab (C-L MT)	0.11 [a]	0.09 [a]	0.0196 [bc]	0.0086 [c]
Ratoon Cane (RC ZT)	ND	0.21 [a]	0.0012 [c]	0.0026 [c]
Stone River (SR) [2]				
Contin. Cane (C-C FT)	0.66 [a]	0.78 [a]	0.0119 [a]	0.1063 [a]
Cane-Soybean (C-S FT)	0.70 [a]	0.74 [a]	0.0010 [c]	0.0806 [a]
Cane-Soybean (C-S MT)	0.42 [a]	0.54 [a]	0.0019 [c]	0.0672 [ab]
Ratoon Cane (RC ZT)	0.10 [a]	0.21 [a]	0.0005 [c]	0.0206 [bc]
Grand Mean	*0.41*		*0.0260*	
	P	LSD	P	LSD
$F_{Treatment \times Time}$ (5%)	0.127	0.093	0.004	0.0478
$F_{Treatment}$ (5%)	<0.001	0.066	0.021	0.0338
F_{Time} (5%)	<0.001	0.041	<0.001	0.0195

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. Isolation of *Chaetomium* spp. from sugarcane roots (*Chaetomium globosum, Chaetomium* spp.)
   ND: Not determined

$F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in *Chaetomium* spp. root isolation.

$F_{Treatment}$ Ingham: RC ZT (0.21 ^{c}) > C-L MT (0.10 ^{d})

$F_{Treatment}$ Stone River: C-C FT (0.72 ^{a}) = C-S FT (0.72 ^{a}) > C-S MT (0.48 ^{b}) > RC ZT (0.16 ^{cd})

F_{Time}: T2 (0.47 ^{a}) > T1 (0.40 ^{b})

4. Relative abundance of *Chaetomium* spp. (rDNA-OTU's) in rhizosphere soil fungal microbiome

   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in abundance of *Chaetomium* spp.

   $F_{Treatment}$ Ingham: C-L MT (0.0141 ^{bc}) = RC ZT (0.0019 ^{c})

   $F_{Treatment}$ Stone River: C-C FT (0.0539 ^{a}) = C-S FT (0.0408 ^{ab}) = C-S MT (0.0345 ^{abc}) = RC ZT (0.0106 ^{bc})

   F_{Time}: T2 (0.0477 ^{a}) > T1 (0.0043 ^{b})

6.3.2. Saprophytic and Root Endophytic *Penicillium* species

Low to moderate frequencies (0.02 – 0.17) of endophytic *Penicillium* spp. were isolated from surface sterilised sugarcane roots (Table 4). *Penicillium* strains (6) were identified as plant-associated saprophytic taxa *P. janthinellum*, *P. ochrochloron*, *P. singorense* and an unidentified *Penicillium* spp. Among all cropping treatments the low overall mean *Penicillium* root isolation frequency (7%) was comparable to the relative abundance of *Penicillium* taxa (OTUs) in the rhizosphere soil fungal microbiome (3%) (Table 4). There were no significant differences in *Penicillium* endophytic root colonisation or rhizosphere soil relative abundance (OTUs) among sugarcane treatments at either site across both cropping seasons (Table 4).

Table 4 Endophytic colonisation of sugarcane roots by *Penicillium* spp. and their relative abundance in the rhizosphere soil fungal microbiome at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld.

Crop Management Treatment	*Penicillium* spp.			
	Root Isolation Frequency [3]		OTU Relative Abundance [4]	
	T1	T2	T1	T2
Ingham (IHCPSL) [1]				
Cane-Lablab (C-L MT)	0.07 ^{a}	0.06 ^{a}	0.0374 ^{a}	0.0125 ^{a}
Ratoon Cane (RC ZT)	ND	0.03 ^{a}	0.0264 ^{a}	0.0061 ^{a}
Stone River (SR) [2]				
Contin. Cane (C-C FT)	0.10 ^{a}	0.08 ^{a}	0.0315 ^{a}	0.0206 ^{a}
Cane-Soybean (C-S FT)	0.04 ^{a}	0.03 ^{a}	0.0464 ^{a}	0.0368 ^{a}
Cane-Soybean (C-S MT)	0.17 ^{a}	0.16 ^{a}	0.0147 ^{a}	0.0283 ^{a}
Ratoon Cane (RC ZT)	0.02 ^{a}	0.05 ^{a}	0.0032 ^{a}	0.0151 ^{a}
Grand Mean	*0.07*		*0.0232*	
	P	LSD	P	LSD
$F_{Treatment\ x\ Time}$ (5%)	0.646	0.050	0.089	0.0228
$F_{Treatment}$ (5%)	<0.001	0.035	0.005	0.0161
F_{Time} (5%)	0.783	0.022	0.158	0.0093

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. Isolation of *Penicillium* spp. from roots (*P. janthinellum*, *P. ochrochloron*, *P. singorense*, *Penicillium* spp.) ND: Not determined

$F_{Treatment \times Time}$: Letters in superscript indicate significant differences in *Penicillium* spp. root isolation.

$F_{Treatment}$ Ingham: RC ZT C-L MT (0.07 ^{b}) > RC ZT (0.03 ^{c})

$F_{Treatment}$ Stone River: C-S MT (0.17 ^{a}) > C-C FT (0.09 ^{b}) > C-S FT (0.03 ^{c}) = RC ZT (0.03 ^{c})

F_{Time}: Not significant

4. Relative abundance of *Penicillium* spp. (rDNA-OTU) in rhizosphere soil fungal microbiome

$F_{Treatment \times Time}$: Letters in superscript indicate significant differences in abundance of *Penicillium* spp.

$F_{Treatment}$ Ingham: C-L MT (0.0249 ^{bc}) = RC ZT (0.0162 ^{bc})

$F_{Treatment}$ Stone River: C-S FT (0.0416 ^{a}) = C-C FT (0.0261 ^{ab}) = C-S MT (0.0215 ^{bc}) = RC ZT (0.0091 ^{c})

F_{Time}: Not significant

6.3.3. Saprophytic and Root Endophytic *Mortierella* species

Low to high frequencies (0.08 – 0.91) of endophytic *Mortierella* spp. were isolated from surface sterilised sugarcane roots (Table 5). Among all cropping treatments, the overall mean *Mortierella* isolation frequency was 0.36 (36%) (Table 5). *Mortierella* strains from rhizosphere soil (28) and sugarcane roots (37) were identified as recognised plant-associated saprophytic taxa *M. alpina, M. chlamydospora, M. elongata, M. exigua, M. sarnyensis* and *M. sclerotiella*. Each *Mortierella* spp. was isolated from all sugarcane treatments at both sites.

In 2016 (T1) there were no significant differences in *Mortierella* root isolation frequencies at both sites (Table 5). Following an addition year of cane growth (T2) significant (P<0.001) increases in *Mortierella* root colonisation were observed in all Stone River sugarcane cropping treatments (Table 5), greater colonisation (P<0.001) observed in 1st ratoon continuous cane compared with cane-soybean and longer-term (5th) ratoon cane (Table 5). There were no significant differences in *Mortierella* root colonisation among Ingham sugarcane cropping treatments at either sampling time, with colonisation in 2017 (T2) being significantly lower than at Stone River (Table 5).

The overall mean relative abundance of *Mortierella spp.* (OTUs) in the sugarcane rhizosphere soil fungal microbiome was 3% (Table 5). There were no significant differences in relative abundance (OTUs) of *Mortierella* spp. among the sugarcane treatments across cropping seasons at either site (Table 5).

Across both sites, the overall mean level of viable *Mortierella* rhizosphere soil inoculum was 248 propagules g^{-1} soil (Table 5). In 2016 (T1) *Mortierella* inoculum was significantly (P<0.001) higher under full tillage cane-soybean rotation (Stone River) compared with all other cropping treatments at both sites (Table 5). Another year of cane growth (T2) resulted in significant declines in rhizosphere soil inoculum (P<0.001) and uniformity of *Mortierella* inoculum among all sugarcane cropping treatments in 2017 (T2) (Table 5). The contrast between declining rhizosphere soil inoculum and increasing root colonisation across cropping seasons suggests selection of root endophytic *Mortierella* genotypes by the actively growing sugarcane crop.

Table 5 Endophytic colonisation of sugarcane roots by *Mortierella* spp. and their relative abundance in the rhizosphere soil fungal microbiome at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and

Ingham, Qld. *Mortierella* spp. abundance in rhizosphere soil was determined by culture-dependent (*i.e.* viable soil inoculum) and culture-independent (*i.e*. rDNA sequencing) approaches.

Crop Management Treatment	*Mortierella* spp.					
	Propagules (g^{-1} soil) [3]		Root Isolation Frequency [4]		OTU Relative Abundance [5]	
	T1	T2	T1	T2	T1	T2
Ingham (IHCPSL) [1]						
Cane-Lablab (C-L MT)	395 [b]	60 [d]	0.15 [def]	0.24 [d]	0.0404 [a]	0.0336 [a]
Ratoon Cane (RC ZT)	325 [b]	90 [cd]	ND	0.20 [de]	0.0836 [a]	0.0366 [a]
Stone River (SR) [2]						
Contin. Cane (C-C FT)	370 [b]	135 [cd]	0.08 [f]	0.91 [a]	0.0119 [a]	0.0026 [a]
Cane-Soybean (C-S FT)	490 [a]	140 [cd]	0.13 [ef]	0.74 [b]	0.0081 [a]	0.0030 [a]
Cane-Soybean (C-S MT)	395 [b]	100 [cd]	0.11 [ef]	0.60 [c]	0.0163 [a]	0.0226 [a]
Ratoon Cane (RC ZT)	320 [b]	160 [c]	0.13 [ef]	0.64 [c]	0.0708 [a]	0.0104 [a]
Grand Mean	*248*		*0.36*		*0.0283*	
	P	LSD	P	LSD	P	LSD
$F_{Treatment\ x\ Time}$ (5%)	0.031	89.4	<0.001	0.095	0.063	0.036
$F_{Treatment}$ (5%)	0.027	63.2	<0.001	0.067	<0.001	0.016
F_{Time} (5%)	<0.001	36.5	<0.001	0.042	0.007	0.015

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. *Mortierella* inoculum in sugarcane rhizosphere soils (*M. alpina, M. chlamydospora, M. elongata, M. exigua, M. sarnyensis, M. sclerotiella*)
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in viable inoculum of *Mortierella* spp.
   $F_{Treatment}$ Ingham: C-L MT (228 [b]) = RC ZT (208 [b])
   $F_{Treatment}$ Stone River: C-S FT (315 [a]) = C-C FT (253 [ab]) = C-S MT (248 [b]) = RC ZT (240 [b])
   F_{Time}: T1 (383 [a]) > T2 (114 [b])
4. Isolation of *Mortierella* spp. from sugarcane roots
   ND: Not determined
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in *Mortierella* spp. root isolation.
   $F_{Treatment}$ Ingham: C-L MT (0.20 [d]) = RC ZT (0.20 [d])
   $F_{Treatment}$ Stone River: C-C FT (0.49 [a]) = C-S FT (0.43 [ab]) = RC ZT (0.38 [bc]) = C-S MT (0.36 [c])
   F_{Time}: T2 (0.63 [a]) > T1 (0.12 [b])
5. Relative abundance of *Mortierella* spp. (rDNA-OTU) in rhizosphere soil fungal microbiome
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in abundance of *Mortierella* spp.
   $F_{Treatment}$ Ingham: RC ZT (0.0601 [a]) > C-L MT (0.0370 [b])
   $F_{Treatment}$ Stone River: RC ZT (0.0406 [b]) > C-S MT (0.0195 [c]) = C-C FT (0.0072 [c]) = C-S MT (0.0055 [c])
   F_{Time}: T1 (0.0385 [a]) > T2 (0.0181 [b])

6.3.4. Plant Pathogenic *Fusarium* species

Low to high frequencies (0.09 – 0.66) of plant pathogenic *Fusarium* spp. were isolated from surface sterilised sugarcane roots (Table 6). Among all cropping treatments, the overall mean *Fusarium* isolation frequency was 0.30 (30%) (Table 6). *Fusarium* strains (23) were identified as recognised sugarcane and maize plant pathogenic taxa *F. kyushuense*, *F. oxysporum, F. sacchari* and *F. verticillioides* (Egan *et al.*, 1997; Fa´varo *et al.,* 2012; Petrovic *et al*., 2013; Viswanathan & Rao, 2011; Wang *et al.*, 2014)

At Stone River in 2016 (T1), *Fusarium* root infection was significantly (P<0.001) higher in replant continuous cane compared with cane-soybean rotations and 4th ratoon cane (Table 6). Ratoon cane exhibited the lowest (P<0.001) root infection frequency among all treatments (Table 6). Following an addition year of cane growth (T2) significant (P<0.001) increases in *Fusarium* root infection were observed in all treatments except continuous replant cane, the latter exhibiting a significant decline (Table 6). At both sites *Fusarium* root infection of longer-term (T2) ratoon cane remained significantly (P<0.001) lower than in 1st ratoon cane following rotation with either soybean (Stone River) or lablab bean (Ingham) (Table 6).

The overall mean relative abundance of *Fusarium spp.* (rDNA OTUs) in the fungal microbiomes of sugarcane rhizosphere soils across sites was 1% (Table 6). There were no significant differences in *Fusarium* relative abundance (OTUs) among the sugarcane tillage and rotation treatments at either site, thereby contrasting with culture dependent analyses of *Fusarium* root isolation frequencies (Table 6). The dissimilarity between *Fusarium* root colonisation (30%) and low relative abundance in rhizosphere soils (1%) implies selection of *Fusarium* by the actively growing sugarcane crop.

Table 6 Endophytic colonisation of sugarcane roots by recognised plant pathogenic *Fusarium* spp and their relative abundance in the rhizosphere soil fungal microbiome at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld.

