Studies in the ecology and biocontrol of *Burkholderia pseudomallei*

Thesis submitted by

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ETHICS / SAFETY DECLARATION

All experimental work with *B. pseudomallei* was carried out in Australia at James Cook University. The Australian/New Zealand Standard 2243.3:2010 (Safety in Laboratories) was adhered to, with work on live organisms carried out in a BSL3 laboratory to BSL3 standard. All protocols were approved by the Institutional Biosafety Committee and biological samples were handled in a C2 cabinet. Confirmation of sterilization was carried out on duplicate samples prior to any processed, killed organism being removed from the laboratory to a BSL2 laboratory. Human and animal ethics were not requied.

STATEMENT ON THE CONTRIBUTION OF OTHERS

Nature of Assistance	Contribution	Names and Affiliations of Co-Contributors
Intellectual support	Proposal writing	Dr Jennifer Elliman, Assoc Prof Jeffrey Warner, conceptual encourage
	Statistical support	Assoc Prof Leigh Owens, Suggestion on critical point
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Data collection	Plant identification and native seed collecion	Mr Christopher Gardiner and me ut some case confirm with Ms Nanette Hooker

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ABSTRACT

Burkholderia pseudomallei is a saprophytic bacterium that causes melioidosis, the most common cause of fatal bacterial community acquired pneumonia in the tropics. The disease is endemic in North Queensland and has been studied extensively, yet the ecology of the bacterium remains uncertain. The organism must persist and multiply in the environment to maintain a clinically significant reservoir, This study examined the questions of whether *B. pseudomallei* was maintained in plants in an endemic region, whether local and imported rice species could be infected in the laboratory, and if so what effect the bacterium had on the growth of the plants and whether biocontrol agents could decrease bacterial loads of *B. pseudomallei* in a plant infection model.

Two rice models of infection with *B. pseudomallei* were developed, a domestic rice grown in Australia which was then examined by qPCR and fluorescent antibody microscopy and a native (wild) rice found in the local endemic region, also examined using qPCR. Rice cultivar differences in susceptibility to *B. pseudomallei* and near-neighbor species were identified with another cultivar of rice identified as resistant to any growth inhibition.

Two possible agents, a bacteriocin-like compound derived from a Papua New Guinea strain of *Burkholderia ubonensis* and a bacteriophage cocktail were developed in two different rice species models of infection (one native and one introduced rice species) and a biofilm model. The first biocontrol candidate, a previously reported bacteriocin-like compound extracted from *B. ubonensis*, was partially purified and chacterised. The second biocontrol agent was a bacteriophage cocktail developed from previously isolated bacteriophages at James Cook University, which were screened for activity against *B. pseudomallei* isolates of interest, amplified, measured by transmission electron microscopy, quantified and combined. Individual phage samples were all partially effective against the bacterial isolate of interest. However, on combination they completely cleared growth on bacterial lawns. Both the bacteriocin-like compound and the bacteriophage cocktail were equally able to significantly inhibit biofilm

formation in a 96 well plate model over 24 hr. The bacteriocin was less effective by 48 hr, while the bacteriophage cocktail, due to self replication activity, maintained its ability to keep the biofilm from growing. As the bacteriocin lost activity over time, it was not used directly in the rice models and *B. ubonensis* was used instead to identify whether interaction with *B. pseudomallei* would result in less inhibition of growth. *B. ubonensis* did not act as an effective biocontrol agent, probably because it was also somewhat inhibitory at the load applied. The bacteriophage cocktail was effective, with significant increases in growth in treatment groups relative to infected groups. The domestic rice model of infection produced partial recovery of infection load while the wild rice model of infection produced complete recovery and a two log drop in bacterial load. The wild rice species likely has a long exposure to *B. pseudomallei* and has natural defences which, in combination with phage, are able to control the high *B. pseudomallei* infection.

A spatial / botantical analysis of Castle hill in the dry season (a previously described melioidosis-endemic site in Townsville, Queensland) identified particular plant species most commonly found near *B. pseudomallei* positive soil, however a wet season analysis failed to find *B. pseudomallei* in roots, although it was found in low levels around the roots and at increased numbers at 10 and 30 cm below ground level. Low exposure and natural resistance of native species to *B. pseudomallei*, or simply infection below detection limits are probable explanations and it is likely that at this endemic site, plants may play less of a role in survival of *B. pseudomallei* than does the physio-chemical attributes of soil.

This study has developed rice models of *B. pseudomallei* infection and used these to examine the viability of a biocontrol approach with two different types of biocontrol agents. This approach has shown promise in the laboratory. In addition, this study examined the role played by plants in the ecology of melioidosis in an endemic region and how those plants related to *B. pseudomallei* presence in the soil.

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LIST OF ABBREVIATIONS

<	less than
>	greater than
°C	degree Celsius
^{o}N	north latitude
^{o}S	south latitude
µl	micro litre
µm	micro meter
AHLs	N-acyl-homoserine lactones
APS	Ammonium persulfate
ATP	adenosine triphosphate
ANOVA	analysis of variance
B. cepacia	*Burkholderia cepacia*
B. cenocepacia	*Burkholderia cenocepacia*
B. pseudomallei	*Burkholderia pseudomallei*
B. thailandensis	*Burkholderia thailandensis*
B. ubonensis	*Burkholderia ubonensis*
B. vietnamiensis	*Burkholderia vietnamiensis*
BLIS	bacteriocin-like inhibitory substance
bp	base pair
BPSA	*Burkholderia pseudomallei* selective agar
BSA	bovine serum albumin
CD	cluster of differential
CF	cystic fibrosis
CFU	colony forming unit
CI	confidence interval
CLSM	confocal laser scanning microscopy
cm	centimeter
df	degree of freedom
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EM	effective microorganisms
FISH	fluorescent in situ hybridization
g	gram
g	gravity
G-CSF,	granulocyte colony-stimulating factor
GIS	geographic information system
hr	hour(s)
i.n.	Intranasal
i.p.	intraperitoneal
i.v.	Intravenous
IFA	immunofluorescence assay
IFN-γ	Interferon gamma
Ig	immunoglobulin
IHA	Indirect haemagglutination
IPTG	isopropyl-β-d-thiogalactopyranoside

kDa	kilodalton
l	liter
LB	Luria Bertani
LPS	Lipopolysaccharides
m	meter
mg	milligram
min	minute
ml	millilitre
mm	millimeter
MPN	most probably number
MLST	multi locus sequence typing
NCTC	the national collection of type cultures
nm	nanometer
O.C.T.	optimum cutting temperature
O. australiensis	*Oryza australiensis*
O. meridionalis	*Oryza meridionalis*
O. rufipogon	*Oryza rufipogon*
OD	optical density
p	probability
P. aeruginosa	*Pseudomonas aeruginosa*
P. pseudomallei	*Pseudomonas pseudomallei*
PBS	phosphate buffered saline
pfu	plaque forming unit
PC 2	the physical containment level 2
PC 3	the physical containment level 3
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PGP	phosphatidylglycerol phosphatase
PHB	poly-b-hydroxybutyrate
PNG	Papua New Guinea
PRRs	pathogen recognition receptors
qPCR	quantitative polymerase chain reaction
QS	quorum sensing
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
spp	species
TAE	tris acetate
TBSS	threonine-basal salt solution
T.E.M	transmission electron microscopy
TEMED	tetramethylethylenediamine
TLR	toll like receptor
TTSS	type three secretion system
UMB	ultramicrobacteria
UV	ultraviolet
VBNC	viable but non culturable

VOCs	volatile organic compounds
XGaI	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
ZOI	zones of inhibition

CHAPTER 1: INTRODUCTION

Burkholderia pseudomallei is a Gram negative bacteria that is the causative agent of melioidosis, a disease of the tropics. *Burkholderia pseudomallei* has been recognized as an antibiotic resistant organism with a high fatality rate (White, 2003; Wiersinga *et al.*, 2012). One high risk factor of *B. pseudomallei* infection is environmental exposure (Warner *et al.*, 2008; Baker *et al.*, 2011b). *Burkholderia pseudomallei* is often isolated in rice field soil (Rattanavong *et al.*, 2011) and recently, *B. pseudomallei* has been isolated from exotic plants in the Northern Territory in Australia (Kaestli *et al.*, 2012). An endemic site in Townsville (Queensland, Australia) has also been identified, with links between human infections and proximity to the area (Baker *et al.*, 2011b), although studies of plants in this area have not been done. Bacteriocin and bacteriophage with biological control activities against *B. pseudomallei in vitro* have been reported, including some which were isolated from the Papua New Guinea and Townsville endemic site (Marshall *et al.*, 2010; McRobb, 2010). Despite this, little attention has been paid to testing activity against these agents againist *B. pseudomallei in vivo* in biocontrol capacity.

Is there a relationship with survival of *B. pseudomallei* in particular environments and the plants present? Can a biological control approach be used to limit *B. pseudomallei* in plants and therefore control it in endemic areas? The aim of this thesis was to investigate the effectiveness of biological control agents to control *B. pseudomallei* infection in plants and to further characterise the endemic site in Townsville. These aims were achieved by first developing rice models of environmental infection, including protocols to identify the location of the *B. pseudomallei.* With a model available, potential biological control agents including both a previously reported bacteriocin-like compound (Marshall *et al.*, 2010) and previously isolated bacteriophage (McRobb, 2010), unpublished data, were developed. These were assessed for effectiveness in a biofilm model while the bacteriophage and the bacteria producing the bacteriocin-like compound were assessed in plant models. Finally, a comparative study was carried out between a known melioidosis-endemic site

(Townsville: Castle Hill) againist a reported negative site, which included plant diversity and presence of *B. pseudomallei* in plants and soil.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Melioidosis is caused by *B. pseudomallei*, which is a saprophytic pathogen and distributed in tropical zones (Smith *et al.*, 1987). melioidosis incidence is significantly increased in North Queensland when the monsoon season begins, especially in Townsville (Hanna *et al.*, 2010) as well as in Darwin in the Northern Territory (Currie and Jacups, 2003; Parameswaran *et al.*, 2012). Populations, especially in developing countries and remote areas (Warner *et al.*, 2008; Limmathurotsakul and Peacock, 2011; Rac and McLaughlin, 2013), have an increased risk to contracting *B. pseudomallei* because it has been persistent in the environment for a long period (Baker *et al.*, 2011a).

The impact on humans of melioidosis remains a cause for concern. Firstly, there is a high fatality rate of melioidosis in patients who have developed septicaemias (Leelarasamee, 2004) as there is no vaccine licensed to control disease (Scott *et al.*, 2013). Secondly, melioidosis is a great mimicker, which presents an array of clinical signs and symptoms (Wiersinga *et al.*, 2006). Thirdly, *B. pseudomallei* is difficult to treat due to it being an intracellular organism, which survives and multiplies in phagocytic cells (Adler *et al.*, 2009) and is inherently resistant to the antibiotics typically used for community acquired pneumonia and sepsis (White, 2003; Wiersinga *et al.*, 2012). Fourthly, formation of biofilms is suggested as a possible cause for antimicrobial resistance and chronic infection (Vorachit *et al.*, 1993; Vorachit *et al.*, 1995). Fifthly, *B. pseudomallei* is an ideal agent for biowarfare and is designated a group B bioterrorism agent (Cheng *et al.*, 2005; Pappas *et al.*, 2006). Finally, high isolation of *B. pseudomallei* in soil and water are known to correlate with a prevalence of melioidosis (Vuddhakul *et al.*, 1999; Thaipadungpanit *et al.*, 2014).

The genus *Burkholderia* has a wide range of ecological niches with most research focused on the pathogenic species (clinical importance) and ecology in soil and water

(Suarez-Moreno *et al.*, 2012). Due to its disease causing potential, bioterrorism status and persistence in the environment, *B. pseudomallei* has been extensively studied in recent years (Gilad *et al.*, 2007; Wiersinga *et al.*, 2012). *Burkholderia pseudomallei* is considered saprophytic in normal environmental niches and is found in cultivated and irrigated agricultural sites in Southeast Asia (Dance, 2000). Undisturbed soil in grassy areas has been associated with *B. pseudomallei* as has disturbed soil associated with livestock animal in Australia (Kaestli *et al.*, 2009). Mayo *et al.* (2011) found one third of unchlorinated bore water sites tested in Northern Australia to be positive for *B. pseudomallei.* One of the isolates found was also a sequence type match to a clinical sample. Of even more concern for public health was when *B. pseudomallei* was found in tap water in Northeastern Thailand (Limmathurotsakul *et al.*, 2014).

Biological control, in which live organisms are used to reduce pathogen bacterial loads (Eilenberg *et al.*, 2001), could be a tool to control persistence of *B. pseudomallei* in the environment. Some agents are currently being researched for new ways to approach the *B. pseudomallei* problem. For example, natural enemies for *B. pseudomallei* could be bacteriophages (McRobb, 2010; Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011) and bacteria producing antimicrobial substances (Marshall *et al.*, 2010; Lin *et al.*, 2011) which have been found to have potential to inhibit *B. pseudomallei* in the planktonic state. However, little work has been done to examine the biocontrol efficiency against *B. pseudomallei* in other presentations such as biofilms and plant infections, Thus, the purpose of this literature review is to review our current understanding of the ecology of *B. pseudomallei* and the possible devices for *B. pseudomallei* biocontrol.

2.2 History of *B. pseudomallei*

Burkholderia pseudomallei was first described by Captain Whitmore (Figure 2.1) in 1911 from the body of an opiate addict in Rangoon, Burma (Whitmore, 1913). He isolated a Gram negative bacillus which then produced luxuriant growth on peptone agar, and a wrinkling colony on glycerin agar, appeared on a gelatin stab culture, displayed long filamentous bacilli on salt agar and was motile from post-mortem

examination; all this suggested that it was a new disease and it was named Glander like disease (Whitmore, 1912). In Kuala Lumpur, 1913, several animals were affected by a severe distemper like illness, which was recognized as melioidosis several years later (Jayaram, 2005).

The word melioidosis which combines the Greek word Melis and edios, meaning distemper of asses and resemblance, respectively, was defined by Stanton and Fletcher (1932), according to (Jayaram, 2005). Numerous nomenclature changes have occurred, resulting in the names *Bacterium whitmori, Bacillus whitmori, Pfeifferella whitmori, Pfeifferella pseudomallei, Actinobacillus pseudomallei, Lofflerella whitmori, Flavobacterium pseudomallei, Malleomyces pseudomallei, and Pseudomonas pseudomallei*, before being altered to *B. pseudomallei* (Vietri and Deshazer, 2007). *Burkholderia pseudomallei* is divided from Genus *Pseudomonas* by rRNA homologous group II (Yabuuchi *et al.*, 1992a). The genus *Burkholderia* is credited to Burkholder who first reported sour skin on an onion bulb caused by *Pseudomonas cepacia* (Burkholder, 1950).

The French and American militaries suffered infection with *B. pseudomallei* in the Indochina War and Vietnam War, according to Smith *et al.* (1987). In Australia, Cottew (1952) first reported that Whitmore's bacillus was isolated from sheep in 1949, near Winton, Queensland, whereas the first reported human case of melioidosis occurred in 1950 in Townsville, North Queensland (Rimington, 1962). Ashdown (1979) reported the development of a selective agar technique for *B. pseudomallei*, which was more useful than conventional methods for screening clinical specimens. Further, Leakey *et al.* (1998) suggested that the mice models BALB/c and C57BI/6 could be excellent for acute melioidosis and chronic human melioidosis respectively.

Figure 2.1: Alfred Whitmore (1876-1941) who first discovered *B. pseudomallei* from postmortem of an opiate addict at Rangoon, Burma (Dvorak *et al.*, 2005)

2.3 Taxonomy *B. pseudomallei*

Burkholderia. pseudomallei organisms are classified as: Kingdom *Procaryotae*, Phylum *Proteobacteria,* Class *Betaproteobacteria*, Order *Burkholderiales,* Family *Burkholderiaceae* and Genus *Burkholderia* (Garrity *et al.*, 2005). At present, the 16S rRNA sequences are tools of phylogenetic analysis for Genus *Burkholderia* differentiation. The characteristic of genus *Burkholderia* was well reviewed by Palleroni (2005) in Bergey's mannal of systemic bacteriology: *B. pseudomallei* is a saprophyte bacteria, with Gram negative straight or curved rods (0.5-1 X 1.5-4 μm). It is non spore forming, motile, catalase positive, has rough surface colonies and also has polar flagella that looks like a safety pin when Gram stained (Brindle and Cowan, 1951). They grow well in aerobic conditions (Wetmore and Gochenour, 1956), but can utilize nitrate under anaerobic conditions. Moreover, *B. pseudomallei* can utilize many organic compounds as energy sources (Redfearn *et al.*, 1966). For example, *B. pseudomallei* utilized a carbon reserve material, poly-b-hydroxybutyrate (PHB), for growth, (Stanier *et al.*, 1966).

2.3.1 Biogeography of *B. pseudomallei*

The major prevalence of melioidosis, is located between 20 ^{o}N and 20 ^{o}S (Smith *et al.*, 1987), and it is widely distributed in the tropical zone. High endemic areas are mainly recognized as Northeast Thailand, Northern Australia, Singapore and parts of Malaysia (Currie *et al.*, 2008). The endemic region includes India, Indochina region, Papua New Guinea and the Pacific nation of New Caledonia whereas the Caribbean, Central, South America countries and also West and East countries show sporadic instances of melioidosis. However, temporary outbreaks of melioidosis have appeared in France, Southeast Queensland and Southwest Western Australia, which are located outside the tropical zone (Figure 2.2).

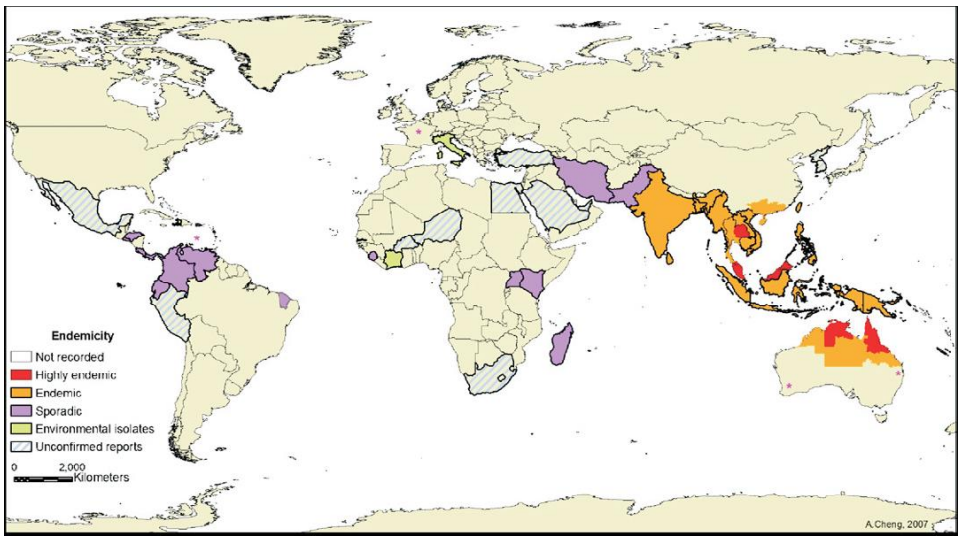


Figure 2.2: The global map, showing distributed locations of melioidosis along the tropical zone; asterisks indicate temporary outbreak in France, Southeast Queensland and Southwest Western Australia (Currie *et al.*, 2008).

Baker *et al.* (2011a) found that the isolation of Southeast Asia and Australia of *B. pseudomallei* are significant in differentiating two clusters in a phylogenetic tree and these have been persistent for a long period in the environment (Figure 2.3). The data of two clusters overly the biogeography separation. This evidence suggested that two cluster group have some relationship between biogeographic boundary and independent evolution of the organism.

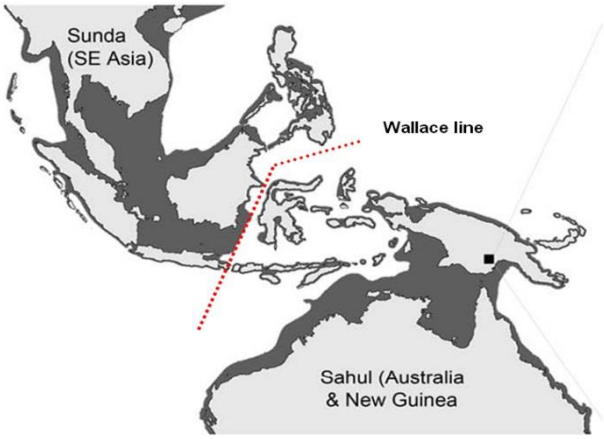


Figure 2.3: A shade of gray represents dry land during the last glacial era (21,500 years ago). Southeast Asia (Sunda) and Australia and Papua New Guinea (Sahul) was linked by land bridges (Baker *et al.*, 2011a). Wallace line separates terrestrial Asia from Australia (Barber *et al.*, 2000).

2.3.1.1 Biogeography in Southeast Asia

Burkholderia pseudomallei infection from Indonesia and the Philippines is rarely reported in world literature, however the majority of reports are from returning travelers (Leelarasamee, 2000). Although *B. pseudomallei* has been reported sporadically in Indonesia, after the tsunami disaster hit Banda Aches, four survivors were confirmed with infection of *B. pseudomallei* similar to reports in Thailand (Athan *et al.*, 2005; Chierakul *et al.*, 2005). A farmer was the first report for *B. pseudomallei* infection in the Philippines (Ereno *et al.*, 2002). In Singapore, the majority of *B. pseudomallei* infections are not associated with wet soil exposure and *B. pseudomallei* is isolated from only 1.8% of soil samples (Lo *et al.*, 2009).

On the other hand, Wuthiekanun *et al.* (2006) studied *B. pseudomallei* serology in an adult population at Myanmar and found that people are commonly exposed to this bacteria. *Burkholderia pseudomallei* are commonly isolated from soil samples in

Cambodia (Wuthiekanun *et al.*, 2008), Laos (Rattanavong *et al.*, 2011), Vietnam (Parry *et al.*, 1999), Malaysia (Strauss *et al.*, 1969) and Thailand (Vuddhakul *et al.*, 1999). In Thailand, Vuddhakul *et al.* (1999) found that the clinical incident of melioidosis is related to environmental prevalence in soil of *B. pseudomallei* and they have a spacial clustering of disease in the northeast (Table 2.1a and 2.1b).

Table 2.1: (a) Prevalence of *B. pseudomallei* isolated from soil samples (b) Incident of *B. pseudomallei* infection in humans (Vuddhakul *et al.*, 1999).

a)

Region	No. of soil samples	No. of positive samples	No. of collection sites	No. of positive sites
North	720	32 (4.4%)	180	25 (13.8%)
Central	196	12 (6.1%)	49	12 (24.5%)
Northeast	1,428	291 (20.4%)	357	179 (50.1%)
South	1,241	73 (5.9%)	310	57 (18.4%)

b)

Region	Total no. of inpatients	Patients infected with *B. pseudomallei*	Infection rate per 100,000 inpatients
North	609,775	110	18
Central	946,353	127	13.4
Northeast	761,562	1,050	137.9
South	458,743	66	14.4

2.3.1.2 Biogeography in Australia

As described above (Section 2.3) *B. pseudomallei* is prevalent within 23°S of the equator (The latitude of the tropics). Southwest Western Australia, Brisbane, Alice Springs and Mackey are outside the epidemic boundary in Australia (Cheng and Currie, 2005). Northern Australia, including Darwin and Townsville, is a high epidemic area of *B. pseudomallei* infection (Cheng *et al.*, 2003). In Darwin, *B. pseudomallei* has been found close to streams and in the rhizosphere of spear grass in undisturbed soil, whereas the it is found in association with animal activity areas and irrigation in disturbed soil (Kaestli *et al.*, 2009). Moreover, *B. pseudomallei* is common in

unchlorinated bore water supplies in Darwin (33%). One molecular epidemiology analysis identified common isolates from environmental, bore water and clinical samples of *B. pseudomallei* (Mayo *et al.*, 2011).

Townsville has a high incidence of melioidosis and poorly drained soil when compared to Innisfail and Cairns, which has low incidence of infection (Corkeron *et al.*, 2010). The infection rate of indigenous adults in the Torres Strait and Northern Peninsula Area are approximately 40 cases per 100,000 (Hanna *et al.*, 2010). That means indigenous people have environmental exposure with potential of diabetes mellitus, alcoholism, cirrhosis and chronic disease or immunosuppression, resulting in increased risk of melioidosis infection.

2.3.2 Isolation of *B. pseudomallei* from environmental sources

Soil isolation of *B. pseudomallei* has usually depended on enrichment techniques. Ashdown agar was first established by Ashdown (1979) and modified Ashdown broth with Colistin was used to isolate *B. pseudomallei* from soil (Ashdown and Clarke, 1992). Later, Wuthiekanun *et al.* (1995) used threonine-basal salt solution (TBSS) with Colistin. Researchers found TBSS+ Colistin was a good enrichment media to isolate *B. pseudomallei* from soil in the dry season. The amount of soil and depth tested has varied in many publications (Thomas and Forbesfaulkner, 1981; Wuthiekanun *et al.*, 1995; Brook *et al.*, 1997; Kaestli *et al.*, 2007). Not all techniques reported use amplification steps, Larsen *et al.* (2013) soaked soil in a extraction buffer and the buffer was plated directly on Ashdown agar with Colistin and incubated at 37 °C for 2 to 14 days. *Burkholderia pseudomallei* speciation was confirmed by real-time PCR. However no determination of sensitivity with this protocol was reported. A consensus protocol has also been developed (Limmathurotsakul *et al.*, 2013) which includes sampling frequency and amount of soil to collect as well as what enrichment buffers and plating media to use as well as detection techniques (Table 2.2)

Table 2.2: Environmental guidelines recommended to normalize *B. pseudomallei* soil sampling.

	Sampling (meters)	Depth (cm)	Amount of soil	Enrichment buffer	Plating media	Detection
Consensus	2.5-5	30	10	Threonine-basal salt plus colistin 50 mg/l	Ashdown agar	Culture or PCR

Isolation from water has been done using two general steps; 1. concentration and 2. detection using culture or animal inoculation (Limmathurotsakul *et al.*, 2013) Recently, samples from soil and water were enriched by culture, with detection carried out using qPCR on extracted DNA. This was found to be very sensitive for detection of *B. pseudomallei* from environmental samples (Knappik *et al.*, 2015). *Burkholderia pseudomallei* has also been found in the aerosphere using a qPCR method. Although Ong *et al.* (2013) could not find *B. pseudomallei* in air and rain water using a culture method in an epidemic area, Chen *et al.* (2014) was able to find *B. pseudomallei* in air with a filtration/real-time qPCR method, especially in the typhoon season. This is of public health interest as Thomas *et al.* (2012) demonstrated that Balb/c mice could be infected when only the nose was exposed to *B. pseudomallei* aerosols for 10 min.

2.3.3 Identification

2.3.3.1 Selective agars

B.pseudomallei characteristically appears rugose, or like cornflower heads when grown on Ashdown's selective medium. (Ashdown, 1979), which is a cost effective and powerful media that guarantees reliable isolation of *B. pseudomallei* from normal flora (Walsh and Wuthiekanun, 1996). Howard and Inglis (2003) used a relatively small sample size (50) to develop *B. pseudomallei* selective agar (BPSA) and claimed BPSA inhibits *Pseudomonas aeruginosa* and *Burkholderia cepacia* and easily recognizes *B. pseudomallei* due to colony morphology. Using a larger samples size, Ashdown medium (524), *B. cepacia* medium (524) and BPSA (526) were shown to have equivalent sensitivity to detect *B. pseudomallei* in clinical samples (Peacock *et al.*, 2005). However, Ashdown's or *B. cepacia* medium are significantly more selective than BPSA to prevent growth of other species such as *P.aeruginosa.* As a result, Peacock *et al.* (2005) proposed Ashdown's medium should remain the standard for *B. pseudomallei* differentiation in endemic regions whereas *B. cepacia* medium is an alternative method in non-endemic regions for isolation of *B. pseudomallei*.

According to consensus guidelines for environmental sampling of *B. pseudomallei* as noted above (Limmathurotsakul *et al.*, 2013), TBSS + Colistin was proposed as one broth choice for enrichment of samples to isolate *B. pseudomallei* and Ashdown broth with Colistin was the alternative choice. After enrichment, plating on Ashdown agar and incubation at 40 ^{o}C for a week, checked every 24 hr for typical colonies was suggested. The use of 40 ^{o}C was based on Chen *et al.* (2003), who demonstrated that optimal growth of *B. pseudomallei* could be at 40 ^{o}C, which could inhibit the growth of other soil borne organisms.

2.3.3.2 Biochemical tests

The API20NE is based on 8 conventional tests and 12 assimilation tests for bacterial identification. Dance *et al.* (1989) suggested that the API20NE biotype could be helpful

to delineate the geographical distribution of melioidosis, since the API20NE test is cost effective, simple and accurate (97.5%). Inglis *et al.* (2005) reported that the API20NE method identified only 37% of isolated *B. pseudomallei*, however, three groups of researchers, which included Amornchai *et al.* (2007), Kiratisin *et al.* (2007) and Deepak *et al.* (2009) suggested API20NE was reliable and accurate for identification of *B. pseudomallei.* Amornchai *et al.* (2007) suggested the low identification rate of Inglis *et al.* (2005) study could be the result of misinterpretation. It is important to be aware of *B. cepacia* when using API20NE application for the identification of *B. pseudomallei* (Kiratisin *et al.*, 2007), since the phenotype of *B. cepacia* is very similar to *B. pseudomallei* (Deepak *et al.*, 2009).

2.3.3.3 Antibody detection

Rapid latex agglutination (Bps-L1 monoclonal antibody) has been reported to have 100% sensitivity and specificity on blood culture test to identify *B. pseudomallei* (Dharakul *et al.*, 1999). A combination latex agglutination (lipopolysaccharide and exopolysaccharide monoclonal antibody) has also been shown to detect *B. pseudomallei* in soil samples (100% accuracy) when it was compared with culture and able to distinguish between *B. pseudomallei* and *Burkholderia thailandensis* prior to culture being able to distinguish the two (Wuthiekanun *et al.*, 2002). Duval *et al.* (2014) reported that latex agglutination (capsular polysaccharide monoclonal antibody) had a 98.7% sensitivity to *B. pseudomallei* and 100% sensitivity to *Burkholderia mallei*. Only one *B. thailandensis* isolate tested positive when it was tested alongside other related *Burkholderia species* (97.2% specificity).

2.3.3.4 Molecular detection

Polymerase chain reaction (PCR) has been used to identify *B. pseudomallei* for more than two decades (Lew and Desmarchelier, 1994). Target genes have included 16S (Brook *et al.*, 1997) and 23S rRNA (Kunakorn and Markham, 1995), *rapsU*, *fliC* (Tomaso *et al.*, 2005), rec A (Payne *et al.*, 2005) and the Tat domain protein gene (Lau

et al., 2014). Environmental samples have also been examined for *B. pseudomallei* using the TTS gene and analysed by real time PCR (Novak *et al.*, 2006), which has been reported to be more sensitive and specific than culture methods (Kaestli *et al.*, 2007). Location analysis has also been done using *B. pseudomallei* specific fluorescent probes to detect *B. pseudomallei in situ* in plants with detection via a confocal laser microscope (Kaestli *et al.*, 2012).

Strain and isolate variation analysis has also been carried out. Pulsed-field gel electrophoresis (PFGE) with *Xba*I has been used to identify the ribotype pattern in *B. pseudomallei* isolates (Inglis *et al.*, 2004). Multi-locus sequence typing (MLST) has been applied to identify *B. pseudomallei* for more than a decade (Godoy *et al.*, 2003) and has been used in epidemiological analyses to identify the origins of *B. pseudomallei* isolates (Baker *et al.*, 2011a; McRobb *et al.*, 2014). Recently, McRobb *et al.* (2015) also used full genome sequences to study epidemiologic chronology.

2.4. Melioidosis

2.4.1 Inhalation

Due to helicopter crews becoming infected with *B. pseudomallei* during the Vietnam War, the infection route of inhalation inoculums was suggested with helicopter rotors distributing infectious dust particles of *B. pseudomallei* (Howe *et al.*, 1971). Using a mouse model, intranasal (i.n.) and aerosol was determined as the inhalation route. Intranasal (i.n.), *B. pseudomallei* colonized in both the upper and lower respiratory tracts and lung, due to the alveolar macrophage engulfing *B. pseudomallei,* which in turn destroyed the macrophage (Goodyear *et al.*, 2009), then spreading to the liver and spleen (Owen *et al.*, 2009). Although the lung was the primary colonization site of *B. pseudomallei* in aerosol inoculums, low numbers of bacteria were observed in the spleen and kidney. Other colonization sites were the blood, kidney and brain (Lever *et al.*, 2009).

2.4.2 Percutaneous and intraperitoneal

Percutaneous inoculation of *B. pseudomallei* occurred when people were exposed to wet soil and water contamination in Northern Australia (Currie *et al.*, 2000) and also in rice paddy fields in Northeast Thailand (Chaowagul *et al.*, 1989). The Intravenous (i.v.) route was used to study the acute form of *B. pseudomallei* (Hoppe *et al.*, 1999). After mice were intravenously injected with *B. pseudomallei*, fatalities rapidly occurred between two to four days (Leakey *et al.*, 1998). On the other hand, the intraperitoneal (i.p.) *B.* pseudomallei route test showed that although the number of bacteria inoculated was higher than for i.v., about 10^2 cells, the disease's symptom could be developed after day six. For both studies it was shown that bacteria played a major colonization role in liver and spleen (Santanirand *et al.*, 1999). From these results, Warawa (2010) suggested that the animal inoculation method could be traumatic percutaneous inoculation which leads to disease development.

2.4.3 Oral

Although *B. pseudomallei* may be transmitted by the oral route, high dosage of *B. pseudomallei* was required for inoculation in the animal model. BALB/c and C57BL/6 mice were administered 10^3 and 10^6 CFU of *B. pseudomallei.* Both mice breeds survived for six weeks. When inoculated with the higher dose, approximately 50% of both breeds presented with symptoms of hunching for the duration. (West *et al.*, 2010). This may have been due in part to endotoxin at this high dose. Therefore, this evidence suggested that the oral route was not a natural common source.

2.4.4 Animal models

The route of animal infection includes intranasal (i.n.), aerosol, intravenous (i.v.), intraperitoneal (i.p.) and oral inoculations. Currently, animal models have been used to study the infection route of *B. pseudomallei* including BALB/c, C57BL/6, SWISS, CBA, CD-1, DBA/2, C3H/HeN, Namru Albino, Taylor Outbred (TO), 129/SvEv, and SCID mice (Warawa, 2010). Each strain used a different state of disease. For example,

BALB/c is a mouse model for acute disease experiment, whereas C57BL/6 is a mouse model for chronic disease (Leakey *et al.*, 1998).

2.5 Clinical Manifestations

2.5.1 Typical manifestations

A key clinical characteristic of melioidosis is abscesses formation. Although clinical presentation of melioidosis presents a broad spectrum of symptoms, which include an acute fulminant septicaemia to a chronic debilitating localized infection (White, 2003), the most severe case is septic shock that disseminates bacteria to other organs such as the liver, spleen, and lung. The lung is a common organ where *B. pseudomallei* is localized, where abscess form and empyema and pulmonary infection occurs (Wiersinga *et al.*, 2006), leading to acute pneumonia, a common cause of high mortality of patients (Leelarasamee, 2004).

2.5.2 Atypical manifestations

Moreover, atypical melioidosis have been reported in intrathoracic subclavian artery pseudoaneurysm (Schindler *et al.*, 2002), abscess at the root of the mesentery (Kiertiburanakul *et al.*, 2002), abscess in the prostatic (Tan *et al.*, 2002) , and iliac mycotic aneurysm (Luo *et al.*, 2003).

2.5.3 Latency

The longest latent period of melioidosis recorded was a 62 year old; the infection likely occurred when he was taken prisoner by Japanese soldiers during World War II (Ngauy *et al.*, 2005).

2.5.4 Clinical assessment

The classification for clinical assessment of melioidosis was developed by in terms of Leelarasamee and Bovornkitti (1989) antibiotic administration, since it is straightforward and is associated to the severity of sepsis or mortality - a major proposition of the classification as illustrated in Table 2.3. By 2004, the classification data was updated, thus, for the purposes of this article Leelarasamee's (2004) classification has been utilized.

Table 2.3: Clinical assessment classification of melioidosis (Leelarasamee, 2004).

	No. organs involved[a]	**Blood culture**	**Severity of illness**	**Mortality rate[b] (%)**
Melioidosis with septic shock	Any	Positive	Fulminating sepsis/septic shock	80-95
Disseminated septicemic melioidosis: e.g.bloodstream pneumonia from liver abscess or pyopericardium or septic arthritis from splenic abscess	>1	Positive (most)	Sepsis to severe sepsis	40-50
Septicemic melioidosis: e.g. lobar pneumonia	1	Positive	Sepsis to severe sepsis	10-40
Localized melioidosis: e.g. lymphadenitis, prostatitis	1	Negative	Fever to sepsis	0-10
Bacteremic melioidosis	0	Positive	Nil to fever	0
Asymptomatic melioidosis[c]	0	Negative	Nil or healthy	0

[a]Classified by clinical assessment. [b]Cases treated with ceftazidime. [c]Cases with a positive serological result for melioidosis.

2.6 Treatment and Vaccine

2.6.1 Antibiotic treatment

Burkholderia pseudomallei displays resistance to many antibiotic include cephalosporins, penicillins, rifamycins, aminoglycodides, quinolones and macrolides (Cheng and Currie, 2005). Although ceftazidime and imipenem are antibiotics of choice for melioidosis treatment (White, 2003), the mortality rate is high (80-95%) when the patient develops a septic shock condition (Leelarasamee, 2004). Ceftazidime and imipenem could be most effective against the planktonic state of *B. pseudomallei*, however; Vorachit *et al.* (1993) found that a *B. pseudomallei* biofilm was resistant to ceftazidime treatment. From a clinical trial currently ongoing in Thailand, Leelarasamee (2004) suggested ceftazidime, imipenem or meropenem, activated protein C, and granulocyte colony-stimulating factor (G-CSF), which increases the number and function of neutrophils, are useful for combination treatment.

Maximum doses of all these factors directly destroy both extra- and intracellular *B. pseudomallei* to stop secretion of exogenous mediators to prevent coagulopathy and endothelial injury. The cost of melioidosis treatment, which is difficult to treat and has a high rate of relapse, is still expensive (approximately US$ 100 per day), which could have a large impact for developing countries (White, 2003).

2.6.2 Vaccine development

CpG ODN adjuvants or liposome-DNA adjuvant vaccines produce strong cellular immune responses, which produce IFN-γ production, whereas TLR 9 vaccine activates innate immune responses, mostly Th1, against *B. pseudomallei* in the animal model (Estes *et al.*, 2010). Many attempts have been made to develop a *B. pseudomallei* vaccine and these are reviewed by Silva and Dow (2013). Unfortunately, *B. pseudomallei* vaccines are still not commercially available. Recent literature has proposed the use of direct comparison of current vaccine candidates in well

characterized mouse models to select candidates for use in nonhuman primate models and then human clinical trials (Limmathurotsakul *et al.*, 2015).

2.7 Pathogenesis of *B. pseudomallei* in Mammals

Burkholderia pseudomallei is a facultative intracellular pathogen, which is resistant to phagocytic cells (Jones *et al.*, 1996), complement activation, effective opsonization, or complement-mediated immune responses (Egan and Gordon, 1996) and it produces proteases (Sexton *et al.*, 1994), haemolysin and cytotoxin (Haussler *et al.*, 1998). *Burkholderia pseudomallei* can survive and multiply in phagocytic cells such as neutrophils and monocytes (Pruksachartvuthi *et al.*, 1990) as well as epithelial cells (Ahmed *et al.*, 1999). The *B. pseudomallei* life cycle diagram, as showed in Figure 2.4, identifies the initial step of *B. pseudomallei* infection of host cells as adhesion, with incubation temperature of 30 ^{o}C more effective than at 37 ^{o}C (Brown *et al.*, 2002). Type IV pili play a role in adhesion and virulence (Essex-Lopresti *et al.*, 2005). After uptake, bacteria enter primary phagosomes and then escape from phagosome into the host cell due to a type III secretion which disrupts the membrane (Stevens *et al.*, 2002). *Burkholderia pseudomallei* then activate pathogen recognition receptors (PRRs) and BimA-dependent actin-based motility and evade killing by host autophagy. The Type VI secretion system promotes replication and actin polymerization of bacteria and induces plasma membrane fusion (Galyov *et al.*, 2010), permitting spread to adjacent cell.

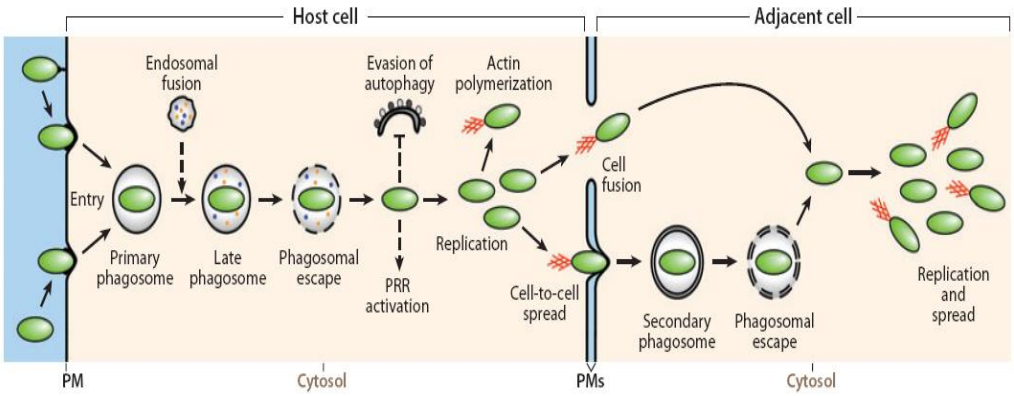


Figure 2.4: *Burkholderia pseudomallei* invade and replicate inside phagocyte (PM = plasma membrane and PRRs = pathogen recognition receptors) (Galyov *et al.*, 2010)

2.8 Virulence Factors of *B. pseudomallei*

2.8.1 Overview

The paucity of the putative virulent factors of *B. pseudomallei* is mainly based, in part, on the knowledge of other virulent pathogens, especially Gram negative bacteria or on experimental animal models. Some important virulent factors described in these sections include quorum-sensing, the secretion system, capsule, polysaccharide and flagella.

2.8.2 Quorum sensing

Quorum-sensing (QS), which is the cell to cell communication process of bacteria, involves the production and detection of autoinducers (bacterial pheromones), which diffuse signaling molecules (Lazdunski *et al.*, 2004). *Burkholderia pseudomallei* quorum-sensing, which secretes mostly N-acyl-homoserine lactones (AHLs) molecules for intra-species communications, has been examined by eliminating the signals of quorum-sensing on multiple luxIR genes, which decreased pathogenicity of BALB/c mice (Ulrich *et al.*, 2004).

2.8.3 Secretion systems

All bacteria have secretion systems, which signal macromolecules across cellular membranes secreting essential molecules for virulence and survival (Fronzes *et al.*, 2009). Secretion systems involved in the *B. pseudomallei* virulence include Type II, III, IV and VI secretion (Galyov *et al.*, 2010). For example, type III protein secretion apparatus (Bsa) of *B. pseudomallei* involved actin protrusions, which helped *B. pseudomallei* escape from macrophages. The inability of actin protrusions to escape from macrophage of *B. pseudomallei* were illustrated when the Bsa was inhibited (Stevens *et al.*, 2002).

2.8.4 Structures

Burkholderia pseudomallei produce two forms - (PS-I, PS-II) of Capsule or O-antigenic polysaccharide (Knirel *et al.*, 1992). This is a major virulence determinant in *B. pseudomallei* (Atkins *et al.*, 2002) since it enhances resistance to the intracellular killing mechanism (macrophage) (Wikraiphat *et al.*, 2009) and also reduces the complement factor C3b that is essential in the phagocytosis process (Reckseidler-Zenteno *et al.*, 2005).

The lipopolysaccharide of *B. pseudomallei* plays a role as a virulence factor and is similar to Enterobacteriaceae lipopolysacharides, it exhibits weak pyogenic activity in rabbits, lethal toxicity in galactosamine-sensitized mice and murine macrophage activation (Matsuura *et al.*, 1996). However, lipopolysaccharide of *B. pseudomallei* and *B. thailandensis* strain E264 , which has a similar structure to *B. pseudomallei* lipopolysaccharide were inoculated in a mouse model and protected against a lethal dose of *B. pseudomallei* strain K96243 (Ngugi *et al.*, 2010).

Flagella are important virulence determinants in *B. pseudomallei,* research showed BALB/c mice infected by flagella mutants did not succumb to infection (Chua *et al.*, 2003). Wikraiphat *et al.* (2009) reported that flagella are involved in intracellular virulence, ridding the body of polymorphonuclear cells and macrophages and they are also important to biofilm formation (Tunpiboonsak *et al.*, 2010). The Flagellin protein, extracted from flagella (Brett *et al.*, 1994), acts as an invasion mode of non-phagocytic cells (Chuaygud *et al.*, 2008).

2.9 The *Burkholderia* species Interaction with Plants

Bacteria living in the soil can have both positive and negative effects on the plants that they are associated with, particularly those in the rhizosphere of plants. Relationships have been described as saprophytic, symbiotic, pathogenic or mutualistic (Kobayashi and Crouch, 2009). These relationships can stunt the growth of plants, promote plant growth or even induce plant resistance to other pathogens or predators. Figure 2.5 provides a simple diagram of the development of a healthy relationship (Spence and Bais, 2013). Among the Burkholderias, there are a range of relationships including both growth promoting species as well as phytopathogenic species. For example, *B. glumae* and *B. plantarii* are plant pathogens while *B. phymatum* and *B. kirkii* are symbiotic and *B. ambifaria* has been reported as a biocontrol agent (Weisskopf and Bailly, 2013). *B. phytofirmans* strain PsJN has been reported to enhance plant growth (Barka *et al.*, 2000), induce chilling resistance (Barka *et al.*, 2006) and also enhance tolerance to other pathogens (Sharma and Nowak, 1998). *B. vietnamensis* has been shown to promote rice growth (Van *et al.*, 1996) while *B. cepacia* has been shown to stunt the growth of alfalfa (Bernier *et al.*, 2003).

Mechanisms of activity in the Burkholderias include the production of volatile organic compounds (VOCs) which can both promote or inhibit growth of plants (Weisskopf and Bailly, 2013). It is also likely that biofilm formation is important in these relationships as all plant associated *Burkholderia* species process a unique and highly conserved N-acyl-homoserine lactones (AHL) system, which is used for bacterial communication in biofilm formation (Angus and Hirsch, 2013).

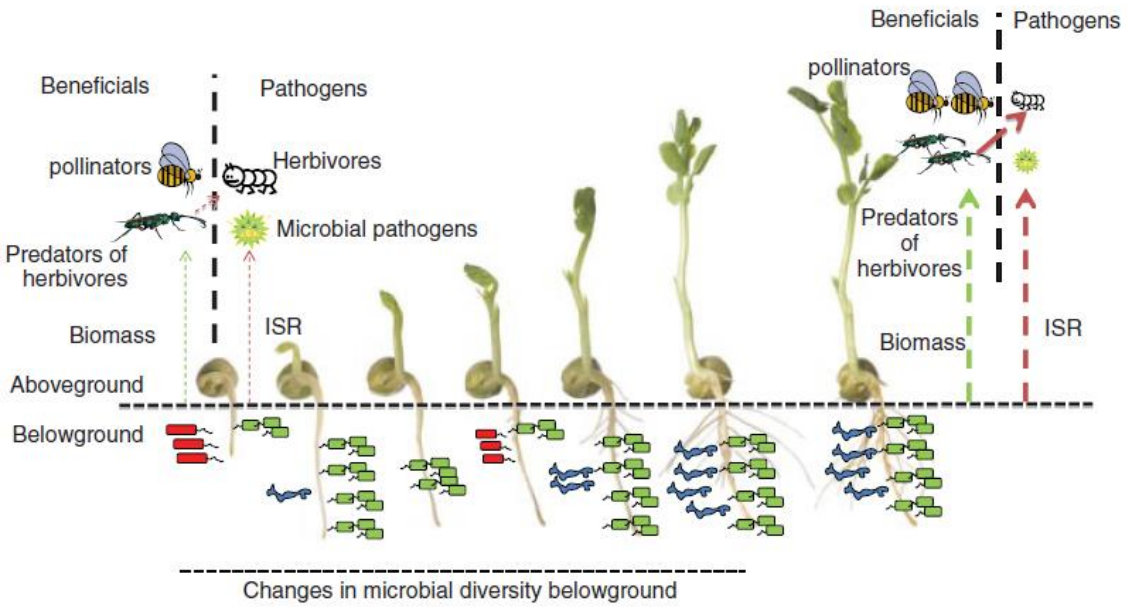


Figure 2.5: Diagram of interactions between plants and microbes where microbial pathogens can stun root and leaf growth (red color) while plant growth promoting bacteria (blue and green color) can promote plant growth and induced systemic resistance (ISR) against microbial pathogens and predators (herbivores) (Spence and Bais, 2013).

2.9.1 *B. pseudomallei* interaction with plants

There are few studies on the interactions of *B. pseudomallei* with plants. Some work has been carried out examining the rhizosphere soil and this is reviewed in section 2.10.3. Root and foliage samples of native grasses which were surveyed in the Northern Territory (Australia) were *B. pseudomallei* positive in 10% of cases (13/126) which was lower than in exotic grasses (47%; 43/91) (Fisher's Exact, $P < 0.001$) when samples was analysed by real-time PCR. Fluorescent in situ hybridization/confocal laser scanning microscopy (FISH/CLSM) also successfully detected *B. pseudomallei* in the rhizosphere of exotic grasses (Tully, Paspalum, Mission Grass) from a field site (Kaestli *et al.*, 2012). The frequency of detection using FISH/CLSM was not reported. Limited experimental infections have also been attempted. Lee *et al.*, (2010) was unable to stunt the growth of rice (*Oryza sativa* L. cv. Nipponbare) with either *B. pseudomallei* or *B. thailandensis* while tomato plants were affected by both *Burkholderia* species. Kaestli *et al.*, (2012) also infected domestic grasses (*Sorghum intrans* and *Oryza rufipogon*) and was able detect *B. pseudomallei* inside cells with FISH/CLSM, and

some evidence of growth inhibition was reported. Given the importance of this organism as a human pathogen, and the highly diverse roles that other Burkholderias play, this is a clear gap in our understanding of *B. pseudomallei.*

2.10 Persistence of Bacteria in the Environment

Generally, bacteria can persist in environments because they can utilize organic or inorganic matter as nutrient sources (Pelleroni, 2005). If bacteria have continued starvation in extreme environments such as depths of 5,000 ft, the starvation triggers the production of ultramicrobacteria (UMB) which exist in a sleep mode, according to Kjelleberg (Costerton, 2007). Other forms of persistence such as biofilm production, which will be discussed later, also exist.

2.10.1 Persistence of *B. pseudomallei* in water

Carbon, nitrogen, phosphorus and other trace element are nutrients sources for bacteria (Hu *et al.*, 2005) and bacterial biofilm production is a strategy for survival in water (Hall-Stoodley *et al.*, 2004). *Burkholderia pseudomallei* has an optimum temperature between 20 ^{o}C - 40 ^{o}C and can survive at 2 ^{o}C in the laboratory as well as surviving between pH 4-7 in water (Robertson *et al.*, 2010). *Burkholderia pseudomallei* are found in low saline water (12.8-160 ppm), however this bacteria can live in 2.5% of NaCl (25 000 ppm) (Inglis and Sagripanti, 2006; Draper *et al.*, 2010). *Burkholderia pseudomallei* prefer to live in high iron (Fe^{3+}), soft water and with a high coliform count (Draper *et al.*, 2010). There are logical reasons for each of these. *Burkholderia pseudomallei* can produce siderophores to keep Fe^{3+} (Ferric iron) and reduce to Fe^{2+} (Ferrous iron). Bacteria need Fe^{2+} for several function such as reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA and other essential purposes (Neilands, 1995). Soft water can corrode (roughen) surfaces which then promote biofilm formation. Coliform bacteria could provide nutrients for bacteria to grow in bore water. Moreover, *B. pseudomallei* have been found in 47 bore water samples in Northern

Australia (Draper *et al.*, 2010) and can survive in water samples for up to 20 months (Strauss *et al.*, 1969).

2.10.2 Persistence of *B. pseudomallei* in soil

Usually, bacterial growth in soil is limited by nutrient levels therefore bacteria prefers living in organic soil or at the roots of plants (rhizosphere)(Kaestli *et al.*, 2015). Most soil bacteria have an optimum growth in moderate temperature and natural pH (Alexander, 2002). *Burkholderia pseudomallei* prefer to live in a weak acid to a weak base (pH 5.0-7.8) environment and *B. pseudomallei* have a persistence in soil which is associated with soil type and moisture (Thomas *et al.*, 1979; Thomas and Forbesfaulkner, 1981; Palasatien *et al.*, 2008). For example, *B. pseudomallei* persistence in sandy moist soil was less than 6 months whereas clay persistence was between 30-36 months. Numbers of organisms per gram from dry soil are lower than moist soil because moisture content is essential to maintain pore space in the soil and also transfer air, water, nutrient and products of bacteria (Pepper and Gerba, 2004). *Burkholderia pseudomallei* have been shown to die within seven days when moisture content in soil is lower than 10% whereas *B. pseudomallei* have survived up to 726 days when water content was more than 40 % (Tong *et al.*, 1996).

2.10.3 Persistence of *B. pseudomallei* in the rhizosphere soil

Both pathogen and biocontrol bacteria typically prefer the rhizosphere when living in soil because roots are major nutrient source of bacteria as root secretions result in nutrient enrichment of the soil (Haas and Defago, 2005). Bacterial biofilms surround the root of the plant to take advantage of this (Costerton, 2007). For example, *Pseudomonas spp* forms a biofilm within 11 μm of the root surface (Watt *et al.*, 2006). *Burkholderia pseudomallei* has been reported to be present in the rhizosphere soil of *Acacia colei* in Western Australia (WA) (Inglis *et al.*, 2000). *Burkholderia pseudomallei* was strongly associated with grass and root-rich soil at undisturbed soil sites (Fisher's extract, $P<0.001$ and $P=0.001$ respectively). Spear grass (*Sorghum spp*)

in Northern Territory was also identified as being significantly related to the presence of *B. pseudomallei* in rhizosphere soil as analysed by multivariable logistic regression analysis (Kaestli *et al.*, 2009). One third of soil samples taken from a rice paddy field in Loas also tested positive for *B. pseudomallei* (Wuthiekanun *et al.*, 2005), indicating there may be a link with the growth of rice.

2.10.4 Possible persistence of *B. pseudomallei* in plants.

As noted above, bacteria can be found in environments such as water, bulk soil and rhizosphere soil. While there are no studies specifically looking at persistence of *B. pseudomallei* in plants, it has been proven that some bacteria which can persist in soil have enhanced persistence when it has been grown on the roots of plants. *Escherichia coli* O157:H7 persisted in soil for 25-41 days, persisted on rye roots for 46-96 days and persisted on alfalfa roots for 92 days. Morover, clay soil increased persistence and activity of *E. coli* O157:H7 (Gagliardi and Karns, 2002).

2.11 Biofilm Formation

Costerton *et al.* described the biofilm concept in 1978, which was later accepted by microbiologists (Costerton, 2004). Biofilms are microbial communities that irreversibly attach to a surface or interface and embed in a matrix of extracellular polymeric substances. The biofilms substances include biotic and abiotic components and also other organism byproducts. Moreover, biofilms change the characteristics of growth rates and genes transcription from the planktonic state (Donlan and Costerton, 2002). The basic state of biofilm development (Stoodley *et al.*, 2002) includes the attachment state of bacteria to surface and colonization, then they form exopolysaccharide to stabilise their attachment to the surface as an irreversible process. Biofilm architecture formed in a high shear environments is strong and resistant to breakdown when compared to a low shear environments (Stoodley *et al.*, 1998). Biofilm architecture is typically prolific until the development to mature biofilm. Once

mature, biofilms have been identified as continually dispersing to the environment such as releasing planktonic bacteria or detaching fragments of the biofilm.

2.11.1 Biofilm composition and relevance

The composition of biofilm matrices includes water, (up to 97% of total mass), microbial cells (two to five percent) and polysaccharides (one to two percent), whereas proteins, DNA and RNA form less than one to two percent (Sutherland, 2001). The biofilm matrix is the bacteria's survival strategy, which maintains water in a microenvironment, absorbing organic compounds such as nutrient sources, and inorganic compounds such as ions, and protects bacterial cells from the surrounding environment such as heat stress in hot springs (Costerton, 2007; Flemming and Wingender, 2010). Although biofilms are utilized in wastewater treatment for bioremoval of pollutants (Breisha and Winter, 2010), biofilms remain associated with major problems both in industry and medicine. For example, dental decay and metal corrosion are a focal attack on the surface after the formation of biofilms (Lappin-Scott and Costerton, 1989). Moreover, biofilms are permanent sources of reinfections and are difficult to remove by disinfection (Costerton *et al.*, 1999).

2.11.2 Persistence in the environment due to biofilm formation

Escherichia coli, Acinetobacter calcoaceticus, and *Erwinia stewartii* biofilm mutants were less resistant to storage under dessicated conditions (Ophir and Gutnick, 1994). Indicating biofilm formation is advantageous in survival of organisms in dry soil, possibly due to the matrix of extracellular polymeric substances protecting the bacteria from dehydration. The importance of biofilm formation in *B. pseudomallei* could be supported by evidence of its ability to survive in a desiccator (Strauss *et al.*, 1969), since osmolarity of water (solute stress) and dehydration (matric stress) have been involved in water permeability of bacteria. Bacteria have to adjust intracellular osmotic pressures to maintain cellular pressure. For example, van de Mortel and Halverson (2004) showed some genes were induced by solute stress or matric stress or both of

them to form *Pseudomonas putida* biofilms. Thus, persistence of *B. pseudomallei* in environments may be associated with biofilm formation. *Burkholderia pseudomallei* biofilm experiments in soil and plants have not been report yet.

2.12 Problem of Bacterial Persistence in the Environment

Bacterial persistence in the fields of agriculture, aquaculture, veterinary and food production are of concern because they have impacts on economic losses. For example, *Vibrio harveyi* biofilms can persist in hatchery farms and can resist 50 ppm of chloramphenicol and tetracycline (Karunasagar *et al.*, 1996). They have been causing massive mortalities of early-state hatchery-reared phyllosoma (Crothers-Stomps *et al.*, 2010).

2.12.1 Problem of *B. pseudomallei* persistence that requires control

Several persistence sources of *B. pseudomallei* such as bore water, rice field, detergent, cystic fibrosis and ground water seeps have been identified as points of concern. As noted above, in the Northern Territory *B. pseudomallei* is common in un-chlorinated bore water (33%) which most residents use in homes and agricultural activities and the first case of *B. pseudomallei* from a clinical sample matching to a water sample where the patient lived has been identified (Mayo *et al.*, 2011). Rice fields are classic examples where farmers have been infected with *B. pseudomallei* in Thailand (Chaowagul *et al.*, 1989), Laos (Rattanavong *et al.*, 2011) and Cambodia (Wuthiekanun *et al.*, 2008), however enhancement of *B. pseudomallei* persistence in soil due to rice roots has not been proved yet. Contaminations in hand wash detergent, while in the literature, are an uncommon exposure to *B. pseudomallei* (Gal *et al.*, 2004). Although, cystic fibrosis (CF) has not been reported as risk factor of melioidosis, *B. pseudomallei* have been increased in CF patient (Corral *et al.*, 2008). Moreover, *B. pseudomallei* have been reported form biofilms in melioidosis patients and an animal model (Vorachit *et al.*, 1995).

Baker *et al.* (2011b) found that *B. pseudomallei* from a reservoir source (Castle Hill, Townsville) can be facilitated by groundwater seeps. This evidence has been associated with high clinical incidence from Castle Hill (Corkeron *et al.*, 2010; Hanna *et al.*, 2010). *Burkholderia pseudomallei* has shown a clonal persistence in the environment. For example, a strain of *B. pseudomallei* now submitted to the National Collection of Type Cultures (NCTC 13177) was found 500 km east of the outbreak, from which it was isolated, after severe weather (Inglis *et al.*, 2011).

2.13 Controlling Bacteria in the Environment

Chemical and biological control are both ways to control bacterial contamination in the environment. Chemical agents are classified as oxidising and non oxidising groups (Bott, 2011). Ethylene oxide, chlorine dioxide gas and hydrogen peroxide are common oxidizing agents for cleaning bacteria from environment. The common action involves oxidizing macromolecules such as protein, carbohydrate, lipids and nucleic acids (McDonnell and Russell, 1999). Although ethylene oxide is a highly reactive molecule, it is also flammable, explosive and a human carcinogen. Chlorine dioxide gas is an oxidizing agent which acts on the protein of bacteria and viruses. However, this gas is unstable and killing is effective decreased when humidity drops below 70%. Hydrogen peroxide is a safe chemical, but only of medium decontamination effectiveness against *B. cepacia* compared with enveloped viruses (Niederwöhrmeier and Richardt, 2008). Aldehydes as solution or gas are common non oxidizing agents (Bott, 2011). Formaldehyde has been used as disinfectant, however there is evidence that it is a carcinogen when inhaled (Niederwöhrmeier and Richardt, 2008). Quicklime (calcium oxide) was the first chemical with evidence to control *B. pseudomallei* in soil. Forty percent of calcium oxide inhibits *B. pseudomallei* for up to six weeks (Na-ngam *et al.*, 2004).

From previous descriptions, the chemical agents are not friendly to humans and the environment. Moreover, chemical agents can kill all organisms in the environment therefore they change the ecological system and some surviving organisms have

developed resistance (Hajek, 2004). On the other hand, biological control can be selective as is described below.

2.14 Biocontrol Agents

Biological control (biocontrol) uses live micro-organisms such as a parasites or organisms producing antibiosis agents to decrease specific pests, decrease abundance and lessen the damage of undesirable organisms (Eilenberg *et al.*, 2001). The majority of biocontrol is used in healthy crop production to serve humans. The recorded history of biocontrol began in 324 BC, when the Chinese used ants (*Oecophylla smaragdina*) to control caterpillars and large boring beetles in citrus trees (Hajek, 2004). In 1921, Hartley inoculated thirteen antagonistic fungi; he was the first person to develop an effective biological agent to control *Pythium debaryanum* (Baker, 1987). Hartley used forest nursery soil mixed with fungi and *P. debaryanum to* give 35.8% biocontrol effectiveness, however; autoclaved soil mixed with fungus and *P. debaryanum* was 100% effective in removing *P. debaryanum.* Na-ngam *et al.* (2009) demonstrated that 50% concentration of effective microorganisms (EM) inhibited *B. pseudomallei* in rice field soil, however longer studies identified that the inhibition was only temporary (Wuthiekanun *et al.*, 2013). *Burkholderia thailandensis* E261 did not inhibit *B. pseudomallei* in rice paddy field soil a year after *B. thailandensis* E261 had been introduced to the soil (Wongsuwan *et al.*, 2013), so effective biocontrol agents for soil borne *B. pseudomallei* remain elusive.

2.14.1 Risks of biocontrol

In terms of human health, especially the risk for human infection, biocontrol agents used in the environment are the primary concern, since some pathogens produce secretions to control other bacteria and use of pathogens as biocontrol agents could then affect human health. Whether or not an infection occurs relies on the dose of agents, the characteristic of the agents such as virulence factors or susceptibility of host to the agents. The infection risk factor to humans of biocontrol agents is classified into four groups (Official Journal of the European Communities, 2000): Group one, which is not a cause of human disease, is the selected agent for biocontrol agents. . Group two

causes disease and could be hazardous to workers, however; this organism cannot spread to a community and treatment is available. Group three, which is a severe human disease and is a serious hazard to workers, has a risk of spreading to a community, but there are effective treatments. Group four causes severe human disease and is a serious hazard to workers. There is high risk of spreading to a community and there are no available treatments.

Groups two to four are human pathogens that are not used as biological control agents. Moreover, allergies to some fungi and bacteria are of concern when workers contact high concentrations of biocontrol agents for a long period (Strasser and Kirchmair, 2006). For instance, fungi are well known to cause allergic reactions (Ward *et al.*, 2003). Also, high dose inhalation of Gram negative bacteria biocontrol agents have been of concern with bacterial endotoxin inducing IFN-γ and IL-12 production which developed to allergy and asthma (Renz and Herz, 2002). With respect to *Burkholderia* species, *B. cepacia* complex are widely used as a plant biocontrol agent, biofertilizer and for bioremediation (Chiarini *et al.*, 2006). They are also a cause of sour skin disease of onion bulbs (Burkholder, 1950), and have been isolated in cystic fibrosis in humans (Vandamme *et al.*, 2007). However, these cases of infection were identified in immunocompromised patients and rarely in normally healthy persons. In contrast, lytic bacteriophage (viral) biocontrol agents have no impact on other hosts. (Crothers-Stomps *et al.*, 2010).

2.14.2 Mechanisms of biocontrol of soil borne pathogens

The strategies of biological control against pathogens in soil have been classified to three categories (Chet *et al.*, 1990). These categories include parasitism, antibiosis and competition. Parasitism is living organisms directly attacking their host to obtain nutrients and they may destroy the host organism. For example, Bacteriophages are bacterial viruses that are specific to their hosts and usually have a narrow spectrum of targets. Bacteriophage classification and biology will be discussed in more detail in section 2.15. Bacteriophage have shown potential against *B. pseudomallei* in the laboratory (McRobb, 2010; Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011), however there is no literature covering use of bacteriophage in soil environments against *B. pseudomallei.*

Antagonism is classified into two activities: secretion (antibiosis), and between whole organisms (competition). Antibiosis is substrate secretion from biological organisms containing either broad-spectrum activity (antibiotics), or narrow-spectrum activity (bacteriocins) where the host organisms are usually closely related to the bacterial strain (Riley and Wertz, 2002). For example, *Bacillus subtilis* has multiple antagonistic modes as a biological control agent. *Bacillus subtilis* produces bacteriocins such as bacillin 1115, subtillin and bacilliocin 22 (Zheng and Slavik, 1999; Burianek and Yousef, 2000). Four to five percent of strains produce more than two dozen antibiotics (Stein, 2005). *Bacillus subtilis* 6051 can form biofilms on roots and can protect plant from mortality caused by *Pseudomonas syringae* by approximately 70% in soil (Bais *et al.*, 2004). Further, *B. ubonensis* isolated from Papua New Guinea has shown bacteriocin activity against *B. pseudomallei* in culture (Marshall *et al.*, 2010). Bacteriocin classification and production will be discussed in more detail in section 2.16.

According to Baker (1986) (p. 267), the competition concept was defined by Clark (1968) “as the injurious effect of one organism on another because of the utilization or removal of some resource of the environment”. Normally, the major mechanism of competitive biological agents involves niche exclusion, high reproduction rate, nutrient

uptake and iron chelating compounds (Chet and Chernin, 2002) This means the biological agent is competing with the pathogen for limited nutrients. As the biological agent absorbs the nutrient, the pathogen is in a state of starvation. For example, *E. coli* O78:K80 in poultry are reduced when *B. subtilis* persists in the intestine, since the biocontrol agent acts via competitive exclusion (La Ragione *et al.*, 2001).

