UNDERSTANDING THE INTERACTIONS BETWEEN PHOMA STEM CANKER (*LEPTOSPHAERIA MACULANS* AND *L. BIGLOBOSA*) AND LIGHT LEAF SPOT (*PYRENOPEZIZA BRASSICAE*) PATHOGENS OF OILSEED RAPE (*BRASSICA NAPUS*)

James Fortune, BSc. (Hons), MRSB

Submitted to the University of Hertfordshire in partial fulfilment of the requirements of the degree of Doctor of Philosophy

School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Hertfordshire, AL10 9AB

May 2022

Abstract

The aim of this project was to understand the interspecific interactions between *Leptosphaeria maculans*, *L. biglobosa* and *Pyrenopeziza brassicae* in oilseed rape through identification of interactions at different levels of investigation. The aim was achieved by (1) investigating the interactions between phoma stem canker (*L. maculans* and *L. biglobosa*) and light leaf spot (*P. brassicae*) causal pathogens *in vitro* and *in planta;* (2) examining the interactions between *L. maculans* and *P. brassicae* on different cultivars under field conditions; (3) identifying relationships between weather, prevalence of light leaf spot or phoma leaf spot and stem canker and yield loss. Results of this project show that both direct and indirect interspecific interactions exist between *L. maculans*, *L.* biglobosa and *P. brassicae.*

The results of *in vitro* experiments showed that *L. maculans* produces a phytotoxin called sirodesmin PL when cultured on its own, whereas when *L. biglobosa* or *P. brassicae* were cultured on their own sirodesmin PL was not produced. When secondary metabolite extracts from liquid culture filtrates from these three pathogens cultured on their own were applied to fungal plugs of *L. maculans, L. biglobosa* or *P. brassicae,* the only extract that reduced colony area was the secondary metabolite extract from *L. maculans* applied to *L. biglobosa* and *P. brassicae*. However, when the culture filtrate from *L. maculans* and *L. biglobosa* simultaneously cultured together was applied, there was no reduction in colony area of *L. biglobosa* or *P. brassicae*, nor was sirodesmin PL identified in the secondary metabolite extracts. Interestingly, when *L. maculans* and *L. biglobosa* were sequentially cultured 7 days apart, the secondary metabolite extract decreased the colony area of both *L. biglobosa* and *P. brassicae,* and contained sirodesmin PL. This implies that Sirodesmin PL had an inhibitory effect on *L. biglobosa* and *P. brassicae*. Therefore, these experiments showed that direct interspecific interactions exist between these three pathogens, and that direct interactions when *Leptosphaeria* spp. are simultaneously co-inoculated indirectly influence *P. brassicae* due to inhibition of Sirodesmin PL production that would not inhibit *P. brassicae* growth.

A similar pattern was observed *in planta* when oilseed rape cotyledons were inoculated with *L. maculans*, *L. biglobosa* or a mixture of both *Leptosphaeria* spp. Large, undefined lesions developed when *L. maculans* conidia were applied and smaller, more well-defined dark lesions developed when *L. biglobosa* conidia were applied. However, when a mixture of both *Leptosphaeria* spp. were applied, the phenotype of the lesion was more similar to that of *L. biglobosa* rather than *L. maculans*, suggesting that *L. biglobosa* outcompeted *L. maculans* in a simultaneous infection situation. When secondary metabolite extractions were analysed, sirodesmin PL was found only in the *L. maculans* only treatment, not in the *L. biglobosa* or the *Leptosphaeria* spp. mixture treatments. These findings show that the interspecific interactions found *in vitro* were also found *in planta*. Additionally, *in planta* work using near-isogenic oilseed rape lines with or without the *Rlm7* gene provided preliminary data to suggest that the presence of *Rlm7* may increase the susceptibility of plants to *L. biglobosa*.

The field experiments showed that cultivar resistance and fungicides were effective at reducing phoma stem cankers and *P. brassicae* sporulation as well as reducing the transmission of *Leptosphaeria* spp. inoculum between seasons. The monitoring of ascospore release events showed that there were differences in release timing and relative quantities of inoculum between seasons, including the simultaneous release of *Leptosphaeria* spp. ascospores with *P. brassicae* ascospores. Additionally, due to the long asymptomatic latent period between *P. brassicae* infection and presence of indicative light leaf spot disease symptoms, such as *P. brassicae* sporulation, there is no defined threshold for fungicide application to control *P. brassicae*. So, autumn disease control for phoma stem canker and light leaf spot causal pathogens is often defined by when the *L. maculans* fungicide threshold is met, irrespective of *L. biglobosa* or *P. brassicae* ascospore release events.

In England and Wales, analysis of relationships between weather and light leaf spot disease prevalence or yield loss found the prevalence of *P. brassicae* pod lesions had positive relationships with autumn or winter temperature and a negative relationship with the number of autumn or winter air frosts. This suggests that warmer temperatures and fewer air frosts would result in a greater prevalence of *P. brassicae* pod lesions.

There was also a positive correlation between average precipitation or mean number of rain-days in winter and spring and incidence of light leaf spot pod lesions. Met Office weather data has shown that the 5-year mean temperature average since 1969 for autumn and winter has increased whereas spring precipitation has not changed. Therefore, if this trend continues with autumn and winter getting warmer, this will increase the probability of *P. brassicae* infection.

Therefore, findings from all experiments in this study suggest that;

- Changes in *Leptosphaeria* spp. ascospore release patterns under natural conditions influence direct interspecific interactions between *L. maculans* and *L. biglobosa*, which indirectly affect *P. brassicae.*

- The widespread adoption of effective integrated *L. maculans* control strategies under *P. brassicae* favourable weather conditions may unintentionally make the UK oilseed rape crops more vulnerable to *P. brassicae* infection.

- Development of integrated pest management strategies is required to improve the control of *L. maculans*, *L. biglobosa* and *P. brassicae* together rather than in isolation.

Acknowledgements

List of Publications, Presentations, Events and Awards

1.1 Publications

Fortune, J.A., Qi, A., Ritchie, F., Karandeni Dewage, C.S., Fitt, B.D.L. and Huang, Y.J. (2021) Effects of cultivar resistance and fungicide application on stem canker of oilseed rape (*Brassica napus*) and potential interseasonal transmission of *Leptosphaeria* spp. inoculum. Plant Pathology, 70, 2115-2124

Fortune, J.A., Baker, D., Stanley, J., Karandeni Dewage, C.S., Ritchie, F., Fitt, B.D.L. and Huang, Y.J. (2021) Chemical warfare: the fungal quest to conquer oilseed rape. Management of Disease and Pests of Oilseed Rape. Jellis & Fitt (eds). Agrifood Charities partnership & University of Hertfordshire. ISBN 9798778481145. pp 25-26.

Fortune, J.A. (2021) Why is phoma getting more difficult to control?. The Arable Farmer. September pg 44-45 - https://issuu.com/farmersguardian/docs/arable_farming_september_2021

1.2 Presentations

<u>Internal</u>

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (light leaf spot) and *Leptosphaeria maculans* (phoma stem canker) during leaf infection on oilseed rape. - University of Hertfordshire Life and Medical School Research conference – April 2018 – Poster – (Appendices 1A)

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (light leaf spot) and *Leptosphaeria maculans* (phoma stem canker) during leaf infection on oilseed rape. - University of Hertfordshire Postgraduate Research Conference – October 2018 – Presented

Interspecific interactions between fungal pathogens causing light leaf spot (*Pyrenopeziza brassicae*) and phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) *in vitro* - University of Hertfordshire Life and Medical School Research conference – April 2019 – Presented

Chemical Warfare – The Fungal Quest To Conquer Oilseed Rape - University of Hertfordshire Life and Medical School Research conference – June 2021 – Presented

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (light leaf spot) and *Leptosphaeria maculans* (phoma stem canker) during leaf infection on oilseed rape - Agritech Week 2018: Solving the challenges of crop protection – November 2018 – Presented

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (light leaf spot) and *Leptosphaeria maculans* (phoma stem canker) during leaf infection on oilseed rape - Cereals Event – June 2018 – Presented

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (light leaf spot) and *Leptosphaeria maculans* (phoma stem canker) during leaf infection on oilseed rape. British Society for Plant Pathology Presidential Conference – 2018 – Presented

Interspecific interactions between fungal pathogens causing light leaf spot (*Pyrenopeziza brassicae*) and phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) *in vitro* - Agrifood Charities Partnership Conference – April 2019 – Presented – (Appendices 1C and 2E)

Interspecific interactions between fungal pathogens causing phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) *in vitro* - British Society for Plant Pathology Presidential Conference – Sept 2019 – Presented – (Appendices 1E)

Chemical Warfare – The Fungal Quest To Conquer Oilseed Rape - BCPC 2019: Disease Review at NIAB, Cambridge – Oct 2019 - Presented

Chemical Warfare – The Fungal Quest To Conquer Oilseed Rape - Agri-Food charities partnership oilseed rape conference – June 2021 – Presented

Chemical Warfare – The Fungal Quest To Conquer Oilseed Rape - BCPC 2019: Disease Review at NIAB, Cambridge – Oct 2021 – Presented

Chemical Warfare – The Fungal Quest To Conquer Oilseed Rape - Agritech East – REAP conference – Nov 2021 - Presented

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (light leaf spot) and *Leptosphaeria maculans* (phoma stem canker) during leaf infection on oilseed rape. - International Congress of Plant Pathology – Boston, USA – August 2018 - Presented – (Appendices 1B and 2B)

Interspecific interactions between fungal pathogens causing phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) *in vitro* - International Rapeseed Congress – Berlin, Germany – June 2019 – Presented –(Appendices 1D and 2F)

Interspecific interactions between fungal pathogens causing phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) *in vitro* - European Plant Science Retreat – Nottingham, UK – July 2019 – Presented – (Appendices 2G).

1.3 Events

Internal
University of Hertfordshire Postgraduate Research Conference – October 2017
ADAS conference – December 2017
Hertfordshire Science Partnership launch - March 2018 – (Appendices 2A)

Regional
NFU Farm Walk: Clavering Park Farm – June 2018
AHDB Monitor Farm: Duxford – June 2018
ADAS Open Day: High Mowthorpe – June 2018
Agritech Week 2018: Above, Below and Around – November 2018 – (Appendices 2C)
Agritech Week 2018: REAP Conference – November 2018
Open Farm Sunday: EW Davies Farm – June 2018
Tour of Wimpole Estate – June 2018

National
Elsoms Vegetable Open days – October 2017
CropTec – 29th November 2017
AHDB Agronomist conference – December 2017
Agri-Food charities partnership conference – April 2018
Doctoral Training Alliance Autumn School – November 2018 – (Appendices 2D)
Cereals Event – June 2019
Doctoral Training Alliance Summer School –July 2019
Royal Society of Biology Parliamentary Networking Event – Oct 2019 – (Appendices 2H)
Agrifood charities partnership – Management of Diseases and Pests of Oilseed Rape – June 2021 – (Appendices 2I)
Cereals Event – June 2021 (Appendices 2J)
Doctoral Training Alliance Summer School – July 2021
7th Virtual BCPC Disease Review – October 2021 – (Appendices 2K)
Agritech East – REAP Conference 2021 – Changing Time(s) for Agriculture – November 2021 – (Appendices 2L)

International

European Extension Meeting – Cambridge, UK – March 2019

1.4 Awards

University of Hertfordshire Life and Medical School Deans Awards 2019 – Student Engagement with Research – (Appendices 3A)

Table of Contents

List of Figures

List of Tables

List of Equations

List of Acronyms

ADM	Arthur Daniels Mathews
AHDB	Agricultural and Horticultural Development Board
ANOVA	Analysis of variance
Avr	Avirulence gene
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie growth scale
BBSRC	Biotechnological and Biological Sciences Research Council
CAA	Canola Association of Australia
CE	Controlled Environment
CSFB	Cabbage Stem Flea beetle
CTAB	Cetyltrimethylammonium bromide
Cv.	Cultivar
Cvs	Cultivars
d.f.	Degrees of freedom
DEFRA	Department for Environment, Food and Rural Affaris
DMI	Demethylation inhibitors
dpi	Days post inoculation
DSB	Disulphide bridge
dsDNA	double stranded DNA
EtOAc	Ethyl acetate
ETP	Epipolythiodioxopiperazine
EU	European Union
Expt	Experiment
FAO	Food and Agricultural Organisation
Fig.	Figure
FRAG	Fungicide resistance action group
FSAI	Food Safety Authority of Ireland
GA	Growth away
Genstat	General Statistics
GH	Glasshouse
GRAS	Generally recognised as safe
GT	Growth towards
HPLC	High performance liquid chromatography
IMS	Industrial methylated spirit
IPM	Integrated pest management
L. biglobosa	*Leptosphaeria biglobosa*
L. maculans	*Leptosphaeria maculans*
Lb	*Leptosphaeria biglobosa*
LC-MS	Liquid chromatography - Mass Spectrometry
Leptosphaeria spp.	*Leptosphaeria* species (*Leptosphaeria maculans* and *Leptosphaeria biglobosa*)
LLS	Light leaf spot
Lm	*Leptosphaeria maculans*

Lm&Lb	*Leptosphaeria maculans* and *Leptosphaeria biglobosa* simultaneously inoculated
Lm+Lb	*Leptosphaeria maculans* and *Leptosphaeria biglobosa* sequentially inoculated
LOD	Limit of detection
$\log_e$	Natural Log
LOQ	Limit of quantification
LSD	Least significant difference
m/s	Mass / charge ratio
MBC	Methyl benzimidazole carbamate
MEA	Malt Extract Agar
OREGIN	Defra Oilseed Rape Genetic Improvement Network
OSR	Oilseed rape
P. brassicae	*Pyrenopeziza brassicae*
Pb	*Pyrenopeziza brassicae*
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
pg	Picogram
PLS	Phoma leaf spot
QoI	Quinone outside inhibitor
qPCR	Quantitative PCR
R-genes	Resistance genes
RL	Recommended list
RPM	Rotations per minute
rt	Retention time
S. sclerotiorum	*Sclerotinia sclerotiorum*
SDHI	Succinate dehydrogenase inhibitors
SDW	Sterilised distilled water
STEYX	Standard error of the y- intercept of a regression line
T1	Fungicide timing 1
T2	Fungicide timing 2
TIC	Total ion chromatographs
TL	Target leaf
USDA	United States Department of Agriculture
V. longisporum	*Verticilium longisporum*
V8	V8 agar
V8*	Clarified V8 Agar
V8*B	Clarified V8 Broth
WA	Water Agar
WP	Whole plant
X bp	Base pair

Chapter 1 General introduction

1.1   Why oilseed rape?

Oilseed rape (*Brassica napus*) is the second most widely cultivated oil seed crop globally, with 68.02 million tons produced per year (USDA 2020; Zheng *et al.* 2020) (Figure 1.1), of which the European Union produces 34 % (USDA 2020). It is the third most consumed vegetable oil globally (USDA 2020). The primary product of oilseed rape is oil; either edible cooking oil or biodiesel or engineering fluids. The edible cooking oils derived from oilseed rape are healthy alternatives compared to animal fats, because rapeseed oil contains more unsaturated fatty acids, such as high oleic acid and α-linolenic acid (Dubois *et al.* 2007; Ferguson *et al.* 2016) and these oils can be ethically sourced, unlike palm oil (Carlson *et al.* 2013), which is the most widely consumed vegetable oil globally (USDA 2020). Oilseed rape has previously been estimated to account for 84% of biodiesel production in Europe (Yunus-Khan *et al.* 2014) but it is now widely accepted that it is not feasible to expect bio-renewable sources of energy to completely replace the fossil fuel sources currently used, due to the large volumes required. However, it may be more conceivable to see bio-renewable sources replace more specific and less volumetric petrochemical products such as feedstocks for plastics and nylon, and for replacements of specific industrial chemicals and materials, such as those used for lubrication (Carlson *et al.* 2011; Ruiz-Lopez *et al.* 2017).

Global food productivity must increase by 70% to feed an additional 2.3 billion people by 2050; without an increase in farm productivity, an additional 1.6 billion hectares of arable land is needed (FAO 2009). To further exacerbate this, a 3-4% decrease in global crop production due to increased urban sprawl caused by the growing urban population is predicted (D'Amour *et al.* 2017). The global agricultural community not only needs to produce more food but also must accommodate changing social issues, such as the changing diets in both developing and developed countries (Vranken *et al.* 2014; Henchion *et al.* 2017). Furthermore, within the developed world particularly, there is an ever-increasing demand for improving environmental social responsibilities and welfare.
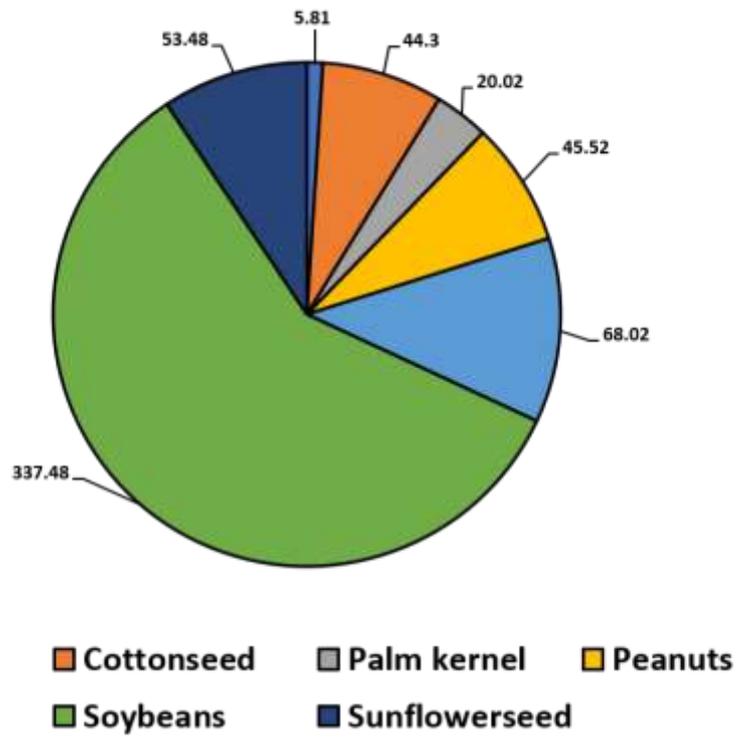
Figure 1.1: Worldwide oilseed production in 2019/2020 by type (in million metric tonnes). Data adapted from USDA 2020.

This has created a strong pressure to reduce the consumption of more environmentally damaging products such as meat and animal-derived products, palm oil and products derived from non-renewable feedstocks such as plastics and crude oil in favour of plant based, sustainable and/or renewable alternatives (Carlson *et al.* 2013; Henchion *et al.* 2014,2017; Vranken *et al.* 2014)

There is currently a nutrition transition occurring in developing countries, including Brazil, Russia, India, China and those within the Middle East (Vranken *et al.* 2014; Henchion *et al.* 2017). Initial improvements to average income per capita have resulted in change in diet from heavy reliance on carbohydrate-rich staple crops such as maize, rice and potatoes towards a diet containing animal products (Delgado 2003; Vranken *et al.* 2014). In the developing world, this nutrition-transition has resulted in expansion of the livestock and animal feed industries (Delgado 2003), which in turn has the potential to increase the importance of growing oilseed rape. After the development of the low erucic acid and low glucosinolate oilseed rape, there was a large demand for oilseed rape meal, a high protein meal containing crude protein, produced as a by-product of oil extraction from oilseed rape (Chen *et al.* 2011). It is used as a food source for many different species used in agriculture and aquaculture, including poultry, pigs, cattle and fish because oilseed rape meal contains higher percentages of essential amino acids than in soybean, sunflower, peanut and sesame meals, whilst maintaining a well-balanced amino acid composition of high nutritional value (Chen *et al.* 2011; Hald *et al.* 2019). There are many arguments, such as food-animal feed or food-biofuel arguments, surrounding the use of food crops for other uses rather than for human consumption (Makkar and Ankers 2014; Yunus-Khan *et al.* 2014). These concerns would be minimised if part of the livestock diet was replaced with oilseed rape meal, as this product is not a traditional food crop, but this may change in the future.

Due to the increases in social responsibility and governmental agreements, particularly within developed countries, to reduce greenhouse gas emissions, there is a greater demand for protein from sustainable plant-based sources in place of traditional animal-derived sources that provide 43% of the global protein supply for human consumption; meat (18%), dairy (10%), fish and shellfish (6%) and other animal products (9%) (van der

Spiegel *et al.* 2013; Henchion *et al.* 2014, 2017). Cattle farming and other livestock farming is a major source of these environmentally damaging emissions (van der Spiegel *et al.* 2013) and therefore this has been targeted as an area to reduce greenhouse emissions. This has encouraged the global agricultural industry to invest in existing source,s such as soya, wheat, vegetables and potatoes, in addition to investigating novel sources of plant-based protein for human consumption (van der Spiegel *et al.* 2013; Henchion *et al.* 2017). Novel sources of plant-based protein include algae, duckweed and oilseed rape (van der Spiegel *et al.* 2013).

By-products from established processes, such as those from the oilseed rape extraction process, are ideal candidates for alternative novel sources of protein because the infrastructure, regulations and space for production of the raw materials have already been established (van der Spiegel *et al.* 2013). These factors have enabled the time taken for oilseed rape to enter the market to be shorter than that of proteins derived from insect *in vitro* meats. This may allow oilseed rape proteins to replace traditional ingredients within the food processing industry (Aluko and Mcintosh 2005; Aider and Barbana 2011). In the USA, oilseed rape protein isolates Supertein® and Puratein® have obtained GRAS pre-market approval for human consumption under GRN000327 (FDA 2016) (Figure 1.2). The intended applications for these products include meat alternatives, dairy alternatives, dressings and sauces, ready to drink beverages and within confectionary, bars and baked goods (Burcon 2019a.2019b). However, in the EU, an application to produce protein preparation was declined. Although the initial assessment done by Food Safety Authority of Ireland (FSAI) concluded that it could be safely consumed, the Dutch committee on safety assessment of novel foods (VNV) over-ruled this decision. They stated that there was a lack of limit values for antinutritional factors, heavy metals, allergens and other undesirable compounds (VNV 2012). These obstacles may be alleviated in future because the use of modern technologies has enabled many of the anti-nutritional factors, such as erucic acids, glucosinolates, phytates and tannates, to be removed (Aider and Barbana 2011; Flores-Jimenez *et al.* 2019; Hald *et al.* 2019). Additionally, the removal of kaempferol 3-O-(2‴-O-sinapoyl-β-sophoroside), the compound responsible for the bitter taste will improve the palatability of oilseed rape protein products (El-Beltagi and Mohamed 2010; Hald *et al.* 2019).

Puratein®

Puratein® has high-quality functional attributes ideal for use in baked goods, protein cereal bars, dressings, sauces and meat substitutes.

Puratein® holds enormous potential in a wide variety of food systems.

Puratein® is a canola protein isolate comprised mainly of globulin proteins. The functional properties of Puratein® include emulsification, gel formation, thickening, formation of heat-stable foams, and water- and ingredient-binding. Applications for Puratein® include dressings, sauces, meat substitutes, baked goods and protein bars, among many others.

Supertein®

Supertein® is a highly soluble canola protein isolate comprised principally of albumin proteins. The functional properties of Supertein® include excellent solubility, an ability to form transparent solutions, high foaming ability and nutritional robustness. Applications for Supertein® include beverages, confectionery, aerated desserts, and protein bars, to name only a few.

Supertein® canola protein isolate is currently under license to Merit Functional Foods Corporation and marketed under the brand name Puratein® HS. For product inquiries and sample requests, please visit meritfoods.com.

Figure 1.2: Novel oilseed rape food products. Puratein and Supertein. Adapted from Burcon (2019ab).

These advances in the processing of oilseed rape meal will help to support future applications for approval of oilseed rape derived proteins and increase the demand for these products as alternative sustainable protein sources, which will subsequently valorise the global oilseed rape industry (Rodrigues *et al.* 2012; van der Spiegel *et al.* 2013; Ravindran and Jaiswal 2016).

Oilseed rape is the third most economically important arable crop in the UK, with a 5-year annual mean value of £669 M (Defra 2019ab) (Figure 1.3). The AHDB planting survey estimates for harvest 2021 is just 0.32M hectares which is less than half of the record planting of 0.76M hectares in 2012 (AHDB 2021a). This due to the increased pressure of cabbage stem flea beetle (CSFB) leading to damaged/ failed oilseed rape crops. This increase in cabbage stem flea beetle numbers is a result of the EU neonicotinoid insecticide ban in 2013 on flowering crops such as oilseed rape (EU 485/2013). The alternate insecticides that are used to control the CSFB are pyrethroids, but the beetles have widespread insensitivity to them, which renders this insecticide control strategy ineffective (Willis *et al.* 2020). Due to the lack of CSFB control options, a large £1.8M BBSRC project has been awarded that will build on previous indicative studies that have identified variations in adult feeding damage and resistance against larval infestation in both laboratory and field experiments. This project hopes to have adapted material for trialling within five years (AHDB 2021b; UKRI 2021). The current price of oilseed rape is very high (£515/t in September 2021, AHDB 2021c). However, this price is very inflated from the price two years ago that was ca. £305/t in the UK (Farmers Weekly, 2019). This inflation in the price is due to a reduced supply of oilseed rape, partly due to CSFB. In addition to the potential development of CSFB resistant varieties, there may also be a future valorisation of by-product streams that may result in an increase in the UK oilseed rape production. Therefore, it is imperative to conserve as much yield in the surviving crop area as possible by reducing yield losses due to plant diseases such as phoma stem canker and light leaf spot, amongst others.
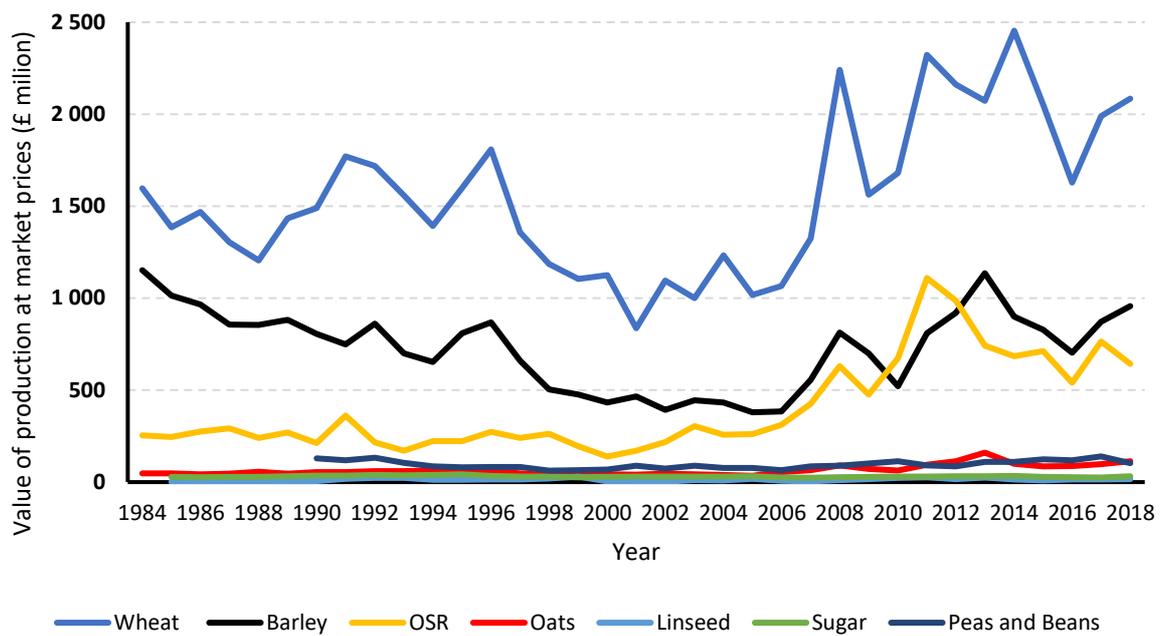
Figure 1.3: Value of production at market prices (£ million) for the six most economically valuable arable crops since 1984 in the UK. Wheat, barley, oilseed rape (OSR), oats, linseed, sugar (beet+ cane), and peas and beans. Adapted from (Defra 2019ab).

1.2   Induction of plant defences

Many plant pathogens (and their related molecules) are unable to infect the host because they are unable to avoid the detection by the plants extracellular membrane receptors, so 'pathogen-associated molecular pattern (PAMP) – triggered immunity' (PTI) is induced (Dangl et al. 2013). However, pathogens that can infect a host have evolved to produce proteins to regulate the PTI response to facilitate infection (Oliva et al. 2010). To combat pathogen infections, some plants have evolved intracellular proteins that can detect and recognise the pathogen effectors using avirulence proteins. The genes that code for the specific pathogen effectors are called avirulence genes. Genotypes of the host plant that contain the receptors to detect the specific effectors, coded for by resistance (*R*) genes, can induce a layer of plant immunity called 'effector-triggered immunity' (ETI), that triggers a hypersensitive response to the infected site to limit further spread and colonisation of the pathogen within the host; an incompatible combination has occurred (Fig. 1.4). Salicylic acid and jasmonic acid accumulation occur simultaneously during the induction of ETI (Liu et al. 2016). Some pathogens genotypes have mutations to the avirulence gene so can code for a mutated effector. If the 'mutated effector' cannot be recognised by the plant resistant proteins then ETI is not induced, and the pathogen can avoid detection to infect and colonise the host; the pathogen is virulent allowing a compatible combination to occur (Fig 1.4). A successful inoculation occurs when a compatible combination occurs under favourable environmental conditions.

1.3   Diseases

Phoma stem canker and light leaf spot are the two most economically important diseases of oilseed rape in the UK, resulting in an estimated > £165M of annual crop losses (Fitt *et al.* 2006ab; Stonard *et al.* 2010; CropMonitor 2020) (Figure 1.5).

		Corresponding receptor genes in the host plant	
		Resistant (R) dominant	Susceptible (r) recessive
Effector genes in the pathogen	Avirulence (A) dominant	AR Incompatible	Ar Compatible
	Virulence (a) recessive	aR Compatible	ar Compatible

Figure 1.4: Genetic basis of *R* gene-mediated resistance in plants. The gene for gene interaction states that when there is a combination of host receptors, coded by *R*- genes, and pathogen effectors, coded by dominant *Avr* genes, then a resistance response is triggered, a resistance phenotype is observed. However, if this combination is not made, then a resistance response is not triggered, and a susceptible phenotype is observed (Karandeni-Dewage *et al.* 2018).
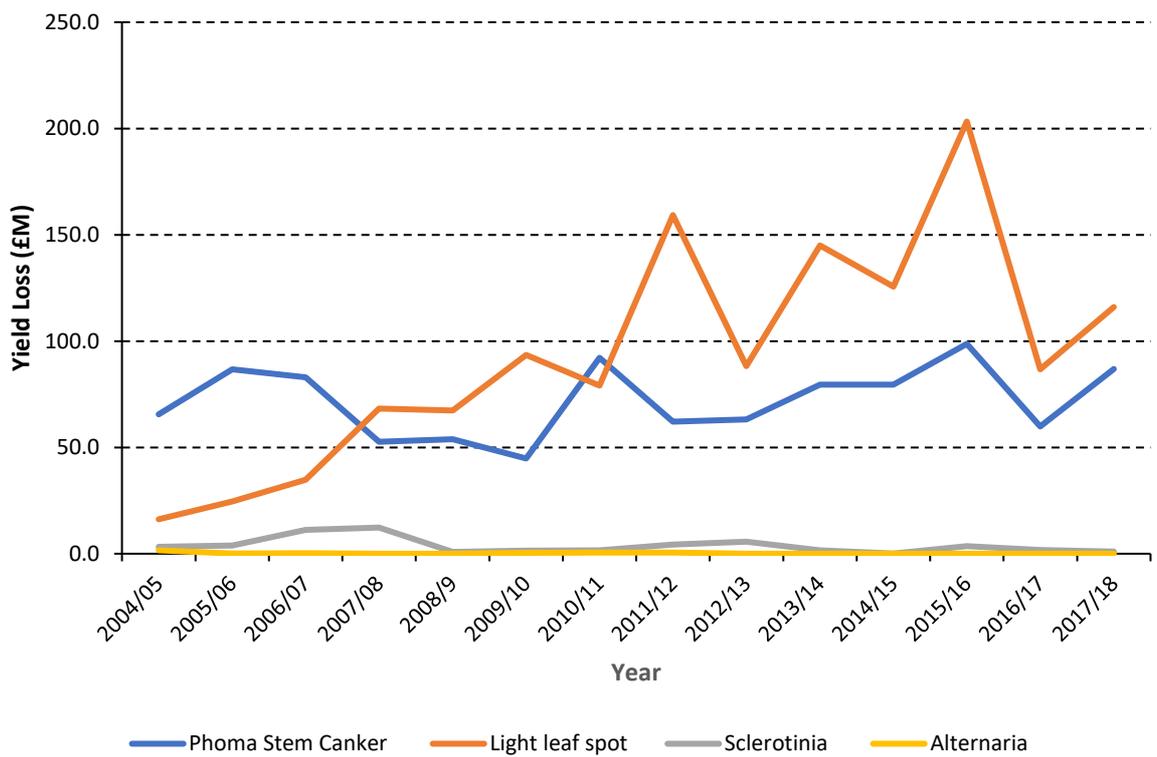
Figure 1.5: Yield loss (£M) to oilseed rape growers in England and Wales since 2005 as a result of the four most economically damaging fungal diseases – Phoma stem canker, light leaf spot, Sclerotinia stem rot and Alternaria leaf spot. Adapted from CropMonitor (2020).

1.3.1 Phoma stem canker

Phoma stem canker is of worldwide importance as it causes economic losses in Europe, North America, Asia, South America, Africa and Australia (Fitt *et al.* 2006ab; Zheng *et al.* 2020).

The disease is caused by two co-existing closely related hemi-biotrophic ascomycete fungal species *Leptosphaeria maculans* and *L. biglobosa* (Shoemaker and Brun 2001: Fitt *et al.* 2006ab)*.* It is widely reported that *L. maculans* is more damaging of the two co-existing causal pathogens (Fitt *et al.* 2006ab). These two pathogens co-exist because they occupy different ecological niches due to slight differences in their biological and epidemiological characteristics (Fitt *et al.* 2006ab). One example is a difference in their temperature optima for fruiting body maturation, which causes a temporal separation of ascospore release in Western Europe (Williams and Fitt 1999; West *et al.* 2002; Huang *et al.* 2003, 2005, 2011; Toscano-Underwood *et al.* 2003; Fitt *et al.* 2006ab; Stonard *et al.* 2010). Phoma stem canker causes yield losses as a result of crop lodging, premature ripening and inhibition of flow of water and nutrients up the plants (Piliponyte-Dzikiene *et al.* 2014). In contrast, *L. biglobosa* is reported to cause upper stem lesions that are generally confined to the outer cortex. However, severe plant infections with *L. biglobosa* can also lead to substantial yield losses (Huang *et al.* 2005; Fitt *et al.* 2008; Dawidziuk *et al.* 2010; Brachaczek *et al.* 2016; Cai *et al.* 2018). Although these two pathogens are found to co-exist in the UK, continental Europe, Australia and Canada, this is not true for China. In China, only the less aggressive *L. biglobosa* has been found in commercial crops (Fitt *et al.* 2008; Liu *et al.* 2014; Zhang *et al.* 2014; Cai *et al.* 2018). However, the more aggressive and damaging co-existing pathogen *L. maculans* has been detected on imported oilseed rape seeds at ports in China (Zhang *et al.* 2014). The establishment of *L. maculans* in China would be very damaging with predicted annual yield losses worth $495M (Fitt *et al.* 2008).

1.3.2 Light leaf spot

Light leaf spot is also of worldwide importance as it causes economic losses in the UK, continental Europe (including France, Germany and Poland), Australia and New Zealand,

Asia (Japan and Philippines) and North America (Carmody *et al.* 2019; Zheng *et al.* 2020). The disease is caused by the ascomycete fungal pathogen *Pyrenopeziza brassicae* (Rawlinson *et al.* 1978). Light leaf spot has become established as the most economically important disease of winter oilseed rape crops in England (King *et al.* 2018; CropMonitor 2020) (Figure 1.5). Within the UK, this disease was predominantly associated with oilseed rape in Northern England and Scotland (Figueroa *et al.* 1995). However, due to the cooler, wetter winters which favour light leaf spot development, the disease is now widespread throughout the UK (Figueroa *et al.* 1995; Boys *et al.* 2007). In 2016, the incidence of light leaf spot on oilseed rape pods and stems was the greatest (95% and 100% crops) since 1987. It was estimated to have caused > £200M of damage to oilseed rape yields in England and Wales in 2016 (Figure 1.5). The symptoms of light leaf spot are reduced leaf photosynthetic area, reduced plant vigour, increased susceptibility to frost damage, premature ripening, increased pod shattering and even plant death (Ashby 1997; Baierl *et al.* 2002; Pielaat *et al.* 2002; Boys *et al.* 2007).

1.4 Taxonomy and trophic nature of the pathogens

1.4.1 Taxonomy

Leptosphaeria maculans (Desm.) Ces. & De Not. 1863 [anamorph *Plendomus lingam* (Tode:Fr.) Desm.] (*L. maculans*; Lm) and *L. biglobosa* sp. No. [anamorph *Plendomus biglobosus* comb. Nov.] (*L. biglobosa*; Lb) De Gruyter et al. 2013; Shoemaker and Brun, 2001) are sibling species. They were believed to belong to the same species but since advancement of molecular and genetic techniques they have been able to be distinguished as two separate species (Fitt et al. 2006a). Therefore, both species belong to the Class *Dothideomycetes*, Order *Pleosporales*, Family *Leptosphaeriaceae* and Genus *Leptosphaeria* (Appendices 4). Phylogenetic analysis of the class *Dothideomycetes* indicates that the two *Leptosphaeria* spp. diverged approximately 22 million years ago (Grandaubert et al. 2014), and *L. maculans* is believed to be the younger species because it contains a greater percentage of transposable elements (Mendes-Pereira et al. 2013). *Pyrenopeziza brassicae* Sutton & Rawlinson [anamorph *Cylindrosporium concentricum*]

(*P. brassicae*; Pb) belongs to the Class *Leotiomycetes*, Order *Helotiales*, Family *Dermateacea* and Genus *Pyrenopeziza.*

1.4.2   Trophic nature

Trophic nature is key to understand the nutritional strategy that a pathogen uses to survive and colonise a plant. There are three main categories for trophic nature; the first is biotrophs whereby nutrients are obtained by the pathogen directly from the host without killing the cell; the second are necrotrophs whereby the pathogen break into the host cell and cell death to obtain nutrients; the third category are called hemi-biotroph, they exhibit a biotrophic nature during their initial phase before transitioning into a necrotrophic strategy later. The stimulus that is responsible for the change in nutritional strategy (trophic nature) remain unknown (Padmathilake and Fernando, 2022). It is important to understand the trophic nature that a pathogen utilises because in the plant system different phytohormones, that are used for signalling to induce plant responses, are linked with the pathogens trophic nature (Huang et al. 2020). In biotrophs and hemi-biotrophs, the induced plant defence responses are regulated by salicylic acid, in contrast to necrotrophs where the defence responses are induced by different phytohormones called jasmonic acid and ethylene (Pieterse et al. 2009; Tsuda et al. 2009;2013; Vlot et al. 2009). Both *Leptosphaeria* spp. and *P. brassicae* are hemi-biotrophic pathogens (Ashby 1997, Shoemaker and Brun 2001: Fitt *et al.* 2006ab). Therefore, this would suggest that salicylic acid is important for the induction of a plant defence response to these pathogens.

1.5   Pathogen life cycles

In a typical winter oilseed rape cropping season in the UK, epidemics of both phoma stem canker and light leaf spot are initiated by airborne ascospores in the autumn (Gladders and Musa 1980; Gilles *et al.* 2000a;2001ab; Toscano-Underwood *et al.* 2003; Huang *et al.* 2007; Khangura *et al.* 2007). The wind-dispersed ascospores land on young oilseed rape plants and infect leaves, causing phoma leaf spot (*Leptosphaeria* spp.) or light leaf spot (*P. brassicae)* (Li *et al.* 2003; Boys *et al.* 2007). The life cycles of these pathogens are different.

1.5.1 *Leptosphaeria maculans* and *L. biglobosa* life cycle

The *L. maculans* and *L. biglobosa* life cycles are similar (West *et al.* 2002) (Figure 1.6A). *Leptosphaeria* spp. have a monocyclic life cycle; this means that these pathogens only undergo one full life cycle within a season. *Leptosphaeria* spp. are heterothallic ascomycetes that can form both sexual spores (ascospores) and asexual spores (conidia) (Shoemaker and Brun, 2001). *Leptosphaeria* spp. disease epidemics are dependent on the release of ascospores from ascocarp structures called pseudothecia. *Leptosphaeria* spp. life cycles are initiated by wind-dispersed ascospore landing on leaves of new oilseed rape crop (Lo-Pelzer *et al.* 2009). The ascospores use germ tubes to enter the plant via stomatal opening or wounds. At this stage the *Leptosphaeria* spp. are endophytically developing within the leaf apoplast (Stotz et al. 2014) to create leaf lesions, termed phoma leaf spots (PLS). The appearance of phoma leaf spots is characterized by grey lesions with black pycnidia for *L. maculans* and smaller black lesions without pycnidia for *L. biglobosa*, observed on leaves in early autumn (Figure 1.6B), during the vegetative growth stages. However, on some genotypes of oilseed rape an incompatible combination occurs (Fig. 1.4), meaning that the host can detect *L. maculans* through the initiation of ETI; ETI has not been identified against *L. biglobosa*. This results in a very dark margin around the lesion forms because of a hypersensitive response to contain the pathogen, stopping the disease lifecycle from progressing any further. This provides a potential explanation for why there is limited correlation between phoma leaf spotting and stem cankers at the end of the season. However, if a compatible combination occurs (Fig. 1.4), a successful initial leaf infection allows the pathogens grow towards the stem via the leaf petiole; at this stage the pathogens are gaining nutrients biotrophically. This is until there is a switch in the trophic nature of the pathogen from biotrophic to necrotrophic. During the necrotrophic phase of the pathogen life cycle, stem lesions develop by invading and killing cells within the stem and continue to develop until harvest (Hammond *et al.* 1985; Huang *et al.* 2006; Sprague *et al.* 2007; Travadon *et al.* 2009; Eckert *et al.* 2010). Outer stem lesions of *Leptosphaeria* spp. appear like the leaf spotting; a grey lesion with black pycnidia.
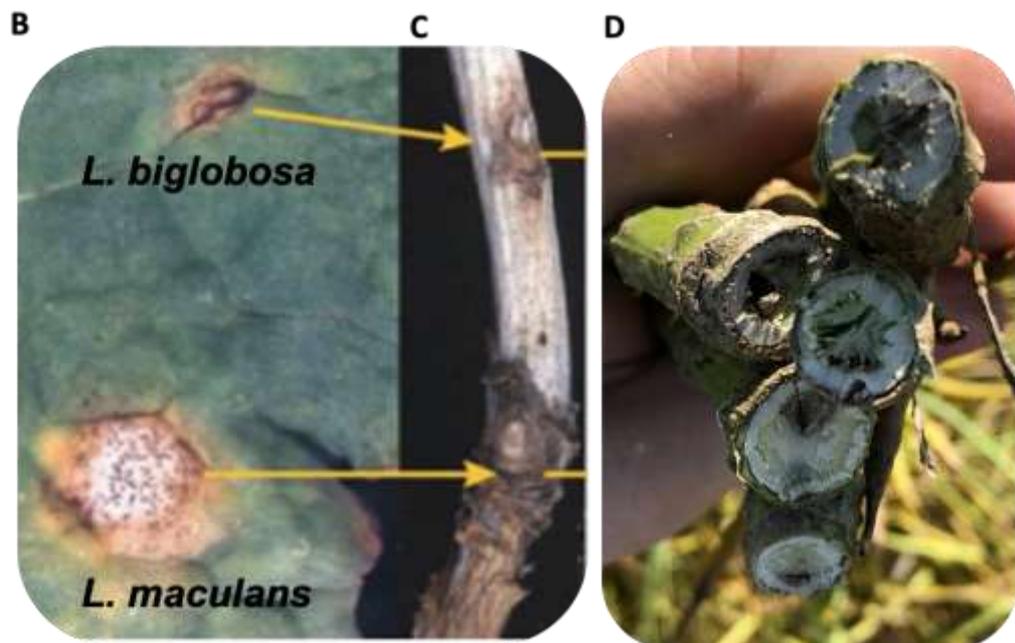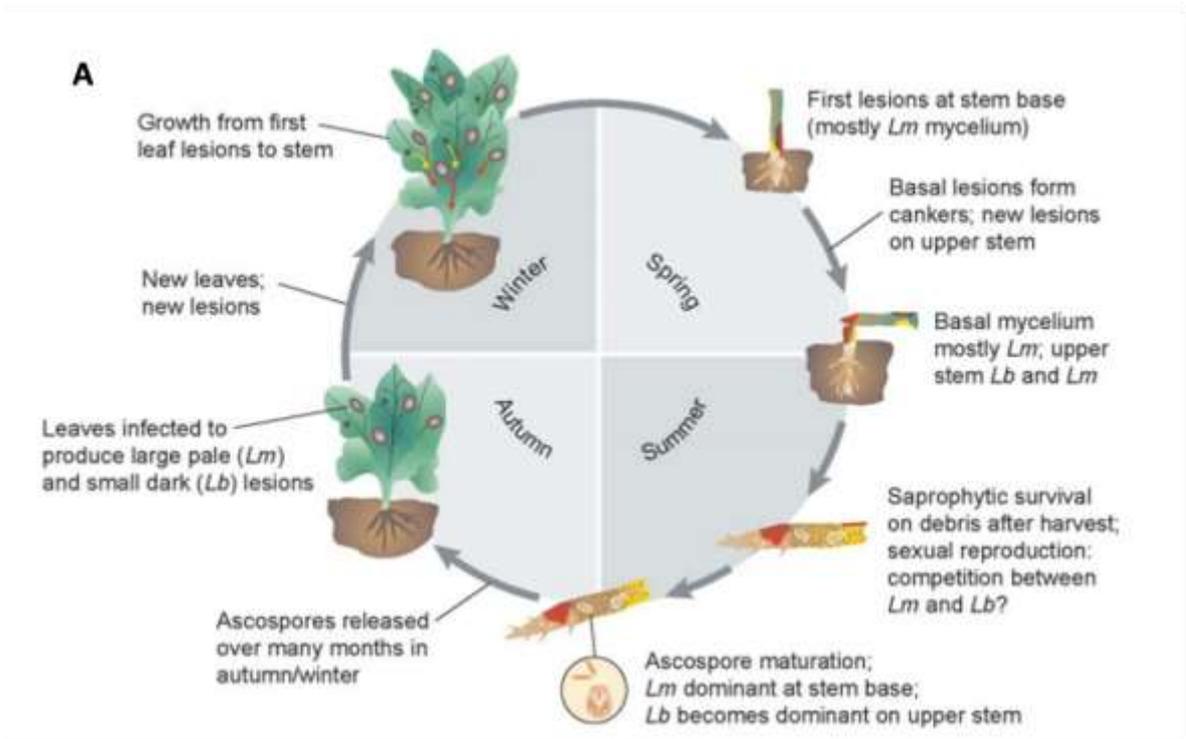
Figure 1.5: A) Life cycles of *Leptosphaeria maculans* and *L. biglobosa*, the causal pathogens of phoma leaf spot and stem canker, adapted from Fitt *et al.* (2006ab). Differences between *L. maculans* and *L. biglobosa* on; B) a leaf and C) the stem, adapted from Fitt *et al.* 2006ab. D) Cross-section of oilseed rape stems at root collar of cv. Charger with stem canker from Terrington St Clement, Norfolk in July 2019.

Lesions found slightly above the root collar are often associated with *L. maculans* in contrast to those upper stem lesions that are associated with *L. biglobosa* (Figure 1.6C). This difference in lesion location has often been attributed to their differences in pseudothecial maturation, where previous studies had shown that *L. biglobosa* ascospores were often released later than those of *L. maculans* (Huang *et al.* 2011) because of specific environmental conditions (Toscano-Underwood et al. 2001, Huang et al. 2007). However, more recent investigations that have used qPCR analysis have reported that ascospores of both species are more frequently released at similar times (Javaid *et al.* 2019) and may provide an explanation as to why *L. biglobosa* is now being found in stem basal regions more frequently.

A severe stem canker will result in a weakening of the stem leading to premature ripening and crop lodging (Huang *et al.* 2003; Eckert *et al.* 2010). Stem basal cankers appear in late spring-early summer (Fitt *et al.* 2006ab), but the necrotic tissue is more clearly visible in horizontal stem cross-sections made in the summer (Figure 1.6D). *Leptosphaeria* spp. can also infect the pods (Zamani-Noor and Knufer 2018). Any infected crop debris (stem stubble, pod debris and infected seed) that is left after harvest can develop pseudothecia (containing ascospores), which allow *L. maculans* to transition from one growing season to the next by releasing airborne ascospores between late September and early March (Fitt *et al.* 2006ab; Huang *et al.* 2005).

Conidia can be released from the pycnidia but they rarely contribute to European phoma stem canker epidemics (West *et al.* 2001), although it has been suggested that they are important in Australian, and Canadian agriculture for *L. maculans* (Barbetti 1976; Hall *et al.* 1996; Travadon *et al.* 2007). They can cause phoma leaf lesions if the plant is wounded under experimental conditions (Huang *et al.* 2001, 2003, 2006, 2011). To maintain oilseed rape as a cost-effective crop, new strategies have been developed such as grazing sheep on oilseed rape and defoliating crops mechanically to combat cabbage stem flea beetle infestations (Sprague *et al.* 2015; White *et al.* 2020). Both practices create wounds on the plant, that may enable conidia of *Leptosphaeria* spp. to infect these damaged crops (West *et al.* 2001), thereby increasing their importance to agriculture.

1.5.2 *Pyrenopeziza brassicae* life cycle

P. brassicae infections are different from those of *L. maculans* and *L. biglobosa* as the pathogen is polycyclic; the pathogen is capable of several infection cycles in a growing season (Figure 1.7A). Additionally, the conidia contribute to the disease epidemic (Fitt *et al.* 1998; Gilles *et al.* 2000ab; 2001ab). After successful leaf infection, there tends to be a long asymptomatic growth stage before the associated early symptoms of white 'conidial ring' and 'leaf crinkling' are observed (Figure 1.7B). The 'conidial ring' symptoms become visible when the acervuli break through the leaf surface (Rawlinson *et al.* 1978a; Gilles *et al.* 2000a; Boys *et al.* 2007; 2012). The change from asymptomatic to symptomatic is not fully understood. However, it has been suggested that it is a result of stress response from biomass and/or space limitations (Ashby 1997). Any precipitation, especially rainfall, causes the acervuli (asexual fruiting bodies) to disperse spores into highly concentrated suspensions of conidia that spread the disease to surrounding leaves and plants via rain-splash causing secondary infections (Gilles *et al.* 2000ab; 2001abd; Papastamati *et al.* 2001; Pielaat *et al.* 2002; Evans *et al.* 2003; Welham *et al.* 2004). A second disease cycle, unlike the *L. maculans* life cycle (Figure 1.5A), can be initiated through the development of sexual fruiting bodies (apothecia) on infected leaves that have senesced. Once they have matured, a new release of ascospores can occur and begin the cycle again (Gilles *et al.* 2000a;2001ab). Meanwhile, the effects of the conidial secondary infection are still occurring from previous infection cycles forming a very complex and 'chaotic' build-up of *P. brassicae* inoculum and infected plants (Evans *et al.* 2017) (Fig. 1.8). The stem lesions associated with *P. brassicae* are purple-brown outer stem lesions with black speckling around the edges (Figure 1.7C). They appear only on the stem surface and tend to appear further up the stem than *L. maculans* lesions, in a similar region to that associated with *L. biglobosa* stem lesions. *P. brassicae* also infects the pods, leading to a purple-brown speckling on pod surfaces. As with *L. maculans,* any infected crop debris that is left after harvest can develop ascocarps (containing ascospores); which will allow both diseases to transition from one growing season to the next by airborne ascospores (Gilles *et al.* 2000ab,2001ab; West *et al.* 2001; Marcroft *et al.* 2004; Hossard *et al.* 2018). *P. brassicae* transmission infected seed is not unanimously agreed upon (Maddock and Ingram, 1981, Majer et al. 1998).
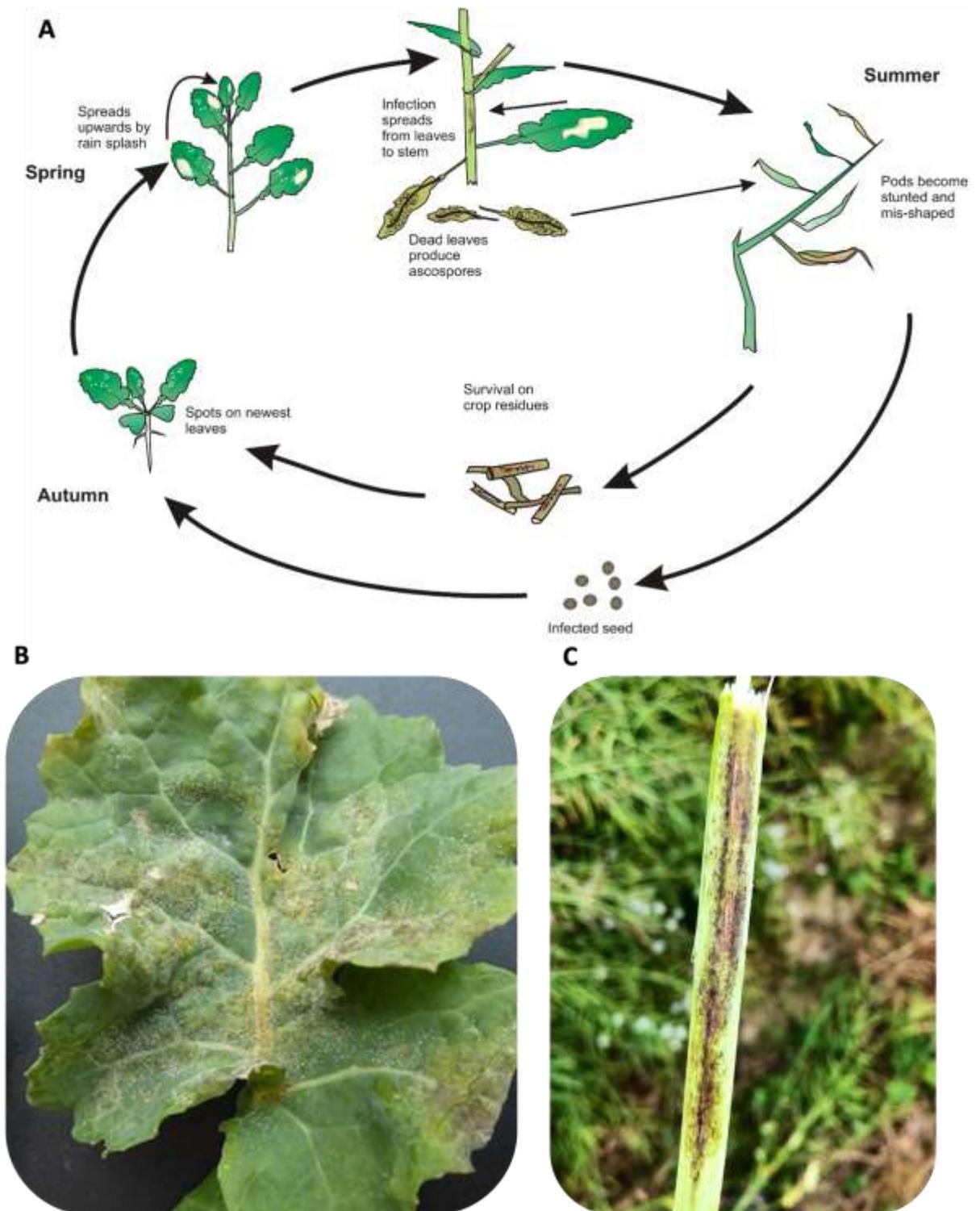
Figure 1.7: A) Life cycle of *Pyrenopeziza brassicae* the causal pathogen of light leaf spot (ADAS and BASF 2009). B) *P. brassicae* sporulation on cv. Bienvenu in April 2018 at Withern, Lincolnshire, courtesy of Dr Yongju Huang. C) Light leaf spot stem lesion on cv. Express in Ebsdorfergrund, Germany in June 2019.
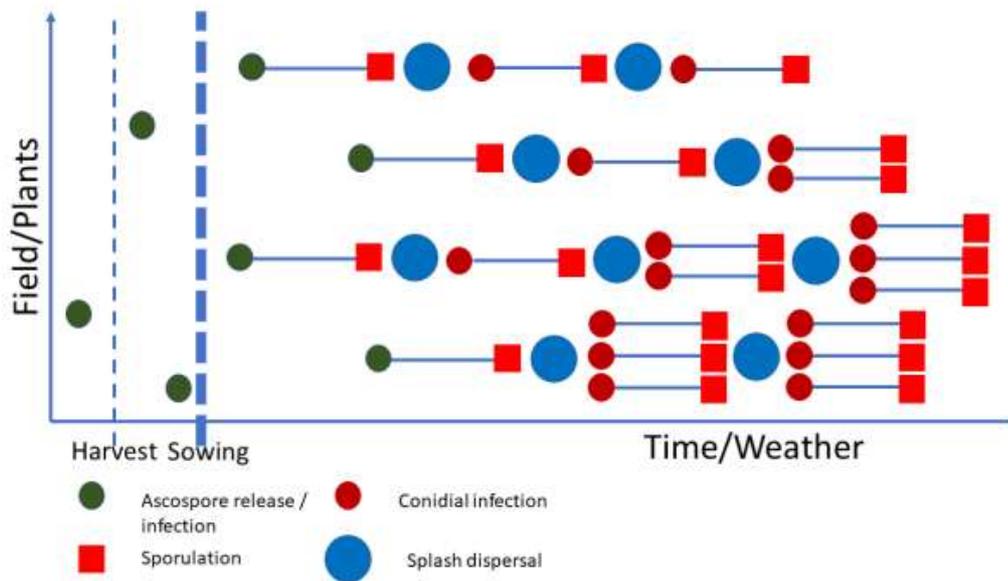
Figure 1.8: A schematic diagram showing the proposed 'chaotic' development of a light leaf spot epidemic on winter oilseed rape in the UK in late autumn to early winter (Evans *et al.* 2017). Ascospore release events (green circles) that happen before the sowing (thick perforated line) are not able to infect the emerging crop. Therefore, the earlier the crops sown, the more ascospore release event the emerging plants will be exposed to. Release events that occur when the crop is emerging can infect the crop. *P. brassicae* has a long latent period (blue horizonal line) before sporulation (red square) of conidia in the form of acervuli are able to be spread by rain splash (blue circles) to infect surrounding plants and leaf tissues in a secondary infection spread (red circles).This then continues whereby conidial infection as a result of splash dispersal all occur at different times and rates depending on the time and weather throughout the season. This results in a very chaotic development of light leaf spot epidemic as at any one point, *P. brassicae* could be in an ascospore release, sporulation, conidial infect or splash dispersal phase.

1.6   Ascocarps

Ascocarps are the fruiting bodies of ascomycete fungi (Sadava *et al.* 2012). The fruiting bodies are crucial to the survival of pathogens as it is these that allow the pathogen to pass from one growing season to the next over periods of unfavourable conditions (Lacey *et al.* 1987; McCartney and Lacey 1990). There are five different fruiting body structures that all serve the same function; the production, maturation and release of ascospores, but the differing structures relate to the biotic and abiotic factors the pathogens require for release of ascospores (Webster and Weber 2007) (Figure 1.9).

L. maculans and *L. biglobosa* form ascocarp structures called pseudothecia (Toscano-Underwood *et al.* 2003; Huang *et al.* 2007; Shoemaker and Brun 2001). This ascocarp is a bitunicate flask-shaped structure with its asci contained in one or more cavities formed within pre-existing ascostroma, protected from the outside environment (Webster and Weber 2007) (Figure 1.9e). This ascocarp structure determines the conditions that are important for *Leptosphaeria* spp. because both *L. maculans* and *L. biglobosa* have been shown to require continual wetness and mean temperature of 5-20°C for successful maturation and dispersal of ascospores (Toscano-Underwood *et al.* 2003). Although they are closely related, there is a difference in the maturation of pseudothecia and development of ascospores between *L. maculans* and *L. biglobosa*; pseudothecial maturation of *L. biglobosa* occurs more slowly at temperatures <10°C compared to that of *L. maculans* (Toscano-Underwood *et al.* 2003)*.* This may provide explanation as to why there are fewer *L. biglobosa* lesions observed on leaves in crops over winter, with an increase in the spring with the increase in temperature (West *et al.* 2002). These differences in pseudothecial maturation contribute to enabling a temporal and spatial separation between the two pathogens so that they occupy different ecological niches to allow co-existence (Toscano-Underwood *et al.* 2003; Fitt *et al.* 2006ab). *Pyrenopeziza brassicae* forms ascocarp structures called apothecia (McCartney and Lacey 1990; Figueroa *et al.* 1995; Gilles *et al.* 2001bc) (Figure 1.9c). This ascocarp is an open saucer shaped structure with its asci present on the hymenium surface exposed to the environment (Webster and Weber 2007).
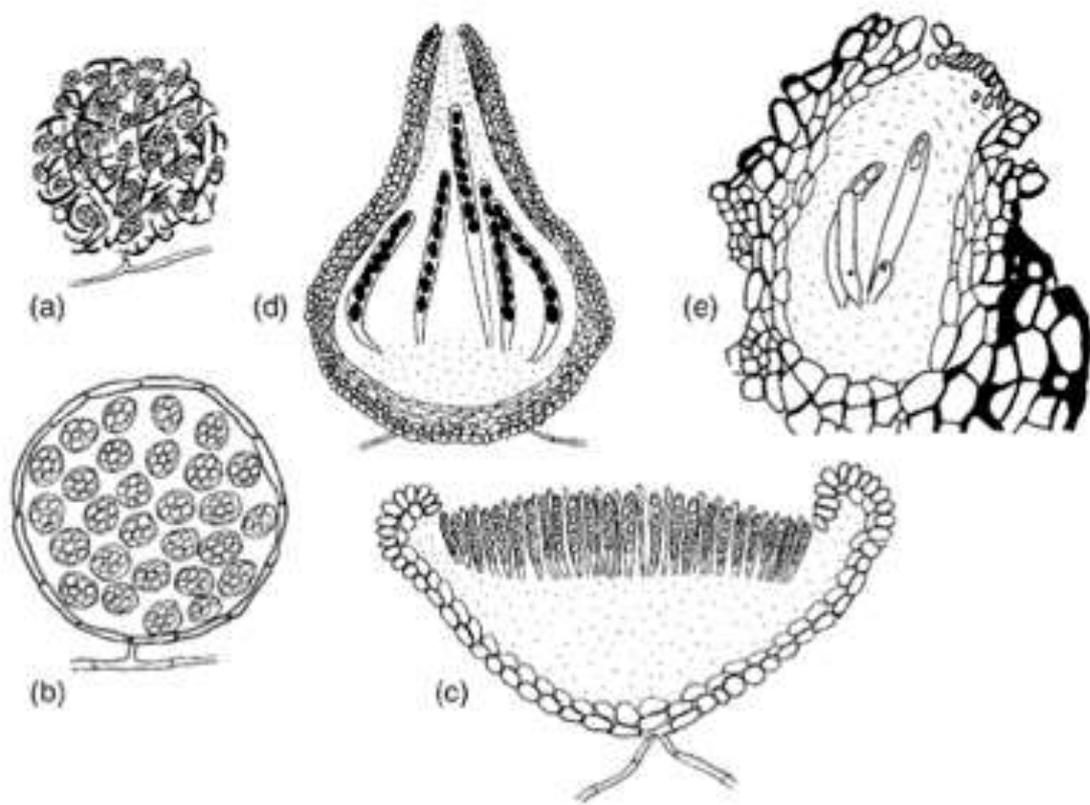
Figure 1.9: Different types of ascomycete fruiting bodies. (a) Gymnothecium, (b) Cleistothecium, (c) Apothecium, (d) Perithecium, and (e) Pseudothecium. Adapted from Webster and Weber 2007.

This ascocarp structure means that the asci are very exposed. Temperature of 18°C and continual wetness are important environmental factors for *P. brassicae* ascospore maturation. Gilles, Fitt and Jeger (2001a) showed that having an interruption in the wetness delayed apothecial development. Therefore, long exposure to the environment has its benefits as well as its costs. The apothecia structure allows water to be trapped in the cup shape and is easily accessible for enabling ascospore production and maturation. However, it is also vulnerable to water loss through evaporation. Therefore, frequent precipitation events and humid conditions favour *P. brassicae* for ascospore maturation (Gilles *et al.* 2001c). For release of ascospores, the exposure to the environment is advantageous for *P. brassicae* because, once mature, the ascospores are ejected and consequently get dispersed via the wind during periods of dryness following precipitation (McCartney and Lacey 1990; Webster and Weber 2007). The very specific conditions that are required for *P. brassicae* ascospore release explain why *P. brassicae* ascospore release events are often very short, sharp, and intense, in contrast to *Leptosphaeria* spp. ascospore release events, where there is a broader range of environmental conditions that ascospore can mature and be released under, so the ascospore release events occur over a longer period.

1.7 Interspecific competition

Resources are defined as the entities which contribute positively to population growth (Grover 1997). The offspring of most living organisms are provided with a limited stock of resources for initial development. However, to produce the fundamental energy to develop, grow, survive and reproduce they must obtain additional resources individually. The acquisition of resources follows conservation rules; the resources that one individual obtains are not immediately available to another (Grover 1997). When a resource is insufficient to supply the combined needs of all the individuals that are supported on it, it is common for competition for resources to arise between genotypes of the same species (intraspecific competition) and between individuals from different species (interspecific competition). This is called resource competition (Grover 1997; Hortal *et al.* 2016; Abdullah *et al.* 2017; Kozanitas *et al.* 2017; Dutt *et al.* 2021). If an essential resource becomes finite, the population that it is supporting is in danger of not

being able to sustain itself. This places strong selection pressures on the individuals involved and can influence the persistence and evolution of the species through the principles of competitive exclusion or resource partitioning (Dutt *et al.* 2021). The principle of competitive exclusion that states that 'no two species can share the same limiting resource indefinitely' may result in the inferior competitor going extinct locally (Gausse 1934; Hardin 1960; Fitt *et al.* 2006a). However, the resulting selection pressures may lead to changes in the way that competing species use the resource; often by partitioning the resource, termed resource partitioning (MacArthur and Levins, 1967; Tilman, 1982). There are two types of resource partitioning that may be used in the host when pathogens are competing. The first is whereby a resource is partitioned using a physical or molecular barrier so that resources either side of the barrier are available only to the pathogens on either side. Alternatively, a resource such as a host plant can have its resources partitioned by being able to access parts of the resource that are not available to competing pathogens using temporal or spatial differentiation, such as delayed ascospore releases to coincide with development of fresh plant tissues that was previously not accessible to a competing pathogen. Pathogens that can effectively partition a resource are able to co-exist. Competition can be minimized by altering the niche the species occupies. A species niche is the set of physical and biological conditions that a species requires to survive, grow and reproduce. The physiological capabilities of the species define its fundamental niche, but the effects of interspecific interactions resulting from competitive exclusion and resource partitioning limit the amount of the fundamental niche that can be occupied by a species when competing species are present, forming the realized niche (Sadava *et al.* 2012; Gerz *et al.* 2018). There are three major mechanisms of competition that occur.

Exploitative competition occurs when a limiting resource is available to all competitors and the outcome of the competition depends on the relative efficiency with which the competitor uses the resource; the species that can colonize the resource fastest or more efficiently outcompetes its competitor to survive (Tan *et al.* 2016; Dutt *et al.* 2021). Tan *et al.* (2016) showed that when a biocontrol strain of *Bacillus amyloliquefaciens* and *Ralstonia solanacera* (a bacterial wilt pathogen) was studied on a tomato plant, the results showed that the biocontrol bacterial strain colonised the root more efficiently

and effectively and formed a biofilm on the root surfaces; this restricted the access of bacterial wilt pathogens to the host plant and therefore suppressed its presence. This showcases the exploitative competitive strategy; *Bacillus amyololiquefaciens* was effective at excluding *Ralstonia solanacera* from accessing the resource.

Host-mediated competition occurs when the development of one pathogen enhances the host plant defence responses against other competing pathogens leading to reduced survival and success of the other competing pathogen (Liu *et al.* 2006; Aime *et al.* 2013; Dutt *et al.* 2021). Liu *et al.* (2006) showed that when oilseed rape plants were exposed to *L. biglobosa* before *L. maculans*, then the plants local and systemic resistance to *L. maculans* could be induced with both short term and long-term effects on the development of phoma leaf spotting and phoma stem canker development 8 months later. This suggests that *L. biglobosa* may use host-mediated competition by enhancing the host plant defence responses to reduce the survival of *L. maculans*. This is important because the salicylic acid induced plant defence response is important for the control of pathogens utilising a biotrophic nature of nutrition. Although, *Leptosphaeria* spp. and *P. brassicae* do not have an obligate biotrophic stage in their life cycle, they do have endophytic stages during the initial stages of their life cycles followed by necrotrophic stages (Stotz et al. 2014). Although, host-mediated competition can arise between *L. biglobosa* and *L. maculans*, these species can co-exist due to the temporal dynamics of ascospore release caused by the differences between *Leptosphaeria* spp. in pseudothecial maturation. This allows a spatial separation of the two *Leptosphaeria* spp. to occur on the host plant.

Interference competition is the other mechanism by which a species interferes with access by another competitor to a limiting resource (Sadava *et al.* 2012; Dutt *et al.* 2021). In beech trees (*Fagus sylvatica*) with dead trunks infected by the basidiomycete porcelain fungus, *Oudemansiella mucida*, the fungus produces an antifungal compound called Mucidin A, which is a strobuliurin, that inhibits the growth of any other fungus within the dead trunks, thereby interfering with access by other fungi to the resource (Von Jagow *et al.* 1986; Jennings and Lysek 1996). An example of this can be found in

Leptosphaeria maculans whereby a non-host-selective phytotoxin called sirodesmin PL is produced (Rouxel *et al.* 1988).

1.8   Sirodesmin PL

Sirodesmin PL is produced by *L. maculans*, but not by *L. biglobosa*. *L. biglobosa* was previously believed to be a weakly virulent form of *L. maculans*. However, many distinctions between the two were identified that resulted in *L. maculans* and *L. biglobosa* being classed as 'A' and 'B' type or Tox+ or Tox- *L. maculans* isolates respectively (Johnson and Lewis, 1990; Williams and Fitt, 1999; West *et al.* 2002). The differentiation between these isolates was determined by the ability to produce sirodesmin PL and water-soluble pigments; with 'A' type or Tox+ (*L. maculans*) isolates described as those that could produce sirodesmin PL, but not the water-soluble pigments in liquid culture, in contrast to 'B' type or Tox- (*L. biglobosa*) that were not able to produce Sirodesmin PL but could produce the water-soluble pigments.

The function of sirodesmin is still unknown. However, studies have shown that mutants without this chemical had less antibacterial and antifungal activity than the wild type but did not have decreased growth or fertility, suggesting that it may be used as an interference competition strategy against competing pathogens. It can cause yellow chlorotic lesions on plants, hence why it is described a phytotoxin and a mycotoxin (Rouxel *et al.* 1988; Gardiner *et al.* 2004). Sirodesmin PL belongs to a class of secondary metabolites that are characterized by the presence of a disulphide bridge, called epipolythiodioxopiperazine (ETP) (Curtis *et al.* 1977; Gardiner *et al.* 2005) (Figure 1.10). The biological activity of the ETPs appears to reside exclusively in the disulphide bridge and the removal of this section inhibits the biological activity (Middleton 1974; Gardiner *et al.* 2005). There are at least two known mechanisms of toxicity of ETPs, either through the generation of reactive oxygen species resulting from redox cycling between disulphide (oxidized) and dithiol (reduced) forms; or through the formation of mixed disulphides with free thiol groups on proteins leading to their inactivation (Gardiner *et al.* 2005). However, the function that is relevant to Sirodesmin PL is still unknown (Chai and Waring 2000).
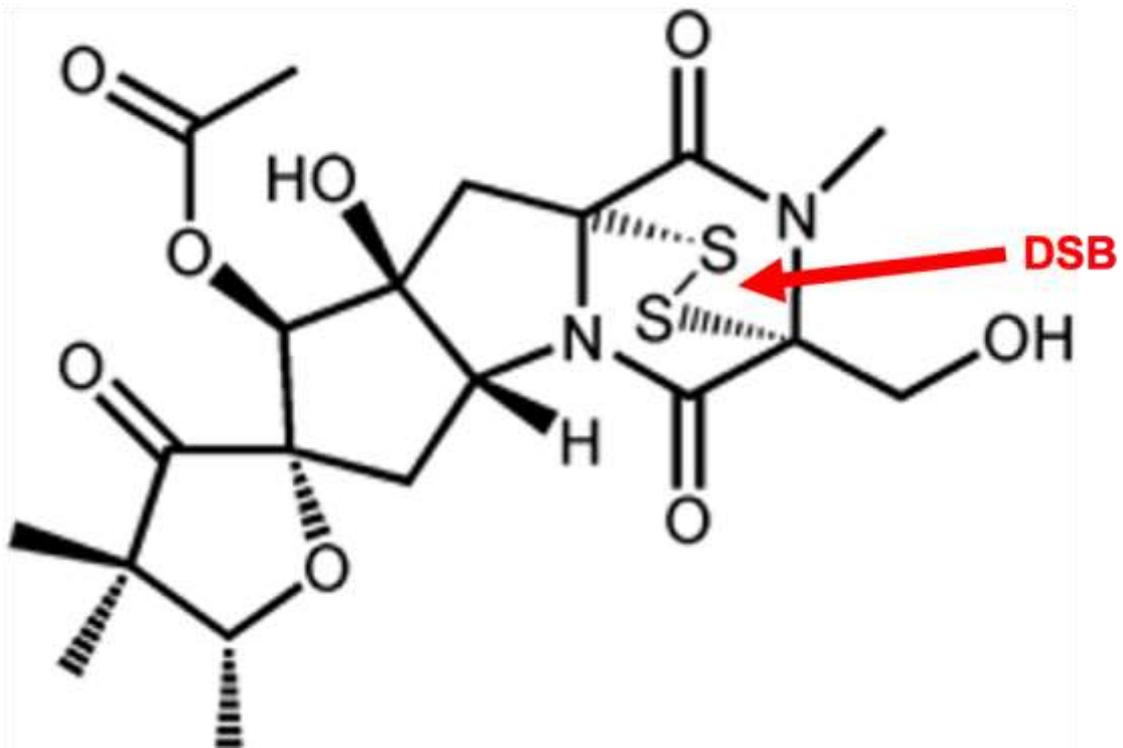
Figure 1.10: Chemical structure of the non-host-selective phytotoxin Sirodesmin PL, produced by _L. maculans_. It belongs to the epipolythiodioxopiperazine class of secondary metabolites characterized by the presence of a disulphide bridge (DSB); modified from Howlett _et al._ (2009).
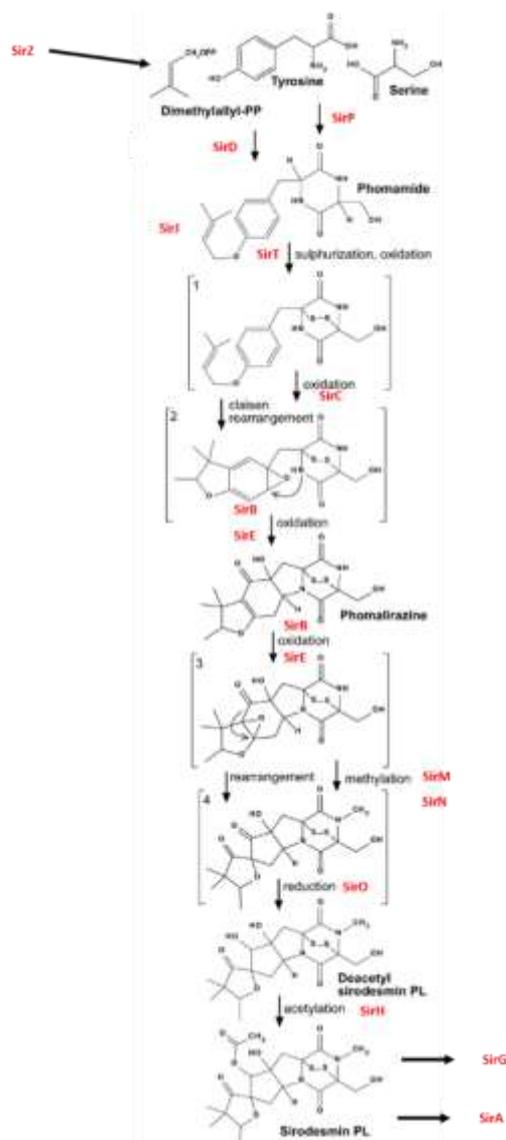
Figure 1.11: Proposed biosynthetic pathway of sirodesmin PL by *L. maculans* with the identified biocluster genes that have putative roles in the sirodesmin PL pathway placed in position of their proposed function. Known compounds are named in bold, intermediates are inside brackets and numbered. Some steps are likely to be multistep reactions catalysed by more than one enzyme. Adapted from Gardiner *et al.* (2004). The three primary molecules, dimethylallyl-P-P, tyrosine and serine, are used to form phomamide (the first stable sirodesmin precursor) catalysed by the *Sir* gene encoded enzymes. After a series of chemical reactions (oxidation, sulpherization, rearrangements), catalysed by different *Sir* gene encoded enzymes, Phomalirazine (the second stable sirodesmin precursor) is formed. Several other *Sir* gene encoded enzymes catalyse further chemical reactions (methylation, reduction, reaarangements and acetylation) allow the formation of Deacetyl sirodesmin (third stable precursor moleculer) and finally Sirodesmin PL.

There are 18 co-regulated genes that have been reported to be involved in the biosynthesis pathway of sirodesmin PL (Gardiner *et al.* 2004) (Fig 1.11). Recently, Urquhart *et al.* (2021) identified two further genes within this bio-cluster that they refer to as *sirK* and *sirX.*

It has been shown that disruption of *sirP* blocks the sirodesmin biosynthesis (Gardiner *et al.* 2004). Its function was hypothesized to be the first step in the sirodesmin PL biosynthesis pathway, but Kremer *et al.* (2010) suggested that *sirD* is responsible for producing one of the precursor molecules that *sirP* uses (Gardiner *et al.* 2004; Fox and Howlett 2008).This suggests that these two genes are both responsible for catalyzing steps very early in the sirodesmin PL formation mechanism. Another very important gene in the cluster is *sirZ*, that encodes a $Zn(II)_2Cys_6$ binding protein that is responsible for regulating the expression of the genes in the *sir* cluster (Fox *et al.* 2008; Elliot *et al.* 2011). The function of sirodesmin PL is still unknown. Although sirodesmin PL causes chlorotic lesions when inoculated on oilseed rape, when plants were inoculated with mutant isolates that did not produce sirodesmin PL they were still able to cause normal disease symptoms on cotyledons, and this only resulted in a mild reduction in stem canker severity and 50% reduction in fungal biomass (Elliot *et al.* 2007), suggesting that it is not required for infection and disease progression. Sirodesmin PL has also been found in stem base lesions as well as in leaf plant tissues (Elliot *et al.* 2007).

