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Enhancing sugarcane growth and yield by biocontrol agents/biofertilizers

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Section 1: Executive Summary

Australian sugarcane industry is facing significant challenges affecting sugarcane growth. Sugarcane diseases and soil degradation are the two major threats. Many sugarcane diseases are caused by pathogenic fungi and bacteria. In order to protect sugarcane against these diseases, enormous efforts have been given on the development and application of chemical-based pesticides. The use of microorganism-based biocontrol agents to protect plant crops attracts increasing research interests due to the environmentally friend nature of the agents. In addition, many biocontrol agents also promote plant growth through enhancing nutrient uptake and improving solid quality.

In this project, a number of microbial strains were screened in terms of their biocontrol activities against two sugarcane pathogens. Growth of two of the identified strains on sugarcane processing by-products (sugarcane bagasse and molasses) was demonstrated in order to produce liquids having biocontrol activities. The results show that it is possible to use these liquid products as biocontrol agents though further optimisation of the production process is required to improve the biocontrol activities. The microorganisms identified in this project have not been previously reported as biocontrol agents. Further development of these biocontrol agents may lead to new IP.

The project has addressed the Key Focus Area in SRA Strategic Plan – Pest, disease and weed management. Further research based on this project may lead to the development and adoption of SRA-developed packages for integrated management of key diseases. Biocontrol agents are environmentally friendly. The use of biocontrol agents will improve sugarcane growth and sugar production while manufacturing biocontrol agent will provide job opportunities in rural and regional areas.

Section 2: Background

A number of microorganisms like strains from *Trichoderma* and *Paneibacillus* can be used as both biocontrol agents and biofertilisers. For example, a Chinese group led by A/Prof Bai has found that a *Paneibacillus polymyxa* could be used as a biocontrol agent against some pathogenic fungi and bacteria (private communication) and as a biofertiliser to improve the growth of tea trees and both the yield and quality of tea.[1] In 2006, the applicant (Dr Zhang) and A/Prof Bai have been granted a patent on the production of *Trichoderma*-based biocontrol agent from solid state fermentation of grape marc and lees.[2]

Till now only a few strains have been tested against sugarcane diseases and/or used as biofertilisers. An Indian research group found that *Trichoderma harizianum* can protect 71% of cane from red rot whereas the control was only 21%.[3] The use of *Trichoderma* as biocontrol agent also increased both macro- and micro-nutrient uptake (N by 27%, P by 65%, K by 44%, Cu by 6%, Fe by 100%, Mn by 79%, Zn by 55% and organic-C by 56%). It was found that *Pantoea dispersa* had biocontrol efficiency of 81%-100% against leaf scald disease.[4] Recently, a project funded by Queensland Government has led to a discovery of a new species of *Burkholderia,* a nitrogen fixation strain, from sugarcane roots although its biocontrol activity is still unknown.[5]

Sugarcane industry produces large amounts of sugarcane bagasse and molasses, which are low cost carbon sources for value-added products by microorganisms. In order to reduce the biocontrol agents production cost, it is important to grow use readily available low cost sugarcane by-products as substrates.

References: (1) S. J. Xu, et. al.,, *Sci Rep*, 2014, 4; (2) Z. H. Bai, et al., *Chinese Patent*, 2006, CN1311068 C; (3) V. Singh, et al., *J Hortic For* 2010, 2, 66-71; (4) L. Zhang and R. G. Birch, *J Appl Microbiol*, 1997, 82, 448-454; (5) C. Paungfoo-Lonhienne, et al., *Microb Biotechnol*, 2014, 7, 142-154.

Section 3: Outputs and Achievement of Project Objectives

Project objectives

The project objectives include (1) identification of potential biocontrol microorganisms, and (2) preliminary investigation of the production of biocontrol microorganisms using sugar mill by-products.

These project objectives have been achieved and the detailed methodology, results and discussion are described as follows.