Crop Management Treatment	*Fusarium* spp.			
	Root Isolation Frequency [3]		OTU Relative Abundance [4]	
	T1	T2	T1	T2
Ingham (IHCPSL) [1]				
Cane-Lablab (C-L MT)	0.15 [ef]	0.51 [b]	0.0158 [a]	0.0274 [a]
Ratoon Cane (RC ZT)	ND	0.33 [d]	0.0007 [a]	0.0066 [a]
Stone River (SR) [2]				
Contin. Cane (C-C FT)	0.66 [a]	0.09 [f]	0.0021 [a]	0.0017 [a]
Cane-Soybean (C-S FT)	0.23 [e]	0.35 [cd]	0.0016 [a]	0.0015 [a]
Cane-Soybean (C-S MT)	0.21 [e]	0.43 [bc]	0.0018 [a]	0.0019 [a]
Ratoon Cane (RC ZT)	0.10 [f]	0.20 [e]	0.0003 [a]	0.0063 [a]
Grand Mean	*0.30*		*0.0056*	
	P	LSD	P	LSD
$F_{Treatment \times Time}$ (5%)	<0.001	0.103	0.920	0.0180
$F_{Treatment}$ (5%)	<0.001	0.073	0.014	0.0127
F_{Time} (5%)	<0.001	0.046	0.229	0.0074

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. Isolation of *Fusarium* spp. (*F. kyushuense*, *F. oxysporum*, *F. proliferatum*, *F. sacchari*, *F. verticillioides*)
   ND: Not determined
   $F_{Treatment \times Time}$: Letters in superscript indicate significant differences in *Fusarium* spp. root isolation
   $F_{Treatment}$ Ingham: C-L MT (0.33 [ab]) = RC ZT (0.33 [ab])
   $F_{Treatment}$ Stone River: C-C FT (0.38 [a]) = C-S MT (0.32 [ab]) = C-S FT (0.29 [b]) > RC ZT (0.15 [c])
   F_{Time}: T2 (0.43 [a]) > T1 (0.16 [b])
4. Relative abundance of *Fusarium* spp. (rDNA-OTU) in rhizosphere soil fungal microbiome
   $F_{Treatment \times Time}$: Letters in superscript indicate significant differences in *Fusarium* spp. OTU abundance.
   $F_{Treatment}$ Ingham: C-L MT (0.0216 a) > RC ZT (0.0036 b)

$F_{Treatment}$ Stone River: RC ZT (0.0033 b) = C-C FT (0.0019 b) = C-S MT (0.0018 b) = C-S FT (0.0015 b)

F_{Time}: Not significant

6.3.5. Plant Pathogenic *Pythium spinosum*

Moderate to high frequencies (0.43 – 0.89) of *Pythium* spp. were isolated from surface sterilised sugarcane roots, the overall mean frequency being 0.64 (64%). Representative strains (34) were identified as plant pathogenic *P. spinosum* by morphology (Van der Plaats-Niterink, 1981) and rDNA sequencing. At Stone River in 2016 (T1), root infection by *P. spinosum* was significantly (P<0.001) higher in full tillage, replant continuous cane compared with cane-soybean rotations and longer-term ratoon cane (Table 7). Following an addition year of cane growth (T2), significant (P<0.001) increases in *P. spinosum* root infection were observed in all treatments except continuous replant cane (Table 7). There were no significant differences in root infection among sugarcane cropping treatments at Ingham (Table 7). Overall, replant continuous cane had significantly (P<0.001) higher levels of *P. spinosum* root infection than cane-legume rotations and longer-term (4^{th}-5^{th}) ratoon cane (Table 7).

Analyses of viable *P. spinosum* inoculum and Pythium Clade-F DNA (PredictaB) in sugarcane rhizosphere soils (see 6.2, above) are included for comparisons with root infection. Despite significant differences in *P. spinosum* root isolation among cropping treatments at Stone River, there were no significant differences in levels of viable rhizosphere soil inoculum (Table 7). Another year of cane growth did however, result in significant (P<0.002) increases in *P. spinosum* rhizosphere inoculum and root infection with time (Table 2). This contrasts with the culture-independent (PredictaB) analysis of *P. spinosum* inoculum dynamics, where the *Pythium* clade_F diagnostic indicated a significant decrease in rhizosphere soil DNA over time (Table 7). High incidences of sugarcane root infection by *P. spinosum* and generally low levels of rhizosphere inoculum, may indicate that the rhizosphere soil communities are comprised of non-pathogenic and saprophytic *P. spinosum* genotypes. This has implications when using existing DNA-based diagnostics (*e.g*. Pythium Clade_F) as predictor of Pythium root disease severity.

Pythium spp. rDNA OTUs were not detected in the rhizosphere microbiome of sugarcane soils at either site, despite high root isolation frequencies and the relatively low levels of viable *P. spinosum* inoculum and *Pythium* Clade-F DNA in rhizosphere soils (Table 7). No other oomycetes were identified in the rhizosphere microbiome of sugarcane or forest soils, implying that alternative sequence methods are required to quantify *Pythium* and *Pachymetra* communites in the rhizosphere microbiome.

Table 7 Rhizosphere soil-borne inoculum and endophytic colonisation of sugarcane roots by *Pythium spinosum* at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld. *Pythium*

Clade_F inoculum was quantified by culture-independent DNA diagnostics (PredictaB, SARDI) and culture-dependent isolation of viable propagules on oomycete-selective medium.

Crop Management Treatment	*Pythium spinosum*					
	Propagules (g^{-1} soil) [3]		Root Isolation Frequency [4]		Clade_F (pg DNA g^{-1} soil) [5]	
	T1	T2	T1	T2	T1	T2
Ingham (IHCPSL) [1]						
Cane-Lablab (C-L MT)	60 ^{a}	140 ^{a}	0.54 ^{efg}	0.58 ^{def}	130 ^{a}	35 ^{b}
Ratoon Cane (RC ZT)	45 ^{a}	110 ^{a}	ND	0.59 ^{def}	6 ^{bc}	34 ^{bc}
Stone River (SR) [2]						
Contin. Cane (C-C FT)	20 ^{a}	105 ^{a}	0.89 ^{a}	0.68 ^{cd}	11 ^{bc}	1 ^{c}
Cane-Soybean (C-S FT)	20 ^{a}	95 ^{a}	0.49 ^{fg}	0.80 ^{ab}	5 ^{bc}	4 ^{bc}
Cane-Soybean (C-S MT)	25 ^{a}	65 ^{a}	0.64 ^{cde}	0.74 ^{bc}	4 ^{bc}	1 ^{c}
Ratoon Cane (RC ZT)	35 ^{a}	80 ^{a}	0.43 ^{g}	0.64 ^{cde}	32 ^{bc}	2 ^{bc}
Grand Mean	*67*		*0.64*		*22*	
	P	LSD	P	LSD	P	LSD
$F_{Treatment\ x\ Time}$ (5%)	0.833	56.7	<0.001	0.124	<0.001	34.1
$F_{Treatment}$ (5%)	0.11	40.1	<0.001	0.087	<0.001	24.1
F_{Time} (5%)	<0.001	23.1	0.002	0.055	0.001	13.9

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. Viable *Pythium spinosum* inoculum in sugarcane rhizosphere soils.
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in viable inoculum of *Pythium* spp.
   $F_{Treatment}$: Not significant
   F_{Time}: T2 (99 ^{a}) > T1 (34 ^{b})
4. Isolation frequency of *Pythium spinosum* from sugarcane roots.
   ND: Not determined
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in *Pythium* spp. root isolation.
   $F_{Treatment}$ Ingham: RC ZT (0.59 ^{cd}) = C-L MT (0.56 ^{cd})
   $F_{Treatment}$ Stone River: C-C FT (0.79 ^{a}) > C-S MT (0.69 ^{b}) = C-S FT (0.64 ^{bc}) > RC ZT (0.53 ^{d})
   F_{Time}: T2 (0.69 ^{a}) > T1 (0.60 ^{b})
5. *Pythium* Clade_F DNA in sugarcane rhizosphere soils (*i.e. P. spinosum*)
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in abundance of *Pythium* spp.
   $F_{Treatment}$ Ingham: C-L MT (83 ^{a}) > RC ZT (20 ^{b})
   $F_{Treatment}$ Stone River: RC ZT (17 ^{b}) = C-C FT (6 ^{b}) = C-S FT (4 ^{b}) = C-S MT (3 ^{b})
   F_{Time}: T1 (31 ^{a}) > T2 (13 ^{b})

6.3.6. **Pathogen Antagonistic *Trichoderma* species**

Moderate to high frequencies (0.32 – 1.00) of endophytic, pathogen antagonistic *Trichoderma* spp. were isolated from surface sterilised sugarcane roots. The overall mean *Trichoderma* isolation frequency from sugarcane roots was 0.76 (76%). Representative *Trichoderma* isolates (44) were identified as *T. asperelloides*, *T. gamsii*, *T. harzianum*, *T. hamatum*, *T. koningiopsis* and *T. virens*. At both sites *Trichoderma* root colonisation was significantly (P<0.001) greater under long-term ratoon cane compared with replant continuous cane and cane-legume rotations (Table 8). Overall, *Trichoderma* root colonisation significantly (P<0.001) increased with another year of cane growth (Table 8).

The overall mean relative abundance of *Trichoderma spp.* (OTUs) in the fungal microbiome of sugarcane rhizosphere soils was 0.0439 (*i.e.* 4%). At Stone River, there were no significant differences in *Trichoderma* abundance among cropping treatments at T1 (Table 8). An additional year of cane growth however, resulted in significantly (P=0.002) greater abundance in the rhizosphere of 5th ratoon cane, compared with cane-legume rotations and replant continuous cane (Table 8).

At Ingham however, *Trichoderma* abundance was significantly (P=0.002) greater in the T1 rhizosphere of 4th ratoon cane compared with cane-lablab rotation, and then significantly declined in the ratoon cane treatment at T2 (Table 6). The lower rhizosphere soil abundance at T2 contrasts with culture-dependent analyses, *Trichoderma* root colonisation being significantly greater under the longer-term ratoon cane (T2) than in the cane-lablab rotation (Table 8).

Among all sugarcane cropping treatments, the overall mean rhizosphere inoculum of potentially pathogen antagonistic *T. harzianum* was 5.01 x 10^4 conidia g^{-1} of soil. Whilst *T. gamsii* was isolated from sugarcane roots, rhizosphere inoculum of this pathogen antagonist were below detection limits of the diagnostic assay in all cropping soils. At Stone River, *T. harzianum* populations (T2) were generally significantly (P<0.001) higher in rhizosphere soils of 5th ratoon cane compared with cane-legume rotations and replant continuous cane (Table 8). This was in accordance with root colonisation and community abundance (OTUs) analyses, implying that *T. harzianum* is the dominant *Trichoderma* species at Stone River.

In contrast, *T. harzianum* populations at Ingham were significantly (P<0.001) lower in T1 rhizosphere soil of ratoon cane compared with the cane-lablab rotation and were not significantly different at T2 (Table 8). The disimilarities between relative abundance of *Trichoderma* spp. (OTUs) and size of *T. harzianum* populations in T1 rhizosphere soils implies that this species does not dominate the *Trichoderma* rhizosphere community at Ingham.

Table 8 Endophytic colonisation of sugarcane roots by *Trichoderma* spp. and their relative abundance (rDNA-OTU) in the rhizosphere microbiome at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld. Rhizosphere soil colonisation by *T. harzianum-T. afroharzanum* was quantified by a culture-independent, species-specific diagnostic (qPCR).

Crop Management Treatment	*Trichoderma* spp.					
	Root Isolation Frequency [3]		OTU Relative Abundance [4]		Log *T. harzianum* Conidia Equivalents (g^{-1} soil) [5]	
	T1	T2	T1	T2	T1	T2
Ingham (IHCPSL) [1]						
Cane-Lablab (C-L MT)	0.81 [e]	0.83 [de]	0.0145 [d]	0.0479 [bcd]	5.39 [a]	4.96 [bc]
Ratoon Cane (RC ZT)	ND	0.93 [abc]	0.0976 [a]	0.0334 [cd]	3.79 [g]	4.85 [bcd]
Stone River (SR) [2]						
Contin. Cane (C-C FT)	0.41 [f]	0.81 [e]	0.0469 [bcd]	0.0246 [d]	4.55 [def]	4.75 [cd]
Cane-Soybean (C-S FT)	0.32 [g]	0.91 [bcd]	0.0431 [cd]	0.0233 [d]	4.32 [f]	4.89 [bc]
Cane-Soybean (C-S MT)	0.47 [f]	0.97 [ab]	0.0380 [cd]	0.0217 [d]	4.42 [ef]	4.68 [cde]
Ratoon Cane (RC ZT)	0.88 [cde]	1.00 [a]	0.0584 [bc]	0.0769 [ab]	4.69 [cde]	5.07 [b]
Grand Mean	*0.76*		*0.0439*		*4.70*	
	P	LSD	P	LSD	P	LSD
$F_{Treatment\ x\ Time}$ (5%)	<0.001	0.090	0.002	0.0383	<0.001	0.032
$F_{Treatment}$ (5%)	<0.001	0.069	<0.001	0.0239	<0.001	0.263
F_{Time} (5%)	<0.001	0.022	0.093	0.0139	<0.001	0.152

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.

2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. Isolation of *Trichoderma* spp. from sugarcane roots (*T. asperelloides*, *T. gamsii*, *T. harzianum*, *T. hamatum*, *T. koningiopsis*, *T. virens*)
   ND: Not determined
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in isolation of *Trichoderma* spp.
   $F_{Treatment}$ Ingham: RC ZT (0.93 ^{a}) > C-L MT (0.82 ^{b})
   $F_{Treatment}$ Stone River: RC ZT (0.94 ^{a}) > C-S MT (0.72 ^{c}) > C-S FT (0.62 ^{d}) = C-C FT (0.61^{d})
   F_{Time}: T2 (0.90 ^{a}) > T1 (0.58 ^{b})
4. Relative abundance of *Trichoderma* spp. (rDNA-OTU's) in rhizosphere soil fungal microbiome
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in abundance of *Trichoderma* spp.
   $F_{Treatment}$ Ingham: RC ZT (0.0655 ^{a}) > C-L MT (0.0312 ^{b})
   $F_{Treatment}$ Stone River: RC ZT (0.0676 ^{a}) > C-C FT (0.0357 ^{b}) = C-S FT (0.0332 ^{b}) = C-S MT (0.0298 ^{b})
   F_{Time}: Not significant
5. Abundance of *Trichoderma harzianum – T. afroharzianum* in sugarcane rhizosphere soil
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in abundance of *Trichoderma* spp.
   $F_{Treatment}$ Ingham: C-L MT (5.18 ^{a}) > RC-ZT (4.32 ^{d})
   $F_{Treatment}$ Stone River: RC ZT (4.88 ^{b}) = C-C FT (4.65 ^{bc}) = C-S FT (4.60 ^{c}) = C-S MT (4.55 ^{cd})
   F_{Time}: T2 (4.87 ^{a}) > T1 (4.53 ^{b})

6.4. Anti-biosis of Sugarcane Root Pathogens by Endophytic *Epicoccum* and *Trichoderma*

Culture-dependent isolation of fungi from sugarcane roots identified abundant *Trichoderma harzianum* strains (6.3.6, above) and rare *Epicoccum nigrum* strains that actively inhibited growth of sugarcane pathogenic *Fusarium* and *Pythium* spp., presumably through the production of secondary metabolites. *T. harzianum* is a recognised antagonist of plant pathogenic fungi and oomycetes (Zhang *et al.* 2015; Mukherjee *et al.,* 2012) and *E. nigrum* has previously reported to have antibiosis activity against the sugarcane root pathogens *Fusarium verticillioides* and *Ceratocystis paradoxa* (Fa´varo *et al.,* 2012).