1.9   Integrated pest management

The European Council define integrated pest management (IPM) as the "careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimise risks to human health and the environment" (EU, 2009/128/EC). Integrated pest management emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms. The European Council encourages the use of resistant/tolerant cultivars, crop rotations and

other adequate cultivation techniques, use of sustainable biological control where appropriate and risk monitoring and forecasting. Anti-insensitivity strategies to maintain/prolong the effective life of chemicals, including the use of different modes of action, are also suggested (EU, 2009/128/EC). It has long been accepted that an integrated approach combining chemical, cultivar and cultural control strategies is the most effective strategy for controlling phoma and light leaf spot epidemics, as for other diseases (Strand 2000; Krupinsky *et al.* 2002; Gladders *et al.* 2006; Sprague *et al.* 2006; Juroszek and von Tiedemann 2011).

1.10   Current control strategies

1.10.1 Chemical control

Chemical control is a fundamental part of any UK oilseed rape disease control programme, especially against these two economically important diseases. A range of fungicide modes of action are used for disease control commercially; however, azole fungicides are the most commonly used in agriculture; in Europe 50% of fungicides used commercially belong to the azole class (Azevedo *et al.* 2015). Examples include prothioconazole and tebuconazole that are commonly found in fungicide formulations such as Proline, Folicur and Kestrel (Eckert *et al.* 2010; Huang *et al.* 2011). The use of these two active ingredients, either as solo or co-formulations, is increasing; they accounted for 34% (2012), 40% (2014), 49% (2016) and 52% (2018) of all fungicides applied on oilseed rape in the UK (Garthwaite *et al.* 2012;2014;2016;2018). Azoles inhibit *CYP51,* which codes for lanosterol 14α-demethylase enzyme, which is responsible for the conversion of lanosterol to ergosterol (Price *et al.* 2015). Ergosterols are fundamental components of the fungal cytoplasmic membrane (Sewell *et al.* 2017). Reduction in lanosterol 14α-demethylase enzyme concentration results in an unstable and permeable plasma membrane and leads to cell death due to the accumulation of toxic sterol intermediates (Price *et al.* 2015; Sewell *et al.* 2017).

Fungicide insensitivity is defined as the acquired, heritable reduction in sensitivity of a fungus to a specific anti-fungal agent (Brent and Hollomon 2007). It is important because once a pathogen that has developed insensitivity to a fungicide/mode of action has

becomes widespread, the fungicide can no longer control the disease effectively. This is particularly pertinent because the development of new modes of actions is slow and very expensive (van de Wouw *et al.* 2017). Thus, there is a need to develop and distribute robust anti-insensitivity strategies, to help retain and maximise the effective lives of these fungicides. Insensitivities can develop if there is a reliance upon a single mode of action for disease control (van de Wouw *et al.* 2017). This was observed for light leaf spot in oilseed rape where benzimidazole (MBC) and triazole fungicides (DMI) were applied excessively. This led to selection for strains possessing insensitivities conferring β-tubulin substitutions E19A and L240F and substitution in *CYP51,* which results in fungicide insensitivities to MBC and DMI fungicides (Russell 2005, Carter *et al.* 2013, 2014). Fungicide insensitivity to DMI fungicides has not been reported in field isolates of *L. maculans* or *L. biglobosa* in the UK (FRAG-UK 2017) but there are reports of field isolates of *L. maculans* with DMI insensitivity in Australia (Van de Wouw *et al.* 2017), through a 275bp insertion or long terminal repeat retrotransposons (Yang *et al.* 2020). Laboratory tests also indicate that *L. biglobosa* isolates were less sensitive to DMI fungicides than *L. maculans* isolates (Eckert *et al.* 2010; Huang *et al.* 2011).

One strategy to prevent unnecessary use of fungicides is to apply sprays only when they are needed at crucial stages of the life cycle of a pathogen. Accurate fungicide application timing is paramount for effective fungicide control (Gladders *et al.* 2006). For phoma stem canker, the timing for treating with fungicide is when there is a threshold of 10 to 20% of plants with phoma leaf spot in autumn when the risk is greatest (October-November), to protect plants from development of cankers in June/July (AHDB 2020a). This is followed by another fungicide application when reinfection is observed (November-December). Most UK oilseed rape fungicide programmes include a fungicide application in late winter-spring, in addition to the two autumn applications, against light leaf spot or sclerotinia stem rot (Garthwaite *et al.* 2012;2014;2016;2018). This is to safeguard the crop from secondary infection from *P. brassicae* conidia that would coincide with stem elongation to minimise the spread of disease up the canopy towards the pods (Figueroa *et al.* 1995; Fitt *et al.* 1998). However, because the latent period of light leaf spot is symptomless, lesions may not be observed until the spring (Fitt *et al.* 1998; Gilles *et al.* 2000ab; Boys *et al.* 2007). This would mean

that a spray to coincide with initial *P. brassicae* ascospore release would need to be applied before any symptoms are present in autumn (Gilles *et al.* 2000ab, Boys *et al.* 2007; Evans *et al.* 2017). If a grower applies a fungicide when light leaf spot symptoms become visible, it may result in an unnecessary application of fungicide that may help to select for fungicide-insensitive strains of *P. brassicae* because fungicides have limited efficacy when utilised curatively (Boys *et al.* 2007, Carter *et al.* 2014; King *et al.* 2018).

However, Evans *et al.* (2017) provided evidence to suggest that due to the polycyclic nature of *P. brassicae* infection, there is a 'chaotic' infection scenario in the crops, resulting in a continuous infection of new oilseed rape leaf material (Figure 1.10). This would suggest that yield responses to fungicides are observed even though light leaf spot is present in the crop, because the fungicides are providing protection during the 'chaotic' light leaf spot epidemic pattern shown in Figure 1.10. To reduce the considerable reliance on azole fungicides, there is a need to increase the use of products with alternative modes of actions such as quinone outside inhibitor (QoI) or succinate dehydrogenase inhibitor (SDHI) fungicides (Sewell *et al.* 2016; FRAG-UK 2017). Reliance on azoles is likely to have contributed to the selection for *P. brassicae* strains with decreased sensitivity to azoles (Carter *et al.* 2014) and this was observed previously for MBC fungicides (Russell 2005; Carter 2013;2014). Widespread field insensitivities to azole or MBC fungicides have not been detected in *L. maculans,* although isolates with azole insensitivity have been detected (Huang *et al.* 2011; van de Wouw *et al.* 2017; FRAG-UK 2017). Insensitivity to SDHIs or QoIs has not been identified in either *P. brassicae* or *L. maculans* (Carter *et al.* 2014; Sewell *et al.* 2016; FRAG-UK 2017; van De Wouw *et al.* 2017). There is evidence to show that applying two fungicides with alternative modes of action in alternation or as a mixture is more effective at slowing the selection for fungicide-insensitive strains than reliance on the repeated use of a fungicide product with a single mode of action (van den Bosch *et al.* 2014). This is a strategy that has been adopted by many agro-chemical companies, such as Bayer, Corteva Agroscience and BASF. These companies have developed commercial fungicide products that are co-formulations or recommended tank mixes of two or more active ingredients that have differing modes of action (e.g. Bayer's Skyway 285 Xpro which contains bixafen (SDHI), prothioconazole (DMI) and tebuconazole (DMI)). One product

that was gaining importance in the market was DuPont Refinzar™, a mixture of picoxystrobin (QoI) and penthiopyrad (SDHI), appearing as the fifth most commonly used formulation in the UK (Garthwaite *et al.* 2016). However, there have been changes in EU legislation that have resulted in the withdrawal of many active ingredients from the pesticide register including picoxystrobin (EU, 2017/1455/EC). Therefore, after 30 November 2018, Refinzar™, along with 11 other fungicides, could not be applied (HSE, 2020). This results in reduced options, from an already limited range of fungicides exploiting alternate modes of action, leaving mainly azole fungicides, such as Proline 275, available to growers for control of oilseed rape diseases, promoting the positive selection towards azole insensitivity and reducing effectiveness of chemical control of diseases in oilseed rape.

1.10.2 Cultivar resistance

Cultivar resistance is an economical and environmentally friendly method to control diseases. There are two types of cultivar resistance; quantitative, which is controlled by multiple genes to give 'partial' resistance and provides protection against the development and progression of the disease throughout the host, whereas major resistance (*R*) gene-mediated qualitative resistance is controlled by a single gene and provides 'complete' resistance, by following a gene for gene interaction (Huang *et al.* 2014a) explained by Flor (1971); therefore *R*- genes are isolate-specific (Huang *et al.* 2018) allowing an incompatible combination to occur (Fig. 1.4). *R*- genes provide resistance by recognising pathogen effectors during the infection process in the leaves to inhibit early infection, whereas quantitative resistance is responsible for reducing further development and progression of the pathogen throughout the plant. Due to the reproductive systems and dispersal ability of pathogens, they have large evolutionary potential (McDonald and Linde 2002). *R*-genes and quantitative resistance have been identified against *L. maculans* (*e.g. Rlm1,2,3,4,6,7,9,11*) or *P. brassicae* (*e.g. PBR1 and PBR2*) but not against *L. biglobosa* (Bradburne *et al.* 1999; Boys *et al.* 2007;2012; Rouxel and Balesdent 2017; Karandeni-Dewage *et al.* 2018). *R*-gene resistance is less durable than quantitative resistance due to its reliance on recognising a specific effector (*Avr*) gene product, so mutation would render the *R*-gene ineffective. (Sprague *et al.* 2006;

Carter *et al.* 2014; Mitrousia *et al.* 2018) (Figure 1.11). These changes in virulence to *R*-genes create 'Boom and Bust' cycles of crop production, whereby cultivars with qualitative durable resistance unexpectedly break down and severe epidemics ensue (Rouxel and Balesdent 2017; Mitrousia *et al.* 2018). In 2000, *Brassica sylvestris*-derived resistant (functionally like *Rlm1*) oilseed rape cultivars were developed and released in Australia. They had an Australian blackleg (phoma) resistance rating of 9, in a 0 – 9 scale whereby 1 is susceptible and 9 is highly resistant (Sprague *et al.* 2006); this resistance was controlled by a single dominant gene. However, by 2002, due to the rapid evolution of virulence of *L. maculans* to the *Brassica sylvestris* derived resistance gene, the resistance broke down and a severe epidemic ensued (Sprague *et al.* 2006). There is good evidence to suggest that *R* gene efficacy and durability can be prolonged by combining *R*-genes and quantitative resistance against *L. maculans in* oilseed rape (Brun *et al.* 2010; Huang *et al.* 2018). Conventional plant breeders have favoured breeding for *R*-gene disease resistance rather than quantitative resistance as it is easy to screen and select for because it has a Mendelian inheritance pattern. Additionally, it provides complete rather than partial control against the pathogen (Karandeni-Dewage *et al.* 2018). Phenotyping of young plants can reliably select *R* gene-mediated qualitative resistance, whereas quantitative resistance screening is more complex and time-consuming (Huang *et al.* 2014a). These methods could be even more effective if they were aligned with *R*-gene deployment strategies (Bousset *et al.* 2018). These policies would enable different *Rlm* genes to be used in space and time, endeavouring to prolong the durability of these *Rlm* genes (van den Bosch and Gilligan 2003; Marcroft *et al.* 2004; Gladders *et al.* 2006; Mitrousia *et al.* 2018).

1.10.3 Cultural control

There are many different cultural control strategies available to growers to reduce their vulnerability to phoma stem canker and light leaf spot. The main four strategies are change of sowing date, longer rotations between oilseed rape crops, removal and decreased proximity to infected crop stubble, and the use of biological control.

Changing the sowing date is a cultural control strategy that helps to reduce the exposure of the crops to ascospores of pathogens that cause the predominant autumn foliar diseases such as phoma leaf spot or light leaf spot. Ascospores of *L. maculans*, *L. biglobosa* and *P. brassicae* are mainly released in the autumn (Huang *et al.* 2005; 2011; Evans *et al.* 2017). Consequently, if light leaf spot is the dominant disease, then a later sowing date may be more favourable because it would avoid exposure of the crop during major periods of *P. brassicae* ascospore release. However, the crop would be more susceptible to the onset of phoma leaf spotting because the crop would not be as well established when *L. maculans* ascospores are released, so there is an increased risk of stem cankers (Aubertot *et al.* 2004; FRAG-UK 2017) because the leaves and petioles are shorter when plants are small in the autumn so the pathogen would reach the stem more rapidly (Sun *et al.* 2001). Additionally, the disease is less damaging and it is easier to control phoma leaf spot on larger plants (FRAG-UK 2017). Susceptibility to pathogen epidemics is not the only factor affecting the choice of sowing date; other factors are also involved, for example UK growers in the east of England are tending to sow their crops earlier to counter likely cabbage stem flea beetle attacks (Farmers Weekly 2016). Also, oilseed rape usually follows a wheat crop, so the sowing date is dependent on the harvest date of the wheat crop. The 2016/2017 northern England wheat harvest was greatly delayed due to rainfall which meant that many oilseed rape crops were sown later than growers would have liked, therefore increasing the susceptibility of the crop to the phoma leaf spot pathogen, but potentially decreasing the susceptibility to the light leaf spot pathogen (Aubertot *et al.* 2004; Welham *et al.* 2004; Farmers Weekly 2015).

The pathogens survive from one growing season to the next using their ascocarp structures on infected debris; pseudothecia and apothecia for *L. maculans and P. brassicae,* respectively; these release the inoculum for initial infections in newly emerging crops (Gladders and Musa 1980; Gilles *et al.* 2001abc; Khangura *et al.* 2007). Therefore, it is of paramount importance to attempt to reduce the inoculum reaching the emerging crop the following season. One strategy is to increase the duration of rotations because oilseed rape rotations are often shorter than ideal (FRAG-UK 2017). Increased length of rotations reduces the probability of infected debris being present

when the next crop is sown (Krupinsky *et al.* 2002). This strategy is especially important for soil-borne diseases, such as clubroot and sclerotinia stem rot, caused by *Plasmodiophora brassicae* and *Sclerotinia sclerotiorum,* respectively (FRAG-UK 2017). Another strategy is to reduce the proximity of the new crop to the previous crop. Ascospores are wind-dispersed, so have the potential to travel large distances; thus it is recommended that new crops are not planted adjacent to the stubble from the previous season and preferably more than 200-500m away (Marcroft *et al.* 2004; FRAG-UK 2017). Additionally, infected crop debris can be buried by ploughing to remove inoculum from subsequent seasons (Bailey and Lazarovits 2003). However, ploughing affects the soil rhizosphere which plays an integral but very complex part in ensuring healthy crop growth. The rhizosphere flora can reduce the amount of pathogen inoculum due to sequestration, antibiosis, competition or direct contact with pathogens (Lemtiri *et al.* 2016).

1.11   Disease modelling and forecasting

Disease models are computational programmes/equations that aim to simulate the epidemic cycle of a disease at a variety of scales and situations using knowledge of the pathogen and its lifestyle and interactions, in addition to information/data inputted into the model, such as number of spores, environmental conditions, cultivar selection and total growing area (van den Bosch and Gilligan 2003). If the data entered are current, relevant and accurate, it is possible to input forecast scenarios for the next season and forecast future disease severities using the outputs. They are therefore valuable methods available to growers that enable them to make well-informed decisions about cultivar selection for the next growing season, as well as assisting with fungicide spray timing throughout the season to ensure fungicides are well timed, to help avoid unnecessary sprays (Gladders *et al.* 2006). There are crop management decision support tools, including disease support, available to growers in the UK, such as AHDB Phoma Leaf Spot Forecast (AHDB 2020a) (Figure 1.12).

This tool provides visual regional risk predictions for phoma stem canker, light leaf spot, alternaria and sclerotinia. Additionally, there is a developing feature that provides local

risk predictions for crop management, including disease, but it also provides accessory information for spraying such as 5-day spray weather forecast and guidance on product selection (Fera 2020a). The AHDB also produces an annual preliminary light leaf spot forecast in the autumn to predict the proportion of the oilseed rape crop (AHDB LLS resistance rating of 5) estimated to have more than 25% of plants affected by light leaf spot in the next spring of the current season (Figure 1.13). This forecast is based on Welham et al. (2004). However, due to the polycyclic nature and the very long latent period there is no standardised threshold for fungicide application, like for phoma. The advice is to apply fungicide when symptoms are apparent, to control the spread of the secondary conidial infection process.

In practice, it is very difficult to create a 'perfect' forecasting model due to the unpredictable nature of the environment. These models are dependent upon the knowledge and understanding of the life cycles of pathogens and their interactions under a range of environmental conditions. It is difficult to know and understand these pathogens completely, so there will always be a degree of unreliability in every model, but more research that helps to build up the bank of knowledge and understanding about the pathogens will help to improve the accuracy and reliability of these models.

1.12   Rationale

There is a wide range of literature and understanding about the two phoma stem canker pathogens (*L. maculans* and *L. biglobosa*) and the light leaf spot pathogen (*P. brassicae*), as well as their development and interactions with oilseed rape (*Brassica napus*) individually.   However, there has been limited work investigating the interactions between those pathogens at key stages of their life cycles even though symptoms caused by the three pathogens can be found together on the same plant throughout the growing season. The interactions between the three pathogens during leaf infection and ascospore production are still unknown. Recent research suggests that cultivars with good resistance against *L. maculans,* particularly those possessing the *Rlm7* resistance gene, are susceptible to *P. brassicae* (Huang *et al.* 2021). These *Rlm7* crops have grown in popularity between 2012/13 and 2015/16, when the percentage of UK cropping area

made up of these cultivars increased from 5% to >15%, respectively (Mitrousia *et al.* 2018). There is a correlation between the increase in UK cropping area with *Rlm7* cultivars and the recent increase in incidence and severity of light leaf spot in England. However, a correlation does not mean a causation, and other factors may be involved, especially accuracy of spray timings.

Consequently, it is fundamental for future integrated control strategies that more work needs to be done to investigate the unknown interactions and identify potential unintentional effects between these economically important fungal pathogens of oilseed rape at key stages of the growing season. Therefore, this PhD project aims to understand the interactions between *Leptosphaeria maculans, L. biglobosa* and *Pyrenopeziza brassicae in-vitro* and *in-planta* to improve integrated pest management strategies against phoma stem canker and light leaf spot diseases on oilseed rape (*Brassica napus*).

1.13   Hypothesis and objectives

There are interspecific interactions between *Leptosphaeria maculans, L. biglobosa* and *Pyrenopeziza brassicae.*

This hypothesis will be tested through the investigation of three related objectives.

1. To investigate the interactions between phoma stem canker (*L. maculans and L. biglobosa*) and light leaf spot (*P. brassicae*) causal pathogens *in vitro* (Chapter 3).

2. To study interactions between phoma stem canker (*L. maculans* and *L. biglobosa*) and light leaf spot (*P. brassicae*) causal pathogens *in planta* (Chapter 4).

3. To examine interactions between *L. maculans* and *P. brassicae* on different cultivars under field conditions (Chapter 5).

Figure 1.12: Phoma leaf spot forecast 2019/20 in the UK. Pointer symbols provide forecast information for that location. There were four colours of pointers to indicate the infection status. Green = No symptoms, Yellow = infection has started, Red = 10% threshold has been met, and grey = No data (Evans et al. 2008; AHDB 2020a).

Figure 1.13: AHDB Light leaf spot forecast. Preliminary 2019/20 forecast in the UK (excluding Northern Ireland) provided in the autumn (left) and the final forecast provided in the spring (right). The map is split into nine region and colour coded from green to red (low-high) depending on the proportion of the oilseed rape crop (AHDB LLS resistance rating of 5) estimated to have more than 25% of plants affected by light leaf spot in the spring of the current growing season (Welham et al. 2004; AHDB 2020b).

Chapter 2 Interactions between phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) causal pathogens *in vitro*.

2.1   Introduction

All organisms require nutrients to live. Initial resources are provided by the parent for early development; but for further development, growth, survival, and reproduction nutrients need to be acquired (Grover 1997). Resource conservation rules state that resources acquired by one organism are not immediately available to another, so if there is a limited supply of resources, competition will arise – resource competition (Grover 1997; Abdullah *et al.* 2017; Dutt *et al.* 2021). Interactions between pathogens and host plants do not occur in isolation; a host is likely to interact with many different species or genotypes within a species (Kozanitas *et al.* 2017). There will be resource competition between individuals of the same species (different genotypes), called intraspecific competition, and competition between different species, called interspecific competition (Hortal *et al.* 2016; Dutt *et al.* 2021). Interspecific competition arises due to the principle of competitive exclusion that states that 'no two species can share the same limiting resource indefinitely', which results in the exclusion of the inferior competitor (Gausse 1934; Hardin 1960; Fitt *et al.* 2006a).

There are three major mechanisms for interspecific competition. One is exploitative competition - when a limiting resource is available to all competitors, the competing species that can most efficiently conquer the resources making it unavailable to the competing pathogen and therefore out-competition occurs (Tan *et al.* 2016; Dutt *et al.* 2021). Another mechanism is interference competition in which a species inhibits its competitors access to the resource using toxins, that may directly inhibit the

development, reproduction or the transmission of the co-infected pathogen on a common host, or by providing a physical barrier such as a biofilm (Dutt *et al.* 2021). The other mechanism is host-mediated, where the host's defence response is induced after the development of one pathogen leading to the competing pathogens having a reduced survival and infection success (Liu *et al.* 2006; Aime *et al.* 2013; Dutt *et al.* 2021).

Phoma stem canker, caused by co-existing fungal pathogens *L. maculans* and *L. biglobosa,* and light leaf spot, caused by *P. brassicae,* produce combined average UK annual yield losses of > £165M (CropMonitor 2020). *L. maculans and L. biglobosa* have been shown to grow in the same region, and light leaf spot is often found on the same plant and in the same region colonised by *Leptosphaeria spp.* (Fig. 2.1). Competition can be minimized if the species occupies a different niche. *L. maculans, L. biglobosa* and *P. brassicae* are all apoplastic pathogens but the niches they occupy are eventually different; *L. maculans and L. biglobosa* colonise space between host mesophyll cells and *P. brassicae* colonises the space under the cuticle (Fitt *et al.* 2006ab; Stotz *et al.* 2014).

L. maculans is known to produce a non-host selective epipolythiodioxopiperazine (ETP) called sirodesmin PL (Rouxel *et al.* 1988), which has been shown to have an inhibitory effect on *L. biglobosa* conidial growth (Elliott *et al.* 2007). This suggests that sirodesmin PL may be used as a form of interference competition by *L. maculans. L. maculans* and *L. biglobosa* lesions are associated with the stem base and upper stem lesions, respectively (Fitt *et al.* 2006b). The difference in stem lesion location has been suggested to be due to differences in ascospore maturation timing (Toscano-Underwood *et al.* 2003). However, *L. maculans* and *L. biglobosa* have both been found at the stem base (Huang *et al.* 2014b), suggesting that *L. biglobosa* has a larger fundamental niche due the physiological capability to infect the same tissues as *L. maculans.* However, interspecific interactions resulting from *L. maculans* and sirodesmin PL may result in a realised niche; *L. biglobosa* forms characteristic upper stem lesions (Fitt *et al.* 2006ab).
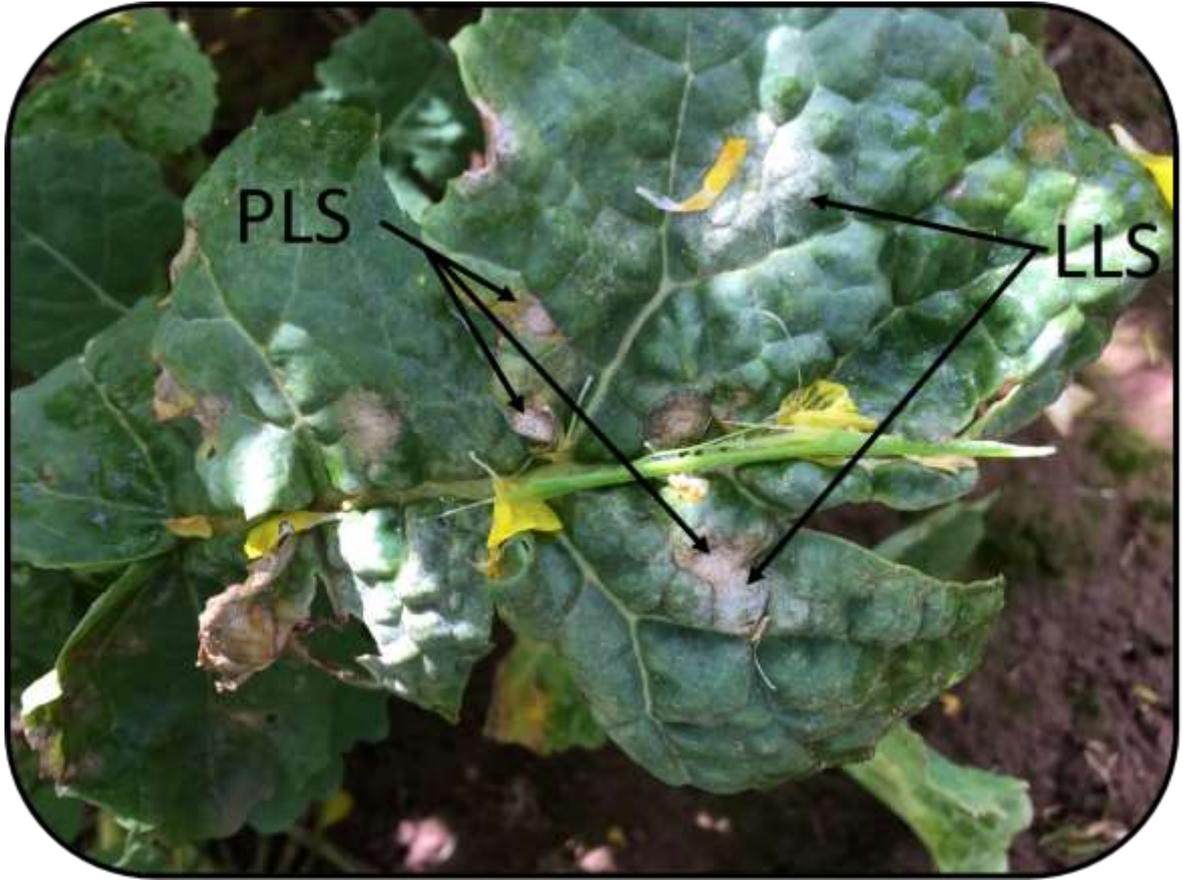
Figure 2.1: Phoma leaf spot (PLS) and light leaf spot (LLS) on the same oilseed rape leaf from a susceptible cultivar (cv. Charger) in Herefordshire in May 2018.

Sirodesmin PL has been shown to be found throughout the plant and at the stem base. It has been suggested that sirodesmin PL is important during the asymptomatic stages of *L. maculans* growth through the plants vascular system via the leaf petiole and stem to the stem base. (Elliot *et al.* 2007). Therefore, if Sirodesmin PL is released whilst moving through the xylem or phloem of the plant then it will be transported around the plant to all tissues so may interact with other competing pathogens despite them occupying different niches (such as *P. brassicae* that colonises the area under the cuticle (Fig 2.1)). This would potentially provide an interspecific competitive advantage against other disease causal pathogens that infect the plant in different tissues and at different times, such as *P. brassicae*, *Sclerotinia sclerotiorum*, *Verticilium longisporum* and others, if they are susceptible to sirodesmin PL.

Recently, simultaneous ascospore release of the two *Leptosphaeria* spp*.* has been recorded. *L. biglobosa* ascospores have been shown to occur at the same time as those of *L. maculans* (Huang *et al.* 2019); the faster colonisation by *L. biglobosa* (Shoemaker and Brun, 2001; Huang *et al.* 2003)*,* which is an example of exploitation competition, may give *L. biglobosa* an advantage over *L. maculans* if ascospore leaf infection started at the same time, since sirodesmin PL takes ~ 3 days to be produced *in vitro* (Gardiner *et al.* 2004) (Fig. 2.2). There has been very limited work investigating sirodesmin PL *in planta,* and it is very difficult to detect sirodesmin PL *in planta*, so sirodesmin PL time series have not been produced.

Even though these pathogens eventually occupy different niches, their life cycles are both initiated by ascospores released in the autumn from fruiting bodies on infected host debris from the previous season (Gladders and Musa 1980; Gilles *et al.* 2001ab; Toscano-Underwood *et al.* 2003). Recently, it has been shown that the release of ascospores of all three fungal pathogens (*L. maculans, L. biglobosa* and *P. brassicae*) can occur at the same time (Evans *et al.* 2017; Huang *et al.* 2019)*.* Thus, albeit temporarily, interspecific competition for host resources between these pathogens may arise on the leaf surface or very early in leaf infection before host colonisation (Dutt *et al.* 2021).
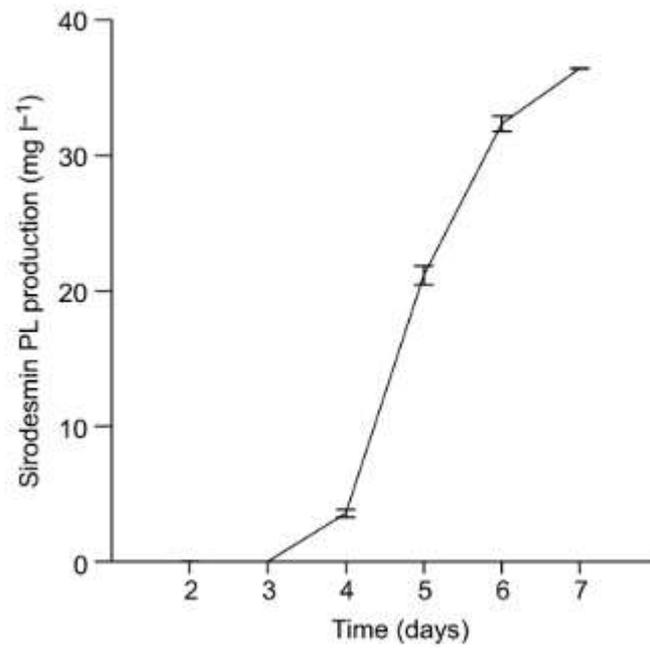
Figure 2.2. Production of sirodesmin PL, the non-host-selective phytotoxin during *in vitro* growth of *Leptosphaeria maculans* in 10% Campbell's V8 juice over 7 days (Gardiner *et al.* 2004).

Therefore, this Chapter aims to identify interspecific interactions between *L. maculans*, *L. biglobosa* and *P. brassicae in vitro* and identify potential consequences of these increased interspecific interactions on other major oilseed rape pathogens.

This study aim to investigate the interactions between phoma stem canker (*L. maculans and L. biglobosa*) and light leaf spot (*P. brassicae*) causal pathogens *in vitro* by testing the following four
hypotheses ;

1. *Leptosphaeria* spp. and *P. brassicae* can be effectively co-cultured on the same medium and incubated at the same temperature.
2. The *P. brassicae* colony morphology is affected by being co-cultured with *Leptosphaeria* spp.
3. The effect of secondary metabolites from either solo, simultaneous, or sequential inoculation when applied to cultures of oilseed rape pathogens differs.
4. The composition of secondary metabolites from either solo, simultaneous, or sequential inoculation are different.

These hypotheses were tested by addressing the following three main objectives:

1. To identify the interspecific competition strategies of *L. maculans* or *L. biglobosa* and *P. brassicae* by assessing changes to colony morphology when combinations of these pathogens are grown under resource competition.
2. To investigate the effects of secondary metabolites on mycelial growth of *L. maculans*, *L. biglobosa* and *P. brassicae* and other oilseed rape pathogens *S. sclerotiorum* and *V. longisporum.*
3. To detect differences in secondary metabolite composition between the three fungal pathogens using HPLC and LC-MS.

2.2   Materials and methods

2.2.1   Inoculum preparation

The four isolates that were used throughout this project are shown in (Table 2.1). To avoid continual / sequential sub-culturing of each isolate (Table 2.1), each isolate was initially cultured on PDA, then multiple fungal plugs were made and stored at 4°C until needed. For *L. maculans* and *L. biglobosa*, cultures were incubated at 20°C in the dark, then fungal plugs were made from the outer colony edge of each initial culture after 10 days using an 8 mm corer. For *P. brassicae* isolates, cultures were incubated at 15°C in the dark, then individual *P. brassicae* colonies were removed using a 6 mm corer after 15 days. Smaller plug sizes were used for *P. brassicae* than for *Leptosphaeria* spp. to reduce the time taken to create enough inoculum because it is a slower growing pathogen than both *Leptosphaeria* spp. For each new experiment, up to three fungal plugs were removed from storage. Plugs were placed mycelial side down on either V8 or V8* agar for 7-12 days to bulk up inoculum. Inoculum required for the experiment was taken from the bulked-up sub-cultured plates.

2.2.2   Identification of optimal growth media for *L. maculans*, *L. biglobosa* and *P. brassicae in vitro.*

The optimal growing media were tested by culturing *L. maculans* (ME24), *L. biglobosa* (WH17 Why-1) and *P. brassicae* (17WOSR and 15WOSR-64-SS1) (Table 2.1) on a range of media; malt extract agar (MEA), potato dextrose agar (PDA), V8 juice agar (V8), clarified V8 juice agar (V8*) and water agar (WA) in triplicate (Table 2.2). Recipes can be found in Appendices 5. Fungal plugs were placed in the centre of the respective agar plate according to the treatment list. For *Leptosphaeria* spp.*,* 10mm diameter fungal plugs from V8 agar were made, whereas for *P. brassicae* 6 mm diameter fungal plugs from MEA were made.

Table 2.1: Isolates of *L. maculans*, *L. biglobosa* and *P. brassicae* used.

Pathogen species	Name of isolate	Origin of isolate	Reason	Acquired from
L. maculans	ME24	Stem canker on cultivar Apex, Darrington, West Yorkshire in 2002	Used in previous work. Avirulent on *Rlm7*	OREGIN isolate collection (https://www.herts.ac.uk/oregin) (Maria Eckert)
L. biglobosa	WH17-Why-1	Leaf lesion on cultivar Whiskey, Woodhall, Hertfordshire in 2017	Fast growing representative isolate that has been used in previous work.	Dr Harika Gajula
P. brassicae	17WOSR-CUI	Single conidial isolate from cultivar Cuillin, Morley, Norfolk in 2017. It was inoculated onto Cuillin and re-isolated.	Isolate found on a resistant cultivar. A lab friendly isolate used in the lab.	Dr Chinthani Karandeni-Dewage
P. brassicae	15WOSR-64-ss1	Single conidial isolate from cultivar Bristol, Hereford in 2015	Isolate that is being used for genome sequencing work. A lab friendly wild type isolate.	Dr Chinthani Karandeni-Dewage

Table 2.2: List of media that were used to identify optimal growth medium for _L. maculans_, _L. biglobosa_ and _P. brassicae_

Treatment	Medium*	Replicates
1	MEA	3
2	PDA	3
3	V8	3
4	V8*	3
5	WA	3

*MEA = Malt extract agar

PDA = Potato dextrose agar

V8 = 20% V8 agar

V8* = 20% Clarified V8 agar

WA = Water agar

(See Appendices 5 for recipes)

Plates were sealed using Parafilm and incubated at the optimal temperatures for each pathogen, with 20 $\pm$ 1°C for *Leptosphaeria* spp. and 16$\pm$ 1°C for *P. brassicae* in continuous darkness (Gilles *et al.* 2001c; Huang *et al.* 2001). The maximum colony diameter on each plate was measured in three different directions at 7- and 23-days post inoculation.

2.2.3 Comparison of morphology of *L. maculans, L. biglobosa* and *P. brassicae* colonies under resource competition *in vitro.*

2.2.3.1 Morphological changes in *L. maculans*, *L. biglobosa* and *P. brassicae* colonies under resource competition at different temperatures.

The colony morphology changes were investigated using PDA and V8* media. Fungal plugs of *L. maculans* (ME24) (Lm) or *L. biglobosa* (WH17) (Lb) (both 10 mm diameter) and squares of *P. brassicae (*17WOSR) (Pb) (5 mm diameter) were used. There were five combinations of either solo or dual cultures (Lm, Lb, Pb, Lm + Pb and Lb + Pb), at two temperatures (15°C and 18°C) and on two different media (V8* and PDA) (Table 2.3). There were 20 treatments. Each treatment was done in triplicate. Therefore, there was a total of 60 plates. Fungal plugs within dual cultures were placed 30 mm apart. The dual cultures were monitored weekly and visual assessments of colony shape were made at 14- and 25-days post inoculation.

Additionally, *P. brassicae* colony shape was assessed by measuring the colony growth from the centre of the fungal plug to the outer edge of the fungal plug towards (GT) or away (GA) from the competing pathogen. A percentage of growth towards the pathogen (%GT) was calculated (%GT = GT ÷ GA x 100). Due to the *L. maculans* and *L. biglobosa* colonies reaching one edge of the plate before the other, symmetry could not be measured so analysis focussed on the *P. brassicae* data.

Table 2.3: List of treatments to investigate interactions between *L. maculans, L. biglobosa and P. brassicae* used for dual culture on PDA and V8*.

Inoculum treatment	Pathogen	Temperature (°C)	Medium*	Replicates
1	Lm only	15	PDA	3
2	Lb only	15	PDA	3
3	Pb only	15	PDA	3
4	Lm vs Pb	15	PDA	3
5	Lb vs Pb	15	PDA	3
6	Lm only	18	PDA	3
7	Lb only	18	PDA	3
8	Pb only	18	PDA	3
9	Lm vs Pb	18	PDA	3
10	Lb vs Pb	18	PDA	3
11	Lm only	15	V8*	3
12	Lb only	15	V8*	3
13	Pb only	15	V8*	3
14	Lm vs Pb	15	V8*	3
15	Lb vs Pb	15	V8*	3
16	Lm only	18	V8*	3
17	Lb only	18	V8*	3
18	Pb only	18	V8*	3
19	Lm vs Pb	18	V8*	3
20	Lb vs Pb	18	V8*	3

*PDA = Potato dextrose agar

V8* = Clarified 20% V8 agar

(See section 2.1.1 for recipes)

2.2.4 Gliotoxin standard curve.

2.2.4.1 Rationale for using gliotoxin

Gliotoxin is produced by *Aspergillus fumigatus*, as well as *A. terreus, A. flavus, A.niger, Penicillium terlikowskii* and *Trichoderma virens*. Gliotoxin is the best characterised epipolythiodioxopiperazine (ETP); the same class of fungal secondary metabolite as sirodesmin PL. Gliotoxin has also been used in other experiments to help identify the presence of sirodemsin PL using a Gliotoxin standard curve and has been shown to have a high recovery efficiency (91±7%) (Gardiner et al. 2004). Assumptions were made that it would act similarly to sirodesmin PL and throughout this experiment using a similar extraction and analytical method.

2.2.4.2 Serial dilutions of gliotoxin samples for HPLC analysis.

Gliotoxin samples were prepared for a 5 mg/mL stock. Using a serial dilution series, 1mL samples with concentrations of 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL were created. Gliotoxin samples were suspended in ethyl acetate. 200 μL from each stock suspension were pipetted into clean HPLC vials containing glass inserts. The maxima areas for each concentration were plotted to create a standard curve. The 0.01 and 0.05 mg/mL points were removed because the maxima were too small to be confidently distinguished from the background noise/maxima (Fig. 2.4).

2.2.5 Identification of secondary metabolite composition using HPLC

The secondary metabolite compositions in the liquid culture extracts were analysed using a Shimadzu Prominence HPLC machine (Japan) with a diode array detector (SPD-M20A) using 20 μL injections modified from Smedsgaard (1997). Separations were done on a C_{18} column (Varian pursuit 5μm 150 x 4.6 mm) (Fig. 2.3). A linear gradient starting from 85% water and 15% acetonitrile going to 100% acetonitrile in 40 min, then maintaining 100% acetonitrile for 3 min before starting a linear gradient back to 85% water over 5 min. It was then held at 85% water before starting the next sample. Each sample had a total run time of 53 min. A flow rate of 1 mL/min was used. Chromatographs were visualized from 190-400 nm.

Figure 2.3: Shimadzu Prominence HPLC machine with various component units. Degassing Unit (DGU-20A5R) (1), Liquid Chromatograph (LC-20AD) (2), Autosampler (SIL-20AC HT) (3), Communications BUS module (CBM-20A) (4), Column oven (CTO-20AC) (5) and a diode array detector (SPD-M20A) (6).
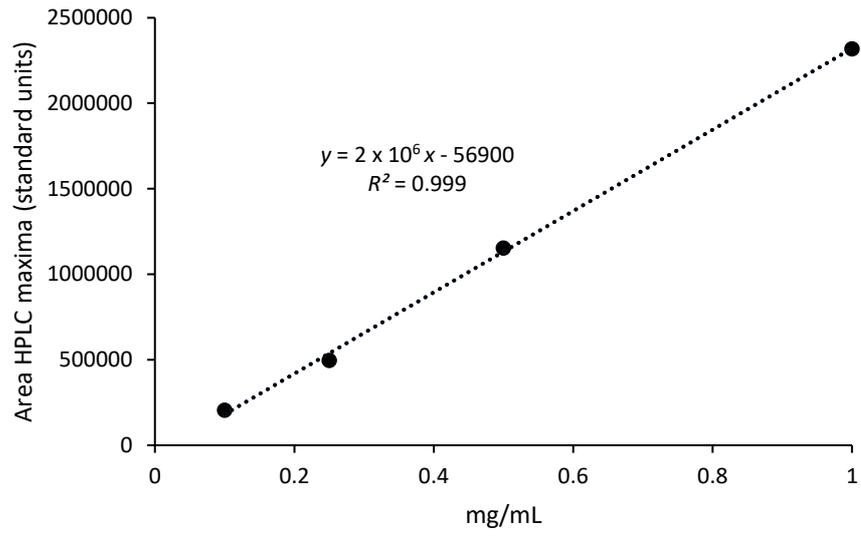
Figure 2.4: Gliotoxin standard curve using area of gliotoxin maximium on HPLC when samples of different concentrations were analysed.

Results were visualized at 254 nm using Lab solution version 5.92 (Shimadzu corporation). All samples and chemicals were passed through a 0.45 µM nylon microfilter to remove any insoluble material before being analyzed.

2.2.6 Identification of maxima of interest found on the chromatograms from extracted secondary metabolites using LC-MS.

Individual 1.5 mL fractions were taken for each of maxima of interest. Fractions identified using a Waters I-Class UPLC system coupled to a Xevo Micro TQ-S mass spectrometer were used for analysis. Chromatographic separation was done on a Waters BEH C18 column (2.1 mm x 50 mm, 1.8 µm) held at 40 °C with mobile phase A = 0.2 % formic acid in water, and mobile phase B = acetonitrile. The flow rate was set at 0.4 mL/min and a gradient separation done by ramping initial starting conditions of 85 % A to 0 % A over 9 min, holding 100 % B for 1 min, returning to starting conditions of 85 % A over 0.1 min, and holding 85 % for 4.9 min to re-equilibrate the column. The injection volume was 10 µL. A full mass scan was done in positive ion mode over the range 50 – 650 m/z using a scan time of 0.2 sec. Probe capillary voltage was 3 kV, cone voltage was fixed at 20 V, and collision energy was fixed at 3 V. Desolvation gas flow was set at 1000 L/Hr, with a temperature of 500 °C, and a cone gas flow of 150 L/Hr was used, with source temperature set at 150 °C.

2.2.6.1 Quantification of gliotoxin samples using HPLC analysis

The prepared samples were run on the HPLC following the same HPLC methodology outlined in section 2.2.5. The area for the maximum of interest that related to gliotoxin was recorded for each concentration three times and an average was calculated. The mean maximum area for each concentration was plotted on a line graph and the equation of the line was calculated being $y = 2E+06x - 56900$ with an $R^2 = 0.999$ (Fig. 2.4). The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations 2.1 and 2.2. The level of detection (LOD) and level of quantification (LOQ) were 70 and 200mg/L, respectively. The expected range concentration of sirodesmin *in vitro* is *c*. 50 mg/L after 7 days (Gardiner et al. 2004). Due to the limited amount of work

in planta, the expected concentration is unknown. However, it is known to be very low (Candace Elliott*, pers. comm.).*

2.2.7 Concentration of sirodesmin PL and its precursors at regular time intervals between 0-14 days post inoculation.

2.2.7.1 - Liquid culture growth conditions, experiment design and secondary metabolite extraction.

Three plugs of *L. maculans* (8 mm diameter) were cultured in 250 mL conical flasks containing 75 mL of clarified 20% V8 juice broth in a rotary shaker at 80RPM and 18°C for 14 days in continuous darkness.

Equation 2.1 - Equation for calculating the limit of detection (LOD)

$$\text{Equation 2.1} \quad LOD = \left(\frac{STEYX}{Slope}\right) \times 3.3$$

Equation 2.2 – Equation for calculating the limit of quantification (LOQ).

$$\text{Equation 2.2} \quad LOQ = \left(\frac{STEYX}{Slope}\right) \times 10$$

STEYX is the standard error of the y-intercept of a regression line.

There were six time points; Day 1, 3, 7, 8, 10 and 14 for Expt 1 and Day 1, 3, 6, 8, 10 and 14 for Expt 2; three samples were assessed at each time and a mean was calculated. The secondary metabolites were extracted as outlined in section 2.2.8.

2.2.8 Secondary metabolite extraction using ethyl acetate

The culture filtrate was separated away from the fungal mycelium using miracloth. Samples of the same treatment were combined into Duran bottles. Then, 75 mL ethyl acetate (EtOAc) was added to the culture filtrate and gently inverted twenty times to increase secondary metabolite uptake, then left so that the liquid phases could settle (1-2 hours). For each treatment, the aqueous phase was pipetted into two 50ml Falcon tubes and centrifuged at 6000 RPM for 5 min then 40 mL of the centrifuged aqueous phase was extracted and combined into two new skirted 50ml Falcon tubes (20 mL in each). This was repeated for each treatment. Once all secondary metabolite extractions were completed, the EtOAc was evaporated under a constant stream of nitrogen using a sample concentrator. The dried metabolites were re-suspended in 1.5 mL of EtOAc. Falcon tubes containing the same treatments were combined into either sterile glass bijoux flasks or sterile plastic sample bottles and kept at 4°C until required (Fig. 2.5).

2.2.9 Identification of secondary metabolite composition using HPLC analysis and LC-MS.

HPLC analysis was done as outlined in section 2.2.5. The expected pattern of maxima for deacetylsirodesmin PL, phomamide and sirodesmin PL were identified using information from Pedras et al. (1998). The two fractions that corresponded to maxima of interest were analysed using LC-MS, as outlined in section 2.2.6, to confirm the identities of deacetylsirodesmin PL, phomamide, and sirodesmin PL. The concentrations of sirodesmin PL were calculated using a gliotoxin standard curve (R^2 =0.999).

2.2.10 Effects of secondary metabolites derived from simultaneously inoculated liquid cultures on the growth of *L. maculans*, *L. biglobosa* and *P. brassicae in vitro*.

2.2.10.1 Liquid culture growth conditions and experiment design

For each treatment, a total of four plugs (8mm diameter) (co-cultures with two plugs of each fungus) were cultured in 250 mL conical flasks containing 75 mL of clarified V8 juice (V8*) broth in a rotary shaker set at 80RPM and 18°C for 14 days in continual darkness. There were seven treatments (Table 2.4).
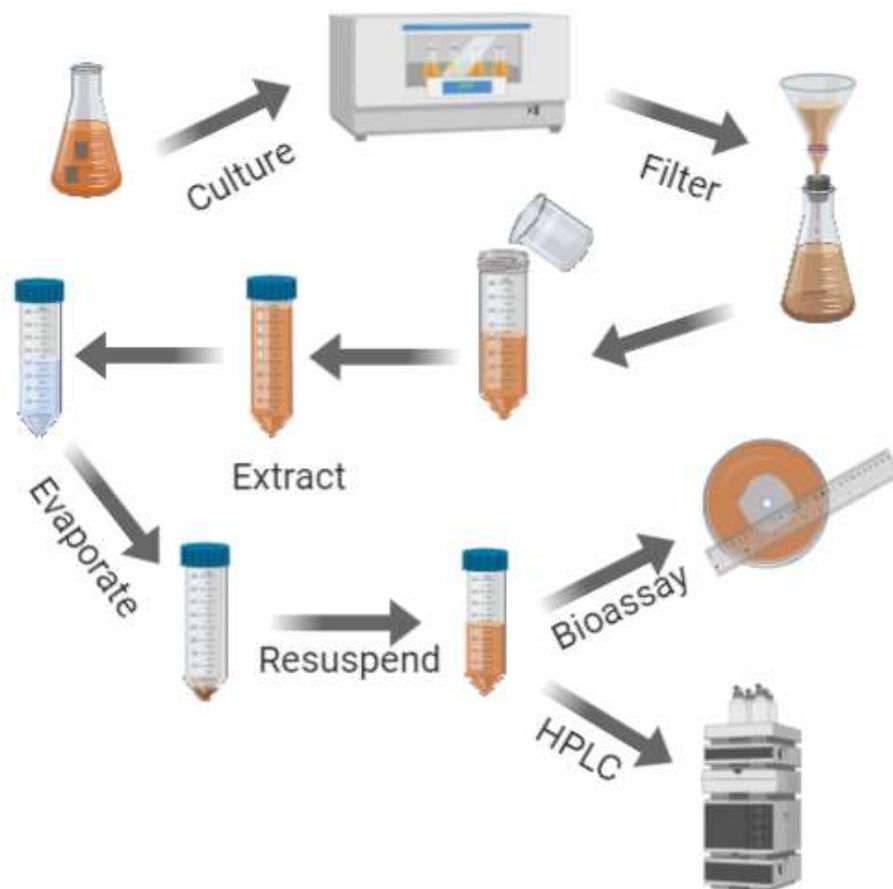
Figure 2.5: Ethyl acetate extraction method used to obtain secondary metabolite extracts for bioassay and HPLC analysis (Created with Biorender.com). Fungal plugs were placed into 75mL conical flasks before being placed into a rotary shaker. After 14 days in the rotary shaker, the mycelial mass was filtered from the culture media using miracloth. Ethyl acetate was added to the culture filtrate and inverted multiple times to extract any secondary metabolites or compounds. The ethyl acetate phase was collected and evaporated under a constant stream of nitrogen. The evaporated sample was resuspended in an equal volume of ethyl acetate. To test the effects of the different metabolites a bioassay was conducted by pipetting 20 uL of this extract on to an agar plate with a fungal pathogen plug. The colony area was calculated. To identify differences in the composition of the extracts they were analysed using analytical chemistry techniques such as HPLC and LC-MS.

Table 2.4: List of treatments for production of secondary metabolites in liquid culture and for plug bioassay for *L. maculans* (Lm), *L. biglobosa* (Lb) or *P. brassicae* (Pb).

Treatment	Description	Replicates	Liquid culture	Plug bioassay
1	Medium only	3	✔	✔
2	Lm only	3	✔	✔
3	Lb only	3	✔	✔
4	Pb only	3	✔	✔
5	Lm&Pb	3	✔	✔
6	Lb&Pb	3	✔	✔
7	Lm&Lb	3	✔	✔
Control	EtoAc	3	✘	✔

Each treatment was done in triplicate and the plates were placed in a randomised position decided using a randomised number generator limited to numbers 1-21.

2.2.10.2 Effects of secondary metabolite extracts from simultaneously co-inoculated liquid cultures on growth of *L. maculans*, *L. biglobosa* and *P. brassicae*

Fungal plugs of *L. maculans, L. biglobosa* and *P. brassicae* on agar (8 mm, 8 mm and 6 mm diameter, respectively) were inoculated onto clarified V8 juice (V8*) agar plates. Smaller plugs were used for *P. brassicae* to reduce the time to obtain enough inoculum because it is a very slow growing pathogen. Each fungal plug was co-inoculated with 20 μL of the corresponding secondary metabolite extract in triplicate. A control of EtoAc was used (Table 2.4). Plates were sealed using Parafilm and incubated at 18°C in continuous darkness.

Due to difficulties in distinguishing mycelium from competing pathogens in liquid culture, work was done on agar plates. Assessments were made at 7- and 10-days post inoculation for *L. maculans* and *L. biglobosa* and 10- days post inoculation for *P. brassicae,* by measuring the maximum colony diameter. Measurements were made later for *P. brassicae* because it is a slower growing pathogen. The maximum colony diameter was measured in three different directions (mm). The mean of these three measurements was used as the mean colony diameter. Using the mean colony diameter, the mean colony area was calculated. Three independent experiments were done.

2.2.11 Effects of secondary metabolites derived from sequentially co-inoculated liquid culture on the growth of *L. maculans* and *L. biglobosa in vitro*

2.2.11.1 Liquid culture growth conditions and experiment design

The growth conditions and experimental design were very similar to those reported in section 2.2.10.1. However, there were only four treatments; Lm only, Lb only, Lm&Lb (simultaneously inoculated) or Lm+Lb (sequentially inoculated). For the Lm+Lb treatment, the liquid media was inoculated with *L. maculans* first, then 7 days later *L. biglobosa* was added. Each treatment was done in triplicate and the plates were placed

in a randomised position decided using a randomised number generator limited to numbers 1-12. Three independent experiments were done.

2.2.11.2 Secondary metabolite extraction using ethyl acetate

The secondary metabolites were extracted as outlined in section 2.2.8.

2.2.11.3 Quantification and identification of unique maxima in secondary metabolites extracts from sequentially co-inoculated liquid cultures of *L. maculans* and *L. biglobosa*

The secondary metabolites were identified and quantified by interpreting results from HPLC (section 2.2.5) and LC-MS (section 2.2.6) analysis and interpreted as outlined in section 2.2.9.

2.2.11.4 Effects of secondary metabolite extracts from sequentially co-inoculated liquid cultures on growth of *L. maculans* and *L. biglobosa*.

The effects of secondary metabolite extracts from sequentially co-inoculated liquid cultures on the growth of *L. maculans* and *L. biglobosa* were investigated as outlined in section 2.2.10.1. However, standardised photographs on each plate were taken and then colony area was calculated using Image J software by drawing round the colony. The calibration factor for number of pixels per cm^2 was calculated using an object of known area photographed using the same standardised settings.

2.2.12 Effects of secondary metabolites derived from simultaneous or sequential co-inoculations of *L. maculans* and *L. biglobosa* on other oilseed rape plant pathogens.

2.2.12.1 Inoculum preparation

S. sclerotiorum was cultured *for* 4 days and *V. longisporum* was cultured for 4 weeks before 8 mm fungal plugs were taken from the leading edge of a colony. The difference in culturing time between pathogens is because *S. sclerotiorum* is a very fast-growing pathogen and starts to for sclerotia after 1 week, whereas *V. longisporum* is a slower growing pathogen. Both pathogens were incubated at 18°C in continuous darkness.

2.2.12.2   Inoculation procedure and experimental design

The growth conditions and experimental design were very similar to those reported in section 2.2.10.1

2.2.12.3   Colony area assessment using ImageJ

The effects of secondary metabolite extracts from solo, simultaneous and sequentially co-inoculated liquid cultures of *L. maculans* and/or *L. biglobosa* (section 2.2.11), on the growth of *S. sclerotiorum* and *V. longisporum* was studied using ImageJ as outlined in section 2.2.11.4.

2.2.13 Statistical analysis

The statistical analysis of the data was done using GenStat (General Statistics) (VSN International 2021). To analyse the difference in colony area between *L. maculans*, *L. biglobosa* and *P. brassicae* on different media, a factorial analysis of variance (ANOVA) was done. To analyse the difference in colony area between *L. maculans*, *L. biglobosa* and *P. brassicae* when different secondary metabolites extracted from simultaneously inoculated liquid cultures were applied, an analysis of variance (ANOVA) was done. To analyse the difference in colony area between *L. maculans* and *L. biglobosa* when different secondary metabolites were extracted from simultaneous or sequentially inoculated liquid cultures were applied, an analysis of variance (ANOVA) was done. To analyse the difference in colony areas between *S. sclerotiorum* and *V. longisporum* when different secondary metabolites extracted from simultaneous or sequentially inoculated liquid cultures were applied, using an analysis of variance (ANOVA). To analyze the differences in phytotoxin production in extracts from simultaneous or sequentially co-inoculated cultures, an analysis of variance (ANOVA) was done. Values $P > 0.05$ were assumed to be significantly different. For balanced and unbalanced designs, a Tukey and Fishers LSD, respectively, post -hoc tests were applied.

2.3 Results

2.3.1 Identification of optimal growth media for *L. maculans*, *L. biglobosa* and *P. brassicae*

There were significant differences in the colony area on different media for *L. maculans* ($F_{4.13}$= 169.37, *P* < 0.001, LSD = 1.63), *L. biglobosa* ($F_{4.13}$= 21.31, *P* < 0.001, LSD = 3.55) and between different isolates of *P. brassicaei*; 15WOSR-64-ssi ($F_{3.10}$= 12.46, *P* =0.003, LSD = 61.0) and 17WOSR-Cul ($F_{3.10}$= 19.05, *P* < 0.001, LSD = 80.8). All three pathogens had significantly larger colony areas when cultured on 20% clarified V8 juice agar than on all other media tested. (Fig. 2.6). For *L. maculans,* the growth medium that resulted in the smallest colony area was water agar (5.6 cm^2), but the colony area was not significantly different from the colony area when cultured on malt extract agar (6.4 cm^2). The colony area when *L. maculans* was cultured on potato dextrose agar (10.4 cm^2) was significantly larger than on WA or MEA; however, it was significantly smaller than when cultured on both V8 agar (15.4 cm^2) and V8* agar (21.2 cm^2), but colony area when cultured on V8 agar was significantly smaller than on V8* agar (Fig. 2.7 & 2.8). The only medium where the colony area was significantly different to when *L. biglobosa* was cultured on MEA (13.6 cm^2) was V8* (23.3 cm^2) that resulted in a significantly larger colony area. Despite there not being a significant difference in colony area between cultures on V8 or MEA, the colony area of *L. biglobosa* on V8 (11.6 cm^2) was significantly smaller than when cultured on WA (15.4 cm*^{2}*) or PDA (15.7 cm*^{2}*) but these colony areas were not significantly different from each other (Fig. 2.6-2.8). It was easier to measure colonies on clarified V8 agar than on V8 agar due to the increased transparency of the media. (Fig 2.7).

For *P. brassicae* isolate 15WOSR-64-ssi, there was no significant difference in colony area when it was cultured on MEA (370 mm^2), PDA (416 cm^2) or V8 (400 cm^2), but the colony area on all three media types were significantly smaller than those on V8* agar (530 cm^2). However, the colony area of 15WOSR-64-ssi isolate on V8* was significantly smaller than the colony area of 17WOSR-Cul isolate when cultured on the same media (661 cm^2). The mean colony area of 17WOSR-Cul when cultured on V8* was larger than on all other growth media that it was cultured on.
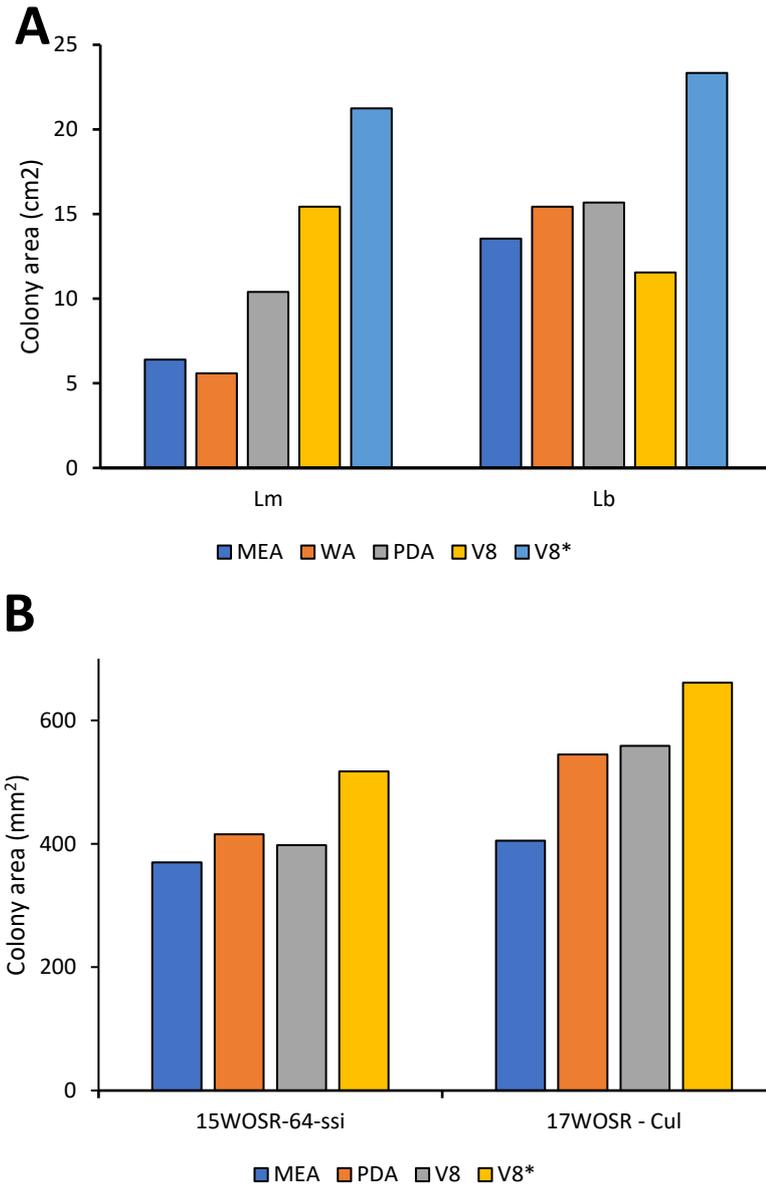
Figure 2.6. Colony area (mm²) of fungal plugs of *L. maculans* (Lm) or *L. biglobosa* (Lb)(A) or *P. brassicae* (Pb) isolates (15WOSR-64-ssi and 17 WOSR-Cul) (B) when cultured on different media types after 7 days. Malt extract agar (MEA), Water agar (WA), Potato dextrose agar (PDA), 20% V8 juice agar (V8) or 20% clarified V8 agar (V8*). (P =0.05, Lm = 13 d.f., Lb = 13 d.f. and Pb = 10 d.f.).
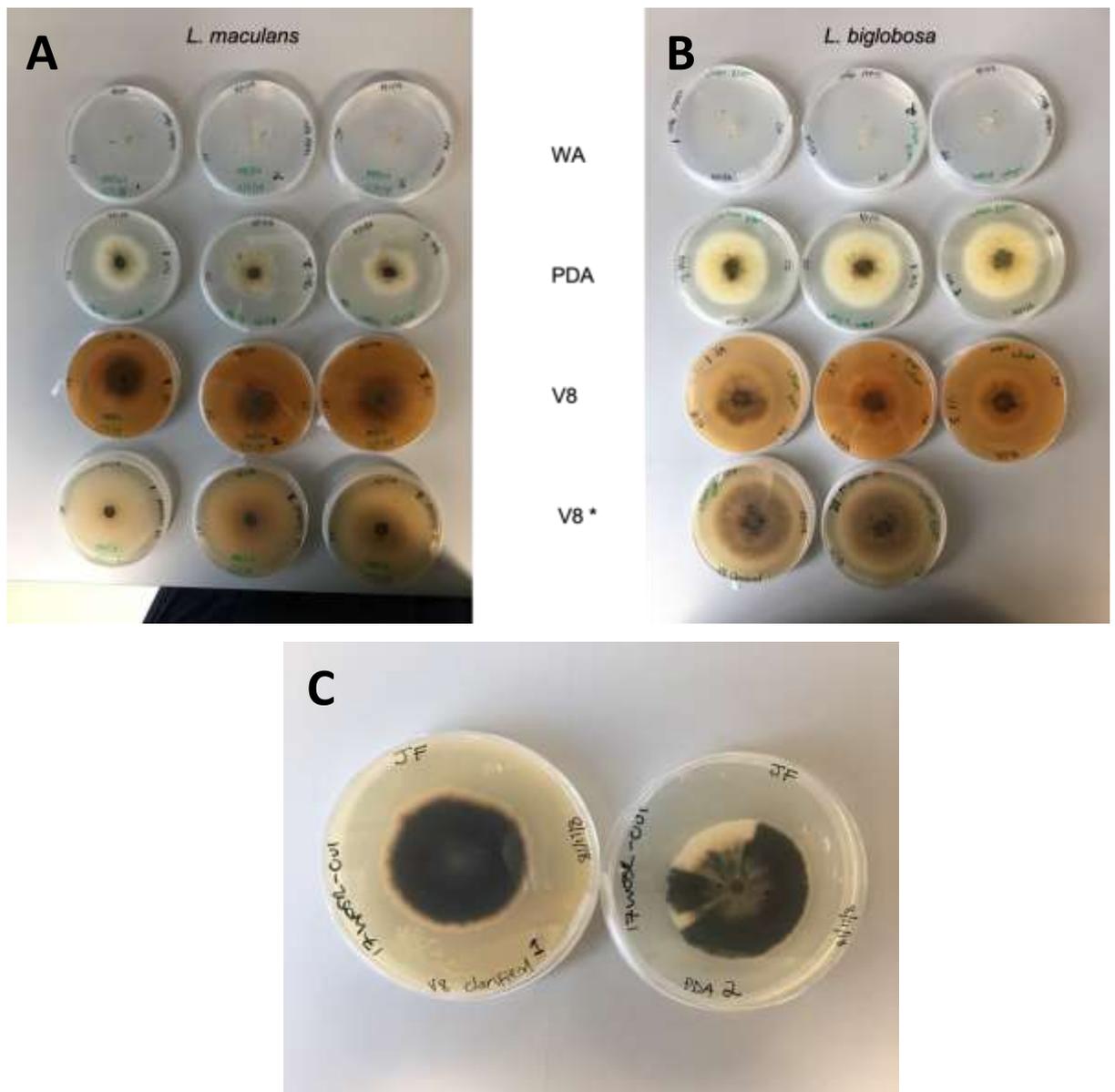
Figure 2.7: Visual comparison between *L. maculans* (A). *L. biglobosa* (B) grown on different media at 10 days post inoculation (dpi). WA = Water agar, PDA = Potato dextrose media, V8 = V8 juice agar, or V8* = clarified V8 juice agar. Visual comparison of *P. brassicae* (C) grown on V8* and PDA after 12 weeks.
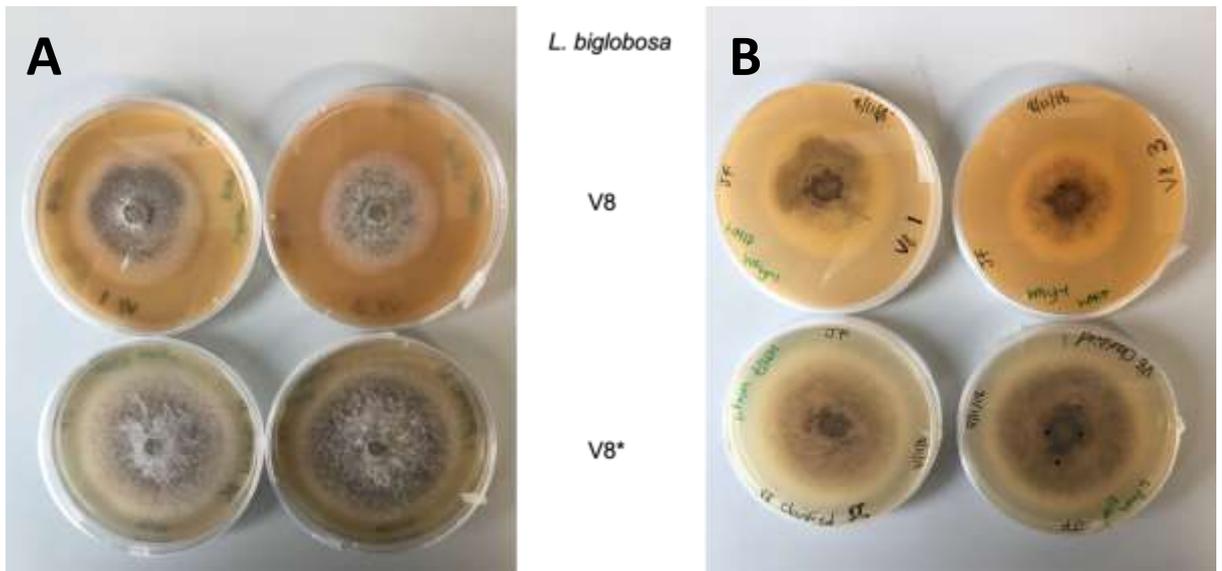
Figure 2.8: Visual comparison from above (A) and below (B) *L. biglobosa* **cultures grown on different media; V8 agar (V8) or clarified V8 agar (V8*) at 10 days post inoculation.**

However, the colony area was significantly smaller when cultured on MEA (410 cm^2) than on PDA (550 cm^2) or V8 agar (560 cm^2). However, the colony areas were not significantly different between PDA and V8 growth media (Fig. 2.6).

2.3.2 Comparison of morphology of *L. maculans, L. biglobosa* and *P. brassicae* colonies under resource competition *in vitro*.

2.3.2.1 Interspecific interactions between *L. maculans*, *L. biglobosa* and *P. brassicae* when co-cultured on PDA or V8* agar.

After 25 days post inoculation, it was apparent that there were interspecific interactions between the fungal species and on different media, due to the reduced growth towards the competitor and a zone of inhibition that is a classic symptom of antibiosis due to the production and release of antagonistic metabolites (Fig. 2.9). It appeared that antagonistic metabolites were released between *L. biglobosa* and *P. brassicae* on V8* agar but not on PDA at both 15°C and 18°C, due to the presence of a small zone of inhibition between the two fungal species. In contrast, antagonistic metabolites appeared to be released from *L. maculans* against *P. brassicae* and they were not affected by medium or temperature; asymmetrical colonies were produced due to reduced growth towards *L. maculans* on both medium types and at both temperatures. However, it appeared that *L. biglobosa* did not release any secondary metabolites against *P. brassicae* at either temperature or on either medium type as a zone of inhibition was not observed and *P. brassicae* colonies were circular in shape. *L. biglobosa* had a faster growth rate than *L. maculans,* so therefore surrounded the *P. brassicae* colony faster, limiting *P. brassicae* colony diameter.

2.3.2.2 *P. brassicae* colony shape changes when in the presence of a competitor

There were no significant differences between temperatures or medium types in *P. brassicae* colony symmetry ($F_{1,35}$ = 0.00, *P* = 0.972, LSD = 9.14; $F_{1,35}$ = 4.10, *P* =0.54, LSD = 9.14, respectively), but there were differences in *P. brassicae* colony symmetry depending on the competing pathogen ($F_{2,35}$ = 68.85, *P* <0.001, LSD = 11.2) (Table 2.5).
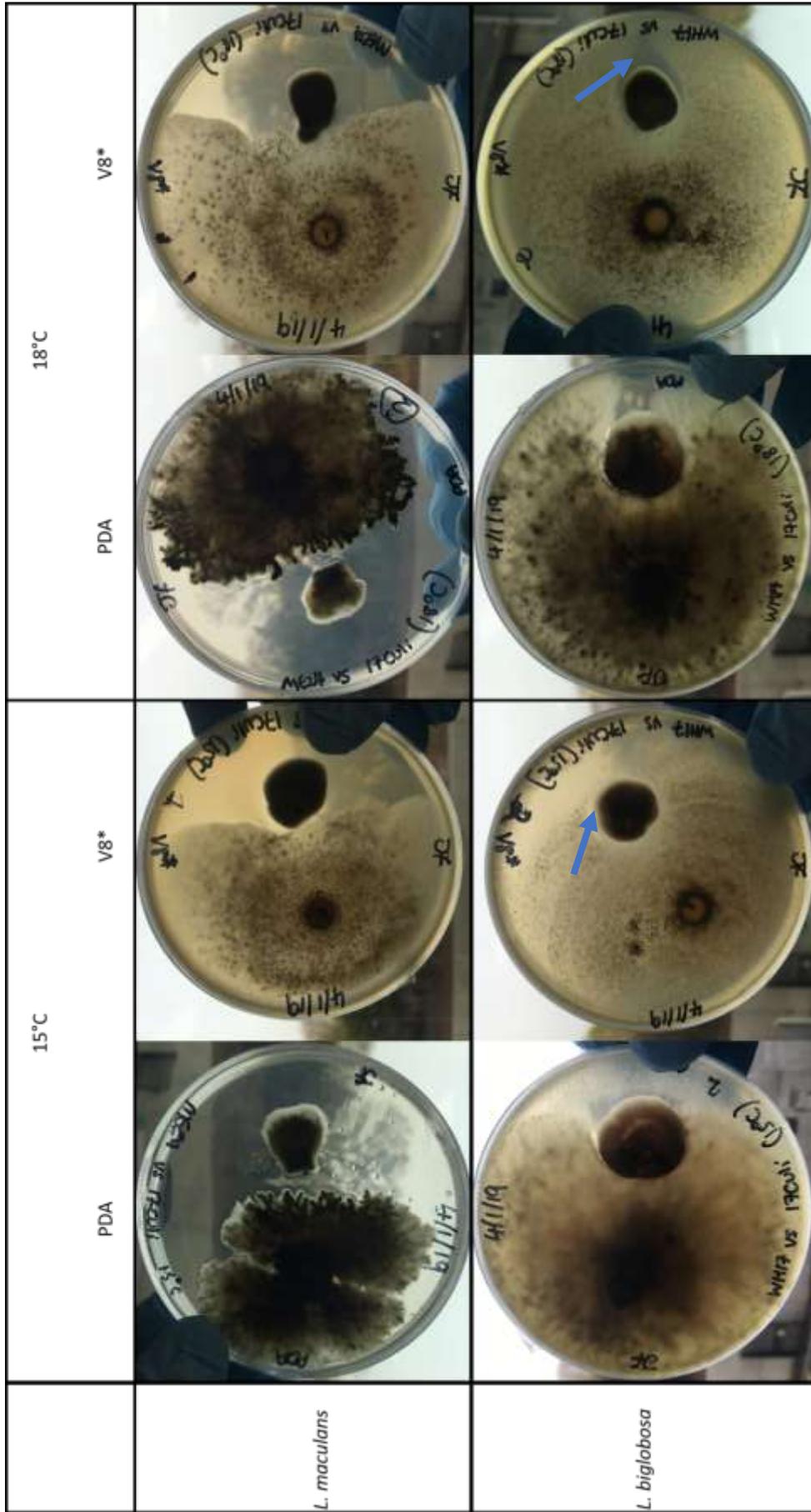
Figure 2.9: Growth of *L. maculans* and *L. biglobosa* with *P. brassicae* on either potato dextrose agar (PDA) or clarified V8 juice agar (V8*) at 15°C or 18°C at 25 dpi. Blue arrows indicate small zones on inhibitions

Table 2.5. Testing output of significant probability levels by fitting a mixed model for the main effects of isolate, media and temperature, the two-way interactions, and the three-way interaction.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Pathogen	2	68.85	35	11.20	<0.001
Media	1	4.10	35	9.14	0.054
Temperature	1	0.00	35	9.14	0.972
Pathogen x Media	2	0.48	35	15.83	0.622
Pathogen x Temperature	2	0.19	35	15.83	0.831
Media x Temperature	1	2.76	35	12.93	0.110
Pathogen x Media x Temperature	2	0.72	35	22.39	0.498

However, there was no influence on competing on different media or at different temperatures, nor was there an influence of different media or temperature or an influence of competing pathogen, medium and temperature on colony symmetry of *P. brassicae* growth (*p*> 0.05) (Table 2.5). When *P. brassicae* grew on its own, it produced a symmetrical colony (97.5%) (Fig 2.10 and 2.11); this was significantly more symmetrical than when *P. brassicae* was cultured with *L. biglobosa* (80.1%). With both treatments, colonies were significantly greater than when *P. brassicae* was cultured with *L. maculans* (35.8%).

When the treatments were compared, the individual treatments that resulted in the most asymmetrical colony shape was when *P. brassicae* was co-inoculated with *L. maculans* at 18°C on clarified V8 agar (31.5%) ; this was not significantly different from all other *P. brassicae* or *L. maculans* treatments on either clarified V8 agar or PDA, or at 15 or 18°C. These treatments had a significantly more asymetrical colony shape than all other treatments, except for *P. brassicae* co-inoculated with *L. maculans* on clarified V8 agar at 18°C (43.3%) that was not significantly different from *P. brassicae* co-inoculated with *L. biglobosa* on either media at 15°C, or on V8* at 18°C. However, it was significantly different from P. brassicae co-inoculated on PDA at 18°C; this treatment did not produce a *P. brassicae* colony that was more asymetrical than when *P. brassicae* was cultured without competition on either medium type or cultured at either temperature (Fig. 2.10) .