2.14.3 Application of biocontrol

Bacteriophage and bacteriocin as control agents are old concepts. The potential of their use for controlling disease are: firstly, lytic phages have multiplication properties within host bacteria, allowing self replication after initial application, while bacteriocin require the presence of the producing bacteria (probiotic) to continue releasing the agent to disrupt targeted cells. Secondly, bacteriophages and bacteriocins are natural elements and distributed throughout the environment, thus more acceptable as a treatment option than the introduction of foreign chemicals. Thirdly, bacteriophages are highly specific to their host whereas a minority of bacteriocins show broad spectrums, similar to antibiotics and the majority of bacteriocins express a narrow spectrum-, meaning more fine-tuned control of selected targets. Finally, bacteriophages' and bacteriocins' application could be cost effective to control bacteria in the environment; currently, both bacteriophages (Crothers-Stomps *et al.*, 2010; Monk *et al.*, 2010) and bacteriocins (Huff *et al.*, 2003; Desriac *et al.*, 2010; Dicks *et al.*, 2011) have been useful for infectious disease control in several fields such as agriculture, aquaculture, veterinary and medicine.

2.14.3.1 Agriculture biocontrol

Bacteriophages are used for controlling agriculture diseases such as fruit spot. *Xanthomonas campestris* pv. *Pruni*, which is causative of fruit spot on peaches, is controlled by spray suspension of bacteriophage (Saccardi *et al.*, 1993). Recently, bacteriophages against plant disease have been applied to commercial products for agriculture management such as AgriPhage™ (Monk *et al.*, 2010). In the same way,

Burkholderia species which produce bacteriocins have been inoculated in agriculture soil as a form of biocontol (Vandamme *et al.*, 2007). A strain of bacteriocin producing *Pseudomonas* species has also been used to inhibit plant pathogens such as *Pseudomonas savastanoi*, which causes olive knot in olive plants (*Olea europaea*) (Rokni Zadeh *et al.*, 2008).

2.14.3.2 Aquaculture biocontrol

Aquaculture is a significant source of foodstuff products for livestock and humans. An essential aquaculture problem is bacterial disease in the hatcheries sector. For example, massive mortality was reported in prawn hatcheries as a result of luminous bacteria disease (Karunasagar *et al.*, 1994). Lytic bacteriophages from prawn farms effectively inhibited *Vibrio harveyi* however, not to 100% elimination. Further, phage cocktails were suggested for farm hatcheries (Crothers-Stomps *et al.*, 2010). Also, *Lactococcus lactis ssp. lactis* produced a strong bacteriocin (bacteriocin-like inhibitory substance) against *Streptococcus iniae*, which is a serious fish pathogen, unpublished (Wright, 2010).

2.14.3.3 Veterinary and food biocontrol

Animal production and food safety has increased awareness about antibiotic residues and antibiotic resistance. As a result, the animal and food industries started using bacteriophages for biological control. For instance, Huff *et al.* (2003) showed a single intramuscular injection of bacteriophage significantly decreased the mortality rate from severe respiratory infection in chickens, from 57% (control) to 10% (experiment). Modi *et al.* (2001) used lytic bacteriophage against *Salmonella enteritidis* in Cheddar cheese, which decreased the bacteria load 10-100 times while the control bacteria load increased 10 times within 99 days. Currently, bacteriophages are approved by the US Food and Drug Administration and/or the US Department of Agriculture to help control bacterial contamination of livestock, crops, and other food (Ross *et al.*, 2009). Bacteriocins have also been used; faecal shedding and colonization of O157:H7

decreased when calves were inoculated with *E. coli* which produces bacteriocins (Colisins), at a dose of 10^8 CFU/day (Schamberger *et al.*, 2004). Nisin (100 ppm) combined with high pressure (350 MPa) and 1% gluco delta lactone extended the 30 day chilled storage shelf life of poultry (Yuste *et al.*, 1998). The Food and Agriculture Organization/World Health Organization (FAO/WHO) has accepted the addition of Nisin to food since 1969 (Chen and Hoover, 2003).

2.15 Bacteriophage

In 1886, E.H. Hinkin observed that water from the Ganges and Jumma rivers in India killed the cholera pathogen according to Stone (2002). It may indeed be the first evidence of bacteriophages in the environment. This was further supported by Frederick William Twort (1915) and Félix d'Herelle (1917) discoveries of phage according to Kropinski and Clokie (2009). Phage originates from the Geek "phagein", which means to eat; therefore bacteriophage means virus killing bacteria (Kropinski and Clokie, 2009).

Although the golden age of bacteriophage research happened early (approximately 1915-1930s), the utilization and application of bacteriophages virtually disappeared from western countries for two main reasons (Eaton and Bayne-Jones, 1934). Firstly, as suggested by the American Medical Association's Council on Pharmacy and Chemistry, phage preparations of bacteriophages were without standardization and criteria for purity, and bacteriophages biology was still unknown. Secondly, during the preparation of antibiotics a high standard and stability was possible and these antibiotics displayed broad spectrum action against bacteria, so antibiotic consumption rapidly increased and replaced phage therapy in western countries.

Furthermore, the evaluation of phage therapy was complicated and in the case of *vibrio* therapy, variable susceptibility to different serotypes of *vibrios* occurred with a low diversity of effectiveness during the second dose of phage therapy (Marcuk *et al.*,

1971). However, emerging bacteriophage research is needed as antibiotic resistant bacteria are significantly increasing, and bacteriophages are an alternative tool which have been successfully used against antibiotic resistant organisms such as phage Sb1 which was effective killing 84% of the 93 clinical MRSA strains tested and phage Ent6 which killed 98% of the 186 vancomycin resistance enterococci tested (Chanishvili *et al.*, 2001).

2.15.1 Classifications of bacteriophages

Since 1965, the cumulative numbers of described bacteriophage have dramatically increased (Ackermann, 2007). They are able to be described because of Brenner and Horne (1959) application of negative staining in high resolution electron microscopy. As of 2007, 5 568 bacteriophage have been identified by way of Brenner and Horne's method (Ackermann, 2007). Bacteriophage taxonomy includes 14 families, with five families of phage still being classified by the International Committee on Taxonomy of Viruses (ICTV) (Ackermann, 2009). Bacteriophage or phages have been classified according to characteristics including a tailed, polyhedral or cubic, filamentous and pleomorphic structure (Table 2.4). Genomes, which include DNA or RNA, are enclosed in a protein or lipoprotein coat, or capsid (Carter and Saunders, 2007).

Tailed phages have been classified as Order *Caudovirales* (Latin cauda, tail) (Ackermann, 2005). They are the largest and most widespread group of bacteria virus that includes 5 360 viruses or 96.2% of total members (Ackermann, 2007). Order *Caudovirales* consists of double stranded DNA (dsDNA) and is divided into three families. Firstly, Myoviridae has a contractile tail that consists of a neck, sheath and a central tube. Myoviridae members include 1,320 viruses or 25% of tailed phages. Secondly, Siphoviridae has a long, flexible and non-contractile tail or rigid tube. Siphoviridae members have been observed in 3,229 viruses (61%), the largest group of tailed phages. Finally, Podoviridae has short and non-contractile tails. Their members comprise 771 viruses or 14.5% of tailed phages. Recently, *B. pseudomallei* phages

have been identified in all three families of tailed phages (McRobb, 2010: Yordpratum *et al.*, 2011; Gatedee *et al,* 2011).

On the other hand, 204 viruses or approximately 4% belongs to the cubic, filamentous and pleomorphic structure (CFP) (Ackermann, 2007). Polyhedral or cubic phages (icosahedra) are bodies with cubic symmetry while filamentous phages have helical symmetry, and pleomorphic phages have non symmetry axes (Ackermann, 1998). Some phages such as *Cystoviridae* and *Lipothrixviridae* have enveloped and lipid contents (Ackermann, 2005), which are sensitive to chloroform that first breaks down lipids and then lyses the capsid of phages (Fortier and Moineau, 2009).

Table 2.4: Classification of bacteriophages (Modified Ackermann, 2005 and 2009)

Morphology	Family	Nucleic Acid	Sample	Numbers
Tailed	*Myoviridae* (Greek mys,myos, "muscle")	Double stranded DNA	T4	1320
	Siphoviridae (Greek siphon, "hollow tube")		λ	3229
	Podoviridae (Greek *pous,* podos, "food")		T7	771
Cubic	*Microviridae* (Greek, *mikros*, "small")	Single Stranded DNA	ØX174	40
	Corticoviridae (Latin *cortex*, "bark, crust")	Double stranded DNA	PM2	3?
	Tectiviridae (Latin *tectus*, "covered")		PRD1	19
	SH1*		SH1	1
	STIV*		STIV	1
	Leviviridae (Latin *levis*, "light")	Single stranded RNA	MS2	39
	Cytoviridae (Greek *kystis*, "bladder, sack")	Double stranded DNA	Ø6	3
Filamentous	*Inoviridae* (Greek *is,inos*, "fiber")	Single stranded DNA	M13	67
	Lipothrixviridae (Greek *lipos*, "fat"; *thrix*, "hair")	Double stranded DNA	TTV1	7
	Rudiviridae (Latin *rudis*, "small rod")		SIRV-1	3
Pleomorphic	*Plasmaviridae* (Greek *plasma*, "shaped product")		L2	5
	Fuselloviridae (Latin *fusellum*, "little spindle)		SSV1	11
	Guttavirridae (Latin *gutta*, "drop")		SNDV	1
	Salterprovirus (Exclude: phage-like bacteriocin)		His1	1
	Ampullaviridae* (Latin *ampul*, "flask")		ABV	1
	Bicaudaviridae* (Latin bi, "twice"; cauda, "tail")		ATV	1
	Globuloviridae* (Latin globus, "ball"		PSV	1

* Archaeal bacteria viruses are mostly found in hot springs and are still classified by ICVT.

2.15.2 Biology of bacteriophages

Sewage, faeces, sea and soil are natural habitats for bacteriophages (Furuse *et al.*, 1983). The host target of phage is a specific cluster of bacteria, however; some bacteriophage can infect a broader range of bacterial species. For example, *Sphaerotilus natans* bacteriophage infected *Sphaerotilus natans, P. aeruginosa, Pseudomonas fluorescens, E. coli, Shigella flexneri, Proteus vulgaris* and *Rhodospirillum rubrum* (Jensen *et al.*, 1998). There are two basic bacteriophage infection types; lytic and lysogenic (Summers, 2001). Typical lytic bacteriophage (virulent) produce virions after destroying their host cell, whereas lysogenic bacteriophage (temperate) does not promote cell death or produce bacteriophage particles after bacteria is infected by lysogenic bacteriophage, except when lysogenic bacteriophage is induced by stressors such as DNA damaging agents.

2.15.2.1 Lytic (virulent) bacteriophage

The process of lytic bacteriophage infection can be divided into three actions: absorption, maturation and lysis. Firstly, absorptions of phages begin when structures such as fibres or spikes attach to particular surface molecules or capsules on their target bacteria. Several of the proteins, oligosaccharides, and lipopolysaccharides may act as receptors for various phages to Gram negative bacteria while the complex murein of Gram-positive bacteria binds with phages (Guttman *et al.*, 2005). Moreover, divalent cation especially Ca^{2+}, Mg^{2+} are specific cofactors for many phages, helping the phage penetrate bacteria (Wollman and Stent, 1952).

Secondly, the maturation phase consists of penetration, transition metabolism and morphogenesis. Penetration starts when phage genomes are inserted into the host cell and the peptidoglycan layer of the bacterial host is digested by enzymes from the tail tip of phages, which releases the nucleic acid directly into the cell (Guttman *et al.*, 2005). Consequently, many phages inhibit the host nucleus when nucleic acid is inserted to the host cell. The transition metabolism between host and phage involves host RNA

polymerase. RNA polymerase induces the transcription of early genes of the phage. The products of these genes may defend the phage genome from the host enzyme. Therefore, the host cell is controlled by phages, which degrade the host DNA, and reprogramme the host cell to produce new bacteriophages (Guttman *et al.*, 2005). Morphogenesis begins when the DNA is packed into icosahedral proteins called procapsids. This process is an interaction between specific scaffolding proteins and the major head structural proteins. The scaffolding, and the N-terminus of head proteins, are cleaved by the proteolytic enzyme. Internal capacities of DNA are enlarged when the head expands and becomes more stable (Guttman *et al.*, 2005).

Thirdly, lysis of the bacteria is the last episode of lytic phage infection. Two molecules are expressed by the phage and allow the new virion breaks out of the bacterial cell. The virion comprise lysin and holin that destroys the cell wall of bacteria (Young *et al.*, 2000). Lysins or endolysins are lytic phage enzymes, which hydrolyse the peptidoglycan component of the bacteria cell wall. Lysin accumulates in the cytoplasm of phage maturation during phage growth inside the infected bacterium. At the same time, small molecules of protein are located on the membrane, and the activation state of holin is insertion of protein into the cytoplasmic membrane. Subsequently, pores' formation allows the lysin in the cytoplasm to contact the peptidoglycan which causes cell lysis and liberates progeny phages (Wang *et al.*, 2000). Moreover, if suitable hosts are not in attendance, lytic bacteriophages can survive freely. Several phages have been shown to maintain their ability to infect for extensive periods and bacteriophages can multiply and reproduce again after they come in contact with their host (Rohwer, 2003).

2.15.2.2 Lysogenic (temperate) bacteriophage

The lysogenic lifecycle has been studied well in relation to the lambda prophage. The lysogenic state is where the genome of the bacteriophage enters the cell and inserts into specific sites of bacterial host chromosome where it remains during cell division. However, some lysogenic phages such as phage Mu insert their genome into non-

specific sites, whereas phage Pi does not insert into the genome, but replicates as a plasmid alongside the host chromosome (Weisberg *et al.*, 2010).

The mode of maintenance of the prophage includes insertion and plasmid formation approaches. The insertion techniques consist of recombination and transposition methods to insert the phage gene into the bacterial chromosome. For example, excisionase enzyme, which is essential to reverse insertion of the genome, is absent after the temperate phage genome is inserted into the host genome (Thyagarajan *et al.*, 2001). This occurrence means the lysogenic bacteriophage genome remains inside the bacterial genome until induced by a DNA damaging agent (Campbell, 2006).

Mitomycin C or UV are DNA damaging agents that induce prophage to the lytic cycle. DNA damaging agents break DNA causing activation of the SOS response and stimulates the RecA protein. Activation of the RecA protein cleaves the LexA protein, inactivating it and stopping repression of DNA repair genes , (Little, 1993). At the same time, RecA cleaves the phage repressor, which mimics LexA therefore; inducing lytic development from bacteria (temperate phage) (Campbell, 2006). Lytic development from prophage is different from lytic bacteriophage, which can be isolated from the environment (previous description), but are not induced by DNA damaging agents or insert their bacteriophage genome into the bacterial cell, According to Guttman *et al.* (2005).

2.15.2.3 Pseudolysogenic bacteriophage

Pseudolysogen (preprophage) present in host cells is an unstable situation, in which the bacteriophage cannot either produce virulent bacteriophage or insert the genome into the host bacterial genome (temperate) (Mann, 2006). The bacteriophage genome remains in a host cell until either lifecycle can be triggered (Miller and Day, 2008). Miller and Day (2008) hypothesized that pseudolysogeny occurred when the bacterial host was starved in the environment and could not afford the energy or nutrients to

prepare a lytic or lysogenic bacteriophage. The relationship between host, lytic bacteriophage, lysogenic bacteriophage and pseudolysogenic bacteriophage (environment habitat) are illustrated in Figure 2.6.

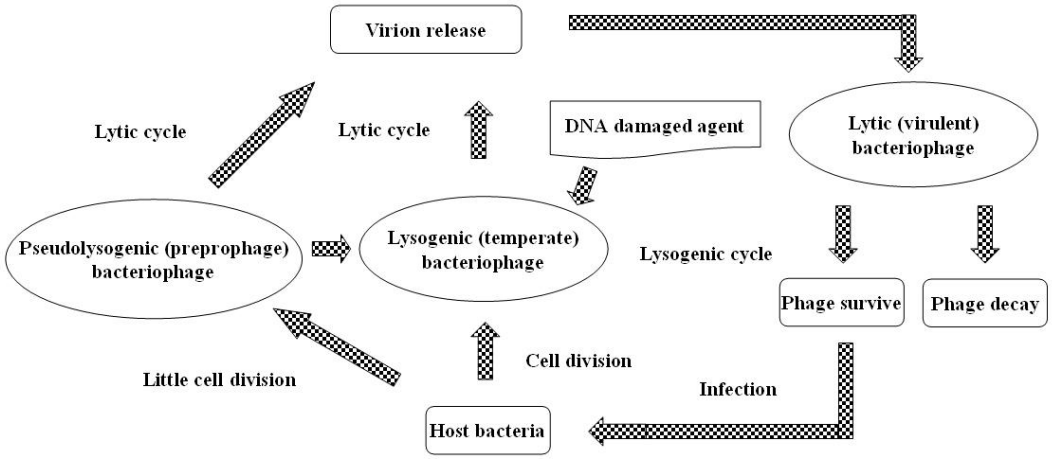


Figure 2.6: The majority of the bacteriophage lifecycle involves lytic and lysogenic cycles, however; pseudolysogenic bacteriophage is possible when starvation in the environment occures (Modified Miller and Day, 2008).

2.15.3 *Burkholderia* bacteriophage

Burkholderia bacteriophage is usually isolated in agriculture soil with high organic content, since *Burkholderia* species have a natural habitat in the rhizosphere (Summer *et al.*, 2007). Most *Burkholderia* bacteriophage can be divided into two groups; Firstly, from *Burkholderia* species that cause disease only in immunocompromised patients, particularly in cystic fibrosis and the second group from species that can cause disease in healthy people i.e. *B. pseudomallei*. For example, lysogenic bacteriophage were induced from 10 of 20 strains of *B. cepacia* complex, and three temperate and five lytic bacteriophage of this organism were isolated from environments such as soil, river sediment and plant root . Six bacteriophages belong in tailed families and two exhibited as lambda-like structure (Langley *et al.*, 2003). Bacteriophages have also been isolated

from *B. thailandensis* which is a non-pathogenic bacteria, closely related to *B.pseudomallei.* One case of disease has been reported; in a 2-year-old male, it caused pneumonia and septicaemia (Glass *et al.*, 2006). Lysogenic phage of *B. thailandensis* infected 9 out of 10 of *B. mallei,* and 3 out of 13 of *B. pseudomallei* (Woods *et al.*, 2002)

According to Yordpratum *et al.* (2011), *Whitmore* bacteriophage or *B. pseudomallei* bacteriophage was first reported by Leclerc & Sureau (1956). The first lytic *B. pseudomallei* phage was isolated from stagnant water in Hanoi, Vietnam and belongs to the *Myoviridae* family, a result similar to Yordpratum *et al.* (2011) who found six lytic bacteriophage from soil at Khon Kaen, Thailand, whereas Gatedee *et al.* (2011) found lytic bacteriophage in the *Podoviridae* family. McRobb (2010) isolated lytic bacteriophage (*Myoviridae* and *Siphoviridae* family) and also *B. pseudomallei* lysogenic phage was identified as a member of family *Myoviridae*, unpublished (Elliman, 2006).

2.16 Bacteriocins

According to Rea *et al.* (2011), the first evidence of media as bacteriocins was suggested by Pasteur and Joubert (1877). They found that bacteria isolated from urine inhibited the growth of *Bacillus anthracis,* and Jacob *et al* (1953) first suggested the term bacteriocin according to, Sharma *et al.* (2010). Bacteriocins are one potential biocontrol agent which counters related bacteria in the environment. Although the main characteristic dividing bacteriocins from antibiotics is the narrow spectrum of related bacteria against which bacteriocins are active (Jack *et al.*, 1995), many Gram positive produced bacteriocins have a broad spectrum of activity and do not follow many of the criteria that used to define Gram negative bacteriocins such as Colicins (Rea *et al.*, 2011). Rea *et al.* (2011) (p. 30) suggested that a new definition of bacteriocin to be "modified or unmodified peptide antimicrobials produced by bacteria which are protected by a dedicated immunity system". This immunity system protects the bacteria

from its own bacteriocin. Further, bacteriocins are split into two major groups based on their production by Gram positive or Gram negative bacteria.

2.16.1 Gram positive bacteriocins

A major Gram positive bacteriocin producer is lactic acid bacteria (LAB) which includes the Genera *Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Pediococcus, Carnobacterium* and some members of *Streptococcus*. Commonly, bacteriocins are utilised for food safety and preservation (Galvez *et al.*, 2007). In addition, lantibiotics, which originated from lanthionine, including antibiotics such as nisin, dramatically reduce numbers of human pathogens such as *Streptococus pneumoniae* (Severina *et al.*, 1998). Table 2.5 illustrates the classification update of Gram positive bacteriocins.

Although bacteriocin extracts have not been used in soil microbial control, whole organisms producing bacteriocins have been inoculated in the soil for plant pathogen control. *Bacillus subtilis* has been used in this fashion. It is widely spread in soil and is convenient for large scale production. *Bacillus subtilis* is non-pathogenic and forms spores, which provide these bacteria with persistence and high tolerance in the environment.

Table 2.5: Current updates classification of Gram-positive bacteriocins and bacteriocin-like peptides and proteins (Modified Rea *et al*, 2011).

Class	Update	Further subdivisions	Example
Class I	Modified peptides (a) Lantibiotics (b) Labyrinthopeptins	 Subclass I and II (modified by LanBC abd LanM proteins and can also be further divided into 12 further subclass based on amino acid sequences. Subclass III and IV (modified by RamC-like and LanL proteins, respectively).	 Nisin, Epidermin and Microbisporicin (against multi-drug-resistant pathogens) Labyrinthopeptins (against herpes simplex
Class II	Non-modifed peptides (a) Pediocin-like (b) Two-peptide bacteriocins (c) Circular	 Four subclasses I-IV Two subclasses, i.e. A and B Two subclasses, i.e. 1 and 2	 Listerocin (food preservative), tolerance at high temperature and broad spectrums. Brochocin C, Lactinocin F and Lactococcocin G.
Bacteriolysins	Non-bacteriocin lytic proteins		Helveticin J, Zoocin A and Enterolysin A

2.16.2 Gram negative bacteria

According to Gratia (2000), Gratia (1925) was a pioneer in the isolation of bacteriocins from Gram negative bacteria. Major bacteriocins, mainly isolated from Enterobacteriaceae such as *E. coli*, are divided into two main groups (Rebuffat, 2011): large molecular weight proteins (30-80 kDa) called Colicins and small molecular weight proteins (1-10 kDa) called Microcins. Colicins are triggered by DNA damaging agents and induce the SOS response to prophage gene in bacteria chromosomes. For example, the Pyrocin gene located in the *P. aeruginosa* chromosomes is induced by DNA damaging agents such as mitomycin C (Michel-Briand and Baysse, 2002). On the

other hand, Microcins, are resistant to proteases, extreme pH and temperature. Bacteriocin-like inhibitor substances (BLIS) of this type have been identified in *B. ubonensis* and have a molecular weight of 5 kDa. Marshall (2007) found heating of cell free suspension of *B. ubonensis* to 50 or 70 ^{o}C or adjusting pH from 7 to 4 or 9 enhanced antagonistic activity against *B. pseudomallei* using an agar diffusion method. *Burkholderia ubonensis* BLIS caused close to 100% inhibition of some strains of *B. pseudomallei* (Marshall *et al.*, 2010). *B. pseudomallei* are also inhibited by *Burkholderia cenocepacia* or *Burkholderia multivorans*, which survived in a broader range of pH, temperature, and salt concentration (Lin *et al.*, 2011).

2.16.3 Mechanism of bacteriocins

Although antibiotics are still the drugs of choice, bacteriocins are increasingly being researched as a form of biocontrol, especially as the antibiotic resistance of microorganisms to conventional antibiotics is increasing (Sang and Blecha, 2008). Bacteriocins, which have a high positive net charge, strongly bind the negative charge on bacterial membranes and take action on polypeptides such as histones and angiotenins (Boman, 2003). This process promotes bacteriocins to flip into a bacterial membrane. This allows entry into the cell at which point the bacteriocin may act by inhibiting DNA and protein synthesis, cell wall synthesis, membrane disruption, autolysis or inhibiting enzyme activity (Figure 2.7). It should be noted that bacteria have the potential to resist bacteriocins. Recently, *Listeria monocytogenes* resistance to bacteriocin (peptidocin like) was determined to be based on down regulation of man-PTS gene expression and shows spontaneous resistance (Kjos *et al.*, 2011).

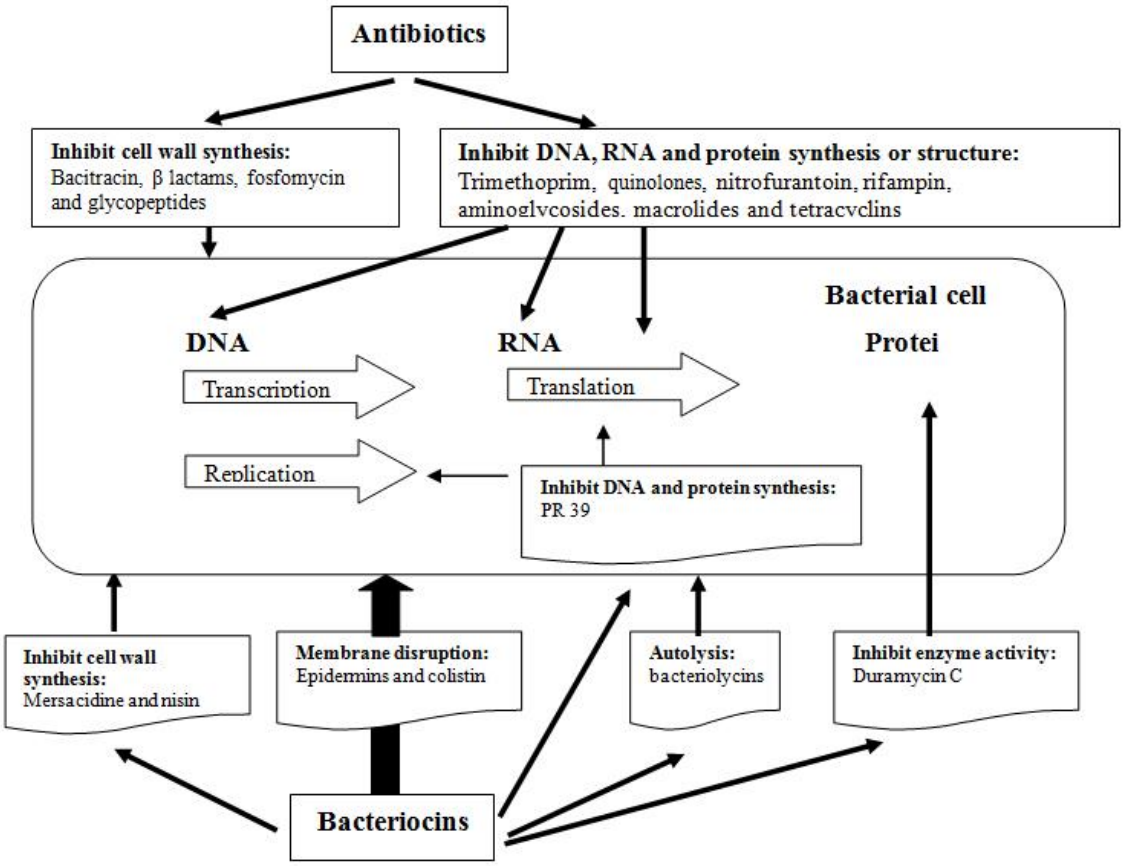


Figure 2.7: Bactericidal mechanisms act on antibiotic and antimicrobial peptides or bacteriocin (modified Sang and Blecha (2008)).

2.16.4 Production of bacteriocin

Bacteriocins made commercially can be produced in large quantities via fermentation biotechnology. One example is carrot, radish and cucumber fermentation with lactobacillus, producing lactic acid and bacteriocins. These bacteriocins antagonised *E. coli, Staphylococcus aureus and Bacillus cereus* (Joshi *et al.*, 2006). However, organisms producing bacteriocin are less effective producers in natural environments, since limitation of nutrients can inhibit bacteriocin production (Hajek, 2004).

2.17 Conclusions

Previous research has effectively documented and highlighted the potential of bacteriophages and bacteriocins as biocontrols against several infectious organisms, including *B. pseudomallei*, for example, reported that lytic bacteriophage found in soil has proved effective to inhibit *B. pseudomallei in vitro*. *Burkholderia ubonensis* produced bacteriocin has the potential to inhibit *B. pseudomallei* in culture media. However, these studies either have been planktonic in nature or have not focused on the environment such as soil or potentially plants where *B. pseudomallei* survive. In this literature review, we introduced the potential of *B. pseudomallei* biocontrol. We found that bacteriophages or bacteriocins could be a method of choice to control the organism. We also identified limitations in the availability of plant models of infection which could be tested. Development of a new plant model and testing with bacteriophage and bacteriocin would be the first investigation as to the effectiveness of biocontrol of *B. pseudomallei* in a plant model. However, some limitations such as nutrition are a concern. The limited nutrient sources in substrates such as soil could affect bacteriocin formation and application of preformed bacteriocin may be needed to overcome this.

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 Bacteriology

3.1.1 Bacterial recovery

All isolates of bacteria used in this study were subcultured from the culture collection at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia. *Burkholderia pseudomallei* were removed from storage (-80° C), were streaked onto Ashdown agar (Appendix A1.1) (Ashdown, 1979) and were incubated at 37 °C for 48 hr in a Physical Containment Level 3 Laboratory (PC3). Near-neighbor *Burkholderia* species were processed as above with the exception that incubation temperatures for Ashdown agar were 30 °C and they were handled in a class 2 cabinet in the Physical Containment Level 2 (PC 2) laboratory. Other bacteria were streaked onto Luria Bertani (LB) agar (Appendix A1.3) and incubated at 37 °C for 24 hr.

3.1.2 Broth cultures

A single 48 hr old colony of *Burkholderia* species from Ashdown agar was subcultured into 10 ml of Luria Bertani (LB) broth. Non *Burkholderia* species were subcultured from 24 hr old colonies on LB agar similarly. Broths were incubated at 37 °C (or 30 °C near-neighbor *Burkholderia* species) with agitation (166 rpm, RATEK, Orbital shaking incubator) overnight (18-20 hr). The suspension were adjusted to 0.4 OD ± 0.05 at 600 nm (about 10^8 CFU/ml count as section 3.1.4).

3.1.3 Bacterial lawns

One ml overnight broth cultures was added to LB agar or Mueller-Hinton (MH) agar and swirled around the plate to ensure coverage of the surface. The excess broth was removed with a transfer pipette (Sarstedt, Germany) and the plate dried in a C2 cabinet for one hour prior to any processing and incubation.

3.1.4 Quantification of bacteria (CFU/ml)

Bacteria were quantified as per the Miles Misra protocol (Miles *et al.*, 1938). Briefly, bacterial culture was tenfold serially diluted 10^{0} to 10^{-8}) and 20 µl of each dilution was spotted onto LB agar plates (duplicate). The plate was dried for about 30 min until all liquid was absorbed. Plates were then incubated overnight at the temperature relevant to the species (30 ^{o}C or 37 ^{o}C). The least diluted countable colonies were then counted, an average determined and original bacterial numbers were calculated. An example calculation is shown.

E.g. 20 µl is the working volume (1000 / 20 = 50)

CFU/ml = colony count × reciprocal of dilution × 50

20 colonies at 10^{-5} dilution

CFU/ml = $20 \times 10^{5} \times 50$

. CFU/ml = 1×10^{8}

3.2 Molecular Technique

3.2.1 DNA extraction of bacterial cultures

DNA extraction of bacterial samples was carried out using the method of (Mogg and Bond, 2003) with modifications. One ml each of overnight bacterial broths were added to 2 ml microfuge tubes with O ring (Scientific Specialties, Inc. (SSI), USA) and were centrifuged for 5 min at 16 000 × *g* (Eppendorf, centrifuge 5415D). Supernatant was discharged and 600 µL of extraction buffer (Appendix A1.5) was added to the pellet and mixed and incubated overnight at 37 °C. 200 µl of 5 M NaCl (Appendix A1.5.3) was added followed by incubation on ice for 5 min. The samples were centrifuged for 5 min at 16 000 × *g*. Supernatants were transferred to new microfuge tubes containing 600 µl of 100% isopropanol (Sigma, USA) and mixed by inversion followed by incubation for 3 min at room temperature. Samples were centrifuged for 5 min at 16

000 × *g* and the supernatant discharged. 600 µl of absolute ethanol (Sigma, USA) was added to the pellet and incubated for 3 min at room temperature. Samples were centrifuged for 5 min at 16 000 × *g*, the supernatant discharged and the sample allowed to dry for 15 min prior to addition of 100 µl of molecular biology-grade H2O (Sigma, USA). Finally, the microfuge tube was incubated for 1 hr at 65 °C or overnight at 4 °C to completely resuspend the DNA

3.2.2 Real time polymerase chain reaction.

The target of the real time PCR assay was a 115 bp stretch in orf2 of the TTS1 of *B. pseudomallei* (Novak *et al.*, 2006). Reactions were made to 20 µl with molecular biology-grade H_2O (Sigma, USA) and consisted of 1x SensiFAST™ Probe No-ROX Kit (Bioline, Australia), 10 µM each of BpTT4176F primer (5'GCT CTC TAT ACT GTC GAG CAA TGC 3'), and BpTT4290R primer (5' CGT GCA CAC CGG TCA GTA TC 3'),10 µM BpTT4208P probe (5' CCG GAA TCT GGA TCA CCA CCA CTT TCC 3') with 5' FAM (6-carboxyfluorescein) -3' Black hole Quencher (BHQ)1), and 2 µl of template . The real-time PCR cycle consisted of an initial denaturation period of 3 min at 95° C followed by 45 cycles at 95 °C for 30 seconds during annealing and 59 °C for 30 seconds during extension. The real-time PCR was performed using a Rotor-Gene 6000 series thermocycler (Corbett Life Science, Australia).

3.2.3 Production of Plasmid standard curve for real time PCR

A standard curve was developed using 10× serial dilutions of a plasmid (pGEM-T Easy Vactor System, Promega, A1360) containing the target DNA in a background of 5 ng/µl of herring sperm DNA (Promega, D1811). This was developed as follows;

3.2.3.1 PCR

Standard end point PCR was carried out using the conditions of section 3.2.2 and *B. pseudomallei* (SA12) template extracted as per section 3.2.1. The product was then run on a gel

3.2.3.2 Gel electrophoresis and extraction of band

Volume of PCR product was run on a 1% TAE gel (1g of agarose added in 100 ml TAE buffer (Appendix A1.6) at 100 volts for about 90 min. The correctly sized band was cut out using a sterile scalpel blade and DNA extracted into of extraction buffer using a gel extraction kit (Wizard® SV Gel and PCR Clean-Up, Promega, A9281). Yield was quantified using absorbance at 260nm on a nanophotometer (IMPLEN, UK)

3.2.3.3 Cloning

Cloning was carried out using a commercial AT cloning kit (pGEM®-T Easy Vector Systems, Promega, A1360) and colonies selectively isolated on LB plates with ampicillin/IpTG/XGaI (Appendix A1.7) using blue white screening. Selected colonies were cultured in LB amp broth as per section 3.1.2. Plasmids were extracted from 5 ml overnight culture using (Wizard® Plus SV Miniprep DNA Purification System, Promega, A1270) and yield quantified using nanophotmeter (IMPLEN, UK)

3.2.3.4 Sequencing confirmation

Plasmids were sent for sequencing at Macrogen and sequences were cleaned and analysed using (SEQUENCHER ^{TM}, Version 4.2) and a Blastn search.

3.2.3.5 Calculation of copy number and dilution series

Copy number/µl was calculated using the URL Genomics & Sequencing Center online copy number calculator (http://cels.uri.edu/gsc/cndna.html).

Total sequence length of plasmid (pGEMT + PCR product) = 3128

Concentration of plasmid: 85 ng/µl

Copy number 2.52×10^{10}/µl

The serial dilution was carried out using 10 µl aliquots of plasmid in 90 µl aliquots of sterile deionized water containing 5 ng/µl herring sperm DNA (Promega, D1811). The final dilution series concentrations are listed in Table 3.1

Table 3.1 Dilution series of Target DNA for standard curve used in TTS1 real time PCR.

Dilution level	**Copy number/ul**
$\mathbf{10^{0}}$	2.52×10^{9}
$\mathbf{10^{-1}}$	2.52×10^{8}
$\mathbf{10^{-2}}$	2.52×10^{7}
$\mathbf{10^{-3}}$	2.52×10^{6}
$\mathbf{10^{-4}}$	2.52×10^{5}
$\mathbf{10^{-5}}$	2.52×10^{4}
$\mathbf{10^{-6}}$	2.52×10^{3}
$\mathbf{10^{-7}}$	2.52×10^{2}
$\mathbf{10^{-8}}$	2.52×10^{1}
$\mathbf{10^{-9}}$	2.52×10^{0}

3.3 Seed preparation

Rice (*Oryza sativa* L. cv Amaroo) seeds were sown, grown and harvested at the College of Public Health, Medical & Vet Sciences, James Cook University during 2012-2013. Wild rice (*Oryza meridionalis*) seed were harvested at Woodstock, Townsville in May 2012 and kept for one year until the dormancy period for the seeds had passed.

3.3.1 Seed cleaning

The seeds were cleaned as per the modified method of Oyebanji *et al.* (2009) by soaking and agitating (200 rpm) in 3.5% bleach (sodium hypochlorite) at 30 °C for 10 min. Disinfectant solutions were discharged and seeds were washed by soaking and agitating in sterile distilled water for three min (washes were repeated four times). The last sterile distilled water was decanted and inoculated onto Ashdown's agar (3 × 20 ul spots) with incubation for 48 hr at 37 °C to confirm no *B. pseudomallei* were present. Seeds were soaked at a depth of approximately one cm of sterile distilled water at 30 °C for two days to encourage uniform imbibition and germination (Handelsman *et al.*, 1990). This sterile distilled water was also tested for contamination with *B. pseudomallei* by incubation on Ashdown's agar for 48 hr as above.

3.3.2 Gnotobiotic plant growth

The surface clean seeds were transferred for propagation based on the method of Hoagland and Arnon (1950), modified by Watt *et al.* (2006) to 1% (w/v) ¼ strength Hoagland agar (Appendix A1.18) using sterile tweezers. The seedlings were grown in Hoagland agar in sealed glass bottles and incubated under cycles of fluorescent light (12 hr) and darkness for (12 hr) for seven days at 30 °C.

3.3.3 Plant measurement and statistical analysis of plant growth determination.

Plantlet were removed from agar using sterile tweezers and photographed alongside a scale. Images were analysed using the area measurement commands in Adobe Photoshop CS6 to calculate area as described by Villar *et al.* (2013). When an analysis consisted only of a control and experimental group, Independent T tests (p=0.05) were performed using IBM SPSS version 20 and where three or more groups are compared, a one way ANOVA was performed (p=0.05) using a post hoc test.

3.4 Testing sterilization of *B. pseudomallei* slides for removal from PC3 laboratory

Burkholderia pseudomallei fixed onto slides as per (Section 4.2.6) were testing for sterility by removal of fixed bacteria from at least three replicate wells. These were removed by scratching the surface with a sterile cotton swab and plating from the swab onto Ashdown agar. Agar plates were incubated at 37 °C for 48 hr. Slides were brought out of the PC3 laboratory if none of the Ashdown agar plate had colonial growth by 48 hr as confirmed by two people.

3.5 Bacteriophage

3.5.1 Bacteriophage amplification (plate technique) modified from

Each phage (20 µl) from College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia. *Burkholderia pseudomallei* were removed from storage (4 °C) were amplified using the same bacterial lawn as per section 3.1.3 and incubated 37 ^{o}C overnight. Each spot were added 200 µl of SM buffer (Appendix A1.9) and using of 10 µl blue loop (SARSTEDT, Germany) blend the area containing plaques. The liquid were removed by transfer pipette and placed in 1.5 ml microfuge tube (Quantum scientific, Australia). The microfuge tube were centrifuged for five min at 10 000 × *g* to discharge pellet bacteria and kept supernatant. This process was repeated until get 10^{7} PFU/ml and the last supernatant was passed through a 0.45 µm (Sarstedt, Filtropur S 0.45) to keep as a bacteriophage working solution in 4 °C.

Comment: In large volume, one ml of bacteriophage were added in lawn bacterial as per section 3.1.3 and swirled around the plate to ensure coverage and let the dry one hour and incubated 37 ^{o}C overnight. Five ml of SM buffer were added to plague formation and using spreader were moved in an arc on the surface of agar with rotation to blend agar. The liquid were added to conical tube. The conical tube were centrifuged for 15 min at 3 000 × *g* (Techcomp refrigerated benchtop centrifuge, CT15RT) to

discharge pellet bacteria and kept supernatant. Samples were filtered using 0.45 µm filters (Sarstedt, Filtropur S 0.45). They were then stored at 4 °C in SM buffer.

3.5.2 Quantification of bacteriophage

Suspension bacteriophage was made a tenfold dilution series (10^0 to 10^8) and 10 µl of each dilution was inoculated to lawn plate as per 3.1.3 (replicate) and let plate dry (about 30 min) and incubated 37 ^oC overnight (Miles *et al.*, 1938).

E.g. 10 µl is a working concentration (1000 / 20 = 50)

CFU/ml = colony count x reciprocal of dilution × 50 20 colony in 10^5 dilution

CFU/ml = $1 \times 10^5 \times 100$

CFU/ml = 1×10^7

Comment: due to pin point plaque formation, we cannot count individual plaque

CHAPTER 4: DEVELOPING A PLANT MODEL AND LOCALIZATION DETECTION OF *B. PSEUDOMALLEI* INFECTION

4.1 Introduction

The genus *Burkholderia* consists of over 60 species and plays roles in human, animal and plant disease. *Burkholderia* species are usually present in in soil and rhizospheres that provide nutrients to support bacterial growth (Barea *et al.*, 2013; Weisskopf and Bailly, 2013). Many *Burkholderia* species have a plant association. For example, *B. cenocepacia* is an endophytic bacteria in *Triticum aestivum* L.(wheat), *Lupinus* sp. (lupine) and *Zea mays* L (maize) (Balandreau *et al.*, 2001). While *Burkholderia vietnamiensis* has been found in in rhizospheres of *Coffea arabica* L. (coffee), *Zea mays* L. (maize) (Estrada-De los Santos *et al.*, 2001) and *Oryza sativa* L (rice) (Van *et al.*, 2000). *Burkholderia ubonensis* has been found in rhizospheres of *Heteropogon contortus* (black spear grass) (Tahani, 2009), unpublished data.

Burkholderia pseudomallei causes melioidosis, a disease that has been significantly associated with rice farmers, especially those with diabetes mellitus or renal failure in Northeastern Thailand (Chaowagul *et al.*, 1989). *Burkholderia pseudomallei* has a high frequency of isolation in rice fields (Rattanavong *et al.*, 2011). During the last outbreak of acute melioidosis in Western Australia, *B. pseudomallei* was isolated from the rhizospheres soil of *Acacia coleii* (Inglis *et al.*, 2000) and it has also been found in rhizosphere soil of *Oryza* sp. (most likely *O. meridionalis* or *O. rufipogun*) and *Pseudoraphis* sp. (most likely *P. spinescens*) in the Balimo region of Papua New Guinea (Nilsson, 2006), unpublished data. Kaestli *et al.* (2009) demonstrated that *B. pseudomallei* positive samples in undisturbed soil were associated with areas of rich grasses such as spear grass (e.g. *Sorghum spp.*).

In contrast, Lee *et al.* (2010) found no effects on a Japanese rice cultivar (*Oryza sativa* L. cv. Nipponbare) and no infection during experimental infection trials. Recently, Kaestli *et al.* (2012) found that *B. pseudomallei* can infect exotic and native grasses in

Darwin such as *Brachiaria humidicola* cv Tully (Tully grass), *Pennisetum pedicellatum* and *polystachion* (Mission grass) and *O. rufipogon* (Wild Rice). An examination of the interaction of *B. pseudomallei* with a rice plant cultivar (*Oryza sativa* L. cv Amaroo) could provide more information about whether domestic rice is in general resistant to *B. pseudomallei* or identify a potential host for an infection model.

Localization in tissues is important in identifying the pathogenesis or interaction of microorganism with the tissue. Electron microscopy and fluorescent in situ hybridization (FISH) have been used to investigate the location of *B. pseudomallei* in plants (Lee *et al.*, 2010; Kaestli *et al.*, 2012). Recently, a monoclonal antibody-based immunofluorescence assay has been used to identify *B. pseudomallei* in clinical samples with a specificity up to 99.8% (Tandhavanant *et al.*, 2013). While there is currently no commercially available antibody against *B. pseudomallei* in Australia, there is one against *B. mallei* lipopolysaccharide (LPS) (MCA2823 AbD Serotec/ BIO-RAD, USA).

According to Yabuuchi *et al.* (1992b) cellular lipid, fatty acid composition, DNA-DNA homology value and 16S rRNA sequence are similar between *B. pseudomallei* and *B. mallei*. These species also have a close relationship on phylogenetic tree based on complete and draft genomes of *Burkholderia* species (Vandamme and Dawyndt, 2011). So it is expected that some epitopes are shared between the two species and antibodies raised against one species might cross-react with the other species? An anti-*B. mallei* monoclonal antibody specific *B. mallei* LPS was demonstrated by Zou *et al.* (2008) not to cross react with *B. pseudomallei*, however Anuntagool and Sirisinha (2002) showed that anti-*B. mallei* monoclonal antibodies against LPS did cross react with *B. pseudomallei* (three of six antibodies produced). As such, a commercial anti-*B. mallei* antibody may be of use for detection of *B. pseudomallei*.

The aim of this chapter was to investigate if *Oryza sativa* L. cv Amaroo was affected by *B. pseudomallei* and whether *B. pseudomallei* was internalized, thus forming a plant model of infection, leading to the use of this rice strain in biocontrol experiments.

4.2 Material and Methods

4.2.1 Bacteria.

B.pseudomallei TSV189 and *B. vietnamensis* (38SP) were subcultured from storage as per section 3.1.1. They were cultured into broth as per section 3.1.2

4.2.2 Optimization of seed cleaning protocol

The seed cleaning protocol of Oyebanji *et al.* (2009) was modified to optimize seed cleaning of *Triticum aestivum* L. (wheat). Seeds were soaked in sterile distilled water for 24 hr. Sterile distilled water was decanted and seeds (100 per group) were added to 100 ml of disinfectant solution consisting of sodium hypochlorite (3.5%). Seeds were soaked in the disinfectant solution for 10, 20 and 40 min with magnetic stirrer agitation (200 rpm at 30 ^{o}C). 100 seeds were also soaked in 70% ethanol for 10 min. Seeds were then washed and processed as per section 3.3.1. The final wash solution was spotted (3 × 20 µl spots) onto Ashdown agar with incubation for 48 hr at 37 ^{o}C to detect any *Burkholderia* or related species. Five cleaned seeds were added to 3 ml of LB broth with incubation for 48 hr at 37 ^{o}C to detect any further bacterial contamination.

4.2.3 Preparation and cleaning of rice seeds.

Rice (*Oryza sativa* L. cv Amaroo) seeds were sown, grown and harvested at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia. during 2012-2013. The seeds were cleaned as per section 3.3.1. Germination rate was determined using 45 seeds.

4.2.4 Survival kinetics of *Burkholderia* species in ¼ strength Hoagland solution

Burkholderia pseudomallei (TSV189) and *B. vietnamensis* (38SP) were grown overnight in 10 ml LB broth as per section 3.1.2. These were centrifuged at 3 000 x *g* (Techcomp refrigerated benchtop centrifuge, CT15RT) for 15 min and the supernatant discharged. To wash the pellet, 10 ml of 0.85% NaCl (Appendix A1.10) was added to the pellet. The pellet was resuspended and the solution centrifuged at 3 000 × *g* for 15 min. The supernatant was discharged and the process repeated three times. Finally, the pellet was made up in 0.85% NaCl to the same original volume (10^8 CFU/ml, 10 ml). One hundred µl of bacterial suspension was added to 9.9 ml of ¼ strength Hoagland solution (Appendix A1.8) and incubated at 30 °C. Twenty µl samples were removed each day from day zero to seven and at 90 days. Bacteria in samples were counted use of the Miles Misra technique (section 3.1.4)**.** Statistical significance was calculated using an ANOVA with post hoc test (p=0.05) from day one to 90. Post hoc test were perform this experiment due to heterogeneity of variance

4.2.5 Infection of seeds for plant growth experiments.

An infection dose trial was carried out based on high dose (10^8 CFU/ml) serial diluted to low dose (10^2 CFU/ml) of *B. pseudomallei* TSV 189 in LB broth (Appendix A1.4). Seeds were added to these broths and allowed to sit for an hour, prior to removal from bacterial broth. Control seeds were treated by soaking in LB broth (Appendix A1.4) for the same amount of time. 45 seeds at each dose and 45 control seeds were used for this study. The seeds were then transferred to experimental chambers as per section 3.3.2 Plant measurements on day seven were carried out as per section 3.3.3. Statistically significant changes in growth based on root and leaf area were calculated using an ANOVA with post hoc test (p=0.05). Germination rate changes were analysed using Odds ratio and Chi-squared (X^2) analysis. In addition, *B. vietnamensis* was tested at the high dose of 10^8 CFU/ml in a similar protocol and compared to control growth of root and leaf using an independent T-test (p=0.05).

4.2.6 Immunofluorescence assay optimization

MCA2823 (AbD Serotec/ BIO-RAD, USA), an anti-*B. mallei* antibody was selected for testing as a tool for detection/identification of *B. pseudomallei*. Isolates of *B. pseudomallei* (isolate TSV 189) were centrifuged at 3 000 × *g* for 15 min and the supernatants were discharged. Pellets were washed three times with 0.85% NaCl and 15 µl of bacterial suspensions were added on slides (Menzel-GmbH & Co KG, Braunschweig, Germany), in duplicate, dried for at least one hour at room temperature (RT) and up to overnight until the sample was completely dry.

To determine the optimum fixation protocol, a liquid blocker (Pro Sci Tech, ID 300) was sued to make wells on microscopes slide. Bacteria was added to the wells and dried on slides by fixing in either absolute ethanol or acetone at -20 ^{o}C or 4% paraformaldehyde in PBS or 10% formalin in water at room temperature for various times (one, two and four hours) then they were dried for one hour at RT. The slides were kept at -20 ^{o}C until confirmation of sterility (section 3.4) and then they were processed for IFA. Slides were soaked in phosphate buffered saline (PBS) (Appendix A1.11) for 5 min at room temperature (RT) and blocked for 30 min at RT in 10% (v/v) goat sera diluted in 1% (w/v) bovine serum albumin (BSA) (Appendix A1.12) in phosphate buffered saline (PBS) (BSA/PBS). After a gentle wash in PBS the bacteria were incubated with various dilutions (1:10, 1:50, 1:250, 1:1250) of mAb MCA2823 in 1% BSA/PBS for two hours at RT or overnight at 4 °C in a humidified chamber. The slides were gently washed in PBS (Appendix A1.11) and the bacteria were incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 595 (Invitrogen, USA) diluted 1/300 in 1% BSA/PBS for 45 min at RT. After a final wash in PBS the slides were mounted using fluorescent mounting media (KPL, USA) and observed under an epifluorescent microscope (AxioImager.Z1, Zeiss, Germany). Pictures were taken using a digital camera (AxioCam MRm, Zeiss, Germany). As a negative control, a mouse mAb of the same isotype (IgG1) (MM1A, Anti CD3 receptor, Washington State University) was used at the same concentration as mAb MCA2823. Fluorescence was scored qualitatively as +4: very strong signal to +1: lowest visible signal while +2 and

+3 graduated between +1 and +4. Slides were scored blind and matched to the processing conditions after scoring (Appendix A2.1).

4.2.6.1 Reactivity of the antibody specific for *B. mallei* with *B. pseudomallei,* and other bacteria.

Various species and strains of bacteria were cultured, washed in PBS and dried on slides as described above for *B. pseudomallei* isolate TSV 189 and processed using the following conditions. The bacteria were fixed in acetone for four hours. Slides were dried and stored at -20 ^{o}C. Duplicate slides were tested for sterility (section 3.4). Once sterility was confirmed, slides were blocked with goat sera and incubated with mAb MCA2823 diluted 1/100 overnight at 4 °C in a humidified chamber. After washing in PBS the bacteria were incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 595 diluted 1/300 for 45 min at RT, washed in PBS and mounted as described before. The reactivity of all species and strains of bacteria with mAb MCA2823 was checked in duplicate. As a negative control, a mouse mAb of the same isotype (IgG1) (MM1A, Anti CD3 receptor, Washington State University) was used at the same concentration (as described previously).

4.2.6.2 *Burkholderia pseudomallei* infection of seeds for testing with IFA.

Seeds were cleaned as per section 3.3.1. Cleaned, primed seeds were incubated in Petri dishes for two days for primary root germination. At this time, roots of half the seedlings were inoculated with about 10^7 CFU *B. pseudomallei* (isolate TSV189) as per the method of Kaestli *et al.* (2012) by dropping 100 µl of 10^8 CFU/ml *B. pseudomallei* in LB directly onto the roots while the other half were uninfected (three seedlings per group). The seeds were transferred for propagation based on the method of Hoagland and Arnon (1950), modified by Watt *et al.* (2006) to 1% (w/v) ¼ strength Hoagland agar (Appendix A1.8). The seedlings were grown in Hoagland agar in sealed glass bottles and incubated under cycles of fluorescent light (12 hr) and darkness for (12 hr) for seven days at 30 °C.

4.2.6.3 Preparation of plant samples for IFA.

Plantlets were gently lifted from the agar surface and roots were washed in 0.85% NaCl (Appendix A1.10) with gentle agitation three times to remove any loose bacteria or agar. Root pieces were cut to 0.5-1 cm long and fixed in acetone at -20 °C for 3 days, until sterility of samples was confirmed. Further, the samples were embedded in Optimum Cutting Temperature (O.C.T.) (Tissue-Tek®, Sakura, Japan) and stored at -80 °C until sections were cut. Cryosections, five micrometer thick, were cut from roots with a cryostat (Leica CM1850, Germany) at -20 °C and transferred to slides (Menzel-GmbH & Co KG, Braunschweig, Germany), Sections were dried overnight at room temperature under a fan.

4.2.6.4 IFA on root sections.

Sections were immersed in PBS and blocked for 30 min at RT in 10% (v/v) goat sera diluted in 1% BSA in PBS. After a brief wash in PBS the sections were incubated at RT for 40 min then overnight at 4 ^{o}C with mAb MCA2823 diluted 1:100 in 1% BSA in PBS (final concentration 10 µg/ml). The slides were washed gently with PBS and the sections were incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 595 (Invitrogen, USA) diluted 1/300 in 1% BSA/PBS for 45 min at room temperature. After three gentle washes with PBS, slides were mounted using fluorescent mounting media (KPL, USA) and observed under an epifluorescent microscope (ZEISS, Axioshop 40)

4.3 Results

4.3.1 Optimization of cleaning protocol

The results obtained from the seed cleaning optimization on wheat is presented in Table 4.1. Untreated seed surfaces were found to be heavily contaminated with both bacteria and fungi. Although these were not speciated, the growth on Ashdown agar was not typical of *B. pseudomallei*. Ethanol cleaning, while effective at sterilization, resulted in the least amount of germination and was rejected for further use. Of the sodium hypochlorite (NaOCl) treatments, the longest treatment resulted in the lowest germination rates while the 10 and 20 min treatments had similar germination rates. Low levels of contamination were observed on Ashdown agar for the 10 min treatment. When rice was available it was determined that a 10 min treatment was typically sufficient to result in no growth on Ashdown agar. A cleaning protocol of 3.5% NaOCl for 10 min, with confirmation of sterility of final wash solution on Ashdown agar was selected for rice. The germination rate of *Oryza sativa* L. cv Amaroo seed was 86.6% (39/45) for these conditions. Multiple batches of rice were processed via this method for later experiments and it was observed that seeds with a healthy appearance were less likely to be rejected due to failing the wash solution step than seeds that appeared damaged or unhealthy.

Table 4.1: Optimization of seed surface cleaning using dehulled *Triticum aestivum* L.(wheat) seed. Fungal observations on Hoagland agar were taken at three days. T* represents turbidity in broth, NT** represents no turbidity observed in broth. ++++ represents heavy bacterial growth, +/- represents limited bacterial growth as a qualitative observation

Experiment	**Mean germination (100 seeds)**	**Bacterial growth from 4th rinse solution**		**Fungus present on seeds on Hoagland agar**
		LB broth	**Ashdown agar**	
Control	38	T *	Yes ++++	Yes
3.5% NaOCl, 10 min	15	T *	Yes +/-	No
3.5% NaOCl, 20 min	16	T *	No	No
3.5% NaOCl, 40 min	5	T *	No	No
70 % Ethanol 10 min	0	NT **	No	No

4.3.2 Survival kinetics in ¼ strength Hoaglands solution

Burkholderia pseudomallei (TSV189) and *B. vietnamensis* (38SP) numbers increased by up to one log over the first day of incubation, after which numbers remained relatively constant with no significant change in numbers between day one and seven. Both species can survive and maintain themselves in ¼ Hoagland broth for more than three months (Figure 4.1). The single most marked observation was that the number of *B. vietnamensis* (38SP) increased at 90 days (Appendix A3.1).

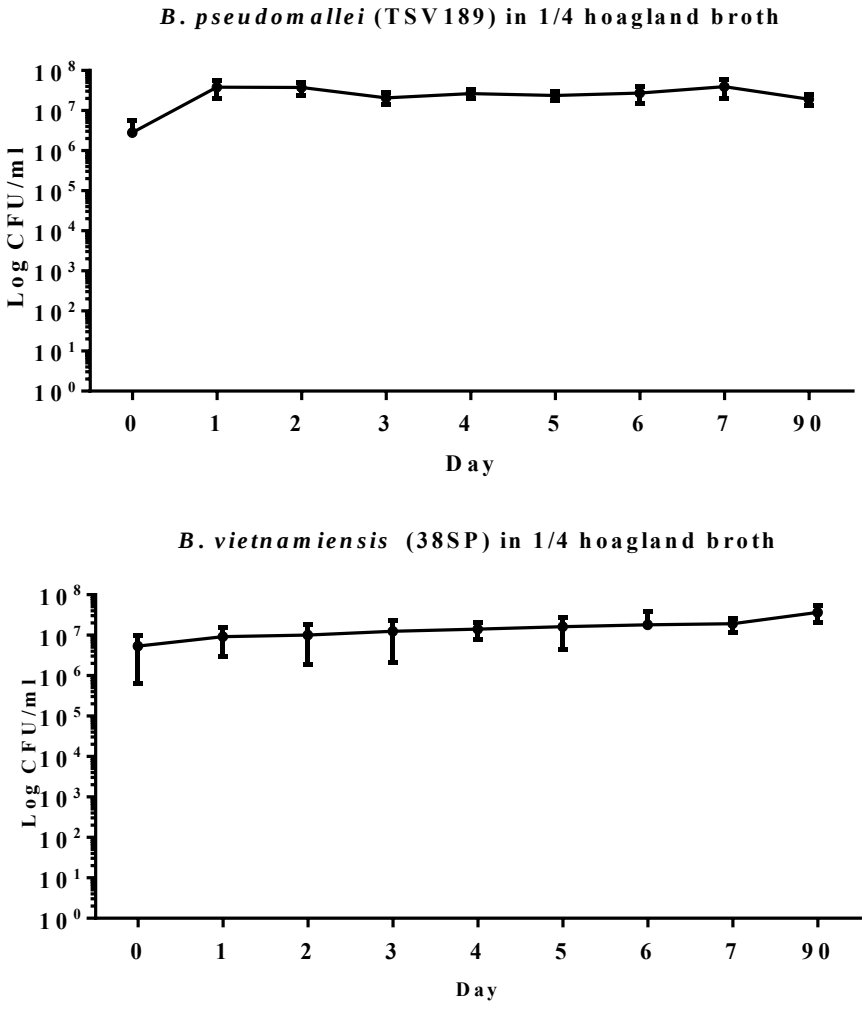


Figure 4.1: Survival data (mean ± 95% CI) obtained in ¼ strength Hoagland solution for (a) *B. pseudomallei* TSV189 and near-neighbor species (b) *B. vietnamensis* 38SP

4.3.3 Dose dependent growth inhibition of rice with *B. pseudomallei*

The dose dependent exposure test revealed that root growth was more inhibited by *B. pseudomallei* (TSV189) than leaf growth (Figure 4.2). Inhibition of growth was significant at 10^6 CFU/ml *B. pseudomallei* (TSV189) (Figure 4.2 a) ($p<0.000$), while leaf growth inhibition was significant at 10^7 CFU/ml *B. pseudomallei* (TSV189) ($p<0.000$) (Figure 4.2b). A trend of decreased germination rate was also observed with higher doses of *B. pseudomallei*, which is supported by an Odds ratio analysis, however this was not significant (Table 4.2, Chi-square ($\boldsymbol{X^2}$) (Appendix A3.2)

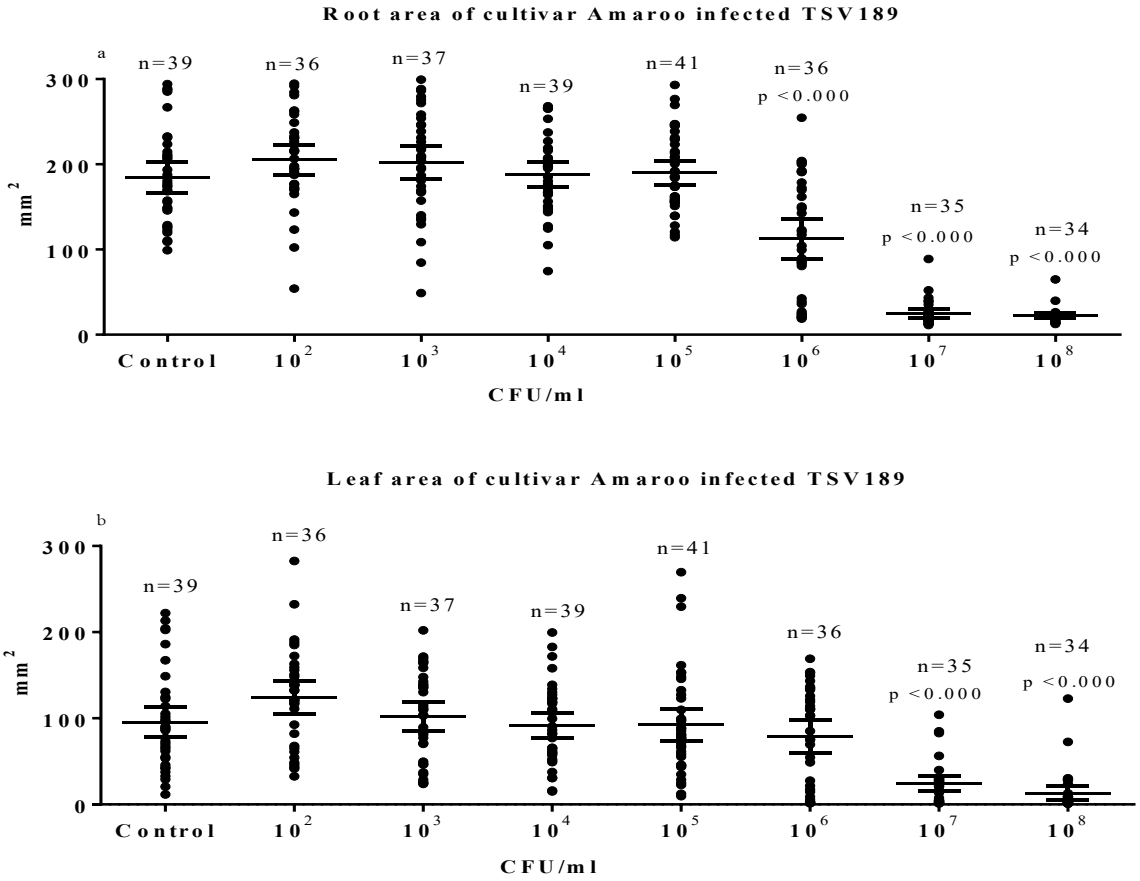


Figure 4.2: Exposure of *Oryza sativa* L. cv Amaroo to low to high dose (102 to 108 CFU/ml) of *B. pseudomallei* TSV 189. There is no significant stunting of the roots until a dose of 10^6 CFU/ml is applied (a). Leaf growth is significantly inhibited at 10^7 to 10^8 CFU/ml (b). n represents the number of germinating plants used in the statistical analysis. Non-germinating seeds were removed prior to analysis as the number of non-germinating seeds due to exposure to *B. pseudomallei* as opposed to cleaning or other factors could not be determined.

Table 4.2: Germination success with different doses of *B. pseudomallei*. Summary report Chi-square (X^2), the degree of freedom (df), and the significance value of each concentration of *B. pseudomallei* (TSV189) with respect to germination success after seven days of growth.

Report	**10^2 CFU/ml**	**10^3 CFU/ml**	**10^4 CFU/ml**	**10^5 CFU/ml**	**10^6 CFU/ml**	**10^7 CFU/ml**	**10^8 CFU/ml**
X^2, df	0.720, 1	0.338, 1	0.0, 1	0.450, 1	0.720, 1	1.216, 1	1.813, 1
P value	0.396	0.561	1.000	0.502	0.396	0.272	0.178
Odd ratio	1.625	1.405	1.000	0.6341	1.625	1.854	2.103

4.3.4 Comparison of growth inhibition with *B. vietnamensis*

B. vietnamensis was used as a control organism to test the effect of dose of bacteria rather than species specific effect. A dose of 10^8 CFU/ml was selected as the highest exposure that was used with *B. pseudomallei* (Figure 4.3**)**. While there was a slight trend of lower growth at exposures of 10^8 CFU/ml with *B. vietnamensis*, this was not significant (root p=0.291, leaf p=0.064) (Appendix A3.3).

