Bio-prospecting for endophytes of Brassica

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Abstract

Disease and insect pests are major limiting factors for crop production worldwide. Farmers are often heavily reliant on synthetic biochemicals and fertilisers to mitigate the negative impact of pests and disease and to increase crop yield. However, the extensive use of chemicals has led to environmental concerns due to contamination of soil and water, human health issues, disturbance of macro and microorganisms balance and the development of resistance by both insects and fungal pathogens. Use of biological control agents including endophytic microorganisms is an alternative control option to combat these problems. Many endophytes are able to provide their host with beneficial traits such as resistance against insect-pests and pathogens and enhance crop performance under abiotic stresses. Although beneficial microorganisms of brassica crops have been discovered, endophytes of wild brassica's, particularly those associated with the seed, have been ignored. In this study, we screened seed of various brassica species with a worldwide distribution and isolated 131 bacterial and two fungal species. Molecular identification of bacterial isolates indicates that most seed accessions harboured endophytic bacteria belonging to 17 species. Among these isolates, two species, identified as *Methylobacterium fujisawaense* and *Me. phyllosphaerae* were dominant and widespread across the majority of accessions sampled, and originated from different species and locations. The inoculation of oilseed rape (*Brassica napus*) root with these endophytic bacteria significantly increased the fresh weight of the seedlings.

The fungal endophyte species identified were *Beauveria bassiana* and *Geomyces pannorum*, isolated from two different accession of a wild brassica species (*B. rapa*). Inoculation of the seeds of three brassica species, *B. napus*, *B. rapa* and *B. oleracea*

with these fungal endophytes resulted in infection of below and above ground tissues of inoculated seedlings but colonisation of the next generation seeds/seedlings did not occur. Seed inoculation, foliar application and soil drenching when the plants were grown on non-sterile soil also did not result in plant colonisation.

A dual culture test was performed to study the antagonistic effect of these bacterial and fungal endophytes against *Leptosphaeria maculans*, the causal agent of phoma stem canker in brassica crops. The highest inhibition rate was recorded for *Stenotrophomonas rhizophila*, *Novosphingobium resinovorum*, *Pseudomonas azotoformans*, *Plantibacter flavus*, *Me. fujisawaense* and *Me. phyllosphaerae* which produced a significant inhibition zone indicating the antagonistic ability of these species. The fungal endophytes also suppressed the growth of the pathogen and created an inhibition zone. *In planta* tests in which the fungal endophytes were inoculated on to seed of a susceptible oilseed rape cultivar were also undertaken. At the cotyledon leaf stage, the leaf was punctured and spore suspension of *L. maculans* was placed on the wound site. Inoculated seedlings particularly *B. bassiana*, significantly decreased phoma stem canker disease symptoms on the cotyledon.

To our knowledge, this is the first study that screen the fungal and bacterial endophytes of wild brassica species associated with the seeds and demonstrate their beneficial characteristic when inoculated to brassica crops.

Key words: Fungal endophyte, Bacterial endophyte, Brassica, Wild brassica species, Seed-associated endophyte, Oilseed rape, Inoculation method, Seed inoculation, Bioactivity, Disease tolerance, Plant growth promotion, *Beauveria bassiana*, *Geomyces pannorum*, *Methylobacterium* sp., Phoma stem canker, *Leptosphaeria maculans.*

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Glossary of abbreviations and symbols

Abbreviations	Full name/meaning
ACC	1-aminocyclopropane-1-carboxylate
ANOVA	analysis of variance
BCA	biological control agent
BLAST	Basic local alignment search tool
cf.	conferatur in Latin, meaning ‘compare’
C-endophyte	Clavicipitaceous endophyte
cfu	colony forming unit
cm	centimetre
cv.	cultivar
©	copyright
EU	European Union
dNTPs	deoxynucleotide triphosphates
DGGE	denaturing gradient gel electrophoresis
rDNA	ribosomal deoxyribonucleic acid
DSV	Deutsche Saatveredelung AG
e.g.	exempli gratia in Latin, meaning ‘for example’
FAR	Foundation for Arable Research

g	gram
GFP	green fluorescent protein
GRIN	Germplasm Resources Information Network
ha	hectare
hr	hour
i.e.	id est in Latin, meaning ‘in other words’
Inc.	incorporated
IPK	Leibniz-Institute of Plant Genetics and Crop Plant Research
ISR	induced systemic resistance
ITS	internal transcribed spacer
L	litre
Ltd.	limited
LSD	least significant difference
min	minute
Mm	millimetre
mM	millimolar
MPI	Ministry for Primary Industries
M&S	Murashige and Skoog

NA	nutrient agar
NCBI	National centre for biotechnology information
NC-endophytes	non-Clavicipitaceous endophyte
NZ	New Zealand
NordGen	The Nordic Genetic Resource Centre
PCR	polymerase chain reaction
PCF	plant frequency
PDA	potato dextrose agar
pH	potential of hydrogen
PGGW	PGG Wrightson Seeds Limited
psi	pounds per square inch
Pty	proprietary limited company
pv.	pathovar
®	registered trade mark
s	second
SAR	systemic acquired resistance
sp.	species
spp.	species (plural)
subsp.	subspecies

TCF	tissue frequency
TM	trade mark
UK	United Kingdom
UN	United Nations
USA	United States of America
USDA	U.S. Department of Agriculture
var.	variant
ver.	version
WA	water agar
µL	microliter
µm	micrometre
µM	micromolar
°C	degrees Celsius
%	percentage
$	dollars (New Zealand, unless otherwise stated)
€	Euro
≤	less than or equal to
≥	greater than or equal to
<	less than
>	greater than

=	equal to
±	plus and minus
&	and

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1. Chapter 1. General introduction

1.1 Introduction

Plant pathogens and insect pests can have a devastating effect on agricultural plant production causing a significant loss to crop productivity and sustainability. Although certain agronomic practices, including crop rotation and resistant cultivars, are used to mitigate the effects of these detrimental organisms, many farmers rely heavily on the application of synthetic chemical pesticides. This reliance on agrochemicals has had an adverse effect on natural fauna and flora neighbouring agricultural systems by killing non-target species. Reliance on agrochemicals can also lead to chemical resistance in pathogen and pest populations, and compromises in the sustainability of crop production (Bass *et al.*, 2015). In addition, there is mounting public concern for both the environment and pesticide residues in human and animal foodstuffs (Bhandari, 2014, Blair *et al.*, 2015). Concerns over the increasing use of chemicals in agricultural systems and the demands for agricultural products with less pesticide residues has increased in the past few decades (Alabouvette *et al.*, 2006). For instance, France has become the first country to ban all five pesticides though to be linked to bee death. The UN announcing that 40% of all invertebrate pollinators are in risk of global extinction (Samuel, 2018). This has encouraged plant researchers to focus on the development of novel, effective tools to manage plant diseases (Lee *et al.*, 2013).

Biological control is one such approach and utilises biological control agents (BCAs), generally living microorganisms, that are able to suppress, deter or kill plant pathogens and/or invertebrate pests (Weber & Gunasekaran, 1995, Lee *et al.*, 2013). Endophytic BCAs are possibly more effective in controlling plant pathogens as they already directly occupy certain niches within the plants environment and do not require an

establishment phase compared with traditional BCAs (Weller, 1988). BCAs generally do not build up a strong association with the plant tissues and need a repetitive application in each growing season.

Some fungi and bacteria able to establish a close association with plants, colonising their internal tissues with no apparent symptoms are termed 'endophytes'. These endophytic organisms are present in a number of plant species bestowing them with many beneficial features including plant growth promotion and insect and disease resistance (Aly *et al.*, 2011). For example, selected animal-friendly strains of the endophytic fungus *Epichloë festucae* var. *lolii*, deter insect pests such as Argentine stem weevil (*Listronotus bonariensis*), from feeding and laying eggs on endophyte-infected perennial ryegrass through the production of secondary metabolites such as peramine and epoxy-janthitrems (Johnson *et al.*, 2013). This genus of endophytic fungi forms host-specific associations with cool-season grasses in subfamily Pooideae by colonising the intercellular spaces of the aboveground host tissues. The asexual species are strictly vertically transmitted via host seed and the endophyte guarantees its transmission to the next generation via this mechanism (Zhang *et al.*, 2017). This novel method of plant protection, using endophytes as BCAs, has generated interest in finding seed-transmitted endophytes in other agricultural crops such as brassica (Card *et al.*, 2015). Associating BCAs with the seeds of many crops eliminate the need for complex formulations and can therefore facilitate its marketing.

Brassica crops, particularly oilseed rape (*Brassica napus* L.), cabbage (*B. oleracea* L.) and turnip (*B. rapa* L.), are extensively cultivated throughout the world. *B. napus* is a major source of edible oils, while *B. oleracea* and *B. rapa* provide leafy vegetables for human consumption and forage (Gómez-Campo, 1980, Rakow, 2004, Dixon, 2007).

These three species were domesticated from wild brassica species originating from Europe and the wider Mediterranean regions (Rakow, 2004). Unfortunately, a wide range of pathogens and pests can significantly damage many brassica crops (Kimber & McGregor, 1995) with few or no control options available (Granér *et al.*, 2003). Phoma stem canker (also termed as blackleg) is one of the most important being a devastating worldwide disease of oilseed rape responsible for huge economic losses in Australia, Canada, Europe and New Zealand (Fitt *et al.*, 2006, Lob *et al.*, 2013). Heavy losses, as much as 95%, have been recorded when fungicide applications are ineffective (Hammoudi *et al.*, 2012).

There are numerous studies documenting the positive traits conferred by bacterial (Wulff *et al.*, 2002, Granér *et al.*, 2003, Xing *et al.*, 2005, Poonguzhali *et al.*, 2006, Lee *et al.*, 2008, Sheng *et al.*, 2008) and fungal endophytes (Raps & Vidal, 1998, Doan *et al.*, 2010, Zhao *et al.*, 2010, Deng *et al.*, 2011, Muhammad *et al.*, 2011) to brassica. Endophytes of brassica, and/or their metabolites, have been demonstrated to improve and promote plant growth; increase yield; reduce disease symptoms caused by plant pathogens; reduce herbivory from insect pests; remove contaminants from soil; improve plant performance under extreme conditions of temperature and water availability; solubilise phosphate and contribute assimilable nitrogen to their hosts (as reviewed by Card *et al.* 2015). Most of these studies have focused on the isolation and culture of endophytes originating from vegetative parts (i.e. leaves, roots and stem) of brassica. Only few studies to date have targeted the reproductive plant tissues (i.e. flowers and seed) or chosen to screen wild brassica accessions with the intention of inoculating selected endophytes into modern brassica cultivars.

Some researchers hypothesise that intense selection pressure during the breeding of new brassica cultivars coupled with the extensive use of chemical pesticides, including fungicides, may have significantly reduced the frequency and diversity of endophytes within brassica (Harvey *et al.*, 1982, Engelhard *et al.*, 2000, Mohandoss & Suryanarayanan, 2009). Seed of wild plant species are viewed as rich sources of endophytic bacteria that may offer advantageous enhancements to their hosts including activity against fungal seed pathogens (Kremer, 1987, Murphy *et al.*, 2015, Abdallah *et al.*, 2016, Yokoya *et al.*, 2017).

1.2 Hypothesis, aims and objectives

In this study we aimed to develop a novel method of isolating bacterial and fungal endophytes associated with the seeds of wild brassica species. We hypothesised that seeds of wild brassica species harbour endophytic microorganisms that could confer some beneficial traits such as plant growth promotion and disease resistance to cultivated brassicas. This research therefore utilised seed accessions of wild brassica species stored in international germplasm centres collected from various regions of the world as well as species growing in New Zealand to be screened for fungal and bacterial endophytes with potential benefit to elite cultivar of brassica and potential vertical transmission; transmission from plants to seeds. Therefore, the objectives were to:

1) Develop an understanding of biological control with microbial endophytes of brassica by undertaking an in-depth literature review (Chapter 1 and 2).

2) Development of an endophyte discovery pipeline for screening the seed of wild brassica accessions, plus the isolation and culture of axenic strains to store in a culture collection (Chapter 3).
3) Species identity of selected bacterial and fungal isolates using morphological characteristics and/or DNA sequencing (Chapter 3).
4) Artificial inoculation of elite brassica cultivars with selected endophyte isolates (Chapter 4).
5) Assessment of the bioactivity of selected fungal endophytes against phoma stem canker *in planta* (Chapter 5).
6) Assess the plant growth promotional traits of selected bacterial endophytes *in planta* (Chapter 5).

2. Chapter 2. Literature review

2.1 Introduction

This chapter reviews the economic importance of brassica to New Zealand and its production on a world scale. The chapter lists the most important domesticated species and provides information regarding the critically important pests and pathogens that effect production of brassica crops. The chapter concludes with information regarding pest and disease control options, including chemical, cultural and biological strategies, with particular emphasis on options for control of phoma stem canker in oilseed rape caused by *Leptosphaeria maculans* with the use of selected endophytic bacteria and fungi.

2.1.1 Importance and world production

Crop brassicas are the most economically important members of the *Brassicaceae* family (also known as the mustard or crucifer family) and includes many of the earliest domesticated plants (Kumar *et al.*, 2015). Domesticated species such as *Brassica napus* and *B. oleracea* are a major source of vegetable oils and leafy vegetables and both are widely cultivated across the world (Warwick *et al.*, 2009). Most brassica plant parts, including the roots, tubers, stems, buds, flowers and seeds, are edible (as forage and food for human consumption) and members of the family are also sources of condiments, lubricants, biofuels, industrial oils, soil conditioners, medicines, green manures and composting crops (Gómez-Campo, 1980, Rakow, 2004, Warwick *et al.*, 2009, Card *et al.*, 2015).

Brassica oil crops, such as oilseed rape (*B. napus*), are globally in high demand as these vegetable oils are deemed healthy due to their unsaturated compounds that are of

high nutritional value. The oil is also utilised as a biofuel in the transport industry and is a preferred substitute for many fossil fuels (Gunstone, 2004). *B. napus* is the third most important source of vegetable oil in the world (by the volume produced) followed by soybean and palm oils, and the world`s second leading source for protein meal (Lob, 2014, Carruthers *et al.*, 2017). Large crops of *B. napus* are being planted increasingly worldwide in countries such as Australia, Canada, China, the United Kingdom (UK) and the United States of America (USA) (Lob, 2014). According to FAO statistics (FAO Stat, 2014), the globally harvested area of oilseed rape increased from 26 million to more than 36 million ha between the years 2000 and 2014, an increase of 72 %.

Vegetable brassicas, those used for fodder and human consumption, can be divided into six main groups according to morphology: cabbage (i.e. headed cabbage, Brussel sprouts and savoy cabbage), kale (i.e. green kale and stem kale), kohlrabi, inflorescence kale (i.e. cauliflower, broccoli and sprouting broccoli), branching-bush kale and Chinese kale (Rimmer *et al.*, 2007).

2.1.2 Brassica industry in New Zealand

Brassica crops are important to the New Zealand (NZ) economy and can be grouped according to four main industries, 1) crops for forage, 2) vegetables for human consumption, 3) vegetable seed and 4) oil for human consumption and biofuels.

Annual cultivation of forage brassicas in NZ is around 300,000 ha, mostly in the Canterbury region, on the east coast of the South Island (Walker, 2009, Walker *et al.*, 2011). About 30-40% of the cultivated area supplies forage brassica to the dairy industry while the remaining 60-70% is used as forage for sheep, beef and deer production (Dumbleton *et al.*, 2012). Swede, kale, turnips and leaf turnips are the

important brassica forage crops in NZ as they provide high quality feed especially during the winter months (Lob, 2014).

The three main vegetable brassica crops grown in NZ are broccoli (*B. oleracea* var. *italica*), cabbage (*B. oleracea* var. *capitata*) and cauliflower (*B. oleracea* var. *botrytis*) (2018). Almost all production is consumed domestically and is grown in the Auckland, Canterbury, Manawatu and Wanganui regions on a combined total of 3,300 ha (Lob, 2014). Over 92,000 tonnes of vegetable brassicas were produced in 2015 in NZ with domestic and export sales valued at $80 million and $2.7 million, respectively (Lob, 2014). In 2011 the NZ horticultural industries provided around $220 million research investment into these three brassica crops (Ghazalibiglar, 2014).

The production of vegetable seeds in NZ started over a century ago and Canterbury has been recognised as the major seed producing region. The production of vegetable seeds has increased from a combined weight of 4.8 million kg in 2010 to 9.2 million kg in 2014 (FAR, 2018). NZ is currently one of the world's most important producers of brassica seed, which is recognised worldwide for its quality (Anonymous, 2018). Brassica seed is a key export seed crop for the country with an average production of 5500 tonnes annually (Lob, 2014); 4100 tonnes forage brassica seeds (FAR, 2018). Two thirds of the brassica seed production is exported to overseas; to the USA, the UK, Asia, Europe and Australia, providing NZ$13 million in trade value (Lob, 2014). According to the Ministry for Primary Industries (MPI), there is a bright future for the NZ seed industry with seed export revenues expecting to reach $240 million by 2022 (2018).

2.1.3 Brassica species

The Brassicaceae family represents 51 genera in which *Brassica* is the most

economically important, comprising 37 plant species. Brassica species originated from the Mediterranean regions of both Europe and North Africa and central Asia. Many species are now cultivated worldwide (Figure 2.1) (Dixon, 2007) while some are considered to be invasive weeds in Australasia and the Americas (Card et al., 2015). The majority of species are annuals or biennials ranging from weedy (wild) plants to domesticated crops. Amongst these species, *B. napus* (oilseed rape), *B. rapa* (turnip rape) and *B. oleracea* (kale and cabbages) are the most widely planted species with a worldwide distribution (Raymer, 2002, Rakow, 2004). *B. rapa* and *B. oleracea* are interesting species as they incorporate a multitude of cultivated subspecies with diverse morphology (Rakow, 2004).

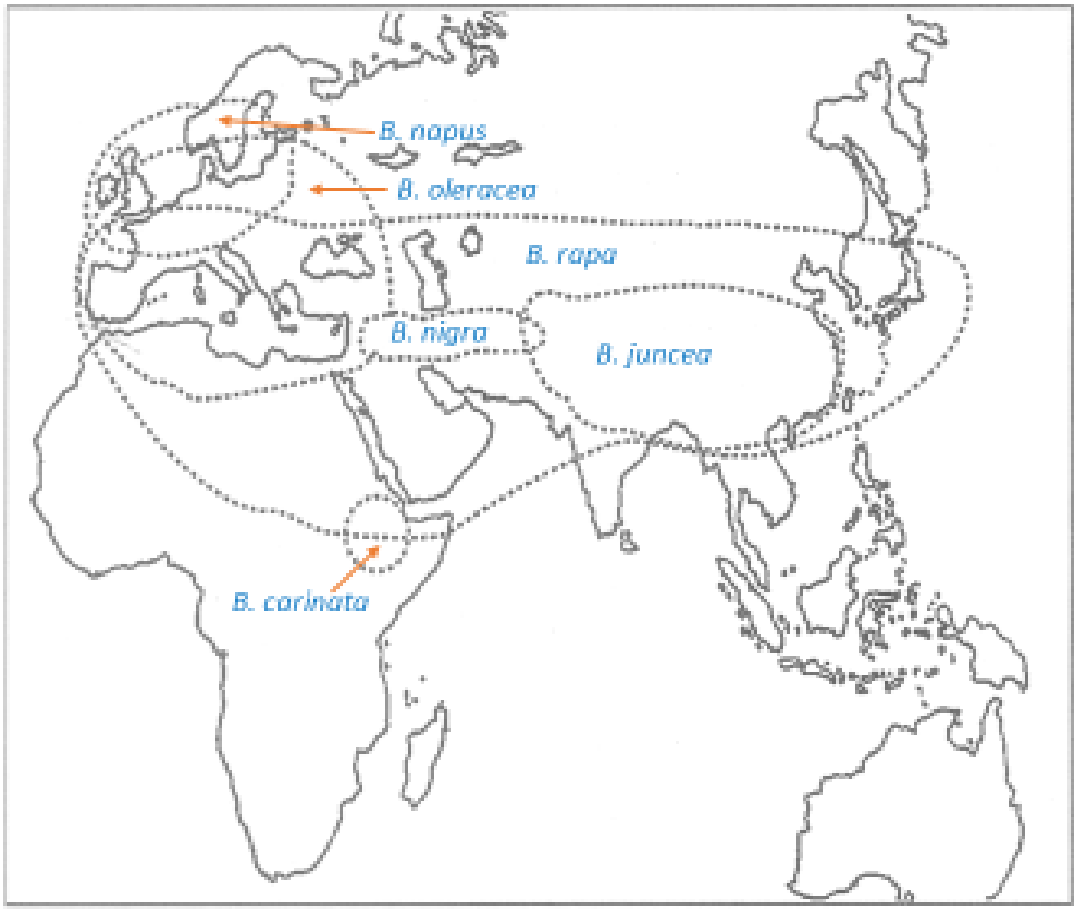

Figure 2. 1 Biogeography of the origins and diversity of the major crop-founding Brassica species (United Nations Food and Agriculture Organisation, Rome) (Nagaharu, 1935, Dixon, 2007).

The cytogenetic study of brassica species revealed that these species are closely related; *B. napus*, *B. juncea* (Indian mustard) and *B. carinata* (Ethiopian mustard) are amphidiploid plants created from inter-crosses between *B. campestris* (*B. rapa*) × *B. oleracea*, *B. campestris* × *B. nigra* (black mustard) and between *B. oleracea* × *B. nigra*, respectively (Figure 2.2) (Nagaharu, 1935, Katam, 2011). The taxonomy and usage of these economically important brassica species is shown in Table 2.1.

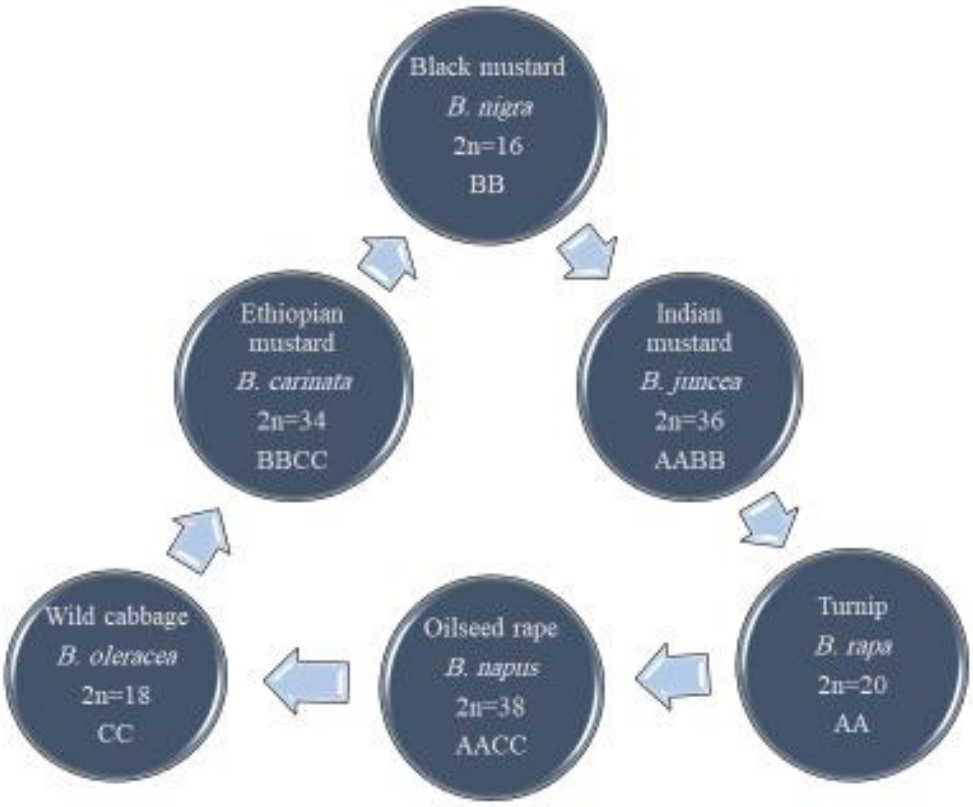

Figure 2. 2 The triangle showing the relationship among different brassica species (Nagaharu, 1935).

Table 2. 1 Taxonomy of crop brassica species (Kumar et al., 2015).

Botanical name	Common name	Usage
B. nigra	Black mustard	Condiment (seed)
B. oleracea		Vegetable fodder (leaves)
var. *acephala*	Kale	Vegetable (head)
var. *capitata*	Cabbage	Vegetable (terminal buds)
var. *sabauda*	Savoy cabbage	Vegetable (head)
var. *gemmifera*	Brussels sprouts	Vegetable, fodder (stem)
var. *botrytis*	Cauliflower	Vegetable (inflorescence)
var. *gongylodes*	Kohlrabi	Vegetable, fodder (stem)
var. *italic*	Broccoli	Vegetable (inflorescence)
var. *fruticose*	Branching bush kale	Fodder (leaves)
var. *alboglabra*	Chinese kale	Vegetable (stem, leaves)
B. rapa		
subsp. *Oleifera*	Turnip rape	Oilseed
var. Brown Sarson	Brown sarson	Oilseed
var. Yellow Sarson	Yellow sarson	Oilseed
var. Toria	Toria	Oilseed
subsp. *Rapifera*	Turnip	Fodder, vegetable (root)
subsp. *Chinensis*	Bok choi	Vegetable (leaves)
subsp. *Pekinensis*	Chinese cabbage	Vegetable, fodder (head)
subsp. *Nipposinica*	-	Vegetable (leaves)
subsp. *Pamchinensis*	-	Vegetable (leaves)
B. carinata	Ethiopian mustard	Vegetable oilseed
B. juncea	Mustard	Oilseed, vegetable
B. napus		
subsp. *Oleifera*	Rapeseed/oilseed rape	Oilseed
subsp. *Rapifera*	Rutabaga, swede	Fodder

Many cultivated vegetable brassica species are direct descendants of wild brassica species (Figure 2.3) and many of these wild species are now being recognised as new sources of industrial oils, soil fumigants and human and animal food crops. Certain wild relatives of brassica may also carry potentially useful agronomic traits that could be used in breeding programs for the development of new cultivars resistant to fungal and/or bacterial plant diseases, insect pests and/or nematodes. Other wild brassica species are being investigated with respect to their male sterility traits, intermediate C3-C4 photosynthetic properties and their anti-carcinogenic activity (Warwick *et al.*, 2009). Many Brassicaceae species also associate with many beneficial microbes

including a plethora of potentially beneficial endophytes that could be exploited in the agricultural and horticultural industries, as reviewed by Card *et al.* (2015).

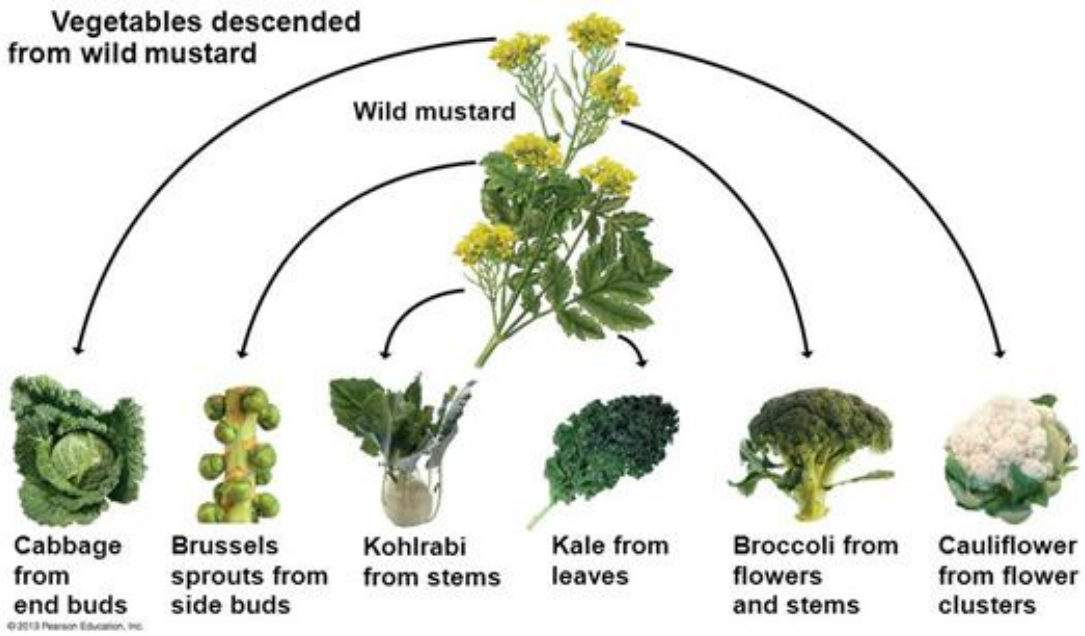

Figure 2. 3 Different brassica vegetables developed from wild mustard (anonymous- picture retrieved form image google search).

2.1.4 Economically important pests and diseases of brassica

All the economically important species of brassica, including oil or leafy types, are vulnerable to attack from a wide range of insect pests. These include the cabbage aphid (*Brevicoryne brassica*), green peach aphid (*Myzus persicae*), diamond back moth (*Plutella xylostella*), flea beetle (*Phyllotreta striolata*), cabbage root fly (*Delia radicum*), cabbage seed weevil (*Ceutorhynchus assimilis*) and pollen beetle (*Meligenthes aeneus*) (Lamb, 1989, Kimber & McGregor, 1995, Dixelius *et al.*, 2004, Williams, 2010). These insects cause huge damage to crop production with increased production costs. For example, a study showed that the estimated worldwide annual

cost of diamond back moth control is between US$ 4-5 billion (Zalucki *et al.*, 2012) with brassica crops grown in temperature zones in NZ and India also particularly vulnerable to this pest (Kimber & McGregor, 1995).