2.3.3 Concentration of sirodesmin PL at time intervals between 0-14 days post inoculation.

When the concentration of sirodesmin PL was recorded at regular time intervals, sirodesmin PL was not detected until Day 3; it then increased until Day 10. At Day 14, there was a lower concentration of sirodesmin PL when compared to Day 10 (Fig. 2.12). This pattern was observed in both experiments. Differences in concentrations may be due to the age of fungal plugs that were applied.
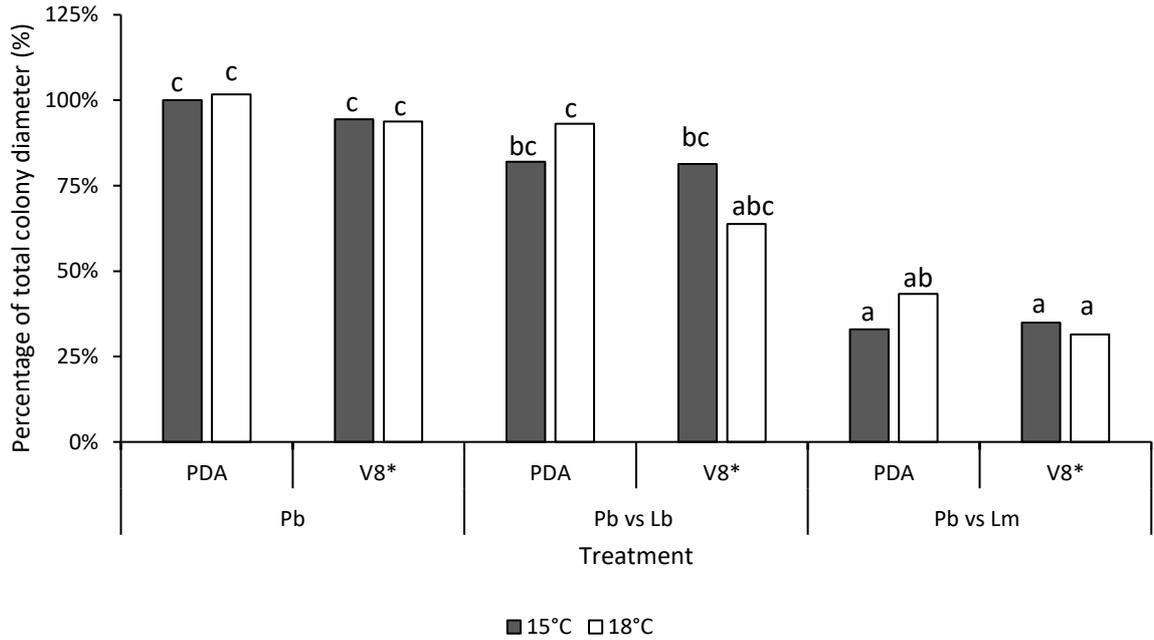
Figure 2.10: Percentage of the total *P. brassicae* colony diameter growing towards the competitor at 15°C and 18°C, when grown in isolation or in dual culture with either *L. maculans* or *L. biglobosa*. Pb = *P. brassicae* in isolation, Pb vs Lb = *P. brassicae* and *L. biglobosa* dual culture, and Pb vs Lm = *P. brassciae* and *L. maculans* dual culture. A post-Tukey test was done. Columns that share the same letter are not significantly different from each other (*P* =0.05, LSD = 22.39, 35 d.f).
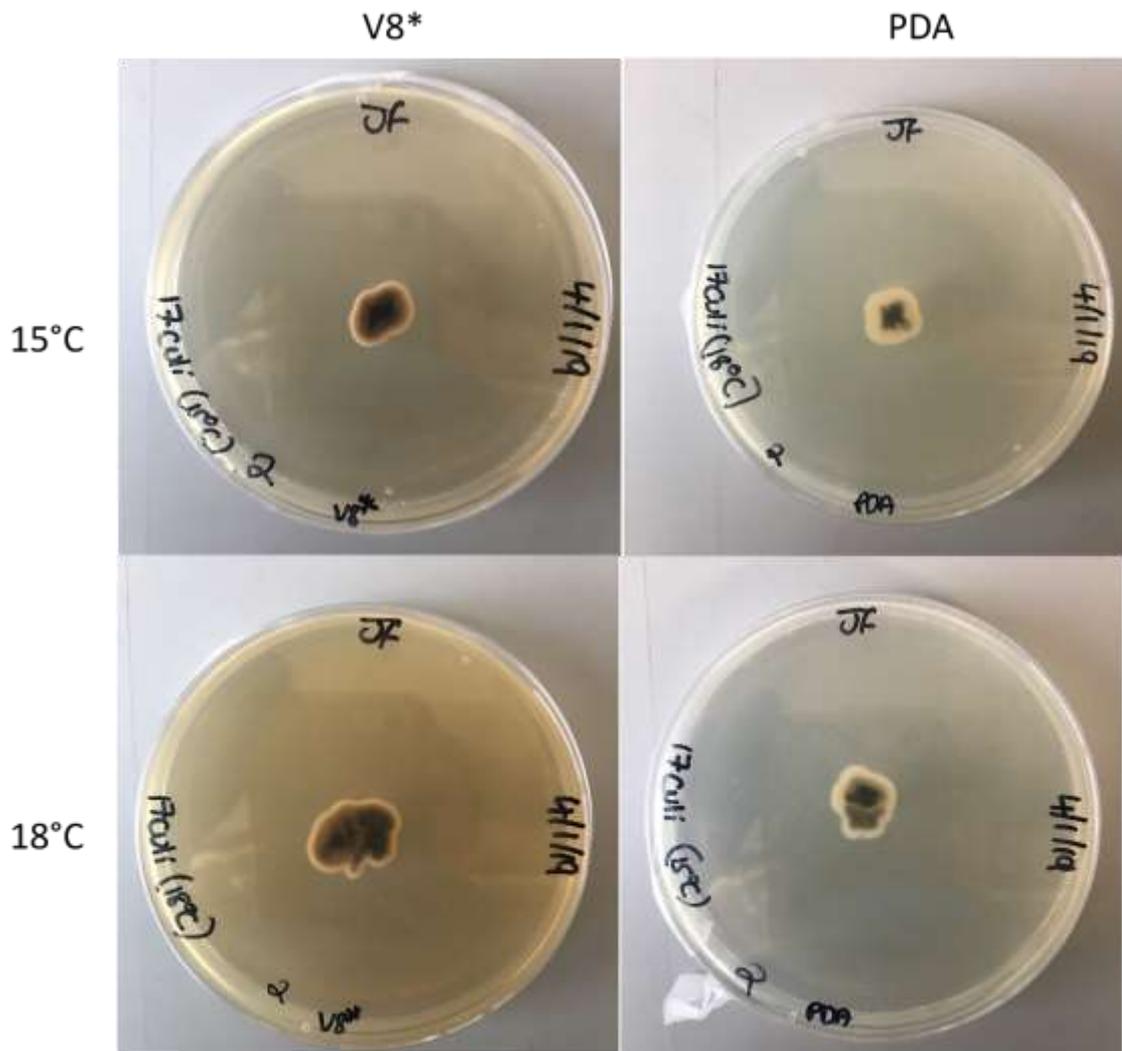
Figure 2.11: Growth of *P. brassicae* on either potato dextrose agar (PDA) or clarified V8 juice agar (V8*) at 15°C or 18°C at 14 dpi.
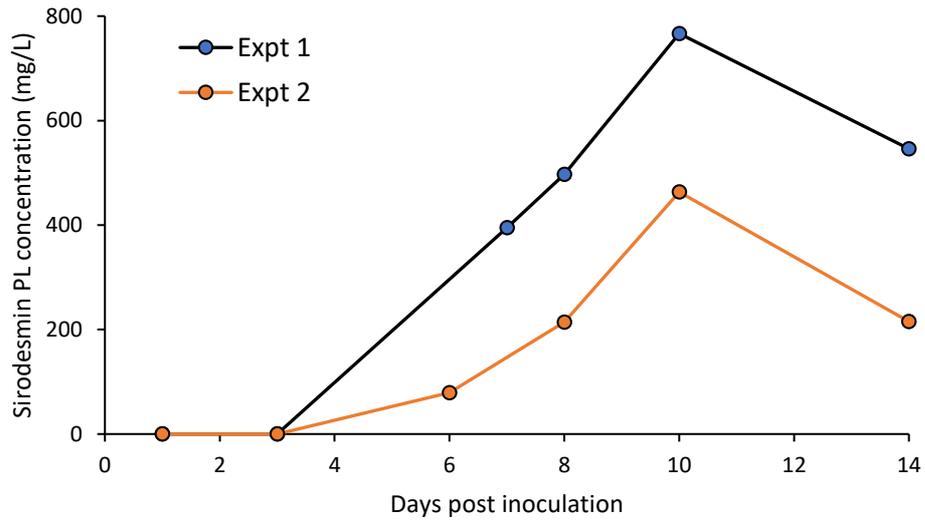
Figure 2.12: Concentration (mg/L) of sirodesmin PL from clarified V8 liquid media inoculated with fungal plugs of *L. maculans* at regular intervals from 0 – 14 days post inoculation. Concentrations were calculated using a gliotoxin standard curve ($y = 2x10^6\ x - 56900$; $R^2 = 0.999$). LOD and LOQ were 70 and 200 mg/L.

2.3.4 Effects of secondary metabolites derived from simultaneously inoculated liquid cultures on the growth of *L. maculans*, *L. biglobosa* and *P. brassicae in vitro*

2.3.4.1 Effect of secondary metabolites on *L. maculans* at 7 days post inoculation.

For the effect of secondary metabolites on colony area of *L. maculans* at 7 days post inoculations, there was a significant difference between the three experiments ($F_{2.71}$= 153.35, *P* < 0.001, LSD = 0.38). The mean colony area was significantly greater in Experiment 3 than in the other experiments, followed by experiment 2 that was significantly greater in colony area than Experiment 1 (Table 2.6 & 2.7). Individual analysis of each experiment can be found in Appendices 6.

There was also a significant difference in the mean colony diameter when different secondary metabolite extracts were applied ($F_{7.71}$= 19.26, *P* < 0.001, LSD = 0.62) (Table 2.6 & 2.7) (Fig. 2.13). The 'EtOAc' (11.63 cm^2) control had the greatest colony area across all three experiments, this was significantly greater than all other treatments except for 'Pb only' (11.08 cm^2). This was followed by when 'Lm only' (10.64 cm^2). The colony area was significantly smaller than when 'EtOAc' was applied but was not significantly different from when 'Pb only' was applied, nor was it significantly different from when the 'Lm&Pb' (9.97 cm^2) was applied. The extract that resulted in the smallest colony area when applied was 'Lm&Lb' (8.75 cm^2); this colony area was not significantly different from when the 'Lb&Pb' (9.25 cm^2) or 'Lb only' (9.59 cm^2). There was no significant difference in colony area when 'Lm&Pb', 'Lb&Pb' or 'Lb only' was applied.

There was also a significant effect of interaction of experiment and secondary metabolite extract ($F_{14.71}$= 3.03, *P* = 0.002, LSD = 1.08) (Table 2.6 & 2.7) (Appendix 6). When the 'EtOAc' and 'Lm&Pb' extracts were applied there was a significantly greater colony area in Experiment 3 than in Experiment 1 but the colony area in Experiment 2 was not significantly different from either Experiment 1 or 3. There was no significant difference between experiments when 'Lm only' was applied. When the 'Lb only' extract was applied there was a significant difference in each experiment; the colony area was greatest in Experiment 3, followed by Experiment 2 and then Experiment 1.

Table 2.6 - Testing output of significant probability for the main effects of Experiment, Treatment and the two-way interactions on colony area for *L. maculans, L. biglobosa* and *P. brassicae* when different secondary metabolites were applied at 7 or 10 days post inoculation.

L. maculans 7 dpi

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	153.35	71	0.38	<0.001
Treatment	7	19.26	71	0.62	<0.001
Experiment x Treatment	14	3.03	71	1.08	0.002

L. maculans 10 dpi

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	31.98	71	2.31	<0.001
Treatment	7	2.06	71	3.78	0.07
Experiment x Treatment	14	0.67	71	3.25	0.002

L. biglobosa 7 dpi

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	41.56	71	0.9	<0.001
Treatment	7	262.29	71	1.50	<0.001
Experiment x Treatment	14	10.92	71	2.50	<0.001

L. biglobosa 10 dpi

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	16.51	62	3.86	<0.001
Treatment	6	12.93	62	5.89	<0.001
Experiment x Treatment	12	1.22	62	10.20	0.304

P. brassicae 7 dpi

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	60.68	69	0.051	<0.001
Treatment	7	262.29	69	0.084	<0.001
Experiment x Treatment	14	3.03	69	0.072	0.536

P. brassicae 10 dpi

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	1.52	60	0.18	0.230
Treatment	6	12.23	60	0.28	<0.001
Experiment x Treatment	12	1.56	60	0.48	0.144

Table 2.7. *L. maculans* colony area (cm^2) when agar plates were inoculated with different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 7 and 10 dpi. A post-hoc Tukey LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each.

L. maculans – 7 dpi

Treatment	Expt 1	Expt 2	Expt 3	Treatment Mean
EtOAc	10.0defghi	12.0ijk	12.9k	11.6e
Lm only	9.3cdef	9.8defgh	9.9defgh	10.6cd
Lb only	7.3abc	9.9defgh	11.5ghijk	9.6ab
Pb only	9.3cdef	10.8efghij	13.1k	11.1de
Lm&Pb	8.7abcd	9.8defgh	11.3fghijk	10.0bc
Lb&Pb	7.2ab	8.9abcde	11.6ghijk	9.3ab
Lm&Lb	6.9a	9.5def	9.868defgh	8.8a
Experimental Mean	8.6a	10.0b	11.9c	

L. maculans – 10 dpi

Treatment	Expt 1	Expt 2	Expt 3	Treatment Mean
EtOAc	21.98abcd	28.61cd	27.97bcd	26.18a
Lm only	18.75abcd	24.60abcd	26.25abcd	23.20a
Lb only	16.15abc	25.43abcd	24.92abcd	22.16a
Pb only	17.29abc	28.48cd	28.51cd	24.76a
Lm&Pb	19.12abcd	26.43abcd	25.56abcd	23.71a
Lb&Pb	15.93ab	23.77abcd	21.70abcd	20.47a
Lm&Lb	14.68a	25.34abcd	27.25bcd	22.43a
Experimental Mean	18.27a	25.87b	26.56b	
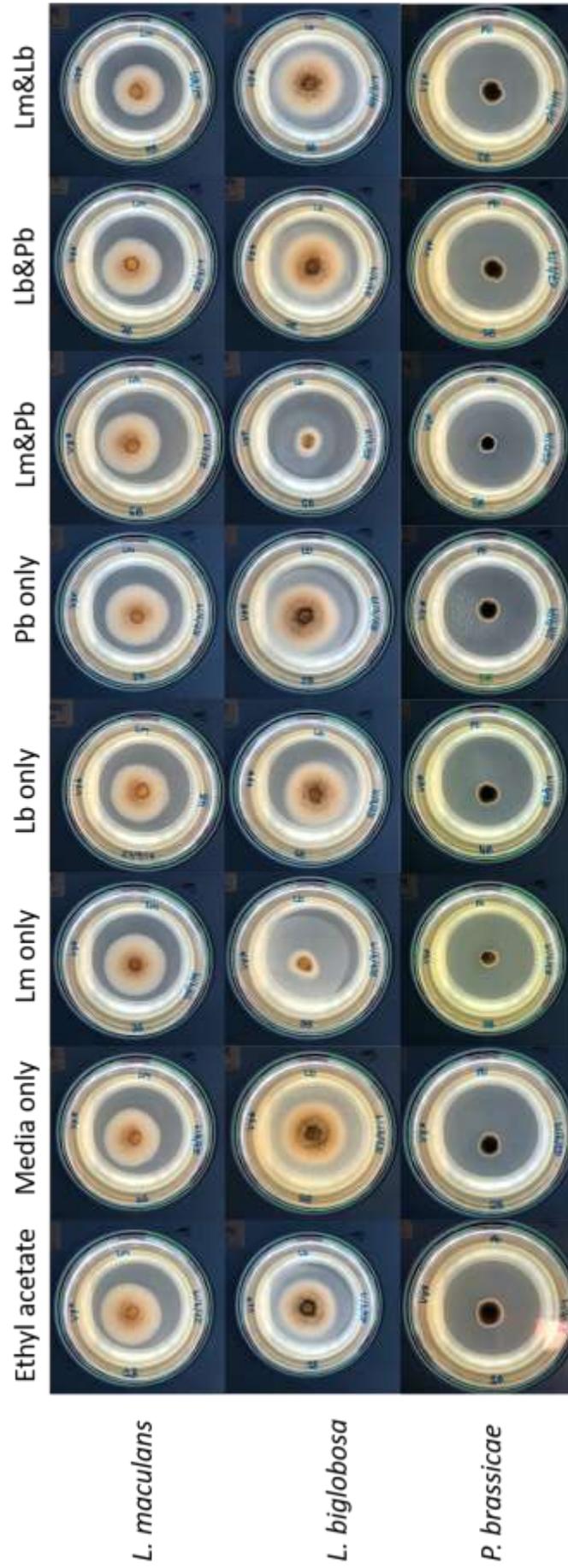
Figure 2.13: Comparison of colonies of *L. maculans*, *L. biglobosa* or *P. brassicae* when different secondary metabolite extracts were applied at 7 dpi for *L. maculans* and *L. biglobosa*, and 10 dpi for *P. brassicae*. Secondary metabolite extracts included two controls Ethyl acetate and Media only, and six metabolite extracts *L. maculans* only (Lm), *L. biglobosa* (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *P. brassicae* inoculated simultaneously or *L. biglobosa* and *P. brassicae* inoculated simultaneously. Colonies were cultured on clarified V8 juice agar at 18°C.

When the 'Lm&Lb' extract was applied there was no significant difference in colony area between Experiment 2 and 3 but both had significantly greater colony areas than in Experiment 1. When the 'Pb only' or 'Lb&Pb' extracts were applied there were no significant differences between Experiment 1 and 2 but both colony areas were significantly smaller than in Experiment 3.

2.3.4.2 Effect of secondary metabolites on *L. maculans* at 10 days post inoculation.

For the effect of secondary metabolites on colony area of *L. maculans* at 10 days post inoculations, there was a significant difference between the three experiments ($F_{2.71}$= 31.98, *P* < 0.001, LSD = 2.312). The mean colony area was significantly smaller in Experiment 1 than in both Experiments 2 and 3; there was no significant difference in colony area between Experiment 2 and 3 (Table 2.6 & 2.7). There was no significant difference in the mean colony diameter when different secondary metabolite extracts were applied after 10 dpi ($F_{7.71}$= 2.06, *P* = 0.066, LSD = 3.776) (Table 2.6 & 2.7). There was also no significant effect of interaction of experiment and secondary metabolite extract ($F_{14.71}$= 0.67, *P* = 0.793, LSD = 3.253) (Table 2.6 & 2.7).

2.3.4.3 Effect of secondary metabolites on *L. biglobosa* at 7 days post inoculation.

For the effect of secondary metabolites on colony area of *L. biglobosa* at 7 days post inoculations, there was a significant difference between the three experiments ($F_{2.71}$= 41.56, *P* < 0.001, LSD = 0.9). The mean colony area was significantly greater in Experiment 2 than in the other experiments, followed by experiment 3 that was significantly greater in colony area than Experiment 1 (Table 2.6 & 2.8). There was also a significant difference in the mean colony diameter when different secondary metabolite extracts were applied ($F_{7.71}$= 262.29, *P* < 0.001, LSD = 1.5) (Table 2.6 & 2.8). There was no significant difference in colony area between the 'EtOAc' control (15.53 cm^2) and all other treatments, except from being significantly larger than when 'Lm only' (5.03 cm^2) or 'Lm&Pb' (6.10 cm^2) or being significantly smaller than when 'Pb only' (16.39 cm^2) was applied. The only treatments that had significantly smaller colony area than when 'Pb only' were 'EtOAc', 'Lm only' and 'Lm&Pb'.

Table 2.8. *L. biglobosa* colony area (cm^2) when agar plates were inoculated with different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 7 and 10 dpi. A post-hoc Tukey LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each.

L. biglobosa – 7dpi

Treatment	Expt 1	Expt 2	Expt 3	Treatment Mean
EtOAc	15.76efghi	16.71hi	14.12d	15.53cde
Lm only	2.51a	8.86c	3.72ab	5.03a
Lb only	14.62de	16.79hi	15.75efghi	15.72de
Pb only	16.39ghi	16.30fghi	16.46ghi	16.39e
Lm&Pb	3.69ab	10.07c	4.53b	6.10b
Lb&Pb	15.08defg	14.92def	16.79hi	15.59cde
Lm&Lb	14.38de	16.71hi	17.10i	16.06de
Experimental Mean	12.22a	14.44c	12.81b	

L. biglobosa – 10 dpi

Treatment	Expt 1	Expt 2	Expt 3	Treatment Mean
EtOAc	32.87 c	40.49c	28.28abc	33.88b
Lm only	10.31a	27.35abc	22.24abc	19.97a
Lb only	31.07bc	41.23c	39.73c	37.35b
Pb only	33.43c	40.10c	33.33c	35.62b
Lm&Pb	13.13ab	31.98bc	22.93abc	22.68a
Lb&Pb	30.66bc	37.76c	41.10c	36.51b
Lm&Lb	31.73bc	40.72c	40.89c	37.78b
Experimental Mean	26.17a	37.09b	32.64b	

The colony area when 'Lm only' was applied was significantly smaller than when 'Lm&Pb' was applied.

There was also a significant effect of interaction of experiment and secondary metabolite extract ($F_{14.71}$= 10.92, P < 0.001, LSD = 2.50) (Table 2.6 & 2.8) (Appendix 6). When the 'EtOAc' was applied the colony area in Experiment 3 was significantly smaller than in Experiment 1 and 2. There was no significant difference in colony area between Experiment 1 or 2. When the 'Lm only' or 'Lm&Pb' extracts were applied the colony area was significantly greater in Experiment 2 than in Experiment 1 and 3; there was no significant difference between Experiment 1 and 3. When the 'Lb only' extract was applied the colony area was significantly greater in Experiment 2 than in Experiment 1. The colony area in Experiment 3 was not significantly different from Experiment 1 and 2. When the 'Pb only' extract was applied there was no significant difference between Experiments. When the 'Lb&Pb' was applied, the colony area in Experiment 1 and 2 were not significantly different from each other. However, the colony area was significantly greater in Experiment 3 than in the other two Experiments. When the 'Lm&Lb' extract was applied there was no significant difference between Experiment 2 and 3, but both were significantly greater than in Experiment 1.

2.3.4.4 Effect of secondary metabolites on *L. biglobosa* at 10 days post inoculation.

For the effect of secondary metabolites on colony area of *L. biglobosa* at 10 days post inoculations, there was a significant difference between the three experiments ($F_{2.62}$= 16.51, P < 0.001, LSD = 3.857) (Table 2.6 & 2.8). The mean colony area was significantly smaller in Experiment 1 than in the other Experiments; there was no significant difference between Experiment 2 and 3 (Table 2.6 & 2.8). There was also a significant difference in the mean colony diameter when different secondary metabolite extracts were applied ($F_{6.62}$= 12.93, P < 0.001, LSD = 5.891) (Table 2.6 & 2.8). The only treatments that had significantly different colony areas to the EtOAc (33.88 cm^2) were 'Lm only' (19.97 cm^2) and 'Lm&Pb' (22.68 cm^2).

There was no significant difference between these two extracts. There was no significant effect of interaction of experiment and secondary metabolite extract ($F_{12.62}$= 1.22, P = 0.304, LSD = 10.203) (Table 2.6 & 2.8) (Appendix 6).

2.3.4.5 Effect of secondary metabolites on *P. brassicae* at 7 days post inoculation.

For the effect of secondary metabolites on colony area of *P. brassicae* at 7 days post inoculations, there was a significant difference between the three experiments ($F_{2.69}$= 60.68, P < 0.001, LSD = 0.0514). The mean colony area was significantly greater in Experiment 2 than in the other Experiments. There was no significant difference between Experiments 1 and Experiment 3 (Table 2.6 & 2.9). There was also a significant difference in the mean colony area when different secondary metabolite extracts were applied ($F_{7.69}$= 39.26 P < 0.001, LSD = 0.0840) (Table 2.6 & 2.9). The 'EtOAc' (1.097 cm^2) control had the greatest colony area across all three experiments. However, the colony are was no significantly difference than when 'Pb only' (0.968 cm^2) and 'Lm&Lb' (1.031 cm^2) extracts were applied; the only treatments that were significantly different from these were when 'Lm&Pb' (0.608 cm^2) and 'Lm only' (0.615 cm^2). There was no significant difference between 'Lm&Pb' and 'Lm only'. There was no significant effect of interaction of experiment and secondary metabolite extract ($F_{14.69}$= 3.03, P = 0.536, LSD = 0.072) (Table 2.6 & 2.9) (Appendix 6).

2.3.4.6 Effect of secondary metabolites on *P. brassicae* at 10 days post inoculation.

For the effect of secondary metabolites on colony area of *P. brassicae* at 10 days post inoculations, there was no significant difference between the three experiments ($F_{2.60}$= 1.52, P = 0.230, LSD = 0.1805) (Table 2.6 & 2.9). There was a significant difference in the mean colony area when different secondary metabolite extracts were applied ($F_{6,60}$= 12.23 P < 0.001, LSD = 0.2758) (Table 2.6 & 2.9) (Fig. 2.13). The colony area when the 'EtOAc' (1.798 cm^2) control was applied was only significantly different from when the 'Lm&Pb' (1.077 cm^2) and 'Lm only' (1.043 cm^2). There was no significant difference between 'Lm&Pb' and 'Lm only'. There was no significant effect of interaction of experiment and secondary metabolite extract ($F_{12,60}$= 1.56, P = 0.144, LSD = 0.4776) (Table 2.6 & 2.9).

Table 2.9. *P. brassicae* colony area (cm^2) when agar plates were inoculated with different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 7 and 10 dpi. A post-hoc Tukey LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each.

P. brassicae – 7dpi

Treatment	Expt 1	Expt 2	Expt 3	Treatment Mean
EtOAc	1.052efgh	1.219h	1.021defgh	1.097c
Lm only	0.503a	0.803bcde	0.539ab	0.615a
Lb only	0.934cdefg	1.070efgh	0.885cdefg	0.963b
Pb only	0.914cdefg	1.133fgh	0.857cdef	0.968bc
Lm&Pb	0.503a	0.760abcd	0.560ab	0.608a
Lb&Pb	0.734abc	1.153gh	0.875cdefg	0.921b
Lm&Lb	0.952cdefgh	1.218h	0.925cdefg	1.031bc
Experimental Mean	0.816a	1.061b	0.818a	

P. brassicae – 10dpi

Treatment	Expt 1	Expt 2	Expt 3	Treatment Mean
EtOAc	2.016c	1.849c	1.528abc	1.798b
Lm only	0.736ab	1.239abc	1.155abc	1.043a
Lb only	1.666c	1.664c	1.767c	1.699b
Pb only	1.851c	1.795c	1.440abc	1.695b
Lm&Pb	0.711a	1.229abc	1.292abc	1.077a
Lb&Pb	1.615bc	1.769c	1.771c	1.719b
Lm&Lb	1.795c	1.904c	1.742c	1.814b
Experimental Mean	1.484a	1.636a	1.528a	

2.3.5 Effects of secondary metabolites derived from sequentially co-inoculated liquid culture on the growth of *L. maculans* or *L. biglobosa in vitro*

For the effect of secondary metabolites on colony area of *L. maculans*, there was a significant difference between the three experiments ($F_{2.73}$= 103.09, *P* < 0.001, LSD = 0.88). The mean colony area was significantly greater in experiment 2 than in the other experiments. The mean colony area was significantly greater in experiment 1 than in experiment 3 (Table 2.10 & 2.11). There was also a significant difference in the mean colony area when different secondary metabolite extracts were applied ($F_{4.73}$= 55.07, *P* < 0.001, LSD = 1.14) (Table 2.10 & 2.11) (Fig. 2.14) (Appendix 7). The 'EtOAc' (25.80 cm^2) control had the greatest colony area across all three experiments, this was significantly greater than all other treatments. This was followed by when 'Lm only' (24.12 cm^2) and 'Lb only' (20.36 cm^2) were applied; they were significantly different from each other. The two extracts than had the smallest colony areas were 'Lm + Lb' (19.23 cm^2) and 'Lm&Lb' (19.37 cm^2); there was no significant difference between these two extracts. There was also a significant effect of interaction of experiment and secondary metabolite extract ($F_{8.73}$= 7.74, *P* < 0.001, LSD = 1.98) (Table 2.10 & 2.11). There was no significant difference between experiments on the colony area of the EtOAc control. For 'Lm only' there was no significant difference in colony area between Experiment 1 and Experiment 3, but both were significantly smaller than in Experiment 2. For the other three treatments, the colony areas were greater in Experiment 2 than in both Experiment 1 and 3. However, the colony areas were greater in Experiment 1 than Experiment 3. Individual analysis of each experiment can be found in Appendices 7.

For the effect of secondary metabolites on colony area of *L. biglobosa*, there was a significant difference between the three experiments ($F_{2.70}$= 113.00, *P* < 0.001, LSD = 0.84). The mean colony area was significantly greater in experiment 2 than in the other experiments. The mean colony area was significantly greater in experiment 3 than in experiment 2 (Table 2.10 & 2.11). There was also a significant difference in the mean colony area when different secondary metabolite extracts were applied ($F_{4.70}$= 343.91, *P* < 0.001, LSD = 1.01) (Table 2.10 & 2.11).

Table 2.10. Testing output of significant probability for the main effects of Experiment, Treatment and the two-way interactions on colony area for _L. maculans_ and _L. biglobosa_ when different sequential secondary metabolites were applied.

L. maculans

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	103.09	73	0.88	< 0.001
Treatment	4	55.07	73	1.14	<0.001
Experiment x Treatment	8	7.74	73	1.98	<0.001

L. biglobosa

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	113.00	70	0.84	<0.001
Treatment	4	343.91	70	1.01	<0.001
Experiment x Treatment	8	10.41	70	1.89	<0.001

Table 2.11. Effects on *L. maculans* and *L. biglobosa* when the application of secondary metabolites extracts from solo or co-inoculated from clarified V8 juice broth inoculated were applied. There were four treatments, *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb) or *L. maculans* and *L. biglobosa* inoculated sequentially (Lm+Lb) (B). For the Lm+Lb treatment, the liquid media was inoculated with *L. maculans* first then seven days later *L. biglobosa* was added. Fisher least significant difference (LSD) tests were used to separate the mean values.

L. maculans

Treatment	Expt 1	Expt 2	Expt 3	Treat Mean
EtOAc	25.43ab	26.93a	25.05abc	25.80a
Lb Only	21.06d	24.72bc	16.18fg	20.36c
Lm + Lb	17.81ef	24.62bc	15.26g	19.23d
Lm and Lb	19.21de	23.95bc	14.96g	19.37d
Lm Only	23.42c	25.82ab	23.11c	24.12b
Experimental Mean	21.39b	25.23a	18.91c	

L. biglobosa

Treatment	Expt 1	Expt 2	Expt 3	Treat Mean
EtOAc	22.11bc	23.97ab	22.68bc	23.04a
Lb Only	15.29d	25.75a	21.94c	21.36b
Lm + Lb	5.68g	13.49de	7.10g	8.76d
Lm and Lb	21.35c	24.77a	21.60c	22.57a
Lm Only	9.85f	13.18e	7.22g	10.08c
Experimental Mean	14.18c	20.23a	15.86c	
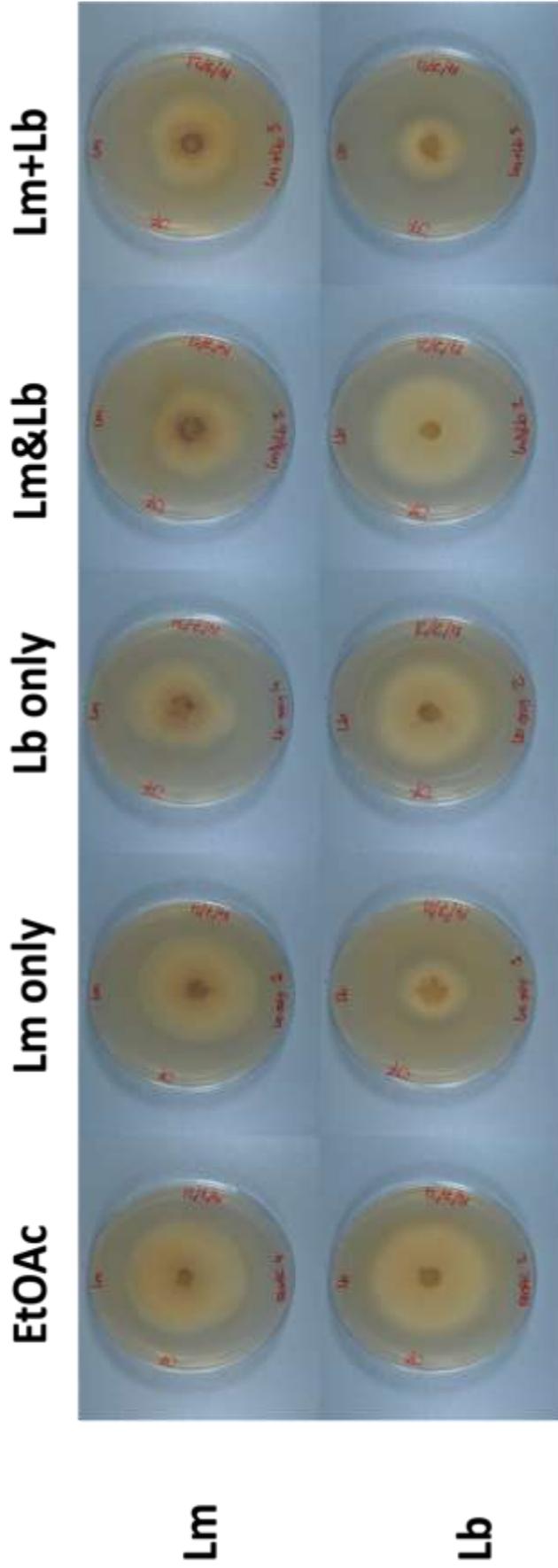
Figure 2.14. *L. maculans* and *L. biglobosa* colony phenotypes when different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) simultaneous and sequentially inoculated co-cultures of these pathogens were applied. Assessments were made at at 7 dpi. Treatments included an Ethyl acetate control (Ethyl Acetate), and four metabolite extracts *L. maculans* only (Lm), *L. biglobosa* only (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially after 7 days (Lm+Lb).

The 'EtOAc' (23.04 cm^2) control had the greatest colony area across all three experiments, this was significantly greater than all other treatments except for 'Lm&Lb' (22.57 cm^2). This was followed by when 'Lb only' (21.36 cm^2). The other two secondary metabolite extracts had significantly smaller colony areas than the other treatments. However, the colony area was significantly smaller in 'Lm+Lb' (8.76 cm^2) than in the 'Lm only' (10.08 cm^2) treatment. There was also a significant effect of interaction of experiment and secondary metabolite extract ($F_{8.70}$= 10.41, $P < 0.001$, LSD = 1.89) (Table 2.9). There was no significant difference between experiments on the colony area of the EtOAc control. For 'Lm only' and 'Lb only' there was a significant difference in colony area between all three experiments, for 'Lm only' the colony area was greatest in Experiment 2, followed by Experiment 1 and then Experiment 3. Whereas for 'Lb only' the colony area was greatest in Experiment 2, followed by Experiment 3 and then Experiment 1. For both treatments that contained metabolites from co-inoculated cultures, there was no significant difference in colony area between Experiments 1 and 3, but both were significantly smaller than Experiment 2. Individual analysis of each experiment can be found in Appendices 7.

2.3.6 Effects of secondary metabolites derived from simultaneous or sequential co-inoculations of *L. maculans* and *L. biglobosa* on other oilseed rape plant pathogens.

2.3.6.1 Effect of secondary metabolites derived from simultaneous and sequential co-inoculation of *L. maculans* and *L. biglobosa* on *Sclerotinia sclerotiorum*

For the effect of secondary metabolites on colony area of *S. sclerotiorum*, there was a significant difference between the three experiments ($F_{2.74}$= 124.22, $P < 0.001$, LSD = 5.64). The mean colony area was significantly greater in experiment 2 than in the other experiments. The mean colony area was significantly greater in experiment 3 than in experiment 2 (Table 2.12 & 2.13). There was also a significant difference in the mean colony area when different secondary metabolite extracts were applied ($F_{4.74}$= 42.37, $P < 0.001$, LSD = 5.64) (Table 2.12 & 2.13) (Fig. 2.15). Individual analysis of each experiment can be found in Appendices 8.

Table 2.12. Testing output of significant probability for the main effects of Experiment, Treatment and the two-way interactions on colony area for *S. sclerotiorum* and *V. longisporum* when different sequential secondary metabolites were applied.

S. sclerotiorum

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	124.22	74	5.64	< 0.001
Treatment	4	42.37	74	5.64	<0.001
Experiment x Treatment	8	4.23	74	9.78	<0.001

V. longisporum

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Experiment	2	20.98	73	0.19	<0.001
Treatment	4	194.04	73	0.24	<0.001
Experiment x Treatment	8	7.72	73	0.42	<0.001

Table 2.13. Effects of the application of secondary metabolites extracts from solo or co-inoculated from clarified V8 juice broth on colony area of *S. sclerotiorium*. *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb) or *L. maculans* and *L. biglobosa* inoculated sequentially (Lm+Lb). For the Lm+Lb treatment, the liquid media was inoculated with *L. maculans* first then seven days later *L. biglobosa* was added. Fisher least significant difference (LSD) tests were used to separate the mean values.

Sclerotinia sclerotiorum

Treatment	Expt 1	Expt 2	Expt 3	Treat Mean
EtOAc	28.65def	19.08bcd	61.38g	35.24c
Lb Only	34.36f	23.94cde	63.07g	39.41c
Lm + Lb	11.49ab	2.97a	18.73bcd	10.64a
Lm and Lb	35.81f	16.81bc	61.81g	36.94c
Lm Only	20.66bcde	2.97a	29.53ef	17.01b
Experimental Mean	26.19b	13.15a	46.90c	
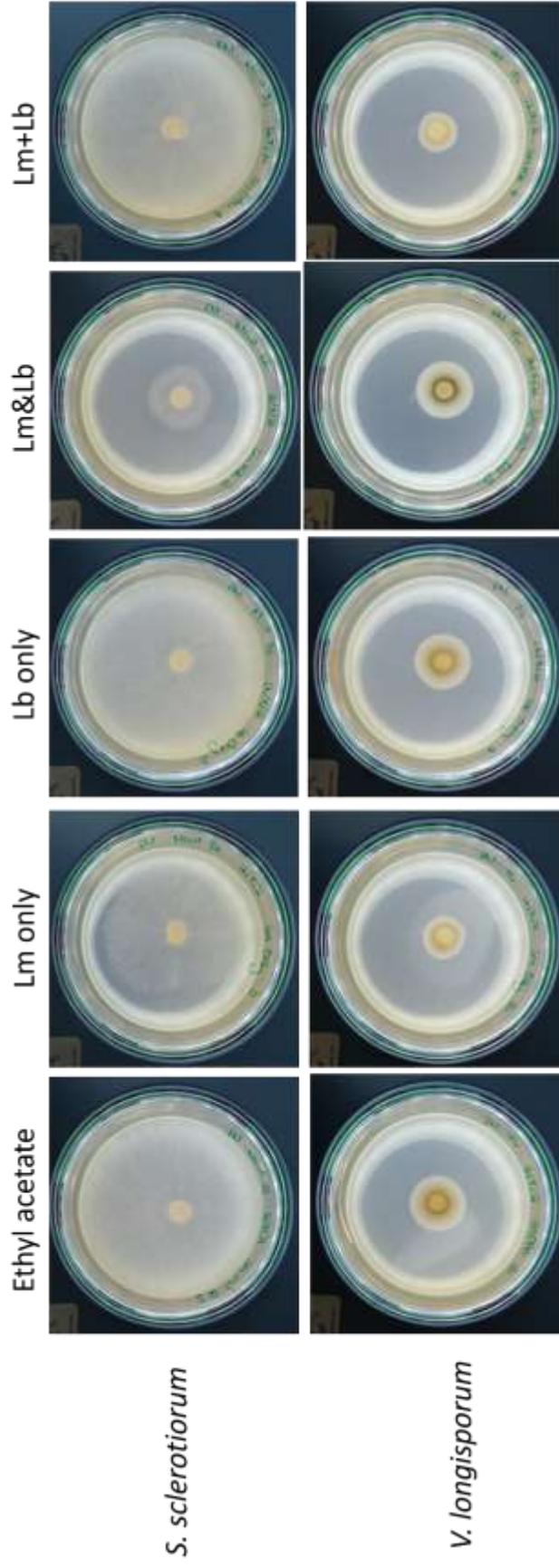
Figure 2.15. *S. sclerotiorum* and *V. longisporum* colony phenotypes when inoculated onto agar plates and different secondary metabolite extract from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) simultaneous and sequentially inoculated co-cultures of these pathogens were applied. Assessments were made at 2 and 10 dpi respectively. Treatments included an Ethyl acetate control (Ethyl acetate), and four metabolite extracts *L. maculans* only (Lm), *L. biglobosa* (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa inoculated* sequentially after 7 days (Lm+Lb).

The 'EtOAc' (35.24 cm^2) control had the greatest colony area across all three experiments, this was significantly greater than all other treatments except for 'Lm&Lb' (36.94 cm^2) and 'Lb only' (39.41cm^2). The other two secondary metabolite extracts had significantly smaller colony areas than the other treatments. However, the colony area was significantly smaller in 'Lm+Lb' (10.64 cm^2) than in the 'Lm only' (17.01 cm^2) treatment. There was also a significant effect of interaction of experiment and secondary metabolite extract ($F_{8.74}$= 4.23, P < 0.001, LSD = 9.78) (Table 2.12 & 2.13). There was no significant difference between experiments on the colony area of the EtOAc control between Experiment 1 and 2, but the colony area was significantly smaller than in Experiment 3. For 'Lb only' and 'Lm&Lb' there was a significant difference in colony area between all three experiments, for both treatments the colony area was greatest in Experiment 3, followed by Experiment 1 and then Experiment 2. For treatment 'Lm+Lb', there was a significant difference in colony area between Experiments 2 and 3, but neither was significantly different from Experiment 1. For treatment 'Lm only' there was no significant difference between Experiments 1 or 3, but both were significantly greater than the colony area in Expt 2. Whereas, when treatment 'Lm+Lb' was applied there was no significant difference between Experiment 1 or 2, both were significantly smaller than in Experiment 3.

2.3.6.2 Effect of secondary metabolites derived from simultaneous and sequential co-inoculation of *L. maculans* and *L. biglobosa* on *Verticillium longisporum*

For the effect of secondary metabolites on colony area of *V. longisporum*, there was a significant difference between the three experiments ($F_{2.73}$= 20.98, P < 0.001, LSD = 0.1886). There was no significant difference in mean colony area between Experiment 2 and 3, but both were significantly greater than in Experiment 1 (Table 2.12 and 2.14) (Fig. 2.15). There was also a significant difference in the mean colony area when different secondary metabolite extracts were applied ($F_{4.73}$= 194.04, P < 0.001, LSD = 0.2435) (Table 2.12 & 2.14) (Fig. 2.15) (Appendix 9). The 'EtOAc' (5.58 cm^2) control had the greatest colony area across all three experiments, this was not significantly greater than when treatments 'Lm&Lb' (5.48 cm^2) or 'Lb only' (5.45 cm^2) was applied. Individual analysis of each experiment can be found in Appendices 9.

Table 2.14. Effects of the application of secondary metabolites extracts from solo or co-inoculated from clarified V8 juice broth on colony area of *S. sclerotiorium*. *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb) or *L. maculans* and *L. biglobosa* inoculated sequentially (Lm+Lb). For the Lm+Lb treatment, the liquid media was inoculated with *L. maculans* first then seven days later *L. biglobosa* was added. Fisher least significant difference (LSD) tests were used to separate the mean values.

V. longisporum

Treatment	Expt 1	Expt 2	Expt 3	Treat Mean
EtOAc	5.35 de	5.45 de	5.93 e	5.58 b
Lb Only	5.13d	5.47 de	5.75 de	5.45 b
Lm + Lb	3.37 bc	3.97 c	2.88 ab	3.40 a
Lm and Lb	5.15 d	5.47 de	5.82 de	5.48 b
Lm Only	2.39 a	3.76 bc	3.56 bc	3.24 a
Experimental Mean	4.28 a	4.79 b	4.82b	

However, the colony ara when these three extracts were applied were all significantly greater than when the 'Lm only' (3.24 cm^2) or 'Lm+Lb' (3.40 cm^2) extracts were applied; there was no significant difference between the colony area when these two extracts were applied. There was also a significant effect of interaction of experiment and secondary metabolite extract ($F_{8.73}$= 7.72, P < 0.001, LSD = 0.4217) (Table 2.12 & 2.14). There was no significant difference between the effect of the secondary metabolite and the experiments on the colony area of the 'EtOAc' control, 'Lb only' and 'Lm&Lb'. Whereas for 'Lm+Lb', the colony area in Experiments 2 and 3 were significantly different; the colony area was greater in experiment 2. However, the colony area in experiment 1 was not significantly different from either experiment 2 or 3. For 'Lm only', there was no significant difference in colony area between Experiment 2 or 3, but both were significantly greater than in Experiment 1.

2.3.7   Identification of secondary metabolite composition using HPLC analysis.

2.3.7.1 Comparison of secondary metabolite chromatograms from simultaneously inoculated samples of *L. maculans*, *L. biglobosa* or *P. brassicae*

Overall, all samples that were tested had a similar baseline to each other, but across the tested samples there were seven detectable maxima between 10-23 min of run time (Fig. 2.15). Maxima that were outside of these times were identical to those of the control and across all samples, so were therefore excluded. Of the seven maxima, there were three (Fig. 2.15) that were present only in the 'Lm only' and 'Lm+Pb' samples (Maximum 1- Rt 11.2; Maximum 2 - Rt 16.2 and Maximum 3 – Rt 19.25), so these maxima were unique to these samples (Fig 2.15). Maximum 1 was a broad maximum (Fig 2.16).

Another maximum was identified at 22.2 min; this was found in all samples tested; a very steep maximum was identified for 'Lm +Pb', 'Lm only' and 'Pb only' samples. When *L. maculans* was cultured in the presence of *L. biglobosa* ('Lm+Lb'), the three maxima unique to 'Lm only' and 'Lm+Pb' were not present and its chromatograph was like that of 'Lb only' (Fig 2.16).
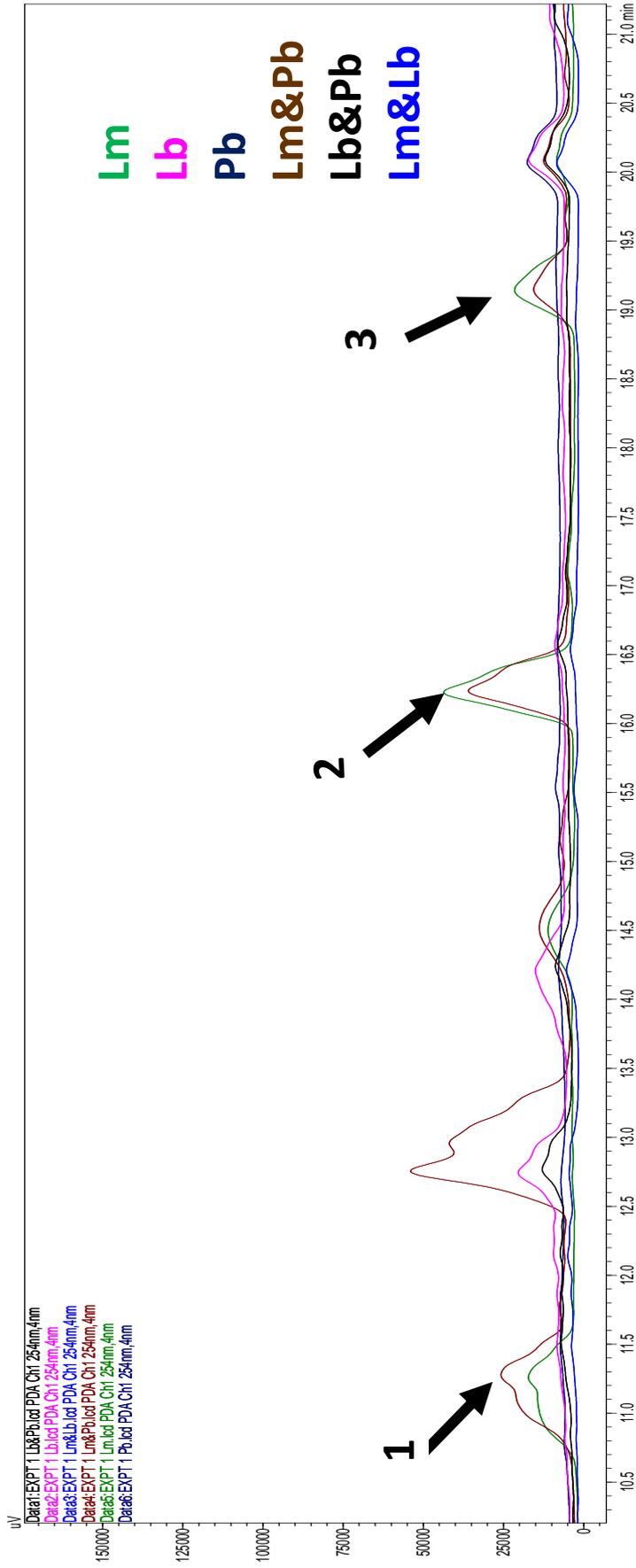
Figure 2.16: HPLC chromatograph of six secondary metabolites extracts from liquid cultures of *L. maculans* (Lm), *L. biglobosa* only (Lb) or *P. brassicae* (Pb). Maxima that were identified only in 'Lm only' or 'Lm&Pb' are labelled 1-3.

2.3.7.2 Comparison of secondary metabolite chromatograms from simultaneously or sequentially inoculated samples of *L. maculans* and *L. biglobosa*

When the samples from simultaneously or sequentially inoculated samples were assessed, the three unique maxima identified for 'Lm only' and 'Lm+Pb' in section 3.3.8.1 were present only in samples 'Lm only' and 'Lm+Lb' (Fig. 2.17). For the other treatments, there were additional maxima, but these were either present in all samples or were less than the level of detection.

2.3.7.3 Identification of maxima found on the chromatograms from extracted secondary metabolites using published literature.

Pedras *et al.* (1998) analysed culture extracts of *L. maculans* using HPLC and the secondary metabolite profile that was published had a similar pattern to the one that has been produced in this work. Using the maxima that were published (Fig. 2.18) it suggests that.

- Maximum 1 = Deacetylsirodesmin PL and Phomamide
- Maximum 2 = Sirodesmin PL
- Maximum 3 = Unknown (potentially Brassinin)

2.3.8 Confirmation of secondary metabolites identified using LC-MS.

The LC-MS analysis of the four fractions that contained the three unique maxima found in the 'Lm only' and 'Lm&Pb' extracts, and maximum D from the HPLC analysis showed; Maximum 1 contained an ion in its LC-MS positive ion spectrograph at *m/z* 445.20 (95%), 446.23 (18%) and 447.21(12%) with a mean m/z 445.5 (Fig 2.19), which corresponds to a monocharged molecule with a molecular weight 444.5 Da; Deacetylsirodesmin PL is a known secondary metabolite produced by *L. maculans* and has a molecular weight of 444.5 Da (Pedras and Yu 2009; https://pubchem.ncbi.nlm.nih.gov). Additionally, the following ions were found; *m/z* 381.24 $[M - S_2 + H]^+$, corresponding to Deacetylsirodesmin PL lacking two sulphur atoms; *m/z* 403.23 $[M - S_2 + Na]^+$, corresponding to the sodium adduct of Deacetylsirodesmin lacking two sulphur atoms (Fig 2.18).
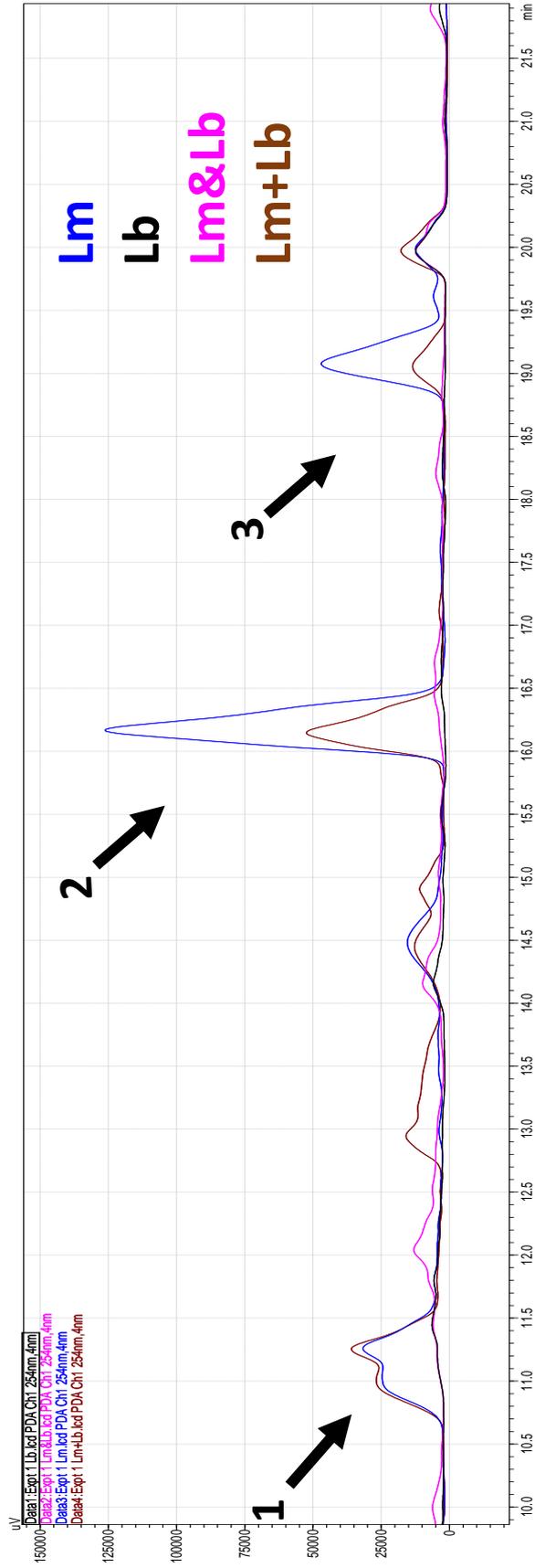
Figure 2.17: HPLC chromatogram of secondary metabolite extracts from liquid cultures *L. maculans* only (Lm), *L. biglobosa* only (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb) and *L. maculans* and *L. biglobosa* inoculated sequentially (Lm+Lb). For the Lm+Lb treatment, the liquid media was inoculated first with *L. maculans* then seven days later *L. biglobosa* was added.
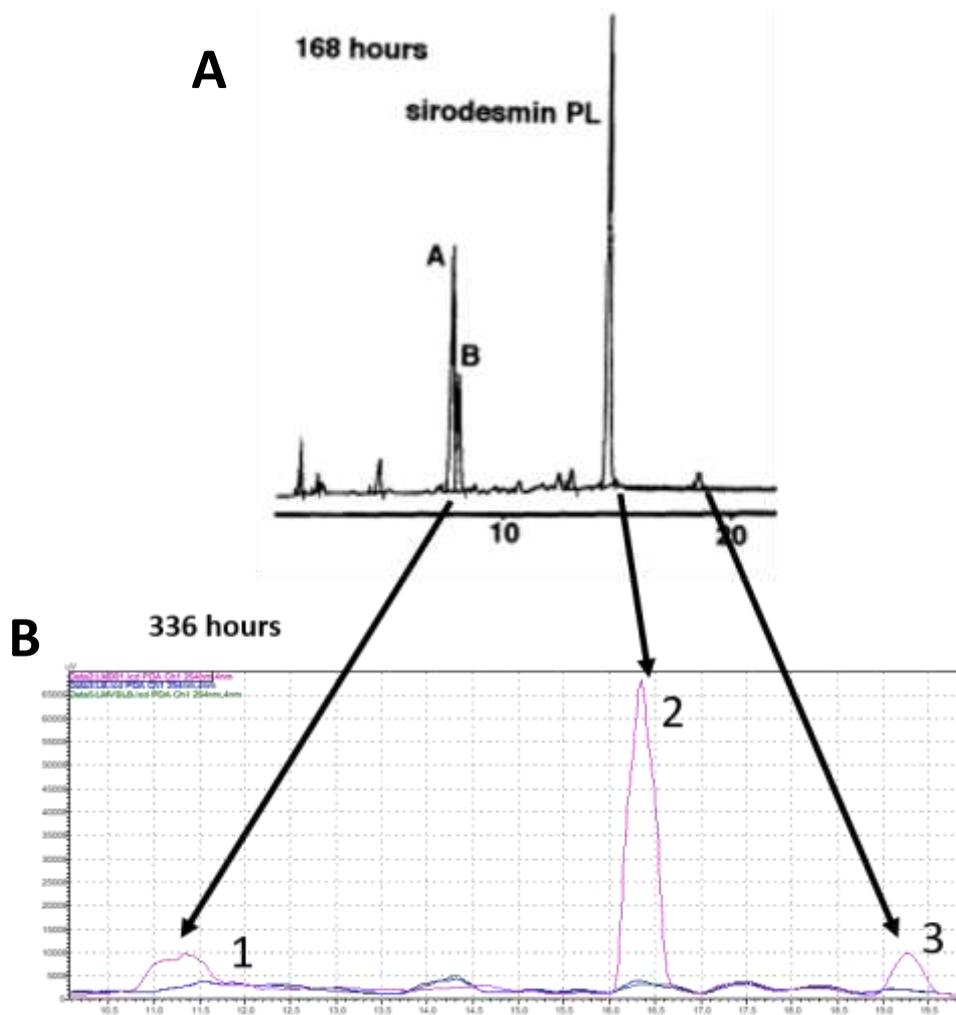
Figure 2.18: Identification of similar maxima found in both the HPLC chromatographs from those found by Pedras *et al.* (1998) (A) and Experiment 3 (B). Differences in Rt between experiments can be a result of using a different coloumn, different flow rates or different instruments (internal diameters).
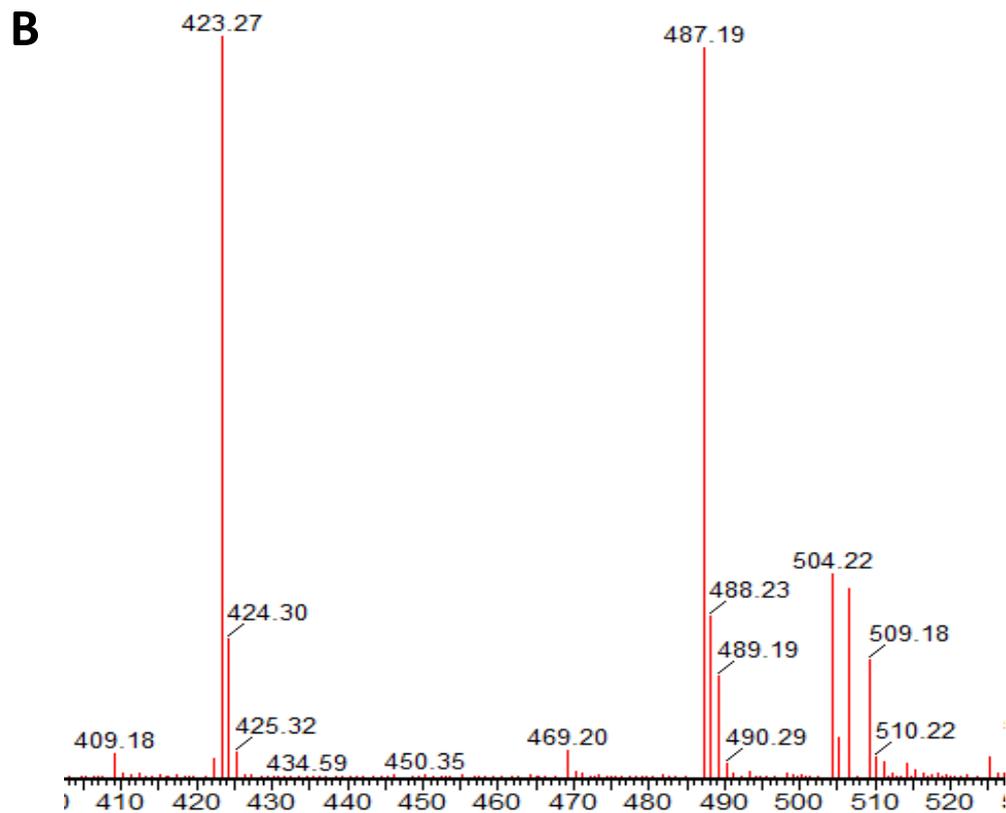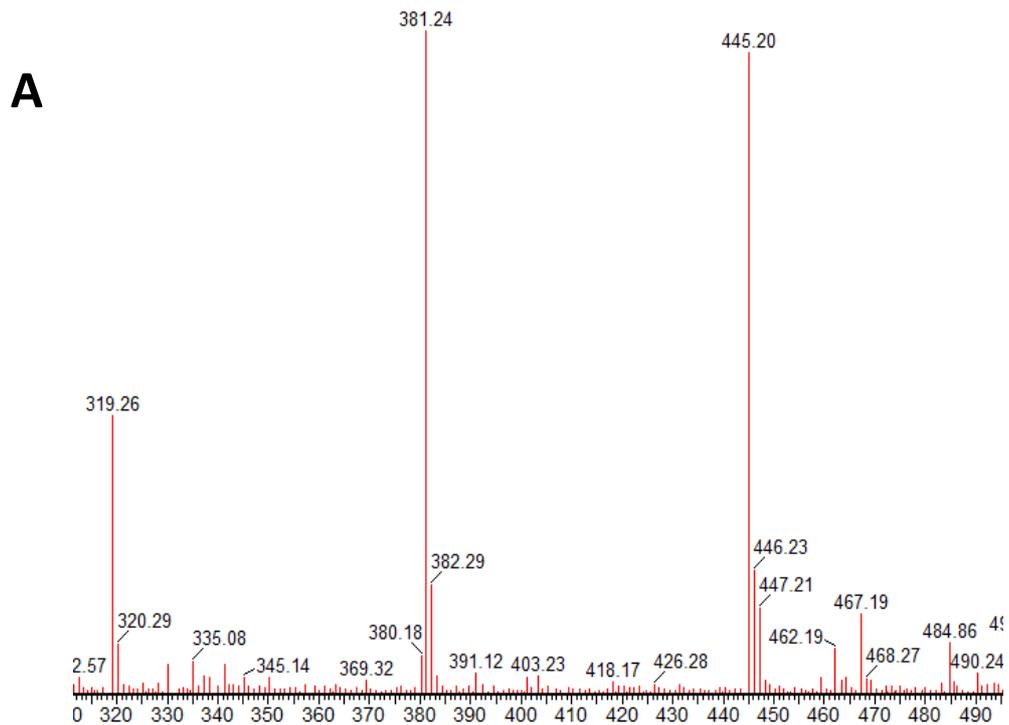
Figure 2.19. ESI- LC-MS spectra of fractions taken for (A) Maximum 1 at Rt 11.2 min and (B) Maximum 2 at Rt 16.2 min.

In addition to these maxima, ions at *m/z* 319.26 (43%) and 320.29 (7%) with a mean m/z of 319.4 were found, which corresponds to monocharged molecule with a molecular weight of 318.4 Da (Fig 2.19); Phomamide is a known secondary metabolite produced by *L. maculans* and has a molecular weight of 318.4 Da (Pedras and Yu 2009; https://pubchem.ncbi.nlm.nih.gov). Maximum 2 from the chromatograph contained ions in its LC-MS positive ion spectrograph at m/z 487.19 (97%), 488.23 (18%), 489.19 (12%) and 490.29 (3%) with a mean m/z 487.6, which corresponds to a monocharged molecule with a molecular weight 486.6 Da (Fig 2.19); Sirodesmin PL is a known secondary metabolite produced by *L. maculans* with a molecular weight of 486.6 Da (Pedras and Yu 2009; https://pubchem.ncbi.nlm.nih.gov). Additionally the following ions were also found: *m/z* 423.27 $[M - S_2 + H]^+$, corresponding to sirodesmin PL lacking two sulphur atoms; m/z 504.22 $[M + NH_4]^+$, corresponding to the ammonium adduct of sirodesmin PL and *m/z* 509.18 $[M+Na]^+$, corresponding to the sodium adduct of sirodesmin PL (Fig 2.19).

Maximum 3 from the HPLC chromatograph could not be identified using LC-MS. The fraction was further concentrated by resuspending in only 0.3mL ethyl acetate but a unique maximum could not be identified from the LC-MS chromatograph. Therefore, the compound responsible for maximum 3 on the HPLC chromatograph remains unknown.

2.3.9   Effect of simultaneous co-inoculation of *L. maculans*, *L. biglobosa* or *P. brassicae* on the production of sirodesmin PL and its precursors

There were significant differences between extracts in the concentrations of sirodesmin PL 'precursors' (Deacetylsirodesmin and Phomamide) ($F_{1.17}$= 13.79, *P* = 0.021, LSD = 138.5) (Fig. 2.19). There were only two extracts that had concentrations of 'precursors' were detected; they were 'Lm only' and 'Lm&Pb' (Fig 2.20). The concentration of the 'precursors' was greatest in the "Lm&Pb" extract (432 mg/L); this was significantly greater than the concentration of 'precursors' in the 'Lm only' extract (246 mg/L).
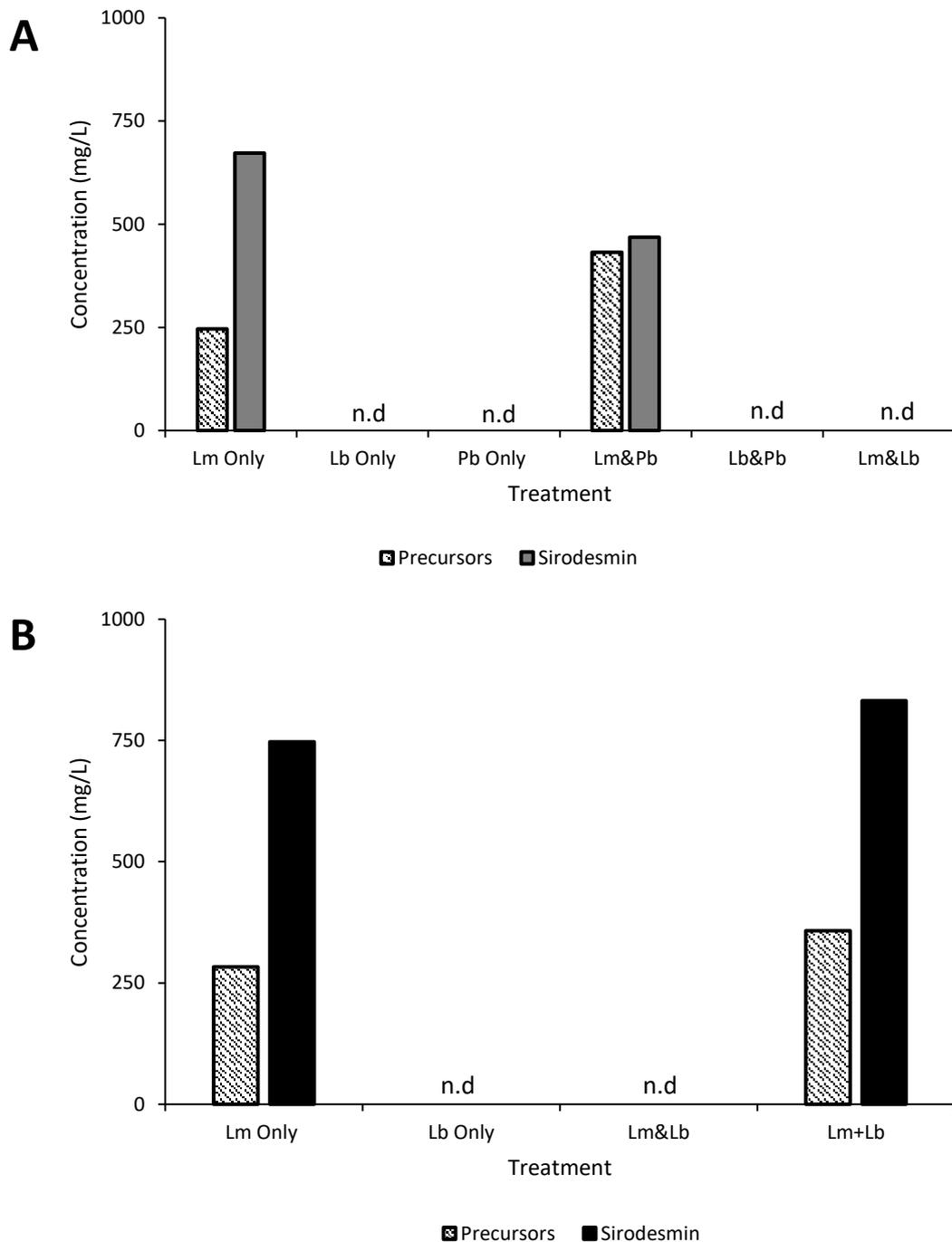
Figure 2.20. Concentration of sirodesmin PL (mg/L) extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) or *P. brassicae* (Pb) or simultaneous co-cultures of these pathogens (A) or liquid culture inoculated with four treatments (*L. maculans* only (Lm), *L. biglobosa* only (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb) and *L. maculans* and *L. biglobosa* inoculated sequentially (Lm+Lb)) (B). For the Lm+Lb treatment, the liquid media was inoculated first with *L. maculans* then seven days later *L. biglobosa* was added. Samples where a maxima corresponding to 'precursors' or 'sirodemsin PL' were not detected are indicated (n.d).

There were no significant differences between extracts in the concentrations of sirodesmin PL ($F_{1.17}$= 1.55, P = 0.281, LSD = 453.2) (Fig. 2.20). There were only two extracts that had concentrations of 'sirodesmin' were detected; they were 'Lm only' (672 mg/L) and 'Lm&Pb' (469 mg/L) (Fig 2.20).

2.4   Discussion

L. maculans used an interference interspecific competition strategy to outcompete *L. biglobosa* and *P. brassicae*, whereas *L. biglobosa* used an exploitation interspecific competition strategy. The direct competition strategy, if any, of *P. brassicae* ascospores in autumn during disease initiation by ascospores remains unknown. However, interspecific competition later in the season is better understood. *P. brassicae* is a polycyclic pathogen, with successive generations of conidia being dispersed; Gilles *et al.* (2001d) estimated that 17 asexual generations of *P. brassicae* can occur between early October until June in a cropping season. Furthermore, there are also successive generations of ascospores produced (Evans et al. 2017). In contrast, to the monocyclic *Leptosphaeria* spp. pathogens, at least in the UK, where there is one generation of ascospores per cropping season with a long release period, lasting several months, September – March (West *et al.* 2001; Huang *et al.* 2005). This means that new plant tissues produced later in the season are available to *P. brassicae* but not to *Leptosphaeria* spp. For example, pod infections by *P. brassicae* are common, pod infections by *Leptosphaeria* spp. are not. Therefore*, P. brassicae* has a competitive advantage over *Leptosphaeria spp*. because *P. brassicae* can exploit the resources of the pods with little competition from *Leptosphaeria* spp., but competition by other oilseed rape pathogens may arise on pods. The 'chaotic' light leaf spot disease cycle in new crops is initiated by successful infection by *P. brassicae* ascospores (Gilles *et al.* 2001b; Evans *et al.* 2017) (Fig. 1.8). Therefore, the identified interspecific interactions between *Leptosphaeria* spp. and *P. brassicae* may be important for understanding changes in susceptibility and vulnerability of oilseed rape crops to *P. brassicae* due to the absence of sirodesmin PL enabling more successful *P. brassicae* infections to occur, allowing the *P. brassicae* disease cycle to start earlier and in more plants to cause a more 'chaotic' and severe *P. brassicae* epidemic.

When *L. maculans* and *L. biglobosa* or *P. brassicae* were co-cultured an antibiosis event occurred, indicated by a zone of inhibition. This supports previous findings from Elliott *et al.* (2007) that showed that *L. maculans* has an inhibitory effect on *L. biglobosa* through the appearance of a zone of inhibition. It appeared that an antagonistic secondary metabolite was released, which interfered with the colony formation by limiting the growth of *L. biglobosa* or *P. brassicae* mycelium towards *L. maculans*, producing a non-symmetrical colony formation. Therefore, *L. maculans* may have limited the access of *L. biglobosa* or *P. brassicae* to the resources available; thus, outcompeting the competing pathogens using an interference interspecific competition strategy (Dutt *et al.* 2021). In contrast, when *L. biglobosa* and *P. brassicae* were co-cultured, *L. biglobosa* colonised the medium more efficiently than *P. brassicae*, reducing the resource available for the competing pathogen. This is supported by previous studies that have shown that *L. biglobosa* has a faster colonisation rate than *L. maculans* (Shoemaker and Brun, 2001; Huang *et al.* 2001; 2003). Therefore, it was concluded that *L. biglobosa* utilises an interspecific exploitation competition strategy (Dutt *et al.* 2021).

There was a small zone of clearing surrounding the *P. brassicae* colony when in competition against *L. biglobosa*. However, this was seen only on clarified V8 agar, not on PDA. However, this zone of clearing was not observed when *P. brassicae* was co-cultured with *L. maculans*. This may be due difference in the identified interspecific competition strategies utilised by *L. maculans* and *L. biglobosa*. Black (2020) found that zones of clearing on V8 media are associated with a starch depletion in the media due to larger colonies digesting the starch, depleting the nutrients available to other competing pathogens in an otherwise relatively energy poor medium compared to PDA. In this study, sucrose, a disaccharide of glucose and fructose, was added at 1g/L to supplement the media. However, due to the potential exploitative interspecific competition strategy of *L. biglobosa* these limited nutrients may have been made unavailable to *P. brassicae* by *L. biglobosa*. This zone of clearing was not observed when competing with *L. maculans* because it relies on an interference strategy rather than exploitation. This explanation could have been confirmed using Benedict's Test and/or iodine solution to identify the presence of reducing sugars and/or starch respectively.

The *L. maculans*-derived antagonistic compound was identified using HPLC and LC-MS analytical chemistry techniques to be sirodesmin PL. The extracts that were shown to contain sirodesmin PL within their HPLC chromatographs were 'Lm only', 'Lm&Pb' and 'Lm+Lb' and these were the only extracts that consistently caused a reduction in colony area when compared to their respective controls for *L. biglobosa*, *P. brassicae*, *V. longisporum* and *S. sclerotiorum.* These findings agree with the identified differences in the interspecific competitive strategies using phenotypic changes in colony morphology between *L. maculans* and *L. biglobosa*. In the secondary metabolite bioassay, none of the extracts that were extracted from a liquid culture containing *L. biglobosa* had a consistent significant antagonistic effect on any of the pathogens tested and there were no maxima found in the HPLC analysis that differed from those of other extracts, confirming that antagonistic metabolites were not produced by *L. biglobosa* as a form of interference competition. This confirms that *L. maculans* exploits an interference competitive strategy, whereas *L. biglobosa* may use an exploitation competitive strategy against competing plant pathogens.

This work also suggested that the compounds from the antagonistic extracts were 'de-toxified' over time because colony growth was delayed rather than permanently inhibited. Colony area when antagonistic extracts were applied at 10 dpi were similar or larger than the Day 7 colony area of those that had not been inoculated with an antagonistic extract. Additionally, metabolites, such as sirodesmin PL, was shown to be produced constitutively, even when not participating in interspecific competition with another pathogen. A limitation of the experiment is that naturally the production of compounds would be gradual, yet continual as opposed to the unique extreme 'hit' like that used in this experiment. However, if metabolites, like sirodesmin PL, were produced constantly at a rate greater than that of detoxification and decomposition this would result in inhibition of growth of the competing pathogen, producing a competitive advantage over the competing pathogen. Another limitation of this study was the very slow rate of colonisation by *P. brassicae;* this may have masked the true extent of the inhibition. Further investigations to understand the effect of sirodesmin PL should be done in liquid culture over a longer period.

Interestingly, when the extract "Lm&Lb" was analysed, the sirodesmin PL maximum was not present, nor were the maxima for its precursors despite the presence of *L. maculans.* Unique detectable maxima were not identified in the chromatograph for extract 'Lm&Lb', suggesting that these compounds were not broken down. Instead, it is believed that the production of pre-cursors was inhibited. This could be due to inhibition of gene expression of 18 co-regulated genes that have been reported to be involved in the biosynthesis pathway of sirodesmin PL (Gardiner *et al.* 2004). It has been shown that the disruption of these genes produced mutants that had significantly reduced production of sirodesmin PL (Gardiner *et al.* 2004; Fox and Howlett 2008). Therefore, if *L. biglobosa* disrupts the expression of products of the genes responsible for the biosynthesis of the sirodesmin PL precursors early in the pathway then all three different compounds will not be produced; that would provide an explanation for the observed results (Fig. 3.35). It has been shown that *O*-Prenyl-L - tyrosine is the first committed precursor for sirodesmin PL biosynthesis; a step catalyzed by prenylase (*SirP*) (Pedras and Yu 2009) has been shown to inhibit the production of sirodesmin PL (Gardiner *et al.* 2004), so denaturation of *sirP* would inhibit the production of the precursors and sirodesmin PL production. The expression of these genes in the presence of *L. biglobosa* and the potential gene inhibitors will need to be investigated further.

Elliott *et al.* (2007) showed that when a 13-day wild-type *L. maculans* colony was overlaid with molten agar containing *L. biglobosa* conidia, a zone of inhibition around *L. maculans* was formed. This inhibition was not observed when a non-sirodesmin producing *L. maculans* mutant was used, implying that this zone of inhibition was caused by sirodesmin PL. This study provides different results to those published by Elliott *et al.* (2007) because in this study *L. maculans* and *L. biglobosa* were cultured simultaneously in liquid culture rather than using a sequential co-inoculation. However, when *L. maculans* and *L. biglobosa* were sequentially inoculated, the secondary metabolite extract caused a reduction in colony area, which supports the Elliott *et al.* (2007) findings. This simultaneous co-inoculation may have allowed *L. biglobosa* to exert its exploitative interspecific competitive strategy, enabling *L. biglobosa* to colonize the medium and exclude *L. maculans* from the resource or potentially disrupt *L. maculans* gene expression for sirodesmin PL precursor production before sirodesmin PL

production is initiated. If *L. biglobosa* minimises or inhibits the production of sirodesmin PL and its precursors, then it would give itself and other pathogens such as *P. brassicae, V. longisporum* and *S. sclerotiorum,* a competitive advantage by limiting the interference competitive advantage of *L. maculans* over them. Also, this may help to explain why the effectiveness of *L. biglobosa* (WV type) in inducing resistance to *L. maculans* (HV) infection was compromised if *L. biglobosa* was added at 64h or later after *L. maculans* (Mahuku *et al.* 1996) because 64 hours or ~ 2.5 days is approximately when sirodesmin PL starts to be produced.

Results of this study may have a practical implication as previous work has shown that *L. maculans* and *L. biglobosa* ascospores can be released simultaneously (Huang *et al.* 2019). If the *Leptosphaeria* spp*.* ascospores are released and infect the host at the same time, then a simultaneous infection will occur; that could result in reduction in sirodesmin PL production. This is in addition to resistant cultivars that select against *L. maculans,* and helps to provide an advantage to *L. biglobosa*, *P. brassicae, V. longisporum* and *S. sclerotiorum*. This may provide an explanation for the increase in yield losses and change in geographical distribution of *P. brassicae* across the UK.

Chapter 3 Interactions between phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) causal pathogens *in planta*.

3.1   Introduction

Phoma stem canker and light leaf spot caused by *L. maculans* and *L. biglobosa*, and *P.brassicae,* respectively, are the two most economically damaging diseases to English and Welsh oilseed rape growers (CropMonitor 2020). The use of resistance in oilseed rape cultivars is well established for control of *L. maculans* but not for control of *P. brassicae* and *L. biglobosa*. The AHDB recommended list field trials report field resistance to *P. brassicae,* but not *L. biglobsa*. However, the underlying mechanisms of that resistance have not been determined. *R*-genes against *L. maculans* (*Rlm1,2,3,4,6,7,9,11*) and genetic traits that appear to confer resistance against *P. brassicae (PBR1 and 2*) have been identified; but none have been identified for *L. biglobosa* (Bradburne *et al.* 1999; Boys *et al.* 2007;2012; Rouxel and Balesdent 2017; Karandeni-Dewage *et al.* 2018). However, commercial cultivars are available with *R* genes against *L. maculans*. A common *R*-gene-mediated resistance that is currently being used in the UK is *Rlm7*. Its use appears to be increasing due to the high proportion of cultivars on the 2021/22 AHDB recommended list known to contain this gene for resistance to *L. maculans* (Mitrousia *et al.* 2017; AHDB 2021d). Recent research suggests that cultivars with good resistance against *L. maculans,* particularly those possessing the *Rlm7* resistance gene, are susceptible to *P. brassicae* (Huang *et al.* 2021*;* Karandeni Dewage *et al.* 2021). It is often reported that *L. maculans* is more damaging than *L. biglobosa* since *L. biglobosa* is often associated with upper stem lesions while *L. maculans* is often associated with stem base cankers (West *et al.* 2009; Fitt *et al.* 2006ab). However, recently *L. maculans* and *L. biglobosa* are both frequently being found at the stem base (Huang *et al.* 2014b). Severe plant infections with *L. biglobosa*

can also lead to substantial yield losses (Huang *et al.* 2005; Fitt *et al.* 2008; Dawidziuk *et al.* 2010; Brachaczek *et al.* 2016; Cai *et al.* 2017). Therefore, the economic importance of *L. biglobosa* and its role in the disease-host complex should not be neglected.