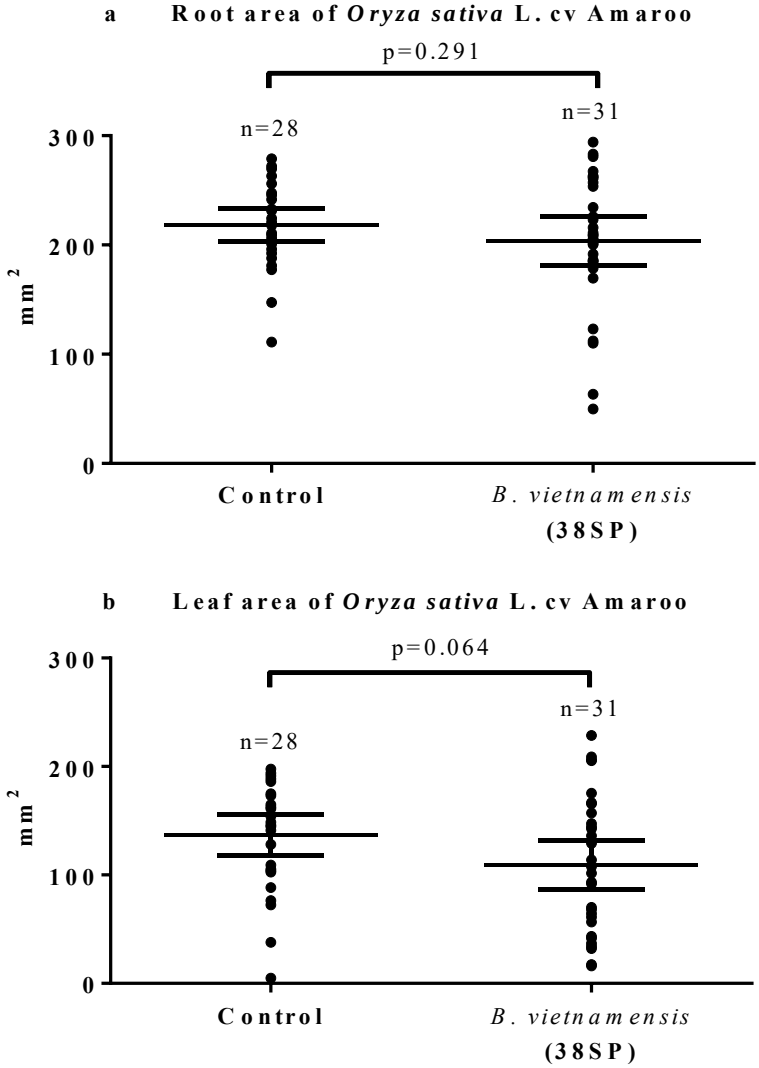


Figure 4.3: The high dose (108 CFU/ml) of *B. vietnamensis* (a -root and b -leaf) does not significantly stunt root or leaf (*B. vietnamensis* root p=0.291, leaf p=0.064) (Independent T test, p=0.05).

4.3.5 IFA optimisation

Acetone and ethanol were identified as better fixatives than 4% paraformaldehyde and 10% formalin as they produced a stronger signal (Table 4.3). Acetone was selected for further work as some samples treated with ethanol were not sterile after the four hour treatment. Although 1:10 dilution of antibody specific for *B. mallei* LPS (AbD Serotec/ MCA2823) produced a high signal, this dilution was deemed uneconomical. Dilutions between 1:50 to 1:250 were identified as producing strong signals while 1:1250 dilution of antibody produced an unacceptably low signal (if you have images for all of these, they go in an appendix and you quote the appendix here). A 1:100 dilution was selected for all further work. Two hour incubations at room temperature were deemed acceptable, however overnight incubation produced the highest signal and was selected for further work.

The anti-*B. mallei* antibody was tested for reactivity to a variety of isolates of *B. pseudomallei* as well as cross-reactivity against other species (see Table 4.4). *Burkholderia mallei* is not found in Australia, where this work was done and was not available for testing. The IFA using this antibody was positive for *B. pseudomallei* and negative for *B. pseudomallei* near-neighbor species and other organisms tested. *Burkholderia pseudomallei* could clearly be seen in infected plant roots in Figure 4.4.

Table 4.3: Qualitative analysis of fluorescent signal under a range of antibody concentrations and fixative techniques. +4 is a very strong signal, +1 would be the lowest visible signal while +2 and +3 a graduated between +1 and +4 (Appendix A2.1).

anti-*B. mallei* (MCA2823)	acetone	ethanol	4% paraformaldehyde in PBS	10% formalin in water
1:10	+4	+4	+4	+4
1:50	+4	+4	+3	+3
1:250	+3	+3	+2	+2
1:1250	+3	+3	+2	+1

Table 4.4: Reactivity of mAb MCA2823 with *B. pseudomallei*, neighbour *Burkholderia* species and other bacteria. Superscript numbers in species column indicate method previously used to identify bacterial species ([1]: API 20 NE, [2]: IHA, EIA IgG/M, [3]: MLST, [4]: PCR TTSS gene, [5]: Full sequence and [6]: recA gene.

Code	Species	Isolated	Present	IFA
4	*Burkholderia pseudomallei* [1]	Human clinical	Mornington Island, Australia	+
8	*Burkholderia pseudomallei* [2]	Human clinical	Cloncurry, Australia	+
C1	*Burkholderia pseudomallei* [3]	Human clinical	Adiba, Sanabase, Papua New Guinea	+
C2	*Burkholderia pseudomallei* [3]	Human clinical	Kimama, Teleme, Papua New Guinea	+
TSV189	*Burkholderia pseudomallei* [4]	Alpaca necropsy	Townsville, Australia	+
14-289	*Burkholderia pseudomallei* [4]	Parrot necropsy	Townsville, Australia	+
14-327	*Burkholderia pseudomallei* [4]	Koala necropsy	Townsville, Australia	+
TSV192	*Burkholderia pseudomallei* [4]	Soil	Townsville, Australia	+
TS5	*Burkholderia pseudomallei* [1]	Soil	Townsville, Australia	+
K96243	*Burkholderia pseudomallei* [5]	Human clinical	Thailand	+
TSV4	*Burkhoderia arboris* [6]	Water seep	Townsville, Australia	-
TSV19	*Burkholderia gladioli* [6]	Bulk water	Townsville, Australia	-
TSV21	*Burkholderia cepacia* [6]	Water seep	Townsville, Australia	-
TSV87	*Burkholderia pyrrocinia* [6]	Bulk water	Townsville, Australia	-
TSV88	*Burkholderia pseudomultivorans* [6]	Bulk water	Townsville, Australia	-
E1	*Burkholderia thailandensis* [3]	Clay	Biula, Papua New Guinea	-
A21	*Burkholderia ubonensis* [6]	Soil	Adiba, Sanabase, Papua New Guinea	-
17sp	*Burkholderia cenocepacia* [6]	rhizosphere soil	Townsville, Australia	-
31sp	*Burkholderia latens* [6]	rhizosphere soil	Townsville, Australia	-
38 sp	*Burkholderia vietnamiensis* [6]	rhizosphere soil	Townsville, Australia	-
A03a	*Bordetella spp.* [6]	rhizosphere soil	Adiba, Sanabase, Papua New Guinea	-
13sp	*Achromobacter xylosoxidans* [6]	rhizosphere soil	Townsville, Australia	-
ATCC27895	*Pseudomonas aeruginosa*	Control		-
ATCC25921	*Escherichia coli*	Control		-
ATCC10876	*Bacillus cereus*	Control		-
ATCC13076	*Salmonella enteritidis*	Control		-

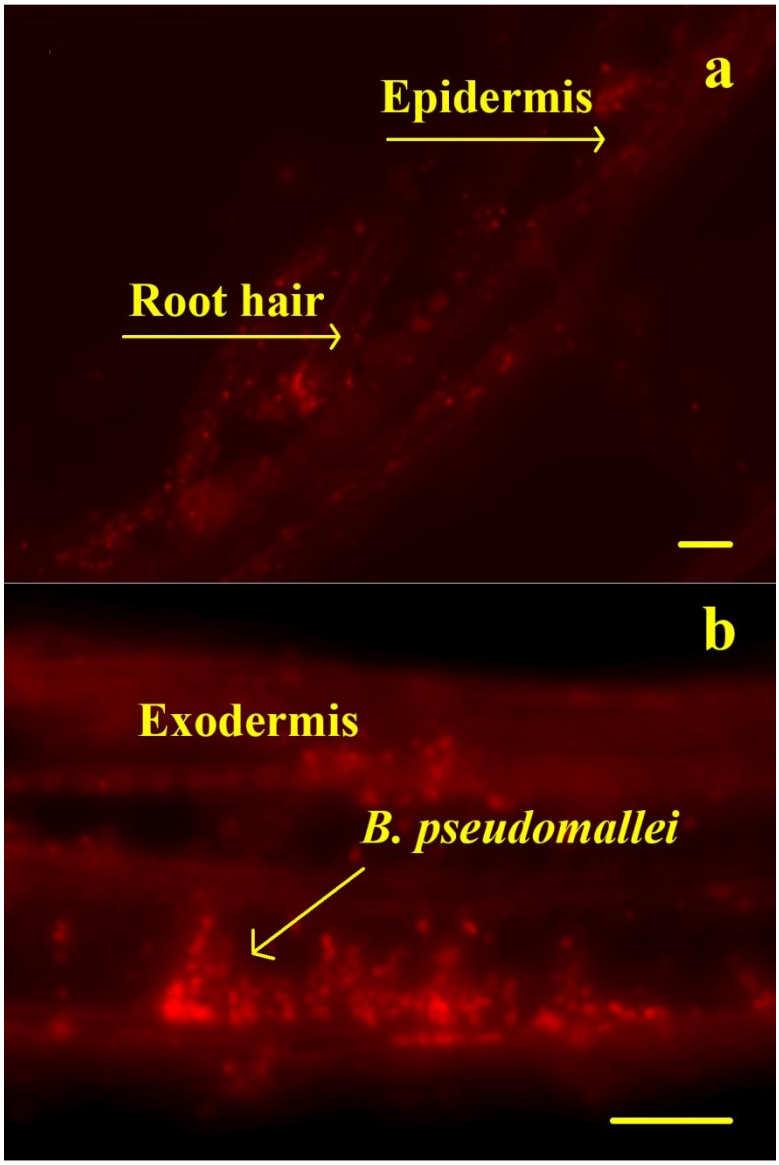


Figure 4.4: 4.4a is a positive longitudinal section which displays *B. pseudomallei* (strong red reaction) with Texas red stain on the epidermis. 4.4b is a positive longitudinal section at higher magnification displaying *B. pseudomallei* (arrow and elsewhere) inside the exodermis of a root hair. Bars display a 10 µm length.

4.4 Discussion

Wheat was used as a proxy for rice to test different cleaning regimes in this study because the rice was still being grown when this work began. Soaking wheat seeds in 3.5% sodium hypochlorite (NaOCl) for 10 min was not sufficient to remove all surface bacterial contaminants, but did heavily lower the contamination level (Table 4.1). As time was increased, germination rate decreased, so the 10 min protocol with a confirmation step was used for rice. The rice had an even higher germination rate than the wheat and the surface cleaning was typically sufficient (data not shown). It was also observed that damaged or unhealthy rice seeds were more likely to remain contaminated at the end of the process. The higher contamination of wheat seeds may be due to the dehulling process causing damage to the seeds and providing protective sites for bacteria (Charkowski *et al.*, 2001; Fandohan *et al.*, 2006). This appears to be the case with the damaged rice seeds as well. The effectiveness of cleaning conforms to previous findings in the literature. Sauer and Burroughs (1986) reported 1-5% of sodium hypochlorite for one min killed almost all spores of *Aspergillus* species on corn and wheat surfaces. Some spores were not killed due to air bubbles, cracks and debris on seed surfaces. Oyebanji *et al.* (2009) demonstrated that cowpea seed, rice and sorghum seed were variably surface sterilized when seeds were soaked for different times in 3.5% sodium hypochlorite. The mechanism of sodium hypochlorite depends on hypochlorous acid (HOCl) and hypochlorite ion ($^{-}$OCl) which is a strong oxidizing agent that reacts with biological molecules such as protein, amino acid, peptide, and DNA so dose is important to avoid damaging the seed tissues and lowering germination rate (Fukuzaki, 2006). Piernas and Guiraud (1997) identified that ethanol reduced the germination rate of rice although 70% ethanol was effective in surface sterilizing seed. Destruction of enzymes required for germination were proposed as the cause of the lowered germination. This also matches with the results found in this study. It should be noted that surface cleaning does not eliminate all endophytic bacteria as shown by Kaga *et al.* (2009) and attempts to kill endophytic bacteria typically have killed the seed, which is counterproductive. At least some of the turbidity in overnight broths of cleaned seeds may be due to endophytic bacteria.

Survival kinetics were carried out to eliminate the possibility of a differential effect of Hoagland agar on the two *Burkholderia* species ability to replicate or survive. The survival of *B. pseudomallei* and *B. vietnamensis* in Hoagland broth (Figure 4.1) is similar, thus eliminating Hoagland agar as being responsible for different bacterial numbers affecting plant growth differently. The number of both species initially increase and have a stable or slightly increased growth over a considerable time in Hoagland solution. Hoagland broth contains a range of carbon sources and the initial growth may be due to energy sources already present in the bacteria as well as use of some of these carbon sources. The sustained numbers may be due to *Burkholderia* species ability to utilize many carbon compounds (Garrity *et al.*, 2005) . It is also important to note that *B. pseudomallei* also has a long stable survival in distilled water, with numbers only starting to drop off around 90 days. (Moore *et al.*, 2008) proposed a survival mechanism involving a gene encoding a putative phosphatidylglycerol phosphatase (PGP) which would be induced when cells were shifted to water as well as lipopolysaccharide (LPS) providing an outer membrane architecture which allows prolonged survival in water.

Oryza sativa L. cv Amaroo seed (domestic rice) has a high germination rate (86.6%) on Hoagland agar after cleaning, probably due to the high quality of seed. Both root and leaf area are inhibited at statistically significant levels when a sufficient dose of *B. pseudomallei* is applied. The possibility that this is due merely to the fact that a high dose of Gram negative bacteria has been applied can be eliminated as the near-neighbor species *B. vietnamensis* can be applied at the highest dose (10^8 CFU/ml) without significantly inhibiting growth (Figure 4.3). Thus the inhibition is *B. pseudomallei* specific.

Lower doses of *B. pseudomallei* were not inhibitory (leaf 10^6 and lower, root 10^5 and lower) which may be due to competition with endophytic bacteria already present in seeds (Figure 4.2). Unfortunately it was not possible to remove all presence of potentially competing bacteria without killing the seeds as noted above. This dose response was also reported by Kaestli *et al.* (2012). In the previous work, Kaestli *et al.* (2012) challenged Tully Grass (*Brachiaria humidicola* cv Tully), Mission Grass (*Pennisetum pedicellatum* and *polystachion*) and wild rice (*O. rufipogon*) with 10^4 and 10^7 CFU of *B. pseudomallei* for 4 weeks by soaking the roots with the *B. pseudomallei* and transferring to soil for growth. The extended time of this experiment can be explained by the use of soil which is of lower nutrient than laboratory growth media and the slow growing species used. All species except wild rice had signs at the higher inoculums (increased deaths of seedlings and a trend of reduced growth).

Other previous studies have looked at developing various plant models for a range of *Burkholderia* species. Alfalfa (*Medicago sativa*) seedlings have had their leaves punctured, followed by infection with 10^1 to 10^5 CFU of a range of species in the *Burkholderia cepacia* group by Bernier *et al.* (2003), with high concentrations of bacteria resulting in a higher percentage of symptoms in seedlings after seven days (yellowing leaves, stunted roots, necrotic regions). Mattos *et al.* (2008) used 10^8 CFU of *B. kururiensis* to inoculate domestic rice roots (*Oryza sativa* L., cultivar not described) over seven days with significant increases in growth measures, indicating the bacteria acted as a growth promoter. In another study, tomato (*Solanum lycopersicum* variety Season Red F1 Hybrid), rice (*Oryza sativa* L. cv. Nipponbare) and rockcress (*Arabidopsis*) roots (with and without wounding) were exposed to *B. pseudomallei and B. thailandensis* by Lee *et al.* (2010) at 10^7 CFU for 7 days, but only tomato plantlets showed signs of disease. Tomato leaves were also exposed directly via wounding of the leaves and bacteria could be found in the xylem vessels. The similar experiments with rice resulted in no signs of infection. It is clear that species in the genus *Burkholderia* can affect the growth of multiple species of plants, however, that affect is not uniform, even using the same bacterial isolate on different plant species.

Of note from the above studies, no domestic rice cultivar has previously been identified as a plant model of growth inhibition. The *Oryza sativa* L. cv amaroo model developed in this study is the first to show growth inhibition that is due to the presence of *B. pseudomallei,* although we have not yet observed bacteria inside the tissues. Further work using the 10^8 CFU/ml dose will be required to clarify whether there is invasion or merely rhizosphere interaction. Given the range of effects in other studies, it would also be valuable to determine whether other rice species are affected by *B. pseudomallei* TSV189 and whether the *Oryza sativa* L. cv amaroo cultivar is equally sensitive to other *Burkholderia* species and *B. pseudomallei* strains.

There is limited research studying the colonisation of plants with *B. pseudomallei*. Lee *et al.* (2010) used transmission electron microscopy to identify *B. pseudomallei* and *B. thailandensis* inside wounded leaves of tomato plants (Solanum lycopersicum variety Season Red F1 Hybrid), while Kaestli *et al.* (2012) used FISH to identify natural infection of *B. pseudomallei* in grasses (*Brachiaria humidicola* cv Tully (Tully grass)), *Paspalum plicatulum* (Paspalum), *Pennisetum pedicellatum* and *polystachion* (Mission grass) as well as experimental infections in *S. intrans* (Native sorghum) and *O. rufipogon* (Wild rice). The bacterial were found not only the rhizosphere and roots (Tully and Mission grass), but also aerial parts of Mission grass, Paspalum, *S. intrans* and *O. rufipogon*.

In this study a *B. mallei* monoclonal antibody was optimised and used to develop an in house IFA tool to localize *B. pseudomallei* presence in rice roots. No commercial anti-*B. pseudomallei* antibodies were able to be sourced at the beginning of our experiments. However, antibodies specific for *B. mallei* are available and considering that some lipopolysaccharides are shared by *B. pseudomallei* and *B. mallei,* a monoclonal antibody specific for *B. mallei* LPS was tested

Interestingly, during optimization, the protocol for confirming sterility of samples prior to removal of bacteria and tissues from PC3 for use in IFA identified flaws in production of sterility in use of 100% ethanol. We expected that the use of ethanol for 4 hr would be sufficient for killing all bacteria, however it was not. It is known that bacteria in biofilms have increased resistance to disinfectant (Peeters *et al.*, 2008). In addition, ethanol is usually diluted to 75-80% for use as a sterilizing agent as 100% ethanol can affect the cell walls, stopping the ethanol entering the cell (Sumbali and Mehrotra, 2009), thus being less effective. This is a salient lesson in not assuming a standard protocol for sterilizing samples will work under all pretreatments for all bacteria.

In this study, the immunofluorescence signals from ethanol and acetone fixatives were better than paraformaldehyde and formalin fixatives when using low concentrations of primary antibody (Table 4.3). Paraformaldehyde is a crosslink fixative. Crosslink fixation changes tertiary and quaternary structures and could make it impossible or difficult at best to form antigen antibody bonds (Montero, 2003; Ramos-Vara, 2005) which may be the cause of lower signals in this case. The signal from the acetone fixative was slightly better than ethanol fixative and a lower detection with ethanol fixation has been previously reported (Walker *et al.*, 1984).

The IFA identified all strains of *B. pseudomallei* tested, including the ones we intended to use in biocontrol assays and did not detect any other bacteria, either in the rice stock we were going to use or in some common soil bacteria and near-neighbor species (Table 4.4). It also effectively identified *B. pseudomallei* on the rice rootlets (Figure 4.4). As such this assay was considered fit for the purpose of experimental infection with known bacterial strains and known rice stocks.

However, this may not always be the case. There are a broad range of *Burkholderia* species and a wider testing regime may identify non *B. pseudomallei* species which will also cross-react with the monoclonal antibody. For example, a lipopolysaccharide (LPS) of *B. thailandensis* E264 has been found to cross react with rabbit and mouse

sera from animals infected with *B. mallei* and *B. pseudomallei*. *Burkholderia thailandensis* E264 has shares similar LPS structures with both *B. mallei* and *B. pseudomallei* (Qazi *et al.*, 2008). This E264 strain of *B. thailandensis* was not available in Australia to test and while a strain of *B. thailandensis* from Papua New Guinea did not react, we cannot exclude the possibility that other strains such as *B. thailandensis* E264 would.

This work identified that high doses (10^7 and 10^8 CFU/ml) of *B. pseudomallei* TSV189 inhibited root and leaf growth in *Oryza sativa* L. cv Amaroo, which means this cultivar or rice can be used for biocontrol studies. The presence of *B. pseudomallei* inside root cells indicates that the bacteria is actually infecting the plant, not just acting at the rhizosphere.

CHAPTER 5: THE EFFECTS OF *BURKHOLDERIA* SPP IN INFECTION ON A RANGE OF *ORYZA* SPP

5.1 Introduction

As discussed in Chapter 4, a single *B. pseudomallei* strain may affect different species of plants in different ways. Lee *et al.* (2010) found that of tomato (*Solanum lycopersicum* variety Season Red F1 Hybrid), Mousear Cress (*Arabidopsis thaliana*) and Domestic Japanese rice (*Oryza sativa* L. cv Nipponbare). Only tomatoes were susceptible to *B. pseudomallei* (10^7 CFU). *Burkholderia pseudomallei* isolated from clinical, soil and animal samples showed similar disease scores in the tomato model. Kaestli *et al.* (2012) demonstrated that high doses of *B. pseudomallei* (10^7 CFU) inhibited growth of Mission Grass (*Pennisetum pedicellatum* and *polystachion*) seedlings significantly and there was also a trend of inhibition of foliage growth in Tully Grass (*Brachiaria humidicola* cv Tully), however wild rice (*O. rufipogon*) was not affected.

Different *Burkholderia* species may affect a single plant host differently. Alfalfa (variety 57Q77) seeds infected with 10^5 CFU *B. cepacia* complex have shown this variation. Signs of disease were observed with *B. cepacia* (genomovar I), *B. cenocepacia* (genomovar III), *B. vietnamiensis* (genomovar V), *B. dolosa* (genomovar VI), *B. ambifaria* (genomovar VII), limited signs of disease with *B. stabilis* (genomovar IV) and no signs of disease with *B. multivorans* (genomovar II) (Bernier *et al.*, 2003; Coenye *et al.*, 2003; Vermis *et al.*, 2004).

Domestic Japanese rice (*Oryza sativa* L. cv Nipponbare) has previously been reported as not being inhibited by *B. pseudomallei* (Lee *et al.*, 2010), however in this study we have produced a domestic rice model (*Oryza sativa* L. cv Amaroo) which is affected by *B. pseudomallei* (TSV 189), but not by *B. vietnamansis*. Other wild rice (*O.rufipogon*) and grass species have also been found to be infected with *B. pseudomallei* (Mission and Tully grass) (Kaestli *et al.*, 2012).

This study aims to test the effects of a variety of *Burkholderia* spp on a range a gramenacious plants including a variety of endemic and exotic rice specie

5.2 Material and Methods

5.2.1 Bacteria.

Burkholderia pseudomallei (TSV189, TSV192 and K96243), *Burkholderia. cenocepacia* (17SP), and *B* (A21) were subcultured from storage as per section 3.1.1. They were cultured into broth as per section 3.1.2.

5.2.2 Germination of wild seed tested

Wild rice species (*Oryza meridionalis* and *Oryza australiensis*) and black spear grass (*H. contortus*) were collected as per section 3.3. Seeds were dried at 37 ^{o}C for a week and kept at room temperature for about one year to break dormancy. Seed surfaces were cleaned as per section 3.3.1. Three hundred seeds of each *O. meridionalis* and *O. australiensis* and 637 seed of *H. contortus* were soaked in sterile water at 30 ^{o}C for 6 days and transferred to Hoagland media as per section 3.3.2. A sample of the germination rate calculation is shown below.

E.g. $\% = \frac{\text{number of germination}}{\text{total seed number}} \times 100$

$\% = (83/300) \times 100,$

$\% = 27.6$

Growth of germinated seeds was observed over seven days to identify whether germinated seeds continued to grow. When germination appeared to stop, the seeds were considered to not grow well.

5.2.3 Survival kinetics of *Burkholderia* species in ¼ strength Hoagland solution

Burkholderia pseudomallei (TSV192 and K96243), *B. cenocepacia* (17SP), *B. ubonensis* (A21) were grown in ¼ strength Hoagland solution (Appendix A1.8) and growth analysed as per section 4.2.4

5.2.4 Comparisons of *Burkholderia* species infecting *Oryza sativa* L. cv Amaroo (rice seeds) for plant growth experiments.

A high dose (10^8 CFU/ml) of each *Burkholderia* species in LB was used to test each plant species as per section 4.2.5. Each experiment was carried out with an uninfected control group to control for between experimental error. All growth measurements were compared to the relevant average control growth. An example of percentage (%) of growth relative to average control (uninfected) growth is shown below.

E.g. $\% = \frac{\text{(each area (root /leaf) experiment)}}{\text{average area (root/leaf) control}} \times 100$

$\mathbf{\%} = \frac{(217.5)}{230.4} \times 100$

$\% = 94.4$

Statistically significant changes in growth were calculated using ANOVA with post hoc test ($p=0.05$) using percentage growth figures.

5.2.5 Effect of *B. pseudomallei* (TSV189 and K96243) on *Oryza sativa* L. cv Koshihikari growth.

Burkholderia pseudomallei (TSV189 and K96243) was used at the high dose (10^8 CFU/ml) to infect *Oryza sativa* L. cv Koshihikari as per section 4.2.5. Comparison between infected and control growth in each case was carried out using independent t tests.

5.2.6 Effect of *B. pseudomallei* (TSV189) infection of different cultivars of rice.

Burkholderia pseudomallei (TSV189) was used at the high dose (10^8 CFU/ml) to infect two different cultivars of rice (*Oryza sativa* L. cv Amaroo and *Oryza meridionalis*) as per section 4.2.5. Uninfected rice of each cultivar was grown as control groups. Statistically significant changes in growth were calculated an using independent t test using percentage growth figures.

5.3 Results

5.3.1 Germination of wild seed

Germination rate and growth of wild seed in hydroponic agar was carried out. *O. meridionalis* had the highest germination rate (27.6%) and *O. australiensis* and *H. contortus* both had lower germination rates. Both *Oryza* species grew well in hydroponic agar, but *H. contortus* did not (Table 5.1). Due to low germination rates and limited access to seeds to overcome this, *O. australiensis* was not used further. Due to low germination rates and poor growth on Hoagland agar *H. contortus* was not used further. *O. meridionalis* was selected for growth inhibition studies.

Table 5.1: Wild species germination test on Hoagland agar**.**

Growth determination is based on observation of continued growth over the seven day observation period.

Wild plant species	**Germination**	**Rate (%)**	**Hoagland agar growth**
O. meridionalis	83/300	27.6	good
O. australiensis	11/300	3.6	good
H. contortus	93/637	14.5	poor

5.3.2 Survival kinetics in ¼ strength Hoaglands solution

The survival kinetics of *B. pseudomallei* (TSV192 and K96243), *B. ubonensis* (A21), and *B. cenocepacia* (17SP) are relatively similar in Hoaglands solution (Figure 5.1). There were no significant changes in the *B. pseudomallei* species numbers between day 1 and 7, the time course of all the planned experiments although by day 90 *B. pseudomallei* K96243 counts had dropped slightly. While there were technically some significant changes in bacterial counts between the first few and last few days in the incubation of *B. ubonensis* (A21), and *B. cenocepacia* (17SP), these required a sensitive post hoc test to find, have no obvious rising or falling trend and are all within one log of growth (Appendix A3.4).

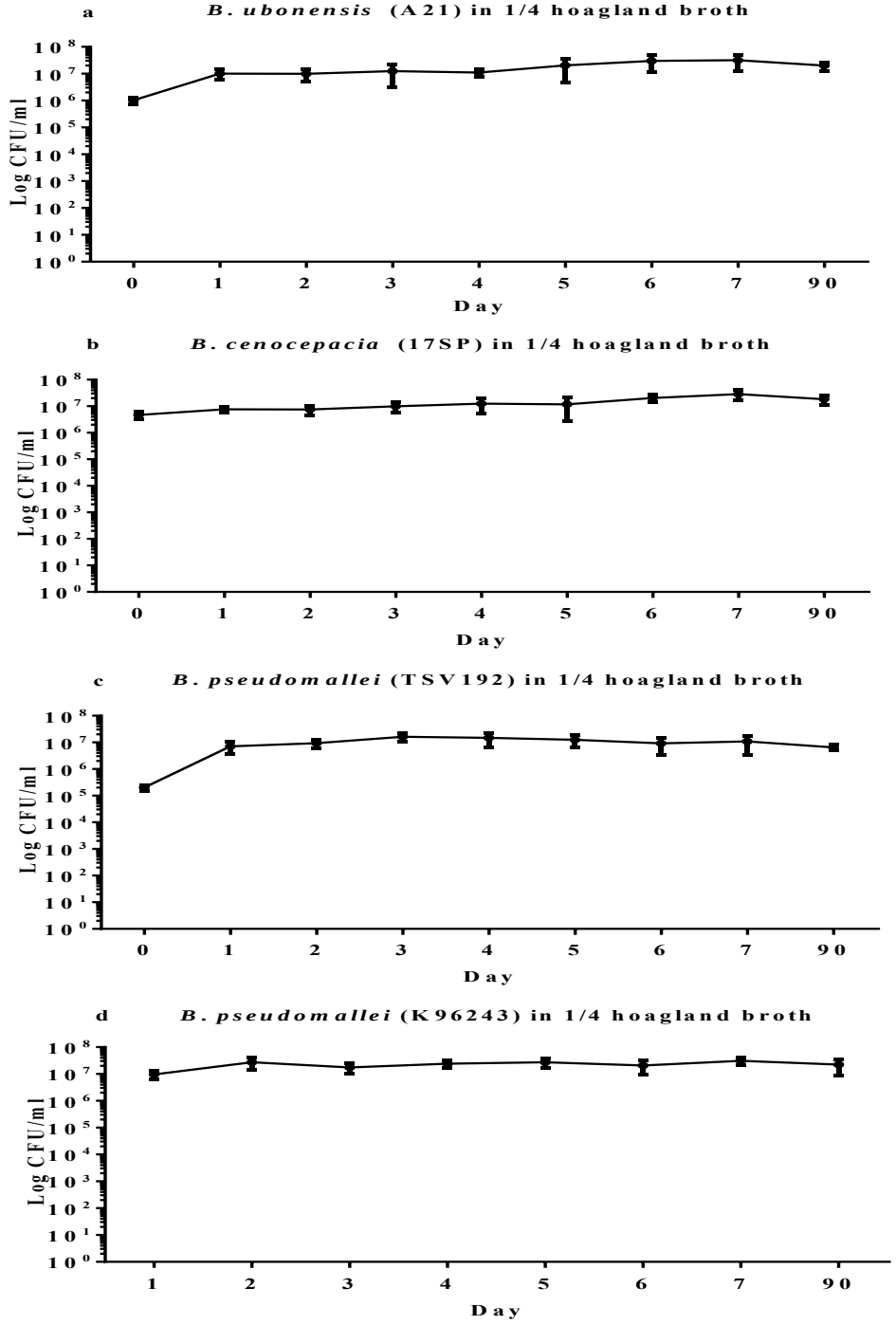


Figure 5.1: Survival kinetics of *Burkholderia* species in ¼ strength Hoaglands broth. ¼ strength Hoaglands broth was inoculated with (a) *B. ubonensis* (A21), and (b) *B. cenocepacia* (17 sp) and *B. pseudomallei* (TSV192(c), K96243(d)), at a concentration of 10^6 CFU/ml in triplicate and incubated at 30°C. Bacteria were counted over 90 days by Miles *et al.* (1938) on LB agar (n=6). Growth curves (mean with 95% CI) over 90 days are presented above.

5.3.3 Response of *Oryza sativa* L. cv Amaroo to a range of *Burkholderia* species

As per section 4.3.4 *B. vietnamensis* did not inhibit growth of *Oryza sativa* L. cv Amaroo at the high dose (10^8 CFU/ml). The inhibition pattern of both root (a) and leaf (b) was similar in each case (percentage of growth relative to average control growth) (Figure 5.2). *Burkholderia pseudomallei* isolates of different origin (TSV189 (animal), TSV192 (soil), and K96243 (human) all inhibited plant growth significantly to a similar amount at the high dose (10^8 CFU/ml). *Burkholderia cenocepacia* also inhibited growth to the same level. *Burkholderia ubonensis* also significantly inhibited plant growth, but the level of inhibition is weaker when it compared with *B. pseudomallei* and *B. cenocepacia*. Significance values are displayed in (ANOVA, post hoc test, (Appendix A3.5).

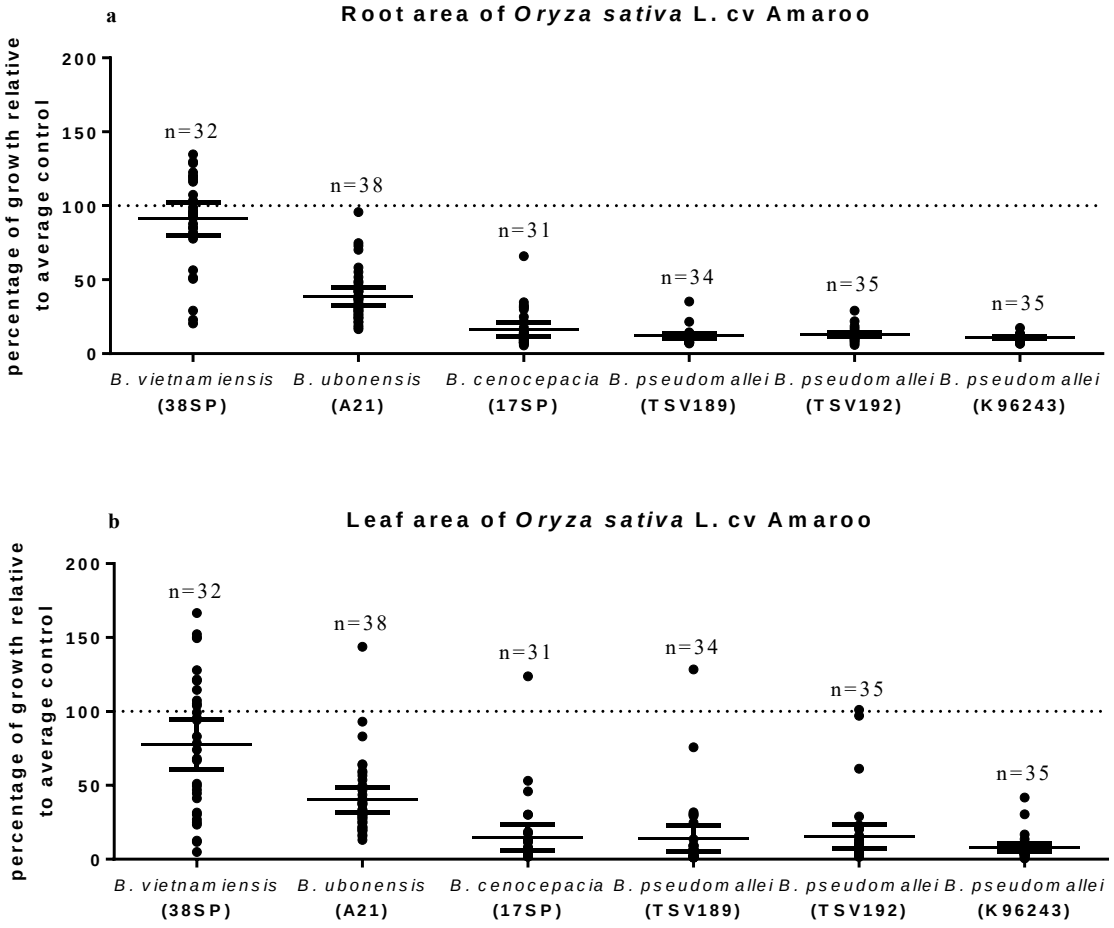


Figure 5.2: Comparison of inhibition of growth of *Oryza sativa* L. cv Amaroo, due to a range of *Burkholderia* species, relative to the uninfected control growth. Percent of root (a) and leaf area (b) ± 95% CI are displayed. *B. vietnamensis* does not significantly inhibit growth (as per section 4.3.4) while all other species do. *Burkholderia ubonensis* inhibition is also significantly different to all other species. *Burkholderia cenocepacia* and the *B. pseudomallei* strains are not significantly different to each other in their inhibition. P values are presented in Appendix A3.5.

5.3.4 Effect of *B. pseudomallei* (TSV189 and K96243) on *Oryza sativa* L. cv Koshihikari growth

Oryza sativa L. cv Koshihikari was not significantly inhibited by either strain when compared to control growth. Leaf growth was actually increased (p=0.038) when *B. pseudomallei* (K96243) was added, however all other growth in each case was not affected (Figure 5.3 and (Appendix A3.6)).

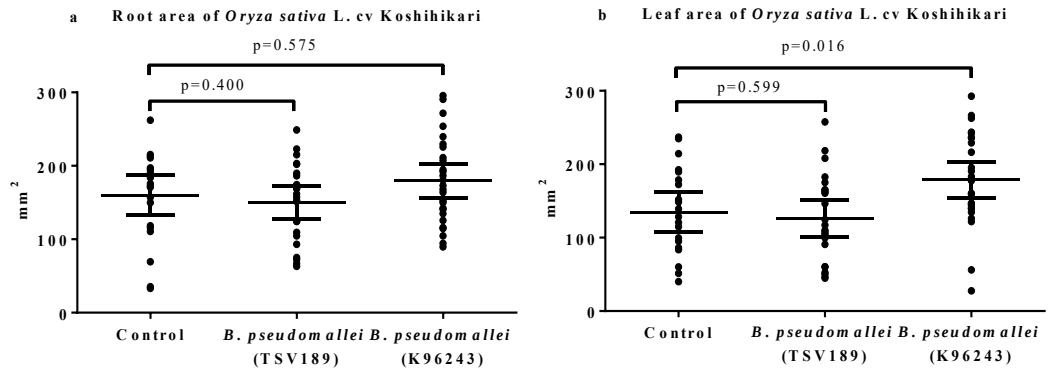


Figure 5.3: Comparison of inhibition of growth of *Oryza sativa* L. cv Koshihikari, due to exposure with *B. pseudomallei* (TSV189 and K96243), Area of leaf/root ± 95% CI are displayed. Cultivar Koshihikari growth is not significantly reduced relative to its control for either *B. pseudomallei* species and leaf growth was increased when exposed to *B. pseudomallei* (K96243) (P=0.016)

5.3.5 *Burkholderia pseudomallei* (TSV189) infection of different cultivars of rice

Burkholderia pseudomallei produced different levels of inhibition with the different rice species (Figure 5.4). *Oryza sativa* L. cv Koshihikari was not inhibited (section 5.3.4) while the other two species were inhibited, however the level of inhibition varied. *Oryza sativa* L. cv Amaroo was most inhibited, while *O. meridionalis* on root area ($p<0.000$) and leaf area ($p<0.000$) had slightly increased resistance to *B. pseudomallei* (TSV189) relative to the Amaroo cultivar. All differences between rice cultivars were significant for both root and leaf (Appendix 3.7).

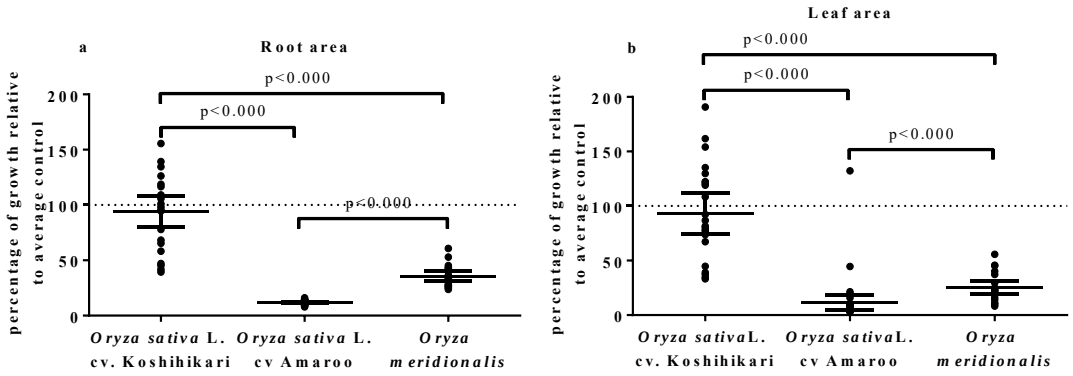


Figure 5.4: Percent of root (a) and leaf (b) area growth relative to control growth ± 95% CI are displayed for each cultivar of rice. Statistical comparison of percent inhibition of growth of the different cultivars is included above the bars.

5.4 Discussion

The results of this chapter have been published in Applied and Environmental Microbiology (Appendix 4). After publishing, a decision was made to standardize the selection criteria for statistical tests across chapters as much as possible. This has not changed the outcomes of the assays, but has changed some of the p values (eg the difference between growth of *Oryza sativa* L. cv. Koshihikari roots with and without *B. pseudomallei* TSV189 goes from p=0.571 to p= 0.400).

Germination rates for the wild species tested were quite low in Hoagland agar. A previous report on the germination rate of *O. meridionalis* described the germination rate in soil as about 30 to 60 % (Williams *et al.*, 2011) however, that experiment did not include a seed cleaning step, which may have lowered the germination rate in this study and breaking of dormancy was done using burning, rather than storage, which may be more efficient. The limited access to viable seed (*O. australiensis*) and the poor growth in hoaglands agar *(H. contortus)* meant that only one wild rice species that was collected (*Oryza meridionalis*) was suitable for further testing. Development of alternate growth medias or better access to wild seed in some cases would be necessary to examine these other local species.

Survival kinetics for all *Burkholderia* species and strains (including those previously tested, Section 4.2.4) in Hoaglands broth were within one log of difference. This

indicated that the effects of Hoagland agar on the bacteria can be excluded as a cause of experimental variation. Even though minor fluctuations were picked up by the most sensitive post hoc test used for *B. ubonensis* and *B. cenocepacia* we can assume that there is no biological relevance to these as there was no trend of rise or fall in counts and actual changes did not exceed a log (Figure 5.1).

The use of percentage growth relative to control unexposed growth also eliminates between experimental error as all exposed groups of plants were grown at the same time as unexposed plants. In addition, this means that different species of plants can be compared to each other, irrespective of the natural growth rate of each plant. Kaestli *et al.* (2012) previously examined inhibition of three plant species and identified whether or not inhibition occurred, but a comparison between species was not carried out. Most other studies either only examined one species of plant, or two species where only one was inhibited (Bernier *et al.*, 2003; Lee *et al.*, 2010).

Burkholderia pseudomallei TSV189 inhibited the plant growth of *Oryza sativa* L. cv Amaroo (an Australian cultivar) and the native Australian wild rice (*Oryza meridionalis*) but not that of Japanese rice (*Oryza sativa* L. cv. Koshihikari) (Figure 5.3 and 5.4). The previous reported attempt to develop a domestic rice model used cultivar *Oryza sativa* L. cv Nipponbare (Lee *et al.*, 2010), which is a similar cultivar to Koshihikari (Yamamoto *et al.*, 2010). It is interesting to note that our results with this similar cultivar support that by Lee *et al.* (2010) regarding the resistance of the Japanese cultivar. Other resistant rice models include the wild rice (*O. rufipogon*) model of Kaestli *et al.* (2012) which was not affected by *B. pseudomallei* inoculation. It is important to note that while our wild rice species was inhibited, these two wild rice plants are different species, rather than different cultivars as in the case for the domestic rice cultivars examined. As such they are more likely to have different outcomes when exposed. Of note, while *O. meridionalis* showed significant inhibition, it was significantly less than that of *Oryza sativa* L. cv Amaroo. The wild rice was collected from an area where *B. pseudomallei* has been found and this species may have a long history of exposure to the bacteria, such that it has some level of accommodation. Alternately, some wild plant species or types can be more resistant to

infection than domestic species due to the R gene (Jones and Dangl, 2006) which may play a role here. The wild rice species infected by Kaestli *et al.* (2012) supports the concept of increased resistance as *B. pseudomallei* number in leaves decreased six days after infection, compared to two wild grasses tested.

The studies by Lee *et al.* (2010) and Kaestli *et al.* (2012) both used other plant models which were susceptible to the *B. pseudomallei* isolates involved. This indicates the *B. pseudomallei* isolates used were able to inhibit at least some host plant species. It can be concluded that there is variation within the *Oryza* genus, and even between cultivar types, which determines the ability of *B. pseudomallei* to inhibit growth.

One interesting outcome was that the resistant *Oryza sativa* L. cv. Koshihikari model actually had increased leaf growth when exposed to the clinical Thai isolate *B. pseudomallei* (K96243), although not when exposed to *B. pseudomallei* (TSV189). There may be some opposing effects of growth promotion with this strain which can be seen when used with a resistant rice model.

Examination of the inhibition of *Oryza sativa* L. cv Amaroo with different *Burkholderias* identified that while all three *B. pseudomallei* strains tested produced the same pattern of inhibition, other species did not (Figure 5.2). The *B. pseudomallei* strains tested were from a variety of sources (soil - Australian, human clinical – Thai, animal clinical – Australian), indicating this effect is likely to be seen in further *B. pseudomallei* strains. This assumption is supported by the work of Lee *et al.* (2010) who also tested a tomato model using *B. pseudomallei* isolates from different sources, with the same outcome. In addition, the *B. pseudomallei* strains were more inhibitory than *B. vietnamensis* and *B. ubonensis*, although *B. cenocepacia* was equally inhibitory. Previous studies using one plant species with multiple *Burkholderias* have also produced variable effects, from obvious signs of disease to no signs (Bernier *et al.*, 2003).

As both clinical and environmental Australian *B. pseudomallei* isolates as well as a clinical Thai isolate equally inhibited *Oryza sativa* L. cv Amaroo, *Burkholderia pseudomallei* may be generally inhibitory to some rice cultivars. This leads to the question of whether different rice cultivars will succeed in *B. pseudomallei* endemic areas.

An examination of rice growth in Thailand based on the iRiceZoning map identifies three major rice group areas in Thailand (http://carsr.agri.cmu.ac.th/projects/iRPZ/MAPRiceVarGroup.aspx). Aromatic rice and glutinous rice are grown in northeast Thailand, a highly endemic area of *B. pseudomallei* (Vuddhakul *et al.*, 1999; Limmathurotsakul *et al.*, 2010a) in contrast to the central part of Thailand where non-aromatic rice is grown and where *B. pseudomallei* is less commonly found (Vuddhakul *et al.*, 1999). There are also other variations between these regions such as rainfall, irrigation patterns and soil types (Boonsompopphan *et al.*, 2008), but it could be hypothesized that *B. pseudomallei* has a role in the success of different cultivars. Rice currently grown in northeast Thailand may need to be more resistant to the effects of *B. pseudomallei*. Rice that does not grow there might not grow because of the more common exposure to *B. pseudomallei.* An infection trial of the different rice types and varieties would be useful to answer this question.

This is the first study to our knowledge to successfully experimentally colonize the roots of a domestic rice cultivar with *B. pseudomallei* and to identify differential inhibition of growth of different species and cultivars of rice. The limitation of this study is that the incubation period is short and the plants have been grown in hydroponic agar. Growth in soil typical of rice paddies, for longer periods, may result in other outcomes. In addition, as discussed in Chapter 4 this experiment used a high dose of bacteria, though other literature also uses relatively high inoculums, and/or wounding of tissue to encourage inoculation or invasion (Bernier *et al.*, 2003; Fujishige *et al.*,

2006; Mattos *et al.*, 2008; Lee *et al.*, 2010; Kaestli *et al.*, 2012). Natural environmental conditions may result in lower exposures. While *B. pseudomallei* can infect the roots of (*Oryza sativa* L. cv Amaroo) via root hairs and retard growth (Chapter 5), it does not retard growth of *Oryza sativa* L. cv. Koshihikari. This cultivar difference could be a factor in the successful or unsuccessful growth of particular cultivars of rice in endemic regions. The relative susceptibility of plants may also affect persistence and thus the biogeographical boundaries of *B. pseudomallei.* A susceptible rice cultivar also means that biocontrol experiments can be carried out with rice.

CHAPTER 6: PARTIAL PURIFICATION AND CHARACTERISTION OF POTENTIAL BIOCONTROL AGENTS AGAINST *B. PSEUDOMALLEI*

6.1 Introduction

Classical biocontrol involves using natural enemies to control pests (Hajek, 2004) and involves the use of one or more organisms to decrease an inoculum or disease (pathogen activity) (Baker, 1987) . There are different approaches which can be taken. Augmentation involves the addition of biocontrol agents to the environment, either because they are not already present, or at higher concentrations than are already present (Hajek, 2004) while conservative approaches involve making alterations to the environment such that already present organisms, which function against the target organism, are encouraged to replicate or act at higher levels (Eilenberg *et al.*, 2001). Irrespective of approach, antagonists of plant pathogens can work either directly or indirectly. Indirect effects include antagonists inducing resistance in the plant. The mechanisms of direct effects are based on competition, parasitism and antibiosis (Hajek, 2004). Examples of biocontrol agents (microbial antagonists) include; bacteria such as *P. fluorescens* (Clarkson and Lucas, 1997), fungi such as *Trichoderma harzianum* (Shakeri and Foster, 2007) and viruses, such as bacteriophages (Balogh *et al.*, 2010). Viruses such as bacteriophages are parasites, infecting bacteria and potentially replicating inside the bacterial cell, rupturing the cell on release (Summers, 2001). Bacteriophage isolated from an epidemic area (Northern Thailand) have been proven to inhibit a range of strains of *B. pseudomallei in vitro*. (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011). McRobb (2010) also found lytic phage mixtures against *B. pseudomallei* from soil and water samples collected from the *B. pseudomallei* endemic area at Castle Hill in Townsville, unpublished data. Myoviridae and Siphoviridae phages were identified in these mixtures. These were further purified by Bilbrey (2011) and showed promise as phage therapy agents in mouse assays (unpublished data).

Bacteria which produce bacteriocins act by antibiosis (Riley and Wertz, 2002). Previous work at James Cook University (Marshall *et al.*, 2010) identified that cell free supernatants from *B. ubonensis* (A21) culture were able to inhibit growth of *B.*

pseudomallei via a well diffusion assay. In the original work, the authors identified that activity was lost using pepsin but not using other proteases, found that it became more active after heating to 70 ^{o}C and that activity was lost at 100 ^{o}C. They described the crude product as a bacteriocin-like inhibitory substance (BLIS).

As both the bacteriophages and BLIS previously identified at James Cook University have shown evidence of biocontrol activity against *B. pseudomallei*, further purification and characterisation with respect to their activity against *B. pseudomallei* would be useful to determine whether they could be effective biocontrol agents against *B. pseudomallei* in plants and this is the focus of this chapter.

6.2 Materials and Methods

6.2.1 Production of cell free supernatant of *B. ubonensis*

One colony of *B. ubonensis* A21 (48 hr growth) was used to inoculate 100 ml of LB broth in a 500 ml conical flask. The culture was incubated at 30 ºC and shaken at 100 rpm (Bioline, Orbital Incubator Shaker), for either 1, 2, 3, 4, 5 or 6 days, followed by centrifugation at 10 000 × *g* for 30 mins. Supernatants were filtered through a 0.45 µm filter (Sarstedt, Filtropur S 0.45) and stored at 4 ^{o}C. Cell free supernatants that were to be heated prior to further experimentation or analysis were heated at 70 ^{o}C for 30 mins in volumes of 1 ml in microfuge tubes in a hot block (RATEK, Australia) and stored at 4 ^{o}C until processed further.

6.2.2 Cell lysis of *B. ubonensis*

Bacterial cells pelleted from section 6.2.1 were washed with 100 ml of a 50 mM potassium phosphate buffer, pH 7.4 (Appendix A1.13) and re-centrifuged at 10 000 × *g* for 30 mins at 4 ^{o}C. Pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4 to an OD_{600} of 5 (+/-0.5). The cells were then disrupted using the following technique modified from Benov and Al-Ibraheem (2002): each bacterial suspension was

added to a 2 ml sterile tube with o ring (SSI, USA), containing one gram of sterile glass beads (2.5 mm, Daintree scientific). Tubes were frozen at -80 ^{o}C for 30 min and then placed in a hot block for 30 min at 30 ^{o}C. The cell suspensions were bead beaten for 1 min (Mini Bead Beater, BIOSPEC). The protocol was repeated six times and the suspensions were filtered through a 0.45 µm syringe filter (Sarstedt, Filtropur S 0.45) and stored at 4 ^{o}C for further analysis.

6.2.3 Agar diffusion assay

Mueller Hinton (MH) agar plates were used to identify any antagonistic activity of cell free supernatants. They were processed according to the method of Marshall *et al.* (2010) as follows: a sterilised metal tube with a sharp rim was used to punch holes 6 mm in diameter in the agar, removing the whole depth of the agar. Six holes were punched in a ring about half way between the centre and the edge of the plate. A seventh hole was punched in the centre of the plate. 100 µl of cell free supernatant, processed sample or control was added to each hole and the solution allowed to absorb into the agar over 2 hr at room temperature in a C2 cabinet. Plates were then moved to a PC3 facility to add *B. pseudomallei* to produce a lawn. Samples were assayed within 48 hr of production to avoid any degradation of the sample affecting antagonistic activity.

Burkholderia pseudomallei isolates from a range of sources were grown in culture overnight as per section 3.1.2. One ml was centrifuged at 10 000 × *g* and the supernatant removed. A *B. pseudomallei* suspension at 0.5 MacFarland was made by addition of 0.85% NaCl solution (Appendix A1.10) to the pellet. A sterile swab (Sarstedt, Forensic swab) was soaked in this solution and cross streaked on the prepared MH agar containing samples. The MH agar plate was dried in the C2 cabinet for one hour and incubated at 37 ºC for 2 days. Zones of inhibition (ZOI) were measured digitally by photographing plates beside a scale and using Adobe Photoshop CS6 to measure the diameter of clearance of any zone around a well. A measurement of 6 mm, the total diameter of the well, was indicative of no inhibition or activity.

6.2.4 Processing of cell free supernatants

6.2.4.1 Ammonium sulphate precipitation of heated cell free supernatants

Ammonium sulphate was added at 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 g/ml to one ml aliquots of heated cell free supernatant. They were mixed by inversion until all ammonium sulphate was dissolved, kept cold on ice for 30 min, and centrifuged at 18 000 x *g* for 20 min at 4 ^{o}C (Thermo scientific, Sorvall). The supernatants were transferred into a second set of microfuge tubes. The pellets were then reconstituted in LB broth to 1 ml by vortexing. Agar diffusion assays (Section 6.2.3) were carried out on all reconstituted pellet and supernatant samples.

6.2.4.2 Temperature stability of ammonium sulphate precipitated samples

One ml of cell free supernatant (section 6.2.1) was processed using 0.3 g/ml $(NH_4)_2SO_4$ to produce a reconstituted pellet. Half of the sample was incubated at 70 ^{o}C for 30 min in a microfuge tube in a hot block (RATEK, Australia) and the other half incubated similarly at 100 ^{o}C. Agar diffusion assays (Section 6.2.3) were carried out with both samples.

6.2.5 Size separation of cell free supernatant components by dialysis

Dialysis through 10 kDa pore size tubing (Snake skin, Pierce) was carried out to determine whether the activity of the heated cell free supernatant could be dialysed at this molecular weight cut off. 100 µl aliquots of sample were removed at each step and tested for activity using an agar diffusion assay. Samples used for testing are noted and numbered at the relevant points in the process. Figure 6.1 describes the dialysis steps and sampling protocol.

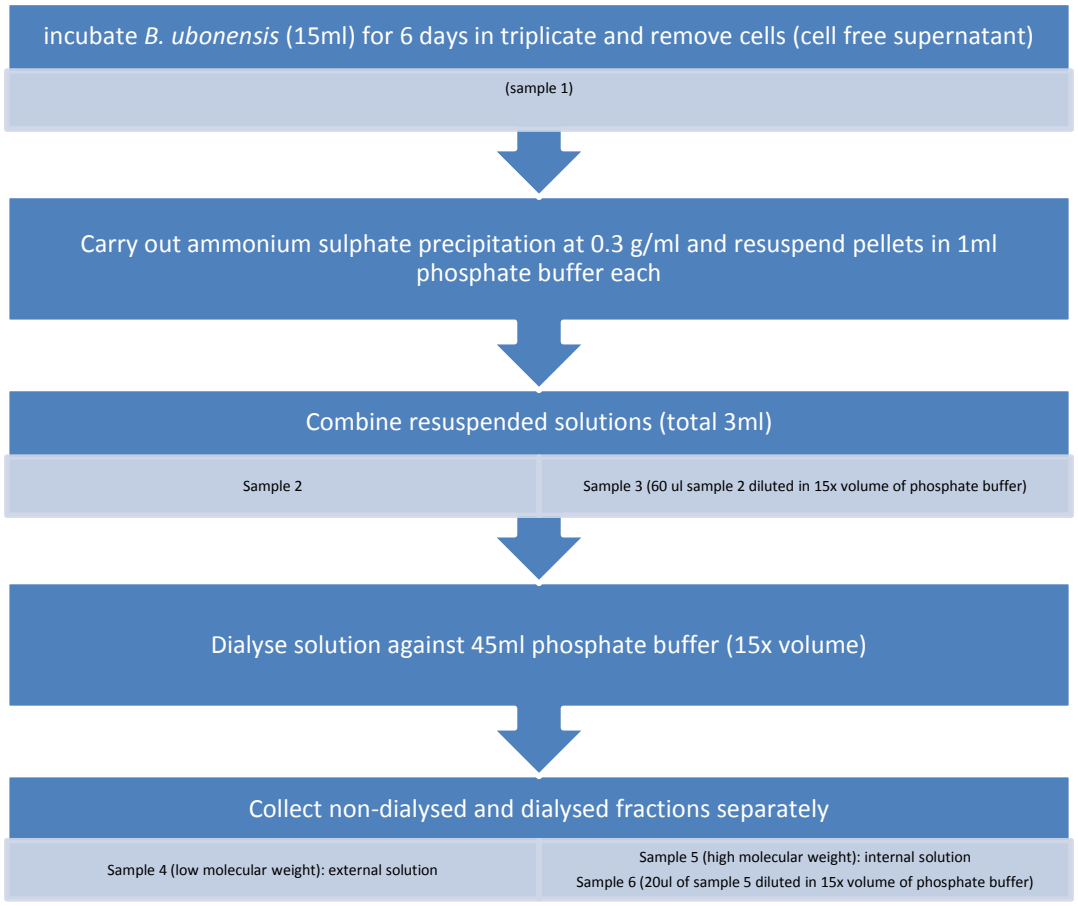


Figure 6.1 Flow diagram of dialysis procedure including points at which samples were taken for analysis. Samples are; Sample 1. positive control (untreated cell free supernatant), Sample 2. concentrated sample, Sample 3. rediluted sample, Sample 4. low molecular weight separation (outside dialysis tube), Sample 5. high molecular weight separation (inside dialysis tube), Sample 6. rediluted high molecular weight sample.

6.2.5.1 Preparation of cell free supernatant for dialysis

Three cultures of 15 ml of cell free supernatant (section 6.2.1) were produced and a sample taken (sample 1: cell free supernatant). Ammonium sulphate precipitation at 0.3 g/ml was carried out (section 6.2.4.1) on each culture using a centrifugation step of 40 mins to pellet the precipitate. Pellets were resuspended in 1 ml each of phosphate buffer (50 mM) pH 7.4. The resuspended pellets were combined (3 ml total) for use in dialysis and a sample was taken (sample 2: ammonium sulphate precipitation). This

resuspension resulted in a supernatant concentration factor of 15×. Another 60ul sample was also aliquoted and mixed with 840 µl phosphate buffer (50 mM) pH 7.4 to create a sample at the same concentration as the original pre-$(NH_4)_2SO_4$ precipitated volume (Sample 3: rediluted sample).

6.2.5.2 Dialysis of cell free supernatant

About 3 ml of the resuspended pellet was dialysed (pore size 10 kDa, Snake skin, Pierce) with 45 ml phosphate buffer (50 mM) pH 7.4 overnight at 4 ^{o}C. This equated to 15× the volume of the resuspended solution. Samples were taken from interior and exterior solutions and the solutions inside the dialysis tubing (sample 5: high molecular weight) and outside (sample 4:low molecular weight) were then stored separately at 4 ^{o}C for an agar diffusion assay (section 6.2.3). The outside solution was effectively diluted back to 15× the resuspended pellet volume, i.e., the pre ammonium sulphate precipitation volume. 20 µl of the interior solution was also samples and diluted back by a factor of 15, by addition of 280 µl phosphate buffer (sample 6: rediluted high molecular weight).

6.2.5.3 Examination of Dialysis samples via agar diffusion assay

All samples collected during the dialysis process, except sample 2, were analysed for the size of ZOI in an agar diffusion assay (section 6.2.2). In addition, the following controls were analysed; negative control buffer (phosphate buffer (50 mM) pH 7.4), $(NH_4)_2SO_4$ negative control broth (LB only processed as per ammonium sulphate precipitation using 0.3 g/ml $(NH_4)_2SO_4$ (section 6.2.4.1) Sample 1 was used as a positive control as the untreated cell free supernatant had already been proved to produce a ZOI.

6.2.6 Size separation of cell free supernatant components by column chromatography

One hundred ml cell free supernatant at day 6 (Section 6.2.1) was added 0.3 g/ml (30 g) $(NH_4)_2SO_4$ with centrifugation at 50 000 × *g* at 4 ^oC for 30 min. The pellet was dissolved using one ml of phosphate buffer (50 mM) pH 7.4 and the solution transferred to a microfuge tube. This was centrifuged at 18 000 × *g*, at 4 ^oC for 40 mins as a precautionary step to remove non soluble material prior to loading onto the column. The final solution was at 100× concentration of the cell free supernatant.

Two hundred µl of concentrated sample was gel purified with a Superdex 200 10/200GL column (GE Healthcare Bio-Sciences AB, Sweden) using a BioLogic DuoFlow Chromatography System (BIO-RAD). The column was equilibrated with phosphate buffer (50 mM) pH 7.4 and sodium chloride (150 mM). The flow rate for the sample run was set to 0.65 ml/min. The initial fraction collection was 8.2 ml after which 0.65 ml fractions were collected to a total of 16 fractions. One hundred µl of each fraction was used in the agar diffusion assay as per section 6.2.3

6.2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Ammonium sulphate reconstituted pellets of cell free supernatant (section 6.2.4.1) with and without heat treatment and samples 2, 4 and 5 from the dialysis testing (section 6.2.5.2) were run on SDS-PAGE gels. Double concentration of 2x loading dry (Appendix A1.15**)** was added to 100 µl of each sample. The sample was then heated at 95 °C for 5 min. Twenty µl of this was then run on a polyacrylamide gel (Appendix A1.14) in an electrophoresis chamber (Mini Trans Blot, BIO RAD) with Tris-glycine-SDS buffer (Appendix A1.16) at 150 V for an hour. A HyperPage (Cat No. BIO-33066) protein ladder was used as a size standard.

6.2.7.1 Coomassie Brilliant Blue staining

SDS gels were washed three times for five mins each with double distilled water with shaking (low speed) at room temperature. Double distilled water was discharged and the gel incubated overnight in Coomassie Brilliant Blue solution (Appendix A1.17) with shaking (low speed) at room temperature. Coomassie solution Brilliant Blue was discharged and gels were washed three times for five min each with distilled water with shaking (low speed) at room temperature. Double distilled water was discharged and gels were destained with destain solution (Appendix A1.18) for an hour, or until the background was clear. The gels were scanned using a HP scanner (HP Deskjet F380 All in one).

6.2.7.2 Silver staining

SDS gels were fixed in silver fixative (Appendix A1.19) for 30 min. They were then washed three times for five min each with double distilled water with shaking (low speed) at room temperature and were sensitized with sensitize (Appendix A1.20) for one min. SDS gels were washed three times for 20 second each with double distilled water and then stained with silver nitrate solution (Appendix A1.21) for 10 min. The gels were then washed three times for five min each with double distilled water with shaking (low speed) at room temperature and developed in a development solution (Appendix A1.22) When development was judged sufficient by eye, the solution was removed and the gels were washed for 20 seconds with double distilled water followed by one min in termination solution (Appendix A1.23). The gels were scanned using a HP scanner (HP Deskjet F380 All in one).

6.2.8 Phage amplification

Stock phage samples (sample labels: 4-55, 4-54-25, 4-56-55, 8-18, 8-55, 8-56-25, 69-27, 69-54, C1-19, C2-27, C2-44) originally isolated from Castle Hill and previously semi-purified and amplified (McRobb, 2010; Bilbrey, 2011) were retrieved from 4 ^{o}C storage. These were tested for activity against *B. pseudomallei* isolates of interest via

phage activity assays (section 6.2.8.1) and were amplified as per section 3.5.1 and quantified as per section 3.5.2. Phage active against *B. pseudomallei* TSV189 were amplified to 10^7 PFU/ml.

6.2.8.1 Phage activity tests

Burkholderia pseudomallei TSV189 was used to make a lawn as per section 3.1.3. Twenty µl of each phage solution or serial dilution of phage solution in SM buffer (Appendix A1.9) was added to the bacterial lawn and dried for an hour before incubation at 37 ^{o}C for 48 hr. Plaques were observed and counted, if required, to the closest log level as per section 3.5.2.

6.2.9 Phage concentration

Phage solutions that produced plaques on the *B. pseudomallei* isolates of interest were filtered using 0.45 µm filters (Sarstedt, Filtropur S 0.45). Ten ml aliquots of each phage solution were ultracentrifuged at 200 000 × *g* for 4 hr at 4 ^{o}C (Optima L-90K ultracentrifuge, Type 50.2 Ti rotor; Beckman Coulter). Supernatants were discarded and 500 µl of distilled water was added using a transfer pipette (Sarstedt, Germany). Each concentrated bacteriophage solution was kept at 4 ^{o}C for further processing.

6.2.10 Characterisation of Bacteriophage (TEM)

Bacteriophage samples were characterised morphologically to the family level via transmission electron microscopy (TEM). Five hundred µl of each concentrated sample (6.2.9) was transported on ice to the University of Queensland, Department of Microscopy and Microanalysis for TEM examination. One percent of ammonium molybdate stain was used and all images were returned and analysed. Analysis of the width of the head and length of the tail was carried out. If a single sample contained bacteriophage with variable measurements, these were manually clustered and assumed to be non-pure samples containing multiple bacteriophage.

6.3 Results

6.3.1 Activity of *B. ubonensis* A21 cell free supernatant against *B. pseudomallei* isolates

Activity of cell free supernatant at a range of incubation times was tested using the well diffusion assay to confirm results of previous reports (Marshall, 2007) regarding activity. A bacteriocin-like inhibitory substance (BLIS) in the cell free supernatant of the *B. ubonensis* culture was first clearly observed at day four when tested against multiple *B. pseudomallei* isolates (Figure 6.2). It was most effective against a Papua New Guinea isolate (C4) and a Townsville isolate (TSV189). Incubation of samples at 70 ^{o}C for 30 min, prior to a well diffusion assay, resulted in activity being clearly present by day three in most *B. pseudomallei* strains and increased zones of clearance for each day were observed.