Methodology

Materials

Two common sugarcane fungal pathogens - *Glomerella tucumanensis* (Gt) and *Ceratocystis paradoxa* (Cp) were kindly provided by Dr Barry Croft and his team (Dr Priyanka Wickramasinghe and Mr Andrew Greet) at SRA. The names of the diseases caused by these pathogens are red rot disease (Gt) and pineapple disease (Cp), respectively. 16 *Trichoderma* strains (filamentous fungi) were purchased from CSIRO Food Fungal Culture Collection and 5 *Paenibacillus* strains were purchased from ATCC, US. Table 1 lists the details of these strains.

Table 1. List of the tested *Trichoderma* and *Paenibacillus* strains.

No.	***Trichoderma***
1	*T. virens* FRR 0419
2	*T. viride* FRR 1544
3	*T. viride* FRR 1565
4	*T. harzianum* FRR 2504
5	*T. harzianum* FRR 2514
6	*T. harzianum* FRR 2533
7	*T. harzianum* FRR 4428
8	*T. virens* FRR 4485
9	*T. atroviride* FRR 4510
10	*T. hamatum* FRR 4512
11	*T. harzianum* FRR 4513
12	*T. koningii* FRR 4514
13	*T. longibrachiatum* FRR 4515
14	*T. pseudokoningii* FRR 4516
15	*T. reesei* FRR 4517
16	*T. saturnisporum* FRR 4518
No.	***Paenibacillus***
1	*P. polymyxa* ATCC 12712
2	*P. polymyxa* ATCC 15970
3	*P. polymyxa* ATCC 21830
4	*P. polymyxa* ATCC 31037
5	*P. polymyxa* ATCC 39564

Sugarcane bagasse and molasses used for cultivation of selected *Trichoderma* and *Paenibacillus* strains were collected from Racecourse Sugar Mill (Mackay).

Screening of biocontrol agents

For antagonistic test with *Triochoderma* strains, dual culture method was used to assess their antagonistic activities against sugarcane pathogens. Both *Trichoderma* strains and fungal pathogens were firstly propagated on potato dextrose agar (PDA) plates at 28 °C for 5 days. The propagated *Trichoderma* strains and fungal pathogens were either directly used for dual culture or stored at 4 °C for later use (no more than 2 weeks).

Agar disc (5 mm) of each *Trichoderma* and each pathogen were taken from the propagation plates and placed onto a fresh PDA plate opposite to each other. Depending on the experiment design, the two discs may be placed onto the fresh agar plate at the same time or at different time. The antagonistic activity assessment was based on (1) the extent of inhibition and (2) days of *Trichoderma* to fully over-grow pathogens. In terms of the extent of inhibition, the radius of the pathogen colony in the direction of the antagonist colony (R2) and the radius of the pathogen colony in the control plate (R1) were used to calculate the percentage inhibition of radial growth (PIPG) using the following formula: PIRG=(R1-R2)/R1 × 100.

For antagonistic test with *Paenibacillus*, co-cultures of 5 *Paenibacillus* strains with each of the 2 pathogens were conducted. *Paenibacillus* strains were firstly propagated on PDA agar at 28 °C for 3 days. Inoculation of *Paenibacillus* to a fresh PDA was conducted by dipping the *Paenibacillus* colony on the propagation plate followed by piecing the side of the fresh PDA plate with the tip. A pathogen disc (5 mm) was placed onto the centre of the fresh PDA plate before or after the growth of *Paneibacillus* strains depending on the types of pathogens. The assessment was based on the size of the inhibition zones. Repeated cultures were conducted with both *Trichoderma* and *Paenibaccillus*. Figure 1 shows the schematic diagram on how the strains were allocated on the plates.