More than 20 economically important fungal, viral and bacterial diseases are recognised in NZ brassica crops (Rimmer *et al.*, 2007). These cause a combined damage of NZ$10 million annually (Ghazalibiglar, 2014). The most important include phoma stem canker caused by *Leptosphaeria maculans* or *L. biglobosa,* Sclerotinia stem rot caused by *Sclerotinia sclerotiorum*, clubroot caused by *Plasmodiophora brassica* and black rot, caused by the seed-borne bacterium *Xanthomonas campestris* pv. *campestris* (Kimber & McGregor, 1995, Dixelius *et al.*, 2004, Dixon, 2009, Ghazalibiglar *et al.*, 2016).

2.2 Phoma stem canker

2.2.1 Importance

Phoma stem canker (or blackleg) is a devastating worldwide disease of all brassica species. It causes serious yield losses in many brassica species and is a major constraint to the production of oilseed rape in Australia, Europe and North America (West *et al.*, 2001, Fitt *et al.*, 2006). The disease is caused by two closely related fungal species, *L. maculans* and *L. biglobosa*. A wide range of brassica species, including *B. juncea*, *B. napus*, *B. nigra*, *B. rapa* and *B. oleracea* and a number of cultivars, can be infected by both species of *Leptosphaeria* (Fitt *et al.*, 2006, Fitt *et al.*, 2006). Some wild brassica, described as weeds, such as wild turnip, shepherds purse and hedge mustard are also reported to be susceptible to these pathogenic fungi (McKenzie & Dingley, 1996). Both species are able to transmit via host seed including within *B. oleracea*, *B. napus* and *B. rapa*, and can therefore be accidentally exported to

many countries via seed. Although both pathogens are responsible for the disease in oilseed rape, *L. maculans* is the primary causal agent of phoma stem canker. *L. maculans* is also associated with a damaging basal stem canker, which can also result in severe yield losses (West *et al.*, 2001). The first record of *L. maculans* in NZ was reported in 1927 (Cunningham, 1927) where the author recovered isolates of *L. maculans* from swede and turnip.

Annual yield losses due to phoma stem canker are estimated at €30M in Australia, €147M in France and €65M in the U.K. (Fitt *et al.*, 2006) with up to 10-20% of the oil seed rape crop lost depending on weather, region and cultivar (Hwang *et al.*, 2016). Phoma stem canker is also responsible for up to a 70% yield loss of cauliflower and broccoli in central Mexico (Moreno-Rico *et al.*, 2001). In NZ both forage brassica and oilseed rape crops can be severely affected by the disease with estimates of 50% of oilseed rape paddocks infected (Lob *et al.*, 2013). Since *L. maculans* is one of the most economically important pathogens of oilseed rape (*B. napus*) the following review focuses on this species.

2.2.2 Disease symptoms

Leptosphaeria species infect different varieties (winter and spring) of oilseed rape and causes cankers that ultimately result in host death. However, the extent of the symptoms varies from one host to another (West *et al.*, 2001). In NZ, forage brassica crops, such as swede and turnip, are also frequently infected causing dry rot disease (Lob, 2014). Symptoms on oilseed rape are large pale lesions with abundant pycnidia present on leaves (Figure 2.4 A), which later lead to basal stem cankers as the disease progresses (Figure 2.4 B) (West *et al.*, 2001). The pathogen is able to produce similar leaf lesions on swede as observed on oilseed rape and dry rot lesions on bulbs (Lob,

2014).

Figure 2. 4 Symptoms of *L. maculans* infection on leaves of oilseed rape (A) and basal stem canker (B) (Lob, 2014).

L. maculans can remain inside the infected stubble of a previous crop and release large amounts of ascospores through sexual reproduction. These propagules can spread widely by means of wind and water splash and infect cotyledons and young leaves of oilseed rape. These spores germinate on leaves, penetrate through openings including stomata and wound sites and after infection lead to initial pale grey spots that turn pale brown with maturity indicating the presence of pycnidia. These spots can reach 1-2 cm in diameter and eventually the lesion centres break apart and create holes in the leaves (Brun *et al.*, 1997, West *et al.*, 2001). Hyphal penetration continues through the leaf laminar to the petiole and along the stem where basal stem cankers are then formed. These cankers are light brown in colour with distinct dark margins that lead to stem weakness, plant lodging and early senescence resulting in dramatic yield losses (West *et al.*, 2001, West *et al.*, 2002, Fitt *et al.*, 2006).

Oilseed rape may be infected by the pathogen at any stage of crop development. Hypocotyl infection causes constriction of the stem growing above the ground and below the first leaves. Hypocotyl lesions can lead to severe seedling blight and a decline in crop establishment. Infection of older plants generally happens at the base of stem or leaf attachment causing stem girdling and ripening of premature plants

(Hwang *et al.*, 2016).

2.2.3 Life cycle

Phoma stem canker is usually considered a monocyclic disease; having one infection cycle per season (West *et al.*, 2001). *L. maculans* survives and develops as a saprophyte on oilseed rape stubble left after harvest and produces fruiting bodies (pseudothecia) containing ascospores which serve as the primary source of inoculum (West *et al.*, 2001, Hwang *et al.*, 2016). The fruiting bodies of *L. maculans* are mainly produced on stubble of Brassicaceae plants and are able to survive more than five years with activity highest in the first three (Kaczmarek & Jedryczka, 2011). The pathogen reproduces sexually and creates many genetically diverse ascospores within the population that can be virulent to new plant varieties (Dilmaghani *et al.*, 2013).

Disease epidemics in oilseed rape are usually initiated in late autumn/winter, following rain or dew which is a requirement for ascospore release (West *et al.*, 2001). The timing of this event varies from region to region but usually coincides with the presence of young seedlings and susceptible plants. In Australia, ascospores are generally common in late autumn and early winter (April-June) after the commencement of rainfall with the disease cycle highly correlated to the susceptible stage of the plant life cycle (McGee & Emmett, 1977). The same pattern is observed in NZ (Lob *et al.*, 2013). After their release, ascospores are able to survive under dry conditions at 5-20°C for as long as 30 days. Ascospores are able to infect surrounding plants within a 500 meters radius of the ejection site but can also be disseminated by air currents as far as 5 km away. Once the ascospores have landed on a suitable host (e.g. cotyledons and young leaves of oilseed rape) they are able to germinate across a wide temperature range of between 5-20°C (Kaczmarek & Jedryczka, 2011). Initial

plant infection results in small spots on the host leaves that become paler forming larger lesions containing pycnidia (Kaczmarek & Jedryczka, 2011). These pycnidia produce abundant conidia, pycnidiospores, on leaf lesions, which in turn can be transmitted by rain splash. These create secondary sources of inoculum for spreading of the disease on the same plant or others in the same vicinity (Figure 2.5) (Kaczmarek & Jedryczka, 2011, Lob, 2014).

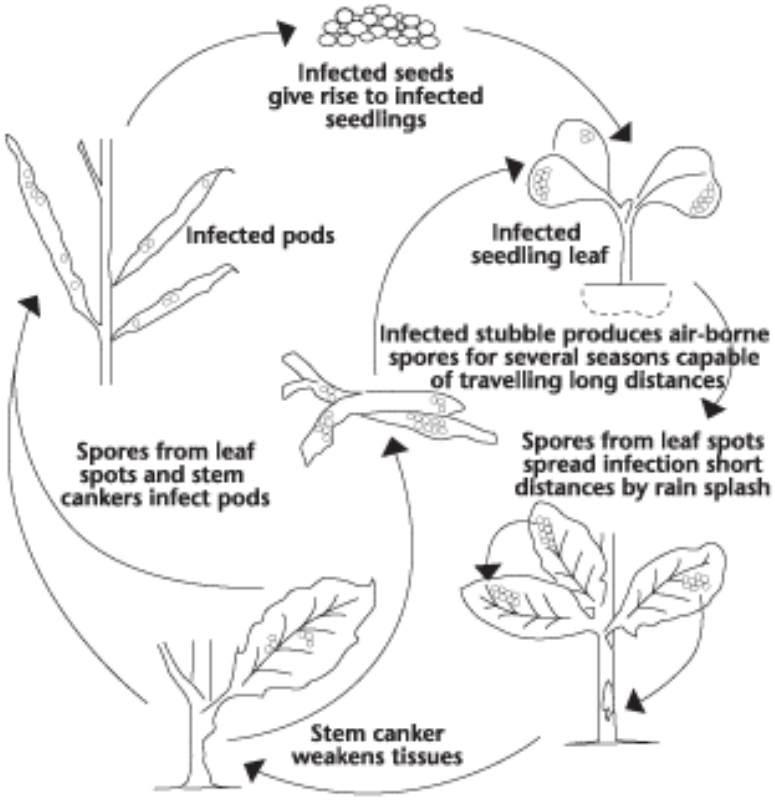

Figure 2. 5 Life cycle disease of phoma stem canker on brassica. (*http://www.canolacouncil.org*).

2.2.4 Management of the disease

Breeding for resistant cultivars is very important for sustainable management of phoma stem canker. Due to the significance of phoma stem canker, in countries with serious epidemics, a minimum resistance standard is now required for the registration of new

B. napus cultivars. This has been an exceptionally effective means of disease management in countries such as Australia, where disease-resistant varieties have saved the oilseed rape industry (West *et al.*, 2001). Other effective cultural control management strategies, such as crop rotation and stubble management, are also implemented to minimise disease infection and postpone any breakdown in plant resistance while fungicide sprays are still routinely used in locations suffering from high infection loads (Salisbury *et al.*, 1995, West *et al.*, 2001). The chemical control of the disease can be made through several ways including seed treatments (Marcroft & Potter, 2008) and/or fungicide sprays on foliage (West *et al.*, 2001). In seed treatments carbathin, thiram and iprodione can be used (West et al., 2001) whilst propiconazole, difenoconazole, carbendazim, or flusilazole alone or in mixtures can be applied as a foliar spray (Gladders *et al.*, 1998, Kharbanda *et al.*, 1999).

2.2.5 Biological control

Biological control of plant diseases can be defined as the use of antagonistic microorganisms, biological control agents (BCAs), or their extracted products, to halt or suppress disease caused by pathogenic microorganisms. This strategy of disease management is considered an environmentally friendly alternative to chemical control if managed properly (Pal & Gardener, 2006). The restriction and/or banning of some pesticides, particularly those leaving a deleterious impact on human health, has created a resurgence in research into this area. Additionally, many BCAs do not suffer from resistance issues as with their chemical counterparts and with a growing public sensitivity on the direct use of synthetic chemicals on foodstuffs and their negative impacts on the environment this has led to a demand from consumers for food products to be free from pesticide residues (Cholerton, 2015). For example, the European Union

(EU) has banned a group of pesticides known as neonicotinoids. These chemicals have been shown to be responsible for negatively effecting the population of many beneficial, non-target insects including honeybees (Samuel, 2018).

There are reports indicating that certain fungal and bacterial BCAs are able to control phoma stem canker in oilseed rape (Kharbanda *et al.*, 1999, Beatty & Jensen, 2002, Danielsson *et al.*, 2007, Abuamsha *et al.*, 2011, Hammoudi *et al.*, 2012, Cholerton, 2015). Hammoudi *et. al* (2012) reported that strains of the bacteria *Serratia plymuthica*, *Pseudomonas chlororaphis*, *P. fluorescens*, and a strain of the fungus *Gliocladium catenulatum* were able to reduce the symptoms of phoma stem canker on leaves of oilseed rape. The reduction of root collar and stem base infestation was more effective when plants were inoculated by *S. plymuthica* and *G. catenulatum* compared to the other species. However, it was noted that the efficacy of these antagonists is highly dependent on their concentration within the seed. Some strains of *Trichoderma* spp. and *Bacillus subtilis* have also been shown to reduce symptoms of the disease when applied to seeds of oilseed rape under glass house conditions (Panjehkeh *et al.*, 2011).

BCAs can employ a variety of antagonistic mechanisms, these include competition for space and nutrients, antibiosis, direct parasitism and induced resistance, although other less known mechanisms also exist such as the modification of leaf wetness (Elad & Chet, 1987, Edwards & Seddon, 1992, Elad, 1996, De Meyer *et al.*, 1998, Meller Harel *et al.*, 2014, Nicot *et al.*, 2016). For competition (an indirect interaction between the antagonist and the target), the BCA can compete for space and/or nutrients when there are insufficient resources for the populations of microorganisms present to multiply, grow and survive (Pal & Gardener, 2006). It is suggested that suppression of *Pythium*

aphanidermatum by *Pseudomonas putida* and *P. cepacia* is due to the competition for nutrients (Elad & Chet, 1987). Antibiosis is the production of toxic secondary metabolites (e.g. antibiotics, enzymes or other lytic agents) produced to kill or supress the growth of the target microbe (Pal & Gardener, 2006). Several biocontrol strains are recognised as producing multiple antibiotics which can suppress one or more pathogens. For example, *Bacillus cereus* strain UW85 is known to produce both zwittermycin (Silo-Suh *et al.*, 1998) and kanosamine (Milner *et al.*, 1996). Parasitism, as observed with many *Trichoderma* spp., is when the BCA directly attacks the pathogen and utilise their nutrients for its metabolic use (Fravel, 1988, Shalini & Kotasthane, 2007, Card *et al.*, 2009). Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are a defensive response from the plant towards a specific pest or pathogen that is initiated by the inoculation of a BCA. The BCA works as an elicitor of the plant's own biochemical defence pathway and restricts pathogen/pest development within the plant (Gerhardson, 2002, Pal & Gardener, 2006). The bacterium *Serratia plymuthica* induces resistance in oilseed rape towards *L. maculans* (Abuamsha *et al.*, 2011). Induction of plant resistance by strains of *Beauveria bassiana* in cauliflower has also been reported (Gautam *et al.*, 2016).

2.3 Endophytes

2.3.1 Definition

Endophytic microorganisms are part of the complex microbial community that associate closely with vascular plants. These organisms exist inside their hosts tissues without causing any symptoms forming a symbiosis (Porras-Alfaro & Bayman, 2011, Card *et al.*, 2016). The term endophyte was first coined by Anton DeBary in 1866 (DeBary, 1879). He used the terms 'endophytes' and 'epiphytes' to describe those

microorganisms living inside plant tissue, and for those living on the outer layer of their host plants, respectively (Azevedo, 1998). The term endophyte, comes from the Greek words, 'endon' meaning inner and 'phyton' meaning plant. The term includes archaea, bacteria, fungi and viruses that live for the whole or part of their lifecycle within the tissues of healthy plants. Microorganisms simply isolated from plant tissues following surface-disinfection should not be termed endophytes and instead should be termed plant-associated microorganisms until they are proven to be endophytic via microscopy (Card *et al.*, 2016).

2.3.2 Modes of endophyte transmission

Endophytic microorganisms are able to transmit to the next generation of plants in two different ways; 1) vertically via host seed and 2) horizontally via propagules such as spores (Figure 2.6) (Rodriguez *et al.*, 2009, Aly *et al.*, 2011). Both modes are displayed by *Epichloë* spp., fungal endophytes of cool season grasses. The asexual species used in agriculture, such as *E. festucae* var. *lolii*, are strictly vertically transmitted after the fungi colonise reproductive tillers, ovules and subsequent embryo tissues (Zhang *et al.*, 2017). Sexual species such as *E. typhina*, which causes a destructive disease of orchard grass (*Dactylis glomerata*) known as choke, can also transmit horizontally via airborne ascospores (Leyronas & Raynal, 2008).

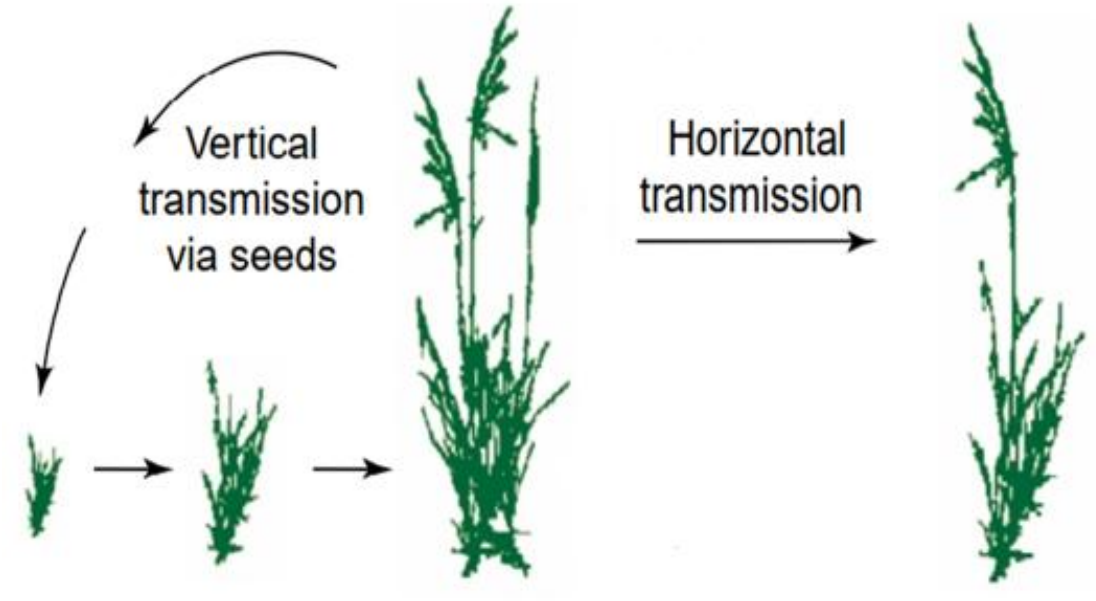

Figure 2. 6 Vertical and horizontal transmission lifecycles of Epichloë fungal endophytes (adopted from Saikkonen et al., 2004).

Unlike vertical transmission, dissemination by horizontal mechanisms do not involve the plants reproductive strategies and instead infect the plant's vegetative tissues after their propagules are spread by wind, rain splash or insect vectors. Once these propagules have located a suitable plant tissue, access to the interior plant cells must be accomplished through natural openings (such as stomata) or wound sites (Salama & Mishricky, 1973).

2.3.3 Plant by endophytes

It is reported that each individual belonging to the of 300,000 plant species that exist on the earth associate with one or more endophytic organisms (Dutta *et al.*, 2014). These endophytes are able to live in nearly all plant tissues including seed, shoots and roots with roots generally possessing higher populations than other types of tissue (Hallmann *et al.*, 1997, Rosenblueth & Martínez-Romero, 2006). Some endophytes are able to penetrate plant tissues from the external environment into the above ground

tissues or into the roots from the rhizosphere or through host seed (Hallmann *et al.*, 1997). Once these microorganisms establish in plant tissues they can remain localised in a particular tissue type or systemically colonise the plant.

Many bacterial endophytes originate from larger rhizosphere communities (Poonguzhali *et al.*, 2006, Rosenblueth & Martínez-Romero, 2006). These are generally termed facultative endophytes as they can move between the soil environment and that of the plant. Germida *et al.* (1998) found that the genetic diversity of bacteria living as endophytes in canola and wheat was less than that of the root associated bacteria. The genera of endophytes were also present in the rhizosphere indicating that the bacterial endophytes of these crops were a subset of the rhizoplane community.

It has been suggested that particular beneficial soil microorganisms are attracted to the roots of certain plant species by specific plant-produced compounds and these bacteria are then allowed access to the internal root tissues of the host in order to benefit the plant microorganisms (Rosenblueth & Martínez-Romero, 2006). For example, certain flavonoids stimulate the of wheat and oilseed rape roots by the beneficial diazotrophic bacterium *Azorhizobium caulinodans* (Webster *et al.*, 1998, O'Callaghan *et al.*, 2000). These bacteria then usually colonise root tissue through cracks formed at the emergence of lateral roots or at the zone of root elongation and differentiation (Dong *et al.*, 2003).

Seed transmitted fungal endophytes such as many *Epichloë* spp. systemically colonise the above ground tissues of their cool-season grass hosts, before infecting flower tissues and then establish themselves within the embryo following plant fertilisation (Zhang *et al.*, 2017). The association between these fungal endophytes and their host

plants is generally mutualistic and has taken place during coevolution of these species providing many beneficial traits to the host plant such as protection against insect pests (Van Heeswijck & McDonald, 1992, Johnson *et al.*, 2013, Card *et al.*, 2016, Lugtenberg *et al.*, 2016, Saikkonen *et al.*, 2016). Rodriguez, et al (2009) has tried to categorise fungal endophytes according to their dispersal mechanism (vertical vs horizontal transmission) and the plant tissue types they colonise. Under this classification system, *Epichloë* spp. are known as Clavicipitaceous or C-endophytes (Table 2.2).

Unlike C-endophytes, non-Clavicipitaceous or NC-endophytes are non-host specific and can be isolated from almost all terrestrial plants and can be classified into three classes based on their host patterns, mode of transmission, their level of species diversity *in planta* and their ecological significance to the environment. Among the NC-endophytes, a particular sub-category known as Class 2 endophytes contains species that are able to transmit vertically after infection of the host via the seed coat (Redman *et al.*, 2002) and/or the rhizome. These Class 2 endophytes can also transmit horizontally, may colonise above and below ground plant host tissues and are able to confer habitat-specific stress tolerance including pH, temperature and salinity. Other sub-categories of endophytes, as classified by Rodriguez et al (2009), namely Class 3 and 4 species, are limited to host shoot and root tissues, respectively, and can only transmit horizontally (Rodriguez *et al.*, 2008, Rodriguez *et al.*, 2009) (Table 2.2). These latter two classes of fungi are capable of colonising their plant hosts extensively, across many tissue types, have low population abundance in the rhizosphere, highly colonise plant hosts occurring in high-stress habitats and are comprised of a diverse species from the Ascomycota and Basidiomycota phyla (Rodriguez *et al.*, 2008, Rodriguez *et al.*, 2009).

Table 2. 2 Criteria used to characterise fungal endophyte classes according to Rodriguez, et al (2009).

	Clavicipitaceous	**non-Clavicipitaceous**		
Criteria	**Class 1**	**Class 2**	**Class 3**	**Class 4**
Host range	Narrow	Broad	Broad	Broad
Host tissue(s) colonised	Shoot & rhizome	Shoot, root & rhizome	Shoot	Root
Type of *in planta*	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical & horizontal	Vertical & horizon	Horizontal	Horizontal
Fitness benefits*	NHA	NHA&HA	NHA	NHA

*Non-habitat-adapted (NHA) benefits include drought tolerance and plant growth promotion are shared among endophyte species regardless of their habitat origin. Habitat-adapted (HA) benefits come from habitat-specific selective pressures such as soil pH, temperature and salinity.

C-endophytes have not been found associated with brassica but many from other class 2, 3 and 4 such as *Trichoderma* sp. (Jiang *et al.*, 2008), *Mucor sp.* (Deng *et al.*, 2011), *Chaetomium globosum* (Morita *et al.*, 2003, Zhang *et al.*, 2014) and *Heteroconium chaetospira* (Hashiba *et al.*, 2003, Morita *et al.*, 2003) have been found associated with this plant genus.

The most recent list of fungal and bacterial endophytes associated with brassica can be found in a review paper published by Card *et al.* (2015) and in Appendix 1.

2.3.4 Seed transmitted/associated endophytes

Seed is crucial for the continuation of many plant populations and serves as the starting point for many agricultural crops. In natural ecosystems, seed also plays an important role in plant dispersal and facilitates the evolution of adaption and persistence traits (Nelson, 2017). Seedlings that emerge during the establishment phase are generally vulnerable to environmental stresses such as drought, extreme temperature, resource limitation and high level of heavy metals and biotic stresses such as insect pests, seed-borne and soil borne pathogens (Leck *et al.*, 2008, Bever *et al.*, 2015). Endophytic microorganisms associated with the seed may offer advantageous traits to counter the challenges facing seed survival and seedling establishment. Therefore, understanding the ecological role of seed-associated microbes and their impact and interaction on plant development during seed to seedling transition is critical (Nelson, 2017, Ruppert *et al.*, 2017). Indication of bacterial and fungal seed associated microorganisms with many plant species has been reviewed in several publications (Rodriguez *et al.*, 2009, Porras-Alfaro & Bayman, 2011, Malfanova *et al.*, 2013, Truyens *et al.*, 2015).

Brassicaceae plants have been reported to be colonised by diverse endophyte species

belonging to phyla of Ascomycota, Basidiomycota and Zygomycota (Card *et al.*, 2015). Many of the genera within the fungal class of ascomycetes such as *Chaetomium globosum* and *Heteroconium chaetospira* are commonly present in soils (Card *et al.*, 2015) where they may transmit horizontally to the seeds before germination and constitute part of the seed microbial community (Nelson, 2017). Although the infected seed zone is not clear in many cases, they may largely colonise the seed coat or reside on the seed surface (Rodriguez *et al.*, 2009). Recent studies indicate that local plant habitat (i.e. plant growing environment) is more strongly correlated to the assembly of fungal seed microorganisms than host genotype (Klaedtke *et al.*, 2016).

Knowledge on seed microbiomes originates from culture based screening studies of microbes that allowed the creation of axenic culture of microbial isolates grown on artificial media (Truyens *et al.*, 2015). However, new advances in DNA sequencing and microscopy techniques have allowed greater elucidation of the seed microbiome, additionally enabling those microbes that cannot be cultured to be elucidated as well (Nelson, 2017). Truyens *et al.* (2015) summarised a list of bacterial endophyte species associated with the seed of 25 plant species including 131 bacterial genera, representing four different fungal phyla. In this list Proteobacteria, especially species of ϒ-Proteobacteria were found to dominate while species of Actinobacteria, Firmicutes and Bacteroidetes were less common.

Bacterial species colonising plant seeds are selected as different communities are found in different plant species, different genotypes of specific plant species, different stages of seed development and in different geographical locations (Nelson, 2017). There is a distinct observational difference between the microbiota associated with the soil and endophytic microbiota in the plants growing in this soil, indicating the plants

largely recruit seed-associated microbes from their mother plant (Van Overbeek *et al.*, 2011). Bacterial species that are conserved in some plant species and provide a species pool that the seedling microbial can recruit in times of stress have also been reported (Johnston-Monje & Raizada, 2011, Links *et al.*, 2014).

Bacterial communities of seed change during seed development. Seed maturation which is accompanied by starch accumulation and water loss favours endophytes able to tolerate environments with high osmotic pressure (Elbeltagy *et al.*, 2000, Mano *et al.*, 2006). Endospore formation can be an important property of bacterial seed associated endophytes as these propagules protect the bacteria from the extreme changes occurring in the seed (Mano *et al.*, 2006, Compant *et al.*, 2011). Possession of amylase enzymes is also a frequent feature of bacterial seed-associated endophytes as this characteristic enables them to utilize starch and resume growth after long-term survival in the seed (Mano *et al.*, 2006). Other properties conferred to the plant host by seed associated bacteria include phosphorous solubilising, acetoin secretion, nitrogen fixation and ACC deaminase production (Johnston-Monje & Raizada, 2011).

It has been reported that seed associated endophyte populations can be varied in size ranging from 55 colony forming units (cfu) in one gram of beans (Rosenblueth *et al.*, 2010) to as high as 10^7 in one gram of oilseed rape (Granér *et al.*, 2003). In general, it is reported that *Bacillus* and *Pseudomonas* are the most commonly identified bacterial genera found in seed of many plant species with *Acinetobacter*, *Micrococcus*, *Paenibacillus*, *Pantoea* and *Staphylococcus* also common residents (Truyens *et al.*, 2015).

There are several reports indicating a wide range of bacterial endophytes from gram-positive and gram-negative groups associated with a many brassica species including

cultivated brassica species of *B. napus, B, rapa* and *B. oleracea* (reviewed by Card *et al.*, 2015). The gram-positive bacteria include a range of bacteria from the group of Actinobacteria such as Streptomyces and Actinobacteria and a group of Fermicutes such as *Bacillus* species while the gram-negative bacteria are mostly dominated by Proteobacteria groups such as *Pseudomonas* and *Enterobacter* species.

2.3.5 Benefits of endophytes to the host plant

Although endophytes were first described in the early 19th century, this group of microorganisms did not gain much attention until their agricultural, pharmaceutical and ecological significance was established (Gunatilaka, 2006, Aly *et al.*, 2011). Recent developments in endophyte screening techniques also revealed that some endophytic microorganisms are endowed with various biologically active compounds of potential use in medicine and agriculture (Aly *et al.*, 2011). Fungal and bacterial endophytes can confer many beneficial characteristics to plants such as greater resistance to biotic and abiotic stresses (Azevedo *et al.*, 2000). In return, endophytes can gain benefits from their host plants through provision of shelter, nutrients and a mechanism for dissemination.

Microbial endophytes have the ability to improve the plant growth, enhance yield, decrease plant damage caused by disease, deter herbivores, remove heavy metal contamination of the soil, promote plant performance under extreme conditions such as high temperatures and drought, fix nitrogen and enhance soil nutrient availability (Raps & Vidal, 1997, Rosenblueth & Martínez-Romero, 2006, Guo *et al.*, 2008, Powell, 2009, Rodriguez *et al.*, 2009).

The beneficial effects of fungal endophytes of brassica was well-studied by Zhang *et al.* (2014). They studied antifungal activity of 97 endophytic fungal isolates from

oilseed rape against *S. sclerotiorum* and reported that twenty-four species presented antagonistic activity against this pathogen *in vitro*. Culture filtrates of some isolates such as *Aspergillus flavipes*, *Chaetomium globosum* and *Clonostachys rosea* exhibited consistent and effective suppression of *S. sclerotiorum* on excised leaves of oilseed rape. However, a cultured filtrate from isolates of *C. globosum* and *C. rosa* exhibited poor activity against *S. sclerotiorum* implying that different strains of the same endophyte species possess mixed bioactivity (Zhang *et al.*, 2014).