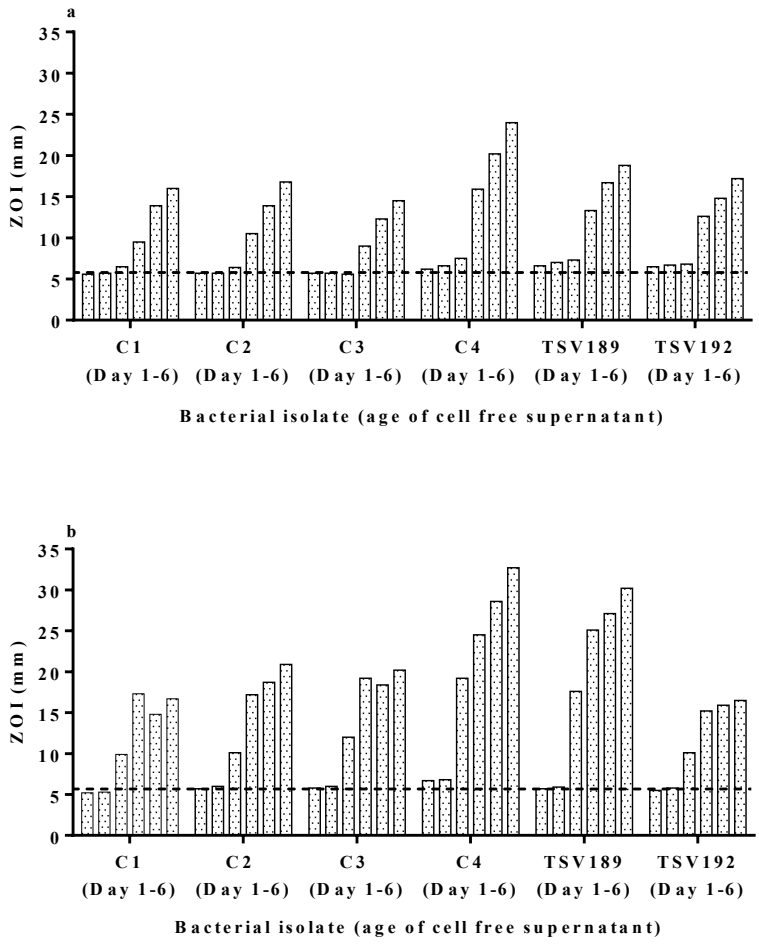


Figure 6.2: Well diffusion activity of cell free supernatants before (a) and after (b) heating at 70 ^{o}C for 30 mins. Activity is measured by the size of the zone of inhibition. The dotted line represents the diameter of the hole punched through the agar The size of hole may vary 1-2 mm depending on manual handling. C1-4 and TSV189 and 192 are the *B. pseudomallei* isolates used to produce lawns. For each lawn isolate, bars represent from left to right the days of incubation prior to harvesting of the cell free supernatant (day 1 to day 6)

6.3.2 Activity of *B. ubonensis* A21 lysed cell contents against *B. pseudomallei* isolates

Cell contents were lysed at various incubation times to identify any patterns of activity of the cell contents over time so comparison to cell free supernatant could be done. The lysed contents of the cells (6.2.2) were tested for inhibitory activity using the well diffusion assay. A bacteriocin-like inhibitory substance (BLIS) was first clearly observed at day two when tested against multiple *B. pseudomallei* isolates (Figure 6.3a). It was most effective against *B. pseudomallei* isolates after it was activated in 70 ^{o}C for 30 min (Figure 6.3b).

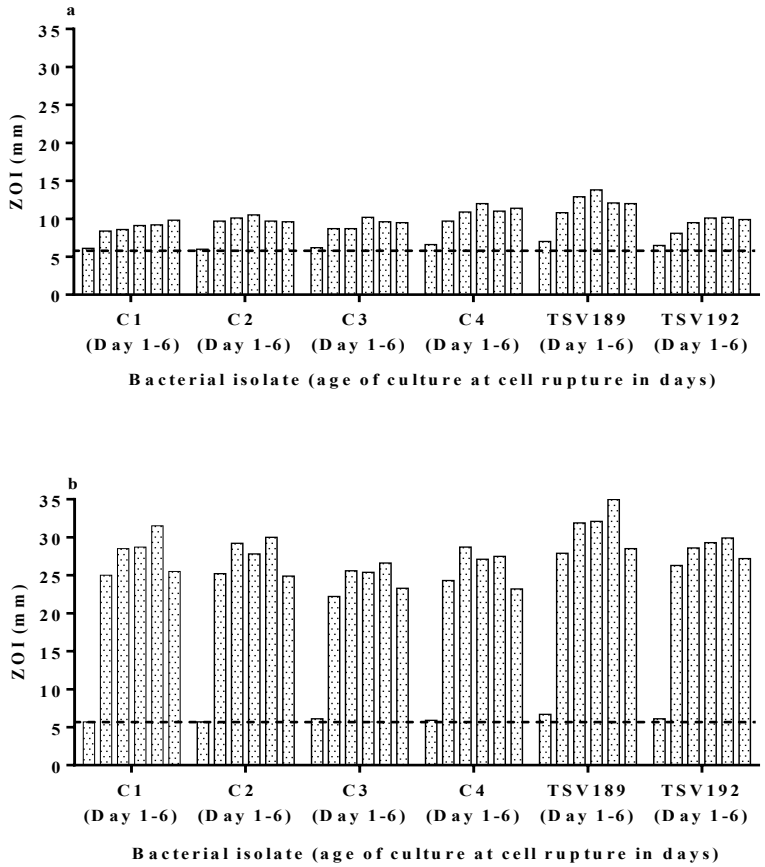


Figure 6.3: Well diffusion activity of the lysed cell contents of bacterial culture before (a) and after (b) heating at 70 ^{o}C. Activity is measured by the size of the zone of inhibition. The dotted line represents the diameter of the hole punched through the agar The size of hole may vary 1-2 mm depending on manual handling. C1-4 and TSV189 and 192 are the *B. pseudomallei* isolates used to produce lawns. For each lawn isolate, bars represent from left to right the days of incubation prior to harvesting of the cell free supernatant (day 1 to day 6).

6.3.3 Effect of $(NH_4)_2SO_4$ precipitation on cell free supernatant using *B. pseudomallei* strain TSV 189 and C4.

Cell free supernatants were precipitated with various concentrations of ammonium sulphate to identify optimum conditions for an initial partial purification of the BLIS. When samples were processed with 0.3 g/ml $(NH_4)_2SO_4$, all activity was removed from the supernatant and found in the resuspended pellet (Figure 6.4). Activity appeared to be present in the supernatant again at 0.5 g/ml, however testing of LB pelleted with 0.5 g/ml $(NH_4)_2SO_4$ and resuspended in LB confirmed this zone of inhibition was also present (data not shown). This indicating clearance at this ammonium sulphate concentration was due to high concentrations of $(NH_4)_2SO_4$. In the case of both bacterial lawns, the largest zones of clearance were at 0.35 g /ml $(NH_4)_2SO_4$.

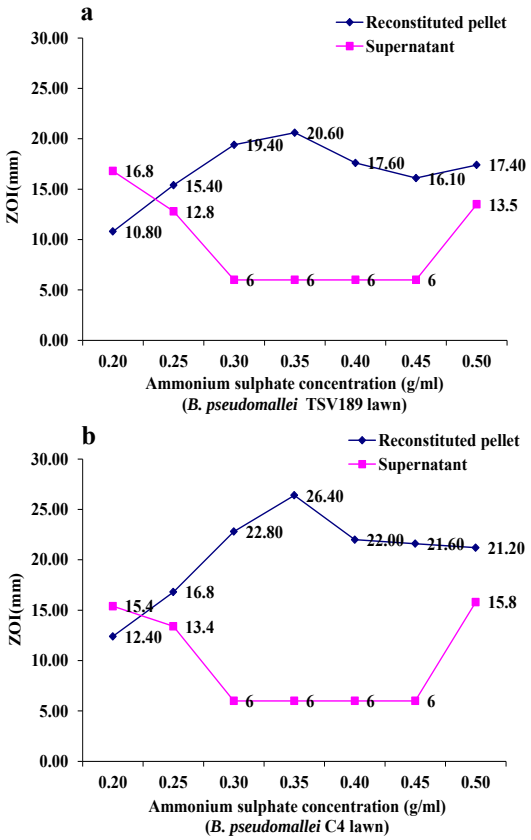


Figure 6.4: Well diffusion assay of ammonium sulphate precipitation optimization experiment on a lawn of *B. pseudomallei* strain TSV 189 (a) and *B. pseudomallei* strain C4 (b). Cell free supernatants were precipitated using 0.2 - 0.5 g/ml $(NH_4)_2SO_4$. The remaining supernatant (pink line) and resuspended pellet (blue line) are displayed. Numbers on each line are actual zones of inhibition measurements in mm.

6.3.4 Effect of ammonium sulphate precipitation and heating on protein purification, examined using Silver and Coomassie Brilliant Blue staining of SDS-page gels.

Coomassie Brilliant Blue and Silver staining of SDS page gels was carried out to identify any bands of interest in heated and non-heated ammonium sulphate treated cell free supernatants. It was hoped that a clear candidate for a BLIS would be identified. Coomassie Brilliant Blue stain on ammonium sulphate treated cell free supernatants produced only a few very faint bands, indicating a low concentration of protein in the samples (figure 6.5a and b). Silver staining produced multiple bands (figure 6.5c and d), with increased density as ammonium sulphate concentrations increased, as expected. Heat treatment increased the density of bands at 0.2 - 0.25 g/ml ammonium sulphate when compared to higher concentration. The number of bands makes identification of any obvious BLIS band difficult. A few candidates are noted on the gels (A-C).

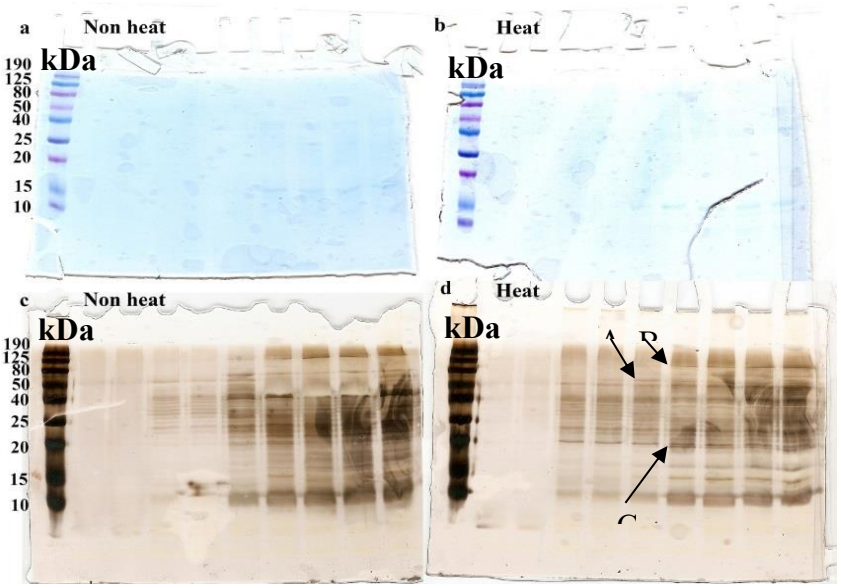


Figure 6.5:Visualisation of ammonium sulphate precipitated cell free supernatants, reconstituted in LB and separated by SDS-PAGE gel via Coomassie Brilliant Blue staining (a and b) and silver staining (c and d). All gels contain the same type of samples in the same order with the exception of heat or non-heat treatment. Gels on the left (a and c) are of samples which have not been heat treated at 70 ^{o}C for 30 min before ammonium sulphate precipitation. Gels on the right (b and d) have been heat treated at 70 ^{o}C for 30 min. Lanes in order are: 1: marker (HyperPage, Cat No. BIO-33066), 2 and 3: LB media, lanes 4-10: samples treated with 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 g/ml ammonium sulphate respectively. Arrows A (0.3 g/ml) B and C (0.35 g/ml) identify bands which may be different from non heated bands at the same concentration of ammonium sulphate and from other concentrations of ammonium sulphate.

6.3.5 Activity of BLIS with dialysis through 10 kDa sized dialysis tubing.

Given the previous literature regarding the BLIS (Marshall, 2007), it was thought that the BLIS would dialyse through a 10 kDa pore size tubing, which would simplify purification. Samples were taken through the course of the dialysis experiment to track activity and ensure concentration or dilution effects were taken into account. All samples taken from the dialysis experiment were analysed using both *B. pseudomallei* strains TSV189 and C4, with similar patterns of ZOI formation on both lawns.(Figure 6.6). The phosphate buffer and LB precipitated with ammonium sulphate and reconstituted (negative control buffer) on their own did not cause any inhibition of the bacterial lawn. There was a very small ZOI in the solution outside the dialysis tubing (sample 4), but most activity remained in the tubing (sample 5 and 6), indicating most activity was in molecules greater than 10 kDa, The high molecular weight sample when diluted 15x (sample 6) had higher activity than its concentrated form (Sample 5).

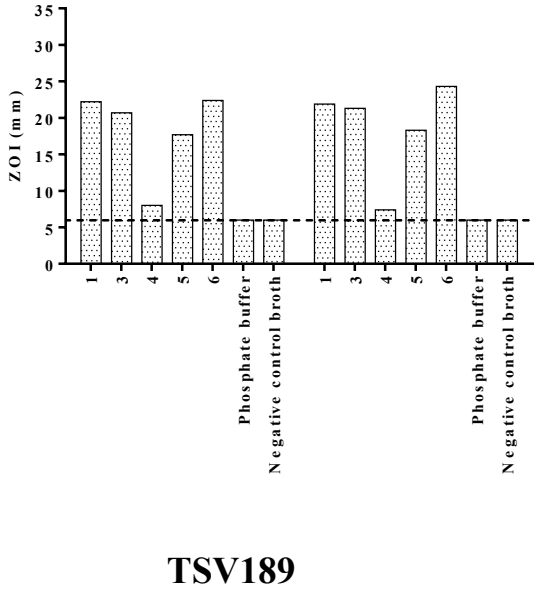


Figure 6.6: Activity of possible BLIS prior to and after dialysis. Activity via well diffusion is tested using both TSV189 (left) and C4 (right) strains of *B. pseudomallei*. Activity of samples 1, 3, 4, 5 and 6 as well as phosphate buffer and ammonium sulphate precipitated (0.3 g/ml) LB (negative control broth) as a negative control are shown. Sample 1 is the original cell free supernatant, sample 3 is the ammonium sulphate precipitated solution after resuspension back to the original volume, sample 4 is the external dialysis solution after dialysis, sample 5 and 6 are the internal dialysis solution after dialysis, with sample 6 being rediluted to the same volume as sample 3.

6.3.6 Activity of BLIS with size separation via Column chromatography.

Given the active agent did not clearly dialyse through the 10 kDa dialysis pores, further purification was carried out using size exclusion column chromatography as this technique could separate a wider size range of molecules. The sample which was loaded onto the column was 200 µl of a 100× concentrated ammonium sulphate precipitated sample. This sample was gel like in texture and the fractionation process diluted this sample into 18.6 ml (8.2 ml+16 x 0.65 ml fractions =18.6 ml); a 1:93 dilution. The agar diffusion assay identified only small ZOI's in spite of this dilution not being more than the original concentration. An example of the agar diffusion assay of the fractions is displayed in Figure 6.7a, with the key to the fractions in Figure 6.7b. The positive control, fraction 4 and fraction 5 (F4 and F5) produced ZOI's. No other fractions produced ZOIs. SDS Page gel analysis with silver staining was carried out on fractions 2 to 8 (Figure 6.7c) with no clear production of a band in fractions 4 and 5 that could not be seen elsewhere. In addition, there is limited size separation according to SDS-page gel analysis. A standard curve was approximated using reported results for this column (Figure 6.8b) under the same conditions as were used in this assay. Active fractions 4 and 5 correspond to a size between 340 and 460 KDa and these fractions are highlighted on the chromatography readout (Figure 6.8a).

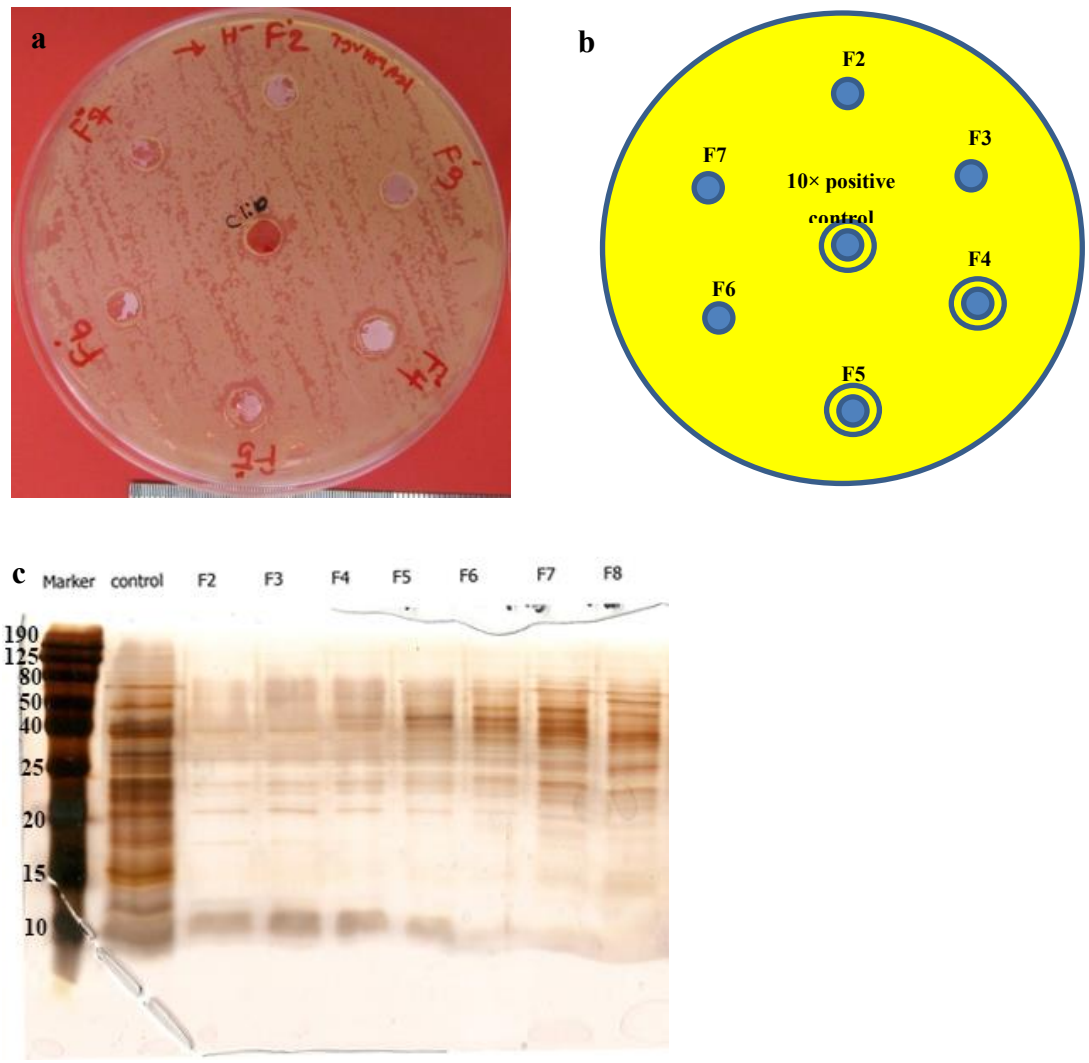


Figure 6.7: Identification of active fractions from column chromatography size separation. Fractions presented are labelled as F2 to F8 for fraction 2 to fraction 8. The well diffusion assay (a: actual and b: key) displays a 10× positive control and fractions 2-7 only. Active fractions are identified in the key by an extra ring around the relevant blue circles. The actual zones (a) can be seen as a zone around the wells. Fractions 4 and 5 have activity as does the positive control. Fractions 8 onward have no ZOI and are not shown. The SDS PAGE gel of the sample prior to addition to the column (control, diluted by a factor of 10) and fractions 2-8 is also displayed (c). Fractions 4 and 5 produce multiple bands, none of which can be clearly seen to be only in these two fractions.

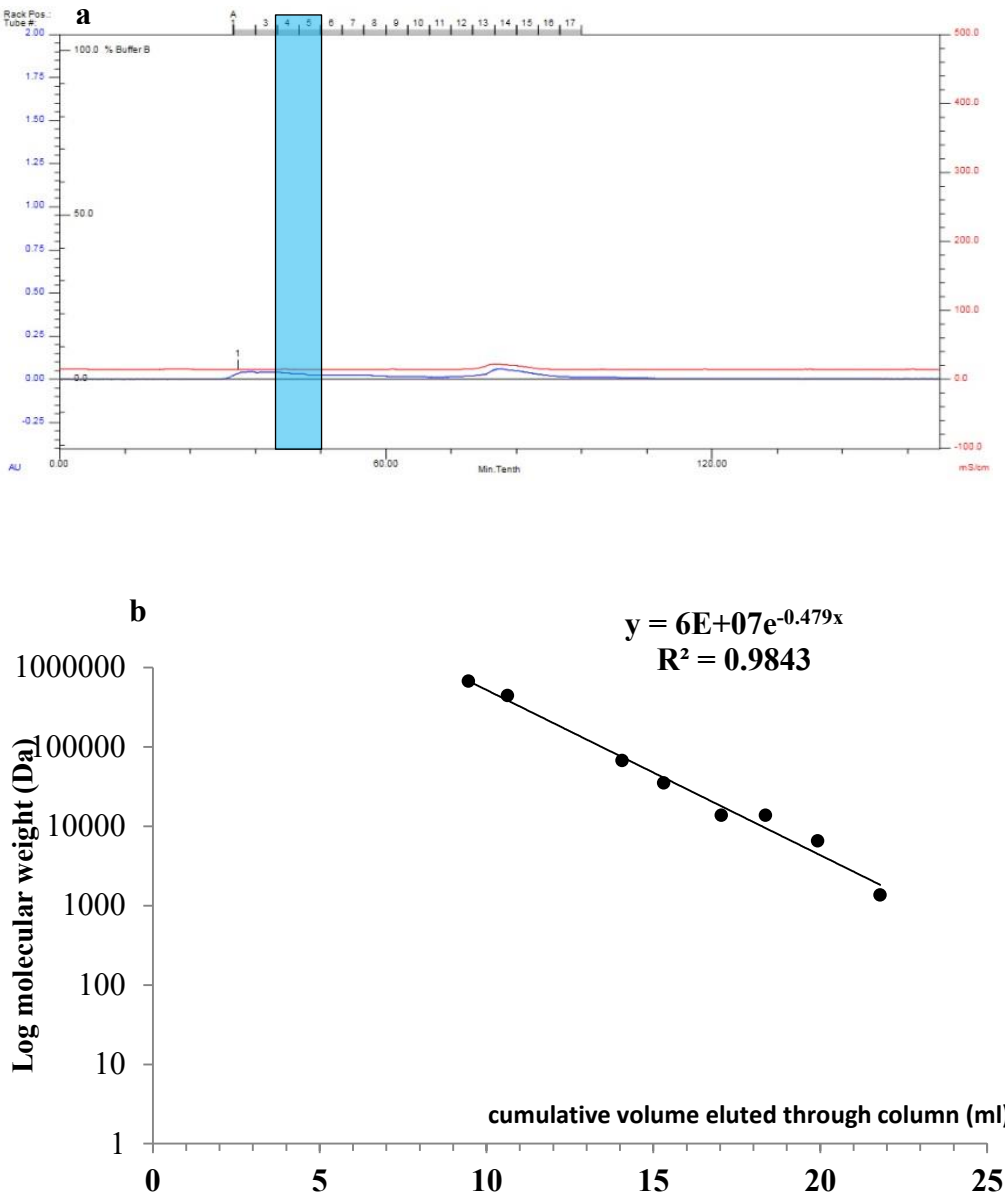


Figure 6.8: Column chromatography run print out (a) showing fractions with activity highlighted in blue. A standard curve (b) calculated from reported results (https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314807262343/litdoc71501796_20150114215337.pdf) using the same conditions as used in this experiment, was used to estimate the size range of the active fractions. This size range was determined to be between 340 and 460 kDa.

6.3.7 Identification of active bacteriophage samples.

Bacteriophage samples at James Cook University had not previously been tested for activity against the *B. pseudomallei* isolate of interest in this study and as such a screening step was required. Of the 11 stock bacteriophage samples tested against *B. pseudomallei* isolate TSV189 by spot on lawn assay (3.1.3), 7 were able to lyse the host and produce plaques (Table 6.1). All plaques were pin prick sized. When these 7 phage samples were combined in a cocktail at 10^7 PFU/ml, clearance could be seen in the spot (Figure 6.9).

Table 6.1: Observation of plaques on *B. pseudomallei* TSV189 lawns using previously isolated and purified bacteriophage stocks. + means plaques were observed, - means no plaques were observed. The top line is the previously used code for the phage sample.

Phage	**4-55.**	**4-54-55**	**4-56-55**	**8-18.**	**8-55**	**8-56-55**	**69-27**	**69-54**	**C1-19**	**C2-27**	**C2-44**
Result	+	-	-	+	+	+	-	-	+	+	+

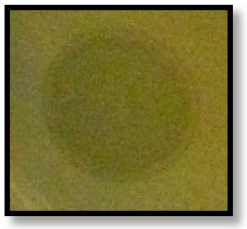


Figure 6.9: Photograph of clearance of *B. pseudomallei* TSV189 in a spot on lawn assay using a phage cocktail at 107 PFU/ml. The spot is approximately 10mm in diameter (20 µl).

6.3.8 Transmission electron microscope (TEM) analysis.

Transmission electron microscopy was carried out to identify purity and family of phages in the 7 samples selected for use in this study. All 7 previously purified bacteriophage samples were identified as having at least two different phages present due to variation in the diameter of the capsid or tail length. Most phage came from the family Myoviridae (Figure 6.10 a and c) and one was identified with an unusually short tail, although this is longer than typical of the Podoviridae (Figure 6.10 b). Average capsid diameters ranged from 67nm to 148nm (Figure 6.11 a) with most tails between 58 and 107 nm (Figure 6.11 b).

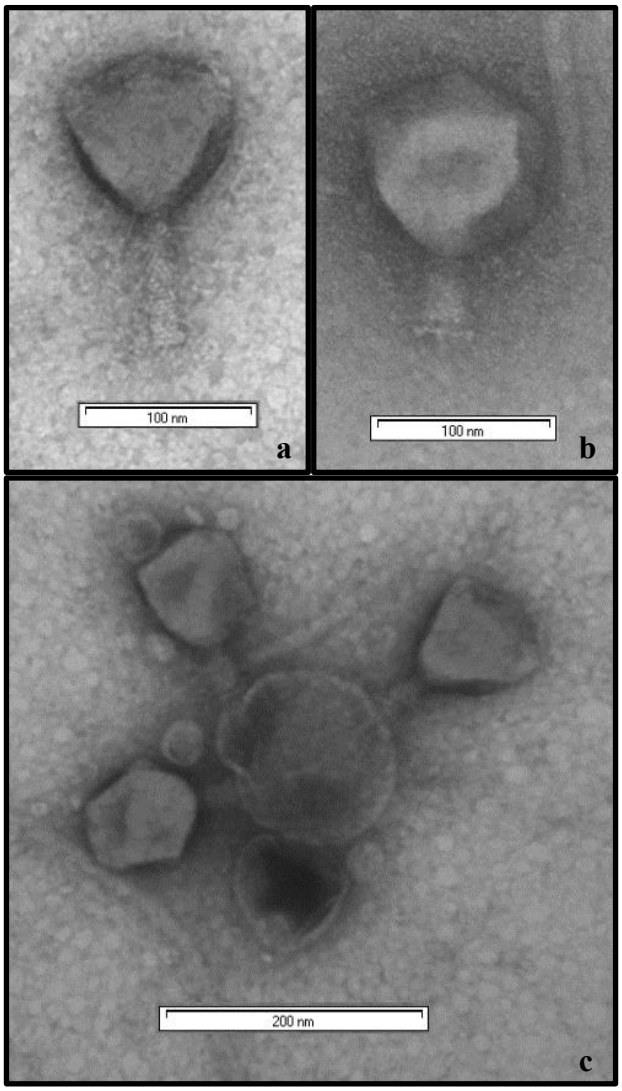


Figure 6.10: Characteristic TEM image of phages in previously purified phage extracts. All phage belonged to the order Caudoviridae in the family Myoviridae (a): with one having an unusually short tail (b): An example of Myoviridae phage attached to cell wall debris of *B. pseudomallei* isolate 8 is also presented (c).

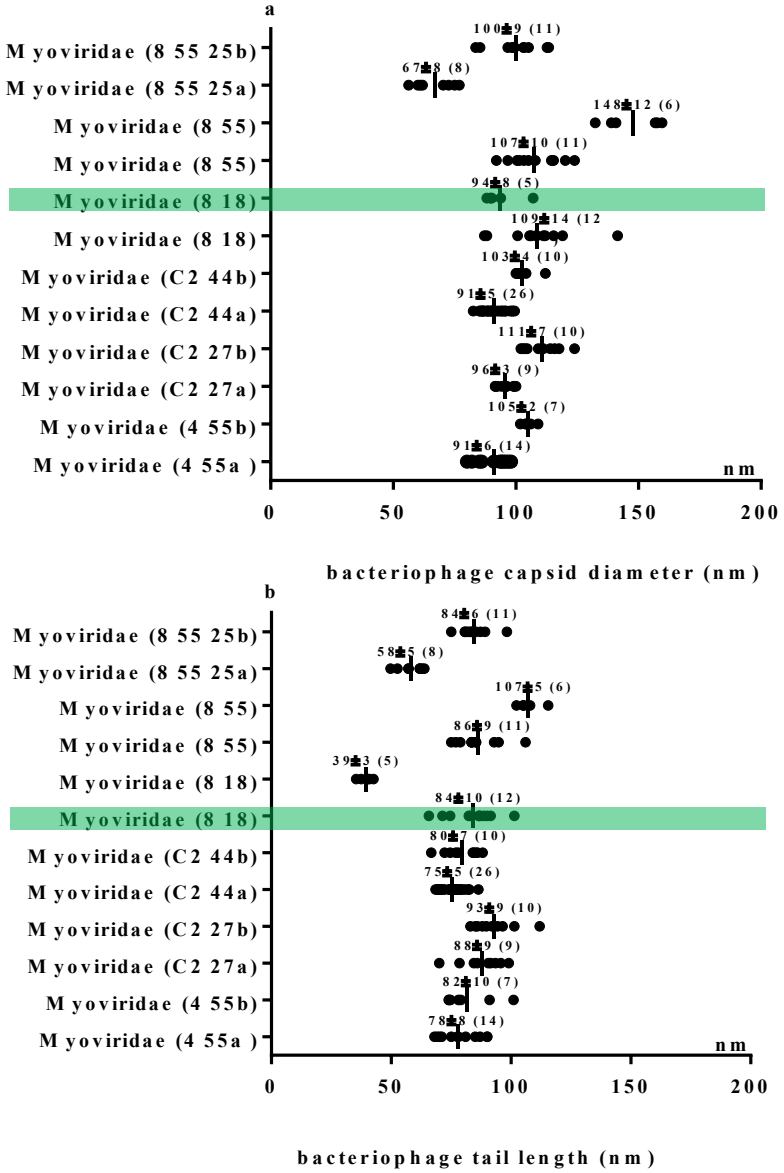


Figure 6.11: Measurement of phages in each sample in nanometers. Phages were separated into different isolates based capsid diameter (a) and if variable, by the tail structure (b). The short tailed phage is highlighted in green. Measurements include the average measurement +/- the range followed in brackets by the number of phage measured.

6.4 Discussion

6.4.1 Bacteriocin analysis

Work in this study confirmed the temperature dependent inhibitory activity of the cell free supernatant and extended the time dependant production of the antagonistic activity of *B. ubonens*is (A21) on *B. pseudomallei* (C1-C4, TSV189, TSV192). In this study, the onset of antagonistic activity was delayed, i.e. 3-4 days as opposed to 21-24 hr (Marshall, 2007). The extended period of culture in this experiment, with increased activity over time, led to the question of whether the active agent was intracellular, with increased activity in cell free supernatant due to lysis of cells releasing the agent. Bacterial cells were lysed to identify what level of activity was present inside cells with the same number of cells (as measured by OD) lysed at each day of testing (day 1 to 6). At day 2, there was some activity from cell contents which was significantly more active after heating at 70 ^{o}C for 30 min. After this time, the activity was relatively similar across all days indicating a possible maximum production of activity retained per cell or solubility. As the cell contents were not resuspended to the same volume as the supernatant, a direct correlation cannot be made between cell contents and cell free supernatant, however at day 6, the volume of cells was half that of the supernatant and the supernatant had similar or greater levels of activity. As incubation time increased, higher activity was found in the supernatant and this in combination with the presence of levels of BLIS in the cells may mean the BLIS is primarilly intracellular and while it may be naturally secreted, it may also be released during cell lysis in the 6 day incubation. While protein secretion is important bacterial pathogenesis, including that of *B. pseudomallei* (eg Type II, III, IV secretion) (Galyov *et al.*, 2010), the experimental design provides insufficient information to hypothesise further regarding the two possibilities. A more thorough study focussing on how the BLIS is released would be required to answer this question.

The earlier presence of activity if the cell free supernatant was heated may mean that heat treatment is producing a change in the active agent, which also occurs naturally over time, this could be a conformational change, to make the agent active site

available, presuming the BLIS is an enzyme (Morton *et al.*, 1958; Marshall *et al.*, 2010). Alternately it could involve removal of an inhibiting component such as exists in some multimeric protein complexes (Lodish *et al.*, 2000) and it is possible that activity does not naturally occur until the agent is secreted from the cell. This may explain why cells which were lysed as well as cell free supernatants, which are likely to contain the lysed products of cells, were not very active until heated. No SDS PAGE gels of cell lysates were attempted as, given the number of bands in the supernatant, the cell lysate bands would be even more difficult to differentiate.

To minimise the amount of intracellular components in our purification protocols, the supernatants were chosen for further assessment. Ammonium sulphate precipitation, and size separation using dialysis and column chromatography were used to fractionate the BLIS. As activity increased to 6 days, 6 day supernatants were used for all analyses. Well diffusion assays were used to test for any activity in fractions. The lowest concentration of ammonium sulphate able to remove all activity from the supernatant was 0.3 g/ml ammonium sulphate and while activity was slightly higher in the reconstituted pellet at 0.35 g/ml, its possible that this extra activity was due to the level of ammonium sulphate used, as can be seen in the supernatant of the 0.5 g/ml samples, so the 0.3 g/ml protocol was selected. The results from Silver and Coomassie Brilliant Blue staining of ammonium sulphate precipitated samples provides evidence that the protein yield of each band was less than one µg as Coomassie staining was not able to pick up any bands and this is the lower limit of this staining method (Simpson, 2010) but more than one ng as this is the lower limit of silver staining (Mortz *et al.*, 2001) (Figure 6.5 a and b). Marshall (2007) was also unable to detect bands with Coomassie Brilliant Blue staining or protein levels via a Bradford protein assay. A silver stain was not attempted in the work by Marshall et al (2007, 2010). In this study, the silver staining a large number of bands were observed. Three possible bands of interest were noted, but given the complex nature of the extract, further purification would be required to identify which if any of these have activity.

Initial expectations based on the work by Marshall (2007) were that the BLIS activity would be due to a low molecular weight compound that would dialyse through the dialysis tubing, however this was not the case. When concentrated samples (inside tubing) were diluted to factor in dilution effects on other samples (outside tubing), the solution inside the dialysis tubing was still far more active than the solution outside and did not appear to have lost any activity compared to the sample prior to dialysis.

Two hypotheses were considered for the very small zone of clearance outside the tubing. Firstly the BLIS was at or around the cut off of the dialysis tubing, Bacteriocins of Gram negative bacteria can be divided into three groups; microcins (<10 kDa), colicin-like (25-80 kDa) and phage tail like bacteriocins (high molecular weight, multimeric peptide assembly) (Chavan and Riley, 2007), so a bacteriocin in this size range would be feasible. The second hypothesis was that there may have been user error with contamination when loading the dialysis tubing. It was concluded that the simplest way to identify the origins of the activity in the low molecular weight sample would be to process the Ammonium sulphate precipitated cell free supernatant through size exclusion chromatography (SEC) as this would provide a more precise size separation.

At this point it was also noted that concentrated samples produced smaller ZOI's than samples rediluted back to the concentration found in the original supernatants. It would be expected that larger ZOIs would be seen with more concentrated samples. While there may be a maximum diffusion range for the BLIS through the agar, the decreased ZOI for the concentrated sample indicated something else was happening. Was it not dissolving, because it was either at saturation already or did not dissolve in phosphate buffer? Were active sites sterically hindered at high concentration by other molecules or by self, possibly neutralising the effect of a conformational change caused by heating? Dilution in phosphate buffer back to the original concentration resulted in increased ZOI's indicating that dissolving in phoshate buffer was unlikely to be an issue, and the protein concentration was low as concluded by lack of bands on

Coomassie staining, however it is still possible that the agent was highly active but minimally soluble (Trevino *et al.*, 2008). This may be supported by the fact that, due to the observed Gel-like appearance of the 100× concentrate, it was centrifuged at very high speed prior to application on the column remove any precipitate. Much of the active agent could have been lost at this point if low solubility was an issue. In addition, if the agent is highly active at low concentrations, but easily hindered at higher concentrations, diffusion through the agar may be insufficient to dilute out this effect. An example of hindrance caused at higher concentrations can be seen in the field of affinity chromatography (Murza *et al.*, 2000), where increasing the concentration of immobilised ligands resulted in lower binding of target proteins as active sites on the ligand became unavailable due to steric hindrance by other ligands.

Based on the active fractions from the size exclusion chromatography, the active agent was very large (340-460 kDa), in the range of phage tail like bacteriocins, although unlike our agent, these are typically heat sensitive (Smarda and Benada, 2005). It was also in the range of some glycoproteins such as the >200 kDa glycoprotein produced by the bacteria *Tannerella forsythia* (Fagan and Fairweather, 2014). There are some indications our active agent may be also be a glycosylated protein.

Both N-linked and O-linked glycosylation pathways are present in eukaryotes, but glycosylation pathways have also occurred in bacteria (Nothaft and Szymanski, 2010). For example, periplasmic and membrane bound proteins in *Campylobacter jejuni* are *N*-glycosylated with a conserved heptasaccharide (Scott *et al.*, 2011) In addition, as noted above, the 100× concentrate was observed to have a "Gel-like" appearance. Although this was not a pure sample and the viscosity may have been due to something other than the active agent, glycosylated proteins have been described as being viscous and these can combine glycans, proteins, lipids and other organic molecules (Ahmad and McPhie, 1980).

The high molecular weight column chromatography purified agent, when analysed on a denatured SDS-PAGE gel, produced a multitude of smaller bands and no visible high molecular weight band, indicating this agent was likely to be multimeric, containing two or more asscociated polypeptide chains. Multiple protein components could also add to number of bands present on the SDS-PAGE gel. Glycoproteins can also have altered charge to mass ratios when run on SDS-PAGE gel electrophoresis and if there is an increased charge to mass ratio, such as is the case with hydrophobic glycoproteins, the glycoprotein will run faster on the gel and have a smaller apparent size. In addition, if there are glycoforms (the same protein with differing numbers of glycosylated sites occupied), these can possibly result in multiple separate bands on SDS-PAGE analysis (Mejanelle *et al.*, 2002) as found in Figure 6.7c.

At this point in the analysis of the BLIS, it was decided that the potentially complex nature of this agent and the difficulty in concentrating it for purification and analysis would mean that further characterisation would require the efforts of a dedicated protein chemist. This was outside the scope of this study. This study has identified that the agent is large, complex, found inside the cells as well as in the supernantant, and able to be precipitated with 0.3 g/ml ammonium sulphate. Increasing activity via concentration of the agent was not successful, but the semipurified ammonium sulphate precipitate was active and can be trialled in biocontrol assays in anticipation of further chemical analysis.

6.4.2 Bacteriophage analysis

The second biocontrol candidate, a selection of bacteriophage isolates, were identified as not being purified single bacteriophage when examined using TEM. However, phage therapy protocols typically require the use of mixed phage (Chan *et al.*, 2013), so this impurity does not affect the use of these in a proof of concept trial. The pinprick sized plaques made quantitation within one log difficult to do accurately and was likely the reason previous work to plaque purify these phages had not been completely successful. However, quantitation within one log was sufficient to judge amplification levels and, on combination, the phage cocktail was able to clear the bacteria via spot on

lawn assay. The small size of the plaques correlated with the unusually large capsids. These were larger than those previously reported for *B. pseudomallei* phage (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011). Phage with large capsid diameters have been reported to have low diffusion through agar, thus producing pinprick sized plaques (Elford and Andrewes, 1932; Abedon and Yin, 2009). While the phage cocktail developed in this study was effective against the *B. pseudomallei* isolate (TSV189) to be trialled in biocontrol assays, it would be advisable for any future work to involve collection of new environmental phage samples from our endemic site to find larger plaque producers. This would simplify processing and may remove any limitations regarding diffusion of the phage when used as a biocontrol agent. As a proof of concept cocktail, for use in a rice model, this cocktail was judged as usable. The plaque size limitation, along with difficulties in purifying each phage and likelyhood that any cocktail designed for environmental trials would not use these particular phage, means that further charactisation would be an excessive use of resources, so characterisation has been limited to T.E.M. measurement.

6.4.3 Conclusion

Although neither of the biocontrol canditates developed in this chapter have been purified and fully characterised, they have shown potential for use as proof of concept agents against the *B. pseudomallei* isolate (TSV189) already found to inhibit growth of rice. As such they can be used to identify what limitations and advantages each approach would have in controlling biofilm formation and plant infection. This will form the basis of the next chapter.

CHAPTER 7: BIOCONTROL OF *B. PSEUDOMALLEI* IN BIOFILMS AND RICE MODELS OF INFECTION

7.1 Introduction

Biological control or biocontrol is a concept whereby live micro-organisms are used to lower the numbers of specific pests, decrease abundance and lessen the damage of undesirable organisms (Eilenberg *et al.*, 2001). Bacteriocin (Marshall *et al.*, 2010) and bacteriophage (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011) active against *B. pseudomallei* have been reported with the ability to kill a range of strains of *B. pseudomallei in vitro*.

Biofilms are a microbial community, which irreversibly attach to a surface or interface and embed in a matrix of extracellular polymeric substances. Bacteria forms biofilms in moist nutrient rich areas of soil such rhizospheres and in areas of animal activity (Costerton, 2007; Knezevic and Petrovic, 2008) *Burkholderia pseudomallei* has also been shown to form biofilms in human and animal tissue (Vorachit *et al.*, 1995). *Burkholderia pseudomallei* presence in a rhizosphere was demonstrated two decades ago, data obtained in previous *in vitro* (plate assay) studies using lytic phage isolated from *B. pseudomallei* endemic areas (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011), indicated the phage were effective against multiple strains of *B. pseudomallei* and did not infect near-neighbour species or other species tested, with the exception of *B. mallei* and *B. thailandensis*. No work has been reported looking at the effectiveness of bacteriophage treatment in a plant model of *B. pseudomallei* infection. In addition, bacteriocin-like compounds have also been identified as effective against *B. pseudomallei* in plate assays (Marshall *et al.*, 2010) and soil (Lin *et al.*, 2011).

In this study, a bacteriocin-like compound and bacteriophage previously developed (Chapter 6) will be used against a biofilm model and the previously developed domestic and wild rice model (Chapter 4 and 5) to determine whether they will be useful as biocontrol agents.

7.2 Materials and Methods

7.2 .1 Biofilm formation in a 96 well plate

A standard method from biofilm development in a 96 well plate was modified from Knezevic and Petrovic (2008). Each well of the flat bottomed 96 well plate (Sarstedt, Germany) had 100 µl double strengthen LB broth plus 0.1% glucose aliquoted and then 10 µl *B. pseudomallei* (TSV189) of overnight culture (Section 3.1.2) at 10^8 CFU/ml (10^6 CFU/well) was added. Wells had bacteriophage and bacteriocin agents added as required (see below, sections 7.2.1.1 and 7.2.1.2), were covered and placed in secondary containers and incubated at 37 ^oC without shaking for times as described in specific biofilm experiments (Knezevic and Petrovic, 2008). Plates were then processed for fixation, staining and measurement by optical density (OD) (Section 7.2.1.3)

7.2.1.1 Crude extract of *B. ubonensis* addition to wells

The crude extract of *B. ubonensis* which had been partially purified using 0.3g/ml ammonium sulphate as per section 6.2.4.1 was used in all biofilm trials. One hundred µl of partially purified solution was added to wells in 96 well plates in 12 replicates at dilution at one time (Section 7.2.1) and 100 µl of PBS (Appendix A1.11**)** was added to wells as a control. This was repeated such that there were two identical experimental layouts, one of which was then incubated for 24 hr and the second incubated for 48 hr.

7.2.1.2 Bacteriophage addition to wells

All bacteriophage isolates previously identified (section 6.2.9) were mixed to create a phage cocktail (10^7 PFU/ml of each phage). A volume of 100 µl of phage cocktail was added to wells in 96 well plates in 12 replicates at 10^6 PFU/well (Section 7.2.1) and 100 µl of SM buffer (Appendix A1.9**)** was added to wells as a control. This was repeated such that there were two identical experimental layouts, one of which was then incubated for 24 hr and the second incubated for 48 hr.

7.2.1.3 Fixing and Crystal violet staining of biofilm

Fixing and staining of biofilms was carried out using a method modified from Knezevic and Petrovic (2008). Supernatant was removed from the wells by multichannel pipette and each well was washed twice with 250 µl of PBS (Appendix A1.11**)**, via pipetting in and out. Flicking of wells and washing under a flow of PBS was not possible in the PC3 laboratory. The plate was left to dry for 10 min. All wells were filled with 250 µl absolute ethanol for 15 min to fix the bacteria and the ethanol removed via pipetting. Each well was then filled with 250 µl 0.4% crystal violet (Appendix A1.24**)**, for 15 min and excess stain removed by pipetting. The plates were then washed 4 times using distilled water by submersion of the plate in a series of tanks with inversion on paper towel between each tank. The outside of the plates were wiped with with 1:50 Trigene, followed by 70% ethanol to remove any potential transferred bacteria.

The plate was then allowed to air dry. All wells were filled with 250 µl of 33% acetic acid for 20 min to allow the crystal violet stain to dissolve. The absorbance was measured at 600 nm using a plate reader (BMG LABTECH, FLUOstar Omega).

7.2.2 Experimental treatment of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo with live *B. ubonensis* (A21)

Seeds were cleaned and primed as per section 3.3.1. Seeds were then separated into four groups. The four experimental groups for each plant species consisted of:

- Untreated control (rice seed only)
- Group A - bacteriocin producing bacteria control (rice seed + 10^7 CFU *B. ubonensis*)
- Group B - experimental treatment (rice seed + 10^7 CFU *B. ubonensis* + 10^7 CFU *B. pseudomallei*)
- Group C - *B. pseudomallei* infection control (rice seed + 10^7 CFU *B. pseudomallei*), Each group used 45 seeds.

Seeds were exposed to bacteria as follows:

- Untreated control seeds were treated by soaking in 10 ml of LB for an hour.
- Group A seeds were soaked in 10 ml of 10^8 CFU/ml *B. ubonensis* for an hour.
- Group B seeds were soaked in 10 ml of 10^8 CFU/ml *B. ubonensis* for an hour, dried for an hour and then soaked in 10 ml of 10^8 CFU/ml *B. pseudomallei* for an hour.
- Group C seeds were soaked in 10 ml of 10^8 CFU/ml *B. pseudomallei* for an hour.

All seeds were then dried for an hour and transferred to experimental propagation chambers for 7 days incubation as per section 3.3.2, and measurement and statistical analysis of plant growth determination at the end of the 7 day experiment was carried out as per section 3.3.3.

7.2.3 Experimental treatment of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo and *O. meridionalis* with a bacteriophage cocktail.

Seeds of both rice species were cleaned and primed as per section 3.3.1. Seeds were then separated into four groups. The four experimental groups for each plant species consisted of:

- Untreated control (rice seed only)
- Group A - phage cocktail only (rice seed + 10^6 PFU phage cocktail)
- Group B - experimental treatment group (rice seed + 10^6 PFU phage cocktail + 10^7 CFU *B. pseudomallei*)
- Group C - *B. pseudomallei* infection only (rice seed + 10^7 CFU *B. pseudomallei*), Each group for the domestic rice experiment used 45 seeds. Each group for the wild rice experiment used 30 seeds.

Seeds were exposed to bacteria as follows:

- Untreated control - seeds were treated by soaking in 10 ml of SM buffer for an hour.
- Group A - seeds had 100µl (10^7 PFU/ml) of phage cocktail directly added onto to each seed (10^6 PFU/seed) by pipette.
- Group B - seeds were soaked in 10 ml of 10^8 CFU/ml *B. pseudomallei* for an hour and dried for an hour and then had 100 µl (10^7 PFU/ml) of phage cocktail directly added to each seed (10^6 PFU/seed) by pipette.
- Group C - seeds were soaked in 10 ml of 10^8 CFU/ml *B. pseudomallei* for an hour.

Seeds from each experimental group were then allowed to dry for one hour. All seeds were then transferred to experimental propagation chambers as per section 3.3.2. The seedlings were grown for seven days and measurement and statistical analysis of plant growth determination at the end of the 7 day experiment was carried out as per section 3.3.3.

7.2.4 Quantitation of bacteria by plate count (phage cocktail treatment).

From each group, three plantlets of median growth (as determined by eye) were selected for plate counts. Plantlets were rinsed three times with 0.85% NaCl (Appendix A1.10) to remove surface bacteria not firmly attached to the plant and then ground in a microfuge tube with 500 µl of 0.85% NaCl using a microfuge pestle (Astral Scientific). Bacterial counts were then determined as per section 3.1.4 on Ashdown agar after incubation at 37 °C for 48 hr. Analysis of bacterial counts comparing infected (Group C) and treatment (Group B) groups for both species of plant were carried out on $\log_{10}$ transformed numbers by an independent t test ($p=0.05$) using SPSS version 20.

7.2.5 Quantitation of bacteria by qPCR (phage cocktail treatment).

7.2.5.1 DNA extraction

DNA was extracted from pure culture as per section 3.2.1 Plantlets were rinsed and stored in acetone until processed. Plantlets were then combined into groups of three for DNA extraction. For each group of three, plantlets were cut at the cotyledon to separate aerial and root parts and the aerial and root parts extracted separately.

7.2.5.2 Total DNA extraction from plant matter

DNA extraction on combined samples was carried out using the method of Mogg and Bond (2003) with modifications. Samples were added to 2 ml microfuge tubes with O ring (Scientific Specialties, Inc. (SSI), USA). 600 µL of extraction buffer (Appendix A1.5) was added and the sample ground using a microfuge pestle prior to addition of 2.5 mm silica beads (1/4 total volume) (Daintree Scientific, Australia). Samples were then chilled at -80 °C for 15 min. The plant samples were disrupted with a Mini-Beadbeater (Biospect products, inc, USA) for 5 min and incubated overnight at 37 °C. 200 µl of 5 M NaCl (Appendix A1.5.3) was added followed by incubation on ice for 5 min. The samples were centrifuged for 5 min at 16 000 × *g*. Supernatants were transferred to new microfuge tubes containing 600 µl of 100% isopropanol and mixed by inversion followed by incubation for 3 min at room temperature. Samples were centrifuged for 5 min at 16 000 × *g* and the supernatant discharged. 600 µl of absolute ethanol was added to the pellet and incubated for 3 min at room temperature. Samples were centrifuged for 5 min at 16 000 × *g*, the supernatant discharged and the sample allowed to dry for 15 min prior to addition of 100 µl of molecular biology-grade H_2O (Sigma, USA). Finally, the microfuge tube was incubated for 1 hr at 65 °C or overnight at 4 °C to completely resuspend the DNA. DNA extracted from the root part of wild rice (*Oryza meridionalis*) was re purified by addition of 10% polyvinyl polypyrrolodine (PVPP) in phosphate buffer (Appendix A1.25) at pH 7.2 (Solaiman and Marschner, 2007) to remove an observed brown colouration in the solution which was noted to inhibit the PCR. Samples were then centrifuged for 5 min at 16000 × *g* and the

supernatant was re extracted using a second round of 5 M NaCl (Appendix A1.5.3) isopropanol and ethanol.

7.2.5.3 Extraction of *B. pseudomallei* from acetone storage solution.

A volume of 1 ml of acetone storage solution was evaporated at room temperature overnight in a microfuge tube. Residual DNA was extracted from the tube as per section 3.2.1. Extracted DNA was quantified as per section 3.2.2 to determine the copy number per ml of *B. pseudomallei* in solution

7.2.5.4 Real time polymerase chain reaction (real time PCR).

The real time PCR was done as per section 3.3.2. A standard curve was developed using 10× serial dilutions of a plasmid (pGEM®-T Easy Vector Systems, Promega, A1360) containing the target DNA in a background of 5 ng/µl of herring sperm DNA (Promega, D1811) as per section 3.3.2. Standards were done in triplicate. PCRs were carried out in a final volume of 20 µl using SensiFAST™ Probe No-ROX Kit (Bioline, Australia), as per manufacturers conditions with no extra addition of $MgCl_2$. Counts were factored back to individual plantlets as follows;

qPCR result (copy/ul extract) × 100 (volume of extract from three plantlets)/3 (number of plantlets in an extract).

7.3 Results

7.3.1 Biocontrol biofilm formation (Crystal violet stain) in 96 well plate.

Biofilm formation, as measured by OD, was reduced by both bacteriocin and phage cocktail treatment (Figure 7.1). Both treatment groups had similar levels of inhibition (p=0.261) at 24 hr (Figure 7.1a). By 48 hr, the inhibition caused by the phage cocktail was significantly greater than that caused by the bacteriocin (Figure 7.1b) (Appendix A3.8). The growth of biofilm significantly decreased at 48 hr for bacteriophage treatment but not for bacteriocin treatment.

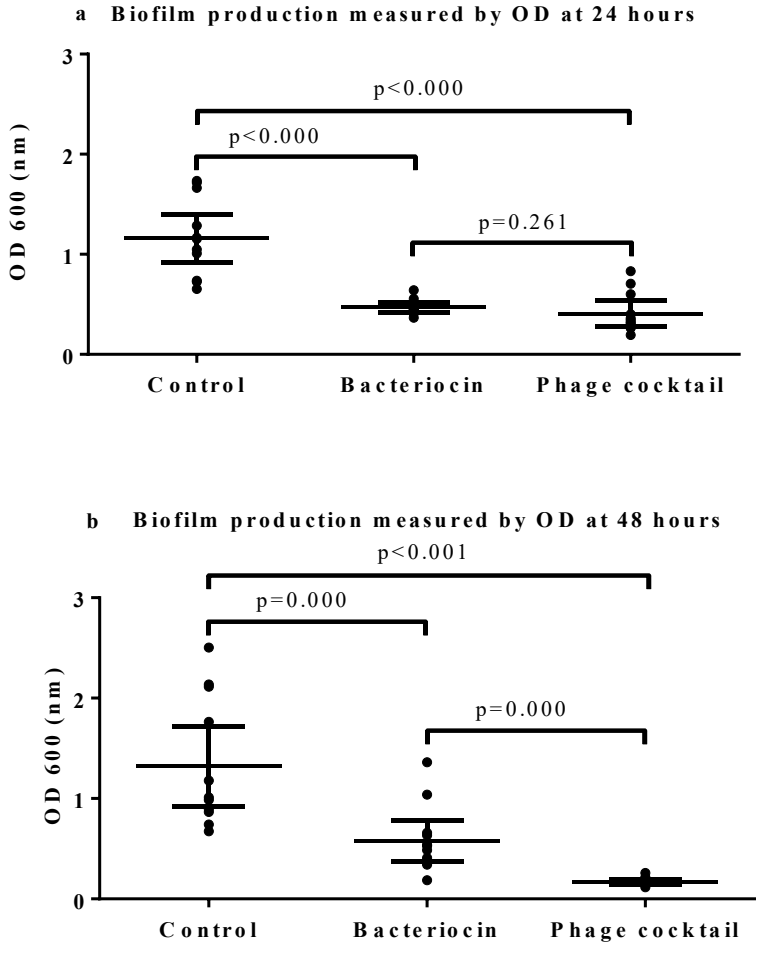


Figure 7.1: Mean OD ± 95 % CI of biocontrol gents (bacteriocin and phage cocktail) inhibition of biofilm formation at 24 hr (a) and 48 hr (b). Data within each treatment were compared by ANOVA at p=0.05.

7.3.2 *Oryza sativa* L. cv Amaroo growth using live *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189).

This first set of analyses investigated the impact of bacteriocin producing *B. ubonensis* (A21) against *B. pseudomallei* (TSV189) infected *Oryza sativa* L. cv Amaroo. Untreated control (rice seed only) had significantly greater growth than all groups where bacteria had been added to the seeds. Group B (experimental treatment with *B. ubonensis* and *B. pseudomallei*) was significant different to Group C (*B. pseudomallei* infection only control) (Figure 7.2a,b) for either root or leaf growth as noted in Chapter 5, *B. ubonensis* was shown to inhibit growth, although not to the extent that *B. pseudomallei* did (Appendix 3.9).

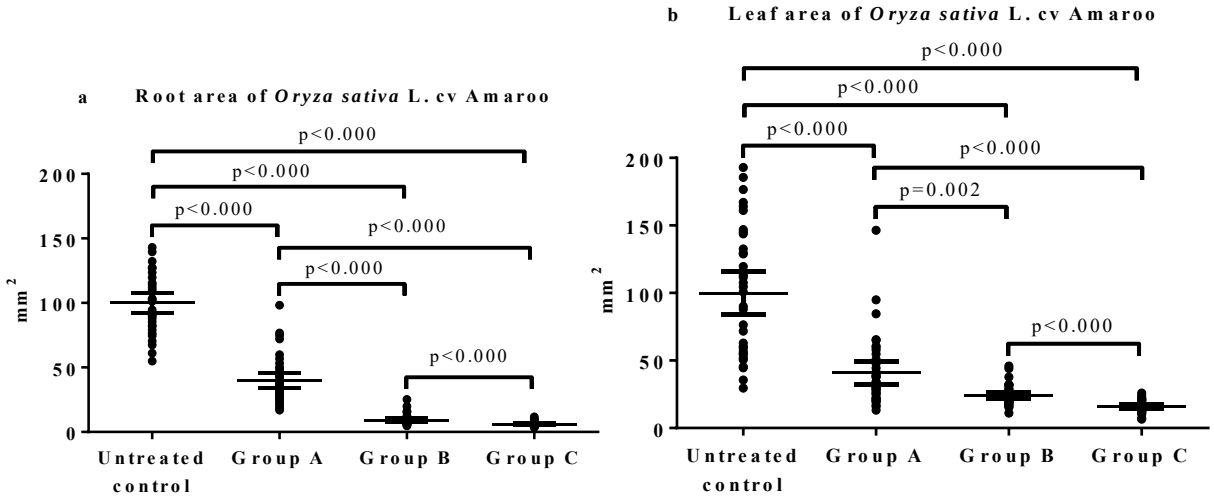


Figure 7.2: Mean mm^2 ± 95 % CI of root and leaf in each treatment including untreated control (rice seed only), Group A (rice soaked with *B. ubonensis*), Group B (rice soaked with *B. ubonensis* and *B. pseudomallei*), Group C (rice soaked with *B. pseudomallei*). Data within each treatment were compared by ANOVA (post hoc test) at p=0.05.

7.3.3 *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

The growth of *Oryza sativa* L. cv Amaroo was visually different depending on the experimental group (Figure 7.3). Statistical analysis of root and leaf growth further supported this initial observation (Figure 7.4). Both root (a) and leaf (b) area in Group A was similar to that of the untreated control. While Group B growth was significantly lower than the untreated control and Group A, it was significantly higher than Group C in root growth, although not in leaf growth. This indicated the bacteriophage cocktail in Group B had effectively improved root growth of *B. pseudomallei* infected rice, although not to the level of uninfected rice (Appendix 3.10).

Untreated control | Group A | Group B | Group C

Figure 7.3: Untreated control (rice seed only) and Group A (rice soaked with phage cocktail) are visually similar. Group B (rice soaked with *B. pseudomallei* phage cocktail and *B. pseudomallei*) appear to have improved growth over *B. pseudomallei* infection (Group C).

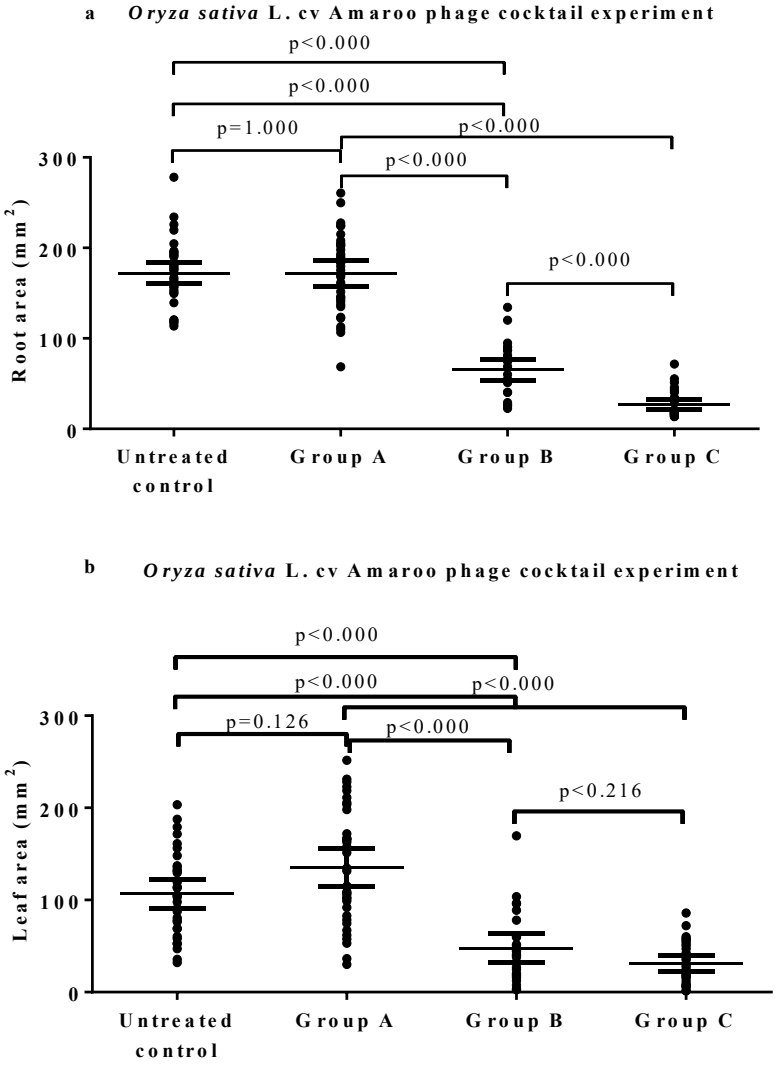


Figure 7.4: Mean mm^2 ± 95 % CI of root and leaf in each treatment including untreated control (rice seed only), Group A (rice soaked with phage cocktail), Group B (rice soaked with cocktail phage and *B. pseudomallei*), Group C (rice soaked with *B. pseudomallei*). Group B (a) improves growth of plantlet ($p<0.001$) when compared to Group C. Data within each treatment was compared by ANOVA (post hoc test) at $p=0.05$. P values directly above Group A, B and C are relative to the untreated control.

7.3.4 *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

As with the growth of *Oryza sativa* L. cv Amaroo, the growth of *O. meridionalis* visually inhibited with exposure to *B. pseudomallei* (Group C) (Figure 7.5). In this case however, the addition of the phage cocktail (Group B) completely eliminates any observable inhibition. This observation was confirmed on analysis of leaf and root growth (Figure 7.6), as both root (a) and leaf (b) area in the untreated control and in Group A and B were statistically similar and had significantly increased growth relative to Group C (the *B. pseudomallei* only infection) (Appendix 3.11).

Untreated control | Group A | Group B | Group C

Figure 7.5: Untreated control (rice seed only), Group A (rice soaked with phage cocktail) And Group B (rice soaked with *B. pseudomallei* and phage cocktail) are visually similar while *B. pseudomallei* infection (Group C) displays inhibited growth.

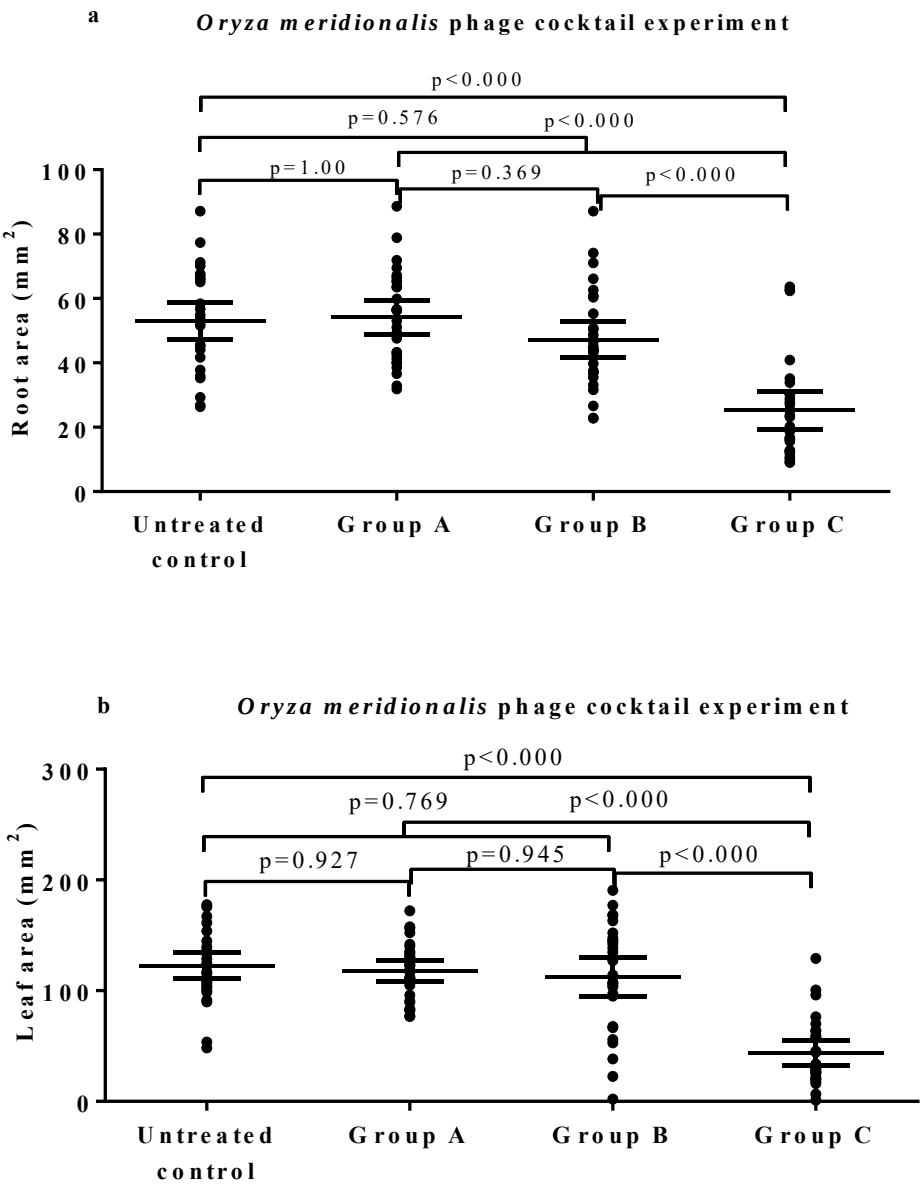


Figure 7.6: Mean mm^2 ± 95 % CI of root and reaf in each treatment including untreated control (rice seed only), Group A (rice soaked with phage cocktail), Group B (rice soaked with phage cocktail and *B. pseudomallei*), Group C (rice soaked with *B. pseudomallei*). Group B (root and leaf) has improved growth of plantlet ($p<0.001$) when compared to Group C. Data within each treatment were compared by ANOVA (post hoc test) at $p=0.05$. P values directly above Group A, B and C are relative to the untreated control.

