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Using *Burkholderia* sp. as a biocontrol agent in kiwifruit plants against *Neofusicoccum parvum*

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Shixi Wu

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science with Honours.

Using *Burkholderia* sp. as a biocontrol agent in kiwifruit plants against *Neofusicoccum parvum*

by

Shixi Wu

Actinidia chinensis (kiwifruit) is an important horticultural crop in New Zealand. A fungal pathogen, *Neofusicoccum parvum* (teleomorph *Botryosphaeria*), was found causing fruit drop symptoms in kiwifruit orchards. To target this issue, a potential bacterial biocontrol agent, *Burkholderia* sp. (isolate W4R11), was used to test its efficacy against *N. parvum in planta*. Molecular approaches and *in vitro* testing were conducted prior to the glasshouse trial to provide insights on understanding the biology of *N. parvum* and *Burkholderia* sp. This work demonstrated the *in vitro* antagonist effect of *Burkholderia* sp. against *N. parvum.* Cultural practices on the two organisms were also conducted in the laboratory, providing inoculant material for the *in planta* testing.

This is the first study testing the use of *Burkholderia* sp. (isolate W4R11) as a wound protectant in one-year old kiwifruit plants against *N. parvum*. It demonstrated an apparent competition between the two organisms and provided suggestions on colonization and movement of *Burkholderia* sp. and *N. parvum* in kiwifruit plants. The *Burkholderia* strain can colonise 15 -30%

of the plants, which had no substantial differences compared to *N. parvum*. When examining the stems further from the wound site, *Burkholderi*a sp. showed suppression on *N. parvum*, resulting in a non-fungal colonization. The result suggested: (1) *Burkholderia* sp. may have a better colonization ability and faster movement than *N. parvum* in kiwifruit plants; (2) *Burkholderia* sp. can be used as a wound protectant after pruning or harvesting of the kiwifruit to inhibit wound-invading pathogens; and (3) to prune the kiwifruit stem 20 cm from the infected site and then applying the biocontrol agent may reduce disease incidence *in planta.*

Future studies should utilize more replicates and field testing to confirm the biocontrol ability of this particular strain.

Keywords: *Neofusicoccum parvum*, *Burkholderia* sp., biocontrol, kiwifruit, *in vitro*, *in planta*

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Chapter 1

General Introduction

1.1 Kiwifruit

1.1.1 Taxa and morphology

The genus *Actinidia* Lindl. contains around 60 plant species . *Actinidia chinensis,* known as kiwifruit*,* is one of the species that have been most commonly commercialized. It was originated from mainland China, and was divided into two varieties: *A. chinensis* Planch. var. *chinensis* and *A. chinensis* var. *hispida*. The latter variety was previously described as *A. chinensis* var. *deliciosa*, which then separated as a distinct species by itself.

Kiwifruit is a woody plant and as a climbing shrub, it may reach 10 meters in length. It is dioecious, with white to cream-colored flowers. Branches are reddish, with oblong lenticels present. Leaves are papery, pale to dark green, broadly obovate or suborbicular shaped. They are often associated with hair on the abaxial surface. In wild *A. chinensis*, the fruit are often small and rounded (Ferguson 1999). Commercially selected cultivars give bigger fruits, which are almost hairless when mature. The fruit has a smooth skin with a beak shape at the stem end. The colors of the flesh vary from green to yellow, and one variety has red inner pericarp flesh. The flavor of the gold kiwifruit, *A. Chinensis*, is sweet and aromatic, which is considered to be more appealing to the market than the green kiwifruit, *A. deliciosa*.

The kiwifruit industry had suffered significant losses between 2010 to 2013, due to the outbreak of one bacterial disease, Psa (*Pseudomonas syringae* pv. *actinidiae*). *Actinidia chinensis* Planch. var*. chinensis*

'Gold3' was found to be more disease resistant. It is now grafted in place of the old variety,“Hort16A”, and been marketed as “SunGold” in New Zealand (PlantandFood 2012).

1.1.2 Distribution and economic role

Kiwifruit can grow under temperate climate. It requires well-drained, fertile soil, irrigation and protection against wind and frost (Ferguson 1991). Kiwifruit growing regions in New Zealand are locating in the coastal areas of the North Island including Auckland, Bay of Plenty, Hawkes Bay area, Wanganui and the top of the South Island near Nelson. The major growing area among which is situated in the Bay of Plenty, with Te Puke known as the kiwifruit capital of the world (KVH 2016).

The global market demand for kiwifruit has increased significantly since the 1980s, with kiwifruit production exceeding 1.1 million tons in 2009. New Zealand, the third largest kiwifruit producing country in the world, yielded 400,000 tons of fruit in 2014. In the same year, the NZ kiwifruit exports earned 930 million dollars, and the growing regions covered 10944 hectares (FreshFacts 2014). Competition from international growers from countries like Chile, exporting duty and tariff levels, have put New Zealand growers and exporters under pressure. They have also been challenged to use less chemicals, due to the minimal chemical residue requirements (MAF 2011). An integrated pest management system that incorporates sustainability and market demands should therefore be considered for the kiwifruit industry (Suckling *et al*. 2003).

1.1.3 Kiwifruit production

A series of processes, from breeding, pollination, harvesting and storage, takes place in the kiwifruit orchard. The production year starts when the vines drop leaves and winter dormancy takes place. Fruited canes should be pruned at this period of time. The break in winter dormancy is indicated by bud break, followed by shoot and flower growth in spring. The timings of which can be affected by weather conditions like frost. In the summer, flowers need to be pollinated and thinned, and successful pollination requires 30-125 pollen grains per stigma (Hopping & Hacking 1982). Damaged fruit and excessively growing vines need to be removed to prevent pest and disease infection. Harvesting takes place in autumn, when the fruit reaches the desired dry matter content. Freshly-picked fruit goes through a storage process where it will be graded, put into trays and placed in cool storage. Kiwifruit suffers from various fungal diseases. Disease management in the production process is therefore important. Various disease control strategies and the understanding of different pathogens are required to achieve an effective control.

1.2 Common fungal disease in kiwifruit

Most fungal diseases show symptoms at the post-harvest stage (Pennycook 1985; Barkai-Golan 2001; Zhou *et al*. 2015). Common pathogens are *Botryosphaeria*, *Botrytis*, and *Sclerotinia* species.

Botryosphaeria species can induce fruit rot and shoot blight in kiwifruit (Pennycook 1985; Thomidis & Exadaktylou 2010). Disease symptoms on the fruit can develop after harvesting or after fruit removal from storage. The disease can deteriorate the flesh quality of the fruit and give an unpleasant smell. Dimples can develop on the fruit surface and grow into lesion during the fruit ripening process. The lesion can expand rapidly into pale brown oval shapes, up to 30 mm long, with a glassy dark green margin. It can develop in all parts of the fruit (e.g. side, distal end or center), resulting in a soft and squashy surface that

is slightly depressed (Koh *et al*. 2005). Inside the lesion, the flesh is macerated and once the skin is peeled off, the oval lesion inside is water-soaked and yellowish or white. *Botryosphaeria* species can also cause canker and shoot blight (Thomidis & Exadaktylou 2010). Common *Botryosphaeria* species that affect kiwifruit are *B. dothidea, Neofusicoccum luteum*, *N. parvum* and *Lasiodiplodia theobromae* (Pennycook 1985).

Botrytis species can cause early infection in the orchard and the symptoms can remain latent until the fruit transferred into the cool storage at 0 to 1^{o}C (Pennycook 1985). This is often caused by one species, *B. cinerea*, and the disease is often known as storage rot or grey mold in kiwifruit. In cool storage, an external darkening at the stem end of the fruit becomes conspicuous. The infected area can develop a defined front, with a darker green color compared to the normal flesh, and the fruit feels soft. The un-infected area of the fruit remains healthy looking. As the rot advances, it may spread all over the fruit, yet often leaves the distal end uninfected. Infected fruit flesh appears water-soaked with a faint pink discoloration (Elmer & Michailides 2004). As the disease progresses, flocculate, dull white mycelium is visible on the outside of the fruit, which will darken into a grey appearance due to dark conidiophores bearing powdery, grey conidia. The mycelium may also form irregular shaped, black sclerotia on the surface of the fruit (Pennycook 1985; Niklis *et al*. 1997).

There are three *Sclerotinia* species that can cause plant disease, and only *S. sclerotorium* has been reported on kiwifruit (Pennycook 1985). It can cause blossom blight that turns blossoms and pedicels pale brown, leaving clusters of blossoms soft and water-soaked in the summer. The symptoms are common in male vines and less in female buds and blossoms (Pennycook 1985). The dead brown tissues can spread onto the pedicel as well. Under dry conditions, secondary spread of infection is less likely to occur, as there are no visible mycelial growth on dead plant tissues. In wet seasons, the rotting blossom can be

covered with aggregated mycelium, which darkens and forms sclerotia. The rot lesions from the pedicels can quickly spread onto the shoots, leaves and petioles, especially in rainy seasons, giving necrotic lesions (Hoyte 2001). Fruit rot symptoms can develop during fruit set but are common in late summer after petal fall. *Sclerotinia* infection give a watery, sunken, and whitish lesion on surface of the fruit, with black sclerotia produced amongst the mycelium. Dried and shrunken whole fruits are observed on the vine and can also fall on the ground (Michailides & Elmer 2000). During dry seasons, shoot blight can also develop, especially on male plants (Pennycook 1985).

1.3 Disease management in kiwifruit

Various methods are applied for disease management in kiwifruit orchard. The following are factors for growers to consider, from planting to the storage process, as a general guideline.

1.3.1 Site selection

Kiwifruit requires well drained soil with regular nitrogen, and phosphorous fertilizer application. Poor drainage of the soil or high rainfall areas can provide favorable conditions for fungal spores to spread and germinate (Carlile *et al*. 2001). At places with standing water (e.g. low point of the field, irrigation overlapped areas or shaded areas), modification to the landscape is required. Besides, when the temperature reaches a preferable range for the pathogen growth, a high incidence of fungal infection may occur (KVH 2017a).

Ensuring adequate air movement and windbreak protection are necessary in disease management. Downwind areas should be avoided, where previous year spores can be trapped and accumulated from

plant debris or volunteer plants (Ogaraku 2010). Adequate spacing between the plants (5-6 metre spacing between rows) is necessary. It allows a good airflow and hence reduces relative humidity that may favor pathogen dispersal and growth (KVH 2017a). Site selection should also consider its practicability for fungicide application. High slope or hilly locations should be avoided for an easy machinery operation.

1.3.2 Resistant cultivars

Selection of disease-resistant cultivars can play a great part in disease management. By understanding the biology of fungal populations and the complicated interaction between the host and pathogen, one may be able to select a better performance plant (Beever *et al*. 2005).

Traditional breeding for resistant cultivar started in the early 21st century. Before which, for a long period of time, the green kiwifruit variety, "Hayward", was the only cultivar commercially grown in New Zealand (Ferguson *et al*. 1990). Nowadays, cultivar selections and breeding programs are conducted all over the world in countries like China, France and New Zealand, offering entries of new *Actinidia* fruit into international markets (Ferguson 1999). However, instead of enhancing the disease resistant ability, most new cultivars are selected to obtain an early maturing and better taste of the fruit. Previous work on "Hayward", in regard to fruit susceptibility of pathogens during cool storage (Pennycook 1985), may lead to studies on the susceptibility of different cultivars to these pathogens.

Genetic studies of host genes has been carried out in several studies, offering insights to the vast genetic sources of this plant, which could hopefully aid future breeding work (Ferguson *et al*. 1990; Beatson *et al*. 2014). Identifying particular desirable genes can be a long process. Take the interactions between *Botrytis cinerea* and kiwifruit for example: biochemical and genetic markers were used in determining host

resistance. By using microarray of gene expression and monitoring biochemical activity of defensive compounds like specific proteins and enzymes, certain genes were correlated with the onset of pathogen infection. Real-time Polymerase chain reaction (PCR) was also used to quantify time of gene expression and its relationship with disease resistance (Beever *et al*. 2005). The use of these genetic markers can be divided into two. Firstly, markers can be used to screen breeding populations. Resistant lines or seedlings containing these genes can then be selected. Secondly, the genetic marker can also be used to develop post-harvest treatments for kiwifruit. Antifungal volatile can be found in particular hosts, whose genetic markers could then be used in identification and screening (Kulakiotu *et al*. 2004).

1.3.3 Cultural management in orchard:

From planting till harvest, several aspects of cultural management should be addressed to reduce fungal disease incidence.

Canopy air flow is important in plant growth and disease management. A metal arch system, developed by NZ growers, can provide headroom under the canopy for pruning and harvesting, and frost protection that allows cold air to flow downward and settle on the ground (Morton 1987). It offers better plant growth, making the host plant less susceptible to pathogens and creating an unfavourable environment for disease establishment. However, pruning and trimming of the shoots can give entry points for infection. Therefore, fungicide application or related treatments on these wounds need to be conducted to reduce disease incidence (Bester *et al*. 2007).

Removal and disposal of diseased material are also crucial in disease control. It can eliminate overwinter inoculum like pycnidia or mycelium that survive in dead plant material (Pennycook 1985). This can be

achieved via cleaning of infected plant materials from previous years and maintaining the general hygiene of the orchard (e.g. machinery). KVH (2017b) offers a detailed infected material disposal protocol that can be applied to fungal disease management. The protocol includes laundry materials, heat treatment, burial and burning of infected materials. Elimination of alternative host plants and weeds are also required for the same purpose (Pennycook 1985).

During the growing season, proper irrigation and fertilization application in managing plant growth also contribute to the disease management. For instance, an overhead irrigator that can help spore dispersal should be avoided (Pennycook & Samuels 1985; Koh *et al*. 2005). Shoot growth management can also reduce disease incidence. Greaves *et al*. (2001) suggested a low carbon treatment on floral shoot. By shading individual shoot, this treatment was able to reduce lesion growth from *B. cinerea* on leaves.

Besides, *Botrytis, Botryosphaeria* or *Sclerotinia* related fungal disease often show latent disease symptoms, and similar cases can be found in *Phomopsis* and *Cryptosporiopsis* species (Fullerton *et al*. 2007). Therefore, cultural practices as such may reduce disease incidence at the post-harvest stage.

1.3.4 Fungicide and biocontrol agents

Numerous fungicides can be used for fungal disease control in kiwifruit. Chemicals like inorganic copper, carboxamide and strobilurin are most commonly used, which can inhibit the early stages of pathogen development like spore production (Novachem 2017). Application of fungicide should strictly follow the specific application rates (e.g. under highest label rate and maximum amount of active ingredients). Toxicity of fungicide to the ecosystem should also be considered (Matthew *et al*. 2014). Most of the fungicide should be applied pre-flowering and pre-harvest to reduce the inoculum levels in the orchard.

For instance, to control *Botrytis* inoculum level, timings are crucial on limiting the buildup of spore population and secondary infection (Pennycook 1985). Additional spraying may also be required, depending on disease severity. Fungicide application should take place under appropriate weather conditions to take full effect. For example, no wind and rain, with suitable temperature, can prevent spray drift. Equipment and individuals that conduct the work should be standardized to achieve a uniform application (Matthew *et al*. 2014).

Scouting is necessary for updating and monitoring disease incidence, and it is a common practice in many crops that can aid on deciding timings of the fungicide application (Coolong & Hanks 2009). By updating climate conditions and disease forecast, a real time monitoring of the orchard can detect disease epidemic in the early stage and allow actions to take place. It can also help to decide the intervals between fungicide applications.