T. harzianum and *E. nigrum* strains isolated from 1st ratoon replant continuous cane (Stone River) and 10th ratoon cane (Ingham) were analysed for *in vitro* antibiosis of sugarcane root pathogens *Fusarium kyushuense*, *F. oxysporum*, *F. verticillioides* and *Pythium spinosum*. Wheat root disease suppressive *T. gamsii* (Stummer *et al.* 2018) was included for comparison. Pathogens were exposed to secondary metabolites produced by antagonistic *Trichoderma* and *Epicoccum* strains by transfer to cell-free culture medium in which the antagonists had been growing for 7 days. Pathogen antibiosis was determined after 7days. This approach was also used to assess antibiosis between pathogen antagonistic *Trichoderma* and *E. nigrum* strains.

Antibiosis of sugarcane root pathogenic fungi ranged from a 22% reduction in biomass to 100% *i.e.* pathogen death (Table 9). All antagonistic strains significantly reduced (P<0.001) the biomass of each pathogen relative to their pathogen-only controls (data not shown). *Trichoderma* strains differed (P<0.001) in their antibiosis efficacy toward each of the *Fusarium* root pathogens, but were equally effective against *P. spinosum* (Table 9). There were no significant differences between *E. nigrum* strains in antibiosis of *F. kyushuense*, *F. oxysporum* or *P. irregulare*. *E. nigrum* strain 7.1 however, had significantly (P<0.001) greater antibiosis toward *F. verticillioides* than strain 96.1.

Among all four pathogens, sugarcane root isolates *T. harzianum* 88.1 and *E. nigrum* 7.1 exhibited significantly (P<0.001) greater antibiosis efficacy than *T. gamsii* A5MH, *E. nigrum* 96.1 and *T. harzianum* 2.1, the latter having the lowest (P<0.001) antibiosis efficacy of all 5 strains. All 4 sugarcane root pathogens differed (P<0.001) in their susceptibility to the antifungal metabolites produced by *Trichoderma* and *Epicoccum* strains, with *P. spinosum* exhibiting the greatest antibiosis (100%) and *F. oxysporum* (58%) the least (Table 9).

Reciprocal antibiosis testing of *Trichoderma* and *Epicoccum* strains showed that *E. nigrum* metabolites significantly (P<0.001) decreased biomass of each *Trichoderma* strain, *T. gamsii* A5MH being most effected (82%) and *T. harzianum* 2.1 (29%) the least (data not shown). Similarly, metabolites of *T. gamsii* strains A5MH and *T. harzianum* 2.1 had significant (P<0.001) antibiosis activity (87% - 97%) toward *E. nigrum* strain 96.1 (data not shown).

Table 9 *In vitro* antibiosis of fungal (*Fusarium* spp.) and oomycete (*Pythium* sp.) pathogens of sugarcane by *Epicoccum nigrum* and *Trichoderma* spp. Antibiosis was quantified as the reduction in pathogen mycelial biomass following exposure to metabolites produced by *E. nigrum* and *Trichoderma* strains.

Antagonist [1]	*In Vitro* Pathogen Antibiosis (% Reduction in Pathogen Biomass) [2]			
	Fusarium kyushuense 12.2	*Fusarium oxysporum* 93.2	*Fusarium verticillioides* 16.1	*Pythium spinosum* 46.1
Epicoccum sp.				
E. nigrum 7.1	77 ^{bc}	54 ^{e}	97 ^{a}	100 ^{a}
E. nigrum 96.1	70 ^{cd}	57 ^{e}	61 ^{de}	100 ^{a}
Trichoderma spp.				
T. harzianum 2.1	59 ^{e}	53 ^{e}	22 ^{g}	100 ^{a}
T. harzianum 88.2	79 ^{bc}	86 ^{b}	78 ^{bc}	100 ^{a}
T. gamsii A5MH	79 ^{bc}	41 ^{f}	72 ^{c}	100 ^{a}
Grand Mean	*74*			
	P		LSD	
$F_{Antagonist \times Pathogen}$ (5%)	P<0.001		11.4	
$F_{Antagonist}$ (5%)	P<0.001		5.7	
$F_{Pathogen}$ (5%)	P<0.001		5.1	

1. Sugarcane root endophytic pathogen-antagonistic fungi were isolated in 2017 (T2) and their origins are shown in parenthesis:
   E. nigrum 7.1 (SR C-C FT), *E. nigrum* 96.1 (IHCPSL RC ZT), *T. harzianum* 2.1 (SR C-C FT), and *T. harzianum* 88.2 (IHCPSL RC ZT)
   Fungal and oomycete disease suppressive inoculant *Trichoderma gamsii* A5MH (CSIRO) was isolated from wheat roots at Avon, South Australia.
2. Sugarcane root pathogens were isolated in 2017 (T2) and their origins are shown in parenthesis:
   F. kyushuense 12.2 (SR C-C FT), *F. oxysporum* 93.2 (IHCPSL RC ZT), *F. verticillioides* 16.1 (SR C-C FT) and *P. spinosum* 46.1 (SR C-S FT)
   $F_{Antagonist \times Pathogen}$: Letters in superscript indicate significant differences in pathogen antibiosis
   $F_{Antagonist}$ *Epicoccum*: *E. nigrum* 7.1 (82 ^{a}) > *E. nigrum* 96.1 (72 ^{b})
   $F_{Antagonist}$ *Trichoderma*: *T. harzianum* 88.2 (86 ^{a}) > *T. gamsii* A5MH (73 ^{b}) > *T. harzianum* 2.1 (58 ^{c})
   $F_{Pathogen}$: *P. spinosum* 46.1 (100 a) > *F. kyushuense* 12.2 (73 b) > *F. verticillioides* 16.1 (66 c) > *F. oxysporum* 93.2 (58 d)

6.5. Fungal Microbiome of Sugarcane Soils

Eco-genomic analyses (rDNA sequencing) of the sugarcane fungal microbiome was conducted on pre-planting (T0) and rhizosphere soils (T1 - T2) isolated from roots used in plant pathological, organic carbon (TOC), culture-dependent (*Pythium*, *Pachymetra*, *Mortierella*) and culture-independent (*Trichoderma*, *Pythium*, *Pachymetra*) soil-inoculum analyses.

The relative abundances of fungal taxa (Order, Family, Genus) in sugarcane soils were used to define impacts of crop rotation and tillage treatments on the taxonomic structures of fungal microbiomes.

This provided information on the effects of agronomic selection pressures (rotation, tillage) on fungal communities and their potential relationships to expression of root disease.

6.5.1. Taxonomic Diversity of Fungal Communities in Sugarcane Rhizosphere Soils

The taxonomic composition of rhizosphere soil fungal communities were inferred from rDNA sequencing, taxa being identified as operational taxonomic units (OTUs). Taxonomic diversity among fungal communities in sugarcane was determined based species richness (S), eveness (J') and the Simpson Index (λ), the latter two indices ranging from a minimum of 0 to a maximum of 1.

Soil fungal communities of sugarcane cropping systems and a natural forest had high levels of taxonomic diversity (λ) and eveness in abundance of these taxa (J'). This is evident in that both indices were >0.95 in all treatments (Table 10). Overall, forest soils and T1 cane following lablab bean had significantly higher species richness, taxonomic eveness and diversity than replant continuous cane and ratoon cane (Table 10). Fungal community diversity and eveness also significantly ($P<0.001$), increased with time (T2) a result of another year (ratoon) of cane growth (Table 10).

At Ingham, significant differences ($P<0.001$) in diversity (all indices) were observed between long-term ratoon cane and cane-lablab rotations at T1 (Table 10). Significant ($P<0.001$) increases in diversity of ratoon cane with time, and corresponding decreases in the rotation treatment, resulted in no significant differences in fungal diversity between these treatments at T2 (Table 10). With the exception of the T1 cane-lab lab rotation, fungal communities in forest soil were significantly more diverse than cane cropping treatments, this diversity increasing over time (T2).

At Stone River, species richness, community eveness and diversity (λ) of replant cane and cane-legume rotations was not significantly different at either T1 or T2 (Table 10). Diversity within all these cropping treatments did however, significantly increase ($P<0.001$) over time (Table 10). Species richness in ratoon cane was significantly ($P<0.001$) different from the other cropping treatments at both T1 and T2, but did not increase with time (Table 10).

Table 10 Diversity of fungal communities within the sugarcane rhizosphere microbiome at 6 weeks post-emergence in 2016 (T1) and 2017 (T2) at Stone River and Ingham, Qld. Fungal taxa were identified as

Operational Taxonomic Units (OTUs) based on rDNA sequencing and their relative abundances calculated as a proportion of the total number of taxa.

Crop Management Treatment	Species Richness [3]		Species Eveness [4]		Simpson Index [5]	
	T1	T2	T1	T2	T1	T2
Ingham (IHCPSL) [1]						
Cane-Lablab (C-C-L MT)	1080 ^{a}	633 ^{cd}	0.96772 ^{b}	0.96385 ^{c}	0.99904 ^{a}	0.99824 ^{b}
Forest (undisturbed)	642 ^{cd}	1128 ^{a}	0.96075 ^{def}	0.97081 ^{a}	0.99826 ^{b}	0.99909 ^{a}
Ratoon Cane (RC ZT)	488 ^{f}	616 ^{cd}	0.95346 ^{h}	0.96342 ^{cd}	0.99734 ^{c}	0.99818 ^{b}
Stone River (SR) [2]						
Contin. Cane (C-C-C FT)	439 ^{f}	778 ^{b}	0.95600 ^{gh}	0.96336 ^{cd}	0.99738 ^{c}	0.99855 ^{b}
Cane-Soybean (C-C-S FT)	402 ^{f}	724 ^{bc}	0.95653 ^{gh}	0.96330 ^{cd}	0.99684 ^{d}	0.99845 ^{b}
Cane-Soybean (C-C-S MT)	500 ^{ef}	772 ^{b}	0.95767 ^{fg}	0.96435 ^{c}	0.99766 ^{c}	0.99854 ^{b}
Ratoon Cane (RC ZT)	648 ^{cd}	606 ^{de}	0.95880 ^{fg}	0.96213 ^{cde}	0.99818 ^{b}	0.99815 ^{b}
Grand Mean	*675*		*0.96137*		*0.99814*	
	P	LSD	P	LSD	P	LSD
$F_{Treatment\ x\ Time}$ (5%)	<0.001	112	<0.001	0.00309	<0.001	0.00045
$F_{Treatment}$ (5%)	<0.001	79	<0.001	0.00218	<0.001	0.00032
F_{Time} (5%)	<0.001	42	<0.001	0.00117	<0.001	0.00017

1. Ingham Herbert Cane Productivity Services Ltd. (IHCPSL): Sugarcane-legume cropping system, Ingham, Qld.
2. Stone River (SR): Sugarcane-legume N management trial, Stone River, (SR) Qld.
3. Species Richness: Numbers of fungal taxa (OTUs) within sugarcane rhizosphere microbiome
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in numbers of fungal taxa
   $F_{Treatment}$ Ingham: Forest (885 a) = C-L MT (856 a) > RC ZT (552 c)
   $F_{Treatment}$ Stone River: C-S MT (636 ^{b}) = RC ZT (627 ^{bc}) = C-C FT (608 ^{bc}) = C-S FT (563 ^{bc})
   F_{Time}: T2 (751 ^{a}) > T1 (600 ^{b})
4. Community Eveness: Similarity in abundance of taxa (OTUs) in the rhizosphere microbiome (range 0 – 1)
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in eveness of fungal communities
   $F_{Treatment}$ Ingham: C-L MT (0.96579 ^{a}) = Forest (0.96578 ^{a}) > RC ZT (0.95844 ^{c})
   $F_{Treatment}$ Stone River: C-S MT (0.96101 ^{b}) = RC ZT (0.96046 ^{bc}) = C-C FT (0.95968 ^{bc}) = C-S FT (0.95841 ^{c})
   F_{Time}: T2 (0.96446 ^{a}) > T1 (0.95828 ^{b})
5. Simpson Diversity Index: Community diversity based on relative abundances of fungal taxa (range 0 – 1)
   $F_{Treatment\ x\ Time}$: Letters in superscript indicate significant differences in taxonomic diversity
   $F_{Treatment}$ Ingham: Forest (0.99868 ^{a}) = C-L MT (0.99864 ^{a}) > RC ZT (0.99776 ^{e})
   $F_{Treatment}$ Stone River: RC ZT (0.99817 ^{b}) = C-S MT (0.99810 ^{bc}) = C-C FT (0.99797 ^{bcd}) > C-S FT (0.99765 ^{e})
   F_{Time}: T2 (0.96446 ^{a}) > T1 (0.95828 ^{b})

6.5.2. Ingham: Similarities among Fungal Communities in Sugarcane and Forest Soils

The mean taxonomic similarity of sugarcane fungal communities in pre-plant (T0) and 6-week post-emergent rhizosphere (T1) soils at Ingham was 38% (Fig. 1). Significant differences (0.001<P<0.004) were observed between fungal microbiomes of cane-lablab bean (C-L) and ratoon cane (RC) treatments in both pre-plant (T0) and rhizosphere (T1) soils (Fig. 1). Fungal communities of both sugarcane treatments were also significantly different (P=0.001) from those in an adjacent forest soil (Fig. 1) at both sampling times. Overall, fungal community structure prior to planting was significantly different (P=0.001) from that in rhizosphere soils 6-week post-emergence.

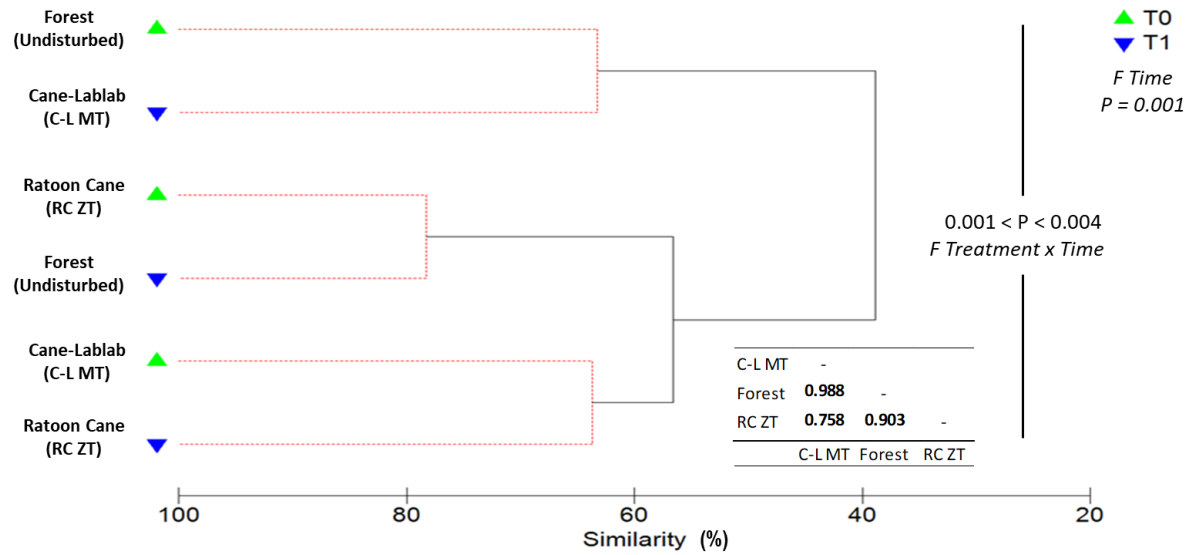


Figure 1 Impacts of lablab bean rotation and tillage treatments on the soil fungal microbiome of 5-week pre-planting (T0) and 6-week post-emergence (T1). sugarcane at Ingham, Qld. Dendrogram illustrates similarities (Bray-Curtis index) in fungal community structure, based on the relative abundance of genera (rDNA-OTU) in soil. Analysis of variance (ANOSIM) was performed on the similarity matrix to test for significance among sugarcane cropping treatments over time. Table (inset) shows pair-wise dissimilarity statistics (R) between treatments, bold text indicating significant differences (P<0.01).