Endophytic fungi can promote plant growth in brassica plants (Cao *et al.*, 2008, Jiang *et al.*, 2008, Khastini *et al.*, 2012, Ansari *et al.*, 2013). *Piriformospora indica*, originally isolated from the Thar desert in India, is a mycorrhizal-like fungus that colonises the intra and inter-cellular root tissues of several plant species. The fungus is capable of promoting plant growth in non-mycorrhizal plants such as brassica. This is unique in the area of plant-microbe interactions as brassica species generally do not form associations with mycorrhizal fungi. *P. indica* establishes a symbiotic association with host plants and can function as a bio-regulator, bio-fertilizer and a bio-protector in both mono and dicot plants (Oelmüller *et al.*, 2009, Ansari *et al.*, 2013).

The plant growth promotion effects conferred by endophytes on brassica plants are suggested to be the result of phytohormone production, ACC deaminase activity and/or siderophore production. For example, growth enhancement expressed in Chinese cabbage (*B. rapa* subsp. *pekinensis*) by *P. indica* and *Neosartorya* sp. is largely due to auxin and gibberellin synthesis respectively (Lee *et al.*, 2011, Muhammad *et al.*, 2011) and promotion of biomass in Chinese kale (*B. alboglabra*) by *Cryptococcus* sp. is probably the result of auxin, ACC deaminase activity and/or siderophore synthesis (Deng *et al.*, 2011).

There are few articles that have been reported on the effect of bacterial endophytes in the control of *L. maculans*. The rhizobacteria *Serratia plymuthica* and *Pseudomonas chlororaphis* show antagonistic activity against *L. maculans* and *Verticillium longisporum* (Abuamsha *et al.*, 2011, Abuamsha *et al.*, 2011, Hammoudi *et al.*, 2012). Another bacterium that has been recovered from the roots of oilseed rape is *Paenibacillus polymyxa* which is able to produce two antifungal substances that supress the growth of *L. maculans in vitro*, on detached leaves, stems and stubble of oilseed rape (Kharbanda *et al.*, 1999).

Bacteria also play important roles in plant growth promotion (Nelson, 2004), which can occur directly through nitrogen fixation, increased nutrient mobilization, production of phytohormones, including auxins and cytokinins, and suppression of ethylene levels in plants under stress. Growth promotion may also be induced through the inhibition of pathogens; via competition for nutrients and space, antibiosis and induction of plant defence mechanisms (Hallmann *et al.*, 1997, Weyens *et al.*, 2009). Strains of *B. subtilis* can colonise the root tissues of oilseed rape and promote growth rates and increase seed yield (Hu *et al.*, 2005, Hu *et al.*, 2011). *Methylobacterium* is another bacterium associated with Brassicaceae capable of plant growth promotion (Idris *et al.*, 2004, Idris *et al.*, 2006). This genus produces plant hormones and improves plant development such as branching, seedling vigour, seed germination, root differentiation and photosynthetic activity (Trotsenko *et al.*, 2001, Prombunchachai *et al.*, 2017). Bacterial species isolated from wild and cultivated oilseed plants have been reported to colonise the roots and stems of cultivated species and promote their growth (Nejad & Johnson, 2000).

The benefits of endophytic microorganisms in agriculture, including in the brassica

seed industry is clear and these microorganisms are important agents in the areas of plant protection, plant growth promotion and bioremediation. Further investigation of specific plant-microbe interactions is required to elucidate their full potential.

3. Chapter 3. Screening wild brassica seed accession for bacterial and fungal endophytes

3.1 Abstract

Plants are commonly colonised by a wide diversity of microbial species and these plant-microbial relationships can range from mutualistic through to parasitic. Microorganisms that typically form symptomless associations with internal plant tissues are termed 'endophytes'. Some endophytes are able to colonise the plant reproductive tissues and this gives many species an efficient mechanism of propagation via plant seed (termed vertical transmission). In this study we screened more than 80 wild brassica seed accessions, with a world-wide coverage, for bacterial and fungal isolates with potential biological control attributes. Over 130 microbial isolates were recovered from the brassica germplasm. These isolates were purified and a culture collection established. Microscopy was used to characterise and separate fungal and bacterial groups while DNA sequencing identified selected isolates to species level. Results indicate most seed accessions harbour endophytic bacteria with *Methylobacterium* species the most dominant across the majority of accessions. Only two fungal endophyte isolates, belonging to the Ascomycota, were recovered from the brassica accessions screened.

3.2 Introduction

Endophytes are a diverse sub-group of microorganisms that reside inside the tissues of nearly every vascular plant and, for at least part of their life cycle, do not cause any immediate symptoms (Wilson, 1995, Porras-Alfaro & Bayman, 2011). However, not all endophytes remain within their plant host throughout their entire life cycle and

these microorganisms may also change their behaviour and become pathogenic during host senescence or when the host is stressed (Fisher & Petrini, 1992, Aly *et al.*, 2011). Endophytes can be found in nearly every type of plant tissue, including the vegetative (i.e. leaves, roots and shoots) and reproductive (i.e. flower and seed) tissues (Rodriguez *et al.*, 2009). The presence of bacterial endophytes within the reproductive tissues has been reported for many different plant species (Mundt & Hinkle, 1976) including rice (Elbeltagy *et al.*, 2000, Okunishi *et al.*, 2005, Mano *et al.*, 2006, Tripathi *et al.*, 2006, Kaga *et al.*, 2009, Hardoim *et al.*, 2012), coffee (Vega *et al.*, 2005), Norway spruce (Cankar *et al.*, 2005), oilseed rape (Granér *et al.*, 2003), tobacco (Mastretta *et al.*, 2009), maize (Rijavec *et al.*, 2007, Johnston-Monje & Raizada, 2011), cotton (Adams, 1996), cucumber (Khalaf & Raizada, 2016) and eucalyptus (Ferreira *et al.*, 2008). These seed-associated bacterial endophytes may be disseminated from one generation to the next and, in some associations, have been shown to persist in the next generation of plants. This is indicative of their vertical transmission ability (López-López *et al.*, 2010). Plant hosts harbouring beneficial endophytes can gain additional advantageous traits, granting them an ecological advantage over individuals lacking endophytes and/or other plant species that occupy a similar ecological niche. These benefits include greater resistance to biotic and abiotic stresses (Hallmann *et al.*, 1997, Mastretta *et al.*, 2006, Rodriguez *et al.*, 2009), and plant growth promotion (Azevedo *et al.*, 2000).

Modern brassica cultivars were originally domesticated from wild species mostly originating from Europe (Rakow, 2004) although now many brassica crops, particularly *B. napus* (oilseed rape), *B. rapa* (turnip) and *B. oleracea* (cabbage), are extensively cultivated throughout the world. These species are a major source of

vegetables for human consumption and for forage, ornamental plants, condiments, medicinal crops, green manure, bioremediation and as very important sources of edible and industrial oils (Gómez-Campo, 1980, Rakow, 2004, Dixon, 2007). A wide range of insect pests, such as aphids (*Brevicoryne brassica*), diamond back moth (*Plutella xylostella*) and flea beetles (*Phyllotreta* and *Psylliodes* spp.) can cause extensive damage to brassica crops. In addition, fungal diseases, such as phoma stem canker (caused by *Leptosphaeria maculans*), clubroot (caused by *Plasmodiophora brassicae*) and sclerotinia stem rot (caused by *Sclerotinia sclerotiorum*) can also cause damage (Kimber & McGregor, 1995) with few or no control options available (Granér *et al.*, 2003).

The majority of studies investigating endophytes of brassica have focused on isolating microorganisms from the vegetative tissues of modern day cultivars (Germida *et al.*, 1998, Narisawa *et al.*, 1998, Sheng *et al.*, 2008, Sunkar & Valli Nachiyar, 2013, Zhang *et al.*, 2014). This strategy may be restrictive as the diversity and frequency of endophytic species found in domesticated crops is assumed to be much lower than in their respective wild relatives (Mousa *et al.*, 2015, Putra *et al.*, 2015). Alternatively, wild plant species are considered good sources of beneficial endophytic bacteria that can confer biotic and abiotic enhancements to their hosts including nitrogen fixation and potential anti-phytopathogenic activity (Kremer, 1987, Engelhard *et al.*, 2000, Elbeltagy *et al.*, 2001, Hung *et al.*, 2007). Additionally, identifying endophyte species that are associated with the reproductive plant tissues (those microorganisms that are seed-borne or seed-transmitted) would greatly aid the marketing of potential commercial products (Card *et al.*, 2015, Card *et al.*, 2016). This particular study focused on developing a strategy for screening wild and landrace brassica species for

mutualistic seed-associated endophytes that may offer beneficial traits to elite brassica cultivars if they can be successfully assimilated into the new host.

3.3 Material and methods

3.3.1 Brassica germplasm

Sixty-four brassica seed accessions (49 wild and 15 landraces), from a diverse set of brassica species with a worldwide distribution (Appendix 2), were obtained (on AgResearch's import permit no. 2015058982) from three international gene banks. These were The United States Department of Agriculture (USDA) via The Germplasm Resources Information Network (GRIN), The Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben) and The Nordic Genetic Resource Centre (NordGen) (Table 3.1).

Table 3. 1 International plant genebanks accessed for Brassicaceae seed accessions.

Institute	Location	Internet web page
Nordic Genetic Resource Center (NordGen)	Sweden	http://www.nordgen.org
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)	Germany	http://www.ipk-gatersleben.de
National Genetic Resources Program (NGRP), United States Department of Agriculture (USDA)	USA	http://www.ars-grin.gov

Seed from a further 19 wild brassica accessions were harvested in 2014 locally from the Manawatu region on the North Island of New Zealand (Appendix 2). These

accessions were catalogued and stored at 0°C and 30% relative humidity in the Margot Forde Germplasm Centre (MFGC), New Zealand's national gene-bank of grassland plants.

Figure 3. 1 Collection of wild brassica species grown in the Manawatu region on the North Island, New Zealand.

3.3.2 Screening of wild brassica seeds

3.3.2.1 Disinfection of plant tissues and isolation of microorganisms

To reduce the possibility of isolating non-target microorganisms (such as saprophytic microorganisms) associated with the seed surface a two-step surface disinfection protocol was developed for wild brassica seed. Initially all 83 accessions were exposed to a 'mild seed-disinfection protocol' as follows: seed were washed for five min in 5% aqueous Tween® 20 solution (Sigma-Aldrich Inc., New Zealand), two min in 70% ethanol, 10 min in 0.5% sodium hypochlorite, one min in 70% ethanol and rinsed three times in sterile tap water. To assess the efficacy of the surface disinfection protocol, 3 × 20 µL drops of tap water from the last rinse were plated onto nutrient agar (NA), (CM003, Oxoid Ltd., UK) and Potato dextrose agar (PDA), (CM0139, Oxoid Ltd.,

UK). Petri plates containing these media were incubated for two weeks at 22°C and inspected daily for microbial growth with the aid of a dissecting microscope (Zeiss, Germany). For those accessions that exhibited some form of microbial growth assumed to be from saprophytic organisms (i.e. those colonised by *Alternaria* spp.), a further 'stringent seed-disinfection protocol' was undertaken whereby the same procedure listed above was repeated with the exception of one modification; seeds were immersed for 10 min in a 2% sodium hypochlorite rather than a 0.5% solution. After disinfection, seeds were dried on filter paper (110mm, Thermo Fisher Scientific Inc, New Zealand) within a laminar flow cabinet and plated onto Petri plates containing 1.5% water agar (WA) with 10 seeds per plate. To break seed dormancy and induce germination, 20 ml sterile 0.2% KNO_3 was also added to the plates before incubating them at 4℃ in the dark for 72 hr. Plates were subsequently transferred to a custom-built growth chamber set at 22-25˚C with a 16/8 hr (light/dark) photoperiod to germinate. Seeds were examined under a dissecting microscope (Zeiss, Germany) and those exhibiting any obvious epiphytic microbial growth were discarded. After 2-3 days, ten seedlings from each accession were transferred to sterile tissue culture pots, 98 mm diameter (2105646, Alto Ltd., New Zealand) containing Murashige & Skoog (M&S) basal salts (Murashige & Skoog, 1962) medium with minimal organics (M6899, Sigma, USA), plus 3% sucrose and 1.5% agar (Ali et al 2007). Pots were placed in the growth chamber (under the same conditions as described earlier) and assessed every day for one month. Plants were discarded if they showed any disease symptoms or any saprophytic microbial growth (Figure 3.2). Four seedlings per accession, showing no symptoms, were selected from each accession and subsequently dissected into two components; shoot and root, using a scalpel. These tissue pieces

were dissected into 2-3 mm^2 pieces and 10 pieces per tissue type from each seedling were transferred to Petri plates containing NA as a growth medium for bacteria and PDA medium for fungi. Petri plates were then incubated for three weeks at 22℃ in the dark and checked daily under a dissecting microscope for microbial and fungal growth. Bacterial and fungal colonies of each accession that appeared frequently and looked morphologically different were selected, sub-cultured and checked for purity. Representative bacterial and fungal isolates were then sub-cultured onto fresh medium and stored in 25% glycerol at -80°C.

Morphological images of fungal isolates were taken with an Olympus BX50 light microscope (Olympus, New Zealand Pty Ltd) and captured with an Olympus ColorView II digital camera and AnalySIS 3.00 image-analysis software.

Figure 3. 2 Growth of wild brassica seedlings on M&S medium.

3.3.2.2 Identification of seed-associated bacterial endophytes

The species identity of bacterial isolates was achieved by the PCR (polymerase change reaction) amplification of partial 16S rDNA gene sequences (Weisburg *et al.,* 1991). PCR was directly applied to suspensions of each purified bacterial colony as follows: each colony was suspended in 10 µL Milli-Q water within a PCR tube (Axygen, USA) and frozen at -20℃ before being thawed and heated to 65℃ for 30 min. Then, 1µL of suspension was added to the PCR reaction containing 5 µL 10X PCR buffer, 1.5 µL $MgCl_2$ (50 mM), forward primer, 27F (AGAGTTTGATCCTGGCTCAG, 1 µL, 10 µM), reverse primer R1497, (CCTATATCGCCGGTAATT, 1 µL ,10 µM,), 0.4 µL dNTPS (25mM), 0.25 µL Taq-polymerase and 39.85 µL sterile Milli-Q water to make a 50 µL PCR reaction. PCR was performed in a thermocycler (Bio-Rad C1000 Touch^{TM}, Bio-Rad Laboratories Inc., USA) with the following conditions: an initial step of 95℃ for 5 min was followed by 36 cycles of 94℃ for 30 s, 56℃ for 60 s, 72℃ for 90 s and a final step of 72℃ for 10 min. The reaction mixture from each sample was electrophoresed on a 1.5% agarose gel containing ethidium bromide. The gel was viewed on a trans-illuminator (Gel Doc™ XR+, Bio-Rad Laboratories Inc., USA) to identify samples with amplification products. PCR amplification products were purified and concentrated using the DNA clean & concentrator kit (Zymo Research Corporation, USA) prior to sequencing (New Zealand Genomics Ltd., New Zealand). DNA sequences were analysed with Geneious software V.8 (Biomatters Ltd., New Zealand). Those greater than 600 bp were used in BLASTn searches against the NCBI non-redundant database and those with greater than 98% identity were selected and named. A phylogenetic tree was generated using Geneious from an alignment of the

nucleotide sequences using the Neighbour-Joining method via bootstrap resampling with support threshold of > 55%.

3.3.2.3 Identification of seed-associated fungal endophytes

Species identification of fungal endophytes was achieved using PCR amplification of the Internal Transcribe Spacer (ITS) of rDNA gene sequences (White *et al.*, 1990). Initially DNA of fungal endophytes, incubated at 22℃ for two weeks, extracted using Quick-DNA™ Fungal/Bacterial Kit (Zymo Research Corporation, USA) and quantified using the Invitrogen Qubit™ 4 Fluorometer (ThermoFisher Scientific Inc., USA). PCR was performed directly on 1 µL of DNA extract suspension (15-20 ng/µL) of each purified fungal colony using the forward primer, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) using the same PCR reaction mixture as used for the bacteria. The thermocycler conditions also were set the same as for bacterial identification procedure with one modification; the annealing temperature was set at 56˚C for 30 s.

Further identification of *Beauveria* sp. was achieved via the 1-alpha (EF1-apha) elongation factor through primers EF-1 (forward) and EF-2 (reverse) using 25 µl of PCR reaction mixture. The reaction mixture consisted of: 15.75 µl sterile water, 2.5 µl buffer (10x) plus $MgCl_2$ (2 mM), 2 µl deoxynucleotide (dNTP's) (2.5 mM), 0.25 µl Fast start polymerase Taq, 1 µl of each primer, 0.5 µl bovine serum albumin (Bio Labs® Inc., New Zealand) and 2 µl of extracted DNA per sample. Thermocycling conditions were set as 95°C for 5 min followed by 40 cycles of 95°C for 45 s, 53°C for 45 s, 72°C for 1 min and final extension of 72°C for 7 min. The elongation factor

sequence was aligned and compared to the sequences of *B. bassiana* reference strains as published by Rehner and Buckley (2005).

3.3.3 Diversity of bacterial endophyte

Simpson`s diversity index (Simpson, 1949) was used as a quantitative measure to reflect how many different bacterial species were in the seed-associated microbial community of the brassica accessions sampled. This was calculated as:

Simpson index of diversity = 1 - D

$$D = \frac{\sum n\,(n-1)}{N\,(N-1)}$$

n = the total number of organisms of a particular species

N = the total number of organisms of all species

3.4 Result

3.4.1 Seed-associated fungal and bacterial isolates

Alternaria spp. were isolated from over 50% of the brassica seed accessions following mild seed-disinfection protocol. The frequency of *Alternaria* was markedly reduced after the application of the stringent seed-disinfection protocol.

Fifty-four accessions (44 wild and 10 landrace), from a total of 83 accessions surface disinfected and sown, resulted in symptomless plants when their seed was germinated on WA. Symptomless seedlings were subsequently transferred to M&S medium. Seed from the remaining 29 accessions all resulted in observable epiphytic fungal growth with the majority of colonies identified as *Alternaria* spp. (Figure 3.3). These were destroyed by autoclaving.

Bacterial colonies were observed growing from the dissected root and shoot tissues of 48 symptomless wild brassica accessions (38 wild and 10 landrace) plated on NA. Fungal colonies were observed growing from the dissected root and shoot tissues of only two symptomless wild brassica accessions, (accessions O2380 and O2377) both *B. rapa*, plated onto PDA (Figure 3.4). Six accessions (O2364, O2369, O2371, O2374, O2384 and O2385) resulted in no bacterial or fungal growth when plated onto NA or PDA, respectively; see Appendix 2 for details of accessions.

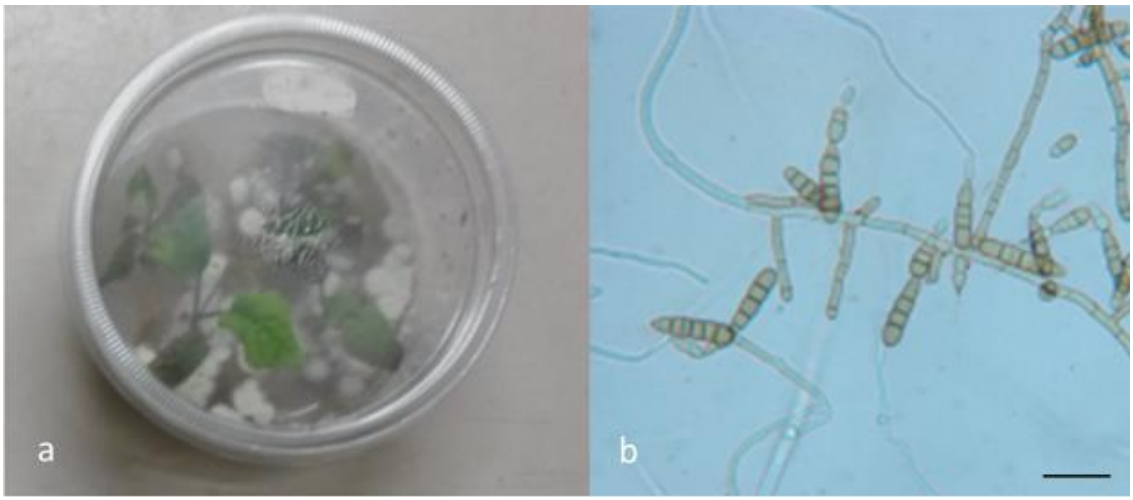

Figure 3. 3 Growth of *Alternaria* sp. colonies within pots containing brassica seedlings growing on M&S media (a) and characteristic *Alternaria* conidia observed under the dissecting microscope (b), bar 20 µm.

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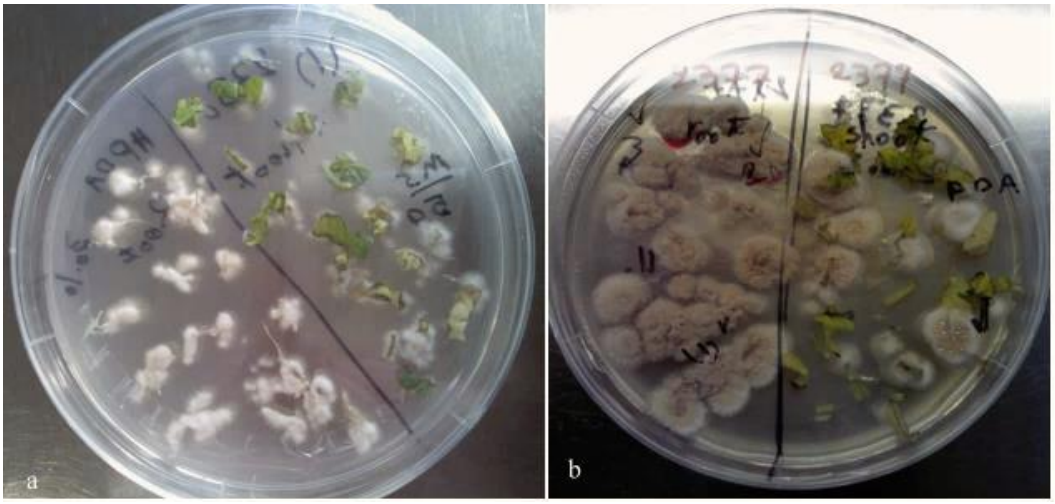

Figure 3. 4 Petri plates showing colonies of two fungal isolates (subsequently identified as a: *Beauveria bassiana*, b: *Geomyces pannorum*) growing from root and shoot tissues of two different wild *B. rapa* accessions previously plated onto PDA.

The fungus isolated from brassica accession 02380 was tentatively identified as *Beauveria* due to the relatively slow growing white colonies formed on PDA after incubation at 22°C and the formation of single-celled hyaline conidia formed on a zig-zag conidiophore or rachis (Figure 3.5 d). The fungus isolated from brassica accession O2377 produced yellow-brown colonies after 2 weeks when cultured at 22°C on PDA. The fungus resembled an ascomycete with single-celled, hyaline conidia produced in short chains on short conidiophores (Figure 3.5 b).

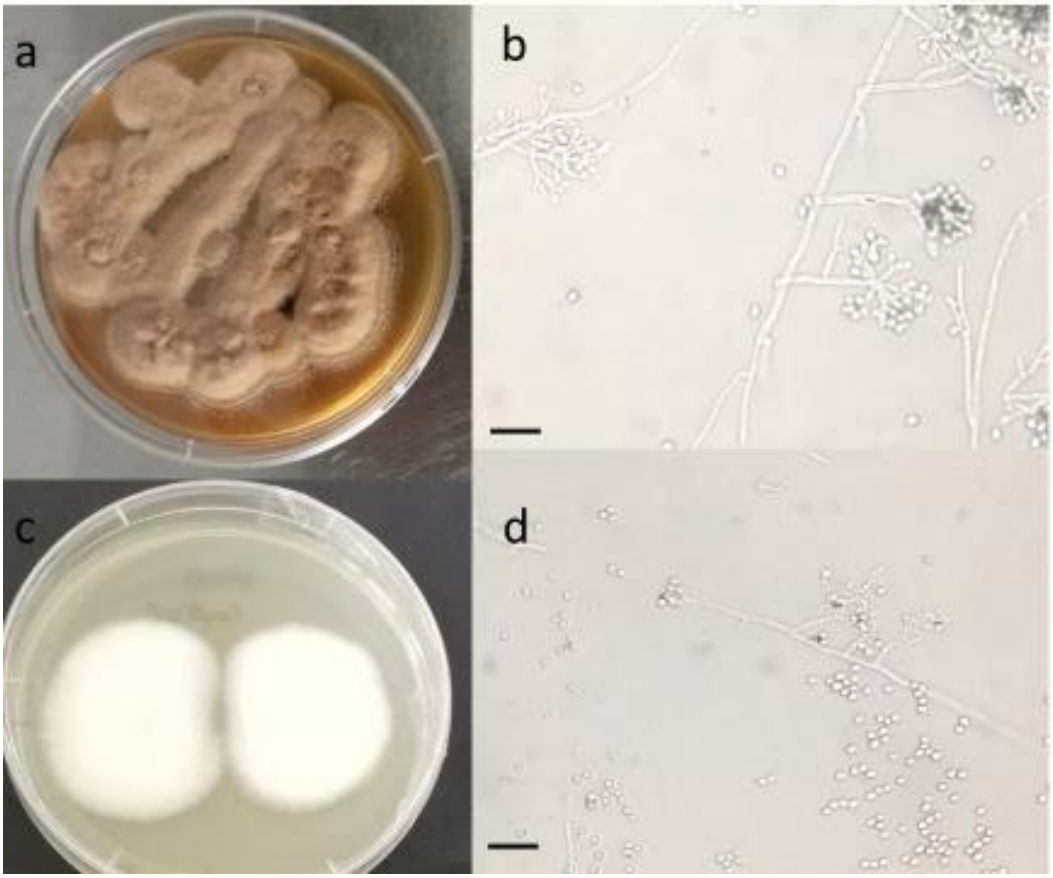

Figure 3. 5 Culture morphology of *Geomyces pannorum* (a) and *Beauveria bassiana* (c) isolated from brassica accessions O2377 and O2380, respectively on PDA. Figures on right hand side show conidia and conidiophores of *G. pannorum* (a) and *B. bassiana* (c) produced on WA under 400x magnification, bar 10 µm.

3.4.2 Species identification of seed-associated fungi

The fungal isolates from accessions O2377 and O2380 were tentatively identified as *Beauveria bassiana* and *Geomyces pannorum*, respectively. Further sequencing of the elongation factor from the *Beauveria* isolate confirmed its identity as *B. bassiana* (Rehner & Buckley, 2005) (Figure 3.7).

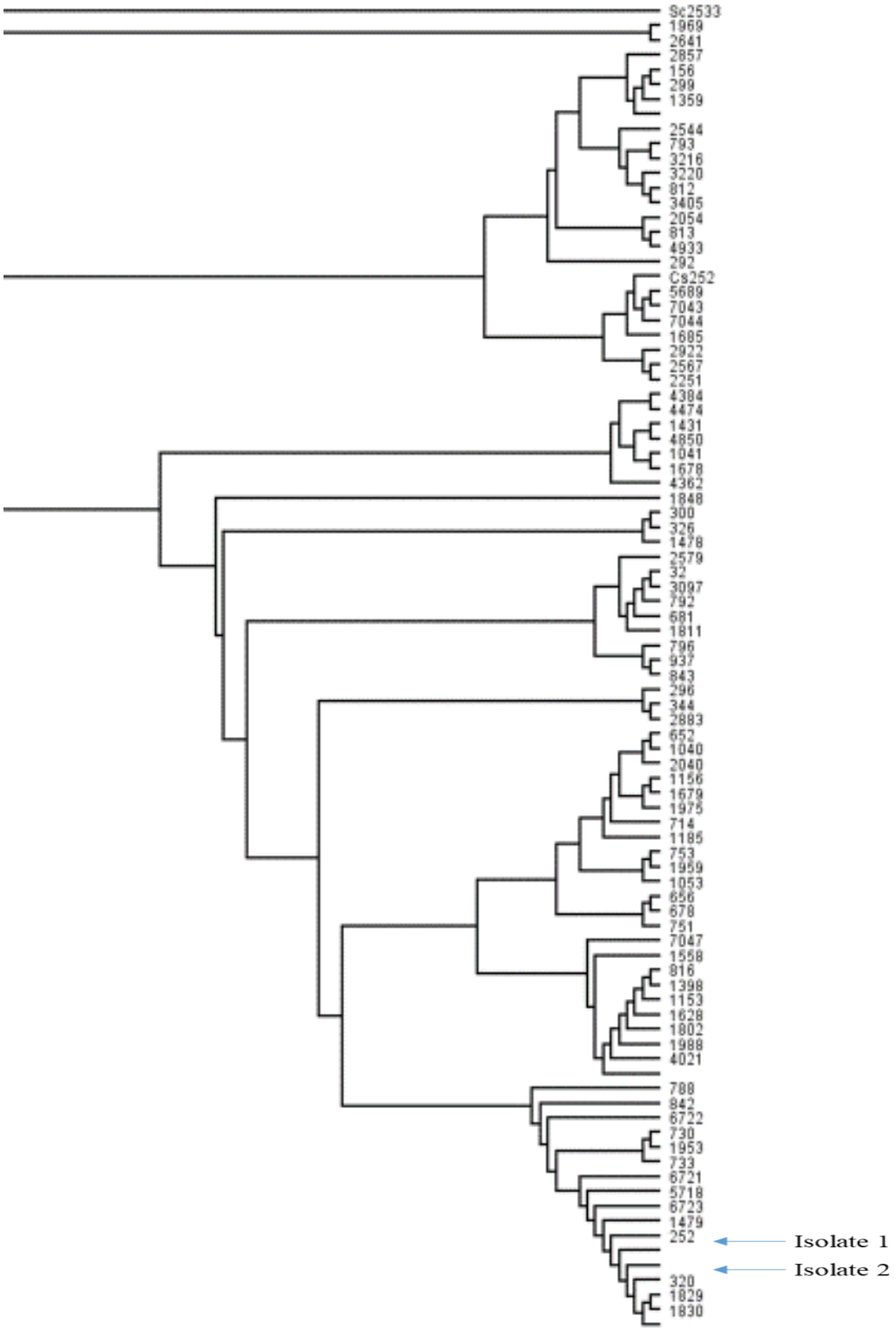

Figure 3. 6 Phylogenetic tree including the alignment of two isolates of *B. bassiana* discovered in this study compared to a further 94 reference isolates sequenced by Rehner & Buckley, 2005.