P. brassicae is a very difficult plant pathogen to control (Karandeni Dewage *et al.* 2021). There are many reasons for this, such as poor timing of fungicide applications (Gilles *et al.* 2000), fungicide insensitivity (Carter *et al.* 2013), the complex, polycyclic disease cycle (Evans *et al.* 2017), legislative and environmental concerns (Hillocks 2012) and limited understanding of host resistance against *P. brassicae* (Karandeni Dewage *et al.* 2021). All these potential reasons may be attributed to the difficulty in studying the true extent of light leaf spot disease severity in controlled-environment or field conditions due to the long asymptomatic period (2-8 months) between leaf infection and the appearance of the most obvious symptom, *P. brassicae* sporulation, when acervuli break through the leaf surface (Rawlinson *et al.* 1978a; Gilles *et al.* 2000a; Boys *et al.* 2007; 2012). The total disease severity can be assessed more accurately by destructively excising leaves and placing these in polyethylene bags at 4-8°C for 5 days (Fitt *et al.*, 1998; Boys *et al.* 2007, 2012; Karolewski *et al.* 2006; Karandeni Dewage *et al.* 2018, 2021).

Recent investigations using qPCR analysis to monitor the ascospore release events of *L. maculans*, *L. biglobosa* and *P. brassicae* have shown that the previously documented ascospore release patterns for these pathogens are changing; ascospores for all three pathogens are being detected in the air at the same time during September and October (Evans *et al.* 2017; Huang *et al.* 2019). Therefore, it is very likely that leaf infections of these pathogens occur simultaneously. *L. maculans* produces a phytotoxin called sirodesmin PL that has an inhibitory effect on the growth of *L. biglobosa,* suggesting that sirodesmin PL may be used as an interspecific interference competition strategy by *L. maculans* (Rouxel *et al.* 1988; Elliott *et al.* 2007; Dutt *et al.* 2021). In contrast, *L. biglobosa* does not produce phytotoxin sirodesmin PL but has been reported to have a faster growth rate than *L. maculans* (Shoemaker and Brun, 2001; Huang *et al.* 2003)*,* suggesting that *L. biglobosa* uses an interspecific exploitation competition strategy (Dutt *et al.* 2021). The competitive strategy at infection for *P. brassicae* unknown but its polycyclic life cycle enables the pathogen to have increased access to other plant tissues,

such as pods, may provide a competitive advantage at other key stages in the oilseed rape growing season. Therefore, these changes in ascospore release sequence may result in changes to the interspecific interactions between these pathogens and their implications on oilseed rape disease control strategies need to be understood. Therefore, there is a need to further investigate the interactions/competition between *Leptosphaeria* spp. and *P. brassicae*.

This study aims to investigate the interactions between *L. maculans*, *L. biglobosa* and *P. brassicae in planta* by testing the following threehypotheses ;

- A robust co-inoculation method for *Leptosphaeria* spp. and *P. brassicae* can be developed.
- The presence of *Rlm7* influences the incidence and severity of disease caused by *Leptosphaeria* spp. and *P. brassicae*.
- The simultaneous inoculation of cotyledons with *L. maculans* and *L. biglobosa* affects the lesion phenotype, secondary metabolite composition and the growth of the two pathogens (e.g. measured by pathogen DNA).

The three hypotheses were tested through the following three objectives.

- To develop a robust inoculation method for investigating co-infection by *L. maculans*, *L. biglobosa* and *P. brassicae*.
- To investigate the effects of *Rlm7* on interactions between *L. maculans*, *L. biglobosa* and *P. brassicae*.
- To study the interactions between *L. maculans* and *L. biglobosa* by assessing lesion development, pathogen DNA and phytotoxin production

3.2 Materials and methods

3.2.1 Preparation of inoculum

3.2.1.1 Preparation of *L. maculans* and *L. biglobosa* inoculum

The *L. maculans* (isolate ME24) and *L. biglobosa* (isolate WH17-Why-1) (Table 2.1) inocula were prepared as outlined in section 2.2.1. Conidial inoculum was prepared as described by Huang *et al.* (2014a). Spores were dislodged from 2-week-old colonies that were cultured on 20% clarified V8 agar plates using an L-shaped spreader and 9 mL of sterilized distilled water. The SDW was poured onto the colony and spores were gently dislodged using the L-shaped spreader, being careful not to damage the agar surface. The suspension containing the dislodged spores was passed through two layers of sterilized Miracloth (Calbiochem, USA) inside a glass funnel into a 15 mL skirted Falcon tube. This was to remove any mycelium and unwanted debris from the suspension. This was repeated for three agar plates for each *Leptosphaeria* spp. The contents of three 15 mL Falcon tubes were combined into one 50 mL Falcon tube. The concentration of the newly formed stock conidial suspension was measured using a haemocytometer slide under a light microscope and was aliquoted equally into three clean 15 mL Falcon tubes. These were stored at -20°C until required.

When required, one aliquot of the stock conidial suspension was defrosted and adjusted to 10^7 conidia mL^{-1} with sterilized distilled water using a haemocytometer slide under a light microscope. This became the working conidial suspension. The working conidial suspension was aliquoted equally into three clean 15 mL Falcon tubes. These were stored at -20°C until required or at 4°C if being used with 48 h.

3.2.1.2 Preparation of *P. brassicae* inoculum

For *P. brassicae*, the initial conidial inoculum was combined by removing spores from plants that had been inoculated with isolate 15WOSR64-SS1 (Table 2.1) because *P. brassicae* conidia become less infective when cultured on medium. Using a leaf wash technique, these *P. brassciae* conidia were washed off by washing individual infected

leaves inside a glass beaker filled with distilled water before being decanted into smaller vessels such as 50mL Falcon tubes. The spore concentration was measured using a haemocytometer slide under a light microscope. Concentrations were adjusted to 1.0 x 10^5 spore mL^{-1} with added 0.01% w/v Tween 20 using sterilized distilled water. These spore suspensions were stored at -20°C until required or at 4°C if being used with 48 h. Lower concentrations of spores were used for *P. brassicae* than for *Leptosphaeria* spp. inoculum because these were concentrations that are routinely used in our laboratory for inoculation work. The difference in concentration may be because *P. brassicae* conidia play a more important role in the life cycle of *P. brassicae* than *Leptosphaeria* spp. conidia play in their respective life cycles.

3.2.2   Preparation of plants

For each experiment, a seed germination success (%) assay was done to ensure seed viability. This was done by moistening a 9 cm diameter Whatmann filter paper placed inside a Petri dish. Pre-wetted 50 seeds were placed on top of the filter paper and placed by a window for 24-48 h. The number of seeds that showed signs of germination were counted and they were deemed to have achieved a successful germination. The percentage of seeds that had successfully germinated was calculated.

The growth media for plants was a mixture of John Innes no. 3 soil and MiracleGro multi-purpose compost mixed in a 1:1 ratio. Seed plug trays (4 x 6 or 8 x 5 wells) or pots (9 cm diameter) were placed on a layer of bench matting inside a seed tray. Seed plug trays or pots were filled with the soil: compost mixture and then moistened with water. Using a finger or the bottom of a 15 mL Falcon tube, indentations (approx. 0.5-1 cm in depth) were made in the centre of the plug tray well or pot for seeds to be sown. The germination success assay was used to determine the method of sowing the seeds. If the germination success of a cultivar was > 90%, then two seeds were sown directly and < 90% of the seeds were pre-germinated, using the same method as used for the germination success (%) assay, before being sown individually. The newly sown seed inside the indentation was gently covered with growth media. Plants were watered from below by pouring water directly onto matting. Plants were watered regularly to ensure

the growth media always remained moist. When the first true leaf was starting to develop, plants were thinned to one plant per well or pot and staked up using a plastic stake and plastic-coated wire to support the plant. For cotyledon experiments, the true leaves were regularly excised using scissors to ensure the maximum longevity of the cotyledon. Plants were grown either in controlled environment cabinets (FITOCLIMA D1200, ARALAB, Rio de Mouro, Portugal) under conditions of 18/16°C and a 12h/12h day/night cycle or in the glasshouse under semi-controlled conditions with heaters to ensure plants did not freeze and lighting for a 12h/12h day/night cycle (Table 3.1)

3.2.3   Plant inoculation with *L. maculans* or *L. biglobosa*

Plants were inoculated with *L. maculans* or *L. biglobosa* using a point inoculation method. For cotyledon inoculation, four inoculation sites were made per plant; one site per lobe of each cotyledon, whereas for true leaves there were four inoculation sites per inoculated leaf; two sites on either side of the leaf vein. For true leaf inoculation, the cuticular wax at the inoculation site was removed by gently rubbing the leaf surface with the eraser at the end of a pencil.

The inoculation sites were made by wounding the plant with a sterilized needle and placing a 11 µL droplet of conidial spore suspension (1×10^7 spores mL^{-1}) onto each wound. Once all plants in a tray were inoculated, a propagator lid pre-misted with water was placed over the plants and sealed using masking tape to increase humidity. The plants were incubated in the dark for 48 hours; either growth cabinet lights were turned off or the propagator lids were covered with black plastic bags to restrict light.

After 24 h, the plants were vented and light was allowed for 1 h; then after a further 24 h (total 48 h) the light was allowed, and the plants were vented again for 1 h to acclimatise before the propagator lid was removed fully. Once all droplets had evaporated, a light mist of water was sprayed above the inoculated plants so that droplets fell onto the plants. The mist was applied above the plants to avoid any cross-contamination between plants.

Table 3.1: Overview of *in planta* experiments done giving the pathogens used, plant growth stage and environment.

Experiment description	Pathogen*	True leaf or cotyledon	Controlled environment (CE) or Glasshouse (GH)	Inoculation method*^
Robust inoculation method	Lm & Pb	True Leaf	CE	Lm, Lb and Pb - Disc, drop or spray (WP)
Suitable environment	Pb	True leaf	CE and GH	Pb - Spray (WP)
Effect of *Rlm7* gene	Lm, Lb & Pb	True leaf	CE	Lm and Lb – Drop (TL) Pb – Spray (TL)
Lm/Lb interactions 17 dpi	Lm & Lb	Cotyledon	CE	Lm and Lb – Drop (TL)
Lm/Lb interactions 26 dpi	Lm & Lb	Cotyledon	CE	Lm and Lb – Drop (TL)

*Pathogens used were *Leptosphaeria maculans* (Lm), *Leptosphaeria biglobosa* (Lb) or *Pyrenopeziza brassicae* (Pb)

^The inoculation method was used either for the whole plant (WP) or target leaf (TL).

3.2.4   Plant inoculation with *P. brassicae*

Plants were inoculated when they had 3-4 true leaves by spraying with *P. brassicae* conidial suspension (1 x 10^5 spores mL^1) using a 50 mL spray bottle until droplets started running off the surfaces of leaves. If only one leaf was to be inoculated, then just that leaf was sprayed with inoculum on the upper and lower sides of the leaf. The plants were then covered in a pre-misted sealed clear polyethylene bag and placed into the controlled environment cabinet in darkness set at 16°C (Fig. 4.1). After 48 h, the bags were removed. Plants were then grown in controlled environment cabinets under conditions of 18/16°C day/night and a 12h/12h day/night cycle.

3.2.5   Assessment of *L. maculans* and *L. biglobosa* lesion severity.

3.2.5.1 Lesion severity score using a 0-9 scale.

The phoma leaf spot lesion severity for *L. maculans* and *L. biglobosa* on true leaves was assessed using a 0-9 severity score. *Leptosphaeria* spp. lesions were assessed, based on the area of the lesions (including yellowing). The scale was 0 = no necrotic lesions around wounds; 1 = lesion diameter ≤ 0.6 mm; 2 = lesion diameter 6-20 mm; 3 = lesion diameter 20 - <40 mm; 4 = lesion diameter 40 - <60 mm; 5 = lesion diameter 60 – <100 mm; 6 = lesion diameter 100 - <300 mm; 7 = lesion diameter 300 mm- <500 mm; 8 = lesion diameter 500-1000 mm; 9 = lesion diameter ≥ 1000 mm; and/or leaf death.

3.2.5.2 Lesion severity using lesion area calculated using ImageJ.

At 17- or 26-days post inoculation, cotyledons were excised from each plant and placed inside a square of known size and a standardised photograph was taken, ensuring the full square was visible in each photograph. This was repeated for all plants. The lesion area was calculated by processing the standardised photographs using ImageJ software. The total number of pixels were converted into cm^2 using the known area of the square.

Figure 3.1: Polyethylene inoculation tent for high humidly during inoculation with *L. maculans, L. biglobosa* or *P. brassicae* on true leaves of oilseed rape.

3.2.6 Assessment of *P. brassicae* severity and sporulation.

3.2.6.1 *P. brassicae* foliar severity score using a 0-7 scale

The *P. brassicae* disease severity scores were assessed using a scale (0-7) (Table 3.2) (Fig. 4.2), then a G-index score was calculated for each plot, using either Equation 3.1 or Equation 4.2. A mean G-index score was calculated for each treatment.

3.2.6.2 Leaf area with *P. brassicae* sporulation

The disease severity caused by *P. brassicae* was also assessed by the amount of sporulation. This was assessed as % of leaf area covered with *P. brassicae* sporulation.

Equation 3.1: Calculation of a 0-9 G-index score from a 0-7 severity score for *L. maculans, L. biglobosa* or *P. brassicae*. n_x = number of samples that scored x.

$$G - index\ severity\ score = \frac{(n_1 + n_2) + 3n_3 + 5n_4 + 7n_5 + 9(n_6 + n_7)}{n_0 + n_1 + n_2 + n_3 + n_4 + n_5 + n_6 + n_7}$$

Equation 3.2: Calculation of a 0-100 G-index score from a 0-7 severity score for *L. maculans, L. biglobosa* or *P. brassicae*. n_x = number of samples that scored x.

$$G - index\ severity\ score = \frac{((n_1 + n_2) + 20n_3 + 40n_4 + 60n_5 + 80n_6 + 100n_7)}{n_0 + n_1 + n_2 + n_3 + n_4 + n_5 + n_6 + n_7}$$

Table 3.2: *Pyrenopeziza brassicae* (light leaf spot) severity score scale (0-7).

Severity score^	Description
0	No disease
1	Trace of disease
2*	Plants with few scattered lesions and/or < 20% of leaf area affected
3*	Plants with several scattered lesions and/or 20-40% of leaf area affected
4	Plants with many small lesions/ scattered large lesions and/or 40-60% of the leaf area affected
5*	Plants with many small lesions/ scattered large lesions and/or 60-80% of the leaf area affected
6	Severely diseased plants > 80% leaf area affected
7*	Plants stunted or dead

^ This assessment scale was modified from the AHDB cereals and oilseeds RL trial light leaf spot assessment key, the assessment scale described by Boys et al. (2012) and Karandeni-Dewage et al. (2021).

*Figure 3.2 for illustrations of same score.
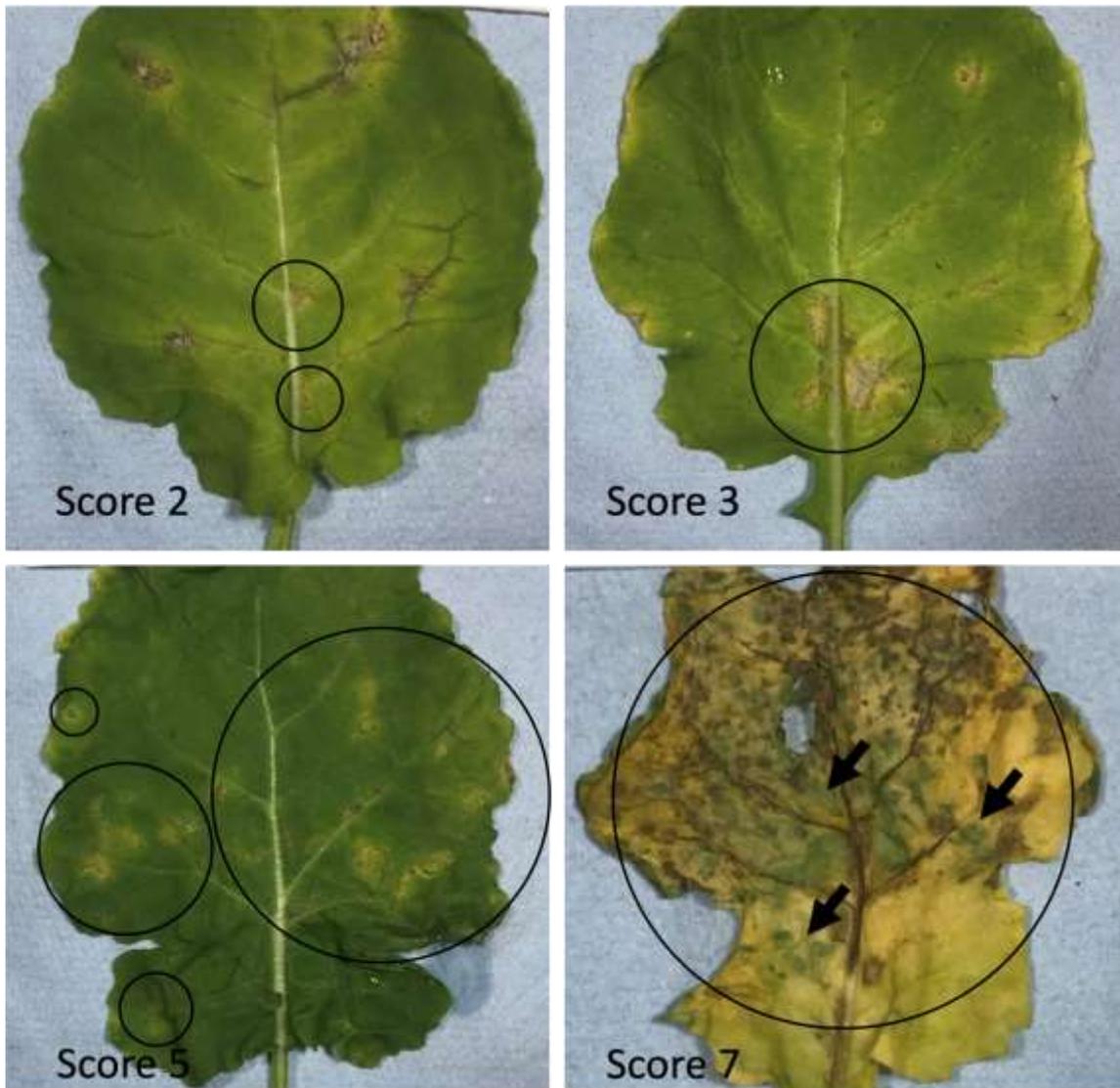
Figure 3.2. *P. brassicae* lesion severity score key. Black circles indicate areas of *P. brassicae* sporulation. Black arrows indicate areas of 'green islands'; a characteristic indication that the dead leaf was extensively colonised by *P. brassicae*. See Table 3.2 for descriptions of these scores.

A mean leaf area with *P. brassicae* sporulation (%) was calculated for each treatment. Plants were incubated to increase sporulation. This was done by either cutting plants at the root collar or excising individual leaves; then they were individually labelled, wrapped in moist blue paper towel, placed inside a zip-lock polyethylene bag, and incubated in a cold storage room (4-5°C) for up to 14 days or until *P. brassicae* sporulation was clearly visible.

3.2.7   Development of a robust inoculation method for investigating co-infection by *L. maculans*, *L. biglobosa* and *P. brassicae*

3.2.7.1 Identification of effective inoculation method for *L. maculans* and *P. brassicae* infection

3.2.7.1.1   Preparation of *L. maculans* and *P. brassicae* inoculum and plant material

Inocula of *L. maculans* and *P. brassicae* were prepared as outlined in section 3.2.1. The plant material was prepared as outlined in section 3.2.2. Pre-germinated seeds of cv. Bristol (good resistance to *L. maculans,* little resistance to *P. brassicae*) and cv. Imola (unknown resistance to *L. maculans*, good resistance to *P. brassicae*) (Table 3.3) were sown into 5 X 8 well plug trays, then transplanted to 5 cm diameter pots when they had two true leaves and placed in a glasshouse until they had three true leaves ready for inoculation. Plants were placed into the CE cabinets set at 20/18°C and a 12h/12h night/day at 24 hours before inoculation.

3.2.7.1.2   Inoculation methods

To investigate the most effective inoculation method, three inoculation methods were used for inoculation of two cultivars cv. Bristol and cv. Imola (Table 3.3) to compare disease symptom development. In total, there were 14 treatments (Table 3.4). For the 'Disc' inoculation method, each plant was point inoculated using 0.5 X 1 cm rectangles of filter paper (soaked in suspensions of either *L. maculans* (10^7 spores per mL) or *P. brassicae* (10^5 spores per mL).

Table 3.3: List of cultivars used in this study and their AHDB resistance ratings to phoma stem canker (Phoma) and light leaf spot (LLS) pathogens (0-9 scale with scores below 5 = little resistance, 6-7 = medium resistance, and 8-9 being good resistance) from AHDB Recommended List (AHDB RL) (AHDB 2020c).

Cultivar	AHDB Phoma rating$^\$$	AHDB LLS rating	Source	Controlled Environment (CE) /Field experiment
Barbados	7	7	AHDB RL 2016/17	Field
Charger	4	4	AHDB RL 2016/17	CE & Field
Django	Medium (6) ^	Medium (6) ^	KWS 2017	Field
Flamingo	4	7	AHDB RL 2017/18	Field
Hunivers (MH13JO76)	Medium- *	Medium (6) *	KWS 2017	Field
Quartz	8	5	AHDB RL 2016/17	Field
Excel	9	5	AHDB RL 2010/11	CE
Imola	Unknown	Good resistance	Boys *et al.* 2012	CE
Bristol	Good resistance	Little resistance	Gladders *et al.* 1998)	CE
Topaz	Little resistance	Unconfirmed	Larkan et al. 2016	CE
Topaz *Rlm7*	Good Resistance	Unconfirmed	Haddadi et al. 2022	CE

^AHDB 2018/19 Winter oilseed not added to the Recommended List
*AHDB 2018/19 Winter oilseed rape candidates
$^\$$ AHDB phoma resistance is for *L. maculans* resistance only.

Table 3.4: List of treatments used to investigate the effects of inoculation method on *L. maculans* or *P. brassicae* disease symptom development on two *Brassica napus* cultivars (Bristol - (good resistance to *L. maculans*, little resistance to *P. brassicae*) and Imola – (unknown resistance to *L. maculans*, good resistance to *P. brassicae*).

Treatment	Inoculum	Cultivar	Method
1	Untreated	Imola	No inoculation
2	*L. maculans*	Imola	Point inoculation with filter paper discs soaked with conidial suspension
3	*L. maculans*	Imola	Point inoculation with a drop of conidial suspension
4	*L. maculans*	Imola	Spray with conidial suspension
5	*P. brassicae*	Imola	Point inoculation with filter paper discs soaked with conidial suspension
6	*P. brassicae*	Imola	Point inoculation with a drop of conidial suspension
7	*P. brassicae*	Imola	Spray with conidial suspension
8	Untreated	Bristol	No inoculation
9	*L. maculans*	Bristol	Point inoculation with filter paper discs soaked with conidial suspension
10	*L. maculans*	Bristol	Point inoculation with a drop of conidial suspension
11	*L. maculans*	Bristol	Spray with conidial suspension
12	*P. brassicae*	Bristol	Point inoculation with filter paper discs soaked with conidial suspension
13	*P. brassicae*	Bristol	Point inoculation with a drop of conidial suspension
14	*P. brassicae*	Bristol	Spray with conidial suspension

Plants were lightly sprayed with sterilized distilled water to retain filter paper-leaf adhesion. For the 'Drop' inoculation method, each true leaf of each plant was point inoculated with a 11 µL droplet of inoculum suspension.

Both 'Disc' and 'Drop' point inoculation methods used a multi-site leaf inoculation plan (Fig. 3.3). All four positions were inoculated with that inoculum. For 'Spray' inoculation, the plants of both cultivars at growth stage BBCH 13-14 (i.e., plants had 3-4 true leaves; Lancashire *et al.* 1991) were sprayed with each of the two inoculum types (*P. brassicae* only or *L. maculans* only). All treatments for cv. Imola were done in triplicate and those for cv. Bristol were done in duplicate for disease symptom development assessments. Each plant was sprayed with approximately 1 mL of either *L. maculans* (10^7 spores per mL) suspension or *P. brassicae* (10^5 spores per mL) suspension or both (0.5 mL of each) using a clean 50 ml spray bottle. Each plant was sprayed individually to ensure that all leaves of each plant were inoculated. All plants were placed into sealed plastic containers sprayed with distilled water (Fig. 3.1) and placed in a controlled-environment cabinet at 18°C /16°C day/night. Each inoculation treatment was placed in a separate plastic container for 48 h. Plants were monitored and watered accordingly, and newly emerging leaves were removed.

3.2.7.1.3   Assessment of *L. maculans* and *P. brassicae* disease symptoms

Symptom development was assessed at 21 dpi. For plants that had been inoculated using a disc or drop inoculation method, the presence/absence of symptoms at each inoculation site (Fig. 3.3) was recorded. For plants that were inoculated using a spray inoculation method, presence, or absence of symptoms on inoculated leaves was recorded. The percentage of successful inoculations was calculated for each treatment.
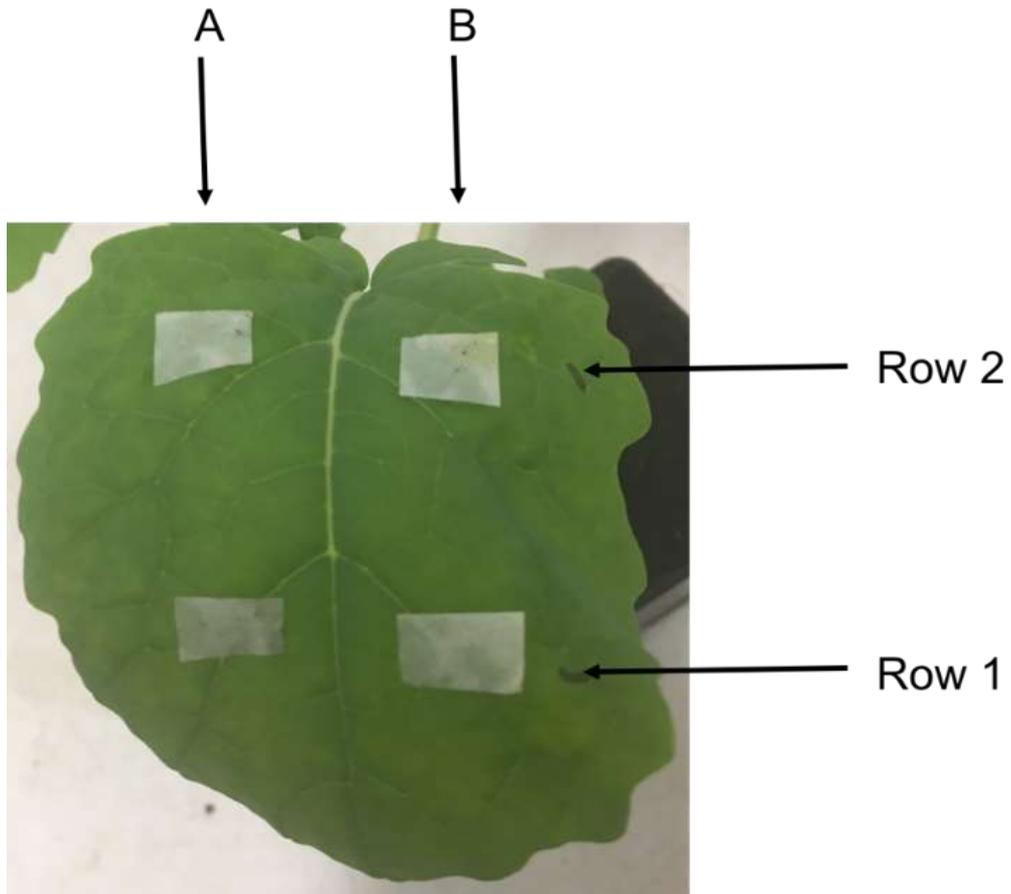
Figure 3.3: Multi-site leaf inoculation plan showing four different inoculation positions, with two positions on each side of the leaf. This photograph shows the 4-point inoculation with filter paper disks soaked with conidial suspensions of *L. maculans* or *P. brassicae* or a mixture of both.

3.2.7.2 Identification of a suitable environment for *P. brassicae* infection

3.2.7.2.1 Inoculum and plant preparation

Inoculum of *P. brassicae* was prepared as outlined in section 4.2.1.2. The plant material was prepared as outlined in section 4.2.2. Cvs Charger (low resistance to both *L. maculans* and *P. brassicae*) and Excel (high resistance to *L. maculans,* low resistance to *P. brassicae*) (Table 3.3) were sown into individual 9 cm diameter pots and placed in a controlled environment cabinet. The CE cabinets were set at 18/16 °C and a 12h/12h night/day.

3.2.7.2.2 Inoculation of plants with *P. brassicae*

Plants at BBCH 13/14 were inoculated with *P. brassicae* conidial suspension by spraying the whole plant as outlined in Section 3.2.4. In total, 24 cv. Excel plants and 12 cv. Charger plants were inoculated. After 48 h, the polyethylene bag was removed, and the plants were split into two halves; half of the plants of each cultivar were placed either in the controlled environment cabinet or placed in the glasshouse to experience semi-controlled conditions (Table 3.5). Soil was watered to ensure that it remained moist.

3.2.7.2.3 Assessment of plants with *P. brassicae*

All plants were assessed for light leaf spot severity at 18 days post inoculation (Section 3.2.6). A G-index was calculated to give a score between 0-100 for each treatment (Equation 3.2). After the severity assessment, plants were incubated (Section 4.2.6.2). After 13 days of incubation, *P. brassicae* foliar severity score (Section 2.4.1) and mean leaf area with *P. brassicae* sporulation (%) (Section 2.4.2) assessments were made and incidence of successful *P. brassicae* inoculation was calculated. Using the 0-7 sporulation severity score, a G-index was calculated to give a score between 0-100 for each treatment (Equation 3.2).

Table 3.5 : List of treatments used to investigate environmental conditions for _P. brassicae_ disease development on _Brassica napus._ Cv. Excel has a LLS RL rating of 5 whereas cv. Charger has a LLS RL rating of 4.

Treatment number	Cultivar	Growth environment	Replicates
1	Charger	CE cabinet	6
2	Charger	Glasshouse	6
3	Excel	CE cabinet	12
4	Excel	Glasshouse	12

3.2.8 Effects of *Rlm7* on interactions between *Leptosphaeria maculans*, *L. biglobosa* and *P. brassicae* on oilseed rape

3.2.8.1 Inoculum and plant preparation

Inocula of *L. maculans, L. biglobosa* and *P. brassicae* were prepared as outlined in section 3.2.1. The plant material was prepared as outlined in section 3.2.2. Seeds of cv. Topaz (susceptible to both *L. maculans* and *P. brassicae*) and its near-isogenic line Topaz-*Rlm7* (resistant to *L. maculans* and susceptible to *P. brassicae*) (Table 2.2) were sown individually into 9 cm diameter pots. Cvs. Topaz and Topaz-*Rlm7* are doubled haploid near isogenic lines that were created through reciprocal back-crossing to produce homozygous single-*R* gene introgression lines (Larkan et al. 2016; Haddadi et al. 2022). They were placed in the controlled environment growth cabinet until they had reached BBCH 14/15. The CE cabinets were set at 18/16 °C and a 12h/12h day/night.

3.2.8.2 Inoculation and experimental design

Plants were inoculated with *L. maculans, L. biglobosa* and/or *P. brassicae* as outlined in Table 4.6. There were 10 treatments. Each treatment was replicated 9 times. Each plant had four-point-inoculation sites. To ensure that inoculation variables were accounted for between treatments, plants that were point-inoculated with *L. maculans* or *L. biglobosa* were sprayed with sterilised distilled water (for Lm only, Lb only) or *P. brassicae* (for Lm and Pb, Lb and Pb). For plants that were spray inoculated with *P. brassicae* only the plant was then wounded and inoculated with 11 μL droplets of sterilised distilled water (for Pb only) (Fig. 3.4). Plants were arranged in a randomised design.

3.2.8.3 Assessment

L. maculans and *L. biglobosa* lesions were assessed at 17 dpi; the assessments were made as outlined in section 3.2.5.1 and 3.2.5.2. For *P. brassicae* symptom assessment, the infected leaves were excised and incubated as outlined in section 3.2.6.1 and 3.2.6.2 at 24 dpi.

Table 3.6. Treatment list for investigating the effect on lesion development and the presence of *Rlm7* when co-inoculated with *L. maculans* (Lm) or *L. biglobosa* (Lb) with *P. brassicae* (Pb)

Treatment number	Cultivar*	Inoculum 1	Inoculum 2	Replicates
1	Topaz	Lm	SDW	9
2	Topaz	Lb	SDW	9
3	Topaz	Pb	SDW	9
4	Topaz	Lm	Pb	9
5	Topaz	Lb	Pb	9
6	Topaz – *Rlm7*	Lm	SDW	9
7	Topaz – *Rlm7*	Lb	SDW	9
8	Topaz – *Rlm7*	Pb	SDW	9
9	Topaz – *Rlm7*	Lm	Pb	9
10	Topaz – *Rlm7*	Lb	Pb	9

* Cv. Topaz is susceptible to both *L. maculans* and *P. brassicae*. Resistance to *L. biglobosa* is unknown.

Cv. Topaz *Rlm7* is resistant to *L. maculans* and is susceptible to *P. brassicae.* Resistance to *L. biglobosa* is unknown.

Figure 3.4: Co-inoculation of a true leaf of oilseed rape using both point inoculation and spray inoculation methods. Arrows to show which is point inoculation (P), followed by spray inoculation (S).

After 13 days of incubation, plants were assessed for *P. brassicae* symptoms as outlined in section 3.2.6.1 and a G-index (0-100) was calculated for each treatment (Equation 3.2).

3.2.9 Effect of interactions between *L. maculans* and *L. biglobosa* on lesion development, quantity of pathogen DNA and phytotoxin production.

3.2.9.1 Inoculum and plant preparation

Inoculum of *L. maculans* and *L. biglobosa* was prepared as outlined in section 3.2.1. For the *L. maculans* and *L. biglobosa* simultaneous inoculation (Lm&Lb), 1 mL of each spore suspension was combined in a 15 mL Falcon tube. Plants were prepared as outlined in section 3.2.2. Seeds of cv. Charger (susceptible to *L. maculans*, resistance to *L. biglobosa* remains unknown) (Table 2.2) were sown into seed plug trays (8 x 5 well). Each tray was placed in the controlled environment growth cabinet as outlined in section 3.2.2. The experiment was replicated four times, with three experiments for lesion phenotype and quantity of pathogen DNA and one experiment for phytotoxin production.

3.2.9.2 Experimental design and inoculation

For lesions phenotype experiments, there were four treatments; plants inoculated with sterilized distilled water (SDW), *L. maculans* only (Lm only), *L. biglobosa* only (Lb only) or *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb). Plants were point inoculated as outlined in section 3.2.3. For quantity of pathogen DNA, a total of 120 plants were inoculated, 30 plants per treatment. There were three seed plug trays used in each experiment. The general layout of the trays is shown in Fig. 3.5. The treatment was assigned to each of the four half tray positions (20 plants) using a random number generator to give a randomised block experimental design. The order for the half tray positions determined the order for the additional quarter tray positions (10 plants). For the phytotoxin production, a total of 160 plants were inoculated with the same four treatments as used in the lesion phenotype experiments. There were four seed trays used (8 x 5 well); one full tray for each treatment. Therefore, there were 40 plants per treatment.
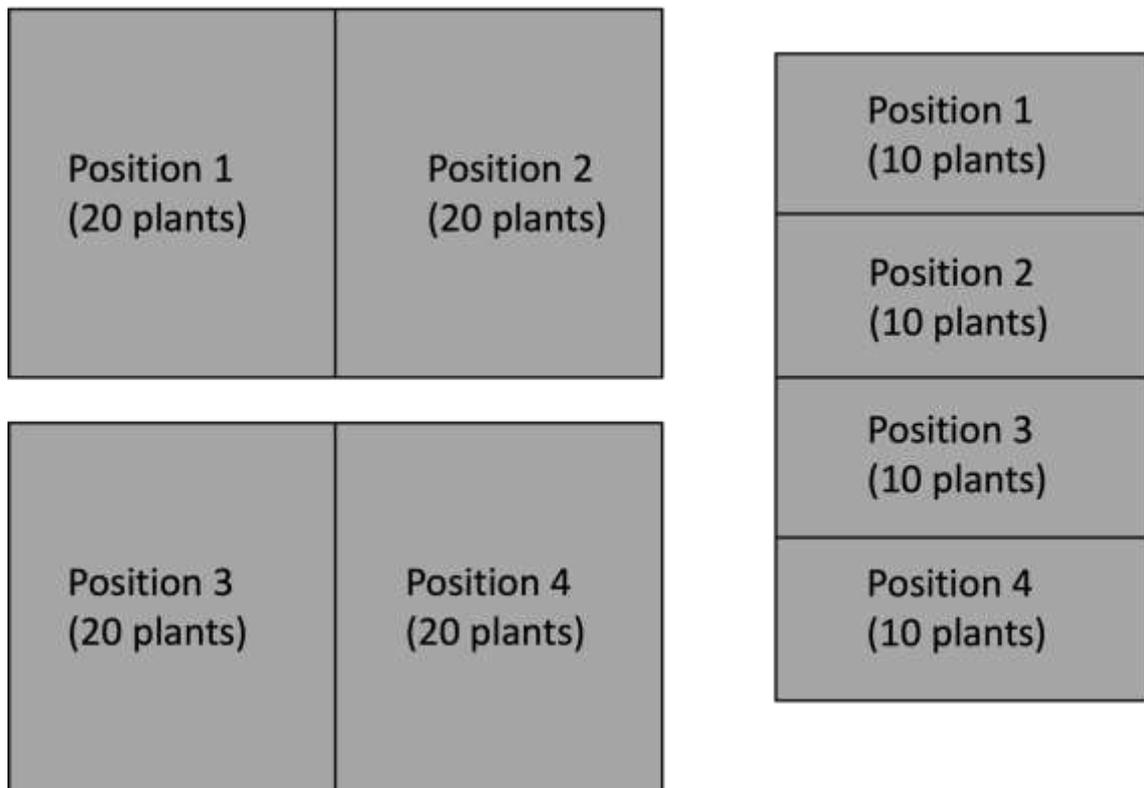
Figure 3.5: Seedling tray layout for when oilseed rape cotyledons were inoculated with *L. maculans* only (Lm only) or *L. biglobosa* only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW). 120 plants were grown in 3 three 40 well seedling trays. Two seedling trays were divided in half, the third seedling tray was split into quarters. Each treatment was randomly assigned to a position. Each treatment contained 30 plants (half tray and quarter tray) using a randomised block design.

3.2.9.3 Assessment

3.2.9.3.1   **Lesion development assessment**

Lesions were assessed at 17 dpi (then cotyledons were used for pathogen DNA quantification) or 26 dpi (then cotyledons were used for detection of phytotoxin production). Assessments were done as outlined in section 3.2.5.2.

3.2.9.3.2   **Quantification of pathogen DNA**

After lesion phenotype photographs were taken, both cotyledons from each plant were put into 2 mL screw cap tubes and stored at -20°C until required. Two experiments were done to investigate the differences in quantity of pathogen DNA between treatments. For the first experiment (experiment 1), the lesions were cut around before being placed into the tubes. The DNA extraction was done using the CTAB DNA extraction method for 10 samples for each treatment. The DNA concentration was calculated using a nanodrop spectrophotometer. The DNA was then diluted to 20ng/μL using nuclease-free water and stored at -20°C until required.

3.2.10 CTAB DNA extraction method

The DNA extraction method was the same as that of Huang *et al.* (2011), which is a slight modification of the CTAB protocol (Kaczmarek *et al.* 2009). Approximately 0.5 g of acid-washed glass beads and 440 μL of DNA extraction buffer were added to each screw cap tube containing the piece of spore tape or leaf tissue. The samples were lysed using a Fast-prep machine (MP24 FastPrep Machine, Classic bead beating grinder and lysis system) set to speed 6.0m/sec for 40 sec. This step was repeated three times with a 5 min incubation period on ice between each run. After lysis, 400 μL of 2% SDS was added to each tube and quickly vortexed. After a 30 min incubation in a water bath set at 65°C, 800 μL of phenol: chloroform: isoamyl alcohol mixture (24:1:1) was added to each sample. The samples were vortexed and centrifuged at 13000 rpm and 4°C for 10 min. The supernatant was pipetted into a new 1.5 mL.

Eppendorf tube containing ammonium acetate (30 µL, 7.5 M), isopropanol (480 µL) and glycogen (1 µL, 20 µg/ µL^{-1}). The samples were inverted and stored at -20°C for 16 h for DNA precipitation. The samples were then centrifuged again at 13000rpm at 4°C for 30 min. The supernatant was removed, and the DNA pellet was washed using 300 µL of 70% ethanol and centrifuged at 13000 rpm at 4°C for 15 min. The ethanol was removed, and excess ethanol could evaporate off. Once dry, the pellet was resuspended into 30 µL of SDW. The extracted DNA suspension was diluted using SDW using a dilution factor of 10. These dilutions were then analysed using qPCR as outlined in section 3.2.11.

3.2.11 Quantitative PCR (qPCR) analysis

The qPCR method used was the same as that of Huang *et al.* (2019). A master mix was prepared according to the number of reactions, for each reaction adding 10 µL of SYBR green master mix, 0.6 µL of 10 µM species-specific forward primer, 0.6 µL of 10 µM species-specific reverse primer and 6.3 µL of HPLC water. Then 17.5 µL of master mix was added to each well of a 96 well PCR plate and 2.5 µL of diluted extracted DNA suspension was added to the well; each DNA sample had two replicated wells. The the 96 well PCR plate was loaded into the qPCR machine (Agilent Stratagene Mx3005P) and run with a specific thermal profile. A set of standards for each pathogen was made using high quality DNA (>1.8 260/280 absorbance ratio on Nanodrop) extracted from pure culture. Standards were produced in a 10x dilution series, with 10,000pg to 1pg DNA for *L. maculans* and *L. biglobosa*. Standards were run alongside the samples to produce a standard curve for *L. maculans* or *L. biglobosa.* Species-specific primers sets were used (Table 3.7) (Appendices 10 and 11). Any value within that range is detectable and can be considered accurate

Table 3.7: Diagnostic primers used for quantitative PCR (qPCR) reactions.

Pathogen	Primer name and sequence	Expected product size (bp)	Reference
L. maculans	Lmac_F (CTTGCCCACCAATTGGATCCCCTA) Lmac_R (GCAAAATGTGCTGCGCTCCAGG)	331	(Liu et al. 2006)
L. biglobosa	WVF1 (CCTTCTATCAGAGGATTGGT) WVR1 (CGTTCTTCATCGATGCCAGA)	237	(Mahuku et al. (1996)
	Lbig_F (ATCAGGGGATTGGTGTCAGCAGTTGA) Lmac_R (GCAAAATGTGCTGCGCTCCAGG)	444	(Liu et al. 2006)
P. brassicae	PbITS-F (TTGAACCTCTCGTCGAAGAAGTTCAGTCT) PbITS-R (AGATTTGGGGGTTGTTGGCTAA)	461	(Karolewski et al. 2006).

The primers used were Lmac_F (CTTGCCCACCAATTGGATCCCCTA) and Lmac_R (GCAAAATGTGCTGCGCTCCAGG) for *L. maculans* and Lbig_F (ATCAGGGGATTGGTGTCAGCAGTTGA) and Lmac_R for *L. biglobosa* (Liu *et al.* 2006). The thermal profiles used were: one cycle of initial denaturation at 95°C for 2 min; 40 cycles of denaturation, annealing and amplification at 95°C for 15 sec, 60°C (for *L. maculans* and *L. biglobosa* for 30 sec, 72°C for 45 sec and 84°C for 15 sec; one cycle of 95°C for 1 min, 60°C for 1 min and 95°C for 15 sec. The samples and standards were duplicated in each run and the resulting total DNA (pg.) per sample was automatically calculated using MxPro QPCR software (Agilent) as explained in Karandeni-Dewage (2019) using the increase in fluorescence level and cycle number for qPCR standards. Mean values of the quantities of *L. maculans* and *L. biglobosa* DNA were calculated. They were used as the quantity of DNA for each pathogen. The mean quantity of pathogen DNA for each treatment was calculated using the means of each sample in the treatment.

These standards were used to form a standard curve to quantify the amount of target DNA in different samples. The performance of qPCR reactions was evaluated using the reaction efficiency and the R^2 value of the standard curve. Although the reaction efficiency varied between runs, the lowest acceptable level that was used was 70% (Appendix 11). The production of the correct amplicon was confirmed using the dissociation curve. The dissociation curve presented in the MxPro qPCR software shows a decrease in fluorescence at a specific temperature. This is because the fluorescence dye dissociates from the dsDNA when the whole amplified product becomes fully dissociated at a certain temperature.

3.2.12 Detection of phytotoxin production

After lesion phenotype photographs were taken, phytotoxins were extracted from lesions from 30 plants of each treatment were excised. Lesions from five plants were grouped together and placed into 2 mL screw cap tubes. There was a total of six full 2 mL screw cap tubes per treatment. The lesions were excised according to their size; for

small or no lesions, an 8mm diameter corer centred over each inoculation site was used. However, for larger lesions a scalpel was used to excise the lesion. After 48 hours of freeze-drying, samples were pushed to the bottom of each tube using a sterile inoculation loop. Three small metal ball bearings and 600 μL of ethyl acetate were added to each tube. Each sample was lysed using a FastPrep machine for 40 s at 6.0 m/s. This step was repeated three times. Samples were placed on ice for 5 min between runs. Lysed samples were centrifuged at 3000 rpm for 5 min. Then 500 μL of ethyl acetate supernatant was removed and pipetted into a sterile 50mL Falcon tube. Supernatants from each treatment were combined together. The four Falcon tubes were placed under a constant stream of nitrogen to evaporate the sample to residue, before resuspending in 500 μL of ethyl acetate. Once completely resuspended, the sample was passed through a 0.45 μm PTFE syringe filter into a 2 mL HPLC vial. This became the stock suspension. Samples were then stored at room temperature until required for HPLC analysis.

Samples were prepared for HPLC by pipetting 150 μL of stock suspension into a 2mL HPLC vial containing a 0.2mL glass insert. The sample was then analysed as outlined in section 2.3.7. Fractions were taken at 11-12.5 min and 16.0-17.5 min. These fractions were then placed under a constant stream of nitrogen to evaporate the sample to residue. The residue was resuspended in 110 μL of ethyl acetate before being analysed using LC-MS as outlined in section 2.3.8.

3.2.13 Statistical analysis

The statistical analysis of the data was done using GenStat (General Statistics) (VSN International 2021). To analyse the differences between treatments applied to either true leaves or cotyledons (severity, lesion area and quantity of DNA) a factorial analysis of variance (ANOVA) was done. Differences with $P < 0.05$ were considered to be significant. To identify differences between individual treatments, post hoc Fisher tests were applied.For balanced and unbalanced designs a Tukey and Fishers LSD, respectively, post -hoc tests were applied.

3.3 Results

3.3.1 Development of a robust inoculation method for investigating co-infection by *L. maculans*, *L. biglobosa* and *P. brassicae*

3.3.1.1 Identification of effective inoculation method for *L. maculans* and *P. brassicae* infection

There was a difference in the effectiveness of infection between different inoculation methods as assessed by disease development for phoma leaf spot and light leaf spot. Overall, the 'Drop' inoculation was the most effective (50% of inoculated plants produced disease symptoms) and the 'Spray' inoculation was the least effective (0% of inoculated plants produced disease symptoms) method for inoculation with *L. maculans*, whereas for *P. brassicae* the 'Spray' inoculation was the most effective inoculation method (28% of inoculated plants produced disease symptoms), (Fig. 3.6). Both point inoculation methods ('Disc' and 'Drop') were less effective for light leaf spot (8% plants produced disease symptoms). There was also a difference in the plant resistance response to the two pathogens. On both cultivars, the resistance response to either *L. maculans* or *P. brassicae* infection occurred at the inoculation site. However, *P. brassicae* also triggered a resistance response known as flecking on the petiole of cv. Imola for the spray inoculation (Fig. 3.7).

3.3.1.2 Identification of a suitable environment for *P. brassicae* infection.

Both cultivars showed 100% of plants displaying light leaf spot symptoms and 100% of plants displaying *P. brassicae* sporulation post-incubation in both controlled environment cabinet and glasshouse experiments. Cv. Charger appeared to be more susceptible to *P. brassicae* than cv. Excel because it produced a greater average light leaf spot symptom severity G-index score than cv. Excel, 75 and 48, respectively (Fig. 3.8). Although there was a difference between cultivars, there was no large difference between the controlled environment cabinet and glasshouse (Figure 3.8).
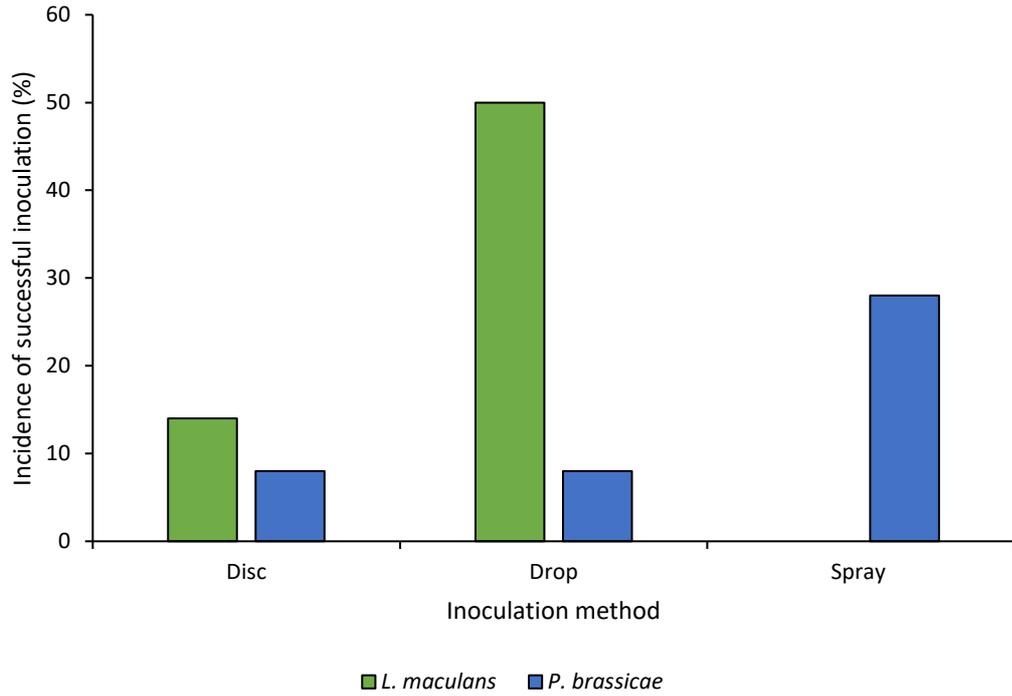
Figure 3.6: Incidence of successful inoculation (% of inoculated plants that produced disease symptoms) with *L. maculans* (phoma leaf spot) or *P. brassicae* (light leaf spot) using different inoculation methods (Disc, Drop and Spray) on cvs Imola and Bristol. The data presented are means of the two cultivars.

Figure 3.7: The black 'flecking' (black circle) symptoms on the petiole of cv. Imola that was inoculated with *P. brassicae* only by spray inoculation (14dpi). Cv. Imola has a strong resistance to *P. brassicae*.
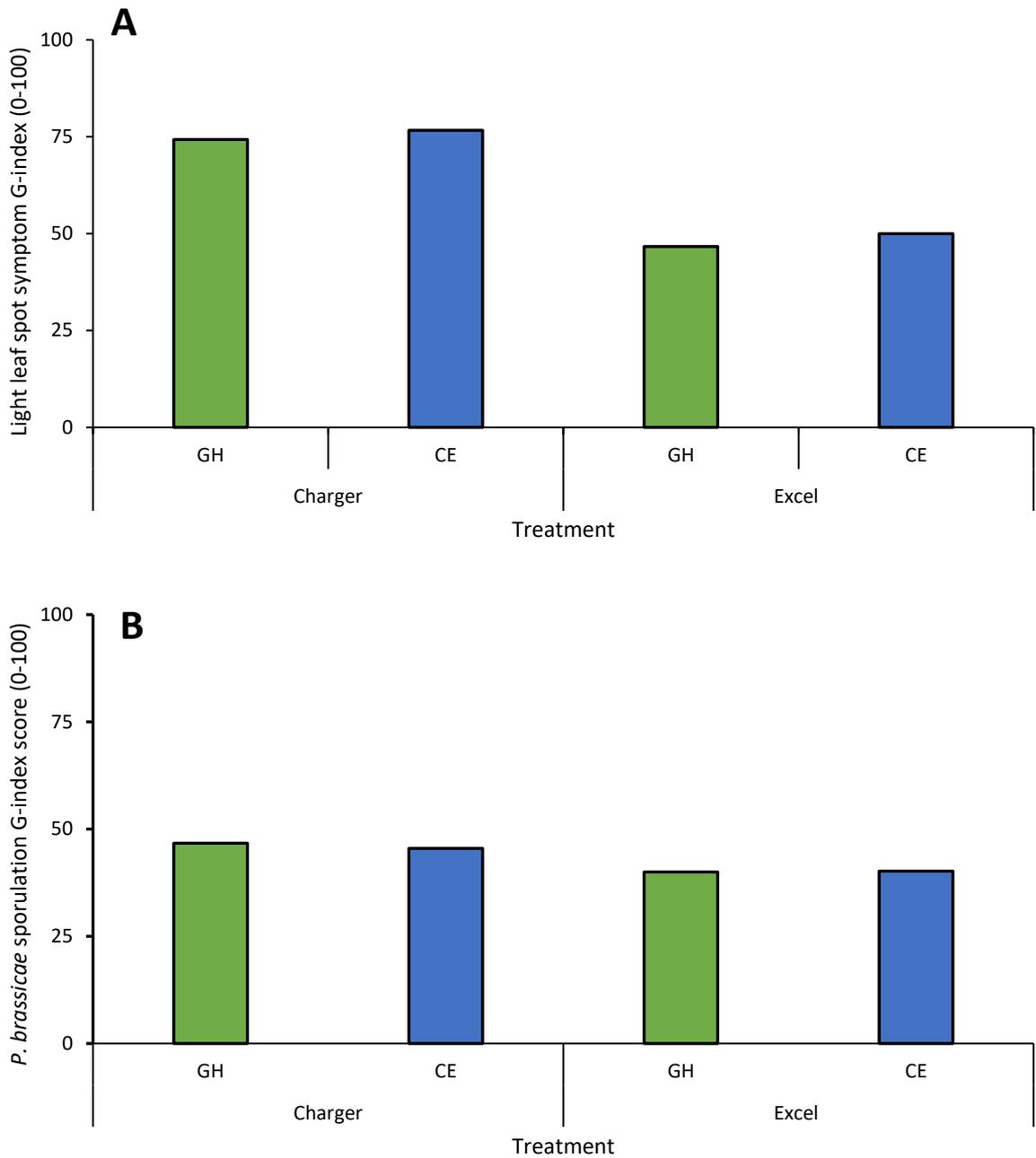
Figure 3.8: Light leaf spot symptom G-index (0-100) severity (A) and *P. brassicae* sporulation G-index (0-100) severity score (B) after incubation at 4°C for 13 days when plants of cultivars Charger or Excel were spray inoculated with *P. brassicae* conidia and then grown either in a semi-controlled glasshouse (GH) or a controlled environment cabinet (CE) facility. Cv. Excel has a LLS RL rating of 5 whereas cv. Charger has a LLS RL rating of 4.

For the sporulation G-index score, there was a similar pattern observed; cv. Charger (46.7) developed slightly more severe *P. brassicae* sporulation symptoms than cv. Excel (40), but there was no difference in the sporulation symptom score between growth environments for each cultivar (Fig. 3.8). There was no difference in the percentage leaf area covered with *P. brassicae* sporulation between cultivars (both cultivars had an average leaf area coverage of 25%), and there was no difference in area of sporulation between the glasshouse or CE cabinet experiments; 28% and 22% and 25% and 24% for cv. Charger and cv. Excel, respectively (Fig. 3.9). However, the symptoms that were observed in the glasshouse were frequently more similar in appearance to those observed in crops than symptoms observed from plants grown under controlled conditions (Fig. 3.10).

3.3.2 Effects of *Rlm7* on interactions between *Leptosphaeria maculans, L. biglobosa* and *P. brassicae* on oilseed rape

3.3.2.1 Lesion severity

There was a significant difference between treatments in *L. maculans* lesion severity score ($F_{1.136}$= 13.59, $P < 0.001$, LSD = 0.16) (Fig. 3.11 and 3.12). The average lesion severity score when plants were inoculated with *L. maculans* only (2.08) was significantly larger than when *L. maculans* was co-inoculated with *P. brassicae* (1.21). Overall, the lesion severity was greater on cv. Topaz (3.42) than on cv. Topaz *Rlm7* (0) ($F_{1.136}$= 425.97, $P < 0.001$, LSD = 0.16). There was a significant difference between treatments when applied on different cultivars ($F_{1.136}$= 15.23, $P < 0.001$, LSD = 0.23). The average lesion severity score when cv. Topaz was inoculated with *L. maculans* only (4.38) was greater than when cv. Topaz was co-inoculated with *L. maculans* and *P. brassicae* (2.56). However, both treatments that were applied to cv. Topaz *Rlm7* did not cause *L. maculans* lesions to form.
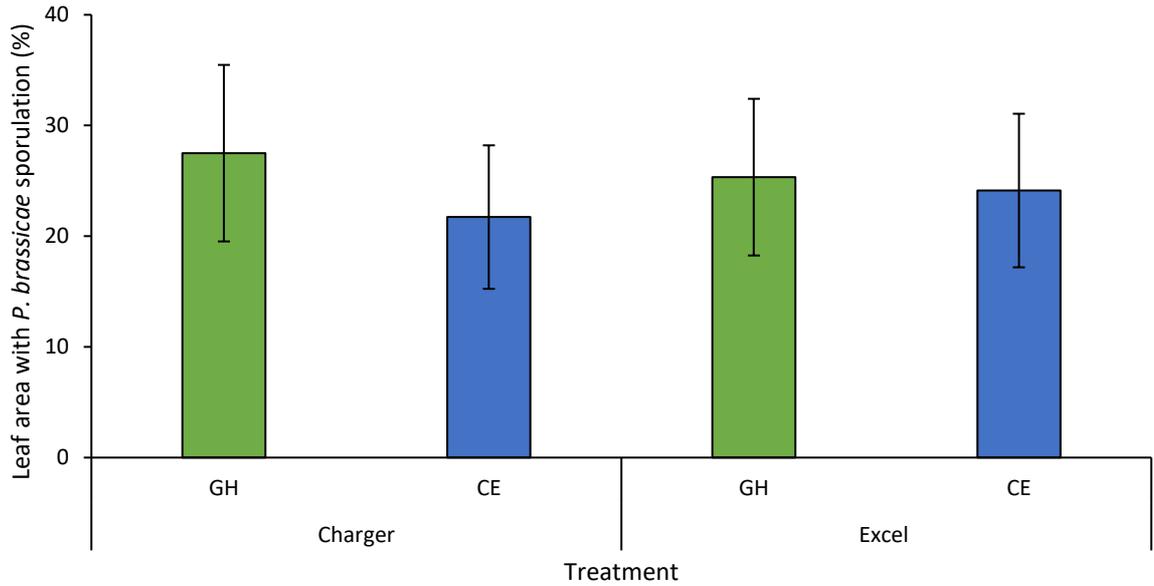
Figure 3.9: Average percentage of leaf area with *P. brassicae* sporulation after incubation at 4°C for 13 days when plants of cultivars Charger or Excel were spray inoculated with *P. brassicae* conidia and then grown either in a semi-controlled glasshouse (GH) or a controlled environment cabinet (CE) facility. Cv. Excel has a LLS RL rating of 5 whereas cv. Charger has a LLS RL rating of 4.

Figure 3.10: Grey *P. brassicae* (light leaf spot) foliar lesions on cv. Charger in the glasshouse (A), and in a winter oilseed rape crop from the field at Terrington St Clement, Norfolk in March 2020 (B). Cv. Charger has a LLS RL rating of 4.
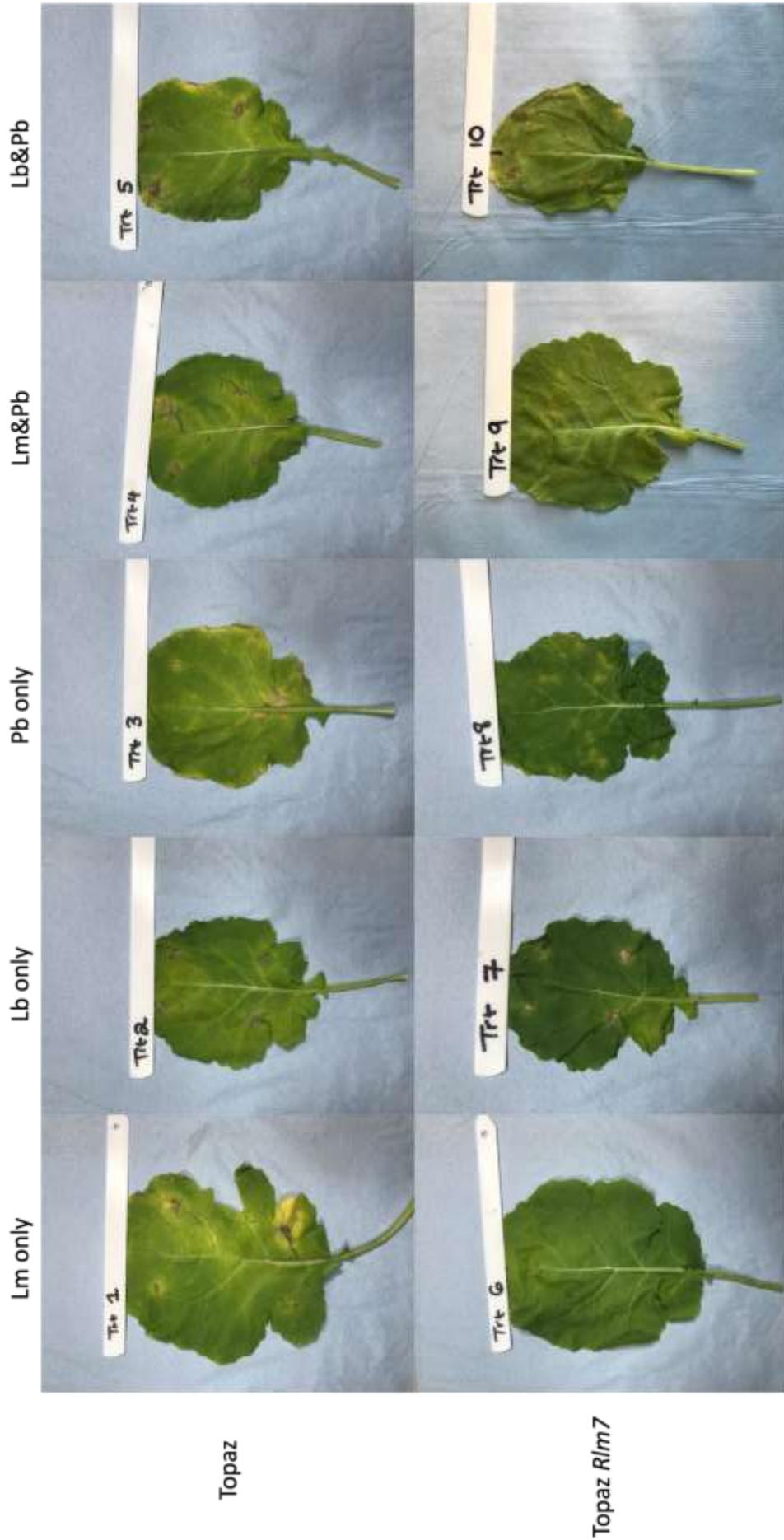
Figure 3.11: Comparison of foliar lesions of when cultivars Topaz and Topaz *Rlm7* were inoculated with different combinations of *L. maculans* (Lm), *L. biglobosa* (Lb) or *P. brassicae* (Pb). There were 10 treatments. Plants were inoculated with *L. maculans* or *L. biglobosa* using a point inoculation method and inoculated with *P. brassicae* using a spray inoculation method. All plants were inoculated using conidia.
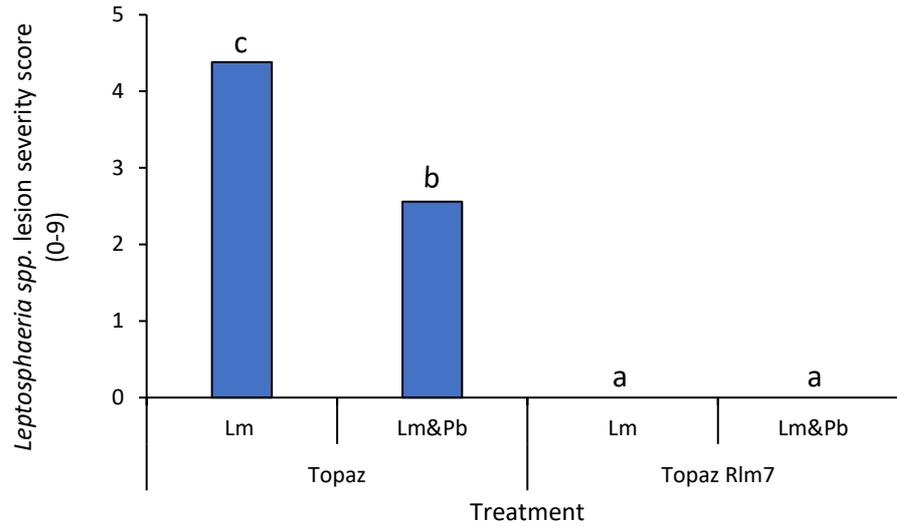
Figure 3.12: *Leptosphaeria* **spp. mean lesion severity score (0-9 scale) when *L. maculans* (Lm) was inoculated alone or co-inoculated with *P. brassicae* (Pb) on two near-isogenic cultivars that differed only by the presence of *Rlm7*. Data were square root-transformed for statistical analysis. Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (*P* < 0.05)**

There was no significant difference in the mean *L. biglobosa* lesion severity score between treatments ($F_{1.138}$= 3.24, *P* = 0.074, LSD = 0.23) (Fig. 3.11 and 3.13). Overall, the lesion severity was greater on cv. Topaz *Rlm7* (1.64) than on cv. Topaz (1.304) ($F_{1.136}$= 8.28, *P* = 0.005, LSD = 0.23)*.* There was no significant difference between treatments when applied on different cultivars ($F_{1.136}$= 0.00, *P* < 0.973, LSD = 0.32) (Fig. 3.13). There were no significant differences in mean *L. biglobosa* lesion severity score between when *L. biglobosa* was inoculated on its own or when it was co-inoculated with *P. brassicae,* when compared on the same cultivar. Although not significantly different from each other, the mean scores of *L. biglobosa* were smaller when it was co-inoculated with *P. brassicae*. However, the lesion severity for the corresponding treatments was greater on cv. Topaz *Rlm7* than on cv. Topaz.

There was no significant difference in the mean *P. brassicae* lesion severity score (0-7) between cultivars Topaz (2.37) and Topaz *Rlm7* (1.88) ($F_{1.52}$= 1.53, *P* = 0.222, LSD = 0.790) (Fig. 3.11 and 3.14A). There was a significant difference in the mean *P. brassicae* lesion severity score between treatments ($F_{2.52}$= 16.54, *P* < 0.001, LSD = 0.967). The *P. brassicae* lesion severity score was significantly greater when plants were inoculated with *P. brassicae* only (3.72) than when co-inoculated with *L. maculans* (1.39) or *L. biglobosa* (1.27). There was no significant difference between treatments applied on the different cultivars ($F_{2.52}$= 0.15, *P* = 0.860, LSD = 0.680). There was no significant difference in *P. brassicae* severity score between Topaz or Topaz *Rlm7* when inoculated with only *P. brassicae* but scores were significantly greater than those of all other treatments. All the co-inoculation experiments with *P. brassicae* with either *L. maculans* or *L. biglobosa* were not significantly different from each other. However, when a *P. brassicae* symptom severity score index (0-100) was calculated between treatments (Fig. 3.11 and 3.14B), the *P. brassicae* lesion severity score was greater on cv. Topaz (40.2) than on Topaz *Rlm7* (31.4). The *P. brassicae* severity was smaller when co-inoculated with either *L. maculans* or *L. biglobosa.* This pattern was observed on both cvs Topaz and Topaz *Rlm7*. The severity index showed that lesion severity on cv. Topaz was smaller when *P. brassicae* was co-inoculated with *L. maculans* than when co-inoculated with *L. biglobosa.* This pattern was also observed on cv. Topaz *Rlm7*. When *P. brassicae* was co-inoculated with *L. maculans*, the *P. brassicae* index was greater on cv. Topaz *Rlm7* than on cv. Topaz.
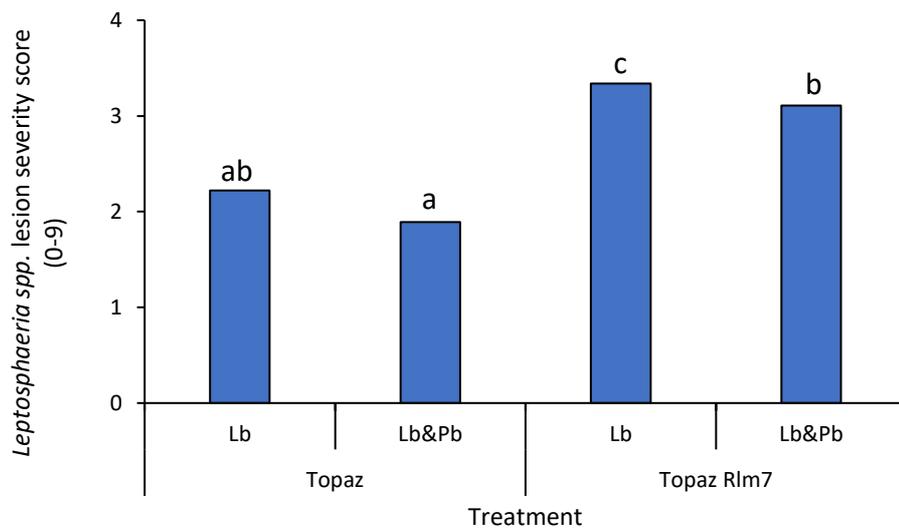
Figure 3.13: *Leptosphaeria* **spp. mean symptom severity score (0-9) when** *L. biglobosa* **(Lb) was inoculated on its own or co-inoculated with** *P. brassicae* **(Pb) on two near-isogenic cultivars that differed only by the presence of** *Rlm7*. **Data were square root-transformed for statistical analysis. Unprotected Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (***P* =0.05)**
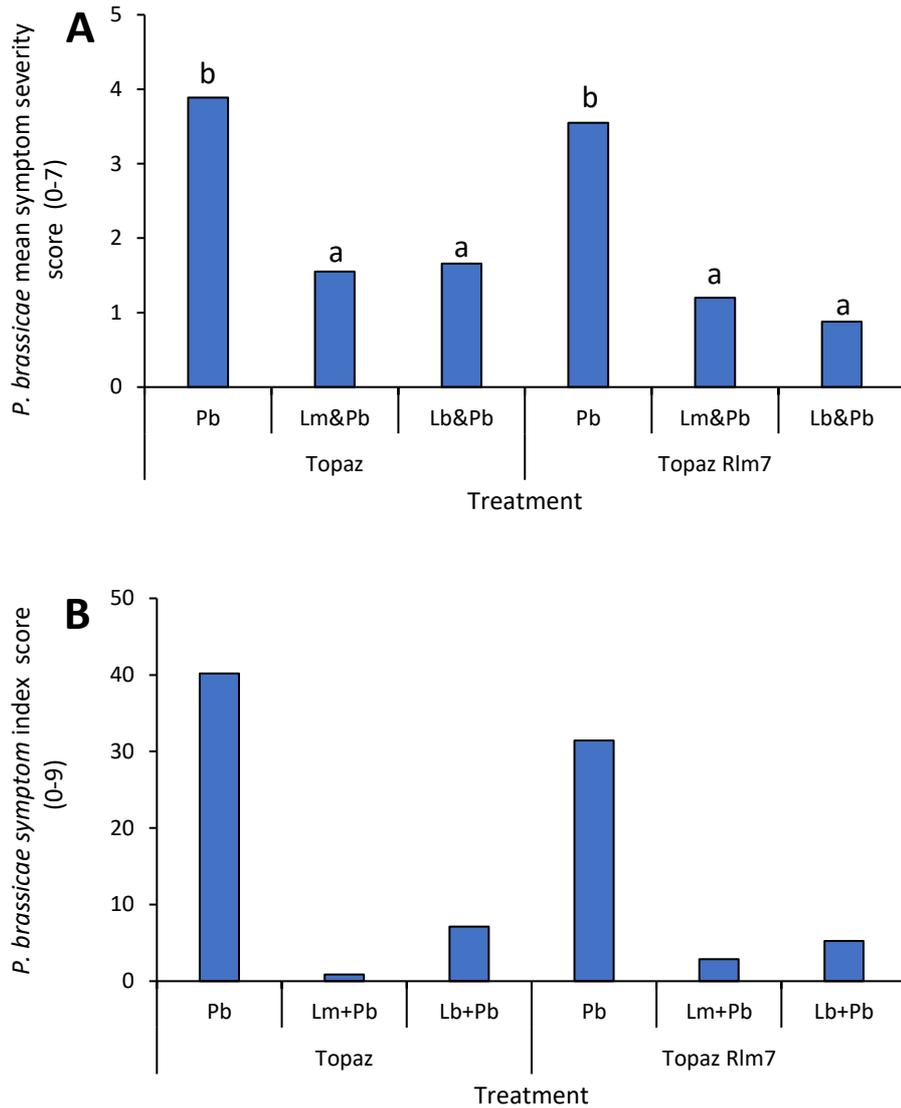
Figure 3.14: *P. brassicae* **mean symptom severity score (0-7) (A) and** *P. brassicae* **index symptom severity score (0-100) (B) when** *P. brassicae* **(Pb) was inoculated on its own or co-inoculated with either** *L. maculans* **(Lm) or** *L. biglobosa* **(Lb) on two near-isogenic cultivars that differed only by the presence of** *Rlm7***. Data were square root-transformed for statistical analysis. Unprotected Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (***P* < 0.05).**

3.3.3 Effect of interaction between *L. maculans* and *L. biglobosa* on lesion development, quantity of pathogen DNA and phytotoxin production.

3.3.3.1 Lesion phenotype and lesion area

3.3.3.1.1 Lesion phenotype and lesion area at 17 days post inoculation

There were differences in lesion phenotypes between the four different treatments (Fig. 3.15). Plants with 'Lm only' inoculation produced large grey, sunken, undefined lesions, while plants with 'Lb only' or 'Lm&Lb' inoculation produced small, dark and defined lesions (Fig. 3.15). There was a significant difference between treatments in lesion area at 17 dpi ($F_{3.1106}$ = 181.43, *P* < 0.001, LSD = 1.603) (Fig. 3.16; Appendix 6. There was no significant difference between experiments ($F_{2.1106}$ = 0.81, *P* = 0.444, LSD = 1.603). The lesion area was significantly greater for the 'Lm only' (18.1 mm^2) than for 'Lb only' (5.3 mm^2) or 'Lm&Lb' (5.3 mm^2) (Fig. 3.16) (Appendix 12). All treatments and lesion assessments were significantly greater than those for 'SDW' control (0.00 cm^2). Individual analysis of each experiment can be found in Appendices 12.

3.3.3.1.2 **Lesion area at 26 days post inoculation**

There were differences in lesion phenotypes between the four different treatments. The lesions were like those identified in section 3.3.3.1.1. However, the lesions were larger (Fig. 3.17). There was a significant difference in lesion area between inoculation treatments ($F_{3.458}$ = 690.08, *P* < 0.001, LSD = 7.35) (Fig. 3.18). The lesion area was significantly greater for the 'Lm only' (153.3 mm^2) inoculation treatment than for all other treatments. The lesion areas for inoculation treatments 'Lb only' (24.6 mm^2) or 'Lm&Lb' (27.1 mm^2) were significantly smaller than for 'Lm only' (Fig. 3.18). Individual analysis of each experiment can be found in Appendices 13.

Figure 3.15: Lesion phenotype on phoma susceptible cv. Charger cotyledons inoculated with *L. maculans* only (Lm only) or *L. biglobosa* only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control at 17 days post inoculation.
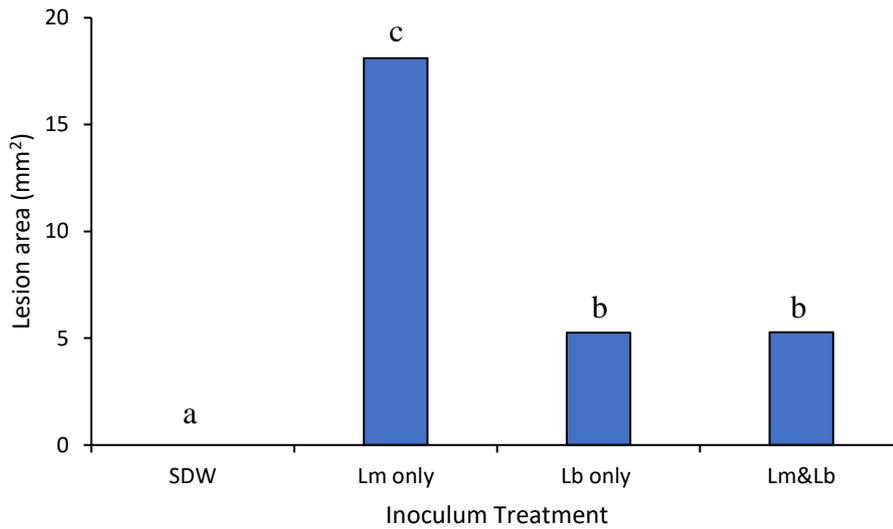
Figure 3.16: Overall lesion area (mm²) on phoma susceptible cv. Charger cotyledons inoculated with *L. maculans* only (Lm only) or *L. biglobosa* only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control for all replicates combined at 17 days post inoculation. Data were square root-transformed for statistical analysis. Unprotected Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (*P* < 0.05)

Figure 3.17: Lesion phenotypes when phoma susceptible cv. Charger cotyledons were inoculated with *L. maculans* only (Lm only) or *L. biglobosa* only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control at 26 days post inoculation.
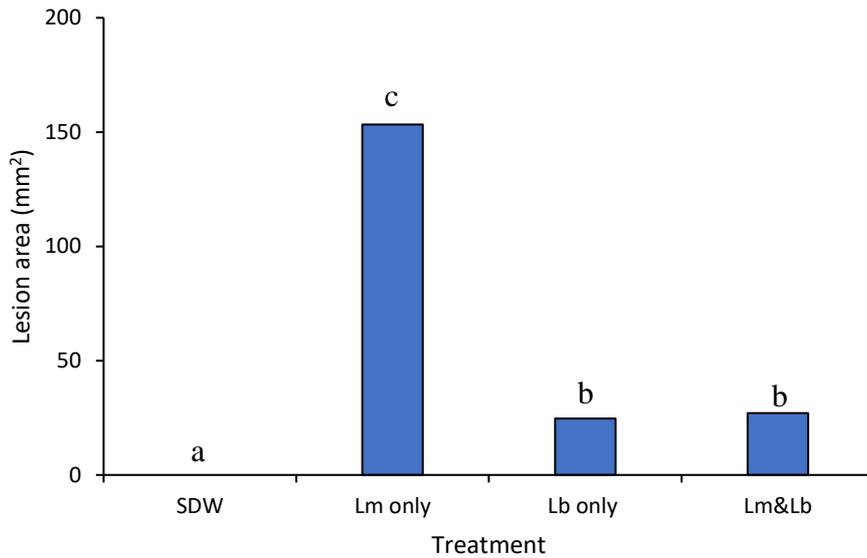
Figure 3.18: Lesion area (mm^2) on phoma susceptible cv. Charger cotyledons inoculated with _L. maculans_ only (Lm only) or _L. biglobosa_ only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control at 26 days post inoculation. Data were square root-transformed for statistical analysis. Unprotected Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (_P_ < 0.05)

3.3.3.2 *L. maculans* and *L. biglobosa* DNA (17 dpi)

There was no significant difference in *L. maculans* pathogen DNA concentration (pg/ng) between experiments ($F_{1.39}$ = 2.30, *P* = 0.138, LSD = 9.729). There was a significant difference in the *L. maculans* pathogen DNA concentration (pg/ng) between treatments ($F_{1.458}$ = 15.27, *P* < 0.001, LSD = 9.729). The 'Lm only' treatments had a significantly greater quantity of *L. maculans* DNA than all other treatments, and there was no significant difference in the quantity of *L. maculans* DNA between the other samples. In both experiments, the quantity of *L. maculans* DNA in the 'Lm&Lb' treatment was >95% smaller than the quantity of DNA recorded in the 'Lm only' treatment (Fig. 3.19 & 3.20). *L. maculans* DNA was not detected in the other treatments.