The overall mean taxonomic similarity of rhizosphere (T2) soil fungal communities at Ingham was 20% (Fig. 2). The low overage average similarity reflecting the significant (P=0.001) differentiation of the forest rhizosphere soil microbiome from those of the sugarcane cropping systems (Fig. 2). The rhizosphere fungal community under 10th ratoon cane (RC ZT) was significantly different from the minimum tillage cane-lablab rotation (P = 0.003), their taxonomic similarity being 58%. The treatment-based differentiation of sugarcane and forest rhizosphere soil fungal communities observed in 2016 (T1) rhizosphere soils was maintained in 2017 (T2).

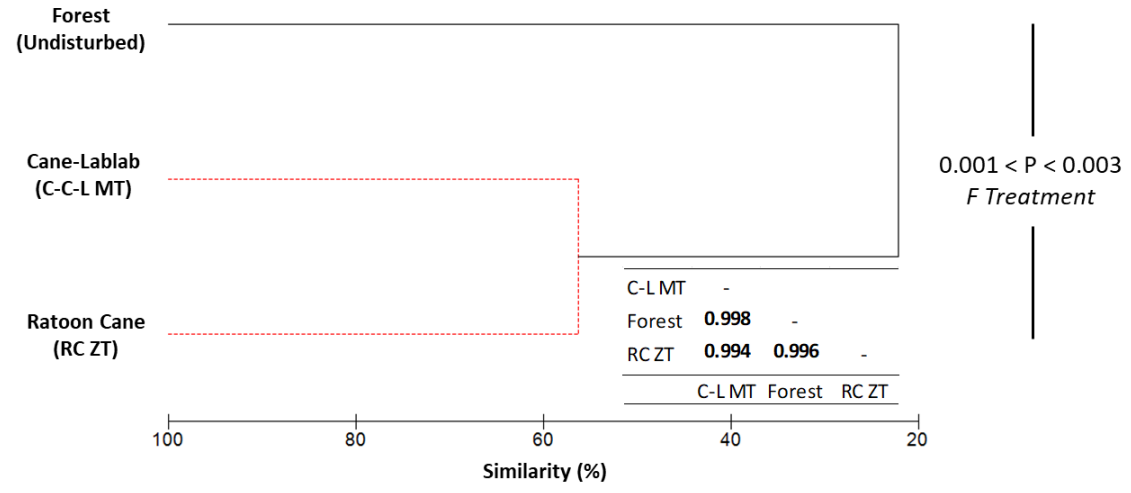


Figure 2 Impacts of lablab bean rotation and tillage treatments on the rhizosphere soil fungal microbiome of 6-week post-emergence (T2) sugarcane at Ingham, Qld. Dendrogram illustrates similarities (Bray-Curtis index) in fungal community structure based on the relative abundance of genera (rDNA-OTU) in rhizosphere soils. Analysis of variance (ANOSIM) was performed on the similarity matrix to test for significance among sugarcane cropping treatments. Table (inset) shows pair-wise dissimilarity statistics (R) between treatments, bold text indicating significant differences (P<0.01).

6.5.3. Ingham: Relative Abundance of Fungal Taxa (rDNA-OTU) in Pre-Plant and Rhizosphere Soils

Thirty three of a possible 60 fungal taxa were identified in the 20 most abundant taxa across the combined sugarcane pre-plant (T0) and 6-week post-emergent (T0, T1) soils (Table 11). Ten of these taxa were highly abundant in soil at all 3 sampling times and included primarily saprophytic *Aspergillus*, *Cladophialophora*, *Mortierella*, *Penicillium* and *Taleromyces* spp., pathogen antagonistic *Trichoderma* and *Humicola* spp. and unidentified taxa with the saprophytic orders Capnodiales, Sordariales and Xylariales (Table 11).

In pre-plant cane and forest soils (T0), significant differences ($0.001<P<0.01$) were observed in relative abundance of primarily saprophytic *Aspergillus*, *Chaetosphaeria*, *Cladophialophora*, *Flagelloscypha* and *Taleromyces* spp., the yeasts *Cryptococcus* and *Trichosporon* spp., anti-biotic producing *Westerdykella* and *Humicola* spp. and unidentified taxa within saprophytic Capnodiales (Table 11). Saprophytic *Chaetosphaeria spp.* and Agaricales, plant growth-promoting *Serendipita* spp. and bacterial-antagonistic *Westerdykella* spp. had high relative abundance (1% - 3%) only in pre-plant (T0) soils, these fungal taxa being suppressed in sugarcane rhizosphere (T1-T2) soils (Table 11).

Conversely, fungi with high relative abundance (1% - 15%) only at T2 included potential saprophytic, plant beneficial and pathogenic taxa within the orders Helotiales and Pleosporales, saprophytic Chaetomiaceae, *Resinicium* and *Trechispora* spp., plant pathogenic *Fusarium* spp., pathogen-antagonistic *Epicoccum* spp. and soil yeasts *Apiotrichum* and *Saitozyma* spp. (Table 11). The higher relative abundance of these fungi under longer-term sugarcane (T2) implies rhizosphere selection of these taxa (Table 11). Taxa within the primarily plant pathogenic order Myriangiales were not ranked in the top 20 most abundant taxa at T2, despite being previously abundant in pre-plant (T0) and T1 rhizosphere soils (Table 11).

Table 11 Ingham: The 20 most abundant fungal taxa (rDNA-OTU) in pre-planting (T0) and 6-week post-emergence rhizosphere soils in 2016 (T1) and 2017 (T2) determined by culture-independent rDNA sequencing.

Analyses included taxa that were identifed to Order, Family and Genus. Fungal endophytes isolated from sugarcane roots (*i.e.* culture-dependent) are shown in bold text.

	Ingham : Relative Abundance of Fungal Taxa (Rank 1 - 20) [1, 2]					
Rank	T0	Grand Mean	T1	Grand Mean	T2	Grand Mean
1	Cryptococcus***	0.1453	Cryptococcus***	0.1475	Saitozyma***	0.1490
2	**Mortierella**	0.1168	Talaromyces***	0.0746	Apiotrichum***	0.1210
3	o_Capnodiales***	0.0857	o_Capnodiales***	0.0681	Resinicium*	0.0757
4	o_Trechisporales	0.0524	**Trichoderma****	0.0503	**Mortierella*****	0.0583
5	Talaromyces***	0.0509	**Mortierella**	0.0497	Talaromyces***	0.0573
6	Flagelloscypha***	0.0275	o_Myriangiales	0.0354	o_Capnodiales***	0.0430
7	o_Agaricales	0.0257	Oidiodendron	0.0291	o_Pleosporales*	0.0397
8	Trichosporon***	0.0199	**Penicillium**	0.0251	**Trichoderma**	0.0336
9	**Trichoderma**	0.0194	Trichosporon***	0.0232	**Penicillium*****	0.0293
10	**Penicillium**	0.0159	o_Hypocreales*	0.0224	o_Hypocreales	0.0281
11	o_Xylariales	0.0148	Flagelloscypha*	0.0187	o_Helotiales	0.0218
12	Aspergillus***	0.0134	o_Trechisporales**	0.0155	f_Chaetomiaceae**	0.0205
13	Serendipita	0.0125	Purpureocillium***	0.0138	**Fusarium**	0.0199
14	o_Myriangiales	0.0120	Humicola**	0.0125	Humicola***	0.0113
15	Bullera	0.0109	Aspergillus***	0.0110	Cladophialophora*	0.0109
16	o_Sordariales	0.0100	Staphylotrichum**	0.0107	Trechispora**	0.0090
17	Westerdykella***	0.0100	o_Xylariales***	0.0100	Aspergillus***	0.0085
18	Humicola***	0.0094	o_Sordariales	0.0098	o_Sordariales*	0.0081
19	Cladophialophora***	0.0093	Bullera**	0.0095	Epicoccum	0.0076
20	Chaetosphaeria**	0.0082	Cladophialophora***	0.0092	o_Xylariales	0.0063
	Fusarium* (37)**	0.0035	**Chaetomium (28)**	0.0071	**Chaetomium (25)**	0.0059
	Chaetomium* (43)	0.0023	**Fusarium (34)**	0.0057		

1. Bold text indicates sugarcane fungal endophytes isolated in culture-dependent analyses (*i.e.* root isolations). Numbers in parenthesis refer to the relative abundance ranking of taxa if lower than 20.
2. Significant differences in relative abundance of fungal taxa among sugarcane cropping treatments and forest soils at each sampling time: * P<0.05, ** P<0.01, *** P<0.001

In 6-week post-emergent cane rhizosphere soils (T1) 15 of the 20 most abundant fungal taxa were significantly different (0.001<P<0.05) among cropping treatments (Table 11, Figs. 3a and 3b). The relative abundance of these genera ranged from 15% (*Cryptococcus* spp.) to 1% (7 taxa, *Purpureocillium* spp. 1.4% - *Cladophialophora* spp. 0.9%). Fungal taxa significantly different in relative abundance among cropping treatments included fungal-antagonistic *Trichoderma*, *Humicola* and *Bullera* spp., saprophytic, Trechisporales, Xylariales, *Aspergillus*, *Cladophialophora*, *Flagelloscypha*, *Purpureocillium*, *Staphylotrichum*, and *Talaromyces* spp., soil yeasts *Cryptococcus* and *Trichosporon* spp., and unidentified functionally diverse taxa within the Capnodiales (*i.e.* saprophytic, plant pathogenic) and Hypocreales (*i.e.* plant pathogenic, disease suppressive). Fungal taxa significantly more abundant in cane-legume compared with ratoon cane rhizosphere communities included saprophytic *Trichosporon, Flagelloscypha, Purpureocillium, Staphylotrichum* and fungal antagonistic *Bullera* spp. (Figs. 3a and 3b). Fungi significantly more abundant in the microbiome of ratoon cane compared with replant cane-legume, included fungal-antagonistic *Trichoderma* and *Humicola* spp., saprophytic *Cryptococcus*, *Taleromyces* and *Aspergillus* spp. and unidentified Capnodiales. Fungal taxa significantly more abundant in forest compared with cane cropping soils included saprophytic Trechisporales, Xylariales, *Cryptococcus* and *Cladophialophora* spp. and unidentified Capnodiales. In contrast, only *Aspergillus* spp. were significantly less abundant in forest soil fungal communities compared with both cane cropping treatments.

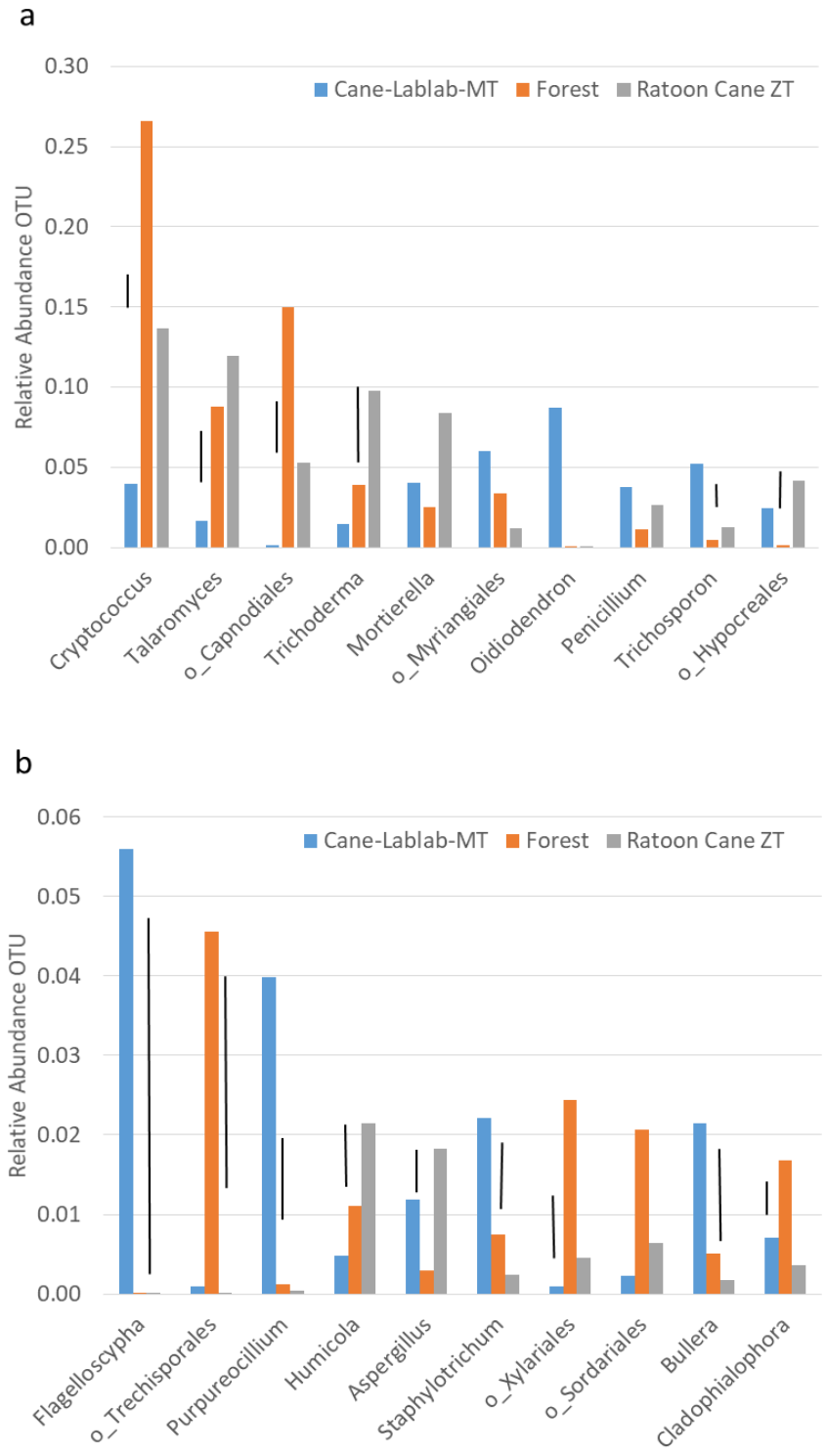
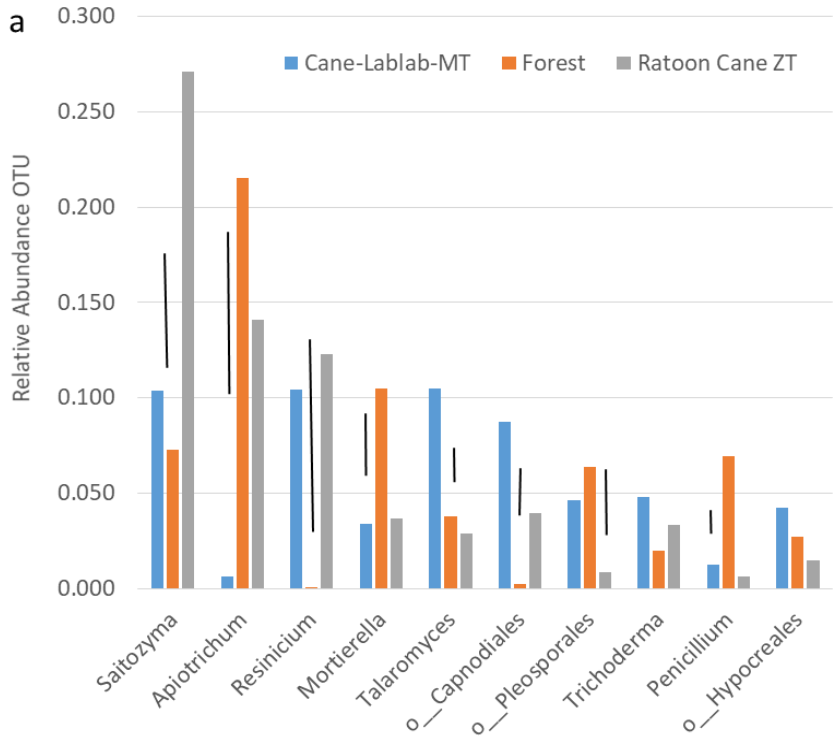