7.3.5 PCR optimization

The PCR used had a reliable detection limit of 2.52 x 10^2 copies/µl (Figure 7.7a), although on occasion lower copy numbers could be detected, and a slope of -3.513 (Figure 7.7b). The *B. pseudomallei* strains tested (TSV189, SA12, 14_289, 14_327) all reacted with this assay. Non- *B. pseudomallei* organisms extracted from pure cultures at 10^8 CFU/ml did not react with this assay confirming it is selective as reported by Kaestli *et al.* (2007). Extraction of total DNA from rice infected with *B. pseudomallei* for seven days resulted in PCR positives (Figure 7.8).

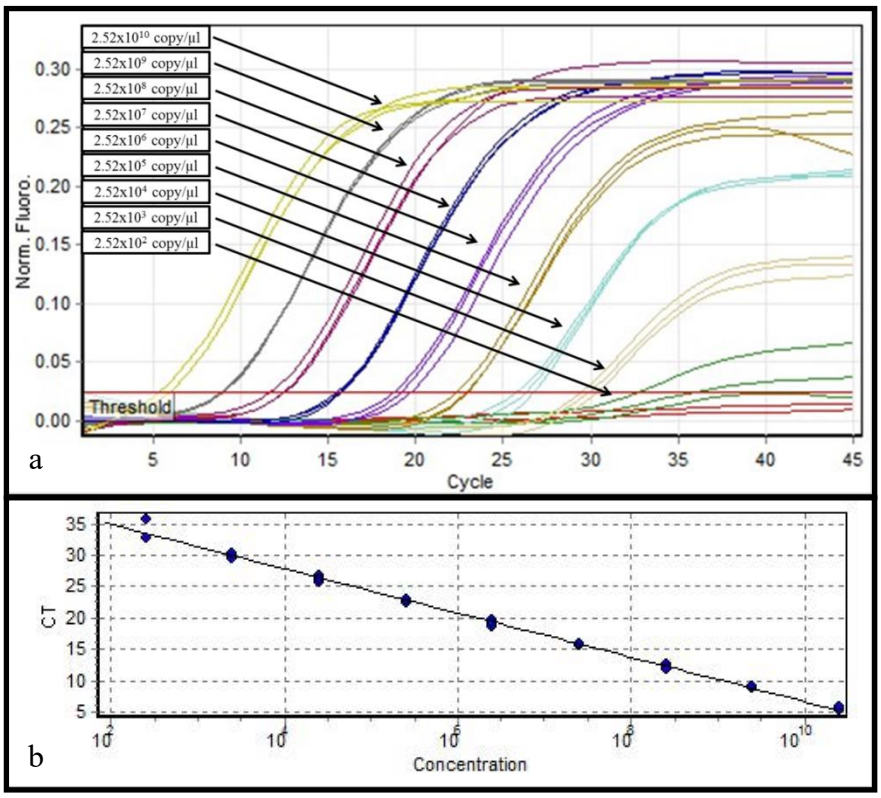


Figure 7.7: a is a standard template amplification table was 10^2 to 10^{10} copy/µl from *B. pseudomallei* which detected using FAM probe label (r^2=0.99557, efficiency =0.93 CT=-3.513*log(conc)+41.162). b is a calculation of PCR number of standard of curve for *B. pseudomallei*.

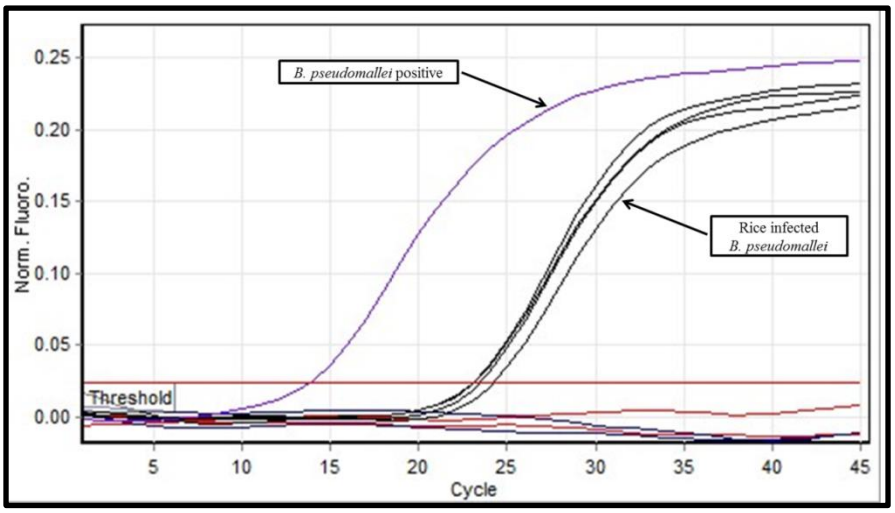


Figure 7.8: quadruplicate run using DNA from rice infected with *B. pseudomallei* SA12.

7.3.6 Quantitation of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) and *O. meridionalis* (wild rice) in with a phage cocktail by qPCR and plate count of whole plant.

The copy number/leaf portion was not significantly different in either species when comparing infection (Group C) and treatment (Group B) (Figure 7.9). The numbers of *B. pseudomallei* in the storage acetone was determined to be at least a factor of 100 lower than that found on leaves (Table 7.1). For both plant species, the bacterial load on the root section was significantly different between the infected and phage treatment groups (Group C and B respectively) when measured by qPCR (Figure 7.10a). Analysis of the whole plantlet by colony count (Figure 7.10b) produced similar results to that of the molecular analysis of the rootlets, with the exception that colony counts were typically a log lower than the qPCR (Appendix 3.12).

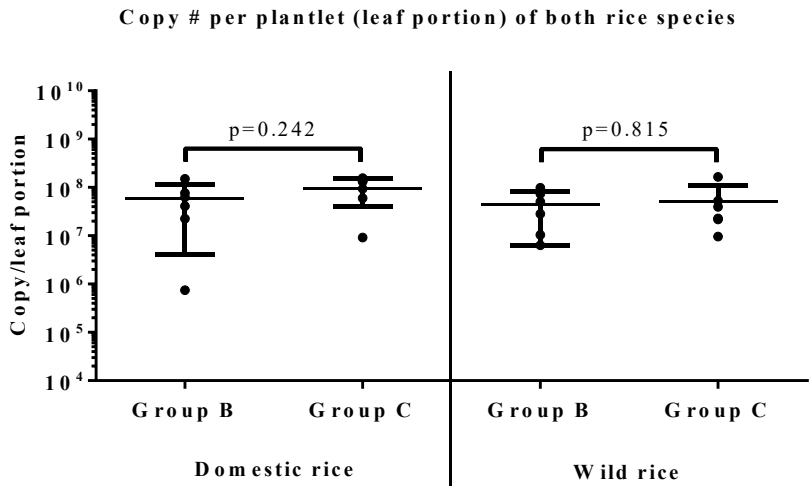


Figure 7.9: Mean copy *B. pseudomallei* ± 95 % CI of leaf portion in Group B (rice soaked with phage cocktail and *B. pseudomallei*), Group C (rice soaked only with *B. pseudomallei*) in different rice species. Both of them have not shown significant difference between Group B and Group C. Data within each treatment were compared by independent T test at p=0.05.

Table 7.1: The copy number per ml of storage acetone of *B. pseudomallei* extracted from acetone used to store rice plantlets after experimentation.

# copy/ml	***Oryza sativa* L. cv Amaroo**	***Oryza meridionalis***
Average group B	7.19E+03	2.93E+03
SD group B	9.48E+02	5.72E+02
Average group C	1.31E+05	6.53E+05
SD group C	5.69E+04	3.41E+05

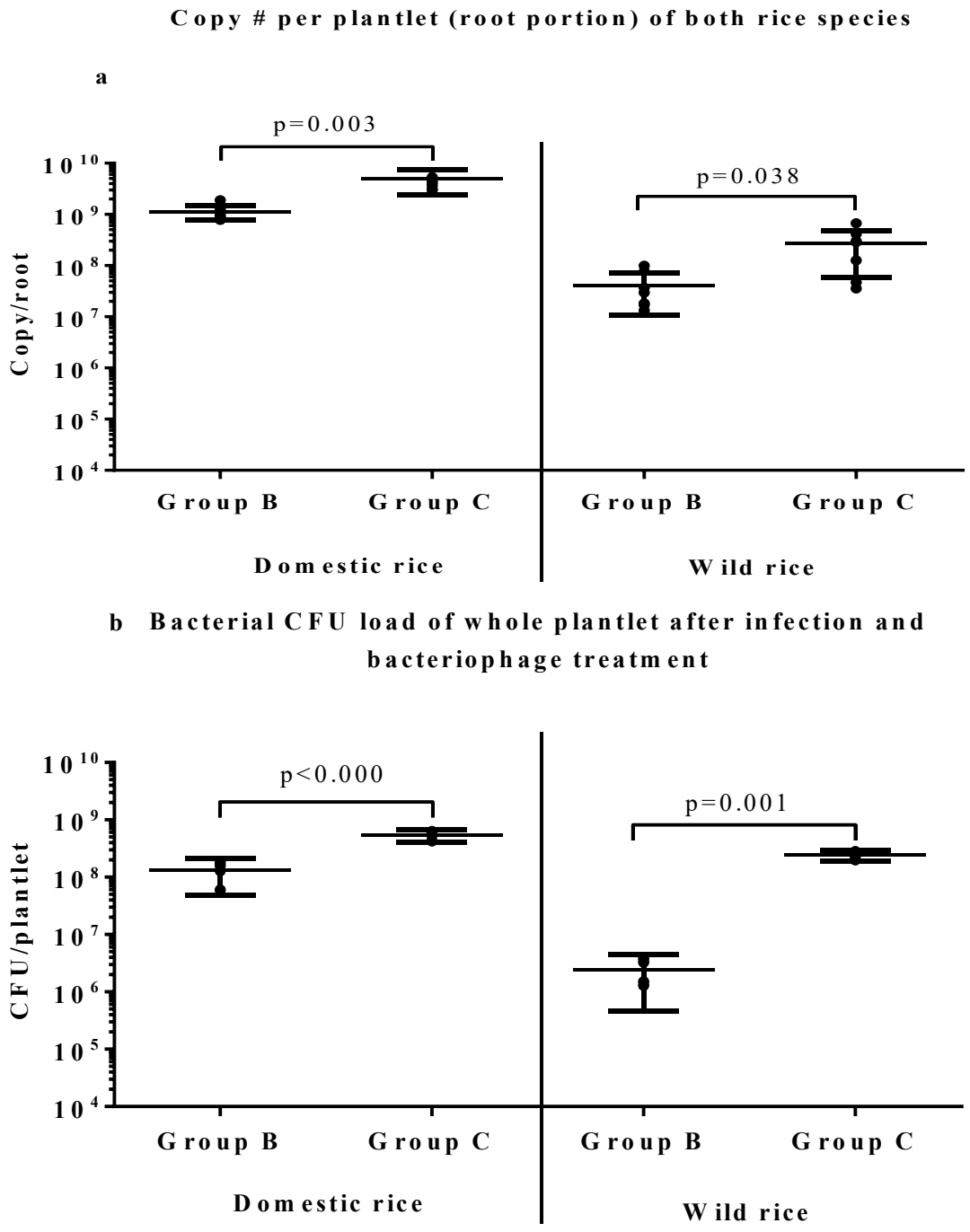


Figure 7.10: Mean copy number of *B. pseudomallei* ± 95 % CI of root portion (a) and CFU/plantlet (b) in treatment Group B (rice soaked with phage cocktail and *B. pseudomallei*) and infection Group C (rice soaked with *B. pseudomallei*) for the two rice species. Both species have a significant difference between Group B and Group C. Data within each treatment were compared by independent t test at p=0.05.

7.4 DISCUSSION

There are two common experimental designs for biocontrol in a 96 well plate, inhibition of development of a biofilm and destruction of a biofilm (Knezevic and Petrovic, 2008). In the first case, as was done in this study, both the bacterial agent and the biocontrol agent are added together, in the second case the bacterial agent is typically added and allowed to form a biofilm prior to addition and incubation with the biocontrol agent. Several articles in literature (Stepanovic *et al.*, 2000; Djordjevic *et al.*, 2002; Fridholm and Everitt, 2005; Knezevic and Petrovic, 2008) that have used both methodologies have identified inhibition of development of a biofilm to produce a greater statistical difference between treatment and control groups. This is reasonable given the increased resistance of biofilms to control agents (Mah and O'Toole, 2001; Sawasdidoln *et al.*, 2010) as well as the increased numbers of bacteria that may be present in a developed biofilm (Donlan and Costerton, 2002) This study used the first model of inhibition of development of a biofilm model only, as it was used to test agents prior to transfer to the rice model. In the plant model, addition of agents after development of the biofilm was not practical due to access to the plantlets in the incubation jars and limited incubation times due to the size of the jars.

Both biocontrol agents were able to equally inhibit production of a biofilm in the 96 well plate, although not completely, at 24 hr. However, by 48 hr it was clear that the phage cocktail was continuing to be effective with the OD being significantly lower than for 24 hr, while the bacteriocin was no longer as effective and the OD was trending higher than for 24 hr (Figure 7.1). These results are reasonable given the agents did not completely kill all the bacteria within 24 hr and surviving bacteria could continue to replicate. The bacteriocin-like compound is a non-reproducing agent while bacteriophage are able to reproduce and continue to infect and kill their host for so long as the host is present (Tait *et al.*, 2002; Sutherland *et al.*, 2004). As the rice model experiment has a seven day incubation period, it was considered unlikely that the bacteriocin would have any effect over this time. Instead, *B. ubonensis*, the bacteria that produces this bacteriocin, was utilised, on the basis it can reproduce and continually produce its bacteriocin over the course of the experiment.

Our qPCR was originally modified from a presence absence PCR from Novak *et al.* (2006). It had also been used quantitatively by Kaestli *et al.* (2007), who identified the linear dynamic range to be 10 copies/µl to 2.5 x 10^5 copies/µl while our linearity result was 2.52 x 10^2 to 2.52 x 10^{10} copy/µl (Figure7.7). The coefficient of determination (r^2) of 0.997 and an amplification efficiency of 92.6% are similar to the levels reported by Kaestli *et al.* (2007). This assay did not pick up near-neighbour and environmental species (Figure 7.8) which matches with the results of Kaestli *et al.* (2007). However, our detection limit was higher than that of Kaestli *et al.* (2007) by a factor of 10. This is probably due to the use of herring sperm DNA in the standards in our work. A log difference in sensitivity in PCRs when comparing standards diluted in a background DNA matrix, relative to no DNA in the matrix has previously been reported along with larger Ct values for standards when herring sperm DNA was added (Siregar *et al.*, 2012). DNA samples from plants contain both target DNA (*B. pseudomallei*) and non target DNA (plant genome). The standards were produced from plasmids containing only the *B. pseudomallei* target sequence and minimal plasmid DNA. This results in matrix differences and may affect the efficiency of the PCR differently for samples and standards, resulting in over or underestimation of the true concentration of target DNA in the samples (Ward and Bej, 2006). Herring sperm DNA was added to the standards to provide non-template DNA to make the matrix in which the plasmid standards were suspended more similar to samples, which would always contain a significant proportion of background, non-template DNA from the plant. While the consequences were a loss of sensitivity, the absolute quantitation of samples was considered more reliable and *B. pseudomallei* was easily detectable in plant samples experimentally infected with bacteria.

In this study, plant models, specifically two rice species examined previously (Chapter 4 and 5), were used. A domestic rice model was selected due to reports of high frequency of isolation of *B. pseudomallei* from rice paddy fields (Wuthiekanun *et al.*, 1995; Rattanavong *et al.*, 2011). The wild rice model was used as this species is found in *B. pseudomallei* endemic areas in Australia (Darwin and Townsville) and Papua New

Guinea (Vaughan and Morishima, 2003; Sotowa *et al.*, 2013) and allows for comparison between a domestic and a wild species of rice.

Burkholderia ubonensis was not found to act as a biocontrol agent (Figure 7.2). Instead, as noted in chapter 5, it also inhibited growth of the rice, although not as much as *B. pseudomallei*. The interaction between the two organisms did not improve growth. It is possible that a lower dose of *B. ubonensis* would permit more subtle interactions to be observed, however, given it can inhibit growth, it is unlikely that this would be considered a good biocontrol agent for use in the environment. Until the bacteriocin-like compound is fully identified and made stable over time, further biocontrol analyses using this agent is probably premature. As the stocks of the wild rice were limited, no tests on the wild rice were undertaken with this organism.

The bacteriophage cocktail treatment which showed promise in the 96 well plate model of biofilm inhibition was also successfully used in both rice models, however it was more effective in the wild rice model. In the domestic *Oryza sativa* L. cv Amaroo model , while the bacteriophage treatment group (Group B) statistically increased growth over that of the infected group (Group C) for root growth, it did not improve growth to the extent of the uninfected control, with the treatment group still being significantly less able to grow than the control groups (Figure7.4a). Leaf growth was not significantly improved (Figure 7.4b) with treatment.

Using the wild *O. meridionalis* model, we found that in the growth experiments, treatment of *B. pseudomallei* infected plants with bacteriophage (Group B), resulted in growth statistically indistinguishable from uninfected plants for both root and leaf (Figure7.6). Similar improvements in other bacteria : plant models have previously been reported, such as control of *Ralstonia solacacearum* infections in tomato plants (Jones *et al.*, 2012).

Overall, the bacterial load analysis by plating (Figure 7.10b) related well to the growth experiments, with fewer bacteria present when bacteriophage treatment was carried out than in infection only groups. Bacterial load after bacteriophage treatment was reduced by about two logs for the *O. meridionalis* model while the reduction was only about one log for the *Oryza sativa* L. cv Amaroo. It is likely that if an alternate bacteriophage cocktail was able to reduce *B. pseudomallei* by two logs on *Oryza sativa* L. cv Amaroo it might also reduce the signs of infection below measureable differences as was the case with *Oryza meridionalis.*

Separation of leaves and roots for PCR analysis identified that *B. pseudomallei* has also travelled to the leaves and treatment of the roots with phage has no significant effect on bacterial loads in the leaves. The plantlets had been stored in acetone, prior to separation of roots and leaves and the possibility that the leaf bacterial loads were transferred from roots to acetone to leaves during storage, rather than transfer during experimentation required consideration. The acetone used in storage was examined and the bacterial loads in the acetone were at least two logs lower/ml than the total load in the leaves (Table 7.1). Given the small surface area of the leaves, any transfer during storage was insignificant compared to levels in the plant matter and this route of transfer can be disregarded.

Removal of *B. pseudomallei* from the leaves of plants may require an alternate application method, such as spraying cocktail onto leaves as was done by Balogh *et al.* (2003), however phage have been reported as having a very short life on plant leaf surfaces, limiting their effectiveness (Jones *et al.*, 2012). Iriarte *et al.* (2012) examined bacteriophage movement and persistence in tomato plants and found that while bacteriophage were sustained on the roots, movement into the leaves was transient and levels declined rapidly. This may mean that bacteriophage have little opportunity to affect bacterial loads in the leaves of the plants on which they are applied. A preinoculation with an avirulent *Burkholderia* species sensitive to the phage may assist with maintaining the phage in the leaves and thus controlling the *B. pseudomallei*

levels. The bacteriophage cocktail used in this experiment also consisted mainly of myoviridae with large heads, on average 100 nm diameter capsid, that produced only pin prick size plaques (Section 6.3.7). This may compromise its ability to diffuse efficiently through the plant tissues and contact the bacteria (Elford and Andrewes, 1932; Abedon and Yin, 2009).

The presence of *B. pseudomallei* on both roots and in leaves of domestic rice is of concern regarding the farming of rice using manual handling in *B. pseudomallei* endemic areas, as this may pose another route of exposure as well as that of exposure to soil and water (Suputtamongkol *et al.*, 1994). However, Thomas and Forbesfaulkner (1981) reported number organism per gram (MPN) of soil was up to 10^5 organisms and Smith *et al.* (1995) demonstrated CUF/ml of *B. pseudomallei* had a median of 230 cfu/ml (range 1-17,000) in an endemic area, both of which are lower by orders of magnitude than the experimental exposure in this study. Low bacterial dose experiments may result in no transmission to the leaves or in better clearance from the leaves with bacteriophage treatment.

Comparison of root load by PCR and plantlet load by colony counts shows a greater discrepancy for the *O. meridionalis* model which can be explained by the need to double extract the DNA for this species. This species had a dark brown seed, containing PCR inhibitors which were not removed by the standard extraction method. If normal extraction resulted in a coloured extract, the PCR was negative. This problem has been reported before with multiple plant species (Schrader *et al.*, 2012). A second extraction using PVPP cleared the colour, resulting in a positive PCR, however, this second extraction also resulted in the loss of some DNA, as measured by spectroscopy, so the yield is expected to be lower. To standardise this result, all *O. meridionalis* root samples were double extracted and, while individual results had higher variability, the load differences between infection and treatment groups were significant and greater than the differences in the *Oryza sativa* L. cv *Amaroo* model (Figure 10a).

One possible explanation for this is that the wild rice may be adapted to live with *B. pseudomallei* in the environment and have developed defences against the organism. As noted previously, this species is distributed in the Northern Territory and North Queensland in Australia and in Papua New Guinea (Office of the gene technology regulator, 2005; Sotowa *et al.*, 2013) and these are endemic areas for *B. pseudomallei* (Wiersinga *et al.*, 2012). Wild plants often have greater disease resistance (R) protein diversity than domestic crop plants (Jones and Dangl, 2006) and combined with the effect of phage cocktail, this could be sufficient to reduce signs of disease with a two log drop in bacterial load. However, there was less than one log difference between the number of *B. pseudomallei* on wild and domestic rice in the *B. pseudomallei* only groups, indicating that any disease resistance effect was either not acting or was insufficient on its own to have an effect on bacterial numbers. An alternate possibility for these bacterial loads is the variation in growth rates of the two plants (Figure 10b). The domestic rice grew more rapidly (data not shown), potentially producing more nutrients for the *B. pseudomallei* via root hair development (Bell-Perkins and Lynch, 2002) and allowing for more rapid bacterial growth able to overcome the effects of the phage cocktail. This argument is also limited by the lack of significant difference between the two plant species *B. pseudomallei* numbers in Group C.

Our finding indicate that use of *B. ubonensis* as a biocontrol agent is not viable and any use of the bacteriocin-like compound would first have to address stability issues. However, phage cocktails can be used to lower bacterial loads of *B. pseudomallei* on plants, and this was particularly effective using the wild rice (*Oryza meridionalis*) model. While bacteria were not completely cleared, all signs of growth inhibition were eliminated, which fits with the principle of successful biocontrol. While any field use of this method of biocontrol would require extensive experimentation relating to dose variation, optimisation of bacteriophage cocktails and field tests, this study provides evidence that such an approach may be viable. Lastly, the evidence of transmission to leaves proposes new questions that are beyond the scope of this study; Can *B. pseudomallei* be transmitted to seeds and hence transmit itself from one field to another

and can consumption of infected leaves result in melioidosis in the animal that consumes it?

CHAPTER 8: THE PREVALENCE OF *B. PSEUDOMALLEI* IN SOIL AND PLANTS AT AN ENDEMIC SITE IN QUEENSLAND, AUSTRALIA

8.1 Introduction

As previously discussed, *B. pseudomallei* is the causative agent of melioidosis, a severe tropical infection which is endemic in Southeast Asia and Northern Australia (Currie *et al.*, 2008; Limmathurotsakul *et al.*, 2010b). The most important reservoir of *B. pseudomallei* is widely considered to be in soil and water rising up from the soil (Baker *et al.*, 2011b; Larsen *et al.*, 2013). The rainy season significantly increases the incidence of melioidosis cases (Currie and Jacups, 2003) About 80% of all incidences of melioidosis in north Queensland occur in the first four months of the year (Hanna *et al.*, 2010). More than 66% of melioidosis cases at Townsville have been found to have an association with wet topsoil (waterlogged on Pleistocene alluvial terraces) and 27.7% of cases were found around Castle Hill (Corkeron *et al.*, 2010).

Previous studies in Townsville have identified that Castle Hill is a location of interest due the presence of *B. pseudomallei* in the soil (Baker *et al.*, 2011b). Early unpublished work by Tahani (2009) found *B. pseudomallei* in one sample at Castle Hill (bulk soil) and one sample (rhizosphere soil) near a drain site at the base of Castle Hill. Later work by Baker *et al.* (2011b) found *B. pseudomallei* in bulk soil and water on the lower ridges of Castle Hill and Larsen *et al.* (2013) also found the organism in soil near an ephemeral creek previously identified by Baker *et al.* (2011b). Unpublished work by Ezzahir (2012), which was carried out at the same time as the preliminary work inthis study, also found *B. pseudomallei* at a depth of 2 m in saturated soil close to this site at the base of a Castle Hill (wet season) and failed to find any *B. pseudomallei* in soil at a drainage site at the Police-Citizens Youth Club (PCYC1) (wet season). See section 8.2.2 for map of site. The focus of most of these studies was soil 30 cm below ground level, not close to plant roots and seeps water, with limited assessment of the relationship of *B. pseudomallei* with plants or comparison of sites on Castle Hill relative to drainage sites. This chapter examines two sites, one considered *B. pseudomallei* prevalent, the other not-prevalent, in terms of a recovery of *B. pseudomallei* from soil

and plants on a site previously examined on Castle Hill, Townsville Queensland to understand the ecology of *B. pseudomallei* in this endemic region.

8.2 Materials and Methods

8.2.1 Preliminary plant survey (July 2012).

During the soil sampling survey of Ezzahir (2012), unpublished data, a visual record of plant species commonly found around soil sampling sites was at taken at Castle Hill (GPS: S19.15259/E146.47339) behind the State Emergency Services base (SES) (Figure 8.1) and at a drain site located next to the Police-Citizens Youth Club (PCYC1) Hugh Street West End, Townsville at (GPS: S19.15242/E146.4768), (Figure 8.1). As analysis of this soil identified the drain site as negative for *B. pseudomallei* and the SES site as positive for *B. pseudomallei*, these sites were allocated as negative and positive sites respectively. Plants found close to sampling holes identified as *B. pseudomallei* soil positive by Ezzahir (2012) were identified as being of further interest. Plant species were then identified with the assistance of Christopher Gardiner from the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia and in some cases by Nanette Hooker, Curator of the JCU herbarium.

Figure 8.1: Contour line map of area of Castle Hill, Townsville and adjacent areas (https://data.qld.gov.au/dataset/queensland-globe/resource/b16dbabe-7173-45e0-94c6-fcb97998e633) identifying the State Emergency Services base (SES) as positive site and the Police-Citizens Youth Club (PCYC1, 2) as drain site (negative site) using yellow pin symbols.

8.2.2 Plant and rhizosphere soil collection in wet season (February 2014).

Each species encountered was identified along the transect lines at each site (SES, PYPC1, PYPC2). Four species including *H. contortus* (black spear grass), *Melinis repens* (red natal grass), *Aristida spp.* (wire grass) and *Scleria rugosa* (sedge) were collected at the Castle Hill site behind the State Emergency Services base (SES) as you can see in Figure 8.2a while at the PCYC1 *Cyperus polystachyos* (sedge) were collected at the drain site in Figure 8.2b. At the PCYC2 drain site (GPS: S19.15244/E146.4722) only *H. contortus* was found and collected. Plants and top soil to a depth of 10 cm surrounding the root of each plant was collected with a sterile shovel, which was wiped with 70% ethanol between each sample. Sample size of plants and rhizosphere soil for detecting *B. pseudomallei* was determined based on percentage of *B. pseudomallei* positive samples in soil at 30 cm depth as per Ezzahir (2012) and based on a 95% confident interval (Cannon and Roe, 1982). Top soil to a depth of 10

cm was defined as rhizosphere soil for the purpose of this study. Samples were collected on dates as stated in Figure 8.4. Roots were washed with running tab water, split into two samples and stored. One sample was kept at room temperature for microbiological analysis and the other kept in acetone at -20^{o}C for qPCR and IFA determination.

Figure 8.2: a) Aerial view of positive site of *B. pseudomallei* at Castle Hill, transect lines in red. b) Aerial view of (negative) drain site at PCYC1 and PCYC2 , transects in red.

8.2.3 Bulk soil collection at 10 and 30 cm depth (March 2014).

Based on previous sampling in the dry season by Ezzahri (2012), Castle Hill behind the State Emergency Services base (SES) (Figure 8.2a) was chosen as a known positive site for *B. pseudomallei* presence in soil and the drain site at the Police-Citizens Youth Club (PCYC1) (Figure 8.2b) was selected as a negative site. Two transect lines (14 meters each) were created at each site, running parallel to one another 5 meters apart. An extra one transect line PCYC2 (7 meters) was also created at the drain site close to an area to be sampled for plants. Sampling took place every meter along the transect lines using a tape measure. Soil was sampled at ten and thirty cm depths using a sterile soil auger (Figure 8.3), wiped with 70% ethanol between each sample. Zip lock bags were used to collect each soil sample and labelled. Where the ground was hard to penetrate with the auger, a shovel was used. A time line (Figure 8.4) comparing collection dates of samples with basic weather data was developed using data gathered from the Bureau of Meteorology (BOM).

Figure 8.3: Collection of soil with an auger.

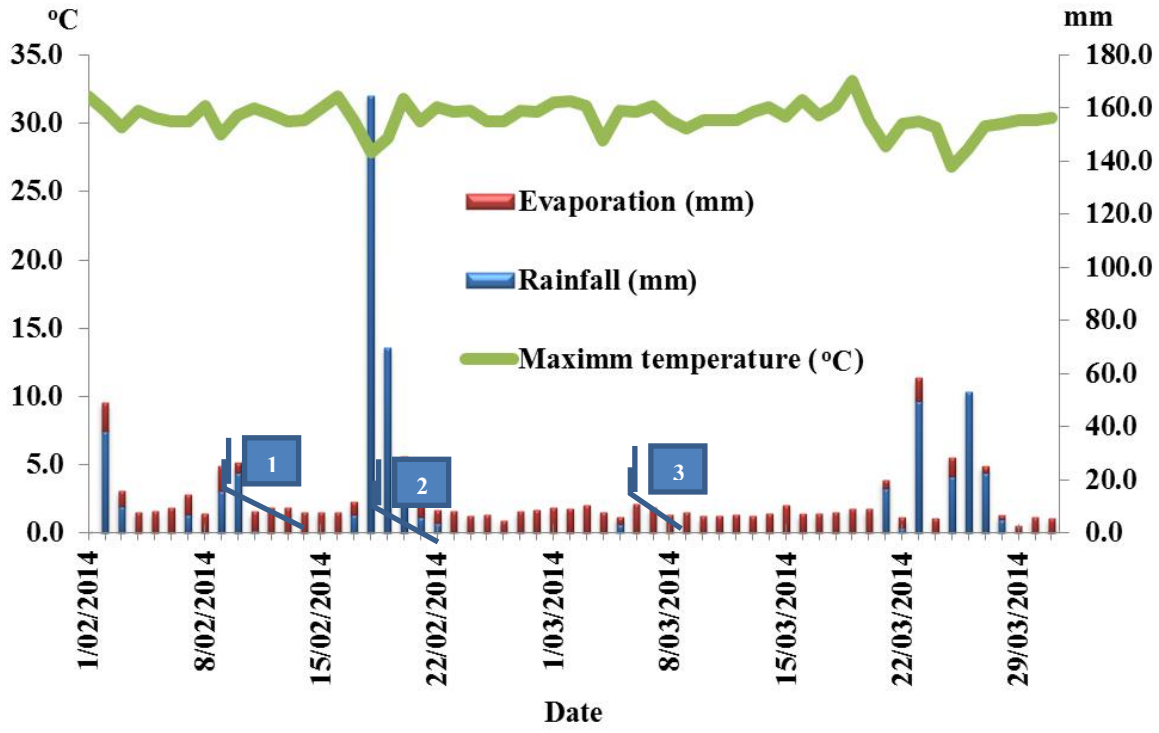


Figure 8.4: Sampling timeline. Representation of rainfall (mm right axis/blue peaks), maximum temperature (°C left axis/green line) and evaporation (mm left axis/red line), data gathered from the Bureau of Meteorology (http://www.bom.gov.au/climate/dwo/201402/html/IDCJDW4128.201402.shtml). Sampling dates are identified with a purple line and numbered based on the sample collected; rhizosphere soil and plant roots SES site (1), rhizosphere soil and plant roots drain site (2) and all free soil at 10 and 30 cm both sites (3).

8.2.4 Enrichment of environmental soil samples.

8.2.4.1 Ashdown protocol

The Ashdown enrichment method was modified from unpublished work by Ezzahir (2012). A mass of 10 g of rhizosphere soil was added to 10 ml double strength Ashdown broth in a sterile container (50 ml sample bottle, Sarstedt) and incubated at 100 rpm, 37 ^{o}C for 24 hr. After incubation, soil was allowed to settle for 20 min. Three ml of supernatant was centrifuged for 5 min at 10 000 × *g* at room temperature. After centrifugation, the supernatant was discarded and the pellet was processed for DNA extraction as per section 3.2.1.

8.2.4.2 Consensus protocol

The consensus guidelines method (Limmathurotsakul *et al.*, 2013) was used as follows; ten ml of Threonine-basal salt plus colistin 50 mg/l (TBSS-C50 broth) (Appendix A1.26) was added to ten grams of environmental soil in McCartney bottles and vortexed for 30 seconds. McCartney bottles were incubated for 48 hr at 40 ^{o}C. Three ml of supernatant was removed and centrifuged for 5 min at 10 000 × *g*. After centrifugation, the supernatant was discarded and the pellet was processed for DNA extraction extraction as per section 3.2.1.

8.2.5 Identification of inhibition of amplification or PCR.

To eliminate the possibility that soils may either inhibit amplification of *B. pseudomallei* or carry over inhibitors of real time PCR, an inhibition experiments was carried out. Inhibition of amplification was tested as follows; a drain soil sample tested negative for *B. pseudomallei* by PCR was weighed into two 10 g samples. One sample was processed directly and the other spiked with 100 µL (10^8 CFU/ml) *B. pseudomallei* (TSV 189). A positive control consisting of 10 ml PBS plus 100 µL (10^8 CFU/ml) was also processed. Samples were processed as per 8.2.4.2 and DNA were extraction as per section 3.2.1.

8.2.6 DNA extraction from root samples.

The root of each plant sample was cut about 1.5 cm from the tip and weight determined to be approximately 130 ± 20 mg. Each plant sample was cleaned as per section 3.3.1 and processed as per section 8.2.4.2, with the exception that this was done with 1 ml media in a microfuge tube. Samples were centrifuged at 3 000 × *g* for 15 min and the supernatant removed. The root and sediment were processed via a plant DNA extraction kit (ZR-96 Plant/Seed DNA kit, ZYMO) for DNA extraction.

8.2.7 Real time polymerase chain reaction.

DNA samples were analysed using the TTS1 realtime PCR (Section 3.2.2). 2 µl of template was used in a total of 20 µl. All samples that crossed the threshold by 40 cycles were considered positive. A standard curve was imported and quantitation was determined (Section 7.3.5). Results of sample types within each location were compared by ANOVA (post hoc test) at p=0.05. Statistical analysis of each sample type between locations was done via independent T tests p=0.05.

8.3 Results

8.3.1 Plant prevalence and relationship with previous soil samples found to be positive for *B. pseudomallei.*

Heteropogon contortus (black spear grass) were the most commonly distributed plants at the Castle Hill site (SES) and *Cyperus polystachyos* (Sedge) was the most frequent plant at the drain site (PCYC1). *Burkholderia pseudomallei* positive soil samples from Ezzahir (2012) were only found at the Castle Hill site, and there were differences in the percent of positive samples found near different plant species on Castle Hill (Table 8.1).

Table 8.1: Plants at the Castle Hill site are different from plants at the drain site, with the most common Castle Hill plants related to *B. pseudomallei* positive soil being black spear grass, red natal grass, wire grass and sedge (samples collected and identified in 2012 dry season, soil samples positive data collected from Ezzahir (2012) unpublished data*).

Castle Hill site (SES)	**Prevalence of plant**	***% Soil sample positive @ 30 cm**	**Drain site (PCYC1)**	**Prevalence of plant**	***% Soil sample positive @ 30 cm**
Heteropogon contortus (Black spear grass)	49%	17%	*Cyperus polystachyos* (Sedge)	29%	0%
Melinis repens (Red Natal grass)	32%	10%	*Urochloa mutica* (Para grass)	26%	0%
Aristida spp. (Wire grass)	9%	22%	*Desmodium* spp. (tick-trefoil)	6%	0%
Scleria rugosa (Sedge)	5%	20%	*Digitaria ciliaris* (Summer grass)	6%	0%
Other species: *Cymbopogon* spp. *Hyptis* spp. *Stylosanthes* spp.	5%	0%	Other species: *Eleusine* spp. *Alysicarpus* spp. *Cynodon* spp. *Axonopus* spp. *Panicum* spp. *Sida* spp. *Sphagneticola* spp. *Ageratum* spp. *Amaranthus* spp. *Hyptis* spp. *Melinis* repens	33%	0%

8.3.2. Examination of soil samples for inhibitory factors.

While the negative soil sample remained negative, both soil and PBS spiked with *B. pseudomallei* resulted in similar curves in the qPCR with minimal differences in where these curves crossed the threshold (Table 8.2). As such, it can be assumed that the soil samples were not causing inhibition in either amplification of *B. pseudomallei* during the enrichment step or in the PCR.

Table 8.2: Examination of soil for inhibitory effects. Group A is a negative control soil sample. Group C is a positive control PBS sample spiked with *B. pseudomallei* and Group B is an experimental soil sample spiked with the same amount of *B. pseudomallei* as the positive control. All samples were processed similarly after spiking.

Experiment	**Group A**	**Group B**	**Group C**
Sample + TBSS-C50 broth	10 g soil	10 g soil + 100µl (10^8 CFU/ml) *B. pseudomallei* (TSV189)	10 ml PBS + 100µl (10^8 CFU/ml) *B. pseudomallei* (TSV189)
Ct value	0	18.66 ± 0.42	19.74 ± 0.06

8.3.3 Detection of *B. pseudomallei* in soil and root samples.

The Ashdown enrichment protocol was used initially to enrich rhizosphere soil samples from *H. contortus* (black spear grass), however, all samples were negative by PCR (Figure 8.4). All further analysis used the consensus guidelines to enrich *B. pseudomallei* in soil and plant samples, including duplicate *H. contortus* rhizosphere soil samples. Enriched counts via qPCR were collated irrespective of plant species and sample types (30 cm soil, 10 cm soil, rhizosphere soil, root) on Castle Hill and at the drain site and these were compared (Figure 8.5). There were no bacteria found in the roots at either site. The bacterial loads at 30 and 10 cm on Castle Hill were not significantly different to each other, but were higher than the rhizosphere bacterial loads at Castle Hill. The pattern of significance was similar for the drain site. Interestingly, while the 10 and 30 cm samples at Castle Hill were significantly higher than at the drain site, the rhizosphere soil bacterial loads at both sites were not significantly different go each other.

At both sites, the samples at 30 cm had a trend of higher yield after enrichment, followed by 10 cm samples then significantly lower rhizosphere samples. No root samples reacted in the qPCR. At 30 and 10 cm, samples from the Castle Hill site yielded higher numbers of *B. pseudomallei* than those at the drain site, (Appendix A3.13). The correlation coefficient (effect size) between Castle Hill and the drain site are represented in Table 8.3 an indicate that there is a strong correlation between finding *B. pseudomallei* at 30 cm at both sites, a medium correlation at 10 cm and a low correlation in the rhizosphere soil.

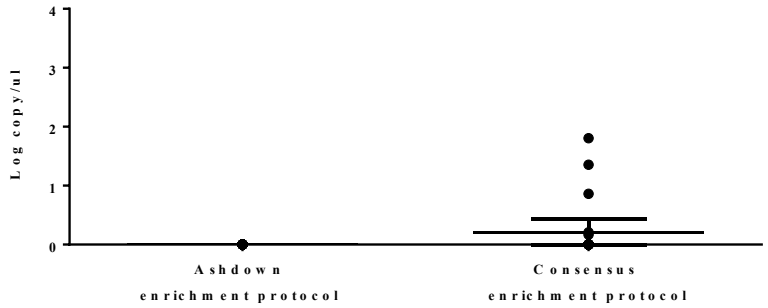
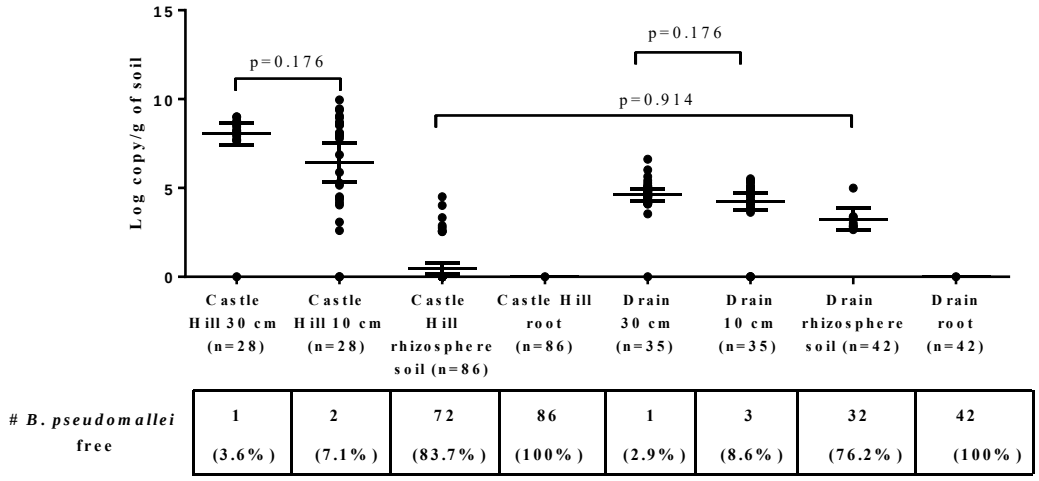


Figure 8.4: Comparison of enrichment techniques using rhizosphere soil from *H. contortus* samples. Mean yield ±95 % CI of *B. pseudomallei* after enrichment of samples in Ashdown and TBSS-C50 broth (Consensus enrichment). Although the consensus protocol can detect *B. pseudomallei*, all rhizosphere soil positives are below the reliable positive detection limit (2.52×10^2 copies/µl) of this assay.



# *B. pseudomallei* free	1 (3.6%)	2 (7.1%)	72 (83.7%)	86 (100%)	1 (2.9%)	3 (8.6%)	32 (76.2%)	42 (100%)

Figure 8.5: Mean yield ±95 % CI of *B. pseudomallei* after enrichment of samples in TBSS-C50 from both sites at two sampling depths as well as from soil around roots and inside roots of plants. Number and percentage of samples which were negative for *B. pseudomallei* in each sample type is displayed under the figure. Sample types within each location were compared by ANOVA (Games-Howell post hoc test) at p=0.05. All not significantly different relationships are identified on the figure.

Table 8.3: Comparison of enriched *B. pseudomallei* counts at each soil depth across the two locations (independent T test, p=0.05).

Experiment	**t**	**df**	**r**
30 cm depth SES to PCYC	10.364	61	0.799
10 cm depth SES to PCYC	3.757	37.635	0.522
rhizosphere soil SES to PCYC	-1.247	64.771	0.153

8.4 Discussion

The selection of sites and plants to examine in this study was based on preliminary soil results produced by Ezzahir (2012), (unpublished data). At the time of the initial Ezzahir (2012) survey, plant species close to soil samples were noted so that target plants could be identified from areas where the soil was positive for *B. pseudomallei*. Since no *B. pseudomallei* had been found at the drain site, two transects were selected, based on the presence of either *Cyperus polystachyos* which is in the same family as *Scleria rugose*, found in positive areas on Castle Hill (Table 8.1) and *H. contortus*, which was also found on Castle Hill and was found near the drain site during the wet season. Soil was also collected at a depth of 30 cm, 10 cm and around the rhizospheres of these plants at the drain site (PCYC). At the Castle Hill site (SES), the roots and rhizosphere soil was collected based on the presence of plant species previously found to be associated with positive soil samples (*H. contortus*, *Melinis repens*, *Aristida* spp., *Scleria rugosa*). The soil at 10 and 30 cm depths were also collected from every meter along the transection.

Baker *et al.* (2011b) used modified Ashdown enrichment broth, plating on modified Ashdown agar, followed by detection of typical colonies and confirmation with a realtime PCR assay to identify *B. pseudomallei* in soil near the end of the wet season, Castle Hill had presented the persistence of *B. pseudomallei*t. In the dry season survey by Ezzahir (2012), the drain site (PCYC1) was found to be negative. In this study a similar enrichment (without colistin), followed by DNA extraction and qPCR was used initially to test the rhizosphere soil around *H. contortus* from the Castle Hill site during the wet season and all results were unexpectedly negative. This was considered to be unlikely given previous positive samples from the area during the dry season (Ezzahir, 2012), and an alternative consensus method for enrichment was tested on these same soil samples and found to identify *B. pseudomallei* positive soil samples (Figure 8.4). All of these soil sample positives were below the reliable detection limit of the qPCR assay (control DNA was not always detected below 10^2 copies/µl extract, Section 7.3.5), so there may also be some non-reacting samples which have very low levels of *B. pseudomallei* present. Further use of this consensus method also identified soil

samples as being positive at the drain site (Figure 8.5). As such it would be worth looking at the soil in the dry season again, using the consensus enrichment protocol.

The lack of identification of *B. pseudomallei* in the dry season as reported by Ezzahir (2012) could be due to insensitivity of the culture method to detect bacteria from the environment when compared with real time PCR. Kaestli *et al.* (2007) illustrated that the rate of detection *B. pseudomallei* using culture on sandy loam to loam soil type at Darwin during the dry season was lower than realtime PCR. In Kaestli's work, at a sampling depth of 30 cm; culture identified 17.6% of samples as positive, while real time PCR identified 23.5%. At 10 cm, none of the samples were positive by culture, while 11.1% were positive by real time assay. Reasons for the lower identification could be that *B. pseudomallei* could have converted to a nonculturable (VBNC: viable but non-culturable) state. *Burkholderia pseudomallei* could have converted to ultramicrobacteria, a survival mechanism where after long starvation periods bacteria shrink and recovery to normal size and replication requires enrichment media (Novitsky and Morita, 1976). Unfortunately, the Ezzahir (2012) experiment used only selective media for culture, which may be insufficient for conversion. Therefore, in the dry season the combined culture method of Ezzahir (2012) might provide a low opportunity to find bacteria in the environment.

Baker *et al.* (2011b) and Ezzahir (2012) and did successfully detect *B. pseudomallei* on Castle Hill. Matching area of Ezzahir (2012) results will be compared to our wet season results, even though the enrichment protocol was not identical. In dry season sampling at 30 cm depth at Castle Hill, a site 33 m above sea level, (google earth programme analysis: https://www.google.com/earth/), 42 % (12 out of 28 holes) of samples were positive with *B. pseudomallei*, but at the same location in the wet season, our data identified more than 96% positive (21 in 28 holes). This matches other studies indicating a higher recovery of *B. pseudomallei* from wetter locations in Thailand (Wuthiekanun *et al.*, 1995), Darwin (Currie and Jacups, 2003b) and Townsville (Thomas *et al.*, 1979; Baker *et al.*,2011b). The percentage of *B. pseudomallei* in native

rhizosphere soil at both sites (16.3-23.8 %) was also similar to previous results of Kaestli *et al.* (2012) in the Northern Territory (9-59% of native rhizosphere soil positive for *B. pseudomallei* during the wet season). Thomas *et al.* (1979) suggested that *B. pseudomallei* is brought up to the surface from lower strata by water during wet seasons and Baker *et al.* (2011b) proved this previous suggestion. The geology surface and soil type properties of Castle Hill are biotile leucogranite, microgranite, minor granophyre and granodiorite (Queensland Goverment, 2014). It is also described as loamy sand soil that consists 81% sand, silt 15.5% and clay 3.5% (Ezzahir, 2012). Sandy soil has good moisture infiltration in wet season but dries out quickly in the dry season. Tong *et al.* (1996) demonstrated that low water content reversed the correlation of survival of *B. pseudomallei* and Kaestli *et al.* (2009), identified that the load of *B. pseudomallei* was dramatically reduced during the dry season in undisturbed soil. It is likely that high numbers of *B. pseudomallei* in the wet season drop off as the environment becomes less conducive to growth.

As noted above, Ezzahri (2012) did not successfully isolate *B. pseudomallei* from the drain site during the dry season with culture methods, while in this study 34 out of 35 holes were positive for *B. pseudomallei* in the wet season. The drain site consists of coastal tidal flats, mangrove flats, supratidal flats, saltspans grass lands – silt, mud and sand, minor salt (Queensland Goverment, 2014) and sandy loam soil type soils (Clark, 2004; Ezzahri, 2012). Given the lack of positive samples in the dry season, and assuming the protocol used would find *B. pseudomallei* that was present, it is likely that *B. pseudomallei* found there in the wet season has transited from the positive Castle Hill site.

Generally, *B. pseudomallei* could move with ground water seeps (Baker *et al.*, 2011b) to ephemeral creek sites (Larsen *et al.*, 2013) in the wet season at Castle Hill therefore, it should be found at the drain site which is lower in the landscape than the Castle Hill site. Recently, Larsen *et al.* (2013) demonstrated that *B. pseudomallei* can be recovered from soil which has desiccated to <10% moisture after three months and as our result

from both sites supported that *B. pseudomallei* was present in undisturbed soil at the waterlogged (drain) site, arrival during the wet season and subsequent desiccation during the dry season could result in *B. pseudomallei* being present and recoverable during the dry season, although Ezzahri (2012) did not find it.

Although, the percentage of soil moisture content at Castle Hill in the dry season (M = 7.4 , SD = 1.95) was lower than at the drain site (M = 15.7 , SD = 11.5) (Ezzahir, 2012), in the wet season, *B. pseudomallei* yield (after enrichment) was higher at Castle Hill than at the drain site, with the exception of rhizosphere soil. Possible reasons include inhibitors of some type in soils at the drain site or lower *B. pseudomallei* numbers reaching the drain site from the Castle Hill site. Possible inhibitors could include bacteriophage, other bacteria producing substances such as bacteriocins and chemical factors such as salt concentration (Kaestli *et al.*, 2015). Both biological agents have a co evolution with bacteria therefore that agent, or possibly both, may have reduced the number of *B. pseudomallei* in the soil, resulting in lower starting numbers to produce lower enrichment numbers, as well as inhibiting reproduction during enrichment. Inhibitors may also kill bacteria over time at the drain site, providing another reason why Ezzahir (2012) could not find *B. pseudomallei* there during the dry season. However, a simple inhibition experiment did not identify any inhibitory effect on the replication of *B. pseudomallei* during enrichment (or inhibition of the PCR), so *B. pseudomallei* numbers reaching the drain site should be considered as an alternative theory to the lower numbers found at the drain site. This study identified that during the wet season at both sites, *B. pseudomallei* is found as frequently at a depth of 10 cm as it is at a depth of 30 cm (Figure 8.5), the consensus depth to find *B. pseudomallei* in soil (Limmathurotsakul *et al.*, 2013). It is reasonable to assume this is due to groundwater seeps bringing *B. pseudomallei* to the top soil surface during the wet season. The enriched yield in the 10 cm depth, while it is not statiscally significant, does have a trend of being less than at the 30 cm depth at each site which may be due to increased exposure to UV radiation or heat at locations closer to the surface. Much lower yields are found around rhizospheres (less than 10 cm from the surface), and this alongside with the similar numbers at both sites supports this concept as any effect

would be stonger closere to surface level. Water moving to the drain site would also likely be exposed to UV radiation, killing some of the bacteria, which may explain the generally lower yields from the drain site samples if there is some death during transit.

Generally, rhizospheres are supportive locations for bacterial growth as many root exudates are released to the soil environment and the provide nutrients for bacteria, so the rhizosphere area should have higher bacterial densities than bulk soil. Molina *et al.* (2000) demonstrated that the number of *P. putida* in rhizosphere soil were one to two orders of magnitude above those in the bulk soil and the density of bacteria decreased in non-vegetative bulk soil. Our results are in contradiction with this. This was unexpected, however there may be reasons for these low numbers; first, it is possible that plants may have more bacteria of other species producing bacteriocins or bacteriophage in the rhizosphere than are present in bulk soil, which was what was used for testing inhibition in this study. Second, root exudates may be inhibitory against *B. pseudomallei*. Third, a physical barrier of protection, due to high ultra violet radiation, temperature and low moisture in rhizosphere top soils may destroy bacteria in this zone as hypothesised above.

A seasonal microbiome, or total 16S rRNA quantitative study of topsoil/rhizosphere soil vs deeper/free soil in Townsville would clarify these possibilities but is not within the scope of this study. It very likely our results prove that *B. pseudomallei* prefers to survive in bulk soil instead of rhizosphere soil and prefers the SES site to the low level drain site during the wet season.

The root system of many plants should be exposed to *B. pseudomallei* during the wet season, since *B. pseudomallei* was commonly found around roots albeit at low levels. Other research has identified several native species carrying *B. pseudomallei* such as trees (*Acacia colei*) (Inglis *et al.*, 2000). The grasses in riparian zone and close to native grasses (*Sorghum* spp.) (Kaestli *et al.*, 2009) and exotic species such as grasses

(*Brachiaria humidicola* cv Tully, *Pennisetum pedicellatum* and *polystachion*, *Paspalum plicatulum*) (Kaestli *et al.*, 2012) None of these studies reported the quantity of *B. pseudomallei* present in tissues, so actual numbers may be very low. Local and alien species may also allow *B. pseudomallei* to be sustained during the dry season when soil samples appear to be negative for this species (Kaestli *et al.*, 2012). However in this study, no *B. pseudomallei* was found in any of the roots examined. While the kit used was specific for extraction of DNA from plants and elimination of inhibitors, the PCR protocol may not have been sensitive enough to pick up very low levels of *B. pseudomallei* from inside the roots. Use of a cellulase to break open plant cells prior to an enrichment step might increase the chances of finding *B. pseudomallei* if it is present.

Our results provide compelling evidence for the movement *B. pseudomallei* from the reservoir area on Castle Hill (average 33 m above sea level) to the drain site waterway (6-7 m above sea level) during the wet season. Future work should include a more sensitive examination of plants and soil in the environment at the epidemic area during both the wet and dry season.

CHAPTER 9: GENERAL DISCUSSION

Burkholderia pseudomallei in the environment is attracting widespread interest due to a high incidence of melioidosis in Southeast Asia and Oceania and a mortality rate of up to 40/100000 person/years (Limmathurotsakul and Peacock, 2011). Particularly, rice farmers working in contaminated soil and water have been reported with a high risk of disease (Suputtamongkol *et al.*, 1994). Most research has studied *B. pseudomallei* associations with soil (Thomas and Forbesfaulkner, 1981; Wuthiekanun *et al.*, 1995; Warner *et al.*, 2008; Kaestli *et al.*, 2015), but fewer studies have looked at plants and their role (Lee *et al.*, 2010; Kaestli *et al.*, 2012). No studies have been carried out looking at biocontrol of *B. pseudomallei* in plants. This study focussed on these gaps in the literature.

To examine these gaps we developed a rice model of infection, developed and partially characterised two biocontrol agents, a bacteriocin-like substance (BLIS) and a bacteriophage cocktail and tested these with a biofilm model as well as testing the *B. ubonensis* BLIS procducer and the bacteriophage with a rice model of *B. pseudomallei*. We also further characterised a local *B. pseudomallei* endemic area with a focus on both flora and soil, with the intent of identifying the potential value of a future biocontrol regime in this area.

Domestic rice (*Oryza sativa*) is an often used plant model (Brkljacic *et al.*, 2011) and is relevant in the case of *B. pseudomallei* given its common presence in *B. pseudomallei* endemic areas. As such, this study utilised several rice strains and species to develop a plant model for testing biocontrol agents to limit plant infection. Previous studies in which domestic rice (*Oryza sativa* L. cv. Nipponbare) was exposed to a high dose (10^7 CFU) of *B. pseudomallei* did not produce signs of disease (Lee *et al.*, 2010). However, in this study we were able to produce growth inhibition in *Oryza sativa* cv. Amaroo using *B. pseudomallei* between 10^7-10^8 CFU/ml. Plantlet viability at10^8 CFU/ml of *B. pseudomallei* was not significantly different to uninfected plantlets (Chi-square, p=0.05), but there was a trend of inhibited rice germination observed. It is interestingly

to note that whatever the origin of the *B. pseudomallei* tested (soil, animal and human) inhibition of growth was similar. An in house immunofluorescence assay (IFA) determined that a biofilm formed around the surface and inside the epidermis of the root. This indicated that *B. pseudomallei* can penetrate rice plant cells in the root. While limited examination of leaf tissue by IFA did not identify any *B. pseudomallei*, an examination of infected plantlets using qPCR did prove the presence of *B. pseudomallei* in leaf tissue, although this was at lower numbers than on root tissue. The presence of *B. pseudomallei* inside plant cells has also been reported in a range of wild and domestic plants, including one wild rice species (Lee *et al.*, 2010; Kaestli *et al.*, 2012) where cells found inside plant, all of which were localised in leaf tissue. No mechanism of infection or path of infection was proposed in these cases, however *B. pseudomallei* is a known intracellular pathogen of vertebrates (Galyov *et al.*, 2010) and mechanisms of infection that are similar for both plant and animal cells have been described for other cross-kingdom pathogens (van Baarlen *et al.*, 2007).

In comparison, another domestic strain of rice (*Oryza sativa* L. cv Koshihikari) that we examined did not show inhibition of growth, a result similar to that of Lee *et al.* (2010) which indicates that there are strain variations in *Oryza sativa* that affect its resistance to *B. pseudomallei*. Both Koshihikiri and Nipponbare strains are considered Japanese rice with similar genetics (Yamamoto *et al.*, 2010), while Amaroo is optimised for Australian conditions (Upadhyaya *et al.*, 2000), however there have been too few strains tested to draw any conclusions regarding what other strains of rice are likely to be resistant or not to *B. pseudomallei.*

Another previous study also produced no inhibition of the wild rice species *O. rufipogon* (Kaestli *et al.*, 2012). Once again, our examination of a different wild rice species (*Oryza meridionalis*) identified inhibition of growth on exposure to *B. pseudomallei* (TSV189). This is not surprising given domestic rice strains can vary and these are not just different strains but actually two different species. *Oryza. meridionalis* was not inhibited as much as the domestic Amaroo strain, which may be

related its wild origin. An important part of the plant immune system is disease resistance (R) protein diversity, which is important in recognising and responding to pathogen effector molecules (Jones and Dangl, 2006). A comparison of wild plants and domestic crops has shown a greater polymorphism at R loci in wild plants than in domestic crops (Dangl and Jones, 2001), inferring more opportunities for wild plants to resist the effects of pathogens.

For the purposes of identifying localisation of bacteria in this study, a monoclonal antibody against *B. mallei* was used, as it was found to also react to *B. pseudomallei* and we did not have access to a commercial anti-*B. pseudomallei* antibody. Access to a confirmed anti-*B. pseudomallei* antibody may be a requirement if this protocol was to be used in environmental surveys in areas where *B. mallei* may be present. Imaging could be also be improved with access to a confocal laser microscope. In our study, limited examination of leaf tissue via immunofluorescence (data not shown) did not identify any *B. pseudomallei*, however leaf tissue was found to be infected via qPCR during biocontrol assays. The protocol for preparing tissue was very time consuming and prone to loss of tissue during washing steps, so to examine leaf tissue more effectively would require more time, practice.

While this study compared the effects of several strains of *B. pseudomallei* on more strains and species of rice than have previously been reported, with evidence of a host variability, further research examining a wider range of commercial rice strains would be useful in identifying patterns of resistance and may be of use for rice farmers in endemic regions. The presence of *B. pseudomallei* in the leaf tissue, as well as in the roots leads us to question whether *B. pseudomallei* could also infect grain and whether this could be a mechanism of transport or infection. Full grow out experiments to determine this would require facilities we do not have at present but could be of value in studying the ecology of *B. pseudomallei*.

Control of bacteria in the soil has been carried out using both chemical and biological agents (Na-ngam *et al.*, 2004; Na-ngam *et al.*, 2009), however chemical application in soil may cause shifts in soil microbial communities (Lancaster *et al.*, 2010). In contrast biocontrol has been widely used to control pathogenic bacteria in various environments (Eilenberg *et al.*, 2001). In terms of chemical agents, calcium oxide has been used to inhibit *B. pseudomallei* in soil (Na-ngam *et al.*, 2004) and effective microorganisms (EM) and *B. thailandensis* have been trialled as biological agents to control *B. pseudomallei* in soil without long term success (Wongsuwan *et al.*, 2013; Wuthiekanun *et al.*, 2013).

Other candidates for biocontrol of *B. pseudomallei* have been reported. Marshall *et al.* (2010) identified a possible bacteriocin produced by *B. ubonensis* that killed *B. pseudomallei in vitro* while, Yordpratum *et al.* (2011), Gatedee *et al.* (2011) and McRobb (2010) have identified bacteriophage capable of killing *B. pseudomallei in vitro*, unpublished data. Both phage and bacteriocin had activity specificity against *B. pseudomallei*, ensuring the soil microbiome diversity could be maintained if used *in vivo*, reducing the possibility of invasive species to colonizing the native microbiome (van Elsas *et al.*, 2012). In our study, the possible bacteriocin produced by *B. ubonensis* (Marshall *et al.*, 2010) and the phage isolated locally (McRobb, 2010) were further characterised and partially purified for use. The size of the bacteriocin-like inhibitory substance (BLIS) was 340-460 kDa. It was tentatively identified as a glycoprotein. The phage used in our cocktail were all characterized in the family Myoviridae. Both candidates had activity against *B. pseudomallei* in agar plate assays. A biofilm assay in microtiter plates showed that both candidates significantly ($p<0.005$) inhibited bacterial biofilm growth within 24 hr. At 48 hr, the phage cocktail continued to be effective at inhibiting biofilm formation ($p<0.005$) while the BLIS had reduced activity. The loss of activity of the BLIS relative to the phage cocktail was not unexpected as the BLIS was a chemical compound which may have been used up or degraded, while phage are self replicating so long as a host is present (Summers, 2001).

To avoid the limitations of the BLIS found in the biofilm assay, the bacteria producing the bacteriocin (*B. ubonensis*) was used in the domestic rice model. *B. ubonensis* was not effective at improving domestic rice growth when co-infected with *B. pseudomallei.* In addition, use of *B. ubonensis* on its own was shown to inhibit rice growth, although not to the extent of *B. pseudomallei*. At this stage, the BLIS of *B. ubonensis* or *B. ubonensis* itself does not appear to be a good biocontrol candidate for rice infections.

The phage cocktail improved rice growth in the domestic rice model, but not to the same rate as rice not infected with *B. pseudomallei.* However, in the wild rice model there was no statistical difference between uninfected rice growth and phage treated *B. pseudomallei* infected growth. Both qPCR and classical counts of bacteria showed a drop in *B. pseudomallei* numbers in both domestic and wild rice when they were treated with the phage cocktail. While these results are promising, further research to identify more effective bacteriophage would be prudent before environmental studies are carried out. These studies could include examining phage effectiveness under environmental conditions where there may be interacting factors such as natural soil environments as well as full grow out experiments to determine life long effects on plants.

The long term aim of our biocontrol experiments were to provide a tool to minimise pathogen numbers in the environment, thus limiting human exposure, although there may also be crop yield advantages. Prior to any field trials, an understanding of the field and the ecological activity of *B. pseudomallei* would be invaluable. Recently, *B. pseudomallei* was found inside exotic grass in the Northern Territory (Kaestli *et al.*, 2012) so it could be present in plants on the Castle Hill site in Townsville. Castle Hill is a partially characterised endemic site and this study was used to further survey the site, including the plants, with respect to types, abundance and *B. pseudomallei* presence. The most common plant found was *H. contortus* and *B. pseudomallei* was commonly found in soil surrounding the roots of these plants. Unfortunately this species was difficult to cultivate and numbers were insufficient for laboratory trials to determine

response to *B. pseudomallei*. If this limitation could be overcome, this would be a very interesting species of plant to study

Although *B. pseudomallei* was detected in rhizosphere soil in both areas, it was not detected inside any plant roots. All plants examined were wild species (*H. contortus, Aristida spp. Scleria rugose, Melinis repens*) which may have natural resistance to *B. pseudomallei* due to R protein diversity (Jones and Dangl, 2006) and selective pressure from long exposure to the organism in the case of *H. contortus, Aristida spp.* and *Scleria rugose*, which are native species, may result in resistance to infection. We cannot however, exclude the possibility that there was *B. pseudomallei* present in roots below the detection level of our method. Although our previous experiment demonstrated a heavy inoculum of *B. pseudomallei* resulted in invasion of the roots of domestic rice, the natural soil exposure on Castle Hill, based on our qPCR rhizosphere soil results, would be at a much lower dose The only reported natural invasion of plant matter with *B. pseudomallei* Kaestli *et al.* (2012) does not report the soil exposure level, frequency of detection of *B. pseudomallei* in plant samples or numbers of *B. pseudomallei* in plant tissue, so it is not possible to compare our data and determine likelihood of finding *B. pseudomallei* based on sample size, soil load or detection limit of the qPCR. A laboratory trial of exposures to *B. pseudomallei* at doses seen on Castle Hill, followed by qPCR, qPCR after enrichment and localisation assays of root and leaf may help to determine whether the negative results in root samples are those of true negatives or due to limitations in the assay.

In addition to examination of plants and rhizosphere soil, soil samples at the standard depth of 30 cm and an interim depth of 10 cm were taken from the plant survey sites. This provided further information on the Castle Hill and nearby drainage area in the wet season. At soil depths of 30 and 10 cm, the *B. pseudomallei* numbers after enrichment were significantly higher than from similar samples taken and enriched from the drain site (PCYC). This drain site had previously been reported as being negative for *B. pseudomallei* in the dry season (Ezzahir, 2012) while the SES site had been reported

positive. The Castle Hill (SES) area is likely to be the source of *B. pseudomallei* that was found at the PCYC area in the wet season with ground seep water (Baker *et al.*, 2011b) transporting bacteria to the drain site (PCYC). Lack of survival during the dry season indicates, assuming there is not an issue with detection protocols, the PCYC environment is not conducive to *B. pseudomallei*, either due to soil type or presence of other inhibitory agents. This seasonal variation could be further assessed by repeating the soil survey work on both sites on a monthly basis over at least a year to identify under what conditions, *B. pseudomallei* is no longer present. A more intensive microbiome survey, although expensive and bioinformatically time consuming, would assist in determining whether there are microbial interactions controlling the *B. pseudomallei* at the drain site.

Our study proved that some strains and species of rice were affected by *B. pseudomallei* and in the case of the domestic rice strain (*Oryza sativa* L. cv. Amaroo), we proved by use of IFA and qPCR that *B. pseudomallei* formed biofilms around the root system and invaded the roots with transport to the leaves. This domestic rice strain can now be used as a plant model of *B. pseudomallei* infection. In addition, a wild rice model (*O. meridionalis*) of infection was identified and was utilised in some of the biocontrol assays, although IFA was not utilised to study this model. We also proved that phage cocktails can be effective at controlling high bacterial loads of *B. pseudomallei* on rice plants, which indicates phage may be a viable tool for the control of *B. pseudomallei* in endemic environments. More work on developing phage and testing them for biocontrol of *B. pseudomallei* in various environments is still needed, including effectiveness and safety of controlling *B. pseudomallei* in the soil. Finally, there is no current evidence that *B. pseudomallei* is infecting the native plant species on Castle Hill, although we have identified that the dry season negative site becomes contaminated during the wet season. The ecology of this site is still not completely understood, but this research has added to the data that we have and has provided direction for future work.