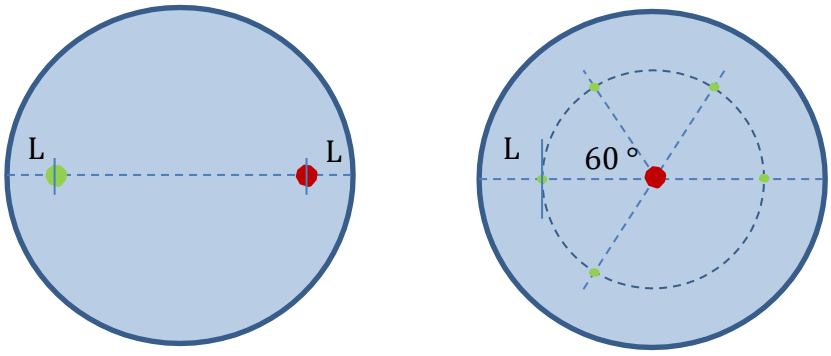

Figure 1. Schematic diagram of strain allocations (red, pathogen strain; green, test biocontrol strain; left, *Trichoderm* vs pathogen; right, *Paenibacillus* vs pathogen).

Antagonistic test with microbial metabolites

Microbial metabolites were prepared by cultivation of selected strains on molasses and processed sugarcane bagasse media. For sugarcane bagasse, 20 kg of raw bagasse (~50% moisture) were firstly pretreated with dilute sulphuric acid in a pilot scale horizontal reactor at QUT Mackay Renewable Biocommodities Pilot Plant. The pretreatment conditions were as follows: a liquid/solid (w/w) ratio of 6, a temperature of 170 °C, a reaction time of 15 min and an acid loading of 2.4 wt% on fibre. After pretreatment, the solid residue was collected and washed with distilled water. The washed solid residue was enzymatically hydrolysed to produce glucose-rich solution at 50 °C for 72 h with a glucan loading of 5% and a cellulase (Accellerase™ 1500) loading of ~20 FPU/g glucan. At the end of hydrolysis, the solid residue and liquid were separated by vacuum filtration. The glucose-rich liquid was collected. Molasses and enzymatic hydrolysis liquid of sugarcane bagasse were diluted to 30 g/L glucose equivalent, respectively, with the addition of 2 g/L $(NH_4)_2SO_4$ and 0.5 g/L KH_2PO_4 to prepare cultivation media. The media were autoclaved at 121 °C for 15 min prior to the inoculation of microorganisms.

One selected *Trichoderma* strain (No. 3, *T. viride* FRR 1565) and one selected *Paenibacillus* strain (No. 3, *P. polymyxa* ATCC 21830) were cultivated in 250 shake flasks containing 50 mL of bagasse-derived glucose-rich media and molasses media. For the growth of *T. viride* FRR 1565, 1 mL of spores solution were inoculated to the cultivation media to start the cultivation with a spores concentration of 2 X 10^5/mL. For the *Paenibacillus* strain, a inoculation loop of *P. polymyxa* was transferred to the growth media to start the cultivaiton. After 72 h cultivation, microbial biomass and cultivation broth were separated by filtration with sterile syringe discs for the cultivation with bagasse-derived media and sterile centrifugation for the cultivation with molasses media since the filtration rate with molasses media was too slow.

The collected cultivation broth was diluted 2 times, 5 times and 10 times. 1 mL of the original bagasse-derived medium or molasses (30 g/L sugar equivalent) medium (controls), original cultivation broth and diluted fermentation broths were mixed with 20 mL molten PDA, respectively, and poured into Petri dishes.

Agar discs (5 mm) of each of the two sugarcane disease strains were transferred on the center of fresh agar plates with and without metabolites. The pathogens on agar plates were incubated at 28 °C for 1 – 4 days. Pictures are taken during incubation. The linear growth of mycelia was measured and the percentage of inhibition was estimated by the following equation: % inhibition = (D1-D2)/D1X100, where D1 was the diameter of the radial growth in control and D2 was the diameter of the radial growth in test Petri dish.

Results and discussion

Screening of Trichoderma strains by dual culture with Cp and Gt

Two batches of dual culture experiment were conducted to test the antagonistic activities of *Trichoderma* strains against *Cp*. Table 2 summarises the observations on the antagonistic activities of *Trichoderma* against Cp for Batch 1 and Batch 2. It was found that the extents of antagonistic activities varied among strains and between batches. *Trichoderma* No. 9 and 10. were the most effective strains because they had the highest PIRGs and shortest times to over-grow on Cp. The other three *Trichoderma* strains showing high antagonistic activities (both inhibition and overgrowth) were *Trichoderma* No. 3, 6 and 13. The rest of the strains were considered less effective because of their low PIRGs and/or no (or less significant) overgrowth on Cp. Figure 2 shows the images of the dual culture of *Trichoderma* No. 9 and Cp at day 2 and day 5.