3.4.3 Species identification of seed-associated bacteria

In total, 98 bacterial strains were sequenced from this study. These strains were isolated from 48 brassica accessions with some accessions producing two or three different (according to morphology) bacterial colonies. 16S rDNA sequencing identified 17 different bacterial species belonging to three phyla, namely Actinobacteria, Firmicutes and Proteobacteria (Table 3.2). According to the phonological tree of bacterial isolates (Figure 3.6) the most frequently isolated species belonged to the Proteobacteria and included the following species; *Brevundimonas vesicularis, Caulobacter mirabilis*, *Methylobacterium fujisawaense*, *Me. extorquens, Me. phyllosphaerae, Novosphingobium resinovorum, Pseudomonas azotoformans, Stenotrophomonas rhizophila, Sphingobium yanoikuyae, Sphingomonas paucimobilis*, *Sphingom. mucosissima* and *Sphingom. insulae*. Three species were identified from the Actinobacteria, namely *Kocuria palustris, Micrococcus luteus, and Plantibacter flavus* while two species were identified from the Firmicutes phylum, namely *Bacillus mycoides* and *Paenibacillus hordei*.

Simpson's diversity index recorded a value of 0.74 indicating that wild brassica species contain a high diversity of seed-associated microorganisms. Nevertheless, two species, *Me. fujisawaense* and *Me. phyllosphaerae* constituted 58% of all the isolated bacterial strains and were common in 77% of all the brassica accessions screened (38 brassica accessions; 28 wild and 10 landrace) in this study. These were also distributed among many brassica species including *B. barrelieri*, *B. elongate, B. gravinae*, *B. indica, B. juncea, B. napus, B. nigra* and *B. rapa* sourced from five continents, namely Africa, Asia, Australasia, Europe and North America.

Figure 3. 7 Phylogenetic tree of bacteria isolated from seed of different brassica species utilising the maximum likelihood method, based on 16S rDNA sequences isolates. Bootstrap values were >55%.

Table 3. 2 Seed-associated bacterial endophytes of wild and landrace brassica.

Endophyte species	**Host species**	**Accessions number***
Bacillus mycoides	*B. juncea*, *Brassica* sp.	O2334, NZ03
Brevundimonas vesicularis	*B. rapa*	O2380
Caulobacter mirabilis	*B. napus*	O2395
Kocuria palustris	*B. napus*	O2393
Methylobacterium fujisawaense	*B. barrelieri*, *B. elongate*, *B. juncea*, *B. nigra*, *B. rapa*, *B. napus*, *Brassica* sp.	NZ02, NZ06, NZ07, NZ08, NZ09, NZ11, NZ12, NZ13, NZ16, NZ18, NZ19, O2331, O2337, O2350, O2357, O2359, O2363, O2370, O2377, O2380
Me. phyllosphaerae	*B. incana*, *B. gravinae*, *B. napus*	NZ10, NZ14, NZ15, O2333, O2367, O2390, O2393, O2401
Me. extorquens	*B. rapa*	O2377
Micrococcus luteus	Unknown	Unknown
Novosphingobium resinovorum	*B. rapa*	O2356
Paenibacillus hordei	*Brassica* sp.	NZ17
Plantibacter flavus	*B. juncea*	O2372
Pseudomonas azotoformans	*B. balearica*, *B. juncea*, *B. nigra*, *B. oleracea*	O2330, O2338, O2351, O2352
Sphingobium yanoikuyae	*B. nigra*, *Brassica* sp.	NZ09, O2350
Sphingomonas paucimobilis	*B. balearica*	O2329
Sphingom. mucosissima	*B. juncea*, *Brassica* sp.	O2334
Sphingom. insulae	*Brassica sp.*	NZ14
Stenotrophomonas rhizophila	*B. juncea*, *B. oleracea*	O2334, O2335, O2338, O2352

* Accession information can be found in Appendix 2.

Table 3. 3 The genera of isolated seed-associated endophytic bacteria of wild and landrace brassica and some of their potential beneficial properties.

Genus	**Phylum**	**Beneficial properties**
Bacillus	Firmicutes	Biocontrol activity (Berg & Hallmann, 2006, Andreolli *et al.*, 2016) plant growth promotion (Rashid *et al.*, 2012) phytoremediation (Rajkumar *et al.*, 2009)
Brevundimonas	Proteobacteria	Plant growth promotion (Sessitsch *et al.*, 2004, Ying *et al.*, 2016)
Caulobacter	Proteobacteria	Plant growth promotion (Rout & Chrzanowski, 2009), seed germination (Naveed *et al.*, 2014)
Kocuria	Actinobacteria	Plant growth promotion (Andreolli *et al.,* 2016)
Methylobacterium	Proteobacteria	Nitrogen fixation (Sy *et al.*, 2001, Lee *et al.*, 2006, Menna *et al.*, 2006), phytohormone production (Meena *et al.*, 2012), pathogenic interaction (Araújo *et al.*, 2002, Lacava *et al.*, 2004), plant growth promotion (Lee *et al.*, 2006, Madhaiyan *et al.*, 2006a, Madhaiyan *et al.*, 2006b, Tani *et al.*, 2012), induce systemic resistance (Madhaiyan *et al.*, 2006b, Ardanov *et al.*, 2012), induce higher photosynthesis activity (Cervantes-Martí & López-Dí, 2004), siderophores production (Lacava *et al.*, 2008), phytoremediation (Rajkumar *et al.*, 2009), stimulate seed germination (Lidstrom & Chistoserdova, 2002)
Micrococcus	Actinobacteria	Biocontrol activity (Berg & Hallmann, 2006)

Genus	Phylum	Beneficial properties
Novosphingobium	Proteobacteria	Plant growth promotion (Zhang *et al.*, 2016, Krishnan *et al.*, 2017, Rangjaroen *et al.*, 2017), biodegradation (Kertesz & Kawasaki, 2010)
Paenibacillus	Firmicutes	Biocontrol activity (Granér *et al.*, 2003, Sessitsch *et al.*, 2004, Ghazalibiglar *et al.*, 2016, Rybakova *et al.*, 2016), phytoremediation (Rajkumar *et al.*, 2009), plant growth promotion (Sessitsch *et al.*, 2004, Anand & Chanway, 2013, Ying *et al.*, 2016)
Plantibacter	Actinobacteria	Bioremediation (Li *et al.*, 2012), plant growth promotion (Ying *et al.*, 2016)
Pseudomonas	Proteobacteria	Biocontrol activity (Sessitsch *et al.*, 2004, Laugraud *et al.*, 2017), plant growth promotion (Rashid *et al.*, 2012), phytoremediation (Rajkumar *et al.*, 2009, Li *et al.*, 2012), seed germination (Naveed *et al.*, 2014)
Stenotrophomonas	Proteobacteria	Antifungal activity, plant growth promotion, bioremediation (Wolf *et al.*, 2002, Kai *et al.*, 2007, Ryan *et al.*, 2009, Berg *et al.*, 2010, Schmidt *et al.*, 2012, Andreolli *et al.*, 2016)
Sphingomonas	Proteobacteria	Biocontrol activity (Berg & Hallmann, 2006), plant growth promotion (Doty *et al.*, 2009, Khan *et al.*, 2014), phytoremediation (Rajkumar *et al.*, 2009, Li *et al.*, 2012, Chen *et al.*, 2014)
Sphingobium	Proteobacteria	Biodegradation (Kertesz & Kawasaki, 2010), plant growth promotion(Rout & Chrzanowski, 2009), seed germination (Naveed *et al.*, 2014)

3.5 Discussion

This research developed and implemented an endophyte discovery pipeline to screen accessions of Brassicaceae (wild, landrace and cultivars) for culturable bacteria and fungi intimately associated with their host seed. The aim was to identify endophytic microorganisms capable of colonising brassica seed that have potential to form new associations with elite brassica cultivars while conferring additional beneficial traits. In addition, microorganisms that rely on the plant's reproductive strategy (i.e. those vertically transmitted via seed) may aid the marketing of any potential plant-endophyte product (Card *et al.*, 2016).

The discovery pipeline was adjusted several times during the preliminary stages of the study. The main change was the incorporation of a harsher seed surface disinfection protocol as the original protocol resulted in a high frequency of samples being overgrown with saprophytic *Alternaria* spp., a fungus commonly associated with the seed coats of many plant species (Neergard, 1977, Harman, 1983). Once the protocol was adjusted, a reduced frequency of *Alternaria* was observed and brassica seedlings could be successfully grown without displaying any symptoms of disease while the seed retained a high germination percentage. Additionally, the majority of accessions, exposed to the harsher protocol, gave rise to multiple isolates of bacteria indicating that the protocol did not sterilise the seed.

Discovery of endophytic communities are generally based on two approaches, being culture-dependent and culture-independent (Stefani *et al.*, 2015). While the first approach uses more traditional mycological techniques and aims to isolate microorganisms from the plant tissue the second approach uses more recent molecular-

based techniques and aims to identify the microbiome of the plant tissue including culturable and non-culturable microorganisms. These approaches have their own specific advantages and disadvantages. In culture-dependant approach only the endophytic microorganisms able to grow on synthetic media could be isolated, and this may result in a subset of the culturable microorganisms due to the media chosen for isolation. In addition, the approach may result in a bias towards fast-growing, ubiquitous species, whereas rare species with minor competitive strength and more specialized requirements may remain undiscovered (Unterseher & Schnittler, 2009, Sun et al., 2011). For culture-independent approaches, the entire microbiome can be uncovered and accompanied with statistical analysis; lists of the most abundant microorganisms can be produced, including culturable and unculturable species. This can be advantageous as information regarding the frequency and biomass of each microorganism living within the plant can be calculated. For example, a culture-independent study, aimed at evaluating fungal diversity, identified 14 operational taxonomic units in leaves of *Magnolia liliifera* and uncovered microbial taxa that had never been associated with this plant host from previous culture-dependant studies (Duong *et al.*, 2006). Therefore, to improve the pipeline discovery it is suggested that the two approaches are applied to enable those requiring microbial specific media to be isolated. However, the discovery pipeline applied in this chapter used a culture-dependent approach for screening brassica for endophytic microorganisms. This enabled the establishment of a viable culture collection composed of axenic endophytes that can be mined further for strains with beneficial traits. In turn, this collection can support further experimentation including the development and characterisation of novel plant-endophyte associations, not just with brassica.

The presence of many diverse endophyte species isolated from different species of Brassicaceae was reviewed by Card *et.al* (2015). This review highlighted that very few publications have reports of beneficial seed transmitted endophytic microorganisms and that most reported endophytes were instead isolated from vegetative tissues rather than reproductive. This study targeted the reproductive tissues, namely seeds of wild brassica, with the rationale that wild germplasm would have a greater diversity of microbial endophytes than cultivated varieties and that those endophytes in seed would also colonise the vegetative seedlings and mature plants potentially conferring beneficial traits throughout the plants lifecycle. In addition, a BCA that is endophytic within the seed is obviously easier to disseminate with the crop as it is built-into the seed product itself compared to a traditional BCA that requires a form of application strategy as with many agrichemicals. Therefore, a BCA present in the seed would also be easier to market for seed companies.

Our results indicate that the diversity of bacterial endophytes in seeds of wild brassica species is relatively high, being composed of 13 bacterial genera from three phyla. The majority of bacterial species (12 out of 17) were identified as genera belonging to the Proteobacteria, a major phylum of gram-negative bacteria. Previous researches investigating the bacterial diversity of oilseed rape cultivars also identified that this was the most dominant phyla present (Rybakova *et al.*, 2017). More interestingly the same authors, using amplicon sequencing, identified that each cultivar of oilseed rape investigated had its own diversity, unique bacterial structure, and proportion of unique microorganisms (Rybakova *et al.*, 2017). Truyens *et al*. (2015) summarised a list of bacterial endophyte species associated with the seed of 25 plant species including 131 bacterial genera, representing four different phyla. In this study Proteobacteria,

especially species of ϒ-Proteobacteria were found to dominate while species of Actinobacteria, Firmicutes and Bacteroidetes were less common.

In this study, we isolated 98 bacterial isolates, from root and shoot tissues of 48 brassica accessions. These isolates, once identified, represented 17 different bacterial species with many of the genera reported in the literature as seed-inhabiting endophytes with several beneficial traits (Table 3.3). For example, many *Bacillus* species are recognised endophytes inhabiting the seed of *Coffea arabica*, *Eucalyptus* spp., *Oryza sativa* and *Phaseolus vulgaris*. *Methylobacterium* and *Paenibacillus* have been identified as a seed inhabiting endophyte of *Eucalyptus* spp., *O. sativa* and *P. vulgaris* while *Sphingomonas* has been documented in its of seed from *P. vulgaris* and *O. sativa* (Vega *et al.*, 2005, Mano *et al.*, 2006, Ferreira *et al.*, 2008, López-López *et al.*, 2010). *Bacillus* and *Pseudomonas* were the most frequently genera found in plant seeds (Truyens *et al.*, 2015). In domesticated Cucurbits, *Bacillus* and *Paenibacillus* are found to be the most dominant seed-endophytic bacteria that showed antagonistic activity against plant pathogens (Khalaf & Raizada, 2018) and in wheat, *Paenibacillus* and *Pantoea* are the seed endophytes showing antagonism against *Fusarium graminearum,* the causative agent of wheat head blight (Díaz Herrera *et al.*, 2016). Information regarding the endophytic communities of brassica seed is limited. Granér, *et al*. (2003) isolated endophytic bacteria from pre-germinated seeds of oilseed rape cultivars that belonged to 10 genera and 16 species of bacteria with the majority identified as *Bacillus* and *Pseudomonas* species. In their study the mode of transmission by endophytic bacteria was not investigated and no fungal endophytes were isolated. However, the technique they used for isolation did not favour the growth of fungi.

Our results indicated that *Methylobacterium* sp. is associated with the seed of several wild brassica species. The presence of *Methylobacterium* as a part of the endophytic community of *Arabidopsis thaliana* seeds has been reported earlier (Truyens *et al.*, 2013) with three species, *M. extorquens*, *M. mesophilicum* and *M. goesingens*e, are associated with *Thlaspi goesingense* from the *Brassicaceae* family (Idris *et al.*, 2006). The intimate associations of *Methylobacterium* with a number of other plant species has been reported previously (Dourado *et al.*, 2015). For example, strains of *Methylobacterium* have been found as endophytes of *Oryza sativa* seed, originating in this tissue before colonising the growing plant (Mano *et al.*, 2006, Kaga *et al.*, 2009). Our results are consistent with other studies like that by Sanchez *et al*., (2018) who demonstrated that *Methylobacterium* were the most dominant community member of *Crotalaria pumila* after members of this bacterial genus was isolated from the three successive plant generations. In the same study, a strain of *Methylobacterium* was able to migrate from the soil to the plant tissue of *A. thaliana*, move through xylem and colonise the seeds. This process of systemic plant by bacterial endophytes has been previously suggested by Compant *et al*. (2010) and Truyens *et al*. (2015). Interestingly, using 16S rDNA gene sequencing of seed samples, a small subset of the seed microbiome has been identified that is conserved across generations of *A. thaliana* (Truyens *et al.*, 2016). The putative bacterial endophytes isolated from the seed of brassica in our study could also be part of a larger bacterial seed community originating from previous plant generations.

The presence of *Methylobacterium* in association with the seed and its subsequent of seedlings may play an ecological role in plant development. This genus is recognised as a producer of plant mimicking hormones and these have the ability to promote plant

development through, improved seedling vigour, increased frequency of seed germination, improved seedling growth, root differentiation and photosynthetic activity (Trotsenko *et al.*, 2001, Prombunchachai *et al.*, 2017). Strains of *Methylobacterium* have also conferred other beneficial traits to their plant hosts including nitrogen fixation (Sy *et al.*, 2001, Lee *et al.*, 2006, Menna *et al.*, 2006), induced systemic resistance (Madhaiyan *et al.*, 2006b), decreased environmental stress (Muller *et al.*, 2011) and immobilisation of heavy metals (Dourado *et al.*, 2012). Cultivated brassica crops, such as oilseed rape (*B. napus*), have a high nitrogen demand (Rathke *et al.*, 2006) and their growth is generally reliant on nitrogen fertilisers that usually have a low nitrogen use efficiency (Bouchet *et al.*, 2014, Bouchet *et al.*, 2016). Many wild brassicas grow in infertile habitats, such as sandy soil like those visited on our collection trips along a river bank in NZ (Manawatu river), and this could explain the frequent isolation of these nitrogen fixing bacteria within these plants. This suggests that this association between *Methylobacterium* and brassica form a mutualistic symbiosis and therefore there is potential for the use of *Methylobacterium* in improving the growth of the cultivated brassica plants. This is the subject of our study in Chapter 5.

In this study fungal seed endophytes were present in a much lower diversity than bacterial endophytes and only two fungal endophytes were isolated. These were identified as *Beauveria bassiana* and *Geomyces pannorum*. The information regarding the seed-associated fungal endophytes in brassica is very limited and there are few reports indicating their presence in other plant species (Fisher *et al.*, 1992, Parsa *et al.*, 2016). For example, a study screened the germinated seed of 11 common bean cultivars and isolated many fungal all belonged to ascomycetes, with exception one

from basidiomycetes. In this study the putative fungal endophytes of *B. bassiana* and *G. pannorum* were isolated from almost all the fragments of root and shoot plated. These seem to potentially have seed transmission ability in the whole plant. The seed transmission ability and potential bioactivity of these endophyte in control of *L. maculans* were studied in Chapter 4 and 5.

To our knowledge this is the first report investigating the culturable endophytic fungal and bacteria originating from seeds of wild and landrace brassica species. In this study we presented a straight forward strategy to screen brassica and cultivate their seed-associated endophytes. The next stage of this research is to characterise selected strains, develop novel associations in cultivars of brassica and assess their beneficial traits with the hypothesis that these endophytes may increase their hosts' resistance against biotic and abiotic stresses.

4. Chapter 4. Inoculation of brassica cultivars with fungal endophytes

4.1 Abstract:

Endophytic microorganisms can be found inside the tissues of many plants species. These endophytic microorganisms once isolated can be inoculated into the same or different host plant species through various methods of inoculation. In this chapter, two putative endophytic fungi that were isolated from wild seeds of brassica accessions, namely *Beauveria bassiana* and *Geomyces pannorum*, were inoculated to three forage brassica species, *Brassica napus*, *B. rapa* and *B. oleracea* and five cultivars of oilseed rape; *B. napus*. The inoculation methods included; seed, foliar and soil inoculation and were applied to three types of plant growth medium; sterile soil, non-sterile soil and vermiculite. Re-isolation of fungal endophytes form the inoculated plant tissues was undertaken at different plant growth stages including cotyledon, one-leaf, three-leaf, four-leaf growth stage and also from the flowering parts and off springs. The results indicate that both fungal endophytes colonise below and above ground tissues of all three brassica species at all vegetative growth stages studied. *G. pannorum* showed a higher rate than *B. bassiana* and root tissues were more infected by *G. pannorum* and *B. bassiana* than shoot tissues in overall. Fungal endophytes were not re-isolated from flowering stems or the off springs from oilseed rape species. Tissue observation of infected plants under light microscope illustrated that the conidial spore of these fungal endophytes can germinate on the surface of plant tissues, penetrate the epidermal cell, and establish a point infection. This implies that these fungal endophytes can establish a horizontal dissemination in the new host species rather than a vertical transmission.

4.2 Introduction

Microorganisms that live inside plant tissues, termed endophytes, commonly associate with the majority of plant species found in natural and managed ecosystems (Card *et al.*, 2016). Many endophytes offer benefits to their host with examples of growth promotion; increased seed yield; reduced disease symptoms caused by plant pathogens; reduced herbivory from insect pests; removal of contaminants from soil; improved plant performance under extreme conditions of temperature and water availability; phosphate solubilisation and nitrogen fixation (Azevedo *et al.*, 2000, Card *et al.*, 2015).

Due to these desirable traits, selected strains of endophytic fungi and bacteria have significant potential in agricultural and horticultural systems (Johnson *et al.*, 2013, Le Cocq *et al.*, 2017). Characterised endophytic strains can be transferred from their original wild plant hosts into elite cultivars via various methods of inoculation. These methods generally include soaking surface disinfected host seed in bacterial cell suspensions, depositing host seed on fungal cultures and allowing them to be colonised, creating small wounds in host seedlings to insert fungal mycelium or drenching the rhizosphere of potential plant hosts with fungal spore suspensions (Latch & Christensen, 1985, Usuki *et al.*, 2002, Bressan & Borges, 2004, Tefera & Vidal, 2009). However, the formation of artificial or novel associations can create a unique set of problems regarding plant-endophyte compatibility. This usually stems from the type of host or organ/tissue specificity exhibited by the particular endophytic species in question (Petrini, 1996). For example, one of the most studied fungal endophytes, *Epichloë* spp., generally exhibit a high level of host-specificity with species of cool-season grasses within the family Poaceae. The original associations have evolved over

many millions of years (Schardl *et al.*, 2008) and therefore it should be of no surprise that newly formed associations might exhibit symptoms of incompatibility (Christensen *et al.*, 1997, Johnson *et al.*, 2013).

While host-specificity is a key behavioural feature of certain species of fungal endophyte belonging to many species within the Clavicipitaceae family (C-endophytes), such as many *Epichloë* spp. (Johnson *et al.*, 2013, Card *et al.*, 2016), in contrast many non-Clavicipitaceous endophytes are capable of colonising a broad spectrum of plant species (Rodriguez *et al.*, 2009). For example, endophytic strains of *Beauveria bassiana* have been observed colonising artichoke, banana, bean, brassica, cocoa, coffee, cotton, date palm, pine, poppy, pumpkin, sorghum and wheat (Vega, 2008, Tefera & Vidal, 2009, Vega *et al.*, 2009, Lohse *et al.*, 2015, Vidal & Jaber, 2015, Gautam *et al.*, 2016).

Aside from host-specificity, there are other factors that have been identified as influencing the initial infection and subsequent host tissue by the newly acquainted endophyte. These include, but are not restricted to, the existing endophytic community within the new host, the plant growth medium, plant growth stage, host organ or tissue at the inoculation site, method of inoculation, plus many environmental factors (Tefera & Vidal, 2009, Truyens *et al.*, 2015). The significance of the host plant growth medium and inoculation method with regards to developing novel plant-endophyte associations with *B. bassiana* and sorghum has been studied by Tefera and Vidal (2009). These authors demonstrated that strains of *B. bassiana* were able to colonise above and below ground tissues of young sorghum seedlings but the rate of was affected by the plant growth medium (sterile soil, non-sterile soil, or vermiculite). They

speculated that this might be due to the presence or abundance of certain antagonistic microorganisms already present in some media types.

This chapter aimed at investigating different inoculation strategies for infecting elite brassica cultivars by two strains of endophytic fungi, belonging to the species *B. bassiana* and *Geomyces pannorum*, both previously isolated from accessions of wild brassica (see Chapter 3 for details).

4.3 Materials and methods

4.3.1 Plant cultivars and fungal material

For the development of artificial endophyte-brassica associations, three forage cultivars (*Brassica* spp.) and five oilseed rape cultivars (*B. napus*) were selected as hosts for inoculation (Table 4.1). Since the original hosts of the two fungal endophytes selected for inoculation were belonged the *B. rapa* species, initially we used the cv. Hunter (the only *B. rapa* cultivar selected as a host) for the inoculation strategies and the successful method were further applied on other species and cultivars of brassica to investigate any host-specificity.

In order to reduce any epiphytic microorganisms attached to the seed coat, all brassica seed was surface disinfected using the stringent seed-disinfection protocol as explained in chapter 3 (section 3.3.2.1). Seeds were then dried on filter paper (110 mm, Thermo Fisher Scientific Inc., New Zealand) within a sterile environment and kept at 4°C in the dark until required.

Table 4. 1 Brassica cultivars used in this study and the suppliers of this seed.

Cultivar	**Common name**	**Species**	**Supplier**	**Inoculation strategy used**
Titan	Forage rape	*Brassica napus*	PGGW, New Zealand	Seed inoculation
Regal	Forage kale	*B. oleracea*	PGGW, New Zealand	Seed inoculation
Hunter	Forage leafy-turnip	*B. rapa*	PGGW, New Zealand	Foliar, rhizosphere and soil inoculation
King	Oilseed rape	*B. napus*	DSV, Germany	Seed inoculation
Ladoga	Oilseed rape	*B. napus*	DSV, Germany	Seed inoculation
Veritas	Oilseed rape	*B. napus*	DSV, Germany	Seed inoculation
Hybrirock	Oilseed rape	*B. napus*	KWS, Germany	Seed inoculation
Turan	Oilseed rape	*B. napus*	KWS, Germany	Seed inoculation

Two fungal endophyte strains, one strain of *Beauveria bassiana* and one strain of *Geomyces pannorum*, originally isolated from wild brassica accessions (Chapter 3), were selected for all inoculation attempts with the brassica cultivars mentioned in Table 4.1. Fungi were recovered from long-term storage (in 20% glycerol at -80°C), defrosted at room temperature and plated onto PDA (CM0139, Oxoid Ltd., UK). Petri plates containing the fungi were then incubated for approximately two weeks at 22°C in the dark to promote sporulation. Spores were dislodged by adding 50 ml of sterile

water to the Petri plate and gently brushing the fungal colony with a sterile loop. The crude suspension was passed through a single layer of sterile Miracloth (Sigma-Aldrich®, USA) to remove mycelial fragments. One drop of Tween-20® as added to the solution to avoid the spores sticking together. The concentration of each spore suspension was estimated using a haemocytometer and adjusted to 10^6 spores per ml for both fungi. The viability of fungal spores was assessed by spraying aliquots of the prepared spore suspensions onto fresh PDA and counting the developing cfu's after 3 days of incubation at 22°C in the dark.

4.3.2 Inoculation strategies

4.3.2.1 Inoculation of brassica seed

Seed of all brassica cultivars (Table 4.1) were first surface disinfected using the stringent seed-disinfection protocol (Chapter 3, section 3.3.2.1) and soaked in a spore suspension of each endophyte strain for 10 min at room temperature. Control seeds were soaked in aqueous Tween-20® solution (1 drop of Tween-20 in 1 L of water). All treated and control seed then were transferred to sterile filter papers and allowed to dry at room temperature for 30 min. Initially non-sterile soil, sterile soil and vermiculite (non-sterile) was used for planting the seeds of cv. Hunter and then only vermiculite was used for sowing the seed of other cultivars as the growth medium proved to be successful in seedling by the fungal endophytes and is easier to work with. The soil potting mix was composed of 50% fine bark, 12.5% compost and 25% pumice plus nutrient, gypsum and Agri lime. Sterile soil was autoclaved twice at 121°C and 15 psi for 20 min. Essential nutrients including necessary macro and micronutrients were supplied using a nutrient solution according to its instruction (Thrive, Yates New Zealand) to support plant growth in vermiculite medium. Ten seeds were sown in each

pot (10 × 15 cm) which were later thinned to three seedlings per pot after germination. Pots were placed in a glasshouse with natural light at 20-25˚C. Plant health, development, and any visible disease symptoms were assessed daily. The recovery of both endophytes was assessed at three-leaf growth stage as described in section 4.3.3.1.

4.3.2.2 Inoculation of brassica foliage

Spore suspensions of *B. bassiana* and *G. pannorum* were prepared as mentioned previously. Seeds of cv. Hunter (Table 4.1) were sown in non-sterile potting mix within plastic pots (10 × 15 cm, 10 seeds in each pot) and transferred to the glasshouse as described in section 4.3.2.1. After germination, seedlings were thinned to three seedlings per pot. At the four-leaf growth stage, the tip of each stem was cut (creating a 10 cm plant height approximately) and the plants were defoliated using a sterile scalpel. A spore suspension of the fungal endophytes was then sprayed on all the above ground plant tissues with a plastic 50 mL hand mist sprayer (Arthur Holmes Ltd, NZ). For equal dispensing of fungal spores, three puffs of spray were applied to each plant. To avoid spraying on the soil, the surface of pots was covered with a plastic sheet. Control plants were only sprayed with and aqueousTween-20® solution. The efficacy of the hand-jet sprayer in the dispensing of fungal spores was assessed by spraying on a fresh PDA plate and daily observing the growth of fungus on the plate after one week of incubation at 22˚C in the dark. One new-emerged leaf was randomly selected and removed from each of the eight inoculated and eight control seedlings and assessed for of endophytes at three-leaf growth stage as described in section 4.3.3.1 (Figure 4.1).

Figure 4. 1 Four-leaf growth stage of cv. Hunter leafy turnip (left) and defoliated plants while spraying (middle) and newly grown leaves (right).

4.3.2.3 Inoculation of brassica rhizosphere

For inoculation of the plant rhizosphere, seeds of cv. Hunter (Table 4.1) were sown in potting mix within plastic pots (10 × 15 cm, 10 seeds in each pot) and transferred to the glasshouse as described in section 4.3.2.1. After germination, seedlings were thinned to three seedlings per pot. At the one-leaf growth stage, 2 ml of a fungal spore suspension, either *B. bassiana* or *G. pannorum*, was applied to the base of plant roots. For control plants, 2 ml aqueous Tween-20® solution was applied. At the three-leaf stage, the recovery of both endophytes from eight inoculated and eight un-inoculated control plants was assessed as described in section 4.3.3.1.