Figure 3 Ingham 2016 (T1): Impacts of lablab bean rotation and tillage treatments on the relative abundance of fungal taxa (rDNA-OTU) in rhizosphere soils of 6-week post-emergence (T1) sugarcane. Analyses of variance was performed on the 20 most abundant taxa among sugarcane cropping treatments (Figure 3a Taxa 1 – 10; Figure 3b Taxa 11 – 20). Black vertical bars indicate statistically significantly LSD values for individual fungal taxa (0.001<P<0.05).

In 6-week post-emergent cane rhizosphere soils (T2), 14 of the 20 most abundant fungal taxa were significantly different (0.001<P<0.05) among cropping treatments (Table 11, Figs. 4a and 4b). The relative abundance of these genera ranged from 15% (*Saitozyma* spp.) to 1% (5 taxa, *Humicola* spp.

1.1% - Sordariales 0.8%). Fungal taxa significantly different in relative abundance among cropping treatments included pathogen-antagonistic *Humicola* spp., saprophytic Sordariales, *Aspergillus,* Chaetomiaceae, *Cladophialophora*, *Mortierella*, *Penicillium*, *Resinicium*, *Talaromyces* and *Trechispora* spp., the soil yeasts *Apiotrichum* and *Saitozyma* spp. and unidentified saprophytic and potentially plant pathogenic Capnodiales and Pleosporales (Table 11, Figs. 4a and 4b). Fungal taxa significantly more abundant in the cane-legume rhizosphere microbiome compared with long-term (10th) ratoon cane included *Aspergillus*, *Humicola*, *Talaromyces*, *Trechispora* spp. and unidentified taxa within the Chaetomiaceae, Capnodiales and Pleosporales. In contrast only the saprophytic soil yeasts *Apiotrichum* and *Saitozyma* spp. were significantly more abundant under ratoon cane. Fungal taxa significantly more abundant in forest compared with cane cropping soils included saprophytic *Aspergillus*, *Mortierella* and *Penicillium* spp. Fungi significantly less abundant in forest soils included functionally diverse saprophytic and plant pathogenic Capnodiales and saprophytic Chaetomiaceae, *Cladophialophora*, and *Resinicium* spp.



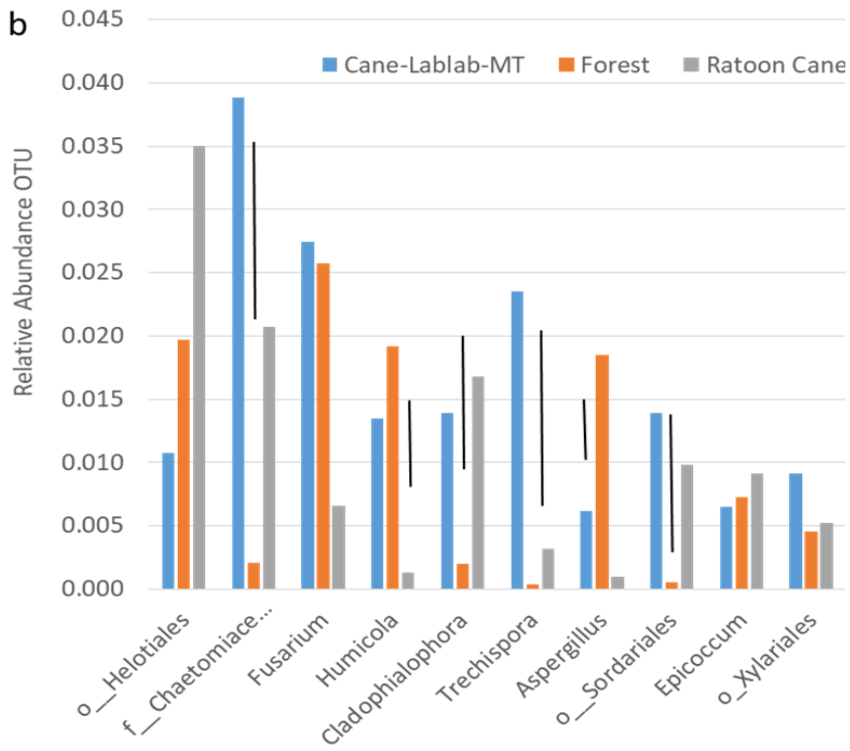


Figure 4 Ingham 2017 (T2): Impacts of lablab bean rotation and tillage treatments on the relative abundance of fungal taxa (rDNA-OTU) in rhizosphere soils of 6-week post-emergence (T2) sugarcane. Analyses of variance was performed on the 20 most abundant taxa among sugarcane cropping treatments (Figure 4a Taxa 1 – 10; Figure 4b Taxa 11 – 20). Black vertical bars indicate statistically significantly LSD values for individual fungal taxa (0.001<P<0.05).

6.5.4. Stone River: Similarities among Soil Fungal Communities in Sugarcane

The mean taxonomic similarity of sugarcane fungal communities in pre-plant (T0) and 6-week post-emergent rhizosphere (T1) soils at Stone River was 45% (Fig. 5). At 5 weeks prior to cane planting (T0), there were no significant differences between soil fungal communities from continous cane or after soybean. Fungal communities from under 3rd ratoon cane were however, significantly different (P<0.01) from those after soybean. At 6-weeks post-emergence (T1) only the fungal microbiome of 4th ratoon cane (RC) was significantly different from the other cropping treatments. There were no significant differences between fungal microbiomes of replant continuous cane and cane-soybean rotations (Fig. 5). Despite the general uniformity among non-ratoon fungal communities within each sampling time, the overall community structure prior to planting (T0) was significantly different (P=0.001) from that in rhizosphere soils (T1) of the actively growing cane crop.

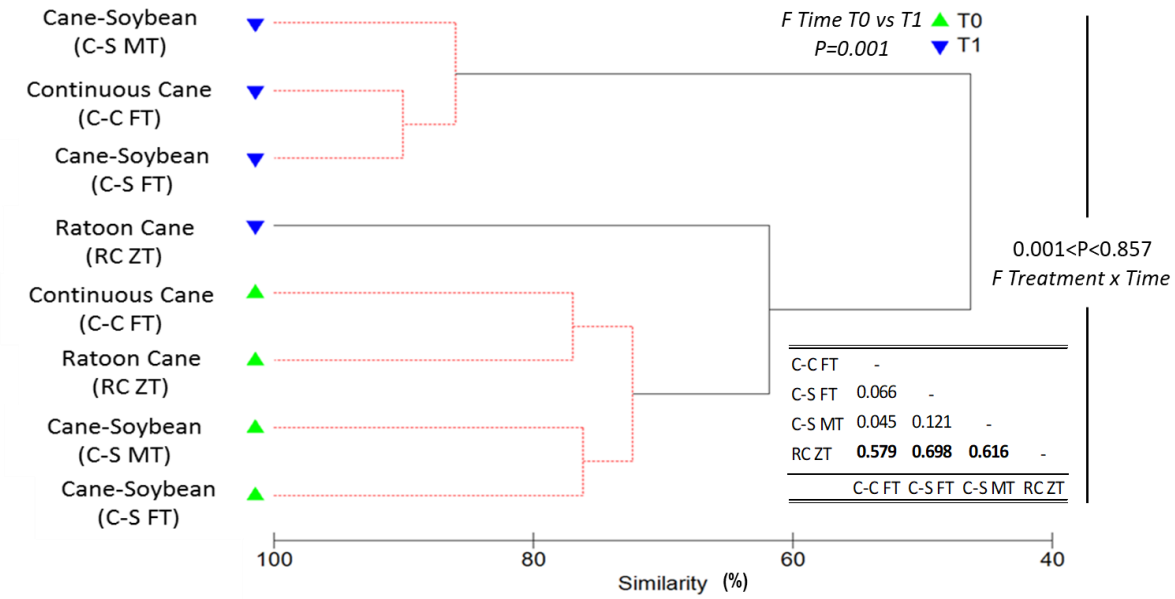


Figure 5 Impacts of soybean rotation and tillage treatments on the soil fungal microbiome of 5-week pre-planting (T0) and 6-week post-emergence (T1) sugarcane at Stone River, Qld. Dendrogram illustrates similarities (Bray-Curtis index) in fungal community structure, based on the relative abundance of genera (rDNA-OTU) in soil. Analysis of variance (ANOSIM) was performed on the similarity matrix to test for significance among sugarcane cropping treatments over time. Table (inset) shows pair-wise dissimilarity statistics (R) between treatments irrespective of sampling time, bold text indicating significant differences (P<0.01).

Among all T2 cropping treatments at Stone River, the overall mean taxonomic similarity of the sugarcane rhizosphere fungal microbiome was 62% (Fig. 6), significantly lower (P=0.003) than the 82% similarity among replant continuous cane (C-C-C FT) and cane-soybean rotations (C-C-S). Fungal community structure of 5th ratoon cane (RC ZT) was significantly different from 1st ratoon continuous cane (P=0.001) and cane-soybean rotations (P<0.002), regardless of tillage (Fig. 6). There were however, no significant differences in fungal community structure among the 1st ratoon continuous cane and cane-soybean rotation treatments. Consequently, the treatment-based differentiation of sugarcane rhizosphere fungal communities established in 2016 (T1) was maintained in 2017 (T2).

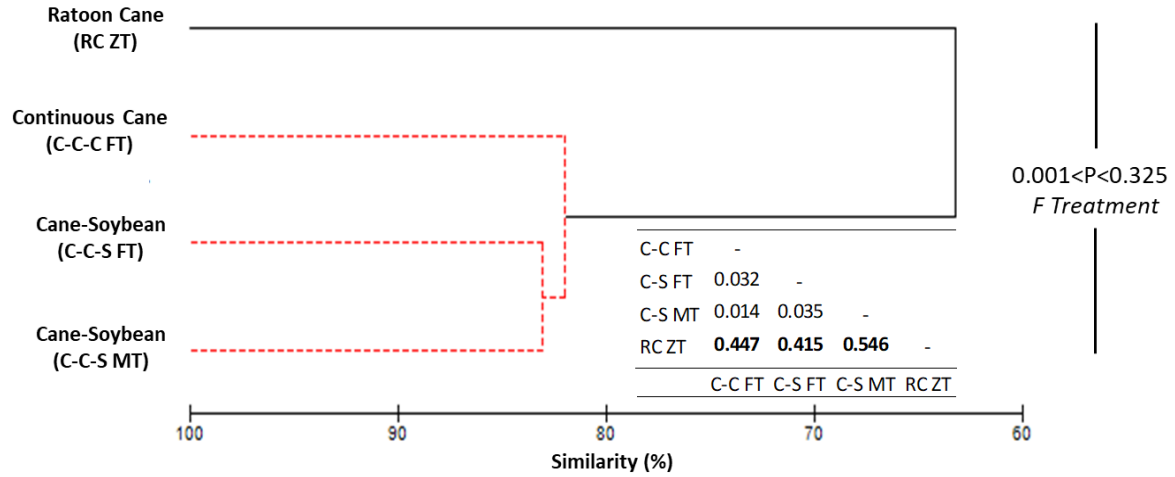


Figure 6 Impacts of soybean rotation and tillage treatments on the rhizosphere soil fungal microbiome of 6-week post-emergence (T2) sugarcane at Stone River, Qld. Dendrogram illustrates similarities (Bray-Curtis index) in fungal community structure based on the relative abundance of genera (rDNA-OTU) in rhizosphere soils. Analysis of variance (ANOSIM) was performed on the similarity matrix to test for significance among

sugarcane cropping treatments. Table (inset) shows pair-wise dissimilarity statistics (R) between treatments, bold text indicating significant differences (P<0.01).

6.5.5. Stone River: Relative Abundance of Fungal Taxa (rDNA-OTU) in Pre-Plant and Rhizosphere Soils

Twenty eight of a possible 60 fungal taxa were identified in the 20 most abundant taxa across the combined sugarcane pre-plant (T0) and 6-week post-emergent (T0, T1) soils (Table 12). Eleven of these taxa were highly abundant in soil at all 3 sampling times and included primarily saprophytic *Cladophialophora*, *Mortierella*, *Penicillium*, *Taleromyces* and *Trechispora* spp., pathogen antagonistic *Trichoderma* spp. and unidentified taxa with the functional diverse orders Agaricales, Capnodiales, Helotiales, Hypocreales and Sordariales. These orders are represented by saprophytic, plant pathogenic, plant beneficial (*e.g.* mycorrhizal) and disease suppressive (*e.g.* mycoparasitic) fungi.

In pre-plant soils (T0), the relative abundance of the saprophytic *Cyphellophera* spp and unidentified taxa within the orders Capnodiales, Hypocreales and Sordariales were significantly different (0.001<P<0.05) among cropping treatments (Table 12). *Cyphellophora* and *Periconia* spp. only had high relative abundance (2%) in pre-plant soils (T0), these saprophytic and mycotoxin producing fungi being suppressed in the sugarcane rhizosphere soils (Table 12).