There was a significant difference in *L. biglobosa* pathogen DNA concentration (pg/ng) between experiments ($F_{1.36}$ = 49.00, *P* < 0.001, LSD = 0.3935). For experiment 1, there was a significant difference between treatments in the quantity of *L. biglobosa* DNA ($F_{1.17}$ = 5.35, *P* = 0.034, LSD = 1.25) (Fig. 3.19A). The 'Lb only' treatments had a significantly greater quantity of *L. biglobosa* DNA (3.0 pg/ng) than all other treatments. The quantity of *L. biglobosa* DNA was significantly greater in the 'Lb only' treatment than in the 'Lm&Lb' (1.6 pg/ng sample DNA) treatment; *L. biglobosa* DNA was not detected in the other treatments. There was 45.4% less *L. biglobosa* DNA in the 'Lm&Lb' treatment than in the 'Lb only' treatment.

For experiment 2, there was no significant difference in the quantity of *L. biglobosa* DNA between treatments when *L. biglobosa* DNA was detected ($F_{1.18}$ = 1.36, *P* = 0.259, LSD = 0.11) (Fig. 3.19B). The 'Lb only' treatments did not have a significantly greater quantity of *L. biglobosa* DNA (0.15 pg/ ng sample of DNA) than the 'Lm&Lb' (0.09 pg/ng). *L. biglobosa* DNA was not detected in the other treatments.

A similar effect was seen when the quantity of *L. maculans* and *L. biglobosa* DNA was analysed at 26 dpi (*L. maculans* - $F_{1.36}$ = 14.76, *P* < 0.001, LSD = 15.49; *L. biglobosa* - $F_{1.36}$ = 1.18, *P* = 0.286, LSD = 0.96) (Fig 3.20).
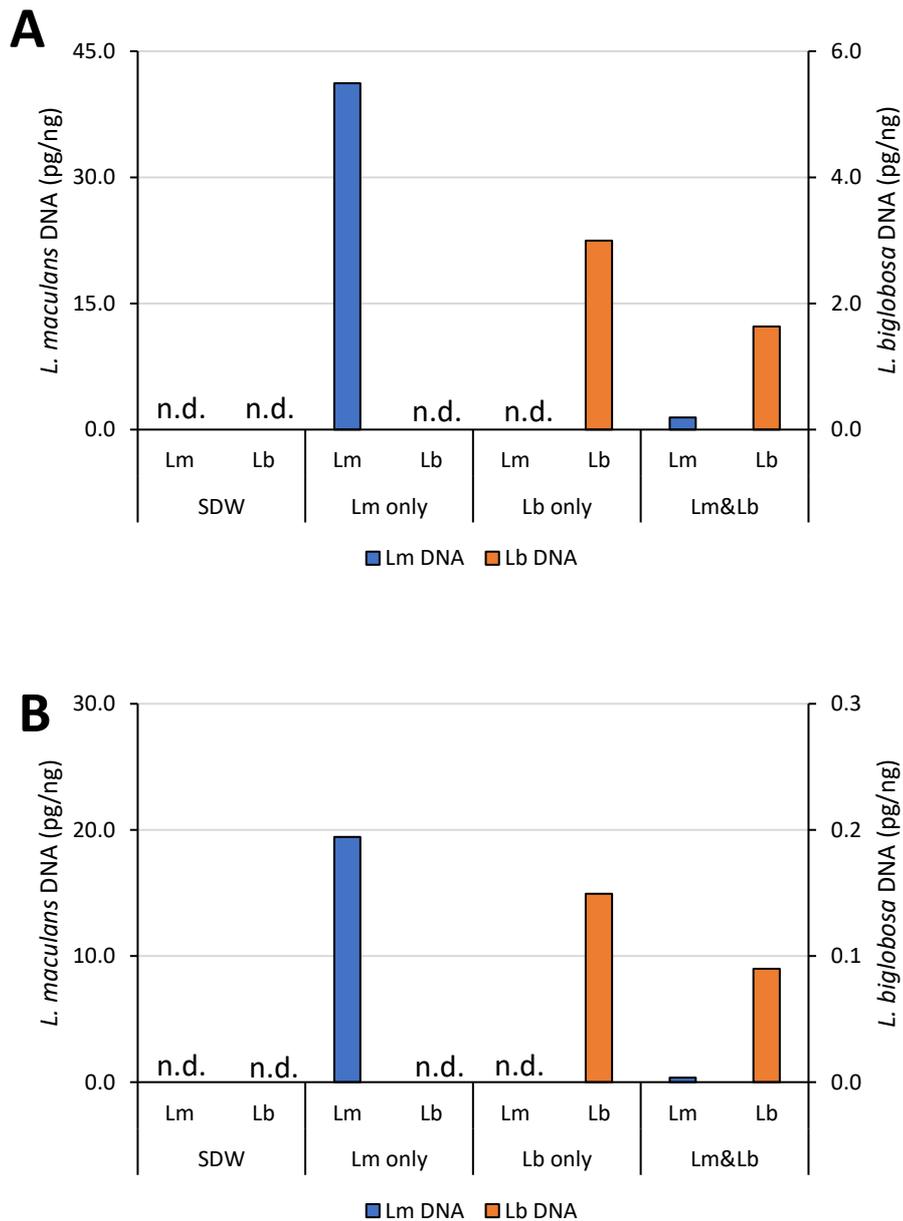
Figure 3.19: Quantities of *L. maculans* **DNA (blue) and** *L. biglobosa* **(orange) (pg/ng) in extracted DNA at 17 dpi for Experiment 1 (A) or Experiment 2 (B) from plant samples when phoma susceptible cv. Charger cotyledons were inoculated with** *L. maculans* **(Lm only) or** *L. biglobosa* **(Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control for all replicates combined. Samples where pathogen DNA was not detected are indicated by n.d. (LSD – Lm = 9.73, Lb = 1.25).**
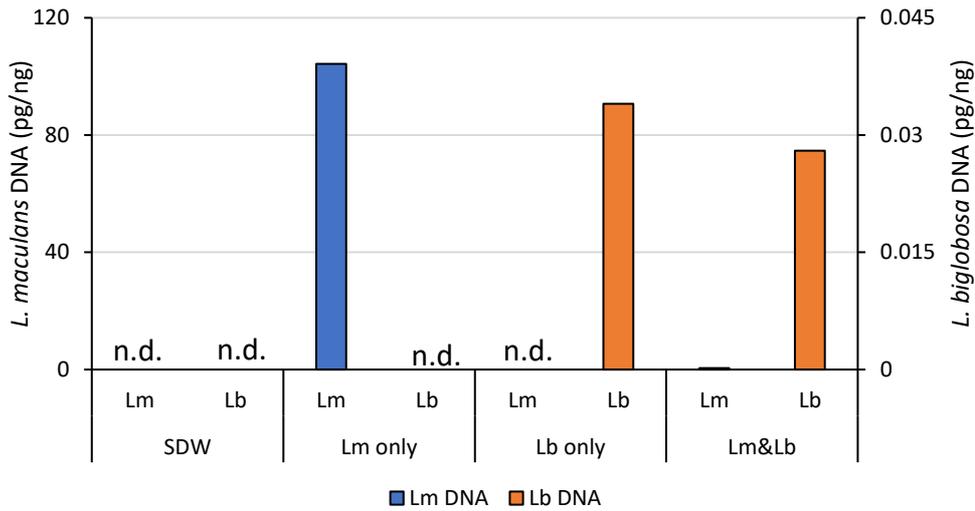
Figure 3.20: Quantities of *L. maculans* DNA (blue) and *L. biglobosa* (orange) (pg/ng) in extracted DNA from plant samples when phoma susceptible cv. Charger cotyledons were inoculated with *L. maculans* (Lm only) or *L. biglobosa* (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control for all replicates combined at 26 dpi. Samples where pathogen DNA was not detected are indicated by n.d. (LSD – Lm = 26.6, Lb = 0.01).

3.3.4 Phytotoxin production

There was a difference in the composition of metabolites found between samples in the HPLC. There were three maxima that were only found in plants with the 'Lm only' inoculation treatment. The three maxima were found at rt 11.6 min, and 16.3 and 16.8 min. However, these maxima were less than the LOD (70mg/L) and therefore less than the LOQ (200mg/L).

The LC-MS chromatographs showed that there were no unique maxima identified in the fraction taken from 11.5-13 min. When the fraction from 16-17.5 min was taken for analysis, there were three unique maxima at retention times 4.95, 5.03 and 5.11 min in the 'Lm only' sample (Fig. 3.21 & 3.22ac) The difference in retention times between using the HPLC and LC-MS was due to the use of different methodologies, columns, mobile phases and flow rates and fundamentally different instruments. At retention time 4.95 min, the positive ion spectrograph contained two ions that were detected at *m/z* 242.29. The identity of these ions remains unknown.

At retention time 5.03 min, the positive ion spectrograph (Fig. 3.22b) contained ions at *m/z* ions at *m/z* 319.26 (100%), which potentially corresponds to a monocharged molecule with a molecular weight of 318.4 Da. Phomamide is a known secondary metabolite produced by *L. maculans* and has a molecular weight of 318.4 Da (Pedras and Yu 2009; PubChem 2021).

At retention time 5.11 min, the positive ion spectrograph (Fig. 3.22d) contained ions at m/z 487.18 (98%), which corresponds to a monocharged molecule with a molecular weight of 486.57 Da. Sirodesmin PL has molecular weight of 486.6 Da (Pedras and Yu 2009; PubChem 2021). The positive ion spectrograph (Fig. 3.22d) also contained ions at m/z 445.24, which potentially corresponds to a monocharged molecule with a molecular weight of 444.24 Da. Deacetyl-sirodesmin PL is a known secondary metabolite produced by *L. maculans* and has a molecular weight of 444.5 Da (Pedras and Yu 2009; PubChem 2021).
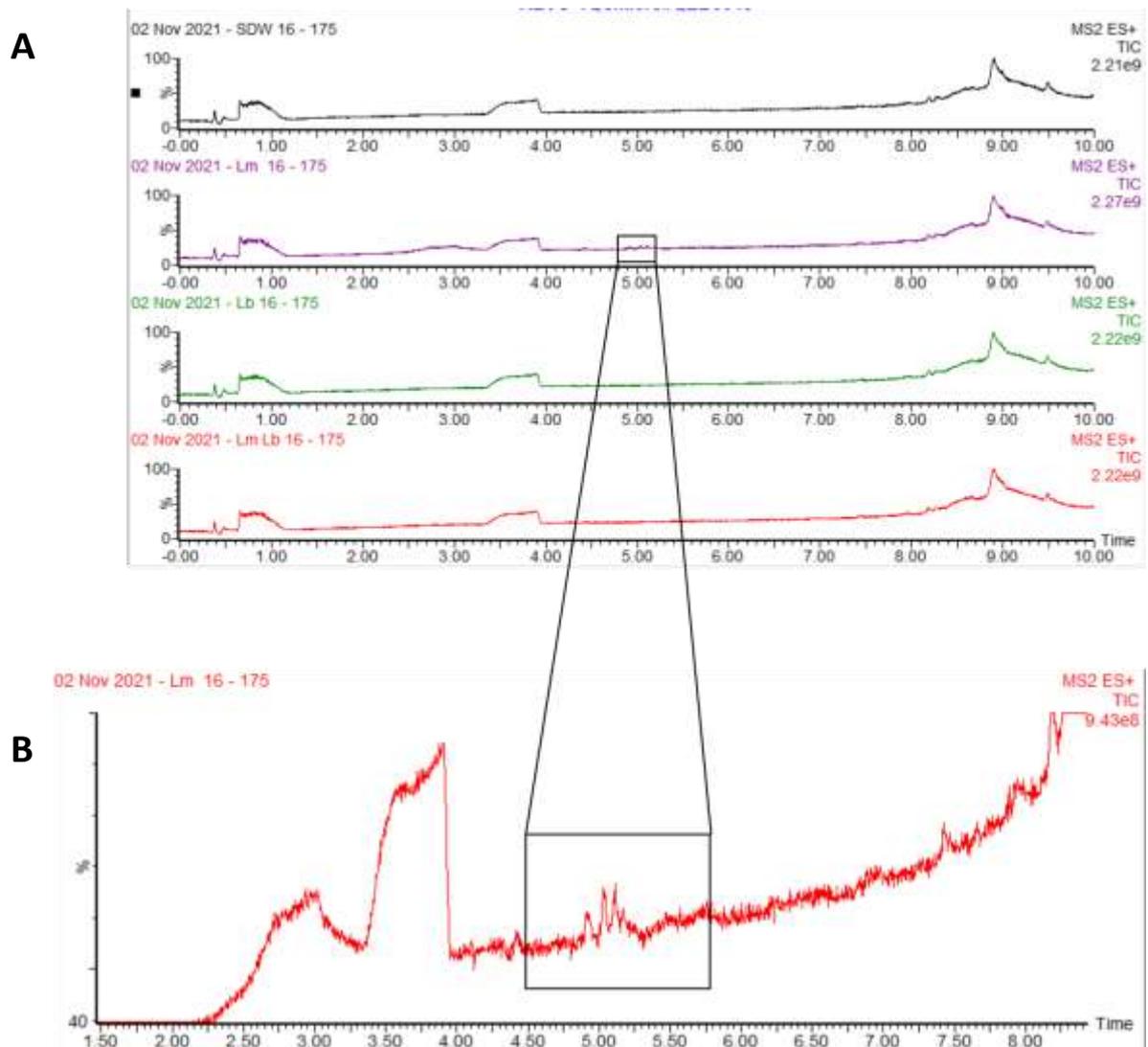
Figure 3.21. LC-MS total ion chromatographs of HPLC fractions of secondary metabolite extracts from oilseed rape phoma susceptible cv. Charger cotyledons inoculated with sterilised distilled water (SDW), *Leptosphaeria maculans* only (Lm only), *L. biglobosa* only (Lb only) and Lm and Lb simultaneously (Lm&Lb). HPLC fractions for Lm only, Lb only, Lm&Lb and SDW were taken from rt 16.0-17.5 min the unique maxima (black rectangle) only found in 'Lm only' extract at retention time (a) and a zoomed in chromatograph of the unique maxima (black rectangle) found at rt 4.95, 5.03 and 5.11 min (b).
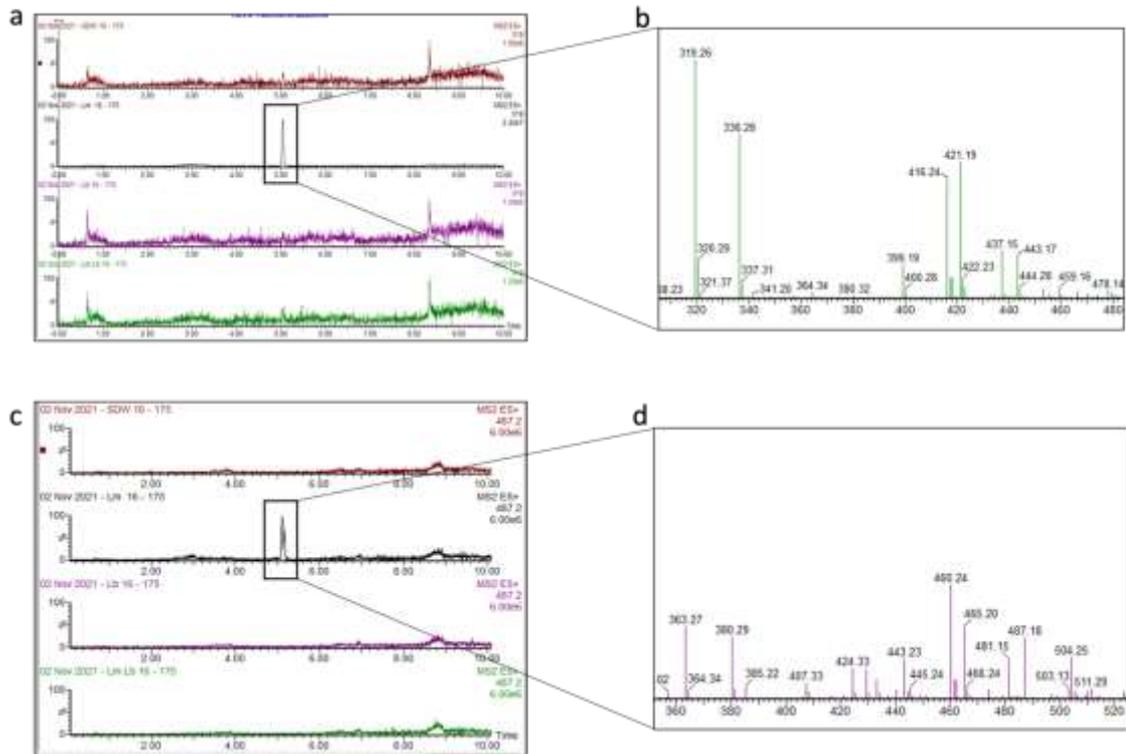
Figure 3.22. LC-MS chromatographs of HPLC fractions of secondary metabolite extracts from oilseed rape phoma susceptible cv. Charger cotyledons inoculated with sterilised distilled water (SDW), *Leptosphaeria maculans* only (Lm only), *L. biglobosa* only (Lb only) and Lm and Lb simultaneously (Lm&Lb). HPLC fractions for Lm only, Lb only, Lm&Lb and SDW were taken from rt 16.0-17.5 min. Unique maxima were found only in the 'Lm only' sample at rt 5.03 min (a), and 5.11 min (c) and their ion spectrographs (b and d, respectively).

Additionally, the following ions were also found in the positive ion spectrograph (Fig 3.22d); m/z 424.32 $[M - S_2 + H]^+$, potentially corresponding to sirodesmin PL lacking two sulphur atoms; m/z 460.24 (98%), 461.30 (18%) and 462.33 (13%) $[M - S_2 + K]^+$, potentially corresponding to the potassium adduct of sirodesmin PL lacking two sulphur atoms; 465.20 (52%) and 466.25 (8%) $[M-S_2 + CH_3CN + H]^+$, potentially corresponding to the acetonitrile adduct of sirodesmin PL lacking two sulphur atoms. m/z 504.25 $[M+ NH_4]^+$, corresponding to the ammonium adduct of sirodesmin PL.

3.4 Discussion

The results demonstrate that the most effective co-inoculation methods for *L. maculans, L. biglobosa* and *P. brassicae* inoculation were to use a 'drop' inoculation method for *L. maculans* and *L. biglobosa* and a 'spray' inoculation method for *P. brassicae.* Due to the similarities in their life cycles and the evidence that *L. maculans* and *L. biglobosa* are sibling species (Fitt *et al.* 2006), the effectiveness of inoculation type was assumed to be the same. However, the initial experiment showed that the incidence of successful inoculation was not very great (50% and 28% for *L. maculans* and *P. brassicae* respectively). The low incidence of successful inoculation for *L. maculans* could be due to the spore type used for inoculation; in this experiment *L. maculans* conidia were used rather than ascospore suspensions. Ascospores have been shown to have a much greater infection efficiency than conidia (Huang *et al.* 2006). However, due to the requirement to confidently distinguish between *L. maculans* and *L. biglobosa*, many attempts to create enough ascospore inoculum from pseudothecia produced *in vitro* were unsuccessful (Balesdent *et al.* 2001); most probably due to isolates of different mating types having low fertility rates. There was evidence that conidia can effectively be used in controlled conditions (Huang *et al.* 2009; 2014). Therefore, *Leptosphaeria* spp. conidia were used as the inoculum type throughout this study. The *P. brassicae* inoculation success may appear less than the true inoculation success due to the inoculated leaf not being incubated to promote sporulation. When inoculated leaves were incubated, the severity of *P. brassicae* was increased. Therefore, it is important to incubate infected leaves to ascertain a more accurate understanding of *P. brassicae* disease severity.

Differences in plant growth conditions between the glasshouse or controlled environment (CE) cabinet had very little effect on severity of light leaf spot. However, it was noted that, although not more severe, the light leaf spot lesions in the glasshouse were more like those observed in field experiments. There was no difference in *P. brassicae* sporulation severity post-incubation between plants grown in the glasshouse or grown in controlled environment cabinets, and although not significantly different the mean leaf area of *P. brassicae* sporulation was less in plants from the CE cabinets. This may provide evidence to support the theory that frosts, or fluctuating temperatures damage the waxy cuticle, consequently enhancing eruption of acervuli. Additionally, it appeared that cv. Charger was more susceptible to *P. brassicae* than cv. Excel because it had greater light leaf spot severity score than cv. Excel. This was expected since cv. Charger has a smaller AHDB RL resistance rating for light leaf spot than cv. Excel (scoring 4 (2016/17) and 5 (2010/11) on the AHDB RL respectively) (Table 2.2). Despite the difference in resistance score there was no difference in the post-incubation *P. brassicae* sporulation scores. However, there was a smaller number of replicates of cv. Charger than of cv. Excel, so the reliability of the scores may be different. Additionally, the appearance of 'flecking', the phenotype of necrosis of epidermal cells (Bradburne *et al.* 1999; Boys *et al.* 2007;2012; Karandeni-Dewage *et al.* 2018) was identified on the *P. brassicae* resistant cv. Imola. 'Flecking' is associated with the presence of the resistance gene *PBR2* (Bradburne *et al.* 1999). The gene does not prevent infection by *P. brassicae*; however, it does inhibit *in planta* proliferation and asexual sporulation (Boys *et al.* 2012; Karandeni-Dewage *et al.* 2018). Therefore, the 'Flecking' trait is something to be aware of when screening future breeding material for resistance against *P. brassicae*.

The presence of *Rlm7* did influence the interactions between *L. maculans*, *L. biglobosa* and *P. brassicae*. No significant increase in the disease severity of *P. brassicae* was found on Topaz *Rlm7* compared to cv. Topaz. This may appear to contradict the findings of recent investigations that found that cultivars carrying the *Rlm7* gene were very susceptible to *P. brassicae* (Huang *et al.* 2021; Karandeni-Dewage *et al.* 2021), but Karandeni-Dewage *et al.* (2021) suggested the presence of isolate-specific resistant interactions since some cultivars varied in leaf area with *P. brassicae* sporulation between isolates. Both this study and Karandeni-Dewage *et al.* (2021) used the same *P.*

brassicae isolate 15WOSR64-SS1. When the cultivars carrying the *Rlm7* gene (cvs Excel, Harper and Hearty) were inoculated with this isolate, they had smaller sporulation scores than cultivars without *Rlm7* (cvs Yudal, Tapidor, Ningyou7 and Dartmor) (Karandeni-Dewage *et al.* 2021). Therefore, the suggested increased susceptibility to *P. brassicae* in the presence of *Rlm7* may not be the case for all isolates. This would provide an explanation as to why there was not an increase in *P. brassicae* severity score in near-isogenic line Topaz-*Rlm7* compared to cv. Topaz without *Rlm7.* Therefore, a range of *P. brassicae* isolates needs to be used to confirm the interaction between *Rlm7* and increased *P. brassicae* susceptibility using near-isogenic lines Topaz and Topaz-*Rlm7*.

As expected, there was a greater *L. maculans* disease severity on cv. Topaz than on Topaz-*Rlm7* due to the presence of *Rlm7.* This is due to the *Rlm7* gene being a source of major gene resistance against *L. maculans* (Mitrousia *et al.* 2017). However, the protection effect of *Rlm7* was not observed for disease severity of *L. biglobosa;* the *L. biglobosa* disease severity was significantly greater on Topaz-*Rlm7* than on cv. Topaz. This implies that the presence of *Rlm7* increases the susceptibility of cv. Topaz to *L. biglobosa.* Therefore, this control strategy against *L. maculans* using *Rlm7* may unintentionally increase the susceptibility of the plant to *L. biglobosa*. This may explain why *L. maculans* and *L. biglobosa* are more frequently being found at the stem base together (Huang *et al.* 2014b). However, it is important to recognise that most breeders, such as DSV, often combine *Rlm7* with quantitative resistance (DSV, 2021) to increase the durability of the resistance gene. It is recommended that growers use an integrated control strategy to incorporate use of fungicides and cultural techniques (FRAG-UK, 2017) that provide non-species-specific protection for control of *L. maculans*, *L. biglobosa* and other oilseed rape pathogens. This is to reduce selection for insensitive populations of *L. maculans* that could render major genes (such as *Rlm7*) ineffective resulting in a complete *R*-gene break down (Delourme et al. 2006; Huang *et al.* 2014a, 2021) such as that seen when *Brassica sylvestris*-derived resistant (*LepR3*) oilseed rape cultivars were developed and released in Australia (Sprague *et al.* 2006).

Despite the widespread adoption of an integrated control strategy in the UK, populations of *L. maculans* virulent against *Rlm7* have been identified at a time when

cultivars with *Rlm7* represented approx. 5% of the UK cropping area. However, this may have increased to >20% of the OSR area by 2016/17 with a shift towards the increased use of the *Rlm7* gene (Mitrousia *et al.* 2018). In France, when 50% of French oilseed rape area was sown with *Rlm7* cultivars, 20% of the *L. maculans* population was virulent against *Rlm7* (Mitrousia *et al*. 2018). Therefore, it is essential that the *Rlm7* gene is used wisely and is not extensively relied on because break down of this important source of resistance would cause substantial losses to the breeding industry and to farmers. There is increased selection for *Rlm7* virulence in France because French growers often rely on host resistance and do not apply fungicides on resistant cultivars, unlike in the UK. Despite these differences, it is still concerning as there is a disproportionate percentage of cultivars on the 2021-22 AHDB RL recommended for use in the UK (not Clubroot or Described cultivars) that publicly promote the inclusion of *Rlm7* resistance in their cultivar (AHDB, 2020d). Of the 24 cultivars recommended for the UK, 11 promote the inclusion of *Rlm7* (46%). Of the other 13 cultivars, one (LSPB cv. Respect) has *RlmS*, one (Syngenta cv. George) has a high resistance rating of 8 but does not state the *R*-gene, but the remaining 11 cultivars have phoma stem canker resistance scores of only 5 (seven cultivars) or 6 (three cultivars) and 7 (one cultivar cv. Barbados). If a grower is wanting to use a 2021/22 recommended list cultivar in an area with a high concentration of *L. maculans* inoculum without additional knowledge about the resistance genes, there would be a strong possibility that a *Rlm7* cultivar would be selected because out of the 13 cultivars on the recommended list with a phoma resistance score > 7, there were 10 (potentially 11) containing *Rlm7*, albeit with differences in quantitative resistance, but there are only three cultivars with good resistance that use alternate resistance strategies (cvs Respect (*RlmS*), George (if different) or Barbados (strong QR) that would not be known about unless each cultivar on the list is researched. Therefore, it would be beneficial for host resistance management strategies and for the growers if resistance genes could be published on the AHDB recommended list. These methods could be even more effective if they were aligned with *R*-gene deployment strategies (Bousset *et al.* 2018). These policies would enable different *Rlm* genes to be used in space and time, endeavouring to prolong the durability of these *Rlm* genes (van den Bosch and Gilligan 2003; Marcroft *et al.* 2004; Gladders *et al.* 2006; Mitrousia *et al.* 2018).

There was a smaller mean disease severity score when cultivars were co-inoculated than when inoculated on their own. These differences were significant on cv. Topaz for *L. maculans* and *P. brassicae* and on Topaz-*Rlm7* for *P. brassicae*. The reduction was not observed on Topaz-*Rlm7* for *L. maculans* because both severity scores were 0. These differences are believed to be due to the initiation of the salicylic acid defence response which is important for the control of pathogens utilising a biotrophic nature, such as *Leptosphaeria* spp. and *P. brassicae*, to induce plant defence responses and changes in regulation of plant defence related genes (e.g. *PR1*, *WYK70*, *ACS2* and *CHI*) that would decrease disease development and therefore result in smaller disease severity scores. Previous work investigating the plant response to co-inoculation of *L. maculans* and *L. biglobosa* suggested that *L. biglobosa* can induce local and systemic resistance to *L. maculans*, thereby reducing its disease severity (Liu *et al.* 2006) suggesting that *L. biglobosa* may be able to compete with other pathogens using a host-mediated interspecific competition strategy. Therefore, further work that investigates the genetic defence response when plants are co-inoculated with *L. maculans* or *L. biglobosa* with *P. brassicae* will need to be done.

There was an interaction between *L. maculans* and *L. biglobosa* in lesion development, pathogen DNA and phytotoxin production when investigated *in planta*. *L. biglobosa* was found to inhibit the production of metabolites, like the phytotoxin sirodesmin PL and its precursors, to increase its competitiveness against *L. maculans*. *L. biglobosa* reduced *L. maculans* lesion area as well as disproportionately reducing *L. maculans* DNA when cotyledons were simultaneously co-inoculated with *L. maculans* and *L. biglobosa*. These findings support previous findings by Liu *et al.* (2006) and Mahuku *et al.* (1996) that found *L. maculans* leaf spot lesions were smaller when weakly virulent *L. maculans* (*L. biglobosa*) was inoculated less than 48 h after highly virulent *L. maculans* (*L. maculans*). These DNA results support Liu et al. (2006) further because the disproportionate reduction in *L. maculans* DNA when co-inoculated with both Leptosphaeria spp. provides evidence that *L. biglobosa* can prime the plants defence response to increase resistance to *L. maculans*, in a form of host-mediated interspecific competition. This study also provides an explanation for why Mahuku *et al.* (1996) found that this effect was not observed if *L. biglobosa* was inoculated at 64 h or ~ 2.5 days, after *L. maculans*.

Gardiner *et al.* (2004) showed that sirodesmin PL takes up to 3 days to be produced. Sirodesmin PL has been shown to have an inhibitory effect on *L. biglobosa* growth (Elliott *et al.* 2007); therefore, in its absence *L. biglobosa* may be able to infect and colonise the host plant without inhibition. This was shown in this study because when cotyledons were co-inoculated with *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb), the lesions were similar and had similar appearance to *L. biglobosa* lesions and no sirodesmin PL was found in the extracts which suggests that the presence of *L. biglobosa* may inhibit the production of sirodesmin PL allowing it to potentially outcompete *L. maculans.* Whereas Mahuku *et al.* (1996) showed that after sirodesmin PL had been started to be produced at ~2.5 days post-inoculation, *L. biglobosa* could not outcompete *L. maculans.* However, further investigation is required to fully understand this phenomenon.

Results of this study have important practical and agricultural relevance because previous studies have shown that *L. maculans* and *L. biglobosa* ascospores can be released simultaneously (Huang *et al.* 2019). If the *Leptosphaeria spp.* ascospores are released and infect the host at the same time, then a simultaneous infection will occur, that could result in reduction in sirodesmin PL production and smaller phoma leaf spot lesions. This study showed that when cotyledons were co-infected with *L. maculans* and *L. biglobosa* at the same time, the *L. maculans* lesions were smaller and had an appearance characteristically like those of *L. biglobosa.* Therefore, in cropping seasons where simultaneous infection of plants with both *Leptosphaeria* spp. occurs, the growers may apply the fungicide later or not at all due to the advised threshold of plants with *L. maculans* lesions not being met. This is because our findings showed that interspecific interactions between these *Leptosphaeria* spp. resulted in reduction of metabolites, such as sirodesmin PL, production by *L. maculans* to the advantage of *L. biglobosa.* The potential increase in susceptibility to *L. biglobosa* due to the presence of *Rlm7* may cause unforeseen and unintended challenges to the UK oilseed rape industry in the future.

Chapter 4 Interactions between phoma stem canker (*Leptosphaeria* spp.) and light leaf spot (*Pyrenopeziza brassicae*) causal pathogens on different winter oilseed rape cultivars under field crop conditions.

4.1   Introduction

Leptosphaeria maculans and *L. biglobosa* are two co-existing fungal pathogens that are responsible for causing Phoma stem canker. *Pyrenopeziza brassicae* is the fungal pathogen responsible for causing light leaf spot (Rawlinson *et al.* 1978; Shoemaker and Brun; Fitt *et al.* 2006ab). These two diseases cause combined average annual yield losses of > £165M to English and Welsh growers (CropMonitor 2020). The current control strategies for these two diseases rely on the use of fungicides and cultivars with resistance to the causal pathogens (West *et al.* 2002; Gladders *et al.* 2006; Huang *et al.* 2011; Evans *et al.* 2017).

The most common fungicidal active ingredients used as solo or co-formulations are prothioconazole and tebuconazole. They both belong to the demethylation inhibitor (DMI) class of fungicides. The reliance on these active ingredients is increasing, as their use increased from 34% to 58% of all fungicides applied on oilseed rape in the UK between 2012 and 2020 (Garthwaite *et al.* 2012;2014;2016;2018;2020). Fungicide

insensitivity to DMI fungicides has not been reported in field isolates of *L. maculans* or *L. biglobosa* in the UK (FRAG-UK 2017) but there are reports of field isolates of *L. maculans* with DMI insensitivity in Australia (Van de Wouw *et al.* 2017), through a 275bp insertion or long terminal repeat retrotransposons (Yang *et al.* 2020). *L. biglobosa* isolates have been shown to be less sensitive to DMI fungicides than *L. maculans* isolates (Eckert *et al.* 2010; Huang *et al.* 2011). However, insensitivity to MBC and DMI fungicides has been reported in *P. brassicae* (Carter *et al.* 2013;2014). In Europe, life cycles of these three pathogens are initiated by ascospores released in the autumn (West *et al.* 1999; Gilles *et al.* 2000a;2001ab; Huang *et al.* 2005; Fitt *et al.* 2006ab). Accurate fungicide application timing is important for effective fungicide control (Gladders *et al.* 2006). Leaf infection by *Leptosphaeria* spp. ascospores initiates the formation of leaf lesions, which is the first easily observable indicator of the presence of the phoma stem canker pathogen. For phoma stem canker, the advised threshold for fungicide application is 10-20% incidence of plants with phoma leaf spotting, as this is when the risk of severe disease is greater than the threshold; it is the best time to spray fungicide to prevent the pathogens spreading to the stems to cause stem cankers (Zhou *et al.* 1999; Gladders *et al.* 2006; AHDB 2020). However, there is no advised threshold for light leaf spot control before stem extension (AHDB 2018), but it is advised that a fungicide application is made when light leaf spot symptoms appear; this is often when *P. brassicae* sporulation is observed in crops. Due to the symptomless latent period of *P. brassicae,* the time between leaf infection and appearance of symptoms can be 3-5 months (Fitt *et al.* 1998; Gilles *et al.* 2001bd; Boys *et al.* 2007). Additionally, the appearance of *P. brassicae* sporulation in crops may be not visible if there are many precipitation events causing rain-splash dispersal of conidia (Gilles *et al.* 2000ab; Evans *et al.* 2003). However, the fungicide applications that are applied when the phoma leaf spot threshold is met, such as Proline 275 (Bayer Crop Science 2019), also provide some protection against *P. brassicae* infection as many of the fungicides provide control against both *Leptosphaeria* spp. and *P. brassicae* infections.

Cultivars with quantitative resistance against *L. maculans, L. biglobosa* and *P. brassicae* are commercially available. However, commercially available cultivars with known *R*-gene resistance are available only for *L. maculans* (Rouxel and Balesdent 2017),

although there may be *R*-gene resistance against *P. brassicae* in commercial cultivars which has not been identified yet. Recent research suggests that cultivars with good resistance against *L. maculans* have increased susceptibility to *P. brassicae* (Huang *et al.*, 2021). Trade-offs in resistance to different pathogens have previously been reported, such as the incorporation of *mlo* resistance into barley where resistance to *Blumeria graminis* (powdery mildew) was increased, but there was a subsequent increase in susceptibility to *Ramularia collo-cygni*, cause of ramularia leaf spot (McGrann *et al.* 2014)*.* Interactions between *L. maculans, L. biglobosa* and *P. brassicae* infection in terms of incidence and severity at key plant developmental stages are still unknown.

This study aims to understand interactions between phoma stem canker (*Leptosphaeria* spp.) and light leaf spot (*Pyrenopeziza brassicae*) causal pathogens on different winter oilseed rape cultivars under field crop conditions by testing the following main hypotheses ;

- The incidence and severity of phoma lesions, light leaf spot lesions and fruiting bodies at key plant developmental stages are affected by the season, resistant cultivars, and the application of fungicide.
- Light leaf spot disease has the greatest effect on crop yield.
- *Leptosphaeria* spp. and *P. brassicae* ascospores are released simultaneously throughout the autumn.
- There are relationships between seasonal weather, disease prevalence and yield in winter oilseed rape in England and Wales.

These hypotheses were tested by the following four objectives.

- To assess the incidence and severity of diseases caused by *L. maculans*, *L. biglobosa* and *P. brassicae* in winter oilseed rape at key plant developmental stages when different control strategies are applied and relate them to their effects on yield.

- To monitor the timings of *L. maculans, L. biglobosa* and *P. brassicae* ascospore release events and relate these to seasonal weather.

- To investigate the changes in pseudothecial density of *Leptosphaeria* spp. on oilseed rape debris when different control strategies are applied.

- To identify relationships between seasonal weather and prevalence of light leaf spot, phoma leaf spot and stem canker or yield loss in winter oilseed rape for England and Wales.

4.2   Materials and methods

4.2.1   Winter oilseed rape field experiments

4.2.1.1 Choice of cultivars

Six cultivars with a range of 'field resistance' to *P. brassicae* (light leaf spot) and *L. maculans* (phoma stem canker) were selected for the field experiments at Terrington St Clement, Norfolk. They were selected based on the AHDB recommended list resistance ratings (0-9 scale with 9 being good resistance). Cv. Barbados was selected because it had good resistance ratings for both phoma stem canker and light leaf spot pathogens (Table 2.2). In contrast, cv. Charger was selected because of its poor resistance ratings for both pathogens. Cvs Flamingo and Quartz were selected because they have a good resistance rating for one of the pathogens that cause light leaf spot or phoma stem canker, respectively, and a susceptibility to the other pathogens. Cvs Hunivers and Django were included in the field experiment because they have moderate resistance ratings for pathogens that cause both diseases (Table 2.2).

4.2.1.2 Field experiment preparation and design

The experiment diary is displayed in Table 5.1. The experiment was sown at the end of August/ start of September in every season (29 August 2017, 24 August 2018, and 05 September 2019) at a seed rate of 120 seeds per m^2 at a depth of 2-3 cm and row width of 18 cm. Each plot was 10 m in length and 3 m wide. Plots were arranged in a

randomised block design (Appendices 14. Each of the six cultivars had an untreated and a prothioconazole treated plot, each with three replicates. There were 12 treatments and a total of 36 plots in each experiment. Other standard farm inputs and treatments were applied uniformly across the experiment (Table 5.2).

4.2.1.3 Choice of fungicide, dose, and spray timings

Proline 275 (® Bayer Crop Science UK; a.i. prothioconazole-desthio 275g/L) was used because it is a triazole fungicide, representing the main chemical group used to control oilseed rape diseases in the UK (Garthwaite *et al.* 2018). A half-rate dose (0.315 L/ha) was used because it has been shown that there is little difference between 50% and 100% full rate in fungicide performance or yield effect for both phoma stem canker and light leaf spot (AHDB 2019). Additionally, the average proportion of full label rate of prothioconazole fungicide formulations used by growers in 2016 was 60% (Garthwaite *et al.* 2018). This figure may be greater due to the use of prothioconazole later in the cropping season during flowering for sclerotinia stem rot control as the required rates are greater (Faye Ritchie, pers. comms.). A two-application (early T1 spray and late T2 spray) (Table 4.1 and 4.2) autumn fungicide spray programme was used to provide effective control of phoma stem canker and light leaf spot. The timing of the early spray was dependent on the timing of 10-20% incidence of phoma leaf spot on the *Leptosphaeria* spp. susceptible cultivar Flamingo and a late spray was applied 4-6 weeks later upon re-infection by *Leptosphaeria* spp.

4.2.1.4 Plot assessments

4.2.1.4.1 **Establishment and vigour assessments**

In November of each cropping season, each plot was assessed for establishment and vigour (Table. 4.1). For establishment assessments, each plot was scored using a 0-9 scale whereby 1 = very thin and 9 = very thick. For vigour assessments, each plot was scored using a 0-9 scale whereby 1 = very weak and 9 = very vigorous. Mean establishment and vigour scores for each treatment were calculated for each season.

Table 4.1: Dates of key events and assessments made throughout the winter oilseed rape field experiments for 2017/18, 2018/19 and 2019/20 cropping seasons.

Task	2017/18	2018/19	2019/20
Drilled	29 August 2017	24 August 2018	05 September 2019
T1 spray applied [a]	25 October 2017	16 November 2018	05 November 2019
Emergence and vigour experiment	2 November 2017	23 November 2018	12 November 2019
Post-T1 phoma leaf spot assessment [b]	27 November 2017	11 December 2018	12 December 2019
T2 spray applied [a]	28 November 2017	11 December 2018	16 December 2019
Post-T2 phoma leaf spot assessment [b]	09 January 2018	10 January 2019	20 January 2020
Light leaf spot foliar assessment	20 April 2018	03 April 2019	01 May 2020
Phoma stem canker and light leaf spot stem assessment	28 June 2018	03 July 2019	02 July 2020
Harvest	26 July 2018	24 July 2019	05 August 2020

[a] T1 = Prothioconazole application 1 (refer to Table 4.2)
T2 = Prothioconazole application 2 (refer to Table 4.2)
[b] Post-T1/T2 phoma leaf spot assessment was 4-6 weeks after fungicide application

Table 4.2: List of treatments in the field experiments for the 2017/18, 2018/19 and 2019/20 seasons. Six cultivars were either untreated or treated with prothioconazole (0.315L/ha) in two-spray programmes.

Trt	Cultivar£	Early spray* T1 (L/ha)	Late spray* T2 (L/ha)
1	Quartz	Untreated	Untreated
2	Quartz	Proline (0.315)	Proline (0.315)
3	Flamingo	Untreated	Untreated
4	Flamingo	Proline (0.315)	Proline (0.315)
5	Barbados	Untreated	Untreated
6	Barbados	Proline (0.315)	Proline (0.315)
7	Charger	Untreated	Untreated
8	Charger	Proline (0.315)	Proline (0.315)
9	Django	Untreated	Untreated
10	Django	Proline (0.315)	Proline (0.315)
11	Hunivers	Untreated	Untreated
12	Hunivers	Proline (0.315)	Proline (0.315)

*For dates of sprays see Table 4.1. For plans of experiments see Appendices.

£ Quartz has good resistance to *L. maculans* and low resistance to *P. brassicae*.

Flamingo has low resistance to *P. brassicae* and good resistance to *P. brassicae*.

Barbados has medium resistance to both *L. maculans* and *P. brassicae*.

Charger has low resistance to both *L. maculans* and *P. brassicae*.

Django has medium resistance to both *L. maculans* and *P. brassicae*.

Hunivers has medium resistance to both *L. maculans* and *P. brassicae*.

4.2.1.4.2 Phoma leaf spot (*L. maculans*) and light leaf spot (*P. brassicae*) foliar assessments

For each cropping season, *L. maculans* phoma leaf spot and light leaf spot assessments were done non-destructively using 10 randomly selected plants. Each plant was assessed for phoma leaf spot caused by *L. maculans* by assigning a severity score (0-3) dependent on the number of foliar lesions per plant. 0 = No lesions, 1 = < 5 foliar lesions, 2 = 5-10 foliar lesions, and 3 = >10 lesions (Mitrousia *et al.* 2018). *L. biglobosa* leaf spots were not presented due to initial focus of the project being on *L. maculans* in 2017/18, and low incidences in the 2018/19 and 2019/20 growing seasons. A G-index score was calculated for each plot. A mean G-index score was calculated for each treatment using Equation 4.1. *L. maculans* disease incidence was calculated. Full disease assessment was made 4 to 6 weeks after each fungicide application. Weekly disease incidence progression graphs for all cultivars were produced by assessing 25 plants per plot from all untreated plots for each cultivar. An additional foliar assessment for light leaf spot was made in April when *P. brassicae* sporulation was observable on the leaf lamina (Section 3.2.6). A G-index score was calculated to give a score between 0-100 (Equation 4.2). A mean G-index score was calculated for each treatment.

4.2.1.4.3 Phoma stem canker and light leaf spot stem assessments

Phoma stem base canker was assessed by destructively sampling 20 randomly selected plants and scoring stem base cankers by cutting the stem at the crown collar.

Equation 4.1: G-index severity score for *L. maculans* phoma leaf spot (Mitrousia *et al.* 2017).

$$\text{G-index} = \frac{(n_1 \times 10) + (n_2 \times 50) + (n_3 \times 100)}{n_1 + n_2 + n_3}$$

$$n_x = Count\ of\ plants\ with\ score\ x$$

Stem cross sections were scored using a 0-7 scoring system (whereby 0 = 0%, 1= <5%, 2= 6-25%, 3=26-50%, 4= 51-75%, 5= 76-99%, 6= 100% cross-section affected and plant still alive; 7= 100% cross-section affected, dead stem with a hollow or severely necrotic pith), modified from the 1–6 scale of Lô-Pelzer *et al.* (2009). Stem basal cankers were classified as cankers at the root crown or < 10 cm above it (Mitrousia *et al.* 2018). Light leaf spot stem lesions were assessed using the same 20 randomly selected plants and assigning a severity score (0-7) for the percentage of a 10 cm section starting from the root crown possessing light leaf spot stem lesions. The disease incidence was determined by calculating the number of plants with disease symptoms out of the 20 plants assessed. For all severity scores, a G index score was calculated for each plot (0-9) (Equation 4.1). A mean G-index score was calculated for each treatment.

4.2.1.4.4   Yield

At the end of each growing season, each plot was harvested for yield. The yield from each plot (kg) was adjusted to 91% moisture and the plot yield (t/ha) was calculated using plot length and grain dry matter using Equation 4.2.

Equation 4.2: Yield calculation equation using grain dry matter (%), adjusted dry matter (%), plot yield (kg), plot area (m^2) to give corrected yield (t/ha).

$$Corrected\ yield = \left(\frac{grain\ dry\ matter}{adjusted\ dry\ matter}\right) \times plot\ yield \times \left(10 \div (plot\ area)\right)$$

4.2.2   Monitoring patterns of release of *Leptosphaeria* spp. and *P. brassicae* air-borne ascospores.

4.2.2.1 Burkard spore tape preparation

As described by Lacey and West (2006), the Burkard drums were cleaned with 70% industrial methylated spirit (IMS) and blue paper towel to minimise any contamination, before Melinex tape was tightly wound around the drum. Double-sided sticky tape

placed at the starting point of the drum was used to affix the Melinex tape to the drum so that both the start and end of the tape covered half of the double-sided tape. Under a fume hood, the drum was secured onto the Burkard drum coating apparatus (Fig. 4.1) to allow the drum to be rotated. The trough on the apparatus was filled with a coating solution (10 g: 20 mL petroleum jelly/hexane mixture). A small roller pin was placed in the trough to provide a smooth contact between the coating solution and the Melinex tape on the drum to allow a uniform coat to be formed. The drums were left to dry for 1-2 h, before being placed in the carrying box that was labelled with the start and finish dates and times.

4.2.2.2 Burkard sampler set up

A Burkard 7-day spore sampler (Burkard Manufacturing Company Ltd, Rickmansworth) was set up as described by Lacey and West (2006) in the field experiment at Terrington St Clement, Norfolk. The sampler was operated from September until March each cropping season (2017-18, 2018-19 and 2019-20). In all seasons, the Burkard sampler was surrounded with oilseed rape stubble from the previous cropping season (Huang *et al.* 2005). The drum was secured into the Burkard spore sampler according to the protocol of the manufacturers. The sampler operated at the rate of 10L air/min and was operated for 7 days before the drum was replaced with a clean drum. Each used drum was processed.

4.2.2.3 Burkard sample processing

The Burkard sample processing is shown in Fig. 4.2. The used tape was separated from the drum using forceps and sharp medical scissors. processed by cutting the Melinex tape into seven 48mm long pieces from left to right (each piece representing one day). Each individual piece was divided equally horizontally. Using fungal mounting fluid, one half of the tape was mounted onto a labelled microscope slide (7.5 x 2.5 cm) so that the start of each tape was positioned nearest the slide label. In the fume hood, 3-5 small drops of cotton blue stain were placed on the mounted tape before covering it with a cover slip (5 x 2 cm).
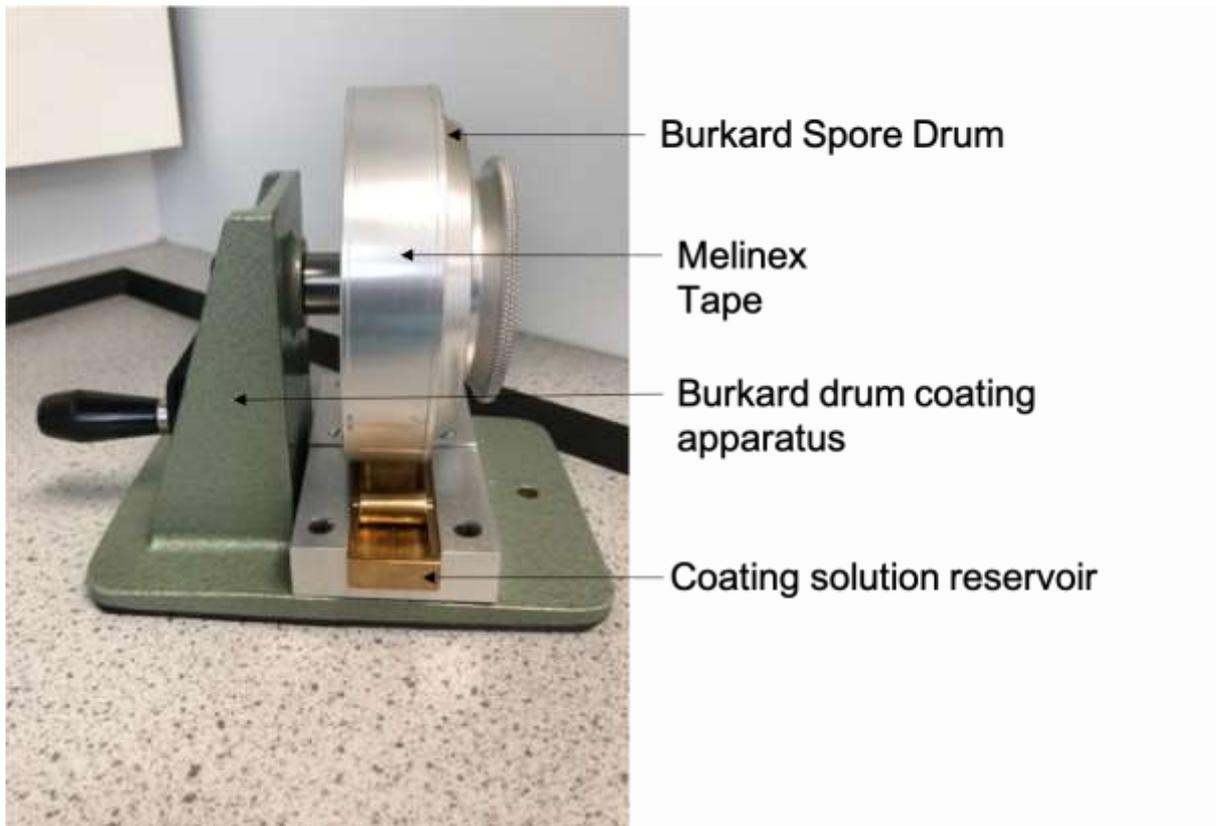
Figure 4.1: Burkard spore drum coating apparatus with the key components identified (Lacey and West, 2006).
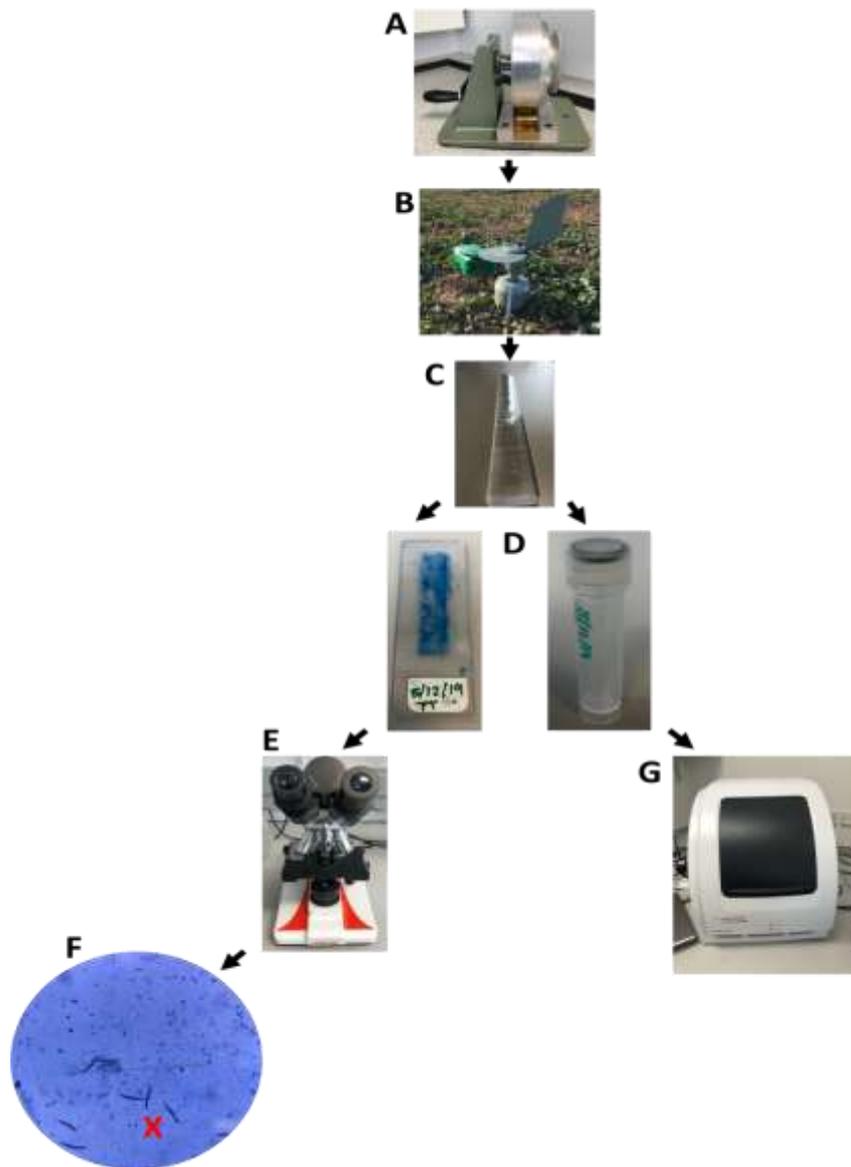
Figure 4.2: Burkard spore drum processing. A) A prepared spore drum is coated with petroleum jelly using a Burkard spore drum coating apparatus. **B)** The drum is placed inside a Burkard spore sampler in the field with additional stubble surrounding the sampler and left to run for 7 days. **C)** The spore drum is taken into the laboratory and the spore tape is placed on the cutting block where the tape is cut into seven equal pieces (each piece represents one day). **D)** Each piece is bisected horizontally, and one half is fixed and stained on a microscope slide, the other half is placed inside a sample tube and stored at -20°C until required. **E)** Using a light microscope, **F)** The prepared microscope slide is viewed at 200X magnification for counting. **G)** After DNA has been extracted from the tape inside the sample tube, it is analysed using qPCR. 'X' indicating the *Leptosphaeria* spp. ascospores.

Under 200X magnification using a light microscope, the numbers of *Leptosphaeria* spp. ascospores were counted (Huang *et al.* 2005; 2011; Lacey and West 2006). The other half of the tape was placed in a screw cap 2.0 mL tube and stored at -20°C for DNA extraction and quantification of *L. maculans*, *L. biglobosa* DNA and *P. brassicae* DNA using quantitative PCR (qPCR) (Huang *et al.* 2011).

The tape was placed Melinex side up onto a pre-marked Perspex cutting ruler block so that the start of the tape was placed on the left-hand side of the block. The tape was

4.2.2.4 DNA extraction from Burkard sampler spore tapes and quantitative PCR (qPCR)

The DNA extraction method is outlined in section 3.2.10. The spore tape samples were extracted the same way as that of Huang *et al.* (2011), which is a slight modification of the CTAB protocol (Kaczmarek *et al.* 2009). The extracted DNA suspension was diluted using SDW using a dilution factor of 10. These dilutions were then analysed using qPCR (section 4.2.2.5) to determine the proportions of *L. maculans* DNA, *L. biglobosa* DNA and *P. brassicae* DNA in the air samples. Each DNA sample was replicated twice in the qPCR analysis. One *Leptosphaeria* spp. ascospore has been determined to account for approximately 5 pg. of DNA (Huang *et al.* 2011). Therefore, amounts of *L. maculans* and *L. biglobosa* DNA can be treated equally, so if a greater amount of DNA was found for *L. maculans* than *L. biglobosa*, then there was a greater number of *L. maculans* ascospores in that sample. However, due to variable amount of stem stubble being placed around the spore sampler the spore release data cannot accurately be related to disease severity. This data is used to identify patterns of release and relative differences between pathogens.

4.2.2.5 Quantitative PCR (qPCR)

The qPCR method used was the same as that of Huang *et al.* (2019) and is outlined in section 3.2.11. The differences were that these qPCR experiments included *P. brassicae* analysis and a different *L. biglobosa* primer set was used in the 2017/18 analysis. The species-specific primer sets that were used were the same as in section 3.2.11; Lmac_F (CTTGCCCACCAATTGGATCCCCTA) and Lmac_R (GCAAAATGTGCTGCGCTCCAGG) for *L.*

maculans for all cropping seasons (2017/18, 2018/19 and 2019/20) ascospore tapes and WVF1 (CCTTCTATCAGAGGATTGGT) and WVR1 (CGTTCTTCATCGATGCCAGA) for *L. biglobosa* for 2017/18 (Mahuku *et al.* 1995), but LbigF (ATCAGGGGATTGGTGTCAGCAGTTGA) and Lmac_R were used for 2018/19 and 2019/20 ascospore tapes (Liu *et al.* 2006, 2014). There was a change between the two primer sets because of discrepancies between WVF1/WVR1 and LbigF/Lmac_R. It was decided that the most appropriate primer set for quantification of *L. biglobosa* was the LbigF/Lmac_R primer set because these were designed using a UK isolate which is *L. biglobosa* brassicae. Whereas for the other primer set it was not known whether the isolate used for primer design belonged to *L. biglobosa* brassicae or another subclade such as *L. biglobosa* canadensis. The species-specific primer set that was used for *P. brassicae* for all three cropping seasons were; PbITS-F (TTGAACCTCTCGTCGAAGAAGTTCAGTCT) and PbITS-R (AGATTTGGGGGGTTGTTGGCTAA) (Karolewski *et al.* 2006) (Appendices 5). The standards were 0.1pg – 10 ng. The thermal profiles that was used for *P. brassicae* was: one cycle of initial denaturation at 95°C for 2 min; 50 cycles of denaturation, annealing and amplification at 95°C for 15 sec, 58°C for 45 sec, 72°C for 45 sec and 83°C for 15 sec; one cycle of 95°C for 1 min, 60°C for 1 min and 95°C for 15 sec (Appendices 10).

4.2.2.6 Mean temperature and precipitation at the field experiment site.

Weather data were obtained from Fox Weather; they use a Met office calibrated weather station to record temperature and precipitation. The weather station was positioned within 500 m of the experimental site. The weather data were arranged to relate with the life cycles of the *Leptosphaeria* spp*.* and *P. brassicae* pathogens. Therefore, the four seasons were made to relate to the stages of plant growth: Autumn (Jul-Sept) for maturation and primary release of ascospores from pseudothecia and apothecia, for *Leptosphaeria spp.* and *P. brassicae,* respectively. Winter (Nov-Feb) for pathogen host colonisation during plant vernalisation and secondary infection, primarily for *P. brassicae*, Spring (Mar-May) for pathogen host colonisation during elongation, bud development and flowering, and secondary infection, primarily for *P. brassicae*, and Summer (Jun and Jul) for pathogen host colonisation during seed and pod development,

and secondary infection, primarily for *P. brassicae*. A separate weather variable called post March (March – July) was used for *L. maculans* and *L. biglobosa* colonisation of stems.

4.2.3 Monitoring *Leptosphaeria* spp. pseudothecial densities on stem debris from the previous cropping season.

4.2.3.1 Stem incubation for pseudothecial maturation

Similar to Huang *et al.* (2005), naturally infected oilseed rape stems from each treatment were collected from the field experiments (Section 4.2.1) every year after harvest. Stems from each treatment were combined together and placed in a free-draining plastic tray to be exposed in natural conditions so that pseudothecia could develop and mature under natural conditions. The trays were placed around the Burkard spore sampler at Bayfordbury in Hertfordshire. When major ascospore release events were recorded for the Burkard spore sampler used in 4.2.2, the stems were collected and dried, because the major release of ascospores indicates that most of the pseudothecia are mature. Once dried, randomly selected stems from each treatment were cut into stubble sticks. Sticks were made by cutting the stem at the root crown then cutting up the stem to form 'stubble sticks' that were 5cm (l) x 0.5cm (w). Total numbers of sticks per stem were between four and five. The 'stubble sticks' from each stem were placed inside a Petri-dish. This was repeated so that there were 'stubble sticks' for five stems for each treatment.

4.2.3.2 *Leptosphaeria* spp. pseudothecial density assessment

Using a bifocal dissecting microscope at 10X magnification, the numbers of mature pseudothecia were counted for each stubble stick. A characteristically shaped and sized *Leptosphaeria* spp. pseudothecium was considered mature when the neck was formed or the ostiole was open (i.e., ascospores were released) (Toscano-Underwood *et al.*, 2003). The average number of pseudothecia per stem was calculated using a mean of the four sticks per stem. The average number of pseudothecia per treatment was calculated using the means of the five stems per treatment (e.g., 20 stubble sticks per treatment were assessed).

4.2.3.3 Stem basal canker score on *Leptosphaeria* spp. against pseudothecial density

To study the effects of cultivar resistance, the six cultivars were classified into three groups depending on their AHDB RL disease resistance ration – Good resistance (8+), Medium resistance (6 or 7) and Susceptible (5 or less). Cultivar Quartz (AHDB resistance rating 8) had good resistance, while cvs Barbados (AHDB resistance rating 7), Django (AHDB resistance rating 6) and Hunivers (AHDB resistance rating 7) had medium resistance and cvs Charger and Flamingo (both AHDB resistance rating 4) were susceptible to *Leptosphaeria* spp. The stem basal canker severity score data were obtained from section 4.2.1.4.3. The relationship between stem canker severity score and the density of pseudothecia on the sampled stems was analysed using linear regression and linear regression with groups.

4.2.4 Identification of relationships between weather, prevalence of light leaf spot, phoma leaf spot and phoma stem canker and yield loss in winter oilseed rape in England and Wales.

4.2.4.1 Collection of data on weather, disease incidence and yield loss

4.2.4.1.1 **Weather data collection and formatting**

Weather data for England and Wales were collected from (https://www.metoffice.gov.uk/research/climate/maps-and-data/uk-and-regional-series). The weather parameters for England and Wales used were monthly mean temperature, mean number of rain days, mean precipitation and mean number of air frosts. Data were collected for the period between August 2007 and March 2020. The data were formatted to fit with the general UK oilseed rape plant growth cycle, with crops being sown in August and harvested in July. Therefore, the four seasons were made to relate to the stages of plant growth: Autumn (Aug, Sept, and Oct) for initial growth, Winter (Nov, Dec, and Jan) for vernalisation, Spring (Feb, Mar, Apr, May) for elongation, bud development and flowering and Summer (Jun and Jul) for seed development and harvest. A mean was calculated for each season for all weather parameters.

4.2.4.1.2   Oilseed rape cropping area data collection

The oilseed rape cropping area data for England and Wales were collected from an AHDB database   (https://ahdb.org.uk/cereals-oilseeds/planting-variety-survey-results).   Any missing values were calculated by subtracting the crop production areas for Scotland (https://www.gov.scot/publications/cereal-oilseed-rape-harvest-2019-final-estimates/pages/3/)   and   Northern   Ireland   (https://www.daera-ni.gov.uk/publications/statistics-crop-production-form-1981 from the total UK cropping area   (https://www.gov.uk/government/statistical-data-sets/agriculture-in-the-united-kingdom). Data for 2008/09 for Northern Ireland were not available so a value of 600 ha, the average cropping area for 2011-2018, was used. Oilseed rape yield loss per 1000 hectares was calculated by either dividing the recorded light leaf spot or phoma stem canker yield loss by the recorded cropping area (Table 4.3). This was done to supress the effect of greater yield losses due to larger oilseed rape cropping areas.

4.2.4.1.3   Disease incidence and yield loss data collection for England and Wales.

The incidences of crops with light leaf spot foliar lesions in spring, pod lesions at harvest, phoma leaf spotting in autumn, phoma stem cankers in the summer and yield loss data were obtained from FERA from 2007-08 and 2017-18 cropping seasons (FERA, 2021). These data are collected by DEFRA by carrying out stratified surveys across England and Wales of the diseases that are commercially managed, including light leaf spot. Between 80-100 randomly selected crops are sampled at three periods during the cropping season: December, March, and June. At each assessment, 25 plants were randomly selected from each crop.

4.2.4.1.4   Identification of patterns using simple regression analysis

The relationships between disease incidence assessments (*P. brassicae* foliar sporulation in autumn and spring, *P. brassicae* pod lesion incidence, phoma leaf spot phoma stem canker incidence) and yield loss (£M) per thousand hectares were plotted against each other. The average seasonal weather parameters vs disease incidence assessments were analysed.

Table 4.3. Annual yield loss (£M) to oilseed rape (OSR) growers due to light leaf spot (LLS) and phoma stem canker per thousand ('000) hectares in England and Wales from 2007-08 until 2017-18.

Year	LLS yield loss (£M)*	Phoma yield loss (£M)*	England and Wales OSR production ('000 ha)^	LLS yield loss /'000 hectares (£M)	Phoma yield loss/'000 hectares (£M)
2008-09	67.3	53.8	526	0.12	0.10
2009-10	93.5	44.8	612	0.15	0.07
2010-11	79.1	92.1	665.9	0.11	0.14
2011-12	159.3	62.1	718.7	0.21	0.09
2012-13	88.2	63.1	681	0.12	0.09
2013-14	145	79.6	637.5	0.21	0.12
2014-15	125.6	79.6	615.4	0.19	0.13
2015-16	203.3	98.7	547.4	0.35	0.18
2016-17	86.8	59.8	527.3	0.15	0.11
2017-18	116	86.9	549.2	0.20	0.16

*Data was obtained from CropMonitor (Fera, 2021)

^Data was calculated from the AHDB (https://ahdb.org.uk/cereals-oilseeds/planting-variety-survey-results). Any missing values were calculated as described in section 5.2.4.1.2.

All data sets were plotted against each other, and a simple regression analysis was done to give a R^2 value.

4.2.5 Statistical analysis

The statistical analyses of the data collected from the field experiments were done using GenStat (General Statistics) (VSN International 2021). To analyse the differences between treatments for the phoma and light leaf spot severity and incidence and yield, a factorial analysis of variance (ANOVA) was done on the three replicate blocks to determine significant effects of cultivar, application of fungicide and interactions between cultivar and the application of fungicide. Differences with $P<0.05$ were considered to be significant. To identify the differences between individual treatments for each assessment, a simple ANOVA was done, with additional post-hoc tests applied. Tukey tests and Fisher LSD were used for balanced and unbalanced designed experiments. For the analysis of *Leptosphaeria* spp. pseudothecia density stem assessments the data were transformed using a square root-transformation to reduce variation.

4.3   Results

4.3.1   Winter oilseed rape field experiments

4.3.1.1 Disease progression in untreated plots – phoma leaf spotting

4.3.1.1.1   **The 2017/18 cropping season**

There were differences in patterns of *L. maculans* phoma leaf spot incidence between the cultivars (Fig. 4.3). Cv. Hunivers was highly variable with large maxima and minima in incidence, whereas cultivars such as Quartz and Flamingo tended to have a relatively stable incidence of phoma leaf spots with large increases in incidence on 25 October and 20 November, respectively. *L. maculans* phoma leaf spot incidence on cv. Charger increased over the cropping season with a period of stabilisation between 25 October and 6 November.
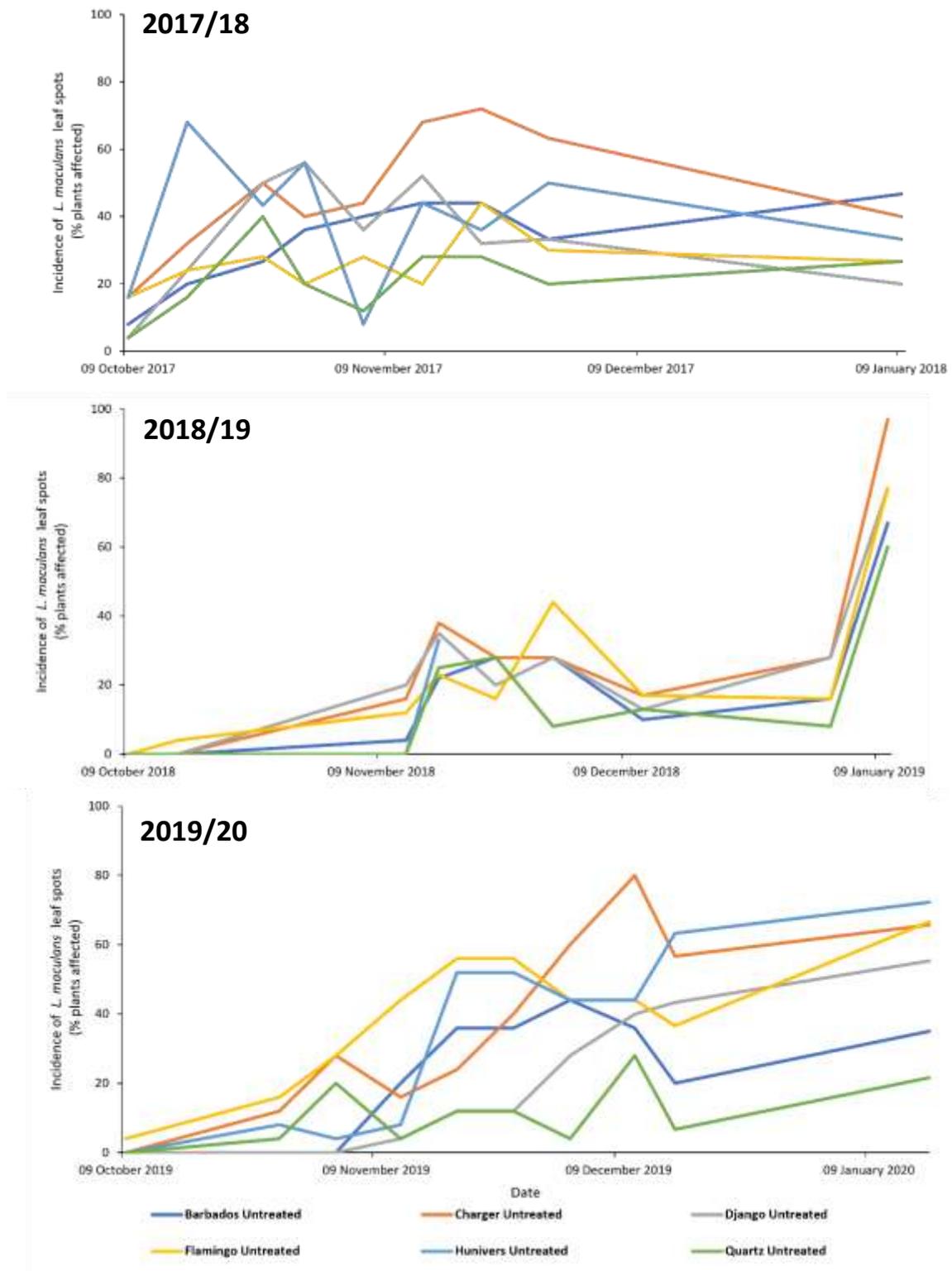
Figure 4.3: Incidence of *L. maculans* leaf spotting (% plants affected) on untreated cultivars from field experiments at Terrington St Clement between 9 October and 9 January during the 2017/18, 2018/19 and 2019/20 cropping seasons. Light blue = Hunivers, yellow = Flamingo, green = Quartz, dark blue = Barbados, grey = Django and orange = Charger.

Cv. Django's pattern of disease progression was a continual rapid increase to a maximum on 30 October and then a fluctuating slow decline, whereas cv. Barbados showed a similar pattern to cv. Django; however, the continual increase was much slower, and the decrease started later than for cv. Django on the 20 November. For most cultivars, the incidence of *L. maculans* phoma leaf spotting was maximal at between 40-56% plants affected (cv. Quartz, cv. Barbados, cv. Flamingo and cv. Django). The cultivars that had the greatest incidence of phoma leaf spotting were cv. Hunivers and cv. Charger at 68 and 72% of plants affected, respectively. The phoma leaf spot threshold (incidence > 10% plants affected) in the experiment was recorded to have been met on 9 October 2018.

4.3.1.1.2 The 2018/19 cropping season

At the start of the 2018/19 cropping season, there was a similar pattern of *L. maculans* phoma leaf spot incidence for all cultivars tested (Fig. 4.3). There were two periods of increase in symptoms; from 15-30 November and near 10 January. At the two periods of increased incidence of symptoms, cvs Charger and Flamingo recorded the greatest incidences of *L. maculans* phoma leaf spots whereas cv. Quartz recorded the smallest incidences during these periods. Phoma leaf spot incidences for the other three cvs (Django, Hunivers and Barbados) fluctuated between the greatest and smallest values. Cv. Django consistently had a greater incidence than the cv. Hunivers and cv. Barbados, which both recorded similar incidences throughout the start of the growing season. The phoma threshold (incidence > 10% plants affected) in the experiment was recorded to have been met on 12 November 2018.

4.3.1.1.3 The 2019/20 cropping season

During the start of the 2019/20 cropping season, *L. maculans* phoma leaf spotting on cv. Hunivers occurred two weeks earlier than on cvs Barbados and Django. Cv. Charger recorded the greatest incidence of phoma leaf spotting on 11 December 2019. Cv. Quartz recorded the smallest incidences of phoma leaf spotting across the whole season. The other cultivars fluctuated between the greatest and smallest values, but cv.

Hunivers had greater *L. maculans* phoma leaf spot incidences than cvs Barbados and Django. The phoma threshold (incidence > 10% plants affected) in the experiment was recorded to have been met on 28 October 2019.

4.3.1.1.4 **Overall disease progression across the three cropping seasons**

Overall, the start of the *L. maculans* epidemic (>10% plants with phoma leaf spots incidence) was recorded much earlier in 2017/18 (before 9 October) than in 2018/19 (12 November) or 2019/20 (28 October). When the maximum incidence recorded for each cultivar in each season was ranked across all three seasons, cv. Charger had the greatest maximum incidence of *L. maculans* leaf spotting, in contrast to Quartz having the smallest. All the other cultivars fluctuated in rank across these seasons, with the greatest fluctuations in position by cvs Flamingo and Hunivers.

4.3.1.2 Establishment and vigour of plots in November

Overall, there was a significant difference in the establishment of plots in November between cropping seasons ($F_{2,107}$ = 1967.09, *P* <0.001, LSD = 0.21). There was a significantly greater November establishment score in the 2017/18 (7.8) and 2018/19 (7.7) cropping seasons than in 2019/20 (2.0) (Fig. 4.4). There was a significant difference in the establishment of plots in November between cultivars ($F_{5,107}$ = 3.35, *P* = 0.009, LSD = 0.30). The cultivar that had the smallest establishment score was cv. Quartz (5.6), but this score was only significantly smaller than the establishment score of cv. Flamingo (6.0). Establishment scores of all other cultivars were not significantly different from those of cvs Quartz or Flamingo. There was no significant difference in the establishment of plots in November between treatments ($F_{11,107}$ = 1.82, *P* = 0.07, LSD = 0.42). There was no significant interaction between cultivar and cropping season ($F_{10,107}$ = 1.61, *P* = 0.126, LSD = 0. 59) or between treatment and cropping season ($F_{22,107}$ = 0.24, *P* = 1.0, LSD = 0.83) for the November establishment score.
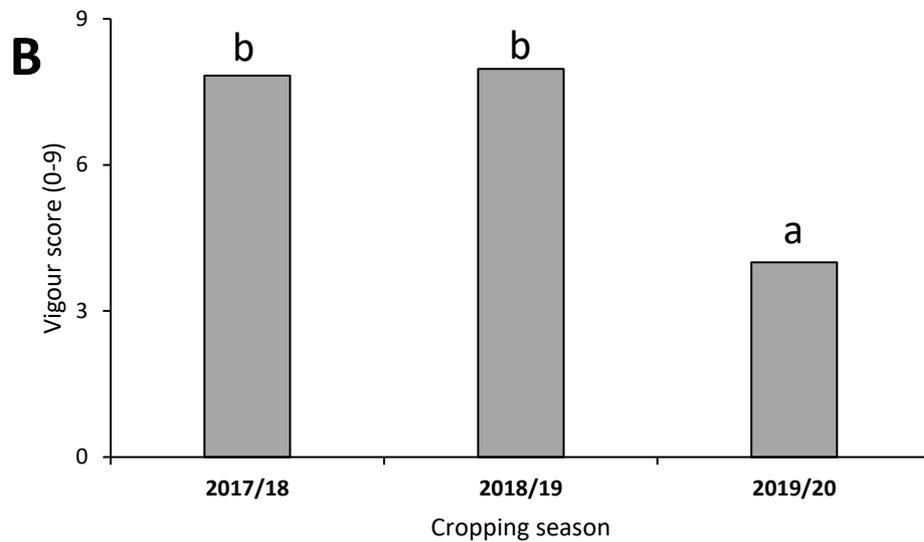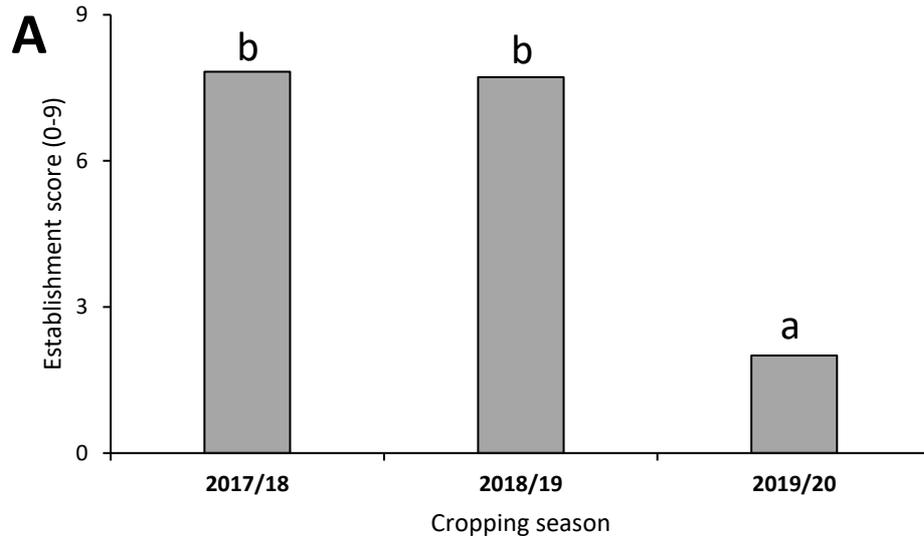
Figure 4.4: A) Establishment score (0-9 scale) and B) vigour score (0-9 scale) in field experiments at Terrington St Clement, Norfolk in the 2017/18, 2018/19 and 2019/20 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (107 d.f.).