4.3.3 Assessment of brassica

4.3.3.1 Assessment of seedling by isolation

The frequencies of brassica seedlings initially inoculated with either of the two fungal endophytes using spore suspensions directly onto seed was determined. Assessments were completed on root and shoot (stem) tissues at four different vegetative plant growth stages; cotyledon, one-leaf, three-leaf and the four-leaf growth stage (Figure 4.2).

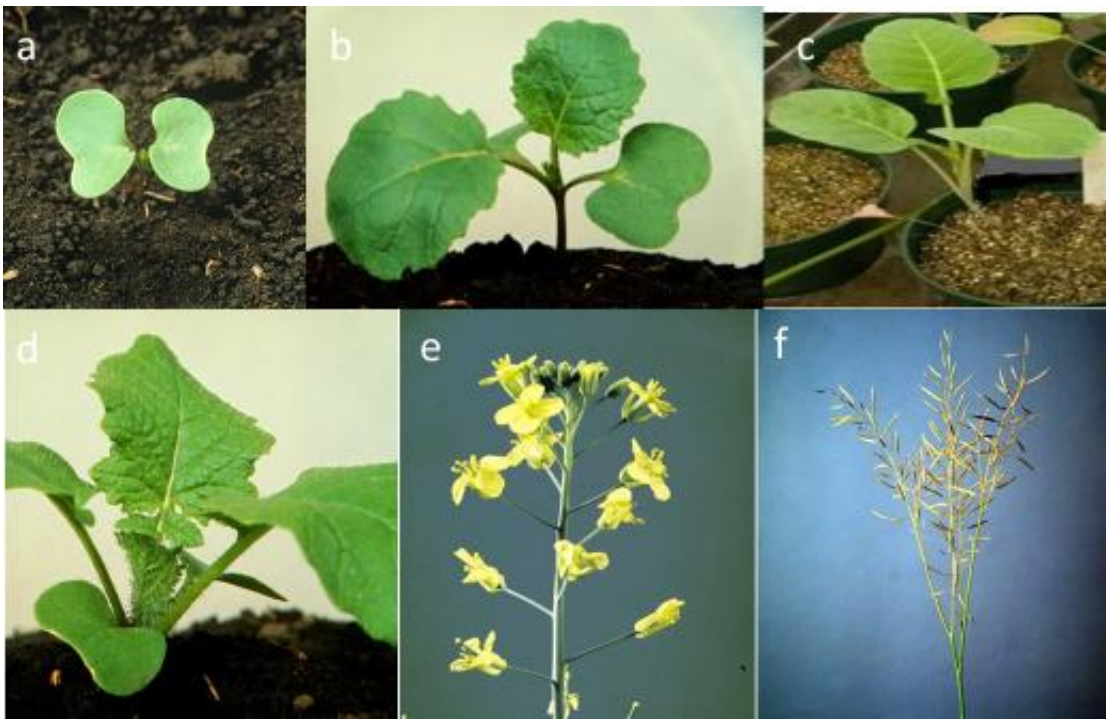

Figure 4. 2 Vegetative and reproductive growth stages of oilseed rape; (a) cotyledon; (b) two-leaf stage; (c) three-leaf stage; (d) four-leaf stage; (e) flowering (f) maturity (Canola-Council-Canada, 2012).

For each of the two endophyte strains, at each growth stage, eight inoculated and eight un-inoculated (control) plants was assessed. Each plant was removed from the growth medium and washed in tap water, primarily to remove any soil from the roots. Seedlings were then surface disinfected by washing in 5% aqueous Tween-20® solution for five min, 70% ethanol for 2 min, 5 min in 1% sodium hypochlorite followed by 70% ethanol for one min and finally three rinses in sterile tap water. (hereafter termed the stringent shoot-disinfection protocol). At the cotyledon and one-leaf stage, a modified surface disinfection protocol (hereafter termed the mild shoot-disinfection protocol) was utilised; as the stringent protocol was proven to be too harsh on the delicate plant tissues. In this protocol 70% ethanol was used for one min and 30 s rather than two min and one min. To assess the efficacy of the surface disinfection protocols, 3 × 20 µL drops of tap water from the last rinse were plated onto PDA and

at 22ºC. These PDA plates were subsequently observed every day for two weeks under a dissecting microscope for microbial growth.

Following surface disinfection, seedlings were dried on filter papers within a sterile environment for 30 min at room temperature. Root and shoot tissues were dissected from each plant with a sterile scalpel and ten 1-2 mm^2 pieces (of both root and shoot tissues) transferred to Petri plates containing PDA. Petri plates were incubated at 22˚C and checked regularly (for up to three weeks) for microbial growth with the aid of a dissecting microscope. Once fungal growth was observed, each fungal endophyte strain was initially identified by characteristic morphological features, particularly colony margin shape and colour. Axenic cultures of the two endophytes, previously grown from long-term storage, were used as a comparison. For further confirmation of endophyte species and strain, the ITS region of rDNA of selected cultures was amplified (see Chapter 3, section 3.3.2.3) and compared with the original endophyte sequences. The number of endophyte colonies that emerged from each tissue piece was recorded (up to three weeks post dissection) and data presented as the tissue frequency (TCF) for each endophyte strain as shown below.

$$\%\ Tissue\ Colonisation\ Frequency = \frac{\text{Number of infected tissue pieces}}{\text{Total number of tissue pieces assessed}}$$

4.3.3.2 Assessment of brassica off-spring following inoculation of mother plants

To determine the vertical transmission frequency of both endophyte strains within oilseed rape cv. King, plants were kept within a glasshouse at 5-15°C and allowed to flower and develop seed when temperatures reached 20-30°C. To initially assess endophyte infection of reproductive tissues, ten plants from each treatment (inoculated with strain of *G. pannorum* or *B. bassiana* or the un-inoculated control) were selected

and one secondary flowering branch from each plant raceme was cut, disinfected by stringent shoot-disinfection protocol, dissected in ten pieces and plated on PDA for fungal recovery as explained in section 4.3.3.1. The selected plants maintained in the glasshouse till the full seed maturity. Seeds were harvested by hand and stored at 4°C until required. To assess endophyte infection, seeds were surface disinfected using the stringent seed-disinfection protocol (Chapter 3, section 3.3.2.1), dried on filter paper within a sterile environment at room temperature and transferred to Petri plates containing 2% water agar (WA) to imbibe. For each seed batch, with either strain of endophyte and control treatment, 12 seedlings were transferred to sterile plastic plant containers (product number 2105646, Alto Ltd., New Zealand) (three seedlings per container) filled with Murashige & Skoog (M&S) basal salts (Murashige & Skoog, 1962) with minimal organics (M6899, Sigma-Aldrich, USA), plus 3% sucrose and 1.5% agar (Ali et al 2007). Seedlings were placed in a temperature controlled growth chamber (A1000, Conviron Asia Pacific Pty Ltd., Australia) set at 22˚C with a 16 hr photoperiod for one month. Seedlings were then dissected into shoot (petiole and stem) and root tissues with the aid of a scalpel. These tissues were further dissected into smaller pieces (1-2 mm^2) and 15 of these smaller pieces per tissue type from each seedling were transferred to Petri plates containing PDA. Petri plates were incubated at 22˚C in the dark for up to three weeks and assessed daily under a dissecting microscope for fungal growth.

4.3.3.3 Assessment of seedling using microscopy

Oilseed rape, cv. King was selected to study the endophytic behaviour of both *B. bassiana* and *G. pannorum*. Seeds of cv. King were surface disinfected and inoculated (as described earlier in section 4.3.2.1) while a control treatment immersed seed in a

sterile aqueous Tween-20® solution. Seed from all treatments was subsequently sown in sterile vermiculite and transferred to plant growth chamber (A1000, Conviron Asia Pacific Pty Ltd., Australia) at 22ºC with a 16/8 hr (light/dark photoperiod). After approximately 2 weeks, at the cotyledon growth stage, eight seedlings per treatment were selected and their stems dissected into 1 mm^2 pieces. These tissues pieces were transferred to a glass slide with a few drops of an aniline blue solution (lactic acid: water: glycerol [1:1:2], plus 0.1% aniline blue). A coverslip was placed on top and the slide was heated gently to aid penetration of the dye into the plant tissue. The slide was examined at 100× and 400× magnification under a light microscope (BX50, Olympus, New Zealand Pty Ltd) and image captured with an Olympus ColorView II digital camera and AnalySIS 3.00 image-analysis software.

4.4 Results

4.4.1 Assessment of forage brassica seedlings following inoculation of brassica seed

Endophytes of *B. bassiana* and *G. pannorum* were recovered at three-leaf stage from above and below ground tissues of cv. Hunter grown in the vermiculite and sterile soil. Under the microscope, hyphae of fungal endophytes were observed emerging from the tissues indicating the of plant tissues by these fungal endophytes (Figure 4.3). Our results showed all control plants were free from *Geomyces* and *Beauveria*.

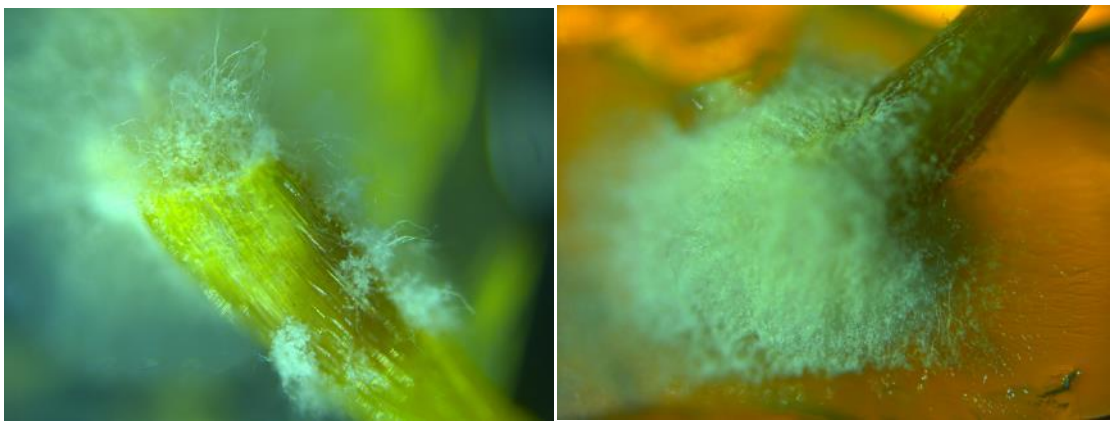

Figure 4. 3 Emergence of *B. bassiana* (left) and *G. pannorum* (right) hyphae on stem piece of inoculated seedling.

The results from re-isolation of fungal endophytes from all three inoculated forage brassica species at three and four-leaf stage grown on vermiculite demonstrated that the fungal endophytes of *G. pannorum* and *B. bassiana* colonise root and shoot tissues of all three species of forage brassica studied (*B. napus*, *B. oleracea* and *B. rapa*), (Appendix 3 and 4). The visual observation of inoculated plants showed that the endophytes does not induce any disease symptoms or change in plant growth and development of brassica plants (Figure 4.4). Control plants and testing plates for assessing of disinfection protocols were not infected with these fungal endophytes.

Figure 4. 4 Healthy forage brassica (cv. Titan rape) plants grown in vermiculite and seed inoculated with *B. bassiana*.

However, the recovery of fungal endophyte at cotyledon and one-leaf growth stage, when stringent shoot-disinfection protocol was applied, showed that none of the species were colonised by fungal endophytes; only one colony of *G. pannorum* in shoot tissue of Titan rape at one-leaf stage was re-isolated. When the re-isolation of endophytes at cotyledon and one-leaf stage was repeated by applying the mild shoot-disinfection protocol, both fungal endophytes were recovered from root and shoot tissues (Appendix 5 and 6).

The results from re-isolation of brassica species inoculated by *B. bassiana* and *G. pannorum* indicate that these fungal endophytes have no preference in of brassica species (*B. napus*, *B. oleracea* and *B. rapa*) despite the fact that their origin host species was *B. rapa*. It also demonstrated that the rate of for both endophytes is higher in shoot tissues than root parts in average of all three species studied (Figure 4.5 and 4.6).

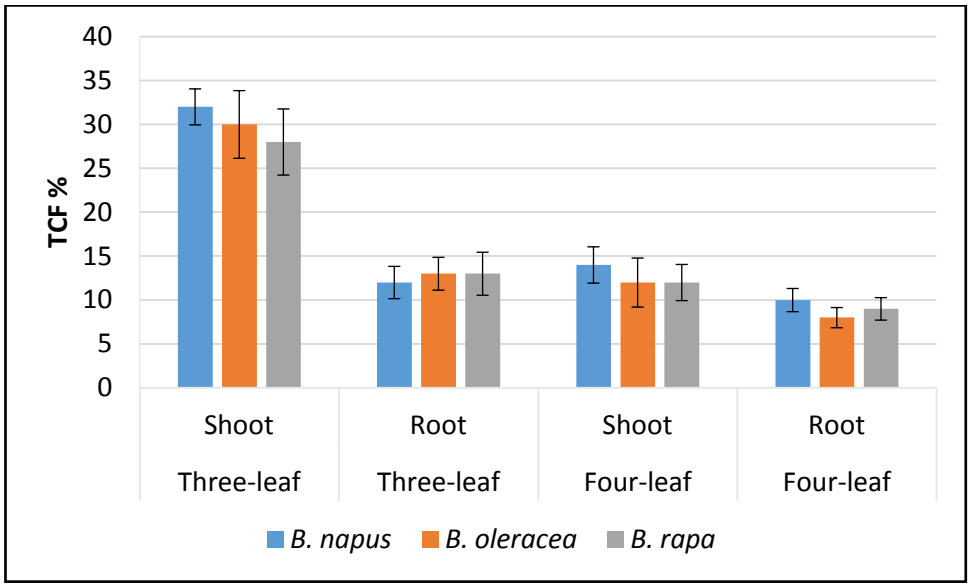

Figure 4. 5 Tissue Frequency (TCF %) of *G. pannorum* in shoot and root tissues of three forage brassica species (*B. napus*, *B. oleracea* and *B. rapa*) at three and four-leaf growth stage.(Bar line in each column shows the standard error).

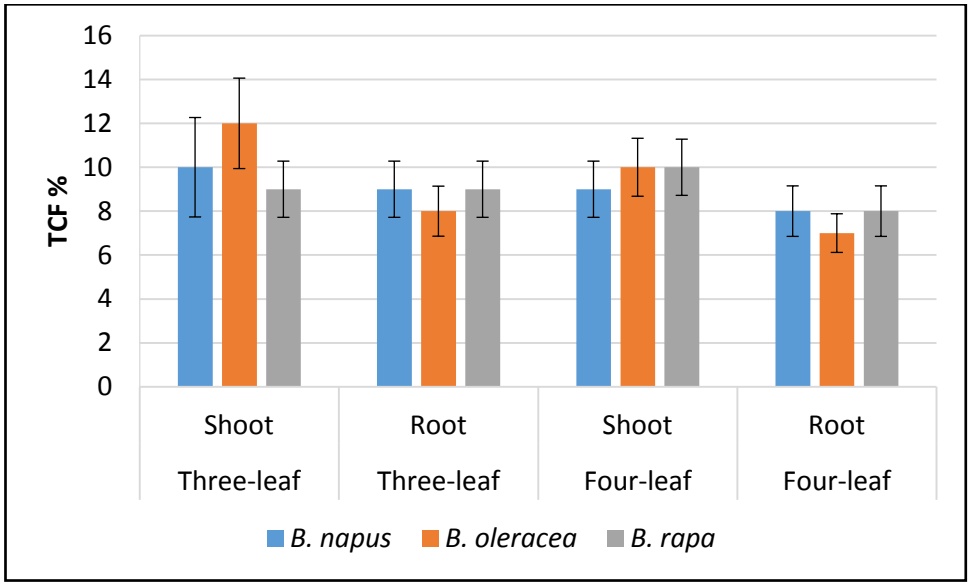

Figure 4. 6 Tissue Frequency (TCF %) of *B. bassiana* in shoot and root tissues of three brassica species (*B. napus*, *B. oleracea* and *B. rapa*) at three and four-leaf growth stage. (Bar line in each column shows the standard error).

4.4.2 Assessment of oilseed brassica seedlings following inoculation of brassica seed

Results from the recovery of *G. pannorum* and *B. bassiana* from above ground tissues of inoculated oilseed rape cultivars indicate that the endophytes colonised the shoot tissues of all 5 oilseed rape cultivars at one and three-leaf growth stage when inoculated to the seeds (Appendix 7 and 8). No fungal endophytes were isolated from untreated seeds.

The recovery of fungal endophytes from reproductive tissues of cv. King inoculated with fungal endophytes revealed that none of these endophytes could colonise the inflorescence stem and the offspring.

The pattern of these fungal endophytes in shoot tissues of oilseed rape cultivars indicates that they do not present any cultivar preference in terms of infection (Figure 4.7 and 4.8).

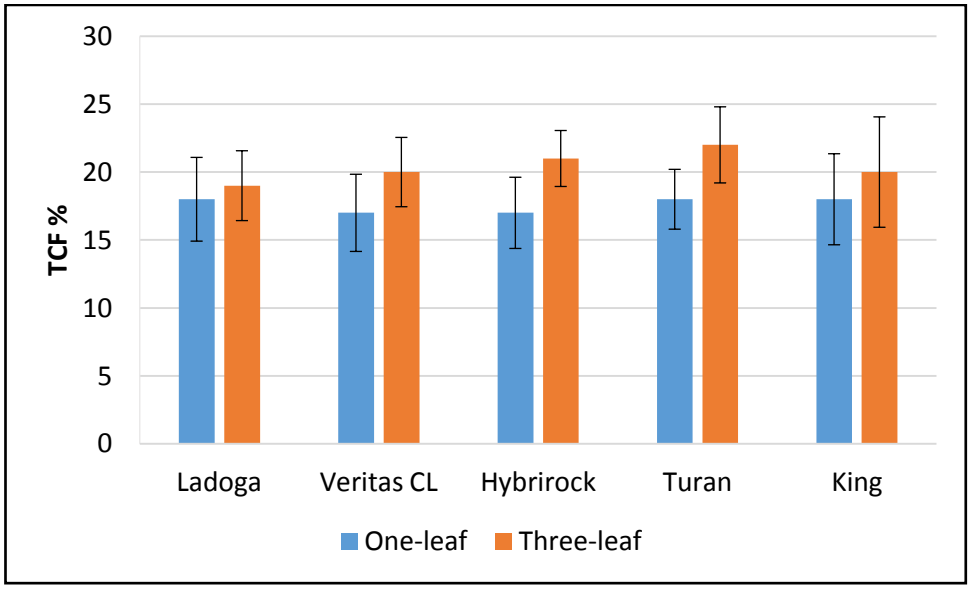

Figure 4. 7 Tissue Frequency (TCF %) of *G. pannorum* in shoot tissues of five oilseed rape (*B. napus*) cultivars at one and three-leaf growth stage. (Bar line in each column shows the standard error).

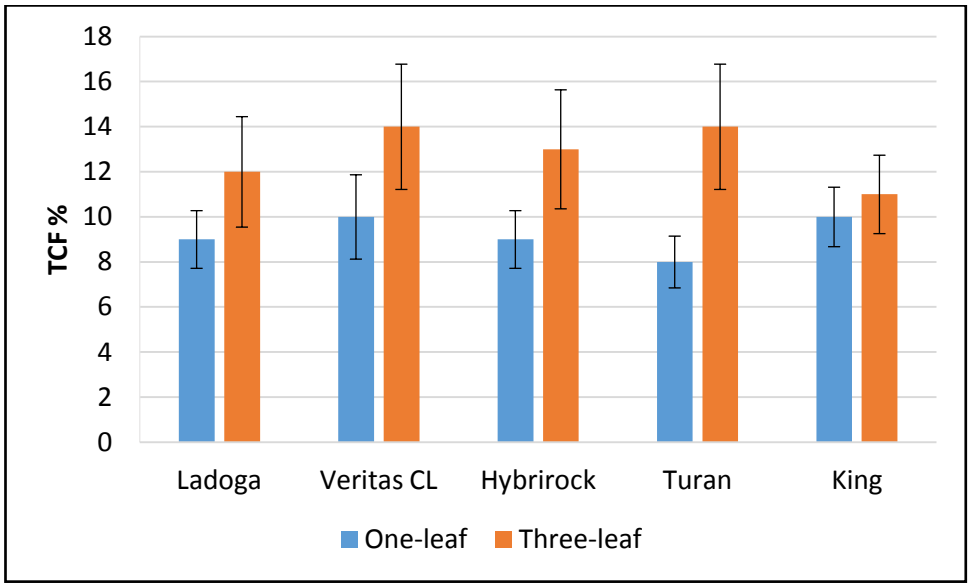

Figure 4. 8 Tissue Frequency (TCF %) of *B. bassiana* in shoot tissues of five oilseed rape (*B. napus*) cultivars at one and three-leaf growth stage. (Bar line in each column shows the standard error).

However, the average rate of infection in all oilseed rape cultivars is higher for *G. pannorum* than *B. bassiana* at both one and three-leaf growth stage (Figure 4.9).

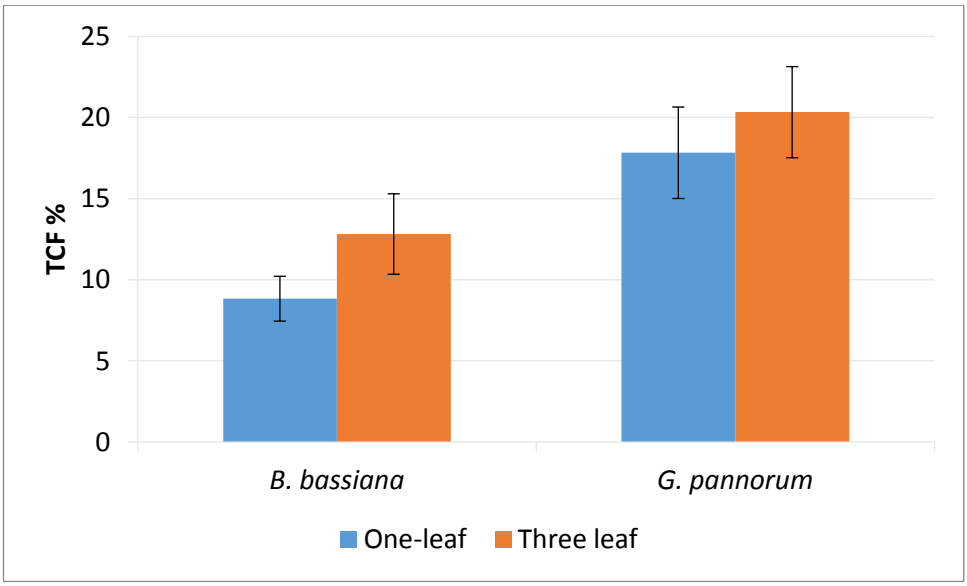

Figure 4. 9 Tissue Frequency (TCF %) of *B. bassiana* and *G. pannorum* in shoot tissues of oilseed rape (*B. napus*) cultivars at one and three-leaf growth stage. (Bar line in each column shows the standard error).

4.4.3 Assessment of brassica seedlings following inoculation of brassica foliage and rhizosphere

Re-isolation of fungal endophytes of *B. bassiana* and *G. pannorum* from the shoot tissues of foliar and soil inoculated forage brassica species (cv. Hunter), grown in non-sterile soil did not result in recovery of the endophytes. No fungal endophyte was also recovered from control plants.

4.4.4 *In planta* of brassica seedlings

When stem pieces of inoculated oilseed rape seedling (cv. King) were studied under the light microscope, large numbers of spores and hyphae were seen spread on the epidermal layers of stem (Figure 4.12). It was also observed that the spread of conidia and mycelium on stem were much more extensive for *G. pannorum* than *B. bassiana*.

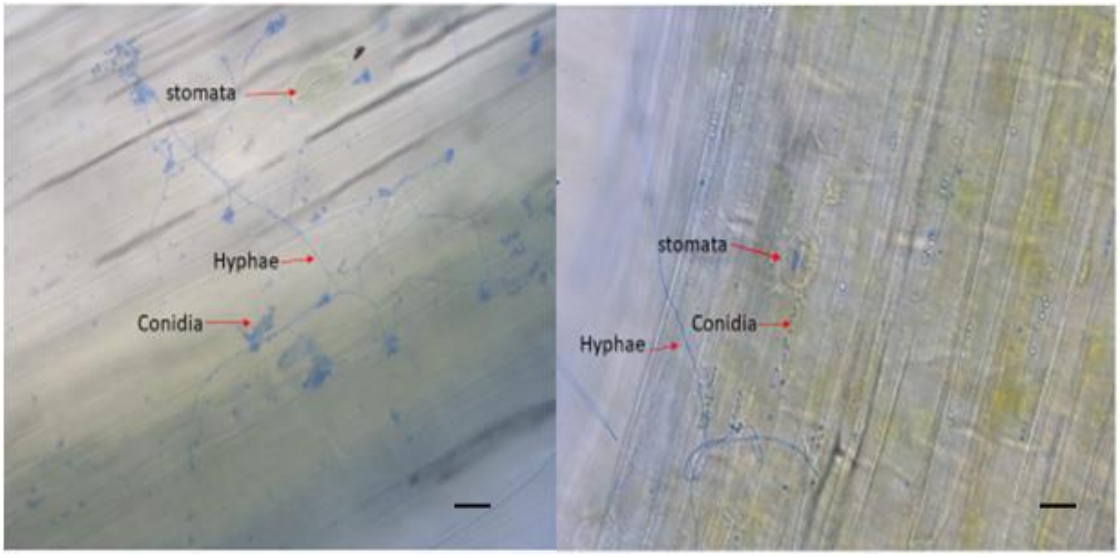

Figure 4. 10 Conidial and hyphal of *B. bassiana* (left), and *G. pannorum* (right) on stem piece of inoculated oilseed rape seedling (bar 10 µm).

These hyphae probably gained entrance through wounds and stomata from where they entered the inside of plant tissues. Unbranched hyphae extension was observed in some segments of the stem tissues (Figure 4.13).

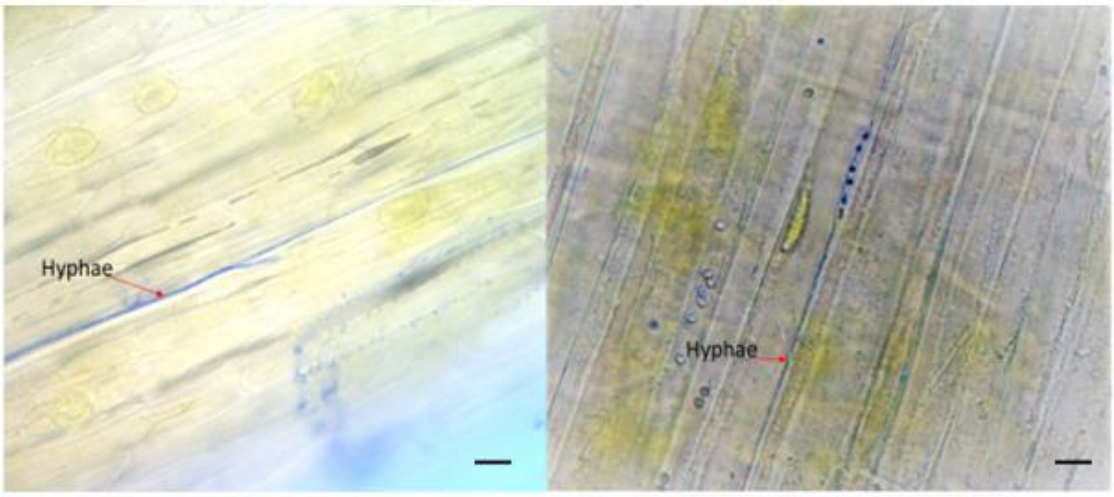

Figure 4. 11 Extension of unbranched hypha of *B. bassiana* (left), and *G. pannorum* (right) inside epidemical layer of inoculated oilseed rape stem (bar 20 µm).

4.5 Discussion

The ability of two putative fungal endophytes of *B. bassiana* and *G. pannorum*, isolated from wild brassica species, in of elite cultivars of forage brassica and oilseed rape species was investigated in this chapter. Seeds of brassica species when inoculated with the endophytes and sown in vermiculite resulted in of plant tissues indicating that these fungal endophytes are able to colonise both root and shoot plant tissues of three species of forage brassica namely, *B. napus*, *B. oleracea* and *B. rapa*. This is consistent with the results of other studies that show *B. bassiana* can establish a endophytic in sorghum (Tefera & Vidal, 2009) cotton and tomato (Ownley *et al.*, 2008) when their seed inoculated by fungal conidia.

Although the origin host of these fungal endophytes was *B. rapa* species, the result from inoculation indicates that neither strain shows any host specificity. This is consistence with the result of another study that showed a strain of *B. bassiana* isolated from another host can colonise other species such as *B. napus* (Vidal & Jaber, 2015) and *B. oleracea* (Gautam *et al.*, 2016). The recovery of these fungal endophytes from

plant tissue of oilseed rape cultivars also emphasises the lack of genotype preference in terms of . Rodriguez et. al. (2009) reported that many non-Clavicipitaceous endophytes (NC-endophytes) have a broad range of host species and are able to colonise both above and below ground tissues of host plant. The fungal species of order Sebacinales of Basidiomycetes such as *Piriformospora indica* is an example of these endophytes. This unique and interesting fungus which is originally isolated from the roots of xerophytic woody shrubs such as *Prosopis juliflora* and *Zizyphus nummularia* lacks host specificity and is cosmopolitan in nature and capable of colonising roots of many plant species (Johnson *et al.*, 2014). However, other studies also indicate some NC-endophytes show host specificity at the species level or host preference within a species and tissue type (Cannon & Simmons, 2002, Sun *et al.*, 2012).