Table 2. Antagonistic activities of *Trichoderma* strains against pineapple disease pathogen (Cp).

No.	*Trichoderma*	PIRG		Days to full overgrowth	
		Batch 1	Batch 2	Batch 1	Batch 2
1	*T. virens* FRR 0419	58.9	57.8	-	7
2	*T. viride* FRR 1544	58.9	55.6	8	-
3	*T. viride* FRR 1565	**63.3**	**56.7**	**8**	**7**
4	*T. harzianum* FRR 2504	48.9	52.2	8	-
5	*T. harzianum* FRR 2514	53.3	55.6	-	-
6	*T. harzianum* FRR 2533	**60.0**	**55.6**	**8**	**7**
7	*T. harzianum* FRR 4428	58.9	45.6	8	-
8	*T. virens* FRR 4485	58.9	57.8	-	-
9	*T. atroviride* FRR 4510	**64.4**	**61.1**	**6**	**5**
10	*T. hamatum* FRR 4512	**63.3**	**61.1**	**6**	**5**
11	*T. harzianum* FRR 4513	47.8	45.6	-	-
12	*T. koningii* FRR 4514	50.0	50.0	-	-
13	*T. longibrachiatum* FRR 4515	**58.9**	**55.6**	**8**	**7**
14	*T. pseudokoningii* FRR 4516	58.9	56.7	-	-
15	*T. reesei* FRR 4517	60.0	55.6	-	-
16	*T. saturnisporum* FRR 4518	53.3	54.4	-	-

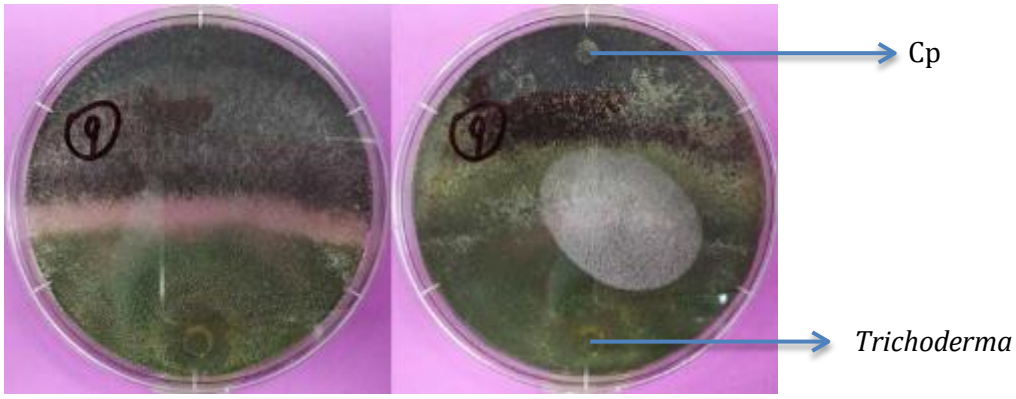

Figure 2. Dual cultures of *Trichoderma* No. 9 and Cp at day 2 (left) and day 5 (right).

A total of three batches of dual culture trials were conducted to test the antagonistic activities of *Trichoderma* strains against Gt. The first batch was conducted with the co-cultivation of *Trichoderma* strains and Gt on the same agar plates at the same time. However, the first batch failed due to the slow growth of Gt. In the second batch, Gt was cultivated for 3 days followed by the inoculation of *Trichoderma* strains. Clear inhibition zones were observed between *Trichoderma* strains and Gt. It was found that Gt still grew very slowly. After 7 days of dual cultivation (10 days for Gt), only the hyphae of *Trichoderma* No.3 and 4 crossed the inhibition zones and contacted Gt, indicating their antagonistic activities against Gt.