Bio-control products are also commercially available. Some derived from natural occurring bacteria and can suppress spore germination, germ tube elongation and penetration. For example, Serenade Max that based on dried *Bacillus subtilis* (var. *amyloliquefaciens* strain D747) can be applied when *Botrytis* infection takes place during later flowering to early fruit set (Novachem 2017), and is found to be effective in disease control in several other crops like apple and strawberry (Toure *et al*. 2004; Hang *et al*. 2005). Some biocontrol agents are also found to induce host resistance. For instance, a yeast biocontrol agent, *Candida sake*, is applied during fruit curing and can inhibit *B. cinerea* activity (Cook *et al*. 1999). Antifungal volatile phenolics derived from *Eucalyptus* has been found to be effective against *Botrytis* and *Botryosphaeria* species in Korea (Oh *et al*. 2008).

1.3.5 Harvest and storage management

Harvesting of kiwifruit requires snapping the fruit off the stalks. The fruit should then be stored at an ambient temperature at 13-15^{o}C for a period of time, which is called “curing”, that can let the fruits soften and sweeten. The fruit is then transferred into cool storage for up to nine months. The picking wounds offer potential entry points (Poole & McLeod 1994) and cool storage provide a favorable environment for the pathogen to infect and establish (Pennycook 1985).

With these risks presenting, some alternative storage conditions were proposed by several studies. Pennycook and Manning (1992) suggested that a curing for up to a week before the cool storage can significantly reduce *Botrytis* conidial infection from 49% to 6%. Poole and McLeod (1994) suggested a cool-storage delay after harvesting, which can reduce pathogen incidence of *Botrytis*. There are also controlled atmosphere treatments using 1-MCP, salicylic acid, CO_2 or calcium prior to the storage process. All showed inhibition of fruit damage (Manning & Lallu 1997; Basiouny & Basiouny 2000).

Treatments on harvested fruit have also been used. In Kim and Yook (2009), gamma radiation on harvested fruit showed less infection from *Botrytis* and *Botryosphaeria* species. However, like most chemical and physical approaches (e.g. soaking in water, chemical wash), these treatment can potentially reduce the marketability of the fruit (Brigati *et al*. 2010). Wurms *et al*. (2011) suggested an alternative volatile treatment. It was based on a salicylate compound that was shown to be effective in reducing fruit ripe riot incidence by 50-75%. Future studies are required to find more effective storage methods and treatments to reduce disease incidence.

1.4 Aims and objectives of this research

In recent years, fruit drop symptoms have occurred in New Zealand kiwifruit orchards prior to harvesting. Field isolates showed several fungal pathogens that may be the cause of the symptom. Among which, *Botryosphaeria* spp. were considered important. The industry therefore seeks biocontrol agents to target this issue. One potential bacterial agent, *Burkholderia* sp., was previously isolated from mānuka plants, and showed antagonist effect to *Botryosphaeria* species *in vitro* (Wicaksono *et al*. 2017).

In this study, the *Burkholderia* sp. isolate will be used in a greenhouse trial, to test its biocontrol efficacy against *Botryosphaeria* infection. The treatment effect will be observed and described. To achieve this aim, objectives were developed as below:

1. To identify and culture the *Botryosphaeria* pathogen and the *Burkholderia* isolate.
2. To observe the *in vitro* interaction between the pathogen and the bacteria, as reference for *in planta* testing.
3. To evaluate the plant inoculation results, which may give indication on the efficacy of the bacteria as a biocontrol agent.

Chapter 2

Identifying and culturing of *Neofusicoccum parvum* from *Actinidia* species

2.1 Introduction

2.1.1 Botryosphaeriaceae species

Phylogeny based on DNA sequences has greatly shaped the taxonomy of Botryosphaeriaceae species over the years (Wikee *et al*. 2013). The order Botryosphaeriales represents several families of fungi that are isolated from woody plants. In the Botryosphaeriaceae family, morphological evolution may have occurred more than once, giving morphological traits that are difficult to use for the identifying phylogenetic signal (Slippers *et al*. 2013). The species within the Botryosphaeriaceae have been redefined based on their anamorphs only (Crous *et al*. 2006). Within the Botryosphaeriaceae family, there are several plant pathogens including *Botryosphaeria*, *Neofusicoccum* and *Dothiorella* that cause damage in kiwifruit production.

The most abundant species of Botryosphaeriaceae family is *Neofusicoccum parvum.* Its teleomorph, *Botryosphaeria*, is seldom seen in culture, whereas the anamorphic stage is common (Pennycook and Samuel 1985). Ascostromata, the teleomorph structure, is often erumpent on stromatic tissues and clustered. Ascomata is 150-250 μm in the diameter and black with white content, inside containing eight-spored, bitunicate asci. Ascospores are often unicellular, hyaline and ellipsoid to fusoid in shape. In the anamorph of *N. parvum*, globose pycnidia are often present. The pycnidia contain conidia that are ellipsoid with an obtuse apex and a flat base. The conidia are often unicellular, hyaline, becoming light brown with 1–2 septa with age. The middle section of the conidia are often dark brown (Pavlic *et al*. 2009).

2.1.2 Cultural morphology of *Neofusicoccum parvum*

In culture, the *N. parvum* mycelium looks white or greenish-white, then changes to blackish-brown on the upper side after 7 days at 25°C (Koh *et al*. 2005). The fluffy, aerial mycelium shows faster growth rate (at around 20 mm/day) than other *Botryospheria* species. It is initially white and turns pale grey from the middle of the colony after 3 to 4 days. The mycelium columns in the middle of the plate can reach the lid. On the reverse side of the plate, a faint yellow tint shows and can persist for up to a week, which will then change to bluish black (Pennycook & Samuels 1985). Pycnidia are formed in stromatic masses. They are globose-shaped and uniformly spread on culture plate. Around 9 days after the formation of pycnidia, conidia are produced (Crous *et al*. 2006; Pavlic *et al*. 2007)

2.1.3 Inducing sporulation

Neofusicoccum species utilize conidia or ascospores to infect their hosts (Pennycook 1985). Therefore, to replicate natural infection, sporulation inducement is important for inoculation. However, many species do not readily produce pycnidia and conidia in culture. For pycnidial formation, a period of stress is often required.

The difficulties in inducing sporulation on agar plate for *N. parvum* can be solved by incorporating stress factors during the culturing process. Cultural media including pine needle agar and prune agar have proved successful in inducing sporulation (Leng *et al*. 2009). These agars may have certain chemical compounds or can create an acidic environment that triggers the pycnidia to be produced. A detached stem assay on grape and blueberry also yielded successful sporogenesis (Amponsah *et al*. 2008; Tennakoon *et al*. 2017). With wounded plant tissue inoculation, sporulation occurred readily after the

infected tissue was incubated on an agar plate. Disruption of mycelium growth was also found to facilitated the production of pycnidia and conidia for *Neofusicoccum* species (Yang *et al*. 2017).

2.1.4 DNA sequence

Studies often combine morphological observation with DNA sequence to identify *Botryosphaeria* species. Molecular approaches in fungal identification often involves using the internal transcribed spacer (ITS) gene region of the rDNA operon (Denman *et al*. 2000; Crous *et al*. 2006). Due to the complicity of the classification and the taxonomy within the *Botryosphaeria* species, ITS regions may not be sufficient in differentiating species level. For example *N. parvum* and *N. ribis* are closely related cryptic species, making it difficult to distinguish if based only on single gene genealogy (Slippers *et al*. 2004; Pavlic *et al*. 2009). Multiple gene regions such as beta-tubulin and alpha elongation factors can be utilised that enable such differentiation (Phillips *et al*. 2008; Ismail *et al*. 2013). This method is also used to determine the taxonomy and phylogeny of fungi that look morphologically like *Botryosphaeria*.

The aim of this chapter is to identify the fungal isolate. The most appropriate culturing methods for the fungal isolate will also be investigated to induce spore production which will be used an inoculum in the *in planta* biocontrol efficacy experiment (Chapter 4).

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2.2 Material and Methods

2.2.1 Isolation of endophytic fungi from plant tissue

The fungal isolate was obtained from the Plant Pathology group, Lincoln University. The isolate was sub-cultured on water agar (Fischer Scientific, Thermofisher Scientific Inc.) with a mycelial discs (Ø 3mm). The processes were conducted in the laminar flow using a scalpel blade and a 3mm core borer, sterilised in 90% ethanol and subsequently flamed prior to sub-culturing. The plates were incubated at 20℃ in dark for two to three days to obtain hyphal growth. A hyphal tip isolation was conducted for each plate, by finding a single hyphae at the edge of the culture under the microscope in the laminar flow. A hyphal tip was cut prior to the last branching point using a sterile scalpel blade. The tips were then transferred on ⅕ Potato dextrose agar (PDA) (Fischer Scientific, Thermofisher Scientific Inc.). The plates were incubated at 20℃ in 24-hour dark.

2.2.2 Fungal culturing

The hyphal-tip plates described in Section 2.2.1 were sub-cultured onto ⅕ PDA agar, prune agar and vegetable juice agar (V8) (Appendix A) and incubated in 20℃ in the dark, as described in Section 2.2.1. Morphology observation were conducted during the incubation process.

2.2.3 Inducing sporulation

Several different media were used to culture isolates described in Section 2.2.1 for the purpose of inducing sporulation. Mycelial discs (Ø 3mm), were sub-cultured on prune agar and pine-needle agar (Appendix A). The plates were incubated at 20℃ in a 12-hour photoperiod for up to a month.

Detached plant stems were also used to induce sporulation following modification of the method described by Tennakoon *et al*. (2017). Plant materials included: young grapevine, kiwifruit and camellia shoots, all collected from Lincoln University, New Zealand. A mycelial disc (Ø 3mm) was placed onto wounds cut in the centres of washed shoots, with 20 shoots per treatment. The base of each shoot was

inserted into a Universal bottle containing sterile distilled water and placed in a completely randomized design within an enclosed transparent chamber for 10 days at around 20℃. Frequent misting of water was applied inside the chamber for the first three days. After incubation, the shoots with visible lesions were surface sterilized in 1% sodium hypochlorite for one minute, then transferred to 70% alcohol for 30 seconds, followed by rinsing in sterile water twice for one minute each. The shoots were dried on sterile tissue paper in the laminar flow for 6 hours. For each dried shoot, it was placed on two toothpicks with moist filter paper at the bottom of a Petri dish (Figure 1). The plates were then incubated for two or three days under 20℃ in a 12-hour photoperiod to induce pycnidial formation. Pycnidia were crushed on a slide and placed under a cover slip to examine for conidia.

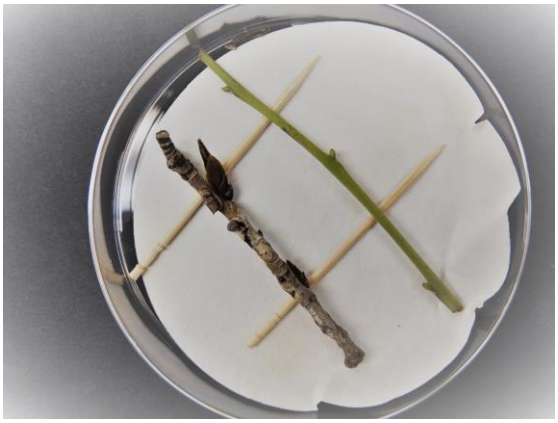

Figure 1: *Neofusicoccum parvum* inoculated young camellia stem sections after surface sterilization and ready for incubation.

Disruption of the mycelium mat was also undertaken. Fungal isolates were grown in ⅕ PDA at 20℃ for a week to allow mycelium growth. Sterile glass rods were used to disrupt the mycelium mat in a perpendicular grid pattern for each plates, with a total of 10 replicates for the isolate. The plates were then rinsed with sterile distilled water, and placed in the laminar flow to dry for an hour. Afterwards, each plate was covered with sterile filter paper (Labserv, Thermofisher Scientific Inc.) on the top of the disrupted mycelium mat. The plates were incubated at room temperature at around 20℃ on the bench

under natural light. Plates were monitored after seven days incubation for sporogenesis. Plates with pycnidia were examined in the same manner as described previously.

2.2.4 DNA extraction and amplifying

A 10% Chelex 100 resin solution (Bio-Rad, Bio-Rad laboratories.) was made and stored at 4℃. For each fungal isolate, sterile forceps were used to remove around 2 mm of the mycelium from the plates described in Section 2.2.2. The mycelium was placed with 200 μl 10% Chelex solution in a sterile 1.7 ml tube, with two replicates for each isolate. The tubes were vortexed and placed on a heating block at 100℃ for ten minutes. They were removed to vortex and replaced on the heating block for a further 10 minutes. Centrifugation (Thermofisher Scientific Inc.) followed for ten minutes at 12300 x g. DNA quality and yield were determined using a Nanodrop 2000 UV-Vis spectrophotomter (Thermo Fisher scientific Inc. New Zealand). Samples were placed at -18°C.

Three gene regions were amplified: ITS, Beta-tubulin and Alpha-elongation factor. Approximately 500-600 bp of the ITS region was amplified with ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Romanelli *et al*. 2014). A 500 bp of Beta-tubulin region was amplified with T1(5'-AAC ATG CGT GAG ATT GTA AGT-3') and T22(5'-TCT GGA TGT TGT TGG GAA TCC-3') (O'Donnell & Cigelnik 1997). A 500 bp of Alpha-elongation factor region was amplified with 983F(5'-GCY CCY GGH CAY CGT GAY TTY AT) and 2218R(5'-ATG ACA CCR ACR GCR ACR GTY TG) (Rehner & Buckley 2005).

For the ITS region, PCR was performed in a thermal cycler (Applied Bio system Veriti, Life Technologies Ltd, New Zealand) in a total volume of 20 μl containing 10 μl of DreamTaq (Life Technologies, Thermo Fisher Scientific Inc., USA), 4 μl of 0.5 μM of each forward and reverse primer (IDT, Integrated DNA Technologies

Inc., Australia), 2μl 10-20 ng/mg DNA template, and nanopure water added to make up to the final volume of 20 μl. The amplifying cycle was initiated with 3 minutes at 95°C, then 35 cycles of 1 minute at 94°C, 30 seconds at 55°C and 1 minute at 72°C, followed by 7 minutes at 72°C and a final hold at 4°C.

For the Beta tubulin region, PCR was performed in a thermal cycler (Applied Bio system Veriti, Life Technologies Ltd, New Zealand) in a total volume of 20μl containing 10μl of DreamTaq (Life Technologies, Thermo Fisher Scientific Inc., USA), 0.2μM of each forward and reverse primer (IDT, Integrated DNA Technologies Inc., Australia), 1μl 10-20 ng/mg DNA template and nanopure water added to make up the final volume of 20μl. The amplifying cycle was initiated with 2 minutes at 94°C, then 36 cycles of 35 seconds at 94°C, 55 seconds at 56°C and 2 minute at 72°C, followed by 10 minutes at 72°C and a final hold at 4°C.

For the alpha elongation factor region, PCR was performed in a thermal cycler (Applied Bio system Veriti, Life Technologies Ltd, New Zealand) in a total volume of 20μl containing 10μl of Reddymix (Life Technologies, Thermo Fisher Scientific Inc., USA), 0.4μl of 100 x Bovine serum albumin (New England Biolabs.Inc.), 1μM of $MgCl_2$ (Life Technologies, Thermo Fisher Scientific Inc., USA), 0.3μM of each forward and reverse primer (IDT, Integrated DNA Technologies Inc., Australia), 1μl 10-20 ng/mg DNA template and nanopure water added to make up the final volume of 20μl . The touchdown amplifying cycle was initiated with 2 minutes at 95°C, then 9 cycles of 30 seconds at 95°C, 30 seconds at 64°C and 90 seconds at 72°C, 36 cycles of 30 seconds at 95°C, 30 seconds at 56°C, 90 seconds at 72°C, followed by 10 minutes at 72°C and a final hold at 4°C.