The visual observation of growth and development of plants infected by *B. bassiana* and *G. pannorum* indicate that these two fungal endophytes present no symptoms of disease infection in plant tissues and the plants did not show any change in growth performance and development. It implies that these endophytes are probably able to colonise the plant tissues through the utilization of plant assimilate to build and extend their structural organs. It has been reported that infection of *B. bassiana* in sorghum (Tefera & Vidal, 2009) and corn (Lewis *et al.*, 2001) have not had any adverse effect on plant growth, seed germination and do not cause any plant disease development.

Although the isolates of *B. bassiana* and *G. pannorum* in the origin host plants completely colonised all root, shoot tissues, and showed a complete infection, the re-isolation of these fungal endophytes from new host plants demonstrated a point infection ability of these endophytes. In this study, none of the new host plants was

completely colonised by the endophytes and potential tissue rate was below 30 percent in all brassica species studied.

In our study, the data from rate of in tissues of root and shoot components indicated that both fungal endophytes have a higher rate in shoot tissue than root and *G. pannorum* presents a higher rate of than *B. bassiana* in general. This is consistent with the result from other studies that show *B. bassiana* can colonise both above ground and below ground tissues of cotton, bean and tomato seedlings (Ownley *et al.*, 2008). They also reported that a strain of *B. bassiana* was only detected down to 4 cm of root and up to 8 cm above the soil line for the aerial portion of cotton seedlings and the rate of in shoot tissues was higher that root tissues (Ownley *et al.*, 2008).

B. bassiana and *G. pannorum* are known as soil fungi (Bidochka *et al.*, 1998, Hayes, 2012) therefore when added to the soil through inoculated seeds, they will probably germinate in the soil and produce many spores which can spread all over the plant tissues. This may partly explain the horizontal dissemination of these endophytes and establishment of a point infection. When the plant tissues of inoculated brassica were examined under a microscope, the presence and spread of many conidial endophytes on the surface of plant tissues was observed (Figure 4.12). Some of these conidia could probably germinate on the epidemical layer of some individual plants, go through the natural openings such as wounds and stomata and gain entrance to the internal part of plant tissues, and established a point (Figure 4.13). Electron microscopy of cauliflower leaves has revealed that *B. bassiana* can randomly germinate on the epidermis cells. Conidia imbibe water from surrounding leaf surface to germinate and exert mechanical pressure, leading to the rupture of the outer cell and the hyphae penetrate into the leaf tissues. After penetration, the primary hyphae branch and

develop multicellular mycelial network. Hyphae can also directly extend into neighbouring epidermal cells and/or grow into the intercellular spaces. (Gautam *et al.*, 2016). In corn plants, *B. bassiana* has been reported that produce extensive mycelium growth on the plant surface and few hyphae could colonise the vascular tissues (Wagner & Lewis, 2000). It has been reported that some endophytic microorganisms may reside in both external and internal tissues simultaneously (Santamaría & Bayman, 2005). Regarding the of plants by *G. pannorum* reports are very limited. It has been shown that the fungus can colonise the root of *Rhododendron* sp. and *Vaccinium* sp. and form intracellular coils in the rhizodermal plant cells in a way similar to certain mycorrhiza (Vohník *et al.*, 2007).

In this study, we could not recover the fungal endophytes of *B. bassiana* and *G. pannorum* from the reproductive tissues of inoculated brassica. This again indicates the lack of systemic transmission of these fungal endophytes in brassica. This is inconsistent with reports of vertical transmission of *B. bassiana* in poppy plants (Quesada-Moraga *et al.*, 2014). In their study *B. bassiana* was detected from surface-sterilised leaf pieces and seeds of 50% of capsules samples of seed-inoculated plants. When these seeds were planted in sterile soil, the fungus also recovered from 42% of the emerged seedlings. However, it is better to trace the presence or lack of internal systematic of fungal endophyte inside the plant tissues through other techniques such as marker genes like green florescence protein (Maor *et al.*, 1998, Zakria *et al.*, 2008).

Regarding *G. pannorum* there is no evidence showing the potential ability of this fungus in of other host plants species. This fungal endophyte has been isolated from leaves of *Colobanthus quitensis*; a dicotyledonous plant that lives in Antarctic regions (Rosa *et al.*, 2010) and associated with the seeds and needles of *Pinus monticola*

(Ganley & Newcombe, 2006). Geomyces fungi have a global distribution and the known species are common soil fungi of temperate and high-latitude ecosystems as well as marine and cold environments but this study provides the first evidence that it has an association with brassica species.

of *B. bassiana* and *G. pannorum* in plant tissues did not occur when inoculated seeds were sown in non-sterile soil or when fungal endophytes applied directly on non-sterile plant rhizosphere. This can probably be due to the interaction of present microorganisms in the soil and fungal endophytes. Tefera and Vidal (2009) reported that *B. bassiana* is able to colonise leaves, stems, and roots of young sorghum seedlings regardless of inoculation method (leaf, seed or soil inoculation). In their study plant growth medium had a great influence on rate of *B. bassiana* in sorghum so that sterile soil and vermiculite increased the rate of endophytic . They suggested the lower endophytic in non-sterile soil could be due to the influence of biotic factors and soil antagonism which inhibit the germination of *B. bassiana* conidia or prevent its penetration to root (Tefera & Vidal, 2009). Common soil fungi such as *Penicillium utricae* (Shields *et al.*, 1981) and *Aspergillus clavatus* (Majchrowicz *et al.*, 1990) have been reported to produce water soluble inhibitor and fungicidal metabolites respectively which control survival of *B. bassiana* in soil. Low persistence of *B. bassiana* in soil medium has also been reported in other studies (Pereira *et al.*, 1993, Vänninen *et al.*, 2000).

Foliar conidial application of *B. bassiana* and *G. pannorum* did not result in of newly formed leaves of brassica plant as no fungal colonies emerged from disinfected tissues pieces. This could either be due to the lack of systemic of these fungal endophytes in brassica or because the spore of endophytes could not survive in the environment. It is

been reported that viability of facultative endophytes such *B. bassiana* is impacted by many environmental conditions such as unfavourable temperature and humidity (Bing & Lewis, 1991). Our result is however inconsistent with others reporting that *B. bassiana* colonises the leave tissues of cauliflower (*B. oleracea* L.) (Gautam *et al.*, 2016), corn (*Zea mays* L.) (Bing & Lewis, 1991, Bing & Lewis, 1992) and poppy (*Papaver somniferum* cv. *nigrum* L.), (Quesada-Moraga *et al.*, 2006) following foliar spraying.

Surface tissue disinfection is a critical issue for recovery of fungal endophytes. It has been reported that disinfection strategy should be adjusted according to tissue type since sterilise solution may diffuse into the tender plant tissue and kill the endophytes (Ownley *et al.*, 2008). In our study, we could not re-isolate the endophytic fungi from the brassica plant tissues at cotyledon and one-leaf growth stage when the stringent shoot-disinfection protocol was applied; they were then recovered when the mild shoot-disinfection protocol was used. Therefore, we recommend setting up a proper disinfection protocol according to plant species and tissue type before recovery of endophytes in inoculation studies.

5. Chapter 5. Bioactivity of endophytic fungal and bacterial of brassica

5.1 Abstract

Endophytic microorganisms are able to confer a range of benefits on their associated plants such as plant disease resistance and plant growth promotion. In this chapter the two fungal endophytes of *Beauveria bassiana* and *Geomyces pannorum* and 13 different species of bacterial endophytes of brassica which were previously isolated from a wide range of wild brassica species were tested for their antifungal activity against the plant pathogenic fungus of *L. maculans*, the causal agent of phoma stem canker in oilseed rape. Also two dominant bacterial endophytes of *Methylobacterium fujisawaense* and *Me. phyllosphaerae* were studied for their plant growth promotion bioactivity in oilseed rape. The fungal endophytes applied as seed inoculation and the bacterial endophytes inoculated through root dipping using bacterial suspension. For antifungal bioactivity a dual culture test carried out and inhibition zone was measured. For *in planta* test (just tested for fungal endophytes), a two times growth chamber experiment was carried out and lesion size caused by *L. maculans* on cotyledon leaves was measured and scored. For the plant growth promotion test, the fresh weight of oilseed rape plants was measured and analysed. Data from inhibition zones indicate that both fungal endophytes and almost half bacterial species (6 out of 13) presented an antifungal activity against *L. maculans* by creating a high inhibition zone. When two fungal endophytes were inoculated into the seed of a susceptible cultivars of oilseed rape and conidia of *L. maculans* was applied on the cotyledon leaves, a smaller lesion size appeared on the cotyledon in comparison to non-endophyte inoculated seedlings. Our results indicated that *B. bassiana* and *G. pannorum* can colonise the oilseed rape seedling and once seed inoculated can significantly increase plant resistance against

phoma stem canker caused by *L. maculans* suggesting they can be used as biological control agents. The results from inoculation of bacterial endophyte to the root of oilseed rape seedlings showed that they significantly enhance plant growth by producing higher fresh weight of the seedlings. This is the first reports that demonstrate the endophytes of wild brassica species confer plant resistance against a plant pathogen in an oilseed rape cultivar and enhance growth.

5.2 Introduction

Endophytic microorganisms are able to confer a range of benefits to host plants including resistance against disease and growth promotion of in return for shelter and nutrition (Raps & Vidal, 1997, Rosenblueth & Martínez-Romero, 2006, Guo *et al.*, 2008, Powell, 2009, Rodriguez *et al.*, 2009). Many beneficial fungal endophytes, including Ascomycetes such as, *Trichoderma* sp. and *Heteroconium chaetospira* that associate with brassica species, can provide resistance to diseases, insect pests, heavy metal toxicity and enhancement of plant growth (Card *et al.*, 2015). Beneficial bacterial endophytes also play important roles in plant growth promotion and health (Nelson, 2004) through, for example, nitrogen fixation, increased nutrient mobilization, production of phytohormones including auxins and cytokinins and suppression of ethylene levels under stress conditions (Card *et al.*, 2015). Growth promotion may also be induced through inhibition of pathogens via competition for nutrients and space, antibiosis and induction of plant defence mechanisms (Hallmann *et al.*, 1997, Weyens *et al.*, 2009). For example, some strains of *B. subtilis* can colonise the root tissues of oilseed rape plants, promoting growth and increasing seed yield under field conditions (Hu *et al.*, 2005, Hu *et al.*, 2011).

Phoma stem canker or blackleg is a serious worldwide disease of brassica species. It

causes large yield losses and is a major constraint to the production of oilseed rape in Europe, Australia, New Zealand and North America (West *et al.*, 2001, Fitt *et al.*, 2006, Lob *et al.*, 2013). The disease is caused by two pathogens namely *Leptosphaeria maculans* and the related *L. biglobosa*. These pathogens are capable of seed transmission in a wide range of brassica species including *B. oleracea, B. napus, B. rapa* and therefore are widely distributed. Although both pathogens are responsible for stem rot in oilseed rape, *L. maculans* is considered the major species responsible for stem canker in this crop in many countries, including New Zealand, causing severe yield loss (West *et al.*, 2001). The first record of *L. maculans* in New Zealand was reported by Cunningham in 1927 who isolated the species from swede and turnip crops (Cunningham, 1927).

Leptosphaeria species infect different types (winter and spring) of oilseed rape varieties and causes cankers that result in death of host plants. In New Zealand, forage brassica crops are also frequently infected by this pathogen, causing dry rot disease in turnips and swedes (Lob, 2014).

Endophytic microorganisms that establish a close association with plants can be used as Biological Control Agents (BCAs) to halt or supress pathogenic microorganisms of brassica (Card *et al.*, 2015). This approach to disease control is considered an environmentally friendly method because of the limited negative side effects and has consequently has received a lot of attention among agricultural scientists looking for novel methods to control plant diseases and reduce the use of synthetic chemical fungicides (Pal & Gardener, 2006).

Some non-endophytic fungal and bacterial BCAs are able to control phoma stem canker in oilseed rape (Beatty & Jensen, 2002, Danielsson *et al.*, 2007, Abuamsha *et*

al., 2011, Hammoudi *et al.*, 2012). However, the patterns of different microorganisms in plants are varied and for successful utilisation of microbial agents for controlling soil-borne pathogens, the plant rhizosphere must be colonised by the antagonists (Weller, 1988). The antagonistic microbes must also able to compete with the other microorganisms present within the biocontrol arena (Milus & Rothrock, 1993).

In this study we isolated two endophytic fungi from wild brassica species; *Beauveria bassiana* and *Geomyces pannorum,* which were subsequently found to form artificial or novel associations with a range of oilseed rape cultivars (Chapter 3 and 4). Although *B. bassiana* is classically described as an entomopathogen there are reports in the literature of strains with antifungal activity against plant pathogenic fungi (Ownley *et al.*, 2004, Ownley *et al.*, 2008). *Geomyces* spp. are common soil fungi of temperate and high-latitude ecosystems as well as marine and cold environments with a global distribution. They have psychrotolerance properties that enable them to thrive in cold and low-nutrient arctic regions. The fungus can form intracellular coils in the rhizodermal plant cells in away similar to ericoid mycorrhiza (Vohník *et al.*, 2007). In response to low temperature the fungus can change its fatty acid composition and metabolic pathway and tolerate cold temperatures (Hayes, 2012). *G. pannorum* produces bioactive metabolites including antimicrobial asterric acid derivatives called "geomycins" which are active against *Aspergillus fumigatus* as well as Gram-positive and Gram-negative bacteria (Li *et al.*, 2008).

Screening wild brassica species for the presence of endophytic bacteria in this study (Chapter 3) resulted in the isolation of 17 bacterial species and almost all of the plant accessions dominantly harboured two bacterial species, *Me. fujisawaense* and *Me. phyllosphaerae* (Roodi *et al.*, 2016). Methylobacterium are associated with a number

of plant species including many within the Brassicaceae (Idris *et al.*, 2004, Idris *et al.*, 2006) and are able to produce plant hormones and influence plant development including branching, seedling vigour, seed germination, root differentiation and photosynthetic activity (Trotsenko *et al.*, 2001, Prombunchachai *et al.*, 2017).

This chapter aimed to test the antifungal bioactivity of the two isolated endophytes of brassica against *L. maculans*, the causal agent of phoma stem canker in oilseed rape. In addition, *Me. fujisawaense* and *Me. phyllosphaerae*, the two dominant bacterial species of wild brassicas, were assessed for potential benefits from increased plant growth in oilseed rape plants.

5.3 Material and methods

5.3.1 Plant growth promotion

To investigate the plant growth promotion bioactivity of *Me. fujisawaense* and *Me. phyllosphaerae*, two strains from the isolated colonies that were previously stored in a -80 ºC freezer were selected based on their high tissue rate in the original plants. The bacteria were plated in a nutrient agar (NA) (CM003, Oxoid Ltd., UK) and incubated for two weeks at 22℃. For each strain, a bacterial suspension of 10^9 cells per ml was prepared using serial dilution and counting the cells with haemocytometer under the light microscope (BX50, Olympus, New Zealand Pty Ltd) to which was added one drop of Tween-20® solution (Sigma-Aldrich, Inc., New Zealand). The oilseed rape (cv. King, *B. napus*, provided by DSV-Germany) was selected as host plant. Seed was surface disinfected using the stringent seed-disinfection protocol (Chapter 3, section 3.3.2.1). After disinfection seed was put on a filter paper (110 mm, Thermo Fisher Scientific Inc., New Zealand) under a laminar flow cabinet to dry and then transferred to Petri plates containing 2% water agar (WA). Petri plates were incubated at 22ºC in a

custom-made lighting room with 18/6 hr (light/dark photoperiod) to initiate germinate. Following germination, the root tip of each seedling was excised with a sterile scalpel, dipped into the prepared bacterial suspension and transplanted into sterile pots (7 × 15 cm) containing potting mix (50% fine bark, 12.5% compost and 25% pumice plus nutrient, gypsum and Agri lime). The potting mix was autoclaved at 121°C, 15 psi, which was repeated after 24 hr to destroy potential bacterial endospores that may have survived the previous round. Control seedlings, after excising the root, were dipped in sterile water containing one drop of Tween-20® per litre. Then 40 ml of sterile water was added to each pot, pots were lidded and placed in the plant growth chamber (A1000, Conviron Asia Pacific Pty Ltd., Australia) at 18ºC with a 16/8 hr (light/dark photoperiod). The experiment was laid out in a completely randomised design with eight replications. Each experimental unit comprised five pots/plants. After one month plants were removed and all soil debris removed by washing and each plant placed on a filter paper for 8 hr at room temperature (25ºC) to completely dry. The seedlings were weighed (0.001 g scale) and the mean weight of five plants in each experimental unit were used for analyse of variance using SPSS software (IBM© SPSS© Statistics, ver. 24, USA).

5.3.2 Antagonistic bioactivity of brassica endophytes and *L. maculans*

5.3.2.1 *In vitro* assay

The antagonistic bioactivity of bacterial and fungal endophytes previously isolated from wild brassica (Chapter 3) was assessed *in vitro* against our target pathogen, *L. maculans*. This assessment included two fungal species, *B. bassiana* and *G. pannorum* and thirteen bacterial species, *Bacillus mycoides, Brevundimonas vesicularis, Caulobacter mirabilis, Kocuria palustris, Me. fujisawaense, Me. phyllosphaerae,*

Novosphingobium resinovorum, Paenibacillus hordei, Plantibacter flavus, Pseudomonas azotoformans, Stenotrophomonas rhizophila, Sphingomonas mucosissima and *Sphingom. insulae*. The *L. maculans* strain utilised is a virulent pathogen of *Brassica* sp. isolated from brassica crops in New Zealand (Personal communication with Eirian Jones, Bio-protection centre). Dual culture tests were carried out, with NA medium for bacterial and potato dextrose agar (PDA) (CM0139, Oxoid Ltd., UK) for fungal strains to assess. Bacterial and fungal endophytes, previously stored at -80°C, were thawed and streaked on NA and potato PDA, respectively, and incubated for two weeks at 22ºC. For each bacterial strain a cell suspension at 10^9 cfu/ml was prepared (see section 5.3.1) and 50 µL of suspension placed in a line at the centre of Petri plate and streaked evenly. Control treatments were streaked with sterile water instead of a bacterial solution. Petri plates were incubated for two weeks at 20ºC and then two mycelial plugs (5mm diameter) of PDA taken from the actively growing region of a two-week old *L. maculans* culture were placed 25 mm from the edge of each plate opposite each other (Figure 5.1); there were 10 replicate plates for each treatment.

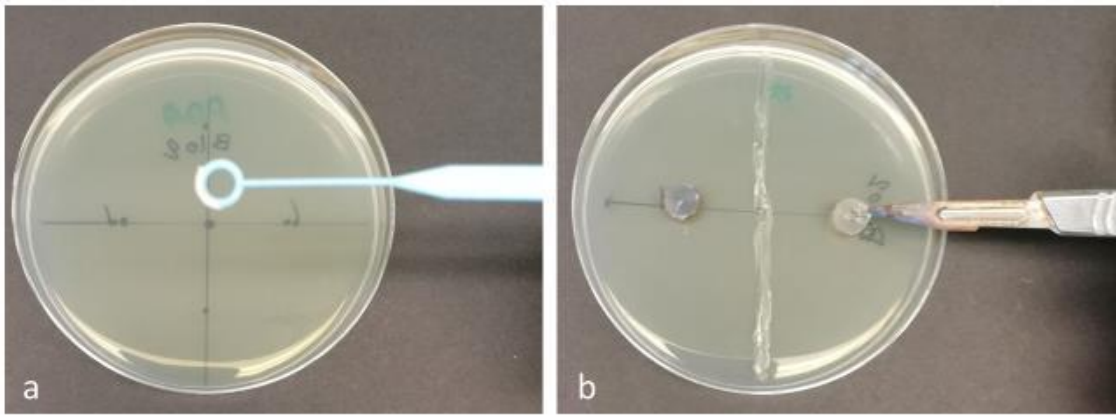

Figure 5. 1 Dual culture test plates. Streaking the bacterial suspension in a line at the centre of the Petri plate (a) and placing two mycelial plugs of *L. maculans* at the edges of plate after two weeks of incubation (b).

When the two fungal colonies of *L. maculans*, placed on opposite sides of the control plates, had grown sufficiently to close the gap between them, the distance between the bacterial colony and the *L. maculans* colony was measured using a digital calliper (Mitutoyo Corporation, Japan) to determine any antagonistic effect of endophytes against the pathogen. The inhibition zone was rated using a 5 points scaling system; 4 (very high): inhibition zone >10 mm; 3 (high): imbibition zone 5-10 mm; 2 (medium): inhibition zone 5mm; 1(low): *L. maculans* growth stopped at the bacterial streak line; 0 (zero): no inhibition zone with *L. maculans* typically growing over the bacterial streak (Hammoudi *et al.*, 2012). To test the antagonistic bioactivity of two endophytic strains and *L. maculans*, one disc of endophyte mycelium (5 mm) and one disc of *L. maculans* mycelium, both grown on two-week old PDA (incubated at 22℃ in the dark), were placed 25 mm from the edge of each plate dish opposite each other. Petri plates incubated at 20℃ and the distance between fungal pathogen and fungal endophyte colonies (antagonism) was scored as described above.

5.3.2.2 *In planta* assay

The antagonistic bioactivity of two fungal endophytes (*B. bassiana* and *G. pannorum*) against *L. maculans* was tested on oilseed rape plants (*B. napus*), cultivar Flash, which is susceptible to *L. maculans* (Lob, 2014, Anonymous, 2018). This was achieved via infecting the cotyledon leaf of seedlings using a spore suspension. To prepare the suspension fungal mycelium of *L. maculans* stored in 20% glycerol at -80°C was defrosted at room temperature and plated onto PDA. Petri plates containing the pathogen were incubated for approximately two weeks at 15-20℃ and 16/8 hr (light/dark) using natural light to promote sporulation. Spores were dislodged by adding 50 ml of sterile water to the Petri plate and gently brushing the fungal colony with a sterile loop. The crude suspension was passed through a single layer of sterile Miracloth (Sigma-Aldrich®, Inc., New Zealand) to remove mycelial fragments. One drop of Tween-20® was added to the solution to prevent the spores from sticking together. The concentration of each spore suspension was estimated using a haemocytometer and adjusted to 10^7 spores per ml. The viability of fungal spores was assessed by spraying aliquots of the prepared spore suspensions on fresh PDA and observing the developing colonies after 5 days of incubation at 18°C in the dark.

Seedlings were produced by planting seeds in a tray containing potting mix (see section 5.3.1 for constituents) and water. The tray was covered with a lid, sealed with plastic tape to keep humidity in the tray elevated and placed in a controlled environment (A1000, Conviron Asia Pacific Pty Ltd., Australia) at 18°C and 16/8 hr (light/dark) lighting conditions to initiate germination. At the cotyledon leaf stage, 10 seedlings were selected, one cotyledon leaf per each seedling was punctured with a sterile needle and 15 µL of the *L. maculans* prepared suspension placed on the wound

site using a pipette and sterile tip. Control plants were treated with sterile aqueous Tween-20® (one drop in a litre of water). Trays were kept in the growth chamber under the same conditions for two weeks to allow any disease symptoms to appear.

Seeds of oilseed rape (cultivar Flash) were disinfected and inoculated with fungal endophytes of *B. bassiana* and *G. pannorum* as described in chapter 4, section 4.3.2.1. Control plants were treated with sterile aqueous Tween-20®. Seeds were then put on sterile filter paper to dry and transferred to sterilise plastic plant containers (product number 2105646, Alto Ltd., New Zealand) containing autoclaved vermiculite (autoclaved at 121°C, 15 psi). Germinated seeds were then planted in seedling trays containing sterile (autoclaved at 121°C, 15 psi and repeated after 24 hr to destroy potential bacterial endospores that may have survived the previous round) potting mix.

A completely randomised trial was set up of three treatments with four replications. Treatments were; inoculation with *B. bassiana*, inoculation with *G. pannorum* and a control treatment. The experiment was repeated two times (during April-June, 2018). Each treatment consisted of a tray containing 15 seedlings. Trays were kept in a plant growth chamber under the conditions described above. To test the effectiveness of the inoculation procedure 10 inoculated and un-inoculated germinated seeds were planted on separate trays. After two weeks (cotyledon stage) seedlings were removed from the trays, surface disinfected with mild shoot-disinfection protocol and assessed for infection using the method described in chapter 4 (section 4.3.3.1).

At the cotyledon leaf stage 10 seedlings from each tray were selected; one cotyledon leaf of each seedling was punctured with a sterile needle and infected with *L. maculans* suspension. The remaining 5 seedlings in each tray were also punctured using the same method but treated 15 µL of sterile aqueous Tween-20® litre. Trays were placed back

in the growth chamber for two weeks; emerging new leaves were removed at regular intervals to prevent plant development (Hammoudi *et al.*, 2012). After two weeks, the severity of phoma stem canker on the cotyledon leaf was assessed and scored (Hammoudi *et al.*, 2012). Absence of symptoms = score of 0: no lesion; 1: lesions on the infection site (diameter) < 1.5 mm; 2: lesions on the infection site 1.5 - 3.0 mm; 3: lesions on the infection site > 3.0 mm; 4: grey to green tissue collapse 3.1 - 5.0 mm; 5: grey to green tissue collapse > 5.0 mm (≤10 pycnidia); 6: grey to green tissue collapse > 5.0 mm (>10 pycnidia). The mean score from 10 infected seedlings from each tray was used in the analysis. ANOVA was undertaken using SPSS (IBM© SPSS© Statistics, ver. 24, USA) statistical software.

5.4 Results

5.4.1 Plant growth promotion

The analysis of variance of weight of plants indicates that inoculated oilseed rape plants significantly increased the growth of seedlings compared with un-inoculated seedlings (Table 5.1, Figure 5.2). However, *Me. fujisawaense* inoculated seedlings had a mean weight of 1.33 gr/plant, significantly higher ($P<0.05$) than *Me. phyllosphaerae* inoculated seedlings (0.88 g/plant) (Figure 5.3). The fresh weight of control plants (0.69 g/plant) was significantly lower than both inoculation treatments ($P<0.05$).

Table 5. 1 Analysis of variance of the fresh weight of oilseed rape plants inoculated with *Me. fujisawaense* and *Me. phyllosphaerae* compared with un-inoculated.

Source of variation	**Sum of squares**	**Degree of freedom**	**Mean square**	**F value**	**Significance**
Between groups	1.738	2	0.869	44.905	<0.0001
Within groups (treatments)	0.406	21	0.019		
Total	2.144	23			

Figure 5. 2 Growth of oilseed rape plants when un-inoculated (top row) and inoculated with *Me. fujisawaense* (middle row) and *Me. phyllosphaerae* (bottom row).

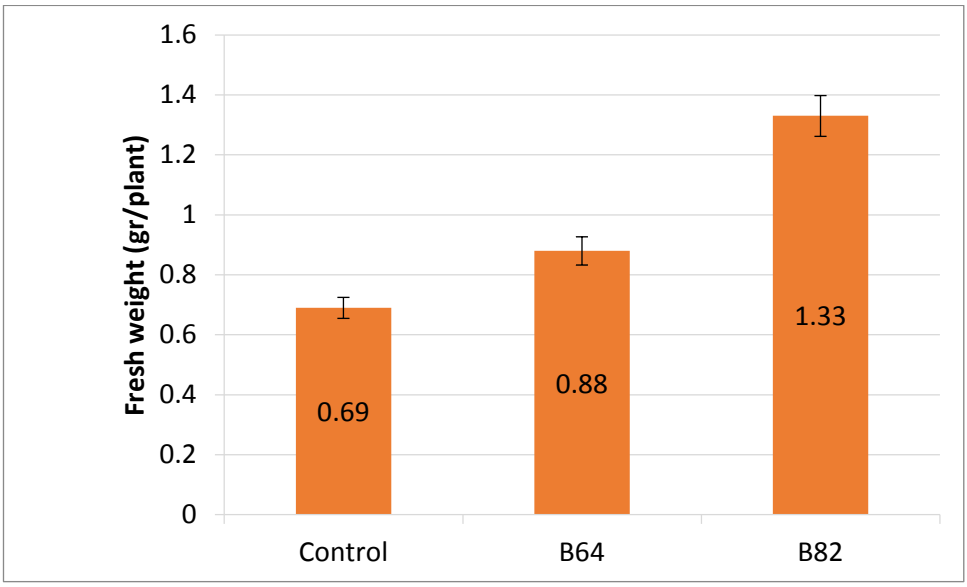

Figure 5. 3 Mean fresh weight of oilseed rape plants inoculated with *Me. fujisawaense* (B82) and *Me. phyllosphaerae* (B64) and un-inoculated (control) plants. (LSD 0.05% = 0.143). (Bar line in each column shows the standard error).

5.4.2 Antagonistic bioactivity of bacterial endophytes and *L. maculans*

5.4.2.1 *In vitro* assay

The results from dual culture test demonstrate that some endophytic bacterial strains and the *L. maculans* have antagonistic behaviour to each other by creation of inhibition zone (Table 5.2). The strongest inhibition effect was observed in *Me. fujisawaense* and *Me. phyllosphaerae N. resinovorum*, *Ps. azotoformans*, *Pl. flavus* and *St. rhizophila* which all showed a large, clear inhibition zones between the edge of the bacterial streak and the pathogen. However, *L. maculans* was not affected by *K. palustris*, *Sphingom. Mucosissima*, *Sphingom. Insulae*, *Ba. mycoides* and *B*r. *Vesicularis*; no inhibition zone produced (Figure 5.4).