APPENDIX 1: AGARS, CULTURE MEDIA AND GENERAL REAGENTS

A1.1 Ashdown agar

Tryptone (Acumedia, Neogen corporation, USA)	12 g
Glycerol (Merck, Germany)	32 m1
Crystal violet (0.1% aqueous - **A1.1.1**) (Sigma, USA)	4 m1
Neutral red (1% aqueous - **A1.1.3**) (Sigma Aldrich, USA)	4 m1
Agar Technical (Acumedia, Neogen corporation, USA)	12 g
Double distilled water	800 m1

Combine ingredients and boil for 15 mins in water bath. Autoclave at 121 ^{o}C for 15 mins, cool to 55°C in water bath and add 2.0 rnl of 5 mg/ml gentamycin sulphate (65.5% pure - **A1.1.2**). Pour into plates and cool.

A 1.1.1 Crystal violet (0.1%)

Crystal violet (Sigma, USA)	0.l g
Double distilled water	Make up to l00 ml

Dissolve crystal violet in double distilled water and autoclave at 121°C for 15 mins.

A 1.1.2 Gentamycin sulphate (0.05 g)

Gentamycin sulphate (G3632, Sigma Chemicals, Australia)	0.05 g
Double distilled water	Make up to l0rnl

Dissolve gentamycin sulphate in double distilled water and filter sterilise through a 0.2 µm filter (Filtropur S 0.2, SARSTEDT, Germany)

A 1.1.3 Neutral red (1% aqueous)

Neutral red (Sigma Aldrich, USA)	1 g
Double distilled water Make up to l00 ml	

Dissolve neutral red in double distilled water and autoclave at 121°C for 15 mins.

A1.2 Ashdown broth

Tryptone (Acumedia, Neogen corporation, USA)	12 g
Glycerol (Merck, Germany)	32 m1
Crystal violet (0.1% aqueous - **A1.1.1**) (Sigma, USA)	4 m1
Neutral red (1% aqueous - **A1.1.3**) (Sigma Aldrich, USA)	4 m1
Double distilled water	800 m1

Combine ingredients and boil for 15 mins in water bath. Autoclave at 121 ^{o}C for 15 mins, cool to 55°C in water bath and add 2.0 ml of 5 mg/ml gentamycin sulphate (65.5% pure - **A1.1.2**). Pour into plates and cool.

A1.3 Luria-Bertani (LB) agar

Tryptone (Acumedia, Neogen corporation, USA)	10 g
Yeast extract (Acumedia, Neogen corporation, USA)	5 g
NaC1 (Univar, Ajax Finechem, Australia)	10 g
Agar Technical (Acumedia, Neogen corporation, USA)	12 g
Double distilled water	Make up to l000 rnl

Make up to 950m1 with double distilled water, adjust pH to 7.0 and bring volume up to l000 ml. Autoclave at 121°C for 15 mins, cool to 55°C in water bath and pour into plates and cool.

A1.4 Luria-Bertani (LB) broth

Tryptone (Acumedia, Neogen corporation, USA)	10 g
Yeast extract (Acumedia, Neogen corporation, USA)	5 g
NaC1	10 g
(Univar, Ajax Finechem, Australia)	
Double distilled water	Make up to l000 rnl

Make up to 950m1 with double distilled water, adjust pH to 7.0 and bring volume up to l000 ml and autoclave at 121°C for 15 mins.

A1.5 Extraction buffer (DNA)

Tris (100 mM) (**A1.5.1**)	10 ml
EDTA (50 mM) (**A1.5.2**)	10 ml
NaCl (500 mM) (**A1.5.3**)	1 0 ml
Sodium dodecyl sulphate (0.7%) (**A1.5.4**)	7 ml
Proteinase K (50 µg/ml)	227.3µl
Double distilled water	Make up to 100 ml

Ingredients except proteinase K were combined and autoclave at 121°C for 15 mins. Reagent was cool down to room temperature and proteinase K was added (Roach, Germany).

A1.5.1 Tris-HCI (1M) (pH 7.5)

Tris (Merck, Germany)	12.14 g
Double distilled water	100 ml

Dissolve tris in 80 ml double distilled water and adjust pH to 7.5. Make up to 100 ml with double distilled water and autoclave at 121°C for 15 mins

A1.5.2 EDTA (0.5M) (pH8.0)

EDTA (Calbiochem, Germany)	186.12 g
Double distiled water	Make up to l000 ml

Combine ingredients and make up to 800m1 with double distilled water. Adjust pH to 8.0. Make up to 1000 ml with double distil1l water when EDTA has dissolved. Autoclave at 121°C for 15 mins

A1.5.3 NaCI (5M)

NaC1 (Univar, Ajax Finechem Australia)	292.2 g
Double distilled water	Make up to l000 ml

Dissolve NaC1 in 800m double distilled water and make up to l000 ml with double distilled water and autoclave at 121°C for 15 mins.

A1.5.4 Sodium dodecyl sulphate (SDS) (10%)

SDS (OmniPur, Merck KGaA , Germany)	10g
Double distilled water	Make up to 100 ml

Dissolve SDS in 80 ml double distiled water. Make up to 100 ml with double distiled water.

A1.6 Tris-acetate (TAE) buffer (50 X)

Tris (Merck, Germany)	242g
Glacial acetic acid (Univar, Ajax Finechem, Australia)	57.l ml
0.5M EDTA(pH8.0) (Calbiochem, Germany)	100 ml
Double distilled water	Make up to l000 ml

Combine ingredients and make up to l000 ml with double distilled water and autoclave at 121°C for 15 mins.

A1.7 LB plates with ampicillin/IPTG/XGal

Make the LB agar with ampicillin without pouring plates; then supplement with 0.5 mM IPTG (**A1.7.1)** and 80 µg/ml X-Gal (**A1.7.2)** and pour the plates. Alternatively, l00 µl of 100 mM IPTG (**A1.7.1)** and 20 µl of 50 mg/ml X-Gal (**A1.7.2)** may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 mins at 37 °C prior to use.

A1.7.1 IPTG stock solution (0.1M)

isopropyl-β-d-thiogalactopyranoside (Astral Scientific, Australia)	1.2 g

Add water to 50rnl final volume. Filter sterilize and store at 4°C.

A1.7.2 X-Gal (2m1)

5-bromo-4-chloro-3-indolyl-β-D-galactoside (Astral Scientific, Australia)	100 mg

Dissolve in 2 m1 N,N'-dimethylformamide. Cover with aluminum foil and store at -20°C.

A1.7.3 LB medium

Tryptone (Acumedia, Neogen corporation, USA)	10 g
Yeast extract (Acumedia, Neogen corporation, USA)	5 g
NaCI (Univar, Ajax Finechem Australia)	5 g

Dissolve ingredient in double distilled water and make up to l000 ml and adjust pH to 7.0 with NaOH

A1.7.4 LB plates with ampicillin

Agar technical (Acumedia, Neogen corporation, USA)	15 g
LB medium.(A1.13)	1000 ml

Mixed and autoclave at 121°C for 15 mins. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100 µg/ml. Pour 30-35 ml of medium into 90 mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

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A1.8 Hoagland nutrient

A1.8.1 ¼ Hoagland solution agar

Solution number 2 (**A1.8.2**)	250 ml
Supplement solution (**A1.8.3**)	250 µl
Agar Technical (Acumedia, Neogen corporation, USA)	10 g
Distilled water	up to 750 ml

Combine ingredients and boil for 15 mins in water bath. Autoclave at 121 ^{o}C for 15 mins, cool to 55°C in water bath and add 250 µl of iron solution (**A1.8.4**). Pour into plates and cool.

A1.8.2 Solution number2

$NH_4H_2PO_4$ (BDH, England)	0.115 g
KNO_3 (Univar, Ajax Chemicals, Australia)	0.607 g
$Ca(NO_3)_2.4H_2O$ (AnalaR, BDH Chemical, Australia)	0.945 g
$MgSO_4.7H_2O$ (Merck, Germany)	0.493 g
Distilled water	l000 ml

Ingredients were combined on magnetic stirrer until dissolved and autoclave at 121°C for 15 mins .

A1.8.3 Supplement solution

H_3BO_3 (Univar, Ajax Chemicals, Australia)	2.86 g
$MnCl_2.4H_2O$ (Merck KGaA , Germany)	1.81 g
$ZnSO_4$ (BDH, England)	0.22 g
$CuSO_4.5H_2O$ (Mallinckrodt, USA)	0.08 g
$H_2MoO_4.H_2O$ (85% MoO_3) (BDH, England)	0.02 g
Distilled water	l000 ml

Ingredients were combined on magnetic stirrer until dissolved and autoclave at 121°C for 15 mins.

A1.8.4 Iron solution (0.5%)

Ferric ammonium citrate (Sigma, USA)	0.25 g
Distilled water	50 ml

Ingredients were combined on magnetic stirrer until dissolved and filter sterilise through a 0.2 µm filter (Filtropur S 0.2, SARSTEDT, Germany). Aliquot 1 ml and keep in dark container at -20 ^{o}C.

A1.9 SM buffer

NaC1 (Univar, Ajax Finechem. Australia)	5.8 g
$MgSO_4.7H_2O$ (Merck, Germany)	2.0 g
1M Tris-HCL (pH 7.5) (2.2) (Merck, Germany)	50 ml
2% Gelatin (A1.19) (Sigma, USA)	5 ml
Double distilled water	1000 ml

Combine ingredients and make up to 1000 ml with double distilled water. Aliquot as required and autoclave at 121°C for 15 mins

A1.9.1 2% Gelatin

Gelatin (Sigma, USA)	0.2 g
Double distilled water	l0 ml

Dissolve gelatin in double distilled water with heating and stirring and autoclave at 121°C for 15 mins.

A1.10 NaCl (0.85 %)

NaC1 (Univar, Ajax Finechem Australia)	8.5 g
Double distilled water	Make up to l000 ml

Dissolve NaC1 in 800m double distilled water and make up to l000 ml with double distilled water and autoclave at 121°C for 15 mins.

A1.11 Phosphate buffered saline (PBS) (pH 7.4)

NaC1 (Univar, Ajax Finechem. Australia)	8 g
Na_2HPO_4 (Univar, Ajax Finechem. Australia)	4 1 .44 g
KH_2PO_4 (LabServ, Thermo Fisher scientific, Australia)	0.24 g
KC1 (Univar, Ajax Finechem. Austrnlia)	0.2 g
Double distilled water	Make up to l000 ml

Make up to 950 ml with double distilled water, adjust pH to 7.2 and make upvolume to l000 ml and autoclave at 121^{O}C for 15 mins.

A1.12 1% (w/v) Bovine serum albumin

Bovine serum albumin (Sigma, USA)	0.1 g
Phosphate buffered saline (A1.21)	10 ml

Dissolve bovine serum albumin in phosphate buffered saline.

A1.13 50 mM potassium phosphate buffer, pH7.4

sodium phosphate, mono-sodium salt (**A1.13.1**)	19 ml
sodium phosphate, di-sodium salt (**A1.13.2**)	81 ml
Double distilled water	100 ml

Mixed and check final pH with pH meter

A1.13.1 0.1 M sodium phosphate, mono-sodium salt

$NaH_2PO_4.H2O$ (Univar, Ajax Chemicals, Australia)	13.8 g
Double distilled water	1000m1

A1.13.2 0.1 M sodium phosphate, di-sodium salt

$Na_2HPO_4.7H_2O$ (Univar, Ajax Finechem, Australia)	14.2g
Double distilled water	1000 m1

A1.14 SDS-PAGE gel

A1.14.1 15 % SDS-PAGE separation gel

1.5 M Tris pH 8.8 (Univar, Ajax Finechem, Australia)	2 ml
40% Acrylamide solution (Amresco, USA)	3 ml
Distilled water	2.84 ml
10% SDS	80 µl
10% APS (Ammonium persulfate) (Sigma, USA)	80 µl
TEMED (Tetramethylethylenediamine) (Amresco, USA)	8.4 µl

Mix quickly and gently (avoid bubbles frothing). Pour immediately till ¾ of height

A1.14.2 SDS-PAGE loading gel

0.5 M Tris pH 6.8 (Univar, Ajax Finechem, Australia)	1 ml
40% Acrylamide solution (Amresco, USA)	0.532 ml (5.32%)
Distilled water	2.44 ml
10% SDS (**A1.9**)	40 µl
10% APS (Ammonium persulfate)	40 µl
TEMED (Tetramethylethylenediamine) (Amresco, USA)	4 µl

Mix quickly and gently (avoid bubbles frothing). Pour immediately and insert comb.

A1.15 2x Loading dye for SDS-PAGE gel

milliQ Water	800 µl
0.5M Tris (pH 6.8) (Univar, Ajax Finechem, Australia)	2 ml
Glycerol (Merck, Germany)	1.6 ml
10% SDS (**A1.9**)	3.2 ml
Bromophenol blue (Sigma, USA)	3 or 4 grains
50 mM EDTA (Calbiochem, Germany)	80 µl
14.2 M β-mercaptoethanol (pure) (Sigma, USA)	50 µl

Mixed and store in aliquots at -20^{o}C

A1.16 10× Tris glycine SDS buffer

SDS (OmniPur, Merck KGaA , Germany)	10 g
Tris (Univar, Ajax Finechem, Australia)	30.3 g
Glycin (Sigma, USA)	144.1 g

Dissolve ingredient in 800 ml distilled water and adjust volume to 1 liter

A1.17 Coomassie stain

Methanol (Univar, Ajax Finechem, Australia)	500 ml
Acetic acid (Univar, Ajax Finechem, Australia)	100 ml
Coomassie Brilliant blue R (Sigma, USA)	0.5 g
Distilled water	350 ml (final 1L vol)

Mix in a 2L beaker until all dissolved (~5-10 min). Filter through whatman filter paper in a fume hood

A1.18 Coomassie Blue destaining solution

Methanol (Univar, Ajax Finechem, Australia)	450 ml
Acetic acid (Univar, Ajax Finechem, Australia)	100 ml
Double distilled water	400 ml

Combine ingredients and make up to l 000 ml with distilled water

A1.19 Silver fixative

Methanol (Univar, Ajax Finechem, Australia)	500 ml
Acetic acid (Univar, Ajax Finechem, Australia)	50 ml
Double distilled water	400 ml

Combine ingredients and and make up to l 000 ml with distilled water

A1.20 Sensitizer

$Na_2S_2O_3$ (Univar, Ajax Finechem, Australia)	200 mg
Double distilled water	600 ml

Dissolve $Na_2S_2O_3$ in 600 ml double distilled water and make up to l000 ml with double distilled water

A1.21 Silver nitrate

$AgNO_3$ (Sigma, USA)	1 g
Double distilled water	800 ml

Dissolve $AgNO_3$ in 800 ml double distilled water and make up to l000 ml with double distilled water

A1.22 Development solution

Na_2CO_3 (Univar, Ajax Finechem, Australia)	20 g
Formalin	400 µl
Double distilled water	400 ml

Dissolve Na_2CO_3 in 800 ml double distilled water and make up to l000 ml with double distilled water

A1.23 Termination solution

Acetic acid (Univar, Ajax Finechem, Australia)	50 ml
Double distilled water	800 m1

Add acetic acid to 800 ml distilled water and mix. Adjust final volume to 1 litre

A1.24 0.4% crystal violet

Crystal violet (Sigma, USA)	0.4 g
Double distilled water	800 ml

Dissolve crystal violet in 800 ml double distilled water and make up to l000 ml with double distilled water

A1.25 Polyvinyl polypyrrolodine (PVPP) in phosphate buffer

Polyvinyl polypyrrolodine (PVPP) (Sigma, USA)	10 g
Phosphate buffer, pH 7.4 (**A1.13**)	800 ml

Dissolve polyvinyl polypyrrolodine (PVPP) in 800 ml Phosphate buffer and make up to l000 ml with phosphate buffer (suspension solution)

A1.26 Threonine basal salt solution (TBSS-C50)

L-Threonine solution (**1.26.3**)	100 ml
Base (**1.26.2**)	900 ml
Distilled water	Make up to 1000 ml

Combine ingredients and mix for 15 minutes. Autoclave at 121 ^{o}C for 15 minutes, cool to 55°C in water bath and add 334.5 µl of colistin sodium methanesulfonate solution (**1.26.4**)(384615.4 Unit or 50 mg/l)

A1.26.1 Solution A

H_3PO_4 (85%) (Sigma, USA)	2.306 ml
$FeSO_4.7H_2O$ (Sigma, USA)	0.55 g
$ZnSO4._7H_2O$ (BDH, England)	0.279 g
$CuSO4.5H_2O$ (Mallinckrodt, USA)	0.0218 g
$MnSO_4.H_2O$ (Merck, Germany)	0.125 g
$Co(NO_3)_2.6H_2O$ (BDH, England)	0.030 g
$Na_2MoO_4.2H_2O$ (Sigma, USA)	0.030 g
H_3BO_3 (Univar, Ajax Chemicals, Australia)	0.062 g
Distilled water	800 ml

Combine ingredients and make up to l 000 ml with distilled water

A1.26.2 Base

KH_2PO_4 (LabServ, Thermo Fisher scientific, Australia)	0.451 g
K2HPO4 (Univar, Ajax Chemicals, Australia)	1.730 g
$MgSO_4._7H2O$ (Merck, Germany)	0.123 g

$CaCl_2.2H_2O$ (Univar, Ajax Chemicals, Australia)	0.0147g
NaCl (Univar, Ajax Chemicals, Australia)	10 g
Nitrilotriacetic acid (Sigma, USA)	0.200 g
Solution A (**A1.25.1**)	20 ml
Distilled water	900 ml

Combine ingredients and adjust pH to 7.2 and make up to l 000 ml with distilled water

A1.26.3 L-Threonine solution

L-Threonine (Astral scientific, Australia)	5.965 g
Distilled water	100 ml

Ingredients were combined until dissolved and filter sterilised through a 0.2 µm filter (Filtropur S 0.2, SARSTEDT, Germany)

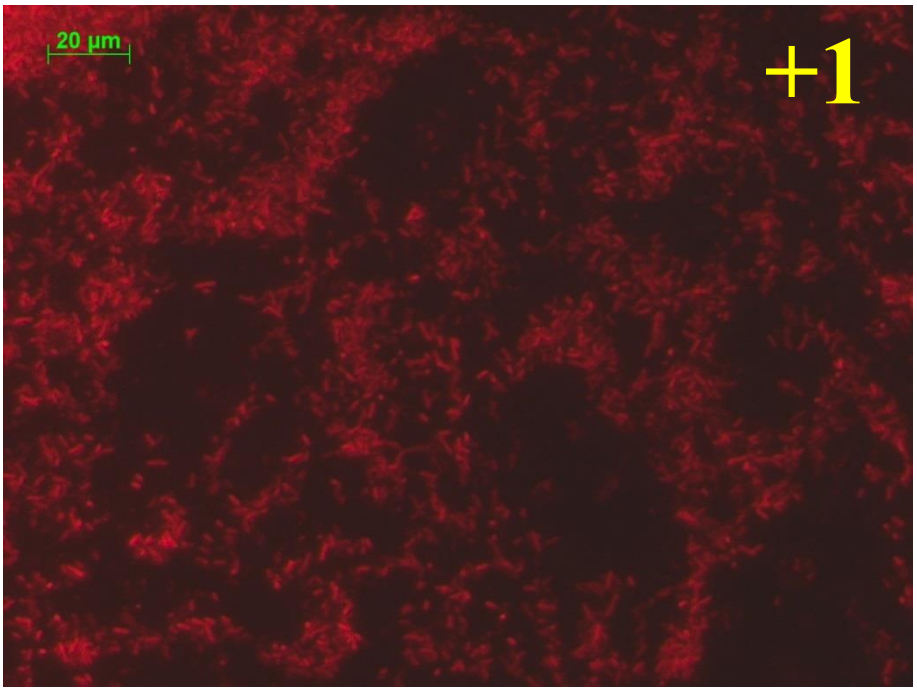
A1.26.4 Colistin sodium methanesulfonate solution

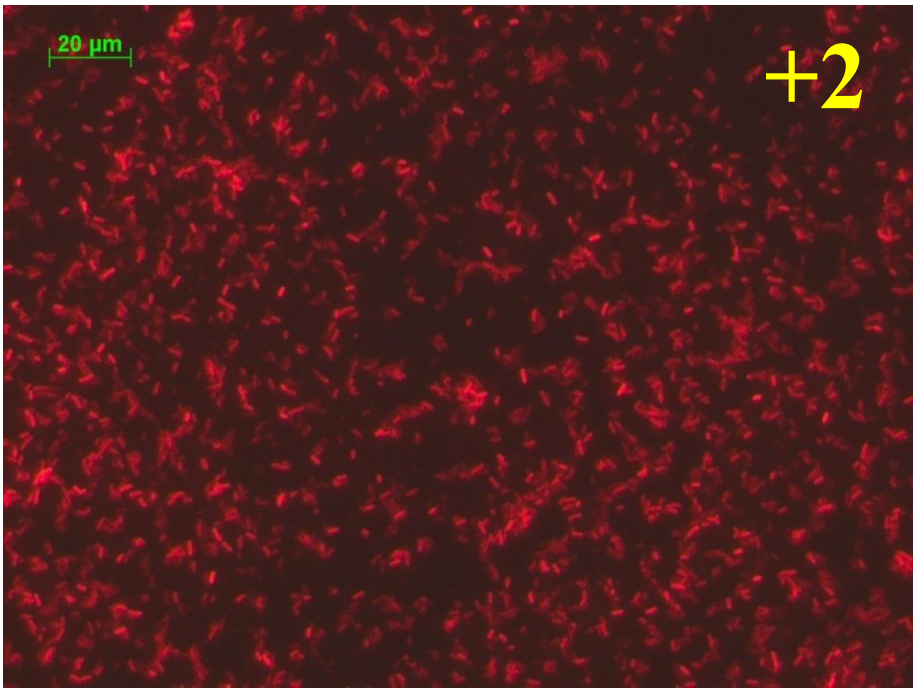
Colistin sodium methanesulfonate (C1511, Sigma, USA)	0.1 g
Sterilised double distilled water	1000 µl

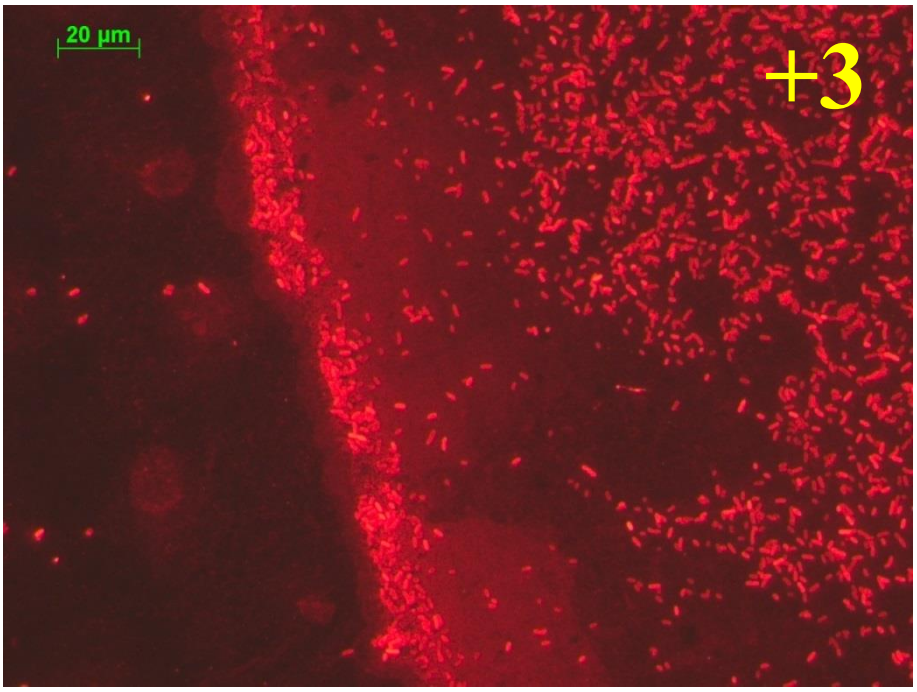
Colistin sodium methanesulfonate was dissolved in 1 ml sterilised double distilled water (1,150,000 U/ml)

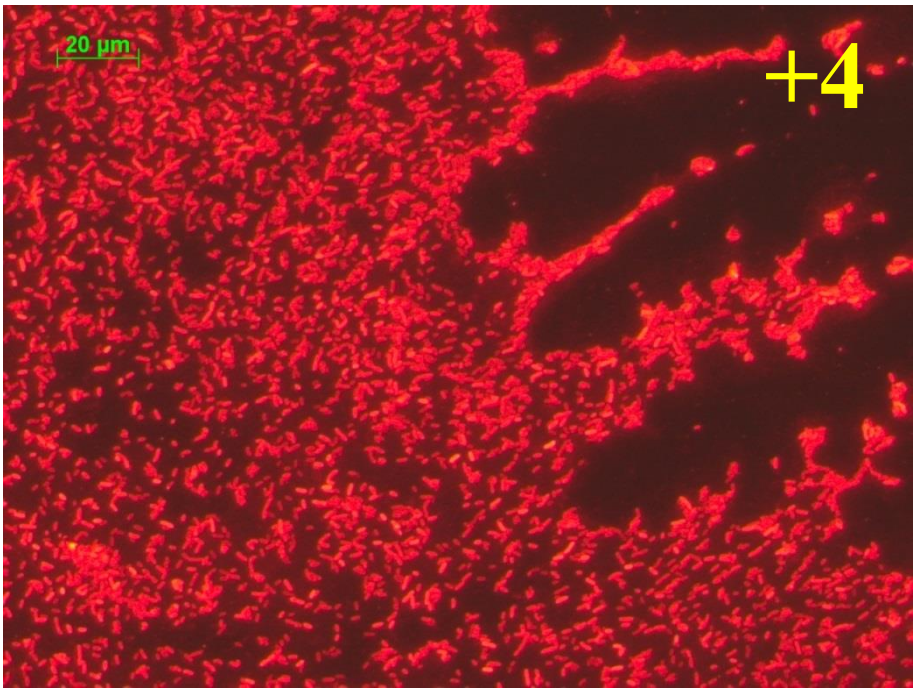
APPENDIX 2: IMMUNOFLUORESCENT ASSAY SCORING IMAGES

A2.1 Examples of fluorescent microscope images of fluorescent monoclonal antibody bound *B. pseudomallei,* scored from 1 to 4 based on the intensity of fluorescence observed under standard conditions as described in Section 4.2.6. Scoring values represented by the yellow numbers on the images.






20 µm
+3


20 µm
+4

APPENDIX 3: RAW DATA AND STATISTICAL ANALYSES

Statistical analyses of data utilised the following criteria. All data was independent. For groups of three or more, if sample sizes were ≥25 normality was assumed (Krithikadatta, 2014). For smaller sample sizes, normality was tested and if data was not normally distributed, a log10 transformation was carried out. Assuming normality was acceptable, an ANOVA was used. If variances were homogeneous, a Gabriel post-hoc test was used. If variances were not homogeneous, a Games-Howell post hoc was used (Field, 2005). When other post hoc tests were used, the reason for doing so was included in the section introduction. For groups of two, if data was not normally distributed, a log10 transformation was carried out. If data was still not normally distributed, a non-parametric Mann-Whitney U test was used, otherwise the most applicable T-test was used.

A3.1 Analysis of Survival data obtained in ¼ strength Hoagland solution for *B. pseudomallei* TSV189 and near-neighbor species *B. vietnamensis* 38SP (as used in chapter 4.3.2). All statistical analysis were carried out using SPSS version 20.

Raw counts of *B. pseudomallei* TSV189 (Table A.3.1.1) were analysed for normality of distribution (Table A3.1.2) and homogeneity of variance (Table A3.1.3). Data was normal and variances were not homogeneous. Bacterial counts were used in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.1.4, post hoc test Table A3.1.5) to determine whether bacterial counts changed between day 1 and day 90. The initial inoculum counts were not included. No significant difference between day 1 and 90 was found.

Similarly, *B. vietnamensis* 38SP raw counts (Table 3.1.6) were analysed for normality of distribution (table A3.1.7) Data was not normally distributed in all cases. Raw counts were then transformed (log10) and data was reanalysed, Log transformed data was normally distributed (Table A3.1.8). Analysis of homogeneity of variance on log transformed data (Table A3.1.9) indicated variances were homogeneous. Log transformed counts were used in a one way ANOVA with Gabriel post hoc test (p=0.05, ANOVA Table A3.1.10, post hoc test Table A3.1.11) to determine whether bacterial counts changed between day 1 and day 90. The initial inoculum counts were not included. Day 90 had significantly higher bacterial numbers than day one and two. No other significant differences were found.

Table A3.1.1 Raw data of replicates of bacterial counts/ml of *B. pseudomallei* TSV189 over 90 days. The top row represents the day after incubation at which counts were made

0	1	2	3	4	5	6	7	90
3.50E+06	5.00E+07	1.50E+07	2.50E+07	2.50E+07	3.00E+07	4.20E+07	5.76E+07	1.00E+07
3.50E+06	4.00E+07	3.50E+07	3.00E+07	3.00E+07	3.00E+07	3.05E+07	5.84E+07	1.50E+07
1.50E+06	4.50E+07	3.50E+07	2.50E+07	3.50E+07	2.00E+07	1.24E+07	5.18E+07	2.00E+07
	3.50E+07	4.00E+07	1.50E+07	3.00E+07	1.53E+07	3.78E+07	2.25E+07	2.00E+07
	5.50E+07	5.00E+07	1.50E+07	2.50E+07	2.08E+07	1.92E+07	2.28E+07	2.50E+07
	5.00E+06	5.00E+07	1.50E+07	1.50E+07	2.65E+07	2.15E+07	2.31E+07	2.50E+07

Table A3.1.2 Test for normality of *B. pseudomallei* TSV189 bacterial counts

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data_sk_tsv189	1 day	.259	6	.200*
	2 day	.257	6	.200*
	3 day	.310	6	.074
	4 day	.237	6	.200*
	5 day	.190	6	.200*
	6 day	.191	6	.200*
	7 day	.313	6	.067
	90 day	.223	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.1.3 Test for homogeneity of variances of *B. pseudomallei* TSV189 bacterial counts

Test of Homogeneity of Variances

data_sk_tsv189

Levene Statistic	df1	df2	Sig.
3.283	7	40	.007

Table A3.1.4 One way ANOVA of bacterial counts of *B. pseudomallei* TSV189 from day 1 to day 90.

ANOVA

data_sk_tsv189

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.796E+15	7	3.995E+14	2.876	.016
Within Groups	5.556E+15	40	1.389E+14		
Total	8.353E+15	47			

A3.1.5 Post hoc analysis of counts of *B. pseudomallei* TSV189 (Games-Howell test).

Multiple Comparisons

Dependent Variable: data_sk_tsv189

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 day	2 day	833333.3333	8983008.652	1.000	-33550459.9	35217126.58
	3 day	17500000.00	7754926.749	.426	-15379043.5	50379043.48
	4 day	11666666.67	7781745.020	.788	-21197347.2	44530680.51
	5 day	14566666.67	7666347.819	.592	-18384642.6	47517975.93
	6 day	11100000.00	8643198.996	.883	-22523711.5	44723711.51
	7 day	-1033333.33	10418231.88	1.000	-40114925.6	38048258.97
	90 day	19166666.67	7646713.165	.335	-13805602.6	52138935.95
2 day	1 day	-833333.333	8983008.652	1.000	-35217126.6	33550459.92
	3 day	16666666.67	5939509.894	.223	-7293084.76	40626418.10
	4 day	10833333.33	5974482.776	.631	-13157928.3	34824594.92
	5 day	13733333.33	5823381.988	.376	-10157549.9	37624216.54
	6 day	10266666.67	7060012.590	.814	-16294986.5	36828319.80
	7 day	-1866666.67	9147592.276	1.000	-36999237.0	33265903.65
	90 day	18333333.33	5797509.044	.152	-5550300.56	42216967.23
3 day	1 day	-17500000.0	7754926.749	.426	-50379043.5	15379043.48
	2 day	-16666666.7	5939509.894	.223	-40626418.1	7293084.763
	4 day	-5833333.33	3890872.510	.793	-20429048.8	8762382.111
	5 day	-2933333.33	3654616.563	.989	-16671274.2	10804607.53
	6 day	-6400000.00	5411859.405	.917	-27803024.1	15003024.14
	7 day	-18533333.3	7944991.434	.396	-52346680.4	15280013.69
	90 day	1666666.667	3613247.231	1.000	-11932736.6	15266069.95
4 day	1 day	-11666666.7	7781745.020	.788	-44530680.5	21197347.17
	2 day	-10833333.3	5974482.776	.631	-34824594.9	13157928.25
	3 day	5833333.333	3890872.510	.793	-8762382.11	20429048.78
	5 day	2900000.000	3711184.297	.990	-11069377.8	16869377.80
	6 day	-566666.667	5450219.160	1.000	-22026967.2	20893633.91
	7 day	-12700000.0	7971170.275	.743	-46495719.2	21095719.15
	90 day	7500000.000	3670452.591	.502	-6337242.33	21337242.33
5 day	1 day	-14566666.7	7666347.819	.592	-47517975.9	18384642.60
	2 day	-13733333.3	5823381.988	.376	-37624216.5	10157549.87
	3 day	2933333.333	3654616.563	.989	-10804607.5	16671274.20
	4 day	-2900000.00	3711184.297	.990	-16869377.8	11069377.80
	6 day	-3466666.67	5284148.202	.996	-24718878.7	17785545.32
	7 day	-15600000.0	7858555.140	.551	-49493077.4	18293077.35
	90 day	4600000.000	3418999.204	.862	-8225327.78	17425327.78
6 day	1 day	-11100000.0	8643198.996	.883	-44723711.5	22523711.51
	2 day	-10266666.7	7060012.590	.814	-36828319.8	16294986.47
	3 day	6400000.000	5411859.405	.917	-15003024.1	27803024.14
	4 day	566666.6667	5450219.160	1.000	-20893633.9	22026967.24
	5 day	3466666.667	5284148.202	.996	-17785545.3	24718878.65
	7 day	-12133333.3	8814130.070	.846	-46562994.2	22296327.51
	90 day	8066666.667	5255621.329	.773	-13161029.9	29294363.26
7 day	1 day	1033333.333	10418231.88	1.000	-38048259.0	40114925.63
	2 day	1866666.667	9147592.276	1.000	-33265903.7	36999236.98
	3 day	18533333.33	7944991.434	.396	-15280013.7	52346680.35
	4 day	12700000.00	7971170.275	.743	-21095719.2	46495719.15
	5 day	15600000.00	7858555.140	.551	-18293077.4	49493077.35
	6 day	12133333.33	8814130.070	.846	-22296327.5	46562994.18
	90 day	20200000.00	7839401.905	.313	-13715434.4	54115434.44
90 day	1 day	-19166666.7	7646713.165	.335	-52138935.9	13805602.61
	2 day	-18333333.3	5797509.044	.152	-42216967.2	5550300.562
	3 day	-1666666.67	3613247.231	1.000	-15266070.0	11932736.62
	4 day	-7500000.00	3670452.591	.502	-21337242.3	6337242.326
	5 day	-4600000.00	3418999.204	.862	-17425327.8	8225327.785
	6 day	-8066666.67	5255621.329	.773	-29294363.3	13161029.93
	7 day	-20200000.0	7839401.905	.313	-54115434.4	13715434.44

Table A3.1.6 Raw data of replicates of bacterial counts/ml of *B. vietnamensis* 38SP over 90 days. The top row represents the day after incubation at which counts were made.

0	1	2	3	4	5	6	7	90
4.50E+06	1.00E+07	5.00E+06	5.00E+06	1.00E+07	5.00E+06	6.00E+06	1.72E+07	4.00E+07
4.00E+06	2.00E+07	5.00E+06	5.00E+06	1.50E+07	1.50E+07	1.22E+07	2.69E+07	3.00E+07
7.50E+06	5.00E+06	2.50E+07	3.00E+07	2.00E+07	3.50E+07	6.20E+06	2.88E+07	4.50E+07
	5.00E+06	5.00E+06	1.50E+07	1.50E+07	5.10E+06	6.30E+06	1.30E+07	3.00E+07
	5.00E+06	1.00E+07	1.50E+07	2.00E+07	1.56E+07	1.92E+07	1.39E+07	6.00E+07
	1.00E+07	1.00E+07	5.00E+06	5.00E+06	2.12E+07	5.85E+07	1.49E+07	1.50E+07

Table A3.1.7 Test for normality of *B. vietnamensis* 38SP bacterial counts

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data_sk_38sp	1 day	.277	6	.168
	2 day	.333	6	.036
	3 day	.276	6	.170
	4 day	.223	6	.200*
	5 day	.186	6	.200*
	6 day	.311	6	.071
	7 day	.276	6	.173
	90 day	.168	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.1.8 Test for normality of log transformed *B. vietnamensis* 38SP bacterial counts.

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
Logtsv38SP	1 day	.293	6	.117
	2 day	.282	6	.147
	3 day	.306	6	.083
	4 day	.282	6	.147
	5 day	.246	6	.200*
	6 day	.267	6	.200*
	7 day	.229	6	.200*
	90 day	.237	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.1.9 Test for homogeneity of variances of log transformed *B. vietnamensis* 38SP bacterial counts

Test of Homogeneity of Variances

Logtsv38SP

Levene Statistic	df1	df2	Sig.
1.263	7	40	.293

Table A3.1.10 One way ANOVA of log transformed bacterial counts of *B. vietnamensis* 38SP from day 1 to day 90. A significant difference was found.

ANOVA

Logtsv38SP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.764	7	.252	3.168	.009
Within Groups	3.181	40	.080		
Total	4.945	47			

A3.1.11 Post hoc analysis of log transformed counts of *B. vietnamensis* 38SP (Gabriel test). Day 90 is different to days 1 and 2.

Multiple Comparisons

Dependent Variable: LOG38SP

Gabriel

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 day	2 day	-.01615	.16281	1.000	-.5568	.5245
	3 day	-.08805	.16281	1.000	-.6287	.4526
	4 day	-.20921	.16281	.995	-.7498	.3314
	5 day	-.20804	.16281	.995	-.7487	.3326
	6 day	-.18479	.16281	.999	-.7254	.3558
	7 day	-.35948	.16281	.555	-.9001	.1811
	90 day	-.62764*	.16281	.011	-1.1683	-.0870
2 day	1 day	.01615	.16281	1.000	-.5245	.5568
	3 day	-.07189	.16281	1.000	-.6125	.4687
	4 day	-.19306	.16281	.998	-.7337	.3476
	5 day	-.19188	.16281	.998	-.7325	.3487
	6 day	-.16864	.16281	1.000	-.7093	.3720
	7 day	-.34333	.16281	.634	-.8840	.1973
	90 day	-.61148*	.16281	.015	-1.1521	-.0709
3 day	1 day	.08805	.16281	1.000	-.4526	.6287
	2 day	.07189	.16281	1.000	-.4687	.6125
	4 day	-.12117	.16281	1.000	-.6618	.4195
	5 day	-.11999	.16281	1.000	-.6606	.4206
	6 day	-.09675	.16281	1.000	-.6374	.4439
	7 day	-.27143	.16281	.919	-.8121	.2692
	90 day	-.53959	.16281	.051	-1.0802	.0010
4 day	1 day	.20921	.16281	.995	-.3314	.7498
	2 day	.19306	.16281	.998	-.3476	.7337
	3 day	.12117	.16281	1.000	-.4195	.6618
	5 day	.00118	.16281	1.000	-.5395	.5418
	6 day	.02442	.16281	1.000	-.5162	.5650
	7 day	-.15027	.16281	1.000	-.6909	.3904
	90 day	-.41842	.16281	.297	-.9591	.1222
5 day	1 day	.20804	.16281	.995	-.3326	.7487
	2 day	.19188	.16281	.998	-.3487	.7325
	3 day	.11999	.16281	1.000	-.4206	.6606
	4 day	-.00118	.16281	1.000	-.5418	.5395
	6 day	.02324	.16281	1.000	-.5174	.5639
	7 day	-.15144	.16281	1.000	-.6921	.3892
	90 day	-.41960	.16281	.293	-.9602	.1210
6 day	1 day	.18479	.16281	.999	-.3558	.7254
	2 day	.16864	.16281	1.000	-.3720	.7093
	3 day	.09675	.16281	1.000	-.4439	.6374
	4 day	-.02442	.16281	1.000	-.5650	.5162
	5 day	-.02324	.16281	1.000	-.5639	.5174
	7 day	-.17469	.16281	1.000	-.7153	.3659
	90 day	-.44284	.16281	.217	-.9835	.0978
7 day	1 day	.35948	.16281	.555	-.1811	.9001
	2 day	.34333	.16281	.634	-.1973	.8840
	3 day	.27143	.16281	.919	-.2692	.8121
	4 day	.15027	.16281	1.000	-.3904	.6909
	5 day	.15144	.16281	1.000	-.3892	.6921
	6 day	.17469	.16281	1.000	-.3659	.7153
	90 day	-.26816	.16281	.927	-.8088	.2725
90 day	1 day	.62764*	.16281	.011	.0870	1.1683
	2 day	.61148*	.16281	.015	.0709	1.1521
	3 day	.53959	.16281	.051	-.0010	1.0802
	4 day	.41842	.16281	.297	-.1222	.9591
	5 day	.41960	.16281	.293	-.1210	.9602
	6 day	.44284	.16281	.217	-.0978	.9835
	7 day	.26816	.16281	.927	-.2725	.8088

*. The mean difference is significant at the 0.05 level.

A3.2 Statistical analysis of dose dependent growth inhibition of rice with *B. pseudomallei* (TSV189) (as used in chapter 4.3.3). All statistical analysis were carried out using SPSS version 20.

Root area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured after exposure to different doses of *B. pseudomallei* (Table A3.2.1). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance (Table A3.2.2) identified that the assumption of homogeneity of variance was violated for ANOVA so a Games-Howell post hoc test was selected for use with the ANOVA as it is advised in cases where variances are not equal (Field, 2005). A one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.2.3, post hoc test Table A3.2.4) was used to determine whether root area of rice changed between 10^2 and 10^8 CFU. Doses up to 10^5 were not significantly different from the control, 10^6 was significantly different to all other doses and 10^7 was not significantly different to 10^8

Similarly, leaf area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) raw counts (Table 3.2.5) Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). As above, issues with variance (Table A3.2.6) were found and a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.2.7, post hoc test Table A3.2.8) was used to determine whether leaf area of rice changed between 10^2 and 10^8 CFU. Doses up to 10^6 were not significantly different from the control and 10^7 was not significantly different to 10^8. 10^2 was significantly different to 10^6-10^8.

Table A3.2.1 Area measurements in mm^2 of rice roots (*Oryza sativa* L. cv Amaroo) infected with different doses of *B. pseudomallei* TSV189. Doses in (CFU/ml) are listed in the top row. Control represents rice which have no *B. pseudomallei* exposure

Control	10^2	10^3	10^4	10^5	10^6	10^7	10^8
110	198	168	176	213	21	17	26
126	233	181	174	162	43	19	18
146	191	209	207	156	36	18	22
129	177	49	195	175	24	13	40
120	165	226	206	277	86	17	23
148	292	209	167	185	19	29	18
267	285	85	205	192	90	23	21
123	263	135	150	184	83	24	24
156	195	130	265	159	85	19	21
266	216	140	203	215	121	25	25
208	295	204	125	174	39	22	21
231	54	169	203	151	25	29	25
99	281	217	197	156	100	14	65
127	259	279	127	186	104	28	26
285	123	272	219	160	123	24	22
170	188	195	185	201	143	39	23
287	215	174	172	139	191	40	14
129	143	258	173	121	192	52	19
294	227	286	145	162	117	19	19
109	223	288	237	208	148	20	21
215	102	208	253	140	27	21	24
224	231	157	105	156	81	16	16
215	195	254	75	239	203	19	23
211	249	239	148	117	201	35	14
168	195	299	265	114	200	13	13
181	216	220	197	209	191	17	14
149	207	167	156	174	172	21	14
178	170	187	167	223	255	13	22
210	173	137	144	229	150	43	22
206	237	247	207	229	162	28	22
157	262	109	216	231	179	89	18
188	231	276	227	270	20	18	18
146	177	231	267	154	21	17	21
233	171	196	180	247	23	11	22
194	189	246	164	293	170	18	
147	172	204	198	157	204		
174		219	199	158			
289			268	247			
175			151	246			
				128			
				245			

Table A3.2.2 Homogeneity of variances test of root area (*Oryza sativa* L. cv Amaroo) infected with different doses of *B. pseudomallei* TSV189.

Test of Homogeneity of Variances

ROOT

Levene Statistic	df1	df2	Sig.
17.011	7	289	.000

Table A3.2.3 One way ANOVA of root area measurements (*Oryza sativa* L. cv Amaroo) with exposure to different doses of *B. pseudomallei* TSV189. A significant difference was found.

ANOVA

ROOT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1520935.666	7	217276.524	92.290	.000
Within Groups	680388.220	289	2354.284		
Total	2201323.886	296			

Table A3.2.4 Post hoc analysis of root area (*Oryza sativa* L. cv Amaroo) with exposure to different doses of *B. pseudomallei* TSV189 (Games-Howell test). Numbers in the data column represent the log exposure. (Control =0, 10^2=2 and so on)

Multiple Comparisons

Dependent Variable: ROOT

Games-Howell

(I) DATA_R	(J) DATA_R	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CONTROL	102	-21.19658	12.44010	.685	-60.0174	17.6243
	103	-17.53292	13.09868	.881	-58.4072	23.3414
	104	-3.28205	11.36904	1.000	-38.7632	32.1991
	105	-5.44590	11.40351	1.000	-41.0159	30.1241
	106	71.88675*	14.69701	.000	25.8900	117.8835
	107	159.50183*	9.18244	.000	130.2847	188.7189
	108	162.12368*	8.98292	.000	133.4219	190.8254
102	CONTROL	21.19658	12.44010	.685	-17.6243	60.0174
	103	3.66366	13.02861	1.000	-37.0349	44.3622
	104	17.91453	11.28823	.756	-17.3708	53.1999
	105	15.75068	11.32295	.858	-19.6234	51.1248
	106	93.08333*	14.63459	.000	47.2445	138.9221
	107	180.69841*	9.08220	.000	151.6852	209.7116
	108	183.32026*	8.88043	.000	154.8197	211.8208
103	CONTROL	17.53292	13.09868	.881	-23.3414	58.4072
	102	-3.66366	13.02861	1.000	-44.3622	37.0349
	104	14.25087	12.01014	.933	-23.3234	51.8251
	105	12.08701	12.04278	.972	-25.5711	49.7452
	106	89.41967*	15.19838	.000	41.8928	136.9465
	107	177.03475*	9.96523	.000	145.2068	208.8627
	108	179.65660*	9.78168	.000	148.2947	211.0185
104	CONTROL	3.28205	11.36904	1.000	-32.1991	38.7632
	102	-17.91453	11.28823	.756	-53.1999	17.3708
	103	-14.25087	12.01014	.933	-51.8251	23.3234
	105	-2.16385	10.13450	1.000	-33.7303	29.4026
	106	75.16880*	13.73573	.000	31.9907	118.3469
	107	162.78388*	7.54886	.000	138.8394	186.7284
	108	165.40573*	7.30486	.000	142.1022	188.7092
105	CONTROL	5.44590	11.40351	1.000	-30.1241	41.0159
	102	-15.75068	11.32295	.858	-51.1248	19.6234
	103	-12.08701	12.04278	.972	-49.7452	25.5711
	104	2.16385	10.13450	1.000	-29.4026	33.7303
	106	77.33266*	13.76428	.000	34.0814	120.5839
	107	164.94774*	7.60068	.000	140.8899	189.0055
	108	167.56958*	7.35840	.000	144.1537	190.9855
106	CONTROL	-71.88675*	14.69701	.000	-117.8835	-25.8900
	102	-93.08333*	14.63459	.000	-138.9221	-47.2445
	103	-89.41967*	15.19838	.000	-136.9465	-41.8928
	104	-75.16880*	13.73573	.000	-118.3469	-31.9907
	105	-77.33266*	13.76428	.000	-120.5839	-34.0814
	107	87.61508*	11.98885	.000	49.1883	126.0418
	108	90.23693*	11.83673	.000	52.1901	128.2838
107	CONTROL	-159.50183*	9.18244	.000	-188.7189	-130.2847
	102	-180.69841*	9.08220	.000	-209.7116	-151.6852
	103	-177.03475*	9.96523	.000	-208.8627	-145.2068
	104	-162.78388*	7.54886	.000	-186.7284	-138.8394
	105	-164.94774*	7.60068	.000	-189.0055	-140.8899
	106	-87.61508*	11.98885	.000	-126.0418	-49.1883
	108	2.62185	2.90227	.985	-6.5084	11.7521
108	CONTROL	-162.12368*	8.98292	.000	-190.8254	-133.4219
	102	-183.32026*	8.88043	.000	-211.8208	-154.8197
	103	-179.65660*	9.78168	.000	-211.0185	-148.2947
	104	-165.40573*	7.30486	.000	-188.7092	-142.1022
	105	-167.56958*	7.35840	.000	-190.9855	-144.1537
	106	-90.23693*	11.83673	.000	-128.2838	-52.1901
	107	-2.62185	2.90227	.985	-11.7521	6.5084

*. The mean difference is significant at the 0.05 level.

Table A3.2.5 Area measurements in mm^2 of leaves (*Oryza sativa* L. cv Amaroo) infected with different doses of *B. pseudomallei* TSV189. Doses in (CFU/ml) are listed in the top row. Control represents rice which have no *B. pseudomallei* exposure

Control	10^2	10^3	10^4	10^5	10^6	10^7	10^8
70	133	159	62	162	6	31	73
62	132	165	83	82	3	26	13
54	151	138	66	72	17	21	9
91	156	35	31	44	2	22	7
42	144	136	49	88	55	29	7
21	159	47	57	46	22	26	6
33	139	37	119	35	9	5	2
12	139	110	65	230	110	2	9
89	188	103	82	133	169	8	6
97	172	135	86	86	135	3	5
86	139	148	158	76	28	3	9
99	61	170	124	81	15	40	2
29	121	172	123	10	49	30	123
38	191	81	127	66	85	31	30
125	49	84	130	25	143	24	8
46	138	116	31	55	153	28	2
123	163	70	66	23	126	83	3
55	54	104	60	29	119	56	2
88	185	90	38	110	133	14	1
54	118	167	106	61	154	2	2
131	33	26	100	45	2	1	4
114	283	29	16	77	149	3	4
102	42	130	15	153	76	2	2
115	82	143	183	58	70	28	29
105	111	113	111	68	103	27	29
67	117	77	172	100	103	29	28
43	120	25	200	146	75	29	24
167	93	71	134	80	111	25	1
213	143	47	117	78	111	85	2
65	122	50	109	98	137	20	1
204	68	24	123	78	122	104	3
149	65	111	139	239	1	5	1
203	43	85	91	270	3	4	1
73	46	140	119	148	3	5	1
77	232	164	77	89	114	3	
96	148	84	51	46	135		
90		202	59	97			
186			60	153			
222			52	127			
				12			
				123			

Table A3.2.6 Homogeneity of variances test of leaf area (*Oryza sativa* L. cv Amaroo) infected with different doses of *B. pseudomallei* TSV189.

Test of Homogeneity of Variances

LEAF

Levene Statistic	df1	df2	Sig.
7.454	7	289	.000

Table A3.2.7 2.3 ANOVA of leaf area (*Oryza sativa* L. cv Amaroo) infected with different doses of *B. pseudomallei* TSV189.

ANOVA

LEAF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	371387.907	7	53055.415	22.370	.000
Within Groups	685431.454	289	2371.735		
Total	1056819.360	296			

Table A3.2.8 Post hoc test of leaf area in mm^2 (*Oryza sativa* L. cv Amaroo) infected with different doses of *B. pseudomallei* TSV189. Numbers in the data column represent the log exposure. (Control =0, 10^2=2 and so on)

Multiple Comparisons

Dependent Variable: LEAF

Games-Howell

(I) DATA_R	(J) DATA_R	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CONTROL	102	-28.64957	12.84570	.347	-68.7471	11.4479
	103	-6.58351	12.03375	.999	-44.1227	30.9557
	104	3.71795	11.41858	1.000	-31.9093	39.3452
	105	3.13634	12.76019	1.000	-36.6086	42.8812
	106	16.68376	12.92364	.899	-23.6599	57.0274
	107	71.39487*	9.74742	.000	40.6630	102.1267
	108	82.58899*	9.73199	.000	51.8984	113.2796
102	CONTROL	28.64957	12.84570	.347	-11.4479	68.7471
	103	22.06607	12.43666	.639	-16.7993	60.9315
	104	32.36752	11.84243	.131	-4.6769	69.4120
	105	31.78591	13.14083	.248	-9.1961	72.7680
	106	45.33333*	13.29961	.023	3.7792	86.8874
	107	100.04444*	10.24067	.000	67.6101	132.4788
	108	111.23856*	10.22598	.000	78.8428	143.6343
103	CONTROL	6.58351	12.03375	.999	-30.9557	44.1227
	102	-22.06607	12.43666	.639	-60.9315	16.7993
	104	10.30146	10.95638	.981	-23.8963	44.4992
	105	9.71984	12.34830	.993	-28.7739	48.2136
	106	23.26727	12.51714	.583	-15.8536	62.3881
	107	77.97838*	9.20165	.000	48.9531	107.0037
	108	89.17250*	9.18531	.000	60.1909	118.1541
104	CONTROL	-3.71795	11.41858	1.000	-39.3452	31.9093
	102	-32.36752	11.84243	.131	-69.4120	4.6769
	103	-10.30146	10.95638	.981	-44.4992	23.8963
	105	-.58161	11.74961	1.000	-37.2222	36.0589
	106	12.96581	11.92693	.957	-24.3498	50.2814
	107	67.67692*	8.38112	.000	41.3565	93.9973
	108	78.87104*	8.36317	.000	52.5995	105.1426
105	CONTROL	-3.13634	12.76019	1.000	-42.8812	36.6086
	102	-31.78591	13.14083	.248	-72.7680	9.1961
	103	-9.71984	12.34830	.993	-48.2136	28.7739
	104	.58161	11.74961	1.000	-36.0589	37.2222
	106	13.54743	13.21703	.969	-27.6745	54.7694
	107	68.25854*	10.13319	.000	36.3414	100.1757
	108	79.45265*	10.11835	.000	47.5753	111.3300
106	CONTROL	-16.68376	12.92364	.899	-57.0274	23.6599
	102	-45.33333*	13.29961	.023	-86.8874	-3.7792
	103	-23.26727	12.51714	.583	-62.3881	15.8536
	104	-12.96581	11.92693	.957	-50.2814	24.3498
	105	-13.54743	13.21703	.969	-54.7694	27.6745
	107	54.71111*	10.33826	.000	21.9594	87.4628
	108	65.90523*	10.32372	.000	33.1917	98.6188
107	CONTROL	-71.39487*	9.74742	.000	-102.1267	-40.6630
	102	-100.04444*	10.24067	.000	-132.4788	-67.6101
	103	-77.97838*	9.20165	.000	-107.0037	-48.9531
	104	-67.67692*	8.38112	.000	-93.9973	-41.3565
	105	-68.25854*	10.13319	.000	-100.1757	-36.3414
	106	-54.71111*	10.33826	.000	-87.4628	-21.9594
	108	11.19412	5.87969	.553	-7.2017	29.5899
108	CONTROL	-82.58899*	9.73199	.000	-113.2796	-51.8984
	102	-111.23856*	10.22598	.000	-143.6343	-78.8428
	103	-89.17250*	9.18531	.000	-118.1541	-60.1909
	104	-78.87104*	8.36317	.000	-105.1426	-52.5995
	105	-79.45265*	10.11835	.000	-111.3300	-47.5753
	106	-65.90523*	10.32372	.000	-98.6188	-33.1917
	107	-11.19412	5.87969	.553	-29.5899	7.2017

*. The mean difference is significant at the 0.05 level.

A3.3 Statistical analysis of inhibition of rice with *B. vietnamensis* (38SP) (as used in chapter 4.3.4). All statistical analysis were carried out using SPSS version 20.

Root area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured after exposure to 10^8 CFU *B. vietnamensis* (38SP) and compared to control unexposed rice (Table A3.3.1). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). An independent T-Test (p=0.05, Table A3.3.2) was used to determine whether root area of rice differed between control and infected samples. Analysis of variance identified that the assumption of variance was accepted. There was no significant difference between the mean of the two sample groups.

Similarly the leaf area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured and compared to control unexposed rice (Table 3.2.3). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). An independent T-Test (p=0.05, Table A3.3.4) was used to determine whether leaf area of rice differed between control and infected samples. Analysis of variance identified that the assumption of variance was accepted. There was no significant difference between the mean of the two sample groups.

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Table A3.3.1 Area measurements in mm^2 of roots (*Oryza sativa* L. cv Amaroo) infected with *B. vietnamensis* (38SP). Control represents rice which have no *B. vietnamensis* exposure

Root	
Control	***B. vietnamensis* (38SP)**
263	262
224	186
201	205
181	257
188	170
177	201
272	267
209	223
196	281
270	254
211	209
210	63
181	226
111	179
147	209
273	112
192	123
218	216
206	200
279	211
221	261
242	50
246	234
248	110
256	185
231	206
224	263
233	185
	192
	283
	294

Table A3.3.2 T-Test of root area of ***Oryza sativa* L. cv Amaroo infected with *B. vietnamensis* (38SP).**

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
R_38sp	Equal variances assumed	2.592	.113	1.066	57	.291	14.43975	13.54897	-12.69160	41.57109
	Equal variances not assumed			1.089	51.764	.281	14.43975	13.26238	-12.17605	41.05554

Table A3.3.3 Area measurements in mm^2 of leaves (*Oryza sativa* L. cv Amaroo) infected with *B. vietnamensis* (38SP). Control represents rice which have no *B. vietnamensis* exposure

Leaf	
Control	***B. vietnamensis* (38SP)**
145	61
72	57
146	129
146	167
163	68
173	166
194	205
189	34
141	92
109	143
161	147
175	17
104	175
38	102
5	229
128	64
76	37
198	157
154	131
105	70
149	209
165	16
188	93
186	32
88	42
149	136
191	144
103	206
	43
	114
	108

Table A3.3.4 T-Test of leaf area (*Oryza sativa* L. cv Amaroo) infected with *B. vietnamensis* (38SP).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
L_38sp	Equal variances assumed	3.094	.084	1.888	57	.064	27.65101	14.64916	-1.68343	56.98546
	Equal variances not assumed			1.910	56.061	.061	27.65101	14.47543	-1.34607	56.64810

A3.4 Analysis of Survival data obtained in ¼ strength Hoagland solution for (a) near-neighbour species *B. ubonensis* (A21) and *B. cenocepacia* (17SP) and (b) *B. pseudomallei* (TSV192 and K96243) (as used in chapter 5.3.2). All statistical analysis were carried out using SPSS version 20.

Raw counts of *B. ubonensis* (A21) (Table A.3.4.1) were analysed for normality of distribution (Table A3.4.2) and homogeneity of variance (Table A3.4.3). Distribution was normal while variances were not homogeneous. Data was analysed in a one way ANOVA with LSD post hoc test was carried out as the ANOVA identified significantly differences but the Games Howell post hoc test was not sensitive enough to pick up where these were (p=0.05, ANOVA Table A3.4.4, post hoc test Table A3.4.5). The initial inoculum counts were not included Day 6 and 7 means were significantly different to day 1 to 4.

Similarly, *B. cenocepacia* (17SP) raw counts (Table A3.4.6) were analysed for normality of distribution (Table A3.4.7). Distribution was normal. Analysis of homogeneity of variance indicated variances were not homogeneous (Table A3.4.8). An ANOVA with LSD post hoc test was carried out as the ANOVA identified significantly differences but the Games Howell post hoc test was not sensitive enough to pick up where these were (p=0.05, ANOVA Table A3.4.9, Post hoc test Table A3.4.10). The initial inoculum counts were not included. Day 1 to 4 are significantly different to day 6, 7 and 90 and there were also other differences noted.

Similarly, raw counts of *B. pseudomallei* TSV192 (Table A.3.4.11) were analysed for normality of distribution (Table A3.4.12) and normal distribution were violated at day 4 and 5, so data was log transformed (Table A3.4.13). All transformed data was normally distributed (Table A3.4.13) Variances of transformed data was homogeneous (Table A3.4.14). Transformed data was used in a one way ANOVA with Gabriel post hoc test (p=0.05, ANOVA Table A3.4.15, post hoc test Table A3.4.16) to determine whether bacterial counts changed between day 1 and day 90. The initial inoculum counts were not included. No significant difference between day 1 and 90 was found.

Similarly, *B. pseudomallei* K96243 raw counts (Table A3.4.17) were analysed for normality of distribution (Table A3.4.18). All data was normally distributed. Analysis of homogeneity of variance determined variances were homogeneous (Table A3.4.19). An ANOVA with Gabriel post hoc test (p=0.05, ANOVA Table A3.4.20, post hoc test Table A3.4.21) was used to determine whether bacterial counts changed between day 1 and day 90. Day 90 had significantly lower bacterial numbers than day one. No other significant differences were found.

Table A3.4.1 Raw data of replicates of bacterial counts/ml of *B. ubonensis* (A21) TSV189 over 90 days. The top row represents the day after incubation at which counts were made.

0	1	2	3	4	5	6	7	90
1.25E+06	1.20E+07	6.50E+06	5.00E+06	1.00E+07	5.00E+06	6.00E+06	3.98E+07	1.00E+07
1.10E+06	1.40E+07	1.15E+07	5.00E+06	1.50E+07	3.50E+07	3.66E+07	5.53E+07	1.50E+07
7.00E+05	1.45E+07	1.85E+07	1.00E+07	1.50E+07	4.00E+07	6.20E+07	5.12E+07	2.00E+07
	5.00E+06	9.00E+06	1.00E+07	7.50E+06	5.10E+06	2.27E+07	1.54E+07	2.00E+07
	5.50E+06	5.50E+06	1.50E+07	1.05E+07	1.04E+07	2.50E+07	1.56E+07	2.50E+07
	9.00E+06	8.50E+06	3.00E+07	8.00E+06	2.65E+07	2.67E+07	1.25E+07	3.00E+07

Table A3.4.2 Test for normality of *B. ubonensis* (A21) bacterial counts.

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data_sk_a21	1 day	.194	6	.200*
	2 day	.244	6	.200*
	3 day	.272	6	.187
	4 day	.227	6	.200*
	5 day	.239	6	.200*
	6 day	.234	6	.200*
	7 day	.295	6	.112
	90 day	.167	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.4.3 Test for homogeneity of variances of *B. ubonensis* (A21) bacterial counts.

Test of Homogeneity of Variances

data_sk_a21

Levene Statistic	df1	df2	Sig.
5.750	7	40	.000

Table A3.4.4 One way ANOVA of bacterial counts of *B. ubonensis* (A21) from day 1 to day 90.

ANOVA

data_sk_a21

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.262E+15	7	4.661E+14	3.229	.008
Within Groups	5.773E+15	40	1.443E+14		
Total	9.036E+15	47			

Table A3.4.5 Post hoc Analysis of *B. ubonensis* (A21) bacterial **counts (Games-Howell test).**

Multiple Comparisons

Dependent Variable: data_sk_a21

LSD

(I) gr_sk_a21	(J) gr_sk_a21	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 day	2 day	83333.33333	6936238.374	.990	-13935327.3	14101994.01
	3 day	-2500000.00	6936238.374	.720	-16518660.7	11518660.68
	4 day	-1000000.00	6936238.374	.886	-15018660.7	13018660.68
	5 day	-10333333.3	6936238.374	.144	-24351994.0	3685327.345
	6 day	-19833333.3*	6936238.374	.007	-33851994.0	-5814672.65
	7 day	-21633333.3*	6936238.374	.003	-35651994.0	-7614672.65
	90 day	-10000000.0	6936238.374	.157	-24018660.7	4018660.679
2 day	1 day	-83333.33333	6936238.374	.990	-14101994.0	13935327.35
	3 day	-2583333.33	6936238.374	.712	-16601994.0	11435327.35
	4 day	-1083333.33	6936238.374	.877	-15101994.0	12935327.35
	5 day	-10416666.7	6936238.374	.141	-24435327.3	3601994.012
	6 day	-19916666.7*	6936238.374	.007	-33935327.3	-5898005.99
	7 day	-21716666.7*	6936238.374	.003	-35735327.3	-7698005.99
	90 day	-10083333.3	6936238.374	.154	-24101994.0	3935327.345
3 day	1 day	2500000.000	6936238.374	.720	-11518660.7	16518660.68
	2 day	2583333.333	6936238.374	.712	-11435327.3	16601994.01
	4 day	1500000.000	6936238.374	.830	-12518660.7	15518660.68
	5 day	-7833333.33	6936238.374	.265	-21851994.0	6185327.345
	6 day	-17333333.3*	6936238.374	.017	-31351994.0	-3314672.65
	7 day	-19133333.3*	6936238.374	.009	-33151994.0	-5114672.65
	90 day	-7500000.00	6936238.374	.286	-21518660.7	6518660.679
4 day	1 day	1000000.000	6936238.374	.886	-13018660.7	15018660.68
	2 day	1083333.333	6936238.374	.877	-12935327.3	15101994.01
	3 day	-1500000.00	6936238.374	.830	-15518660.7	12518660.68
	5 day	-9333333.33	6936238.374	.186	-23351994.0	4685327.345
	6 day	-18833333.3*	6936238.374	.010	-32851994.0	-4814672.65
	7 day	-20633333.3*	6936238.374	.005	-34651994.0	-6614672.65
	90 day	-9000000.00	6936238.374	.202	-23018660.7	5018660.679
5 day	1 day	10333333.33	6936238.374	.144	-3685327.35	24351994.01
	2 day	10416666.67	6936238.374	.141	-3601994.01	24435327.35
	3 day	7833333.333	6936238.374	.265	-6185327.35	21851994.01
	4 day	9333333.333	6936238.374	.186	-4685327.35	23351994.01
	6 day	-9500000.00	6936238.374	.178	-23518660.7	4518660.679
	7 day	-11300000.0	6936238.374	.111	-25318660.7	2718660.679
	90 day	333333.3333	6936238.374	.962	-13685327.3	14351994.01
6 day	1 day	19833333.3*	6936238.374	.007	5814672.655	33851994.01
	2 day	19916666.7*	6936238.374	.007	5898005.988	33935327.35
	3 day	17333333.3*	6936238.374	.017	3314672.655	31351994.01
	4 day	18833333.3*	6936238.374	.010	4814672.655	32851994.01
	5 day	9500000.000	6936238.374	.178	-4518660.68	23518660.68
	7 day	-1800000.00	6936238.374	.797	-15818660.7	12218660.68
	90 day	9833333.333	6936238.374	.164	-4185327.35	23851994.01
7 day	1 day	21633333.3*	6936238.374	.003	7614672.655	35651994.01
	2 day	21716666.7*	6936238.374	.003	7698005.988	35735327.35
	3 day	19133333.3*	6936238.374	.009	5114672.655	33151994.01
	4 day	20633333.3*	6936238.374	.005	6614672.655	34651994.01
	5 day	11300000.00	6936238.374	.111	-2718660.68	25318660.68
	6 day	1800000.000	6936238.374	.797	-12218660.7	15818660.68
	90 day	11633333.33	6936238.374	.101	-2385327.35	25651994.01
90 day	1 day	10000000.00	6936238.374	.157	-4018660.68	24018660.68
	2 day	10083333.33	6936238.374	.154	-3935327.35	24101994.01
	3 day	7500000.000	6936238.374	.286	-6518660.68	21518660.68
	4 day	9000000.000	6936238.374	.202	-5018660.68	23018660.68
	5 day	-333333.333	6936238.374	.962	-14351994.0	13685327.35
	6 day	-9833333.33	6936238.374	.164	-23851994.0	4185327.345
	7 day	-11633333.3	6936238.374	.101	-25651994.0	2385327.345

*. The mean difference is significant at the 0.05 level.