For each PCR product, 8μl aliquot combined with 3 µl loading dye (40% (w/v) sucrose; 0.25% bromophenol blue; 0.25% xylene cyanol) was loaded onto a 1% agarose gel together with a 1 Kb plus DNA ladder

(Invitrogen). Gels were run in 1 x TAE (Tris-acetate-EDTA) buffer for 30 minutes at 90 V. They were then stained for 15 minutes in ethidium bromide (0.5 µg / ml, AMRESCO®, OH, USA), de-stained in water for 15 minutes and observed under ultraviolet light using an UVIreader.

The PCR-amplified products were sequenced at the Lincoln University Sequencing Facility. The sequences obtained were viewed using Chromas Lite 2.1 (Technelysium Pty Ltd, Australia) and trimmed and assembled to approximately 100-500 bp, depending on the gene region and sequence quality, using DNAMAN 5.0 (Lynnon Biosoft, Canada) to give high quality sequences. The edited sequences were compared with sequences database using the Basic Local Alignment Search Tool (BLAST) in "The National Center for Biotechnology Information" (NCBI).

2.3 Results

2.3.1 Cultural description

On agar plates, the white or greyish-white, flocculate mycelium changed to greyish-black on the upper side after around 7 days at 20°C. The mycelium has a growth rate at around 15-20 mm/day. On the reverse side of the plate, there was a faint yellow tint that persisted up to a week and subsequently changed to black. At the edge of the plate, clustered mycelium reached the top of the lid. No pycnidia were observed to be produced on colonies growing on PDA and V8 agar (Figure 2).

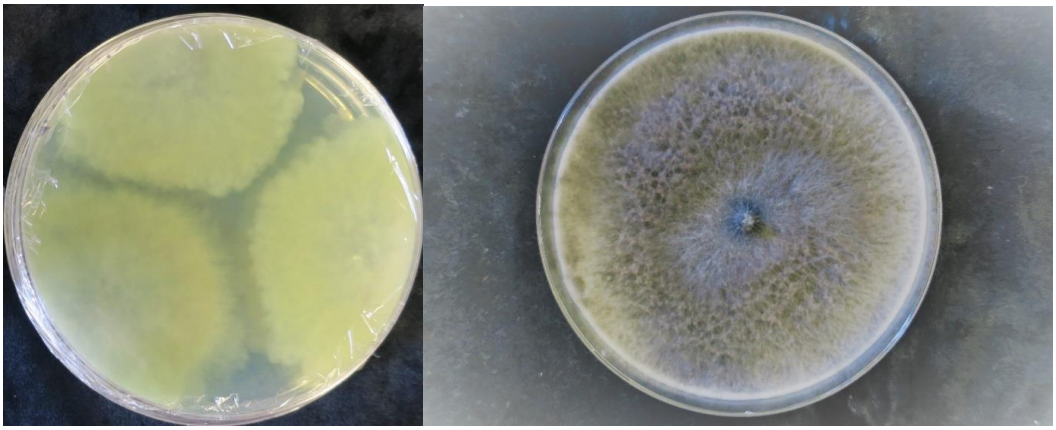

Figure 2: Reverse side of 1/5 PDA plate after 3 days of incubation shows faint yellow (left) and upper side of V8 plate shows mycelium growth of the fungal colony after 7 days of incubation (right).

2.3.2 Sporulation inducing

The prune agar plates showed formation of pycnidia in stromatic masses. The pycnidia were globose-shaped and uniformly spread on the plates. On the cover-slip slides, conidia released from pycnidia were observed. These were hyaline, unicellular, thin-walled and septate (Figure 3). The pine-needle agar failed to induce sporulation and only mycelium growth was observed.

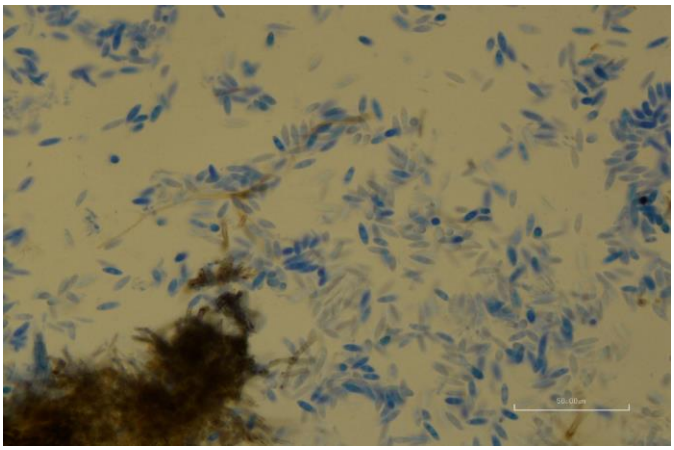

Figure 3: Crushed open pycnidia from prune agar plate shows releasing of conidia. Scale bar represents 50 µm.

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For detached stem inoculation, after ten days inoculation, the grapevine shoot produced black lesions, both upward and downward of the inoculated point. In the Petri dishes, black pycnidia were observed on the shoot (Figure 4). On the cover-slip slides, crushed open pycnidia were empty and no conidia were observed. The kiwifruit and camellia shoot blackened after ten days of incubation, with no visible lesions or pycnidial formation on the shoots.

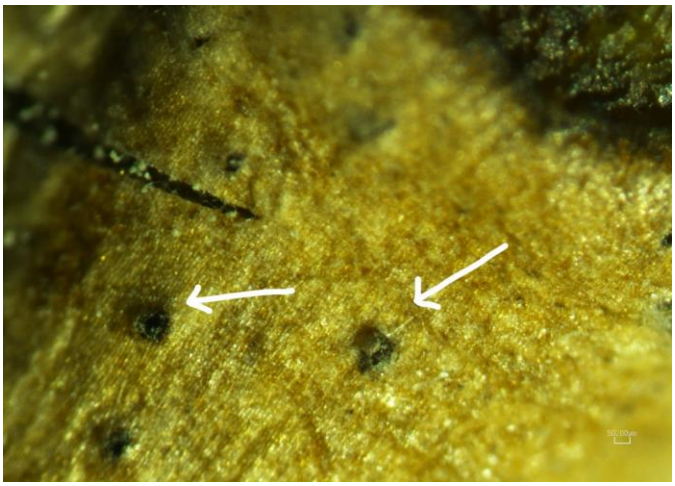

Figure 4: Stereo-microscope examined grapevine tissue shows formation of black pycnidia as indicated by arrows.

2.3.3 Identification of the fungi using DNA sequence

The Chelex DNA extraction yielded a DNA sample with around 10-20 ng/ml, with 260/280 ratio at around 1.7-1.9. PCR products from the DNA extractions of most replicates give bright band on agarose gels. The products were approximately 1000-1200 base pair (Figure 5).

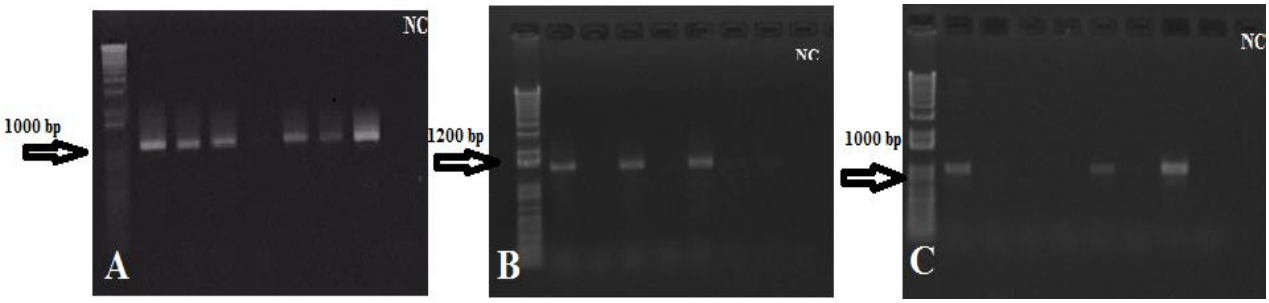

Figure 5: Agarose gel band picture from (A) ITS gene region amplified PCR products with eight replicates showing bright bands and negative control (NC) at the end; (B) Beta-tubulin gene region amplified PCR products with seven replicates showing bright bands and negative control at the end; (C) Alpha elongation factor gene region amplified PCR products with seven replicates showing bright bands and negative control at the end. Labelled band size of the molecular ladder is shown in arrow with base pair units.

The sequences obtained had a high similarity to *Neofusicoccum parvum* sequences from BLAST (Table 1). The ITS region showed ambiguity between identifying *N. parvum* and *N. ribis*, which lead to a lower than 100% similiarity. This problem is solved by amplifying two smaller gene regions (Appendix B). The amplified beta-tubulin gene region showed 100% similarity to *N. parvum*, and the amplified alpha elongation factor region 99%.

Table 1: BLAST results shows three gene region amplified sequences with high similiarity (>98%) to *N. parvum*. From top to bottom, the amplified gene regions comparsion are with ITS, ITS, beta-tubulin, beta-tublin and alpha elongation factor respectively.

Description	Query Cover	Ident	Accession
Neofusicoccum parvum isolate UCR736 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1. 5.8S ribosomal RNA	100%	98%	HQ529767.1
Neofusicoccum parvum strain 121 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1. 5.8S ribosomal RNA	100%	98%	GU944807.1
Neofusicoccum parvum strain GEVB10 beta-tubulin gene, partial cds	100%	100%	KU530157.1
Neofusicoccum parvum strain NpS92 beta-tubulin gene, partial cds	100%	100%	KJ841779.1
Neofusicoccum parvum isolate UCRNP2 putative elongation factor 1-alpha protein mRNA	98%	99%	XM_007579574.1

2.4 Discussion

This study confirmed the identity of the fungal isolate from kiwifruit as *N. parvum.* By using cultural and molecular techniques, the fungal isolate showed morphological features and sequencing result confirming previous studies (Pennycook 1985; Zhou *et al*. 2015). Here the first two steps of Koch's postulate was demonstrated, with successful isolation of a pathogen and the ability for it to grow in pure culture (Koch 1876).

Morphological observation of the pathogen sample showed high similarity to previous studies (Pennycook & Samuels 1985; Zhou *et al*. 2015). Mycelium growth rate, color and formation in agar plates showed typical *Neofusicoccum* morphology. This part of the study facilitated the initial identification of the fungi. As morphological similarity may occur between different *Neofusicoccum* species (Denman *et al.* 2000; Slippers *et al.* 2004), DNA sequencing was required.

In the sporulation inducement, only prune agar was able to induce pycnidial and conidial formation. The 12 hour photoperiod may be the key to pycnidial formation. In Smith and Fergus (1971), different light intensities were examined to test their effects on sporulation. In their study, with a 12-hour photoperiod at 27°C, pycnidial formation occurred after 4 days. Copes and Hendrix (2004) also stated that for *B. dothidea*, maximum sporulation occurred at 24°C. They also found a curvlinear response between temperature and conidial maturation. In this study, natural light was used, which may have fulfilled the light intensity requirement for sporulation of *N. parvum*. However, the time of pycnidial formation was much longer (a month) compared to other studies. This may be due to the lower incubation temperature at 20°C. This factor may prolong the process of pycnidial formation and the development of conidia.

Pine needle agar failed to produce pycnidia and had very sparse mycelium growth. In Su *et al.* (2012), pine needle medium was able to induce sporulation of 40% of the plant pathogens, including *Botryosphaeria* species. In contrast, although Amponsah *et al.* (2008) reported production of pycnidia containing conidia on pine needle agar for isolates of four Botryosphaeriacea species (*Neofusicoccum luteum, N. australis*, *B. obtusa* and *B. stevensii*), the *N. parvum* isolates failed to sporulate, indicating an effect of species on sporulation on this agar. In our experiment, the reason of failed sporulation may also due to the insufficient initial mycelium growth. The level of stress placed on the fungi may be too high and that may have led to starvation; hence stunting further fungal development. Nevertheless, the process of sporogenesis can be affected by various environmental and nutritional factors. Future studies on testing such parameters are required to contribute to the understanding on the development of *N.parvum*.

The detached stem assay also failed to give successful sporulation. In Amponsah *et al*. (2008), *N. parvum* produced fewer number of pycnidia than other *Botryosphaeria* and *Neofusicoccum* species and it did not release any conidia. In this experiment, grapevine shoot did produce pycnidia, yet they were empty with no conidial formation. Therefore, further pathogen identification was not possible. For the other two plant medium, kiwifruit and camellia, no obvious lesion was visible after inoculation. Although studies suggested that these two plants are known as host of *Botryosphaeria* species (Manning *et al*. 2003; Jayawardena *et al*. 2016) and often young plant parts can stimulate sporulation (Su *et al*. 2012), this experiment did not yield satisfying results. Unsuccessful initial inoculation may be the cause. Besides, the mycelium discs may not be sufficient pathogenicity-wise to initiate infection. Additionally, the humid condition for tissue incubation may require adjustment to obtain a more suitable environment for infection. For future work, the pathogenicity of this species, especially its mycelium, may under further examination under different abiotic parameters.

Molecular work in this study yielded a detailed identification of the fungal species. The Chelex DNA extraction offered a cheap and easy way to extract fungal DNA. Different extraction methods using Chelex were mentioned in other studies (Möhlenhoff *et al*. 2001). In this experiment, the incubation time of the DNA samples on a block heater was adjusted and concluded that 20 minutes of heating resulted in a better quality DNA extraction. The PCR cycles of this experiment also underwent several adjustment. The number of cycles was not only based on previous published studies, as described in Section 2.2.4, but also on our own adjustment to suit the specific samples. The agarose gel indicated that the DNA of the samples were sufficiently amplified. Sequence modified and assembled give a BLAST result that leaded to conclusion of the fungal isolate to be *N. parvum*.

In all, the fungal isolate identified in this study, which had previously associated with fruit drop in kiwifruit, was confirmed for the first time. Future fungal identification work to achieve a faster DNA extraction, amplification and sequence process is underway (Schuster 2008). A time and financially economic alternative may be expected.

Chapter 3

Identifying and culturing of the potential bio-control agent *Burkholderia* sp.

3.1 Introduction

3.1.1 The *Burkholderia* family

Burkholderia species are gram-negative, rod-shaped bacteria. They can be found in soil, water, and the rhizosphere of plants. The type specie of this genus is *B. cepaciam*. It was originally described as *Pseudomonas cepacia*, isolated as the causal agent of sour skin of onion (Burkholder 1950). In 1992, this species reclassified from the genus *Pseudomonas* and placed in the new genus *Burkholderia*.Taxonomic evaluation found 128 *B. cepacia* strains, mostly from patients with cystic fibrosis (Vandamme *et al*. 1997). The *B. cepacia* was then split into eight genetic species, including five named species, which in combination, known as the "*B. cepacia* complex" (Bcc). The 16S gene region can be used to differentiate *B. multivorans* and *B. vietnamiensis*, but not for other species. The large genome size that ranges from 4 to 9 Mb is one of the distinguishing characteristics of this bacteria (Mahenthiralingam *et al*. 2008).

3.1.2 Niche filling capacity

The niche filling capacity describes how an organism responds in natural conditions with various distribution of resources and competitors. This ability of the bacteria can assist our understanding of how well it can colonize space in plant and soil, which may indicate a promising biocontrol effect (Coenye & Vandamme 2003).

Burkholderia spp. can occupy various ecological niches, from soils and plants rhizosphere to human and other animals. For example, *B. cepacia* isolates showed growth promotion when colonizing bean and sorghum (Chiarini *et al*. 1998; Peix *et al*. 2001). The *B. cepacia* complex strains are also known to be plant pathogenic, and have been isolated from environmental and clinical sources. For example, the bacteria can be found as a contaminant in water supplies and agricultural products. *Burkholderia mallei* is known to cause glander issues in animals, and *B. pseudomallei* is correlated with human disease (Coenye & Vandamme 2003). *Burkholderia. glathei, B. phenazinium* and *B. graminis* have been isolated from a range of soils and plant rhizospheres (Viallard *et al*. 1998). Its ability to use a large array of compounds as nutrient sources and its metabolic versatility may be the reason why it comprises a large proportion of the endophytic bacterial community in plants (Whitby *et al*. 2006).