It was speculated that the very slow growth of Gt was possibly due to the use of stored Gt (4 °C on agar plate for 1 week). Therefore, a third batch of dual culture of *Trichoderma* strains and freshly prepared Gt was conducted. Fresh Gt discs were directly inoculated on PDA plates and were incubated for 3 days, followed by inoculation of *Trichoderma* strains. The growth of Gt in the third batch was significantly improved. It was found that the growth of Gt and most of the *Trichoderma* strains almost stopped after dual culture for 4 days and only *Trichoderma* No. 3 partly over-grew on Gt. These results confirmed the antagonistic activity of *Trichoderma* No. 3 but also indicate that Gt may be more difficult to control by biocontrol agents compared to Cp. Figure 3 shows the dual cultures of *Trichoderma* No. 3 and Gt at day 2 and day 4. Based on the three batches of dual culture trials with Gt, it was concluded that *Trichoderma* No. 3 had antagonistic activity against Gt.

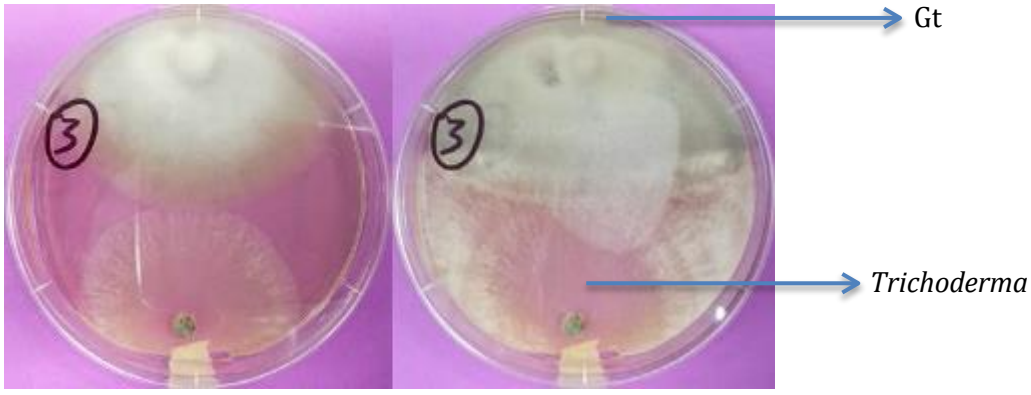

Figure 3. Dual culture of *Trichoderma* No. 3 and Gt at day 2 (left) and day 4 (right).

Screening of Paenibacillus strains by co-culture with Cp and Gt

In order to test the antagonistic activities of *Paenibacillus* strains against *Cp*, each of the *Paenibacillus* strain was firstly cultivated on a fresh PDA plate for 2 days, followed by the inoculation of a Cp disc in the centre of the PDA plate to start the co-culture. However, in terms of the antagonistic activity test against Gt, the first two batches failed due to the slow growth of Gt. The third batch test was conducted with the freshly prepared Gt. A Gt disc was placed to a PDA plate and was cultivated for 3 days followed by the inoculation of *Panebacillus* for co-culture. Table 3 summarises the observation after 2 days co-culture. The results showed that *Paenibacillus* No. 3 and 4 had the highest antagonistic activities against both pathogens, followed by that *Paenibacillus* No. 3. Figure 4 shows the images of co-cultures of *Paenibacillus* strains with pathogens on the PDA plates.

Table 3. Sizes of inhibition zones of *Paenibacillus* strains against pineapple disease (Cp) and red rot (Gt) pathogens after 2 days co-cultivation.