Table A3.4.6 Raw data of replicates of bacterial counts/ml of *B. cenocepacia* (17SP) over 90 days. The top row represents the day after incubation at which counts were made.

0	1	2	3	4	5	6	7	90
5.00E+06	5.00E+06	5.00E+06	5.00E+06	1.00E+07	1.00E+07	1.50E+07	1.44E+07	1.00E+07
5.00E+06	1.00E+07	5.00E+06	1.00E+07	1.00E+07	2.00E+07	2.26E+07	2.19E+07	1.50E+07
4.00E+06	1.00E+07	1.00E+07	1.50E+07	2.50E+07	2.50E+07	2.79E+07	2.22E+07	1.50E+07
	7.00E+06	1.00E+07	5.00E+06	5.00E+06	5.10E+06	1.39E+07	3.00E+07	2.00E+07
	6.50E+06	1.00E+07	1.25E+07	1.00E+07	5.20E+06	1.92E+07	3.80E+07	2.00E+07
	7.50E+06	5.00E+06	1.20E+07	1.50E+07	5.30E+06	2.47E+07	4.62E+07	3.00E+07

Table A3.4.7 Test for normality of *B. cenocepacia* (17SP) bacterial counts

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data_sk_17sp	1 day	.213	6	.200*
	2 day	.319	6	.056
	3 day	.217	6	.200*
	4 day	.308	6	.077
	5 day	.272	6	.186
	6 day	.165	6	.200*
	7 day	.213	6	.200*
	90 day	.237	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.4.8 Test for homogeneity of variances of *B. cenocepacia* (17SP) bacterial counts.

Test of Homogeneity of Variances

data_sk_17sp

Levene Statistic	df1	df2	Sig.
3.071	7	40	.011

Table A3.4.9 One way ANOVA of bacterial counts of *B. cenocepacia* (17SP) from day 1 to day 90. A Significant difference was found.

ANOVA

data_sk_17sp

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.245E+15	7	3.206E+14	6.734	.000
Within Groups	1.905E+15	40	4.762E+13		
Total	4.149E+15	47			

Table A3.4.10 Post hoc Analysis of *B. cenocepacia* (17SP) counts (Games-Howell test).

Multiple Comparisons

Dependent Variable: data_sk_17sp

LSD

(I) gr_sk_17sp	(J) gr_sk_17sp	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 day	2 day	166666.6667	3984119.518	.967	-7885539.24	8218872.576
	3 day	-2250000.00	3984119.518	.575	-10302205.9	5802205.910
	4 day	-4833333.33	3984119.518	.232	-12885539.2	3218872.576
	5 day	-4100000.00	3984119.518	.310	-12152205.9	3952205.910
	6 day	-12050000.0*	3984119.518	.004	-20102205.9	-3997794.09
	7 day	-21116666.7*	3984119.518	.000	-29168872.6	-13064460.8
	90 day	-10666666.7*	3984119.518	.011	-18718872.6	-2614460.76
2 day	1 day	-166666.667	3984119.518	.967	-8218872.58	7885539.243
	3 day	-2416666.67	3984119.518	.548	-10468872.6	5635539.243
	4 day	-5000000.00	3984119.518	.217	-13052205.9	3052205.910
	5 day	-4266666.67	3984119.518	.291	-12318872.6	3785539.243
	6 day	-12216666.7*	3984119.518	.004	-20268872.6	-4164460.76
	7 day	-21283333.3*	3984119.518	.000	-29335539.2	-13231127.4
	90 day	-10833333.3*	3984119.518	.010	-18885539.2	-2781127.42
3 day	1 day	2250000.000	3984119.518	.575	-5802205.91	10302205.91
	2 day	2416666.667	3984119.518	.548	-5635539.24	10468872.58
	4 day	-2583333.33	3984119.518	.520	-10635539.2	5468872.576
	5 day	-1850000.00	3984119.518	.645	-9902205.91	6202205.910
	6 day	-9800000.00*	3984119.518	.018	-17852205.9	-1747794.09
	7 day	-18866666.7*	3984119.518	.000	-26918872.6	-10814460.8
	90 day	-8416666.67*	3984119.518	.041	-16468872.6	-364460.7569
4 day	1 day	4833333.333	3984119.518	.232	-3218872.58	12885539.24
	2 day	5000000.000	3984119.518	.217	-3052205.91	13052205.91
	3 day	2583333.333	3984119.518	.520	-5468872.58	10635539.24
	5 day	733333.3333	3984119.518	.855	-7318872.58	8785539.243
	6 day	-7216666.67	3984119.518	.078	-15268872.6	835539.2431
	7 day	-16283333.3*	3984119.518	.000	-24335539.2	-8231127.42
	90 day	-5833333.33	3984119.518	.151	-13885539.2	2218872.576
5 day	1 day	4100000.000	3984119.518	.310	-3952205.91	12152205.91
	2 day	4266666.667	3984119.518	.291	-3785539.24	12318872.58
	3 day	1850000.000	3984119.518	.645	-6202205.91	9902205.910
	4 day	-733333.333	3984119.518	.855	-8785539.24	7318872.576
	6 day	-7950000.00	3984119.518	.053	-16002205.9	102205.9098
	7 day	-17016666.7*	3984119.518	.000	-25068872.6	-8964460.76
	90 day	-6566666.67	3984119.518	.107	-14618872.6	1485539.243
6 day	1 day	12050000.0*	3984119.518	.004	3997794.090	20102205.91
	2 day	12216666.7*	3984119.518	.004	4164460.757	20268872.58
	3 day	9800000.00*	3984119.518	.018	1747794.090	17852205.91
	4 day	7216666.667	3984119.518	.078	-835539.2431	15268872.58
	5 day	7950000.000	3984119.518	.053	-102205.9098	16002205.91
	7 day	-9066666.67*	3984119.518	.028	-17118872.6	-1014460.76
	90 day	1383333.333	3984119.518	.730	-6668872.58	9435539.243
7 day	1 day	21116666.7*	3984119.518	.000	13064460.76	29168872.58
	2 day	21283333.3*	3984119.518	.000	13231127.42	29335539.24
	3 day	18866666.7*	3984119.518	.000	10814460.76	26918872.58
	4 day	16283333.3*	3984119.518	.000	8231127.424	24335539.24
	5 day	17016666.7*	3984119.518	.000	8964460.757	25068872.58
	6 day	9066666.67*	3984119.518	.028	1014460.757	17118872.58
	90 day	10450000.0*	3984119.518	.012	2397794.090	18502205.91
90 day	1 day	10666666.7*	3984119.518	.011	2614460.757	18718872.58
	2 day	10833333.3*	3984119.518	.010	2781127.424	18885539.24
	3 day	8416666.67*	3984119.518	.041	364460.7569	16468872.58
	4 day	5833333.333	3984119.518	.151	-2218872.58	13885539.24
	5 day	6566666.667	3984119.518	.107	-1485539.24	14618872.58
	6 day	-1383333.33	3984119.518	.730	-9435539.24	6668872.576
	7 day	-10450000.0*	3984119.518	.012	-18502205.9	-2397794.09

*. The mean difference is significant at the 0.05 level.

Table A3.4.11 Raw data of replicates of bacterial counts/ml of *B. pseudomallei* TSV192 over 90 days. The top row represents the day after incubation at which counts were made.

0	1	2	3	4	5	6	7	90
1.50E+05	4.50E+06	1.00E+07	1.50E+07	3.00E+07	5.00E+06	5.00E+06	5.00E+06	8.50E+06
2.50E+05	4.50E+06	1.50E+07	2.00E+07	1.50E+07	1.00E+07	1.00E+07	2.00E+07	7.50E+06
2.00E+05	9.00E+06	1.00E+07	2.50E+07	5.00E+06	2.00E+07	2.00E+07	2.00E+07	7.00E+06
	5.50E+06	7.00E+06	1.20E+07	1.45E+07	1.00E+07	5.00E+06	1.00E+07	6.00E+06
	6.00E+06	7.50E+06	1.20E+07	1.35E+07	1.00E+07	1.00E+07	5.00E+06	6.00E+06
	1.30E+07	6.50E+06	1.30E+07	1.10E+07	2.00E+07	5.00E+06	5.00E+06	4.00E+06

Table A3.4.12 Test for normality of *B. pseudomallei* TSV192 bacterial counts.

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data_sk_tsv192	1 day	.294	6	.115
	2 day	.250	6	.200*
	3 day	.254	6	.200*
	4 day	.325	6	.046
	5 day	.325	6	.047
	6 day	.277	6	.168
	7 day	.286	6	.136
	90 day	.207	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.4.13 Test for normality of log transformed *B. pseudomallei* TSV192 bacterial counts.

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
LOGTSV192	1 day	.247	6	.200*
	2 day	.214	6	.200*
	3 day	.222	6	.200*
	4 day	.236	6	.200*
	5 day	.254	6	.200*
	6 day	.293	6	.117
	7 day	.302	6	.094
	90 day	.253	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.4.14 Test for homogeneity of variances of log transformed *B. pseudomallei* TSV192 bacterial counts

Test of Homogeneity of Variances

LOGTSV192

Levene Statistic	df1	df2	Sig.
1.565	7	40	.174

Table A3.4.15 One way ANOVA of log transformed bacterial counts of *B. pseudomallei* TSV192 from day 1 to day 90. A Significant difference was found.

ANOVA

LOGTSV192

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.805	7	.115	2.670	.023
Within Groups	1.723	40	.043		
Total	2.528	47			

Table A3.4.16 Post hoc analysis of log transformed *B. pseudomallei* TSV192 counts (Gabriel test).

Multiple Comparisons

Dependent Variable: LOGTSV192

Gabriel

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 day	2 day	-.13601	.11982	.999	-.5339	.2619
	3 day	-.37571	.11982	.081	-.7736	.0221
	4 day	-.29869	.11982	.345	-.6965	.0992
	5 day	-.23465	.11982	.750	-.6325	.1632
	6 day	-.08414	.11982	1.000	-.4820	.3137
	7 day	-.13431	.11982	.999	-.5322	.2635
	90 day	.01420	.11982	1.000	-.3837	.4121
2 day	1 day	.13601	.11982	.999	-.2619	.5339
	3 day	-.23970	.11982	.718	-.6376	.1582
	4 day	-.16269	.11982	.990	-.5605	.2352
	5 day	-.09864	.11982	1.000	-.4965	.2992
	6 day	.05187	.11982	1.000	-.3460	.4497
	7 day	.00170	.11982	1.000	-.3962	.3996
	90 day	.15020	.11982	.996	-.2477	.5481
3 day	1 day	.37571	.11982	.081	-.0221	.7736
	2 day	.23970	.11982	.718	-.1582	.6376
	4 day	.07702	.11982	1.000	-.3208	.4749
	5 day	.14106	.11982	.998	-.2568	.5389
	6 day	.29157	.11982	.384	-.1063	.6894
	7 day	.24140	.11982	.708	-.1565	.6393
	90 day	.38990	.11982	.060	-.0080	.7878
4 day	1 day	.29869	.11982	.345	-.0992	.6965
	2 day	.16269	.11982	.990	-.2352	.5605
	3 day	-.07702	.11982	1.000	-.4749	.3208
	5 day	.06404	.11982	1.000	-.3338	.4619
	6 day	.21456	.11982	.859	-.1833	.6124
	7 day	.16438	.11982	.989	-.2335	.5622
	90 day	.31289	.11982	.273	-.0850	.7107
5 day	1 day	.23465	.11982	.750	-.1632	.6325
	2 day	.09864	.11982	1.000	-.2992	.4965
	3 day	-.14106	.11982	.998	-.5389	.2568
	4 day	-.06404	.11982	1.000	-.4619	.3338
	6 day	.15051	.11982	.996	-.2473	.5484
	7 day	.10034	.11982	1.000	-.2975	.4982
	90 day	.24885	.11982	.659	-.1490	.6467
6 day	1 day	.08414	.11982	1.000	-.3137	.4820
	2 day	-.05187	.11982	1.000	-.4497	.3460
	3 day	-.29157	.11982	.384	-.6894	.1063
	4 day	-.21456	.11982	.859	-.6124	.1833
	5 day	-.15051	.11982	.996	-.5484	.2473
	7 day	-.05017	.11982	1.000	-.4480	.3477
	90 day	.09833	.11982	1.000	-.2995	.4962
7 day	1 day	.13431	.11982	.999	-.2635	.5322
	2 day	-.00170	.11982	1.000	-.3996	.3962
	3 day	-.24140	.11982	.708	-.6393	.1565
	4 day	-.16438	.11982	.989	-.5622	.2335
	5 day	-.10034	.11982	1.000	-.4982	.2975
	6 day	.05017	.11982	1.000	-.3477	.4480
	90 day	.14850	.11982	.997	-.2494	.5464
90 day	1 day	-.01420	.11982	1.000	-.4121	.3837
	2 day	-.15020	.11982	.996	-.5481	.2477
	3 day	-.38990	.11982	.060	-.7878	.0080
	4 day	-.31289	.11982	.273	-.7107	.0850
	5 day	-.24885	.11982	.659	-.6467	.1490
	6 day	-.09833	.11982	1.000	-.4962	.2995
	7 day	-.14850	.11982	.997	-.5464	.2494

Table A3.4.17 Raw data of replicates of bacterial counts/ml of *B. pseudomallei* K96243 over 90 days. The top row represents the day after incubation at which counts were made.

0	1	2	3	4	5	6	7	90
3.00E+06	1.30E+07	5.00E+07	5.00E+06	1.50E+07	1.00E+07	5.00E+06	2.50E+07	5.00E+06
3.00E+06	1.35E+07	2.50E+07	1.50E+07	2.50E+07	3.00E+07	1.50E+07	4.50E+07	1.50E+07
3.00E+06	1.15E+07	3.00E+07	2.50E+07	3.00E+07	3.50E+07	3.50E+07	3.00E+07	2.00E+07
	5.50E+06	2.45E+07	1.50E+07	3.50E+07	2.50E+07	1.50E+07	4.00E+07	2.00E+07
	6.00E+06	1.60E+07	2.00E+07	2.50E+07	2.50E+07	3.00E+07	2.00E+07	3.00E+07
	8.00E+06	1.75E+07	2.50E+07	1.50E+07	4.00E+07	2.50E+07	2.50E+07	4.50E+07

Table A3.4.18 Test for normality of *B. pseudomallei* K96243 bacterial counts.

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data_sk_k96243	1 day	.206	6	.200*
	2 day	.242	6	.200*
	3 day	.204	6	.200*
	4 day	.208	6	.200*
	5 day	.238	6	.200*
	6 day	.200	6	.200*
	7 day	.159	6	.200*
	90 day	.255	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.4.19 Test for homogeneity of variances of *B. pseudomallei* K96243 bacterial counts.

Test of Homogeneity of Variances

data_sk_k96243

Levene Statistic	df1	df2	Sig.
1.349	7	40	.254

Table A3.4.20 One way ANOVA of bacterial counts of *B. pseudomallei* K96243 from day 1 to day 90.

ANOVA

data_sk_k96243

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.043E+15	7	2.919E+14	2.709	.021
Within Groups	4.310E+15	40	1.077E+14		
Total	6.352E+15	47			

Table A3.4.21 Post hoc analysis of *B. pseudomallei* K96243 counts (Gabriel test).

Multiple Comparisons

Dependent Variable: data_sk_k96243

Gabriel

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1 day	2 day	-17583333.3	5992732.868	.133	-37482362.0	2315695.316
	3 day	-7916666.67	5992732.868	.993	-27815695.3	11982361.98
	4 day	-14583333.3	5992732.868	.384	-34482362.0	5315695.316
	5 day	-17916666.7	5992732.868	.117	-37815695.3	1982361.983
	6 day	-11250000.0	5992732.868	.806	-31149028.6	8649028.650
	7 day	-19583333.3	5992732.868	.058	-39482362.0	315695.3162
	90 day	-20416666.7*	5992732.868	.040	-40315695.3	-517638.0171
2 day	1 day	17583333.33	5992732.868	.133	-2315695.32	37482361.98
	3 day	9666666.667	5992732.868	.939	-10232362.0	29565695.32
	4 day	3000000.000	5992732.868	1.000	-16899028.6	22899028.65
	5 day	-333333.333	5992732.868	1.000	-20232362.0	19565695.32
	6 day	6333333.333	5992732.868	1.000	-13565695.3	26232361.98
	7 day	-2000000.00	5992732.868	1.000	-21899028.6	17899028.65
	90 day	-2833333.33	5992732.868	1.000	-22732362.0	17065695.32
3 day	1 day	7916666.667	5992732.868	.993	-11982362.0	27815695.32
	2 day	-9666666.67	5992732.868	.939	-29565695.3	10232361.98
	4 day	-6666666.67	5992732.868	.999	-26565695.3	13232361.98
	5 day	-10000000.0	5992732.868	.919	-29899028.6	9899028.650
	6 day	-3333333.33	5992732.868	1.000	-23232362.0	16565695.32
	7 day	-11666666.7	5992732.868	.758	-31565695.3	8232361.983
	90 day	-12500000.0	5992732.868	.652	-32399028.6	7399028.650
4 day	1 day	14583333.33	5992732.868	.384	-5315695.32	34482361.98
	2 day	-3000000.00	5992732.868	1.000	-22899028.6	16899028.65
	3 day	6666666.667	5992732.868	.999	-13232362.0	26565695.32
	5 day	-3333333.33	5992732.868	1.000	-23232362.0	16565695.32
	6 day	3333333.333	5992732.868	1.000	-16565695.3	23232361.98
	7 day	-5000000.00	5992732.868	1.000	-24899028.6	14899028.65
	90 day	-5833333.33	5992732.868	1.000	-25732362.0	14065695.32
5 day	1 day	17916666.67	5992732.868	.117	-1982361.98	37815695.32
	2 day	333333.3333	5992732.868	1.000	-19565695.3	20232361.98
	3 day	10000000.00	5992732.868	.919	-9899028.65	29899028.65
	4 day	3333333.333	5992732.868	1.000	-16565695.3	23232361.98
	6 day	6666666.667	5992732.868	.999	-13232362.0	26565695.32
	7 day	-1666666.67	5992732.868	1.000	-21565695.3	18232361.98
	90 day	-2500000.00	5992732.868	1.000	-22399028.6	17399028.65
6 day	1 day	11250000.00	5992732.868	.806	-8649028.65	31149028.65
	2 day	-6333333.33	5992732.868	1.000	-26232362.0	13565695.32
	3 day	3333333.333	5992732.868	1.000	-16565695.3	23232361.98
	4 day	-3333333.33	5992732.868	1.000	-23232362.0	16565695.32
	5 day	-6666666.67	5992732.868	.999	-26565695.3	13232361.98
	7 day	-8333333.33	5992732.868	.987	-28232362.0	11565695.32
	90 day	-9166666.67	5992732.868	.963	-29065695.3	10732361.98
7 day	1 day	19583333.33	5992732.868	.058	-315695.3162	39482361.98
	2 day	2000000.000	5992732.868	1.000	-17899028.6	21899028.65
	3 day	11666666.67	5992732.868	.758	-8232361.98	31565695.32
	4 day	5000000.000	5992732.868	1.000	-14899028.6	24899028.65
	5 day	1666666.667	5992732.868	1.000	-18232362.0	21565695.32
	6 day	8333333.333	5992732.868	.987	-11565695.3	28232361.98
	90 day	-833333.333	5992732.868	1.000	-20732362.0	19065695.32
90 day	1 day	20416666.7*	5992732.868	.040	517638.0171	40315695.32
	2 day	2833333.333	5992732.868	1.000	-17065695.3	22732361.98
	3 day	12500000.00	5992732.868	.652	-7399028.65	32399028.65
	4 day	5833333.333	5992732.868	1.000	-14065695.3	25732361.98
	5 day	2500000.000	5992732.868	1.000	-17399028.6	22399028.65
	6 day	9166666.667	5992732.868	.963	-10732362.0	29065695.32
	7 day	833333.3333	5992732.868	1.000	-19065695.3	20732361.98

*. The mean difference is significant at the 0.05 level.

A3.5 Statistical analysis of response of *Oryza sativa* L. cv Amaroo to a range of *Burkholderia* species (as used in chapter 5.3.3). All statistical analysis were carried out using SPSS version 20.

The root area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured after exposure to different doses of *Burkholderia species* (Table A3.5.1).). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance (Table A3.5.2) identified that the assumption of homogeneity of variance was violated for ANOVA so a Games-Howell post hoc test was selected for use with the ANOVA as it is advised in cases where variances are not equal (Field, 2005) A one way ANOVA with Games-Howell post hoc test(p=0.05, ANOVA Table A3.5.3, Post hoc test Table A3.5.4) was used to determine whether root area of rice changed due to exposure to various *Burkholderia* species and strains. Significant differences were found and are discussed in Chapter 5

Similarly, leaf area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) raw counts (Table 3.5.5) were analysed. Normality was assumed and analysis of variance (Table A3.5.6) indicated variances were not homogeneous. Data was analysed with a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.5.7, Post hoc test Table A3.5.8) to determine whether leaf area of rice changed after exposure to a range of *Burkholderia* species. Significant differences were found and are discussed in Chapter 5

Table A3.5.1 Area measurements in mm^2 of roots (*Oryza sativa* L. cv Amaroo) infected with a range of *Burkholderia* species as listed in the top row of the table. Control represents rice which have no *Burkholderia* exposure

B. vietnamiensis (38sp)	*B. ubonensis* (A21)	*B. cenocepacia* (17sp)	*B. pseudomallei* (TSV189)	*B. pseudomallei* (TSV192)	*B. pseudomallei* (K96243)
120	73	8	14	19	10
85	24	9	10	22	13
94	49	7	12	14	17
118	96	7	22	12	11
78	37	33	12	15	11
92	48	10	10	13	13
123	52	16	11	12	13
102	35	13	13	11	12
129	70	25	12	12	12
116	26	66	14	15	7
96	19	10	12	14	10
29	29	8	14	17	9
104	37	7	35	13	13
82	32	22	14	29	8
96	29	9	12	12	10
51	31	30	13	11	10
56	33	35	8	12	10
99	18	31	10	11	12
92	42	11	10	11	10
97	36	14	11	12	10
120	17	8	13	12	11
20	29	9	9	10	9
23	21	6	12	9	9
107	42	8	8	7	13
51	42	10	7	6	10
85	58	6	8	14	7
94	75	18	8	8	12
121	32	32	12	13	11
85	32	12	12	14	12
88	55	14	12	12	13
130	22	14	10	14	11
135	31		10	14	13
	38		11	11	10
	25		12	13	13
	39			13	10
	46				
	24				
	27				

Table A3.5.2 Homogeneity of variances test of root area of *Oryza sativa* L. cv Amaroo infected with a range of *Burkholderia* species.

Test of Homogeneity of Variances

Percentage_root_data

Levene Statistic	df1	df2	Sig.
21.622	5	199	.000

Table A3.5.3 One way ANOVA of root area measurements (*Oryza sativa* L. cv Amaroo) with exposure to **a range of *Burkholderia* species.**

ANOVA

Percentage_root_data

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	161931.650	5	32386.330	139.060	.000
Within Groups	46346.183	199	232.895		
Total	208277.833	204			

Table A3.5.4 Post hoc analysis of root area of *Oryza sativa* L. cv Amaroo infected with a range of *Burkholderia* species (Games-Howell test). Species are listed under (J)Group

Multiple Comparisons

Dependent Variable: Percentage_root_data

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
B. vietnamensis (38SP)	B. ubonensis (A21)	52.42080*	6.07623	.000	34.3790	70.4627
	B. cenocepacia (17SP)	74.76277*	5.84953	.000	57.2991	92.2264
	B. pseudomallei (TSV189)	79.05132*	5.44324	.000	62.5787	95.5239
	B. pseudomallei (TSV191)	78.15118*	5.42149	.000	61.7287	94.5737
	B. pseudomallei (K96243)	80.13970*	5.38940	.000	63.7905	96.4889
B. ubonensis (A21)	B. vietnamensis (38SP)	-52.42080*	6.07623	.000	-70.4627	-34.3790
	B. cenocepacia (17SP)	22.34197*	3.64518	.000	11.6446	33.0393
	B. pseudomallei (TSV189)	26.63052*	2.94944	.000	17.8392	35.4219
	B. pseudomallei (TSV191)	25.73037*	2.90911	.000	17.0391	34.4217
	B. pseudomallei (K96243)	27.71890*	2.84886	.000	19.1738	36.2640
B. cenocepacia (17SP)	B. vietnamensis (38SP)	-74.76277*	5.84953	.000	-92.2264	-57.2991
	B. ubonensis (A21)	-22.34197*	3.64518	.000	-33.0393	-11.6446
	B. pseudomallei (TSV189)	4.28855	2.44861	.508	-3.0582	11.6353
	B. pseudomallei (TSV191)	3.38840	2.39988	.720	-3.8397	10.6165
	B. pseudomallei (K96243)	5.37693	2.32648	.220	-1.6791	12.4329
B. pseudomallei (TSV189)	B. vietnamensis (38SP)	-79.05132*	5.44324	.000	-95.5239	-62.5787
	B. ubonensis (A21)	-26.63052*	2.94944	.000	-35.4219	-17.8392
	B. cenocepacia (17SP)	-4.28855	2.44861	.508	-11.6353	3.0582
	B. pseudomallei (TSV191)	-.90015	1.08224	.960	-4.0796	2.2793
	B. pseudomallei (K96243)	1.08838	.90794	.835	-1.6162	3.7930
B. pseudomallei (TSV191)	B. vietnamensis (38SP)	-78.15118*	5.42149	.000	-94.5737	-61.7287
	B. ubonensis (A21)	-25.73037*	2.90911	.000	-34.4217	-17.0391
	B. cenocepacia (17SP)	-3.38840	2.39988	.720	-10.6165	3.8397
	B. pseudomallei (TSV189)	.90015	1.08224	.960	-2.2793	4.0796
	B. pseudomallei (K96243)	1.98853	.76685	.118	-.2829	4.2600
B. pseudomallei (K96243)	B. vietnamensis (38SP)	-80.13970*	5.38940	.000	-96.4889	-63.7905
	B. ubonensis (A21)	-27.71890*	2.84886	.000	-36.2640	-19.1738
	B. cenocepacia (17SP)	-5.37693	2.32648	.220	-12.4329	1.6791
	B. pseudomallei (TSV189)	-1.08838	.90794	.835	-3.7930	1.6162
	B. pseudomallei (TSV191)	-1.98853	.76685	.118	-4.2600	.2829

*. The mean difference is significant at the 0.05 level.

Table A3.5.5 Area measurements in mm^2 of leaves of *Oryza sativa* L. cv Amaroo infected with a range of *Burkholderia* species as listed in the top row of the table.

***B. vietnamiensis* (38sp)**	***B. ubonensis* (A21)**	***B. cenocepacia* (17sp)**	***B. pseudomallei* (TSV189)**	***B. pseudomallei* (TSV192)**	***B. pseudomallei* (K96243)**
45	83	2	76	97	7
41	13	2	14	61	10
94	93	8	9	29	10
122	37	8	7	13	5
50	57	13	7	23	10
121	17	4	6	7	8
150	144	3	2	5	5
25	16	7	9	20	17
67	28	12	6	16	11
104	32	124	5	10	7
107	37	3	9	10	14
13	22	2	2	4	5
128	25	2	128	13	3
74	59	13	32	101	10
167	26	46	9	2	4
47	25	18	2	3	5
27	29	53	3	11	5
115	20	30	2	6	2
96	21	13	1	21	1
51	34	5	2	4	4
152	54	2	4	6	3
12	20	12	4	4	3
5	44	2	2	5	1
68	30	2	31	5	30
23	21	6	30	10	10
30	32	6	29	5	42
99	64	30	25	7	4
105	39	6	1	4	3
150	33	4	2	6	6
32	19	3	1	6	4
83	50	19	3	3	7
78	28		2	5	4
	47		1	3	2
	38		1	2	4
	30			5	8
	64				
	41				
	60				

Table A3.5.6 Homogeneity of variances test of leaf area of *Oryza sativa* L. cv Amaroo infected with a range of *Burkholderia* species.

Test of Homogeneity of Variances

Percentage_leaf_data

Levene Statistic	df1	df2	Sig.
13.097	5	199	.000

Table A3.5.7 One way ANOVA of leaf area measurements (*Oryza sativa* L. cv Amaroo) with exposure to **a range of *Burkholderia* species.**

ANOVA

Percentage_leaf_data

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	116216.523	5	23243.305	30.951	.000
Within Groups	149442.333	199	750.966		
Total	265658.857	204			

Table A3.5.8 Post hoc Analysis of leaf area of *Oryza sativa* L. cv Amaroo infected with a range of *Burkholderia* species (Games-Howell test).

Multiple Comparisons

Dependent Variable: Percentage_leaf_data

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
B. vietnamensis (38SP)	B. ubonensis (A21)	37.28090*	9.16041	.002	10.0459	64.5159
	B. cenocepacia (17SP)	62.67290*	9.24888	.000	35.1919	90.1539
	B. pseudomallei (TSV189)	63.68164*	9.26212	.000	36.1733	91.1899
	B. pseudomallei (TSV191)	62.26078*	9.11744	.000	35.1322	89.3893
	B. pseudomallei (K96243)	69.65539*	8.30651	.000	44.5273	94.7835
B. ubonensis (A21)	B. vietnamensis (38SP)	-37.28090*	9.16041	.002	-64.5159	-10.0459
	B. cenocepacia (17SP)	25.39200*	5.93030	.001	7.9820	42.8020
	B. pseudomallei (TSV189)	26.40074*	5.95092	.000	8.9578	43.8437
	B. pseudomallei (TSV191)	24.97988*	5.72315	.001	8.2167	41.7430
	B. pseudomallei (K96243)	32.37450*	4.31565	.000	19.5317	45.2173
B. cenocepacia (17SP)	B. vietnamensis (38SP)	-62.67290*	9.24888	.000	-90.1539	-35.1919
	B. ubonensis (A21)	-25.39200*	5.93030	.001	-42.8020	-7.9820
	B. pseudomallei (TSV189)	1.00874	6.08623	1.000	-16.8811	18.8985
	B. pseudomallei (TSV191)	-.41212	5.86371	1.000	-17.6479	16.8237
	B. pseudomallei (K96243)	6.98250	4.50039	.634	-6.5566	20.5216
B. pseudomallei (TSV189)	B. vietnamensis (38SP)	-63.68164*	9.26212	.000	-91.1899	-36.1733
	B. ubonensis (A21)	-26.40074*	5.95092	.000	-43.8437	-8.9578
	B. cenocepacia (17SP)	-1.00874	6.08623	1.000	-18.8985	16.8811
	B. pseudomallei (TSV191)	-1.42086	5.88457	1.000	-18.6892	15.8475
	B. pseudomallei (K96243)	5.97375	4.52753	.773	-7.5818	19.5293
B. pseudomallei (TSV191)	B. vietnamensis (38SP)	-62.26078*	9.11744	.000	-89.3893	-35.1322
	B. ubonensis (A21)	-24.97988*	5.72315	.001	-41.7430	-8.2167
	B. cenocepacia (17SP)	.41212	5.86371	1.000	-16.8237	17.6479
	B. pseudomallei (TSV189)	1.42086	5.88457	1.000	-15.8475	18.6892
	B. pseudomallei (K96243)	7.39461	4.22368	.507	-5.2171	20.0064
B. pseudomallei (K96243)	B. vietnamensis (38SP)	-69.65539*	8.30651	.000	-94.7835	-44.5273
	B. ubonensis (A21)	-32.37450*	4.31565	.000	-45.2173	-19.5317
	B. cenocepacia (17SP)	-6.98250	4.50039	.634	-20.5216	6.5566
	B. pseudomallei (TSV189)	-5.97375	4.52753	.773	-19.5293	7.5818
	B. pseudomallei (TSV191)	-7.39461	4.22368	.507	-20.0064	5.2171

*. The mean difference is significant at the 0.05 level.

A3.6 Statistical analysis of inhibition of rice (*Oryza sativa* L. cv Koshihikari) with *B. pseudomallei* (TSV189 and K96243) (as used in chapter 5.3.4). All statistical analysis were carried out using SPSS version 20.

The root area in mm^2 of rice (*Oryza sativa* L. cv Koshihikari) was measured after exposure to 10^8 CFU *B. pseudomallei* (TSV189) (Table A3.6.1). These measurements were analysed for normality of distribution (Table A3.6.2) and data in the control group was not normally distributed. Log 10 transformation of data did not improve normality (Table A3.6.3). A Mann-Whitney U test (p=0.05, Table A3.6.4) was used to determine whether there was a difference between control and infected groups. There was no significant difference between mean of two sample groups.

Similarly, the root area in mm^2 of rice (*Oryza sativa* L. cv Koshihikari) was measured after exposure to 10^8 CFU *B. pseudomallei* (K96243) (Table A3.6.5). These measurements were analysed for normality of distribution (Table A3.6.6) and as the same control group was used for both *B. pseudomallei* strains, data in the control group was not normally distributed. Log 10 transformation of data did not improve normality (Table A3.6.7). A Mann-Whitney U test (p=0.05, Table A3.6.8) was used to determine whether there was a difference between control and infected groups. There was no significant difference between mean of two sample groups.

The leaf area in mm^2 of rice (*Oryza sativa* L. cv Koshihikari) was measured after exposure to 10^8 CFU *B. pseudomallei* (TSV189) (Table A3.6.9). These measurements were analysed for normality of distribution (Table A3.6.10) and data was normally distributed. An independent T-Test (p=0.05, Table A3.6.11) was used to determine whether there was a difference in leaf area of rice between control and infected (*B. pseudomallei* (TSV189) groups. There was no significant difference between mean of two sample groups.

Similarly the leaf area in mm^2 of rice (*Oryza sativa* L. cv Koshihikari) was measured after exposure to 10^8 CFU *B. pseudomallei* (K96243) (Table A3.6.12). These measurements were analysed for normality of distribution (Table A3.6.13) and data was normally distributed. An independent T-Test (p=0.05, Table A3.6.14) was used to determine whether there was a difference in leaf area of rice between control and infected (*B. pseudomallei* (K96243) groups. There was a significant difference between mean of two sample groups.

Table A3.6.1 Area measurements in mm^2 of roots of *Oryza sativa* L. cv Koshihikari infected with *B. pseudomallei* (TSV189). Control represents rice which have no *Burkholderia* exposure. This control group was also used to control for data in table A3.6.5.

Control	*B. pseudomallei* (TSV189)
211	190
195	186
189	109
69	162
262	223
197	125
215	157
171	215
190	169
116	105
157	72
118	75
111	124
197	202
173	203
150	151
176	67
184	153
213	174
35	173
33	249
	93
	169
	63

Table A3.6.2 Normality test of effect of *B. pseudomallei* (TSV189) on *Oryza sativa* L. cv Koshihikari growth.

		Kolmogorov-Smirnov[a]		
	Group_root_189	Statistic	df	Sig.
data_root_tsv189	control	.190	21	.046
	test	.131	24	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.6.3 Normality test of effect of log transformation of root measurements of *Oryza sativa* L. cv Koshihikari exposed to *B. pseudomallei* (TSV189)

		Kolmogorov-Smirnov[a]		
	Group_root_189	Statistic	df	Sig.
LOGDATAROOT189	control	.247	21	.002
	test	.200	24	.014

a. Lilliefors Significance Correction

Table A3.6.4 Mann-Whitney U test of effect of *B. pseudomallei* (TSV189) on *Oryza sativa* L. cv Koshihikari growth.

Test Statistics[a]

	data_root_tsv189
Mann-Whitney U	215.000
Wilcoxon W	515.000
Z	-.842
Asymp. Sig. (2-tailed)	.400

a. Grouping Variable: Group_root_189

Table A3.6.5 Area measurements in mm^2 of roots of *Oryza sativa* L. cv Koshihikari infected with *B. pseudomallei* (K96243). Control represents rice which have no *Burkholderia* exposure. This control group was also used to control for data in table A3.6.1.

Control	*B. pseudomallei* (K96243)
211	240
195	135
189	152
69	126
262	115
197	181
215	141
171	173
190	254
116	226
157	291
118	230
111	272
197	192
173	296
150	195
176	150
184	187
213	90
35	142
33	168
	94
	105
	116
	164
	211
	207

Table A3.6.6 Normality test of effect of *B. pseudomallei* (K96243) on *Oryza sativa* L. cv Koshihikari growth. Group 1 is the control group, group 2 is the experimental group

		Kolmogorov-Smirnov[a]		
	Group root k9	Statistic	df	Sig.
data_root_k96243	1.00	.190	21	.046
	2.00	.086	27	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.6.7 Normality test of effect of log transformation of root measurements of *Oryza sativa* L. cv Koshihikari exposed to *B. pseudomallei* (K96243) Group 1 is the control group, group 2 is the experimental group

		Kolmogorov-Smirnov[a]		
	Group_root_k9	Statistic	df	Sig.
LOGDATAROOTK9	1.00	.247	21	.002
	2.00	.059	27	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.6.8 Mann-Whitney U test of effect of *B. pseudomallei* (K96243) on *Oryza sativa* L. cv Koshihikari growth.

Test Statistics[a]

	LOGDATAROOTK9
Mann-Whitney U	256.500
Wilcoxon W	487.500
Z	-.561
Asymp. Sig. (2-tailed)	.575

a. Grouping Variable: Group_root_k9

Table A3.6.9 Area measurements in mm^2 of leaves of *Oryza sativa* L. cv Koshihikari infected with *B. pseudomallei* (TSV189). Control represents rice which have no *Burkholderia* exposure. This control group was also used to control for data in table A3.6.12.

Control	*B. pseudomallei* (TSV189)
190	183
128	162
95	100
60	165
237	146
172	60
139	106
192	108
235	125
115	50
152	53
149	60
179	91
86	175
100	218
84	104
96	110
214	165
121	208
40	117
51	258
	47
	161
	45

Table A3.6.10 Normality test of effect of *B. pseudomallei* (TSV189) on *Oryza sativa* L. cv Koshihikari growth.

		Kolmogorov-Smirnov[a]		
	Group_leaf	Statistic	df	Sig.
data_leaf_tsv189	control	.109	21	.200*
	test	.115	24	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.6.11 T-Test of effect of *B. pseudomallei* (TSV189) on *Oryza sativa* L. cv Koshihikari growth.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data_leaf_tsv189	Equal variances assumed	.000	.985	.530	43	.599	9.31190	17.56701	-26.11535	44.73916
	Equal variances not assumed			.530	42.320	.599	9.31190	17.55502	-26.10762	44.73143

Table A3.6.12 Area measurements in mm^2 of leaves of *Oryza sativa* L. cv Koshihikari infected with *B. pseudomallei* (K96243). Control represents rice which have no *Burkholderia* exposure. This control group was also used to control for data in table A3.6.12.

Control	*B. pseudomallei* (K96243)
190	140
128	178
95	126
60	195
237	28
172	191
139	147
192	122
235	229
115	243
152	135
149	183
179	237
86	144
100	216
84	293
96	56
214	159
121	146
40	236
51	263
	139
	179
	180
	260
	244
	266

Table A3.6.13 Normality test of effect of *B. pseudomallei* (K96243) on *Oryza sativa* L. cv Koshihikari growth.

Tests of Normality

		Kolmogorov-Smirnov[a]		
	Group root k9	Statistic	df	Sig.
data_leaf_k96243	1.00	.109	21	.200*
	2.00	.104	27	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.6.14 T-Test of effect of *B. pseudomallei* (K96243) on *Oryza sativa* L. cv Koshihikari growth.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data_leaf_k96243	Equal variances assumed	.003	.957	-2.501	46	.016	-44.00476	17.59300	-79.41764	-8.59189
	Equal variances not assumed			-2.520	44.232	.015	-44.00476	17.46378	-79.19548	-8.81404

A3.7 Statistical analysis of *B. pseudomallei* (TSV189) infection of different cultivars of rice (as used in chapter 5.3.5). All statistical analysis were carried out using SPSS version 20.

The percent of growth of three different rice cultivars (*Oryza sativa* L. cv Koshihikari, *Oryza sativa* L. cv Amaroo and *Oryza meridionalis*) infected with *B. pseudomallei* (TSV189) relative to average growth of control rice was calculated with respect to root area (Table A3.7.1). The percent growth measurements were analysed for normality of distribution (Table A3.7.2) and homogeneity of variance (Table A3.7.3). Data was normally distributed. Variances were not homogeneous (Table A3.7.3) and % growth of roots of rice was used in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.7.4, post hoc test Table A3.7.5) to determine whether the relative growth of roots differed with different cultivars. All rice cultivars were significantly different.

Similarly, the relative leaf growth of the cultivars was measured (Table 3.7.6) and the percent growth of leaves was analysed for normality of distribution (Table A3.7.7) As group which was small (< 25 data points) was not normally distributed, a log transformation was attempted (Table A3.7.8). This group was now normally distributed. One other group was still not normally distributed according to the test, however the large data set compensates in the case of this group, so we can assume normality. Analysis of homogeneity of variance on log transformed data (Table A3.7.9) indicated variances were homogeneous, and the log transformed percent leaf growth was used in a one way ANOVA with Gabriel post hoc test (p=0.05, ANOVA Table A3.7.10, post hoc test Table A3.7.11) to determine whether leaf area of rice changed in different cultivars. All cultivars had significantly different growth.

Table A3.7.1 Raw data and calculations of relative % growth of root area (mm^2) of rice infected with *B. pseudomallei* (TSV189) for three cultivars of rice. Average measurements for uninfected samples are; *Oryza sativa* L. cv Koshihikari: 160.0 mm^2; *Oryza sativa* L. cv Amaroo: 144.4 mm^2; *Oryza meridionalis*: 46.7 mm^2

raw area measurements of control roots			raw area measurements of infected roots			% growth of infected roots		
Oryza sativa L. cv. Koshihikari	*Oryza sativa* L. cv Amaroo	*Oryza meridionalis*	*Oryza sativa* L. cv. Koshihikari	*Oryza sativa* L. cv Amaroo	*Oryza meridionalis*	*Oryza sativa* L. cv. Koshihikari	*Oryza sativa* L. cv Amaroo	*Oryza meridionalis*
210.8	118.1	44.9	189.9	16.1	19.1	118.7	11.1	40.9
194.5	174.6	35.1	186.4	19.3	15.4	116.5	13.4	33.0
188.6	191.5	24.5	109.3	18.2	15.6	68.3	12.6	33.4
69.3	56.1	57.9	161.6	13.3	12.6	101.0	9.2	27.0
262.1	71.2	59.7	222.9	16.7	17.1	139.3	11.6	36.6
197.2	196.1	42.8	124.6	18.6	14.1	77.9	12.9	30.2
215.4	227.9	89.4	157.3	17.7	14.3	98.3	12.3	30.6
170.8	159.4	46.5	215.2	13.2	11.9	134.5	9.1	25.5
190.4	171.9	25.8	169.3	16.6	11.9	105.8	11.5	25.5
115.5	119.8	32.9	104.7	16.4	11.1	65.4	11.4	23.8
157.2	90.5	55.4	72.3	13.1	24.7	45.2	9.1	52.9
118.3	139.3	38.7	75.2	15.8	18.7	47.0	10.9	40.0
110.5	103.3	14.9	124.2	16.8	14.7	77.6	11.6	31.5
196.9	118.9	12.4	202.1	17.6	12.7	126.3	12.2	27.2
172.7	160.1	58.6	203.1	16.7	28.4	126.9	11.6	60.8
149.9	149.3	51.8	151.3	19.3	16.5	94.6	13.4	35.3
175.5	133.8	43.8	66.8	20.7	21.1	41.8	14.3	45.2
183.9	173.6	45.3	152.9	20.7	20.2	95.6	14.3	43.3
213.1	80.9	56.7	174.4	17.6	20.1	109.0	12.2	43.0
35.3	89.5	59.9	173.3	16.1	13.1	108.3	11.1	28.1
33.1	164.7	90.6	248.8	17.9		155.5	12.4	
	125.8	56.3	93.1	16.4		58.2	11.4	
	177.8	38.7	169.2	16.2		105.8	11.2	
	96.3	38.7	63.1	17.9		39.4	12.4	
	157.4			17			11.8	
	191.2			15.1			10.5	
	191.2			16.3			11.3	
	115.2			23.2			16.1	
	240.8			19.6			13.6	
				16.6			11.5	
				20.2			14.0	
				19.6			13.6	
				17.4			12.0	
				15.9			11.0	
				16.9			11.7	
				19.4			13.4	
				19.5			13.5	
				11.4			7.9	

Table A3.7.2 Normality test of percent growth of root area of cultivars infected with *B. pseudomallei* (TSV189).

	Group_percentage_cultivar	Kolmogorov-Smirnov[a]		
		Statistic	df	Sig.
data_percentage_cultivar_root	"Oryza sativa L. cv. Koshihikari"	.131	24	.200*
	"Oryza sativa L. cv Amaroo"	.130	38	.106
	"Oryza meridionalis"	.142	20	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.7.3 Homogeneity of variances test of percent growth of root area of cultivars infected with *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

data_percentage_cultivar_root

Levene Statistic	df1	df2	Sig.
49.160	2	79	.000

Table A3.7.4 One way ANOVA of percent growth of root area of cultivars infected with *B. pseudomallei* (TSV189)

ANOVA

data_percentage_cultivar_root

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	100041.273	2	50020.637	146.366	.000
Within Groups	26998.351	79	341.751		
Total	127039.624	81			

Table A3.7.5 Post hoc Analysis of percent growth of root area of cultivars infected with *B. pseudomallei* (TSV189). (Games-Howell test).

Multiple Comparisons

Dependent Variable: data_percentage_cultivar_root

Gabriel

(I) Group_percentage_cultivar	(J) Group_percentage_cultivar	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
"Oryza sativa L. cv. Koshihikari"	"Oryza sativa L. cv Amaroo"	82.06314*	4.82007	.000	70.3903	93.7359
	"Oryza meridionalis"	58.35123*	5.59707	.000	44.7225	71.9799
"Oryza sativa L. cv Amaroo"	"Oryza sativa L. cv. Koshihikari"	-82.06314*	4.82007	.000	-93.7359	-70.3903
	"Oryza meridionalis"	-23.71191*	5.10696	.000	-36.0055	-11.4183
"Oryza meridionalis"	"Oryza sativa L. cv. Koshihikari"	-58.35123*	5.59707	.000	-71.9799	-44.7225
	"Oryza sativa L. cv Amaroo"	23.71191*	5.10696	.000	11.4183	36.0055

*. The mean difference is significant at the 0.05 level.

Table A3.7.6 Raw data and calculations of relative % growth of leaf area (mm^2) of rice infected with *B. pseudomallei* (TSV189) for three cultivars of rice. Average measurements for uninfected samples are; *Oryza sativa* L. cv Koshihikari: 135.0 mm^2; *Oryza sativa* L. cv Amaroo: 63.1 mm^2; *Oryza meridionalis*: 102.7 mm^2;

raw area measurements of control leaves			raw area measurements of infected leaves			% growth of infected leaves		
Oryza sativa L. cv. Koshihikari	*Oryza sativa* L. cv Amaroo	*Oryza meridionalis*	*Oryza sativa* L. cv. Koshihikari	*Oryza sativa* L. cv Amaroo	*Oryza meridionalis*	*Oryza sativa* L. cv. Koshihikari	*Oryza sativa* L. cv Amaroo	*Oryza meridionalis*
189.6	25.4	108.4	182.7	13.5	30.3	135.3	21.4	131.8
128.3	72.2	96.7	162.1	1.8	14.3	120.1	2.9	13.9
95.2	44	103.7	99.6	2.4	8.4	73.8	3.8	8.2
60.2	20.4	84.2	165.1	2.5	10.4	122.3	4.0	10.1
236.8	30.3	100.9	146.3	2.4	25.7	108.4	3.8	25.0
172.4	60.9	73.1	60.4	2.9	20.4	44.7	4.6	19.9
139.1	122.8	90.8	105.7	2.3	38.3	78.3	3.6	37.3
192.4	71.1	97.4	108.4	2.6	20.1	80.3	4.1	19.6
234.9	150.52	76.4	124.6	3.6	23.2	92.3	5.7	22.6
114.9	33.6	77.1	50.4	4.2	23.9	37.3	6.7	23.3
151.9	24.5	98.2	52.7	3.1	46.5	39.0	4.9	45.3
148.5	84.6	68.4	60.1	2.6	20.6	44.5	4.1	20.1
178.7	26.4	5.7	90.9	5.9	41.4	67.3	9.4	40.3
86.3	57.7	7.8	175.1	5.9	19.4	129.7	9.4	18.9
99.5	59.4	22.4	218.3	3.7	57.3	161.7	5.9	55.8
83.5	74.7	105.1	103.5	4.3	46.9	76.7	6.8	45.7
95.6	40.1	142.5	110.2	4.6	22.8	81.6	7.3	22.2
214.4	42.3	182.9	164.5	6.1	19.9	121.9	9.7	19.4
120.5	14.33	151.7	208.1	4.5	16.5	154.1	7.1	16.1
40.1	14.8	104.6	117.1	6.6	11.3	86.7	10.5	11.0
51.4	74.6	126.5	257.6	3.6		190.8	5.7	
	44.3	203.7	46.6	2.6		34.5	4.1	
	121.8	168.8	160.7	4.1		119.0	6.5	
	15.21	168.3	44.9	4.1		33.3	6.5	
	19.1			2.23			3.5	
	126.6			2.9			4.6	
	100.1			83.5			132.3	
	139.4			9.9			15.7	
	118.6			5.8			9.2	
				2.6			4.1	
				3			4.8	
				12.5			19.8	
				6.1			9.7	
				4.2			6.7	
				4.7			7.4	
				28.1			44.5	
				5.1			8.1	
				6.6			10.5	

Table A3.7.7 Normality test of percent growth of leaf area of cultivars infected with *B. pseudomallei* (TSV189)

	Group_percentage_cultivar	Kolmogorov-Smirnov[a]		
		Statistic	df	Sig.
data_percentage_cultivar_leaf	"Oryza sativa L. cv. Koshihikari"	.115	24	.200*
	"Oryza sativa L. cv Amaroo"	.389	38	.000
	"Oryza meridionalis"	.209	20	.023

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.7.8 Normality test of log transformed percent growth of leaf area of cultivars infected with *B. pseudomallei* (TSV189)

	Group_percentage_cultivar	Kolmogorov-Smirnov[a]		
		Statistic	df	Sig.
LOGPERCENTAGELEAF	"Oryza sativa L. cv. Koshihikari"	.133	24	.200*
	"Oryza sativa L. cv Amaroo"	.184	38	.002
	"Oryza meridionalis"	.127	20	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.7.9 Homogeneity of variances test of log transformed percent growth of leaf area infected with *B. pseudomallei* (TSV189)

Test of Homogeneity of Variances

LOGPERCENTAGELEAF

Levene Statistic	df1	df2	Sig.
.558	2	79	.575

Table A3.7.10 One way ANOVA of log transformed percent growth of leaf area infected with *B. pseudomallei* (TSV189)

ANOVA

LOGPERCENTAGELEAF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.360	2	8.180	107.784	.000
Within Groups	5.995	79	.076		
Total	22.355	81			

Table A3.7.11 Post hoc analysis of log transformed percent growth of leaves infected with *B. pseudomallei* (TSV189) (Gabriel test).

Multiple Comparisons

Dependent Variable: LOGPERCENTAGELEAF

Gabriel

(I) Group_percentage_cultivar	(J) Group_percentage_cultivar	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
"Oryza sativa L. cv. Koshihikari"	"Oryza sativa L. cv Amaroo"	1.05187*	.07183	.000	.8779	1.2258
	"Oryza meridionalis"	.56979*	.08341	.000	.3667	.7729
"Oryza sativa L. cv Amaroo"	"Oryza sativa L. cv. Koshihikari"	-1.05187*	.07183	.000	-1.2258	-.8779
	"Oryza meridionalis"	-.48208*	.07610	.000	-.6653	-.2989
"Oryza meridionalis"	"Oryza sativa L. cv. Koshihikari"	-.56979*	.08341	.000	-.7729	-.3667
	"Oryza sativa L. cv Amaroo"	.48208*	.07610	.000	.2989	.6653

*. The mean difference is significant at the 0.05 level.

A3.8 Statistical analysis of biocontrol of *B. pseudomallei* TSV189 biofilm formation (Crystal violet stain) in 96 well plate (as used in chapter 7.3.1). All statistical analysis were carried out using SPSS version 20.

Optical density was measured after bacteriocin and bacteriophage exposure to of *B. pseudomallei* TSV189 at 24h incubation (Table A3.8.1). These measurements were analysed for normality of distribution (Table A3.8.2). Normality was violated and a log10 transformation was carried out after which data was normally distributed (Table A3.8.3). Analysis of homogeneity of variance on log transformed data (Table A3.8.4) determined variances were not homogeneous. The log transformed optical density was used in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.8.5, post hoc test Table A3.8.6) to determine whether optical density (and thus biofilm growth) changed with exposure to biocontrol agents after 24 hr. Both agents significantly reduced biofilm formation after 24 hr and were not significantly different to each other.

Similarly, optical density was measured after bacteriocin and bacteriophage exposure to of *B. pseudomallei* TSV189 at 48h incubation (Table A3.8.7)and analysed for normality (Table 3.8.8). Normality was violated and a log10 transformation was carried out after which data was normally distributed (Table A3.8.9). Analysis of homogeneity of variance on log transformed data (Table A3.8.10) determined variances were not homogeneous. The log transformed optical density was used in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.8.11, post hoc test Table A3.8.12) to determine whether optical density (and thus biofilm growth) changed with exposure to biocontrol agents after 48 hr. Both agents significantly reduced biofilm formation after 48 hr and were significantly different to each other.

To determine what effect the time to testing (24 or 48 hr) had on the amount of biofilm, the log transformed data was analysed in paired T tests for bacteriocin (pair 1: 24 and 48 hr) and bacteriophage (pair 2: 24 and 48 hr) (Table A3.8.13). Significant differences were found and are discussed in Chapter 7.

Table A3.8.1 Absorbance readings (OD600) of crystal violet staining as a measure of biofilm formation at 24 hr in a 96 well plate format. The top row describes the treatment. Control represents untreated biofilm.

Control	Bacteriocin	Phage cocktail
0.726	0.438	0.345
1.009	0.483	0.325
1.734	0.641	0.195
1.716	0.556	0.265
1.665	0.513	0.294
1.287	0.408	0.268
1.155	0.415	0.361
0.737	0.367	0.312
0.653	0.444	0.707
1.016	0.479	0.601
1.167	0.436	0.401
1.048	0.442	0.832

Table A3.8.2 Normality test of absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 24 hr

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
Data_24	control	.159	12	.200*
	bacteriocin	.213	12	.137
	cocktail phage	.266	12	.019

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.8.3 Normality test of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 24 hr

Tests of Normality

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
LOGDATA24	control	.145	12	.200*
	bacteriocin	.197	12	.200*
	cocktail phage	.197	12	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.8.4 Homogeneity of variance of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 24 hr

Test of Homogeneity of Variances

LOGDATA24

Levene Statistic	df1	df2	Sig.
3.987	2	33	.028

Table A3.8.5 One way ANOVA of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 24 hr

ANOVA

LOGDATA24

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.487	2	.744	36.960	.000
Within Groups	.664	33	.020		
Total	2.151	35			

Table A3.8.6 Post hoc analysis of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 24 hr (Games-Howell test).

Multiple Comparisons

Dependent Variable: LOGDATA24

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	bacteriocin	.37627*	.04589	.000	.2573	.4953
	cocktail phage	.47050*	.06841	.000	.2979	.6431
bacteriocin	control	-.37627*	.04589	.000	-.4953	-.2573
	cocktail phage	.09423	.05723	.261	-.0561	.2445
cocktail phage	control	-.47050*	.06841	.000	-.6431	-.2979
	bacteriocin	-.09423	.05723	.261	-.2445	.0561

*. The mean difference is significant at the 0.05 level.

Table A3.8.7 Absorbance readings (OD600) of crystal violet staining as a measure of biofilm formation at 48 hr in a 96 well plate format. The top row describes the treatment. Control represents untreated biofilm.

Control	Bacteriocin	Phage cocktail
0.74	0.36	0.143
1.179	0.185	0.145
2.136	0.485	0.201
2.503	0.41	0.169
1.764	0.381	0.259
2.113	0.633	0.161
0.674	0.528	0.157
0.986	0.341	0.117
1.011	0.538	0.137
0.895	0.658	0.193
0.865	1.04	0.159
0.988	1.361	0.217

Table A3.8.8 Normality test of absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 48 hr

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Data_48	control	.272	12	.014	.839	12	.027
	bacteriocin	.235	12	.066	.851	12	.037
	cocktail phage	.192	12	.200*	.933	12	.413

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.8.9 Normality test of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 48 hr

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
LOGDATA48	control	.232	12	.072	.895	12	.136
	bacteriocin	.143	12	.200*	.968	12	.883
	cocktail phage	.156	12	.200*	.972	12	.933

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.8.10 Homogeneity of variance of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 48 hr

Test of Homogeneity of Variances

LOGDATA48

Levene Statistic	df1	df2	Sig.
2.913	2	33	.068

Table A3.8.11 One way ANOVA of log transformed absorbance readings of l *B. pseudomallei* TSV189 biofilm formation at 48 hr

ANOVA

LOGDATA48

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.408	2	2.204	66.780	.000
Within Groups	1.089	33	.033		
Total	5.497	35			

Table A3.8.12 Post hoc analysis of log transformed absorbance readings of *B. pseudomallei* TSV189 biofilm formation at 48 hr. (Games-Howell test).

Multiple Comparisons

Dependent Variable: LOGDATA48

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	bacteriocin	.37429*	.08653	.001	.1565	.5920
	cocktail phage	.85491*	.06258	.000	.6935	1.0164
bacteriocin	control	-.37429*	.08653	.001	-.5920	-.1565
	cocktail phage	.48062*	.07139	.000	.2949	.6664
cocktail phage	control	-.85491*	.06258	.000	-1.0164	-.6935
	bacteriocin	-.48062*	.07139	.000	-.6664	-.2949

*. The mean difference is significant at the 0.05 level.

Table A3.8.13 Paired T-Test of biofilm formation measured by log transformed absorbance readings after incubation with biocontrol agents for 24 and 48 hr. Pair 1 is bacteriocin treatment and pair 2 is bacteriophage treatment

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	LOGBAC24 - LOGBAC48	-.03885	.24627	.07109	-.19532	.11763	-.546	11	.596
Pair 2	LOGPHAGE24 - LOGPHAGE48	.34755	.20794	.06003	.21543	.47966	5.790	11	.000

A3.9 Statistical analysis of *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189) (as used in chapter 7.3.2). All statistical analysis were carried out using SPSS version 20.

Root area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured after exposure to bacteria (Table A3.9.1). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance on data indicated variances were not homogeneous (Table A3.9.2). Root area (mm^2) were used in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.9.3, post hoc test Table A3.9.4) to determine whether root area (mm^2) changed in difference treatment. Significant difference all treatment and *B. ubonensis* (A21) cannot recovery rice from *B. pseudomallei* (TSV189) infection.

Similarly the leaf area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured and compared to control unexposed rice (Table 3.9.5). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance on data indicated variances were not homogeneous (Table A3.9.6). Root area (mm^2) were used in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.9.7, post hoc test Table A3.9.8) to determine whether leaf area (mm^2) changed in difference treatment. Significant difference all treatment and *B. ubonensis* (A21) cannot recovery rice from *B. pseudomallei* (TSV189) infection.

Table A3.9.1 Root area (mm^2) of *Oryza sativa* L. cv Amaroo after 7 days growth with exposure to *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189). Group A (rice soaked with *B. ubonensis*), Group B (rice soaked with *B. ubonensis* and *B. pseudomallei*), Group C (rice soaked with *B. pseudomallei*).

Untreated control	Group A	Group B	Group C
67	75	8	6
124	24	9	6
112	50	9	5
91	98	5	5
76	38	10	5
85	49	25	6
103	53	10	4
103	36	20	5
127	72	7	6
75	27	11	6
143	20	8	6
114	30	7	6
83	38	7	5
91	33	7	4
95	30	6	7
140	32	7	12
127	34	7	8
82	18	9	5
114	43	12	6
124	37	15	4
132	17	8	10
108	30	8	6
91	22	9	4
61	43	12	8
70	43	8	5
103	60	11	6
116	77	9	8
70	33	11	6
115	33	16	4
120	57	8	5
110	22	7	7
115	32	7	6
88	39	10	6
101	26	5	7
90	40	8	7
55	47	7	5
79	25	7	10
	28	7	7
		7	
		9	

Table A3.9.2 Homogeneity of variance of root area of *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

Root_data

Levene Statistic	df1	df2	Sig.
39.132	3	149	.000

Table A3.9.3 One way ANOVA of root area of *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189).

ANOVA

Root_data

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	214028.894	3	71342.965	344.681	.000
Within Groups	30840.405	149	206.983		
Total	244869.299	152			

Table A3.9.4 Post hoc analysis of root area *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189) (Games-Howell test). Group A (rice soaked with *B. ubonensis*), Group B (rice soaked with *B. ubonensis* and *B. pseudomallei*), Group C (rice soaked with *B. pseudomallei*).

Multiple Comparisons

Dependent Variable: Root_data

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreat	Group A	60.28054*	4.70612	.000	47.8888	72.6723
	Group B	90.73429*	3.75560	.000	80.6454	100.8232
	Group C	93.89712*	3.71423	.000	83.8993	103.8950
Group A	Untreat	-60.28054*	4.70612	.000	-72.6723	-47.8888
	Group B	30.45375*	2.96965	.000	22.4972	38.4103
	Group C	33.61658*	2.91716	.000	25.7768	41.4563
Group B	Untreat	-90.73429*	3.75560	.000	-100.8232	-80.6454
	Group A	-30.45375*	2.96965	.000	-38.4103	-22.4972
	Group C	3.16283*	.68318	.000	1.3519	4.9737
Group C	Untreat	-93.89712*	3.71423	.000	-103.8950	-83.8993
	Group A	-33.61658*	2.91716	.000	-41.4563	-25.7768
	Group B	-3.16283*	.68318	.000	-4.9737	-1.3519

*. The mean difference is significant at the 0.05 level.

Table A3.9.5 Leaf area (mm^2) of *Oryza sativa* L. cv Amaroo after 7 days growth with exposure to *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189). Group A (rice soaked with *B. ubonensis*), Group B (rice soaked with *B. ubonensis* and *B. pseudomallei*), Group C (rice soaked with *B. pseudomallei*).

Untreated control	Group A	Group B	Group C
145	85	32	22
90	13	17	20
177	95	27	16
144	38	23	15
108	58	28	16
54	17	44	12
36	146	25	14
45	16	46	16
57	29	27	26
45	33	25	14
129	38	21	15
101	23	29	22
186	25	22	15
72	60	22	21
161	27	22	19
52	25	18	11
167	29	21	15
147	20	27	12
193	22	32	10
164	34	32	18
55	55	22	24
105	20	32	17
129	44	19	13
72	31	24	18
113	22	17	15
88	33	16	20
112	65	19	17
59	40	22	19
51	34	38	7
63	19	23	18
76	51	15	7
51	28	23	12
30	48	21	14
60	39	21	13
133	30	20	16
120	65	22	16
113	41	20	21
	61	20	16
		19	
		11	

Table A3.9.6 Homogeneity of variance of leaf area of *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

Leaf_data

Levene Statistic	df1	df2	Sig.
49.529	3	149	.000

Table A3.9.7 One way ANOVA of leaf area of *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189).

ANOVA

Leaf_data

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	161792.529	3	53930.843	75.432	.000
Within Groups	106528.822	149	714.959		
Total	268321.352	152			

Table A3.9.8 Post hoc analysis of leaf area of *Oryza sativa* L. cv Amaroo growth using *B. ubonensis* (A21) to control growth inhibition caused by *B. pseudomallei* (TSV189) (Games-Howell test).

Multiple Comparisons

Dependent Variable: Leaf_data

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreat	Group A	59.01921*	8.77497	.000	35.7780	82.2604
	Group B	75.87761*	7.80196	.000	54.9071	96.8481
	Group C	83.94499*	7.74772	.000	63.0937	104.7963
Group A	Untreat	-59.01921*	8.77497	.000	-82.2604	-35.7780
	Group B	16.85840*	4.32813	.002	5.2865	28.4303
	Group C	24.92578*	4.22959	.000	13.5755	36.2760
Group B	Untreat	-75.87761*	7.80196	.000	-96.8481	-54.9071
	Group A	-16.85840*	4.32813	.002	-28.4303	-5.2865
	Group C	8.06738*	1.32660	.000	4.5666	11.5681
Group C	Untreat	-83.94499*	7.74772	.000	-104.7963	-63.0937
	Group A	-24.92578*	4.22959	.000	-36.2760	-13.5755
	Group B	-8.06738*	1.32660	.000	-11.5681	-4.5666

*. The mean difference is significant at the 0.05 level.

A3.10 Statistical analysis of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189) (as used in chapter 7.3.3). All statistical analysis were carried out using SPSS version 20.

The root area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured after exposure to each treatment and unexposed control (Table A3.10.1). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance on data indicated variances were not homogeneous (Table A3.10.2). Data was analysed in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.10.3, post hoc test Table A3.10.4) to determine whether root area (mm^2) changed after different treatments. The control group and group A (rice and phage cocktail) were not-significantly different but were different to both other groups There were significant differences between phage treatment and *B. pseudomallei* infection groups.

Similarly the leaf area (mm^2) of rice (*Oryza sativa* L. cv Amaroo) was measured after exposure to each treatment and unexposed control (Table 3.10.5). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance on data indicated variances were not homogeneous (Table A3.10.6). Data was analysed in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.10.7, post hoc test Table A3.10.8) to determine whether leaf area (mm^2) after different treatments was affected. The control group and group A (rice and phage cocktail) were not-significantly different but were different to both other groups. There was no significant difference between phage treatment and *B. pseudomallei* infection groups.

Table A3.10.1 Raw data of root area *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189). Untreated control is not exposed to any bacteria or bacteriophage. Groups are; Group A (rice soaked with phage cocktail), Group B (rice soaked with *B. pseudomallei* phage cocktail and *B. pseudomallei*), Group C (*B. pseudomallei* infection only).