With the wide host range, some *Burkholderia cepacia* complex strains are potentially able to control diseases or enhance plant fitness. Application time, amount and methods may affect its ability to colonize and persist in inoculated plants or soil. Seedling inoculation with "Bcc" at 10^6 to 10^8 colony forming units (CFU) per seed at planting is often used (Coenye & Vandamme 2003). Soil drenches and drip irrigation delivery systems are also common practice. However, plant colonization by such application cannot be granted for all strains. Moreover, there are cases of an introduced strain being replaced by the indigenous *Burkholderia* species occurred during seedling treatments (Parke & Gurian-Sherman 2001). Therefore, during the entire growing season, to maintain a stable population of specific strains that can secure an initial biocontrol ability, selection for specific strains and molecular techniques in tracing the introduced species are necessary.

3.1.3 Analyzing *Burkholderia* spp. biocontrol abilities

Numerous antibiotics are produced by *Burkholderia* species, including cepacin, cepaciamide, xylocandins, pseudanes and phenazine (Parke & Gurian-Sherman 2001). Antibiotics activity is analyzed via assessing its inhibition on fungal growth. Although it is uncertain whether to attribute the results to antifungal metabolites production, fungal pathogens like *Rhizoctonia solani* and *Fusarium* spp. were shown to be sensitive to such antibiotics (Quan *et al*. 2006). For example, there was a 50 to 80% reduction in incidence of Fusarium wilt in tomato seedlings treated with *B. cepacia* (Larkin & Fravel 1998). Repeated dipping of bananas in a bacterial solution of 10^{10} CFU per millilitre was used to control crown rot caused by *Colletotrichum* spp. with high efficacy (De Costa & Erabadupitiya 2005). *Burkholderia cepacia* can also produce lipopeptide. For example, one lipopeptide, AFC-BC11, is largely responsible for effective control of damping-off of cotton caused by *R. solani* (Kang *et al*. 1998).

Siderophores, which are iron chelating compounds, are also produced *in vitro* by *Burkholderia* species. Compounds produced by *Burkholderia* spp. which have siderophore activity include ornibactins, pyochelin, and aerobactin (Sokol *et al*. 1999), which possess antifungal growth characteristics. For example, salicylic acid accumulation is essential for expression of disease resistance in plants (Delaney *et al*. 1994). Ornibactin production by some *Burkholderia* species demonstrated biocontrol ability against several fungal diseases such as *Rhizoctonia, Helminthosporium, Fusarium* and *Pythium* (Meyer *et al*. 1995). *Burkholderia tropica* showed fungal growth inhibition of *Fusarium culmorum, F. oxysporum* and *Sclerotium rolffsi*, which may related to siderophore production, such as acetic acid, isobutylether and toluene (Tenorio-Salgado *et al*. 2013).

In the absence of disease, *Burkholderia* species also show plant growth promotion, such as induction of plant hormones such as auxins and suppression of deleterious rhizosphere bacteria (Kurepin *et al*. 2015). *B. phytofirmans* on *Arabidopsis thaliana* plants showed increasing of several growth parameters and

growth rate of the plants, which may due to the bacterial inducement of plant auxin and gibberellin pathways (Poupin *et al*. 2013). The same species of *Burkholderia* was also found to be able to induce endogenous changes in plant growth hormone levels on other plant species like potato (Kurepin *et al*. 2015). This ability of the bacteria may promote a disease resistant in plants and hence achieve indirect biocontrol.

Although most *in vitro* testing shows promising results, *in vivo* testing can yield different outcomes. A rhizosphere strain of *Burkholderia,* which was found as an antagonist of *Alternaria panax* on ginseng, showed poor survival on leaf surfaces (Parke & Gurian-Sherman 2001), indicating instability of certain strains. Whereas the application of the bacteria on postharvest disease in fruit production showed effective control. For instance, control of *B. cinerea* on grape (Reglinski *et al*. 2005), and various diseases on citrus (Scuderi *et al*. 2009) were recorded.

The aim of this section is to understand the biocontrol ability of *Burkholderia* (isolate W4R11) *in vitro*. Through identification and culturing processes, a more comprehensive knowledge of this bacteria is required which will be used to assist *in planta* testing.

3.2 Material and Methods

3.2.1 Bacteria isolation

The endophytic bacterial isolate, *Burkholderia* sp. W4R11, was selected due to previous evidence of its *in vitro* activity against *Botryosphaeria* spp. (Wicaksono *et al*. 2017). This particular strain was obtained from the Plant Pathology group, Lincoln University. For long term preservation, a single colony of the bacteria

was taken from the original King agar B (Duchefa Biochemie) and transferred into 500 μl of nutrient broth (NB; Difco, Becton, Dickinson and Company) in a 1.7 ml sterile tube. Two replicates were made for the bacteria and the tubes were placed in a shaking incubator (Labnet, Labnet International Inc.) at 28℃ and 200 rpm. After around 14 hours of growth, 500 μl of 50% glycerol was added to the broth culture and the tubes were stored at -80℃. Before each experimental assay, the bacteria was recovered from the -80℃ by taking 10 μl of the broth culture and spreading on King agar B (Duchefa Biochemie). The plates were incubated at 25℃ in the dark until used in the assays.

3.2.2 Production of spontaneous mutant using chloramphenicol

To enable the selective reisolation of the *Burkholderia* sp. isolate W4R11 from inoculated plant tissue, a chloramphenicol spontaneous mutant strain, *Burkholderia* sp. isolate W4R11C, was produced. The spontaneous mutant was developed by repeated sub-culturing *Burkholderia* sp. W4R11 onto King agar B containing incremental increases in chloramphenicol concentration. At the start, the *Burkholderia* sp. isolate W4R11 was plated onto King agar B containing 10 ppm chloramphenicol (Sigma-Aldrich, Sigma-Aldrich Co. LLC.). The plate was incubated at 25℃ in dark for a week until single colonies formed. A single colony was then sub-cultured onto King agar B plate with 20 ppm and incubated again at 25℃ in dark for a week. The same processes was repeated with a 10 ppm increment for each subculture until the chloramphenicol concentration reached 125 ppm. To determine the stability of the mutant strain (W4R11C), a single colony was taken from each of the plates and went through two subsequent sub-culturing on King agar B without antibiotics. It was then sub-cultured on 125 ppm chloramphenicol agar plates. The single colony from each plate was preserved in glycerol at -80℃ as described in Section 3.2.1.

3.2.3 Testing Bacterial growth rate

An optical density test was carried out to estimate the growth rate of the chloramphenicol mutant strains of *Burkholderia* sp. W4R11 (strain W4R11-C) obtained in Section 3.2.2. For the strain, a single colony from the agar plate was taken and put into 1 ml nutrient broth (NB; Difco, Becton, Dickinson and Company) in a 1.7 ml sterile tube. Two replicates were made for the bacteria strain and the tubes were placed in a shaking incubator (Labnet, Labnet International Inc.) at 28℃ and 200 rpm overnight. This is called the mother culture. The mother culture was then stored in 4℃. At the same time, 10 μl of the mother culture was taken from each tube and put into 10 ml nutrient broth in a sterile Falcon® tube. The Falcon® tubes were put in a shaking incubator (Labnet, Labnet International Inc.) at 28℃ and 200 rpm. During the incubation process, samples were removed from the Falcon® tubes after 4, 6, 8, 12, 19 and 24 hours incubation. Optical density test and serial dilution plating were carried out each time interval.

An optical density test was carried out by using a spectrophotometer. The OD reading was taken at OD_{600nm} for the bacteria isolate. A 1 ml aliquot of the mother culture was put in a cuvette and optical density measured, thus acting as a calibration for other samples. Samples from the shaking incubation procedures were processed in the same manner.

At the same time when testing optical density, a serial dilution plating was carried for the sample at each assessment time. A 100 μl aliquot of the sample from each falcon tube was put into 900 μl of phosphate buffer saline (PBS) pH 7.2 in a 1.7 ml sterile tube. The tubes were then vortexed and 100 μl of the mixture from the tube were taken and put into 900 μl of PBS in a second 1.7 ml sterile tube. The original sample was diluted in 10^{-1} increments until the final solution reached10^{-7}. A 100 μl aliquot of the 10^{-5},10^{-6} and 10^{-7} dilutions were pipetted and spread onto King agar B, with 3 replicates for each dilution. The plates were

then incubated at 25℃ in the dark for 1 to 2 days, after which the number of colonies were counted. An equation as follows was made to calculate the CFU per ml of the original mixture:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{number of single colony X dilution factor}}{\text{volume}} \text{ of bacteria on each plate}$$

A growth curve correlating the optical density and CFU per ml from the different assessment times was then made.

3.2.4 DNA extraction and sequencing

The DNA extraction of the *Burkholderia* sp. strains W4R11C from Section 3.2.2 was made, using PUREGENE DNA isolation kit (PUREGENE, ProGENZ Limited, New Zealand) according to manufacturer's instruction. Around 800-900 bp of the 16S rRNA gene was amplified with F27 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R1494 (5'-CAT CGG YTA CCT TGT TAC GAC-3') (Heuer *et al*. 1997). PCR was performed in a thermal cycler (Applied Bio system, Veriti, Life Technologies Ltd, New Zealand) in a total volume of 20 μl containing 12.5 μl of DreamTaq (Life Technologies, Thermo Fisher Scientific Inc., USA), 0.3 μM of each forward and reverse primer (IDT, Integrated DNA Technologies Inc., Australia), 0.5 μl 100ng/mg DNA template, and nanopure water added to make up the final 20 μl. The amplifying cycle was initiated with 3 minutes and 30 seconds at 95℃, then 35 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 1 minute at 72℃, followed by 7 minutes at 72℃ and a final hold at 4℃. The PCR products were then loaded on agarose gel and send to sequenced as described in Section 2.2.4.

3.3 Results

3.3.1 Bacteria culturing and production of spontaneous chloramphenicol mutant

Single colonies of *Burkholderia* sp. isolate W4R11 were observed to develop on the Kings B agar plates after around 5 days incubation. A chloramphenicol-resistant mutant, *Burkholderia* sp. isolate W4R11C, was developed successfully, which was able to grow on 125 ppm chloramphenicol agar. During the process, on the 50 ppm, the mutant strain took two weeks to form colonies, compared to one week for the other chloramphenicol concentrations.

3.3.2 Bacterial growth rate

By correlating the optical density with CFU count from the plates and using the equation to calculate the CFU/ml, an approximate CFU/ml in a pre-determined incubation time frame was able to be obtained. Firstly, there was a linear relationship between optical density (OD) reading and incubation time. As the incubation time increased, the OD reading increased (Figure 6). Then the OD reading and CFU/ml was correlated and showed a linear relationship. As the OD increased, the CFU/ml also increased (Figure 7). These two linear relationships can therefore be used to predict that after a specific incubating time, how much CFU/ml one can obtain from the mixture.

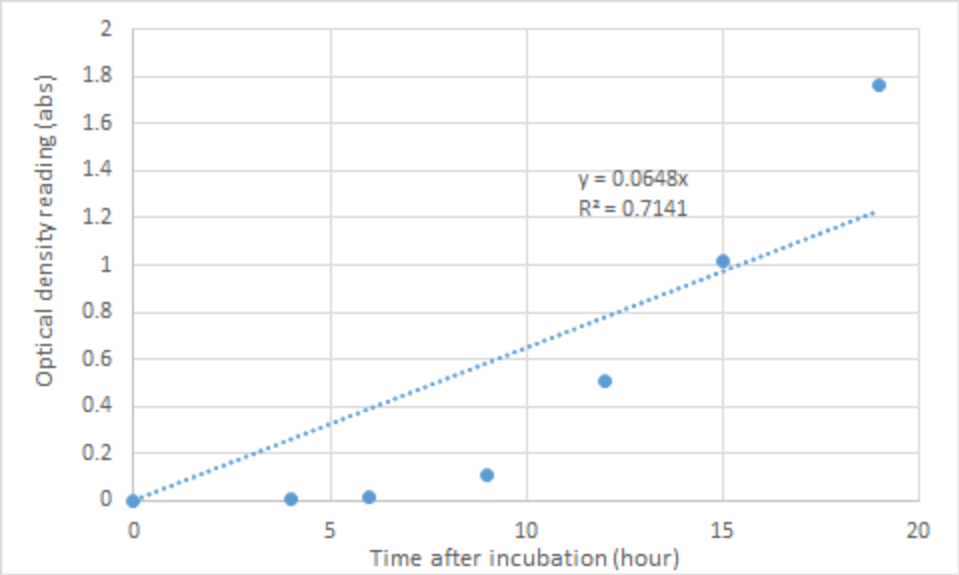

Figure 6: A linear relationship between optical density reading (abs) and time of the incubation (hour).

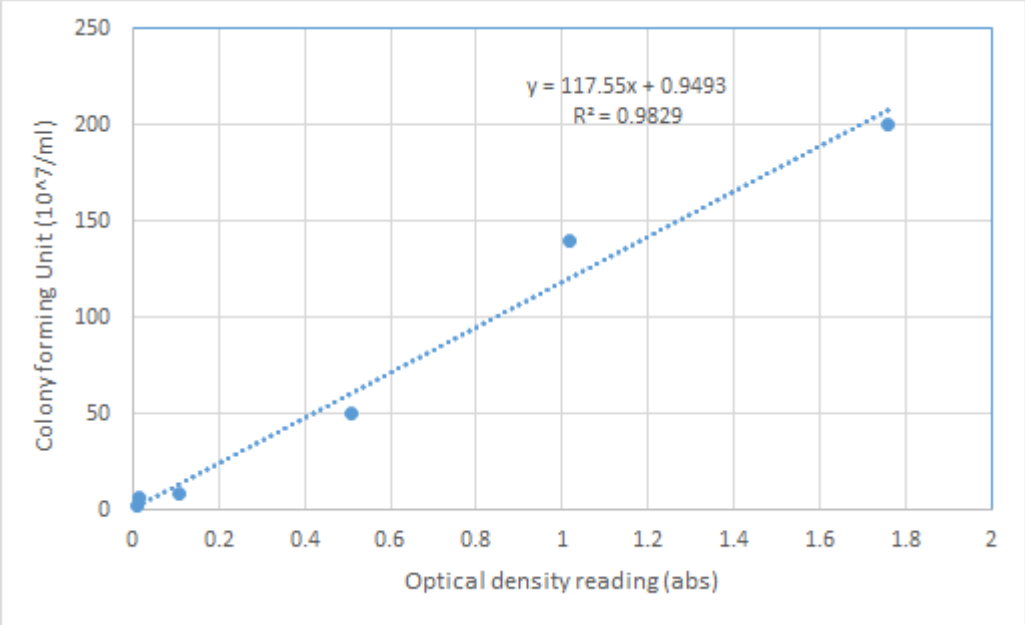

Figure 7: Bacteria growth rate indicated by a linear relationship between optical density (taken at OD_{600nm}) and CFU (10^7/ml).

3.3.3. DNA sequencing results

The PCR products from amplification of 16S region showed bright bands from two replicates of the bacteria strain, *Burkholderia* sp. W4R11C. The product was approximately 1500 base pair (Figure 8).