Overall, there was a significant difference in the vigour of plots in November between cropping seasons ($F_{2,107}$ = 823.29, P <0.001, LSD = 0.22). There was a significantly greater November vigour score in the 2017/18 (7.8) and 2018/19 (7.9) cropping seasons than in 2019/20 (4.0) (Fig. 4.4). There was no significant difference in the vigour of plots in November between cultivars ($F_{5,107}$ = 1.14, P = 0.349, LSD = 0.31). There was no significant difference in the vigour of plots in November between treatments ($F_{11,107}$ = 0.07, P = 1.0, LSD = 0.22). There was no significant interaction between cultivar and cropping season ($F_{10,107}$ = 1.29, P = 0.257, LSD = 0. 55) or between treatment and cropping season ($F_{22,107}$ = 0.20, P = 1.0, LSD = 0.77) for the November vigour score.

4.3.1.3 Effects of fungicides on foliar disease incidence and severity – *Leptosphaeria maculans*

4.3.1.3.1   Incidence and severity of *L. maculans* phoma leaf spotting at T1 + 4-8 weeks

4.3.1.3.1.1   Incidence of *L. maculans* phoma leaf spotting at T1 + 4-8 weeks.

When all three seasons were analysed for the incidence of *L. maculans* lesions at 4-8 weeks after T1 treatment, there was no significant difference in incidence of lesions between the 2017/18 (28.6%), 2018/19 (15.6%) or 2019/20 (19.4%) cropping seasons ($F_{2,107}$ = 7.37, P =0.001, LSD = 6.96) (Table 4.4 & 4.5). However, there was a significant difference between cultivars ($F_{5,107}$ = 5.21, P < 0.001, LSD = 9.84). Cultivar Quartz (9.4%) had the smallest incidence of *L. maculans* lesions but was not significantly different from cvs Flamingo (17.2%) or Barbados (18.3%); the incidences on both cvs Django (22.8%) and Hunivers (27.8%) were not significantly different from the incidence on cv. Barbados, but the *L. maculans* lesion incidence was greater on cv. Hunivers than on cv. Flamingo. There was a significant difference in *L. maculans* incidence between untreated and treated plots ($F_{1,107}$ = 36.49, P < 0.001, LSD = 5.68). The incidence of *L. maculans* lesions was greater on untreated plots (29.8%) than on those plants that have had been treated with prothioconazole (12.6%).

Table 4.4. Testing output of significant probability levels for the main effects of Cultivar, season and Fungicide, the two-way interactions, and the three-way interaction on incidence and severity of *L. maculans* leaf spotting

Incidence of *L. maculans* leaf spotting at T1 + 4-8 weeks.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	7.37	107	6.96	0.001
Cultivar	5	5.21	107	9.84	<0.001
Fungicide	1	36.49	107	5.68	<0.001
Season x Cultivar	10	0.60	107	17.05	0.812
Season x Fungicide	2	17.48	107	9.84	< 0.001
Cultivar x Fungicide	5	1.84	107	13.92	0.116
Cultivar x Season x Fungicide	10	1.82	107	24.11	0.073

Severity of *L. maculans* leaf spotting at T1 + 4-8 weeks.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	2.65	107	2.018	0.078
Cultivar	5	3.32	107	2.854	0.009
Fungicide	1	17.92	107	1.648	<0.001
Season x Cultivar	10	0.43	107	4.944	0.929
Season x Fungicide	2	8.04	107	2.854	<0.001
Cultivar x Fungicide	5	1.81	107	4.037	0.122
Cultivar x Season x Fungicide	10	1.03	107	6.992	0.431

Table 4.5: Incidence (% plants affected) and severity score (0-100) of *L. maculans* leaf lesions from winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The six winter oilseed rape cultivars were either 'Untreated' or 'Treated' with the fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the mean incidence or severity score between the various treatment combinations in the two-way interactions.

Cultivar*	Treatment	Incidence of *L. maculans* leaf spotting at T1 + 4-8 Weeks (% plants affected)				Severity of *L. maculans* leaf spotting at T1 + 4-8 Weeks (0-100)			
		2017/18	2018/19	2019/20	Mean	2017/18	2018/19	2019/20	Mean
Barbados (7)	Untreated	33.3	10.0	20.0	18.3abc	6.0	1.00	3.3	2.5a
	Treated	16.7	26.7	3.3		1.7	2.7	0.3	
Charger (4)	Untreated	63.3	16.7	56.7	31.7d	14.3	3.0	15.0	6.5b
	Treated	23.3	26.7	3.3		2.3	4.0	0.3	
Django (6)	Untreated	33.3	13.3	43.3	22.8bcd	3.3	1.3	8.3	3.17a
	Treated	26.7	20.0	0.0		4.0	2.0	0.0	
Flamingo (4)	Untreated	30.0	16.7	36.7	17.2ab	3.0	1.7	9.0	2.61a
	Treated	10.0	10.0	0.0		1.0	1.0	0.0	
Hunivers (7)	Untreated	50.0	10.0	63.3	27.8cd	6.3	1.0	9.0	3.67ab
	Treated	20.0	23.3	0.0		2.0	3.7	0.0	
Quartz (8)	Untreated	20.0	13.3	6.7	9.4a	2.0	1.3	0.7	0.94a
	Treated	16.7	0.0	0.0		1.7	0.0	0.0	
	Untreated	38.3c	13.3b	37.8c	29.8b	5.8b	1.6a	7.6b	4.98b
	Treated	18.9b	17.8b	1.1a	12.6a	2.1a	2.2a	0.1a	1.48a
		28.6b	15.6a	19.4a		4.0a	1.9a	3.8a	

*AHDB recommended list (0-9 scale with 9 being good resistance).

[a-d] Tukey post hoc tests are represented as letters. Values that do not share a letter are significantly different.

The cultivar that had the greatest *L. maculans* incidence was cv. Charger (31.7%), but this was not significantly greater than the *L. maculans* lesion incidence on cvs Hunivers or Django. There was no interaction between cultivar and application for fungicide on *L. maculans* lesion incidence ($F_{5,107}$ = 1.84, *P* = 0.116, LSD = 13.92). For all cultivars, the mean *L. maculans* lesion incidence was greater on untreated plots than on treated plots. However, there were significant differences between untreated and prothioconazole treated plots only for cvs Charger, Flamingo and Hunivers. Whereas for cvs Barbados, Django and Quartz, the mean incidence was greater on untreated plots than the treated plots, but not significantly (Fig. 4.5). However, there was a significant difference between treatments ($F_{11,107}$ = 4.29, *P* < 0.001, LSD = 17.08) (Fig. 4.5). The treatment that had the smallest incidence score was treated cv. Quartz (5.6%). However, this score was not significantly smaller than that on all other treated treatments or untreated cvs Quartz (13.3%) and Barbados (21.1%). The treatment that had the greatest incidence was untreated cv. Charger (45.6%); this was significantly larger than for all other treatments, except untreated cvs Django (30.0%) and Hunivers (41.1%). The incidence score on treated cv. Charger (17.8%) was significantly different only from untreated cvs Hunivers and Charger. There was no significant interaction between cultivar and year ($F_{10,107}$ = 0.60, *P* = 0.812, LSD = 17.05). There was a significant interaction between application of prothioconazole and year ($F_{2,107}$ = 17.48, *P* < 0.001, LSD = 9.84). The incidence of *L. maculans* lesions was smaller on the treated plots than on untreated plots in the 2017/18 and 2019/20 cropping seasons, but in the 2018/19 season there was no significant difference between treated and untreated plots. There was a significant difference in the mean incidence on untreated plots at the post T1 assessment between the seasons. For *L. maculans* lesion incidence on untreated plots, there was no significant difference between 2017/18 (38.3%) and 2019/20 (37.8%), but they were significantly greater than that in 2018/19 (13.3%). For the *L. maculans* lesion incidence on treated plots, there were no significant differences between 2017/18 and 2018/19 (18.9% and 17.8%) but these incidences were significantly greater than that in 2019/20 (1.1%). There was no significant interaction between cultivar, application of fungicide and year on severity of *L. maculans* at the post T1 assessment ($F_{10,107}$ = 1.92, *P* = 0.057, LSD = 24.49). Individual season analysis can be found in Appendices 15.
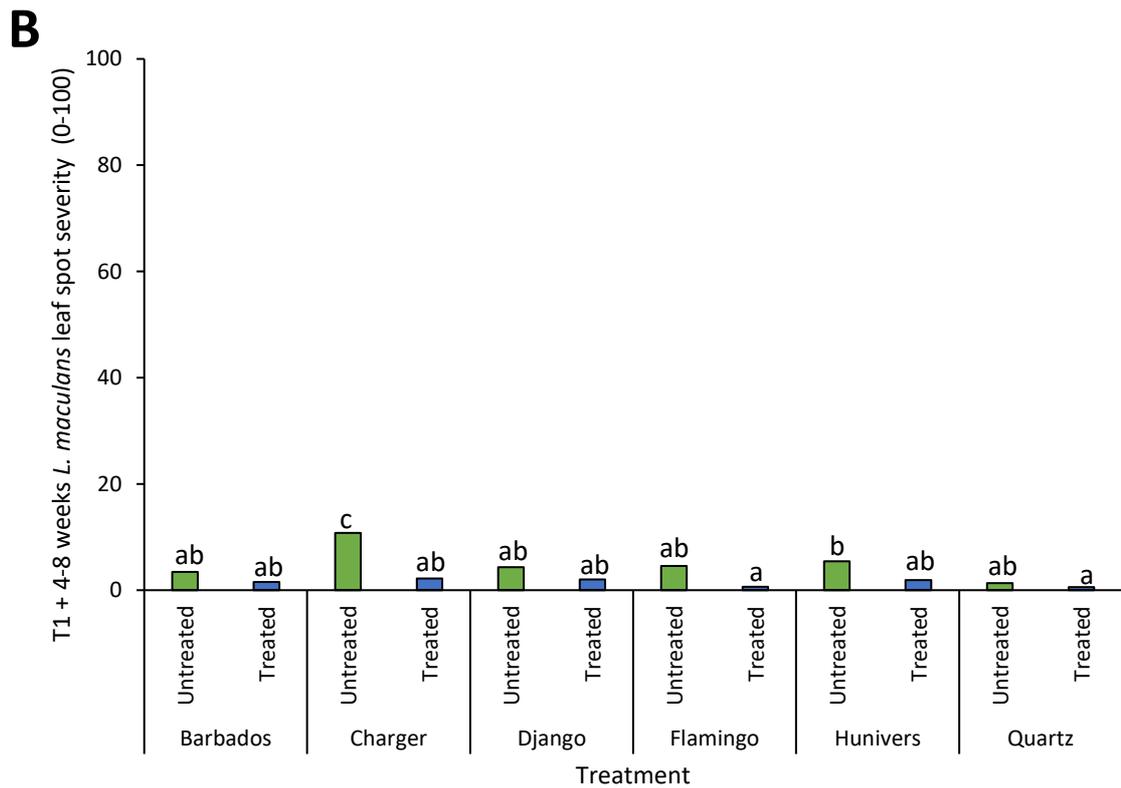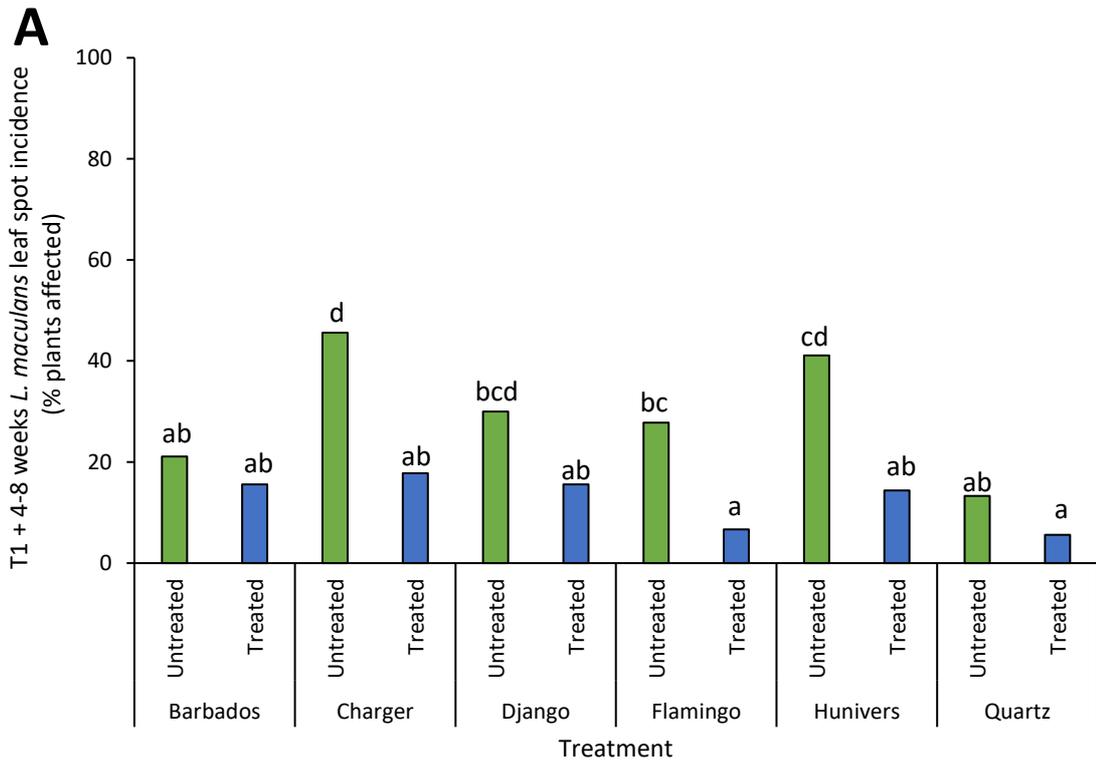
Figure 4.5: Incidence (A) and severity (B) of *L. maculans* leaf spots an T1 + 4 weeks (27 November 2017, 11 December 2018, 12 December 2019) at Terrington St Clement during the 2017/18, 2018/19 and 2019/2020 cropping seasons. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (107 d.f.).

4.3.1.3.1.2 Severity of phoma leaf spotting at T1 + 4-8 weeks.

For the three growing seasons, when the severity of *L. maculans* leaf spotting was compared, there was no significant difference between 2017/18 (3.97), 2018/19 (1.89) or 2019/20 (3.83) cropping seasons ($F_{2,107}$ = 2.018, *P* = 0.078, LSD = 3.876) (Table 4.4 & 4.5). There was a significant difference between cultivars ($F_{5,107}$ = 3.32, *P* = 0.009, LSD = 2.854) (Table 4.4 & 4.5). Cv. Quartz (0.94) had the least severe *L. maculans* lesions, but it was only significantly less severe than the lesions on cv. Charger (6.50) that were significantly more severe than on all other cultivars, except cv. Hunivers (3.7). The severity of *L. maculans* lesions was not significantly different between other cultivars. There was a significant difference in *L. maculans* lesion severity between treated and untreated plots ($F_{1,107}$ = 17.92, *P* < 0.001, LSD = 1.648). The severity of *L. maculans* lesions was significantly greater on untreated plots (4.98) than on treated plots (1.48).

There was no significant interaction between cultivar and application of fungicide ($F_{5,107}$ = 1.81, *P* = 0.122, LSD = 4.037) (Table 4.4) (Table 4.4 & 4.5).. However, there was a significant difference between treatments ($F_{5,107}$ = 1.93, *P* < 0.100, LSD = 3.91) (Fig. 4.5). Untreated cv. Quartz had the least severe untreated severity score but was only significantly less severe than untreated cvs Charger (10.78) and Hunivers (5.44). However, untreated cv. Quartz was not significantly different from any of the treated cultivars (Fig. 4.5). There was no significant interaction between cultivar and year on *L. maculans* severity score at the post T1 assessment ($F_{10,107}$ = 0.43, *P* = 0.929, LSD = 4.944) (Table 4.4 & 4.5). For all cultivars, there was no significant difference in cultivar severity score across the three seasons. There was a significant interaction between fungicide application and year on *L. maculans* severity score ($F_{2,107}$ = 8.04, *P* < 0.001, LSD = 2.854) (Table 4.4). In the 2017/18 and 2019/20 cropping season, the untreated plots had a significantly greater severity score than treated plots, in contrast to the 2018/19 cropping season where there was no significant difference between untreated and treated plots. There was no significant interaction between cultivar, fungicide application and year on *L. maculans* severity score at the post T1 assessment ($F_{2,107}$ = 1.03, *P* = 0.431, LSD = 6.992) (Table 4.4). Individual season analysis can be found in Appendices 15.

4.3.1.3.2 Incidence and severity of *L. maculans* phoma leaf spotting at T2 + 4-8 weeks

4.3.1.3.2.1 Incidence of phoma leaf spotting at T2 + 4-8 weeks

When all three seasons were analysed for the incidence of *L. maculans* lesions at 4-8 weeks after the T2 treatment, there was a significant difference in incidence of lesions between the 2017/18 (23.3%), 2018/19 (44.2%) or 2019/20 (38.1%) cropping seasons ($F_{2,107}$ = 16.58, *P* < 0.001, LSD = 7.42) (Table 4.6 & 4.7). There was a significant difference between cultivars ($F_{5,107}$ = 5.87, *P* < 0.001, LSD = 10.49) (Table 4.6 & 4.7). Cultivar Quartz (22.22%) had the smallest incidence of *L. maculans* lesions at the T1 + 4-8 weeks assessment, but this was not significantly different from cvs Barbados (30.6%) or Django (31.1%); the incidences on these two cultivars were significantly different only from the incidences of phoma leaf spotting found on cv. Charger (47.8%). There was a significant difference in *L. maculans* incidence at the post T2 assessment between untreated and treated plots ($F_{1,107}$ = 145.74, *P* < 0.001, LSD = 6.05) (Table 4.6 & 4.7). The incidence of *L. maculans* lesions was greater on untreated plots (53.5%) than on those plots that had been treated with prothioconazole (16.9%). There was no significant interaction between cultivar and application of fungicide on *L. maculans* lesion incidence ($F_{5,107}$ = 0.32, *P* = 0.899, LSD = 14.83) (Table 4.6). For all cultivars, the mean *L. maculans* lesion incidence was significantly greater on untreated plots than on treated plots. There was a significant difference in *L. maculans* leaf spotting between treatments ($F_{11,107}$ = 9.44, *P* < 0.001, LSD = 19.26) (Fig. 4.6). The treatment with the smallest incidence was treated cv. Quartz (7.8%); this was significantly smaller than all untreated treatments and treated cv. Charger (27.8%). The treatment with the greatest incidence score was untreated cv. Charger (67.8%); this was significantly greater than all treated treatments and untreated Quartz (36.7%). There was no significant interaction between cultivar and year ($F_{10,107}$ = 1.23, *P* = 0.287, LSD = 18.16).

Table 4.6. Testing output of significant probability levels for the main effects of Cultivar, season and Fungicide, the two-way interactions, and the three-way interaction on incidence and severity of *L. maculans* leaf spotting at T2 + 4-8 weeks.

Incidence of *L. maculans* leaf spotting at T2 + 4-8 weeks.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	16.58	107	7.42	<0.001
Cultivar	5	5.87	107	10.49	<0.001
Fungicide	1	145.74	107	6.05	<0.001
Season x Cultivar	10	1.23	107	18.16	0.287
Season x Fungicide	2	16.09	107	10.49	<0.001
Cultivar x Fungicide	5	0.32	107	14.83	0.899
Cultivar x Season x Fungicide	10	1.37	107	25.69	0.211

Severity of *L. maculans* leaf spotting at T2 + 4-8 weeks.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	2.65	107	2.018	0.078
Cultivar	5	3.32	107	2.854	0.009
Fungicide	1	17.92	107	1.648	<0.001
Season x Cultivar	10	0.43	107	4.944	0.929
Season x Fungicide	2	8.04	107	2.854	<0.001
Cultivar x Fungicide	5	1.81	107	4.037	0.122
Cultivar x Season x Fungicide	10	1.03	107	6.992	0.431

Table 4.7: Incidence (% plants affected) and severity score (0-100) at reinfection T2 + 4-8 weeks from winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The six winter oilseed rape cultivars were either 'Untreated' or 'Treated' with the fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the mean incidence or severity score between the various treatment combinations in the two-way interactions.

Cultivar *	Treatment	Incidence of *L. maculans* leaf spotting at T2 + 4-8 Weeks (% plants affected)				Severity of *L. maculans* leaf spotting at T2 + 4-8 Weeks (0-100)			
		2017/18	2018/19	2019/20	Mean	2017/18	2018/19	2019/20	Mean
Barbados (7)	Untreated	46.7	66.7	36.7	30.6ab	4.7	13.7	10.7	5.4ab
	Treated	10.0	6.7	16.7		1.0	0.7	1.67	
Charger (4)	Untreated	40.0	96.7	66.7	47.8c	4.0	38.7	16.5	11.5c
	Treated	23.3	36.7	23.3		2.3	3.7	3.7	
Django (6)	Untreated	20.0	76.7	56.7	31.1ab	2.0	19.7	17.3	7.3b
	Treated	10.0	3.3	20.0		1.0	0.3	3.3	
Flamingo (4)	Untreated	26.7	76.7	70.0	39.4bc	2.7	15.7	6.5	5.6ab
	Treated	16.7	20.0	26.7		1.7	2.0	5.3	
Hunivers (7)	Untreated	33.3	66.7	73.3	40.0bc	3.3	20.3	23.7	9.2bc
	Treated	26.7	16.7	23.3		2.7	1.7	3.7	
Quartz (8)	Untreated	26.7	60.0	23.3	22.2a	2.7	7.3	2.1	2.4a
	Treated	0.0	3.3	20.0		0	0.3	2.0	
	Untreated	32.2b	73.9d	54.4c	53.5b	3.2a	19.2c	12.8b	11.75b
	Treated	14.4a	14.4a	21.7a	16.9a	1.4a	1.4a	3.3a	2.06a
		23.3a	44.2b	38.1b		2.3a	10.3b	8.0b	

*AHDB recommended list (0-9 scale with 9 being good resistance).

a-d Fisher's protected LSD post hoc tests are represented as letters. Values that do not share a letter are significantly different.
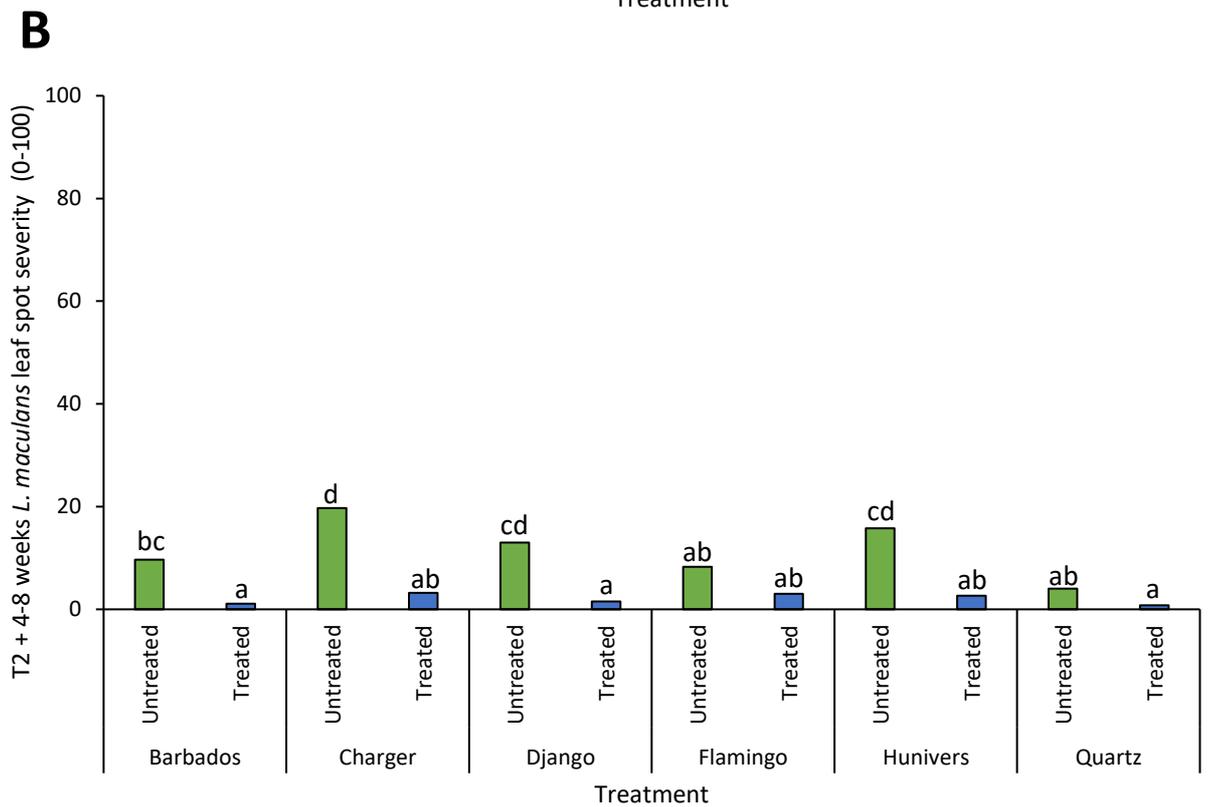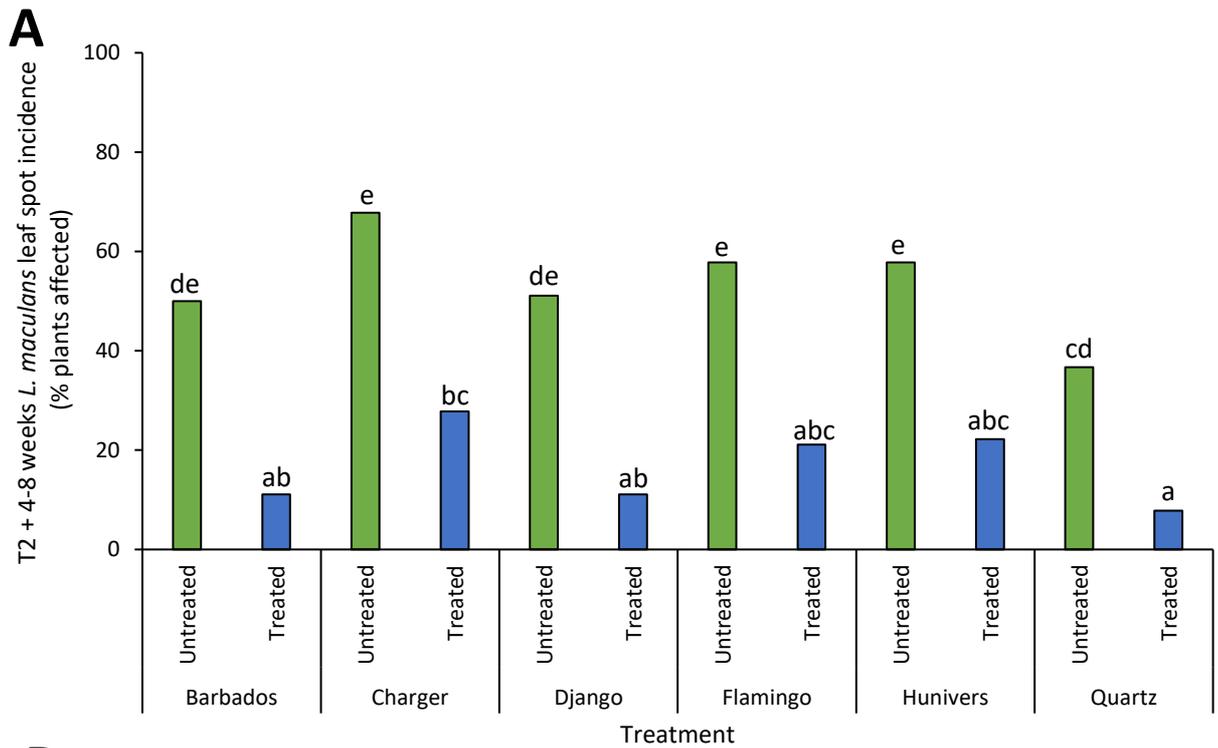
Figure 4.6: Incidence (A) and severity (B) of *L. maculans* leaf spots at T2 + 4 weeks (09 January 2019, 10 January 2019 ,20 January 2020) at Terrington St Clement during the 2017/18, 2018/19 and 2019/2020 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (107 d.f.).

There was a significant interaction between application of prothioconazole and year ($F_{2,107}$ = 16.09, P < 0.001, LSD = 7.44). The incidence of *L. maculans* lesions was smaller on the treated plots than untreated plots in all three cropping seasons. There was also a significant difference between the seasons in the mean incidence on untreated plots at the post T1 assessment. The incidence was significantly smaller in 2017/18 than in 2018/19 and 2019/20 cropping seasons; the incidence was significantly smaller in 2019/20 than in 2018/19. However, when the *L. maculans* lesion incidences on treated plots were compared, there were no significant difference between all three cropping seasons (2017/18, 2018/19 and 2019/20). There was no significant interaction between cultivar, application of fungicide and year on incidence of *L. maculans* at the post T1 assessment ($F_{10,107}$ = 1.37, P = 0.211, LSD = 25.69). Individual season analysis can be found in Appendices 15.

4.3.1.3.2.2 Severity of phoma leaf spotting at T2 + 4-8 weeks

For the growing seasons, when the overall severity of *L. maculans* leaf spotting was compared, there was a significant difference between the 2017/18 (2.3), 2018/19 (10.3) or 2019/20 (8.0) cropping seasons at the post T2 assessment ($F_{2,107}$ = 16.95, P < 0.001, LSD = 2.82) (Table 4.6 & 4.7). The severity *of L. maculans* leaf spots was significantly smaller in 2017/18 than in 2018/19 or 2019/20 cropping seasons. The *L. maculans* leaf spot severity was not significantly different between the 2018/19 and 2019/20 cropping season. There was a significant difference between cultivars ($F_{5,107}$ = 5.04, P < 0.001, LSD = 3.99) (Table 4.6 & 4.7). Cv. Quartz (2.4) had the least severe *L. maculans* lesions but it was significantly less severe only than the lesions on cvs Charger (11.5), Hunivers (9.2) and Django (7.3). Cv. Charger had significantly more severe phoma leaf spots than Cv. Django, but the phoma leaf spotting on neither cultivar was significantly different from that on cv. Hunivers. The only cultivar to have significantly different phoma leaf spotting from cvs Barbados (5.4) and Flamingo (5.6) was cv. Charger. There was a significant difference in *L. maculans* lesion severity between treated and untreated plots ($F_{1,107}$ = 70.34, P < 0.001, LSD = 2.30) (Table 4.6 & 4.7). The severity of *L. maculans* lesions was significantly greater on untreated plots (11.75) than on treated plots (2.06). There was

a significant interaction between cultivar and application of fungicide ($F_{5,107}$ = 3.08, P < 0.014, LSD = 5.64). For all cultivars, the *L. maculans* lesion severity score was significantly greater on untreated plots than on the fungicide treated plots, except for on cvs Flamingo and Quartz (Fig. 4.6).

There was a significant difference in *L. maculans* lesion severity score between treatments ($F_{11,107}$ = 5.62, P < 0.001, LSD = 7.527) (Fig. 4.6). The treatment that had the smallest severity score was treated cv. Quartz (0.8); however, the only treatments that had significantly greater severity scores were untreated cvs Barbados (9.7), Django (13.0), Hunivers (15.8) and Charger (19.7). Untreated cv. Charger had the greatest severity score; this was significantly greater than that of all treated treatments and untreated cvs Quartz (4.0), Flamingo (8.3) and Barbados (9.7). The only treatment that was significantly different from untreated cv. Flamingo (8.3) was untreated cv. Charger. There was no significant interaction between cultivar and year on *L. maculans* severity score at the post T2 assessment ($F_{10,107}$ = 1.82, P = 0.073, LSD = 6.91) (Table 4.6). For all cultivars, there was no significant difference in cultivar severity score across the three seasons. There was a significant interaction between fungicide application and year on *L. maculans* severity score ($F_{2,107}$ = 15.99, P < 0.001, LSD = 5.64) (Table 4.6). In the 2018/19 and 2019/20 cropping seasons, the untreated plots had a significantly greater severity score than treated plots. However, in the 2017/18 cropping season, there was no significant difference between untreated and treated plots. There was no significant interaction between cultivar, fungicide application and season on *L. maculans* severity score at the post T2 assessment ($F_{2,107}$ = 1.63, P = 0.114, LSD = 9.77) (Table 4.6). Individual season analysis can be found in Appendices 15.

4.3.1.4 Foliar disease incidence and severity – light leaf spot (*P. brassicae*)

4.3.1.4.1 Overall light leaf spot incidence and severity across the three cropping seasons

4.3.1.4.1.1 Overall light leaf spot incidence across the three cropping seasons

For the overall incidence across the three growing seasons, there was no significant difference in the incidence of plants with *P. brassicae* sporulation during the April assessment between cultivars ($F_{5.35}$ = 1.70, *P* = 0.140, LSD = 10.45) (Table 4.8 &4.9). However, there was an overall significant difference between treatments that received an application of prothioconazole (13.9%) and those that had not (29.1%) ($F_{1.35}$ = 25.18, *P* < 0.001, LSD = 7.39). There was a significant difference between seasons ($F_{2.35}$ = 41.53, *P* < 0.001, LSD = 6.03). The overall *P. brassicae* sporulation incidence was significantly greater in the 2018/19 (40.8%) season than in both 2017/18 (13.9%) and 2019/20 (9.7%) cropping seasons (Table 4.6 & 4.7). There was a significant interaction between cultivar and application of fungicide ($F_{5.35}$ = 3.43, *P* = 0.008, LSD = 14.78). There was a significant difference in *P. brassicae* sporulation between untreated and treated plots for cvs Charger and Flamingo, but there was no significant difference between cultivars Hunivers, Quartz, Barbados and Django (Fig. 4.7). There was a significant difference between treatments ($F_{11.35}$ = 2.00, *P* = 0.036, LSD = 22.36). The treatment that had the smallest *P. brassicae* sporulation incidence was treated cv. Charger (7.9%), but this was only significantly smaller than untreated cvs Quartz (34.4%), Flamingo (38.9%) and Charger (38.9%). These three treatments were not significantly different from each other, nor were they different from untreated cvs Hunivers (30.0%) and Barbados (17.8%), and treated cv. Barbados (23.3%). There was an interaction between cultivar and year for the *P. brassicae* sporulation ($F_{10.35}$ = 2.17, *P* = 0.029, LSD = 18.10) (Table 4.6). In all cultivars except for cv. Barbados, the disease incidence in the 2018/19 cropping season was significantly greater than in 2017/18 and 2019/20, but the incidences in these two seasons were not significantly different from each other. For cv. Barbados, there was no significant difference in incidence of *P. brassicae* sporulation between the 2017/18 and 2018/19 seasons. However, the *P. brassicae* incidence was significantly greater in 2018/19 than in 2019/20.

Table 4.6. Testing output of significant probability levels for the main effects of Cultivar, Season and Fungicide, the two-way interactions, and the three-way interaction on incidence and severity of light leaf spot on foliar leaves in Spring.

Incidence of light leaf spot

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	41.53	107	7.39	<0.001
Cultivar	5	1.70	107	10.45	0.147
Fungicide	1	25.18	107	6.03	<0.001
Season x Cultivar	10	2.17	107	18.10	0.029
Season x Fungicide	2	19.06	107	10.45	<0.001
Cultivar x Fungicide	5	3.43	107	14.78	0.008
Cultivar x Season x Fungicide	10	0.68	107	25.59	0.741

Severity of light leaf spot

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	41.52	107	7.39	<0.001
Cultivar	5	1.88	107	10.45	0.109
Fungicide	1	18.88	107	6.03	<0.001
Season x Cultivar	10	2.15	107	18.10	0.031
Season x Fungicide	2	14.67	107	10.45	<0.001
Cultivar x Fungicide	5	1.41	107	14.78	0.229
Cultivar x Season x Fungicide	10	0.37	107	25.59	0.954

Table 4.7. Incidence (% plants affected) and severity score (0-100) for spring assessment of *P. brassicae* sporulation from winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The six winter oilseed rape cultivars were either 'Untreated' or 'Treated' with the fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P* =0.05 and used to separate the mean incidence or severity score between the various treatment combinations in the two-way interactions.

Cultivar *	Treatment	Incidence of *P. brassicae* sporulation (% plants affected)				Severity of *P. brassicae* leaf sporulation (0-100)			
		2017/18	2018/19	2019/20	Mean	2017/18	2018/19	2019/20	Mean
Barbados (7)	Untreated	13.3	40.0	0.0	20.6a	0.1	0.6	0.0	0.2a
	Treated	30.0	23.3	16.7		0.3	0.2	0.2	
Charger (4)	Untreated	23.3	76.7	16.7	23.3a	0.3	1.2	0.1	0.3a
	Treated	3.3	3.3	16.7		0.0	0.0	0.2	
Django (6)	Untreated	6.7	33.3	3.3	12.8a	0.1	0.6	0.0	0.2a
	Treated	3.3	20.0	10.0		0.1	0.2	0.1	
Flamingo (7)	Untreated	26.7	86.7	3.3	27.2a	0.3	1.5	0.0	0.5a
	Treated	6.7	40.0	0.0		0.1	0.8	0.0	
Hunivers (6)	Untreated	26.7	60.0	3.3	21.7a	0.3	0.9	0.0	0.3a
	Treated	10.0	23.3	6.7		0.1	0.4	0.1	
Quartz (5)	Untreated	13.3	70.0	20.0	23.3a	0.2	1.0	0.2	0.3a
	Treated	3.3	13.3	20.0		0.0	0.3	0.2	
	Untreated	18.3bc	61.1d	7.8a	29.1b	0.2ab	1.0c	0.1a	0.4b
	Treated	9.4ab	20.6c	11.7abc	13.9a	0.1a	0.3b	0.1a	0.2a
		13.9a	40.8b	9.7a		0.2a	0.7b	0.1a	

*AHDB recommended list (0-9 scale with 9 being good resistance).

a-d Fisher's protected LSD post hoc tests are represented as letters. Values that do not share a letter are significantly different.
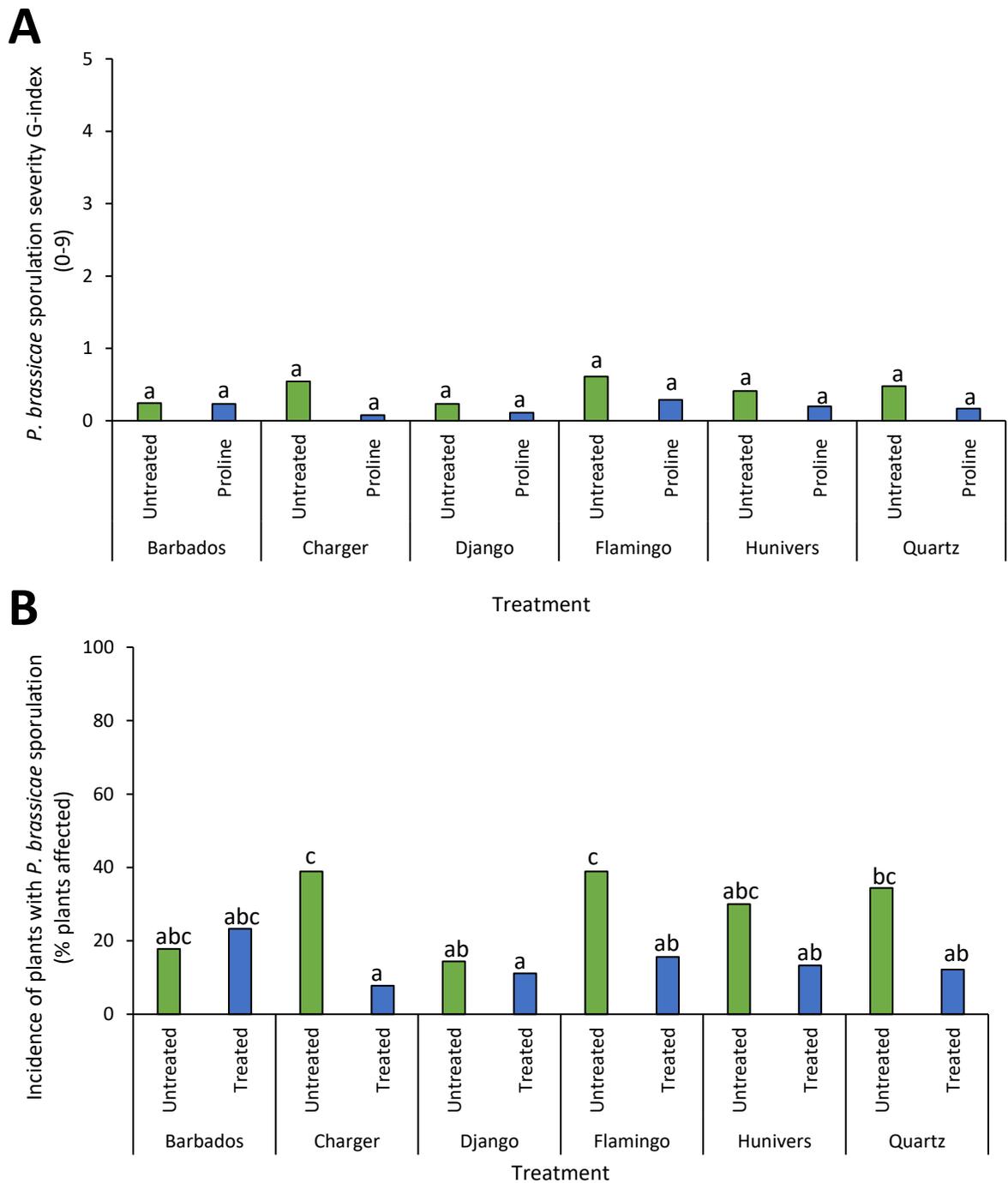
Figure 4.7: A) *P. brassicae* sporulation severity G-index (0-9) and **B)** average incidence (% of plants with symptoms) of *P. brassicae* in the field experiment (01 May 2020) at Terrington St Clement in the 2019/2020 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different P = 0.05 (107 d.f.).

There was a significant interaction between application of fungicide and the cropping season on the incidence of *P. brassicae* sporulation ($F_{2.35}$ = 19.06, *P* < 0.001, LSD = 10.45). In both 2017/18 and 2019/20 seasons, there was no significant difference in *P. brassicae* incidence between untreated and fungicide treated plots, but there was a significantly smaller *P. brassicae* incidence when a fungicide was applied than when untreated in 2018/19. There was no significant interaction between cultivar, application of fungicide and cropping season ($F_{10.35}$ = 0.68, *P* = 0.741, LSD = 25.59) (Table 4.6). Individual season analysis can be found in Appendices 16.

4.3.1.4.1.2 Overall light leaf spot severity across the three cropping seasons

For the overall severity, there were no significant differences in the severity of *P. brassicae* sporulation during the April assessment between cultivars ($F_{5.35}$ = 1.88, *P* = 0.109, LSD = 0.19) (Table 4.6 & 4.7). However, there was an overall significant difference between treatments that received an application of prothioconazole (0.18) and those that had not (0.42) ($F_{1.35}$ = 18.88, *P* < 0.001, LSD = 0.11) (Table 4.6 & 4.7). There was a significant difference between seasons ($F_{2.35}$ = 41.52, *P* < 0.001, LSD = 0.14) (Table 4.6 & 4.7). The *P. brassicae* sporulation severity was significantly greater in the 2018/19 (0.66) season than in both 2017/18 (0.15) and 2019/20 (0.09) cropping seasons. There was no significant difference in the *P. brassicae* severity between 2017/18 and 2019/20.

There was no significant interaction between cultivar and application of fungicide ($F_{5.35}$ = 1.41, *P* = 0.229, LSD = 0.27). There was no significant difference between treatments ($F_{11.35}$ = 1.47, *P* = 0.155, LSD = 0.40) (Fig. 4.7). There was an interaction between cultivar and season for the *P. brassicae* sporulation ($F_{10.35}$ = 2.15, *P* = 0.031, LSD = 0.17). In all cultivars except from cv. Barbados, the disease severity in the 2018/19 cropping season was significantly greater than in 2017/18 and 2019/20, but the incidences in these two seasons were not significantly different from each other. For cv. Barbados, there was no significant difference in *P. brassicae* sporulation severity between the 2017/18 and 2018/19 seasons; however, the *P. brassicae* incidence was significantly greater in 2018/19 than in 2019/20. There was a significant interaction between application of

fungicide and cropping season in the incidence of *P. brassicae* sporulation ($F_{2.35}$ = 19.06, *P* < 0.001, LSD = 10.45). In both 2017/18 and 2019/20 seasons, there was no significant difference in *P. brassicae* severity between untreated and fungicide treated plots, but there was a significantly smaller *P. brassicae* incidence when a fungicide was applied than when untreated. There was no significant interaction between cultivar, application of fungicide and cropping season ($F_{10.35}$ = 0.37, *P* = 0.954, LSD = 0.47). Individual season analysis can be found in Appendices 16.

4.3.1.5 Disease incidence and severity on stem

4.3.1.5.1 Incidence and severity of phoma stem basal canker

4.3.1.5.1.1 Incidence of phoma stem cankers

For the overall incidence of the three seasons, there was an overall significant difference between cultivars in the incidence of plants with phoma stem canker during the July assessment ($F_{5.107}$ = 36.80, *P* < 0.001, LSD = 9.60) (Table 4.8 & 4.9). The cultivar that had the smallest phoma stem canker incidence was cv. Barbados (25.6%). This was not significantly smaller incidence than that on cv. Quartz (37.5%) or Django (39.4%). The cultivar that had the greatest incidence was cv. Charger (79.2%); this was significantly greater than incidence on all other cultivars, except from cv. Flamingo (68.3%). The incidences for cvs Hunivers (58.9%) and Flamingo (68.3%) were not significantly different from each other. There was an overall significant difference between treatments that received an application of prothioconazole (43%) and those that had not (60%) for incidence of plants with phoma stem canker ($F_{1.107}$ = 37.01, *P* < 0.001, LSD = 5.5) (Table 4.8). There was a significant difference between seasons ($F_{2.107}$ = 5.71, *P* = 0.005, LSD = 6.8) (Table 4.8 & 4.9). The phoma stem canker incidence in 2017/18 was not significantly different between 2018/19 or 2019/20. There was no significant interaction between cultivar and application of fungicide ($F_{5.107}$ = 5.71, *P* = 0.572, LSD = 13.60) (Table 4.8). However, there was a significant difference between treatments ($F_{11.107}$ = 18.22, *P* < 0.001, LSD = 14.3) (Fig. 4.8). The treatment that had the smallest incidence was treated cv. Barbados (18.3%); this was significantly smaller than all other

treatments except for untreated cv. Barbados (32.8%), and treated cvs Django (28.3%) and Quartz (31.7%).

Table 4.8. Testing output of significant probability levels for the main effects of Cultivar, Season and Fungicide, the two-way interactions, and the three-way interaction on incidence and severity of phoma stem cankers.

Phoma stem canker Incidence

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	5.71	107	6.8	0.005
Cultivar	5	36.80	107	9.6	<0.001
Fungicide	1	37.01	107	5.5	< 0.001
Season x Cultivar	10	1.65	107	16.6	0.110
Season x Fungicide	2	0.97	107	9.6	0.383
Cultivar x Fungicide	5	0.77	107	13.5	0.572
Cultivar x Season x Fungicide	10	0.59	107	23.4	0.818

Phoma stem canker severity

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	13.50	107	0.18	<0.01
Cultivar	5	38.75	107	0.26	<0.01
Fungicide	1	61.62	107	0.15	<0.01
Season x Cultivar	10	2.88	107	0.44	<0.01
Season x Fungicide	2	7.04	107	0.26	<0.01
Cultivar x Fungicide	5	3.52	107	0.36	<0.01
Cultivar x Season x Fungicide	10	1.35	107	0.63	0.22

Table 4.9. Incidence (% plants affected) and severity score (0-9) for phoma stem cankers from winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The six winter oilseed rape cultivars were either 'Untreated' or 'Treated' with the fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the mean incidence or severity score between the various treatment combinations in the two-way interactions.

Cultivar[*]	Treatment	Incidence of phoma stem cankers (%)				Severity of phoma (0-7)			
		2017/18	2018/19	2019/20		2017/18	2018/19	2019/20	
Barbados (7)	Untreated	33	33	32	26a	0.683	0.417	0.467	0.389a
	Treated	18	10	27		0.250	0.117	0.400	
Charger (4)	Untreated	98	87	87	79c	3.783	2.017	1.583	1.867c
	Treated	70	57	77		1.533	1.083	1.200	
Django (6)	Untreated	55	45	52	39a	1.183	0.567	0.900	0.628a
	Treated	23	18	43		0.350	0.217	0.550	
Flamingo (4)	Untreated	65	82	90	68bc	1.883	1.650	2.017	1.494c
	Treated	43	63	67		1.033	1.217	1.167	
Hunivers (7)	Untreated	70	48	72	59b	1.700	0.800	1.017	1.019b
	Treated	53	50	60		0.833	0.900	0.867	
Quartz (8)	Untreated	50	37	43	38a	1.367	0.717	0.833	0.728ab
	Treated	47	15	33		0.783	0.233	0.433	
	Untreated	62c	55bc	62c	60b	1.767c	1.028b	1.136b	1.310b
	Treated	42ab	36a	51bc	43a	0.797ab	0.628a	0.769ab	0.731a
		52ab	45a	57b		1.28b	0.83a	0.95a	

*AHDB recommended list (0-9 scale with 9 being good resistance).

[a-d] Fisher's protected LSD post hoc tests are represented as letters. Values that do not share a letter are significantly different.
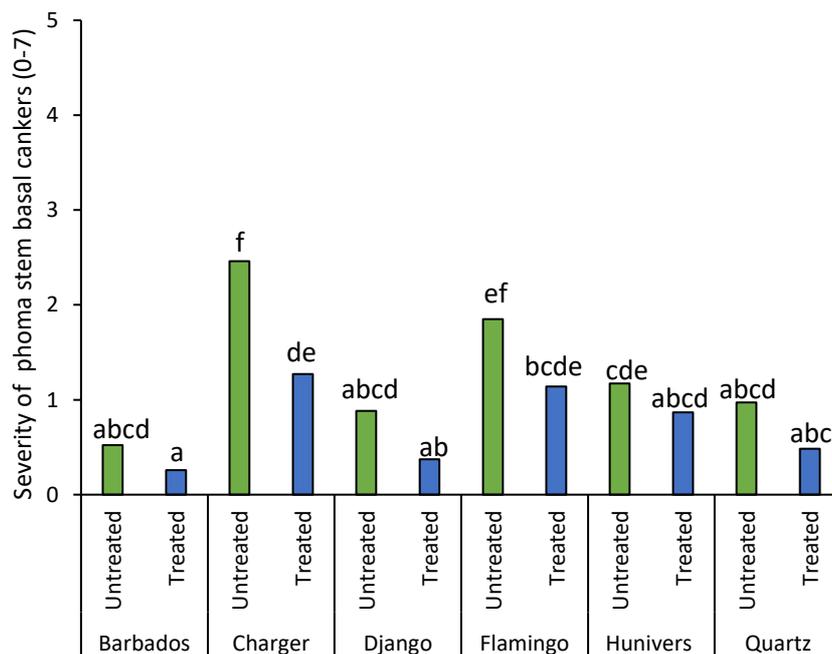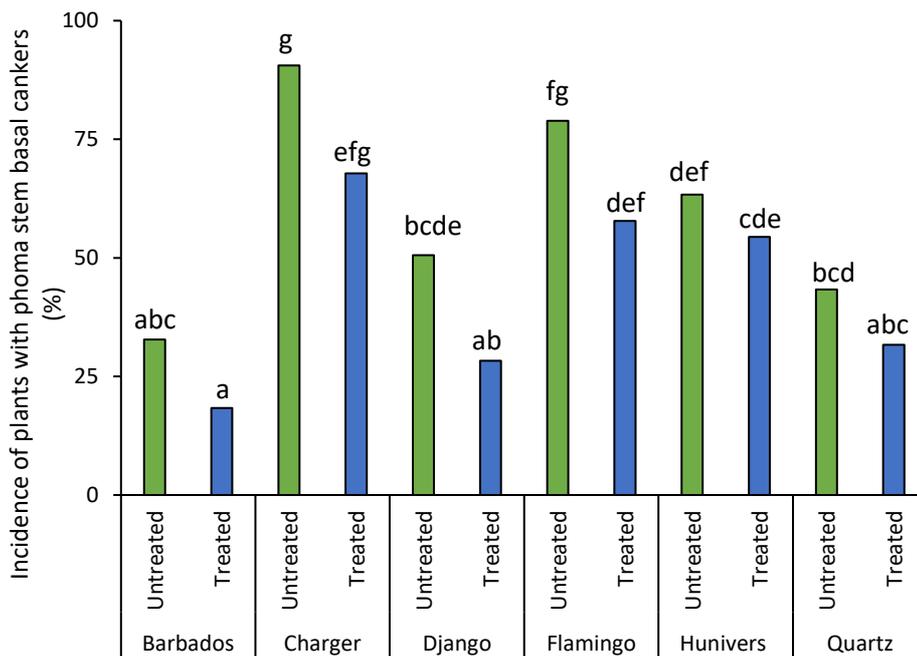
Figure 4.8: Incidence and severity of phoma stem canker at the stem base in field experiment at Terrington St Clement, Norfolk for all three cropping seasons (2017/18, 2018/19 and 2019/2020). Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (107 d.f.).

The treatment that had the greatest incidence score was untreated cv. Charger (90.6%); this was significantly greater than all other treatments except for untreated cv. Flamingo (78.9%) and treated cv. Charger (67.8%). For all cultivars, there was no significant difference in incidence between treated and untreated plots (Fig. 4.8). There was no interaction between cultivar and year for phoma stem canker incidence ($F_{10.107} = 1.65$, $P = 0.110$, LSD = 16.6). There was no significant interaction between the application of fungicide and the season for the incidence of phoma stem canker ($F_{2.107} = 0.97$, $P = 0.383$, LSD = 9.6). There was no significant interaction between cultivar, application of fungicide and cropping season ($F_{10.107} = 0.59$, $P = 0.818$, LSD = 23.4). Individual season analysis can be found in Appendices 17.

4.3.1.5.1.2 Severity of phoma stem cankers

For the overall severity, there was a significant difference between cultivars in the severity of phoma stem basal cankers during the July assessment ($F_{5.107} = 38.75$, $P < 0.001$, LSD = 0.29) (Table 4.8 & 4.9). The cultivar that had the smallest phoma stem canker severity score was cv. Barbados (0.39), but this was not significantly different only from cvs Django (0.63) and Quartz (0.73). The severity score on cv. Quartz was not significantly different from that on cv. Hunivers (1.02). The severity score on cvs Charger (1.87) and Flamingo (1.49) were significantly greater than all other cultivars, but they were not significantly different from each other. There was an overall significant difference between treatments that received an application of prothioconazole and those that did not ($F_{1.107} = 61.14$, $P < 0.01$, LSD = 0.15) (Table 4.8 & 4.9); untreated plots had a significantly greater severity score (1.3) than treated plots (0.7). There was a significant difference in phoma severity between cropping seasons ($F_{2.107} = 13.40$, $P < 0.01$, LSD = 0.18). The *L. maculans* stem canker severity was significantly greater in the 2017/18 (1.3) season than in 2018/19 (0.8) or 2019/20 (1.0) cropping seasons but the severity scores in 2018/19 and 2019/20 were not significantly different from each other. There was a significant interaction between cultivar and application of fungicide ($F_{5.107} = 3.50$, $P < 0.01$, LSD = 0.42) (Table 4.8). For all cultivars, except for cvs Charger and Flamingo there was not a significant difference between untreated and treated plots. There was a significant difference between treatments ($F_{11.107} = 15.28$, $P < 0.001$, LSD =

0.46) (Fig. 4.8). The treatment that had the smallest phoma stem canker severity score was treated cv. Barbados (0.26), but this was only significantly different from untreated and treated cvs Charger and Flamingo, and untreated cv. Hunivers. The treatment that had the greatest phoma stem canker severity score was untreated cv. Charger (2.46); this was significantly greater than that for all other treatments, except from untreated cv. Flamingo. There was an interaction between cultivar and cropping season for *L. maculans* stem canker incidence ($F_{10.107} = 4.44$, $P < 0.01$, LSD = 0.56) (Table 4.8). In all cultivars, except for cvs Charger, the phoma severity score was not significantly different between cropping seasons. In cv Charger the severity of stem canker score was significantly greater in the 2017/18 cropping season than in 2018/19 or 2019/20, but the severity scores in these two cropping seasons were not significantly different from each other.

There were no significant interactions between resistance × fungicide × cropping season(P=0.78), nor was there a significant interaction between resistance × cropping season (P=0.52) when the cultivars were split into the three resistance groups. There were significant interactions between resistance, fungicide and cropping season and the effects of the other two two-way interactions of resistance × fungicide and fungicide × cropping season (P<0.05). In all three resistance groups the application of prothioconazole resulted in a significantly smaller mean stem canker score when compared to the untreated comparison. However, there was no significant difference between the severity of stem canker score on "Good" and "Medium" resistant groups that had received an application of prothioconazole.

There was a significant interaction between application of fungicide and the cropping season for severity of phoma stem canker ($F_{2.107} = 10.51$, $P < 0.001$, LSD = 0.32) (Table 4.8 & 4.9). In both 2017/18 and 2018/19 seasons, there was a significant difference in phoma stem canker severity score between untreated and fungicide treated treatments, but there was a significantly smaller disease severity in 2019/20 season when a fungicide was applied than when untreated. There was no significant interaction between cultivar, application of fungicide and cropping season ($F_{10.107} = 0.63$, $P = 0.2$, LSD = 0.63). Individual season analysis can be found in Appendices 17.
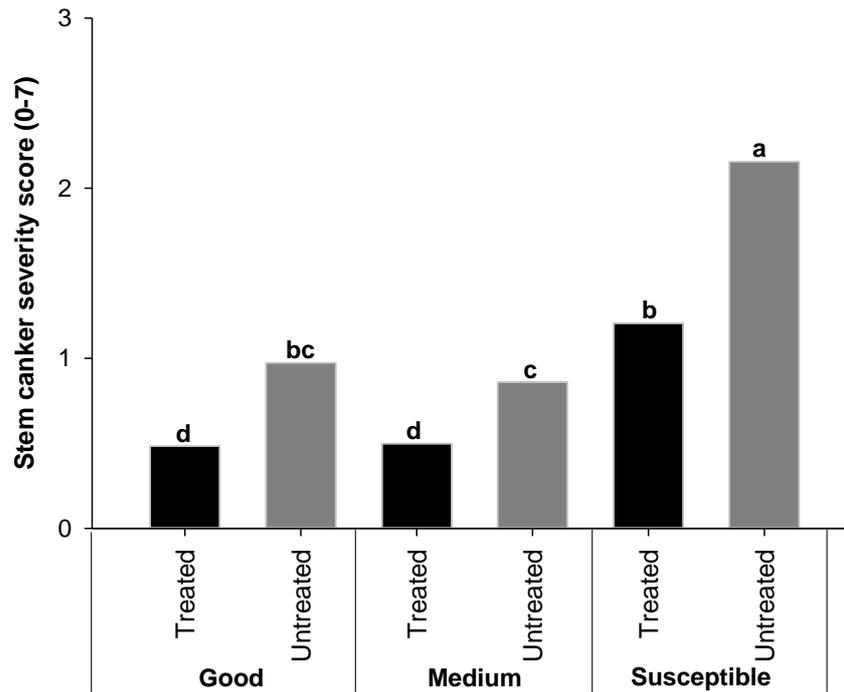
Figure 4.9: Mean severity of phoma stem canker of different fungicide treatments within resistance groups from winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The resistance group "Good" contained cv Quartz, "Medium" contained cvs Barbados, Django and Hunivers, and "Susceptible" contained cvs Charger and Flamingo. They were either 'Untreated' or 'Treated' with fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the mean stem canker score between different treatment combinations in the two-way interaction of resistance × fungicide. Bars that do not share a common letter are significantly different at *P* =0.05 (Fortune *et al.* 2021).

4.3.1.5.2 Light leaf spot disease on stems

4.3.1.5.2.1 Incidence of light leaf spot disease on stems

For the three growing seasons, there was an overall significant difference in the incidence of *P. brassicae* stem lesions during the July assessment between cultivars ($F_{5.107}$ = 3.42, *P* = 0.008 LSD = 10.91) (Table 4.10). The cultivar that had the smallest *P. brassicae* stem lesion incidence was cv. Barbados (39.7%). All other cultivars were not significantly different from cvs Barbados and Charger. There was an overall significant difference between treatments that received an application of prothioconazole or not ($F_{1.107}$ = 7.59, *P* = 0.007, LSD = 6.3); untreated plots had a significantly greater incidence (55.3%) than treated plots (46.6%). There was a significant difference in *P. brassicae* stem lesion incidence between cropping seasons ($F_{2.107}$ = 127.43, *P* < 0.001, LSD = 7.7). The *P. brassicae* stem lesion incidence was significantly greater in the 2018/19 (85.4%) season than in 2019/20 (41.5%) and 2017/18 (25.8%) cropping seasons; the *P. brassicae* stem lesion incidence was significantly greater in 2019/20 than in the 2017/18 cropping season.

There was no significant interaction between cultivar and application of fungicide for incidence ($F_{5.107}$ = 0.58, *P* = 0.718, LSD = 15.4). There was no significant difference between treatments ($F_{11.107}$ = 0.64, *P* = 0.791, LSD = 30.4). There was an interaction between cultivar and cropping season on *P. brassicae* stem lesion incidence ($F_{10.107}$ = 3.89, *P* < 0.001, LSD = 18.9). For cvs Barbados, Django and Hunivers there were no significant differences in light leaf spot incidence between 2017/18 and 2019/20 cropping seasons and the light leaf spot incidence in 2018/19 was significantly greater. For cvs Quartz and Charger, there were a significant difference in incidence of light leaf spot on stem between 2017/18 and 2018/19 cropping seasons but the incidence in 2019/20 was not significantly different than in 2017/18 and 2018/19. For cv. Flamingo, there was no significant difference in light leaf spot stem incidence between the 2018/19 and 2019/20 cropping seasons, but both were significantly greater than the light leaf spot stem incidence in 2017/18.

Table 4.10. Testing output of significant probability levels for the main effects of Cultivar, Season and Fungicide, the two-way interactions, and the three-way interaction on incidence and severity of light leaf spot on the stem.

Incidence of light leaf spot

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	127.43	107	7.713	<0.001
Cultivar	5	3.42	107	10.907	0.008
Fungicide	1	7.59	107	6.297	0.007
Season x Cultivar	10	3.89	107	18.892	<0.001
Season x Fungicide	2	2.25	107	10.907	0.113
Cultivar x Fungicide	5	0.58	107	15.425	0.718
Cultivar x Season x Fungicide	10	0.62	107	26.718	0.793

Severity of light leaf spot

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	80.94	107	0.527	< 0.001
Cultivar	5	5.26	107	0.745	< 0.001
Fungicide	1	2.00	107	0.430	0.162
Season x Cultivar	10	5.28	107	1.291	<0.001
Season x Fungicide	2	0.50	107	0.745	0.607
Cultivar x Fungicide	5	0.62	107	1.054	0.687
Cultivar x Season x Fungicide	10	0.24	107	1.825	0.991

Table 4.11 Incidence (% plants affected) and severity score (0-9) for *P. brassicae* stem lesions from winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The six winter oilseed rape cultivars were either 'Untreated' or 'Treated' with the fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the mean incidence or severity score between the various treatment combinations in the two-way interactions.

Cultivar *	Treatment	Incidence of *P. brassicae* stem lesions (%)				Severity of *P. brassicae* stem lesions (0-9)			
		2017/18	2018/19	2019/20		2017/18	2018/19	2019/20	
Barbados (7)	Untreated	25.0	98.3	8.3	39.7a	0.4	4.0	0.7	1.5a
	Treated	13.3	83.3	10.0		0.1	2.7	0.8	
Charger (4)	Untreated	35.0	90.0	65.0	60.3b	0.7	3.2	5.8	3.1c
	Treated	28.3	78.3	65.0		0.3	3.2	5.5	
Django (6)	Untreated	36.7	85.0	33.3	48.0ab	0.5	2.9	2.6	1.8ab
	Treated	35.0	76.7	21.7		0.6	2.3	1.9	
Flamingo (7)	Untreated	16.7	83.3	45.0	48.3ab	0.2	2.3	3.7	2.3abc
	Treated	10.0	80.0	55.0		0.1	2.8	4.8	
Hunivers (6)	Untreated	46.7	93.3	43.3	53.9ab	0.6	3.6	3.7	2.3abc
	Treated	15.0	85.0	40.0		0.3	2.5	3.1	
Quartz (5)	Untreated	45.0	90.0	55.0	55.3bc	0.8	3.6	4.8	2.8bc
	Treated	3.3	81.7	56.7		0.0	2.7	4.8	
	Untreated	34.2b	90.0c	41.7b	55.28b	0.5a	3.3b	3.6b	2.5b
	Treated	17.5a	80.8c	41.4b	46.57a	0.2a	2.7b	3.5b	2.1a
		25.8a	85.4c	41.5b		0.4a	3.0b	3.5b	

*AHDB recommended list (0-9 scale with 9 being good resistance).

a-d Fisher's protected LSD post hoc tests are represented as letters. Values that do not share a letter are significantly different.

There was no significant interaction in incidence of *P. brassicae* stem lesions between the application of fungicide and the cropping season ($F_{2.107}$ = 2.25, *P* = 0.113, LSD = 10.91). There was no significant interaction between cultivar, application of fungicide and cropping season for the *P. brassicae* stem lesion incidence ($F_{10.35}$ = 1.53, *P* = 0.146, LSD = 2.15). Individual season analysis can be found in Appendices 18.

4.3.1.5.2.2   Severity of light leaf spot disease on stems

For the severity on stems over the three seasons, there was an overall significant difference in the severity of *P. brassicae* stem lesions during the July assessment between cultivars ($F_{5.107}$ = 5.26, *P* < 0.001, LSD = 0.75) (Table 4.10). The cultivar that had the smallest *P. brassicae* stem lesion incidence was cv. Barbados (1.5), but this severity score was not significantly different from all other cultivars, except for cv Quartz (2.8) and Charger (3.1). However, the light leaf psot severity of cv. Quartz was only significantly different from cv. Barbados. The light leaf spot severity on Cvs Hunivers and Flamingo were not significantly different from any other cultivar. There was no overall significant difference between cultivars that did or did not receive an application of prothioconazole ($F_{1.107}$ = 2.00, *P* = 0.040, LSD = 0.13). There was a significant difference in *P. brassicae* stem lesion severity between cropping seasons ($F_{2.107}$ = 80.94, *P* < 0.001, LSD = 0.527). The *P. brassicae* stem lesion severity was significantly smaller in 2017/18 (0.38) season than in 2019/20 (3.0) and 2018/19 (3.5) cropping seasons; the *P. brassicae* stem lesion severity was not significantly different between the 2018/19 and 2019/20 cropping seasons. There was no significant interaction between cultivar and application of fungicide ($F_{5.107}$ = 0.62, *P* = 0.687, LSD = 1.05). There was no significant difference in *P. brassicae* stem lesion severity score between treatments ($F_{11.107}$ = 0.94, *P* = 0.503, LSD = 1.82). There was an interaction between cultivar and cropping season for *P. brassicae* stem lesion severity ($F_{10.107}$ = 5.28, *P* < 0.001, LSD = 1.3). For cv. Django there was no significant difference in severity across different seasons. For cvs Quartz and Hunivers, the light leaf spot stem severity scores were significantly smaller in 2017/18 than in both 2018/19 or 2019/20; there was no significant difference between the 2018/19 or

2019/20 seasons. For cv. Charger, the light leaf spot stem severity was significantly smaller in 2017/19 than in 2018/19 or 2019/20. The severity score was significantly greater in 2019/20 than in 2018/19. For cv. Flamingo, there was no significant difference between the light leaf spot severity scores in 2017/18 and 2019/20, and the severity score was significantly smaller than in the 2018/19 cropping season. However, there was no significant difference in the severity score between the 2018/19 and 2019/20 cropping season. For cv. Barbados, there was no significant difference in the light leaf spot stem lesion severity between the 2017/18 and 2019/20 cropping season. The stem lesion severity was significantly greater in the 2018/19 than in the other two seasons. There was no significant interaction between the application of fungicide and the cropping season for the severity of *P. brassicae* stem lesions ($F_{2.107}$ = 0.5, *P* = 0.607, LSD = 0.75). There was no significant interaction between cultivar, application of fungicide and cropping season on the *P. brassicae* stem lesion incidence ($F_{10.107}$ = 1.83, *P* = 0. 991, LSD = 1.83). Individual season analysis can be found in Appendices 18.

4.3.1.6   Yield

4.3.1.6.1   **Overall of three growing seasons**

For the overall yield of the three seasons, there was an significant difference in yield between cultivars ($F_{5.86}$ = 5.69, *P* < 0.001, LSD = 0.22) (Table 4.12 & 4.13). The cultivar that had the smallest yield was cv. Flamingo (3.70t/ha), but this yield was only significantly less than that of cvs. Quartz (4.07 t/ha) and Django (4.25t/ha). The cultivar that had the greatest yield was cv. Django (4.25 t/ha); this was only significantly greater than the yield of cvs. Flamingo and Charger (3.87 t/ha). The yields of cvs Barbados (3.99t/ha) and Hunivers (4.00 t/ha) were not significantly different from all other cultivars. There was an overall significant difference between treatments that did or did not receive an application of prothioconazole ($F_{1.86}$ = 51.16, *P* < 0.001, LSD = 0.13); untreated plots (3.75 t/ha) had a significantly smaller yield than treated plots (4.21 t/ha) (Table 4.12 & 4.13). There was an average yield benefit of 0.46 t/ha.

Table 4.12. Testing output of significant probability levels for the main effects of Cultivar, Season and Fungicide, the two-way interactions, and the three-way interaction on yield.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	148.57	86	0.157	< 0.001
Cultivar	5	5.69	86	0.222	< 0.001
Fungicide	1	51.16	86	0.128	<0.001
Season x Cultivar	10	9.63	86	0.385	<0.001
Season x Fungicide	2	2.27	86	0.222	0.114
Cultivar x Fungicide	5	2.58	86	0.315	0.037
Cultivar x Season x Fungicide	10	0.89	86	0.545	0.551

Table 4.13. Yield for winter oilseed rape field experiments in the 2017/18, 2018/2019 and 2019/20 cropping seasons. The six winter oilseed rape cultivars were either 'Untreated' or 'Treated' with the fungicide prothioconazole. Least Significant Differences (LSD) were calculated at *P* =0.05 and used to separate yield between the various treatment combinations in the two-way interactions.

Cultivar *	Treatment	Yield (t/ha)			
		2017/18	2018/19	2019/20	Mean
Barbados	Untreated	4.98	3.94	2.77	3.99a**bc**
	Treated	5.51	3.93	2.83	
Charger	Untreated	4.39	3.28	2.61	3.87**ab**
	Treated	5.18	3.76	4.02	
Django	Untreated	4.40	4.01	3.81	4.25**c**
	Treated	4.85	4.23	4.20	
Flamingo	Untreated	4.37	3.38	2.94	3.70**a**
	Treated	4.52	3.73	3.24	
Hunivers	Untreated	4.18	3.37	3.61	4.00a**bc**
	Treated	4.46	3.91	4.45	
Quartz	Untreated	4.84	3.31	3.33	4.07**bc**
	Treated	5.33	3.52	4.11	
	Untreated	4.53**c**	3.55**b**	3.18**a**	3.75**b**
	Treated	4.98**d**	3.84**b**	3.81**b**	4.21**a**
		4.75**c**	3.70**b**	3.49**a**	

*AHDB recommended list (0-9 scale with 9 being good resistance).

[a-d] Fisher's protected LSD post hoc tests are represented as letters. Values that do not share a letter are significantly different.

There was a significant difference in yield between cropping seasons ($F_{2.86}$ = 148.57, P < 0.001, LSD = 0.08). The yield was significantly smaller in the 2019/20 (3.50 t/ha) season than in the 2018/19 (3.70 t/ha) and 2017/18 (4.75 t/ha) cropping seasons; the yield was significantly different between the 2018/19 and 2019/20 cropping seasons (Table 4.12 & 4.13).

There was a significant interaction between cultivar and application of fungicide on yield ($F_{5.86}$ = 2.58, P = 0.037, LSD = 0.32). For all cultivars, except for cvs Charger and Hunivers, there was no significant increase in yield in the treated plots compared to the untreated plots. There was no significant difference in yield between treatments ($F_{11.86}$ = 1.45, P < 0.169, LSD = 0.695). There was an interaction between cultivar and cropping season for yield ($F_{10.86}$ = 9.63, P < 0.001, LSD = 0.39). For cvs Flamingo, Charger and Quartz, the yield was greater in 2017/18 than in both 2018/19 and 2019/20 seasons; there was no significant difference between the yield in 2018/19 and 2019/20. For cvs Hunivers and Django there was no significant difference in yield between seasons. For cv. Barbados the yield was greatest in 2017/18; this was significantly greater than in the other two seasons. However, the yield in 2018/19 was significantly greater than in 2017/18. There was no significant interaction between application of fungicide and cropping season on yield ($F_{2.86}$ = 2.27, P = 0.114, LSD = 0.22). Individual season analysis can be found in Appendices 19.

4.3.1.7 *L. maculans* incidence or severity against yield

There was no correlation between untreated and treated mean incidence of *L. maculans* leaf spotting at T1 + 4-8 weeks and yield (2017/18 – Untreated R^2= 0.32, Treated - R^2= 0.01; 2018/19 – Untreated R^2= 0.19, Treated R^2= 0.39; 2019/20 – Untreated R^2= 0.02, Treated R^2= 0.22). There was no strong correlation between untreated and treated mean severity of *L. maculans* leaf spotting severity at T1 + 4-8 weeks and yield (2017/18 – Untreated R^2 = 0.06, Treated - R^2= 0.00; 2018/19 – Untreated R^2= 0.21, Treated R^2= 0.17; 2019/20 – Untreated R^2= 0. 05, Treated R^2= 0.22).

There was no strong correlation between untreated or treated mean incidence of *L. maculans* leaf spotting at T2 + 4-8 weeks and yield (2017/18 – Untreated R^2= 0.14, Treated - R^2= 0.35; 2018/19 – Untreated R^2= 0.03, Treated R^2= 0.05; 2019/20 – Untreated R^2= 0.05, Treated R^2= 0.08). There was no strong correlation between untreated or treated mean severity of *L. maculans* leaf spotting severity at T2 + 4-8 weeks and yield (2017/18 – Untreated R^2= 0.14, Treated - R^2= 0.05; 2018/19 – Untreated R^2= 0.05, Treated R^2= 0.05; 2019/20 – Untreated R^2= 0. 11, Treated R^2= 0.01).

There was no strong correlation between untreated or treated mean incidence of phoma stem basal canker and yield. However, there was a negative correlation between the incidence of phoma stem basal canker on untreated plots against yield in the 2017/18 cropping season (2017/18 – Untreated R^2= 0.50, Treated - R^2= 0.05; 2018/19 – Untreated R^2= 0.28, Treated R^2= 0.05; 2019/20 – Untreated R^2= 0.05, Treated R^2= 0.08). There was no strong correlation between untreated or treated mean severity of phoma stem canker severity and yield (2017/18 – Untreated R^2= 0.24, Treated - R^2= 0.05; 2018/19 – Untreated R^2= 0.43, Treated R^2= 0.07; 2019/20 – Untreated R^2= 0. 10, Treated R^2= 0.01).

4.3.1.8 *P. brassicae* foliar sporulation against yield

There were differences in the correlation with yield between treated or untreated *P. brassicae* incidence in the 2017/18 and 2018/19 cropping seasons. For untreated plots, there was a small negative correlation between foliar *P. brassicae* sporulation in April and untreated yield in 2017/18, and a stronger negative correlation in 2018/19 (R^2= 0.31 and R^2= 0.82, respectively) (Fig. 4.10) However, there was a much stronger negative correlation between untreated *P. brassicae* sporulation and yield when the data from both 2017/18 and 2018/19 cropping seasons were combined (R^2 = 0.86). For treated samples, there was a no correlation between foliar *P. brassicae* sporulation in April and untreated yield in both 2017/18 and 2018/19 (R^2= 0.17 and R^2= 0.01). There was no correlation between untreated and treated *P. brassicae* incidence in the 2019/20 cropping season (R^2= 0.03 and R^2= 0.00, respectively).
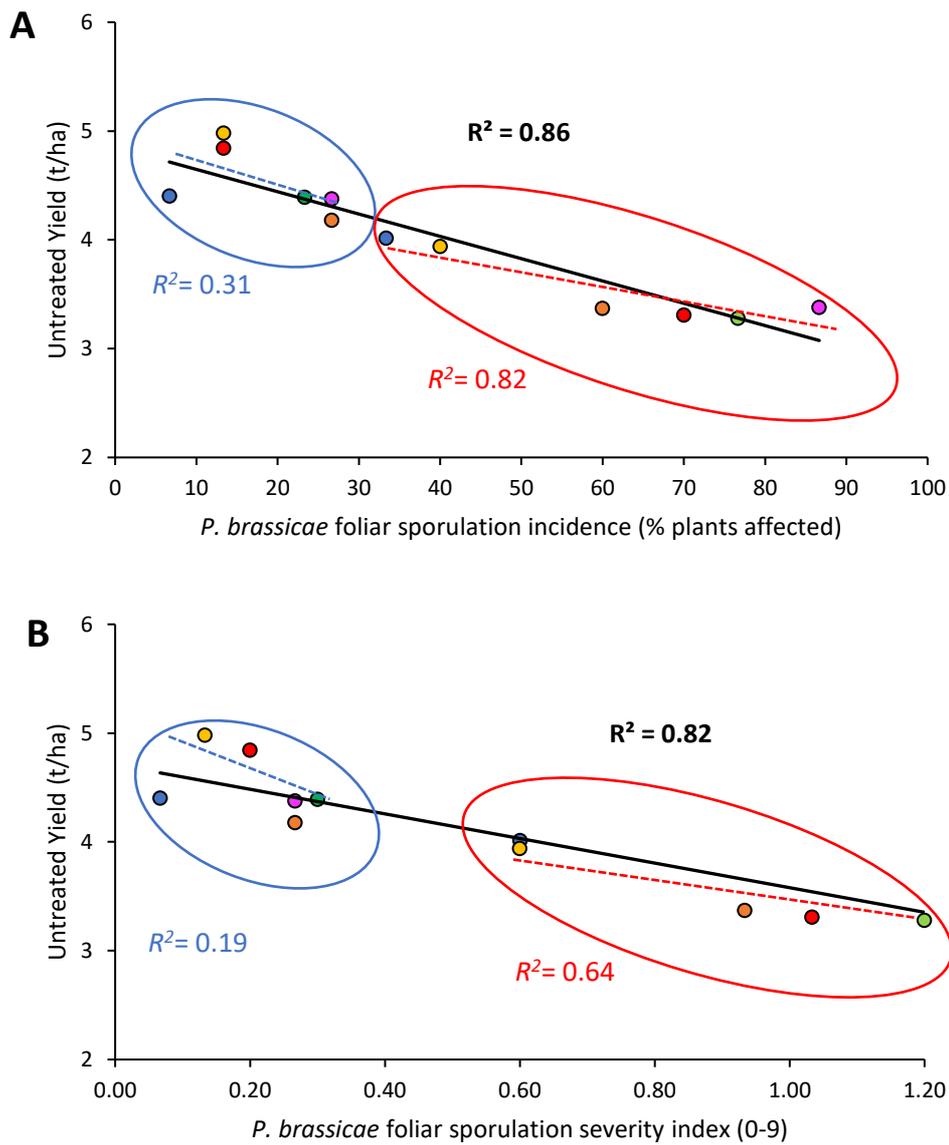
Figure 4.10: A) *P. brassicae* **sporulation incidence (% plants affected) and B) severity index (0-9) were plotted against untreated yield from 2017/18 (Blue circle) and 2018/19 (Red circle). A simple regression was applied for each year individually in addition to a combined simple regression line. Red = Quartz, Pink = Flamingo, Yellow = Barbados, Green = Charger, Blue = Django and Orange = Hunivers.**

For the April *P. brassicae* sporulation severity score in the 2017/18 and 2018/19 cropping seasons, there was no correlation between untreated severity and untreated yield in the 2017/18 cropping season. However, there was a strong negative correlation between untreated *P. brassicae* severity and yield in the 2018/19 cropping season (R^2= 0.19 and R^2= 0.64). When the data points for untreated *P. brassicae* severity score and yield from both cropping seasons were combined, there was a negative correlation (R^2= 0.82) (Fig. 4.10). There was no correlation between treated severity score and treated yield in the same cropping seasons (R^2= 0.17 and R^2= 0.04). There was no correlation between untreated or treated severity score in the 2019/20 cropping season (Untreated R^2= 0.03, Treated R^2= 0.00).