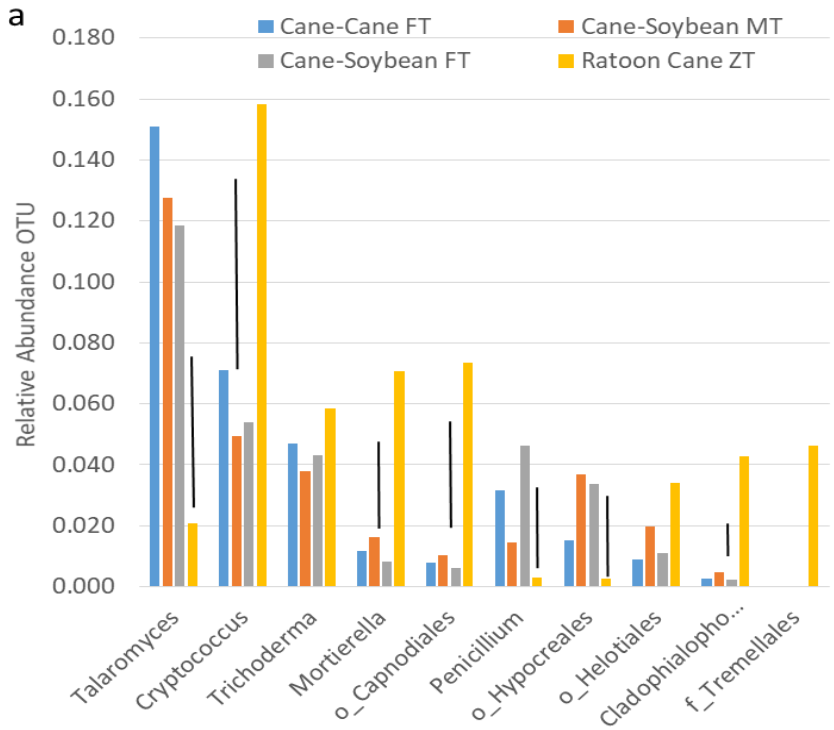
Conversely, taxa that only had high relative abundance (1% - 22%) at T2 included root associated saprophytic Chaetosphaeriaceae and *Chaetomium*, *Coniosporium*, *Scytalidium* spp., pathogen-antagonistic *Epicoccum* spp., mycoparasitic *Tremella* spp. and *Saitozyma* spp. This high relative abundance at T2 potentially indicates rhizosphere selection of these taxa by sugarcane (Table 12).

Table 12 Stone River: The 20 most abundant fungal taxa (rDNA-OTU) in pre-planting (T0) and 6-week post-emergence rhizosphere soils in 2016 (T1) and 2017 (T2) determined by culture-independent rDNA sequencing. Analyses included taxa that were identifed to Order, Family and Genus. Fungal endophytes isolated from sugarcane roots (*i.e.* culture-dependent) are shown in bold text.

	Stone River : Relative Abundance of Fungal Taxa (Rank 1 - 20) [1, 2]					
Rank	T0	Grand Mean	T1	Grand Mean	T2	Grand Mean
1	Cryptococcus	0.1026	Talaromyces***	0.1045	Saitozyma**	0.2188
2	Talaromyces	0.0781	Cryptococcus**	0.0831	o_Capnodiales	0.1136
3	o_Capnodiales***	0.0765	**Trichoderma**	0.0466	**Chaetomium**	0.0687
4	**Mortierella**	0.0666	**Mortierella*** **	0.0268	Talaromyces	0.0546
5	**Trichoderma**	0.0510	o_Capnodiales***	0.0244	o_Helotiales*	0.0389
6	o_Sordariales*	0.0495	**Penicillium***	0.0239	o_Pleosporales	0.0379
7	Trechispora	0.0324	o_Hypocreales*	0.0221	**Trichoderma*** **	0.0366
8	Chaetosphaeria	0.0280	o_Helotiales	0.0184	**Penicillium**	0.0252
9	o_Helotiales	0.0233	Cladophialophora***	0.0131	Cladophialophora	0.0247
10	**Penicillium**	0.0220	f_Tremellales	0.0116	Trechispora	0.0214
11	Cladophialophora	0.0218	o_Chaetothyriales***	0.0094	o_Agaricales*	0.0184
12	o_Myriangiales	0.0190	o_Agaricales*	0.0083	o_Hypocreales	0.0168
13	o_Hypocreales***	0.0169	f_Nectriaceae	0.0064	Scytalidium	0.0119
14	Periconia	0.0163	o_Xylariales***	0.0064	Epicoccum***	0.0118
15	o_Xylariales	0.0162	Trechispora***	0.0061	Coniosporium*	0.0115
16	o_Chaetothyriales	0.0137	o_Sordariales	0.0055	**Mortierella**	0.0097
17	Cyphellophora*	0.0115	o_Myriangiales	0.0038	Tremella	0.0078
18	Bullera	0.0115	Clonostachys**	0.0036	f_Nectriaceae	0.0077
19	o_Pleosporales	0.0099	Chaetosphaeria	0.0033	o_Sordariales	0.0076
20	o_Agaricales	0.0083	Bullera***	0.0033	f_Chaetosphaeriaceae*	0.0069
	Chaetomium (39)	0.0042	**Fusarium (24)**	0.0014	**Fusarium* (33)**	0.0028
	Fusarium (54)	0.0015	**Chaetomium (39)**	0.0012		

1. Bold text indicates sugarcane fungal endophytes isolated in culture-dependent analyses (*i.e.* root isolations). Numbers in parenthesis refer to the relative abundance ranking of taxa if lower than 20.
2. Significant differences in relative abundance of fungal taxa among sugarcane cropping treatments and forest soils at each sampling time: * P<0.05, ** P<0.01, *** P<0.001

In 6-week post-emergent cane rhizosphere soils (T1), 13 of the 20 most abundant fungal taxa were significantly different (0.001<P<0.05) among cropping treatments (Table 12, Figs. 7a and 7b). The relative abundance of these taxa ranged from 10% (*Talaromyces* spp.) to 0.3% (*Bullera* spp.). Fungal taxa significantly different among the rhizosphere communities of cropping treatments included saprophytic Agaricales, Chaetothyriales, Xylariales, *Cladophialophora*, *Cryptococcus* (yeasts) *Mortierella*, *Penicillium*, *Taleromyces* and *Trichospora* spp., potentially fungal-antagonistic *Bullera* and *Clonostachys* spp. and unidentified saprophytic, beneficial (disease suppressive) and potentially plant pathogenic Capnodiales and Hypocreales (Table 12, Figs. 7a and 7b). *Bullera*, *Cladophialophora, Cryptococcus*, *Mortierella*, and *Trichospora* spp. and unidentified Agaricale, Capnodiale, Chaetothyriale and Xylariale taxa were significantly more abundant in the rhizosphere soil microbiome of long-term (5th) ratoon cane (Figs. 7a and 7b). In contrast, saprophtyic *Taleromyces* and *Penicillium* spp., potentially pathogen antagonistic *Clonostachys* spp., and unidentified functionally diverse Hypocreale taxa were significantly more abundant in the rhizosphere microbiome of replant cane and cane-legume rotations (Figs. 7a and 7b).



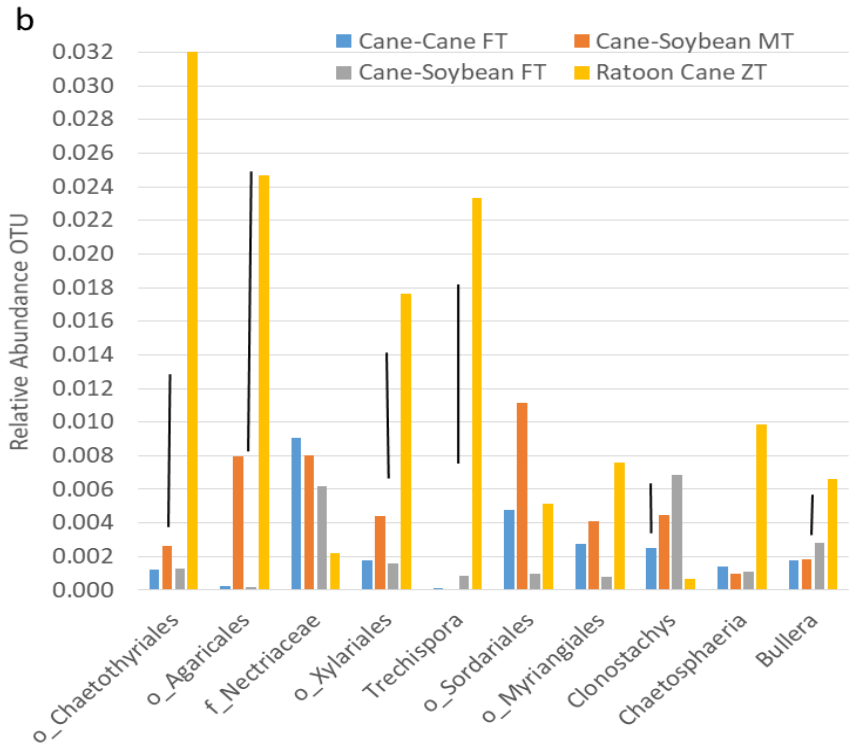


Figure 7 Stone River 2016 (T1): Impacts of soybean rotation and tillage treatments on the relative abundance of fungal taxa (rDNA-OTU) in rhizosphere soils of 6-week post-emergence (T1) sugarcane. Analyses of variance was performed on the 20 most abundant taxa among sugarcane cropping treatments (Figure 7a Taxa 1 – 10; Figure 7b Taxa 11 – 20). Black vertical bars indicate statistically significantly LSD values for individual fungal taxa (0.001<P<0.05).

In 6-week post-emergent ratoon cane rhizosphere soils (T2), 7 of the 20 most abundant fungal taxa were significantly (0.001<P<0.05) different among cropping treatments (Table 12, Figs. 8a and 8b). The relative abundance of these taxa ranged from 22% (*Saitozyma* spp.) to 1% (Chaetosphaeriaceae, *Epicoccum* and *Coniosporum* spp.). Fungal taxa significantly different among the rhizosphere communities of cropping treatments included pathogen antagonistic *Trichoderma* and *Epicoccum* spp., saprophytic Agaricales, Chaetosphaeriaceae, *Coniosporum* and *Saitozyma* spp. (yeasts) and unidentified saprophytic and potentially plant pathogenic Heliotales (Table 12, Figs. 8a and 8b). *Trichoderma*, *Epicoccum* and *Saitozyma* spp., Chaetosphaeriaceae taxa and Heliotales taxa were significantly more abundant in the rhizosphere soil microbiome of long-term (5th) ratoon cane, this being most apparent in comparisons with cane-legume rotations (Figs. 8a and 8b). In contrast, saprophtyic Agaricales and *Coniosporum* spp. were significantly more abundant in the rhizosphere microbiome of replant cane (1st ratoon) and cane-legume rotations (Figs. 8a and 8b). Taxa within the primarily plant pathogenic order Myriangiales were not ranked in the top 20 most abundant taxa at T2, despite being previously abundant in pre-plant (T0) and T1 rhizosphere soils (Table 12).

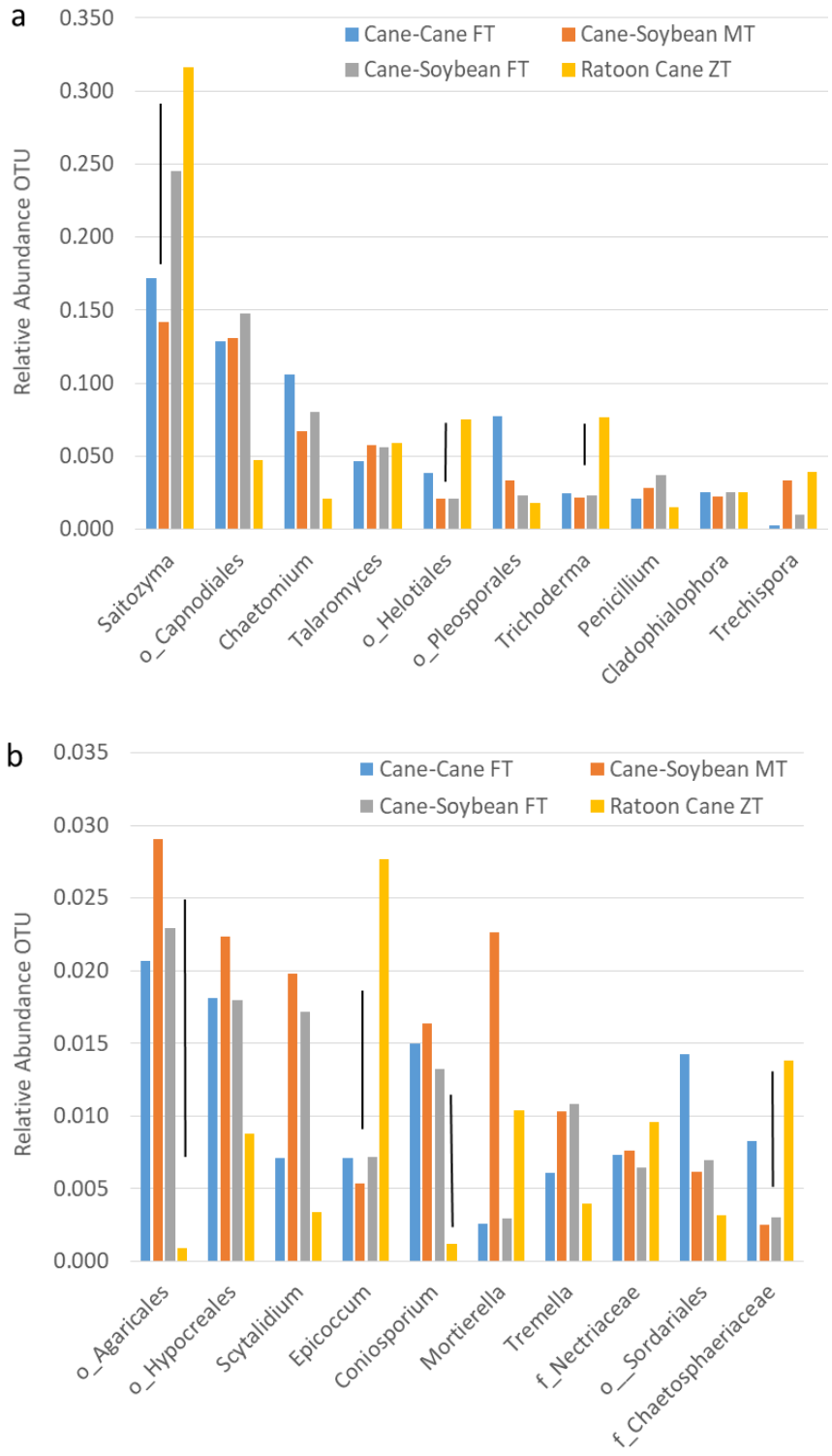


Figure 8 Stone River 2017 (T2): Impacts of soybean rotation and tillage treatments on the relative abundance of fungal taxa (rDNA-OTU) in rhizosphere soils of 6-week post-emergence (T2) sugarcane. Analyses of variance was performed on the 20 most abundant taxa among sugarcane cropping treatments (Figure 8a Taxa 1 – 10; Figure 8b Taxa 11 – 20). Black vertical bars indicate statistically significantly LSD values for individual fungal taxa (0.001<P<0.05).