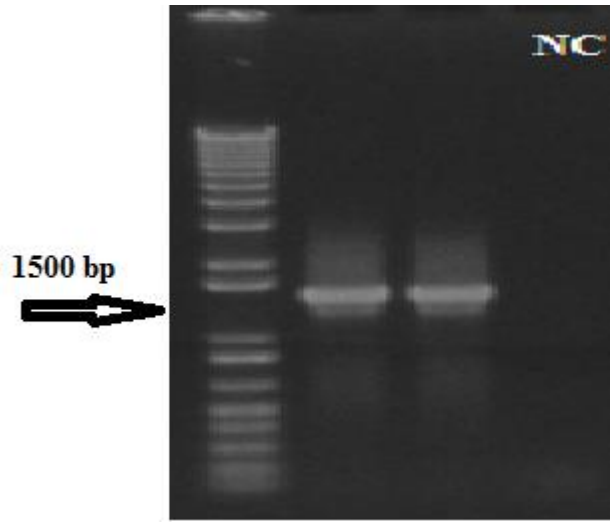

Figure 8: Agarose gel band picture from 16S gene region amplified PCR products with two replicates showing bright bands and negative control (NC) at the end.

The bacterial isolates had high similiarity to the genus *Burkholderia* when compared with sequences from the BLAST (Table 2), which conferred with the genus of the original strain obtained from the Plant Pathology group, Lincoln University (Appendix B).

Table 2: BLAST results show bacterial isolate with high similiarity to *Burkholderia* species(=100%).

Description	Query Cover	Ident	Accession
Burkholderia sp. Strain D-99 16S ribosomal RNA gene, partial sequence	100%	100%	KY906999.1
Burkholderia cenocepacia strainVC7848, complete genome	100%	100%	CP019668.1
Burkholderia cepacia JBK9 chromosome 1, complete sequence	100%	100%	CP013730.1

3.4 Discussion

The work demonstrated the basic techniques with culturable bacteria and provided insights on further usage of this bacteria. The aim of producing a bacterial mutant was to make it traceable for further inoculation process. By applying high antibiotic concentration into the agar plates, the growth of other bacterial isolates can be suppressed during later reisolation process. Whereas if the mutant strain was present inside the plant, it can then be able to reisolated onto the antibiotic amended agar. A clean technique and strictly following of the protocols were necessary in the bacterial culturing, which eliminated confounding artifacts.

The basic protocol of testing bacterial growth rate was designed by Contois (1959). It provided a detailed procedure from bacteria culturing to measurement of the colony density. In our experiment, the O.D. reading from each time interval was a long process, due to the uncertainty on the sampling times. The final time intervals were adjusted several times before consistent data could be obtained. The correlation between time and CFU/ml can be obtained from the results, which can give an approximate predication of the bacterial growth as the unit of CFU/ml under the same incubating environments.

Using less selective agar aided in the isolation and identification of this bacterial isolate. Studies have reported that accurate identification of Bcc bacteria is difficult to achieve and suggested to combine commercial biochemical analysis kits with other specific test (Mahenthiralingam *et al*. 2008). Moreover, partial 16S rRNA gene sequencing may not be sufficient to discriminate all the species in the Bcc. For the purpose of this study, since the original isolates had already been identified, understanding its function and biological characteristics should be the primary targets instead. Identifying to species level is not the aim of this part of the study, yet it may be useful in future work on screening and selection of biocontrol bacteria.

Chapter 4

Using *Burkholderia* to control kiwifruit fungal disease

4.1 Introduction

Bacterial biocontrol agents are often available in horticultural practices. An example of a product available in New Zealand is Blossom Bless™, a wettable powder containing the bacterium (*Pantoea agglomerans* P10c). It can be used as a foliar spray on pipfruit plants against fire blight infection (Vanneste et al. 2002a). The product is known for producing plant elicitors that can induce disease resistant in kiwifruit (Vanneste et al. 2002b). Serenade Max, containing a bacterium *Bacillus subtilis* QST 713, was found to control *Botrytis* related disease and has been reported to result in a slight decrease in Psa population in kiwifruit (Gould *et al*. 2014). Overseas studies have also used *Bacillus* species like *Bacillus subtilis* OSU142 and *Bacillus* RC03 in horticultural production. These are plant growth promoting rhizo-bacteria that can be applied in general nursery potting mix, to enhance root growth of kiwifruit stem cutting (Erturk *et al*. 2010). For kiwifruit production, which suffers from post-harvest disease, due to wound-invading pathogens like *Botrytis* and *Botryosphaeria*, biological control may offer an effective alternative compared to the traditional chemical control. Antagonists can be applied directly to target areas like fruit wounds. A single application of *Bacillus*-based product was found to reduce fruit decay (Jacobsen *et al*. 2004).

Bacterial biocontrol agents are usually isolated from plants and soil. Their biocontrol ability can be achieved via acting against disease infection, or supporting plant growth. These traits are supported by the production of antifungal or antibacterial metabolites, volatile compounds like siderophores and/or by inducing of systematic resistance of the plant (Santoyo *et al*. 2012). A vast number of bioassays were used to screen biocontrol traits of the bacteria. For example, siderophore production can be detected by

Chrome Azurol S (CAS) agar plates. Antagonist effect against pathogens can also be assessed by dual plating using Waksman agar. Field testing of the biocontrol agent for its effect on selected pathogens and host plants is also necessary, before considering its marketability (Utkhede 1996).

A few points should be noted when applying such products. Wojciech and Korsten (2002) suggested that rapid colonization by the antagonist may not be necessary for the control of fruit rot caused by latent pathogen infection, such as *Botryrosphaeria* related fungal disease. This is because fast growing antagonists may only provide a protective defense rather than being curative against the pathogen, which offers little effect on latent infection. Although most biocontrol bacterial strains were found to be effective for *in vitro* studies, field trial may fail to achieve satisfying results (Parke & Gurian-Sherman 2001). These limitations can be addressed by enhancing biocontrol through manipulation of the application environment, using mixtures of beneficial organisms, physiological and genetic enhancement of the biocontrol mechanisms, manipulation of formulations, and integration of biocontrol agents with other alternative control methods (Wilson & Wisniewski 1989). For example, to control grey mold caused by *B. cinerea* in kiwifruit, an integrated pest management system and cold storage using non-chemical methods has been a success. This involved a combination of cultural practices and post-harvest curing (Michailides & Elmer 2000). Besides, it may be unfair to equate or compare biological control agents to chemical treatments, since they each have their own limitation and merits. The post-harvest diseases of kiwifruit usually take place in a well-defined environment. This enables a great opportunity to utilize microorganisms like bacterial biocontrol agents to take effect.

In this study, *Burkholderia* sp., (isolate W4R11), has shown a biocontrol effect *in vitro* again *N. parvum* from kiwifruit in a previous study (Wicaksono *et al.,* 2017). In this chapter, the isolate was used as a wound

protectant in one-year old kiwifruit plants to determine its efficacy on disease control of *N. parvum*. The colonization and movement of the *Burkholderia* sp. and *N. parvum* were also examined.

4.2 Materials and Methods

4.2.1 Bioactivity assays

4.2.1.1 Production of siderophores

Siderophore production by the chloramphenicol mutant strain of *Burkholderia* sp. W4R11C, using Chrome Azurol S (CAS) agar plates (Schwyn & Neliands 1987), was tested to ensure that the mutant strain was still able to produce siderophores to the same level as the original strain. The agar plate was divided into four equal sections and in each section, a loop of single colony bacteria (described in section 3.2.2) was inoculated. The plates were incubated at 25℃ in the dark for seven days. The ability of the bacteria to produce siderophores was determined by measuring the clear zone around the colony.

The clear zone (X) and colony (Y) diameters (mm) were measured in two perpendicular directions using a digital caliper. The average size (mm) of the clear zone was substracted from the average colony size (X-Y) (Figure 9). This yielded a final value (mm) to indicate the siderophore production.

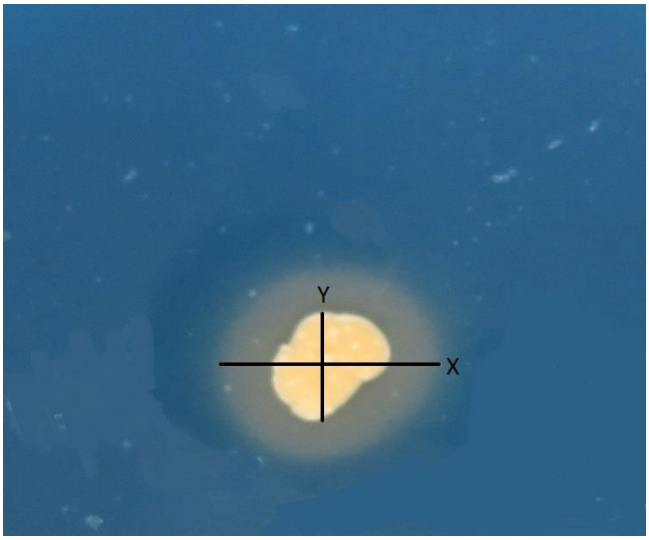

Figure 9: Closed view from CAS agar showing clear zone around the bacteria colony. X and Y represent diameter (mm) of the clear zone and bacterial colony, respectively.

4.2.1.2 Dual culture assay against the *Neofusicoccum parvum*

The ability of the chloramphenicol mutant strain of *Burkholderia* sp. W4R11C from Section 3.2.2 to inhibit the growth of the kiwifruit pathogen, *N. parvum*, from Section 2.2.3 was tested using a dual culture assay, to show that the mutant strain acts the same way as the original type and has not lost any of its activity. A 6 mm diameter agar disc was taken from the edge of a 7-day old culture of *N. parvum*, as described in Section 2.2.2 and placed in the centre of a Waksman agar plate. The bacterial inoculation was done onto the same agar by placing a loop of bacteria at equidistant points around the pathogen colony, making four bacterial inoculation sites on the same plate. A total ten replicates were set up. The plates were incubated at 25℃ in a 12-hour photoperiod for seven days. Antagonist activity of *Burkholderia* sp. W4R11C was determined by measuring the average inhibition zone (mm) between the *Burkholderia* sp. W4R11C and *N. parvum* colonies in two perpendicular directions using a digital caliper (Figure 10).

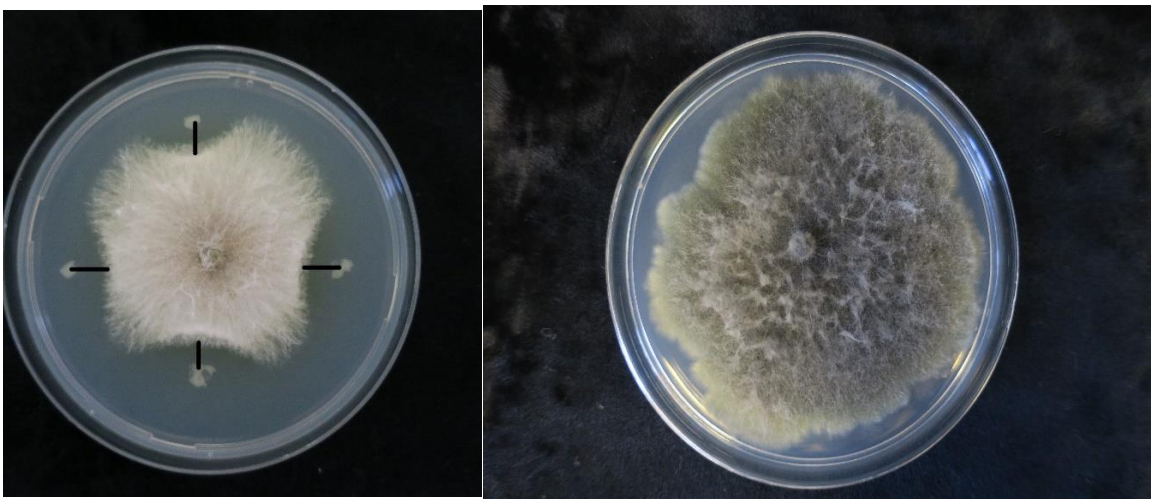

Figure 10: Inhibition of the growth of *N. parvum* by *Burkholderia* sp. Isolate W4R11C (left) compared to normal growth *of N. parvum* (right) after 3 days of incubation. Black lines indicated measurement for inhibition zone.

4.2.2 Fungal and bacterial cultures

The *Botryosphaeria* species used in this experiment is *N. parvum*. The conidia was prepared by harvesting the pycnidia from the prune agar plates, as described in Section 2.2.3. The pycnidia were crushed on a sterile glass slide and washed off with distilled water to obtain a spore suspension. A spore count was then conducted by using haemocytometer for each spore suspension. An adjustment was made by crushing and washing more pycnidia to obtain the final concentration of 6 x 10^4 spore per ml.

Burkholderia sp. W4R11C was sub-cultured from stocks (described in Section 3.2.2) stored at -80°C onto King agar B (Duchefa Biochemie) containing 125 ppm chloramphenicol at 25°C for two days. Once bacterial colonies appeared, a single colony was taken and placed in 1 ml nutrient broth (NB; Difco, Becton, Dickinson and Company) in separate 1.7 ml sterile tube. A total three replicates were set up. The tubes were placed in a shaking incubator (Labnet, Labnet International Inc.) at 28°C and 200 rpm for 14-15

hours. Bacterial cells were then harvested by centrifugation at 4000 x g for 10 minutes in a refrigerated centrifuge. The supernatant was then discarded and the pellets at the bottom of the tubes were resuspended in 10 ml phosphate buffered saline (PBS) pH 7.2 to achieve the final concentration of the suspension to 10^6 CFU/ml based on OD measurements (Section 3.3.2).

4.2.3 Antagonism of *Burkholderia* spp. against *N. parvum in planta*

The experiment was conducted during late winter from June 2017 till August 2017. Forty-two dormant *Actinidia chinensis* Planch. var*. chinensis* 'Gold3' rootstock (one year old) were delivered in a 6L plastic potting bags containing potting mix from Waimea Nurseries Ltd., New Zealand. The plants were placed in 4°C for 2 weeks and then transferred into a greenhouse with a 12-hour photoperiod till the end of the experiment; this ensure plant dormancy was broken.

Inoculants of *N. parvum* and chloramphenicol resistant *Burkholderia* sp. Isolate W4R11C were prepared as described in Section 4.2.2. There were six treatments in total: (1) *Burkholderia* sp. inoculation and 48 hours later *N. parvum* inoculation; (2) *N. parvum* inoculation and 48 hours later *Burkholderia* sp. Inoculation; (3) Single inoculation of *Burkholderia* sp.; (4) Single inoculation of *N. parvum*; (5) *N. parvum* and *Burkholderia* inoculation at the same time and (6) Negative control (water only). Plants were placed in an unbalanced randomized block design, with eight replicates for Treatments 1, 2, 4, 5 and five replicates for Treatments 3 and 6.

To commence the inoculation, a wound was created on the top of the main stem for each plant using sterile secateurs. A 10 μl aliquot of a *Burkholderia* W4R11-C1 cell suspension or *N. parvum* spore suspension at the required concentrations as described in Section 4.2.2 was pipetted onto the wound. For

the negative control, 10 μl of sterile water was applied onto the wound. All the inoculated plants were placed in a 12-hour photoperiod in the greenhouse and watered as required.

One month after inoculation, all plants were harvest. Each inoculated stem, was divided into 4 sections, each 5 cm in length, from the top (the wound site) to the bottom of the stem. Each section was labeled from 1 to 4, indicating the top to the bottom of the stem, respectively (Figure 11).

Figure 11: Inoculated kiwifruit stem showing sections 1, 2, 3 and 4, indicating the top (wound site) to the bottom of the stem respectively. Each section was 5 cm in length.

All plant tissue sections were surface sterilized, prior to placing on agar plates. The stems were debarked and soaked in a 25% sodium hypochlorite solution for 2 minutes, followed by rinsing 2 times in sterile water for 1 minute each time. Each section of the branch was then divided vertically into two and one half pressed gently into the surface of a ⅕ PDA (Difco, Becton Dickinson and Company) plate and the other half onto the surface of a King B agar amended with 125 ppm chloramphenicol. To validate the surface sterilization, randomly selected sterilized plant tissue (around 15% of the sample) were pressed onto the

surface of King agar B and ⅕ PDA, prior to planting on the isolation media (1/5 PDA and King agar B amended with 125 ppm chloramphenicol).