No.	*Paenibacillus*	Cp plate 1 (mm)	Cp plate 2 (mm)	Gt plate 1 (mm)	Gt plate 2 (mm)	Gt plate 3 (mm)
1	*P. polymyxa* ATCC 12712	6	8	0	0	0
2	*P. polymyxa* ATCC 15970	0	0	0	0	0
3	*P. polymyxa* ATCC 21830	12	12.5	5	6	5
4	*P. polymyxa* ATCC 31037	12	11.5	5	6	5
5	*P. polymyxa* ATCC 39564	7.5	5	5	7	4

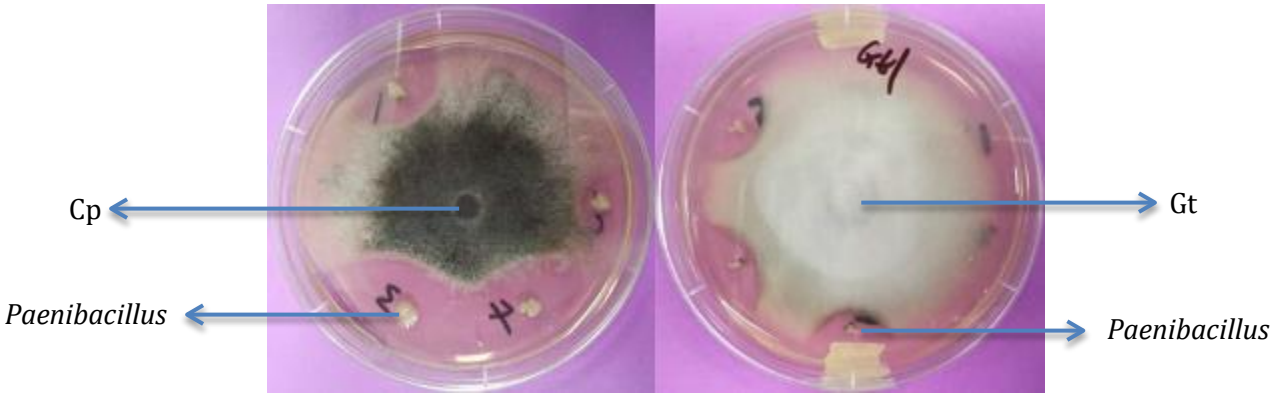

Figure 4. Co-culture of *Paenibacillus* strains with pineapple disease (Cp, left) and red rot (Gt, right) pathogens at day 2.

Antagonistic test with Trichoderma metabolites

T. viride FRR 1565 was selected to produce metabolites for antagonistic test since this strain showed antagonistic activities against both sugarcane pathogens in the screening trial. The strain grew well with both bagasse-derived and molasses media. The 72 h biomass concentrations with the two media reached 4.0 g/L and 6.4 g/L, respectively.

However, the dual culture test showed that both bagasse hydrolysate and molasses broths did not have antagonistic activities against both Cp and Gt. Figure 5 shows the growth of Cp on undiluted molasses cultivation broth in comparison with control. Cp grew rapidly and after 3 days of cultivation, Cp completely grew over the petri dishes.

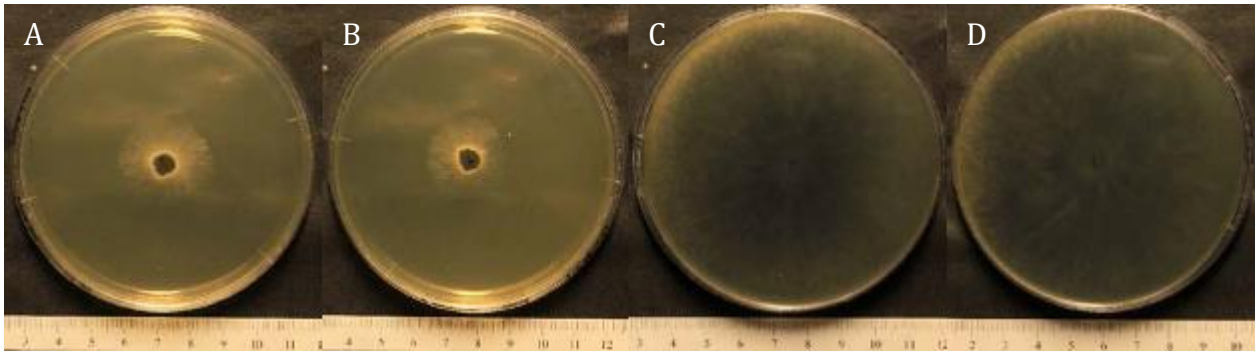

Figure 5. Growth of Cp on day 1 (A and B) and day 3 (C and D) with molasses medium. A and C, control; B and D, undiluted cultivation broth.