Table 5. 2 Inhibition zone (scoring*) created in dual culture between bacterial and fungal endophytes of brassica and *L. maculans*.

Inhibition zone	**Bacterial endophytes**	**Fungal endophytes**
0 (Zero)	*Bacillus mycoides* *Brevundimonas vesicularis* *Kocuria palustris* *Sphingomonas insulae* *Sphingomonas mucosissima*	
1 (Low)	*Paenibacillus hordei*	
2 (Medium)	*Caulobacter mirabilis*	
3 (High)	*Methylobacterium fujisawaense* *Methylobacterium phyllosphaerae* *Novosphingobium resinovorum* *Pseudomonas azotoformans* *Plantibacter flavus* *Stenotrophomonas rhizophila*	*Beauveria bassiana* *Geomyces pannorum*
4 (Very high)		

* 4: inhibition zone >10 mm; 3: imbibition zone 5-10 mm; 2: inhibition zone < 5mm; 1: growth stopped at the bacterial streak line; 0: no inhibition zone or *L. maculans* was growing over the bacterial streak.

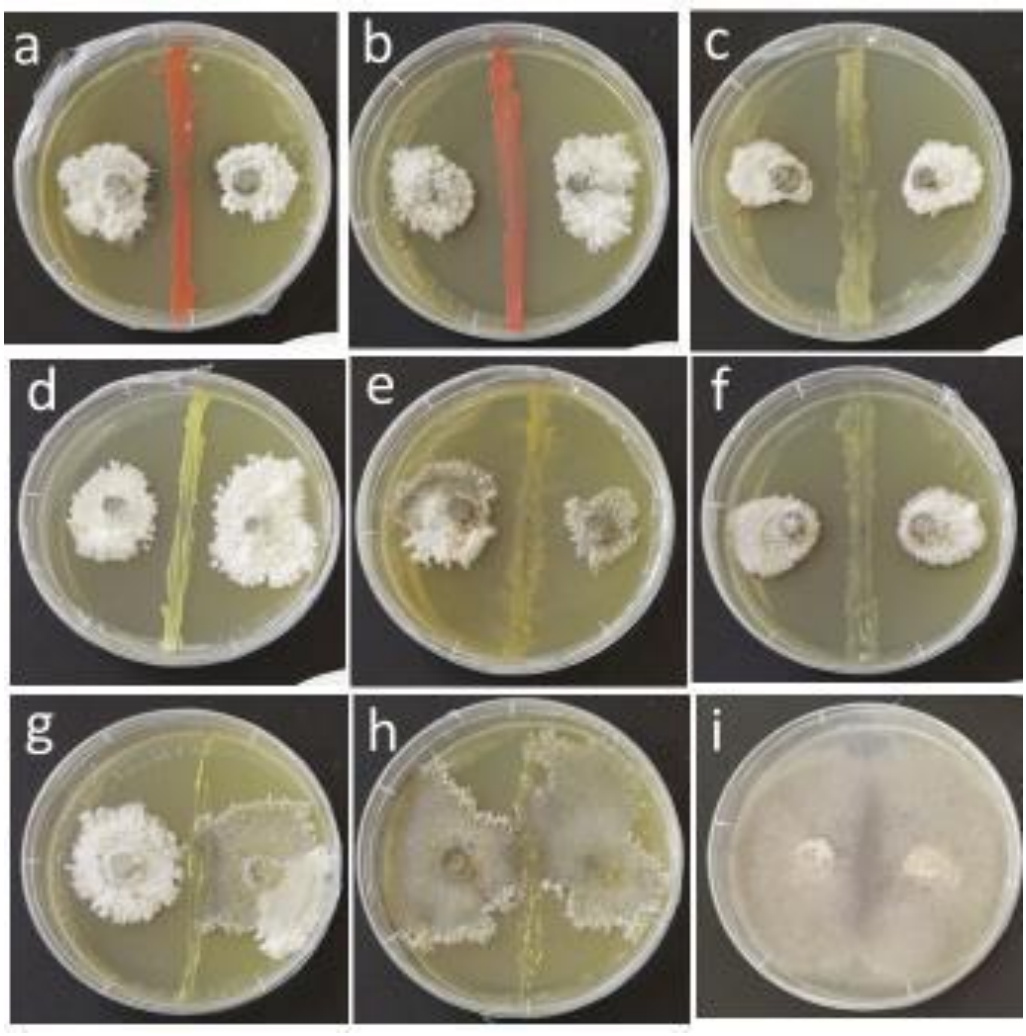

Figure 5. 4 Inhibition zone between bacterial endophytes of brassica (a= *Me. fujisawaense*, b= *Me. phyllosphaerae*, c= *N. resinovorum*, d= *Pl. flavus*, e= *St. rhizophila*, f= *Ps. azotoformans*, g= *Sphingom. insulae*, h= *Sphingom. mucosissima*) and *L. maculans* in a dual culture test and control plate (i).

Both *B. bassiana* and *G. pannorum* produced an antagonistic interaction with *L. maculans* in PDA medium. This is evident in the large inhibition zones between the endophytes colonies and the pathogen (Figure 5.5).

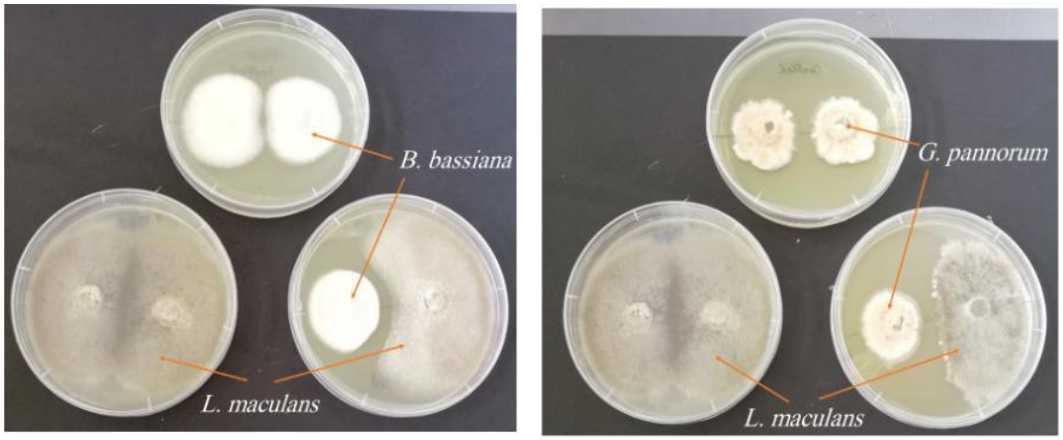

Figure 5. 5 Inhibition zone created between brassica endophytes and *L. maculans* in a dual culture test; *B. bassiana* (left) and *G. pannorum* (right).

5.4.2.2 *In planta* assay

The ANOVA of phoma stem canker scores (two trails combined) reveals that inoculation of oilseed rape seed with *B. bassiana* and *G. pannorum* resulted in seedlings with significantly lower disease scores compared with un-inoculated seedlings (Table 5.3).

Table 5. 3 Analysis of variance of disease scores of oilseed rape seedlings after inoculation with *B. bassiana* and *G. pannorum* compared with un-inoculated.

Source of variation	**Sum of squares**	**Degree of freedom**	**Mean square**	**F value**	**Significance**
Between groups	9.491	2	4.745	116.045	<0.0001
Within groups (treatments)	0.859	21	0.041		
Total	10.350	23			

Seedlings inoculated with either endophyte species and *L. maculans* produced smaller lesions on the cotyledon leaves compared with seedlings infected with the pathogen only (Figure 5.6).

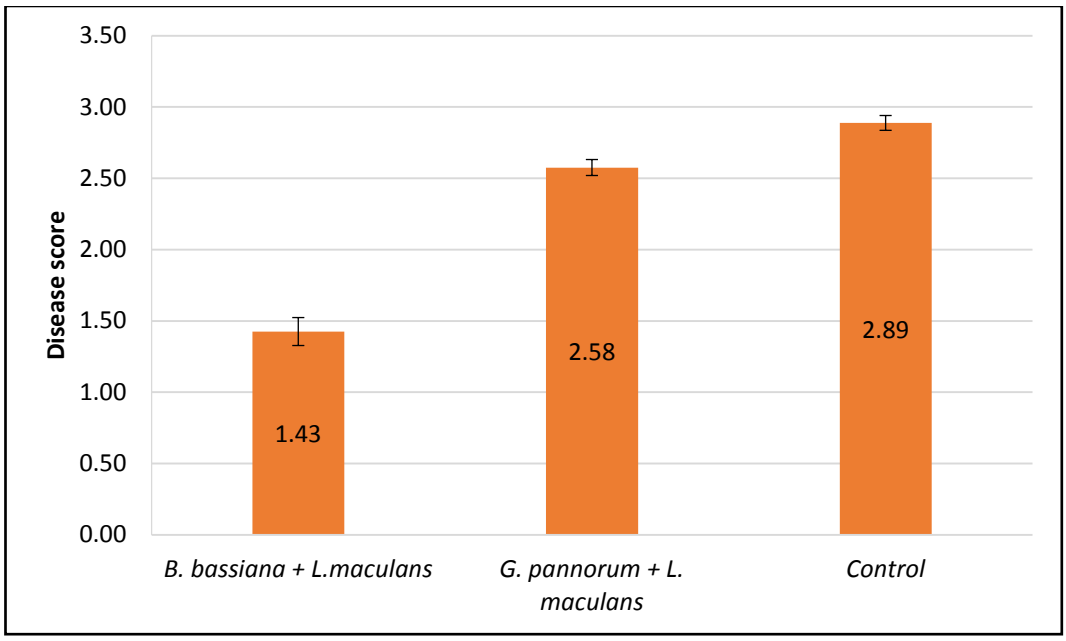

Figure 5. 6 Mean score of disease symptoms on cotyledons of oilseed rape seedlings when inoculated with *B. bassiana* and *L. maculans* or *G. pannorum* and *L. maculans* or inoculated with *L. maculans* only (control); (LSD 0.05% = 0.21). (Bar line in each column shows the standard error).

Lesions were centred on the pathogen wound site and were generally grey in colour. Lesions on the control seedlings were clearly larger than those on inoculated seedlings; seedlings inoculated with *B. bassiana* produced smaller lesions than those inoculated with *G. pannorum* (Figure 5.7).

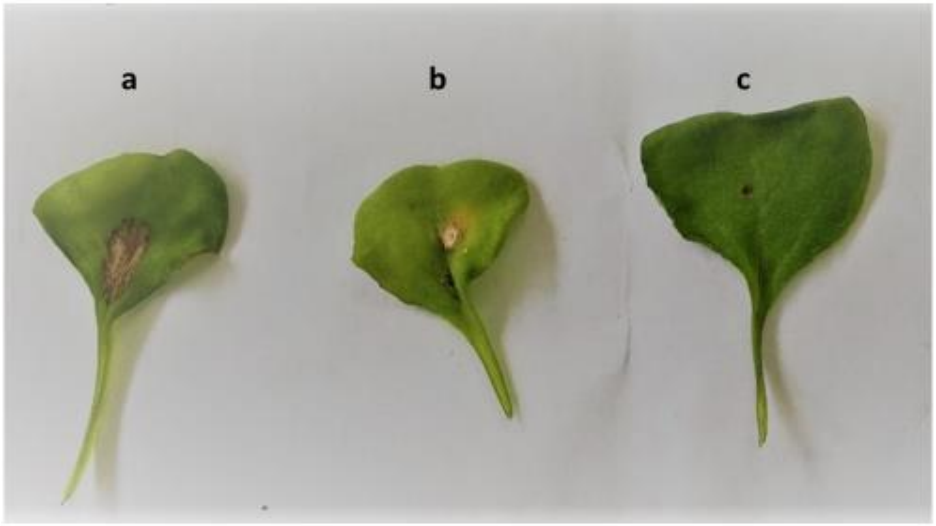

Figure 5. 7 Oilseed rape cotyledons from pathogen-only control (a) (disease score 3), *G. pannorum* (b) (disease score 2) and *B. bassiana* (c) (disease score 1) inoculated seedlings.

The recovery endophytes from the inoculated seedlings indicate that both fungal endophytes are able to colonise oilseed rape seedlings but that the rate of *G. pannorum* seems to be higher than *B. bassiana* (Figure 5.8).

Figure 5. 8 of *B. bassiana* (top row) and *G. pannorum* (bottom row) in oilseed rape seedlings (cv. Flash) at the cotyledon leaf stage.

5.5 Discussion

Endophytic bacterial and fungal microorganisms isolated originally from wild brassica species were studied in vitro and in planta (within novel association with brassica cultivars) to gain an understanding of the ecological importance of these associations and to assess potential benefits for plant growth and development. Two Methylobacterium species (*Me. fujisawaense* and *Me. phyllosphaerae*) commonly associated with brassicas and two fungal endophytes (*B. bassiana* and *G. pannorum*) were selected to investigate their ability to promote plant growth and to provide bio-control of phoma stem canker disease (*L. maculans*) in brassicas.

5.5.1 Plant growth promotion

Our results indicate that both Methylobacterium species were able to colonise new hosts and enhance the growth of oilseed rape seedlings. These bacteria were found to have a high rate in wild brassica species (Chapter 3) indicating that their ability to colonise brassica seedlings is relatively high. The frequent presence of *Methylobacterium* species in wild brassica plants (Idris *et al.*, 2004, Idris *et al.*, 2006, Roodi *et al.*, 2016) which are typically found growing in low fertility soil suggests that this symbiosis can improve plant growth rate. Delmotte *et al* (2009) found that *Methylobacterium, Sphingomonas* and *Pseudomonas* were the most abundant in the phyllosphere of *A. thaliana* grown in the field at one site.

Depending on species Methylobacterium is able to enhance plant development through nitrogen fixation (Sy *et al.*, 2001, Madhaiyan *et al.*, 2004, Lee *et al.*, 2006, Menna *et al.*, 2006), and production of phytohormones such as cytokinins and auxins (Trotsenko *et al.*, 2001, Madhaiyan *et al.*, 2006a, Meena *et al.*, 2012). These hormones are able to enhance plant growth and development including branching, seedling

vigour, seed germination, root differentiation and photosynthetic activity (Trotsenko *et al.*, 2001, Prombunchachai *et al.*, 2017). It has been shown (Lee *et al.*, 2006) that rice seed treated with methylotrophic bacterial strains improved seed germination and vigour and increased the biomass of the seedlings and the vegetative stage of rice plant. The activity of hormone production (cytokinns) and acetylene reduction activity (nitrogen fixation) evidenced by the inoculated bacteria was demonstrated in Lee et al. (2006) study which suggested they stimulate the germination and increase the biomass production. Inoculation of Crambe (*Crambe abyssinica*), species from Brassicaceae, with a strain of *Methylobacterium komagatae* significantly increased the yield and root biomass of the plant and suggest more studies are needed to elucidate the mechanisms of this association in other Brassicaceae (De Aquino *et al.*, 2018). Indication of non-pigmented *Methylobacterium* that form root-nodulating and non-nodulating nitrogen-fixing symbiosis has been demonstrated in other studies (Sy *et al.*, 2001, Raja *et al.*, 2006). We suggest that the plant growth ability of the Methylobacterium species studied in this research when associated with oilseed rape cultivars is a significant advantage for this crop which typically demand high nitrogen input for its productivity (Rathke *et al.*, 2006) and requires further studies.

5.5.2 Antagonistic Bioactivity of endophytes and *L. maculans*

As revealed by the dual culture assay, half of the isolated bacteria (six out of 13 isolates) and two fungal endophytes (*B. bassiana* and *G. pannorum*) were able to provide good control of *L. maculans* under laboratory conditions. Bacterial endophytes have been shown to provide biological control against soil borne fungal pathogens in cultivated brassica crops (Nejad & Johnson, 2000).

The mechanism of this antagonistic characteristic was not determined in this study and

needs further research. Antagonism may be related to the production of metabolic compounds in the growth medium, competition for space or parasitism of the pathogen by the endophytes. For example, the hyphae of *B. bassiana* are able to coil around the hyphae of *Pythium myriotylum*, controlling its extension (Ownley *et al.*, 2008).

Although dual culture assays are commonly used to identify potential antagonists against pathogenic microorganisms, it is important to confirm later whether they are able to have protective effects on plants. There have been many instances where antifungal activity is apparent in a dual culture test but not when tested *in planta*. For example, a strain of endophytic bacterium, *Pseudomonas viridiflava* which inhibit the growth of *L. maculans* in dual culture tests did not reduce the growth of this pathogen in whole oilseed rape seedlings (Romero *et al.*, 2019).

Inoculation of oilseed rape seedlings with *B. bassiana* and *G. pannorum* greatly reduced the disease severity of phoma stem canker on the cotyledon leaves when compared with un-inoculated seedlings, particularly *B. bassiana* which was able to prevent the formation of lesions after seedlings were exposed to *L. maculans*. The increase resistance to *L. maculans* by *B. bassiana* and *G. pannorum* is probably the result of of the brassica seedlings by these fungi (Figure 5.8). These fungal endophytes are able to establish a point infection in plant tissues (Chapter 4), consequently induced systemic resistance might also be another possibility for the facilitation of disease resistance in this relationship (Ownley *et al.*, 2008, Gautam *et al.*, 2016). Another possible explanation for the disease resistance observed in this study may be the production of secondary metabolites in the plant tissue. Both *B. bassiana* and *G. pannorum* can produce bio-active secondary metabolites that are able to suppress pathogens (Bing & Lewis, 1991, Ownley *et al.*, 2004, Li *et al.*, 2008,

Ownley *et al.*, 2008, Gautam *et al.*, 2016). All of these possibilities need to be investigated to fully understand the mechanisms involved in control of plant pathogens by these fungal endophytes.

There is little knowledge on the complicated interactions between endophytes (Mercado-Blanco, 2015). Since most plant species can associate with more than one endophyte, indirect competition between these species is expected (Müller, 2003, Liu *et al.*, 2011), as well as direct competition between the species and strains occupying the same ecological niche. In addition, fungal endophytes have been found to show an extremely antagonistic interaction to each other in the culture (Yan *et al.*, 2015) so if they stabilised a systemic infection it is unlikely to be found simultaneously in the same location. On the other hand, many endophytic species may work together in harmony to present more benefits for themselves and their shared host. For example, the interaction between *P. indica* and rhizobia has been only understood in the last ten years (Molitor & Kogel, 2009). Therefore, fungal endophytes particularly those with localised infection possibly can be inoculated together in the new host. It has been found that there are several endophytic fungi in the forb *Silene dioica* that can establish a localised infection and they suggest any systemic effects of these fungi on other organisms are likely to be due to chemical movement (Yan *et al.*, 2015). *B. bassiana* and *G. pannorum* are able to form a localised infection within tissues of brassica (Chapter 4) so possibly they can be inoculated simultaneously to the host and their beneficial properties added to the plant. Co-inoculation with multiple microorganisms rather than with a single inoculant can improve plant yield (O'Callaghan, 2016). However, interaction of the endophytes with other plant microbial symbiont also needs to be considered since the endophyte inoculation may change the growth, physiology and metabolisms of other beneficial microbial agents and break the balance between

endophyte and the host plant (Gao *et al.*, 2010). It has been demonstrated that isolates of *Bacillus* and *Pseudomonas* when applied individually supress the growth of *L. maculans* in oilseed rape but when they were co-inoculated, no control was observed (Cholerton, 2015).

B. bassiana is an entomopathogen fungi with literature citing its infection ability in many plant species including brassica and its benefits in control of pest insects (Vidal & Jaber, 2015). *G. pannorum* is a sychotolerant fungus that has been found in association with plants growing in arctic regions (Hayes, 2012). These advantages coupled with their plant protection against fungal pathogens confer additional traits to the host plant. Furthermore, little or lack of host specificity of these endophytes (see chapter 4) could probably provide an opportunity for production of BCAs suitable for various crop species. For example, *B. subtilis* which forms a symbiotic association with many plant species such as brassica (Wulff *et al.*, 2003, Hu *et al.*, 2011, Hu *et al.*, 2014) and a great number of BCA products based on this species has been commercially introduced in many countries (Kabaluk *et al.*, 2010).

To our knowledge this is the first study that report *B. bassiana* and *G. pannorum* are able to control *L. maculans* in oilseed rape following seed inoculation. However, there are reports of other non-endophytic fungal microorganisms providing protection against phoma stem canker of brassica's. The fungus *Gliocaldiom catenulatum* (commercially produced under the trade name Prestop® (Verdera, Finland)) can reduce the intensity of this disease by 51 %. (Hammoudi *et al.*, 2012). Microbial agents must be able to colonise rhizosphere of plants and compete with other microbes present if they are going to successfully control plant pathogens (Weller, 1988, Milus & Rothrock, 1993). Endophytic microorganisms which are sheltered in host plants are more protected against environmental stress and less affected by competition of other

microbes (Romero *et al.*, 2019). Both *B. bassiana* and *G. pannorum* were able to colonise the plant tissue of oilseed rape seedlings in this study (also see Chapter 4) indicating that they are promising candidates for biological control agents of *L. maculans* in oilseed rape. However, further investigation needs to be carried out to study the efficacy of this control option in natural and agricultural environments. It has been noted that many bacterial strains exhibit beneficial traits *in situ*, but much lower number are successful in the greenhouse and even lower number effectively function under field condition (Lugtenberg & Kamilova, 2009).

6. Chapter 6. General discussion

6.1 Introduction

Endophytes are critical components of every plant-based community with each of the 300,000 existing plant species hosting from one to several hundred endophytic species (Strobel & Daisy, 2003). They are regarded as important plant partners and their absence can result in a lack of host tolerance to a number of environmental stresses (Card *et al.*, 2016, Rukshana Begum, 2016). Plant domestication by humans can cause morphological as well as physiological changes and these can be used to differentiate domesticated individuals from their wild ancestors (Hancock, 2005). During the plant breeding process and through the use of systemic fungicides, communities of microorganisms associated with wild plants are overlooked and inadvertently removed (Lugtenberg *et al.*, 2016). Consequently, domesticated plants often lack these endophytes and their conferred benefits. Lack of endophytes is a possible explanation for the reliance of many domesticated plants on agrochemicals for survival in the face of biotic stresses. Although wild crop relatives have for years provided plant breeders with genes for pest and disease resistance as well as abiotic stress tolerance (Hajjar & Hodgkin, 2007, McCouch *et al.*, 2007), targeting these wild relatives for beneficial endophytes offers new opportunities for crop production.

Unlike traditional biological control agents (BCAs), endophytes in addition to offering beneficial traits, are able to establish a close association with the host. Host plant species provide shelter for these microorganisms, protecting them from environmental variables. Once the endophyte synchronises its extension with plant development and disseminates through vertical transmission (dissemination through offspring) as observed in *Epichloë* endophyte, opportunities for commercialisation of BCAs arise

(Zhang *et al.*, 2017). In addition, the association of the endophyte with seeds increase the shelf life of the BCA; both the seed and endophyte can be stored as a one component. This eliminates the repetitive application of endophytes, a common practice in the utilization of BCAs, in each growing season. In addition, the obligate endophytes, such as asexual endophytes of cool season grasses, have advantage over facultative endophytes because they either depend on their host for all of their needs and have no free-living life stage or, spend the majority of their life within the plant (Card *et al.*, 2016). This helps to produce a BCA better able to protect the IP for the targeted market. These initial considerations assist us in creating a pipeline for discovering useful endophytic microorganisms that may result in the introduction of endophytic BCA's. This would be potentially valuable for brassica crops which are challenged by many biotic and abiotic stresses. However, there are many critical points in the development of a BCA; these are briefly discussed later in this chapter.

This study focused on the development of an endophyte discovery pipeline with the purpose of ultimately producing novel brassica-endophyte associations with useful traits for the oilseed rape, forage and/or vegetable brassica industries. The pipeline comprised genetically diverse accessions of wild *Brassicaceae* obtained from international gene banks and local collections undertaken in NZ. The beneficial traits conferred from the novel associations identified in the study were investigated under controlled conditions. The following sections provide a brief general summary and discussion of the research outcomes, possible future research direction, and the utilization of these endophytes in agriculture, and conclusions.

6.2 Summary of outcomes and discussion

There are reports indicating seed of some wild plant species are good sources of various endophytic bacteria that can offer biotic and abiotic enhancements to their host's plants (Kremer, 1987, Nejad & Johnson, 2000, Card *et al.*, 2015, Murphy *et al.*, 2015, Murphy *et al.*, 2018). In this study 83 seed accessions were screened comprising 13 brassica species from over 27 countries (Chapter 3). Over 130 microbial isolates were recovered. Initially species were tentatively identified via partial DNA-sequencing and indicated that the majority of the screened accessions harboured cultivable endophytic bacteria from 12 species belonging to the Proteobacteria and included; *Brevundimonas vesicularis, Caulobacter mirabilis Methylobacterium fujisawaense, Me. extorquens, Me. phyllosphaerae, Novosphingobium resinovorum, Pseudomonas azotoformans, Stenotrophomonas rhizophila, Sphingobium yanoikuyae, Sphingomonas paucimobilis, Sphingom. mucosissima* and *Sphingom. insulae*. Three species were identified from the Actinobacteria; *Kocuria palustris, Micrococcus luteus, and Plantibacter flavus* while two species were identified from the Firmicutes phylum; *Bacillus mycoides* and *Paenibacillus hordei*. These bacterial genera are beneficial microorganisms for several plant species (Table 3.3). The microbiome communities of wild brassica species identified in this study includes species such as *Methylobacterium* sp. that are frequently associated with many wild species with wide geographic distribution. Although the location influences the endophytic population and the variation observed may be attributed to many factors such as seasonal changes, plant tissue, plant species, cultivar and soil factors (Mocali et al., 2003), the dominant presence of Methylobacterium species in wild brassica species indicates the possible preservation of this species during their evolution. Methylobacterium species are not widely found in cultivated species (Granér *et al.*, 2003, Card *et al.*, 2015, Roodi *et al.*,

2016, Rybakova *et al.*, 2017). This implies the significance of endophytes studies in wild species of domesticated plants (Murphy *et al.*, 2015, Abdallah *et al.*, 2016, Yokoya *et al.*, 2017). It has been proposed that during evolution endophytes have been adapted to their special microenvironments through genetic variation, including uptake of some plant DNA into their own genomes (Germaine *et al.*, 2004). This probably resulted in the ability of certain endophytes to biosynthesize phytochemical compounds that are characteristic of the respective host species (Stierle *et al.*, 1993).

Bacterial endophytes are important plant growth promoters which act through a number of mechanisms (Glick, 2012). Endophytic species are able to enhance growth through production of plant hormones and antimicrobial metabolites as well as through nutrient availability, for example nitrogen fixation, and solubilisation and mobilization of soil nutrients (Canbolat *et al.*, 2006, Li *et al.*, 2010, Meena *et al.*, 2012, Hu *et al.*, 2013 , Lins *et al.*, 2014). Among the isolated bacterial species identified in this study, *Me. fujisawaense* and *Me. phyllosphaerae* were the most dominant species present in a majority of accessions with both potentially possessing plant growth promotion abilities (Trotsenko *et al.*, 2001, Prombunchachai *et al.*, 2017). This was tested by inoculating oilseed rape seedlings with both species separately and comparing their growth with that of non-inoculated seedlings (Chapter 5). The fresh weights of inoculated seedlings were significantly higher than non-inoculated seedlings (Table 5.1) indicating that both bacterial species promoted the growth under controlled conditions. The possible mechanism of plant growth promotion was not determined in this study but previous research has indicated that Methylobacterium species are able to enhance plant development through nitrogen fixation (Sy *et al.*, 2001, Lee *et al.*, 2006, Menna *et al.*, 2006) and/or phytohormone production including cytokinins and auxins (Trotsenko *et al.*, 2001, Madhaiyan *et al.*, 2006a, Meena *et al.*, 2012). Nitrogen

is a major nutrient for plant growth and requires in large quantities. Although it is abundantly available in air, plants are unable to exploit this atmospheric nitrogen, therefore synthetic nitrogen fertilizer is typically utilised. This increases crop production costs and may result in contamination of the environment, posing a health hazard to humans and environmental degradation (Bhardwaj *et al.*, 2014). In such situations use of endophytic bacteria as a biofertilizer able to provide nitrogen to host plants is a suitable alternative approach (Gupta *et al.*, 2012, Audipudi *et al.*, 2017). Brassica crops such as oilseed rape (*B. napus*), have a high nitrogen requirement for high yields (Rathke *et al.*, 2006). Oilseed rape also has low nitrogen use efficiency (Bouchet *et al.*, 2014, Bouchet *et al.*, 2016). The frequent presence of *Methylobacterium* species in wild brassica plants which are usually found growing in low fertility soils, for example those collected from a sandy river bank in New Zealand (Manawatu river), suggest that these symbionts can be used as biofertiliser. The increasing usage of microbial biofertilizers improves the potential for the development of sustainable farming methods and increased food safety. The Grand View Research (https://www.grandviewresearch.com/industry-analysis/biofertilizers-industry) reported nitrogen fixing microbes are the largest segment of biofertilizers used in agriculture, accounting for over 75.0% of global revenue share of biofertilizers in 2014 and those used as seed treatment are the most common method of application. The convenience in adoption of seed treatment is expected to drive future growth in this sector.

Screening wild brassica accessions also resulted in the isolation of two putative fungal endophytes tentatively identified as *Beauveria bassiana* and *Geomyces pannorum* through partial DNA-sequencing. Identication of *B. bassiana* was then confirmed through partial DNA-sequencing of elongation factor (Rehner & Buckley, 2005).