The plates were incubated in 20℃ with a 12-hour photoperiod. The plates were monitored daily to record any microbial growth. To further confirm the species grow on the plates, glass slides were made when sporulation occurred and morphological structures were recorded after around 2 weeks of incubation. Hyphal tipping and sub-culturing, as described in Section 2.2.1, were also conducted from plates that shown fungal/bacterial growth. Plates that show fungal growth resembling *N. parvum* were sub-cultured, identity confirmed using DNA sequencing as described in Section 2.2.4.

4.3 Results

4.3.1 Bioactivity assays

Burkholderia strain W4R11C inhibited the radial growth of *N. parvum* in the dual cultural assay. The fungal hyphae were observed to be deformed and hyphal tip lysis was noted. The inhibition area ranged from 4.6 to 9.8 mm. The replicate value was compared to the mean value obtained from all replicates, which is 7.2 mm. The data is homogenous, with no substantial differences between each replicate (P=0.941) (Table 3).

Table 3: Inhibition area of each replicate from dual plating of *Neofusicoccum parvum* and *Burkholderia* sp. isolate W4R11C.

Replicates	Inhibition area (mm)	Expected value
1	4.61	7.2
2	5.76	7.2
3	8.61	7.2
4	5.61	7.2
5	8.65	7.2
6	7.2	7.2
7	9.77	7.2
8	6.11	7.2
9	7.64	7.2
10	8.17	7.2
	P value =	0.947364387

In the siderophore production assay, clear zones occurred around *Burkholderia* sp. isolate W4R11C colonies in the replicates, ranging from 9.35 to 12.76 mm. The replicate value was compared to the mean value obtained from all replicates, which is 10.87 mm. The data is homogenous, with no substantial differences between each replicate (P=0.99) (Table 4).

Table 4: Clear zone measurement from CAS agar plates of Burkholderia sp. isolate W4R11C colony.

Replicates	clear zone (mm)	Expected value
1	9.84	10.87
2	11.38	10.87
3	11.52	10.87
4	11.36	10.87
5	10.42	10.87
6	10.36	10.87
7	11.18	10.87
8	12.76	10.87
9	9.35	10.87
10	10.5	10.87
	P value =	0.999790663

4.3.2 Glasshouse inoculation

Upon visual inspection, there was no macroscopic evidence of fungal or bacterial growth nor any visible lesion on the wounded plant stem. However, isolation from the stem confirmed that inoculation was successful with both *Burkholderia* sp. isolate W4R11C and *N. parvum* being recovered based on growth on chloramphenicol and colony morphology, respectively. On the 125 ppm chloramphenicol amended King B agar plates, creamy yellowish white bacterial colonies were present from Treatments 1, 2 3, 5. These resembled the morphological features of *Burkholderia* strain W4R11C used for inoculation (Figure 12).

Figure 12: Bacterial colonies growing from the kiwifruit stem tissue plated onto King agar B amended with 125 ppm chloramphenicol from Treatment 1 after 3 days incubation at 20℃ in 12-hour photoperiod.

Colonies morphologically identified as *N. parvum* were reisolated from the plant material. Reisolation plates showed mycelium growth that resembled the morphological features of *N. parvum* (Figure 13). Colonies with greyish mycelium were found on several plates from Treatments 1, 2, 4 and 5, which had a fast growth that covered the whole agar plates in seven days after incubation.

For the uninoculated negative control, no fungal colonies morphologically identified as *N. parvum*, and no bacterial colonies resistant to chloramphenicol were recovered.

Figure 13: Fungal colonies morphologically identified as *Neofusicoccum parvum* growing from the kiwifruit stem tissue plated onto 1/5 PDA agar plate from Treatment 2 showing greyish white fungal mycelium growth after 3 days incubation at 20℃ in 12-hour photoperiod.

The sequence results, from DNA extractions of samples resembled typical *Botryosphaeria* morphological look, indicated a high similarity to *N. parvum* in BLAST **(**Table 5**)**.

Table 5: BLAST results shows alpha elongation factor gene region amplified sequences with high similiarity to *N. parvum* (99%).

Description	Query Cover	Ident	Accession
Neofusicoccum parvum UCRNP2 putative elongation factor 1-alpha protein mRNA	99%	99%	XM_007579574.1
Diplodia mutila isolate AFTOL-ID 1572 translation elongation factor 1-alpha (EF1a) gene, partial cds	99%	97%	DQ677907.2
Diplodia corticola elongation factor 1-alpha (BKCO1_4100086, partial mRNA	99%	97%	XM_020275852.1

Successful inoculation and colonization results were shown in Table 6, as a percentage of the total replicates within each treatment. The data yielded an abnormal distribution due to a large number of unsuccessful inoculation (e.g. non-presence of the inoculants on the agar plates). Therefore, statistical analysis was confined to examining successful inoculation results only.

Table 6: Successful inoculation of *Burkholderia* sp. W4R11C and *N. parvum* as a percentage of the total number of replicates for the six different treatments.[1]

	Treatments	*Burkholderia*	*N. parvum*
1	*Burkholderia* 1st & *N. parvum* 2rd	13.33%	27.66%
2	*N. parvum* 1st & *Burkholderia* 2rd	15.63%	15.63%
3	*Burkholderia* only	0%	25%
4	*N. parvum* only	36.84%	0%
5	*Burkholderia* & *N. parvum* same time	18.75%	6.25%
6	Negative control	0%	0%

[1] 1st and 2rd indicated sequences of inoculants application, with 1st being first inoculated and 2rd being inoculated 48 hours after the first one.

Results indicated an interaction between the two organisms in regard to their colonization ability in the host plant. From the successful reisolation of the two inoculants, a significant difference (P=0.04) between numbers of *Burkholderia* colonization compared with that of *N. parvum* was found in Treatment 5), where the *Burkholderia* sp. was recovered three times more colonization than the *N. parvum*. In Treatments 1 and 2, no substantial differences were found between the colonization ability of the two isolates.

Section-wise inspection of the inoculum colonization was also conducted, which indicates an organisms' movement within plant tissue. In Treatment 1, 2 and 5, *Burkholderia* sp. isolate W4R11C colonization was less than *N. parvum* at the top section of the stem. Proceeding down to section 2 (10cm from the wound) and 3 (15 cm from the wound), the *Burkholderia* sp. colonization often exceeded the *N. parvum* colonization. At section 4 (20cm from the wound), all three treatments showed no *N. parvum* present. Detailed inoculant movement and colonization of the inoculants from each treatment are presented.

4.3.2.1 Treatment 1: *Burkholderia* sp. isolate W4R11C inoculation and 48 hours later *N. parvum* inoculation

The colonization abilities of *Burkholderia* sp. and *N. parvum* were indicated by the recovery of the two organisms from cultural plates. It changed between different sections of the stem (Figure 14). *Burkholderia* sp. were not found to colonize the top two sections of the plant, whereas *N. parvum* showed high colonization rate that reached nearly 50% of the total replicates. At the last two section at the bottom of the stem, the proportion of *Burkholderia* colonization increased and non-fungal colonization can be found.

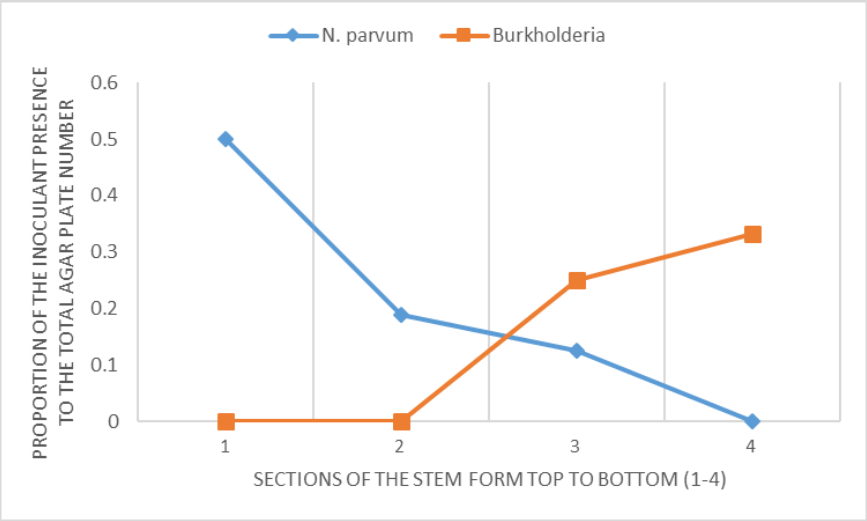

Figure 14: Changes in recovery of *Burkholderia* sp. W4R11C and *N. parvum* in kiwifruit plant sections from Treatment 1. The colonization abilities were indicated by the proportions of replicates where the isolate was recovered on the agar plates within the treatment.

Each section from Treatment 1 was examined to compare the changes of two inoculants recovery (Figure 15). The top two section showed fungal colonization only (Figure 15A and B). In section 3, *Burkholderia* sp. colonization emerged, yet did not make a substantial higher colonization than *N. parvum* (Figure 15C). In section 4, the *Burkholderia* colonization reached 30% of all the treatment plants, and no fungal colonization can be found (Figure 15D).

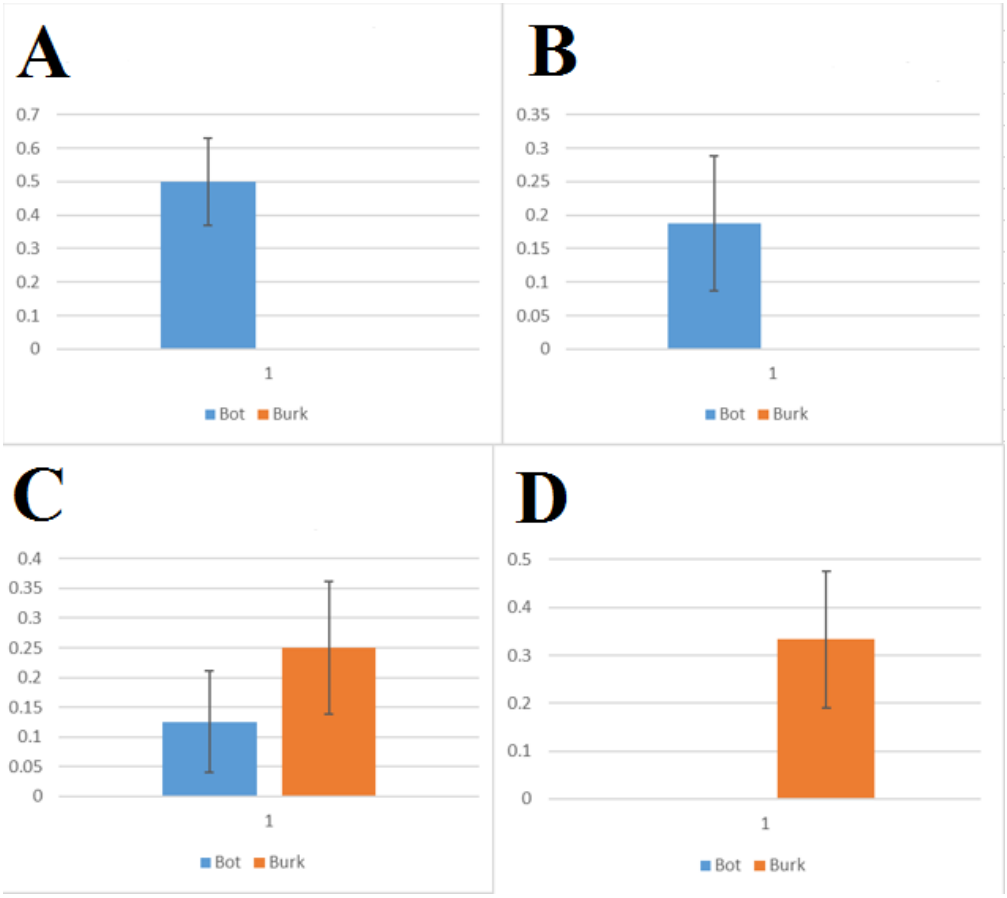

Figure 15: Section-wise comparison between the presence of *N. parvum* (Bot) and *Burkholderia* sp. (Burk) (as a proportion of the total number of replicates) from Treatment 1. (A) Section 1 (0-5 cm from wounded site). (B) Section 2 (5-10 cm from wounded site). (C) Section 3 (10-15 cm from wounded site). (D) Section 4 (15-20 cm from wounded site). Error bars indicate the standard deviation of the mean.

4.3.2.2 Treatment 2: *N. parvum* inoculation and 48 hours later *Burkholderia* sp. isolate W4R11C inoculation

The colonization abilities of *Burkholderia* sp. and *N. parvum* changed between different sections of the stem, as indicated by their recovery (Figure 16). The *Burkholderia* was found to have 52% less colonization than *N. parvum* in the top two sections of the plants. At section 3 and 4, the proportion of *Burkholderia* colonization increased to 25% of the replicates and fungal colonization underwent a continuous decreasing and cannot be found at the last section of the stem.

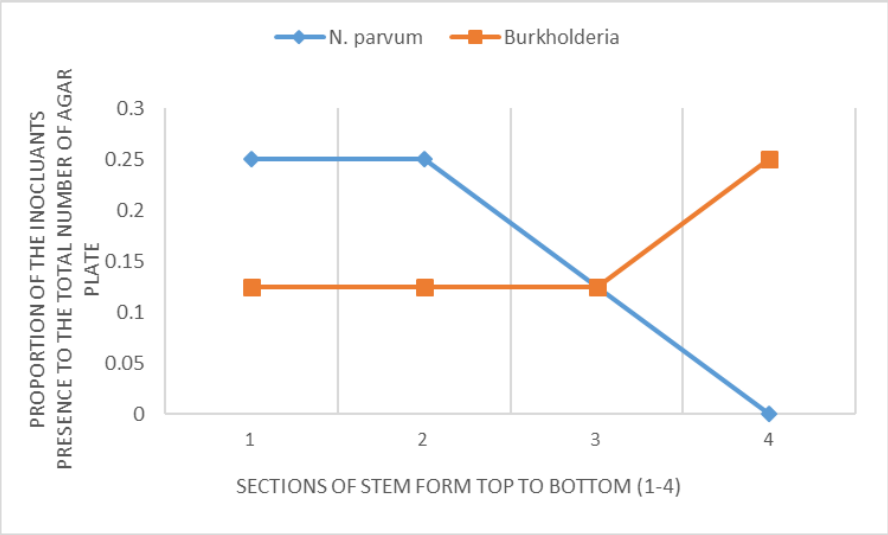

Figure 16: Changes in recovery of *Burkholderia* sp. W4R11C and *N. parvum* in kiwifruit plant sections from Treatment 2. The colonization abilities were indicated by the proportions of replicates where the isolate was recovered on the agar plates within the treatment.

Each section from Treatment 2 was examined to compare the two inoculants colonization changes (Figure 17). The top three sections (up to 15 cm from the wound) showed an increasing of *Burkholderia* sp. colonization and a decreasing of *N. parvum* (Figure 17A,B and C). At the fourth section (20cm from the

wound), *Burkholderia* sp. colonized 25% of the treated plants and no fungal colonization can be found (Figure 17D).