7. DISCUSSION AND CONCLUSIONS

Culture–dependent and culture-independent analyses of sugarcane rhizosphere fungal and oomycete communities were conducted across two cropping seasons at two sites. Integrating these analyses has delivered novel information on sugarcane rhizosphere-fungal interactions and factors impacting on root disease incidence and suppression.

Analyses of total nitrogen (TN) and non-purgeable organic carbon (TOC) in pre-plant (T0) and 6-week post-emergent rhizosphere soils investigated linkages between nutrient and microbial community dynamics in sugarcane cropping treatments. There were no significant differences in soil TN 5 weeks prior to cane planting among replant continuous cane, ratoon cane or cane-legume rotations at either site, indicating no residual excess soil N remaining from legume rotations. Soil TOC was greater ($P<0.001$) in rhizosphere soils of the actively growing sugarcane crops than in pre-plant soils and increased ($P<0.001$) with each year of cane cropping, regardless of the agronomic treatment (Table 1). Lower levels of TOC in pre-plant and rhizosphere soils of replant cane is likely due to recent tillage increasing microbial mineralisation of the labile organic fraction, thereby reducing TOC relative to the less disturbed ratoon cane treatments. In comparison, TOC levels in rhizosphere soils of 10th ratoon cane (Ingham) were 40-fold lower than those reported in ratoon cane of a similar duration at Bundaberg, Qld. (Paungfoo-Lonhienne *et al*., 2017).

Rhizosphere inoculum of the oomycete root pathogens *Pythium* spp. and *Pachymetra chaunorhiza* in sugarcane and forest (Ingham) soils were quantified over 2 consequtive cropping seasons by culture-independent DNA diagnostics (PredictaB) and culture-dependent isolation of viable inoculum on oomycete-selective medium.

Culture-dependent and DNA-diagnostic methods indicated that oomycete communities in sugarcane rhizosphere soils were dominated by plant pathogenic *Pythium spinosum.* Whilst there were no significant differences in *P. spinosum* rhizosphere inoculum among the sugarcane tillage and rotation treatments or the adjacent forest soil, inoculum of the pathogen increased ($P<0.001$) following another year of cane growth (Table 2).

No *Pachymetra chaunorhiza* strains were isolated from rhizosphere soils at either trial site, despite the species-specific diagnostic assay (SRA project 2016/047) detecting the pathogen in all sugarcane and forest soils (Table 2). Whilst there was an overall increase ($P=0.016$) in *Pachymetra* rhizosphere soil DNA with the next cane crop, there were no site-specific significant differences in *Pachymetra* rhizosphere soil DNA among the sugarcane tillage and legume rotation treatments (Table 2).

Assuming a minimum copy number of 1 target sequence per *Pachymetra* genome and given the oospores are uninucleate, the minimum soil-borne inoculum detected approximates to 100,000 oospores per kg^{-1} soil. This would place the all cane treatments at a medium to high risk of *Pachymetra* root rot (Magarey, 2013). Despite this, no *Pachymetra* strains were isolated from sugarcane rhizosphere soils or from cane roots on oomycete-selective medium.

The relative abundances of fungal taxa (Order, Family, Genus) in sugarcane pre-plant and rhizosphere soils were used to define impacts of crop rotation and tillage treatments on the taxonomic structures of the fungal microbiome. This provided information on the effects of agronomic selection pressures (rotation, tillage) on fungal communities and their potential relationships to expression of root disease.

Sugarcane soil fungal communities had high levels of taxonomic diversity and eveness (Table 10), both indices being >0.95 in all treatments (range 0 – 1) and increasing ($P<0.001$) overall following another cane crop (Table 10). Forest soils and cane following a lablab bean rotation (Ingham) had greater ($P<0.001$) species richness, taxonomic eveness and diversity than replant continuous cane and ratoon

cane (Table 10). At Stone River, replant continuous cane and cane-soybean rotations resulted in greater ($P<0.001$) species richness than longer term ratoon cane (Table 10).

Collectively, analyses of the sugarcane rhizosphere soil fungal microbiome indicated that fungal communities of longer-term (5^{th} – 10^{th}) ratooning cane were significantly different ($P<0.003$) from those replant continuous cane and cane-legume rotations, regardless of site or tillage treatment (Figs. 1 and 2, Figs. 5 and 6). There were however, no significant differences in fungal community structure among the replant continuous cane and cane-legume rotation treatments (Figs. 5 and 6). At the Ingham site, the forest soil fungal microbiome was significantly differentiated ($P<0.001$) from those of the adjacent (~10 m) sugarcane cropping treatments (Figs. 1 and 2). The significant differentiation among rhizosphere soil fungal communities with increased duration of cane cropping supports that observed in comparisons of short term (2-3 year) disease conducive and continuous (>10 year) disease suppressive wheat cropping systems (Penton *et al.*, 2013).

Among the 20 most abundant fungal taxa at Ingham, 10 were highly abundant in pre-plant and 6-week post emergent sugarcane rhizosphere soils (Table 11). This included 8 primarily saprophytic taxa and pathogen antagonistic *Trichoderma* and *Humicola* spp. At Stone River, 11 of the 20 most abundant taxa were present at all 3 sampling times. Similarly, this included 8 saprophytic taxa (6 present at both sites) and *Trichoderma* spp. (Table 12). Unidentified taxa within functionally diverse Helotiales and Hypocreales were more abundant at Stone River, these orders know to contain sugarcane pathogenic (Viswanathan & Rao, 2011), mycorrhizal and mycoparasitic species (Kirk *et al.,* 2008; Zhang *et al.,* 2009). Taxa within the primarily plant pathogenic order Myriangiales (Cannon *et al.,* 2012; Fan *et al.,* 2017) were not ranked in the top 20 most abundant taxa at T2, despite being previously abundant in pre-plant (T0) and T1 rhizosphere soils (Tables 11 and 12). The Hypocreales and Myriangiales contain the economically important sugarcane wilt (*Cephalosporium sacchari*) and red rot (*Colletotrichum falcatum*) pathogens, respectively (Cannon *et al.,* 2012; Patel *et al.*, 2018; Viswanathan & Rao, 2011). Neither of these fungal genera were however, ranked in the 20 most abundant taxa at either site (Tables 11 and 12).

At Ingham in 2016, 15 of the 20 most abundant rhizosphere soil fungal taxa were significantly different ($0.001<P<0.05$) among cropping treatments (Figs. 3a and 3b). Those significantly more abundant in the cane-legume rotation included 4 saprophytic taxa and fungal antagonistic *Bullera* spp. Fungi significantly more abundant in the microbiome of ratoon cane included another 4 saprophtic taxa and the fungal antagonists *Trichoderma* and *Humicola* spp. Yet another 5 saprophytic taxa were significantly more abundant in forest soil compared with those in the cane rhizosphere.

Following another ratoon (2017), 14 of the 20 most abundant fungal taxa at Ingham were significantly different ($0.001<P<0.05$) among cropping treatments (Figs. 4a and 4b). Those significantly more abundant in the cane-legume rhizosphere microbiome included 5 saprophytic taxa, fungal-antagonistic *Humicola* spp. and unidentified Pleosporales, a functionally diverse order containing unidentified dematiacous fungal pathogens of sugarcane (Magarey *et al*., 1997;Magarey *et al.,* 2005) and other important root and foliar pathogens of grain crops (Kirk *et al.,* 2008; Zhang *et al.,* 2009). Only the 2 saprophytic yeast genera were significantly more abundant under ratoon cane, whilst yet another 3 saprophytic genera were significantly more abundant in forest than cane rhizosphere soils.

At Stone River in 2016, 13 of the 20 most abundant rhizosphere soil fungal taxa were significantly different ($0.001<P<0.05$) among cropping treatments (Figs. 7a and 7b). Eight primarily saprophytic taxa and fungal antagonistic *Bullera* spp. were significantly more abundant in the rhizosphere soil microbiome of long-term (5^{th}) ratoon cane. In contrast, another 2 saprophtyic genera, potentially pathogen-suppressive *Clonostachys* spp. and unidentified plant pathogenic and fungal antagonistic Hypocreale taxa (Kirk *et al.,* 2008; Penton *et al.* 2013) were significantly more abundant in the rhizosphere microbiome of replant cane and cane-legume rotations.

Following another cropping season (2017), only 7 of the 20 most abundant rhizosphere soil fungi at Stone River were significantly (0.001<P<0.05) different among cropping treatments (Figs. 8a and 8b). Two saprophytic taxa, pathogen suppressive *Trichoderma* and *Epicoccum* spp. and unidentified functionally diverse Helotiales taxa were significantly more abundant in the rhizosphere soil microbiome of 5th ratoon cane. In contrast, another 2 saprophtyic taxa were significantly more abundant in the rhizosphere microbiome of 1st ratoon replant continuous cane and 1st ratoon cane after soybean.

Significant differences in sugarcane fungal microbiomes may primarily be driven by local shifts in abundance of root-associated saprophytic and fungal-antagonistic taxa among treatments within each cropping season. Longer-term ratoon cane is expected to apply additional rhizosphere selection over multiple cropping seasons, potentially accounting for the increased abundance of endophytic, pathogen-antagonistic *Trichoderma* (Figs 3a and 8a) and *Epicoccum* spp (Fig. 8b) and decreased abundance of unidentified potentially plant pathogenic Myriangiales (Tables 11 and 12), Pleosporales (Fig 4a) and Hypocreales (Fig 7a) taxa (Magarey *et al*., 2005; Kirk *et al.,* 2008; Cannon *et al*., 2012; Fan *et al.,* 2017; Zhang *et al.,* 2009).

No oomycetes (OTUs) were detected in the rhizosphere microbiome of sugarcane or forest soil adjacent to sugarcane treatments by rDNA sequencing. Despite this, PredictaB diagnostics detected the oomycete taxa *Pachymetra chaunoriza* and *Pythium* clade-F (Lévesque & De Cock, 2004) DNA in rhizosphere soils of all sugarcane treatments at both sites (Table 2). Viable *Pythium spinosum* (clade_F) inoculum was also isolated from all rhizosphere soils, with *P. spinosum* the most abundant (64%) plant pathogen isolated from sugarcane roots (Table 7). No *Pachymetra* strains or other oomycete taxa were isolated from sugarcane rhizosphere soils or roots. This disconnect between culture-dependent and culture-independent quantification of *Pachymetra* and *P. spinosum* indicates that alternative sequence methodologies (Ritt *et al.,* 2016; Schlatter *et al.*, 2018) are required to quantify these and other unidentified oomycetes within the rhizosphere microbiome of sugarcane and legume rotation crops (Coffua *et al.,* 2016).

Culture-dependent and culture-independent techniques were used to determine the taxonomic structure of the sugarcane rhizosphere fungal microbiome. Analyses focused on the dominant endophytic colonists of sugarcane roots and their relative abundance (OTUs, diagostic qPCR) in sugaracane rhizosphere soil in response to crop rotation and tillage treatments.

The high (41%) overall mean isolation frequency of saprophytic *Chaetomium* spp. from sugarcane roots is supported by the relatively high abundance of *Chaetomium* OTUs in rhizosphere soil (3%). Endophytic root colonisation and rhizosphere abundance of *Chaetomium* spp. were generally greater (P<0.001) in recenly tilled replant continuous cane and cane-legume rotations than in zero tillage longer-term ratoon cane (Table 3).

The overall mean isolation frequency of *Mortierella* spp. from sugarcane roots was 36%, endophytic colonisation (P<0.001) and rhizosphere inoculum (P=0.027) of this saprophytic taxa being greater in full tillage replant compared with zero tillage longer-term ratoon cane (Table 5). *Mortierella* OTUs were also highly abundant in sugarcane (3%) and forest (10%,) soil microbiomes, ranked in the top 20 most abundant fungi in all pre-plant and rhizosphere soils (Tables 11 and 12). Their greater (P<0.001) abundance in forest soil (Fig. 4a) supports *Mortierella* being designated as a key root-associated saprophytic taxon in rhizosphere metacommunities of natural ecosystems (Toju *et al.,* 2018).

The low overall mean isolation frequency of *Penicillium* spp. among cropping treatments (7%) contrasts with the relative abundance (2%) in the rhizosphere soil fungal microbiome, *Penicillium* spp. being ranked in the top 20 most abundant fungal OTUs in all pre-plant and rhizosphere soils (Tables 11 and 12). Whilst endophytic root colonisation by *Penicillium* spp. was not significantly different among sugarcane treatments (Table 4), the relative abundance of this primarily saprophytic taxon was

significantly greater (P<0.001) in forest soil (Fig. 4a) and lower (P<0.05) in longer-term ratoon cane (Fig. 7a).

Primarily saprophytic *Chaetomium*, *Mortierella* and *Penicillium* OTUs were all highly abundant (2% - 3%) in the rhizosphere soil fungal microbiome of sugarcane, but differed greatly in their endophytic colonisation of sugarcane roots. *Mortierella* and *Chaetomium* spp. were active root colonists (36% - 41% isolation frequency) and may be considered root adapted saprophytic species. In contrast, *Penicillium* spp. were infrequent root colonists (7%), likely outcompeted in the rhizoplane and proliferating in rhizosphere soil. Endophytic root colonisation and rhizosphere abundance of saprophytic fungal taxa were generally greater (P<0.001) in recenly tilled replant continuous cane and cane-legume rotations than in zero tillage longer-term ratoon cane.

The high overall mean isolation frequency of *Fusarium* spp. among cropping treatments (30%) contrasts with their low relative abundance (0.5%) in the rhizosphere soil fungal microbiome, the taxon being only once ranked in the top 20 most abundant fungal taxa (*i.e.* Ingham, 2017) (Table 11). *Fusarium* root infection was generally greater (P<0.001) in recenly tilled replant continuous cane and cane-legume rotations than in zero tillage longer-term ratoon cane (Table 5). In contrast, there were no significant differences in *Fusarium* relative abundance (OTUs) among rhizosphere soils of sugarcane tillage and rotation treatments. These results support the effects of long-term maize cultivation and rotation crops on *Fusarium* rhizosphere abundance (Harvey *et al.,* 2008:Wakelin *et al.,* 2008).

Pythium spinosum, a recognised pathogen of sugarcane (Viswanathan & Rao, 2011), had the highest overall mean sugarcane root isolation (64%) among all plant pathogenic and saprophytic taxa (Table 7). *P. spinosum* root infection was generally greater (P<0.001) in replant continuous cane and cane-legume rotations compared with longer-term ratoon cane (Table 7). Whilst no *Pythium* spp. OTUs were identified within the sugarcane rhizosphere microbiome, DNA-based diagnostics (PredictaB) and microbiological approaches detected *P. spinosum* inoculum in all rhizosphere soils (Table 7).