Antagonistic test with Paenibacillus metabolites

P. polymyxa ATCC 21830 was selected to produce metabolites for antagonistic test, which was one of the two *Panenibacillus* strains having strongest antagonistic activities against both Cp and Gt (Table 3). The growth of *P. polymyxa* ATCC 21830 with bagasse-derived medium was poor and the biomass concentration was less than 1.4 g/L after 72 h cultivation. The cultivation broth of bagasse-derived medium did not show the antagonistic activity against both Cp and Gt pathogens.

The growth of *P. polymyxa* ATCC 21830 with molasses medium was better and the bacterial biomass reached ~0.8 g/L after 72 h cultivation. The undiluted cultivation broth showed obvious antagonistic activities against both Cp and Gt as shown in Figure 6 and Figure 7, respectively. However, diluted cultivation broths did not show the antagonistic activities against both pathogens.

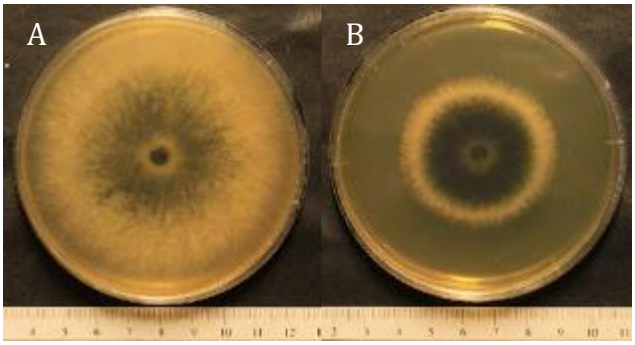

Figure 6. Growth of Cp on molasses medium. A, control (unused medium); B, undiluted cultivation broth.

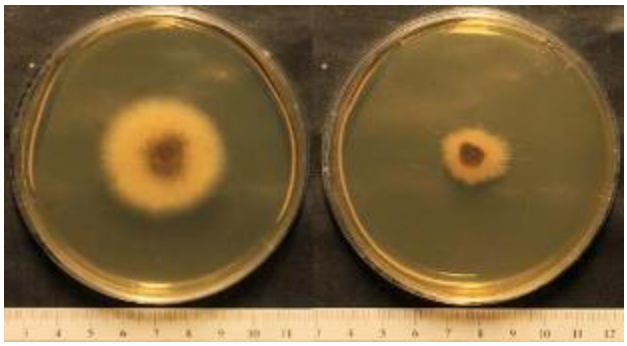

Figure 7. Growth of Gt on molasses medium. A, control; B, undiluted cultivation broth.

Table 4 shows the antagonistic activities of molasses broth following cultivation of *P. paenibacillus* ATCC 21830. The cultivation broth inhibited the growth of Cp (48 h) and Gt (96 h) by 42.7% and 52.6%, respectively.

Table 4. Antagonistic activity of molasses cultivation broth against Cp and Gt.

Pathogen	Radial growth diameter, cm		Inhibition, %
	Control	Test	
Cp at 48 h	8.2 ± 0.1	4.7 ± 0.0	42.7
Gt at 96 h	3.8 ± 0.0	1.8 ± 0.1	52.6