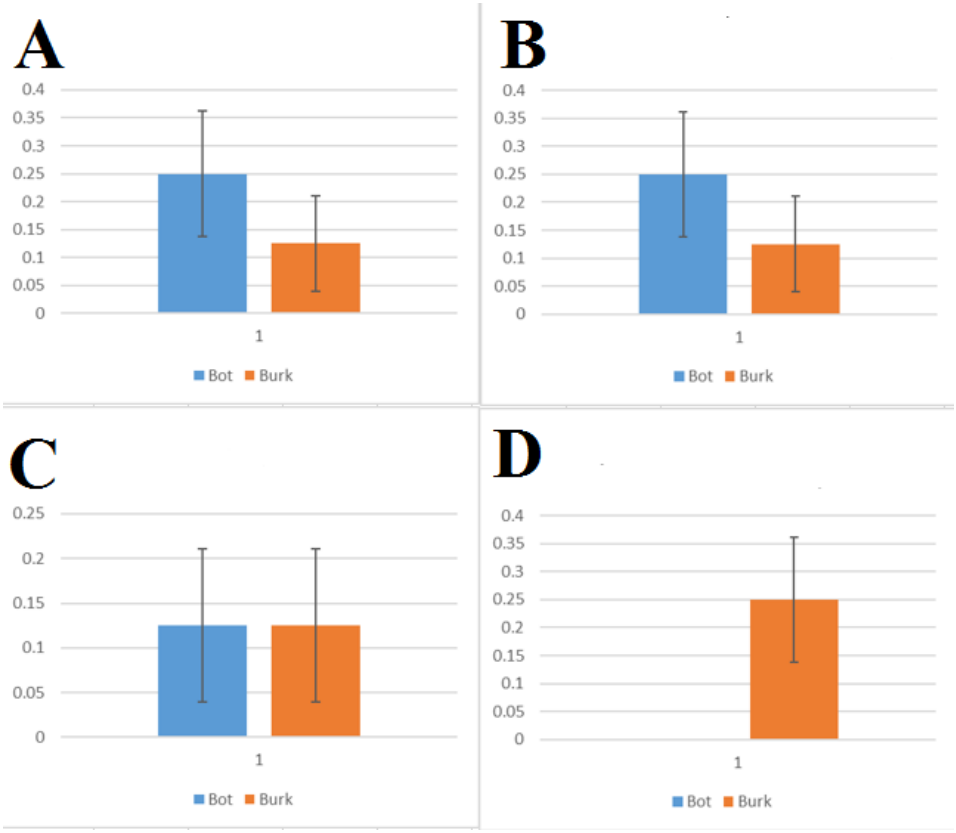

Figure 17: Section-wise comparison between the presence of *N. parvum* (Bot) and *Burkholderia* sp (Burk) (as a proportion of the total number of replicates) from Treatment 2. (A) Section 1 (0-5 cm from wounded site). (B) Section 2 (5-10 cm from wounded site). (C) Section 3 (10-15 cm from wounded site). (D) Section 4 (15-20 cm from wounded site). Error bars indicate the standard deviation of the mean.

4.3.2.3 Treatments 3 and 4: single inoculation of *Burkholderia* sp. W4R11C and *N. parvum*, respectively

The inoculants colonization as proportion to the replicates from treatment 3 and 4 was shown in Figure 18 and 19 respectively, which was indicated by the inoculant's recovery. The *Burkholderia* sp. colonization

declined from 60% to 20% in Treatment 3. In treatment 4, single inoculation of *N. parvum* showed high colonization at around 50% in the first section of the stem, and it decreased to around 10% at last section. Both treatments showed persistent colonization from the organisms from the top to the bottom of the stem.

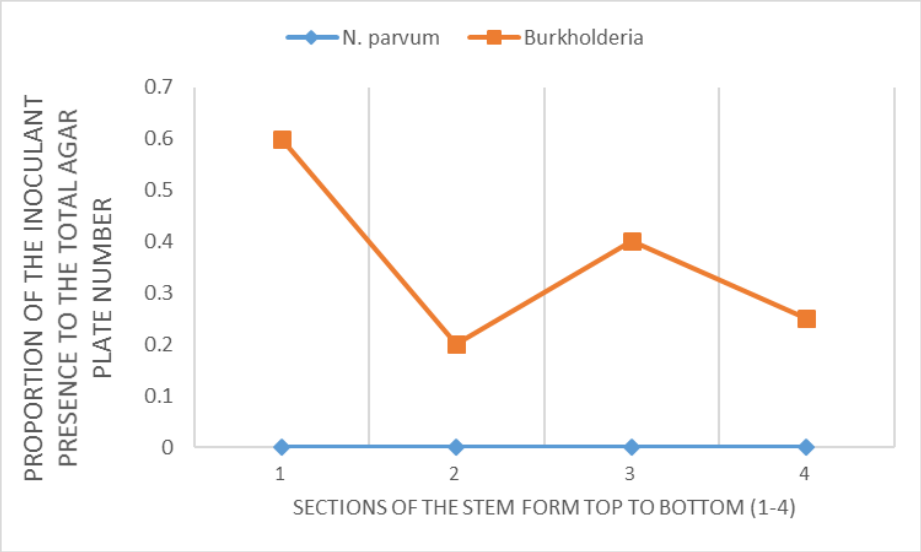

Figure 18: Changes in *Burkholderia* sp. W4R11C recovery in kiwifruit plant tissues of Treatment 3. The colonization ability was indicated by the proportions of replicates where the isolate was recovered on the agar plates within the treatment.

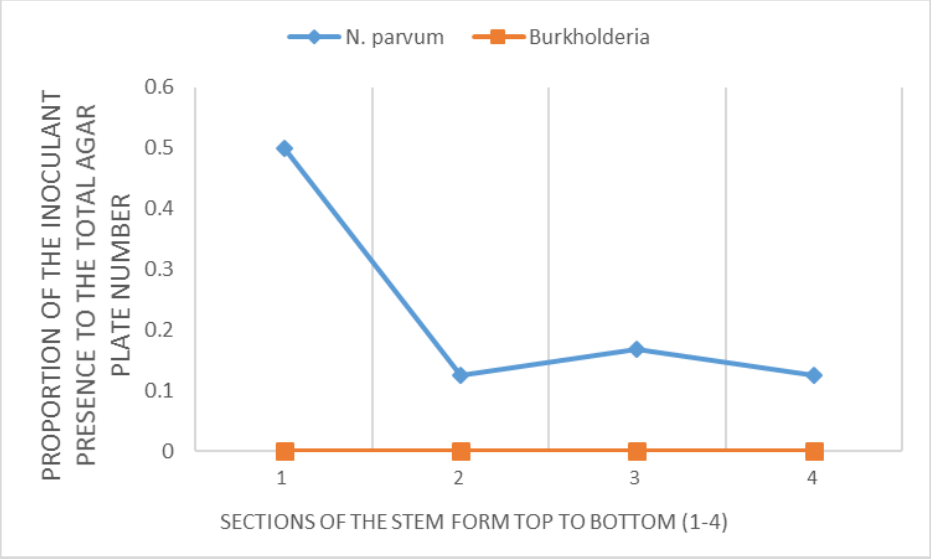

Figure 19: Changes in *N. parvum* recovery in kiwifruit plant tissues of Treatment 4. The colonization ability was indicated by the proportions of replicates where the isolate was recovered on the agar plates within the treatment.

4.3.2.4 Treatment 5: *Burkholderia* sp. W4R11C and *N. parvum* inouculation at the same time

The inoculants colonization, indicated by recovery of the two isolates as proportion to the replicates in Treatment 5, was shown in Figure 20. The *Burkholderia* sp. and *N. parvum* had similar colonization at around 20% in the first section of the stem. At section 2, 3 and 4, the proportion of the *Burkholderia* colonization outcompete *N. parvum*, leaving non-colonization of the fungi.

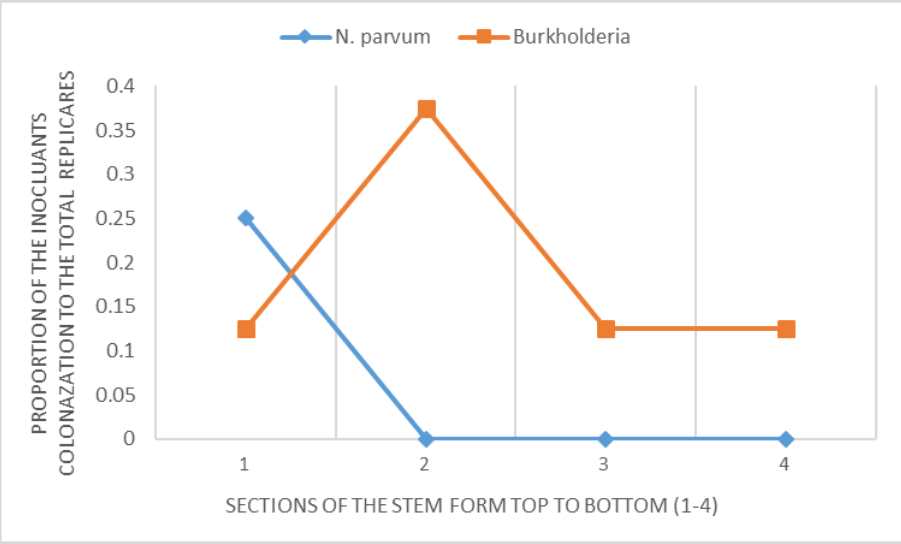

Figure 20: Changes of recovery of *Burkholderia* sp. W4R11C and *N. parvum* within plant sections from Treatment 2. The colonization abilities is indicated by proportions of inoculants presences to the total number of agar plates within the treatment.

Each section from Treatment 5 was then examined to compare the two inoculants colonization changes (Figure 21). The first sections showed a similar colonization proportion-wise between the two inoculants, as indicated by the error bar (Figure 21A). Section 2, 3 and 4 showed non-colonization of *N. parvum* and around 12-30% colonization of *Burkholderia* sp.(Figure 21B, C and D).

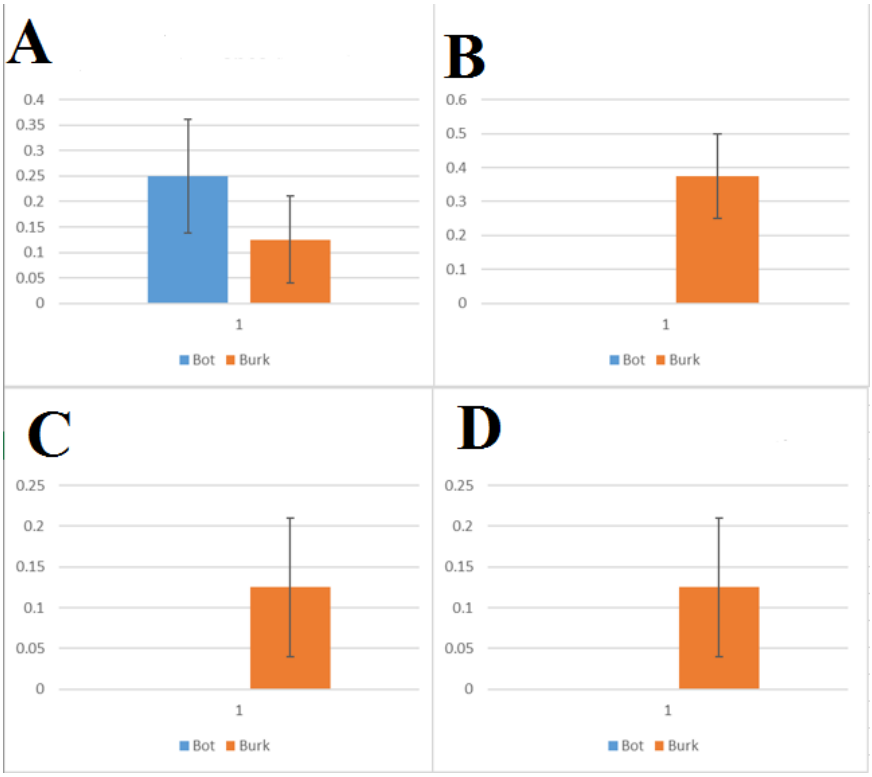

Figure 21: Section-wise comparison between the presence of *N. parvum* (Bot) and *Burkholderia* sp (Burk) (as a proportion of the total number of replicates) from Treatment 5. (A) Section 1 (0-5 cm from wounded site). (B) Section 2 (5-10 cm from wounded site). (C) Section 3 (10-15 cm from wounded site). (D) Section 4 (15-20 cm from wounded site). Error bars indicate the standard deviation of the mean.

4.3.3 Other organism

Besides the presences of the two inoculants, other organism was consistently isolated from the plant tissue. Cover-slip slides showed spores that resemble *Arthrinium* spp. (Figure 22).

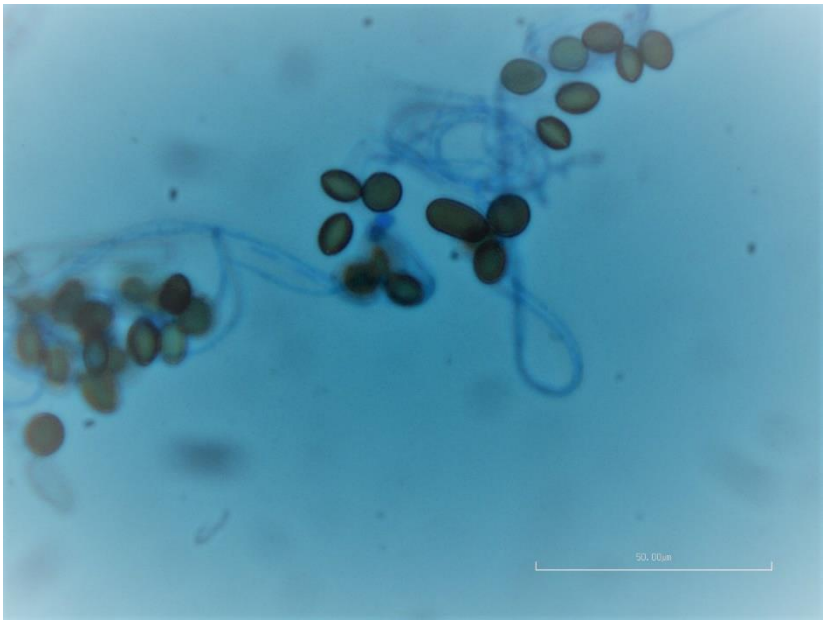

Figure 22: *Arthrinium* spores showing typical morphology being dark brown, oval shaped and a germ slit in the middle.

4.4 Discussion

This work has demonstrated for the first time the apparent competition between *N. parvum* and *Burkholderia* sp. isolate W4R11C, with the *Burkholderia* sp. appearing to have an antagonistic effect on *N. parvum in planta*. *In vitro* assessment had also shown antagonist effect conferred with previous studies *(*Wicaksono *et al.* 2017*)*. A study by Tenorio-Salgado et al. (2013) also reported some *Burkholderia* strains were able to produce volatile compounds and inhibit hyphal growth of phytopathogenic fungi

Both *Burkholderia* sp. W4R11C and *N. parvum* were able to colonizes the one year old *A.chinesis* "Gold3" plants in the glass house experiment. The colonization of *Burkholderia* sp. W4R11C on the kiwifruit plants was confirmed in previous study (Wicaksono *et al*. 2017). The same study also indicated that deliberately wounded tissues provided suitable inoculation courts for the bacteria. To create a stable concentration that being effective, an upper and lower inoculum threshold was defined, namely as the Allee effect

(Etienne *et al*. 2002).In this experiment, 10^6 CFU/ml was used and achieved some successful colonisation of the tissue (at around 15-30%). Besides, the wounding treatment mimics the common agronomic practices such as pruning in the orchard, which provide a similar condition for the inoculation as to natural infection. Several studies that utilized wounding treatments for bacterial inoculation also showed effective colonization results (Wulff *et al*. 2002; Rezgui *et al*. 2016). Future studies on applying biocontrol agents should consider the use of different concentrations of the bacterial suspension to achieve a higher colonization of host plant tissue.

The movement and interaction between *Burkholderia* W4R11C and *N. parvum* were also examined. From the results, the colonization of *N. parvum* decreased from section2, 3 and 4 of the stem (5-20 cm from the wound), where *Burkholderia* sp. colonization persisted. This finding conferred with the common practice in the kiwifruit industry, which suggested to prune 20 cm off the stem from the infected site to eliminate inoculum sources in the plant (Honer *et al*. 2015).