4.3.2 Monitoring patterns of release of *Leptosphaeria* spp. and *P. brassicae* air-borne ascospores

4.3.2.1 Numbers and patterns of ascospore release

Only *Leptosphaeria* spp. ascospores were counted using a microscope. However, qPCR data analysis was done for *L. maculans*, *L. biglobosa* and *P. brassicae*.

4.3.2.1.1 **The 2017/18 season**

The Burkard spore sampler indicated that *Leptosphaeria* spp. ascospores were released from early September 2017 until February 2018 (Fig. 4.11). The pattern of ascospore release was very variable with maxima being recorded intermittently between 29 September and 28 January, with a period of more frequent spore release between 4 November and 7 December. The first major ascospore release (>11 ascospores/m^3) was on the 28 September 2017. The largest maximum was recorded on 3 December 2017, with 144 spores/ m^3. Ascospore release events occurred on the 29 September 9, 18 and 23 October before the first prothioconazole application (T1) was made on the 25 October 2017.

4.3.2.1.2 **The 2018/19 season**

The Burkard spore sampler indicated that *Leptosphaeria* spp. ascospores were released from early October 2018 until March 2019 (Fig. 4.11). The pattern of ascospore release was very consistent, but at a relatively small concentration. The first major maximum was observed on 22 October 2018. The largest maximum was recorded on 3 December 2018 with 32.2 ascospores/ m^3. The first application of prothioconazole was applied (16 November) before any major maxima were recorded (Fig. 4.12).

4.3.2.1.3 **The 2019/20 season**

The Burkard spore sampler indicated that *Leptosphaeria* spp. ascospores were released from late September 2019 until February 2020 (Fig. 4.11). The pattern of ascospore release showed that there was a period of more frequent spore release between 15 October and 27 November. The timing of the first major ascospore release (>11 ascospores/m^3) was on the 15 October 2017. The largest maximum was recorded on 24 November 2019 with 145 spores/ m^3. Ascospore release events occurred before the first prothioconazole application (T1) was made on the 7 November 2019, but it provided protection against the two largest ascospore release events.

4.3.2.2 qPCR Analysis

4.3.2.2.1 **Summary**

The amounts of ascospore release were different in each of the three seasons for *L. maculans*, *L. biglobosa* and *P. brassicae* (Fig. 4.12 – 4.14). There was a significant difference in average daily detected *L. maculans* DNA between seasons ($F_{2.303}$ = 24.14, *P* < 0.001, LSD = 81.7). The greatest average daily detected *L. maculans* DNA was in the 2017/18 (297 pg) cropping season; this was significantly greater than in 2018/19 (85 pg) or 2019/20 (18 pg) cropping seasons. Although the mean amount of daily detected *L. maculans* DNA was greater in 2019/20 than in 2018/19, there was no significant difference between them.
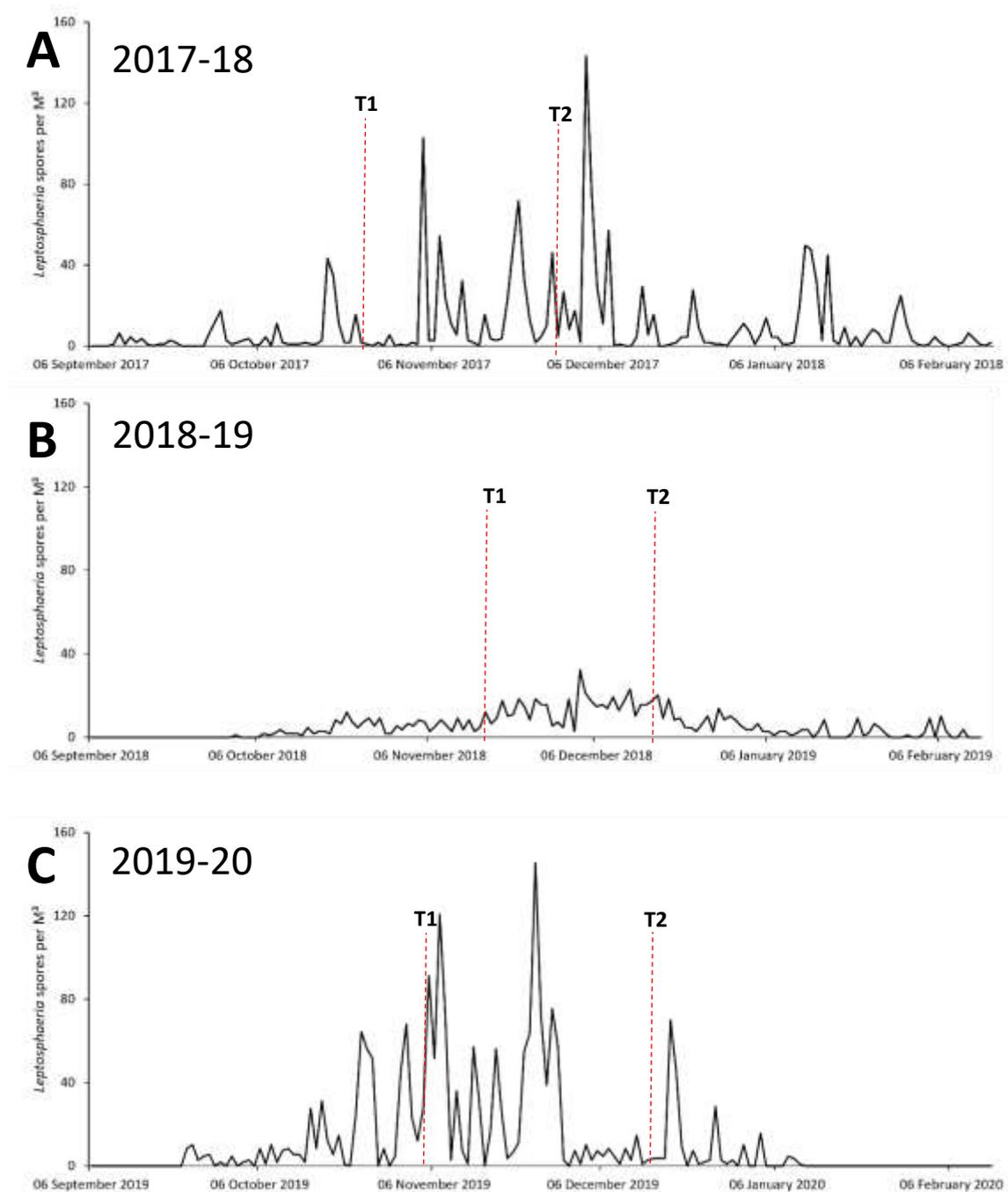
Figure 4.11: Daily release of *Leptosphaeria* spp. ascospores per m³ of air at Terrington St Clement between September and March for 2017/18 (A), 2018/19 (B) and 2019/20 cropping seasons. The early application of prothioconazole was when >10% of plants had phoma leaf spots caused by *L. maculans* (T1) and the second application of prothioconazole when *L. maculans* re-infected the crop (T2), these dates are overlaid on the ascospore release data.
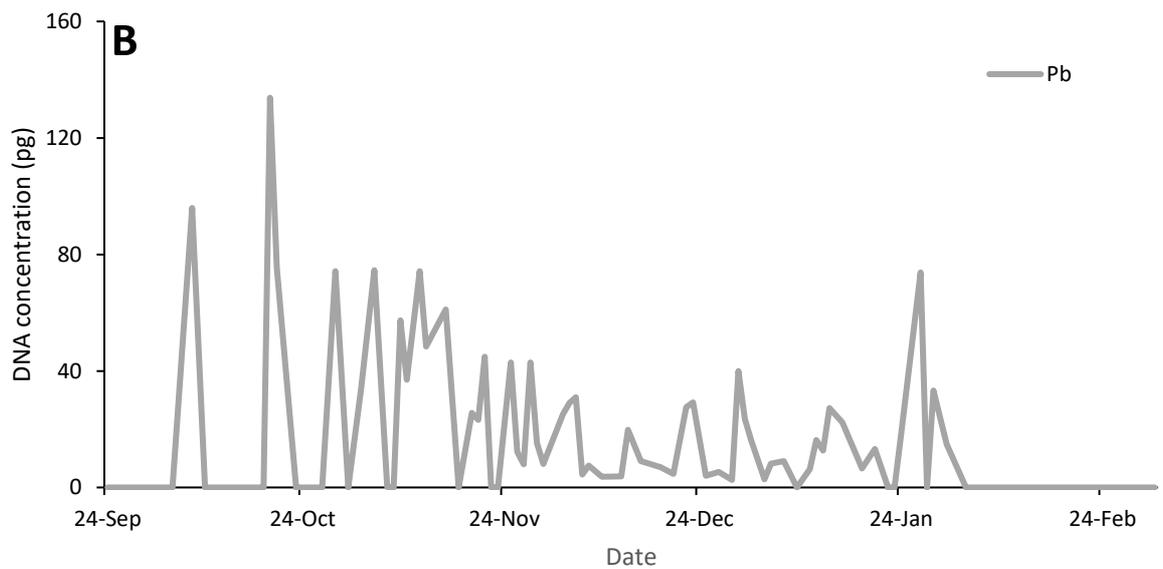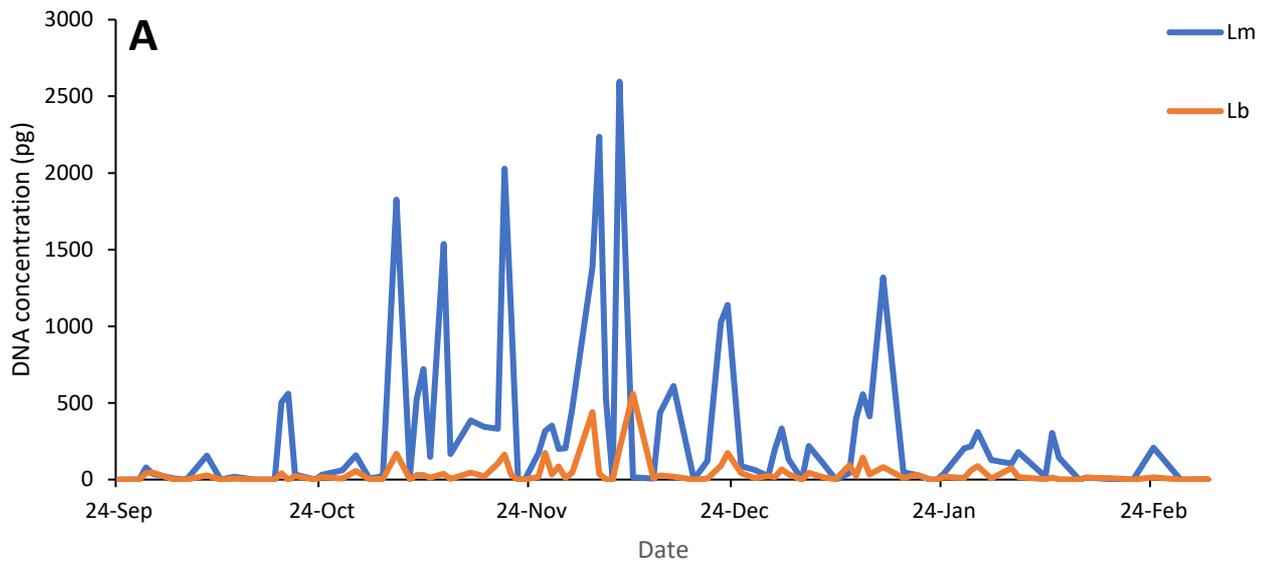
Figure 4.12: The amounts (pg.) of DNA quantified in 30µL of final volume using qPCR from spore tapes from the 2017/2018 cropping season. Lm = *L. maculans***, Lb =** *L. biglobosa* **(A) and Pb =** *P. brassicae* **(B).**
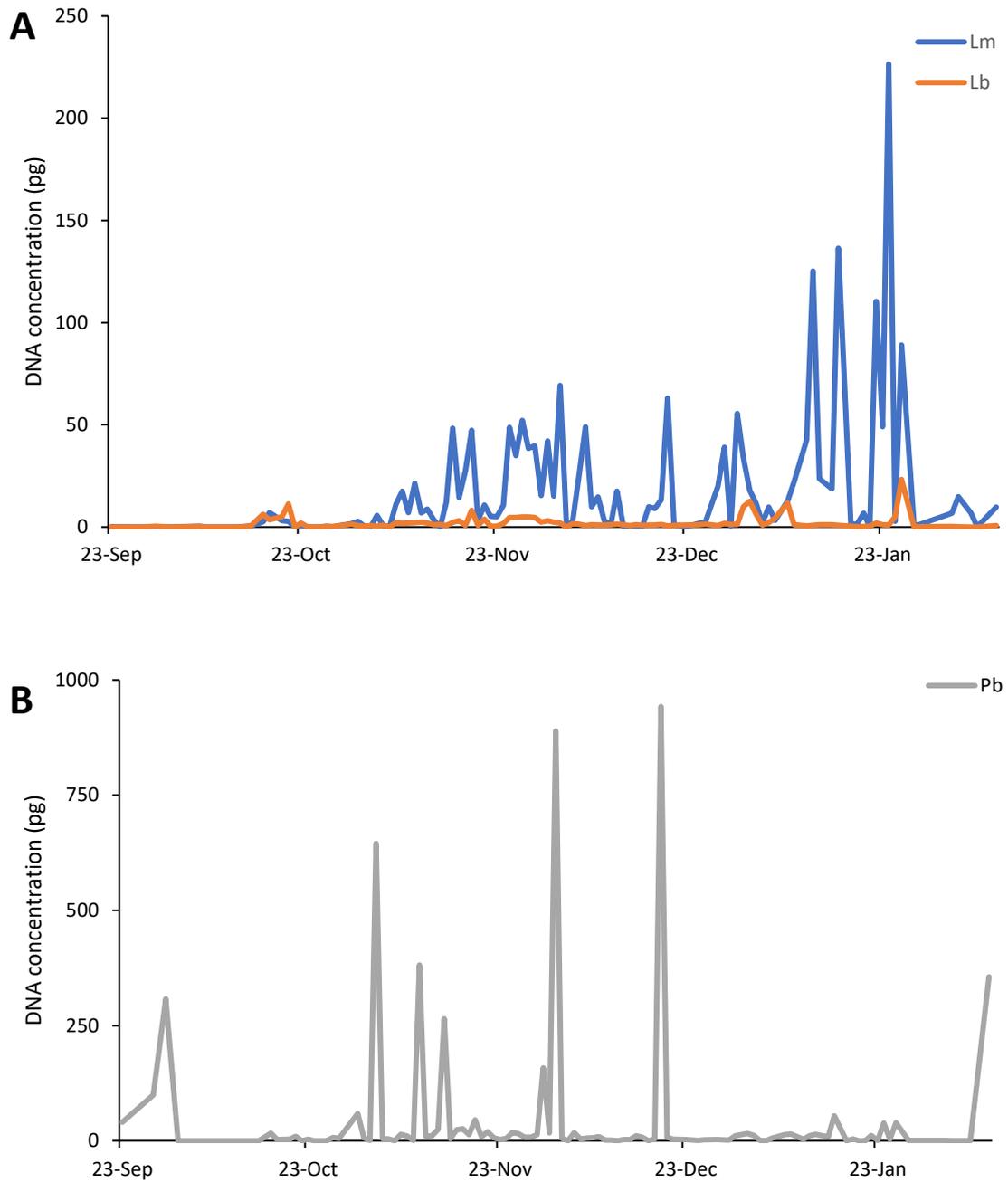
Figure 4.13: The amounts (pg.) of DNA quantified in 30 µL of final volume using qPCR from spore tapes from the 2018/2019 cropping season. Lm = *L. maculans*, Lb = *L. biglobosa* (A) and Pb = *P. brassicae* (B).
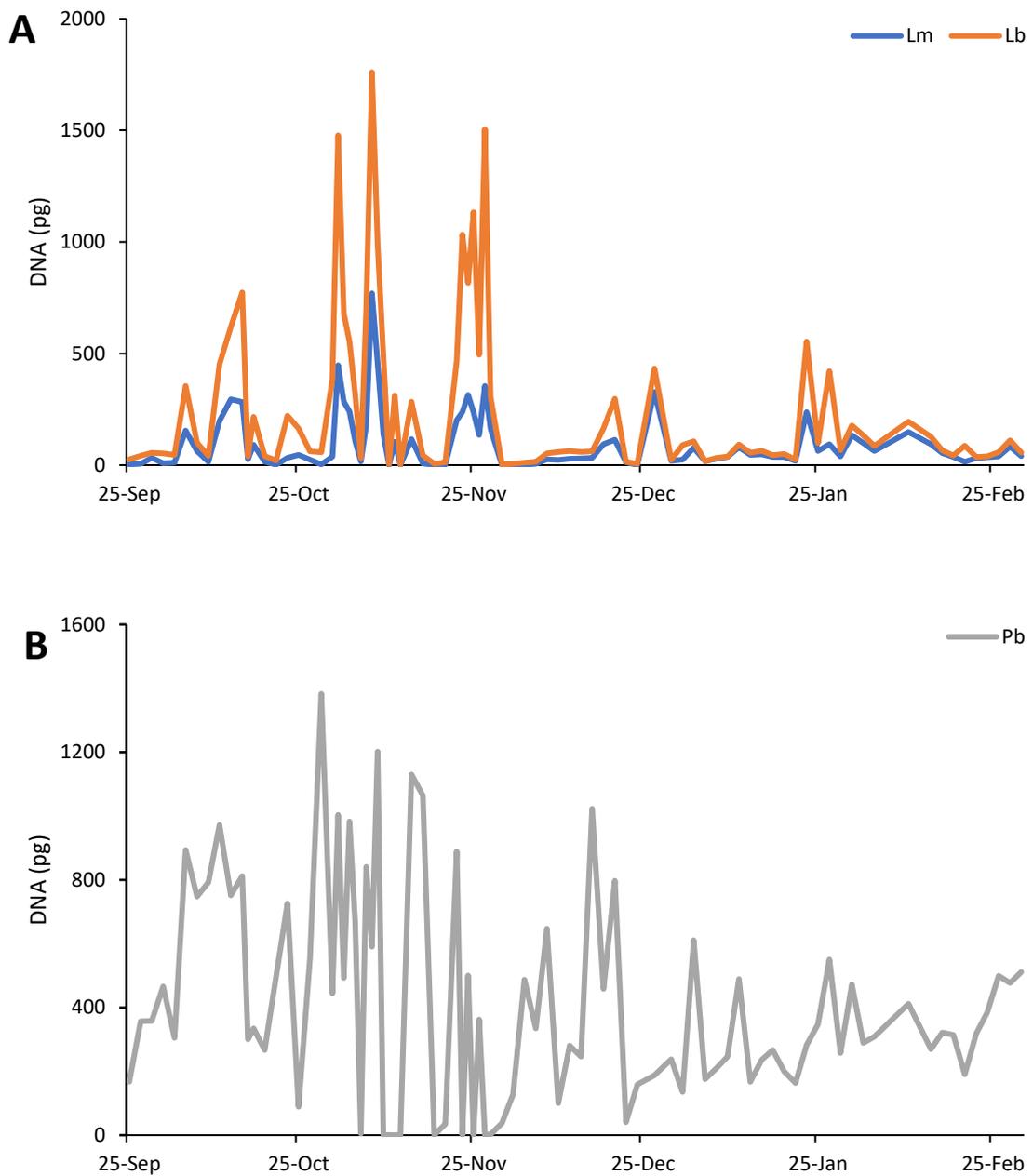
Figure 4.14: The amounts (pg.) DNA quantified in 30 µL of final volume using qPCR from spore tapes from the 2019/2020 cropping season. Lm = _L. maculans_, Lb = _L. biglobosa_ (A) and Pb = _P. brassicae_ (B).

There was a significant difference in the average daily detected *L. biglobosa* DNA between seasons ($F_{2.303}$ = 22.23, *P* < 0.001, LSD = 39.5). The greatest average daily detected *L. biglobosa* DNA was in 2019/20 (131 pg) cropping season; this was significantly greater than in 2017/18 (41 pg) or 2018/19 (2pg) cropping seasons. Although the average daily detected *L. biglobosa* DNA was greater in the 2017/18 cropping season than in the 2018/19 cropping season, there was no significant difference between them.

There was a significant difference in the average daily detected *P. brassicae* DNA between seasons ($F_{2.303}$ = 105.87, *P* < 0.001, LSD = 56.8). The greatest average daily detected *P. brassicae* DNA was in the 2019/20 (400 pg) cropping season; this was significantly greater than in 2017/18 (23 pg) or 2018/19 (49 pg) cropping seasons. Although the average daily detected *P. brassicae* DNA was greater in the 2018/19 cropping season than in the 2017/18 cropping season, there was no significant difference.

Although the total detected amount of *Leptosphaeria* spp. DNA of each respective pathogen was different in each season, the greatest maximum for *L. maculans* DNA occurred earlier than the greatest maximum for *L. biglobosa* in each of all three cropping seasons. For *P. brassicae*, the greatest maximum occurred earlier than the greatest maximum of both *Leptosphaeria* spp. in all three cropping seasons. In all three cropping seasons, the main ascospore release events for *L. maculans* and *L. biglobosa* occurred at similar times to each other in each season, whereas *P. brassicae* ascospore release events occurred throughout the whole season.

For *Leptosphaeria* spp., the start of the main ascospore release events occurred at similar times in 2017/18 and 2019/20 in mid-October but occurred later in the 2018/19 cropping season in early November. Despite this, the greatest maximum for *L. maculans* occurred a month earlier in 2019/20 (7 November 2019) than in 2017/18 (7 December 2017); the greatest *L. maculans* maximum in the 2018/19 cropping season was on 24 January 2019. In all three seasons the first detection of *L. maculans* or *L. biglobosa* was at similar times and in similar amounts. However, the first detection of *P. brassicae* was

at a greater concentration and at the start of sampling. Individual season analysis can be found in Appendices 20.

4.3.2.3 Mean temperature and precipitation weather data

In 2017/18, the average temperature for *Leptosphaeria* spp. pseudothecia and *P. brassicae* apothecia to develop from stem debris (July-Sept) from previous harvest was 16.5°C and average precipitation was 70.9mm per month with 17.7 days of rainfall per month (Figs. 4.15-4.17). The average winter (Nov-Feb) temperature was 5.3°C and average precipitation was 60.8mm per month with 17.5 days of rainfall per month. A result of note was that there was 103.5 mm of precipitation in July. The average spring (March-May) temperature was 10.2°C and average precipitation of 56mm per month and 15.7 days of rainfall per month. The average summer (June-July) temperature was 18.7°C and average precipitation was 22.8 mm per month and there was 6.5 days of rainfall per month.

In 2018/19, the average temperature for *Leptosphaeria* spp. pseudothecia and *P. brassicae* apothecia to develop from stem debris from the previous harvest was 17.9°C and average precipitation was 40.9mm per month with 9.3 days of rainfall per month (Fig. 4.15-4.17). A result of note was that the average temperature in 20.6°C in July. The average winter (Nov-Feb) temperature was 6.8°C and average precipitation of 42.5mm with 13.8 days of rainfall per month. The average spring (March-May) temperature was 10.2°C and average precipitation was 131 mm per month and 12.3 days of rainfall per month. A result of note was that total precipitation in May was 381.1mm. The average summer (June-July) temperature was 17.5°C and average precipitation was 94.4 mm with 15.5 days of rainfall per month.
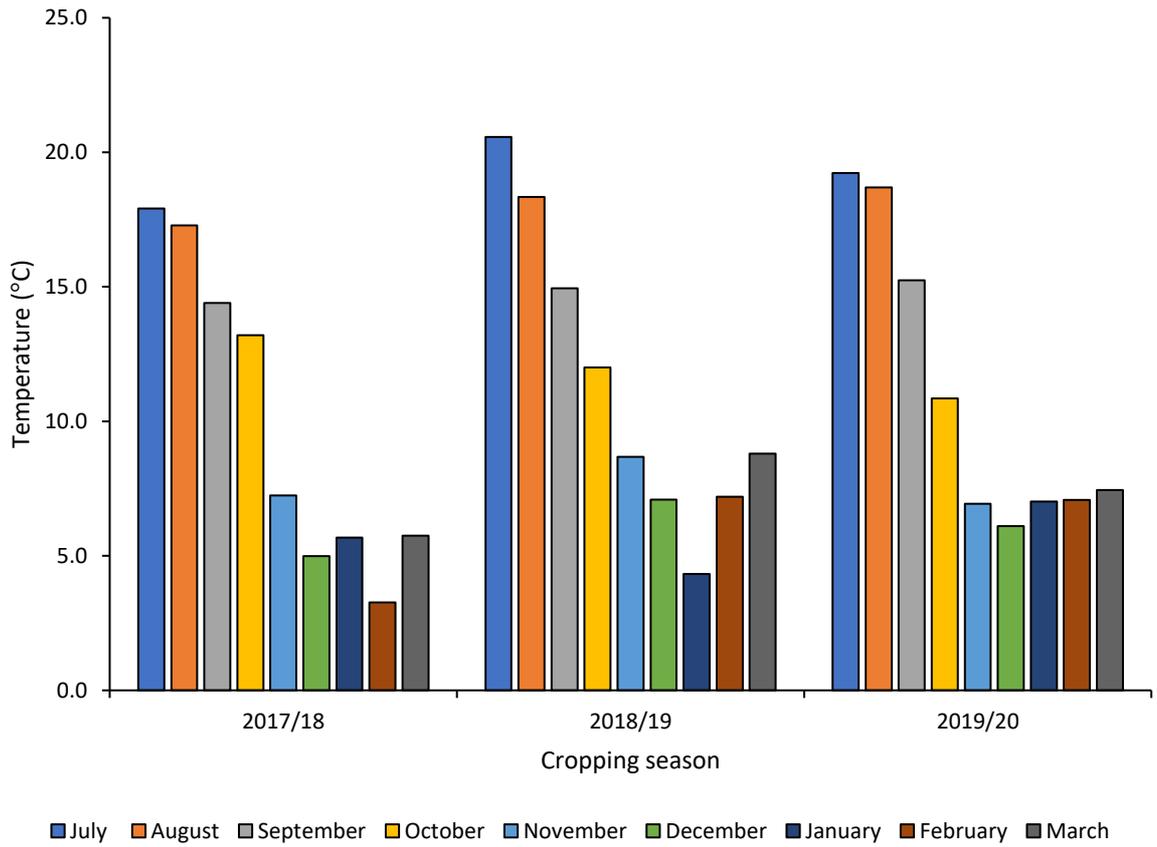
Figure 4.15: Mean monthly temperature at the field experiment site in Terrington St Clement, Norfolk, UK from July until March for the three cropping seasons (2017/18, 2018/19 and 2019/20). The mean monthly temperature was calculated using the daily mean temperature.
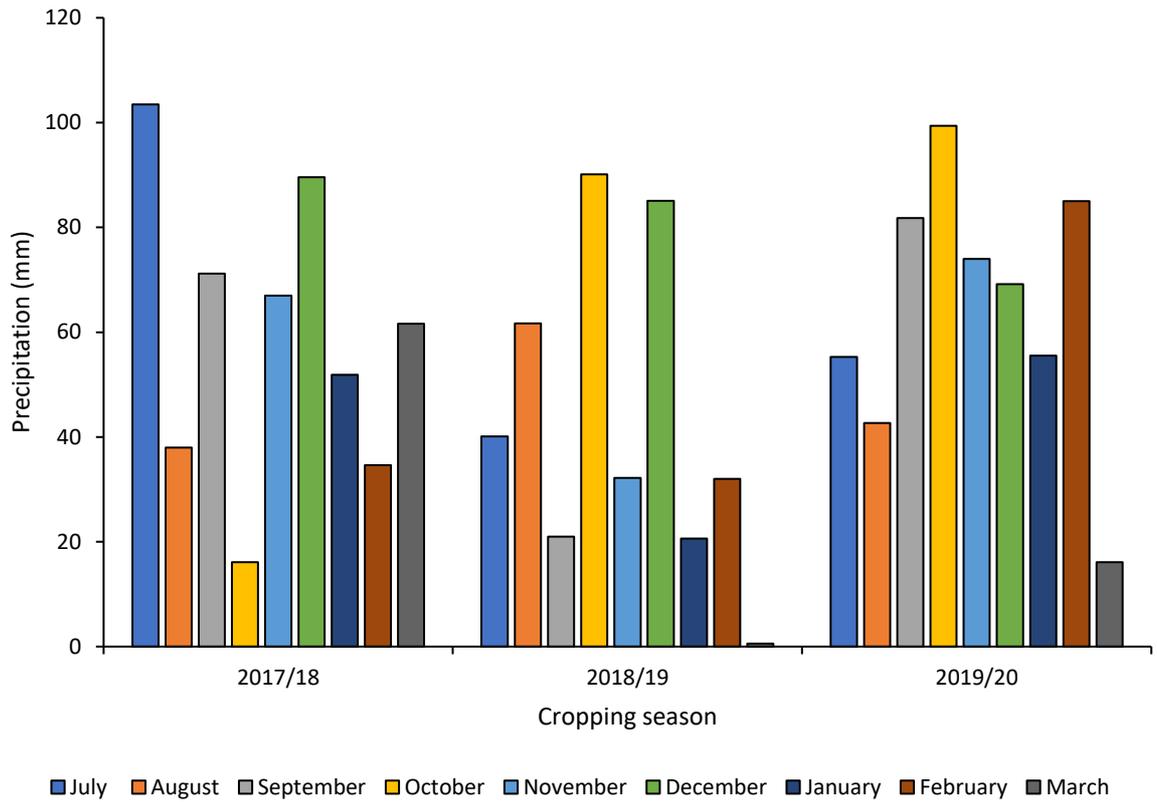
Figure 4.16: Mean monthly precipitation at the field experiment site in Terrington St Clement, Norfolk, UK from July until March for the three cropping seasons (2017/18, 2018/19 and 2019/20). The mean monthly precipitation was calculated using the daily mean precipitation.
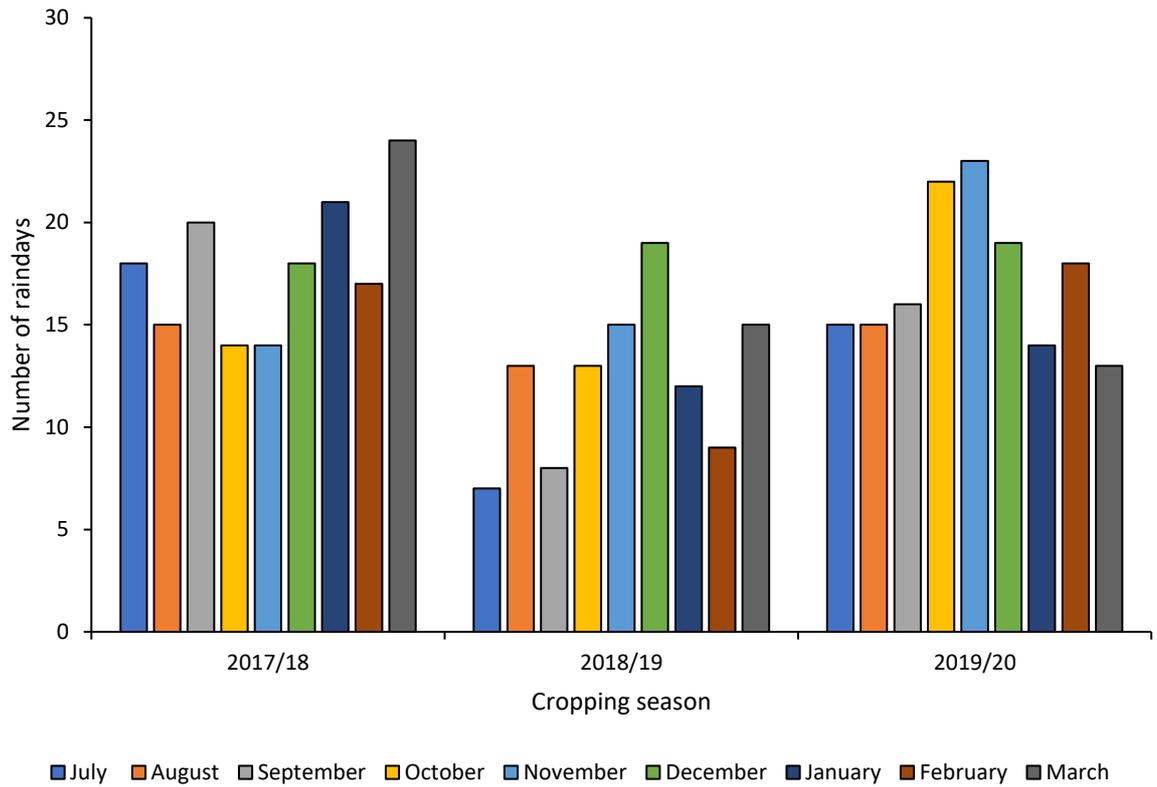
Figure 4.17: Number of days of rainfall per month at the field experiment site in Terrington St Clement, Norfolk, UK from July until March for the three cropping seasons (2017/18, 2018/19 and 2019/20).

In 2019/20, the average summer temperature for pseudothecia and apothecia to develop from stem debris from the previous harvest was 17.7°C and average monthly precipitation was 59.9mm and there was 15.3 days of rainfall (Fig. 4.15-4.17). The average winter (Nov-Feb) temperature was 6.8°C and average monthly precipitation was 71 mm with 18.5 days of rainfall per month. The average spring (March-May) temperature was 10.9°C and average precipitation was 13.3 mm with 7.3 days of rainfall per month. The average summer (June-July) temperature was 17.0°C and average monthly precipitation was 68.1 mm with 15.5 days of rainfall per month.

4.3.3 Monitoring of *Leptosphaeria* spp. pseudothecial densities on stem debris from the previous season.

4.3.3.1 Psuedothecial density of stem debris

There was a significant effect of cultivar, fungicide and cropping season on pseudothecial density on stubble. There were also significant two-way interactions between cultivar × fungicide and cultivar × cropping season ($P<0.01$) on the pseudothecial density on stubble (Table 4.14 & 4.15). There was no significant difference in the two-way interaction of fungicide × year, nor was there a significant three-way interaction of cultivar × fungicide × cropping season ($P=0.10$ and, $P=0.06$ respectively). The significant interaction of cultivar × fungicide meant that the differences between the application of fungicides was influenced by the cultivar (Table 4.14 & 4.15). Cvs. Barbados, Charger, Hunivers and Quartz had significantly smaller pseudothecial densities in treated than in untreated plants but for cvs Django and Flamingo there was no difference between the untreated and treated plants (Fig. 4.14 & 4.15). The significant interaction of cultivar × cropping season meant that the differences within cultivars in pseudothecial density was influenced by the cropping season (Table 4.14 & 4.15). Cvs Barbados and Charger had a significantly greater pseudothecial density in 2017/18 and 2018/19 than in 2019/20. Whereas in cvs Django, Hunivers and Quartz, the pseudothecial density in 2018/19 and 2019/20 cropping seasons were not significantly different but were both significantly smaller than in the 2017/18 cropping season.

Table 4.14. Testing output of significant probability levels for the main effects of Cultivar, Season and Fungicide, the two-way interactions, and the three-way interaction on yield. These statistics were applied on Loge transformed data.

Factor	df_{num}	F statistic	df_{den}	LSD	F probability
Season	2	25.46	124	0.28	<0.01
Cultivar	5	44.17	124	0.40	<0.01
Fungicide	1	33.69	124	0.23	<0.01
Season x Cultivar	10	4.96	124	0.69	<0.01
Season x Fungicide	2	2.41	124	0.40	0.10
Cultivar x Fungicide	5	3.24	124	0.57	<0.01
Cultivar x Season x Fungicide	10	1.86	124	0.98	0.06
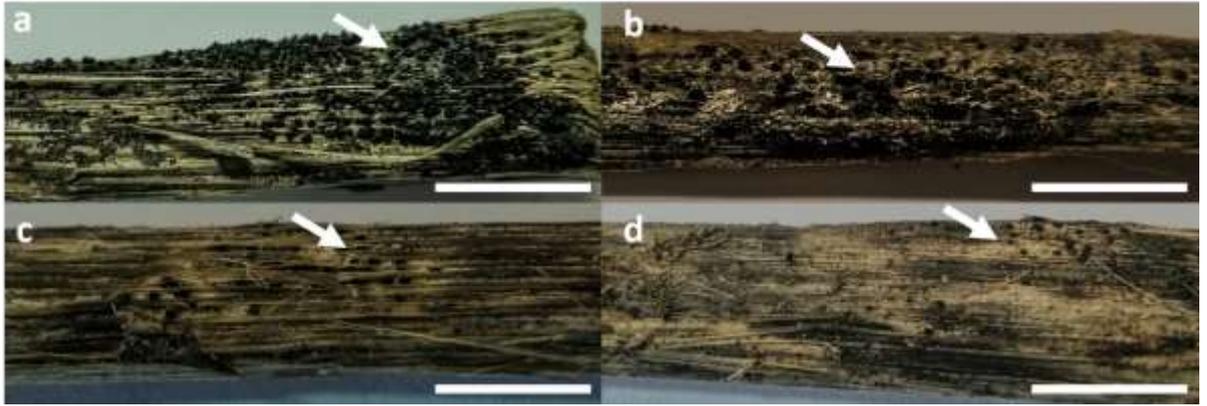
Figure 4.19. *Leptosphaeria* **spp. pseudothecia on oilseed rape stem stubble of two cultivars with different levels of 'field resistance'. Stem stubble from susceptible cv. Charger (a, b) and resistant cv. Quartz (c, d) from plots that were untreated (a, c) or treated with fungicide prothioconazole (b, d), from the 2019/20 cropping season. Stubble was collected after harvest and placed in free draining trays to allow pseudothecia to mature under natural conditions. Scale bars represent 0.5cm. White arrows indicate the pseudothecia (Fortune *et al.* 2021).**

Table 4.15: *Leptosphaeria* spp. pseudothecial densities on stubble of six cultivars from the field experiments in the 2017/18, 2018/19 and 2019/20 oilseed rape cropping seasons [!] (Fortune *et al*. 2021).

| Cultivar [*] | Treatment | Pseudothecial density (No. of pseudothecia /cm^2) ^ | | | |
		2017/18	2018/19	2019/20	Mean of Cultivar × Fungicide
Barbados (7)	Untreated	4	3.89	0.27	2.89[f]
	Treated	1.62	1.65	0.03	1.10[g]
	Mean of Cultivar × Year	2.81[ef]	2.77[ef]	0.14[g]	-
Charger (4)	Untreated	39.07	20.31	20.1	26.02[a]
	Treated	13.06	9.24	7.6	10.01[bc]
	Mean of Cultivar × Year	24.62[a]	15.39[abc]	13.15[bcd]	-
Django (6)	Untreated	5.93	1.7	3.25	3.59[ef]
	Treated	13.59	3.14	1.74	5.13[ef]
	Mean of Cultivar × Year	9.76[cd]	2.43[f]	2.49[f]	-
Flamingo (4)	Untreated	14.26	16.38	14.67	15.04[b]
	Treated	8.62	14.57	15.43	13.07[bc]
	Mean of Cultivar × Year	11.75[cd]	15.47[ad]	15.09[ad]	-
Hunivers (7)	Untreated	33.78	4.74	5.99	15.47[bc]
	Treated	9.2	3.46	3.51	5.24[de]
	Mean of Cultivar × Year	22.85[ab]	4.10[ef]	4.75[ef]	-
Quartz (8)	Untreated	19.35	9.44	3.57	10.17[cd]
	Treated	3.73	2.22	2.48	2.83[ef]
	Mean of Cultivar × Year	10.67[d]	5.83[e]	3.09[ef]	-

[*]Descriptor in bracket following cultivar name indicates the cultivar AHDB recommended list phoma resistance rating (0-9 scale; resistance rating 8-9 was classed as good resistance, 6-7 as medium resistance and < 5 as susceptible).

^ Values that do not share a common letter are significantly different at *P* =0.05.

[!] Stems of the six cultivars were collected after harvest from plots that were 'Untreated' or 'Treated' with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. "Mean of Cultivar ´ Fungicide" represents the pseudothecial densities across three seasons of the two-way interaction between cultivar and fungicide. "Mean of Cultivar ´ Year" represents the mean pseudothecial densities across two treatments of the two-way interaction between cultivar and year. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the pseudothecial densities between the various treatment combinations in the two-way interactions.

Cv. Flamingo was the only cultivar that's pseudothecial density was not significantly different between all three cropping seasons between 2017-2020. Untreated cv. Charger (26.02) had the greatest psuedothecial density when the mean cultivar × fungicide interactions were compared. This was significantly greater than that of all other treatments (Table 4.14 & 4.15).

The treatment that had the second greatest pseudothecial density was untreated cv. Hunivers (15.47), this was significantly greater than all other treatments except from the untreated (15.04) and treated cv. Flamingo (13.07) treatments. Although significantly different from untreated cv. Flamingo, untreated cv. Quartz (10.17) and treated cv. Charger (10.01) were not significantly different from treated cv. Flamingo or treated cv. Hunivers (5.24). However, the pseudotheical densities were significantly different between treated cvs Flamingo and Hunivers. There was no significant difference between the pseudothecial densities on untreated cv. Django (3.59) and treated cvs Django (5.13) and Quartz (2.83); they were not significantly different from either treated cv. Hunivers or untreated cv. Barbados (2.89) but the pseudothecial density was significantly smaller on untreated cv. Barbados than on treated cv. Hunivers. The treatment that had the smallest pseudothecial density was treated Barbados (1.10) (Table 4.15). Individual season analysis can be found in Appendices 21.

There was a significant effect of resistance, fungicide and cropping season and the effect of the two-way interactions of resistance × year ($P<0.05$) on pseudothecial density when the six cultivars were divided into three resistance groups – 'Good' - cv. Quartz; 'Medium' - cvs Barbados, Django and Hunivers; 'Susceptible' - cvs Charger and Flamingo. The pseudothecial density was significantly greater in the susceptible cultivars than those in the 'Good' and 'Medium' resistance groups; but there was no significant difference in pseudothecial densities between 'Medium' or 'Good' resistance groups (Fig. 4.20). There were no significant three-way interaction of resistance × fungicide × year, nor was there a significant or in the two-way interactions of resistance × fungicide, and resistance × year ($P=0.36$, $P=0.39$ and $P=0.25$, respectively)
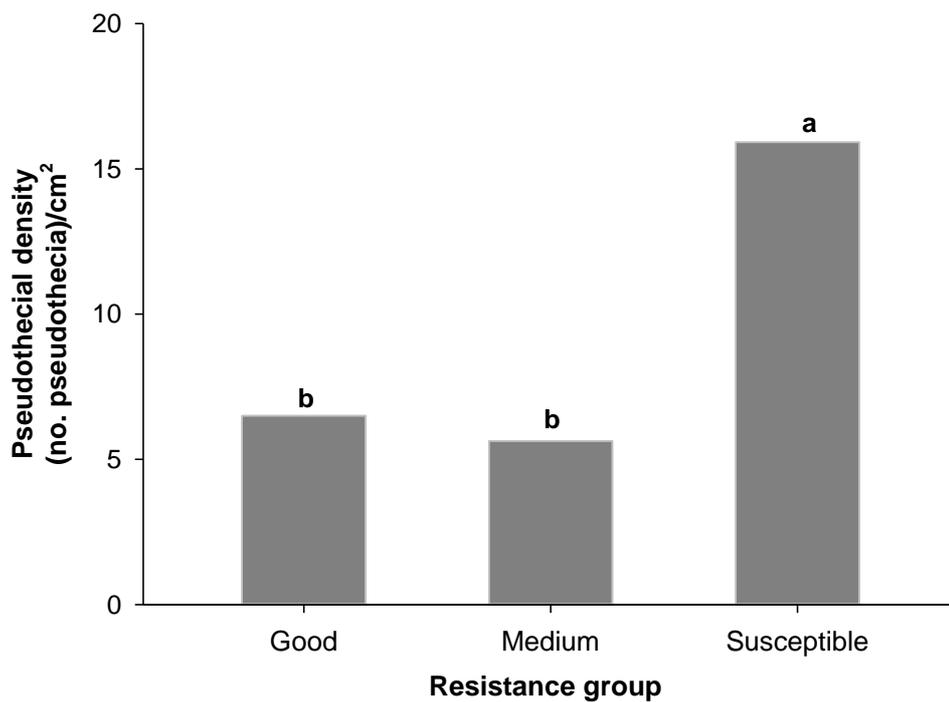
Figure 4.20. Mean *Leptosphaeria* spp*. pseudothecial densities on stubble of cultivars from different resistance groups from winter oilseed rape field experiments in the 2017/18, 2018/19 and 2019/20 cropping seasons. The resistance group "Good" contained cv Quartz, "Medium" contained cvs Barbados, Django and Hunivers and "Susceptible" contained cvs Charger and Flamingo. Stems of these six winter oilseed rape cultivars were collected after harvest from plots that were 'Untreated' or 'Treated' with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. Least Significant Differences (LSD) were calculated at *P*=0.05 and used to separate the mean *Leptosphaeria* spp*. pseudothecial densities between resistance groups because the two-way interaction of resistance × fungicide was not significant (*P*=0.39). Bars that do not share a common letter are significantly different at *P* =0.05 (35 d.f.) (Fortune *et al.* 2021).

4.3.3.2 Stem canker score on *Leptosphaeria* spp. against pseudothecial density

The relationship between stem canker score and pseudothecial density traits was analysed by regressing stem canker severity score against *Leptosphaeria* spp. pseudothecial density; (i.e., $\log_e$ transformed (pseudothecia per cm^2)). The correlation coefficient (r) between stem canker severity and pseudothecial density was r=0.85, which supported a good simple linear relationship between the two traits (p<0.01). Untreated cv. Charger had the greatest stem canker severity score and produced the greatest pseudothecial density (Table 4.9 and Table 4.15).

Greater stem canker severity scores and pseudothecial densities were observed on untreated stems than on fungicide treated stems of the same cultivar. Cultivars in 'Medium' or 'Good' resistance groups had smaller stem canker severity scores and smaller pseudothecial densities than cultivars in the 'Susceptible' resistance group. Therefore, the cultivars that had the smallest phoma stem canker severity score, and subsequently the smallest *Leptosphaeria* spp. pseudothecial density were treated cultivars from either a medium or good resistance group. A common simple linear relation was fitted to the combined data; it accounted for 71.9% of the variance in the observed phoma stem canker severity scores by the pseudothecia densities (i.e., the coefficient of determination R^2=0.719, df=34) (Fig. 4.21). Alternate methods of analysis, including grouping cultivars into their respective resistance groups, were tested but they did not significantly improve the common fitted simple linear line.

4.3.4   Identification of relationships in winter oilseed rape in England and Wales between weather, prevalence of light leaf spot or phoma leaf spot and stem canker and yield loss

4.3.4.1 Identification of national relationships between disease prevalence and yield loss for England and Wales

There was no correlation between *P. brassicae* foliar prevalence in the autumn and yield loss attributed to light leaf spot disease (£/ha) at harvest (R^2=0.14).
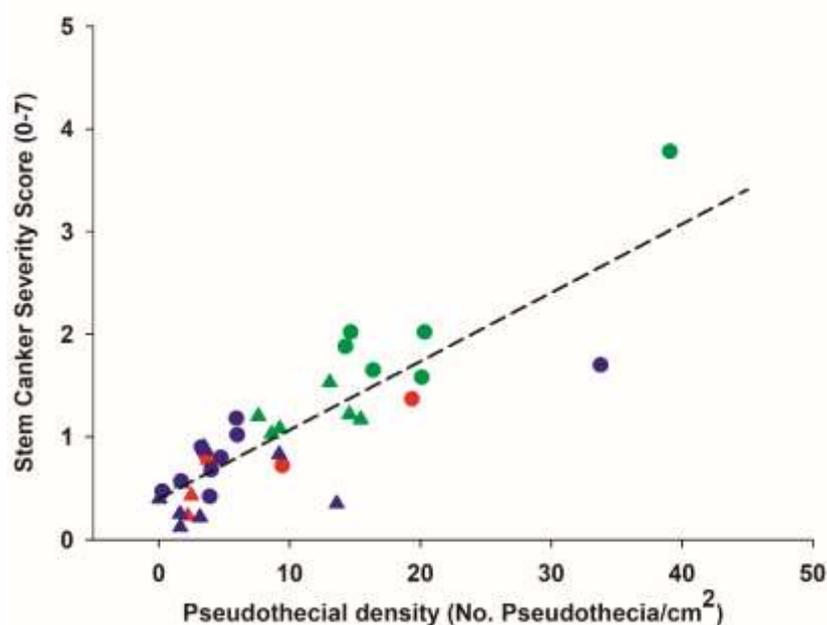
Figure 4.21: Relationship between phoma stem canker severity score and pseudothecial density of *Leptosphaeria* spp. on six different cultivars from the field experiments in 2017/18, 2018/19 and 2019/20 cropping seasons, for untreated samples (●) and fungicide treated samples (▲). The colours represent field resistance rating groups: Red = Good (cv. Quartz), Blue = Medium (cvs Barbados, Django and Hunivers) and Green = Susceptible (cvs Charger and Flamingo). The fitted relation is: *Y*=0.40 +0.067*X* (*R²*=71.9%, df=34) in which "*Y*" is the stem canker severity score (0-7 scale) and "*X*" the pseudothecial density (No. pseudothecia/cm²) (Fortune *et al.* 2021).

There was no correlation between *P. brassicae* foliar prevalence in spring and yield loss attributed to light leaf spot disease (£/ha) at harvest (R^2=0.11). There was very strong positive correlation between prevalence of *P. brassicae* lesions on pods in the summer and yield loss attributed to light leaf spot disease (£/ha) at harvest (R^2=0.75) (Fig. 4.22). There was weak positive correlation between phoma leaf spotting prevalence in the autumn and yield loss attributed to phoma stem canker disease (R^2=0.27). However, when the data point for 2010/11 was removed there was strong positive correlation between these two factors (R^2=0.50) (Fig. 4.23). There was no correlation between phoma stem canker severity and yield loss attributed to phoma stem canker (R^2=0.06).

4.3.4.2 Identification of relationships between seasonal weather and disease prevalence for England and Wales, 2008/09 -2017/18

4.3.4.2.1   Mean seasonal temperature regressed on *P. brassicae* pod prevalence

There was positive correlation between mean autumn temperature and *P. brassicae* pod prevalence in July (R^2 =0.45) (Fig. 4.24). There was a relationship between autumn temperature and an increased prevalence of *P. brassicae* pod lesions. There was very strong positive correlation between mean winter temperature and *P. brassicae* pod prevalence in July (R^2 =0.91) (Fig. 4.24). There was a relationship between winter temperature and an increased prevalence of *P. brassicae* pod lesions. There was no correlation between mean spring or summer temperature and *P. brassicae* pod prevalence (R^2 = 0.00 *and* 0.01, respectively).

There appeared to be a negative correlation between total number of air frosts in autumn and *P. brassicae* pod prevalence in July (R^2 = 0.52) (Fig. 4.25). There appeared to be two groups within this relationship; for the first group, when there were fewer than an average of one day of air frost in England and Wales in the autumn, there was no correlation. However, when there was more than an average of one day of air frosts in England and Wales during the autumn there appeared to be a stronger negative correlation for the second group. There was a negative relationship between number of air frosts in autumn and prevalence of *P. brassicae* pod lesions in the following summer.
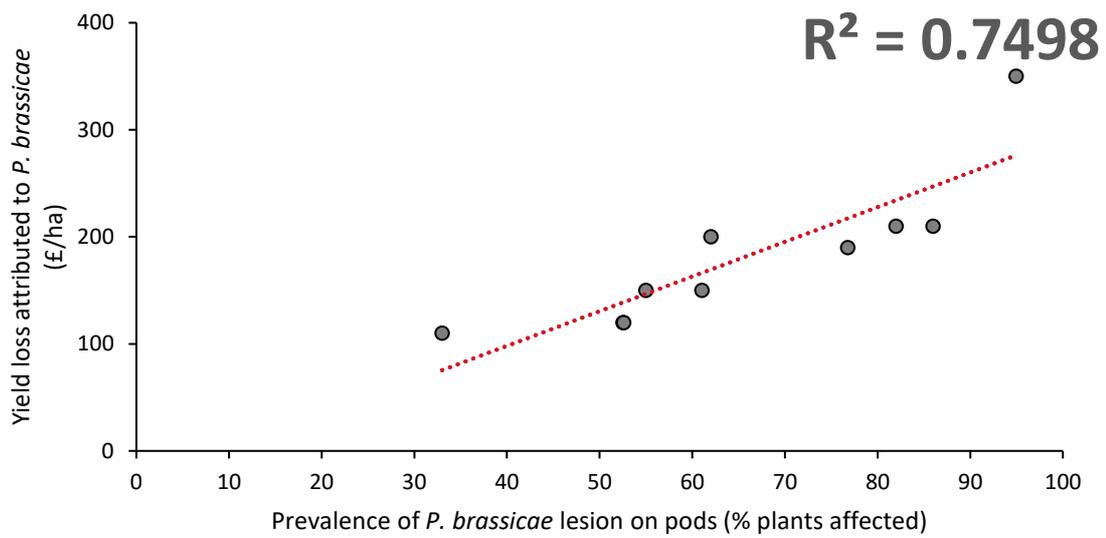
Figure 4.22: Relationship between prevalence of *P. brassicae* lesions on pods (% plants affected) in July and yield loss at harvest attributed to light leaf spot in England and Wales for the cropping seasons 2008/09 to 2017/18. The fitted relation is: *Y*= 3.24*X*-31.52 (*R²*=75.0%, df=8) in which "*Y*" is the yield loss attributed to *P. brassicae* (£/ha) and "*X*" the prevalence of *P. brassicae* lesions on pods (%). Prevalence was determined as the incidence of crops affected.
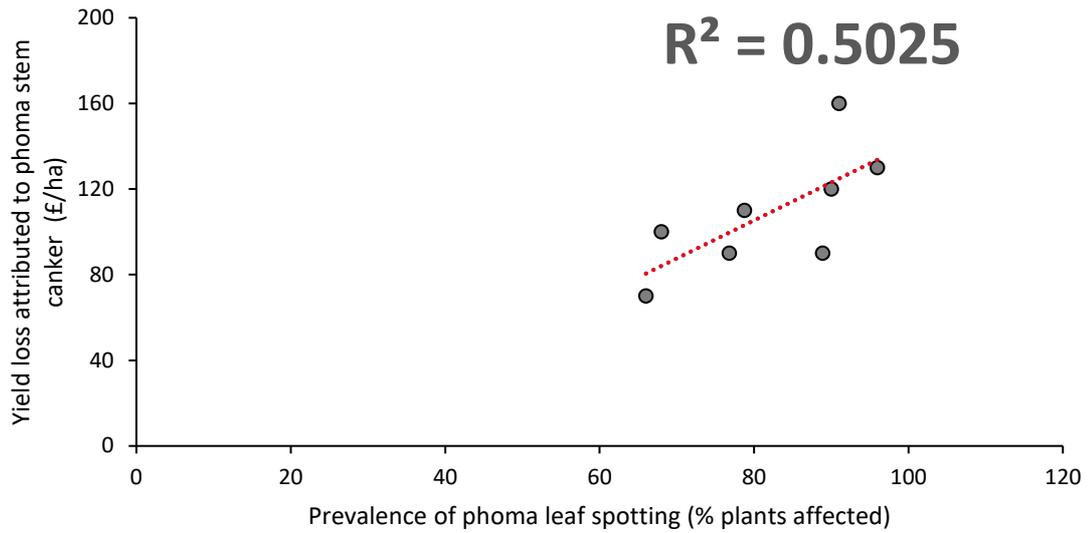
Figure 4.23: Relationship between prevalence of phoma leaf spotting lesions in autumn (% plants affected) and yield loss at harvest attributed to phoma (£/ha) in England and Wales for the cropping seasons 2008/09 to 2017/18 at the end of the season. The data point for 2010/11 cropping season was removed. The fitted relation is: $Y= 1.77X$-365.49 (R^2=50.3%, df=6) in which "Y" is the yield loss attributed to phoma (£/ha) and "X" prevalence of phoma leaf spotting (%). Prevalence was determined as the incidence of crops affected.
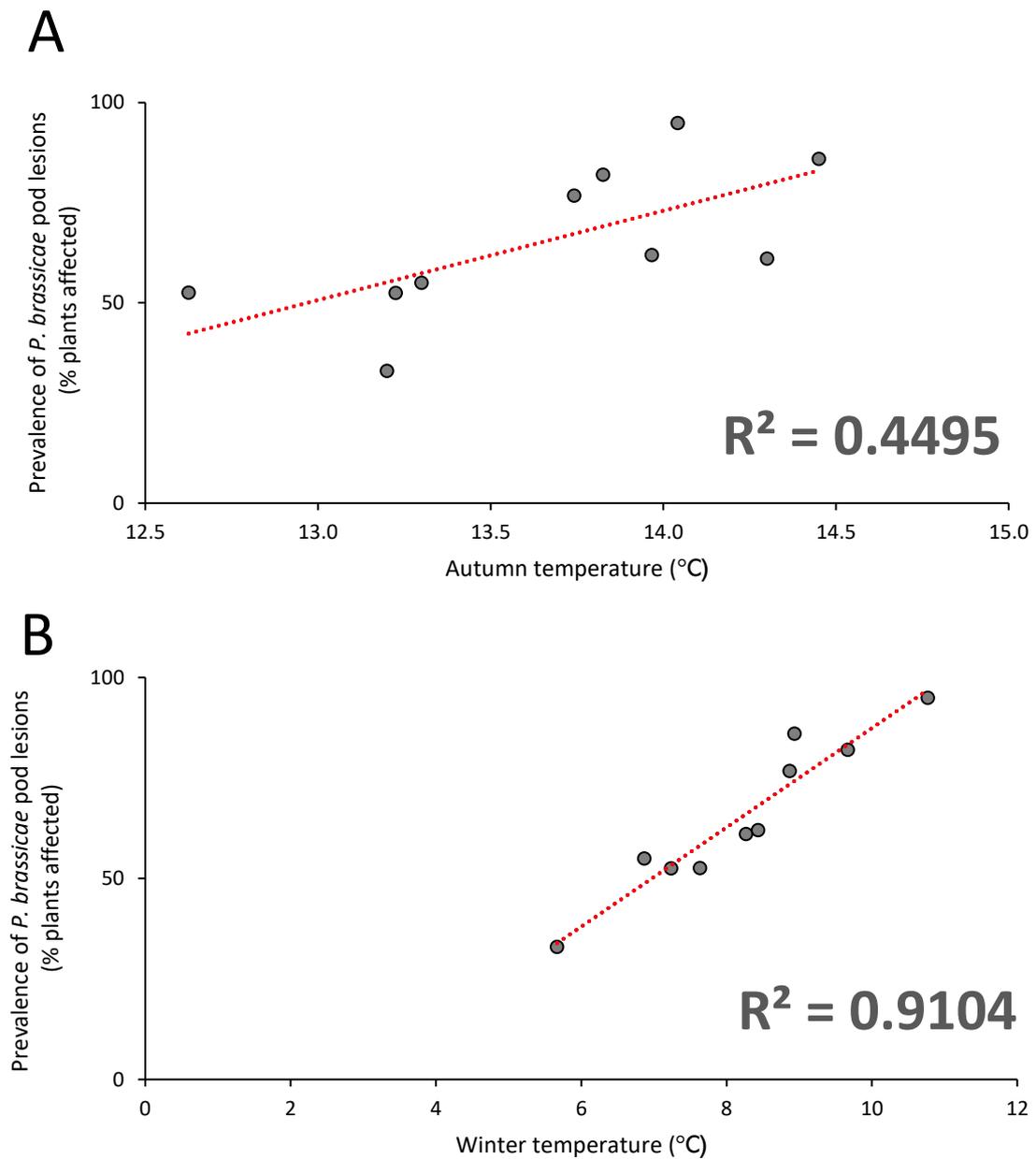
Figure 4.24. Relationship between prevalence of *P. brassicae* pod lesions (%) in July and autumn (A) and winter temperature (B) (°C). The fitted relation for autumn temperature is *Y*= 22.31*X*-239.33 (*R²*=45.0%, df=8) and for winter temperature is: *Y*= 12.35*X*-36.09 (*R²*=91.0%, df=8) and for winter in which "*Y*" is the prevalence of *P. brassicae* pod lesions and "*X*" is either the mean autumn (Aug-Oct) or winter (Nov-Jan) temperature (°C). Prevalence was determined as the incidence of crops affected.
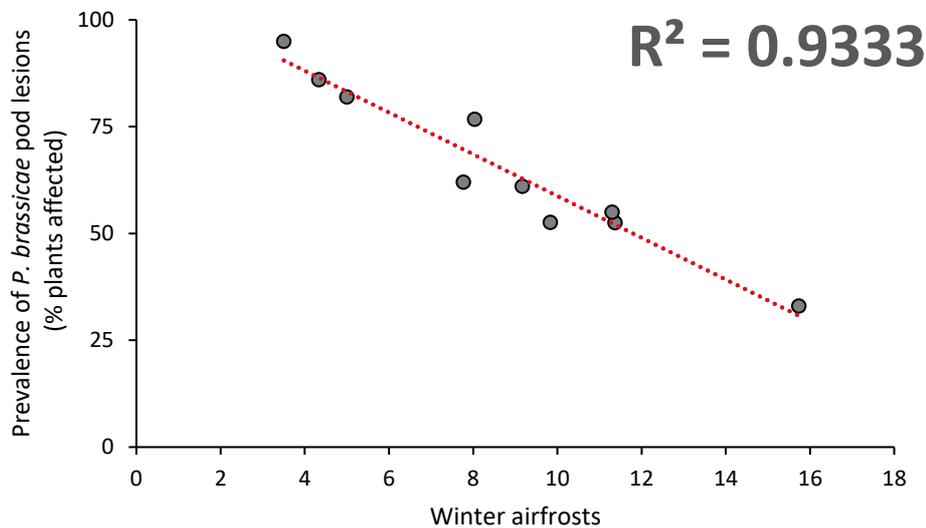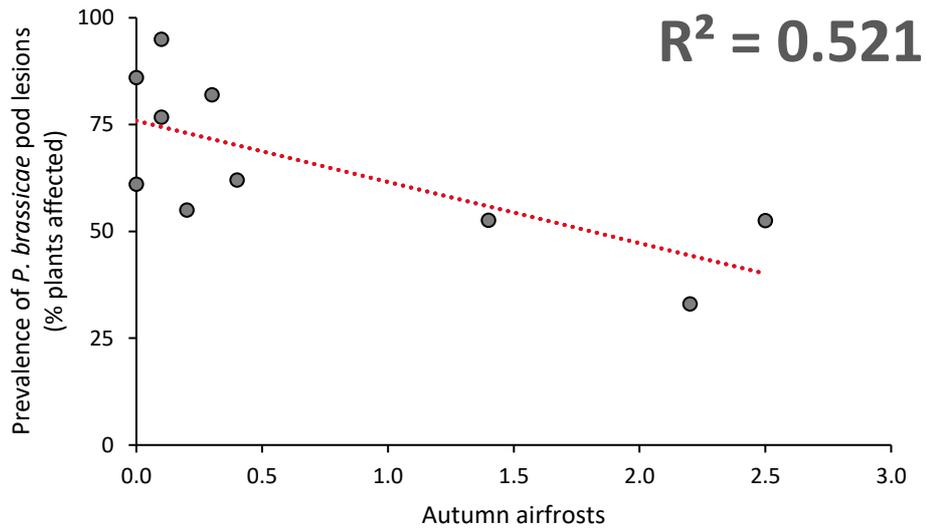
Figure 4.25 Relationship between prevalence of *P. brassicae* pod lesions (% plants affected) in July and number of air frosts in autumn (A) and winter (B). The fitted relation for autumn air frosts is *Y*= -14.32*X* + 75.89 (*R*²=52.1%, df=8) and for winter air frosts is: *Y*= -4.89*X*+ 107.63 (*R*²=93.3%, df=8) in which "*Y*" is the prevalence of *P. brassicae* pod lesions and "*X*" is either the number of autumn (Aug-Oct) or winter (Nov-Jan) air frosts (°C). Prevalence was determined as the incidence of crops affected.

There was a very strong negative correlation between total number of air frosts in winter and *P. brassicae* pod prevalence in July (R^2 = 0.93) (Fig. 4.25). There was a negative relationship between number of air frosts in winter and prevalence of *P. brassicae* pod lesions in the following summer. There was no correlation between total number of air frosts in either spring or summer and *P. brassicae* pod prevalence in July (R^2 = 0.02 and 0.00, respectively).

4.3.4.2.2 Mean seasonal rainfall and rain-days on *P. brassicae* pod prevalence

There was no correlation between average monthly rainfall in autumn or winter and *P. brassicae* pod prevalence (R^2 =0.09 and 0.25, respectively). There was positive correlation between average monthly rainfall in spring and *P. brassicae* pod prevalence (R^2 = 0.59) in July (Fig. 4.26). There was a relationship between greater average monthly rainfall in spring and an increased prevalence of *P. brassicae* pod lesions in July. There was no correlation between number of rain-days in summer and *P. brassicae* pod prevalence (R^2 = 0.02) in July.

There was no correlation between number of rain-days in autumn and *P. brassicae* pod prevalence (R^2 =0.06) in July. There was weak positive correlation between number of rain-days in winter and *P. brassicae* pod prevalence (R^2 =0.32) in July (Fig. 4.27). There was a positive relationship between the number of rain-days in winter and prevalence of *P. brassicae* pod lesions in July. There was positive correlation between number of rain-days in spring and *P. brassicae* pod prevalence (R^2 = 0.51) in July (Fig. 4.27). There was a positive relationship between the number of rain-days in spring and prevalence of *P. brassicae* pod lesions in July. There was no correlation between the number of rain-days in summer and *P. brassicae* pod prevalence (R^2 = 0.03) in July.
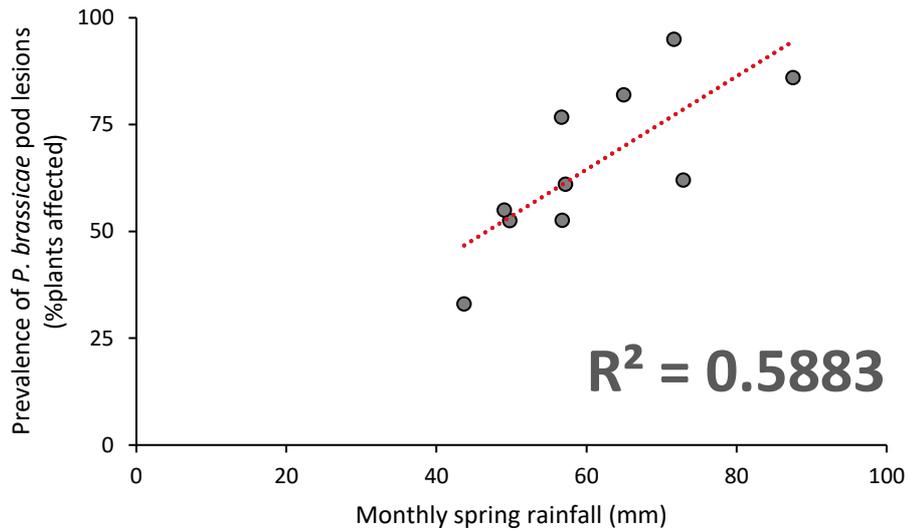
Figure 4.26. Relationship between prevalence of *P. brassicae* pod lesions (%) in July and average monthly rainfall (mm) in spring. The fitted relation for average monthly rainfall (mm) in spring is: *Y*= 1.09*X*- 0.92 (*R²*=58.8%, df=8) in which "*Y*" is the prevalence of *P. brassicae* pod lesions and "*X*" is the average monthly rainfall (mm) in spring (Feb-May). Prevalence was determined as the incidence of crops affected.
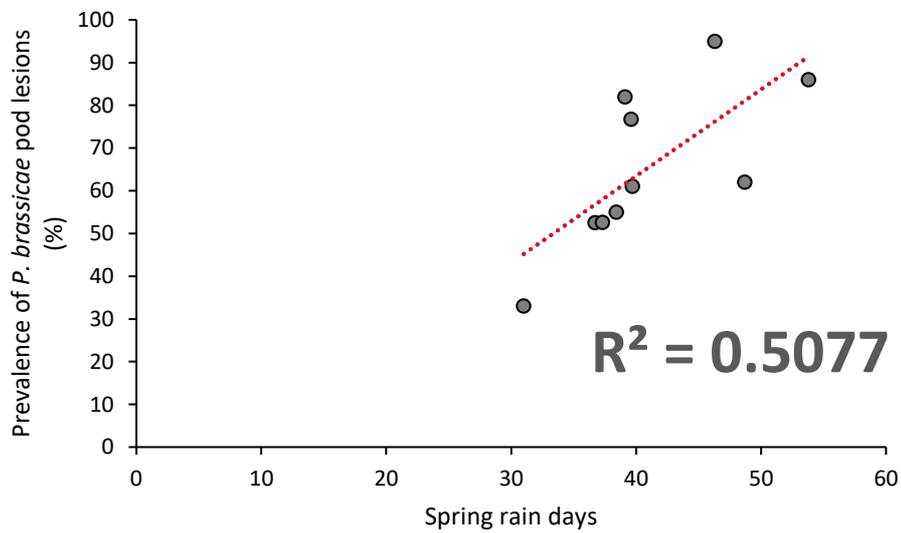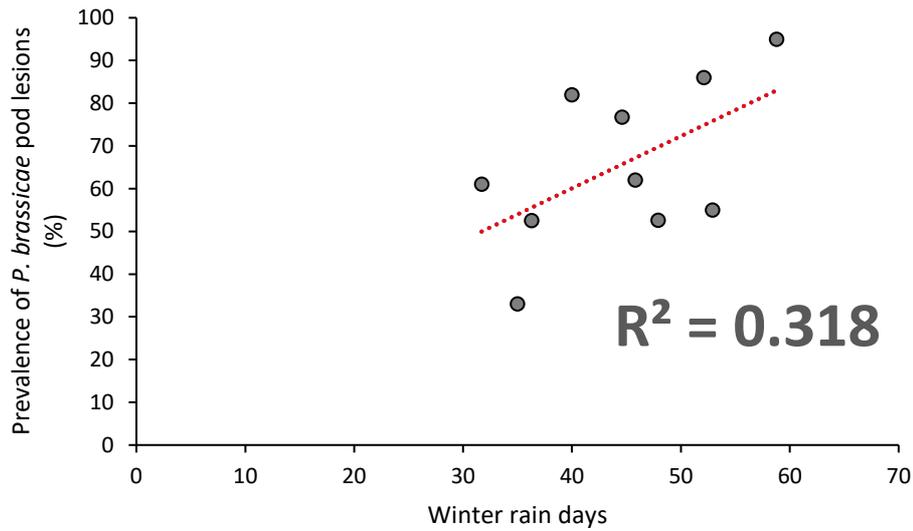
Figure 4.27. Relationship between prevalence of *P. brassicae* pod lesions (%) in July and number of rain-days in winter (A) and spring (B). The fitted relation for winter rain-days is *Y*= 1.22*X* + 11.27 (R^2=31.8%, df=8) and for spring rain-days is: *Y*= 2.028*X*-17.69 (R^2=50.8%, df=8) in which "*Y*" is the prevalence of *P. brassicae* pod lesions and "*X*" is either the number of winter (Nov-Jan) or Spring (Feb-May) rain days. Prevalence was determined as the incidence of crops affected.

4.3.4.2.3   **Mean autumn weather on prevalence of phoma leaf spotting**

There was no correlation between mean autumn (Aug-Dec) temperature and phoma leaf spotting prevalence in December (R^2 = 0.07). There was no correlation between number of air frosts in autumn and phoma leaf spotting prevalence in December (R^2 = 0.08). There was no correlation between number of autumn rain days and phoma leaf spotting prevalence (R^2 = 0.03) in December. There was no correlation between average monthly rainfall in autumn and phoma leaf spotting (R^2 = 0.02) in December.

4.4   Discussion

The incidence of *P. brassicae* sporulation on untreated plants in spring (April-May) had the greatest effect on oilseed rape seed yield. There was a very strong correlation between the incidence of *P. brassicae* foliar sporulation and yield in untreated plants in the 2017/18 and 2018/19 cropping seasons. *P. brassicae* conidia are rain splash dispersed from the leaves up to the pods (Gilles *et al.* 2001c). This study found very strong correlation between pod infection and annual yield losses; therefore if there was a greater incidence of *P. brassicae* sporulation on leaves there would be a greater probability that conidia would be dispersed up to infect the pods and therefore have a greater impact on yield. However, plants in the 2019/20 cropping season did not fit the correlation between *P. brassicae* sporulation and yield loss; this was mainly due to the poor establishment of plants in the 2019/20 cropping season that resulted in fewer and smaller plants in the early season and resulted in reducing the chance of a successful early infection on untreated (before T1 fungicides were applied) plants during maximum *P. brassicae* ascospore release. This is different to the previous seasons when the plants were more established and larger than in 2019/20, so there was a greater chance of a successful *P. brassicae* infection. Additionally, the infection may have occurred earlier in the 2019/20 season and multiple *P. brassicae* infection cycles could have occurred (Evans *et al.* 2017). There was a large amount of *P. brassicae* inoculum in 2019/20 since consistent ascospore release events were observed from September – March 2020. This suggests that the *P. brassicae* sporulation in spring 2020 would have been widespread

if the plant establishment in the autumn 2019 was good in the 2019/20 cropping season. This was supported by results from Bayer's SpotCheck initiative that identified *P. brassicae* infections almost one month earlier in the 2019/20 season than in the previous season 2018/19.

These results from 2017/18 and 2018/19 showed that the use of an azole fungicide reduced the incidence of *P. brassicae* sporulation between untreated and fungicide-treated plots; albeit significantly only in the 2018/19 cropping season. This was believed to be due to the low *P. brassicae* inoculum concentration situation in the 2017/18 cropping season rather than because of the fungicide insensitivity that has been reported in the UK in *P. brassicae* (Carter *et al.* 2014, 2016; King *et al.* 2021). Additionally, cultivar resistance to *P. brassicae* influenced the efficacy of fungicide application on the incidence of *P. brassicae* sporulation in spring. This was evidenced by the two most resistant cultivars to *P. brassicae* cvs Barbados and Django not having a significant difference in incidence of *P. brassicae* sporulation in any of the three cropping seasons, even in 2018/19 when the *P. brassicae* inoculum concentration was high. These findings support Fortune *et al.* (2021) that suggested that the most sustainable and effective integrated control strategy for oilseed rape diseases in seasons with low quantities of inoculum is to use more resistant cultivars and apply fungicide only when required.

It is important to acknowledge that fungicides are applied not only for the control of phoma stem canker; the use of prothioconazole also provides control against other oilseed rape diseases such as light leaf spot. This is particularly interesting because in the 2018/19 cropping season when the incidence of *P. brassicae* sporulation was the greatest among the three seasons, the first fungicide application (T1) was applied late (16 November 2018) due to the *L. maculans* fungicide application threshold (10% crop incidence of *L. maculans* lesions) being met later in this season than other two seasons. Four major *P. brassicae* ascospore release events were observed before 16 November 2018; this resulted in the crop being exposed to four *P. brassicae* ascospore release events without the protection by prothioconazole, whereas in the 2017/18 cropping season the T1 application was applied on 25 October 2017 and only two smaller *P. brassicae* ascospore release events were observed before 25 October 2017, this resulted

in crops being exposed to only two smaller *P. brassicae* ascospore release events without protection by prothioconazole. The T1 application was made on the 5 November 2019 and eight *P. brassicae* ascospore release events were observed before 5 November 2019; this would have meant that if the crop was fully established it would have been exposed to eight *P. brassicae* ascospore release events without protection by prothioconazole. This provides further support for the hypothesis that the *P. brassicae* foliar incidence would be great if the establishment of the crop is good and is co-incident with *P. brassicae* ascospore release. However, these findings provide evidence for indirect inter-specific interactions between *L. maculans* and *P. brassicae.* The late *L. maculans* ascospore release and subsequent late fungicide applications may increase the vulnerability of the crop to *P. brassicae* infection if *P. brassicae* ascospores are released before the fungicide application, because the crop will be exposed to *P. brassicae* ascospores without fungicide protection. This highlights the need for robust disease monitoring to ensure fungicide applications to the appropriate pathogen risk (*Leptosphaeria* spp. or *P. brassicae*). This work has shown that microscopic counts for *Leptosphaeria* spp. can identify the general pattern of ascospore release. However, due to the high level of similarity in phenotype of *L. maculans* and *L. biglobosa,* it would be very difficult to accurately distinguish ascospores between the two species. It is also very difficult to identify and count *P. brassicae* ascospores due to their shape/size and lack of distinguishing features. Therefore, this work suggests that microscopic counts can be used to gain a general understanding of *Leptosphaeria* spp. ascospore release events but molecular techniques are required to distinguish between *L. maculans* or *L. biglobosa* ascospores, and to identify *P. brassicae* release events. Therefore, further work is needed to investigate the indirect interspecific interactions between *L. maculans* and *P. brassicae* through variation in timing of fungicide application in relation to both *L. maculans* and *P. brassicae* ascospore release events.