Discussion

Trichoderma strains are the most studied microorganisms as biocontrol agents. In this study, among the tested *Trichoderma* strains, only *T. harzianum* FRR 2533 (IMI 206050) has been previously reported for its antagonistic activity. However, the screening trial showed that this strain was not the most effective one against the tested pathogens. The results showed there were more effective biocontrol agents in terms of their antagonistic activities against the two tested sugarcane fungal pathogens. *T. atroviride* FRR 4510 and *T. hamatum* FRR 4512 were the two most effective biocontrol agents against pineapple disease pathogen while *T. viride* FRR 1565 showed antagonistic activity against both pineapple disease and red rot pathogens. The strain *T. viride* FRR 1565 grew with both bagasse hydrolysate and molasses media. However, the cultivation broths did not show antagonistic activities against both pathogens. It has been reported that cell wall degrading enzymes such as cellulase and pectinase play a significant role in controlling plant fungal pathogens. These enzymes are induced in the presence of corresponding carbon sources like cellulose and pectin. Cultivation of *T. viride* FRR 1565 was conducted on simple sugars (bagasse-derived glucose rich medium and sucrose-rich molasses medium) in this study, which might not induce the production of sufficient levels of cell wall degradation enzymes. As a result, the cultivation broth did not show the antagonistic activity against the two fungal pathogens.

Two bacterial strains *P. polymyxa* ATCC 21830 and ATCC 31037 showed strongest antagonistic activities against the two pathogens among the five tested bacterial strains. The broth of *P. polymyxa* ATCC 21830 cultivation on molasses had obvious antagonistic activities against the two pathogens. However, no obvious antagonistic activity was observed with the diluted molasses cultivation broth and the bagasse-derived cultivation broth, which was possibly due to the low levels of metabolites. It has been reported that some *P. polymyxa* strains can produce antibiotic compounds like polymyxins and fusaricidins. In addition, these strains may also produce cell wall degradation enzymes like cellulase and pectinase. The antagonistic activity of *P. polymyxa* ATCC 21830

is likely associated with these metabolites. In addition, some *P. polymyxa* strains are known to produce high value polysaccharide. For example, it has been reported that *P. polymyxa* ATCC 21830 produces polysaccharide curdlan, a gelling agent used in the food, construction and pharmaceutical industries.

Section 4: Outputs and Outcomes

Outputs

- Several *Trichoderma* strains and a couple of *Paenibacillus* strains having antagonistic activities against two sugarcane pathogens were identified.
- Growth of some of these selected strains was demonstrated with sugarcane bagasse and molasses media.

Outcomes

- In the long term, further development and application of biocontrol agents will bring significant environmental, economic and social benefits to the Australian sugarcane industry and society through the reduction of chemical pesticide usage, improvement of sugarcane and sugar yields and increasing job numbers from manufacturing of biocontrol agents, respectively.

Section 5: Recommendations and Future Industry Needs

Based on the observations and findings of this study, it is recommended to focus on the following aspects for future studies:

1. Identification of key metabolites produced by the selected *Paenibacillus* strains. Some of these metabolites may also be high value antibiotics. Since the selected *Paenibacillus* strains have not been reported regarding their antagonistic activity and antibiotics production capacity, the research in this field is likely to generate new IP.
2. Optimisation of cultivation conditions to improve the metabolite production by the selected *Paenibacillus* strains. This study has shown that the antagonistic activity of the molasses cultivation broth is very significant although the biomass concentration is only ~0.8 g/L. It is expected that much stronger antagonistic activity can be achieved with the optimisation of cultivation conditions to improve biomass concentration and metabolite production.
3. Growth of Trichoderma using partially hydrolysed sugarcane bagasse to produce antagonistic agent. This will lead to the production of cell wall degradation enzymes and improve the antagonistic activity of Trichoderma strains.
4. Testing the antagonistic activities of the identified strains against a broader range of sugarcane pathogens. Despite the antagonistic activities of the identified strains against the two tested sugarcane pathogens, it is worth testing against a broader range of sugarcane pathogens like smut disease (caused by *Sporisorium scitamineum*) and pachymetra root rot (caused by *Pachymetra chaunorhiza*) pathogens. However, some of these pathogens can not grow easily on agar plates and direct pot trials are recommended. In addition, biocontrol agents may also induce the resistance of plant against pathogens, which can not be tested with dual culture or co-culture trials and has to be studied in pot and field trials.
5. Field trials required for verification of antagonistic activities of the identified strains. The conditions of laboratory trials are significantly different from the sugarcane field conditions. Therefore, more trials are required to verify the antagonistic activities of selected strains under field conditions.