The interaction between the *Burkholderia* sp. and *N. parvum* confirm the antagonism of the bacteria. In cases with the successful inoculation, the *Burkholderia* sp. colonization outnumbered *N. parvum* further down of the stem (Section 4.2.3.1-4.3.2.4). This may indicate a suppression effect on *N. parvum* growth by the *Burkholderia* sp. W4R11C. Although some may argue that *N. parvum* inoculation may result in a weak colonization, the positive control from Treatment 4 suggested the persistency of *N. parvum* throughout the host tissue. Therefore, one should be able to conclude that the non-recovery of *N. parvum* from sections 3 and 4 of the stem was due to the presence and inhibitory action of *Burkholderia* sp. W4R11C.

Some research has indicated that a biocontrol product can be more effective in disease inhibition if applied prior to pathogen arrival (Halleen *et al*. 2016). However, it is contradictory to our finding of a

better colonization from the *Burkholderia* sp. W4R11C when inoculated at the same time as *N. parvum*. Different fungal species can have varied sensitivity to biocontrol bacteria, which may explain this variation in results. For instance, Mazzola & Cook (1991) reported that different *Pythium* species can affect the colonization rate of bacteria in wheat. Besides, the *N. parvum* inoculant has a high spore concentration at 10^4 spore/ml, which should give a more readily colonization. Under such conditions, *Burkholderia* sp. W4R11C still maintained its colonization ability, which showed similiar colonization levels as the non-disease circumstances(Treatment3). The fact that the *Burkholderia* sp. W4R11C has a strong colonization and survival regardless of the presence of the pathogen *N. parvum,* is therefore clear. It indicated a potentially stronger biocontrol ability from this particular strain.

There are a few points that should be considered to improve this experiment. First of all, *N. parvum* incoulated plant material yielded very few reisolations. The low disease incidence in this experiment was contradictory to those reported by Michailides (1991) and Milholland (1972). A study focused on different spore concentrations may explain the low fungal colonization rate. In Biggs (2014), there seems to be a great increase in disease incidence from around 40% to 70%, when the spore concentration was increased from 10^6 to 10^7 spore/ml. For future work to increase inoculation success, a different spore concentration may be considered.

Secondly, the bacterial movement was examined in a previous study on cotton, which showed limited movement (none beyond 5 cm) *in planta* (Chen *et al*. 1995). This is contradictory to our results that indicated a rapid colonization of the plant tissues by *Burkholderia* sp. W4R11C. Yet it may explain the inconsistency of *Burkholderia* sp. colonisation between the replicates. Limited movement of the bacteria may occur in our experiment that restrict its access to plant nutrients. The result of our experiment was not sufficient in supporting the colonization ability of *Burkholderia* sp. W4R11C, and further studies are

required. Alternative methods in analyzing the locality of the bacteria in plant tissue can be applied. For instance, fluorescence *in situ* hybridization confocal laser scanning microscopy can be used (Cardinale 2014).

Nevertheless, from these results, we can conclude that the colonization and movement of *Burkholderia* sp. W4R11C may contribute to its biocontrol ability and can be affected by application time. The inhibition effect from the bacteria may take place more readily during pruning time, serving as a wound protectant during seasons when *N. parvum* inoculum level are high in the orchard. Due to the limited number of replicates, this advice may not be suitable for all kiwifruit orchards. Further studies on examining the interaction between *N. parvum* and the *Burkholderia* sp. are required.

Chapter 5

Concluding discussion

The overall objective of this thesis was to test the biocontrol ability of one *Burkholderia* strain, as a wound protectant, against the pathogen *Neofusicoccum parvum in planta*. This work presented information about a colonization and movement of the bacterial agent, which offered a comprehensive understanding of its biocontrol ability in kiwifruit. *Actinidia chinensis* is an important horticultural crop in New Zealand. The outcome of this study may contribute to an integrated pest management for *N. parvum* for the kiwifruit industry.

The culturing and identifying of the fungal pathogen, *Neofusicoccum parvum*, was performed in Chapter 2. Morphological observation and molecular approaches based on sequencing several gene regions were used. The highlight of this part of the study was these multiple approaches on examining and understanding the fungal biology of *N. parvum*. The morophological identification, such as color, shape and growth rate of the fungi, can aid in initial identification to genus level. It was then followed by DNA extraction and sequencing to distinguish morphologically alike species within the genus. The ITS regions cannot distinguish between different *Botryosphaeria* species. Two other gene regions, beta-tubulin and alpha elongation factors were used, which which did enable the differentiation between two closely associated species *N. parvum* and *N. ribis.* For future studies, species-specific primers should be designed to enable quick and accurate identification of these two species. Induction of sporulation by the *N. parvum* isolate using different agar media and detached plant tissue was also assessed in this part of the study. Production of conidia was only observed on prune agar, with these being used as inoculum for the *in planta* biocontrol experiment in Chapter 4.

In Chapter 3, the aim was to culture, identify and develop an antibiotic resistant mutant of the bacterial isolate, W4R11C, that was previously identified as a potential biocontrol agent. The *Burkholderia* sp. grows well in liquid nutrient broth and King agar B in solid form. For identification, the16S gene was used, which identified the isolate aa a member of the genus *Burkholderia*, but was unable to differentiate *Burkholderia* species. An antibiotic resistant mutant strain was created to enable the selective isolation and thereby traceability of *Burkholderia* sp. W4R11to be determined from the inoculated plant tissue in Chapter 4. Further studies to create alternative methods to enable the colonization of plant tissue by *Burkholderia* sp. should be considered, particularly methods such as fluorescent in situ hybridization which would enable colonization of plant tissue by this isolate to be visualizedwhich may offer a more definite correlation between *in vitro* performance and *in planta*.

In Chapter 4, the biocontrol ability of the *Burkholderia* strain against *N. parvum* was tested. *Burkholderia* sp. W4R11C, the chloramphenicol resistant mutant strain, was used as a wound protectant. This study showed the ability of *Burkholderia* sp. W4R11C to colonize the stem of kiwifruit plants. Of particular note was that *Burkholderia* sp. inhibited the *N. parvum* colonisation *in planta*. *Burkholderia* sp. W4R11C was seen to colonise further down the stem from the inoculation point than *N. parvum*, when both isolates were present. When both *Burkholderia* sp. W4R11C and *N. parvum* were applied to the stem, only the *Burkholderia* sp. colonised the plant stem tissue 10 cm from the inoculation point; no *N. parvum* could be reisolated. These results may give a better understanding of specific circumstances in using the bacteria. For example, since the bacterial biocontrol agent can colonize further than the pathogen in the stem, the standard practice of cutting infected kiwifruit vine 20 cm from the visible lesion and then applying the biocontrol agent may eliminate the pathogen. Since the inoculation was not successful in a large number of plants, future studies should use a greater number of replicates (maximum 8 replicates in current

study). Future work could also focus on seeking a more effective inoculation method, which could be tested on mature plants in the field.

In summary, this work combined the understanding of the biology of the microbial organisms and *in planta* testing, to elucidate the capacity of the bacterial biocontrol agent, *Burkholderia* sp. W4R11, for the control of the kiwifruit pathogen, *N. parvum*. *Burkholderia sp.* W4R11 could potentially be utilized as part of an integrated pest management program for better disease control. Further work on testing the bacterial biocontrol ability and understanding the pathogenicity of *N. parvum* are required to progress this research further.

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Appendix A

Recipes of media types used for culturing and functionality assay

Chrome Azurol S (CAS) agar (Schwyn & Neilands 1951)

The following solutions were prepared separately.

Blue Dye:

a. Solution 1: Dissolve 0.06 g of CAD (Fluka Chemicals) in 50 ml of distilled water.

b. Solution 2: Dissolve 0.0027 g of FeCl3-6H2O (LabServ, Thermofisher Scientific Inc.) in 10 ml of 10 mM HCl (FIsher Scientific, Thermofisher Scientific Inc.).

c. Solution 3: DIssolve 0.073 g of HDTMA in 40 ml of distilled water.

d. Mix Solution 1 with 9 ml of Solution 2. Then mix with Solution 3.

The solution should be blue color and autoclaved.

Mixture Solution

a. Minimal Media 9 (MM9) Salt solution stock: Dissolve 15 g KH2PO4, 25 g NaCl and 50 g NH4Cl in 500 ml of distilled water.

b. 20% Glucose stock: Dissolve 20 g glucose in 100 ml of distilled water.

c. NaOH Stock: Dissolve 25 g of NaOH in 150 ml of distilled water. Its pH should be around 12.

d. Casamino Acid solution: Dissolve 3 g of Casamino acid in 27 ml of distilled water. The solution should then be filter sterilized.

To make the CAS agar:

a. Add 100 ml of MM9 solution to 750 ml of distilled water.

b. Dissolve 32.24 g piperazine-N, N'-bis (2- ethanesulfonic acid) PIPES (Sigma-Aldrich, Sigma-AldrichCo. LLC). Noted that PIPES will not dissolve below pH of 5. Bring pH up to 6 and slowly add PIPES while stirring. The pH should drop as PIPES dissolves. While stirring, slowly bring pH up to 6.8. Do not exceed pH of 6.8.

c. Add 15 g Bacto agar.

d. The MM9/PIPES mixture should be autoclaved and cool to 50℃.

e. Add 30 ml of sterile Casamino acid solution add 10 ml of sterile 20% glucose soluton to MM9/PIPES mixture.

f. Slowly add 100 ml of Blue Dye solution along the glass wall with enough agitation to mix thoroughtly

Prune extract agar

Prune extract:

Destone the dried prune to get 50 g of the flesh in 1 litre of distilled water. Place the mixture to boil and simmer for 30 minutes. The solution should then be strained by a sterile muslin cloth. The strained solution need to add distilled water to bring up to 1 litre.

To make around 1 L of the prune extract agar:

prune extract	100 ml
sucrose (LabServ, Thermofisher Scientific Inc.)	5 g
Difco yeast (Difco, Becton, Dickinson and Company)	1 g
agar	30 g

distilled water		1 L

Pine needle agar		per litre
agar		20 g
distilled water	q	1 L

Autoclave the above mixtures. Place 1-2 previously autoclaved pine needle in the plates and pour the water agar mixture in the same plates.

Vegetable juice agar	per litre
V8 juice	200 mL
Davis agar	15 g
1.0 M NaOH	6 mL
distilled water	800 mL

Waksman agar	per litre
Bacteriological pepton (Difco, Becton, Dickinson and Company)	5 g
Beef extract (Acumedia, Neogen)	5 g
Sodium Chloride (LabServ, Thermofisher Scientific Inc.)	5 g
Glucose (Scharlau, Scharlab S. L.)	10 g
Difco agar (Difco, Becton, Dickinson and Company)	15 g

pH adjust to 7.2

Appendix B

DNA sequence results

***Neofusicoccum parvum* isolate**:

(1): the ITS region

AGGACCATAAAACTCCAGTCAGTGAACTTCGCAGTCTGAAAAACAAGTTAATAAACTAAAACTTTCAACAACGGATCT

CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA

ATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGGCATGCCTGTTTCGAGCGTCATTTTCAACCCTCAAGCT

CTGCTTTGGGTATTGGGCCCCGTCCTCCACGGACCGCGCCTTAAAGACCTCGGGGGTGGGCGTCTTGCCTCAAGCGT

AGTAGAAAACACCTCGCTTTGGAGCGCACGGCG

(2) the Beta-tubulin region

TTCACCTCCAGACCGGCCAATGCGTAAGTCTCCTCGCATCCGCTGCACTCGCTGCACCGCGCTGACTTTGCCCAGGGT

AACCAAATCGGTGCCGCCTTCTGGTTTGTTGCCAAAACACTCCCGCTCCCGCG

(3) the Alpha-elongation factor region (Section 2.2.4)

AACTGGTGAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTGCTCGCCTACACCCTGGGTG

TCAAGCAGCTCATCGTCGCCATCAACAAGATGGACACCACCAAGTGGTCTGAGGAGCGTTACCAGGAGATCATCAAG

GAGACCTCCAACTTCATCAAGAAGGTCGGCTACAACCCCAAGACCGTTCCCTTCGTCCCCATCTCCGGCTTCAACGGC

GACAACATGATCGAGGCCTCCACCAACTGCCCCTGGTACAAGGGCTGGGAGAAGGAGACCAAGACCAAGTCCACCG

GCAAGACCCTCCTCGAGGCCATCGACTCCATCGATACCCCCGTCCGCCCCTCGGACAAGCCCCTCCGTCTTCCCCTCCA

GGACGTCTACAAGATTGGCGGTATTGGCACGGTCCCCGTCGGCCGTGTCGAGACTGGTGTTATCAAGGCCGGCATG

GTCGTCACCTTCGCCCCCGCTGGTGTCACCACTGAGGTCAAGTCCGTCGAGATGCACCACGAGCAGCTTGTCGAGGG

TGTCCCCGGTGACAACGTCGGCTTCAACGTCAAGAACGTCTCCGTCAAGGAGATCCGTCGTGGCAACGTCGCCGGTG

ACTCCAAGAACGACCCCCCCAAGGGCTGCGACTCCTTCAACGCCCAGGTCATCGTCCTCAACCACCCCGGTCAGGTCG

GTGCTGGCTACGCTCCCGTCCTGGACTGCCACACTGCCCACATTGCTTGCAAGTTCTCTGAGCTGCTCGAGAAGATCG

ACCGCCGTACCGGCAAGTCTATTGAGAACAGCCCCAAGTTCATCAAGTCTGGTGATGCCGCCATCGTCAAGATGATT

CCCTCCAAGCCCATGTGCGTTGAGGCTTTCACCGAGTACCCCCCTCTTGGCCGTTTCGCCGTCCGTGACATGGTATGT

CCCTC

(4)the Alpha-elongation factor region (Section 4.2.3)

ACTCATTGCCGCCGTACTGGTGAGTTCGAGGCTGGAATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTGCTCGC

CTACACCCTGGGTGTCAAGCAGCTCATCGTCGCCATCAACAAGATGGACACCACCAAGTGGTCTGAGGAGCGTTACC

AGGAGATCATCAAGGAGACCTCCAACTTCATCAAGAAGGTCGGCTACAACCCCAAGACCGTTCCCTTCGTCCCCATCT

CCGGCTTCAACGGCGACAACATGATCGAGGCCTCCACCAACTGCCCCTGGTACAAGGGCTGGGAGAAGGAGACCAA

GACCAAGTCCACCGGCAAGACCCTCCTCGAGGCCATCGACTCCATCGATACCCCCGTCCGCCCCTCGGACAAGCCCCT

CCGTCTTCCCCTCCAGGACGTCTACAAGATTGGCGGTATTGGCACGGTCCCCGTCGGCCGTGTCGAGACTGGTGTTAT

CAAGGCCGGCATGGTCGTCACCTTCGCCCCCGCTGGTGTCACCACTGAGGTCAAGTCCGTCGAGATGCACCACGAGC

AGCTTGTCGAGGGTGTCCCCGGTGACAACGTCGGCTTCAACGTCAAGAACGTCTCCGTCAAGGAGATCCGTCGTGGC

AACGTCGCCGGTGACTCCAAGAACGACCCCCCCAAGGGCTGCGACTCCTTCAACGCCCAGGTCATCGTCCTCAACCA

CCCCGGTCAGGTCGGTGCTGGCTACGCTCCCGTCCTGGACTGCCACACTGCCCACATTGCTTGCAAGGTTCTCTGAGC

TGCTCGAGAAGATCGACCGC

***Burkholderia* sp.**:

16S region

GAAAGCCGGATTAATACCGCATACGATCTACGGATGAAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGGTTGGCC

GATGGCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACCA

GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG

CCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTTGAC

CCTAATACGGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC