Biological control of *Leptosphaeria maculans* on *Brassica napus* and quantification of the microbes *in planta* using qPCR

Linda Jane Cholerton Bsc. (Hons.)

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ABSTRACT

Brassica napus is a commercially important crop worldwide and its use is quickly increasing due to its beneficial oil products and biofuel demands. Yield can be lost through infection by a fungal pathogen, *Leptosphaeria maculans*, the causal agent of stem canker (blackleg). An early indication of the presence of stem canker is a lesion (leaf spot) on the cotyledons or early leaves. The leaf spot stage of the disease was used in this work to ascertain if biological control agents applied both individually and in combination decreased the lesion area and also to quantify the amount of *L. maculans* DNA present using quantitative polymerase chain reaction (qPCR).

The natural production of antibiotics by some bacteria is a commonly found form of antagonistic biological control. *Bacillus amyloliquefaciens* and *Pseudomonas chlororaphis* spp*. aureofaciens* 30.84 evaluated in this work both produce antibiotics and were assayed for their ability to provide control of *Leptosphaeria maculans*.

Known active strains and field isolates of *Bacillus* and *Pseudomonas* were tested as potential biocontrol agents *in vitro* and then used in *in planta* assays. The *in planta* assays using bacterial isolates applied individually indicated that all the bacteria gave statistically significant control of *L. maculans* at the leaf spot stage. Those isolates with highest activity were further evaluated in combination, to determine if improved control of leaf spot occurred. Firstly, however, it was important to confirm the two bacteria would be compatible and antibiotics would be produced. To this aim, an *in vitro* assay using mutant *Chromobacterium violaceum* confirmed *Pseudomonas chlororaphis* spp. *aureofaciens* upregulated antibiotic production using acyl-homoserine lactones, signalling molecules. Consequently, it was vital that the *Bacillus* applied with it did not produce lactonase which would denature these molecules. PCR was used to confirm the enzyme was not present. It was, however, shown using *in planta* assays that combinations of *Bacillus* and *Pseudomonas* did not halt the infection or growth of *L. maculans,* but appeared to lead to increased lesion size.

Colonisation of the cotyledons by the bacterial biological control agents applied onto the cotyledons was monitored by washing recovery, serial dilution, plating and colony counting along with qPCR of the DNA. All bacteria

colonised successfully when applied individually. However, the populations decreased from the quantity at time zero when they were applied in combination, indicating they were unable to colonise the cotyledons successfully under those circumstances.

To quantify *Leptosphaeria* infection, the concentration of ergosterol, a fungal sterol, was quantified to measure the colonisation of cotyledons. Concentrations were assessed using high performance liquid chromatography (HPLC). This assay was not successful no free ergosterol could be detected. This was probably due to *L. maculans* either having small amounts of ergosterol in its cell membranes, or having most of the ergosterol esterfied and unsuitable for quantification using this method.

Polymerase chain reaction (PCR) was used to ascertain the presence of fungal hyphae within asymptomatic regions of cotyledons. It was found that the fungal DNA was detected within all areas of the cotyledon irrespective of whether the leaf spot could be seen. This result highlights the unreliability of the common method of visually assessing the presence and/or severity of *L. maculans* infection using leaf spot area.

To monitor the populations of bacteria and the fungus in real time, DNA was extracted from the cotyledons and quantified using quantitative PCR (qPCR). The amount of *L. maculans* DNA isolated decreased when the BCAs were applied individually, and increased when the BCAs were applied in combination (when compared with the amount isolated from the control cotyledons). These results confirmed earlier, non-molecular assessments.

To provide a benchmark for biocontrol activity, fungicides used in the control of leaf spot on oilseed rape were tested under the standard experimental conditions. Whilst control was obtained, it was not as effective as when used in the field, probably due to the formulations being optimised for field conditions. Fungicides targeted at wheat pathogens were also tested for control against *L. maculans*. Field application rates of these fungicides were not successful, as all damaged the epidermis of the cotyledon, resulting in death of the plant. Application of ¼ field rate still induced epidermal damage in all cotyledons except those sprayed with Q8Y78 (now called Refinzar®), where a necrotic lesion could be seen without pycnidia, at day 15 after inoculation.

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ABBREVIATIONS

AHL	Acyl-homoserine lactones
AI	Auto-inducers
ATP	Adenosine triphosphate
BCA	Biological control agents
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Centigrade
C_t	Threshold cycle
CTAB	Hexadecyltrimethylammonium bromide
d	Days
dp	Decimal places
ds	Double stranded (DNA)
Da	Dalton
DAI	Days after inoculation
DMI	Demethylase inhibitors
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
ETDA	Ethylenediaminetetraacetic acid
ETP	3,6-epipolythiodioxopiperazine
EU	European Union
g	Gram
GFP	Green fluorescent protein
h	Hour
HL	Hyperladder
HR	Hypersensitive response
HSL	Homoserine lactones
ISR	Induced systemic resistance
IPM	Integrated pest management
JPEG	Joint photographic experts group
Kb	kilobase
L	Litre
LB	Luria-Bertoni medium
MBC	Methyl benzimidazole carbamates
mL	Millilitre

mm	Millimeter
min	Minutes
mM	Micromolar
MRD	Maximum recovery dilutent
mRNA	Mitochondrion ribonucleic acid
MT	Metric tonnes
NA	Nutrient agar
ng	Nanogram
Nm	Nanometer
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDA^{+}	Potato dextrose agar with antibiotics
PDB	Potato dextrose broth
pH	Potential Hydrogen
QoI	Quinone outside inhibitors
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
QTL	Qualitative trait locus
R	Resistance (genes)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
s	Seconds
SAR	Systemic acquired resistance
SD	Standard deviation
SDHI	Succinate dehydrogenase inhibitor
TSB	Tryptone soya broth
wt	Wild type
µL	Microlitre
x g	Centrifugal force

CHAPTER 1 – GENERAL INTRODUCTION

1.1. OILSEED RAPE

Brassica oilseed varieties have been in cultivation for over 4000 years. Originally grown in mainland Europe, the crop was introduced into the UK in the 17th century, providing lighting oil and animal fodder (Rakow, 2004). During World War II there was an increase in the use of oilseed rape oil, as demand for lubricant for ships needed satisfying (Kimber & McGregor, 1995). Demand declined after the war and new uses were sought to take up the higher levels of production (Snowdon *et al*., 2007).

Early varieties contained high concentrations (over 50%) of erucic acid (high erucic acid, HEAR, varieties) in the oil. This is a toxic compound known to cause human heart damage (Snowdon *et al*, 2007). They also contained high amounts of glucosinolates, astringent tasting compounds making the meal produced after pressing unpalatable to animals (Shahidi, 1991). Traditional plant breeding was used to produce oilseed rape varieties with less than 5% erucic acid (LEAR) and low glucosinolates (e.g. cv. Bronowski), which encouraged farmers to again grow the crop in the late 1960s (Shahidi, 1991).

Agricultural growth of *B. napus* has increased more than 260% worldwide in the last 40 years, as both demand and price increased (Hammoudi *et al*., 2012). In 2011, 61 million metric tonnes (MT) of oilseed rape oil was produced, with China being the largest producer (14.6 million MT) (HGCA, 2014). The United Kingdom grew in excess of 621,000 ha of winter oilseed rape in 2014 (DEFRA, 2014). Rape seed is cultivated world-wide today due to its high value (£300 t^{-1} in 2013 Huang *et al.*, 2014) and its ability to grow in varying climate and soil conditions. There are well established agronomic techniques for the crop. Agrochemicals are available to control pests and diseases, and forecasting systems e.g. Crop Monitor are sometimes available, advising when to spray. Together these make oilseed rape a reliable and popular crop, which fits in well with rotations centred on winter wheat production. With harvesting and oil production infrastructure already in place in most countries, oilseed rape is a vital and important crop for the future.

One of the major uses of oilseed rape is for biofuel. Global energy consumption is expected to rise by about 40% by 2030, and biofuels are expected to contribute by providing 20% of the demand for fuel. The European Union has committed to reduce vehicle fuel emissions to 20% below the 1990 levels by 2020, and one way they propose to do this is increasing the use of renewable fuels. Supply of fossil based oil is limited and as prices are continuing to rise, biofuels as an alternative can provide a level of supply security. Biodiesel is safe to handle as it is non-toxic and has a high flash point. It is similar in composition to fossil based diesel and can be used without the modification of modern engines.

An increase in public demand for products with perceived health benfits has given rise to other uses for oilseed rape including vegetable oils, margarines and salad dressings (Hasan *et. al*., 2006). Rapeseed oil has been shown to be low in saturated fats (palmitic acid) and high in the unsaturated essential fatty acids Omega 6 (Oleic Acid) and 3 (linoleic acid), potentially reducing cholesterol and heart problems (HGCA, 2013). Oilseed rape varieties high in glucosinolates can be used as fertilizer, whilst others are be used as cattle food, with the seeds producing a meal containing 40% protein (Shahidi, 1991).

With this higher level of production, there had been an increase in fungal diseases of oilseed rape. The main pathogens affecting oilseed rape are white stem rot (*Sclerotinia sclerotiorum*), light leaf spot (*Pyrenopeziza brassicae*), club root (*Plasmodiophora brassicae*), white rust (*Albugo candida*) and stem canker (*Leptosphaeria maculans*). Stem canker is the most important oilseed rape fungal disease with €56M annual losses worldwide in 2002 (Fitt *et al*., 2006), and is still able to decimate crops when epidemics occur.

1.2. STEM CANKER (*LEPTOSPHAERIA MACULANS*)

Initially recorded as early as 1791 by Tode on the dried stems of *Brassica oleracea* (red cabbage), *Leptosphaeria*, a fungal pathogen of nearly all members of the Brassica family, was first given the name *Sphaeria lingam*. It was renamed *Phoma lingam* when the asexual form was found in 1849, with the sexual stage named *Leptosphaeria maculans* by Tulasne and

Tulasne in 1863. At this point, it was still thought to be one species (Rouxel & Balesdent, 2005). Common names include stem canker and blackleg.

Stem canker is now known to be a mix of two closely related fungi, *L. maculans* (anamorph *Phoma lingam*) and *L. biglobosa* (Williams & Fitt, 1999). Both are usually found on the same *B. napus* plant (West *et al*., 2002) but separated by space and time (Fitt *et al*., 2006). It has been determined using quantitative polymerase chain reaction (qPCR) that in England *L. maculans* is detected more at the base of the stem than *L. biglobosa* (Sprague *et al*., 2010, Stonard *et al.*, 2010) and as such, it is *L. maculans* that girdles the stem restricting water transport. The consequential water loss to the developing pods cause the seeds to ripen too early (decreasing yield) whilst lodging caused by the lesion makes the plant un-harvestable. *Leptosphaeria biglobosa* still causes lesions, but they are higher up the plant and less damaging.

Whilst being phenotypically and microscopically similar (Howlett, 2004), there are differences. Both *L. maculans* and *L. biglobosa* have asexual and sexual mating types (bipolar) and require two compatible partners to produce sexual spores but cannot cross with each other (Howlett *et al*., 2001). Each has a different shaped ascus, with *L. biglobosa* having a beak-like protrusion at the top (Somda *et al.,* 1997). It is now known that there are many sub-species of both *L. maculans* and *L. biglobosa* that infect *B. napus* (Vincenot *et al*., 2008). Even more isolates of *L. maculans*, genetically related to those found on *B. napus,* are found on cruciferous weeds (Howlett *et al*., 2001).

From observations of disease gradient, sexual ascospores are the primary inocula in the autumn (Figure 1.1.). Eight ascospores are formed within each ascus and the asci are contained by a flask shaped pseudothecium (West *et al.,* 2001). Sexual mating can occur at this stage. After 20 or more rain events with an average air temperature of 12ºC, the spores are released in large quantities from infected oilseed rape stubble and carried by rain or on the wind. How far they travel depends on wind speed, topography and the filter of the crop canopy (West & Fitt, 2005). They are also found in the upper 5 cm of the soil profile (Sosnowski *et al*., 2006) where they can reach a concentration of 10^8 to 10^9 spores h^{-1} (Marcroft *et al*., 2003). It is the

sexual spores that initiate epidemics (McGee, 1977) as their release coincides with the crop sowing season. Ascospores of *L. maculans* are frequently released before those of *L. biglobosa* in the UK as the spores mature earlier (Huang *et al*., 2011).

Figure 1.2. shows the life cycle of *L. maculans*, the causal agents of stem canker. Spores land on the cotyledons and early leaves of young plants in autumn and germinate within a day. Each spore produces an appressorium-like structure from which an infection peg grows along the cotyledon or leaf towards stomata and wounds, through which it enters. The hyphae then grow in a short biotrophic phase (5-6 days after infection) in the apoplast between the leaf
mesophyll cells, producing sirodesmin PL, a non host-specific toxin (3,6-epipolythiodoixopiperazine, ETP) (Hammond *et al*., 1985, Fox & Howlett, 2008). This stops the hypersensitive response of the plant. Sirodesmin PL also causes degradation of the chloroplasts, visualised on the leaf as a chlorotic area (Rouxel & Balesdent, 2005, Howlett, 2004, Rouxel *et al*., 1988, Brewer *et al*., 1966). Whilst contributing to the pathogenicity of *L. maculans*, sirodesmin is not a virulence factor (Sacristan, 1985, Howlett, 2004, Elliot *et al.*, 2007, Howlett *et al*., 2010) as mutants unable to produce the toxin and *L. biglobosa* (classified as tox^-) still infect leaves (Balesdent *et al.,* 1992, Shoemaker & Brun, 2001). The fungus utilises manitol, naturally found in the plant apoplast (Oliver & Ipcho, 2004) and induces apoptosis (programmed cell death) of the plant cells, which results in a release of nutrients used for fungal growth (Voegele *et al*., 2005, Li *et al*., 2008).
At 7–9 days after infection, a metabolic shift occurs in the fungus, when the biotrophic phase changes to a necrotrophic phase, inducing the asexual spore production stage of its lifecycle. Proteases, host protein degrading enzymes (e.g. SP2, Wilson & Howlett, 2005) and toxins are produced to kill the host cells. The plant cell membranes rupture, resulting in a necrotic lesion in the leaf or cotyledon epidermis (leaf spot), 4-8 mm in width (*L. maculans*) and 2-3 mm (*L. biglobosa*) and within which a large number of pycnidia are produced (11-12 days after infection). The pycnidia contain countless asexual pycnidiospores (Marra *et al.*, 2010). When mature, (15-17 days after infection) the pycnidia burst through the

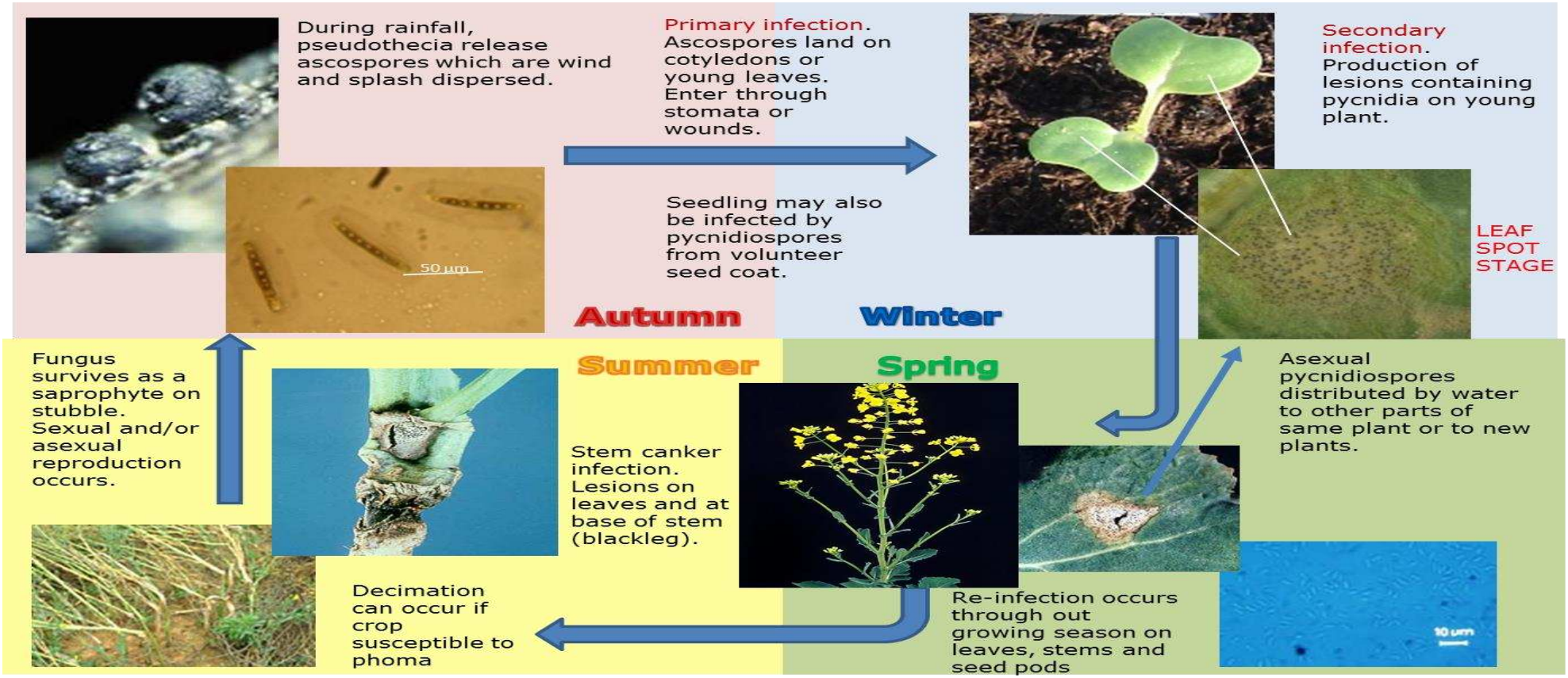


Figure 1.1. Lifecycle of *L. maculans*, the causal agent of stem canker on *Brassica napus*. Images: L. Cholerton www.apsjetorg/edcenter/intropp/lessions/fungi, www.agf.gov.bc.ca/cropprot/images/blackleg

necrotic epidermal layer and the spores ooze onto the lesion surface. The spores are spread a short distance by rainsplash and may be a source of secondary inoculum, as the lesions occur throughout the growing season (Howlett *et al*., 2001). If the oilseed rape plant is susceptible, the mycelia (now highly branched due to abuntant nutrients from the necrotic cells) then grow as a symptomless biotroph by again producing sirodesmin PL to stop the plants hypersensitive response. The fungus grows through the apoplastic spaces of the cotyledon mesophyll, down the petiole to the stem. Here, it enters its first quiescent phase (Rouxel *et al.*, 1988, Li *et al*., 2008). It then reactivates, and grows without symptoms within the stem xylem, moving through the sieve plates. *Leptosphaeria biglobosa* stays high up the plant within the stem, where it produces a necrotic sunken lesion. *Leptosphaeria maculans* grows down to the base of the plant (which is at its rosette stage), where it has a second quiescent period until the plant matures and bolts for flowering (Huang *et al*., 2009). The fungus then returns to being necrotrophic, producing the characteristic purple bordered, sunken lesion in the stem at the base of the plant (stem canker, blackleg). The necrotic cells block or sever the vascular system in the plant leading to early senescence, lodging and/or little or no seed production (West *et al*., 2001). Epidemics of stem canker were prevalent at the end of the 19th and in the 20th century in Australia, North America and Europe, with losses in yield of up to 90% in certain areas (Rouxel & Balesdent, 2005). The pathogen nearly destroyed the entire crop in Western Australia during an epidemic in 1972 (Madacsy *et al.*, 1975, Sivasithamparam *et al.*, 2005).

Stem canker is still considered a serious world-wide fungal disease of crucifers, especially winter oilseed rape, with each season enduring an economic loss of more than £1,000 million world-wide (Huang *et.al*., 2014). It can also infect other important oilseed crops e.g. *B. rapa*, *B. juncea* and *B. carinata*. Colonisation by *L. maculans* is increasing in areas where previously only the less damaging *L. biglobosa* was found. China grows large amounts of brassicas, and currently only has the less aggressive, *L. biglobosa*. Should *L. maculans* become dominant in China, significant crop loss will follow (Huang *et al*., 2014).

Figure 1.2. Devastation of oilseed rape crop caused by stem canker (Rothamstead Research, 2013)

Climate change may also exacerbate the problem of stem canker, as it has been shown, under experimental conditions, that higher temperatures decrease spore germination time and increase the efficiency of stomatal penetration by mycelia. More frequent rain events will also hasten germination (Toscano-Underwood *et al.,* 2001, Huang *et al.,* 2003).

1.3. RESISTANCE OF OILSEED RAPE TO STEM CANKER

The disease resistance of oilseed rape to stem canker is provided by a combination of intrinsic mechanisms and responses induced by the infection. These decrease the symptoms on the plant (West *et al.*, 2001).

The plant has to be able to recognise pathogenic organisms with different lifestyles (Ellis *et al*., 2006) and resist them at minimal cost to themselves. Primary defence responses are already in place; the tough structural barriers provided by the waxy cuticle and lignified epidermal cell walls form a strong barrier which can only be breached using special structures (appressorium) or by entering through stomata and damaged areas (e.g. as used by stem canker). Once the pathogen is inside leaf, it has to overcome the chemical defences (antimicrobial proteins, detoxifying chemicals) already in place to halt its growth (de-Wit, 2007). The pathogen produces a range of conserved proteins (effectors, toxins, suppressors, enzymes, binding proteins) to suppress the defence responses and aid its growth and progress within the

leaf. Along with the presence of fungal cell wall components e.g. chitin, the proteins can act as elicitors for receptors (pattern recognition receptors, PRRs) in the plant cell membrane, the first tier of a two tier response system (de-Wit, 2007). When bound, these either directly activate cellular changes (antimicrobial compounds and proteins, cell wall strengthening, cell death), or via intracellular protein kinase cascades, change gene expression to increase the plant's defence mechanisms (Baker *et al*., 1997).

The first visible symptoms of leaf infection by *L. biglobosa* occur within a few hours after hyphal penetration. The plant causes rapid death of its own cells (hypersensitive response, HR) by up-regulating production of reactive oxygen species (ROS) (Fitt *et al*.,2006). This process causes necrosis of the stomatal guard cells adjacent to the entry site. As *L. biglobosa* hyphae grow and branch intercellularly, the plant responds further by using ROS to increase oxidative cross-linkage, strengthening the cell wall to isolate the hyphae in the cotyledon where it entered (Jindřichová *et al*., 2011). The response also induces the plants mesophyll cytoplasm to shrink causing loss of cell to cell contact usually seen with apoptosis (Li *et al*., 2008).

Cotyledons infected with *L. biglobosa* produce dark brown irregular shaped lesions (leaf spot) containing a few pycnidia. However, if susceptible, the plant does not always successfully contain *L. biglobosa* and necrotic stem lesions can be produced.

The more aggressive species when entering susceptible *B. napus*, *L. maculans* is used in this work. This is the species that produces sirodermin PL as they hyphae grows inside the leaf suppressing the plants HR by inhibiting plant RNA synthesis and stops production of its defence chemicals and proteins allowing the fungal hyphae to continue to grow unhindered.

1.4. BREEDING FOR RESISTANCE TO *LEPTOSPHAERIA MACULANS*

The second tier of resistance to stem canker is using a different set of membrane receptors, encoded by *R* (resistance) genes. Sustainable management of *L. maculans* includes the breeding of plants for resistance using the R genes at both the seedling and adult stages (West *et al*., 2001). Both quantitative (major gene) and qualitative resistance is important in the breeding programmes of *B. napus* cultivars, as it decreases fungicide

applications and reduces yield loss (Fitt *et al*., 2006). It has been shown that quantitative resistance can increase the durability of qualitative resistance (Brun *et al*., 2010). The use of mixed cultivars with different resistance genes helps to stabilise resistance (Gladders *et al*., 1998).

Oilseed rape varieties have a stem canker resistance rating of 1 - 9 (Home Grown Cereal Authority, HGCA) with 1 being susceptible and 9 being resistant. Varieties 'Winner' and 'Compass', used in this work, have a HGCA resistance value of 4 and are thus moderately susceptible.

1.4.1. Qualitative resistance

Qualitative (major gene) resistance in *B. napus* is initiated immediately after penetration through the stomata of the hyphal germ tube. *Leptosphaeria maculans* is prevented from fully colonising leaves by the plant recognising specific *Leptosphaeria* races, resulting in the upregulation of its hypersensitive response.

Quality breeding for stem canker resistance between *Brassica napus* and *Brassica rapa* initially produced winter rapeseed variety Lesira (containing Rlm1 resistance gene) which was widely used in France, and also the spring varieties Oro and Erglu (Rouxel *et al*., 2003). Later, other disease and drought resistance cultivars were produced (Salisbury *et al*., 1995).

Each *B. napus R*-gene product reacts with the product of the corresponding avirulence gene (*AvrLm*) in the *Leptosphaeria* (Parlange *et al.*, 2009). This gene for gene recognition, although initially effective, is not durable as *L. maculans* can evolve into races the plant cannot recognise (Delaney, 1997, Fitt *et al.,* 2006). This can happen quickly, within three growing seasons (Huang *et al*., 2009). For example, it occurred when the *Rlm1* resistance gene in oilseed rape cultivars broke down (Van de Wouw *et al.*, 2010) resulting in a stem canker epidemic. The ability of *L. maculans* to overcome major gene resistance is aided by the the production of a large number of sexual spores which can travel long distances on the wind.

The incorporation of genes from closely related species e.g. *B. rapa* ssp. sylvestris, *Arabidopsis thaliana* Heynh, into *B. napus* can also be broken down by *Leptosphaeria* (Li & Cowling, 2003, Howlett, 2004). Placing multiple resistance genes into the plant (pyramiding) gives durable resistance by

increasing the chance that the genes present will correspond to the *Leptosphaeria* species avirulence genes.

Higher temperatures and wetter conditions, which are forecast to become more frequent due to global warming, have been shown to adversely affect the durability of major gene resistance with an increase in epidemics (Huang *et al*., 2005).

1.4.2. Quantitative resistance

Quantitative resistance (many resistance genes, each with a small effect) occurs during the symptomless growth between the fungi reaching the petiole and when the canker on the stem occurs. It is imparted by multiple quantitative trait loci (QTLs), regions of DNA associated with phenotypic traits. These work in combination with the effect of the environment (Xu *et al*., 2005, Xu *et al*., 2013).

Quantitative resistance to stem canker in *Brassica* is durable as it slows down *Leptosphaeria* growth from the leaf to the stem (Fitt *et al*., 2006) resulting in less severe cankers. This resistance can be overcome in epidemics when the *Leptosphaeria* inoculum pressure is extremely high (Xu *et al*., 2005).

1.5. CULTURAL CONTROL OF STEM CANKER ON OILSEED RAPE

Control can also be aided by management practices, alongside breeding practices. Stubble management (raking, deep ploughing, burning), or flooding speed up the breakdown processes and stop ascospores being released into the air (Turkington *et al*., 2000, Gladders *et al*., 2006). Sanitising farm machinery is employed to stop the spread of the fungus. Early sowing of winter *B. napus* ensures the plant has produced true leaves, which have a strong epidermis. These are better able to resist entry of the germ tube when spores mature and are released (West *et al*., 2000). Crops planted later have tender cotyledons which can be easily damaged allowing fungal entry. Crop rotation employed to keep the field free of inoculum does not always work, due to the large distances *L. maculans* airborne spores can

travel (Gladders *et al*., 2006). Thus cultural control alone is often ineffective and other methods are required to support it.

1.6. CONTROL OF STEM CANKER USING FUNGICIDES

Current farming methods in developed countries have a high dependence on the input of synthetic fertilizers and pesticides. This has helped to overcome many of the crop pests which ravaged crops in the past. As such, the production of chemical pesticides is a profitable business, allowing large quantities of good quality produce to be grown (Gerhardson, 2002).

The main way of controlling stem canker today is to use chemical fungicide sprays in conjunction with resistant cultivars (Fitt *et al*., 2006). Fluquinconazole (a DMI) can be applied to oilseed rape stubble to delay spore maturation and discharge, whilst a mix of DMI and succinate dehydrogenase inhibitors (SDHIs) are applied as a foliar spray (Huang *et al*., 2014). The healthy plants are protected against stem canker before the disease becomes established, thus ensuring a cost effective way of achieving high yields. Growers apply the fungicide in response to a forecast, a decision support mechanism, which predicts when the initial symptoms (leaf spot) will be seen on 10-20% of oilseed rape plants (Gladders *et al*., 2004). The fungicide application will differ between geographical regions and in individual crops. This timing is critical to stop *L. maculans* spreading to the stem base causing stem canker (Huang *et al*., 2011). The spray is repeated one month later. The two main forecasting systems in this country are PASSWORD and Crop Monitor. PASSWORD is a collaboration of Rothamstead Research, the Perry Foundation and Anhui Agricultural University, China. The model is based on previous epidemics, temperature and rainfall along with data on ascospore maturation, (Rothamstead Research, 2012). The information on Crop Monitor (www.cropmonitor.co.uk) is collated from a number of monitored sites and reports up to date information on the severity of stem canker. It takes into account cultivars and their resistance rating, seasonal variation and issues alerts if a threat occurs. Crop Monitor is a collaboration between Department for Environment for Food and Rural Affairs, Food and Environment Research Agency and is sponsored by BAYER A.G. and the Home Grown Cereals Authority.

Fungicides are grouped by mode of action and similarities in their chemical composition. Site specific fungicides (e.g. MBCs, QoIs) disrupt a single metabolic process or structure in the fungus (e.g. sterol synthesis, mitochondrial respiration). These are highly specific in their toxicity, and are often systemic. Fungicides which act on multiple sites in fungal metabolism impede many processes, are less specific and generally effect many classes of fungi. The fungicides used to control stem canker on oilseed rape generally have site specific modes of action (Table 1.1.).

Table 1.1. Fungicides controlling stem canker currently in use and their modes of action. Adapted from (Fraaije *et al*., 2007).

Fungicides	**Active ingredient**	**Mode of action**
Methyl benzimidazole carbamates (MBC)	Benzimidazole	Inhibits mitosis by disrupting the formation of β-tubulin, part of fungal microtubules
Quinone outside inhibitors (QoIs)	Strobulin	Inhibits mitochondrial respiration by disrupting the cytochrome bc1 in complex III
Sterol demethylases inhibitors (DMIs)	Prothioconazole	Inhibits the biosynthetic pathway of ergosterol required for fungal cell membranes

1.6.1. Fungicide resistance

Fungicide control breaks down for a number of reasons, including incorrect application rate, improper timing of application, excessive rainfall and resistance. Reduced sensitivity (resistance) of a fungus to a fungicide is due to a genetic mutation, influenced by the biology number of reproductive cycles, how the active ingredients in the fungicide work on the fungus and excessive application of the fungicide. Genetic mutations occur naturally in fungal populations. These become important when the mutation becomes dominant, due to selection pressures. If the fungicide imparts control on a single gene, resistant fungal strains may arise rapidly, resulting in a sharp decline in control. Botrytis has become resistant to MBC fungicides due to point mutations in codon 198/200, whilst septoria currently has resistance to QoI fungicides due to a single nucleotide polymorphism mutation in codon 143 (Leroux, 2007). Resistance in fungi sprayed with fungicides using multiple control mechanisms results in a slow rate of resistance progression, as it is unlikely all the genes affected will mutate. Resistant pathogens are

usually still present when the fungicide is no longer in use, therefore the chemicals cannot be reintroduced into the same area.

1.6.2. *Leptosphaeria maculans* resistance to fungicides

An increase in fungicide resistance in plant pathogens has been observed. The predicted increase in human population and the consequent rise in the demand for food, without a comparable increase in farmland, may also result in an intensification of diseases, as farmers try to enhance crop yield by increasing the use of pesticides (Emmert & Handelsman, 1999).

Leptosphaeria maculans has become resistant to some fungicides, possibly due to their overuse (selection pressure) or by naturally occurring mutants arising via sexual recombination. As the fungus is haploid, mutations may have an effect on phenotype. Demethylation inhibitor fungicides (DMIs) remove the methyl from the carbon 14 (C14) from the target enzyme sterol demethylase (CYP51), involved in ergosterol biosynthesis. This results in ergosterol, a major constituent of fungal membranes, not being produced and so the fungus cannot grow (Wyand & Brown, 2005). Whilst not totally resistant, the genome of *L. maculans* does have the *Cyp51* gene encoding the target enzyme, which, if mutated, may reduce fungicide efficacy. Over-expression in fungal membrane pumps (ATP binding cassette and major facilitator superfamily transporters) also render these fungicides less effective, as they pump the fungicide out of the fungal cell (Bean *et al*., 2009).

Quinone outside inhibitors (QoIs, e.g. azoxystrobin) fungicides inhibit the splicing of DNA encoding for the cytochrome bc1 of complex III, in the membrane of the mitochondrion electron transport chain (ETC). Cytochrome bc1 couples the transfer of electrons to the generation of a proton gradient using a redox reaction, driving the synthesis of adenosine triphosphate (ATP). As no splicing occurs, mRNA cannot be produced and as such cytochome b cannot be made. This results in a lethal mutation as the fungus is unable to produce ATP. The superoxide products released (free radicals) cause major damage in the DNA (Crofts *et al*., 2004). A single mutation in the fungal genome at codon 143A (GGT to GCT, glycine to alanine) enables the fungus to acquire resistance to QoIs (Gisi *et al.*, 2002).

Methyl benzimidazole carbamate (MBC) fungicides (e.g. benzimidazoles) interrupt the formation of ß-tubulin in microtubules utilised in mitosis, stopping hyphal growth. Point mutations at the allele containing codon 200 (TTC to TAC, phenylalanine to tyrosine) and codon 198 (GAG to GCG, glutamic acid to alanine) giving stem canker resistance to MBC fungicides, with codon 198 also conferring sensitivity to diethofencarb (carbomate) (Koenraadt & Jones, 1993).

1.6.3. Next generation fungicides

Next generation succinate dehydrogenase inhibitor (SDHI) fungicides not currently widely used on phoma leaf spot, were assayed in this work for control (Table 1.2.). It was useful to obtain a comparison between the new and old fungicide under high inoculum pressure in the model method used in this work. These fungicides have a strong and durable affinity to both the pathogen and to the epidermal wax on the cotyledon or leaf (double binding) and work by inhibiting complex II of the tricarboxylic acid (TCA) cycle, halting fungal respiration (Gudmestad *et al*., 2013). There is a mix of DMIs and SDHIs in some products.

Table 1.2. Next generation fungicides, their active ingredients and target crop.

Name	**Manufacturer**	**Active ingredients**	**Current target crops**
Adexar®	BASF	Bixafen Prothioconazole	Wheat, barley, oat, rice
Aviator®	BAYER	Fluxapyroxide Epoxiconazole	Wheat, barley
Seguris®	Syngenta	Isopyrazam Epoxiconazole	Wheat, barley, rye
Vertisan®	DuPont	Penthiopyrad (applied with prothioconazole)	Cereals,soybean, Sugarbeet,citrus, peanut
Q8Y78	DuPont	Penthiopyrad (applied with picoxystrobin	None

1.7. BIOLOGICAL CONTROL OF PLANT PATHOGENS

Biological control on plants is the use of antagonistic microbes (or their extracted products) to suppress pathogens. It is becoming increasingly important as it is widely considered to be environmentally friendly, to have low side effects and may reduce the amount of synthetic chemicals used (Pal & McSpadden Gardener, 2006). Biological control agents (BCAs), the organisms that actually suppress the disease, are a powerful tool as they are non-toxic to birds, bees, fish and other wildlife, and the produce can be eaten on the same day as spraying. BCAs may also be useful when fungicide resistance has occurred or when legislation states that the usual method of control is banned. The European Pesticide Legislation is in the process of recommending banning or restricting the use of many fungicides (e.g. triazoles) in response to studies (Taxvig *et al*., 2007) showing that they may have a detrimental effect on human hormones as *CYP51* of plants may be analogous to mammal *cyp 17*. (EU directive 91/414). The ban may decrease yield of oilseed rape crop by up to 38%, and increase resistance pressure on other fungicides (Adas, 2013). The combination of resistance and withdrawal of registration opens up a stronger market for biological control. There is also public demand for organically grown products, where fungicides are not used or at least their use is limited. The market acknowledges this demand and is putting pressure on growers to reduce the amount of fungicides applied to crops and instead use alternative methods of pest control. The ban on some fungicides along with a change in public opinion of their environmental dangers leaves a gap, one which biological controls can fulfil.

The sensitivity of some people to the use of synthetic pesticides may also be beneficial to a natural method of disease control e.g. environments such as areas of high civic use and glasshouses (Harman, 2000). Biological control can also be a major part of integrated pest management, where a 'natural' control is applied in conjunction with a synthetic one. They are also certified for use in organic systems (Whipps, 1997).

Published definitions of biological control invite debate in the scientific world, as today modern technological developments (e.g. modified organisms or gene products) may be taken into account (Pal & McSpadden Gardener, 2006). At its basic form, the antagonist microbe population is artificially

increased, then applied onto the host plant to directly attack or compete with the pathogen (Gerhardson, 2002). Microbial biological control in sustainable agriculture must occur without detrimental effects on non-target organisms in the environment. It is preferable to apply naturally occurring enemies already adapted to the ecosystem, which have a better chance of survival in what may be hostile surroundings. Any microbe used as a biological control agent must be rhizoplane or phylloplane competent and can impart a durability not always found with the application of chemicals (Ahmad & Baker, 1987, Harman, 2000, Gerhardson, 2002, Pal & McSpadden Gardener, 2006).

The mechanisms employed by biological control agents are diverse, complex and vary between pathogen, host plant and the biological control agent. As such, it is sometimes difficult to ascertain what interactions are actually occurring (Whipps, 2000). The main mechanisms of antagonism are competition, antibiosis, lysis, predation and parasitism. (Pal & McSpadden Gardener, 2006)

Competition is an indirect interaction, with the biocontrol agent competing for space and nutrients, decreasing growth, activity and fecundity using exudates, leachates, and siderophores. The suppression of disease relies on the ratio of carbon to nitrogen as a nutrient source (Pal & McSpadden Gardener, 2006), Guetsky *et al*., 2002) resulting in halting or slowing the growth of nearby colonies (Be'er *et al*., 2010). The success of competition between micro-organisms for nutrients and space is usually determined by rapid germination of spores, fast growth and the ability to utilise the released nutrients as a food source.

Organisms that employ parasitism and predation (direct antagonism) (e.g. *Trichoderma* spp.) directly mechanically attack, penetrate and kill the pathogen, releasing nutrients for metabolic use (Fravel, 1988, Raaijmakers *et al*., 1994). *Trichoderma harzianum* also secretes chitinases and hydralases, which degrade the cell wall, releasing nutrients for its own metabolic use (Chet & Henis, 1975, Elad *et al*., 1999).

Siderophores, secreted by many bacteria e.g. *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, chelate ferric (Fe^{3+}) ions, result in a deficiency for other micro-organisms, causing growth restriction (Guetsky *et al*., 2002.

Antimicrobial substances (e.g. ammonia, carbon dioxide and hydrogen cyanide) may be produced by bacteria, again to increase competitive fitness in their environment. Only a very small amount of these substances is required to block metabolic pathways, resulting in toxicity to micro-organisms. For example, hydrogen cyanide produced by *P. fluorescens* CHA0 (which also produces antibiotics and siderophores) blocks the cytochrome oxidase pathway of the mitochondria, resulting in no energy being produced and causing the death of aerobic organisms (Laville *et al*., 1998).

Antibiotics are secondary metabolites toxic to other microbes at low concentrations which must be produced near the target pathogen for direct effect (Pal & McSpadden Gardener, 2006). A number of different antibiotics may be produced, a primary role of which supresses competition, imparting fitness on the producer (Fravel, 1988, Yim *et al*., 2007) Table 1.3. shows the antibiotic, producing organism and the target pathogen.

Table 1.3. Example BCAs, antibiotic produced and target pathogen. Adapted from Pal & McSpadden Gardener, 2006.

Biological control agent	**Antibiotic**	**Target pathogen**	**Reference**
Bacillus amyloliquefaciens FZB42	Bacillomycin, fengycin	*Fusarium oxysporum*	Koumoutsi *et al.*, 2004
B. subtilis, AU195	Bacillomycin D	*Aspergillus flavus*	Moyne *et al.*, 2001
B. subtilis QST713	Iturin A	*Botrytis cinerea*, *Rhizoctonia solani*	Paulitz & Belanger (2001) Kloepper *et al.,* 2004
Burkholderia cepacia	Pyrrolnitrin, pseudane	*R, solani Pyricularia oryzae*	Homma *et al*., 1989
Pantoea agglomerans C9-1	Herbicolin	*Erwinia amylovora*	Sandra *et al*., 2001
Pseudomas fluorescens F113	2,4-diacetyl-phloroglucinol	*Pythium* spp.	Shanahan *et al.*, 1992
P. fluorescens 2-79 and 30.84	Phenazines	*Gaeumannomyces graminis*	Thomashow *et al*., 1990
Trichoderma virens	Gliotoxin	*R. solani*	Wilhite *et al.,* 2001

Determinants produced by some microbes may also impart biological control by inducing localised and/or systemic plant resistance (Table 1.4.). Induced

systemic resistance (ISR) is a localised broad spectrum resistance induced in the plant mediated by its production of jasmonic acid and/or ethylene by non-pathogenic bacteria and fungi. ISR is upregulated by *Pseudomonas* and *Bacillus* antibiotics (Pal & McSpadden Gardener, 2006). Systemic acquired resistance (SAR) occurs in the whole plant when it has been already had localised exposure to a pathogen (Mahuku *et al*., 1995a). In response to pathogen infection, the plant produces exogenous salicylic acid which accumulates. When salicylic acid binds to membrane receptors in the plant cells, signal transduction pathways are activated inducing the up-regulation of a wide range of pathogenesis related genes even in other parts of the plant.

Table 1.4. Examples of BCA bacteria identifying the determinant produced which imparts resitance in the host plant. 2,3-butanediol is one of the end products of anaerobic fermentation. 2,4-diacetylphloroglucinol is a broad spectrum antibiotic. Z,3-hexenal is an aldehyde. Adapted from Pal & McSpadden Gardener, 2006.

Bacteria	**Determinant**	**Defence mechanism**	**Reference**
B. subtilis	2,3-butanediol	ISR	Ryu *et al*., 2004
P. fluorescens	2,4-diacetylphloroglucinol	ISR	Lavicoli *et al*, 2003
	Siderophore	SAR	Maurhofer *et al*., 1994
	Siderophore	ISR	Leeman *et al*., 1995
	Lipopolysaccharide	ISR	Leeman *et al*., 1995
	Lipopolysaccharide	ISR	Van Wees *et al*., 1997
	Lipopolysaccharide	ISR	Duijff *et al*., 1997
	Lipopolysaccharide	ISR	Van Peer & Schippers, 1992
P. putida	Lipopolysaccharide	ISR	Meziane *et al*., 2005
	Siderophore	ISR	Meziane *et al*., 2005
	Z,3-hexenal	ISR	Ongena *et al*., 2004

The upregulation of ISR and SAR can be advantageous in biological control if a weaker pathogen or non-pathogenic micro-organism induces SAR by producing its own salicylic acid or has a specific side chain on its outer membrane which is recognised by the plant membrane receptors, stopping the infection of a more virulent pathogen species (Gerhardson, 2002). Heads Up® (Bayer, 2013) contains toxic compounds (saponins) extracted from the plant *Chenopodium quinoa*. When this product is applied to soybean seed, the plant defence mechanisms are triggered as soon as germination occurs.

Serenade® (Basf, 2013) containing *B. subtilis* QST elicits both SAR and ISR in the plant (Poleatewich *et al.*, 2012).

1.8. COMMERCIAL APPLICATION OF BIOLOGICAL CONTROL AGENTS

Although extensive research has been carried out, the biological control of plant diseases, very few products are commercially available. The cost in developing, manufacturing and registering biological pesticides is comparable to synthetic pesticides. The biological control agents must also be conducive to large scale production. Heat and desiccation resistant spores produced by *Bacillus* species have been produced commercially for many years. *Pseudomonas* spp. do not produce spores and may have to be pelleted for commercial production (Emmert & Handelsman, 1999).

There may be a larger market for biological control use in environmentally controlled glasshouses and users of integrated pest management (IPM) and organic gardening. The European Union (EU) has limited the use of most synthetic pesticides by 2014 and provides incentives for registration and use of alternative, lower risk substances (Glare *et al*., 2012). Biological control agents will only become mainstream if their cost and efficiency are comparable to currently used methods. However, there are currently biological controls commercially available. Kodiak® (Bayer) is a bio-fungicide which contains *B. subtilis* isolate GB03, used as a seed treatment to control *Fusarium* and *Rhizoctonia* (Emmert & Handelsman, 1999). It is detrimental to spore attachment and disrupts germ tube growth by producing an antibiotic of the iturin type. Iturins interact with cytoplasmic membranes forming large pores through which ions can leak (Arrebola *et al*., 2010). *Bacillus subtilis* strain MBI 600 (NCIMB12376) (BASF) is currently licensed for sale for the suppression of seed-borne diseases and is applied in conjunction with fungicides e.g. for the control of the root pathogen *Aspergillus*. Phylloplane diseases are controlled by Serenade® (BASF) which contains *B. subtilis* QST 713. (Poleatewich *et al.*, 2012).

PlantShield®HC (BioWorks) contains *T. harzianum* which reduces *Sclerotinia sclerotiorum* inoculum in the soybean rhizosphere (Zeng *et al.,* 2012). Trichodex PW (AMC Chemical, Columbia) another *T. harzianum* containing product, is used to control *Botrytis cinerea*.

1.9. BIOLOGICAL CONTROL AGENTS IN THIS WORK

The bacteria used in this work have all been previously shown to work as biological control agents when applied individually. *P. chlororapis* spp. *aureofaciens* was supplied by Dr. R. Fray, University of Nottingham. All *Bacillus amyloliquefaciens* and *Trichoderma harzianum* isolates were supplied by Dr. S. Rossall, University of Nottingham.

1.9.1. *Pseudomonas chlororaphis* spp. *aureofaciens* 30.84

Pseudomonas interact with the plant in many different niches, on the root, the leaf epidermis and intercellularly. *Pseudomonas* bacteria that land on the waxy cuticle of the leaf find the fluctuating environment stressful and may quickly enter the leaf (Preston, 2004).

Pseudomonas chlororaphis spp. *aureofaciens* 30.84 is a rod shaped, aerobic bacterium. The bacterium produces phenazine antibiotics (secondary metabolites) which give an antifungal environment on the phylloplane (Pierson & Thomashow, 1992, Whistler & Pierson, 2003). Antibiotics, produced in the late exponential and stationary growth phases via the shikimic acid pathway (Turner & Messenger, 1986), possibly function as a survival mechanism (Wood & Pierson, 1996). Populations of mutant bacteria unable to produce phenazine ($PhzI^-$) decline rapidly (Pierson & Thomashow, 1992). Although antibiosis is a primary role of antibiotics, their primary functions are to remove or accept mitochondrial elections (electron shuttles), to modify redox states and to be involved in cell signalling and biofilm production (Bisschop, 1979).

1.9.2. *Bacillus amyloliquefaciens*

The *Bacillus* genus comprises endospore-forming, Gram-positive aerobic bacteria. They are commonly saprophytic, occurring in air, soil and water, mainly present in the spore-form and are as such, non-active.

Some *B. subtilis* isolates have been reclassified using the DNA encoding for the 16S ribosomal RNA as *B. amyloliquefaciens (*Stackebrandt *et. al.,* 1987*)*. However, because *B. subtilis* and *B. amyloliquefaciens* have similar

phenotypes, many studies and literature continue to refer to the bacteria as *B. subtilis*.

Bacillus subtilis is known to be useful as a biological control of both root and leaf fungal plant pathogens, mainly by antibiosis (Fiddaman & Rossall, 1993, Raaijmakers *et al*., 2010). It is an attractive biological control agent as its thermostable metabolites can be purified from broths fermented on a large scale. It is safe for human consumption as it is not a human or animal pathogen and it is used as an additive to fermented soybean human food in Japan.

The bacteria initially live as single celled organisms within complex communities. As population density increases in the community, the quorum sensing (QS) mechanisms co-ordinate bacterial behaviour (swarming) and produce many different compounds, including antibiotics (von Bodman *et al*., 2003, Stein, 2005, Jayaraman & Wood, 2008). Antibiotics are non-ribosomally synthesised lipopeptides, produced by enzyme complexes (Katz & Demain, 1977). The differences in fatty acid branching and amino acid sequence gives the different compounds, which can be placed into three families, fengycins, iturins and surfactins (Vanittanakom *et al*., 1986, Vitullo *et al.,* 2011).

Over seven hundred wild-type *B. amyloliquefaciens* isolates producing many different antibiotics have been analysed (Stein, 2005). Approximately 7.5% (6 operons) of the *B. amyloliquefaciens* genome is involved in the production of antibiotics, and the ability to produce so many may suggest it is a recent acquisition, possibly by DNA uptake (horizontal transfer) (Stein, 2005). Antibiotic production is energy intense and they are produced only at stationery phase of growth in conjunction with other starvation induced activities such as sporulation (Marahiel *et al*., 1993, Koumoutski *et al*., 2004). Whist the primary function of antibiotics produced by *Bacillus* is the same as those produced by *Pseudomonas*, their primary role may aid fitness in its natural niche, or kill non-sporulating bacteria (altruism) (Stein, 2005). Antibiotics produced by Gram positive bacteria are predominately peptides but can also be polyketides or phospholipids, and are involved in quorum sensing, programmed cell death (Stein, 2005), and may be signalling molecules (Lazazzera, 2001, Yim *et al*., 2007).

1.9.3. Quorum sensing

In their natural environment such as on plant surfaces, individual microbes do not live in isolation. Bacteria can interact with the same species (cross talk), between species (interspecies) and with higher organisms (inter-kingdom) (Fuqua *et al.,* 1994, Winzer *et al*., 2002). QS molecules have been identified in both Gram positive (e.g. *Bacillus* spp.) and Gram negative (e.g. *Pseudomonas* spp.) bacteria (Fray, 2002).

QS molecules are chemically diverse, small (<1000 Da) organic molecules of 5-9 amino acids. Typically, they are secreted and diffuse through the environment, entering the membranes of neighbouring bacteria (Newton & Fray, 2004). As reproduction increases the density of the bacterial population, more signalling molecules (auto-inducers, AI) are produced and accumulate resulting in a corresponding increase in concentration in the external environment (Newton & Fray, 2004). When a critical AI density is reached a synchronised up regulation of target genes in the bacterial population occurs. QS enables individual bacteria to sense the density of the surrounding bacterial population and employ behavioural co-ordination to respond to an ever changing environment (Williams, 2007).

The target genes upregulated by quorum sensing function in diverse processes including pathogenicity, conjugation, and antibiotic production (Newton & Fray, 2004), and are also found in bacteria which have a beneficial role to plants (e.g *Rhizobium leguminosarum*) (Fray, 2002). A futher role of AIs may be to enable the bacteria to sense local diffusion rates (Redfield, 2002) as the number of cells required to achieve the critical level of QS-molecule production varies according to the environment (Newton & Fray, 2004, Williams, 2007).

The main forms of QS signalling molecule in plant associated Gram negative bacteria are *N*-acyl homoserine lactones (AHLs) (Cha *et al*., 1998). All AHLs have the same basic ring structure with their specificy imparted by the composition and length of the side chains (McClean *et al.,* 1997). AHLs with a small chain (6 carbons or less) may pass through membranes of the neighbouring bacteria by diffusion, whilst longer chain AHLs may require a transport system (Pierson *et al.*, 1994). *Pseudomonas chlororaphis* spp.

aureofaciens 30.84 used in this work has a short hexanoyl (HHL) side chain (Williams, 2007, Tjeerd van Rij *et al*., 2004).

AHLs are derived from the metabolism of fatty acids and amino acids in the shikimic pathway (Parsek *et al.,* 1999) and are generated by the Lux I operon and its homologues (Williams, 2007). The AHL either bind directly to their cognate LuxR transcriptional regulator in neighbouring bacteria (Fray *et al.,* 1999) giving a reversible LuxR/AHL complex, or they bind to a histidine kinase sensor, inducing a phosphorelay cascade (Smith *et al*., 2008). The AHL produced by *P. chlororaphis* spp. *aureofaciens* 30.84 binds to the PhzR transcriptional regulator in a neighbouring *Pseudomonas*. They both bind as a dimer to the 20 bp lux box promotor, inducing a signalling cascade which up-regulates the production of secondary metabolites, including phenazine (Pierson *et al*., 1994, Loh *et al*., 2002, Williams, 2007). The *P. chlororaphis* spp*. aureofaciens PhzI-* mutant used in this work was engineered using a Tn5 leucine insertion so it cannot produce the signal and as such phenazine is not produced (Pierson *et. al*., 1994).

Gram positive bacteria use peptides as a QS signal. The peptide is produced as a precursor then modified into a functioning signal*. Bacillus amyloliquefaciens* uses a number of QS signals. The peptide signal ComX, binds to the transmembrane kinases ComA-ComP. The phosphorylation is transferred downstream, up-regulating the genes for production of antibiotics. A second system is also used; PapR peptide is recognised by the PlcR transcriptional regulator (Osilizo *et al*., 2014).

The AI2 system, found in both Gram negative and Gram positive bacteria may be used for interspecies talk (Osilizo *et al*., 2014).

1.10. COMBINATIONS OF BIOLOGICAL CONTROL AGENTS

Bacillus amyloliquefaciens and *P. chlororaphis* spp. *aureofaciens* employ different survival strategies on plants. They produce diverse molecules which work in different ways to control the target disease. Biological control in the past has favoured using a mix of synergistic microbes, enhancing the range of control with a longer window of activity when compared with the same agents used singly (Mishra *et al*., 2013). It is vital that the two bacteria can work together synergistically. A potential problem in mixed population of *Bacillus* and *Pseudomonas* bacteria is that many bacilli can produce

lactonase enzymes which target and inactivate the N-acyl-homoserine lactones (AHLs) produced by *Pseudomonas* (Pana *et al*., 2008, Williams, 2007). *Pseudomonas* use the AHL molecules to upregulate the antibiotics used in antibiosis. The enzyme lactonase, produced by the *aiiA* gene, hydrolyses the ester bond of the lactone ring of the *Pseudomonas* AHL QS signalling molecule (Dong *et al*., 2001, Williams, 2007), changing its shape and de-activating it. This results in phenazine antibiotics not being produced.

1.11. MONITORING POPULATIONS OF *LEPTOSPHAERIA MACULANS*.

It is vital that populations of *L. maculans* are monitored to enable the grower to apply the fungicides at the correct time. The forecasting systems currently available rely mainly on visual leaf assessments at the leaf spot stage (Figure 1.3.). These forecasting systems are imperfect and more accurate systems are being developed.

1.11.1. Visual assessment of lesions of *Leptosphaeria maculans*

A greyish area 4-8 mm wide caused by the disintegration of the leaf tissue is seen approximately 7 d after initial infection (Huang *et al*., 2003). The lesion occurs behind the hyphal front, so there is more fungus present than the lesion would suggest. The current method used in forecasting systems to ascertain when spraying will be most effective is to assess the lesion size seen in the leaf spot stage of the disease. Visual assessments are subjective, requiring skill and experience to accurately identify the correct lesion area. Whilst this method does work (Eckart *et al*., 2010) it is not an accurate assessment of the amount of fungus present due to the symptomless growth of the hyphae.

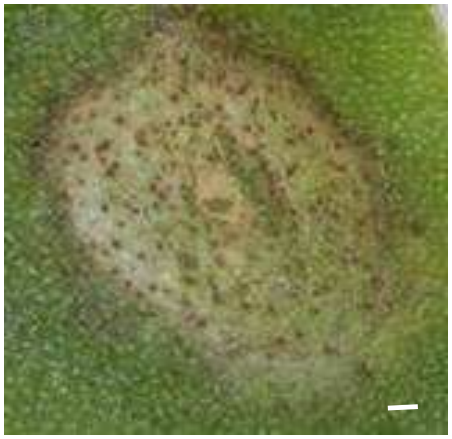


Figure 1.3. Stem canker at the leaf spot stage on oilseed rape cotyledons. Pycnidia containing asexual spores can be seen (brown spots). Photograph, Linda Cholerton. Scale bar – 1mm.

1.11.2. Molecular methods of monitoring populations

Precise detection of plant pathogens is crucial for all aspects of plant pathology especially in agriculture. Molecular diagnostic techniques are now used commonly to quantify microbes, evaluate the genes they express and quantify resistance (McCartney *et al*., 2003, Kakar *et al*., 2014). Diagnostic techniques traditionally have been microscopic observations, isolation, bioassays and pathogenicity tests. Technologies such as microarrays and isothermal amplifications (loop mediated isothermal amplification, nucleic acid sequence based amplification) have potential but many are still in development and are not ready for use in a practical environment (López *et al*., 2009).

The use of Nucleic acid based Polymerase Chain Reaction (PCR) amplification techniques to diagnose and study pathogenic fungi and their biological control has advantages over the traditional methods (Wattiau *et al*., 2001). PCR does not require expertise in visually assessing the disease, the organisms do not necessarily require *in vitro* culturing, and a specific target can be detected within a complex mix of DNA. However, the procedure requires visualisation (gel electrophoresis) to distinguish amplicons in multiplex reactions. It is also not reliable for quantitative analysis (Ginzinger, 2002, Mahuku *et al*., 1995b).

Quantitative PCR has been used in this work and has many advantages over PCR. It can be automated enabling large scale analysis and allows minor changes to be detected whilst being reproducible (Schena *et al.*, 2004). It has allowed insite into population dynamics and the detection of a specific target DNA is proof of an organism being present. Cell viability and their metabolic processes can be determined by the presence of rRNA and mRNA respectively (López *et al*., 2009). As such, qPCR allows detection and monitoring of plant diseases to an accuracy not provided by other molecular methods (Lees *et al*., 2002, Schmit *et al*., 2002, Shannon *et al*., 2007). The risk to the environment and operators is reduced due to the use of the dye SYBR® Green instead of the toxic ethidium bromide used in PCR. SYBR® Green emits fluorescent light when it binds and intercalates with double-stranded (ds) DNA, and does not fluoresce when unbound in solution. As the amount of fluorescence is proportional to the amount of dsDNA of interest

present, primer design and optimisation of conditions are vital to avoid non-specific amplicons, thus obtaining an accurate result. The reaction is heated to 95ºC at the end of each PCR cycle, causing a fluorescent peak which allows the products to be observed in 'real time'. Each amplicon melts at different temperatures, so melt curve analysis can identify different amplicons in a multiplex reaction.

1.12. AIM OF THIS WORK

The aim of this study was to ascertain if a combination of biological control agents is effective in controlling the fungus *L. maculans*, the causative agent of fungal stem canker, on oilseed rape plants, with an objective to obtain information on dynamics *in planta*.

- *Bacillus amyloliquefaciens* and *P. chlororaphis* spp. *aureofaciens* were assayed for ability to control the initial stages of stem canker, the leaf spot stage. Oilseed rape cotyledons were pre-inoculated with biological control agents, then inoculated with *L. maculans.*

- Fungal and bacterial DNA was extracted and quantified per 0.01 mg of leaf material. QPCR was used to determine a correlation between the amount of fungus and control bacteria present with the size of the leaf spot lesion. This was verified by assaying the ergosterol content of the leaves, and colony forming unit counts of the bacteria present. In *Pseudomonas* antibiotic production is upregulated using quorum sensing AHL signalling molecules. A T-streak assay was used to confirm quorum sensing molecules were produced by the *Pseudomas* species used.

- Some species of *Bacillus* produce enzymes which de-activate the *Pseudomonas* AHL signalling molecule. This would cause a problem if a combination of the two bacterial biological control agents were applied as the *Pseudomonas* used in this study, produces antibiotics with AHL-dependent expression. It was therefore advantageous to find a *Bacillus* without lactonase production.

- Quantitative PCR was developed to monitor the biological control populations to ascertain if combinations of biological control agents react in a positive manner and therefore could be used together in biological control to good effect.

CHAPTER 2 MATERIALS AND METHODS

2.0. INTRODUCTION

Where appropriate, laboratory work using micro-organisms was performed under aseptic conditions, using a laminar flow cabinet and flame sterilised instruments. All growth media were sterilised by autoclaving at 120°C for 20 min. Antibiotics were sterilised by filtering through 0.45 µm Millipore membranes. Details of media ingredients are given in Appendix A, and consumables in Appendix B. Primers are stated in the appropriate text.

Data, preliminary analysis and figures were produced using Microsoft Excel. Genstat was used to perform statistical analyses. The raw qPCR sequences were visualised using Roche LightCycler® 480 software v 1.5.

2.1. OILSEED RAPE (*BRASSICA NAPUS*)

This work was carried out using cotyledons of *Brassica napus* cv. Winner and Compass. Seeds were supplied by Dr. S. Rossall, University of Nottingham. These were sown directly in freely draining modular trays (60 x 40 x 10 cm) or in 9 cm diameter pots, using Levingtons M3 peat-based potting compost (Appendix B). For preliminary work, sown seeds were placed under a propagator in a glasshouse at approximately 20ºC until emergence, then the lid was removed and the seedlings left to grow for one week. They were then placed into a glasshouse under natural daylight with a temperature regime maintained at 16ºC day, 12ºC night. Typical natural light provided a photosynthetic photon flux density of approximately 400 µmol m^{-2} s^{-1} Plants were thinned to one seedling per well and left to acclimatise for three days before manipulation. The apex was removed to retain cotyledons (Figure 2.1.). Further assays were carried out in a controlled environment room, using the above temperature criteria and a photosynthetic photon flux density of approximately 200 µmol m^{-2} s^{-1}. Plant apices were continuously removed to keep the cotyledons *in situ*.

(a)

(b)

Figure 2.1. Oilseed rape seedlings at 5 days after sowing.Pictured before removal of apex (a) and after (b).

2.2. *LEPTOSPHAERIA MACULANS*

Leaves with visible symptoms of phoma leaf spot were collected from fields in Bramcote and Sutton Bonington, Leicestershire.

Under aseptic conditions, a small amount of the tissue surrounding the lesion was excised. These were surface sterilised by placing into a 7% Domestos solution (Appendix B) for 2 min, washed with sterile distilled water five times, then left to soak for 2 min. After a final rinse, the explants were left to dry on sterile filter paper for 2 min, then plated onto Petri dishes containing 10-15 mL of 39 g L^{-1} potato dextrose agar (PDA, Appendix A) amended with penicillin and streptomycin (stock concentrations 30 mg mL^{-1}, 133 mg mL^{-1} respectively) antibiotics (PDA^{+}, Appendix A). The cultures were incubated in a controlled environment room at 18 ºC until mycelia were seen growing from the explants.

A sterile scalpel was used to remove hyphal tips which were placed onto fresh amended media. After 3 d, the procedure was repeated resulting in uncontaminated pure cultures. Isolate identification was obtained by PCR and BLAST analysis. Glycerol stocks of mycelia and spores were produced and stored at -80°C.

2.2.1. Routine growth

Leptosphaeria isolates were grown on sealed Petri dishes containing PDA^{+} and incubated in a controlled environment room at 17°C with 24 h fluorescent lighting and 12 h near UV (~360 nm) (to stimulate sporulation) at an intensity of approximately 20 µmol m^{-2} s^{-1}.

2.2.2. Subculturing

Subculturing was performed under aseptic conditions by cutting a 5 mm diameter plug out of the leading edge of an actively growing colony. Plugs were placed mycelia side down onto Petri dishes containing fresh PDA^+. The pathogenicity is not affected by this method of subculturing (Bansal *et al*., 2002).

2.2.3. Purification of cultures

Isolates were sub-cultured onto PDA^+ medium in 9 cm Petri dishes and incubated at 20°C until mycelial growth established. If the fungi showed bacterial contamination, a small piece of mycelium was excised and placed into a 1.5 mL Eppendorf tube with 1 mL sterile distilled water. This was macerated into a suspension, 100 µL removed, 900 µL of sterile distilled water added and mixed using a sterile pipette. A 500 µL aliquot of the mix was placed onto PDA^+ (Appendix A) and incubated at 15°C. Single uncontaminated colonies were excised using a flamed scalpel and placed onto PDA^+ for further growth.

2.2.4. Ascospores (Sexual spores)

Stubble with pseudothecia containing sexual ascospores was placed in plastic Stewart containers (Appendix B) and soaked in sterile distilled water for 30 min at room temperature until the pseudothecia were observed to be swollen.

Explants containing swollen pseudothecia were attached to the underside of a Petri dish lid using petroleum jelly (Appendix B). The lids were flooded with water to swell the asci, left for one minute then drained. Distilled water was placed into the Petri dish and the lids replaced. These were placed into an incubator at 22°C (section 2.2.1.) and left for 24 h for the spores to discharge. The water containing the spores was filtered through 1 layer of sterile muslin into a Falcon tube (Appendix B).

2.2.5. Pycnospores (asexual spores)

Pycnospore cultures were grown at 18ºC, as described in section 2.2.1., on, PDA^+ (Appendix A). When the mycelia filled the growth medium surface, plates were placed in an incubator under UV, until sporulation could be seen. If abundant sporulation did not occur, the mycelia were overlaid with 10 mL

of distilled water agar (DWA) (Appendix A) and incubation continued for a further 7 d.

The cultures were flooded with sterile distilled water and left at room temperature under aseptic conditions for 20 min. A sterile loop was used to gently rub the culture surface to assist release of spores from the pycnidia. The resulting suspension was filtered using a funnel containing 2 layers of autoclaved muslin into sterile Falcon tubes (Appendix B). The suspensions were centrifuged at 1000 x g for 5 min to pellet spores. The supernatant was removed and the pellet re-suspended in 10 mL sterile distilled water and vortexed to mix.

2.2.6. Spore suspension concentration

Leptosphaeria maculans spore concentration was ascertained using a haemocytometer. A spore suspension droplet (20 µL) was placed onto the particle counting chamber of a haemocytometer, and observed under a light microscope at x400 magnification. The numbers of spores present in five large squares were counted and the mean calculated. The required concentration to inoculate the oilseed rape leaves was 10^6 cm^{-2}. The concentration was adjusted by dilution or centrifugation and re-suspension as appropriate.

2.2.7. Storage and maintenance of mycelia

Clean actively growing cultures were placed in screw-top glass universal tubes containing PDA^+ (Appendix A) and grown at 20°C in a controlled environment room under 12 h UV as described in section 2.2.1. When the fungus filled the surface agar, the cultures were placed into storage at 4°C. Cultures were revived by excising a plug and placing it onto fresh PDA^+ media.

2.2.8. Storage and maintenance of spores

Conidial suspensions were used for long term storage. A spore pellet was suspended in 0.5 mL of 80% sterile glycerol and stored at -80°C. The spores were revived by defrosting at room temperature, plating onto fresh growth media and incubated at described above.

2.3. ISOLATION OF BACKGROUND LEAF BACTERIA

Under aseptic conditions, leaf discs were excised using a 5 mm sterile corer. Ten explants were placed into each autoclaved flask containing an aliquot of sterile maximum recovery dilutant (MRD, Appendix B) (to minimise physiological shock and reduce organism multiplication for up to 1 hr), plus 20 µL of Tween 80, a polyethylene sorbitol ester surfactant used to aid removal of bacteria (Appendix B). Flasks were sealed with sterile cotton wool and covered with silver foil and placed in an orbital shaker at 25°C for 20 min at 200 g to remove the surface microflora. Serial dilutions were prepared up to 10^{-9}. An aliquot of undiluted solution was Pasteurised by heating to 80°C for 20 min, to assist recovery of spore-forming bacteria.

Serial dilutions of Pasteurised and untreated bacteria were pipetted onto plates containing NA (Appendix B) growth medium and placed in an incubator at 30°C for 24 h. The cultures were examined after 24 h, single colonies subcultured onto fresh media to obtain pure colonies and identified using PCR amplification and BLAST database searches.

2.4. *PSEUDOMONAS CHLORORAPHIS* SPP. *AUREOFACIENS* AND *BACILLUS AMYLOLIQUEFACIENS*

Pseudomonas chlororaphis spp. *aureofaciens* 30.84 (a known suppressor of take-all of wheat) and PhzI- mutant (unable to produce phenazine antibiotic) were donated by Dr. R. Fray, University of Nottingham. *Bacillus amyloliquefaciens* isolates were provided by Dr. S. Rossall, University of Nottingham and had known control of botrytis. *B. amyloliquefaciens* MBI600 was isolated by Dr. Rossall, with 62p and 66p isolated by R. Laboh.

All bacteria were grown on nutrient agar (NA) for *Bacillus* spp. and *Pseudomonas* CFC selective SR 103 for *Pseudomonas* spp. (Appendix A).

For liquid cultures, *Bacillus amyloliquefaciens* was grown in tryptone soya broth (TSB) and *P. chlororaphis* spp. *aureofaciens* in LB broth in conical flasks with the necks sealed with cotton wool and foil. These were placed onto an orbital shaker and grown at 25ºC at 100 rpm. The cultures were prepared in triplicate, such that inocula for each replicate had a unique source.

2.4.1. Removal of metabolites from bacteria grown in liquid culture

Bacteria were grown as above in liquid culture to stationery phase (72h). A bench centrifuge (1000 x g for 10 min) was used to sediment the bacteria into a pellet, the supernatant containing metabolites was removed and the pellet re-suspended in maximum recovery dilutant (MRD) to maintain osmotic balance.

2.4.2. Storage of bacteria

Under aseptic conditions, an aliquot (900 µL) of sterile 80% glycerol was placed into a sterile Eppendorf tube, mixed with (100 µL) bacterial culture produced in liquid media then stored at -80°C. The bacteria were reactivated by scraping off a small amount of ice with a flamed sterile metal loop. The ice containing the bacteria was plated onto relevant media, and incubated at the appropriate temperature.

2.5. TRICHODERMA HARZIANUM

Trichoderma harzianum was produced by Dr. S. Woodward of Aberdeen University and had broad spectrum suppression.

Mycelia were grown in a controlled environment room with conditions as section 2.2.1.

2.6. DNA EXTRACTION

Six 5 mm diameter plugs of *L. maculans* hyphae were excised from actively growing mycelia under aseptic conditions into 10 mL of potato dextrose broth (PDB) in a 100 mL bottle. Cultures were incubated in a dark growth cabinet at 25ºC for approximately 28 d. Mycelium was harvested by decanting into 15 mL centrifuge tubes and centrifuged at 1000 x g for 10 min. The supernatant was removed, and the mycelium either frozen at -80°C for 24 h or used fresh.

Bacteria were grown in liquid culture, and centrifuged at 3000 g for 10 min after which the supernatant was removed. Various methods were attempted to extract DNA to obtain quality within acceptable parameters for use as standards.

2.6.1. Dneasy Plant Mini kit

Dneasy Plant Mini kit (Appendix B) was used. Liquid nitrogen was added to tissue and ground to a fine powder using a mortar and pestle. The liquid nitrogen was then evaporated at room temperature. Buffer AP1 (400 µl) and 4 µl of RNase A (100 mg/ml) was added to produce 100 mg net weight of ground tissue and then vortexed. The mixture was incubated for 10 min at 65°C in a water bath and inverted 3 times during this time to mix. 130 µl of Buffer AP2 was added to the lysate, mixed, then placed on ice for 5 min following which centrifuged at 18,000 x g for 5 min. Supernatant was added to each QIA shredder spin column placed in a 2 ml collection tube and centrifuge for 2 min at 18,000 x g. The flow-through fraction was placed into an Eppindorf and 1.5 volumes of Buffer AP3 was added and mixed using a pipette. The lysate was added to the mini spin column and centrifuged for 1 min at 6000 x g. The flow-through was discarded. The column was placed into a new 2 ml collection tube and 500 µl Buffer AW was added. This was centrifuged for 1 min at 6000 x g and the flow-through discarded. Another 500 µl Buffer AW was added to the column and centrifuged for 2 min at 18,000 x g to dry the membrane. The flow-through was discarded and the column was transferred to a microcentrifuge tube where 50 µl of Buffer AE applied directly onto the column membrane. This was incubated at room temperature for 5 min, then centrifuged at 6000 x g for 1 min to elute the DNA.

This method produced bacterial DNA of adequate quantity and purity; however it only generated a small amount of fungal DNA.

2.6.2. Hexadecyltrimethylammonium bromide (CTAB)

Mycelia grown in liquid culture were macerated in 500 µL CTAB extraction buffer (2% CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCL, 1.4 mM NaCl and 20 mM EDTA (ethylenediaminetetraacetic acid) and 10 µL ß mercaptoethanol), vortexed and incubated at 70°C for 60 min. Extracts were then centrifuged for 15 min at 1000 x g. The supernatant was removed and an equal volume of a 25:1 chloroform:isoamyl alcohol mix added, vortexed, then centrifuged for 15 min at 16,000 x g. Aliquots (450 µL) of the upper layer were collected. Sodium acetate (45 µL, 3M pH 5.2) and 900 µL of ice cold absolute ethanol was added, mixed by inversion and

placed at -20°C for 60 min to precipitate the DNA. The DNA was pelleted by centrifugation at 16,000 x g for 10 min and the supernatant discarded. The DNA pellet was washed with 1 mL ice-cold 70% ethanol, dried at 37°C for 15 min after which 100-200 µL TE buffer (10 mM Tris HCl pH 7.5, 1mM EDTA) was added to dissolve the DNA. Whilst this method gave some good quality DNA, it was not consistent.

2.6.3 Bio-Nobile PickPen® extraction

Fungal DNA was extracted using a PickPen® and PickPen® kit (Appendix B). Fresh mycelium (100 mg) was placed into extraction bags and homogenised using a tissue grinder (Appendix B). Lysis buffer (400 µL) and 10 µL of Proteinase K were added and the liquid removed. This was placed into a water bath at 65°C for 30 min, after which it was centrifuged at 18000 x g for 5 min. The supernatant was transferred into tube 2 containing magnetic particles and DNA binding buffer. This was continually mixed, whilst being incubated at room temperature for 10 min. The PickPen® (Appendix B), with a tip collected the magnetic particles which were placed into a wash solution. The wash process was repeated twice. Finally, the magnetic particles were placed into elution buffer and left for 5 min, continually being gently shaken. The magnetic particles were removed leaving a small amount of DNA. Qualification using a spectrophotometer indicated good DNA purity.

2.7. DNA CLEAN-UP PROCEDURE.

Extracted DNA was cleaned up to obtain purity within acceptable parameters for use as standards. The DNA was purified using a GenElute™ PCR Clean-up Kit (Appendix B). A miniprep binding column was placed in a collection tube. Column preparation solution (0.5 mL) was added into each column and centrifuged for 1 min at high speed. The elutate was discarded. The amount of PCR product remaining was multiplied by five which gave the quantity of binding solution required in each PCR tube. The solution was mixed using a sterile pipette, placed into the clean-up tubes and centrifuged for 1 min at high speed. The elutate was discarded, leaving the DNA adhered to the column. Wash solution plus ethanol (0.5 mL) was added to each column and centrifuged for 1 min at 16000g. The elutate was discarded and the tubes centrifuged for 2 min at 16000g to dry the DNA. The binding columns containing the DNA were transferred to fresh tubes and 50 µL of elute

solution added, left for 1 min, then centrifuged at 16000g for 1 min. The columns were discarded, leaving the eluate containing the DNA in the bottom of the tube.

2.8. POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) was carried out using 25 µL volumes containing 1 µL genomic DNA as a template. Each reaction was completed using 12.5 µL mango mix (Bioline), 10.5 µL sterile distilled water and 1 µL each relevant forward and reverse oligonucleotide primer (standard concentration 10 µM). The sequences for *L. maculans* primers were obtained from Liu *et al*., 2006, amplifying 5.8S RNA from the large subunit of the ribosome (forward primer *5'*-CTTGCCCACCAATTGGATCCCCTA-*3',* reverse primer *5'*-GCAAAATGTGCTGCGCTCCAGG-*3'*) giving a product size of 331 bp. *Leptosphaeria biglobosa* forward primer sequence (Liu *et al*., 2006) *5'*-ATCAGGGGGATTGGTGTCAGGAGTTGA -3' with the reverse primer the same sequence as *L. maculans* (product size of 444 bp). The *Psudomonas* primer sequences were obtained from Matsuda, 2007 and amplify 16s rRNA of the 30S small ribosomal subunit (forward primer *5'*-CAAAACTACTGAGCTAGAGTACG-*3',* reverse primer *5'*-TAAGATCTCAAGGATCCCAACGGCT-*3'*) giving a product size of 215 bp. Generic bacterial primers were used for *Bacillus* (Anthony *et al*., 2000) amplifying the 23S ribosomal spacer region (forward primer *5'*GCGATTTCYGAAYGGGGRAACCC-*3'*, reverse primer *5'*-TTCGCCTTTCCCTCACGGTACT-*3'*) and giving a product size of 420 bp. Trichoderma harzianum primer sequences were obtained from Glass & Donaldson, 1995 which amplify the highly conserved histone 3 protein involved in the structure of chromatin (forward primer *5'*-ACTAAGCAGACCGCCCGCAGG-*3'*, reverse primer *5'*-GCGGGCGAGCTGGATGTCCTT-*3'*) and giving a product size of 500 bp.

PCR amplification was carried out on a Flexigene PCR unit. The thermal cycling parameters, unless otherwise stated, were: 94°C for 2 min; 30 cycles of 94°C for 30 s, 53°C for 1 min (annealing), 72°C for 1 min; extension 72°C for 10 min, then held at 10°C.

2.9. SEPARATION AND VISUALISATION OF POLYMERASE CHAIN REACTION AMPLICONS

PCR products were separated using electrophoresis with a 1% agarose gel stained with ethidium bromide (0.5 µg mL^{-1}) (Appendix B). A ladder containing DNA fragments of known size and quantity was run on the gel at the same time (Appendix B), and the bands visualised under an ultraviolet light transilluminator. Band fluorescence was imaged using Genesnap Analysis Software with the images recorded by digital camera and saved as Joint Photographic Experts Group (JPEG) files.

2.10. DNA QUANTIFICATION

DNA was quantified using Nanodrop, PCR and QPCR.

2.10.1. Nanodrop

The total DNA was quantified using a NanoDrop-1000 spectrophotometer to confirm DNA was present (Appendix B). A 2 µL aliquot of undiluted genomic DNA was placed onto the measurement pedestal. The concentration of DNA was automatically calculated at 260 nm.

2.10.2. Quantification of *Leptosphaeria maculans* DNA using PCR

Confirmation of the presence of *L. maculans* within the cotyledon was ascertained using PCR. Half a cotyledon was cut into 3, top, middle and bottom, which included petiole (Figure 2.2.). Each portion was placed into PCR using *L. maculans* specific primers. Aliquots were placed into gel electrophoresis and visualised using UV light.

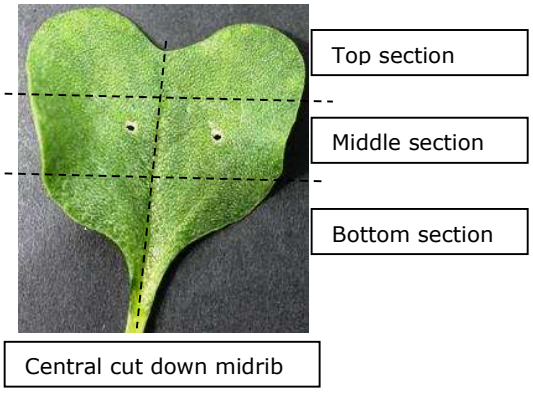


Figure 2.2. Separation of cotyledon into six separate parts.

2.10.3. Quantitative (q) polymerase chain reaction

Primers from published papers were used for *L. maculans and Pseudomonas* (section 2.7.).

Quantitative PCR primers used for *Bacillus* were designed by the author. The nucleotide FASTA sequence for *Bacillus amyloliquefaciens* was obtained from the National Centre for Biotechnology information (NCBI) database (www.ncbi.niti.gov/blast). This sequence was placed into PRIMER3 (www.Geneious.com) to obtain primer sequences (Rozen, 2000). The sequences obtained were placed back into the NCBI database and searched using the Basic Local Alignment Search Tool (BLAST) against *L. maculans* and *Pseudomonas* DNA sequences. This was to ascertain if the primers for *Bacillus* would anneal to the DNA of *L. maculans* and/or *Pseudomonas* as some DNA sequences are conserved throughout different species. Primers which did align were discarded. Primers which didn't align were tested under the experimental conditions of the quantitve time assay to prove they did not bind to any other organism in the assay other than *Bacillus amyloliquefaciens*. Primers from the highly conserved purine gene (GenBank:EF626944.1) gave the least hairpins, dimers and the most GC pairs (forward primer *5'*-ATCATCATGGGAAGCACCTC-*3',* reverse primer *5'*-GCGGATACCACCTGTTTTTC-*3'*, and produced a 98 bp product.

All primers were placed into gradient PCR assays to ascertain the optimum annealing temperatures. Each primer was tested for specificy. It was determined that the Sigma-Aldridge SYBR® Green used was not optimal for these assays, therefore Bioline iQ SYBR® Green Super mix (Appendix B) was tested and proved more effective. Nuclease free water was used instead of DNA for the no template controls. The quantities of the PCR reactants (DNA, SYBR® Green Jump Start Ready Mix (Appendix B), forward and reverse primers and distilled water) were 6 µL distilled water, 12.5 µL SYBR Green and 0.75 µL each of forward and reverse primer (standard concentration 10 µL). The amplification conditions were 2 min 95ºC, 40 cycles of 95ºC 30s, 56 ºC 30s (anneal), followed by 72ºC 1 min (extension), decreasing to and held at 40ºC. A melting (dissociation) curve was constructed after the last amplification cycle to ascertain the PCR specificity. This was analysed using Roche LightCycler® 480 software v 1.5. Each organism had a specific melt temperature.

A comprehensive standard curve was generated by plotting the C_t value against threshold cycle for each sample of a standard series of genomic DNA concentrations using the amplification of two fold serially diluted DNA samples (Figure 2.3.). Regression equations were produced to quantify the amount of DNA in 'unknown' samples.

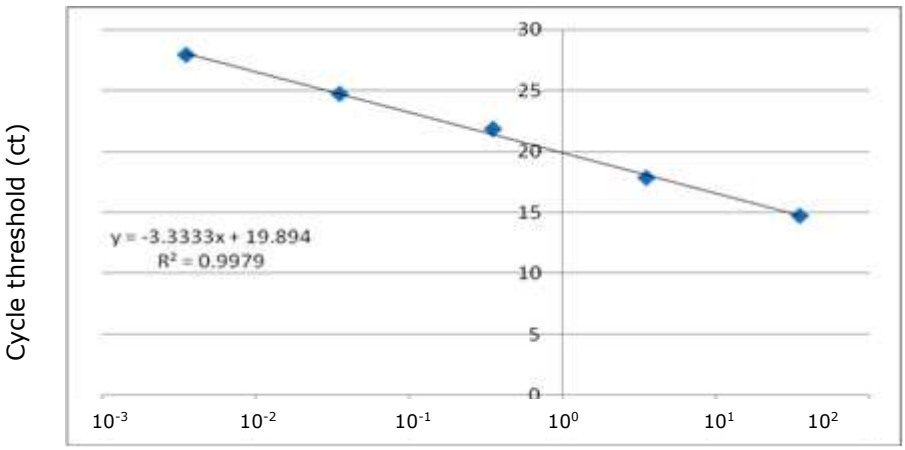


Figure 2.3a. Standard curve of *B. amyloliquefaciens* produced using regression equations using data from 2 fold serial diluted DNA.

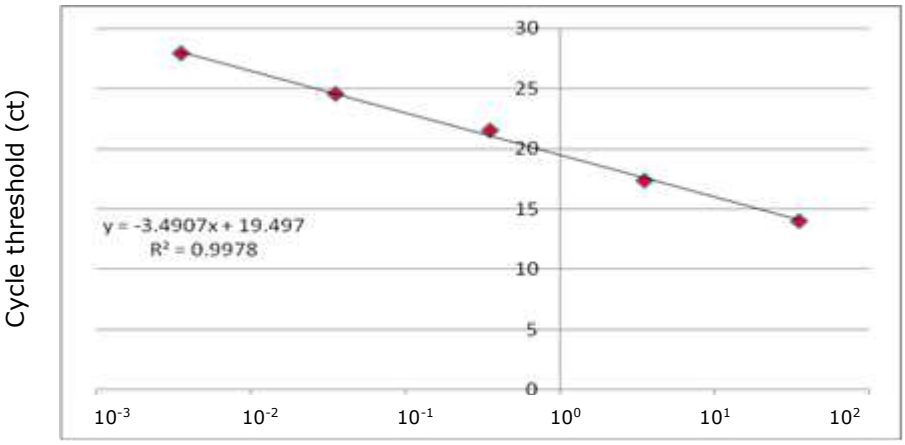


Figure 2.3b. Standard curve of *L. maculans* produced using regression equations using data from 2 fold serial diluted DNA.

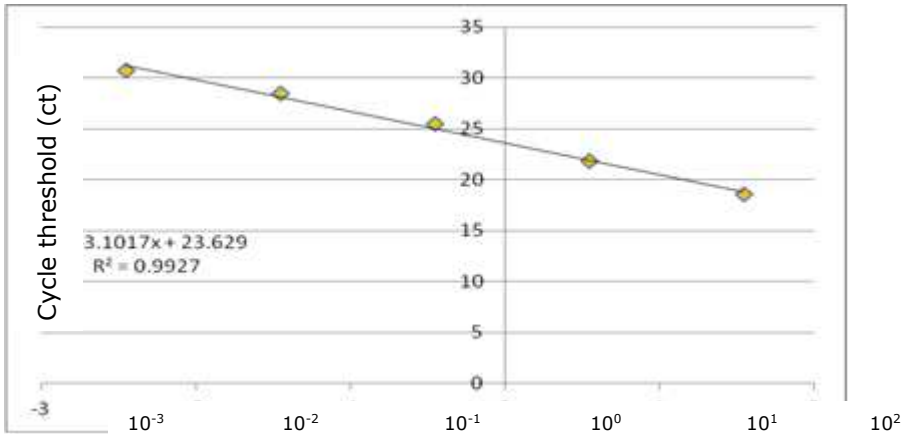


Figure 2.3c. Standard curve of *P. chlororaphis* ssp. *aureofaciens* 30.84 produced using regression equations using data from 2 fold serial diluted DNA.

2.11. DATA ANALYSIS

Data, preliminary analysis and graphs were produced using Microsoft Excel with in depth analysis using Genstat. Raw sequences were visualised using Chromas v2.33 software (© 1998 Conor McCarthy, Australia).

2.12. *IN VITRO* BIOASSAY OF BIOLOGICAL CONTROL AGENTS

A small piece of *Leptosphaeria* mycelium was excised from the leading edge of the colony using a sterile cork-borer. This was placed into the centre of a Petri dish containing PDA and placed in a controlled environment room for 72 h at 20°C with white light illumination for 24 h and near UV for 12 h each day. Possible biological control bacteria were point inoculated onto the agar. A template was used to ensure each bacterium was placed exactly the same distance away from the fungus. Three replicates were carried out using 3 bacterial liquid cultures identically prepared; thus inocula for each assay had a unique source. Fungal growth was measured from the leading edge to the inoculation point at 15 d after inoculation. Those bacteria which inhibited fungal growth were used in *in planta* assays.

2.13. *IN PLANTA* BIOASSAY OF BIOLOGICAL CONTROL AGENTS

The oilseed rape seedlings were thinned out to one in each cell of the seed tray. The seed trays were then cut into individual units (Figure 2.3.). Twenty seedlings were sprayed until run-off with either a single putative biological

control agent or a combination of *Pseudomonas* and *Bacillus.* The negative control was sprayed with MRD only. The cotyledons were left to air dry in the controlled environment room for 4 h.

Figure 2.4. Large seed trays of 16 cells were cut into individuals with one oilseed rape seedling per cell.

2.14. *IN PLANTA* FUNGICIDE ASSAY

Fungicides used to control *L. maculans* on oilseed rape (Proline, Genie) along with fungicides used to control other pathogens on crops were tested for efficacy under these experimental conditions. *Brassica napus* seeds were grown in pots, thinned to one per pot, and their growing point continually removed as it emerged to keep the cotyledons on the plant. To obtain a field rate application concentration (Table 2.1.), five plants were placed in a 1 m^2 square and sprayed with the appropriate fungicide using a hand held spray, at a spray-volume equivalent to 200 L ha^{-1} (1^{st} degree approximation of field rate). A second assay used ¼ field rate. Individual plants were placed in large trays. Triazole fungicides Proline and Genie were the positive control, with a water only negative control. The plants were placed in a controlled environment room (Figure 2.5.) at 16°C day, 12°C night, inoculated, sampled as described previously.

Figure 2.5. Layout of fungicide experiment in controlled environment room.

2.15. INOCULATION PROTOCOLS

After pre-inoculation with fungicides or the putative biological control bacteria, the cotyledons of *B. napus* were inoculated with either *L. maculans* mycelial plugs (primary assay) or a spore suspension (subsequent assays), at a concentration of 10^{-6} conidia cm^2.

2.16. RE-ISOLATION OF *LEPTOSPHAERIA MACULANS* TO OBTAIN A VIRULENT PATHOGENIC STRAIN

As *L. maculans* had been kept in storage for a number of years, it exhibited reduced pathogenicity. To re-activate it, the fungus was re-isolated from inoculated, infected leaves (passage). The waxy cuticle on each *B. napus* cotyledon was gently rubbed with a glass rod and wounded with a thin sterile needle. A plug of fungal hyphae was excised from the leading edge and placed over the wound, fungal side down and placed under a propagator lid to keep high humidity for 72 h. After three days necrosis could be seen, the classic symptoms of phoma leaf spot development. Infected leaves were excised and, under aseptic conditions, the infected area plus a small amount of the surrounding tissue was removed. The samples were surface sterilised in 7% Domestos solution for 2 min, washed with sterile distilled water five times, then left to soak for 2 min. After a final rinse, the explants were left

Table 2.1. Fungicides used as triazole benchmarks and those assayed for control of stem canker of oilseed rape. Field application rates are also shown**.** (Bayer, 2013, BASF, 2013, Syngenta, 2013, DuPont, 2013).

Fungicide name	**Active ingredients (content concentration)**	**Crop**	**Target pathogens**	**Field application rate**
Proline (Triazole benchmark)	Prothioconazole 275 g L^{-1}	Oilseed rape	Stem canker	1.0 L ha^{-1}
Genie (Triazole benchmark)	Flusilazole 250 g L^{-1}	Oilseed rape	Stem canker	1.0 L ha^{-1}
Adexar	Fluxapyroxad and epoxiconazole 62.5 g L^{-1} and 62.5 g L^{-1}	Wheat, barley	Septoria, net blotch	1.25 L ha^{-1}
Aviator	Bixafan and Prothioconazole 75 g L^{-1} and 150 g L^{-1}	Wheat, rye, triticale	Wide range incl. eye spot, rust	1.25 L ha^{-1}
Seguris	Isopyrazam and epoxiconazole 125 g L^{-1} and 90 g L^{-1}	Wheat, rye barley	Wide range incl. leaf spot, rust, blotch	1.0 L ha^{-1}
LEM 17	Penthiopyrad 160 g L^{-1} Tank mix with prothioconazole	Wide range of crops incl. soybean, sugarbeet	Wide range incl. powdery mildew, rhizoctonia	1.0 L + 1.4 L ha^{-1} (tank mix)
Q8Y78	Penthiopyrad with picoxystrobin (160 g and 80 g L^{-1})	Garlic, fruit trees	Rust, Alternaria	1.0 L ha^{-1}

to dry on sterile filter paper for 2 min then transferred onto PDA^{+} media to grow.

2.17. PYCNOSPORE INFECTION

A small area of cotyledon was gently rubbed with a glass rod then wounded with a sterile needle. The hole was immediately covered with 10 µL of spore plus MRD suspension at a spore concentration of 1 x 10^6 cm^2 conidia (Figure 2.7.). The inoculated plants were covered with a propagator lid to maintain high humidity and left for 72 h, when the lid was removed and the inoculum allowed to air dry.

To avoid positional effects, each set of seedlings was moved within the tray and each tray containing a set of treatments was moved within the controlled environment room daily. The cotyledons were randomly destructively harvested at 3, 7, 11 and 15 d after inoculation (DAI).

2.18. ASSESSMENT OF FUNGAL AND BACTERIAL COLONISATION

The lesion width and length was measured and the area calculated in mm^2. Each cotyledon was then aseptically cut in half down the midrib using sterilised blades. Each side was weighed to 2 d.p. and each half was placed into sterile Eppendorf tubes. The left half was used for washing and colony forming unit count, the right half was used for qPCR. The Eppendorf containing half a cotyledon for qPCR was frozen in liquid nitrogen, and stored at -80ºC. The Eppendorfs containing the half cotyledons for colony forming unit (CFU) assays were used immediately.

2.18.1 Colony forming unit count (CFU)

Half a cotyledon (average 0.01 g) was left in 1 mL MRD containing 10 µL of Tween (Appendix B) for 45 min and intermittently shaken to remove the bacteria. The supernatant was used in three assays.

Assay 1. 100 µL was serially diluted then plated onto PDA (Appendix B).

Assay 2. 100 µL was placed onto *Pseudomonas* selective medium which enabled the quantity of *Pseudomonas* present to be ascertained.

Assay 3. 100 µL was Pasteurised in a water bath at 80ºC for 20 min, then plated onto PDA, enabling aerobic spores to be counted (effectively *Bacillus spp*.). Each aliquot were 2 fold serially diluted up to 10^{-9}.

All bacterial plates were incubated at 25ºC overnight before the colonies were counted.

CHAPTER 3 – METHOD DEVELOPMENT

3.1. INTRODUCTION

In order to evaluate the feasibility of biological control using microbial agents singularly and in combination, it is essential to confirm identification of the pathogen and select the most pathogenic strain. The identity of the biological control agents must be confirmed. Background bacterial count must be determined to ascertain any effect on the experiment. Fungicide assays will be used as a benchmark of biological control.

3.2. CONFIRMATION OF THE IDENTITY OF *L. MACULANS* AND *L. BIGLOBOSA*.

The identity of *L. maculans* and *L. biglobosa* was confirmed using PCR amplication with specific primers. (section 2.8.) Amplicons of 331bp and 444bp were produced, indicative of *L. maculans* and *L. biglobosa*, respectively (Figure 3.1.).

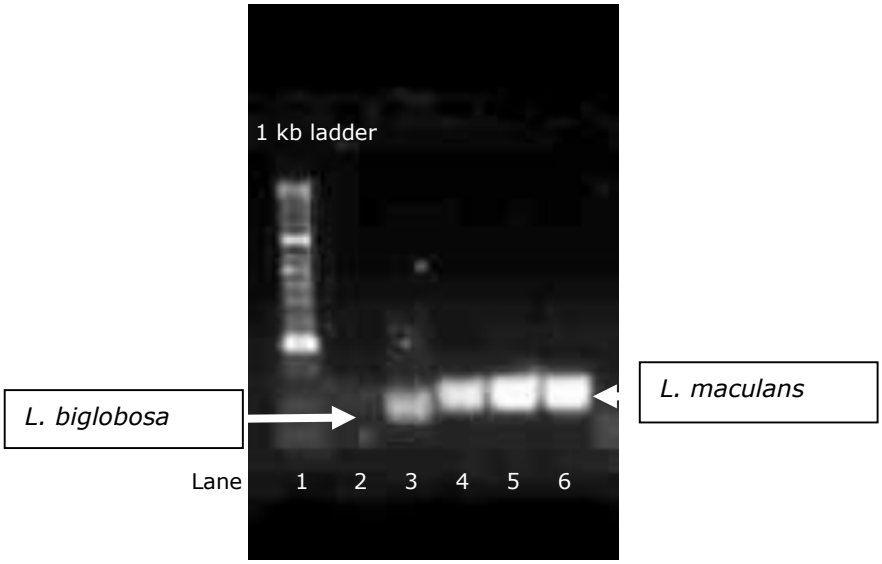


Figure 3.1. Visualisation of L. biglobosa (lane 3, 444 bp) and *L. maculans*, lanes 4-6 (331 bp) amplicons following PCR and gel electrophoresis. Lane 2 is no template control.

3.3. CONFIRMATION OF THE IDENTITY OF THE BIOLOGICAL CONTROL BACTERIA.

3.3.1. *Bacillus amyloliquefaciens*

The *Bacillus* isolates used in this work were initially identified by conventional methods many years ago. PCR amplification with generic bacterial primers (section 2.8.) followed by visualisation using gel-electrophoresis (Figure 3.2.) and a BLAST search on the amplified DNA sequence confirm identity. All strains of the *Bacillus* DNA used in this work indicated greater homology with *B. amyloliquefaciens* in the *B. subtilis* group and it was considered that *B. amyloliquefaciens* was the more accurate name.

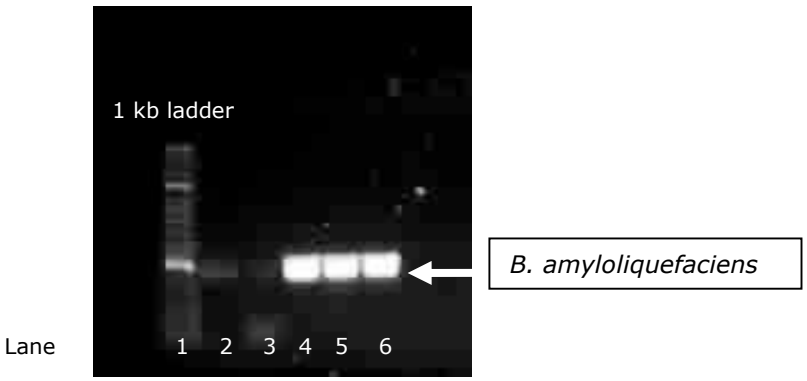


Figure 3.2. Visualisation of *B. amyloliquefaciens* amplicons (lanes 4-6, 420 bp) following PCR and gel electrophoresis.

Bacteria, isolated by washing oilseed rape, cabbage and brussel sprout leaves, were also identified using generic primers (section 2.8.) PCR amplification, visualisation and a BLAST search.

An aliquot of the bacteria in MRD was Pasteurised and plated onto PDA media. Aerobic growth from the spores, which survived heat treatment occurred, which was further confirmation the bacteria was *Bacillus* (Figure 3.3.).

Figure 3.3. Growth of bacterial colonies produced from an aliquot of Pasteurised bacteria indicating that the organism was *Bacillus* spp.

3.3.2. *Pseudomonas chlororaphis* spp. *aureofaciens* 30.84

The identity of *P. chlororaphis* spp*. aureofaciens* was confirmed using PCR amplification with species specific primers (section 2.8.).

Amplification was followed by visualisation using gel electrophoresis which gave an amplicon of 215 bp, indicative of *Pseudomonas* (Figure 3.4.).

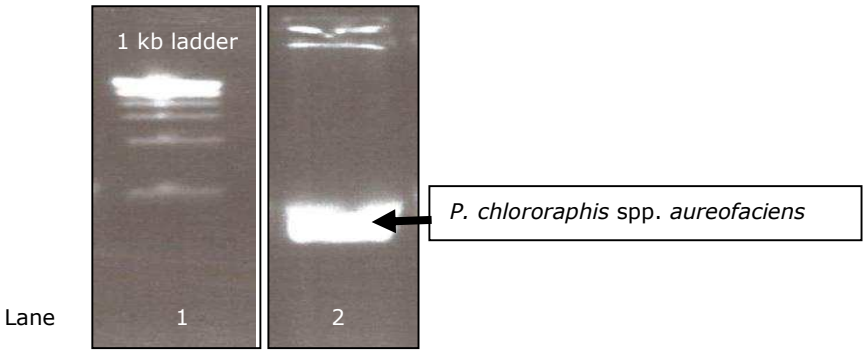


Figure 3.4. Visualisation of *P. chlororaphis* spp. *aureofaciens* amplicon (215 bp) on agarose gel following PCR.

A BLAST search was carried out to confirm identity. Best match (99%) for *P. aureofaciens* was indicated as *P. chlororaphis* spp. *aureofaciens* and it was considered that *P. chlororaphis* spp. *aureofaciens* was the more accurate name.

3.3.3. *Trichoderma harzianum*

The identity of *T. harzianum* was confirmed using primers as section 2.8. followed by visualisation using gel electrophoresis under UV light (Figure 3.5.).

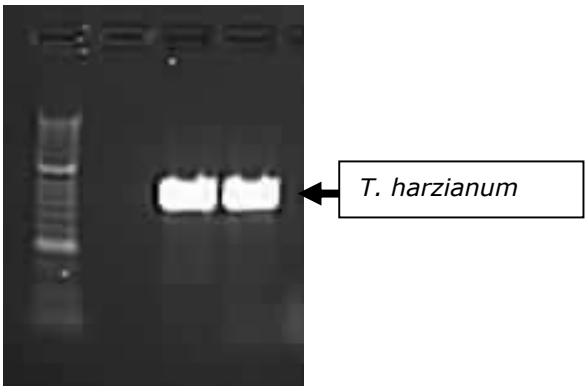


Figure 3.5. Visualisation of *T. harzianum* amplicon (500 bp) on agarose gel following PCR. Hyperladder II (HL II) was used.

A BLAST search on the amplified DNA was carried out which confirmed identity as *T. harzianum.*

3.4. SELECTION OF PATHOGENIC ISOLATE OF *L. MACULANS* AND DEVELOPMENT OF INOCULATION METHODS

Before the inoculation methods could be developed, criteria had to be met to ensure the assays would be consistent and could be replicated. Very little growth or sporulation was seen when *L. maculans* was grown on PDA, but when amended with antibiotics (PDA^+, Appendix A) the best growth and sporulation rate was obtained.

3.4.1. *Leptosphaeria maculans* isolate with the best growth

Several isolates of *L. maculans* were assayed *in vitro* on PDA^+ for their growth potential, as an isolate fast growing was required. Results given in Figure 3.6. show that isolate 31.13 gave the best growth at 18ºC. Over 15 d, this grew three times more than the 30.13 strain and twice as much as isolate LM06N83.2. *L. maculans* isolates ME19, UK29 and UK9 were very slow growing. Isolate 31.13 was tested for pathogenicity.

3.4.2. *Leptosphaeria maculans* pathogenicity

It was important to establish that the pathogen isolate used was consistently virulent on the rape variety used. A reliable virulent isolate (31.13) of *L. maculans* was obtained by inoculating oilseed rape plants and allowing symptoms to develop for 15 d, then re-isolating onto PCA *in vitro* (passage). This was carried out four times to maximise pathogenicity (Figure 3.7.)

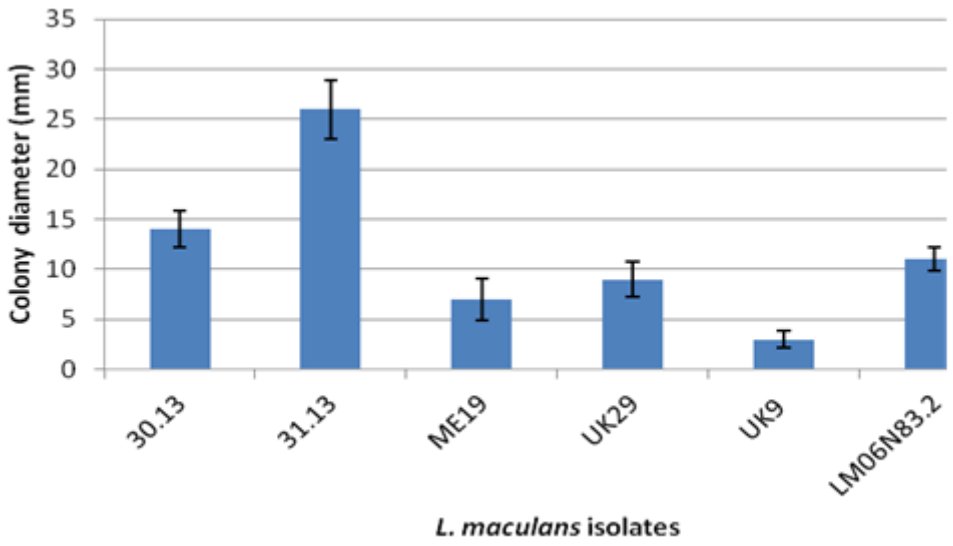


Figure 3.6. Mean diameter of *L. maculans* isolates after 15 d at 18°C, with visible light 24 h and 12 h near UV (N = 20). Error bars – SD

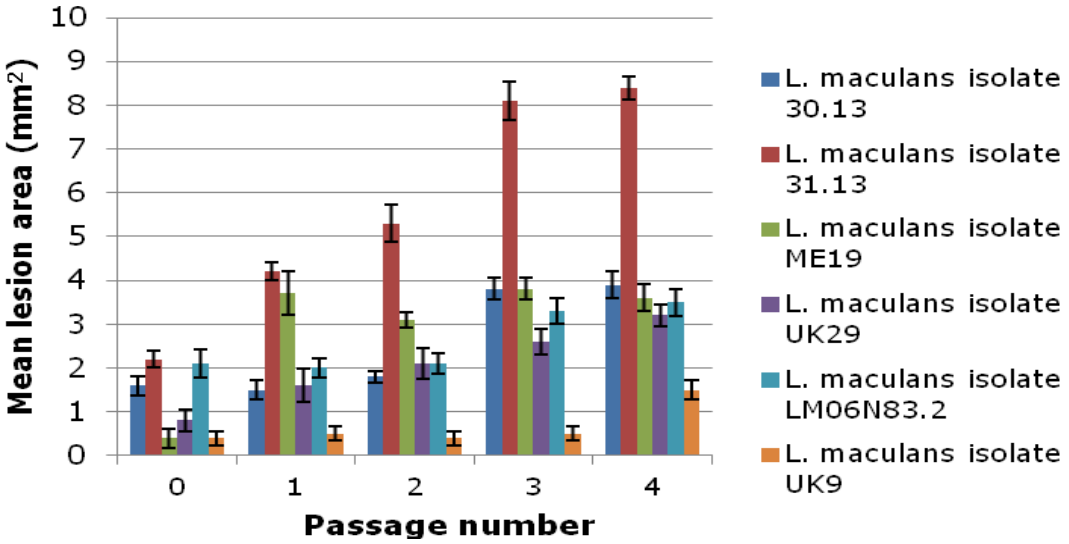


Figure 3.7. Pathogenicity of *L. maculans* isolates before and after passage through B. napus (N = 20). Error bars - SD

3.5 BACKGROUND MICROBIAL POPULATIONS

The background population of microbes on the cotyledons before the assay was ascertained (Figure 3.8.). It was clear that only a very low initial population of culturable bacteria and no fungi were detected. It was considered that these small populations would not adversely affect the subsequent assay results.

After application of washed bacterial suspensions to cotyledons, the recoverable population was determined hourly for five hours (data not shown). The optimum time for bacteria adhering to the cotyledon surface and for their population to increase after the initial application onto the cotyledons was shown to be 4 h. Thus, sprayed cotyledons were incubated for 4 h before inoculation with *L. maculans*.

The bacteria were grown in liquid nutrient broth for application onto the cotyledons. As such, the amounts of bacteria present in liquid culture at 48 h (exponential growth phase) and 72 h (stationary growth phase) were quantified. Figure 3.9. shows more CFU present at 72 h than 48 h (dilution 10^{-9}) for all isolates except *P. chlororaphis* spp. *aureofaciens* PhzI^{-} and *B. amyloliquefaciens* isolate 62p. The viable population of *B. amyloliquefaciens* 62p decreased by over half from the amount quantified at 48 h when quantified at 72 h. There were almost twice as many *B. amyloliquefaciens* 66p and 3 times more *B. megaterium* at 72 h than 48 h.

Figure 3.10 shows the sporulation rates of *Bacillus* grown in liquid culture over 48 and 72 h. *B. amyloliquefaciens* MBI600 sporulated more than any other isolate at 48 h *B. amyloliquefaciens* 62p had more spores at 72 h than 48 h but *B. amyloliquefaciens* 66p produced the largest number of spores at 72 than other isolates did at either 48 or 72 h. *B. amyloliquefaciens* 66p after 72 h growth in liquid culture was deemed the best for the assays in this work.

It was also ascertained that MRD plus 5 µL Tween X80, a polyethylene sorbitol ester non ionic surfactant (1% aqueous solution), removed more bacteria in 20 min on an orbital shaker at 25°C than MRD on its own, therefore Tween X80 was routinely used in the assays (Figure 3.11).

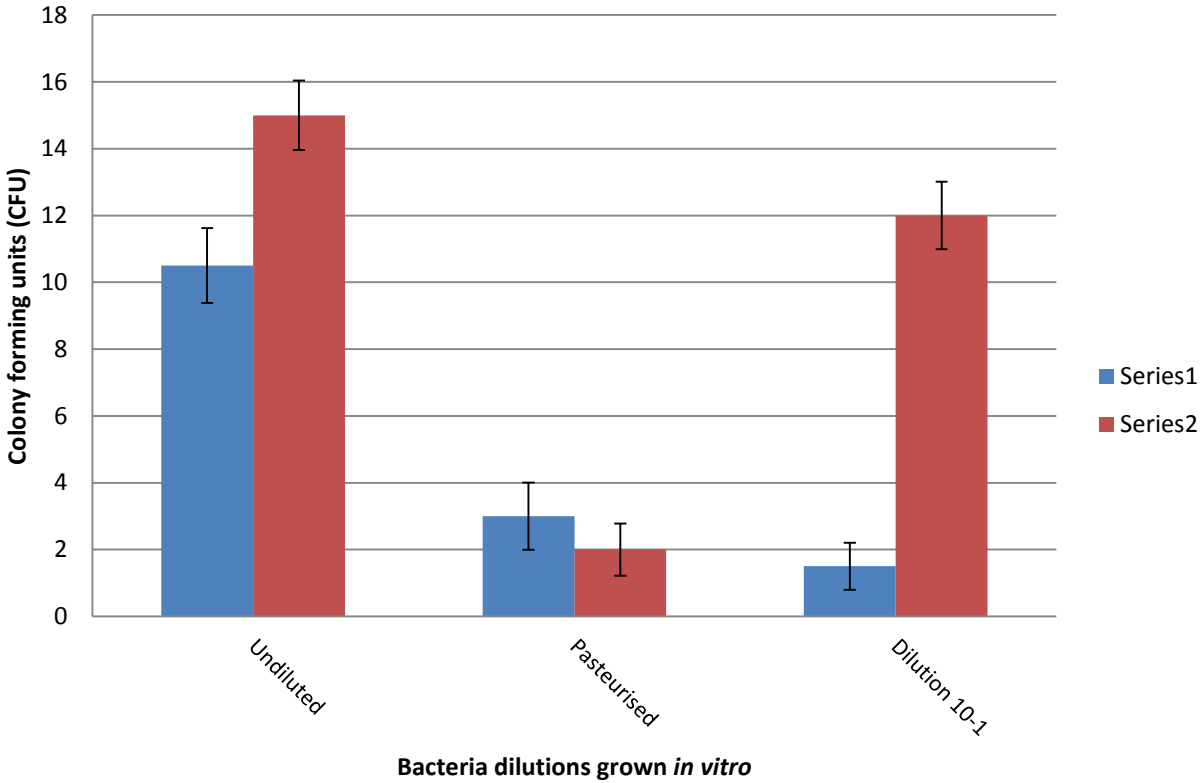


Figure 3.8. Background bacteria removed by washing with Tween 80X from 0.01g of tissue (½ cotyledon). No fungi were detected. Error bars - SD.

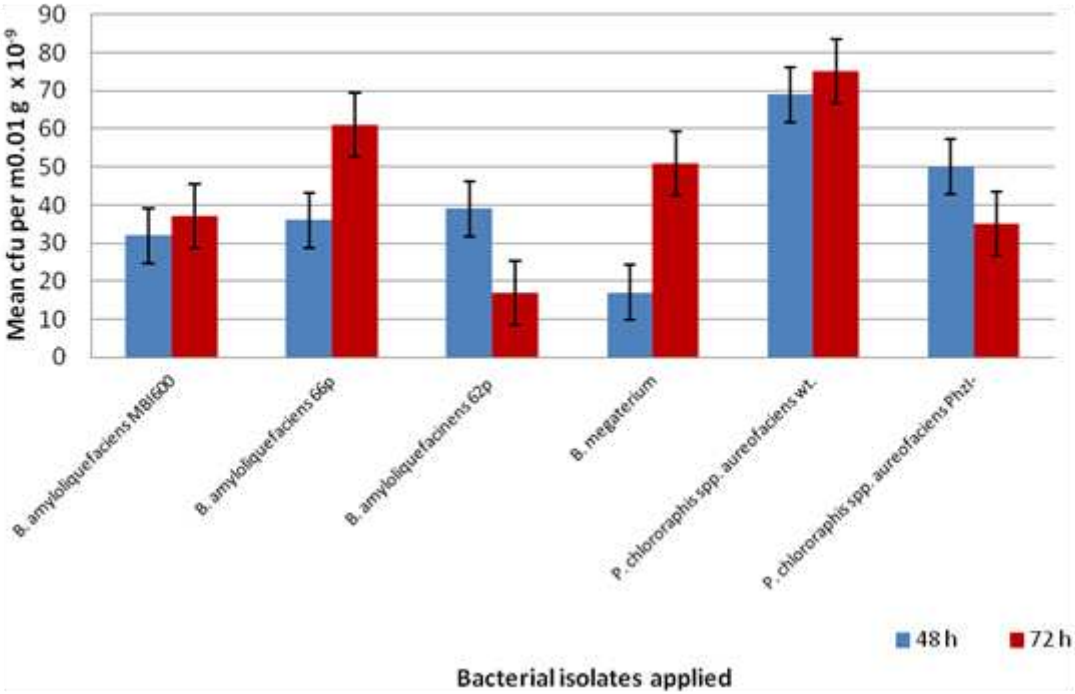


Figure 3.9. Concentration of bacterial isolates grown in liquid culture at 48 and 72 h after application (applied at 10^6). (N = 20). Error bars - SD

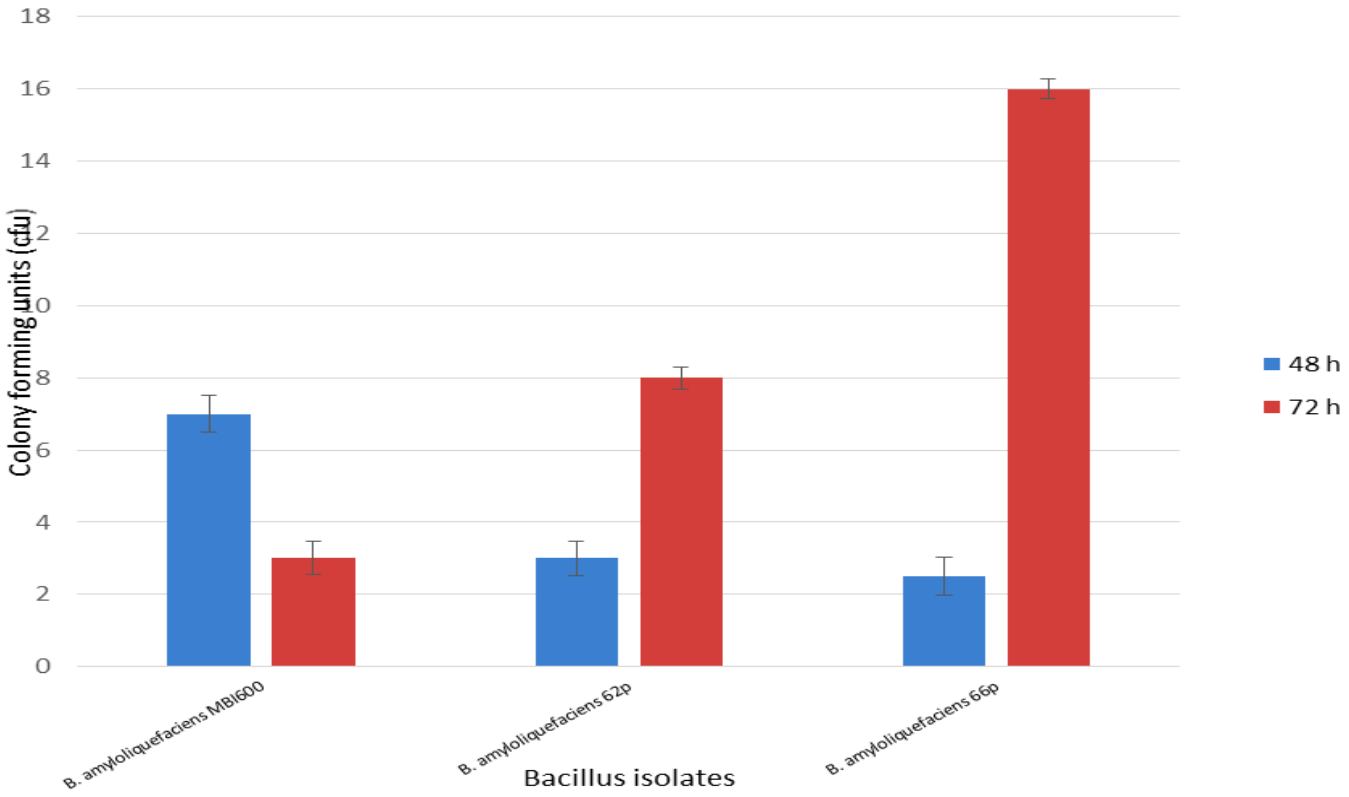


Figure 3.10. Sporulation of *Bacillus* isolates in liquid culture over 48 and 72 h. Plated at serial dilution 10^{-9}. (N = 20). Error bars – SD

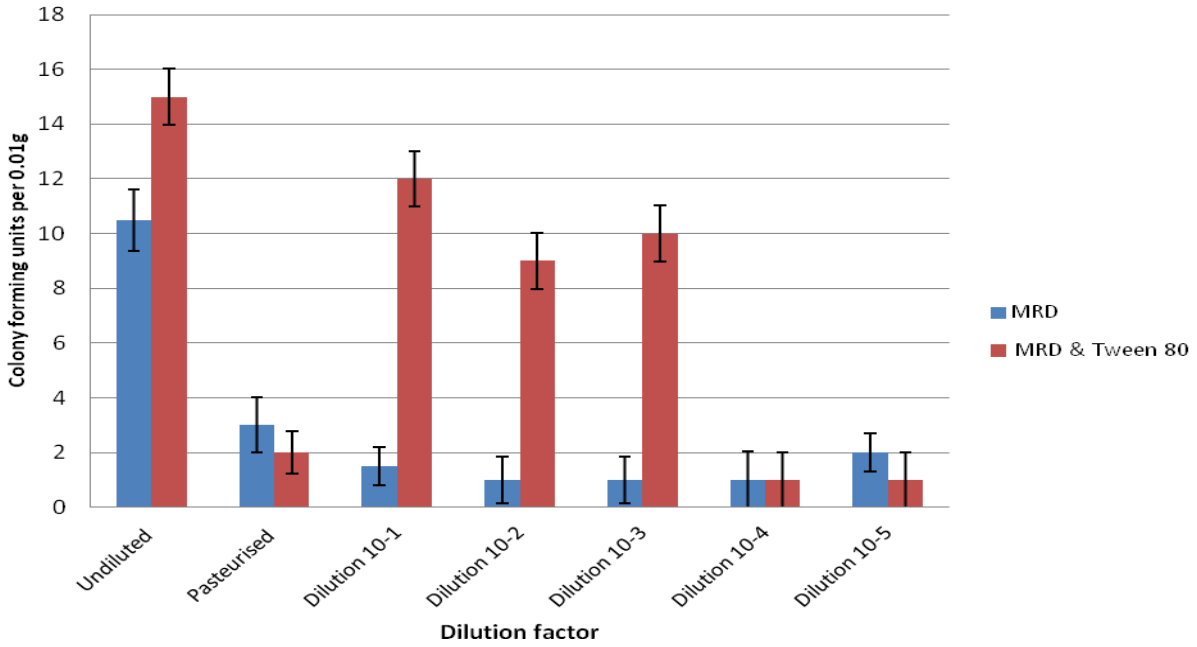


Figure 3.11. The efficacy of using Tween 80 to recover bacteria from the cotyledons. (N = 20). Error bars – SD

3.6. FUNGICIDES

Fungicides were assayed to set the benchmark for the biological control assays under the experimental conditions.

3.6.1. Evaluation of triazole fungicides used in the field for control of stem canker

Triazole fungicides regularly used in the field to control stem canker were assayed under these experimental conditions. Figure 3.13. shows the severity of the leaf spot stage of *L. maculans* decreased with field strength application of triazole fungicides Geni 25 and Proline.

3.6.2. Evaluation of succinate dehydrogenase inhibitor (SDHI) fungicides

Initially, the experimental fungicides were applied at full field rate. After 2 h, damage to the epidermis could be seen (Figure 3.12.). After 24 h, all of the cotyledons wilted, and so the experiment was not continued.

The fungicides were therefore applied at ¼ field rate. This dilution still caused some wilting of cotyledons, but they survived for the duration of the experiment.

Figure 3.13. shows that the *L. maculans* lesions on the Q8Y78 (since the research was completed, this material has now been launched as the product Refinzar®) sprayed cotyledons were similar in size to the unsprayed control at 3 DAI (Figure 3.14.), but had a larger lesion than both triazoles tested. Both Proline and Genie resulted in smaller lesions than the unsprayed control, with Proline having the smallest lesion of the two. At 7 DAI and 11 DAI, the leaf spot lesion produced on the cotyledon sprayed with Q8Y78 was larger than all three controls (Figure 3.14.). At 15 DAI, the experimental fungicide cotyledon had a leaf spot lesion similar in size to the no fungicide control and had no statistical difference $P = 0.058$; $F_{1,23} = 2.71$. The lesions were significantly larger than both triazole controls $P = 0.007$, $F_{1,12} = 3.56$ (Figure 3.14.).

The fungicides assayed did not give good control in this experimental system, despite being competent in the field. All the SDHI fungicides damaged the *B. napus* epidermis at full field rate. All except Q8Y78 damaged the epidermis when applied at ¼ field rate. When placed into an assay alongside triazole controls (Proline and Genie 25), Q8Y78 did not lessen the lesion area (comparable with control where no fungicides were applied). A necrotic lesion

was observed but no pycnidia were produced within the experimental time frame.

(a) Adexar (b) Seguris (c) Lem17 (d) Aviator

Figure 3.12. Damage to cotyledon epidermis caused by full strength fungicides at day 1 AI.

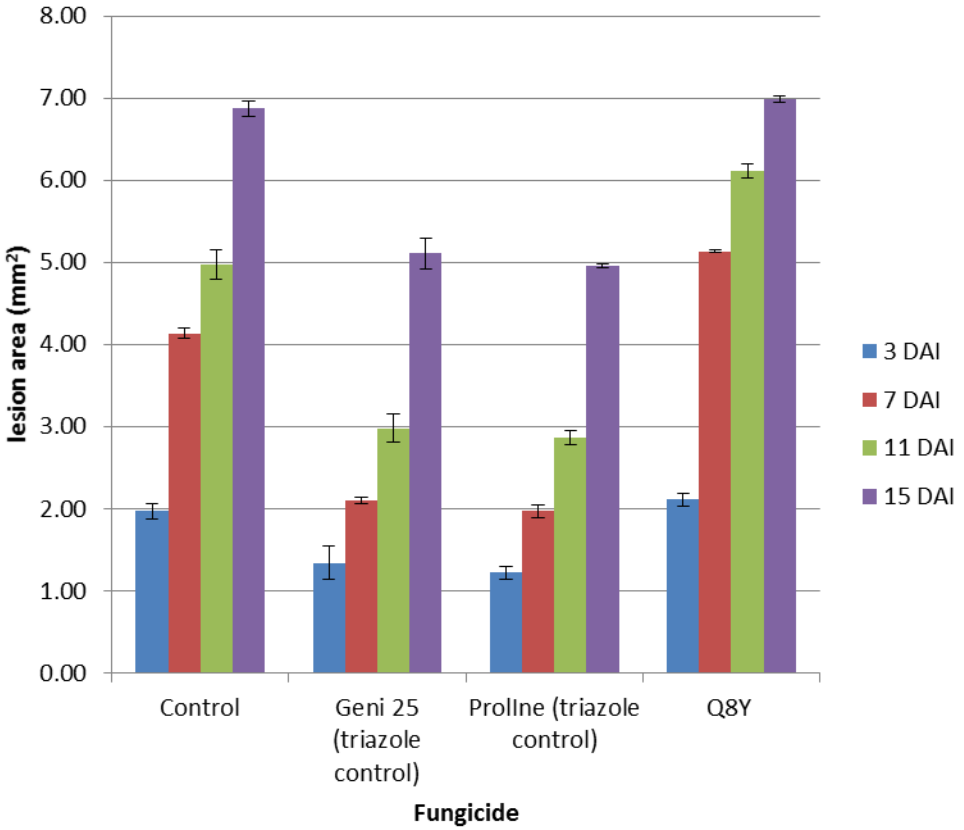


Figure 3.13. Efficacy of Q8Y fungicide sprayed at ¼ field strength for control of *L. maculans* leaf spot on cotyledons. Triazole controls were at field strength (N = 20) Error bars – SD

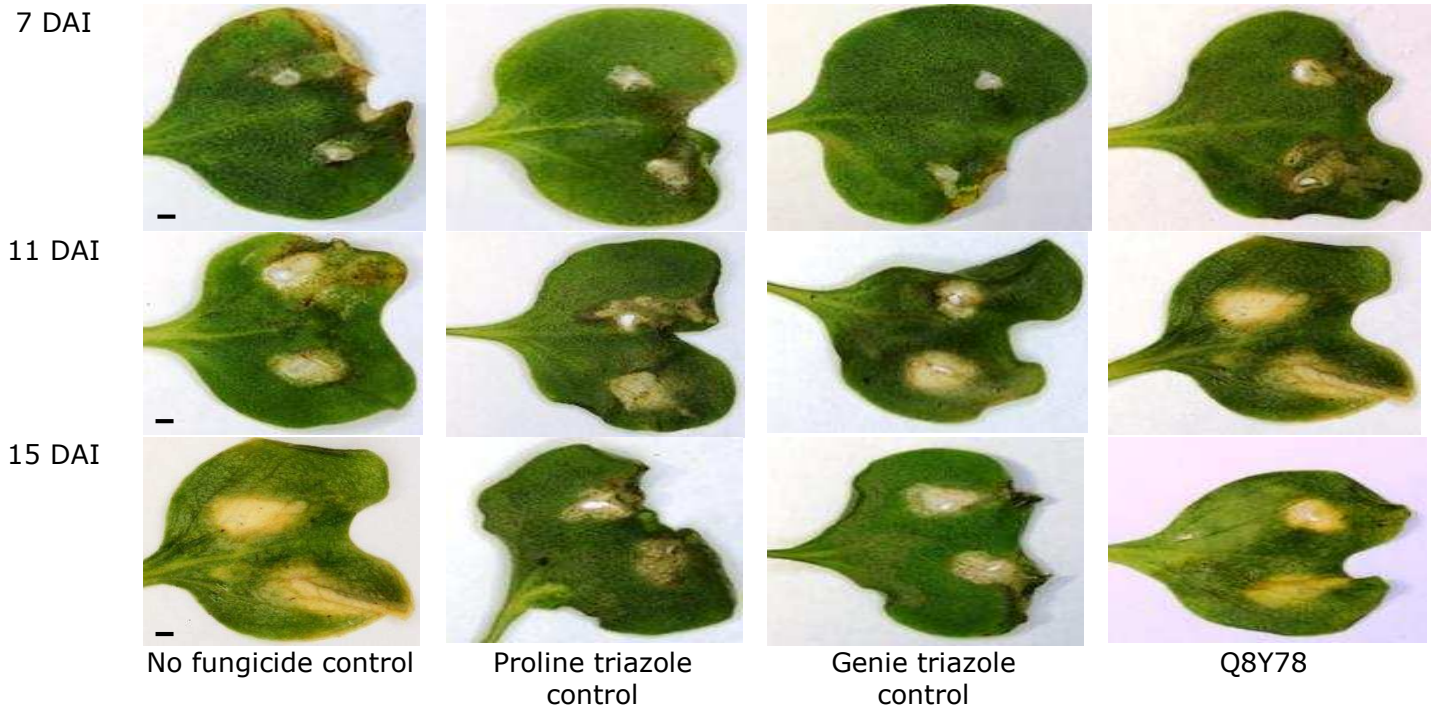


Figure 3.14. Fungicide assays at ¼ strength concentration showing stem canker leaf spot lesions produced at days 7, 11 and 15 after infection. Scale bar – 1 mm.

3.7. DISCUSSION

The identity of *L. maculans*, *L. biglobosa*, *B. amyloliquefaciens*, *P. chlororaphis* spp. *aureofaciens* and *T. harzianum* was confirmed using specific primers in a PCR amplification followed by visualisation using gel electrophoresis, sequencing then a BLAST search. *Bacillus* was further confirmed by Pasteurisation which resulted in growth of surviving spores.

L. maculans isolate 30.13 gave the best growth on PDA+ and this was passaged through an oilseed rape plant for five generations to enhance its pathogenicity.

Recovery of bacteria from oilseed rape cotyledons was found to be more effective when MRD was amended with 0.01% Tween 80.

The culturable background microbial population on the cotyledons was determined as very low and as such would have no effect on the experiments. All the isolates except for *B. amyloliquefaciens* 62p peaked in growth at 72 h when in liquid culture. The optimum time for incubation of the biological control bacteria before inoculation with the pathogen was found to be 4 h.

Field rate applications of fungicides, with known activity against stem canker at the leaf spot stage, were to be used as a benchmark. Under these experimental conditions, however, they did not give good control, probably due to their optimisation for field conditions. Evaluation of SDHI fungicides used on stem canker at the time of the experiments resulted in ineffective control, the lesions being larger than on untreated controls.

CHAPTER 4 - BIOLOGICAL CONTROL OF STEM CANKER ON OILSEED RAPE USING ANTAGONISTS APPLIED INDIVIDUALLY

4.1. INTRODUCTION TO BIOLOGICAL CONTROL

Many micro-organisms applied as a single species are known to give control of diverse plant pathogens and the diseases they cause (section 1.4.). *T. harzianum* controls many pathogens including fungal pathogens of wheat (Hasan *et al*, 2012), *Botrytis*, *Pseudoperonospora*, *Sclerotina*, *Sphaerotheca* (Elad, 2000) and *Fusarium* (Matarese *et al*., 2012). *Serratia plymuthica* inhibits fungal strawberry diseases (Kurze *et al*., 2001), whilst *Glomus mosseae* (Hassan *et al.,* 1997) and *Streptomyces* spp. (de Vasconcellos & Cardoso, 2009) give good control against *Fusarium* wilt. *Streptomyces* also suppreses damping off on seedlings of alfalfa (Jones & Samac, 1996). However, because the control works on one crop does not mean it will be successful on another even with the same, or similar, pathogen.

Antibiotics and other substances e.g. proteases, surfactants, produced by *Bacillus* and *Pseudomonas* result in the suppression of pathogens (section 1.4.). Different isolates of these bacteria have been widely used to control a variety of plant diseases, and each isolate appears to produce one or more antibiotics which may be detrimental to other organisms in the proximate environment. *Bacillus* spp. are able to supress many different pathogens, examples being *Phytophthora* spp. (Lee *et al*., 2011, Jang *et al*., 2011), *Fusarium* spp. (Cavaglieri *et al*., 2005), *Rhizoctonia solani* (Fiddaman & Rossall, 1995, Asaka & Shoda, 1996, Mizumto *et al*., 2007), fungal diseases on grapes (Furuya *et al*., 2011), *Botrytis cinerea* (Paulitz & Belanger, 2001), *Pythium aphanidermatum* (Leclere *et al*., 2005), *Ralstonia solanacearum* (Hyakumachi *et al*., 2013) and many others. *B. amyloliquefaciens* isolates have known supression of banana crown rot fungal pathogens (Alvindia & Natsuaki, 2009), *Colletotrichum gloeosporioides* (Mochizuki *et al*., 2012), *Fusarium oxysporum* (Wang *et al*., 2013), and *P. fuscovaginae* (Kakar *et al.,* 2014). *Pseudomonas* species controls many plant pathogens, including *Gaeumannomyces graminis* (Thomashow *et al*., 2002, Raaijmakers *et al*., 2010,), *Pythium* (Howell & Stipanovic, 1980) and *Erwinia carotovora* (Kloepper *et al*., 1980).

4.1.1. Biological control of stem canker on oilseed rape

Stem canker is a disease which initiates on the leaf surface. There are two main mechanisms of biological control on the phylloplane in temperate conditions, competition and antibiotic production. The use of competition as a method of controlling stem canker is unlikely to work as the stem canker spores quickly germinate and their hyphae grow inside the leaf due to the surface being a stressful environment (Cullen & Andrews, 1984). The spore is at its most vulnerable before or at germination prior to penetration, and so this is the stage antibiotics synthesised by the bacteria applied in this work, should be most effective. BCAs must also be applied directly to the site of infection as they are not systemic (Mathre *et al*., 1999).

Previously, other groups have applied organisms individually to the phylloplane to obtain control of *L. maculans*. Partial suppression of *L. maculans* was obtained using the ascospores of the weakly virulent isolate *L. biglobosa* (Petrie, 1988, Liu *et al.*, 2006). *Cyathus striatus* (birds nest fungus) has also been used in biological control of stem canker on oilseed rape stubble, producing cellulases and lignases to speed up decomposition, consequently decreasing available nutrients (Tewari *et al*., 1997, Maksymiak & Hall, 2000). *Paenibacillus polymyxa,* a Gram positive soil living bacterium, which produces polymixin, a fusaricidin type antibiotic, has been used to inhibit *L. maculans* growth (Beatty & Jensen, 2002). A mycotoxin metabolite (related to citrinin) produced by *Penicillium verrucosum*, isolated from culture filtrates has been used to inhibit *L. maculans* (Karbanda & Dahiya, 1990). Activators of plant chemical defences, e.g. acibenzolar-s-methyl (a fungicide) and menadione sodium bisulphate (synthetic vitamin k) have been used to decrease *L. maculans* severity by increasing the time from infection to the visible symptoms of leaf lesions (lag phase) (Liu *et al.,* 2006). *Gliocladium catenulatum* J1446 (Prestop® www.Lallemand.com) is a fungus used to control *Pythium*, *Phytophthora*, *Rhizoctonia*, *Fusarium*, *Didymella* and *Botrytis* and was found to decrease the intensity of stem canker when compared with untreated controls (Hammoudi *et al.,* 2012).

4.2. CONTROL AGENTS USED IN THIS WORK

4.2.1. *Trichoderma harzianum in vitro* assay

Trichoderma harzianum fungus employs competition to control plant pathogens and can be applied to the phylloplane or rhyzosphere of the plant (section 1.4.).

4.2.1.1. Methods

To ascertain in *T. harzianum* was detrimental to *L. maculans*, a mycelial plug was placed onto a Petri dish containing PDA^{+}. A mycelial plug of *L. maculans* was placed on the same Petri dish 6 cm from the *Trichoderma* plug. These were incubated at conditions as section 2.11. for one week.

4.2.1.2. Results

T. harzianum did not halt the growth of *L. maculans*, and the pathogen grew at a similar rate to that observed in uninoculated control plates.

4.2.1.3 Conclusion

As *Trichoderma* did not work *in vitro* under ideal conditions, it was assumed it would not work *in planta*. Therefore it was not used *in planta* assays, which focussed on the use of bacterial BCAs.

4.3. BACTERIAL BIOLOGICAL CONTROL AGENTS APPLIED INDIVIDUALLY

Bacteria with known biological control activity and bacteria isolated from leaves were assayed *in vitro* for supression of *L. maculans*.

4.3.1. *In vitro* assay of isolates with previously reported biological control activity

4.3.1.1. Methods

Conditions for the experiment were as described in section 2.11. The proposed inoculation sites were indicated by red numbered dots on the Petri dishes. Each bacterial isolate was point inoculated at four sites on each Petri dish (blue numbered dots on Figure 4.1b) with 10 replicate Petri dishes. The inhibition zone was measured from the bacterial inoculation point to the

leading edge of the fungal growth after 10 d (Figure 4.1b). All experiments were carried out 10 times.

4.3.1.2. Results

The control plate, without bacterial inoculation (Figure 4.1a), confirmed that the fungus grew normally on the Petri dish under the conditions described in section 2.11. *B. amyloliquefaciens* isolates 66p, MBI600, 62p produced good inhibition zones (not pictured) with *P. chlororaphis* spp*. aureofaciens* 30.84 produced a strong inhibition, indicating fungal growth was limited (Figure 4.1b.). Figure 4.2. confirms that *P. chlororaphis* spp. *aureofaciens* 30.84 had the largest inhibition zone at 15 mm, followed by *B. amyloliquefaciens* MBI 600 (11.5 mm), *B. amyloliquefaciens* 66p (11 mm) then *B. amyloliquefaciens* 62p (10 mm). *P. chlororaphis* spp. *aureofaciens* PhzI- imparted inhibition (9 mm) but was 6mm less than 30.84.

4.3.1.3. Conclusion

Growth of *L. maculans* was inhibited by all bacterial isolates. Those with the greatest i*n vitro* activity were used for *in planta* assays.

4.3.2. *In vitro* assays of unknown bacterial isolates

Thirty bacterial isolates isolated from Brassica leaves (section 2.11.) were tested *in vitro* for control of *L. maculans*

4.3.2.1. Method

Assay and conditions as described in section 2.11.

4.3.2.2. Results

Bacterial field isolates 3.4, 4.2, 4.5, 4.8, 4.9 and 3.2 showed inhibition (Figure 4.3.) comparable to the bacteria with known inhibitory effect. Isolates 4.8 (1 mm) and 4.9 (6 mm) (isolated from oilseed rape leaves), 3.4 (6.5 mm) and 4.2 (7 mm) (isolated from cabbage leaves) showed weak inhibition and so were discounted as possible biological control agents.

Two strains of bacteria isolated from cabbage, 3.2 and 4.5 showed strong inhibition, indicating a possible use for biological control. Isolate 3.2 with an inhibition zone of 11 mm was investigated by PCR (generic bacterial primers

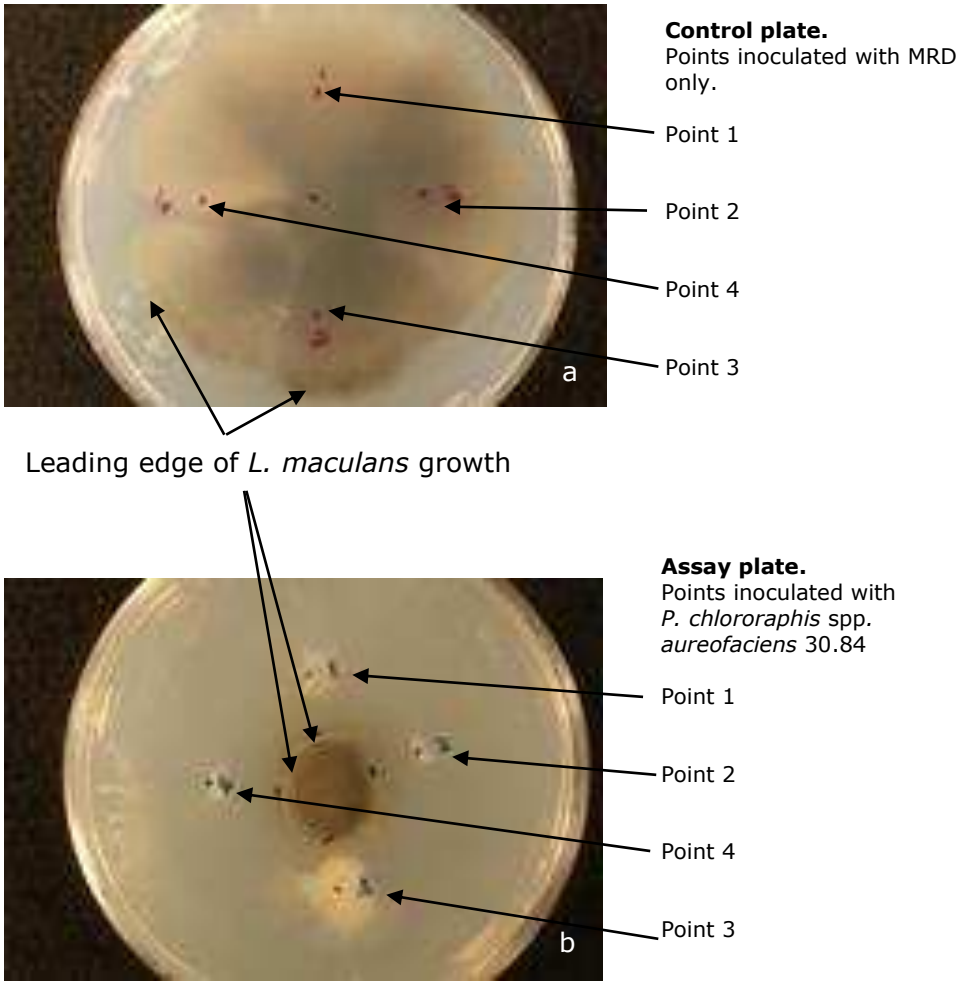


Figure 4.1. *In-vitro* assay of *L. maculans* (a) without and (b) with biological control agents applied (15 days growth). The numbers in red on plate a in indicate proposed inoculation points on the control plate, showing *L. maculans*, without any control, grew over these points. The numbers in blue on plate b indicate actual inoculation points. The creamy growth of bacteria can be seen around each inoculation point on plate b. The extent of fungal growth (leading edge) has been marked, plate b having the least growth.

as section 2.7.) then BLAST searched. It was identified (98% identity) as *Acinetobacter baumanii*, a human pathogen and as such could not be used as a biological control agent. Isolate 4.5 showed strong inhibition (10 mm). This isolate was grown in liquid culture then Pasteurised by heating at 80°C for 20 min to kill any non-spore forming bacteria and those in a vegetative state. Its identity was confirmed as *Bacillus megaterium (*95% identity*)* by PCR (using generic bacterial primers as section 2.7.) and BLAST search.

4.3.2.3. Conclusion

Although some control was seen in the isolates isolated, none were considered to be as effective as the isolates with known control and as such these were not placed into further research.

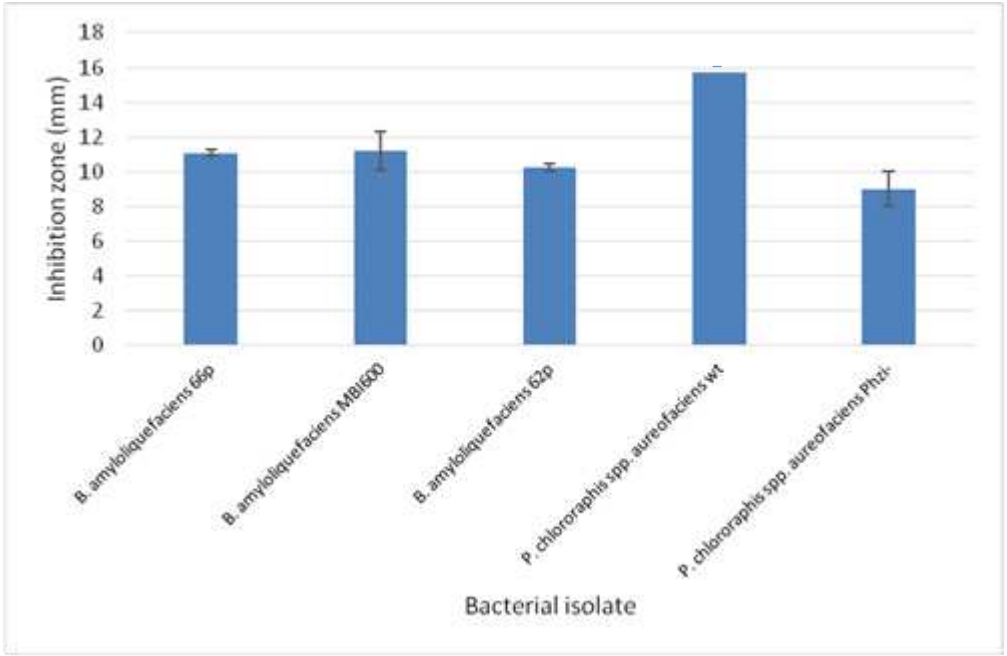


Figure 4.2. *In vitro* assays of inhibition of *L. maculans* growth imparted by bacteria with previously reported biological control activity. N = 20. Error bars – SD.

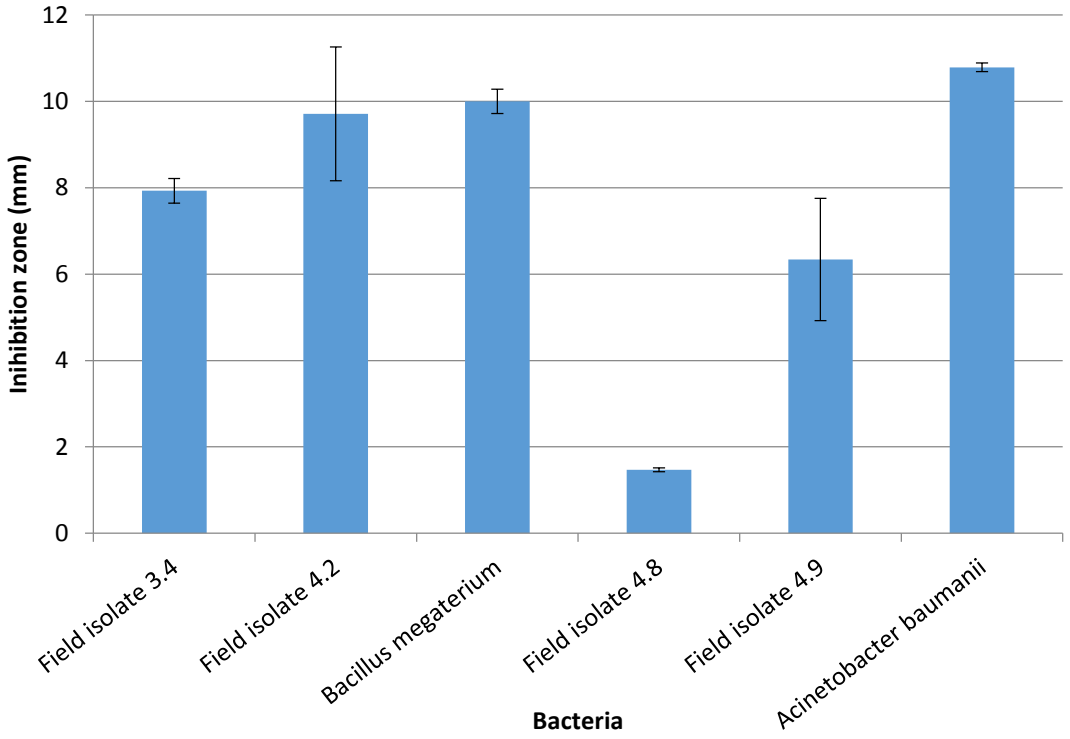


Figure 4.3. *In vitro* assays of inhibition of *L. maculans* growth using bacteria isolated from leaves of brassica species. N = 20. Error bars – SD.

4.4. BIOLOGICAL CONTROL *IN PLANTA*

Bacterial isolates which showed biological control activity during *in vitro* assays were placed into secondary screening using *in planta* assays. Cotyledons were treated with potential bacterial biological control agents, inoculated with pycnospores of *L. maculans,* as described previously, and assessed for lesion development at 3, 7, 11, 15 DAI.

4.4.1. Visual symptoms of leaf spot lesions

4.4.1.1. Methods

Spores were isolated as section 2.2.5. and *in planta* assays carried out under conditions as 2.13.

4.4.1.2. Results

The pycniospores germinated approximately 1 day after being inoculated onto the cotyledon. Pale chlorotic lesions could be seen on all the bacterial inoculated plants at 3 DAI. At day 7 after inoculation, the chlorotic lesion had become necrotic as extensive cell death occurred due to *L. maculans* becoming necrotrophic. Immature pycnidia could be seen developing inside the necrotic lesion (leaf spot stage of blackleg). A visual difference in lesion size could be seen between cotyledons pre-inoculated with bacteria and the control plants (Figure 4.4.).

At day 11, lesions, containing immature pycnidia, were larger (average area 7.3 mm²) on the control plants and the plants pre-inoculated with the biological controls (Figure 4.4.) (average area 5.3 mm²).

Day 15 AI, each cotyledon on the control plants had a circular pale grey/brown lesion averaging an area of 8 mm², with a large number of pycnidia on the outside of the epidermis. The plants pre-inoculated with bacteria had smaller lesions but still contained pycnidia.

Species specific and generic primers in PCR followed by BLAST search confirmed that the fungus recovered from the cotyledon was *L. maculans* and the bacteria present were *B. amyloliquefaciens* and *P. chlororaphis* spp. *aureofaciens*.

DAY 7

Control

P. chlororaphis spp. *aureofaciens*

B. amyloliquefaciens isolate 66p

DAY 11

Control

P. chlororaphis spp. *aureofaciens*

B. amyloliquefaciens isolate 66p

Figure 4.4. Sample pictures of visual symptoms of *L. maculans* leaf spot showing the smaller lesions imparted by the best biological control agents. Scale bar 1 mm.

Figure 4.5. shows the changes in lesion sizes over time. The lesions on the cotyledons pre-inoculated with biological control agents were smaller than the control lesion. The best biological control activity appeared to be imparted by *B. amyloliquefaciens* isolate 66p at 3, 7 and 11 d AI. There is no control of the pathogen by day 15 AI.

A two way ANOVA was used to determine whether pre-inoculation with bacterial biological control decreased the area of the *L. maculans* lesion on the oilseed rape cotyledons.

There was a significant decrease in lesion area over all the pre-inoculated bacterial treatments regardless of isolate compared with the control cotyledon over all the experimental period ($F_{5,68}$ = 2.79, P = 0.024).

All the lesions grew significantly over time whether pre-inoculated or not ($F_{3,68}$ = 520.08, P = <0.001).

When bacterial pre-inoculation and time were taken into account, the change in lesion area just failed to achieve 5% significance ($F_{15,68}$ = 1.77, P = 0.058).

A Tukey pairwise comparison test indicated that on day 11 after inoculation, the lesion areas on cotyledons treated with *B. amyloliquefaciens* before inoculation with *L. maculans* were significantly smaller (P = 0.0069) than on cotyledons not treated with bacteria (Figure 4.6.). A similar result was obtained with *P. chlororaphis* spp. *aureofaciens* 30.84 (P = 0.0082). However, there was no significant difference in lesion area between the two pre-inoculated bacterial isolates (P = 0.125).

4.4.1.3. Conclusion

All bacterial biological control isolates gave good control *in planta* especially at day 11, with *P. amyloliquefaciens* 66p and *P. chlororaphis* spp. *aurefaciens* 30.84 giving the best control, with no difference between them. These were put into combination assays.

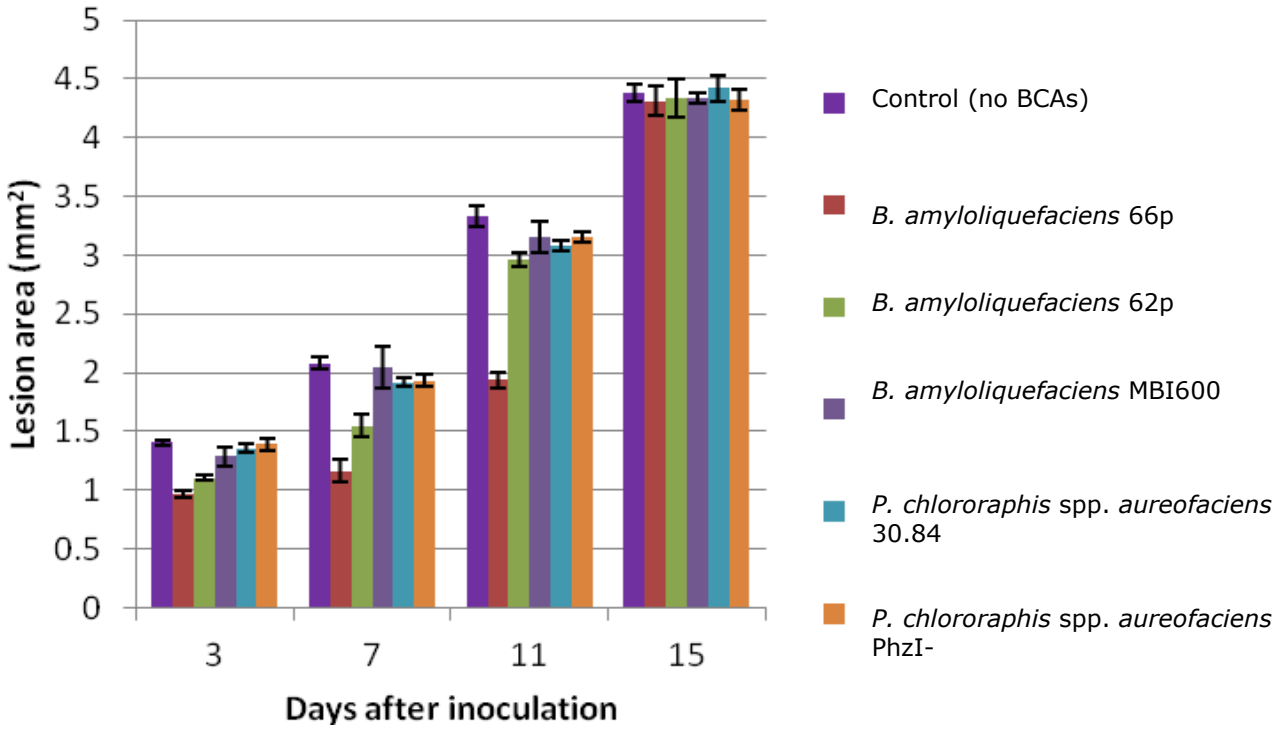


Figure 4.5. Efficacy of BCAs against *L. maculans* lesion development on oilseed rape cotyledons applied individually. N = 20. Error bars – SD.

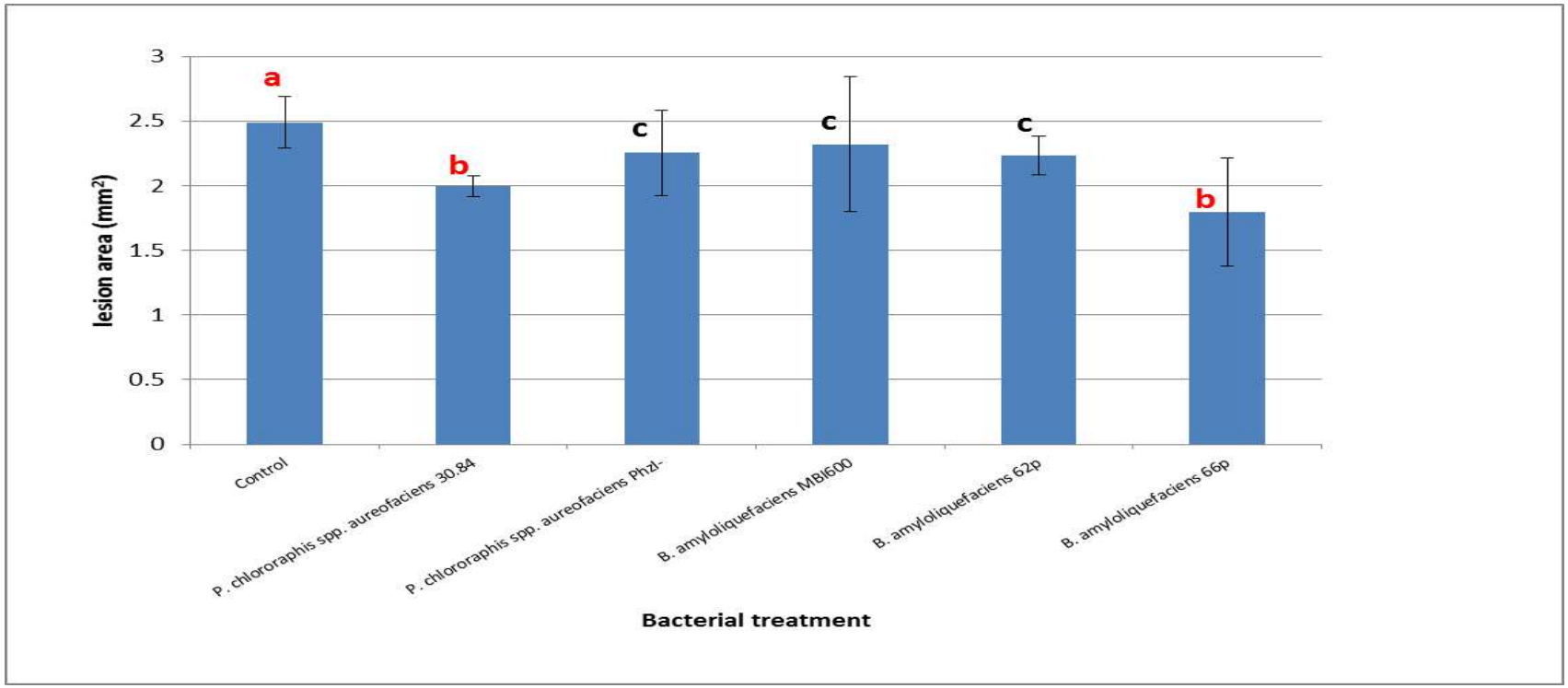


Figure 4.6. Leaf spot lesion area at day 11 after pre-inoculation with bacterial biological controls. Error bars – SD. Means with the same letter are not significantly different from each other (Tukey test, P = <0.05).

4.5. QUANTIFICATION OF *LEPTOSPHAERIA MACULANS* ERGOSTEROL

4.5.1. Ergosterol content quantification in *L. maculans* cotyledons

Determining the quantity of *L. maculans* ergosterol present in the cotyledon samples using HPLC (section 2.18.2) would be advantageous to enable a comparison with the quantified DNA from qPCR assays.

4.5.1.1 Methods

Cotyledons were harvested and the left hand side of each used for ergosterol analysis. Standards of pure ergosterol were prepared and a calibration curve produced. The basic protocol for the ergosterol extraction was adapted from Genney *et al*. 2000. The half cotyledons were rapidly frozen in liquid nitrogen to prevent ergosterol loss (Davis & Lamar, 1992) and weighed. Tissues were placed into bead beater tubes containing sterile glass beads. Methanol (1 mL) was added and the tissue disrupted in a Fast Prep tissue disrupter for 40 s at 10000 x g. The tubes were left at room temperature for 30 min, then centrifuged at 1000 x g for 5 min to pellet any solid material.

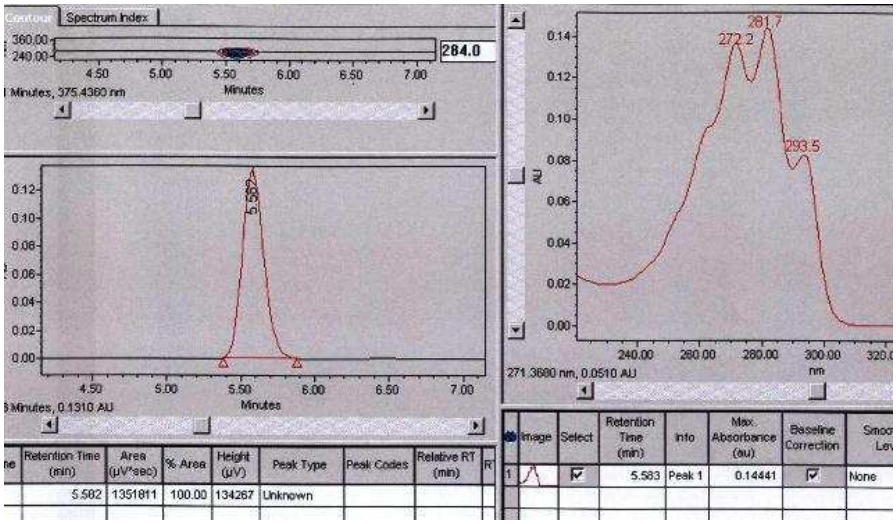
Five different extraction techniques were tested (Padgett & Posey, 1993, Padgett *et al*., 2000).

1. The tubes were sonicated in water at room temperature for 30 min.
2. 100 µL polyvinylpyrrolidone (PVP) (10% w/v in methanol) was added to each tube to precipitate the ergosterol.
3. 100 µL PVP was added, then sonicated in water at room temperature for 30 min.
4. 100 µL potassium hydroxide (10% w/v in methanol) was added to each tube (saponification). The tubes were heated to 60°C for 2 min. Distilled water (3 mL) and 2 mL hexane was added, left for 2h, then vortexed and centrifuged for 5 min at 700 x g. The hexane upper layer, containing the ergosterol, was removed.
5. Same method as 4 but sonicated for 2 h.

The samples were injected into a SPECTRA series P100 HPLC system (Thermoseparations Products, Stone, UK) equipped with a Spectromonitor 3000 detector, using a Phenomanex (www.phenomanex.co.uk) Luna®

octadecyl carbon chain (C18) reverse-phase analytical column, bonded silica particle size 5µm, pore size 100 Å, 250 cm x 3 mm. The UV detector was set at a wavelength of 282 nm. A flow rate of 1.5 mL min^{-1} was used and the system was isocratically run with 100% methanol.

A 20 µL standard of pure ergosterol in methanol eluted at a retention time of 6 min (Figure 4.7a) A U.V. spectrum was produced showing three peaks (Figure 4.7b) at 272.2nm, 281.7 nm and 293.5 nm which is characteristic of ergosterol.



(a) (b)

Figure 4.7. Chromatographic detection of pure ergosterol with an elution time of 5.5-6.0 minutes after separation by HPLC (a) and its absorption spectrum confirming ergosterol (b).

Numerous methods were tested to extract the ergosterol from *L. maculans*. Larger numbers of cotyledons were assayed, as were infected leaves from the field. Cotyledons of diverse ages and stages of visible infection were tested. Different isolates *L. maculans* grown *in vitro* of were assayed. Five extraction protocols were tried (section 3).

4.5.1.2. Results

Ergosterol was not found in any of the samples tested, but was identified in the standard (pure ergosterol).

Other substances were present in the assay (Figure 4.8.) which were not ergosterol as there was no peak at 5-5.6 mins (indicative of ergosterol).

To ensure the protocol was working, the samples were re-tested having been spiked with 100 µL of 20 µL standard. A peak was obtained which

corresponded with the peak obtained from the standard. This indicated the protocol was working correctly.

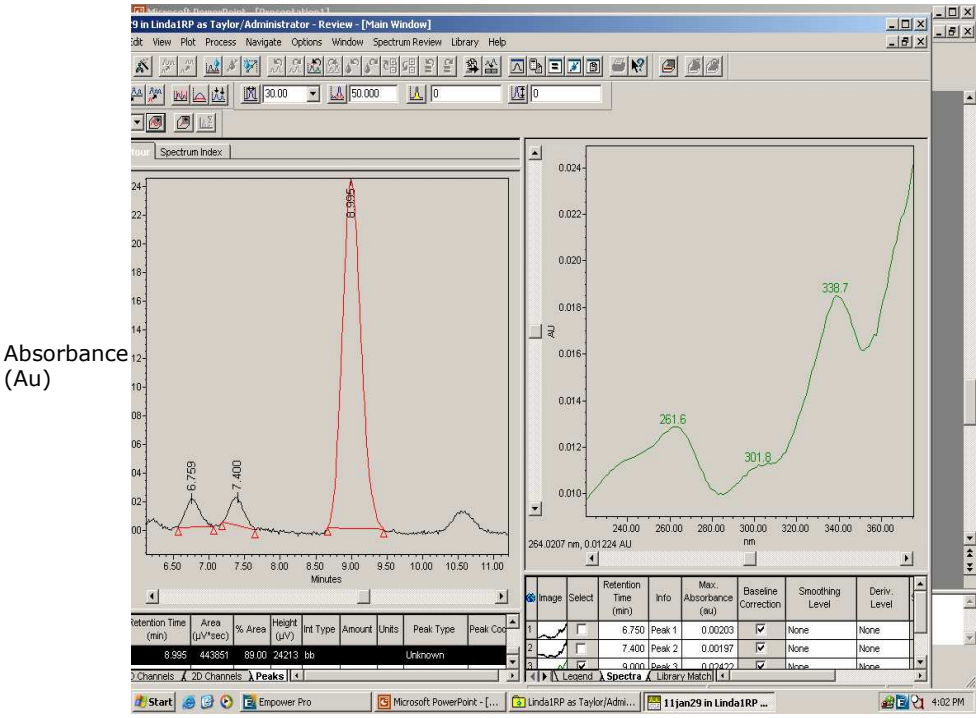


Figure 4.8. Retention time peaks (a) and spectra (b) of substances other than ergosterol obtained from DNA extracted from ½ (0.01g) cotyledon.

To test the protocols further, ergosterol was extracted from 1 g of hyphae from other fungal cultures grown *in vitro*. Ergosterol was detected using the basic protocol in *Fusarium* spp. fungus (methanol only) and in *Botrytis* spp. using amended protocols 1, 2, 3 and 4 (section 3).

4.5.1.3. Conclusion

L. maculans was present in all of the cotyledons, as chlorosis could be seen from 3 DAI and a lesion from 7 DAI. However, the HPLC assay did not find ergosterol. Thus, determination of ergosterol concentration was not a viable method for quantifying *L. maculans* infection.

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4.6. ASSESSMENT OF *L. MACULANS* DNA USING PCR

4.6.1. Assessment of spread of *L. maculans* in oilseed rape cotyledons using PCR.

It would be advantageous to ascertain if *L. maculans* had entered the cotyledon and to see where in the cotyledon the mycelia had reached at each of the sampling days.

4.6.1.1. Method

Each cotyledon was cut into half, then dissected into three sections, top (a), middle (b) and bottom (c), which included the petiole (section 2.17.). Total DNA was extracted and *L. maculans* DNA was isolated and amplified using PCR with *L. maculans* specific primers (section 2.7.). The product was run on a 1% gel, with visualisation under UV light.

4.6.1.2. Results

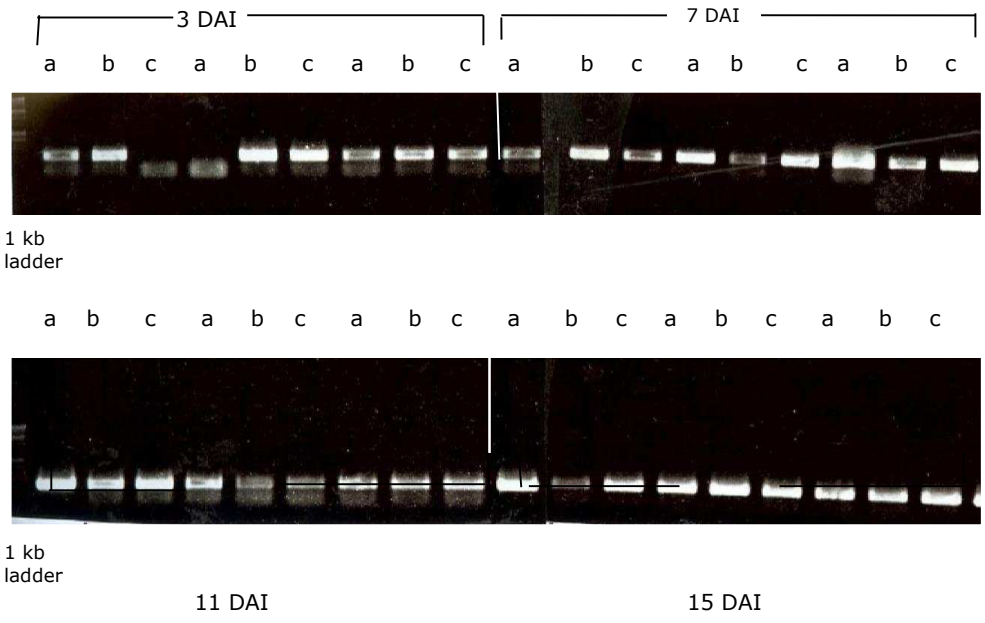


Figure 4.9. UV image from agarose gel electrophoresis (1%, 5 µg mL^{-1} ethidium bromide) of *L. maculans* DNA isolated from the top (a) middle (b) and bottom (c) of a cotyledon harvested at 3, 7, 11 and 15 DAI. Ladder 1kb.

Amplicons indicating the presence of *L. maculans* DNA could be seen in all parts of the cotyledon from day 3 AI to day 15 AI (Figure 4.9.). The amount of DNA present in the separate parts of the cotyledon, indicated by the intensity of band, was inconsistent.

4.6.1.3. Conclusion

The inconsistency of the band intensity when compared indicated that the fungus grew through the cotyledon erratically.

4.7. SURVIVAL OF BIOLOGICAL CONTROL BACTERIA *IN PLANTA*

4.7.1. Survival of bacteria from in planta assay

It is advantageous to ascertain the survival of the control bacteria after application onto the cotyledons throughout the experiment.

4.7.1.1. Methods

The bacteria were grown, applied to the cotyledons and counted as section 2.18.

4.7.1.2. Results

Figure 4.10. shows that all the biological control bacteria had colonised the cotyledon surface, increasing in number from 10^6 to 10^8. The growth of all isolates peaked at day 11 AI, followed by a decrease at day 15 AI to an amount similar to day 7 AI.

4.7.1.3. Conclusion

All bacterial isolates increased 100 x fold at day 11, indicating exponential growth until this time. After day 11, the fitness of the bacteria decreased back down to a similar amount to day 7.

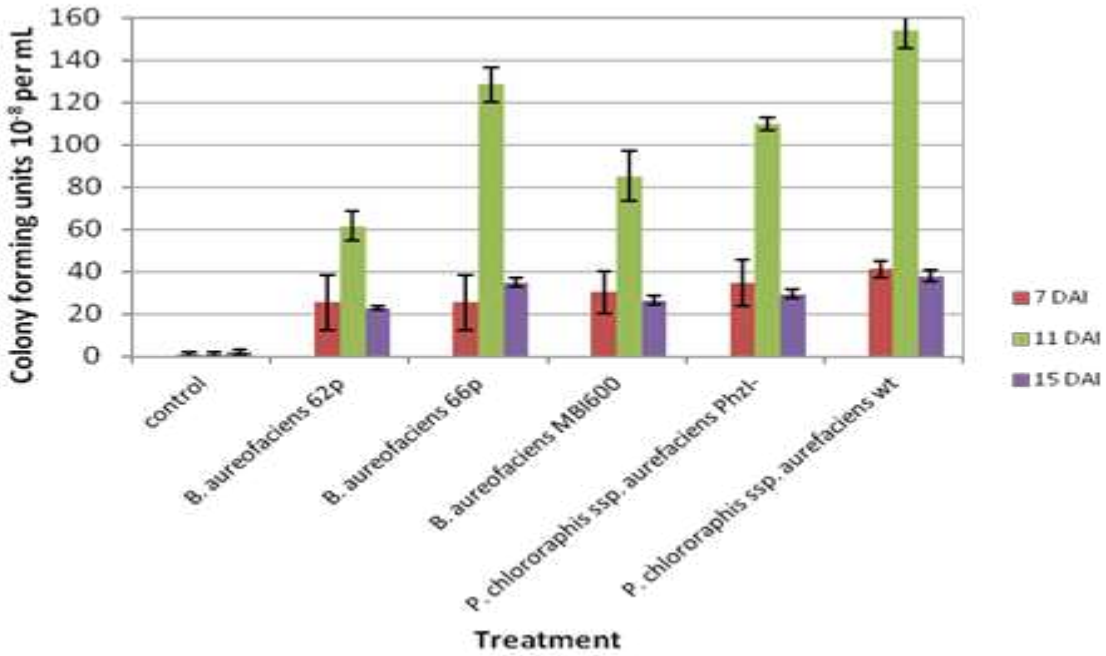


Figure 4.10. Bacterial survival as obtained from washed *B. napus* cotyledons and CFU assays. N = 20. Error bars SD.

4.8 DISCUSSION

Trichoderma harzianum did not inhibit *L. maculans in vitro* indicating that no control occured. *Trichoderma* has been shown to antagonise other fungi by a number of methods, including antibiosis, enzyme production, mycoparasitism and nutrient competition (Howell, 2003). It is perhaps surprising that no antagonism was detected in this work. This may reflect the unsuitability of the growth medium, the method or the isolate used. Guetsky *et al*. (2002) found that *T. harzianum* strain T39 only gave control against *Botrytis cinerea* when applied at a spacially separate site. Although *T. harzianum* is a well known biocontol agent of many pathogens, Yenjerappa *et al*. (2013) found no control was observed over *Xanthomonas axanopodis* pv *punicae*, a bacterial pathogen of pomegranate. As no activity was detected, this biological control agent was not used in *in planta* assays.

All the isolates of *B. amyloliquefaciens* and *P. chlororaphis* spp. *aureofaciens* tested in this work showed good control against *L. maculans*. Their survival was good up to day 11, after which the bacteria began to die resulting in a level similar to day 7. This may have been due to all nutrients being used up on the surface of the cotyledon.

Three phenazine antibiotics are known to be produced by *Pseudomonas chlororaphis* spp. *aureofaciens* 30.84, phenazine-1-carboxylic acid (PCA), 2-hydroxy phenazine-1-carboxylic axid (2OHPCA) and 2-hydroxy-phenazine (2OHPZ) (Pierson & Thomashow, 1992, Pierson *et al*., 1994). Less than 10% of PCA is converted into the 2OHPCA by a monoxygenase enzyme located downstream of the phenazine genes, and it is this phenazine that is antifungal (Maddula *et al*., 2008, Delaney *et al*., 2001).

The antibiotics produced by *Bacillus* spp. can be isolate specific (e.g. subtilin), or be produced by many isolates (e.g. surfactin). Antibiotics produced by *B. amyloliquefaciens/subtilis* are bacillase, fengycin and macrolactin (Yuan *et al.*, 2012, Ongena & Jacques, 2007). Bacillomycin, which disrupts fungal spore attachment and germ tube growth, and fengycin, with haemolytic activity, are the substances effective against fungi, and as such, *L. maculans*.

Antibiotic production in both *Pseudomonas* and *Bacillus* and their secretion onto the cotyledon, can also lead to an upregulation in the oilseed rape plant of broad spectrum resistance genes (section 1.4.), resulting in induced host

resistance (Pal & McSpadden Gardener, 2006). This confirmed work using *B. subtillis,* in the biofungicide Serenade, completed by Lahali *et al*. (2014) which found this was partially the mechanism of suppression of clubroot on oilseed rape. The products of the genes slow the development of pathogens, including *L. maculans*, when they have entered the plant (Verhagen *et al*., 2004). Some control must take place inside the plant as well as on the phylloplane as *L. maculans* spends most of its lifecycle intracellularly. The ability of phenazines to generate reactive oxygen species (ROS) in other organisms results in beneficial effects in the oilseed rape plant by upregulating both ethylene and jasmonic acid pathways.

Although biological control was consistently seen *in planta* under controlled environment conditions, this may not be the case if field trials are carried out. Both Handelsman & Stabb, 1996 and Tomashow & Weller, 1988 found that *P. chlororaphis* PCL 1391 did not give reliable results which may have been due to the variable conditions of a field environment (Tomashow *et al*., 1990, Duffy & Défago, 1999).

It would be interesting to determine whether the biological control agents used in this work elicited the expression of host defence genes.

In conclusion, when used as a single isolate application, both *Pseudomonas chlororaphis* spp. *aureofaciens* and *Bacillus amyloliquefaciens* provided effective control of *L. maculans* in these experiments.

CHAPTER 5 – BIOLOGICAL CONTROL OF STEM CANKER ON OILSEED RAPE USING ANTAGONISTS APPLIED IN COMBINATION

5.1. INTRODUCTION

Today it is common to combine organisms with biological control of crop diseases, resulting in varying degreees of success. *Pichia guilermondii* (yeast) has been applied with *B. mycoides* to successfully control *Botrytis* on strawberry and indeed worked better than when each organism was applied alone (Guetsky *et al*., 2002). *Pichia guilermondii* has been successfully applied in combination with *Candida membraniciens* and *B. subtilis* to control *Botrytis cinerea* on apple trees (Zangoei *et al*., 2014). *B. subtilis* plus *Saccaromyces cerevisae* controlled *Fusarium solani* (Abo-Ghalia & Hussain, 2002). Combinations of various *Bacillus* and *Pseudomonas* isolates have been successfully applied to control *Fusarium* on *Solanum melongena* (Altinok & Dikilitas, 2001). Domenech *et al.*, (2006), found a combined application of *B. subtilis, B. amyloliquefaciens* and *P. fluorescence* worked against *Fusarium* and damping off. Manjukarunambika *et al*., (2013) claim that the field experiments using combinations of *T. atroviride* and *P. fluorescens* gave favourable results compariable to fungicide usage.

Whilst some groups have been successful with combinations, others have found that combinations of biocontrol agents are not as effective as when individually applied. Domenech *et al*., (2014), found that whilst three different strains of *B. subtilis* and and one species of *Chryseobacterium* controlled mixed diseases on bell peppers, while a combination gave less control. Indeed, the strain of control organism is important when being applied in combination with other organisms, as some are antagonistic whilst others not (Ruano-Rosa *et al*., 2014). Raziq & Fox, 2012 found that two different strains of *T. harzianum,* Th1 and Th2 performed differently as a control of *Armillaria mellea* on strawberry. Th2 gave good control when applied alone, but no suppression when applied with Th1. Th1 gave no control when applied individually.

Little research has been undertaken to date to biologically control *L. maculans* on oilseed rape using organisms applied in combination. Panjehkeh, 2011 observed that a combination of *Trichoderma* and *Bacillus* was antognistic to *L. maculans*, but as *Trichoderma* gave no control in this work *in vitro*, any biocontrol seen by that group may have been due to the

isolate of control agents applied. Abuamsha *et al*., (2011) found that whilst applying *Serratia plymuthica* strain HRO-C48 onto oilseed rape seed gave good control of *L. maculans*, when applied in combination with *P. chlororaphis* strain MA 342 control was reduced.

Theoretical modelling has been carried out on the combined use of two foliar biocontrol agents, leading to the conclusion that a combination of organisms is only as good as the best individual agent. It also ascertained that a combination including one competitive organism gave the better control (Xu & Jeger, 2013) Begum *et al*., (2010), saw this *in planta* where the more virulent *T. harzianum* and *T. virens* gave the same control *as T. harzianum* alone.

In this work good control of leaf spot lesion development with all bacterial isolates was indicated *in planta* when they were used singularly. *B. amyloliquefaciens* 66p, which gave the best control, and *P. chlororaphis* spp*. aureofaciens* were used in combination assays at ratios of 1:1, 1:9 and 9:1. It may be beneficial in the use of these biological control agents to apply them in combination of *Bacillus* (producing durable spores) and *Pseudomonas* with previously-noted ability to colonise the leaf. However, for *Bacillus* and *Pseudomonas* to be used in combination successfully, it must first be ascertained if they have a detrimental effect on each other or have a synergistic effect.

5.2. *IN PLANTA* EFFICACY OF BIOLOGICAL CONTRON AGENTS USED IN COMBINATION.

5.2.1. Introduction

A mix of bacteria may be used in biological control to enhance the range of activity (Pal & McSpadden Gardener, 2011). A potential problem in mixed populations of *Bacillus* and *Pseudomonas* bacteria is that many *Bacillus* can produce lactonase enzymes which target and inactivate the AHL signalling molecules produced by the *Pseudomonas* to upregulate their antibiotic production.

A T-streak assay using *Chromobacterium violaceum* was employed to ascertain if *P. chlororaphis* spp *aureofaciens* 30.84 produced the AHL quorum sensing signalling molecule. A PCR was carried out on *Bacillus* DNA using specific primers to determine if the AHL degrading enzyme lactonase was present.

5.2.1.1. T-streak assay introduction

Chromobacterium violaceum is bacterial reporter strain used in quorum sensing assays. The CV026 mutant contains a *Tn5* transposon insertion which prevents synthesis of the purple colour of violacein (an antioxidant), produced by the wild type. This is complemented if AHLs are being produced by another bacteria resulting in the upregulation of violacein visualised as a purple colour.

5.2.1.2. Methods

Both the CV026 and *P. chlororaphis* spp. *aureofaciens* 30.84 were placed into liquid LB medium (Appendix B), then cultured at 28°C overnight. The cultures were centrifuged at 6000 g for 5 min, then the supernatant was removed and filtered through a 0.20 µm Millipore filter to remove any violacein produced (Appendix B).

Plates of LB were buffered to pH 7.0. Two buffers were tested, phosphate buffer (15mM) (Morohoshi *et al*., 2009) and Tris buffer (15 mM) (Carlier *et al*., 2003), with Tris buffer being more effective for this assay.

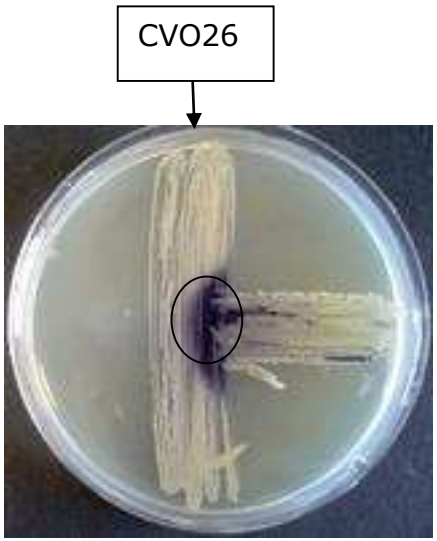
The CV026 was streaked vertically on LB plates buffered to pH 7.0 with the *P. chlororaphis* spp*. aureofaciens* 30.84 streaked horizontally on ten Petri dishes and *P. chlororaphis* spp*. aureofaciens* PhzI- mutant on another 10 Petri dishes. The cultures were incubated at 30°C for 24 h.

5.2.1.3. Results

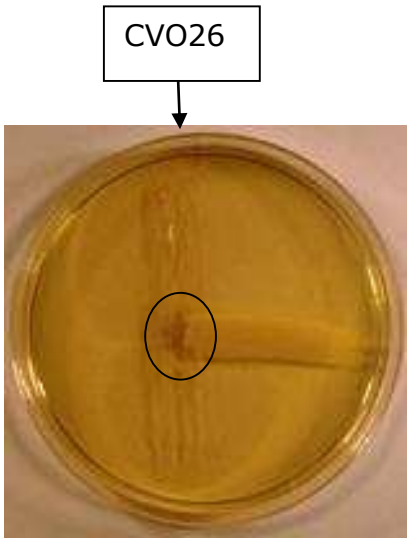
Production of the *Pseudomonas* signalling molecule AHL induced by quorum sensing was confirmed in a T streak assay using a mutant of the bacteria *C. violaceum*. After incubation, a faint vertical purple line could be seen at the intersection (Figure 5.1.).

5.2.1.4. Conclusion

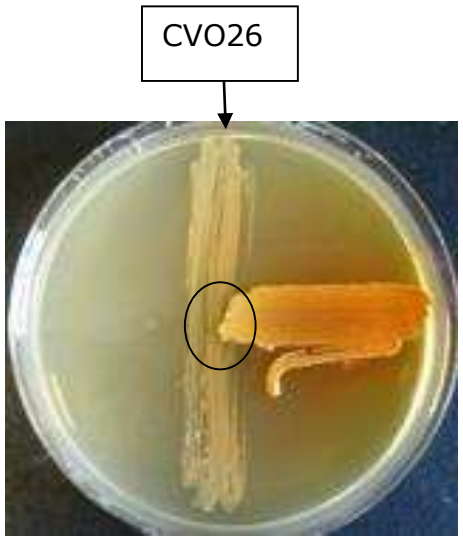
This indicated that complementation had occurred and phenazine was being produced. The CV026 streaked with *P. chlororaphis* spp*. aureofaciens* $PhzI^-$ did not produce the purple pigment.



a) Control. Strong purple line where exogenous AHLs meet the CV026 streaked vertically (circled).



(b) *P. chlororaphis* spp*. aureofaciens* 30.84 streaked horizontal. Purple seen at the intersection with CV026.



(b) *P. chlororaphi*s spp*. aureofaciens* PhzI- streaked horizontal. No purple at the intersection (circled).

Figure 5.1. T-streak assay to determine the presence of AHLs in *P. chlororaphis* spp*. aureofaciens* 30.84 and PhzI- (phenazine minus mutant) by inducing the production of violacein in mutant *Chromobacterium violaceum*, unable to produce the purple pigment.

5.2.1.5. Lactonase amplifying PCR introduction

Lactonase, produced by the *aiiA* gene, hydrolyses the ester bond of the cyclic ester lactone ring of the *Pseudomonas* AHL QS signalling molecule (Dong *et al*., 2001, Williams, 2007) changing its shape and de-activating it.

5.2.1.6. Methods

To ascertain the presence of lactonase in the *Bacillus*, specific primers (forward *5'*-ATGACAGTAAAGAAGCTTTATT-*3'*, reverse *5'*-CTATATATATTCTGGGAACACT-*3'* Yin *et al.*, 2010,) were used in a PCR reaction, followed by gel electrophoresis and visualisation under UV light. The primers for the AHL lactonase gene (aiiA) were designed using *B. amyloliquefaciens* genomic DNA and based on the *aiiA* gene sequences obtained from GenBank (Yin *et al*., 2010).

5.2.1.7. Results

No band was seen on the gel in lane 2 (Figure 5.2.) (*B. amyloliquefaciens* MBI600), lane 3 (*B. amyloliquefaciens* 66p) or lane 4 (*B. amyloliquefaciens* 62p) at 750 bp, which would indicate the presence of the aiiA gene, but it could clearly be seen on the control (lane 6) which was *Erwinia carotovora*, known to contain the lactonase gene. Lane 5 was *B. megaterium* a field isolate which also did not have the aiiA gene.

5.2.1.8. Conclusion

There was no evidence that the *Bacillus* isolates used would inhibit quorum sensing in *Pseudomonas*.

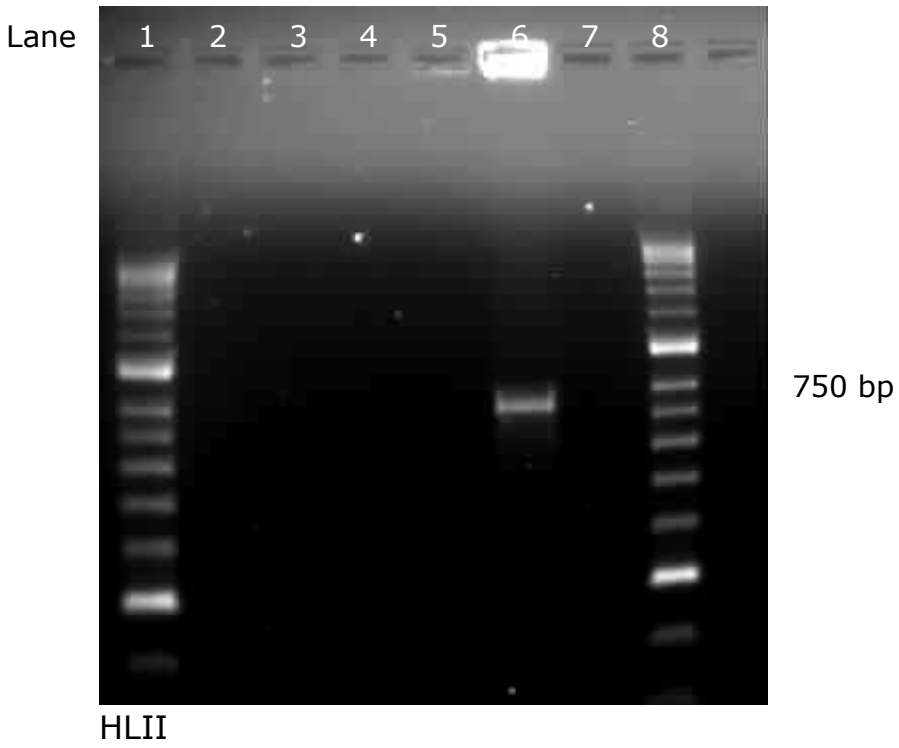


Figure 5.2. Gel electrophoresis results of PCR probing for aiiA lactonase gene. No amplicon in lanes 2,3, 4 and 5 indicate the aiiA gene not present in the *Bacillus* tested. An amplicon at 750 bp in lane 6 indicates the presence of the aiiA gene in the positive control (*E. carotovora*). Lane 7 negative control. Lane 1, hyperladder II.

Further assays were conducted to ascertain if the lactonase gene could be found in any field isolates of *Bacillus*. Soil was placed into distilled water (DW) and Pasteurised at 80ºC for 30 min. The supernatant was placed onto NA (Appendix A) and cultures incubated at 30 ºC overnight.

Single colonies of *Bacillus* grown from Pasteurised soil samples were placed into a PCR with aiiA specific primers (Appendix C). Figure 5.3. shows the primers anneal to the aiiA gene in some *Bacillus* isolates (see lanes 6, 10, 15, 16, 18) but not in others. *E. coli* aiiA used as a positive control (lane 20) with distilled water as a negative control (lane 19).

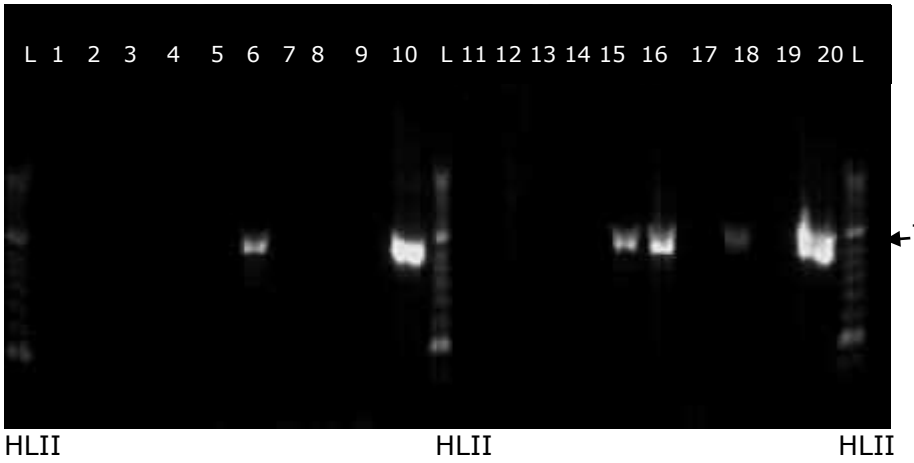


Figure 5.3. Gel electrophoresis results probing *Bacillus* isolates isolated from soil samples with aiiA (lactonase gene) specific primers. No band at 750 bp in lanes 1, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14 and 17 indicate the gene is not present. Bands at 750 bp in lanes 6, 10, 15, 16 and 18 indicates presence of the lactonase fragment in those *Bacilli*. Lanes 19 and 20 negative and positive control respectively. Lane L contains hyperladder II.

Field isolates of *Bacillus* were tested in a PCR (Figure 5.4.). Lane 13 contains *E. coli* aiiA positive control, Lane 14 shows negative control.

Figure 5.4. Gel electrophoresis results of field isolates of *Bacillus*. Bands at 750 bp in lanes 1, 3, 4, 5, 6, 7 and 8 indicate the *Bacillus* field isolates which contained the lactonase gene. No bands were seen in lanes 2, 9, 10, 11 and 12, indicating the *Bacillus* field isolates did not contain the gene. Lane L contained hyperladder II with lanes 13 and 14 the positive and negative controls respectively.

Lanes 1, 3, 4, 5, 6, 7 and 8, had a band at 750 bp which indicated the presence of the lactonase gene. Lane 13 contained *Erwinia carotovora* which is known to have the lactonase gene (positive control). Lane 14 was the negative control which contained DW instead of DNA.

A phylogenic tree was produced by searching the BLAST database for the presence of the AHL lactonase (aiiA) gene sequence within the genomes (Figure 5.5.). The lactonase gene was not found in the *B. subtilis* species group (highlighted green) whilst it was found in the genome sequences of *B. cereus, B. anthracis, B. thuringiensis and B. weihenstephanensis* species groups (highlighted red).

The gene was also not found in *B. megaterium* (highlighted green). The phylogenic tree is just an indication as many of the *Bacillus* genus did not have full genome sequences (highlighted yellow).

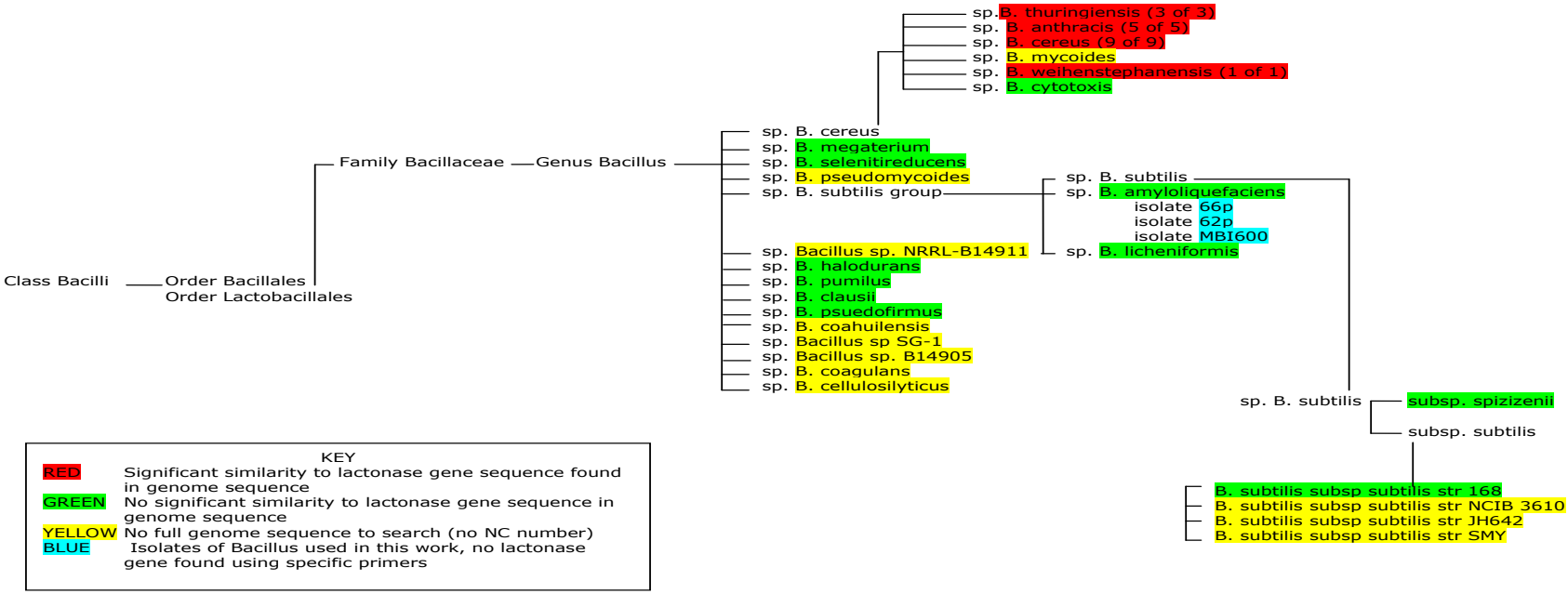


Figure 5.5. Phylogenic tree of order Bacillales showing presence or absence in the genome of the lactonase gene sequence (aiiA) (Accession no. DQ00064). Credit: Linda Cholerton.

5.3. *IN PLANTA* ASSAYS OF BIOLOGICAL CONTROL BACTERIA WHEN APPLIED IN COMBINATION

Bacterial isolates which indicated biological control activity in *in vitro* assays were placed into secondary screening using *in planta* bioassays. Good leaf spot lesion control with all bacterial isolates was indicated *in planta* when they were used singularly. These isolates were then placed into *in planta* combination assays.

The *Bacillus* and *Pseudomonas* combination treatments were used to attempt to control phoma leaf spot and symptoms were monitored from 3-15 DAI as described previously.

Mean lesion areas are given in Figures 5.6. - 5.8. In short, no combination treatment provided effective control of leaf spot symptom formation.

The failure of effective biocontrol is also illustrated in the following sample images of cotyledons at day 7 (Figure 5.9.) and day 11 (Figure 5.10.).

It can be clearly seen from these photographs that lesion development was higher in the presence of the combinations of BCAs.

A two way ANOVA was used to determine whether pre-inoculation with bacterial biological control in a 1:1 combination increased the area of the *L. maculans* lesion on the oilseed rape cotyledons.

There was a significant increase in lesion area compared with the control cotyledon over the experimental period ($F_{2,56} = 3.14$, $P = 0.012$).

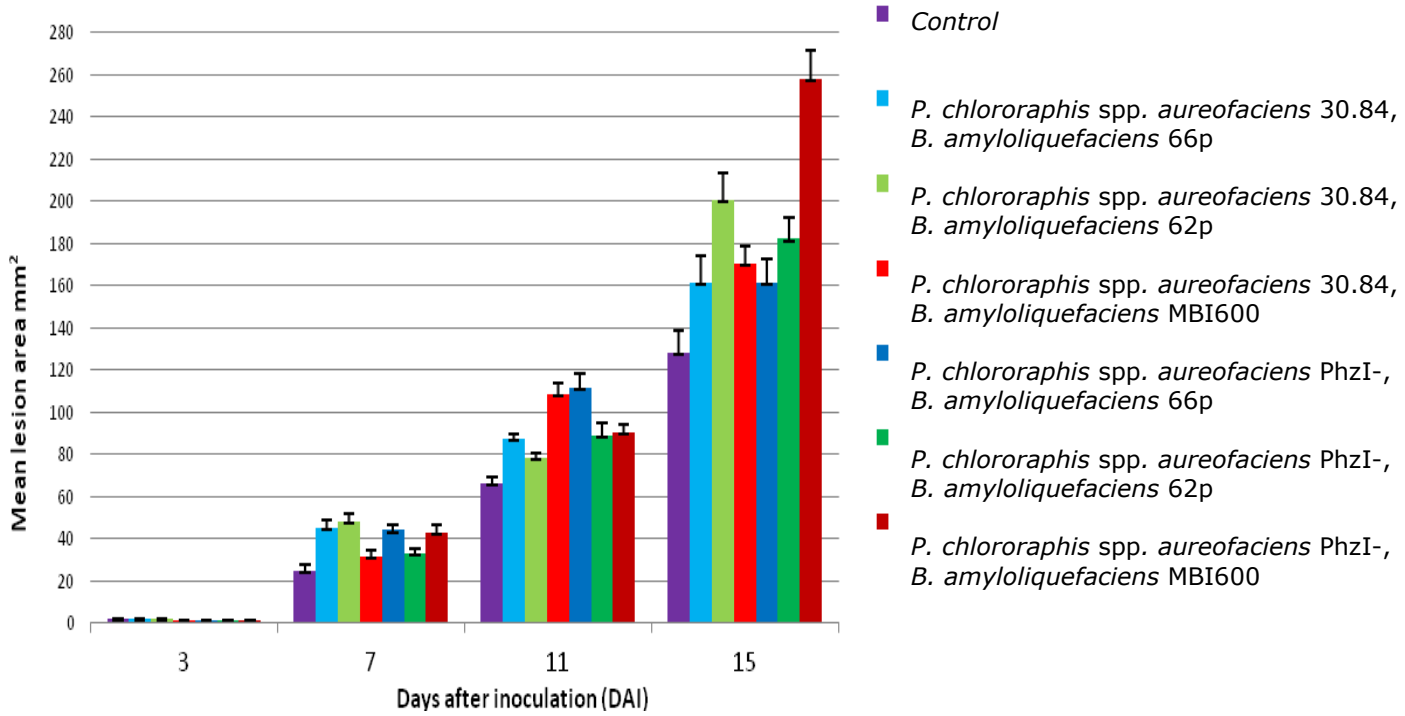


Figure 5.6. Mean *L. maculans* lesion area on cotyledons pre-inoculated with *P. chlororaphis* spp. *aureofaciens* and B. *amyloliquefaciens* isolates applied in a ratio of 1:1. N = 20. Error bars SD.

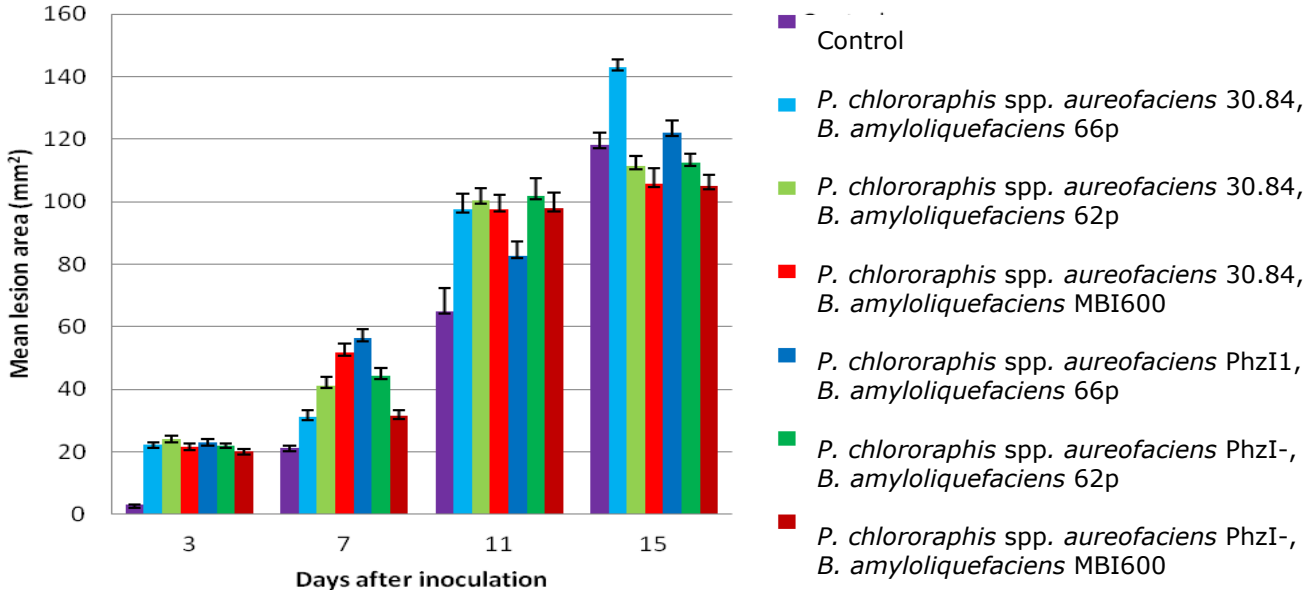


Figure 5.7. Mean *L. maculans* lesion area on cotyledon pre-inoculated with *P. chlororaphis* spp. *aureofaciens* and *B. amyloliquefaciens* isolates applied in a ratio of 1:9. N = 20. Error bars – SD.

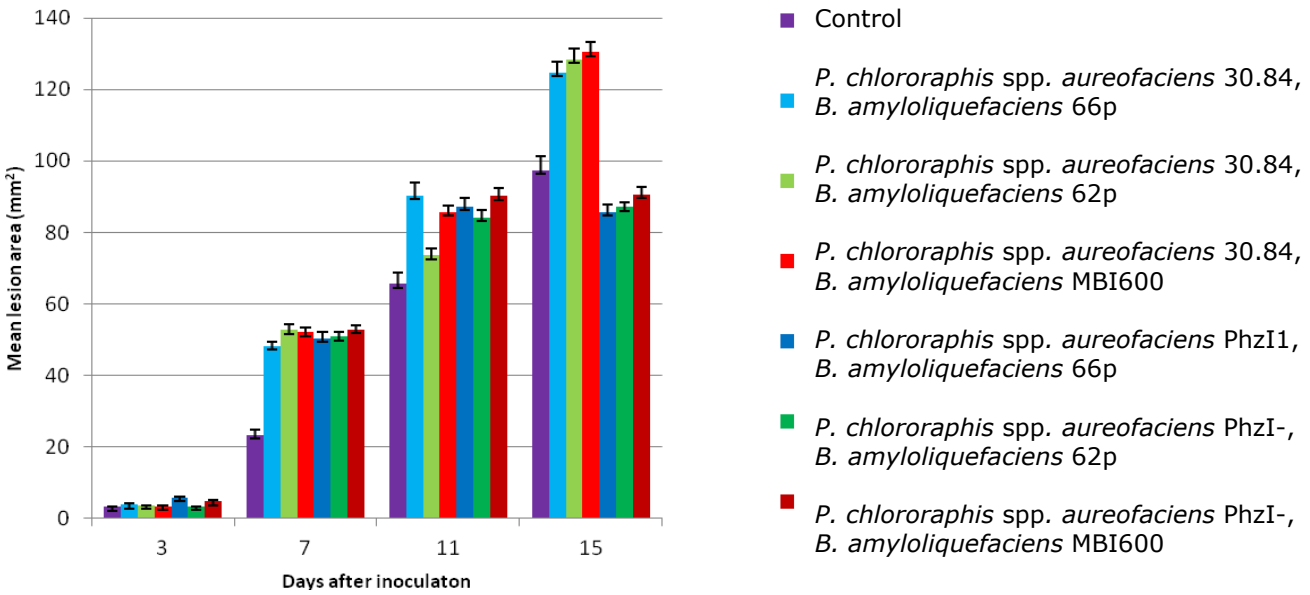


Figure 5.8. Mean *L. maculans* lesion area on cotyledon pre-inoculated with *P. chlororaphis* spp*. aurefaciens* and *B. amyloliquefaciens* isolates applied in a ratio of 9:1. N = 20. Error bars – SD.

Control

Pseudomonas 30.84/
Bacillus MBI600

Pseudomonas 30.84/
Bacillus 66p

Pseudomonas 30.84/
Bacillus 62p

Pseudomonas Phzi-/
Bacillus MBI600

Pseudomonas Phzi-/
Bacillus 66p

Pseudomonas Phzi-/
Bacillus 62p

Figure 5.9. Phoma leaf spot lesions day 7 after pre inoculation with *P. chlororaphis* spp*. aureofaciens* and *B. amyloliquefaciens* applied in a ratio of 1:1. Lesions produced were necrotic. Scale bar 1 mm.

Control

Pseudomonas 30.84/
Bacillus MBI600

Pseudomonas 30.84/
Bacillus 66p

Pseudomonas 30.84/
Bacillus 62p

Pseudomonas Phzi-/
Bacillus MBI600

Pseudomonas Phzi-/
Bacillus 66p

Pseudomonas Phzi-/
Bacillus 62p

Figure 5.10. Phoma leaf spot lesions day 11 after pre inoculation with *P. chlororaphis* spp*. aureofaciens* and *B. amyloliquefaciens* applied in a ratio of 1:1. Lesions produced were all necrotic with immature pycnidia. Scale bar 1mm.

5.4. SURVIVAL OF BIOLOGICAL CONTROL BACTERIA WHEN APPLIED IN COMBINATION

As the efficacy of biocontrol was extremely poor when *Bacillus* and *Pseudomonas* species were combined, the possibility that the bacteria were inhibiting each other was explored. The survival of the biological control agents applied at a concentration of 10^6 cm^2 was ascertained by removing as many of the bacteria present as possible, plating onto solid media and counting the colonies produced. The number of CFUs recovered from cotyledons are given in figures 5.11. - 5.13. which show a decrease in concentration to 10^2 cm^{-2}.

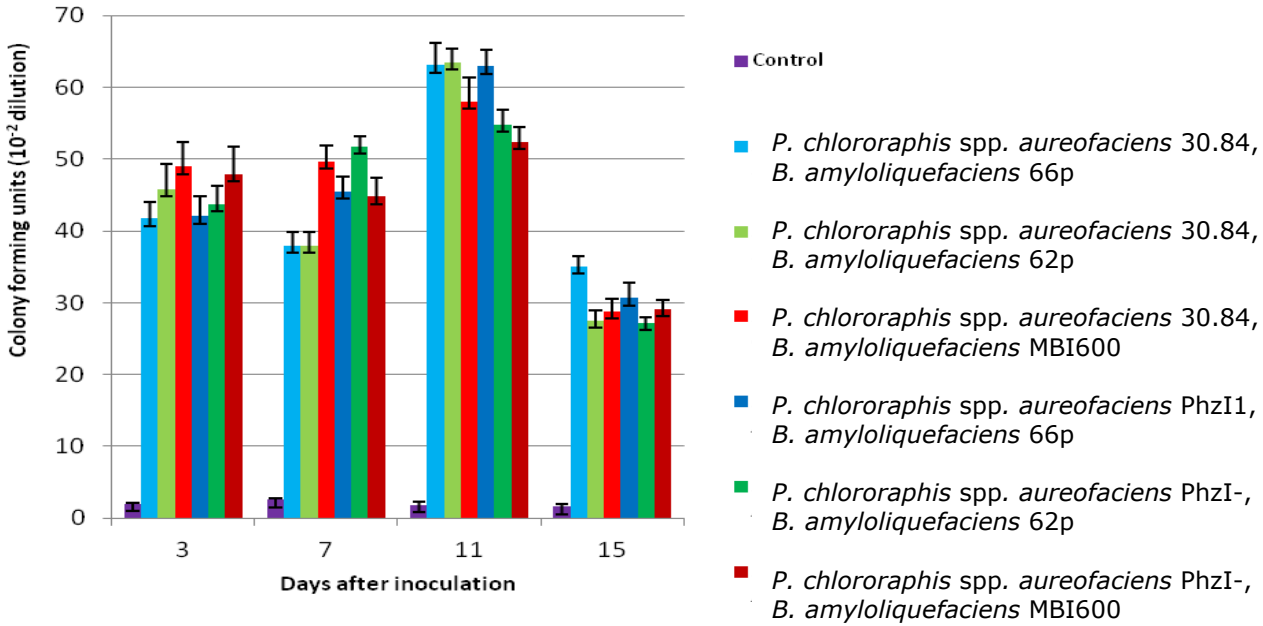


Figure 5.11. Bacterial colony forming units retrieved from *B. napus* cotyledons pre-inoculated with *P. chlororaphis* spp*. aureofaciens* and *B. amyloliquefaciens* isolates applied in a ratio of 1:1. Fewer bacteria were counted when applied in combination than when applied individually. N = 20. Error bars – SD.

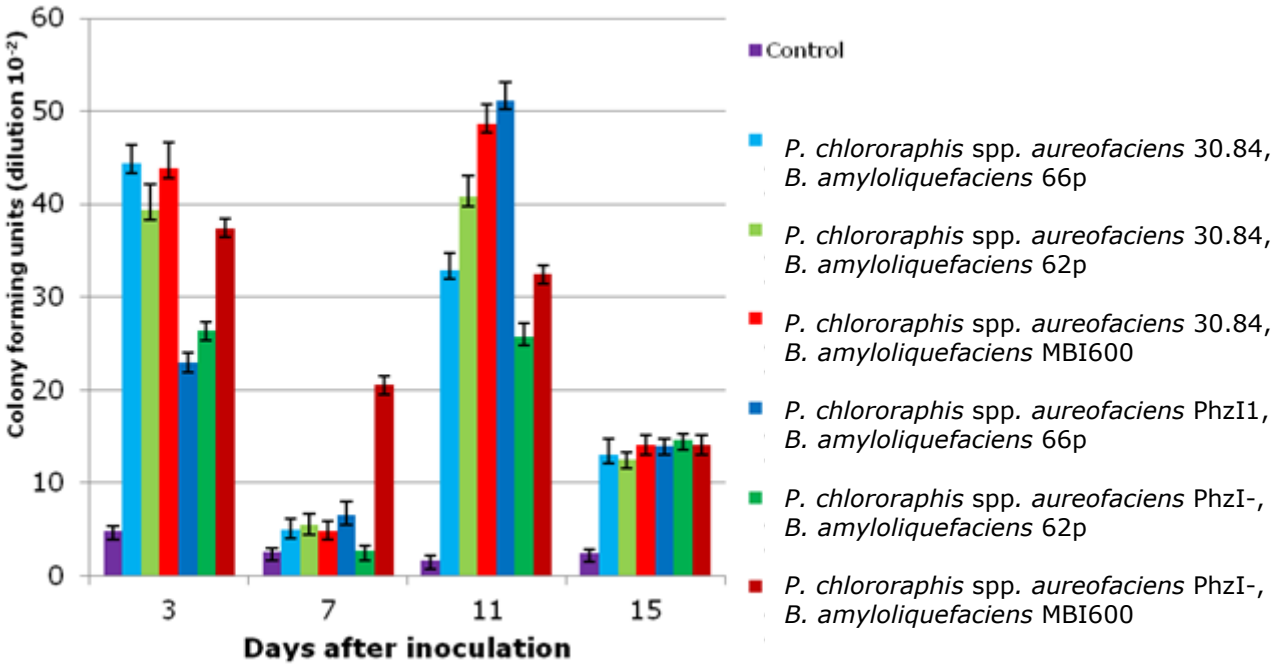


Figure 5.12. Bacterial colony forming units retrieved from *B. napus* cotyledons pre-inoculation with *P. chlororaphis* ssp. *aureofaciens* and *B. amyloliquefaciens* isolates applied in a ratio of 1:9. Fewer bacteria were counted when applied in combination than when applied individually. N = 20. Error bars – SD.

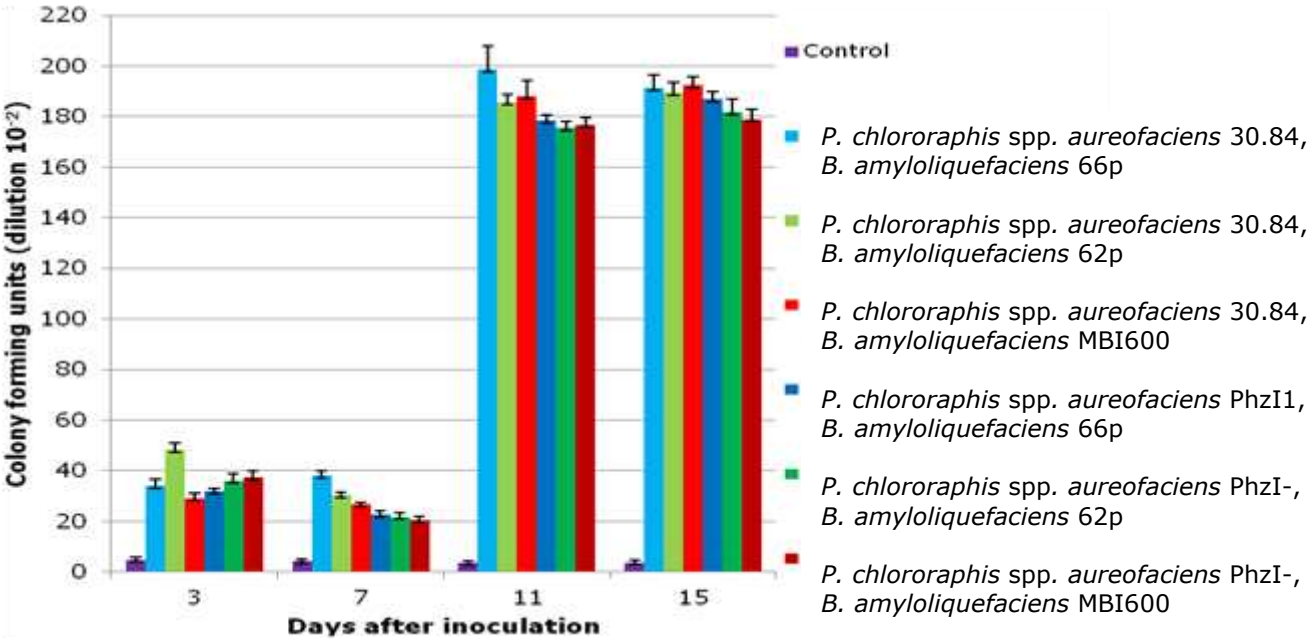


Figure 5.13. Bacterial colony forming unit count retrieved from cotyledons pre-inoculation with *P. chlororaphis* ssp. *aureofaciens* and *B. amyloliquefaciens* isolates applied in a ratio of 9:1. Fewer bacteria were counted when applied in combination than when applied individually. N = 20. Error bars – SD.

5.5. DISCUSSION

Bacillus and *Psuedomonas* used in this work appear to be antagonistic to each other. The bacterial populations decreased from the application rate of 10^6 cm^2 to 10^2 cm^2 regardless of the interaction. Investigations confirmed *Bacillus amyloliquefaciens* did not produce lactonase, indicating it should have been compatible with the *Pseudomonas* when applied in combination. A phylogenic tree produced suggested that those *Bacillus* which were adept at biological control did not contain this enzyme. The decrease may have been partially due to any nutrients present on the cotyledon were utilised quickly. The absence of lactonase in this *Bacillus* may have meant that the strain could not denature AHLs produced by the *Pseudomonas*, and use them as a nutrient source (Yang *et al*., 2005). The lactonase quorum quenching enzyme appears to be an important enzyme for survival in the natural environment for both plant and mamalian species and is highly conserved within each order (Chen *et al*., 2013). Bacteria which lyse release endotoxins (Beifuss & Tietze, 2005), toxic compounds which would also further decrease the live cell population.

A primary role of antibiotics produced by bacteria is to be detrimental to other micro-organisms, thus aiding survival in the environment e.g. lysis of bacterial cells (Pierson *et al.*, 1994), and as such, the bacteria may have been attacking each other. Although being of different species, both *Pseudomonas* and *Bacillus* secrete signalling molecules which can be detected by each other. The detection would increase antibiotic production. However, the resulting low amount of bacterial colonies (as retrieved by CFU and qPCR) may have disrupted the ability of the bacteria to produce quorum sensing molecules, as the quorum amount is not fixed. The upregulation of substances vital to survival, e.g. biofilms may not have occurred. Biofilms are produced to allow the bacteria to attach to surfaces as a community (Chen *et al*., 2002, Maddula *et al*., 2004, Maddula *et al*., 2006, Ramos *et al*., 2010), enabling them to carry out functions e.g. antibiotic resistance, not imparted when functioning as a single colony (Maddula *et al*., 2008) which ensures survival (Davies, 2002). As such, no immunity to the antibiotics produced by the other species would have been obtained. The mutant *P. chlororaphis* spp. *aureofaciens* PhzR/I- cannot make biofilms as a fully functioning QS system is required (Maddula *et al.*, 2006).

Pseudomonas phenazine has been proven to induce apoptosis in *B. subtilis* by producing ROS, hydrogen cyanide (Bisschop, 1979, Li *et al*., 2008, Pierson & Pierson, 2010). Hydrogen cyanide is produced by the *Pseudomonas* primarily for use in cell signalling, but a secondary effect results in the uncoupling of oxidative phosphorylation in the *Bacillus* electron transport chain. The resulting non production of adenosine triphosphate (ATP) would detrimentally affect new cell production. The apoptitic cells may have been used as a food source for both the *L. maculans* and the *Pseudomonas*. However, some *Bacillus* can produce nitric oxide which converts the ROS into inert superoxide dismutase (Tjeerd *et al*., 2004) and therefore the *Bacillus* cells may not have been affected. *Bacillus* have previously been shown to halt growth of nearby bacterial colonies using substances, which inhibit prokaryotic sysnthesis, e.g. bacillase (Be'er, 2010, Yuan *et al*., 2008, Chen *et al*., 2013) enabling resouces to be preserved for themselves. However, nutrients obtained this way would probably be used up quickly. The lack of nutrients may have meant that the *Bacillus* did not reach stationary phase (at approx. 72 h) resulting in an inability or a reduction in sporulation. Bacteria gather around nutrients leached from wounds on the cotyledon, and bacterial survival and competition has been correlated with the amount of nutrients present (Pekkonen *et al*., 2013). The concentration of nitrogen, used as an energy source for the bacteria, may have initially increased in these assays due to the apoptisis of the plant cells but rapidly utilised

No control of *L. maculans* was observed in *in planta* assays regardless of percentage combination of *Bacillus* and *Pseudomonas*, indeed the lesions were visually larger than those of the control plants. The increase in lesion area may have been due to a lack of biological control by the bacterial combination on the epidermis of the plant and/or a subsequent increase in nutrients for a proliferation of fungal hyphal growth. Biological control on the epidermis in this instance is partially based on competition for nutrients with *L. maculans*, before the fungus enters the leaf. Control in this way can be nullified in the nitrogen concentration increases (Guetsky *et al*., 2002) or if the ratio of carbon to nitrogen changes (Howlett, 2006, Gao *et al*., 2007) which could happen with the amount of dead cells in the phyllosphere. Whilst Luti & Yonis, (2013) showed that *Pseudomonas* increased phenazine production in the presence of *Bacillus*, regardless of the cells being dead or alive, this may have been offset by the shikimic pathway using the depleting

nutrients for the increase in antibiotic production (Mavrodi, 2006), resulting in there being little left for cellular growth. Only 10% of the phenzine 1 carboxylic acid produced by *Pseudomonas* is the antifungal agent, 2 hydroxy carboxylic acid (2.OH PCA) which is detrimental to *L. maculans* (Toohey *et al*., 1965). Altering the ratio of the phenazine adversely affects fungal inhibition (Maddula *et al*., 2008) and its loss reduces inhibition under the stressful conditions of combined bacterial application, 2.OH PCA may not have been produced, or in insufficient quantities to up-regulate induced acquired resistance in the plant resulting in no control when the fungus was inside the plant.

When inside the plant, *L. maculans* utilises mannitol within the apoplast found between the cells of oilseed rape, as a nutrient. Mannitol (a sugar alcohol) is naturally produced by the plant as a carbohydrate sink and to overcome cellular osmotic stress (Voegele 2006). It has also been suggested that *L maculans* is able to utilise nutrients released from apoptotic plant and bacterial cells (Howlett, 2006). Hyphal quantity does increase as the available nutrients increase (Howlett, 2006) which could be a reason why the lesion area increased.

Overall, however, it was noted that using combinations of *Bacillus* and *Pseudomonas* was not a viable biocontrol option with the isolates used in this work.

CHAPTER 6 APPLICATION OF QUANTITATIVE PCR TO QUANTIFY PLANT INFECTION AND COLONISATION.

6.1 INTRODUCTION

Quantitative PCR is a powerful tool which allows the quantification in real time of microbes and their dynamics, in many different crops. The optimisation of quantitiative PCR protocols has produced a diagnostic system with sensitivity, reliablility and accuracy (section 1.12.3). Specifity is imparted by primers, designed using many different conserved regions within the microbe genome.

Fusarium spp. can now be studied in diverse crops and environments such as melons (Haegi *et al*, 2013), maize (Atoui *et al*., 2012) and wheat (Horevai *et al*., 2011). The presence of *Erwinia amylovora* can be established (Bereswill *et al*., 1992, Llop *et al*., 2006). *Xanthomonas* spp. is routinely studied (Hartung *et al*., 1996, Verdier *et al*., 1998, Cubero *et al*., 2005, Berg *et al*., 2006, Hill *et al*., 2014). *Rhizoctonia* spp. can now be quantified in potato (Woodhall *et al*., 2007, Woodhall *et al*., 2013), sugar beet and maize (Abbas *et al*., 2014), canola and tobacco (Zhou *et al*., 2014). The presence of *Pseudomonas* spp., is established in crops such as tobacco (Xin *et al*., 2013,) apples (Pujol *et al*., 2006), soil (Xiang *et al*., 2010) and manure (Colinon *et al*., 2013). Many *Bacillus* species can now be routinely found using qPCR (Providenti *et al*., 2009, Bushon *et al*., 2010, Holmberg *et al*., 2012, Lahali *et al*., 2014, Hill *et al*., 2014, Kakar *et al*., 2014, Park *et al*., 2014, Xu *et al*., 2014). Quantitative PCR has previously been used to ascertain the presence of *L. maculans* at different stages of its life cycle (Kenyon *et al.,* 2004, Liu *et al.,* 2006, Stonard *et al*., 2008, Persson *et al*., 2009, Kaczmarek *et al*., 2012, Van de Wouw *et al*., 2010, Zhu *et al*., 2011).

In this work, DNA extracted from the cotyledons inoculated with *B. amyloliquefaciens* 66p and *P. chlororaphis* spp. *aureofaciens* 30.84 separately and in a 1:1 combination was placed into qPCR assay. The population dynamics of *Bacillus, Pseudomonas* and *Leptosphaeria* was examined with and without biological controls. It was determined if qPCR could be an alternative method of monitoring populations in real time.

6.1.1. *Leptosphaeria maculans* DNA isolated from *B. napus* cotyledons when biological control is applied individually

Figures 6.1. and 6.2. show that the *L. maculans* DNA, without biological control agents applied, increased over the time period. This indicates an increase in pathogen colonisation. The *L. maculans* DNA isolated from the cotyledons when *B. amyloliquefaciens* 66p was applied as a biological control agent decreased, indicating growth suppression. The amount of DNA extracted was similar at 0.08 ng in 1 g of tissue at days 7 and 11, whilst at day 15, DNA extracted decreased to 0.02 ng in 1 g of tissue (Figure 6.1.). However, there appeared to be more DNA present than the lesion would suggest, probably due to the endotrophic stages of *L. maculans* growth.

A similar pattern of pathogenic growth inhibition was also observed when *P. chlororaphis* spp. *aureofaciens* was used as the BCA. The lesion area decreased indicating suppression of fungal growth when compared with the control cotyledon tissue. The amount of *L. maculans* DNA extracted increased over time, 0.02 ng at 7 DAI, 0.05 ng at 11 DAI 0.09 ng at 15 DAI which was less than the DNA extracted from the control plant tissue (Figure 6.2.).

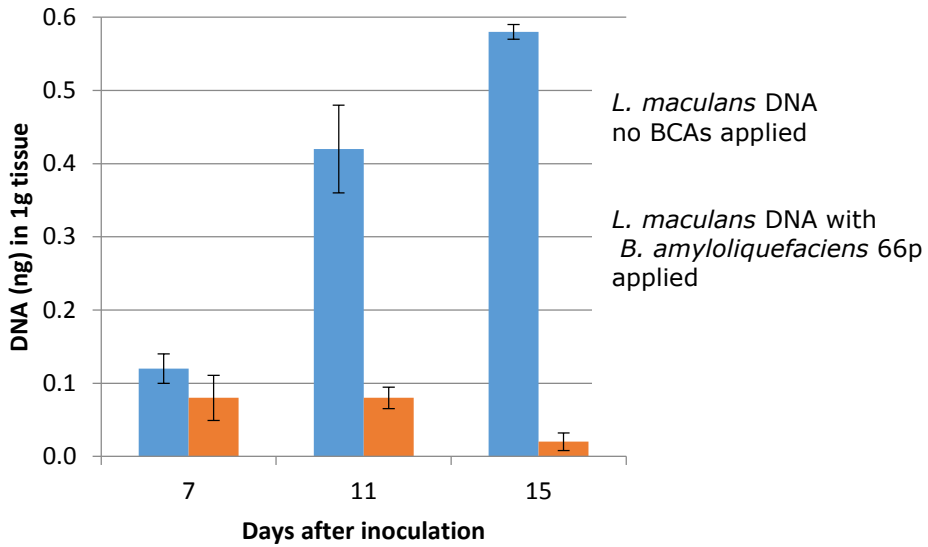


Figure 6.1. *Leptosphaeria maculans* DNA as quantified by qPCR isolated from *B. napus* cotyledon tissue pre-inoculated with *B. amyloliquefaciens* 66p. N = 20. Error bars – SD.

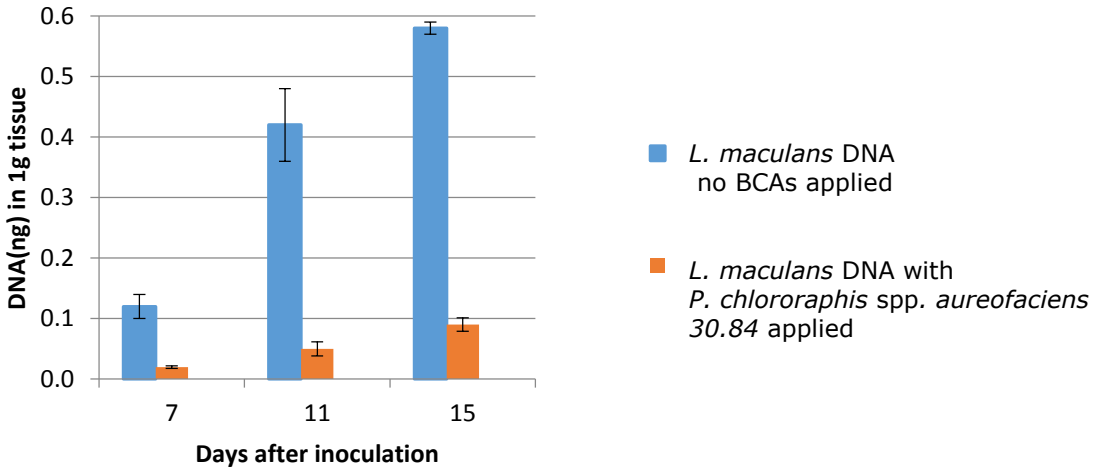


Figure 6.2. *Leptosphaeria maculans* DNA as quantified by qPCR from *B. napus* cotyledon tissue pre-inoculated with *P. chlororaphis* spp. *aureofaciens* 30.84. N = 20. Error bars – SD.

6.1.2. *Leptosphaeria maculans* DNA isolated from *Brassica napus* cotyledons after pre-inoculation with biological control agents applied in a 1:1 ratio.

Preliminary experiments were done using qPCR to examine *L. maculans* DNA when BCAs were either omitted or applied in a 1:1 combination.

Figure 6.3. shows the increase in *L. maculans* DNA in the cotyledons pre-inoculated with the BCAs in a 1:1 ratio. The *L. maculans* DNA increased over time in the control cotyledons and the inoculated cotyledons. The DNA obtained was much greater in the presence of the combined bacteria than in their absence. The lesion areas were visually larger (Chapter 4).

6.1.3 Bacterial DNA isolated from *B. napus* cotyledons after pre-inoculation with biological control applied individually and in 1:1 ratio

Experiments were done using qPCR to quantify bacterial colonisation of oilseed rape cotyledons when *Bacillus* and *Pseudomonas* were applied singly and in a 1:1 combination.

Figure 6.4. clearly indicates that more *Bacillus* DNA was present when the biological control was applied individually than when it was applied with *P. chlororaphis* spp. *aureofaciens* 30.84 in a 1:1 ratio regardless of DAI. There was less *Bacillus* DNA isolated at 11 DAI (0.03 ng) than 7 DAI (0.05 ng), but approximately 6 times more at 15 DAI.

Figure 6.5. shows that the when applied singularly, the *Pseudomonas* DNA remained low, but relatively constant (0.009-0.005 ng DNA in 1 g tissue) When applied in conjunction with the *Bacillus* in a 1:1 ratio, there is more *Pseudomonas* DNA present than when applied singularly. The amount of Pseudomonas DNA isolated at day 11 had increased 2 fold from the amount isolated at 7 DAI. At 15 DAI, the amount of DNA isolated from 1 ng cotyledon tissue was only 0.002 ng less than 7 DAI.

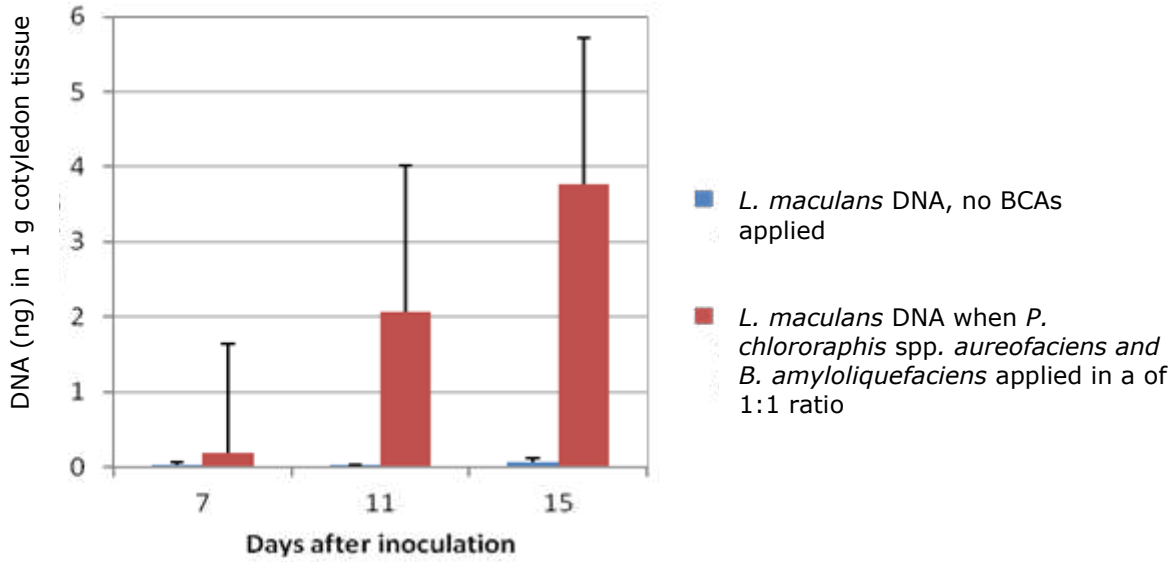


Figure 6.3. *Leptosphaeria maculans* DNA as quantified by qPCR isolated from *B. napus* cotyledon tissue inoculated with *B. amyloliquefaciens* 66p and *P. chlororaphis* spp*. aureofaciens* in a 1:1 ratio. N = 20. Error bars – SD.

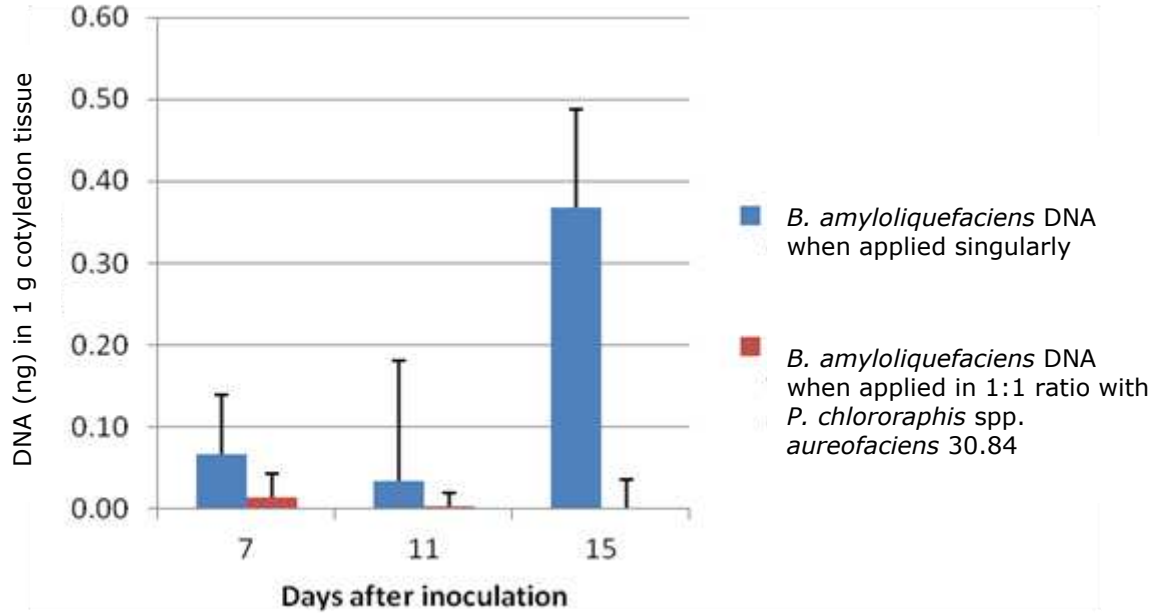


Figure 6.4. *Bacillus amyloliquefaciens* 66p DNA as quantified by qPCR isolated from *B. napus* cotyledon tissue pre-inoculated with *B. amyloliquefaciens* 66p/*P. chlororaphis* .spp*. aureofaciens* 30.84 in a 1:1 ratio. N = 20. Error bars – SD

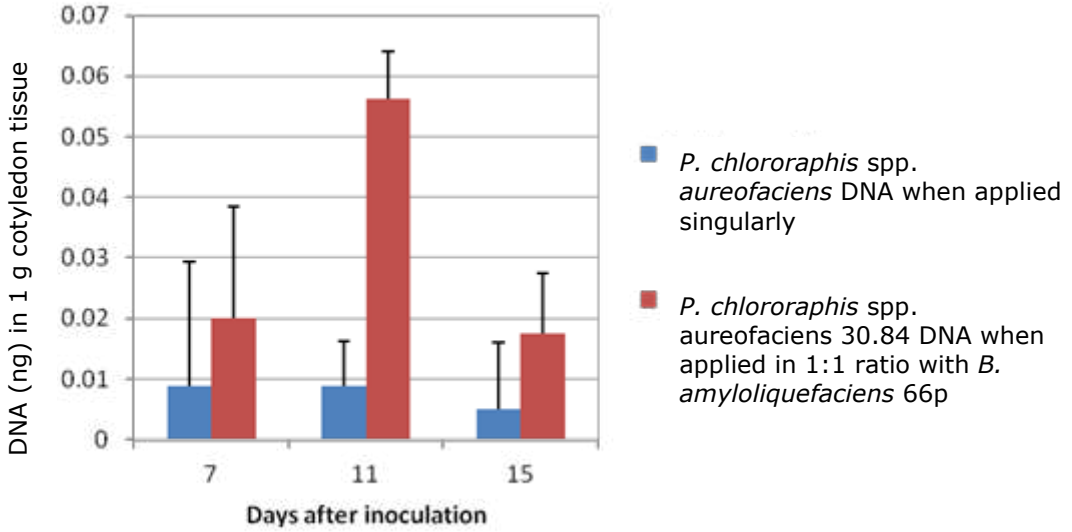


Figure 6.5. *Pseudomonas chlororaphis spp. aureofaciens* DNA as quantified by qPCR isolated from *B. napus* cotyledon tissue pre-inoculated with *P. chlororaphis spp. aureofaciens* 30.84/*B. amyloliquefaciens* isolate 66p in a 1:1 ratio. N = 20. Error bars – SD

6.2. DISCUSSION

This work has demonstrated that quantitiative PCR, using specific primers and SYBR® Green, can quantify both bacterial and fungal DNA when extracted from plant tissue. The method may be useful for large throughput screening for the disease.

The amount of *L. maculans* DNA extracted was less than the amount extracted from the control cotyledons when the BCAs were applied individually, with more being extracted when the BCAs were applied in combination. Whilst the size of the lesions observed gave an indication of such, it was confirmed using qPCR. Due to the irregular intracellular growth of *L. maculans* mycelia, the amount of DNA varied greatly, also noted by Kenyon, 2004. Using qPCR will detect *L. maculans* when in asymptomless growth intercellullarly (Huang *et al*., 2014) before the lesion appears. QPCR assays undertaken after the lesion is seen would quantify the amount of fungal hyphae which had spread to the vascular system, before it reaches its quiescent stage at the crown of the plant.

Comparable to other groups (Schena, 2004), the bacterial populations in this work were quantified successfully. However, dead cells are also quantified alongside live cells, so results may not be conclusive (Hierro, 2006). It must be borne in mind that DNA is stable and survives a long time and as such may detrimentally affect the results when trying to quantify live cells. This could be counteracted by the use of CFU count for bacteria (where only live cells grow), but this assay cannot be used for fungi.

Whilst conventional PCR assays can give an indication of the presence of the organisms, a more sensitive and accurate

approach is beneficial due to the lifecycle of *L. maculans* within the plant. However, the extreme sensitivity of the qPCR assay may cause problems in field use, as many exogenous parameters have adverse effects on the results. The same qPCR machine must be used over all assays to allow a comparison of the data, as primers specific on one are not specific on another, and each machine had different optimisation of temperature and cycles. An estimation of DNA quantity is only obtained as the DNA value is compared with the standard curve. As such, the efficacy of the standard curve is important, but the efficiency of the curve can easily change with SYBR® Green from different providers and even between batches. The quality due to contaminants from the plant DNA easily causes inconsistency as degraded DNA gave late readings, also encounted by Schriewer *et al*., (2011).

QPCR should be effective in detecting *L. maculans* in the field, but technical difficulties may ensue. An alternative may be to use Loop-mediated Isothermal Amplification of DNA (LAMP) which is a robust quantifiable DNA quantification. Kaczmarek *et al*., (2014) found this to be not as sensitive as qPCR when quantifying DNA taken from *L. maculans* spores, whilst Jedrycka *et al.,* (2013) found LAMP to be equal in sensitivity to qPCR, much easier, quicker and lower cost.

CHAPTER 7 DISCUSSION

This study has demonstrated that isolates of *Bacillus* and *Pseudomonas* when applied individually to oilseed rape cotyledons give good control of the leaf spot stage of *L. maculans* and as such can be described as biological control agents. However, when applied in combination, no control is observed or quantified. The main conclusions from this work are:

The *in planta* assays were completed using oilseed rape cotyledons which gave good infection. The cotyledon has been used extensively in other work (Huang *et al*. 2011) when the method was used to evaluate fungicide efficacy. Although *L. maculans* ascospores and conidia infect leaves in the field, the assay using conidia only on cotyledons was considered to be valid as ascospores cannot be grown *in vitro*, an essential requirement for these experimental purposes. Wounding was an initial concern as it was thought that generating a wound may bypass the initial reaction on the epidermis where bacteria inhibited germination. However, as in nature *L. maculans* uses wounds and stomata to enter the plant, this was not deemed a problem.

Prior work on fungicides controlling *L. maculans* on *B. napus* has been previously completed both on cotyledons and in the field (Kaczmarek & Jedryczka, 2010, Huang *et al*., 2011). The fungicides used in this work under these experimental conditions gave poor control, possibly reflecting extremely high disease pressures used in this experiment. This indicated that biological control was worth testing, as the amount of spores used could be possible in epidemics (West *et al.*, 2001). However, the fungicide results were a little supprising as triazole based fungicides has been used extensively to control *L. maculans* under field conditions and it was expected to give good control.

The lesions produced at the leaf spot stage on the cotyledons sprayed with experimental fungicides except for Q8Y78 were larger than the control and the cotyledons had epidermal damage. The epidermis is the first barrier of infection on the plant and the phytotoxic damage caused by the fungicdes may have allowed the pathogen increased access to the inside of the cotyledon causing a larger lesion. This may have been due to the oilseed rape cultivars, Winner and Compass used in this work being very susceptible to *L. maculans* (HGCA susceptible level of 4). However, the epidermal damage did result in amplified access for *L. maculans* and may be one of the causes of the rise in stem canker in the field today, as surrounding crops are

routinely sprayed with the fungicides not targeted at stem canker. Q8Y78, comprising penthiopyrad and piroxistrobin, did provide some control and indeed has now been launched by DuPont™ as Refinzar®, a fungicide controlling stem canker on oilseed rape.

Trichoderma harzianum gave no control *in vitro*, and it was thus not used for *in planta* assays. This observation is is counter to the findings of Starzycki *et al*. (1997) who reported suppression of *L. maculans* using *Trichoderma* spp. This may reflect the use of different antagonistic pathogen isolates used in the work reported. Biological control using *Bacillus* and *Pseudomonas* isolates applied separately, using *in vitro* and *in planta* assays gave good suppression of leaf spot stage of stem canker on oilseed rape, confirming work carried out by many other groups (Panjehkeh *et al*., 2011, Lahali *et al*., 2013, Hammoudi *et al*., 2012, Fernando & de Kievit, 2011, Danielsson *et al*., 2007, Ramarathnam & Fernando, 2006). Some biological control was seen in the *P. chlororaphis* spp *aureofaciens* PhzI- isolate, a mutant unable to produce the antibiotic phenazine. The observed biological control may therefore reflect a different mechanism to that associated with phenazine production, possibly involving competition for exogenous nutrients in the phyllosphere (Lindow & Brandl, 2003). A second quorum sensing system has been found in the *Pseudomonas* genome which partially restores phenazine production (Zhang & Pierson, 2001). However, no presence of phenazine was seen in the T streak assay as no purple pigment was produced at the intersection point. Samvat *et al*., (2014) found the mutant *Pseudomonas* gave control of damping off of cotton seedling and suggests that whilst phenazine is involved in antagonism, siderophores and metabolites produced by both isolates are also important.

The survival of the biological control bacteria was monitored from the cotyledons using leaf washings, dilutions and platings. This form of enumeration has routinely been used by many groups and is still in use today. In recent work, Sivakumar *et al*., (2014) used CFU to express the population of *B. megaterium* in the rhizosphere, whilst Fit *et al*., (2014) used the same procedure to investigate the microbial effect of honey treated with heat or UV radiation. Pavlova *et al*., (2014) enumerated the bacteria present on vegetables. The CFU count undertaken in this work found that the concentration of 10^6 cm^{-2} at time 0 had increased to 10^8 cm^{-2} by 24 h post treatment for both bacterial genera. The increase in growth of the bacteria was further confirmed by an increase in bacterial DNA quantified using qPCR.

These results indicate that the bacterial population was viable and increased when applied individually onto the cotyledons. Sivakumar *et al*., (2014) found the population of *B. megaterium* in the rhizosphere increased from the amount applied as a seed treatment, root dip, as a soil or leaf application.

Biological control using *Bacillus* and *Pseudomonas* isolates applied in combination *in planta* gave no suppression of leaf spot, and indeed the area of the leaf spot lesion increased from the control where no biological control was applied. This suggests that the biological control agents applied have somehow increased the severity of the disease. This may reflect the provision of exogenous nutrients from lysed bacteria which could provide a more conducive microenvironment for *L. maculans* growth. The lack of supression was suprising as it was determined that, using lactonase gene specific primers, the *Bacillus* isolates used would not have directly inhibited phenazine synthesis by *Pseudomonas chlororaphis* spp. *aureofaciens* 30.84, and both bacteria had been proven to give control when applied individually. Thus, in principle the *in planta* assay should have worked. The survival of the biological control bacteria was monitored using leaf washings, serial dilutions and plating out which found that the application concentration of 10^6 cm^{-2} had decreased dramatically to 10^2 cm^{-2}. This reduction in bacteria was again confirmed by a decrease in bacterial DNA as quantified by qPCR. After the initial decline, live cells should have recovered but this did not happen. A toxic environment could have been produced as the dead cells degraded. Under the stressful conditions of being applied in combination, it appeared the two species of bacteria used antagonised each other. In conclusion, using these isolates in these combinations they do not work as a biological control of the leaf spot stage of stem canker of *B. napus*. Naik & Shivaprakash (2013), found that different isolates of *Trichoderma*, *Bacillus* and *Pseudomonas* were both compatible and incompatable depending of the isolate used. It may be that the isolates used in this work were incompatible with each other and it would be interesting to look at efficacy of other bacterial isolates. Fukui *et al.,* (1999) suggest selecting an effective mix of biological control agents first, then screen out ineffective strains may be a more efficient method. Colony forming unit counts monitor only live cells whilst qPCR monitors populations in real time and care must be taken in interpreting data as DNA is still viable shortly after the death of the organism. As such, it is prudent that CFU counts are used in conjunction with the qPCR results.

Although it was possible to enumerate bacterial populations by recovery, planting and counting CFUs to validate qPCR assessment, such counting methods are not feasible with fungi. To confirm the validity of qPCR as a method to quantify fungal colonization of plant tissue, a second indirect method was used. This was based on an attempt to quantify the fungal sterol erogosterol. The same assay had been previously successfully carried out by Griffiths *et al*., (2003) who used the assay to establish the sterol composition of *L. maculans*. Despite using different extraction protocols, over many attempts, the procedure failed to detect ergosterol from *L. maculans* in plant extracts. *L. maculans* may not have enough ergosterol in its membranes for this assay, or compounds co-extracted in the procedure could have interfered with the detection of ergosterol. However, the presence of *L. maculans* was quantified using DNA placed into PCR and qPCR assays. The results indicate *L. maculans* was present in all areas of the leaf even when no visible symptoms were seen on the cotyledon.

An alternative indirect assessment of fungal cononisation could be the quantification of the fungal cell wall polymer chitin, which is not present in plant cell walls. Assays to use the chitin monomer N-acetyl-glucosamine to assess the level of fungus present have been used as a means to determine the extent of fungal colonisation by Wouw *et al*., (2009) on *L. maculans*, and by Lopez-Fernandez *et al*., (2013), who used the presence of chitin as an aid in determining the cross talk between *Fusarium oxysporum* and its host. Ouda (2014) used the presence of N-acetyl glucosamine in *Alternaria alternate* and *Botrytis cinerea* to determine the antifungal activity of nanoparticles.

Quantitative PCR confirmed the efficacy of the biological control as *L. maculans* DNA concentration was reduced when the biological control agents were applied individually, and increased when applied in combination. As such, it was ascertained qPCR data could provide information on the effect of the biological control applied on the pathogen levels. Thus, the DNA based method could be a valuable tool to detect asymptomless infection of *L. maculans* and be useful in optimising control programmes based on fungicides albeit allowing timing of application to coincide with infection and not visible symptoms. This is important as early detection could lead to enhanced fungicide efficacy. Quantitative PCR using SYBR® green has previously been used to establish the presence of *L. maculans* by Kaczmarek *et al*., (2010) and Huang *et al*., (2011). No correlation was seen between

the lesion size and the DNA extracted, probably due to the asymptomless stages of the disease. The usefulness of the qPCR assay should, however, be tested in the field as it is unknown what the presence of other organisms and conditions found there would have on qPCR results. This may be improved if field-based detection is carried out using LAMP based isothermal assay.

Biological control using combinations of organisms could be further investigated using different isolates of *Bacillus*, *Pseudomonas*, and *Trichoderma*. It is possible that it is just these particular isolates that do not work in combination. The efficacy of biological control agents in combination with low doses of fungicides could be determined. This would be feasible using bacterial antagonists but could be problematic if fungi were used as they would inhibit the biological control agent. Juliatti *et al*., (2014) found that antibiotics (Beznalkonium chloride and kasugamycin) isolated from *Penicillium minor* gave control of *Phaeospaheria* spp. on corn when applied in combination with a mix of azoxystrobin and cyproconazole. Work described by Aydn & Turhan (2013) showed that *Rhizoctonia solani*, a fungal disease of potato, was controlled under greenhouse and laboratory conditions by a combination of different *Trichoderma* isolates and low doses of fungicides already extensively used to control *R. solani.* Enhanced control of *Rhizoctonia cerealis* on wheat was obtainedby Peng *et al*., (2014) when applied in a combination of *B. subtilis* NJ-18 and either flutolanil and difenoconazole.

In the field, stem canker development can reflect combined infection by *L. maculans and L. biglobosa*. It would be interesting to repeat the work reported here using *L. biglobosa* to ascertain whether any of the inhibitors used would also inhibit this pathogen. Previous research has ascertained that *L. biglobosa* is affected differently by fungicides than *L. maculans* (Eckert *et al*., 2010) and this may be the case when biological control is used. The interaction of the *L. biglobosa*/*L.maculans* complex must be also investigated, as in the field these two species are found on the same plant (Mendes-Pereira *et al*., 2003). There also may be a number of pathogen isolates present in the field which the primers used cannot differentiate between. To aid this, the specifity of the qPCR assay could be increased to species level using e.g. minisatallite markers for the *L. maculans* primer sequences (Jedryczka *et al*., 2010) or by cloning the internal transcribed spacer (ITS) region from each *L. maculans* species into *E. coli*, followed by

purifying then sequencing (Locatelli *et al*., 2002, Takenaka *et al*., 2008). *L. maculans* hyphae may be ascertained when growing inside the oilseed rape by using primers based on *sp2* gene which encodes for a serine-like protease (SP2). This enzyme is produced by the hyphae in the apoplast between the mesophyll cells to hydrolyse proteins as a nutrient source and and such would be present on active growth (Wilson & Howlett, 2005).

In summary, the aims of this project to establish a biological control of *L. maculans* and to investigate microbial interaction *in planta* have been achieved. The evidence shows some scope for biological control using individual isolates of *Bacillus* and *Pseudomonas* when applied individually has been seen in this work as a reduction of *L. maculans* DNA in cotyledon tissue of oilseed rape plants indicated they were successful in reducing colonisation.

Further investigation is necessary for the selection of isolates which are compatible for use in combination *in planta*. Further research must also be completed to ascertain if the control at the leaf spot stage is carried out resulting in a lesser infection at the stem canker stage. Moreover, the experiment could be completed under field conditions to determine whether biological control agents supress *L. maculans* under the more challenging environmental conditions found in the field. The DNA fingerprint of the lesion may be compared with the DNA fingerprint of the stem canker using qPCR utilising minisatellites (Jedrycza *et al*., 1999, Eckert *et al*., 2005). The use of microbes expressing green fluorescent proteins (GFP) and visualised using confocal laser scanning microscopy (Takenaka *et al*., 2008, Filho *et al*., 2013) could determine if the biological control agents and *L. maculans* co-exist at the infection point on the cotyledon, by active competition.

This work has determined that qPCR could be useful to monitor microbial growth. The use of qPCR to assess the amount of *L. maculans* DNA present even in the asymptomless stages of the disease could be used as a valuable and more reliable predictive tool (compared with lesion size) for growers to assess the correct time to apply control measures.

Although the use of combination treatments fail in this research this approach should not be discarded under crop protection systems. These results raise many new research opportunites, and a step forward has been achieved to contribute to the development of a sustainable biological control of the leaf spot stage of stem canker on oilseed rape caused by *L. maculans*.

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APPENDIX A MEDIA COMPOSITION

All growth media were prepared using distilled water (pH 6.0) in a sterile HEPA-filtered cabinet, then sterilised in an autoclave at 121ºC and 0.1 MPa for 15 minutes. Media was cooled to 55°C then poured into 9 cm Petri dishes in a laminar flow cabinet. They were left to set for 1 hour then stacked and stored at 4ºC until ready to use.

A.1. BACTERIAL GROWTH MEDIA

Luria-Bertoni (LB) liquid medium purchased from Oxoid and prepared according to manufacturer's instructions.

Luria-Bertoni (LB) agar purchased from Oxoid and prepared according to manufacturer's instruction.

Luria-Bertoni (LB) buffered to pH 7.0 5 g yeast extract, 10 g tryptone, 10 g sodium chloride 7.5 g agar in 1L distilled water. Buffered using 1M potassium phosphate solution

Tryptone Soya Broth (TSB) medium purchased from Oxoid and prepared according to manufacturer's instructions.

Nutrient agar (NA) medium purchased from Oxoid and prepared according to manufacturer's instructions.

A.2. FUNGAL GROWTH MEDIA

Potato Dextrose Agar (PDA) and PDA+. 39 g PDA was placed in 1L sterile distilled water and autoclaved, then supplemented with antibiotics penicillin and streptomycin making PDA+ media.

Potato Dextrose Broth (PDB) medium purchased from Oxoid and prepared according to manufacturer's instructions.

Distilled water agar (DWA), 20 g Technical grade agar no. 3, 800 g distilled water.

A2 Antibiotics

All antibiotics were filter sterilised through a 0.45 µm Milliex-HA filter unit. Unless stated otherwise, the antibiotics used in fungal growth media were:

Streptomycin (133 mg L^{-1}) dissolved in sterile distilled water.

Penicillin (30 mg L^{-1}) dissolved in sterile distilled water.

Buffers

TE Buffer 10 mL Tris EDTA Buffer + 990 mL DNA free water

APPENDIX B: CONSUMABLES

	Supplier	Cat. No.
1. Antibiotics		
Penicillin G sulphate	Melford Labs	P0142
Streptomycin G sulphate	Melford Labs	S0148
2. Chemicals and stains		
1 N (β ketocaproyl) L- homoserine lactone	Sigma-Aldrich	K3007
Acetonitrile	Sigma-Aldrich	190659
Ethanol, absolute	Haymans Ltd.	UKAA0169
Ethyl acetate	Sigma-Aldrich	270989
Glycerol	Riedel de Haën	33229
Hexane	Sigma-Aldrich	296090
Methanol, HPLC grade	Fischer	4056/PB17
Polyvinylpyrrolidone (PVP)	Sigma-Aldrich	234257
Potassium hydroxide (KOH)	Sigma-Aldrich	278651
SYBR® Green	Sigma-Aldrich	S4438
iQ™ SYBR® Green Supermix	Bioline	170-8880
Tris HCl	Sigma-Aldrich	T-3253
3. Commercial kits		
GenElute PCR clean up kit	Sigma-Aldrich	NA1020
Dneasy Plant mini kit	Quiagen	69106
QuickPick® kit	Bionobile	53022
4. Gel electrophoresis		
Agarose, molecular biology grade	Lonza	50004
Ethidium bromide	Promega	H5041
Hyperladder II	Bioline	BIO-33039
Hyperladder V	Bioline	BIO-33031
MangoMix	Bioline	BIO-25033
TBE, 10X	National Diagnostics	EC860
5. Media and media ingredients		
Agar, technical grade no. 3	Oxoid	LP0013
LB agar	Q-BioGene	3002-221
Maxiumum recovery dilutent (MRD)	Oxoid	CM0733
Murashige and Skoog Basal Medium	Sigma	M5519
Nutrient agar	Oxoid	CM0003
Potato dextrose agar (PDA)	Oxoid	CM0139
Pseudomonas growth media	Oxoid	CM0559
P. CN selective supplement	Oxoid	SR102
P. CFC selective supplement	Oxoid	SR103
Tryptone soya broth	Oxoid	CM0129
Tween x 80	Sigma-Aldridge	P8074
M3 compost	Levingtons	

6. **Miscellaneous**

Coverslip, 22x22mm	FisherScientific	MNJ350
Epindorf tube, 0.5 mL and 1.5 mL	Eppendorf 5417, Germany	
Falcon tube	SLS	352070
Filter tips 20µl	Starlab	S11201810
Filter tips 1000 µl	Starlab	S11267810
QuickPick® tips	Bionobile	34096
QuickPick® pen	Bionobile	23001
Filter Unit, Millex-HA, 0.45 µm	Anachem	ALG424A
Filter Unit, Millex-HA, 0.20 µm	Anachem	AG422A
Glass beads	SLS	59200-U
Haemocytometer	Hirschmann	8100103
Inoculation loops 1 µm	SLS	G731161
Microscope slide 76x26x0.8mm	SLS	MIC3810
Muslin, white	A. Holt & Sons Ltd.	n/a
PCR plate ultra clear cap strips	Thermofisher Scientific	AB0866
PCR plate, thermo-fast, 96 well	Thermofisher Scientific	AB0600
PCR tube, attached flat cap, 0.2 mL	Starlab	I14028100
Petri dish, 9cm	SLS	PET7002
Scalpel blade no.10	SLS	INS4610
Scalpel handle, No.2 steel/nickel	SLS	INS4603
Skirted tubes, 2ml graduated	Starlab	E14801899
Screw top 2 mL	Greiner Bio-One Ltd.	188271
Microscope slide 76x26x0.8mm	SLS	MIC3810
96 well skirted rtPCR plates	Clent Life Science	4ti-0961

8. Software

Biorad iQ5™ V2.0 for Real time PCR	www.bio-rad.com
Roche LightCycler® 480 sw 1.5	www.roche.com
Blast	www.ncbi.nih.gov/BLAST/
Chromas V2.33 (© 1998 Conor McCarthy, Australia)	
	www.technelysium.com/au
Empower Chromatography data software	www.waters.com/hplc
Genstat 12.1 edn (2009)	www.genstat.co.uk
Primer 3	www.Geneious.com