Root disease complexes caused by *Pythium* and *Fusarium* spp. have previously been reported in sugarcane (Magarey, 1996; Magarey *et al*., 1997), irrigated maize (Harvey *et al*., 2008) and cereal cropping systems (Stummer *et al*., 2018). In this study sugarcane root infection by plant pathogenic *Fusarium* and *P. spinosum* was high frequency and dynamic in comparison to their low, relatively stable abundance in the rhizosphere soil microbiome. This suggests selection of plant pathogenic genotypes of these taxa by actively growing sugarcane and temporal stability of saprophytic or weakly pathogenic genotypes within rhizosphere soil communities. The disconnect between high root infection and low soil inoculum has implications when using soil DNA-based diagnostics (*e.g*. Pythium Clade_F) as predictors of *Pythium* and *Fusarium* root disease severity.

Endophytic pathogen antagonistic *Trichoderma* spp. were the most frequently isolated taxa (76%) from sugarcane roots and were also highly abundant (4%) in the sugarcane rhizosphere soil microbiome (Table 8), ranked in the top 20 most abundant fungal OTUs in pre-plant and rhizosphere soils (Tables 11 and 12). *Trichoderma* root colonisation, relative abundance (OTUs) and rhizosphere inoculum of pathogen antagonistic *T. harzianum* were all greater (P<0.001) under long-term ratoon cane compared with replant continuous cane and cane-legume rotations (Table 8, Figs. 3a and 8a).

At Stone River there was a positive association between root colonisation, rhizosphere community abundance (OTUs) and *T. harzianum* rhizosphere inoculum (Table 8) in long-term ratoon cane, implying that *T. harzianum* is the dominant *Trichoderma* species at this site. In long-term ratoon cane at Ingham (Table 8), *Trichoderma* spp. abundance (OTUs) decreased (P<0.001) in rhizosphere soil with the additional ratoon crop, whereas rhizosphere inoculum of *T. harzianum* increased (P<0.001). This suggests that root-associated *T. harzianum* may have undergone selection from a diverse *Trichoderma* rhizosphere community, resulting in increased (P<0.001) root colonisation in longer-term ratoon cane.

Pathogen antagonistic *Epicoccum* spp. were only rarely isolated from sugarcane roots and had an overall low relative abundance (0.05%) in the rhizosphere soil microbiome of sugarcane. *Epicoccum* OTUs were more abundant (P<0.001) in rhizosphere soils of longer-term ratoon cane (Fig. 8b) and ranked in the top 20 most abundant fungal taxa in 2017 (Tables 11 and 12).

Metabolites of *T. harzianum* (Mukherjee *et al.,* 2012) and *E. nigrum* (Fa´varo *et al.,* 2012; Fatima *et al.,* 2016) strains were analysed for antibiosis of sugarcane root pathogenic *Fusarium* and *P. spinosum* strains *in vitro*. The *Pythium* and *Fusarium* root disease suppressive inoculant *T. gamsii* strain A5MH (Stummer *et al.* 2018) was included for comparison. Metabolites of all *Trichoderma* and *E. nigrum* strains inhibited (P<0.001) *Fusarium* growth and were lethal to *P. spinosum* (Table 9). Sugarcane root isolates *T. harzianum* 88.1 and *E. nigrum* 7.1 exhibited greater (P<0.001) antibiosis efficacy toward *F. oxysporum* and *F. verticillioides* that the root disease suppressive inoculant *T. gamsii* A5MH (Table 9).

Trichoderma spp are recognised antagonists of plant pathogenic fungi and oomycetes (Rinu *et al.*, 2014; Zhang *et al.* 2015), with genotypes of *T. harzianum* and *T. gamsii* registered for the suppression of root and foliar diseases. Sugarcane root endophytic *E. nigrum* have previously reported to have antibiosis activity against sugarcane root pathogenic fungi *F. verticillioides* and *Ceratocystis paradoxa* (Fa´varo *et al.,* 2012). Consequently, the root endophytic, pathogen-antagonistic *T. harzianum* and *E. nigrum* genotypes isolated in this study and the secondary metabolites they produce require investigation as potential disease suppressive treatments in sugarcane (Mukherjee *et al.,* 2012; Fatima *et al.,* 2016; Stummer et al., 2017).

In summary culture-dependent and culture-independent analyses indicated that communities of saprophytic *Chaetomium* and *Mortierella* spp. and plant pathogenic *Fusarium* and *Pythium* spp. were generally more abundant (P<0.001) in roots and rhizosphere of early ratoon replant continuous cane and cane-legume rotations compared with longer-term ratoon cane. In contrast, abundance of endophytic fungal antagonistic *Trichoderma* and *Epicoccum* spp. increased (P<0.001) with the time under cane cropping, being greatest in longer-term (5^{th} – 10^{th}) ratoon cane.

As rhizosphere abundance and root colonisation of pathogen antagonistic *Trichoderma* and *Epicoccum* significantly increased, frequencies of root pathogenic *Fusarium* and *Pythium* and root-associated saprophytic *Chaetomium* and *Mortierella* significantly decreased. Dynamics of these root and rhizosphere fungal and oomycete communities were most evident in the comparisons of replant cane and longer-term ratoon cane. Secondary metabolites produced by *Trichoderma* and *Epicoccum* spp. actively inhibited fungi and oomycetes *in vitro* and act as a mechanism to enhance rhizosphere competition and root colonisation. Multiple years of ratoon cane is expected to apply relatively uniform rhizosphere selection over time, increasing the abundance of these fungal antagonists and suppressing root-associated saprophytes, plant pathogenic *Fusarium* and *Pythium* spp. and unidentified potentially sugarcane pathogenic Pleosporales, Hypocreales and Myriangiales (Magarey *et al*., 2005; Kirk *et al.,* 2008; Cannon *et al.,* 2012; Fan *et al.,* 2017; Zhang *et al.,* 2009).

8. RECOMMENDATIONS FOR FURTHER RD&A

1. *Improve understanding of the agro-ecological processes affecting root-pathogen-antagonist interactions in sugarcane production systems, via comparative eco-genomic analyses of disease suppressive and conducive soils.*

Analyses of the sugarcane rhizosphere soil fungal microbiome indicated that fungal communities of longer-term (5^{th} – 10^{th}) ratooning cane were significantly different from those replant continuous cane and cane-legume rotations. The majority of these differences were attributed to 33 taxa, including saprophytic and pathogen antagonistic genera and unidentified taxa within the functionally diverse orders Helotiales, Hypocreales, Myriangiales and Pleosporales, all reported to contain economically important plant pathogens (Kirk *et al.,* 2008; Cannon *et al.,* 2012; Magarey *et al*., 2005;Viswanathan & Rao, 2011; Zhang *et al.,* 2009).

Recognised plant pathogenic *Fusarium* and and *Pythum* spp. and unidentifed potentially plant pathogenic taxa within the orders Helotiales, Hypocreales, Myriangiales and Pleosporales (*e.g.* dematiaceous fungi) were all more abundant in rhizosphere soils of 1st ratoon replant continuous cane and cane-legume rotations. Rhizosphere abundance and or root infection frequencies of all these plant pathogenic taxa declined in longer-term (5th-10th) ratoon cane as pathogen antagonistic *Trichoderma* and *Epicoccum* increased.

Further research is required to isolate and identify fungal taxa within these orders and to define their pathogenicity characteristics on sugarcane and alternative hosts used in rotation with cane. Novel culture-dependent approaches will be required for their selective isolation from sugarcane roots and rhizosphere soils, as all root fungal isolates in present study were identified to at least genus level, these genera also detected in the rhizosphere soil microbiome (rDNA OTUs).

Comparative culture-dependent and culture-indepenent analyses of replant and long-term ratoon cane, targeted to isolate and identify taxa within the Helotiales, Hypocreales, Myriangiales and Pleosporales, will assist with identification of previously unreported sugarcane root pathogens, their role in root disease complexes and potential impacts on sugarcane productivity.

2. *Determine host-based selection of fungal communities (beneficial and deleterious) in a sugarcane-legume rotation, enabling identification of cropping strategies to enhance disease suppression.*

Root disease suppressive *Trichoderma* and *Epicoccum* inoculant treatments

Trichoderma and *Epicoccum* strains are competitive rhizosphere colonists and potent antagonists of plant pathogens with potential to develop as root disease suppressive inoculants (Fa´varo *et al.,* 2012; Rinu *et al.,* 2014; Fatima *et al*., 2016; Stummer *et al*., 2018; Zhang *et al*. 2015).

The link between increasing *T. harzianum* and *E. nigrum* abundance in sugarcane roots and rhizosphere soils with decreasing *Pythium* and *Fusarium* root infection suggests that these taxa play a significant role in root disease supression. Root endophytc strains of both fungi produced metabolites that significantly inhibited *Fusarium* growth and were lethal to *Pythium spinosum.* These rhizosphere competent pathogen antagonistic genotypes and the secondary metabolites they produce therefore require investigation as potential root disease suppressive treatments in sugarcane. The reciprocal antibiosis between these strains does however, have implications when considering using mixed *Trichoderma-Epicoccum* formulations for disease management.

Culture-dependent approaches will be integrated with quantitative species and strain-specific diagnostics (Stummer *et al.* 2013, Stummer *et al.* 2017) to define the size, spatial distribution and dynamics of *Trichoderma* and *Epicoccum* inoculants in rhizosphere soils. Greater understanding of inoculant rhizosphere ecology of will help develop targeted crop intervention strategies to enhance root disease suppressive efficacies in sugarcane cropping systems.

Novel inoculant formulations (CSIRO) and delivery systems have been used to enhance ease, uniformity and frequency of crop inoculation (Stummer *et al.* 2018). Inoculant delivery uses an emulsion-based (liquid) formulation, providing opportunities for pre- and post- planting treatments during the growing season. The formulation has been shown to increase rhizosphere colonisation and efficacies of a disease suppressive *Trichoderma* inoculant treatment (Stummer *et al.* 2017; Stummer *et al.* 2018).

3. *Improve capacity to predict the disease suppressive status of soils and manage plant pathogenic and beneficial fungal communities.*

Sugarcane root infection by plant pathogenic *Fusarium* spp. and *Pythium spinosum* was high frequency and dynamic in comparison to their low, relatively stable abundance in the rhizosphere soil microbiome. The disconnect between high root infection and low soil inoculum has implications when

using higher taxonomic (non species–specific) DNA-based diagnostics in soil as predictors of *Pythium* and *Fusarium* root disease severity in sugarcane.

In constrast, there was a positive association between root colonisation and abundance of pathogen antagonistic *T. harzianum* rhizosphere soil inoculum with a species-specific DNA diagnostic. The link between increasing *T. harzianum* abundance and decreasing *Pythium* and *Fusarium* root infection suggests that the *T. harzianum*-specific diagnostic may be a predictor of root disease suppression.

Correlating soil-borne inoculum, root colonisation and culture-independent molecular diagnostic (PredictaB, CSIRO) data sets will assist with determining relationships among plant pathogenic (*Pythium*, *Fusarium*) and beneficial (*Trichoderma*) inoculum levels, incidence of root infection and disease severity in sugarcane. Further field-based epidemiological research is required to refine existing (*Pythium* clade_F, *T. harzianum*) and developmental (*Pachymetra, Fusarium*) diagnostics for root disease prediction and management in sugarcane.

4. *Provide resources (DNA extracts) for subsequent eco-genomic and quantitative diagnostic analyses of sugarcane soil microbiota (i.e. bacteria, invertebrates).*

Existing DNA extracts provide a resource to conduct targeted taxa-specific analyses of oomycete (*Pythium*, *Pachymetra*) and unidentified potential sugarcane pathogenic species (orders Helotiales, Hypocreales, Myriangiales and Pleosporales) within the rhizosphere microbiome of sugarcane. These extracts can also be analysed to investigate dynamics of bacterial (Pankhurst *et al.*, 2000) and dematiaceous fungal communities (Magarey *et al.*, 2005), enabling identification of potential pathogens and examining microbial interactions in the rhizosphere that impact on root disease expression.

No oomycetes (OTUs) were detected in the rhizosphere microbiome of sugarcane or forest soil adjacent to sugarcane treatments. However, PredictaB diagnostics detected the oomycete taxa *Pachymetra chaunoriza* and *Pythium spinosum* in rhizosphere soils of all sugarcane treatments. Viable *P. spinosum* inoculum was also isolated from all rhizosphere soils and *P. spinosum* was the most abundant pathogen isolated from cane roots. No *Pachymetra* strains or other oomycete taxa were isolated from sugarcane rhizosphere soils or roots. This disconnect between culture-dependent and culture-independent quantification of *Pachymetra* and *P. spinosum* indicates that oomycete-specific sequencing methods are required to quantify these and unidentified oomycetes within the rhizosphere microbiome of sugarcane cropping systems (Coffua *et al.,* 2016; Ritt *et al.,* 2016; Schlatter *et al.*, 2018). Correlating oomycete rhizosphere abundance with exisiting data on soil-borne inoculum and root disease incidence will improve capacity to predict and manage oomycete diseases in sugarcane cropping systems (8.3 above)

Primers designed to specifically target taxa within the Helotiales, Hypocreales, Myriangiales and Pleosporales (*e.g.* dematiaceous fungi) will assist with identification of currently unidentified sugarcane root pathogens, determine their dynamics in sugarcane cropping soils and examine linkages between rhizosphere abundance and root disease incidence in sugarcane (8.1 above).

A combined bioinformatic analyses of the existing pre-plant (T0) and rhizosphere soil (T1 and T2) sugarcane fungal microbiome data sets may also be undertaken to more clearly define the levels of community differentiation among cropping treatments within and between sites over the 2 consecutive cropping seasons.

9. PUBLICATIONS

To date, no research publications have arisen from the project.

Following submission of the final report, articles summarising research outputs will be submitted to CaneConnection (Pfeffer), HCPSL (Di Bella) and Terrain Natural Resource Management (Waring) newsletters.

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12. APPENDIX

12.1. Appendix 1 METADATA DISCLOSURE

Table 13 Metadata disclosure: Sugarcane fungal and oomycete microbial ecology data sets

Data	DNA sequence data of rhizosphere soil fungal microbiomes. Soil nutrient status, rhizosphere soil inoculum, root colonisation and pathogen antagonism data sets. Fungal and oomycete culture collection (soil and root isolates) and DNA sequence data (ITS-rDNA) of fungal and oomycete strains.
Stored Location	CSIRO Server\\FSSA2-ADL\CLW-SHARE1\Pythium DNA sequence data of fungal microbiomes is also stored on the CSIRO Bowen High performance and long-term storage facility.
Access	Restricted to CSIRO staff working on project CSI001.
Contact	Drs Paul Harvey and Belinda Stummer