Subsequently, we concentrated on the development of novel associations of these fungal endophytes and elite brassica species and cultivars and found that these fungal endophytes were able to asymptomatically infect the root and shoot tissues of three brassica species; *B. napus*, *B. oleracea* and *B. rapa*. The recovery of these fungal endophytes from tissue plant of oilseed rape cultivars also showed there was no genotype preference regarding . Although there are reports indicating some non-Clavicipitaceous endophytes show host specificity at the species level or host preference within a species and tissue type (Cannon & Simmons, 2002, Sun *et al.*, 2012), many fungal species from this endophyte class generally don't show host specificity and are able to colonise a broad range of plant hosts (Rodriguez *et al.*, 2009). In this study, the isolated strains of *B. bassiana* and *G. pannorum* in the origin host plants (wild species of *B. rapa*) completely colonised all root, shoot tissues, producing a systemic infection. However, the recovery of these fungal endophytes from the new host species demonstrated the ability of these endophytes to infect the host plant locally at least. It demonstrated that none of the new host plants were completely colonised by the endophytes and tissue frequency was below 30% in all brassica species assessed. This indicates that the isolated endophytes probably have host preference characteristics and seem to be more adapted to the original host that the new hosts. A close adaptation between the host plant and its fungal partner generally create host specificity, suggesting a mutual interaction arising from an ancient cohabitation and co-evolution. In the long term, this association becomes permanently imbedded in the genetic constitution of both organisms that finally develop complementary genetic systems (Moricca & Ragazzi, 2008). For example, *Taxomyces andreanae*, the first paclitaxel-producing endophytic fungus discovered, has not been isolated from any yew other than the original tree from Glacier National

Park, Montana (Strobel *et al.*, 1996a, Strobel *et al.*, 1996b). Some observations indicate that endosymbionts within a given host are phylogenetically uniform in many cases (Herre *et al.*, 1999). Co-evolution may lead to failure of plant-defence reactions against the presence of microbial endophytes (Christensen *et al.*, 2002) as well as the ability of endophytes to produce the same bioactive metabolites as their host plants (Stierle *et al.*, 1993, Puri *et al.*, 2005). Further evidence of the co-evolution is the synchronisation of fungal and host reproduction system, as occurs in the case of endophytic fungi with vertical transmission where both partners benefit from successful host reproduction system (Schardl *et al.*, 1991, Zhang *et al.*, 2017).

Lack of systemic of *B. bassiana* and *G. pannorum* in new brassica host species also was confirmed when inoculated plant tissues were examined under a light microscope. This revealed that conidia of both fungal endophytes can germinate on the surface of plant tissue and then enter the sub-epidermal tissue. The lack of recovery of endophytes from the inflorescence and the off-spring from the inoculated brassica plants in our study also indicates that *B. bassiana* and *G. pannorum* are not able to colonise the plant systematically so they cannot transmit from the mother plant to the next generation seeds. Although a systemic transmission would be an advantageous trait for commercialisation of a BCA, the successful infection of brassicas with *B. bassiana* and *G. pannorum* through seed inoculation is also an advantage commercially.

This study revealed that *G. pannorum* had a higher rate than *B. bassiana* and that root tissue was more readily infected by *G. pannorum* than *B. bassiana*. This may be the general characteristic of these fungal endophytes. Endophytic fungi may exhibit organ and/or tissue specificity as a result of their adaptation to different physiological

conditions in plants (Rodrigues & Samuels, 1999). The set of selective pressures by the plant occurring in a certain plant tissue type influence its endophytic composition and may differ from that present in other tissue types. Consequently, different fungi are found to dominate in distinctive above-ground organs forming characteristic communities specific to each tissue type (Arnold, 2007, Gazis & Chaverri, 2010). However, there are other factors that may change the interpretation of the pattern of the , consequently investigations of patterns of fungal endophytes based only on isolation data must be interpreted carefully. For example, dimensions of sampling units are critical given the microscopic scale of fungal distributions (Stone *et al.*, 2004). The serial dissection and plating of host tissue pieces provides only approximate information about host patterns; it may be impossible to differentiate between systemic of contiguous tissue by a single or multiple infections on the same pieces where the domain of infection is very small in relation to the size of the sample unit (Stone *et al.*, 2004). Therefore, observations from direct examination of infected tissue should be used to confirm patterns of . A better approach may be the green fluorescence tag protein technique for detection and tracking of the patterns in plant tissues and study of systemic (Maor *et al.*, 1998).

Fungal diseases greatly influence agro-productivity. The use of indigenous endophytic microorganisms is considered to be environmentally friendly and ecologically sustainable. Microbial endophytes in association with a host can produce a wide range of organic compound some of which are classified as antibiotics, antioxidants, anticancer agents, volatile antimicrobial agents, immunosuppressive compounds, plant growth promoting agents, and insecticides (Strobel & Daisy, 2003, Lugtenberg *et al.*, 2016, Strobel, 2018). Endophytic actinomycetes can potentially produce antimicrobial compounds with one extract from *Streptomyces*, isolated from *Allium fistulosum*, able

to suppress the infection of *Alternaria brassicicola* in Chinese cabbage seedlings (Igarashi *et al.*, 2000, Igarashi *et al.*, 2002). The fungal endophytes of *B. bassiana* and *G. pannorum*, isolated in this study, are also able to produce secondary compounds (Li *et al.*, 2008, Ownley *et al.*, 2008, Ownley *et al.*, 2009). Secondary metabolites are a group of compounds that play an important role in adaptation of plants to environmental stress (Gao *et al.*, 2010). Some of these secondary metabolites such as antifungal and antimicrobial compounds can strongly inhibit the growth of other microorganisms including plant pathogens (Gunatilaka, 2006). Consequently, we set up *in vitro* and *in planta* trials to study the antifungal bioactivity of these endophytes against the *L. maculans*. Both endophytes were able to colonise the oilseed rape seedlings and suppress the disease intensity of phoma stem canker caused by *L. maculans in vitro* and *in planta* (Chapter 5). Endophytes colonise the plant surface by producing enzymes including β-1,3-glucanases, chitinases and cellulases which hydrolyse plant cell walls. These enzymes also can also suppress plant pathogen activities and degrade the cell wall of fungi and Oomycetes (Gao *et al.*, 2010). Inhibition of *L. maculans* by *B. bassiana* and *G. pannorum* in our study is probably due to antibiosis through production of secondary metabolites. Reports indicate *B. bassiana* and *G. pannorum* produce bio-active secondary metabolites that can suppress other fungal pathogens (Bing & Lewis, 1991, Ownley *et al.*, 2004, Li *et al.*, 2008, Ownley *et al.*, 2008, Gautam *et al.*, 2016). However, other mechanisms such as competition and induced resistance may also have been involved because both fungal endophytes colonized the plant tissues. Induced resistance from *B. bassiana* has also been identified in cauliflower (*Brassica oleracea*) (Gautam *et al.*, 2016).

The introduction of fungal endophytes can provide an eco-friendly method of increasing the resistance of plants to abiotic and biotic stress. Use of Clavicipitaceous

endophytes such as *Epichloë* endophytes have been widely adopted by farmers growing temperate grasses helping to improve pasture productivity when exposed to insect herbivory and drought (Johnson *et al.*, 2013). Research into the production and commercialisation of non-Clavicipitaceous (NC)-endophytes is on-going. Class 2 NC-endophytes includes fungi able to offer disease, salt and heat tolerance in grasses (Rodriguez *et al.*, 2008). These tolerances can be transferred to agricultural plants to improve tolerance to drought, cold and salinity stress (Rodriguez *et al.*, 2008, Redman *et al.*, 2011). Currently, Adaptive Symbiotic Technologies' BioEnsure®, is the only fungal seed treatment available; it is used in okra, maize, wheat, and millet crops (https://securingwaterforfood.org/innovators/adaptive-symbiotic-technologies-bioensure). The fungi remain dormant on the seed until germination when they establish a symbiotic association with seedlings and confer water-related stress tolerance. BioEnsure R®-Corn is promoted as being able to increase yield by 25%–80% under heavy drought stress. BioEnsure R®-Rice is promoted as being able to increase yield under drought and salt stresses as well as reduce water use by 25%–40% (Lugtenberg *et al.*, 2016).

6.3 Future research

In this study we screened and isolated the bacterial and fungal endophytes of wild brassica species through conventional culturing technique. The isolated fungal species created a novel association with elite cultivars of forage and oilseed brassica. This association significantly increased the resistance of the host against phoma stem canker. Inoculation of brassica seedlings with some bacterial species also enhanced growth. However, issues not addressed that require investigation are outlined here.

- Our study applied a culture-dependent approach for screening endophytic microorganisms of brassica plants. Although this approach is necessary to produce axenic cultures needed for further experimentation, with this approach only the endophytic microorganisms able to grow on synthetic media could be isolated, and this may include a subset of the cultivable microorganisms due to our choice of media for isolation. In addition, bias towards fast-growing, abundant species, whereas rare species with minor competitive strength and more specialized requirements may remain undiscovered (Unterseher & Schnittler, 2009, Sun et al., 2011). Therefore, beside the culture-dependent technique we suggest that a culture-independent approach be utilised to uncover the entire microbiome, including cultivable and uncultivable species. The information gained could be useful to make specific/optimised culture media that may assist in capturing those species detected only by alternative approaches. Culture-independent methods such as environmental PCR is also recommended for recovery of some vertically transmitted fungi as they would probably be intractable to cultivation if they have an obligate association with their hosts. (Arnold, 2007).
- We screened the seeds of brassica species stored in international seed banks with a broad range of storage durations (see chapter 3). As the age of the seed and seed storage factors such as temperature, relative humidity and seed moisture content during the storage affect the viability of endophytes within the seed (Rolston *et al.*, 1986, Cankar *et al.*, 2005, Freitas, 2017), we suggest collecting/harvesting fresh seed produced under natural conditions. This will increase the chance of isolating any surviving endophytes inhabiting the seed.

- Since the dissection and plating of host tissue pieces provides only approximate information about host patterns; (Stone *et al.*, 2004) it would be more appropriate to use green fluorescence tag protein technique for detection and tracking of the pattern in plant tissues (Maor *et al.*, 1998). This approach is also very useful for tracking the endophytes in the off-spring as well.
- The results of the inoculation study indicated that the fungal endophytes *B. bassiana* and *G. pannorum*, originally isolated from the wild species of *B. rapa*, are able to establish a point infection rather that a systemic infection. Further research is required to inoculate the endophytes into an endophyte-free plant of the original host to check if they can infect the wild host in a systemic way. This will elucidate the level of host-specificity of the endophytes.
- Root and shoot inoculation of brassica seedlings with fungal spore suspension did not result in of the plant tissues in this study. However, this trial was undertaken when the seedling was planted in non-sterilised potting soil. The study needs to be repeated to test the inoculation methods in sterilised soil.
- The Methylobacterium species isolated in this study promote the growth of brassica seedlings in a controlled environment. Follow on research is required under field conditions. Depending on species, Methylobacterium are able to enhance plant development through nitrogen fixation (Sy *et al.*, 2001, Lee *et al.*, 2006, Menna *et al.*, 2006) and/or phytohormone production such as cytokinins and auxins (Trotsenko *et al.*, 2001, Madhaiyan *et al.*, 2006a, Meena *et al.*, 2012). To elucidate the mechanisms involved in plant growth promotion by Methylobacterium the influence of nutrient supply on growth response should be determined.

- Representative species of isolated bacteria were tested for their antifungal bioactivity against *L. maculans* in a dual culture test; the results indicated that some bacterial species are able to supress the growth of the pathogen in the agar culture. This bioactivity needs to be tested *in planta* as there is not always a positive correlation between the results of in vitro and in vivo tests. *L. maculans* is one of the brassica pathogens that cause phoma stem canker disease. Other important pathogens of brassica crops including *Sclerotinia sclerotiorum* and *Plasmodiophora brassicae* should be assessed for their susceptibility to these endophytic bacteria both *in vitro* and *in vivo*.
- There are additional beneficial traits cited in the literature attributed to the endophytic bacteria isolated in this study including bioremediation, biodegradation and siderophore production. More research could be done to test the potential beneficial characteristics related to these endophytes.
- As part this study, the antifungal bioactivity of *B. bassiana* and *G. pannorum* was tested against *L. maculans* and indicated that both species are able to control this pathogen under controlled conditions. This experiment needs to be repeated under glasshouse and field conditions and the pathogen control mechanisms identified, in particular the presence of any secondary compounds (Li *et al.*, 2008, Ownley *et al.*, 2008, Ownley *et al.*, 2009).
- *B. bassiana* is an entomopathogen fungi with the ability to control insect pests (Vidal & Jaber, 2015) and *G. pannorum* is a sychotolerant fungi able to protect host plants against cold temperatures (Hayes, 2012). The potential benefits of these traits in brassica crops should be assessed.

6.4 Application of endophytes in agriculture

To introduce endophytes as BCA's in agricultural systems it is critical to fully understand its behaviour under a range of conditions, its complete life cycle and its genome plasticity to assess its risk of becoming a pathogenic agent, either through transition in abiotic conditions or adaptation to an alternative host (Redman *et al.*, 2001). Other potential ecological problems must also be considered. For example, inoculation of an endophyte to the new host plant which is not its original host, may significantly change the pattern of the metabolites produced (Gimenez *et al.*, 2007). For example, the herbicidal activity of *Phyllostica capitalensis* differs depending on the host species (Suryanarayanan *et al.*, 2009). This means that the plant host and/or its metabolism influence the synthesis of metabolites in the endophyte. It also indicates that inoculated new host plants must be evaluated for endophyte security and the potential presence of unknown endophytic compounds (Gimenez *et al.*, 2007). In addition, the interaction of endophytes with other plant microbial symbionts also needs to be considered. It has been noted that inoculation of a new endophyte in the host may change the growth, physiology and metabolisms of other present beneficial microbial agents in the plant and alter the balance between endophyte and host metabolism (Gao *et al.*, 2010).

Although scientific research in multidisciplinary microbial plant science has led to the commercial production of biological formulations (Fravel, 2005) it only represents about 1 % of agrochemical sales currently. The reasons for this include poor cost-effectiveness and the short life span of biological agents. Cost of production is probably the major constraint on the commercialisation of biological control agents (Fravel *et al.*, 1999, Fravel, 2005). Currently liquid and solid fermentation are two most common methods used to produce bio-control agents. Despite the development of new equipment and procedures, large-scale production of bio-control agents is not

very successful because of the specificity and complexity of each individual agent and unexpected quality and quantity issues associated with large-scale production (Fravel *et al.*, 1999). Other factors include lack of understanding of the impact on environment and farming system, complexity of registration and marketing (Lidert, 2001, Fravel, 2005).

Understanding the biology of endophytes and their interactions with host plants can definitely assist the commercialisation of an endophyte as BCAs. Behavioural and biological factors of endophytes including endophyte lifecycle, type of plant-endophyte interaction, the level of intimacy, *in planta* pattern, degree of host specificity, means of propagation and mechanisms of action are key elements that must be investigated before introduction of an endophyte BCA in the market (Card *et al.*,2016).

In sustainable agriculture the use of biological microbes must occur without a damaging effect on non-target organisms present in the environment. They must also be a naturally occurring microbiome of plant ecosystems, rhizoplane or phylloplane and able to resist tough environmental conditions (Cholerton, 2015). With this in mind, endophytic microorganisms which have naturally co-evolved with their hosts including those that can transmit vertically through seed are ideal candidates as BCAs. This is evidenced by commercialised endophytic ryegrass and tall fescue cultivars in New Zealand, Australia and the United States. In New Zealand, grass endophytes make an estimates $200 million contribution to the economy annually (Evans, 2007, Johnson *et al.*, 2013).

If the potentially useful endophytes do not transmit vertically inside the inoculated plants, inoculants must be applied at each sowing event. The most obvious method of

endophyte application in agricultural system is addition of inoculants to the soil or to the seed (Le Cocq *et al.*, 2017). There are some successful examples of this approach in sugarcane (Da Silva *et al.*, 2012). To market endophytic microorganisms as a seed treatment/seed primer, it is important to formulate them in an appropriate way to ensure efficacy, storability and compatibility with current agricultural practices and technologies (Koch & Roberts, 2014). We could not find evidence of fungal hyphae in the offspring of inoculated plants suggesting lack of vertical transmission however, other technical approaches, such as application of fungal spores directly on the inflorescence during seed development might facilitate the incorporation of endophytes into seeds through colonisation of the embryo or the seed coat.

6.5 Final conclusion

In this study we aimed at screening seed of wild brassica species for putative bacterial and fungal endophytes. This resulted in isolation of over 130 bacterial isolates belonged to 17 different species. *Methylobacterium* sp. constituted the dominant bacteria infected many brassica accessions. The fungal endophytes of *B. bassiana* and *G. pannorum* also identified in two seed accessions. The selected isolates of these fungal endophytes were then inoculated into cultivated forage and oilseed brassica species to study the ability of the fungi in the new hosts which shows a non-systemic in plant tissues. Infected oilseed raped seedlings by these fungal endophytes suppressed the growth of *L. maculans* under controlled environment. *Methylobacterium* improved the growth of oilseed rape when inoculated to the seedling. The results of this study suggest that the seed of wild brassica species are a valuable source of putative endophytic microorganisms able to impart beneficial traits to the new host. These endophytic microorganisms could be used as BCAs to decrease

the use of agrochemicals in brassica farming and approach the sustainable crop production. Therefore, natural ecosystems and particularly the habitats of wild relatives of crops must be considered as the rich biodiversity of unique beneficial endophytic microorganisms an immediate preservation is required. Discovery of potentially beneficial endophytes is only the first step in the process which includes verification of the ability to infect non-host species, beneficial effects of the endophyte that have commercial value and confirmation of vertical transmission.

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Appendices

Appendix 1 Beneficial endophytic microorganisms of brassica cited in literature in 2016-2019 (post- Card *et al*. 2015)

Species	**Type of microorganism**	**Host Species**	**Benefits**	**References**
Arthrobacter sp.	Bacteria	*B. napus*	Heavy metal resistance, root development	(Montalbán *et al.*, 2016)
Bacillus sp.	Bacteria	*B. napus*	Antifungal activity against plant pathogens, plant growth promotion	(Etesami & Alikhani, 2018),(Afzal *et al.*, 2017)
B. subtilis	Bacteria	*B. oleracea*	Disease resistance against *Sclerotinia sclerotiorum*	(Tozlu *et al.*, 2016)
Burkholderia phytofirmans	Bacteria	*B. napus*	Plant growth promotion, heavy metal immobilization in soil	(Nafees *et al.*, 2018)
Humicola sp.	Fungi	*Fragaria vesca*	Water use efficiency	(Yokoya *et al.*, 2017)
Paenibacillus polymyxa	Bacteria	*B. napus*	Nitrogen fixation, plant growth promotion	(Puri *et al.*, 2016)
Piriformospora indica	Fungi	*B. napus*	Phosphorous mobilisation and uptake, plant growth promotion, increasing seed yield and quality	(Wu *et al.*, 2018)

Species	Type of microorganism	Host Species	Benefits	References
Pseudomonas sp.	Bacteria	*B. napus*	Heavy metal resistance, plant growth promotion, antifungal activity	(Montalbán *et al.*, 2016), (Afzal *et al.*, 2017)
Pseudomonas fluorescens	Bacteria	*B. napus*	Plant growth promotion	(Wassermann *et al.*, 2017)
Pseudomonas viridiflava	Bacteria	*B. napus*	Disease resistance against *Xanthomonas campestris* and *Sclerotinia sclerotiorum*, plant growth promotion	(Romero *et al.*, 2019)
Streptomyces humidus	Bacteria	*B. oleracea* var *capitata*	Suppression of *Alternaria brassicicola* in cabbage	(Hassan *et al.*, 2017)
Serratia sp.	Bacteria	*B. napus*	Heavy metal resistance, root development	(Montalbán *et al.*, 2016)
Serratia plymuthica	Bacteria	*B. napus*	Antifungal activity against *Verticillium longisporum*	(Wassermann *et al.*, 2017)

Appendix 2 Wild and landrace Brassicaceae accessions at MFGC screened for endophytic fungi and bacteria.

Accession number	Country of origin	Species	Harvest date	Altitude (m)
O2329	unknown	*B. balearica*	unknown	unknown
O2330	unknown	*B. barrelieri*	unknown	unknown
O2331	Portugal	*B. barrelieri*	unknown	888
O2332	Italy	*B. incana*	unknown	74
O2333	unknown	*B. incana*	unknown	13
O2334	Slovakia	*B. juncea*	unknown	144
O2335	unknown	*B. juncea*	unknown	unknown
O2336	Poland	*B. juncea*	unknown	134
O2337	India	*B. juncea*	unknown	248
O2338	Thailand	*B. juncea*	unknown	93
O2350	Italy	*B. nigra*	unknown	234
O2351	Bulgaria	*B. nigra*	unknown	121
O2352	Germany	*B. oleracea*	unknown	202
O2353	Germany	*B. oleracea*	unknown	76
O2354	Spain	*B. oleracea*	unknown	594
O2355	China	*B. rapa*	unknown	1857
O2356	Russia	*B. rapa*	unknown	unknown
O2357	Slovakia	*B. rapa*	unknown	1843
O2358	Georgia	*Brassica sp.*	unknown	1450
O2359	Spain	*B. barrelieri*	1/01/1995	2069
O2360	Spain	*B. barrelieri*	1/01/1996	27
O2361	Turkey	*B. deflexa*	1/01/1995	940
O2362	Morocco	*B. densnottesii*	1/01/1996	2015
O2363	Iran	*B. elongata*	1/01/1995	1301
O2364	Spain	*B. fruticulosa*	1/01/1997	206
O2365	Algeria	*B. fruticulosa*	1/01/1994	306
O2366	Algeria	*B. fruticulosa*	1/01/1994	140
O2367	Algeria	*B. gravinae*	1/01/1994	1749
O2368	India	*B. juncea*	1/01/1999	251
O2369	Zambia	*B. juncea*	1/01/1994	1090

Accession number	Country of origin	Species	Harvest date	Altitude (m)
O2370	Zambia	*B. juncea*	1/01/1994	1257
O2371	Germany	*B. juncea*	1/01/1997	202
O2372	Mongolia	*B. juncea*	1/01/2000	1335
O2373	Spain	*B. nigra*	1/01/1994	19
O2374	Ethiopia	*B. nigra*	1/01/1994	1320
O2375	Germany	*B. nigra*	1/01/1996	73
O2376	Israel	*B. nigra*	1/01/1999	503
O2377	USA	*B. rapa*	1/01/2010	23
O2378	USA	*B. rapa*	1/01/2010	23
O2379	USA	*B. rapa*	1/01/2010	23
O2380	USA	*B. rapa*	1/01/2010	23
O2381	USA	*B. rapa*	1/01/2010	23
O2382	USA	*B. rapa*	1/01/2010	15
O2383	USA	*B. rapa*	1/01/2010	15
O2384	Egypt	*B. rapa*	1/01/1996	3
O2385	India	*B. rapa*	1/01/2000	176
O2386	Spain	*B. repanda*	1/01/2011	8
O2387	Spain	*B. repanda*	1/01/2007	400
O2388	India	*Brassica sp.*	1/01/2010	279
O2389*	Finland	*B. napus*	1/01/1983	158
O2390*	Finland	*B. napus*	1/01/1991	158
O2391*	Sweden	*B. napus*	1/01/2000	16
O2392*	Iceland	*B. napus*	1/01/1988	733
O2393*	Iceland	*B. napus*	1/01/1988	733
O2394*	Iceland	*B. napus*	1/01/2010	733
O2395*	Iceland	*B. napus*	1/01/1988	733
O2396*	Iceland	*B. napus*	1/01/1988	733
O2397*	Iceland	*B. napus*	1/01/1988	733
O2398*	Iceland	*B. napus*	1/01/1988	733
O2399*	Iceland	*B. napus*	1/01/1988	733
O2400*	Iceland	*B. napus*	unknown	733

*Land race species

Accession number	Country of origin	Species	Harvest date	Altitude (m)
O2401*	Norway	*B. napus*	1/01/1996	759
O2402*	Sweden	*B. napus*	1/01/2000	16
O2403*	Sweden	*B. napus*	1/01/1999	16
NZ01	New Zealand	*Brassica sp.*	1/05/2014	28
NZ02	New Zealand	*Brassica sp.*	1/05/2014	28
NZ03	New Zealand	*Brassica sp.*	1/05/2014	28
NZ04	New Zealand	*Brassica sp.*	1/05/2014	28
NZ05	New Zealand	*Brassica sp.*	1/05/2014	28
NZ06	New Zealand	*Brassica sp.*	1/05/2014	28
NZ07	New Zealand	*Brassica sp.*	1/05/2014	28
NZ08	New Zealand	*Brassica sp.*	1/05/2014	28
NZ09	New Zealand	*Brassica sp.*	1/05/2014	28
NZ10	New Zealand	*Brassica sp.*	1/05/2014	28
NZ11	New Zealand	*Brassica sp.*	1/05/2014	28
NZ12	New Zealand	*Brassica sp.*	1/05/2014	28
NZ13	New Zealand	*Brassica sp.*	1/05/2014	28
NZ14	New Zealand	*Brassica sp.*	1/05/2014	28
NZ15	New Zealand	*Brassica sp.*	1/05/2014	28
NZ16	New Zealand	*Brassica sp.*	1/05/2014	28
NZ17	New Zealand	*Brassica sp.*	1/05/2014	28
NZ18	New Zealand	*Brassica sp.*	1/05/2014	28
NZ19	New Zealand	*Brassica sp.*	1/05/2014	28

*Land race species

Appendix 3 Tissue Frequency (TCF%) of endophytic fungus *G. pannorum* in three brassica species following the stringent shoot-disinfection at different plant growth stages after seed inoculation.

Growth stage	Titan rape (*B. napus*)		Regal kale (*B. oleracea*)		Hunter leafy turnip (*B. rapa*)	
	Shoot	**Root**	**shoot**	**Root**	**shoot**	**Root**
Cotyledon stage	0	0	0	0	0	0
One-leaf stage	6 (0.24)*	0	0	0	0	0
Three-leaf stage	31 (2.05)	12 (1.84)	30 (3.85)	13 (1.87)	28 (3.76)	13 (2.45)
Four-leaf stage	14 (2.06)	10 (1.32)	12 (2.79)	8 (1.15)	12 (2.06)	9 (1.28)

* Numbers in the brackets are the standard error.

Appendix 4 Tissue Frequency (TCF%) of endophytic fungus *B. bassiana* in three brassica species following stringent shoot-disinfection at different plant growth stages after seed inoculation.

Growth stage	**Titan rape (*B. napus*)**		**Regal kale (*B. oleracea*)**		**Hunter leafy turnip (*B. rapa*)**	
	Shoot	**Root**	**shoot**	**Root**	**shoot**	**Root**
Cotyledon stage	0	0	0	0	0	0
One-leaf stage	0	0	0	0	0	0
Three-leaf stage	10 (2.27)*	9 (1.28)	12 (2.06)	8 (1.14)	9 (1.28)	9 (1.28)
Four-leaf stage	9 (1.28)	8 (1.15)	10 (1.32)	7 (0.88)	10 (1.28)	8 (1.15)

* Numbers in the brackets are the standard error.

Appendix 5 Tissue Frequency (TCF%) of endophytic fungus *G. pannorum* in three brassica species following mild shoot-disinfection at two plant growth stages after seed inoculation.

Growth stage	Titan rape (*B. napus*)		Regal kale (*B. oleracea*)		Hunter leafy turnip (*B. rapa*)	
	Shoot	**Root**	**Shoot**	**Root**	**shoot**	**Root**
Cotyledon stage	22(2.8)*	16 (2.94)	19 (2.99)	14 (2.06)	21 (2.48)	15 (2.56)
One-leaf stage	17 (2.73)	14 (2.69)	18 (3.94)	13 (1.69)	17 (2.76)	12 (2.5)

* Numbers in the brackets are the standard error.

Appendix 6 Tissue Frequency (TCF%) of endophytic fungus *B. bassiana* in three brassica species following mild shoot-disinfection at two plant growth stages after seed inoculation.

Growth stage	Titan rape (*B. napus*)		Regal kale (*B. oleracea*)		Hunter leafy turnip (*B. rapa*)	
	Shoot	**Root**	**Shoot**	**Root**	**shoot**	**Root**
Cotyledon stage	11 (1.5)*	7 (0.88)	11 (1.5)	9 (1.28)	13 (1.7)	7 (0.88)
One-leaf stage	11 (1.78)	7 (0.88)	12 (1.35)	8 (1.12)	13 (1.77)	7 (0.87)

* Numbers in the brackets are the standard error

Appendix 7 Tissue Frequency (TCF%) of endophytic fungus *G. pannorum* in five oilseed rape cultivars (*B. napus*) following mild shoot-disinfection protocol at one-leaf stage and stringent shoot-disinfection protocol at three-leaf stage after seed inoculation.

Growth stage	**Ladoga**	**Veritas CL**	**Hybrirock**	**Turan**	**King**
One-leaf stage	18 (3.08)*	17 (2.84)	17 (2.62)	18 (2.2)	18 (3.35)
Three-leaf stage	20 (2.57)	20 (2.55)	21 (2.06)	22 (2.8)	20 (4.06)

* Numbers in the brackets are the standard error.

Appendix 8 Tissue Frequency (TCF%) of endophytic fungus *B. bassiana* in five oilseed rape cultivars (*B. napus*) following mild shoot-disinfection protocol at one-leaf stage and stringent shoot-disinfection protocol at three-leaf stage after seed inoculation.

Growth stage	**Ladoga**	**Veritas CL**	**Hybrirock**	**Turan**	**King**
One-leaf stage	9 (1.28)*	10 (1.87)	9 (1.28)	8 (1.15)	10 (1.32)
Three-leaf stage	12 (2.45)	14 (2.78)	13 (2.64)	14 (2.78)	11 (1.74)

* Numbers in the brackets are the standard error.