Untreated control	Group A	Group B	Group C
192.5	203.4	86.9	17.3
181	135.2	134.4	17.7
167	145	74.5	16.1
196.5	260.6	90.3	15.5
161.2	249.9	22.7	32.7
156.7	137.3	78.9	19.8
149.5	175.3	51.1	17.1
149.8	193	120	13.7
194.5	159.2	60	14.2
177.8	161.7	77.7	55.4
182	183.8	68.8	71.6
116.6	184.4	40.4	31.7
120.7	188.8	27.4	13.7
225.9	224.6	81.3	40.4
157.5	208	52.2	17.8
195.3	202.2	86.9	34.2
177.2	205.2	54.6	21.2
278	68.7	75.4	17.8
219.6	112.8	94.8	16.3
119.9	190.7	29.2	15.6
196.5	179.5	90.3	16.4
121.1	146.2	54.4	18.7
175.1	140.5	26.1	51.3
176.4	106.6	53.6	45.9
174.6	215	40.3	34.4
153.3	170.2	25.1	14.3
204.6	168.1		20.7
176.1	224.3		31.2
190	197.9		43.2
113.5	184.1		
139.3	151.6		
118.3	122.3		
161	123.3		
151	142.8		
191.8	227.6		
164.8	110.1		
234.2			
179			

Table A3.10.2 Homogeneity of variance of root area of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

Data_root

Levene Statistic	df1	df2	Sig.
7.107	3	125	.000

Table A3.10.3 One way ANOVA of root area of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

ANOVA

Data_root

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	530754.260	3	176918.087	160.066	.000
Within Groups	138159.988	125	1105.280		
Total	668914.248	128			

Table A3.10.4 Post hoc analysis of root area of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189) (Games-Howell test).

Multiple Comparisons

Dependent Variable: Data_root

Games-Howell

(I) Group_phage_amaroo	(J) Group_phage_amaroo	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreatment control	Group A	-.11944	9.16147	1.000	-24.2498	24.0109
	Group B	106.81923*	8.09579	.000	85.4222	128.2162
	Group C	145.34483*	6.36103	.000	128.4693	162.2203
Group A	Untreatment control	.11944	9.16147	1.000	-24.0109	24.2498
	Group B	106.93868*	9.16757	.000	82.7112	131.1661
	Group C	145.46427*	7.67889	.000	124.9810	165.9475
Group B	Untreatment control	-106.81923*	8.09579	.000	-128.2162	-85.4222
	Group A	-106.93868*	9.16757	.000	-131.1661	-82.7112
	Group C	38.52560*	6.36983	.000	21.3788	55.6723
Group C	Untreatment control	-145.34483*	6.36103	.000	-162.2203	-128.4693
	Group A	-145.46427*	7.67889	.000	-165.9475	-124.9810
	Group B	-38.52560*	6.36983	.000	-55.6723	-21.3788

*. The mean difference is significant at the 0.05 level.

Table A3.10.5 Raw data of leaf area *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189). Untreated control is not exposed to any bacteria or bacteriophage. Groups are; Group A (rice soaked with phage cocktail), Group B (rice soaked with *B. pseudomallei* phage cocktail and *B. pseudomallei*), Group C (*B. pseudomallei* infection only).

Untreated control	Group A	Group B	Group C
187.6	203.7	60.5	16.4
156.3	36.3	169.7	29.3
113.1	171.9	38	7.2
105.1	61.9	51.7	20.1
76.4	131.6	88.9	36.9
129.5	91.8	45.4	42.5
58.2	78.4	24.6	19.1
35.5	53.2	96.4	26.9
87.6	67.1	19	52.6
103.2	106.2	96.1	72
104.8	205.6	40.8	85.8
60.4	166.5	32.5	34.9
80.5	102.6	15.9	15.4
119.3	162.8	50.5	47.1
161.2	157.6	59.6	18.1
156.9	198.2	78.1	58.3
32.1	165.4	17.7	6.1
179.2	30.1	44.2	11.9
188.1	115.1	103.5	7.5
97.8	151.4	7.5	1.6
52.3	57.6	20.4	5.3
68.8	98.9	27.1	8.9
171.5	231	2.7	56.8
203.4	134.7	39.8	60.1
60.2	107.6	12.4	21.3
52.9	82.9	3.6	1.4
69.1	228.1		23.6
131.5	218.5		55.1
58.3	211.1		50.3
47.1	166.5		
137.1	101.5		
148.2	108.9		
114	74.5		
78.2	114.5		
69.9	251.7		
132.4	222.9		
90.9			
135.5			

Table A3.10.6 Homogeneity of variance of leaf area of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

Data_leaf

Levene Statistic	df1	df2	Sig.
10.988	3	125	.000

Table A3.10.7 One way ANOVA of leaf area of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189)

ANOVA

Data_leaf

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	229596.984	3	76532.328	35.835	.000
Within Groups	266959.018	125	2135.672		
Total	496556.002	128			

Table A3.10.8 Post hoc analysis of leaf area of *Oryza sativa* L. cv Amaroo growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189) (Games-Howell test).

Multiple Comparisons

Dependent Variable: Data_leaf

Games-Howell

(I) Group_phage_amaroo	(J) Group_phage_amaroo	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreatment control	Group A	-28.54371	12.79359	.126	-62.2715	5.1840
	Group B	58.74069*	10.75694	.000	30.3127	87.1687
	Group C	75.91098*	8.74538	.000	52.7608	99.0612
Group A	Untreatment control	28.54371	12.79359	.126	-5.1840	62.2715
	Group B	87.28440*	12.76683	.000	53.5317	121.0371
	Group C	104.45469*	11.12483	.000	74.8120	134.0974
Group B	Untreatment control	-58.74069*	10.75694	.000	-87.1687	-30.3127
	Group A	-87.28440*	12.76683	.000	-121.0371	-53.5317
	Group C	17.17029	8.70619	.216	-6.1703	40.5109
Group C	Untreatment control	-75.91098*	8.74538	.000	-99.0612	-52.7608
	Group A	-104.45469*	11.12483	.000	-134.0974	-74.8120
	Group B	-17.17029	8.70619	.216	-40.5109	6.1703

*. The mean difference is significant at the 0.05 level.

A3.11 Statistical analysis of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189) (as used in chapter 7.3.4). All statistical analysis were carried out using SPSS version 20.

The root area (mm^2) of rice (*Oryza meridionalis*) was measured after exposure to each treatment and unexposed control (Table A3.11.1). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance on data indicated variances were homogeneous (Table A3.11.2). Data was analysed in a one way ANOVA with Gabriel post hoc test (p=0.05, ANOVA Table A3.11.3, post hoc test Table A3.11.4) to determine whether root area (mm^2) changed after different treatments. The control group, group A (rice and phage cocktail) and group B (phage treatment of *B. pseudomallei* infection) were not-significantly different to each other but were different to group C (*B. pseudomallei* infection).

Similarly the leaf area (mm^2) of rice (*Oryza meridionalis*) was measured after exposure to each treatment and unexposed control (Table 3.11.5). Samples sizes were ≥25 so normality was assumed (Krithikadatta, 2014). Analysis of homogeneity of variance on data indicated variances were not homogeneous (Table A3.11.6). Data was analysed in a one way ANOVA with Games-Howell post hoc test (p=0.05, ANOVA Table A3.11.7, post hoc test Table A3.11.8) to determine whether leaf area (mm^2) after different treatments was affected. The control group, group A (rice and phage cocktail) and group B (phage treatment of *B. pseudomallei* infection) were not-significantly different to each other but were different to group C (*B. pseudomallei* infection).

Table A3.11.1 Raw data of root area *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189). Untreated control is not exposed to any bacteria or bacteriophage. Groups are; Group A (rice soaked with phage cocktail), Group B (rice soaked with *B. pseudomallei* phage cocktail and *B. pseudomallei*), Group C (*B. pseudomallei* infection only).

Untreated control	Group A	Group B	Group C
77.4	63.6	87.1	30.3
53.6	49.3	62.6	40.9
65.9	32.9	74.1	33.9
71.2	56.3	66.1	12.5
70.1	38.5	55.3	23.2
45.6	43.2	50.4	16.5
56.7	39.9	60.4	63.6
67.6	43.3	22.9	62.8
87.1	41.2	36.9	28.9
67.1	51	33.1	27.2
29.3	47.6	31.6	10.7
35.3	31.9	45.1	12.8
67.8	67.2	46.7	10.1
53.7	48.5	71	9.2
45.1	63.5	43.2	25.1
44.1	88.6	39.8	35.1
41.7	78.9	46.8	62.4
26.4	65.5	37.5	27.9
37.8	56.5	50.8	20.2
66.2	69.5	35.5	15.6
35.9	71.9	43.9	23.6
45.3	36.6	48.7	16.1
45.2	53	47.3	16.7
45.5	40.1	60.9	25.5
54.9	65.1	26.6	30.3
51.6	42.2	37.1	11.9
65.1	59.9	44.4	9
58.4	56.4	43.7	12.7
26.8	56.5	43.9	18.7
52.6	66.5	22.8	

Table A3.11.2 Homogeneity variance of root area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

Data_root

Levene Statistic	df1	df2	Sig.
.114	3	115	.952

Table A3.11.3 One way ANOVA of root area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

ANOVA

Data_root

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15870.253	3	5290.084	23.524	.000
Within Groups	25861.175	115	224.880		
Total	41731.428	118			

Table A3.11.4 Post hoc analysis of root area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189) (Gabriel test).

Multiple Comparisons

Dependent Variable: Data_root

Gabriel

(I) Group_phage_meridionalis	(J) Group_phage_meridionalis	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreatment control	Group A	-1.13667	3.87195	1.000	-11.4939	9.2206
	Group B	5.82667	3.87195	.576	-4.5306	16.1839
	Group C	27.74368*	3.90518	.000	17.2979	38.1895
Group A	Untreatment control	1.13667	3.87195	1.000	-9.2206	11.4939
	Group B	6.96333	3.87195	.369	-3.3939	17.3206
	Group C	28.88034*	3.90518	.000	18.4345	39.3261
Group B	Untreatment control	-5.82667	3.87195	.576	-16.1839	4.5306
	Group A	-6.96333	3.87195	.369	-17.3206	3.3939
	Group C	21.91701*	3.90518	.000	11.4712	32.3628
Group C	Untreatment control	-27.74368*	3.90518	.000	-38.1895	-17.2979
	Group A	-28.88034*	3.90518	.000	-39.3261	-18.4345
	Group B	-21.91701*	3.90518	.000	-32.3628	-11.4712

*. The mean difference is significant at the 0.05 level.

Table A3.11.5 Raw data of leaf area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189). Untreated control is not exposed to any bacteria or bacteriophage. Groups are; Group A (rice soaked with phage cocktail), Group B (rice soaked with *B. pseudomallei* phage cocktail and *B. pseudomallei*), Group C (*B. pseudomallei* infection only).

Untreated control	Group A	Group B	Group C
108.4	130.3	142.2	63.8
161.3	141.8	145.1	63.7
175.5	122.5	190.7	22.1
153.9	83.2	107.1	45.3
167.2	95.9	66.6	27.1
124.5	76.9	95.1	25.5
116.8	81.8	134.7	59.3
136.7	111.7	55.9	100.6
122.4	104.9	163.1	57.4
100.4	134.4	52.9	76.3
144.8	90.5	112	29.6
104.1	123.4	127.2	26.4
98.7	118.2	103.4	26.3
91.3	109.5	95.6	31.9
133.3	83.3	144.7	44.3
160.7	152.3	114.2	129.1
139.6	156.3	177	96.1
106.9	132.3	152.1	70
123.4	130.4	168.3	30.3
89.9	118.8	168.2	33.3
177.6	110.4	128.7	33.5
48.4	111.8	104.6	20.4
99.2	123.3	147.4	16.2
144.9	140.2	138.4	31.1
115.3	172	97.1	57.1
102.6	76.8	22.6	20.2
114.4	117	67.6	18.3
128.7	89.5	107	6.7
124.6	135.7	2	1.2
53.7	157.5	38.3	

Table A3.11.6 Homogeneity of variance of leaf area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

Test of Homogeneity of Variances

Data_leaf

Levene Statistic	df1	df2	Sig.
3.869	3	115	.011

Table A3.11.7 One way ANOVA of leaf area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189).

ANOVA

Data_leaf

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	121300.154	3	40433.385	34.093	.000
Within Groups	136386.384	115	1185.969		
Total	257686.537	118			

Table A3.11.8 Post hoc analysis of leaf area of *O. meridionalis* growth using phage cocktail to control growth inhibition caused by *B. pseudomallei* (TSV189) (Games-Howell test).

Multiple Comparisons

Dependent Variable: Data_leaf

Games-Howell

(I) Group_phage_meridionalis	(J) Group_phage_meridionalis	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreatment control	Group A	4.55333	7.39150	.927	-15.0221	24.1288
	Group B	9.98000	10.32828	.769	-17.4575	37.4175
	Group C	78.75149*	7.95690	.000	57.6934	99.8095
Group A	Untreatment control	-4.55333	7.39150	.927	-24.1288	15.0221
	Group B	5.42667	9.77140	.945	-20.6468	31.5001
	Group C	74.19816*	7.21937	.000	55.0729	93.3234
Group B	Untreatment control	-9.98000	10.32828	.769	-37.4175	17.4575
	Group A	-5.42667	9.77140	.945	-31.5001	20.6468
	Group C	68.77149*	10.20579	.000	41.6332	95.9098
Group C	Untreatment control	-78.75149*	7.95690	.000	-99.8095	-57.6934
	Group A	-74.19816*	7.21937	.000	-93.3234	-55.0729
	Group B	-68.77149*	10.20579	.000	-95.9098	-41.6332

*. The mean difference is significant at the 0.05 level.

A3.12 Statistical analysis of qPCR and plate count quantitation of *B. pseudomallei* (TSV189) exposed to *Oryza sativa* L. cv Amaroo (domestic rice) and *O. meridionalis* (wild rice) with and without a phage cocktail (as used in chapter 7.3.6). All statistical analysis were carried out using SPSS version 20.

Bacterial counts in leaves of infected rice (*Oryza sativa* L. cv Amaroo, *B. pseudomallei* (TSV189)) was measured by qPCR after treatment with phage cocktail (group B) and *B. pseudomallei* (TSV189) infection only (group C) (Table A3.12.1). Data was normally distributed(Table A3.12.2). An independent T-Test (p=0.05, Table A3.12.3) was used to determine whether bacterial counts in leaves of rice differed between treatment (group B) and infected samples (Group C). Variances were assumed to be equal, there was no significant difference between the mean of the both groups. Bacterial counts in leaves of infected rice (*Oryza meridionalis*, *B. pseudomallei* (TSV189)) was measured by qPCR after treatment with phage cocktail (group B) and *B. pseudomallei* (TSV189) infection only (group C) (Table A3.12.4). Normality was violated (Table A3.12.5), so data was then log transformed and was found to be normal (Table A3.12.6). An independent T-Test (p=0.05, Table A3.12.7) was used to determine whether bacterial counts in leaves of rice differed between treatment (group B) and infected samples (Group C). Variances were assumed to be equal, there was no significant difference between the mean of both groups.

Bacterial counts in roots of infected rice (*Oryza sativa* L. cv Amaroo, *B. pseudomallei* (TSV189)) was measured by qPCR after treatment with phage cocktail (group B) and *B. pseudomallei* (TSV189) infection only (group C) (Table A3.12.8). Data was normally distributed (Table A3.12.9). An independent T-Test (p=0.05, Table A3.12.10) was used to determine whether root area of rice differed between treatment (group B) and infected samples (Group C). Variances were homogeneous, there was a significant difference between the mean of the both groups.

Bacterial counts in roots of infected rice (*Oryza meridionalis*, *B. pseudomallei* (TSV189)) was measured by qPCR after treatment with phage cocktail (group B) and *B. pseudomallei* (TSV189) infection only (group C) (Table A3.12.11). Data was normally distributed (Table A3.12.12). An independent T-Test (p=0.05, Table A3.12.13) was used to determine whether root area of rice differed between treatment (group B) and infected samples (Group C). Variances were homogeneous, there was significant difference between the mean of the both groups.

Bacterial counts of whole rice plants (*Oryza sativa* L. cv Amaroo) were measured by plate count after treatment with phage cocktail with *B. pseudomallei* (TSV189) infection (group B) and *B. pseudomallei* (TSV189) infection (group C) (Table A3.12.14). Data was normally distributed according to the Shapiro-Wilk test (the Kolmogorov-Smirnov could not be used with this data set) (Table A3.12.15). An independent T-Test (p=0.05, Table A3.12.16) was used to determine whether total rice plant counts differed between treatment (group B) and infected samples (Group C). Variances were assumed to be equal, there was a significant difference between the mean of the both groups.

Bacterial counts of whole rice plants (*O. meridionalis* (wild rice)) were measured by plate count after treatment with phage cocktail with *B. pseudomallei* (TSV189)

infection (group B) and *B. pseudomallei* (TSV189) infection (group C) (Table A3.12.17). Data was normally distributed according to the Shapiro-Wilk test (the Kolmogorov-Smirnov could not be used with this data set) (Table A3.12.18). An independent T-Test (p=0.05, Table A3.12.19) was used to determine whether total rice plant counts differed between treatment (group B) and infected samples (Group C). Variances were assumed to be equal, there was a significant difference between the mean of the both groups.

Table A3.12.1 Raw data of quantitation of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail by qPCR (leaf portion). Group B (phage cocktail with *B. pseudomallei* (TSV189), Group C (*B. pseudomallei* (TSV189)).

Group B	Group C
4.11E+07	9.38E+07
7.49E+05	9.18E+06
2.26E+07	1.56E+08
7.64E+07	5.98E+07
6.28E+07	1.32E+08
1.50E+08	1.34E+08

Table A3.12.2 Normality test of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail (leaf portion).

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data	Group B	.202	6	.200*
	Group C	.235	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.12.3 T-Test of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail (leaf portion).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data	Equal variances assumed	.108	.750	-1.243	10	.242	-38521833.3	30992941.56	-107578411	30534743.90
	Equal variances not assumed			-1.243	9.971	.242	-38521833.3	30992941.56	-107605272	30561605.03

Table A3.12.4 Raw data of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail (leaf portion).

Group B	Group C
5.06E+07	3.96E+07
7.41E+07	2.17E+07
1.04E+07	2.29E+07
6.36E+06	1.65E+08
2.85E+07	9.57E+06
9.78E+07	5.26E+07

Table A3.12.5 Normality test of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail (leaf portion).

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data	Group B	.171	6	.200*
	Group C	.328	6	.042

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.12.6 Normality test of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) in with a phage cocktail log transformed data (leaf portion) .

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
LOG_DATA	Group B	.186	6	.200*
	Group C	.163	6	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.12.7 T-Test of quantitation of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail log transformed data (leaf portion).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LOG_DATA	Equal variances assumed	.272	.614	-.240	10	.815	-.06229	.25926	-.63996	.51538
	Equal variances not assumed			-.240	9.844	.815	-.06229	.25926	-.64120	.51662

Table A3.12.8 Raw data of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail (root portion).

Group B	Group C
7.78E+08	3.65E+09
1.06E+09	5.33E+09
9.40E+08	1.08E+10
8.87E+08	2.99E+09
1.90E+09	4.16E+09
1.28E+09	4.38E+09
9.45E+08	3.10E+09

Table A3.12.9 Normality test of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) iwith a phage cocktail (root portion).

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data	Group B	.269	7	.134
	Group C	.297	7	.063

a. Lilliefors Significance Correction

Table A3.12.10 T-Test of quantitation of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail (root portion).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data	Equal variances assumed	4.463	.056	-3.668	12	.003	-3802857143	1036630212	-6061480349	-1544233936
	Equal variances not assumed			-3.668	6.236	.010	-3802857143	1036630212	-6316293438	-1289420848

Table A3.12.11 Raw data of quantitation by qPCR of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail (root portion).

Group B	Group C
9.92E+07	1.26E+08
1.32E+07	3.55E+07
1.84E+07	4.13E+08
1.71E+07	3.01E+08
7.70E+07	4.73E+07
2.97E+07	2.87E+08
3.70E+07	6.74E+08

Table A3.12.12 Normality test of quantitation of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail by (root portion).

		Kolmogorov-Smirnov[a]		
	Group	Statistic	df	Sig.
data	Group B	.270	7	.133
	Group C	.164	7	.200*

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.12.13 T-Test of quantitation of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) in with a phage cocktail log transformed data (root portion).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data	Equal variances assumed	8.173	.014	-2.619	12	.022	-227457143	86864668.38	-416718997	-38195289.0
	Equal variances not assumed			-2.619	6.258	.038	-227457143	86864668.38	-437899455	-17014830.7

Table A3.12.14 Raw data of quantitation by plate count of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail (whole plantlet)

Group B	Group C
1.30E+08	5.60E+08
1.80E+08	6.30E+08
1.60E+08	4.30E+08
6.00E+07	5.40E+08

Table A3.12.15 Normality test of quantitation by plate count of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail (whole plantlet).

Shapiro-Wilk		
Statistic	df	Sig.
.924	4	.562
.962	4	.792

Table A3.12.16 T-Test of quantitation by plate count of *B. pseudomallei* (TSV189) exposed *Oryza sativa* L. cv Amaroo (domestic rice) with a phage cocktail by (whole plantlet).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data	Equal variances assumed	.330	.587	-8.307	6	.000	-407500000	49053542.18	-527529694	-287470306
	Equal variances not assumed			-8.307	5.075	.000	-407500000	49053542.18	-533035391	-281964609

Table A3.12.17 Raw data of quantitation by plate count of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail by (whole plantlet).

Group B	Group C
3.30E+06	2.50E+08
3.80E+06	2.80E+08
1.50E+06	2.30E+08
1.30E+06	2.00E+08

Table A3.12.18 Normality test of quantitation by plate count of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) in with a phage cocktail by (whole plantlet).

Shapiro-Wilk		
Statistic	df	Sig.
.854	4	.239
1.000	4	1.000

Table A3.12.19 T-Test of quantitation by plate count of *B. pseudomallei* (TSV189) exposed *O. meridionalis* (wild rice) with a phage cocktail (whole plantlet).

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data	Equal variances assumed	7.631	.033	-14.101	6	.000	-237525000	16844305.42	-278741531	-196308469
	Equal variances not assumed			-14.101	3.008	.001	-237525000	16844305.42	-291046388	-184003612

A3.13 Statistical analysis of comparison of bacterial counts after enrichment using soil, rhizosphere soil and plant roots at SES (Castle Hill) and Drain site (as used in chapter 8.3.3). All statistical analysis were carried out using SPSS version 20.

Soil, rhizosphere soil and plant roots were measured for *B. pseudomallei* counts at different sites and depth by qPCR (Table A3.13.1). While normality could be assumed due to high sample size, a log transformation was carried out on the data due to the broad quantitation range. Analysis of homogeneity of variance on log transformed data indicated variances were not homogeneous (Table A3.13.2). Log transformed data was used in a one way ANOVA with Games-Howell Post hoc test (p=0.05, ANOVA Table A3.13.3, post hoc test Table A3.13.4) to determine whether bacterial counts changed at different site and depths. There were some statistical differences which are discussed in Chapter 8.

Table A3.13.1 Raw data of comparison of enriched soil rhizosphere soil and plants roots at SES (Castle Hill) and Drain site. Where enriched soil samples produced no bacteria (negative samples), a count of one was used to allow log transformed comparisons All data points representing negative samples are highlighted grey. *There were a total of 86 samples in SES soil rhizosphere and SES root. This table is cut off at 42 samples. All remaining samples were also negative and are included in the statistical analysis.

SES soil depth 30 cm	SES soil depth 10 cm	SES soil rhizosphere	SES root	Drain soil depth 30 cm	Drain soil depth 10 cm	Drain soil rhizosphere	Drain root
4.03E+08	3.26E+04	4.92E+01	1.00E+00	3.17E+04	5.36E+04	9.84E+04	1.00E+00
1.01E+08	7.63E+05	5.40E+01	1.00E+00	5.55E+04	6.76E+03	4.53E+02	1.00E+00
6.03E+08	1.91E+05	2.42E+02	1.00E+00	4.18E+06	2.55E+05	6.70E+02	1.00E+00
4.97E+07	1.19E+03	2.12E+03	1.00E+00	6.46E+04	2.42E+05	2.47E+03	1.00E+00
1.46E+08	1.44E+05	7.54E+02	1.00E+00	1.04E+06	1.88E+05	2.80E+02	1.00E+00
3.47E+08	4.63E+08	2.14E+01	1.00E+00	1.08E+05	5.80E+04	6.65E+02	1.00E+00
8.17E+07	1.09E+04	5.68E+02	1.00E+00	1.26E+05	1.11E+05	1.09E+02	1.00E+00
5.43E+07	2.28E+04	3.47E+02	1.00E+00	6.83E+04	1.27E+05	2.27E+03	1.00E+00
3.73E+08	2.40E+09	3.87E+02	1.00E+00	3.53E+04	1.06E+05	8.05E+02	1.00E+00
2.69E+08	2.79E+04	2.94E+02	1.00E+00	1.96E+04	4.98E+04	1.06E+03	1.00E+00
1.28E+08	6.13E+07	3.18E+04	1.00E+00	1.26E+04	4.17E+04	1.00E+00	1.00E+00
4.40E+07	1.22E+08	3.63E+02	1.00E+00	3.15E+04	2.55E+05	1.00E+00	1.00E+00
4.97E+08	4.10E+02	1.05E+04	1.00E+00	2.61E+05	1.28E+04	1.00E+00	1.00E+00
2.70E+08	1.50E+04	1.13E+02	1.00E+00	1.63E+04	3.31E+05	1.00E+00	1.00E+00
9.27E+07	3.50E+08	1.00E+00	1.00E+00	1.31E+04	6.88E+04	1.00E+00	1.00E+00
1.66E+08	3.97E+08	1.00E+00	1.00E+00	3.26E+04	1.77E+04	1.00E+00	1.00E+00
1.41E+08	4.87E+08	1.00E+00	1.00E+00	1.54E+04	4.23E+03	1.00E+00	1.00E+00
6.83E+08	8.47E+07	1.00E+00	1.00E+00	3.43E+03	2.96E+04	1.00E+00	1.00E+00
1.83E+08	9.27E+07	1.00E+00	1.00E+00	2.13E+04	1.79E+04	1.00E+00	1.00E+00
3.43E+08	1.26E+08	1.00E+00	1.00E+00	6.23E+04	1.63E+04	1.00E+00	1.00E+00
3.33E+08	2.90E+09	1.00E+00	1.00E+00	1.35E+05	8.20E+03	1.00E+00	1.00E+00
9.93E+08	9.20E+09	1.00E+00	1.00E+00	3.15E+04	6.01E+04	1.00E+00	1.00E+00
9.73E+08	7.40E+06	1.00E+00	1.00E+00	2.17E+04	1.56E+04	1.00E+00	1.00E+00
4.93E+08	1.12E+09	1.00E+00	1.00E+00	7.67E+04	1.53E+04	1.00E+00	1.00E+00
8.00E+07	3.37E+08	1.00E+00	1.00E+00	4.40E+05	4.12E+04	1.00E+00	1.00E+00
1.67E+08	8.93E+08	1.00E+00	1.00E+00	1.32E+04	1.74E+04	1.00E+00	1.00E+00
3.60E+08	1.00E+00	1.00E+00	1.00E+00	1.85E+05	1.01E+05	1.00E+00	1.00E+00
1.00E+00	1.00E+00	1.00E+00	1.00E+00	8.30E+04	6.19E+04	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	6.37E+04	1.16E+05	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	5.43E+04	6.97E+04	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	9.41E+04	1.18E+04	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	1.43E+04	1.91E+04	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	1.04E+05	1.00E+00	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	2.36E+04	1.00E+00	1.00E+00	1.00E+00
		1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00
		1.00E+00	1.00E+00			1.00E+00	1.00E+00
		1.00E+00	1.00E+00			1.00E+00	1.00E+00
		1.00E+00	1.00E+00			1.00E+00	1.00E+00
		1.00E+00	1.00E+00			1.00E+00	1.00E+00
		1.00E+00	1.00E+00			1.00E+00	1.00E+00
		1.00E+00	1.00E+00			1.00E+00	1.00E+00
		1.00E+00*	1.00E+00*			1.00E+00	1.00E+00

Table A3.13.2 Homogeneity of variance comparison of log transformed data of enriched samples using soil, rhizosphere soil and plant roots at SES (Castle Hill) and Drain site.

Test of Homogeneity of Variances

LOGDATA

Levene Statistic	df1	df2	Sig.
32.549	7	374	.000

Table A3.13.3 One way ANOVA of comparison of log transformed data of enriched samples using soil, rhizosphere soil and plant roots at SES (Castle Hill) and Drain site.

ANOVA

LOGDATA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2777.207	7	396.744	268.445	.000
Within Groups	552.747	374	1.478		
Total	3329.954	381			

Table A3.13.4 Post hoc analysis of log transformed data of enriched samples using soil, rhizosphere soil and plant roots at SES (Castle Hill) and Drain site (Games-Howell test).

Multiple Comparisons

Dependent Variable: LOGDATA

Games-Howell

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SES soil depth 30 cm	SES soil depth 10 cm	1.60928	.61384	.176	-.3453	3.5638
	SES soil rhizosphere	7.61349*	.32602	.000	6.5624	8.6646
	SES root	8.04175*	.30635	.000	7.0371	9.0464
	Drain soil depth 30 cm	3.43993*	.34939	.000	2.3268	4.5531
	Drain soil depth 10 cm	3.79765*	.38753	.000	2.5757	5.0196
	Drain soil rhizosphere	7.31645*	.37174	.000	6.1418	8.4911
	Drain root	8.04175*	.30635	.000	7.0371	9.0464
SES soil depth 10 cm	SES soil depth 30 cm	-1.60928	.61384	.176	-3.5638	.3453
	SES soil rhizosphere	6.00421*	.54349	.000	4.2333	7.7751
	SES root	6.43246*	.53192	.000	4.6881	8.1769
	Drain soil depth 30 cm	1.83064*	.55782	.045	.0252	3.6361
	Drain soil depth 10 cm	2.18837*	.58247	.012	.3203	4.0565
	Drain soil rhizosphere	5.70717*	.57209	.000	3.8663	7.5481
	Drain root	6.43246*	.53192	.000	4.6881	8.1769
SES soil rhizosphere	SES soil depth 30 cm	-7.61349*	.32602	.000	-8.6646	-6.5624
	SES soil depth 10 cm	-6.00421*	.54349	.000	-7.7751	-4.2333
	SES root	.42825*	.11153	.006	.0817	.7749
	Drain soil depth 30 cm	-4.17357*	.20164	.000	-4.8049	-3.5422
	Drain soil depth 10 cm	-3.81584*	.26223	.000	-4.6454	-2.9863
	Drain soil rhizosphere	-.29704	.23828	.914	-1.0434	.4493
	Drain root	.42825*	.11153	.006	.0817	.7749
SES root	SES soil depth 30 cm	-8.04175*	.30635	.000	-9.0464	-7.0371
	SES soil depth 10 cm	-6.43246*	.53192	.000	-8.1769	-4.6881
	SES soil rhizosphere	-.42825*	.11153	.006	-.7749	-.0817
	Drain soil depth 30 cm	-4.60182*	.16799	.000	-5.1439	-4.0598
	Drain soil depth 10 cm	-4.24409*	.23733	.000	-5.0099	-3.4783
	Drain soil rhizosphere	-.72530*	.21057	.026	-1.3975	-.0531
	Drain root	.00000	.00000	.	.0000	.0000
Drain soil depth 30 cm	SES soil depth 30 cm	-3.43993*	.34939	.000	-4.5531	-2.3268
	SES soil depth 10 cm	-1.83064*	.55782	.045	-3.6361	-.0252
	SES soil rhizosphere	4.17357*	.20164	.000	3.5422	4.8049
	SES root	4.60182*	.16799	.000	4.0598	5.1439
	Drain soil depth 10 cm	.35773	.29077	.920	-.5547	1.2702
	Drain soil rhizosphere	3.87652*	.26937	.000	3.0362	4.7169
	Drain root	4.60182*	.16799	.000	4.0598	5.1439
Drain soil depth 10 cm	SES soil depth 30 cm	-3.79765*	.38753	.000	-5.0196	-2.5757
	SES soil depth 10 cm	-2.18837*	.58247	.012	-4.0565	-.3203
	SES soil rhizosphere	3.81584*	.26223	.000	2.9863	4.6454
	SES root	4.24409*	.23733	.000	3.4783	5.0099
	Drain soil depth 30 cm	-.35773	.29077	.920	-1.2702	.5547
	Drain soil rhizosphere	3.51880*	.31728	.000	2.5282	4.5094
	Drain root	4.24409*	.23733	.000	3.4783	5.0099
Drain soil rhizosphere	SES soil depth 30 cm	-7.31645*	.37174	.000	-8.4911	-6.1418
	SES soil depth 10 cm	-5.70717*	.57209	.000	-7.5481	-3.8663
	SES soil rhizosphere	.29704	.23828	.914	-.4493	1.0434
	SES root	.72530*	.21057	.026	.0531	1.3975
	Drain soil depth 30 cm	-3.87652*	.26937	.000	-4.7169	-3.0362
	Drain soil depth 10 cm	-3.51880*	.31728	.000	-4.5094	-2.5282
	Drain root	.72530*	.21057	.026	.0531	1.3975
Drain root	SES soil depth 30 cm	-8.04175*	.30635	.000	-9.0464	-7.0371
	SES soil depth 10 cm	-6.43246*	.53192	.000	-8.1769	-4.6881
	SES soil rhizosphere	-.42825*	.11153	.006	-.7749	-.0817
	SES root	.00000	.00000	.	.0000	.0000
	Drain soil depth 30 cm	-4.60182*	.16799	.000	-5.1439	-4.0598
	Drain soil depth 10 cm	-4.24409*	.23733	.000	-5.0099	-3.4783
	Drain soil rhizosphere	-.72530*	.21057	.026	-1.3975	-.0531

*. The mean difference is significant at the 0.05 level.

APPENDIX 4: PUBLISHED MANUSCRIPTS

The contributions of all authors to the co-authored accompanying paper depended on each authors expertise. The first author carried out the research and the statistical analysis. The first author also designed the experiments and discussed these with Dr Jennifer Elliman. Mr Christopher Gardiner suggested the techniques for preparation of rice seed. Assoc Prof Jeffrey Warner provided the bacterial isolates and suggested using rice as a model and Dr Constantin Constantinou sourced the monoclonal antibody and trained the first author in use of immunofluorescense. All authors proof read the manuscript and provided opinions on focus and content.

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Effect of root colonization of domestic rice (*Oryza sativa* L. cv Amaroo) by *Burkholderia pseudomallei.*

Running title: colonization of rice by *B. pseudomallei*

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ABSTRACT

Burkholderia pseudomallei is a saprophytic bacterium that causes melioidosis and is often isolated from rice fields in southeast Asia where infection incidence is high among rice field workers. The aim of this study was to investigate the relationship between this bacterium and rice through grow out experiments where the effect of colonization of *B. pseudomallei* in roots of domestic rice (*Oryza sativa* L. cv Amaroo) could be observed. When *B. pseudomallei* was exposed to surface sterilised seeds, growth of both root and aerosphere was retarded compared to controls. The organism was found to localise in the root hairs and endodermis of the plant. A biofilm formed around the root and root structures which were colonized. Grow out experiments of a wild rice species (*Oryza meridionalis*) produced similar retardation of growth while another domestic cultivar, (*Oryza sativa* L. cv Koshihikari) did not result in retarded growth. This is the first report where *B. pseudomallei* infected and inhibited *Oryza sativa* L. cv Amaroo and might provide insights into plant and microbe interactions of the important human pathogen.

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INTRODUCTION

Melioidosis is caused by the aerobic Gram-negative bacillus *Burkholderia pseudomallei* which is common in Northern Australia and Southeast Asia where the mortality rate can be as high as 40% (1) *B. pseudomallei* is recognized as a soil and water borne pathogen and is commonly isolated from the soil of rice fields and other environmental sources where the organism is endemic (2-7). Exposure to the organism in rice paddies is a significant risk to rice farmers, particularly those who are also diabetic who are generally more predisposed to infection (8).

The rhizosphere is a rich habitat for bacteria. Microbial interaction with plants can result in a variety of effects from pathogenesis to growth promotion (9) and plants may facilitate persistence of the microorganism (10). Bacteria in the *Burkholderia* genus are well known to be plant endophytes, some with plant growth promotion capacity and some as pathogens (11). As *B. pseudomallei* has a broad host range (12-17) and has been shown to infect other species of plants (18, 19), it may be possible that it will infect some cultivars of rice and a plant-microbe interaction may assist in persistence of *B. pseudomallei* in the rice fields where it is commonly found. However no studies have so far identified any growth promotion or pathogenic relationship between the *B. pseudomallei* and rice. Lee, et al. (19) found no effects on a Japanese rice cultivar (*Oryza sativa* cv. Nipponbare) while Kaestli, et al. (18) found no effects on an Australian wild rice species (*Oryza rufipogon*). An examination of the interaction of *B. pseudomallei* with more cultivars and species of rice could provide more information about whether rice is in general resistant to *B. pseudomallei* or identify a

potential host for an infection model. The development of a rice model of *B. pseudomallei* infection has the potential to assist in further studies of plant-microbe interaction and in development of biocontrol measures.

The aim of this project was to investigate if *B. pseudomallei* could infect domestic rice (*Oryza sativa* L. cv Amaroo) and to demonstrate the effect this infection had on plant growth; this effect was also compared with other species of *Burkholderia* and other cultivars of rice.

MATERIALS AND METHODS

Bacteria. All isolates of bacteria used in this study were subcultured from College of Public Health, Medical and Veterinary Sciences collection, James Cook University, Australia. *B. pseudomallei* were removed from storage (-80°C) were streaked onto Ashdown agar incubated at 37°C for 48 h in a Physical Containment Level 3 Laboratory (PC3). A single colony was subcultured into 10 ml of Luria Bertani (LB) broth. Culture was incubated at 37 °C with agitation (166 rpm) overnight. Other *Burkholderia* species were processed as above with the exception that incubation temperatures for both Ashdown agar and LB broth were 30 °C. Other non-*Burkholderia* species were streaked onto LB agar, incubated at 37 °C for 24 h and subcultured into LB broth at 37 °C for 24 h.

Biosafety Protocols. All experimental work with *B. pseudomallei* was carried out in Australia at James Cook University. The Australian/New Zealand Standard 2243.3:2010 (Safety in Laboratories) was adhered to, with work on live organisms carried out in a BSL3 laboratory to BSL3 standard. All protocols were approved by the Institutional Biosafety Committee and biological samples were handled in a C2 cabinet with all waste autoclaved. Confirmation of sterilization was carried out on duplicate samples prior to any processed, killed organism being removed from the laboratory to a BSL2 laboratory.

Preparation and cleaning of seeds. Rice (*Oryza sativa* L. cv Amaroo) seeds were sown, grown and harvested at the School of Veterinary and Biomedical Sciences, James Cook University during 2012-2013. Wild rice (*Oryza meridionalis*) seeds were harvested at Woodstock, Townsville in May 2012 and kept for one year until the dormancy period for the seeds had passed (20, 21). A Japanese rice cultivar (*Oryza sativa* L. cv. Koshihikari) was provided by a local supplier. The harvested and provided seeds were cleaned as per the modified Oyebanji, et al. (22) by soaking and agitating (200 rpm) in 3.5% bleach (sodium hypochlorite) at 30 °C for 10 minutes. Disinfectant solutions were discharged and seeds were washed by soaking and agitating in sterile distilled water for three minutes (washes were repeated four times). The last sterile distilled water was decanted and inoculated onto Ashdown's agar to confirm no *B. pseudomallei* were present. A selective agar was used as surface cleaning does not remove all bacteria from inside seeds and typically other bacteria will still replicate in non-selective media (23, 24). Seeds were soaked at a depth of approximately one cm in sterile distilled water at 30 °C for two days to encourage uniform imbibition and germination (25) This sterile distilled water was also tested for contamination with *B. pseudomallei* by incubation on Ashdown's agar for 48 hours.

Infection of seeds for plant growth experiments. A dose trial for infection was carried out based on the lower dose used by Kaestli, et al. (18) and higher dose used by Mattos, et al. (26). Cleaned, primed seeds of *Oryza sativa* L. cv Amaroo, were infected using a protocol modified from Kaestli, et al. (18) by placing in 10 ml of 10^4 and 10^8 CFU/ml *B. pseudomallei* TSV 189 in LB for an hour, prior to removal from bacterial broth. Control seeds were treated by soaking in LB for the same amount of time. 45 seeds at each dose and 45 control seeds were used for this study. For comparison of bacterial strains and species, 45 control seeds and 45 infected seeds soaked in 10^8 CFU/ml of each bacterial strain and species were used. The other bacteria included two strains of *B. pseudomallei* (TSV192

environmental, Australia; K96243 clinical, Thailand), as well as bacteria in the same genus (*Burkholderia cenocepacia* (17sp), *B. vietnamensis* (38 sp) and *B. ubonensis* (A21). The seeds were then transferred to experimental chambers. The seeds were transferred for propagation based on the method of Hoagland and Arnon (27), modified by Watt, et al. (28) to 1% (w/v) ¼ strength Hoagland agar. Survival kinetics in ¼ strength Hoaglands broth were also carried out and bacteria were not inhibited in this media (supplementary material). The seedlings were grown in Hoagland agar in sealed glass bottles and incubated under cycles of fluorescent light (12 h) and darkness for (12 h) for seven days at 30 °C. *B. pseudomallei* TSV189 was also used at the higher dose as above to infect Australian wild rice (*Oryza meridionalis,* 30 seeds per group), and Japanese rice (*Oryza sativa* L. cv. Koshihikari, 45 seeds per group). All experiments included a control uninfected rice group to control for between experiment error.

Plant measurement and statistical analysis of plant growth determination. Rice seedlings were removed from agar and photographed alongside a scale. Images were analysed using the area measurement commands in Adobe Photoshop CS6 to calculate area as described by Villar, et al. (29). When an analysis consisted only of a control and experimental group, Independant t tests (p=0.05) were performed using IBM SPSS version 20 and where three or more groups are compared, a one way ANOVA was performed (p=0.05) using a Gabriel post hoc test. Where results are compared across different experiments, actual areas are converted to percent of the average within experiment uninfected control area. This was done to control for between experiment variation.

Reactivity of the antibody specific for *B. mallei* with *B. pseudomallei,* and other bacteria. No commercial anti-*B. pseudomallei* antibodies were could be obtained at the beginning of our experiments. However, antibodies specific for *B. mallei* are available and considering that some lipopolysaccharides are shared by *B. pseudomallei* and *B. mallei* a

monoclonal antibody specific for *B. mallei* LPS (MCA2823 AbD Serotec/ BIO-RAD, USA) was tested.

Various species and strains of bacteria (Table 1) were cultured, centrifuged at 3000 g for 15 min and the supernatants were discharged. Pellets were washed three times with 0.85% NaCl and 15 µl of bacterial suspensions were added on slides (Menzel-GmbH & Co KG, Braunschweig, Germany), in duplicate, and let dry for one hour at room temperature (RT).

Fixation, antibody concentration and incubations were optimised using *B. pseudomallei* TSV189 and *B. ubonensis* A21. After this, all bacteria were fixed in acetone for four hours, blocked with goat sera and incubated with mAb MCA2823 diluted 1/100 overnight at 4°C in a humidified chamber. After washing in PBS the bacteria were incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 595 diluted 1/300 for 45 min at RT, washed in PBS and mounted using fluorescent mounting media (KPL, USA) and observed under an epifluorescent microscope (AxioImager.Z1, Zeiss, Germany). Pictures were taken using a digital camera (AxioCam MRm, Zeiss, Germany). As a negative control, a mouse mAb of the same isotype (IgG1) (MM1A, Anti CD3 receptor, Washington State University) was used at the same concentration as mAb MCA2823. The reactivity of all species and strains of bacteria with mAb MCA2823 was checked in duplicate.

***B. pseudomallei* infection of seeds for IFA.** Cleaned, primed seeds were incubated in Petri dishes for two days for primary root germination. At this time, roots of three seedlings were inoculated with about 10^7 CFU *B. pseudomallei* (isolate TSV189) (100ul of 10^8 CFU/ml) as per the method of Kaestli, et al. (18) while another three were not infected. The six seedlings were then transferred for propagation and incubated as per the plant growth experiment.

Preparation of plant samples for IFA. Plantlets were gently lifted from the agar surface and roots were washed in 0.85% NaCl three times to remove any loose bacteria or agar. Root pieces were cut to 0.5-1 cm long and fixed in acetone at – 20 °C for 3 days, until sterility of samples was confirmed. Further, the samples were embedded in Optimum Cutting Temperature (O.C.T.) (Tissue-Tek®, Sakura, Japan) and stored at -80 °C until sections were cut. Five micrometer thick cryosections were cut from roots with a cryostat (Leica CM1850, Germany) at -20 °C and transferred to slides (Menzel-GmbH & Co KG, Braunschweig,Germany). Sections were dried overnight at room temperature under a fan.

IFA on root sections.

Sections were immersed in PBS and blocked for 30 min at RT in 10% (v/v) goat sera diluted in 1% BSA in PBS. After a brief wash in PBS the sections were incubated at RT for 40 min then overnight at 4°C with mAb MCA2823 diluted 1:100 in 1% BSA in PBS (final concentration 10µg/ml). Negative controls using mAb MM1A as the primary antibody were processed similarly. The slides were washed gently with PBS and the sections were incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 595 (Invitrogen, USA) diluted 1/300 in 1% BSA/PBS for 45 minutes at room temperature. After three gentle washes with PBS, slides were mounted using fluorescent mounting media (KPL, USA) and observed under an epifluorescent microscope (AxioImager.Z1, Zeiss, Germany) as above.

RESULTS

Preparation of seeds. None of the seeds used showed any evidence of prior contamination with *B. pseudomallei,* so all *B. pseudomallei* found was deemed to be present due to infection. No uninfected plantlets showed any evidence of *B. pseudomallei* presence, further supporting this conclusion.

The pilot dose study determined that growth was inhibited at 10^8 CFU/ml but not at 10^4 CFU/ml (Figure 1 a & b) A dose of 10^8CFU/ml was selected for plant infection trials. Rice infected with *B. vietnamensis* displayed growth that was not significantly different to the uninfected control rice, indicating that the 10^8CFU/ml dose of Gram negative bacteria selected was not in and of itself able to stunt growth (Figure 1 c & d).

***Oryza sativa* L. cv Amaroo infection trials with *Burkholderia* species.** When germination of seeds infected with all strains of *B. pseudomallei* did occur, the majority of plantlets did not show fully expanded leaves and had shorter roots than the controls. An example is shown in figure 2 (a and b). Infection with all *Burkholderia* species and strains except *B. vietnamensis* showed statistically significant inhibition of growth relative to the control (Table 2). Of the species tested, *B. pseudomallei* and *B. cenocepacia* caused the most inhibition, followed by *B. ubonensis* (figure 3a and b, Table 3).

Bacterial inhibition of other strains and species of rice. While wild Australian rice (*Oryza meridionalis*) was inhibited by *B. pseudomallei* TSV189, the Japanese rice (*Oryza sativa* L. cv. Koshihikari) was not inhibited in either root or leaf growth (Figure 4).

***B. pseudomallei* Immunofluorescence Assay (IFA).** The reactivity of mAb MCA2823 was tested with a variety of isolates of *B. pseudomallei* as well as other species of bacteria that are closely related to *B. pseudomallei* or can be found in the soil. *B. mallei* is not found in Australia, where this work was done and was not available for testing due to restrictions on importation of potentially pathogenic microorganisms. The IFA using this antibody was positive for *B. pseudomallei* and negative for *B. pseudomallei* near neighbour species and other organisms (see Table1). In addition, no bacteria fluoresced in the roots of uninfected plants when IFA was used, thus it was possible to use mAb MCA2823 as a tool to detect *B. pseudomallei* in our plants.

Examination of infected plants using IFA showed the presence of *B. pseudomallei* as a multilayered structure around the epidermis and root hairs (figure 5a b and c). Individual bacteria were also found inside the root hair (figure 5 d, e and f), inside the exodermis (figure 5g, h and i), indicating infection of the plant rather than just surface colonisation.

DISCUSSION

The plant microbe interaction between rice and *Burkholderia pseudomallei* is of interest as *B. pseudomallei* is often found in rice fields and workers are exposed (2-4). Understanding more about the ecology of any interaction could improve our understanding about the persistence of the bacterium in this environment. Our study identified that *B. pseudomallei* TSV189 retarded the plant growth of Australian domestic rice (*Oryza sativa* L. cv Amaroo) and the native Australian wild rice (*Oryza meridionalis*) but not that of Japanese rice (*Oryza sativa* L. cv. Koshihikari), The resistance of Japanese rice has previously been reported for the cultivar *Oryza sativa* L. cv Nipponbare by Lee, et al. (19), which is a similar cultivar to Koshihikari (30). The wild rice (*Oryza rufipogon*) model of Kaestli, et al. (18) was also not affected by *B. pseudomallei* inoculation, however this is a different wild rice species than the one we tested. Both of these other studies also utilised other models which were affected by *B. pseudomallei*, indicating the *B. pseudomallei* isolates used were able to inhibit at least some host plant species. It is clear that there is a species and cultivar variation within the *Oryza* genus which determines the ability of *B. pseudomallei* to inhibit growth.

We also determined that both clinical and environmental Australian *B. pseudomallei* isolates as well as a clinical Thai isolate equally inhibited *Oryza sativa* L. cv Amaroo, indicating that *B. pseudomallei* may be generally inhibitory to some rice cultivars. This leads

to the question of whether different rice cultivars will succeed in *B. pseudomallei* endemic areas.

As an example, the iRiceZoning map has three major rice group areas that are produced in Thailand (http://carsr.agri.cmu.ac.th/projects/iRPZ/MAPRiceVarGroup.aspx). Aromatic rice and glutinous rice are produced in northeast Thailand (a highly endemic area of *B. pseudomallei* (2, 31) while non-aromatic rice grows in the central part of Thailand, where *B. pseudomallei* is less commonly found (31). While there are differences in rainfall, irrigation patterns and soil types in these regions (32), it is interesting to postulate that *B. pseudomallei* may also play a role in the success of different cultivars. Rice currently grown in northeast Thailand may be more resistant to the effects of *B. pseudomallei* and an infection trial of the different rice types and varieties would be useful to answer this question.

Low doses of *B. pseudomallei* did not inhibit growth of (*Oryza sativa* L. cv Amaroo), which may be due to competition with endophytic bacteria already present in seeds. Unfortunately it was not possible to remove all presence of potentially competing bacteria without killing the seeds. Previous experiments in which cleaning of seeds was studied, have also identified this limitation (24). The high dose selected was designed to skew any potential interaction in favour of *B. pseudomallei* and was a similar dose used in other plant: *B pseudomallei* experiments (18, 19, 26). *B. pseudomallei* has been found in soil at levels up to 10^5CFU/g (6), so growth retardation in natural soils may not be as obvious or may allow for persistent interactions between the plant and microbe. The length of this experiment (7 days) and growth in Hoagland agar, while also commonly used (26, 33, 34), is too short to allow for a full growth of rice and may not mimic growth in soil. It would be interesting to study *B. pseudomallei*: *Oryza sativa* L. cv Amaroo interactions at this bacterial load, over the life of the rice, in a soil environment, however this was not possible with our facilities.

Rhizospheres usually produce nutrient sources for soil bacteria such as root exudates (sugar, amino acid, and oxygen etc), border cells (root cap cell), and root debris (cell contents, lysates etc) (35). This can result in biofilms forming around the epidermis and roots hairs. Root hairs develop during plant growth and autolyse, resulting in proliferation of bacteria which can then invade the epidermis and cortex (36). Using IFA, we found that in root sections, *B. pseudomallei* formed multiple layers around the epidermis. These were not removed by washes in 0.85% NaCl, indicative of a biofilm, although the presence of exopolysaccharide was not tested to confirm this. *B. pseudomallei* were also found inside root hairs and in the endodermis, however images of the cortex were insufficient to clearly confirm the presence of *B. pseudomallei* in the cortex (data not shown). This is similar to results by Kaestli, et al. (18) who reported presence of *B. pseudomallei* in root hairs and cortex of grasses in epidemic areas.

This study successfully experimentally colonized the roots of a domestic rice cultivar with *B. pseudomallei* and identified differential inhibition of growth of different species and cultivars of rice. However, the incubation period is short and the plants have been grown in hydroponic agar. Growth in soil typical of rice paddies, for longer periods, may vary the results. In addition, this experiment used a high dose of bacteria, though other literature also uses relatively high inoculums, and/or wounding of tissue to encourage inoculation or invasion (18, 19, 26, 34, 37). Natural environmental conditions may result in lower exposures. While *B. pseudomallei* can infect the roots of (*Oryza sativa* L. cv Amaroo) via root hairs and retard growth, it does not retard growth of *Oryza sativa* L. cv. Koshihikari. This cultivar difference could be a factor in the successful or unsuccessful growth of particular cultivars of rice in endemic regions. The relative susceptibility of plants may also affect persistence and thus the biogeographical boundaries of *B. pseudomallei*. A susceptible

rice cultivar also means that biocontrol experiments can be carried out with rice and work is currently underway in this area.

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FIGURE LEGENDS

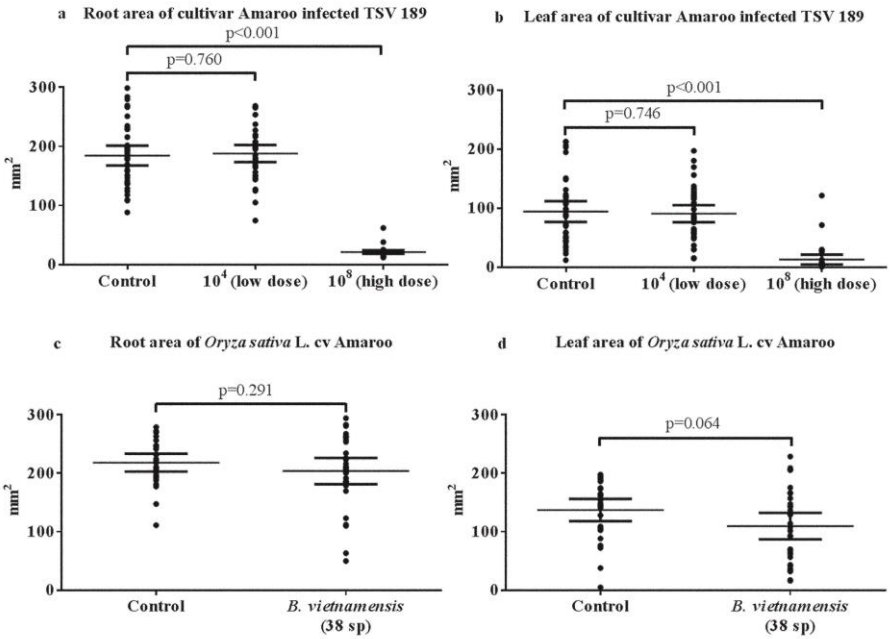
Figure 1: Infection of *Oryza sativa* L. cv Amaroo with low (10^4 CFU/ml) and high (10^8 CFU/ml) doses of *B. pseudomallei* TSV 189 (root (a) and leaf (b)), and a high dose of *B. vietnamensis* (38sp) (10^8 CFU/ml) (root (c) and leaf (d)) as well as control uninfected groups for each infection. Measurement of area (mean with 95% CI). The low dose of *B. pseudomallei* does not significantly stunt root or leaf. (*B. pseudomallei* root p=0.760, leaf p=0.746) while the high dose

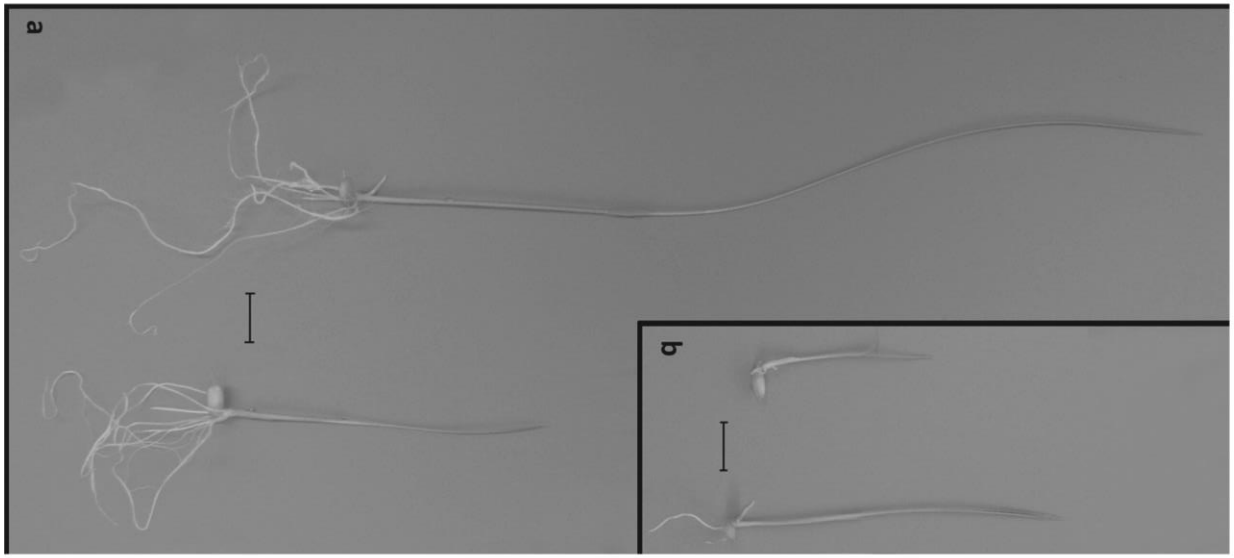
of *B. pseudomallei* produces significant stunting of growth ($p<0.001$) of both root and leaf. The high dose of *B. vietnamensis* does not significantly stunt root or leaf. (*B. vietnamensis* root $p=0.291$, leaf $p=0.064$) (Independent T tests, $p=0.05$).

Figure 2: Effect of *B. pseudomallei* (TSV 189) on rice seedlings. Growth of seedlings after 7 days without *B. pseudomallei* (a) and with *B. pseudomallei* (b). Seedlings exposed to *B. pseudomallei* have a shorter leaf and, fewer, shorter roots compared to the unexposed control (a). Bar indicates 1 cm.

Figure 3: Comparison of inhibition of growth of *Oryza sativa* L. cv Amaroo, due to a range of Burkholderia species, relative to the uninfected control growth. Percent of root (a) and leaf area (b) ± 95% CI are displayed as are significance values of inhibition of growth. *B. vietnamensis* does not significantly inhibit growth (leaf $p=0.064$, root $p=0.291$) while all other species do (p values also presented in Table 2) (independent *t* tests, $p=0.05$). *B. ubonensis* inhibition is also significantly different to all other species (Table 3). *B. cenocepacia* and the *B. pseudomallei* strains are not significantly different to each other in their inhibition (Table 3) (ANOVA, Gabriel post hoc test, $p=0.05$).

Figure 4: Inhibition of growth, due to *Oryza sativa* L. cv Amaroo, of three rice species/cultivars relative to the control growth of each rice species/cultivar. Percent of root (a) and leaf area (b) ± 95% CI are displayed as are significance values of inhibition of growth. Cultivar Koshihikari growth is not significantly reduced relative to its control (root $p=0.571$, leaf $p=0.599$), while both other rice species/cultivars are; *Oryza sativa* L. cv Amaroo (root $p<0.001$, leaf $p<0.001$), *Oryza meridionalis* (root $p<0.001$, leaf $p<0.001$). (Independent T tests, $p=0.05$).

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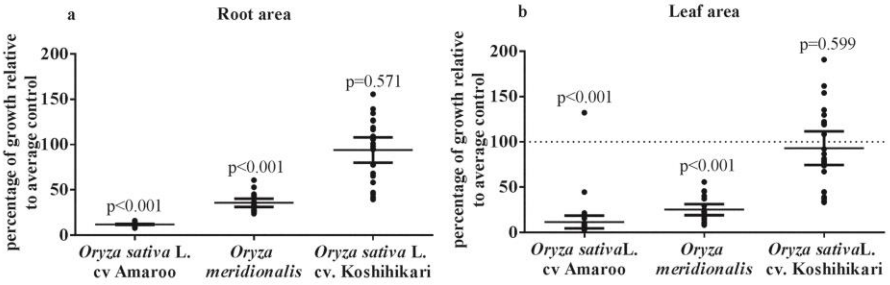

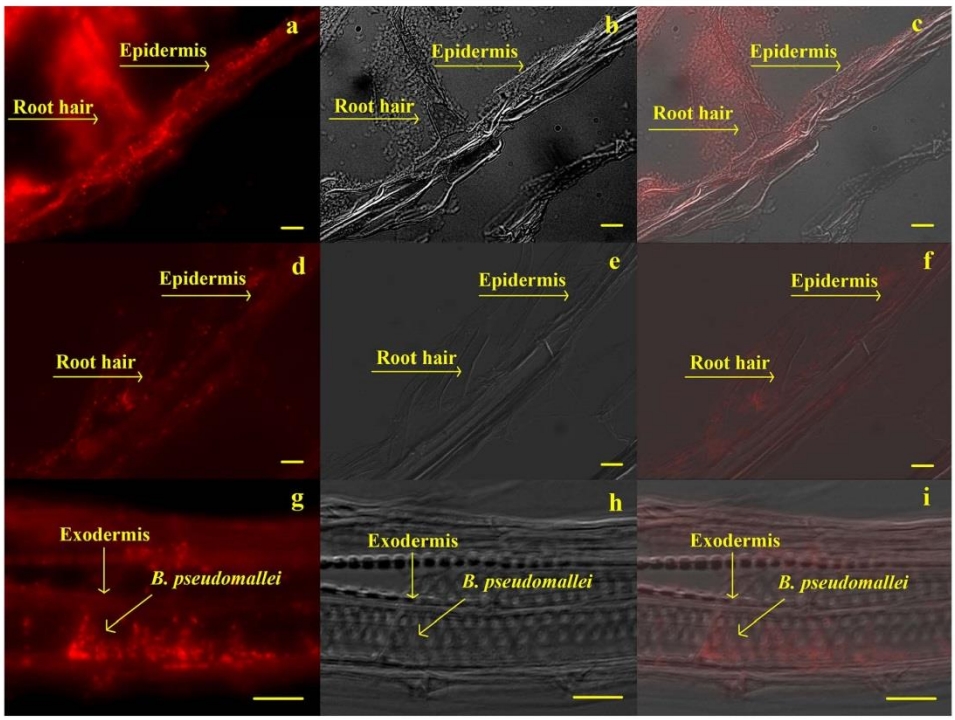

Table 1: Reactivity of mAb MCA2823 with *B. pseudomallei*, neighbour *Burkholderia* species and other bacteria. Superscript numbers in species column indicate method previously used to identify bacterial species ([1]: API 20 NE, [2]: IHA, EIA IgG/M, [3]: MLST, [4]: PCR TTSS gene, [5]: Full sequence and [6]: recA gene.

Code	Species	Isolated	Present	IFA
4	*Burkholderia pseudomallei* [1]	Human clinical	Mornington Island, Australia	+
8	*Burkholderia pseudomallei* [2]	Human clinical	Cloncurry, Australia	+
C1	*Burkholderia pseudomallei* [3]	Human clinical	Adiba, Sanabase, Papua New Guinea	+
C2	*Burkholderia pseudomallei* [3]	Human clinical	Kimama, Teleme, Papua New Guinea	+
TSV189	*Burkholderia pseudomallei* [4]	Alpaca necropsy	Townsville, Australia	+
14-289	*Burkholderia pseudomallei* [4]	Parrot necropsy	Townsville, Australia	+
14-327	*Burkholderia pseudomallei* [4]	Koala necropsy	Townsville, Australia	+
TSV192	*Burkholderia pseudomallei* [4]	Soil	Townsville, Australia	+
TS5	*Burkholderia pseudomallei* [1]	Soil	Townsville, Australia	+
K96243	*Burkholderia pseudomallei* [5]	Human clinical	Thailand	+
TSV4	*Burkhoderia arboris* [6]	Water seep	Townsville, Australia	-
TSV19	*Burkholderia gladioli* [6]	Bulk water	Townsville, Australia	-
TSV21	*Burkholderia cepacia* [6]	Water seep	Townsville, Australia	-
TSV87	*Burkholderia pyrrocinia* [6]	Bulk water	Townsville, Australia	-
TSV88	*Burkholderia pseudomultivorans* [6]	Bulk water	Townsville, Australia	-
E1	*Burkholderia thailandensis* [3]	Clay	Biula, Papua New Guinea	-
A21	*Burkholderia ubonensis* [6]	Soil	Adiba, Sanabase, Papua New Guinea	-
17sp	*Burkholderia cenocepacia* [6]	rhizosphere soil	Townsville, Australia	-
31sp	*Burkholderia latens* [6]	rhizosphere soil	Townsville, Australia	-
38 sp	*Burkholderia vietnamiensis* [6]	rhizosphere soil	Townsville, Australia	-
A03a	*Bordetella spp.* [6]	rhizosphere soil	Adiba, Sanabase, Papua New Guinea	-
13sp	*Achromobacter xylosoxidans* [6]	rhizosphere soil	Townsville, Australia	-
ATCC27895	*Pseudomonas aeruginosa*	Control		-
ATCC25921	*Escherichia coli*	Control		-
ATCC10876	*Bacillus cereus*	Control		-
ATCC13076	*Salmonella enteritidis*	Control		-

Table 2 Summary statistics of infection of *Oryza sativa* L. cv Amaroo with various Burkholderias. Root and leaf area of infected samples are compared to uninfected root and leaf area in each case. Area measurements were compared by independent T test (p=0.05)

Species	Root/Leaf	*t*	df	p value	sized effect (r)
***B. vietnamensis* (38 sp)**	Root	1.066	57	**0.291**	**0.140**
	Leaf	1.888	57	**0.064**	**0.243**
***B. ubonensis* (A21)**	Root	11.565	60.958	<0.001	0.829
	Leaf	6.396	52.259	<0.001	0.663
***B. cenocepacia* (17sp)**	Root	20.336	57	<0.001	0.937
	Leaf	10.651	46.409	<0.001	0.842
***B. pseudomallei* (K96243)**	Root	14.846	28.128	<0.001	0.942
	Leaf	7.477	28.693	<0.001	0.813
***B. pseudomallei* (TSV192)**	Root	14.968	32.779	<0.001	0.934
	Leaf	5.695	28.925	<0.001	0.727
***B. pseudomallei* (TSV189)**	Root	15.373	31.618	<0.001	0.939
	Leaf	5.918	28.134	<0.001	0.745

Table 3 Gabriel Post Hoc test results of one way ANOVA comparing the relative growth (root and leaf) of *Oryza sativa* L. cv Amaroo on inoculation with different species and strains of *Burkholderia*. P values for % leaf area presented in the bottom left hand corner and for % root area in the top right hand corner.

P value	*B. vietnamensis* (38 sp)	*B. ubonensis* (A21)	*B. cenocepacia* (17sp)	*B. pseudomallei* (K96243)	*B. pseudomallei* (TSV192)	*B. pseudomallei* (TSV189)	
B. vietnamensis (38 sp)		<0.001	<0.001	<0.001	<0.001	<0.001	
B. ubonensis (A21)	<0.001		<0.001	<0.001	<0.001	<0.001	
B. cenocepacia (17sp)	<0.001	0.003		0.914	0.999	0.988	
B. pseudomallei (K96243)	<0.001	<0.001	0.995		1.000	1.000	
B. pseudomallei (TSV192)	<0.001	0.002	1.000	0.988		1.000	
B. pseudomallei (TSV189)	<0.001	0.001	1.000	0.999	1.000		
Leaf area							Root area

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