Although there were no relationships between the incidence and severity of *L. maculans* leaf lesions or phoma stem basal canker severity and yield, there was a relationship between the stem canker severity and the subsequent *Leptosphaeria* spp. pseudothecial density in the following season. The severity of stem basal cankers was directly affected by the application of fungicide and the use of resistant cultivars. Cultivars that had

medium or good phoma resistance scores had less severe stem cankers and fewer pseudothecia than susceptible cultivars, which agrees with previous studies (Marcroft *et al.* 2004; Lo-Pelzer *et al.* 2009). Similarly, the application of fungicide effectively reduced the severity of stem cankers and the subsequent pseudothecial density in the following season. Epidemics of phoma stem canker are initiated by ascospores discharged from mature pseudothecia, so reducing the density of pseudothecia by use of integrated pest management strategies such as applying fungicides and the use of cultivar resistance would reduce the inter-seasonal transmission of *Leptosphaeria* spp. inoculum to the subsequent season. Interestingly, the cultivar that had the greatest stem canker severity score (cv. Quartz) produced more pseudothecia than cultivars that had a medium resistance rating to the phoma stem canker pathogen. This may be due to cv. Quartz relying on major gene resistance due to the presence of *Rlm7,* whereas cv. Barbados utilises quantitative resistance. This difference would explain the result because *Rlm7* is species-specific to *L. maculans*, whereas quantitative resistance is not; therefore, the pseudothecia produced on the stubble of cv. Quartz may predominately be *L. biglobosa*. These findings are supported by the work by Sidique (2015) that showed that more *L. biglobosa* ascospores were discharged than *L. maculans* ascospores from stem stubble from cultivars containing the *Rlm7* gene, and Kerdraon *et al.* (2020) that showed cultivars with resistance genes against *L. maculans* promote the colonisation by *L. biglobosa* (Kerdraon *et al.* 2020). *L. biglobosa* is less sensitive to triazole fungicides (such as prothioconazole) than *L. maculans* (Eckert *et al.* 2010; Huang *et al.* 2011), so the selection for *L. biglobosa* could be further promoted. This suggests that the adoption of *L. maculans* control strategies using *L. maculans* resistant cultivars with azole fungicides may result in changes in the predominance of the two species in a local pathogen population. Further investigations and monitoring of pseudothecia that distinguish *L. maculans* and *L. biglobosa* pseudothecia need to be done to confirm these suggestions. The timings of the ascospore release of the three pathogens were strongly related to the weather, in particular precipitation and temperature. This is supported by the differences in the *Leptosphaeria* spp. pseudothecial density on stubble between the three cropping seasons in this study and by previous studies that have found these two environmental factors to be important for pseudothecial and apothecial maturation and

therefore ascospore release (Gilles *et al.* 2001a, 2001c; Toscano-Underwood *et al.* 2003; Huang *et al.* 2007; Evans *et al.* 2007).

For the *Leptosphaeria* spp., the main ascospore release events occurred at similar times to each other in each season. Both *L. maculans* and *L. biglobosa* have been shown to require continual wetness and mean temperature of 5-20°C for successful maturation and dispersal of ascospores (Toscano-Underwood *et al.* 2003). In the 2017/18 and 2019/20 cropping seasons, the main ascospore release events occurred in mid-October but they occurred later in the 2018/19 cropping season with the main ascospore release events occurring in early November due to dry weather in September/October. In all three cropping seasons, the mean monthly temperature and its patterns were similar. However, there were much greater fluctuations in monthly precipitation and rain days between the cropping seasons. Therefore, the differences in ascospore release events may be explained by the differences in the mean precipitation, particularly in September and October. In the 2017/18 and 2019/20 cropping seasons, there was a greater mean monthly precipitation in September (71 mm and 82 mm, respectively) than in the 2018/19 cropping season (21 mm). Also, there were fewer rain days in the 2018/19 cropping season (8 days) than in the 2017/18 and 2019/20 cropping seasons (20 and 16 days, respectively). The reduced precipitation and rain days in the 2018/2019 season slowed the pseudothecial maturation and led to delayed ascospore release.

Previous work has shown that *L. biglobosa* ascospores are released later than those of *L. maculans* (Huang *et al.*, 2011). This is supported by observation that the maximum *L. maculans* ascospore release occurred earlier than the maximum *L. biglobosa* ascospore release in the three cropping seasons. Despite this, the ascospore release events of the *Leptosphaeria* spp. started at the same time in both the 2018/19 and 2019/20 cropping seasons and there was a greater number of *L. biglobosa* ascospores released than *L. maculans* ascospores. Although closely related, there is a difference in the maturation of pseudothecia and development of ascospores between *L. maculans* and *L. biglobosa*; pseudothecial maturation of *L. biglobosa* occurs more slowly at temperatures <10°C compared to that of *L. maculans* but they mature at similar times at higher temperatures (15-20°C) under continual wetness (Toscano-Underwood *et al.* 2003). The mean

monthly temperatures for July-September in this investigation were > 10°C in the 2018/19 (17.9°C), 2019/20 (17.7°C) and 2017/18 (16.5°C) cropping seasons. Although these temperatures were all between 15-20°C, the stems were incubated under natural conditions with fluctuations of dry and wet periods, while the debris was incubated under continual wetness in the previous study (Toscano-Underwood *et al.* 2003).

Large maxima for amount of *P. brassicae* DNA were detected throughout September and March in each cropping season, and the greatest maxima for amount of *P. brassicae* DNA in the 2018/19 and 2019/20 cropping seasons occurred in the month that had the greatest number of rain days (December and October, respectively), suggesting that the release of *P. brassicae* ascospores is affected by rainfall. In the 2017/18 cropping season, the greatest maxima for amount of *P. brassicae* DNA were not in the month with the greatest number of rain days, but this was most probably due to the major ascospore release event occurring before *P. brassicae* ascospore monitoring started (24 September) because July-August 2017 had 60% more rain than in both 2018/19 and 2019/20 cropping season (163mm, 102mm and 98mm), and 20 rain days in September 2017. This suggests that frequent precipitation events are very important for *P. brassicae* ascospore release. This is supported by Gilles *et al.* (2001c) that confirmed this in controlled conditions. The *P. brassicae* life cycle is polycyclic; it does not have a beginning and an end, but rather a continuation of the previous ascospore or conidial infection cycles all occurring at the same time but at different stages (Evans *et al.* 2017). Frequent precipitation events throughout the season enable ascospore release events that can occur throughout the season to cause a very chaotic epidemic, particularly if frequent precipitation events (rain days) occur after harvest (when a lot of debris is produced) or after leaf senescence (Gilles *et al.* 2001a). To obtain a better understanding of the *P. brassicae* ascospore release pattern to understand the seasonal differences in *P. brassicae* ascospore release, monitoring of ascospore release would need to be done before the start of the cropping season, during the season and in the following cropping season.

There was a positive relationship between prevalence of phoma leaf spotting in the autumn and yield loss attributed to phoma stem canker disease when the data point

from 2010/11 was removed. This data point was removed because there was an abnormally high number of air frosts over the winter (10-year ave = 16, 2010/11 = 47). Therefore, it is likely that there were leaves had senescenced or dropped off before sample collection which affected the accuracy of the prevalence of autumn phoma leaf spotting measurement. Surprisingly, there was no relationship between phoma stem canker severity and yield loss attributed to phoma stem canker. There were no relationships between the autumn weather (temperature, air frosts, precipitation, or rain days) and the prevalence of phoma leaf spotting in the autumn. This may have been due to the widespread use of *L. maculans* resistant cultivars and targeted applications of fungicide that would provide protection from further disease development.

There was a strong positive relationship between the prevalence of *P. brassicae* lesions on pods and national yield loss attributed to *P. brassicae* for England and Wales; *P. brassicae* attributed yield loss was greater as the prevalence of *P. brassicae* lesions on pods increased. Therefore, control strategies need to be designed to stop *P. brassicae* spreading to the pods. However, the lack of *P. brassicae* resistant cultivars, a complex epidemic structure and long asymptomatic phases between infection and appearance of symptoms result in a less well refined control strategy that reduces the efficacy of *P. brassicae* control. This places a great importance on influence of *P. brassicae* favourable weather conditions on inoculum concentration to ultimately determine the prevalence of *P. brassicae* lesions on pods (%) at the end of the season. There were positive relationships between autumn or winter temperature and a negative relationship between the number of autumn or winter air frosts against the prevalence of *P. brassicae* pod lesions. This suggests that warmer temperatures and fewer air frosts would result in a greater prevalence of *P. brassicae* pod lesions. This is supported by previous work that a greater incidence of light leaf spot was noticed after mild winters than after colder winters (Karolewski *et al.* 1999). Increased temperature increased the germination rate of conidia (Karolewski *et al.* 2004) and allowed *P. brassicae* to colonise the host more effectively and lead to greater incidence of plants affected (Figueroa *et al.* 1995). By contrast, frequent air-frosts may damage the apothecia reducing the spread of *P. brassicae* ascospore inoculum from senescing leaf debris over the winter (Webster and Weber, 2007).

The identification of strong positive correlation between average precipitation or mean number of rain-days in spring and incidence of light leaf spot pod lesion is important because rainfall is important for two aspects of the *P. brassicae* life cycle: conidial dispersal and apothecial development. Greater precipitation and more days of rainfall favour the rain-splash of conidia of *P. brassicae* to upper parts of the plant (e.g. the pods) and to neighbouring plants (Gilles *et al.* 2000ab), therefore increasing the incidence of *P. brassicae* lesions on pods causing yield losses. Although there was only a small positive correlation between winter rainfall and incidence of light leaf spot pod lesions, a wet winter is important for the development of a light leaf spot epidemic (Boys *et al.* 2007). Met Office weather data has shown that the mean 5-year mean temperature average since 1969 for autumn and winter has increased whereas spring precipitation has not changed. Therefore, if this trend continues with autumn and winter getting warmer, this will increase the probability of *P. brassicae* infection. The patterns identified within this work will provide new opportunities for growers to make informed decisions about the potential risk of light leaf spot and whether it would be cost effective to apply a fungicide. Additionally, this information will allow future light leaf spot disease forecast model development to disproportionately assign importance to temperature in autumn and winter, and rainfall in spring. This is particularly important to be considered due to the changing climate that has seen the autumn and winter temperatures increase, that may influence future crop losses from *P. brassicae.*

The identified indirect interspecific interaction between *Leptosphaeria* spp. and *P. brassicae* has important agricultural and practical relevance. These results suggest that when favourable weather conditions for *P. brassicae*, such as increased autumn and winter temperature and frequent winter and spring precipitation events coincide with delayed fungicide application for control of *L. maculans,* this may increase the vulnerability of crops to *P. brassicae* infection. This means that the crops are exposed for longer without fungicide protection against *P. brassicae* infection, if ascospore release or early conidial rain-splash events occurred before fungicide applications for control of *L. maculans*. Therefore, this study suggests that if there is a high *P. brassicae* inoculum concentration, a high green area index on a *P. brassicae* susceptible cultivar

and the environmental conditions are favourable for *P. brassicae* infection*,* even though the *L. maculans* fungicide threshold has not been met, an application of fungicide should be made for protection against *P. brassicae* rather than *L. maculans* in autumn*.* This is supported by previous work that a single autumn fungicide application was more effective than a single spring application against *P. brassicae* (Jeffrey *et al.* 1994). However, if there is still a need and it is economically viable, another fungicide application should be made in the spring to provide protection against *P. brassicae* conidial infection (Evans *et al.* 2003). Therefore, there is a need to identify key factors for the development of co-ordinated strategies for controlling *P. brassicae* and the *Leptosphaeria* spp. simultaneously (Fitt *et al.* 1997, 1998; Gladders *et al.* 1998ab). The current changes to the climate in England and Wales increasingly favour the *P. brassicae* life cycle and highlight the need for the development of commercially available *P. brassicae* resistant cultivars to reduce the fragility of the UK oilseed rape industry.

Chapter 5 General discussion

The overall aims of this project were to understand the interactions between *L. maculans*, *L. biglobosa* and *P. brassicae in vitro* and *in planta* to improve integrated pest management strategies against phoma stem canker and light leaf spot diseases on oilseed rape (*Brassica napus*). These aims were achieved by investigating the interactions between phoma stem canker (*L. maculans* and *L. biglobosa*) and light leaf spot (*P. brassicae*) causal pathogens *in vitro* (Chapter 3) and *in planta* (Chapter 4), and examining the interactions between *L. maculans* and *P. brassicae* on different cultivars under field conditions (Chapter 5). This chapter discusses the overall findings of the project and suggests developments in the integrated pest management strategies for effective control of *L. maculans*, *L. biglobosa* and *P. brassicae* using the current literature and the novel findings from this investigation.

5.1 Changes in *Leptosphaeria* spp. ascospore release patterns under natural conditions influence direct interspecific interactions *between L. maculans* and *L. biglobosa*, which indirectly affect *P. brassicae*.

The current advised integrated control strategies for phoma stem canker in the UK are designed for *L. maculans*, not for *L. biglobosa*. These strategies for controlling *L. maculans* are mainly using resistant cultivars with effective *R* genes and fungicide application thresholds that are timed specifically for control of *L. maculans*; both strategies are used to intentionally control *L. maculans,* not *L. biglobosa.* This study provides evidence that these control strategies may cause changes in predominance of *L. maculans* and *L. biglobosa* in local populations or at regional and national scales. In fact, changes in the pattern of *L. maculans* and *L. biglobosa* ascospore release have been detected in this study and other recent studies (Javaid *et al.* 2019). Both studies show that *L. maculans* and *L. biglobosa* ascospores were frequently released simultaneously rather than showing that *L. maculans* ascospores were released earlier than *L. biglobosa* ascospores as previously reported (West *et al.* 2001; Huang *et al.* 2011). Previous work showed that *L. biglobosa* pseudothecia matured more slowly than *L. maculans* at > 10°C,

but pseudothecia of both species matured at similar rates at 15-20°C (Toscano-Underwood *et al.* 2003). If infected debris are continually wet due to increases in autumn and winter temperatures (https://www.metoffice.gov.uk/), it is possible that *L. maculans* and *L. biglobosa* pseudothecia will mature at similar rates and their ascospores will be released simultaneously in future.

This study found that *L. biglobosa* can outcompete *L. maculans* through the inhibition of sirodesmin (a non-host selective secondary metabolite) production by *L. maculans*. This study provides strong evidence that the production of sirodesmin is used as a form of interference competition to give *L. maculans* an interspecific competitive advantage against other pathogens because *in vitro* studies showed that sirodesmin had inhibitory effects on both *L. biglobosa* and *P. brassicae*. Additionally, when cotyledons of oilseed rape were inoculated with *L. maculans* and *L. biglobosa* simultaneously, there was an inhibition of sirodesmin production, which increased the competition of *L. biglobosa* (disproportionately smaller quantities of *L. maculans* DNA compared to *L. biglobosa* DNA) and produced small *L. biglobosa* type lesions. This suggests that *L. biglobosa* can outcompete *L. maculans* in these circumstances. This implies that the direct interspecific interactions between *L. maculans* and *L. biglobosa* may indirectly make the crops more susceptible to *P. brassicae* due to the inhibition of sirodesmin production, because sirodesmin has inhibitory effects on *P. brassicae*.

5.2 The widespread adoption of effective integrated *L. maculans* control strategies under *P. brassicae* favourable weather conditions may unintentionally make the UK oilseed rape crops more vulnerable to *P. brassicae* infection

These direct interspecific interactions between *L. maculans* and *L. biglobosa* on simultaneously inoculated plants caused *L. biglobosa* type of phoma leaf spot lesions (e.g. small dark lesions) rather than *L. maculans* type of phoma leaf spot lesions (e.g. large grey lesions). These results suggests that if *L. maculans* and *L. biglobosa* ascospores are released simultaneously, *L. biglobosa* type of phoma leaf spot lesions will be observed on crops which will delay the time in the season when the threshold of 10% plants with *L. maculans* lesions is met, which will delay fungicide applications. In

addition, use of resistant cultivars will reduce the number of *L. maculans* lesions that would also delay the timing of the fungicide applications, or in low *L. maculans* inoculum concentrations seasons they may prevent the threshold from being reached. Fungicides are not only effective against *L. maculans*, but also provide protection against *L. biglobosa* and *P. brassicae* and other autumn oilseed rape diseases. So, if growers follow the advised *L. maculans* threshold for fungicide applications, this will mean that the crops would be vulnerable for longer to infection by other autumn oilseed rape pathogens. This study showed that *P. brassicae* ascospore release events occur throughout the autumn and winter, so a later fungicide application will increase the probability of more *P. brassicae* ascospore release events occurring before protective fungicides are applied, which will increase the probability of infection by *P. brassicae*. Therefore, the timing of the autumn fungicide application needs to be based not only on the date when 10% of plants have *L. maculans* leaf spot lesions but also on *P. brassicae* ascospore release events, so that fungicide application can be targeted for control of both *L. maculans* and *P. brassicae.*

The indirect effect of the *L. maculans* incidence threshold being met later on *P. brassicae* infection is likely to be exacerbated in seasons that have weather favourable for *P. brassicae* at key stages of its life cycle. The long asymptomatic latent period between *P. brassicae* infection and the appearance of conidial rings or acervuli, the most characteristic and unambiguous symptom of *P. brassicae* infection, makes it difficult to control *P. brassicae* early in the cropping season. In the UK, these structures often appear in early winter and are present throughout spring and up to harvest in July/August. These structures are concentrated accumulations of *P. brassicae* conidia awaiting dispersal by rain splash. Therefore, frequent precipitation events throughout the winter and spring will increase the likelihood of conidia being dispersed to neighbouring plants and to upper parts of the plant including the pods, causing pod infection. This study showed a very strong relationship between prevalence of *P. brassicae* incidence on pods and yield loss attributed to *P. brassicae.*

This investigation provides evidence that the increased economic importance of *P. brassicae* to the UK oilseed rape industry in recent years may be attributed to the

widespread adoption of the integrated *L. maculans* control strategies that use *L. maculans* resistant cultivars and accurate fungicide application timings based on the *L. maculans* incidence thresholds, as advised by the Agricultural and Horticultural Directive Board (AHDB). This advice has successfully provided robust protection against *L. maculans*. However, this strong focus on *L. maculans* control may detract from the control of *L. biglobosa* and *P. brassicae* and may indirectly make UK oilseed rape crops more vulnerable to *P. brassicae* infection leading to yield losses.

5.3 Development of integrated pest management strategies is required to improve the control of *L. maculans*, *L. biglobosa* and *P. brassicae* together rather than in isolation.

Results of this study suggested a requirement for new strategies to be developed to reduce yield losses caused by *L. maculans*, *L. biglobosa* and *P. brassicae* using new information on timings of *L. maculans*, *L. biglobosa* and *P. brassicae* ascospore release and new information on weather conditions.

This can be achieved in the short term to medium term by;

- Increasing the real-time efforts to monitor *L. maculans, L. biglobosa* and *P. brassicae* ascospore release events to provide growers with reliable in-season data so that they can make accurate fungicide application decisions based on the relevant threat.

- Incorporation of the phoma stem canker (*L. maculans*) and light leaf spot risk forecasts to identify situations where there is a high *P. brassicae* inoculum concentration, a high green area index, on a *P. brassicae* susceptible cultivar and the environmental conditions that are favourable for *P. brassicae* infection and development, even if the *L. maculans* fungicide threshold is not met or is going to be met later in that season; then an application of fungicide should be prioritised for protection against *P. brassicae* rather than *L. maculans* in autumn.

- Revising the current advised fungicide application threshold (10% incidence of plants with *L. maculans* leaf spot lesions) to integrate the appearance of *L. biglobosa* leaf spot lesions to protect the crops from both *Leptosphaeria* spp. and consider the situations when plants are simultaneously exposed to ascospores of both *L. maculans* and *L. biglobosa*.

It is fully acknowledged that this strategy is not easily implemented and would require further research but the most effective, sustainable, and robust strategy for controlling all these three diseases would be the development of resistant cultivars that incorporate complete resistance, in a quantitative resistance background, against *L. maculans*, *L. biglobosa* and *P. brassicae*.

5.4   Summary of main findings in relation to epidemiology of *Leptosphaeria* spp. and *P. brassicae*

The main finding of this thesis was that the direct interspecific interactions between *Leptosphaeria* spp. may cause indirect interspecific interactions with *P. brassicae* and other oilseed rape pathogens. These newly identified direct interactions between the similar sibling species *L. maculans* and *L. biglobosa* when simultaneous infection may cause increased competition between them due to the similarities in their life cycle.

When such events occur the important temporal and spatial separation for niche differentiation and resource partitioning will not occur because of similar rates of *Leptosphaeria* spp. psuedotheical maturation that results in simultaneous *Leptosphaeria* spp. ascospore release events, Therefore, due to both pathogens' life cycles being reliant on ascospore infection events then these pathogens will be competing for the same resource. However, due to the loss of the temporal separation, *L. maculans* is not afforded the time to produce sirodesmin PL as a form of interference competition to provide the initial barrier to inhibit the growth of competing pathogens. Interestingly, this thesis found that *L. biglobosa* can inhibit the production of sirodesmin by *L. maculans* in simultaneous co-inoculation situations. Also, due to *L. biglobosa* having a faster rate of growth, it will be able to colonise the resource better so symptom-

less growth through the leaf petiole to the stem base via the hosts vascular system by *L. biglobosa* will be promoted. Additionally, because it is the faster growing of the two pathogens *L. biglobosa* has the advantage of becoming more established before triggering the induction of the plant's salicylic acid – induced defence response; this is responsible for controlling biotrophic pathogens, such as *L. maculans* and *P. brassicae,* as a form of host mediated competition.

Despite other competing pathogens, such as *P. brassicae*, *S. sclerortiorum* or *V. longisporum*, occupying different ecological niches interactions may arise in situations where *L. maculans* can produce sirodemsin PL when growing throughout the vascular system. Once in the vascular system, the fungitoxic secondary metabolite sirodesmin PL can be transported around the plant to many different uninfected plant tissues that other competing pathogens require, such as the roots, stem, new leaves, and pods. However, due to the polycyclic life cycle of *P. brassicae* and the efficacy of secondary conidial infection then there will be a far greater chance of a successful infection because of its ability to infect different tissues. This is particularly apparent when investigating pod infections because *Leptosphaeria* spp. pod infection is rare, whereas *P. brassicae* pod infections are more common, and are the most important factor for causing *P. brassicae* yield loss.

The findings that when co-inoculation of *L. maculans* and *L. bigobosa* occurs the lesion phenotypes are more like *L. biglobosa* than *L. maculans,* or that *Rlm7* can provide complete control of *L. maculans,* this may cause unforeseen and unintentional indirect effects on other pathogens because phoma (*L. maculans*) thresholds for fungicide applications may be met later or not at all. This means that fungicides applications may be made later which is important because fungicides are not just effective against *Leptosphaeria* spp., they are effective against other pathogens such as *P. brassicae*. Although, fungicide applications are recommended when *P. brassicae* symptoms (often sporulations) are observed, this is providing control against *P. brassicae* rain splashed secondary conidial infections. However, this study showed that ascospores of *Leptosphaeria* spp. and *P. brassicae* can all be released at the same time, so infections can occur throughout the autumn so a later fungicide application would leave crops

more vulnerable to *P. brassicae* ascospore and early conidial infection for longer. The severity of *P. brassicae* disease cycles are reliant on the number of disease cycles that can occur in a season. Therefore, if fungicide application is applied later or not at all in the autumn then this will allow an increased number of disease cycles to occur within the season than if a fungicide was applied earlier.

Therefore, an improved understanding that direct and indirect interspecific interactions between *Leptosphaeria maculans*, *L. biglobosa* and *P. brassicae* are related to the epidemiology of the pathogens would help to maximise the efficiency of controlling of these pathogens.

6.4 Conclusion

This investigation found that interspecific interactions exist between *L. maculans*, *L. biglobosa* and *P. brassicae.* In seasons when weather conditions are favourable to *P. brassicae* infection, effective control of *L. maculans* by fungicide and cultivar resistance may increase the vulnerability and susceptibility of crops to *L. biglobosa* and *P. brassicae.* The inclusion of winter oilseed rape cultivars with combined resistance to *L. maculans, L. biglobosa* and *P. brassicae* into a well informed and novel integrated control strategy should be what the UK oilseed rape stakeholders (growers, researchers, breeders, agronomists, agricultural consultants, and independent and governmental advisory organisations) strive towards together to reduce yield losses and increase the productively and profitability of UK oilseed rape, whilst implementing a strong fungicide and resistance management strategy to control these pathogens together, rather than in isolation.

References

Abdullah, A.S., Moffat, C.S., Lopez-Ruiz, F.J., Gibberd, M.R., Hamblin, J. and Zerihun, A. (2017) Host-multi-pathogen warfare: pathogen interactions in co-infected plants. Frontiers in Plant Science, 8, 1806.

ADAS, & BASF. (2009). The Encyclopaedia of Oilseed Rape Diseases. Available at: https://www.yumpu.com/en/document/read/11259087/the-encyclopaedia-of-oilseed-rape-diseases-basf-crop-protection- [Accessed 05/03/2022].

AHDB. (2018). Light leaf spot | AHDB. Disease Control Measures. https://ahdb.org.uk/lightleafspot [Accessed 05/03/2022].

AHDB. (2019). Fungicide performance 2019 update for oilseed rape. https://projectblue.blob.core.windows.net/media/Default/Imported%20Publication%20Docs/AHDB%20Cereals%20&%20Oilseeds/Disease/Fungicide%20performance/Fungicide%20performance%202019%20(6.12.19).pdf [Accessed 05/03/2022].

AHDB. (2020a). Phoma leaf spot and stem canker | AHDB. https://ahdb.org.uk/phoma [Accessed 14/09/2020].

AHDB. (2020b). Light leaf spot forecast | AHDB. https://ahdb.org.uk/light-leaf-spot-forecast [Accessed 14/09/2020].

AHDB. (2020c). Recommended List Archive | AHDB. https://ahdb.org.uk/rlarchive [Accessed 24/09/2021].

AHDB. (2021a). Spring planting and variety survey| https://ahdb.org.uk/cereals-oilseeds/planting-variety-survey-results [Accessed 24/09/2021].

AHDB. (2021b). Varietal resistance to feeding (herbivory) by the cabbage stem flea beetle (CSFB) in oilseed rape. https://ahdb.org.uk/varietal-resistance-to-feeding-herbivory-by-the-cabbage-stem-flea-beetle-csfb-in-oilseed-rape. [Accessed 24/09/21].

AHDB. (2021c). UK Delivered Prices| AHDB. https://ahdb.org.uk/cereals-oilseeds/uk-delivered-prices [Accessed 24/09/2021].

AHDB. (2021d). AHDB Recommended List. Winter oilseed rape 2021/22.|AHDB. https://projectblue.blob.core.windows.net/media/Default/Imported%20Publication%20Docs/AHDB%20Cereals%20&%20Oilseeds/Varieties/RL2021-22/15.%20Winter%20oilseed%20rape%20Recommended%20List%202021%E2%80%9322.pdf . [Accessed 26/10/21].

Aider, M. and Barbana, C. (2011). Canola proteins: Composition, extraction, functional properties, bioactivity, applications as a food ingredient and allergenicity - A practical and critical review. Trends in Food Science and Technology, 22, 21-39.

Aimé, S., Alabouvette, C., Steinberg, C. and Olivain, C. (2013) The endophytic strain Fusarium oxysporum Fo47: a good candidate for priming the defense responses in tomato roots. Molecular Plant-Microbe Interactions, 26, 918–926.

Aluko, R. E. and McIntosh, T. (2005). Limited enzymatic proteolysis increases the level of incorporation of canola proteins into mayonnaise. Innovative Food Science and Emerging Technologies, 6, 195-202.

Ashby, A. M. (1997). A molecular view through the looking glass: the *Pyrenopeziza brassicae-Brassica* Interaction. Advances in Botanical Research, 24, 31-70.

Aubertot, J. N., Pinochet, X. and Doré, T. (2004). The effects of sowing date and nitrogen availability during vegetative stages on *Leptosphaeria maculans* development on winter oilseed rape. Crop Protection, 23, 635-645.

Azevedo, M. M., Faria-Ramos, I., Cruz, L. C., Pina-Vaz, C. and Gonçalves Rodrigues, A. (2015). Genesis of azole antifungal resistance from agriculture to clinical settings. Journal of Agricultural and Food Chemistry, 63, 7463-7468.

Balesdent, M.H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M. and Rouxel, T. (2001) Genetic control and host range of avirulence towards *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans.* Phytopathology, 91, 70–76

Baierl, A., Evans, N., Steed, J. M., Fitt, B. D. L. and Sutherland, K. G. (2002). Effects of light leaf spot (*Pyrenopeziza brassicae*) infection on winter survival and yield of oilseed rape (*Brassica napus*). Proceedings Brighton Crop Protection Conference – Pests and Diseases 2022, 629-634.

Bailey, K. L. and Lazarovits, G. (2003). Suppressing soil-borne diseases with residue management and organic amendments. Soil and Tillage Research, 72, 169-180.

Barbetti, M. J. (1976). The role of pycnidiospores of *Leptosphaeria maculans* in the spread of blackleg disease in rape. Australian Journal of Experimental Agriculture, 16, 911-914.

Bayer Crop Science. (2019). Proline 275. https://cropscience.bayer.co.uk/our-products/fungicides/proline/ [Accessed 05/03/2022].

Bergmann, C. W., Ito, Y., Singer, D., Albersheim, P., Darvill, A. G., Benhamou, N. *et al.* (1994). Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris L.* in response to wounding, elicitors and fungal infection. The Plant Journal, 5, 625-634.

Berry, P. M. and Spink, J. H. (2009). Understanding the effect of a triazole with anti-gibberellin activity on the growth and yield of oilseed rape (*Brassica napus*). Journal of Agricultural Science, 147, 273-285.

Black WD (2020) A comparison of several media types and basic techniques used to assess outdoor airborne fungi in Melbourne, Australia. PLOS ONE 15: e0238901.

Bousset, L., Sprague, S. J., Thrall, P. H. and Barrett, L. G. (2018). Spatio-temporal connectivity and host resistance influence evolutionary and epidemiological dynamics of the canola pathogen *Leptosphaeria maculans*. Evolutionary Applications, 11, 1354–1370.

Boys, E. F., Roques, S. E., Ashby, A. M., Evans, N., Latunde-Dada, A. O., Thomas, J. E. *et al.* (2007). Resistance to infection by stealth: *Brassica napus* (winter oilseed rape) and *Pyrenopeziza brassicae* (light leaf spot). European Journal of Plant Pathology, 118, 307-321.

Boys, E. F., Roques, S. E., West, J. S., Werner, C. P., King, G. J., Dyer, P. S. and Fitt, B. D. L. (2012). Effects of *R* gene-mediated resistance in *Brassica napus* (oilseed rape) on asexual and sexual sporulation of *Pyrenopeziza brassicae* (light leaf spot). Plant Pathology, 61, 543-554.

Brachaczek, A., Kaczmarek, J. and Jedryczka, M. (2016). Monitoring blackleg (*Leptosphaeria* spp.) ascospore release timing and quantity enables optimal fungicide application to improve oilseed rape yield and seed quality. European Journal of Plant Pathology. 145, 643-657.

Bradburne, R., Majer, D., Magrath, R., Werner, C. P., Lewis, B. and Mithen, R. (1999). Winter oilseed rape with high levels of resistance to *Pyrenopeziza brassicae* derived from wild *Brassica* species. Plant Pathology, 44, 550-558.

Brent, K. J., & Hollomon, D. W. (2007). Fungicide Resistance in Plant Management: How can it be managed?. Fungicide Resistance Action Committee 2007.

Brun, H., Chèvre, A. M., Fitt, B. D.L., Powers, S., Besnard, A. L., Ermel, M. *et al*. (2010). Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytologist, 185, 285-299

Burcon. (2019a). Puratein - Burcon. https://www.burcon.ca/products/canola-proteins/puratein/ [Accessed 23/07/2019].

Burcon. (2019b). Supertein - Burcon. https://www.burcon.ca/products/canola-proteins/supertein/ [Accessed 23/07/2019].

Cai, X., Huang, Y., Jiang, D., Fitt, B. D. L., Li, G. and Yang, L. (2018). Evaluation of oilseed rape seed yield losses caused by *Leptosphaeria biglobosa* in central China. European Journal of Plant Pathology, 150, 179–190.

CABI. (2022a). Leptosphaeria maculans – Phoma stem canker . - https://www.cabi.org/isc/datasheet/31468 [Accessed 02/07/2022]

CABI (2022b) – Leptosphaeria biglobosa – Phoma stem canker. - https://www.cabi.org/isc/datasheet/109525 [Accessed 02/07/2022]

CABI (2022c) – Pyrenopeziza brassicae – Light leaf spot - https://www.cabi.org/isc/datasheet/46123 [Accessed 02/07/2022]

CABI (2022d) – Verticillium longisporum – Verticilium stem stripe - https://www.cabi.org/isc/datasheet/108100 [Accessed 02/07/2022]

CABI (2022e) – Sclerotinia sclerotiorum – Cottony stem rot - https://www.cabi.org/isc/datasheet/49124 [Accessed 02/07/2022]

CABI (2022f) – Alternaria brassicicola – dark leaf spot of cabbage - https://www.cabi.org/isc/datasheet/4483 [Accessed 02/07/2022]

Carlson, A. S., Yilmaz, J. L., Green, A. G., Stymne, S. and Hofvander, P. (2011). Replacing fossil oil with fresh oil - with what and for what? European Journal of Lipid Science and Technology, 113, 812–831.

Carlson, K. M., Curran, L. M., Asner, G. P., Pittman, A. M. D., Trigg, S. N. and Marion Adeney, J. (2013). Carbon emissions from forest conversion by Kalimantan oil palm plantations. Nature Climate Change, 3, 283-287.

Carmody, S. M., King, K. M., Ocamb, C. M., Fraaije, B. A., West, J. S. and du Toit, L. J. (2019). A phylogenetically distinct lineage of *Pyrenopeziza brassicae* associated with chloratic leaf spot of *Brassiaceae* in North America. Journal of Chemical Information and Modelling, 53, 1689–1699.

Carter, H. E., Cools, H. J., West, J. S., Shaw, M. W. and Fraaije, B. A. (2013). Detection and molecular characterisation of *Pyrenopeziza brassicae* isolates resistant to methyl benzimidazole carbamates. Pest Management Science. 69, 1040-1048.

Carter, H. E., Fraaije, B. A., West, J. S., Kelly, S. L., Mehl, A., Shaw, M. W., & Cools, H. J. (2014). Alterations in the predicted regulatory and coding regions of the sterol 14α-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae.* Molecular Plant Pathology, 15, 513-22

Chai, C. L. L. and Waring, P. (2000). Redox sensitive epidithiodioxopiperazines in biological mechanisms of toxicity. Redox Report, 5, 257-64.

Chen, G. L., Zhang, B., Wu, J. G. and Shi, C. H. (2011). Non-destructive assessment of amino acid composition in rapeseed meal based on intact seeds by near-infrared reflectance spectroscopy. Animal Feed Science and Technology, 165, 11-119.

CropMonitor. (2020a). CropMonitor. http://www.cropmonitor.co.uk/?_ga=2.42957989.1876731776.1582020773-1696467554.1575973747 [Accessed 09/07/2021].

Curtis, P. J., Greatbanks, D., Hesp, B., Cameron, A. F. and Freer, A. A. (1977). Sirodesmins A, B, C, and G, antiviral epipolythiopiperazine-2,5-diones of fungal origin: X-ray analysis of sirodesmin a diacetate. Journal of the Chemical Society, Perkin Transactions 1, 180–189.

D'Amour, C. B., Reitsma, F., Baiocchi, G., Barthel, S., Güneralp, B., Erb, K. H. *et al.* (2017). Future urban land expansion and implications for global croplands. Proceedings of the National Academy of Sciences of the United States of America. 114, 8939-8944.

Dangl, J. L., Horvath, D. M. and Staskawicz, B. J. (2013). Pivoting the plant immune system from dissection to deployment. Science. 341, 746-751.

Dawidziuk, A., Kasprzyk, I., Kaczmarek, J. and Jędryczka, M. (2010). Pseudothecial maturation and ascospore release of *Leptosphaeria maculans* and *L. biglobosa* in south-east Poland. Acta Agrobotanica. 63, 107-120.

DEFRA. (2019a). Agriculture in the United Kingdom data sets - GOV.UK. https://www.gov.uk/government/statistical-data-sets/agriculture-in-the-united-kingdom [Accessed 19/12/2019].

DEFRA. (2019b). Farming Statistics – provisional arable crop areas at 1 June 2019 England. www.statistics.gov.uk. [Accessed 19/12/2019].

De Gruyter, J., Woudenberg, J. H. C., Aveskamp, M. M., Verkley, G. J. M., Groenewald, J. Z. and Crous, P. W. (2013) Redisposition of Phoma-like anamorphs in Pleosporales. Studies in mycology, 75, 1-36.

Delgado, C. L. (2003). Rising consumption of meat and milk in developing countries has created a new food revolution. The Journal of Nutrition. 133, 3907S-3910S.

Delourme, R., Chèvre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., Dias. *et al.* (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). European Journal of Plant Pathology, 114, 41–52.

DSV (2021) Dart oilseed rape. https://www.dsv-uk.co.uk/sorte/5998 [Accessed 10/10/2021].

Dubois, V., Breton, S., Linder, M., Fanni, J. and Parmentier, M. (2007). Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. European Journal of Lipid Science and Technology 109, 710-732.

Dutt, A., Andrivon, D. and Le May, C. (2021) Multiple-infections, competitive interactions, and pathogen co-existence. Plant Pathology, 71, 5-22.

Eckert, M. R., Rossall, S., Selley, A., & Fitt, B. D. L. (2010). Effects of fungicides on *in vitro* spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans*

and *L. biglobosa* (phoma stem canker of oilseed rape). Pest Management Science, 66, 396-405.

El-Beltagi, H.E.D.S, & Mohamed, A. A. (2010). Variations in fatty acid composition, glucosinolate profile and some phytochemical contents in selected oil seed rape (*Brassica napus* L.) cultivars. Grasas y Aceites. 61, 143-150.

Elliott, C. E., Gardiner, D. M., Thomas, G., Cozijnsen, A., Van De Wouw, A. and Howlett, B. J. (2007). Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus*. Molecular Plant Pathology, 8, 791-802.

Elliott, C.E., Fox, E. M., Jarvis, R. S. and Howlett, B. J. (2011). The cross-pathway control system regulates production of the secondary metabolite toxin, sirodesmin PL, in the ascomycete, *Leptosphaeria maculans*. BMC Microbiololgy, 11, 169.

EU, 2009/128/EC. (2009). Establishing a framework for Community action to achieve the sustainable use of pesticides. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0071:0086:en:PDF#:~:text=This%20Directive%20establishes%20a%20framework,non%2Dchemical%20alternatives%20to%20pesticides.&text=1.,-This%20Directive%20shall [Accessed 24/09/2021].

EU, 2013/485/EC (2013). Commission implementing regulation (EU) No 485/2013 of 24 may 2013 amending Implementing Regulation (EU) No 540?2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances. - https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:139:0012:0026:EN:PDF – [Accessed 24/09/2021].

EU, 2017/1455/EC. (2017). Commission implementing regulation (EU) 2017/ 1455 - of 10 August 2017 - concerning the non-renewal of approval of the active substance picoxystrobin, in accordance with Regulation (EC) No 1107 / 2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) No 540/2011. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R1455&from=EN [Accessed 24/09/2021].

Evans, N., Baierl, A., Brain, P., Welham, S. J. and Fitt, B. D. L. (2003). Spatial aspects of light leaf spot (*Pyrenopeziza brassicae*) epidemic development on winter oilseed rape (*Brassica napus*) in the United Kingdom. Phytopathology. 93, 657-665.

Evans, N., Ritchie, F., West, J., Havis, N., Matthewman, C. and Maguire, K. (2017). Investigating components of the oilseed rape light leaf spot epidemic responsible for increased yield loss to the UK Arable Industry. AHDB. Report number. PR587.

FAO. (2009). How to Feed the World in 2050. http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf [Accessed 05/03/2022].

Farmers Weekly. (2015). West: Oilseed rape drilling delayed after late harvest - Farmers Weekly. https://www.fwi.co.uk/arable/west-oilseed-rape-drilling-delayed-late-harvest [Accessed 05/03/2022].

Farmers Weekly. (2016). Tips on beating beetles in OSR - Farmers Weekly. https://www.fwi.co.uk/arable/osr/cereals-2016-tips-beating-beetles-osr [Accessed 05/03/2022]

Farmers Weekly. (2019). Oilseed rape prices hold steady on smaller crop – Farmers Weekly. https://www.fwi.co.uk/business/markets-and-trends/crop-prices/oilseed-rape-prices-hold-steady-on-smaller-crop [Accessed 05/03/2022]

FDA (2016). FDA: GRAS Notification: the use of canola/rapeseed protein isolate as a nutritive and function ingredient in human food. Available at: https://www.fda.gov/media/103212/download [Accessed 05/03/2022]

Fera. (2020a). Fera - Winter oilseed rape case study - Decision Support - Local. https://map.cropmonitor.co.uk/map/pestcharts [Accessed 05/03/2022].

Fera. (2021). Cropmonitor survey graphs. https://secure.fera.defra.gov.uk/cropmonitor/wosr/surveys/surveygraphs/index.cfm [Accessed 05/03/2022].

Ferguson, J. J. A., Stojanovski, E., MacDonald-Wicks, L. and Garg, M. L. (2016). Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs. Progress in Lipid Research, 64, 16-29.

Figueroa, L., Fitt, B. D. L., Welham, S. J., SHAW, M. W. and McCartney, H. A. (1995). Early development of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) in relation to temperature and leaf wetness. Plant Pathology, 44, 641-654.

Fitt, B. D. L., Doughty, K. J., Gladders, P., Steed, J. M. and Sutherland, K. G. (1998). Diagnosis of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) in the UK. Annals of Applied Biology, 133, 155-166.

Fitt, B. D. L., Huang, Y.-J., Van Den Bosch, F. and West, J. S. (2006a). Coexistence of related pathogen species on arable crops in space and time. Annual Review of Phytopathology, 44, 163-82.

Fitt, B. D. L., Brun, H., Barbetti, M. J. and Rimmer, S. R. (2006b). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed Rape (*Brassica napus*). European Journal of Plant Pathology, 114, 3-15.

Fitt, B. D. L., Hu, B. C., Li, Z. Q., Liu, S. Y., Lange, R. M., Kharbanda, P. D. *et al*. (2008). Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits. Plant Pathology. 57, 652-664.

Flor, H. H. (1971). Current status of the gene-for-gene concept. Annual Review of Phytopathology. 9, 275-296.

Flores-Jiménez, N. T., Ulloa, J. A., Silvas, J. E. U., Ramírez, J. C. R., Ulloa, P. R., Rosales, P. *et al.* (2019). Effect of high-intensity ultrasound on the compositional, physicochemical, biochemical, functional and structural properties of canola *(Brassica napus L.*) protein isolate. Food Research International. 121, 947-956.

Fortune, J.A., Qi, A. Ritchie, F., Karandeni Dewage, C.S., Fitt, B.D.L. and Huang, Y. J. (2021) Effects of cultivar resistance and fungicide application on stem canker of oilseed rape (*Brassica napus*) and potential interseasonal transmission of *Leptosphaeria* spp. inoculum. Plant Pathology. 70, 2115-2124.

Fox, E. M., & Howlett, B. J. (2008). Biosynthetic gene clusters for epipolythiodioxopiperazines in filamentous fungi. Mycological Research, 112, 162-169.

FRAG-UK. (2017). Fungicide Resistance Management in Oilseed Rape. https://media.ahdb.org.uk/media/Default/Imported%20Publication%20Docs/AHDB%20Cereals%20&%20Oilseeds/Disease/Fungicide%20resistance%20management%20in%20oilseed%20rape%20(2017).pdf [Accessed 05/03/2022].

Gardiner, D. M., Cozijnsen, A. J., Wilson, L. M., Pedras, M. S. C. and Howlett, B. J. (2004). The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans.* Molecular Microbiology, 53, 1307-1318.

Gardiner, D. M., Waring, P. and Howlett, B. J. (2005). The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. Microbiology. 151, 1021-1032.

Garthwaite, D. G., Hudson, S., Barker, I., Parrish, G., Smith, L. and Pietravalle, S. (2012). Pesticide Usage Survey Report 250: Arable Crops in the United Kingdom 2012 (including aerial applications 2012). 1–89. http://www.ons.gov.uk/ons/index.html

Garthwaite, D., Barker, I., Laybourn, R., Huntly, A., Parrish, G. P., Hudson, S. and Thygesen, H. (2014). PESTICIDE USAGE SURVEY REPORT 263 ARABLE CROPS IN THE UNITED KINGDOM 2014. https://pusstats.fera.co.uk/upload/kxZtSlyLVGQl5Wj0oFpHmPtX3PI3i6ZOF7L8Ep8Q.pdf [Accessed 05/03/2022].

Garthwaite, D., Barker, I., Ridley, L., Mace, A., Parrish, G., MacArthur, R. and Lu, Y. (2016). PESTICIDE USAGE SURVEY REPORT 271 ARABLE CROPS IN THE UNITED KINGDOM 2016.

https://pusstats.fera.co.uk/upload/M2XiV6STmN8rwZJfPZ6OAH6l6dYlfFMDDJYvfto9.pdf [Accessed 05/03/2022].

Garthwaite, D. G., Barker, I., Parrish, G., Smith, C., Chippindale, C. and Pietravalle, S. (2018). Pesticide usage survey report 284: arable crops in the United Kingdom. https://pusstats.fera.co.uk/upload/6zUxOeKmvvo0IECrlhACY6poov1U2g4IXEmOmclq.pdf [Accessed 05/03/2022].

Garthwaite, D. G., Mace, A., Stroda, E., Parrish, G., Rainford, J. and MacArthur, D. (2020). Pesticide usage survey report 295: arable crops in the United Kingdom. https://pusstats.fera.co.uk/upload/fERxvRulVrwRsvVQVIgL3nDvXfW3oxvLeiISwzGA.pdf [Accessed 10/07/2022].

Gausse GF. 1934. The struggle for existence. Baltimore: Williams and Wilkins. Reprinted in 2003 as Dover Phoenix Editions, Dover Publ.

Gerz, M., Bueno, C. G., Ozinga, W.A., Zobel, M. and Moora, M. (2018) Niche Differentiation and expansion of plant species are associated with mycorrhizal symbiosis. Journal of Ecology, 106, 254-264.

Gilles, T., Fitt, B. D. L., Kennedy, R., Welham, S. J. and Jeger, M. J. (2000). Effects of temperature and wetness duration on conidial infection, latent period and asexual sporulation of *Pyrenopeziza brassicae* on leaves of oilseed rape. Plant Pathology. 49, 498-508.

Gilles, Tijs, Evans, N., Fitt, B. D. L. and Jeger, M. J. (2000). Epidemiology in relation to methods for forecasting light leaf spot (*Pyrenopeziza brassicae*) severity on winter oilseed rape (*Brassica napus*) in the UK. European Journal of Plant Pathology, 106, 593-605.

Gilles, T., Fitt, B. D. L. and Jeger, M. J. (2001a). Effects of environmental factors on development of *Pyrenopeziza brassicae* (light leaf spot) apothecia on oilseed rape debris. Phytopathology, 91, 392-398

Gilles, T., Fitt, B. D. L., McCartney, H. A., Papastamati, K. and Steed, J. M. (2001b). The roles of ascospores and conidia of *Pyrenopeziza brassicae* in light leaf spot epidemics on winter oilseed rape (*Brassica napus*) in the UK. Annals of Applied Biology, 138, 141-152.

Gilles, T., Ashby, A. M., Fitt, B. D. L. and Cole, T. (2001c). Development of *Pyrenopeziza brassicae* apothecia on agar and oilseed rape debris. Mycological Research, 105, 705-714.

Gilles, T., Fitt, B. D. L., Welham, S. J., Evans, N., Steed, J. M. and Jeger, M. J. (2001d). Modelling the effects of temperature and wetness duration on development of light leaf spot on oilseed rape leaves inoculated with *Pyrenopeziza brassicae* conidia. Plant Pathology, 50, 42-52.

Gladders, P, and Musa, T. M. (1980). Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. Plant Pathology. 29, 28-37.

Gladders, P., Freer, B., Green, M., Hardwick, N.V., Jones, O.W. and Sutherland, K.G. (1998). Roles of varieties and fungicides in managing light leaf spot and canker in winter oilseed rape. HGCA. Project Report no. OS28.

Gladders, P., Symonds, B. V., Hardwick, N. V. and Sansford, C. E. (1998). Opportunities to control canker (*Leptosphaeria maculans*) in winter oilseed rape by improved spray timing. International Organisation for Biological Control Bulletin 21, 111-120.

Gladders, P., Evans, N., Marcroft, S. and Pinochet, X. (2006). Dissemination of information about management strategies and changes in farming practices for the exploitation of resistance to *Leptosphaeria maculans* (phoma stem canker) in oilseed rape cultivars. European Journal of Plant Pathology, 114, 117-126.

Grandaubert, J., Lowe, R. G., Soyer, J. L., Schoch, C. L., Van de Wouw, A. P., Fudal, I., Robbertse, B., Lapalu, N., Links, M. G., Ollivier, B. and Linglin, J. (2014) Transposable elements-assisted evolution and adaption to host plant within the Leptosphaeria maculans- Leptosphaeria biglobosa species complex of fungal pathogens. BMC genomics, 15, 891-918.

Grover, J. P. (1997). Resource competition. Chapman & Hall.

Haddadi, P., Larkan, N. J., Van de Wouw, A., Zhang, Z., Neik, T. X., Beynon, E., Bayer, P., Edwards, D., Batley, J. and Borhan, M. H. (2022) Brassica napus genes Rlm4 and Rlm7, conferring resistance to Leptosphaeria maculans, are alleles of the Rlm9 wall-associated kinase-like resistance locus. Plant Biotechnology Journal. 20, 1229-1231.

Hald, C., Dawid, C., Tressel, R. and Hofmann, T. (2019). Kaempferol 3- O-(2- O-Sinapoyl-β-sophoroside) causes the undesired bitter taste of canola/rapeseed protein isolates. Journal of Agricultural and Food Chemistry, 67, 372-378.

Hall, R., Chigogora, J. L. and Phillips, L. G. (1996). Role of seedborne inoculum of *Leptosphaeria maculans* in development of blackleg on oilseed rape. Canadian Journal of Plant Pathology, 18, 35-42.

Hammond, K. E., Lewis, B. G. and Musa, T. M. (1985). A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. Plant Pathology, 34, 557-565.

Hardin, G. (1960). The competitive exclusion principle. Science, 131, 1292–1297.

Henchion, M., McCarthy, M., Resconi, V. C., & Troy, D. (2014). Meat consumption: trends and quality matters. Meat Science. 98, 561-568.

Henchion, M., Hayes, M., Mullen, A., Fenelon, M., & Tiwari, B. (2017). Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. Foods, 6, 53.

Hillocks, R.J. (2012) Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. Crop Protection, 31, 85–93.

Hortal, S., Powell, J. R., J. Plett, M., Simonin, A. and Anderson, I.C. (2016) Intraspecific competition between ectomycorrhizal *Pisolithus microcarpus* isolates impacts plant and fungal performance under elevated CO_2 and temperature. *FEMS Microbiology Ecology*, 92, fiw113,

Hossard, L., Souchere, V., & Jeuffroy, M. H. (2018). Effectiveness of field isolation distance, tillage practice, cultivar type and crop rotations in controlling phoma stem canker on oilseed rape. Agriculture, Ecosystems and Environment. 252, 30-41.

Howlett, B. J., Fox, E. M., Cozijnsen, A. J., Van de Wouw, A. P., & Elliott, C. E. (2009). The secondary metabolite toxin, sirodesmin PL, and its role in virulence of the blackleg fungus. The Role of Plant Pathology in Food Safety and Food Security, 8, 791-802.

HSE. (2020).GB PIC Regulatory Outcome information for Listed Chemicals; Chemical summary information on final regulatory action taken by the exporting country. https://www.hse.gov.uk/pic/assets/docs/pic-regulatory-outcome-listed-chemicals.pdf [Accessed 05/03/2022]

Huang, Y. J., Toscano-Underwood, C., Fitt, B. D. L., Todd, A. D., West, J. S., Koopmann, B. and Balesdent, M. H. (2001). Effects of temperature on germination and hyphal growth from ascospores of A-group and B-group *Leptosphaeria maculans* (phoma stem canker of oilseed rape). Annals of Applied Biology, 139, 193-207.

Huang, Y. J., Toscano-Underwood, C., Fitt, B. D. L., Hu, X. J. and Hall, A. M. (2003). Effects of temperature on ascospore germination and penetration of oilseed rape (*Brassica napus)* leaves by A- or B-group *Leptosphaeria maculans* (phoma stem canker). Plant Pathology, 52, 245-255.

Huang, Y. J., Fitt, B. D. L., Jedryczka, M., Dakowska, S., West, J. S., Gladders, P. et al. (2005). Patterns of ascospore release in relation to phoma stem canker epidemiology in England (*Leptosphaeria maculans)* and Poland (*Leptosphaeria biglobosa*). European Journal of Plant Pathology, 111, 263-277.

Huang, Y. J., Li, Z. Q., Evans, N., Rouxel, T., Fitt, B. D. L. and Balesdent, M. H. (2006). Fitness cost associated with loss of the *AvrLm4* avirulence function in *Leptosphaeria maculans* (phoma stem canker of oilseed rape). European Journal of Plant Pathology, 114, 77-89.

Huang, Y. J., Liu, Z., West, J. S., Todd, A. D., Hall, A. M. and Fitt, B. D. L. (2007). Effects of temperature and rainfall on date of release of ascospores of *Leptosphaeria maculans*

(phoma stem canker) from winter oilseed rape (*Brassica napus*) debris in the UK. Annals of Applied Biology, 151, 99-111.

Huang, Y. J., Pirie, E. J., Evans, N., Delourme, R., King, G. J. and Fitt, B. D. L. (2009). Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). Plant Pathology, 58, 314–323.

Huang, Y. J., Hood, J. R., Eckert, M. R., Stonard, J. F., Cools, H. J., King, *et al.* (2011). Effects of fungicide on growth of *Leptosphaeria maculans* and L*. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). Plant Pathology, 60, 607-620.

Huang, Y.J., Qi, A., King, G. J. and Fitt, B. D. L. (2014a). Assessing Quantitative resistance against *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) in young plants. PLoS ONE, 9, e84924.

Huang, Y J, Karandeni-Dewage, S. and Fitt, B. D. L. (2014b). Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. Aspects of Applied Biology, 127, 117-122.

Huang, Y. J., Mitrousia, G. K., Sidique, S. N. M., Qi, A. and Fitt, B. D. L. (2018). Combining R gene and quantitative resistance increases effectiveness of cultivar resistance against *Leptosphaeria maculans* in *Brassica napus* in different environments. PLoS ONE. 13, e0197752.

Huang, Y.J., Paillard, S., Kumar, V., King, G. J., Fitt, B. D. L., & Gine Delourme, R. (2019). Oilseed rape (*Brassica napus*) resistance to growth of *Leptosphaeria maculans* in leaves of young plants contributes to quantitative resistance in stems of adult plants. PLoS ONE, 14, e0222540.

Huang, Y., Javaid, A. & Fitt, B.D.L. (2019) Understanding risks of severe phoma stem canker caused by *Leptosphaeria biglobosa* on winter oilseed rape in the UK. AHDB. Project Report No. 615

Huang, S., Zhang, X. and Fernando, D. (2020) Directing Trophic Divergence in Plant-pathogen interactions: Antagonistic phytohormones with no doubt?. Frontiers. Plant Sci. 11:600063

Huang, Y., Karandeni Dewage, C. S. and Fitt, B., 2021, *Management of Diseases and Pests of Oilseed Rape.* Jellis, G. J. & Fitt, B. D. L. (eds.) Current understanding of phoma stem canker and light leaf spot in oilseed rape in the UK., 1-12.

Javaid, A., Huang, Y. and Fitt, B. D. L. (2019). Importance of *Leptosphaeria maculans* and *L. biglobosa* in causing phoma stem canker on winter oilseed rape in England. PhD thesis, University of Hertfordshire, Hatfield.

Jeffery, D. C., Jenkins, P.D. and Gareth Jones, D. (1994). Comparative studies of light leaf spot (*Pyrenopeziza brassicae*) epidemics on the growth and yield of winter oilseed rape. Annals of Applied Biology 124, 19-25.

Jenks, M. A., Joly, R. J., Peters, P. J., Rich, P. J., Axtell, J. D. and Ashworth, E. N. (1994). Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L.) Moench. Plant Physiology, 105, 1239–1245.

Jennings, D. H., and Lysek, G. (1996). Fungal Biology: Understanding the Fungal Lifestyle. Bios Scientific Publishers Ltd.

Johnson, R.D. and Lewis BG, 1994. Variation in host range, systemic infection and epidemiology of *Leptosphaeria maculans*. Plant Pathology, 43, 269–77

Juroszek, P., and Von Tiedemann, A. (2011). Potential strategies and future requirements for plant disease management under a changing climate. Plant Pathology, 60, 100-112.

Kaczmarek, J., Jedryczka, M., Fitt, B. D. L., Lucas, J. A., and Latunde-Dada, A. O. (2009). Analyses of air samples for ascospores of *Leptosphaeria maculans* and *L.biglobosa* by light microscopy and molecular techniques. Journal of Applied Genetics, 50, 411–419.

Karandeni Dewage, C.S., Stotz, H.U. and Fitt, B.D.L. (2019). Understanding oilseed rape (Brassica napus) resistance against *Pyrenopeziza brassicae* for control of light leaf spot. PhD thesis, University of Hertfordshire, Hatfield.

Karandeni-Dewage, C. S. ., Klöppel, C. A., Stotz, H. U., & Fitt, B. D. L. (2018). Host-pathogen interactions in relation to management of light leaf spot disease (caused by *Pyrenopeziza brassicae)* on *Brassica* species. Crop and Pasture Science. 69, 9-19.

Karandeni Dewage, C.S., Qi, A., Stotz, H.U., Huang, Y.J., Fitt, B.D.L. (2021). Interactions in the *Brassica napus–Pyrenopeziza brassicae* pathosystem and sources of resistance to *P. brassicae* (light leaf spot). Plant Pathololgy, 70, 2104-2114.

Karolewski, Z. 1999. The occurrence of light leaf spot on winter oilseed rape in Western Poland in 1991–96 and the characteristics of *Pyrenopeziza brassicae* isolates. *Phytopathologia Polonica,* 18, 113– 21.

Karolewski, Z., Evans, N., Fitt, B.D.L., Baierl, A., Todd, A.D., Foster, S.J. (2004). Comparative epidemiology of *Pyrenopeziza brassicae* (light leaf spot) ascospores and conidia from Polish and UK populations. Plant Pathology, 53, 29–37.

Karolewski Z, Fitt BDL, Latunde-Dada AO, Foster SJ, Todd AD, Downes K, Evans N (2006) Visual and PCR assessment of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) cultivars. Plant Pathology, 55, 387–400.

Kerdraon, L., Barret, M., Balesdent, M.-H., Suffert, F. & Laval, V. (2020) Impact of a resistance gene against a fungal pathogen on the plant host residue microbiome: the case of the *Leptosphaeria maculans–Brassica napus* pathosystem. Molecular Plant Pathology, 21, 1545– 1558.

Khangura, R., Speijers, J., Barbetti, M. J., Salam, M. U., & Diggle, A. J. (2007). Epidemiology of blackleg (*Leptosphaeria maculans*) of canola (*Brassica napus*) in relation to maturation of pseudothecia and discharge of ascospores in Western Australia. Phytopathology, 97, 1011-1021

King, K. M., Krivova, V., Canning, G. G. M., Hawkins, N. J., Kaczmarek, A. M., Perryman, S. A. M. *et al.* (2018). Loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Pyrenopeziza brassicae* (light leaf spot of brassicas). Plant Pathology. 67, 167-174

King, K.M., Bucur, D.E., Ritchie, F., Hawkins, N.J., Kaczmarek, A.M., Duan, Y., Kildea, S., West, J.S. and Fraaije, B.A. (2021), Fungicide resistance status and chemical control options for the brassica pathogen *Pyrenopeziza brassicae*. Plant Pathology, 70, 2086-2103.

Kozanitas, M., Osmundson, T. W., Linzer, R., and Garbelotto, M. (2017). Interspecific interactions between the Sudden Oak Death pathogen *Phytophthora ramorum* and two sympatric *Phytophthora* species in varying ecological conditions. Fungal Ecology, 28, 86–96.

Kremer, A. and Li, S.M. (2010) A tyrosine O-prenyltransferase catalyses the first pathway-specific step in the biosynthesis of sirodesmin PL. Microbiology, 156, 278–286.

Krupinsky, J. M., Bailey, K. L., McMullen, M. P., Gossen, B. D. and Turkington, K.T. (2002). Managing plant disease risk in diversified cropping systems. Agronomy Journal. 94, 198-209.

Kutcher, H. R., Warland, J. S., and Brandt, S. A. (2010). Temperature and precipitation effects on canola yields in Saskatchewan, Canada. Agricultural and Forest Meteorology. 150, 161-165.

Lacey, M. E., Rawlinson, C. J. and McCartney, H. A. (1987). First record of the natural occurrence in England of the teleomorph of *Pyrenopeziza brassicae* on oilseed rape. Transactions of the British Mycological Society. 89, 135-140.

Lacey, M. E., West, J.S (2006) The Air Spora. Springer, Boston, MA, USA
Lancashire, P. D., Bleiholder, H., Boom, T. van den, Langeluddeke, P., Stauss, R., Weber, E., and Witzenberger, A. (1991). A uniform decimal code for growth stages of crops and weeds. Annals of Applied Biology, 119, 561–601.

Larkan, N. J., Yu, F., Lydiate, D. J., Rimmer, S. R. and Borhan, M. H. (2016) Single *R* gene introgression lines for accurate dissection of the *Brassica-Leptosphaeria* pathosystem. Frontiers in Plant Science. 7:17771

Lemtiri, A., Degrune, F., Barbieux, S., Hiel, M. P., Chélin, M., Parvin, N., *et al.* (2016). Crop residue management in arable cropping systems under temperate climate. Part 1: Soil biological and chemical (phosphorus and nitrogen) properties. A review. Biotechnology, Agronomy and Society and Environment, 20, 236-244.

Li, D., Ashby, A. M. and Johnstone, K. (2003). Molecular evidence that the extracellular cutinase Pbc1 is required for pathogenicity of *Pyrenopeziza brassicae* on oilseed rape. Molecular Plant-Microbe Interactions, 6, 545-552.

Link, K. P. and Walker, J. C. (1929). The isolation of protocatechuic acid from pigmented onion scales and its significance in relation to disease resistance in onions. Journal of Biological Chemistry, 81, 369–375.

Liu, S. Y., Liu, Z., Fitt, B. D. L., Evans, N., Foster, S. J., Huang, Y. J. *et al.* (2006). Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. Plant Pathology, 55, 401–412.

Liu, S., Liu, R., Latunde-Dada, A. O., Cools, H. J., Foster, S. J., Huang, Y. and Fitt, B. D. L. (2007). Comparison of *Leptosphaeria biglobosa*-induced and chemically induced systemic resistance to *L. maculans* in *Brassica napus.* Chinese Science Bulletin, 52, 1053–1062.

Liu, Z., Latunde-Dada, A. O., Hall, A. M. and Fitt, B. D. L. (2014). Phoma stem canker disease on oilseed rape (*Brassica napus*) in China is caused by *Leptosphaeria biglobosa 'brassicae.'* European Journal of Plant Pathology, 140, 841-857.

Liu, L., Sonbol, F-M., Huot, B., Gu, Y., Withers, J., Mwimba, M., Yao, J., Yang He, S. and Dong, X. (2016) Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector triggered immunity. Nature communications. 7:13099.

Lô-Pelzer, E., Aubertot, J. N., Bousset, L., Pinochet, X. and Jeuffroy, M. H. (2009). Phoma stem canker (*Leptosphaeria maculans/L. biglobosa*) of oilseed rape (*Brassica napus*): Is the G2 disease index a good indicator of the distribution of observed canker severities? European Journal of Plant Pathology, 125, 515-522.

MacArthur, R. and Levins, R. (1967) The limiting similarity, convergence, and divergence of coexisting species. American Naturalist. 101, 377-385

Maddock, S. E. and Ingram, D.S. (1981) Studies of survival and longevity of the light leaf spot pathogen of brassicas, *Pyrenopeziza brassicae*. Transactions of the British Mycological Society. 1, 153-159.

Majer, D., Lewis, B. G. and Mithen, R. (1998) Genetic variation among field isolates of *Pyrenopeziza brassicae*. Plant Pathology. 47, 22-28.

Mahuku, G. S., Goodwin, P. H. and Hall, R. (1995) A competitive polymerase chain reaction to quantify DNA of *Leptosphaeria maculans* during blackleg development in oilseed rape. Molecular Plant-Microbe Interactions, 8, 761-767.

Mahuku, G. S., Hall, R. and Goodwin, P. H. (1996). Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oilseed rape. Physiological and Molecular Plant Pathology, 49, 61-72.

Makkar, H. P. S. and Ankers, P. (2014). Towards sustainable animal diets: A survey-based study. Animal Feed Science and Technology, 198, 309-322.

Malhi, S. S., Johnston, A. M., Schoenau, J. J., Wang, Z. H. and Vera, C. L. (2007). Seasonal biomass accumulation and nutrient uptake of canola, mustard, and flax on a black chernozem soil in Saskatchewan. Journal of Plant Nutrition, 30, 641–658.

Marcroft, S. J., Sprague, S. J., Pymer, S. J., Salisbury, P. A. and Howlett, B. J. (2004). Crop isolation, not extended rotation length, reduces blackleg (*Leptosphaeria maculans)* severity of canola (*Brassica napus*) in south-eastern Australia. Australian Journal of Experimental Agriculture, 44, 601-606.

McCartney, H. A. and Lacey, M. E. (1990). The production and release of ascospores of *Pyrenopeziza brassicae* on oilseed rape. Plant Pathology, 39, 17-32.

McDonald, B. A. and Linde, C. (2002). The population genetics of plant pathogens and breeding strategies for durable resistance. Euphytica, 124, 163-180.

McGrann, G. R. D., Stavrinides, A., Russell, J., Corbitt, M. M., Booth, A., Chartrain, L., Thomas, W. T. B. and Brown, J. K. M. (2014). A trade-off between mlo resistance to powdery mildew and increased susceptibility of barley to a newly important disease, Ramularia leaf spot. Journal of Experimental Botany, 65, 1025–1037.

Mendes-Pereira, E., Balesdent, M. H., Brun, H. and Rouxel, T. (2003). Molecular phylogeny of the Leptosphaeria maculans -L. biglobosa species complex. Mycological research, 107, 1287-1304.

Middleton, M. C. (1974). The involvement of the disulphide group of sporidesmin in the action of the toxin on swelling and respiration of liver mitochondria. Biochemical Pharmacology, 23, 811-820.

Mitrousia, G. K., Huang, Y. J., Qi, A., Sidique, S. N. M. and Fitt, B. D. L. (2018). Effectiveness of *Rlm7* resistance against *Leptosphaeria maculans* (phoma stem canker) in UK winter oilseed rape cultivars. Plant Pathology. 67, 1339-1353.

Oliva, R., Win, J., Raffaele, S., Boutemy, L., Bozkurt, T.O., Chaparro-Garcia, A., Segretin, M.E., Stam, R., Schornack, S., Cano, L.M., et al. (2010). Recent developments in effector biology of filamentous plant pathogens. Cell. Microbiol. 12, 705–715.

Padmathilake, K., and Fernando, W. (2022). *Leptosphaeria maculans-Brassica napus* Battle: A Comparison of Incompatible vs. Compatible Interactions Using Dual RNASeq. *International journal of molecular sciences*, *23*, 3964.

Papastamati, K., Welham, S. J., Fitt, B. D. L. and Gladders, P. (2001). Modelling the progress of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) in relation to leaf wetness and temperature. Plant Pathology, 50, 154-164.

Pedras, M.S. C. and Biesenthal, C. J. (1998). Production of the host-selective phytotoxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. Canadian Journal of Microbiology, 44, 547-553.

Pedras, M.S.C. and Yu, Y. (2009) Mapping the sirodesmin PL biosynthetic pathway - A remarkable intrinsic steric deuterium isotope effect on a ^{1}H NMR chemical shift determines beta-proton exchange in tyrosine. Canadian Journal of Chemistry, 87, 564–570.

Pielaat, A., Van Den Bosch, F., Fitt, B. D. L. and Jeger, M. J. (2002). Simulation of vertical spread of plant diseases in a crop canopy by stem extension and splash dispersal. Ecological Modelling, 151, 195-212.

Pieterse, C.M.J.; Leon-Reyes, A.; Ent, S.V.d.; Wees, S.C.M.V. (2009) Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5, 308–316.

Piliponytė-Dzikienė, A., Kaczmarek, J., Petraitienė, E., Kasprzyk, I., Brazauskienė, I., Brazauskas, G. and Jędryczka, M. (2014). Microscopic and molecular detection of *Leptosphaeria maculans* and *L. biglobosa* ascospore content in air samples. Zemdirbyste, 101, 303-312.

Price, C. L., Parker, J. E., Warrilow, A. G., Kelly, D. E. and Kelly, S. L. (2015). Azole fungicides - understanding resistance mechanisms in agricultural fungal pathogens. Pest Management Science. 71, 1054-1058.

Ravindran, R. and Jaiswal, A. K. (2016). Exploitation of food industry waste for high-value products. Trends in Biotechnology, 34, 58–69).

Rawlinson, C. J., Sutton, B. C. and Muthyalu, G. (1978). Taxonomy and biology of *Pyrenopeziza brassicae sp.nov.* (*Cylindrosporium concentricum),* a pathogen of winter oilseed rape (*Brassica napus ssp. oleifera*). Transactions of the British Mycological Society. 71, 425-439.

Rodrigues, I. M., Coelho, J. F. J. and Carvalho, M. G. V. S. (2012). Isolation and valorisation of vegetable proteins from oilseed plants: Methods, limitations and potential. Journal of Food Engineering. 109, 337-346.

Rouxel, T., Chupeau, Y., Fritz, R., Kollmann, A. and Bousquet, J. F. (1988). Biological effects of sirodesmin PL, a phytotoxin produced by *Leptosphaeria maculans*. Plant Science. 57, 45-53.

Rouxel, T. and Balesdent, M. H. (2017). Life, death and rebirth of avirulence effectors in a fungal pathogen of *Brassica* crops. New Phytologist. 214, 526-532.

Ruiz-Lopez, N., Broughton, R., Usher, S., Salas, J. J., Haslam, R. P., Napier, J. A., & Beaudoin, F. (2017). Tailoring the composition of novel wax esters in the seeds of transgenic *Camelina sativa* through systematic metabolic engineering. Plant Biotechnology Journal, 15, 837–849.

Russell, P. E. (2005). A century of fungicide evolution. Journal of Agricultural Science. 143, 11-25.

Sadava, D., Hillis, D. ., Heller, H. and Berenbaum, M. (2012). Life: The Science of Biology (10th ed.). Sinauer Associates.

Sewell, T. R., Moloney, S., Ashworth, M., Ritchie, F., Mashanova, A., Huang, Y. J., Stotz, H. U. and Fitt, B. D. L. (2016). Effects of a penthiopyrad and picoxystrobin fungicide mixture on phoma stem canker (*Leptosphaeria spp.*) on UK winter oilseed rape. European Journal of Plant Pathology. 145, 675-685.

Sewell, T. R., Hawkins, N. J., Stotz, H. U., Huang, Y., Kelly, S. L., Kelly, D. E., Fraaije, B. and Fitt, B. D. L. (2017). Azole sensitivity in *Leptosphaeria* pathogens of oilseed rape: the role of lanosterol 14α-demethylase. Scientific Reports. 7, 15849.

Shoemaker, R. A., & Brun, H. (2001). The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans.* Canadian Journal of Botany, 79, 412–419.

Sidique, S.N.M. (2015) Effects of host resistance on colonisation of *Brassica napus* (oilseed rape) by *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker). PhD thesis, University of Hertfordshire, Hatfield.

Smedsgaard J. (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. Journal of Chromatography A. 760, 264–270.

Sprague, S. J., Balesdent, M. H., Brun, H., Hayden, H. L., Marcroft, S. J., Pinochet, X. *et al*. (2006). Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. European Journal of Plant Pathology. 114, 33-40.

Sprague, S. J., Watt, M., Kirkegaard, J. A. and Howlett, B. J. (2007). Pathways of infection of *Brassica napus* roots by *Leptosphaeria maculans.* New Phytologist. 176, 211-222.

Sprague, S. J., Kirkegaard, J. A., Graham, J. M., Bell, L. W., Seymour, M. and Ryan, M. (2015). Forage and grain yield of diverse canola (*Brassica napus*) maturity types in the high-rainfall zone of Australia. Crop and Pasture Science, 66, 260-274.

Stonard, J. F., Latunde-Dada, A. O., Huang, Y. J., West, J. S., Evans, N. and Fitt, B. D. L. (2010). Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans*/ *L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). European Journal of Plant Pathology, 126, 97.

Stotz, H. U., Mitrousia, G. K., de Wit, P. J. G. M. and Fitt, B. D. L. (2014). Effector-triggered defence against apoplastic fungal pathogens. Trends in Plant Science, 19, 491–500).

Strand, J. F. (2000). Some agrometeorological aspects of pest and disease management for the 21st century. Agricultural and Forest Meteorology, 103, 73-82.

Sun, P., Fitt, B. D. L., Steed, J. M., Underwood, C. T. and West, J. S. (2001). Factors affecting development of phoma canker (*Leptosphaeria maculans)* on stems of winter oilseed rape (Brassica napus) in southern England. Annals of Applied Biology, 139, 227-242.

Tan, S., Gu, Y., Yang, C., Dong, Y., Mei, X., Shen, Q. *et al.* (2016). *Bacillus amyloliquefaciens* T-5 may prevent *Ralstonia solanacearum* infection through competitive exclusion. Biology and Fertility of Soils, 52, 341–351.
Tilman, D. (1982). Resource competition and community structure. Princeton, NJ, USA: Princeton University Press.

Toscano-Underwood, C., Huang, Y. J., Fitt, B. D. L. and Hall, A. M. (2003). Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. Plant Pathology, 52, 726-736.

Travadon, R., Bousset, L., Saint-Jean, S., Brun, H. and Sache, I. (2007). Splash dispersal of *Leptosphaeria maculans* pycnidiospores and the spread of blackleg on oilseed rape. Plant Pathology, 56, 595–603.

Travadon, R., Marquer, B., Ribulé, A., Sache, I., Masson, J. P., Brun, H., Delourme, R., and Bousset, L. (2009). Systemic growth of *Leptosphaeria maculans* from cotyledons to hypocotyls in oilseed rape: influence of number of infection sites, competitive growth and host polygenic resistance. Plant Pathology. 58, 461-469.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J. and Katagiri, F. (2009) Network properties of robust immunity in plants. PLoS Genet. 5, e1000772.

Tsuda, K., Mine, A., Bethke, G., Igarashi, D., Botanga, C., Tsuda, Y., Glazebrook, J., Sato, M. and Katagiri, F. (2013). Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in Arabidopsis thaliana. PLoS Genet. 9, e1004015

UKRI. (2021). Flea beetle fight gets fresh funding injection. https://www.ukri.org/news/flea-beetle-fight-gets-fresh-funding-injection/ [Accessed 24/09/21]

Urquhart, A.S., Elliott, C.E., Zeng, W. and Idnurm, A. (2021) Constitutive expression of transcription factor SirZ blocks pathogenicity in Leptosphaeria maculans independently of Sirodesmin production. PLOS ONE. 16, e0252333.

USDA. (2020). Palm Oil Exports Constrained by Lower Exportable Supplies. https://downloads.usda.library.cornell.edu/usda-esmis/files/tx31qh68h/c247f928p/h415pt06n/oilseeds.pdf [Accessed 24/09/21]

van De Wouw, A. P., Elliott, V. L., Chang, S., López-Ruiz, F. J., Marcroft, S. J. and Idnurm, A. (2017). Identification of isolates of the plant pathogen *Leptosphaeria maculans* with resistance to the triazole fungicide fluquinconazole using a novel *in planta* assay. PLoS ONE, 12, e0188106.

van Den Bosch, F., and Gilligan, C. A. (2003). Measures of durability of resistance. Phytopathology, 93, 616-625.

van den Bosch F., Oliver R., van den Berg F. and Paveley N. (2014) Governing principles can guide fungicide-resistance management tactics. Annual Review of Phytopathology, 52, 175–195

van der Spiegel, M., Noordam, M. Y. and van der Fels-Klerx, H. J. (2013a). Safety of novel protein sources (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their application in food and feed production. Comprehensive Reviews in Food Science and Food Safety, 12, 662-678.

Vlot, A.C., Dempsey, D.A. and Klessig, D.F. (2009). Salicylic Acid, a multi-faceted hormone to combat disease. Annu. Rev. Phytopathol. 47, 177–206.

VNV. (2012). Raapzaad- en koolzaadeiwit Rapeseed protein. https://www.cbg-meb.nl/documenten/rapporten/2012/12/03/nv-raapzaad-en-koolzaadeiwit [Accessed 05/03/2022].

Von Jagow, G.; Gribble, G. W. and Trumpower, B. L. (1986). Mucidin and strobilurin A are identical and inhibit electron transfer in the Cytochrome bc_1 complex of the

mitochondrial respiratory chain at the same site as Myxothiazol. Biochemistry, *25*, 775– 780,

Vranken, L., Avermaete, T., Petalios, D. and Mathijs, E. (2014). Curbing global meat consumption: Emerging evidence of a second nutrition transition. Environmental Science and Policy. https://doi.org/10.1016/j.envsci.2014.02.009

VSN International (2021). Genstat *for Windows* 21st Edition. VSN International, Hemel Hempstead, UK.

Webster, J. and Weber, R. (2007). Introduction to fungi (Issue 3). Cambridge University Press, UK.

Welham, S. J., Turner, J. A., Gladders, P., Fitt, B. D. L., Evans, N. and Baierl, A. (2004). Predicting light leaf spot (*Pyrenopeziza brassicae*) risk on winter oilseed rape (*Brassica napus*) in England and Wales, using survey, weather and crop information. Plant Pathology, 53, 713-724.

West, J. S., Biddulph, J. E., Fitt, B. D. L. and Gladders, P. (1999). Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. Annals of Applied Biology, 135, 535-546.

West, J. S., Kharbanda, P. D., Barbetti, M. J. and Fitt, B. D. L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. In Plant Pathology, 50, 10-27.

West, J. S., Fitt, B. D. L., Leech, P. K., Biddulph, J. E., Huang, Y. J. and Balesdent, M. H. (2002). Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on phoma leaf spot and phoma stem canker development on winter oilseed rape (*Brassica napus*) in southern England. Plant Pathology, 51, 454-463.

White, S., Ellis, E., Pickering, F., Leybourne. D., Corkley, I., Kendall, S., *et al.* (2020) Integrated pest management of cabbage stem flea beetle in oilseed rape. AHDB Project report No. 623. https://projectblue.blob.core.windows.net/media/Default/Research%20Papers/Cereals%20and%20Oilseed/2020/PR623%20Final%20Project%20Report%20(coded).pdf – [Accessed 24/09/2021].

Williams, R. H. and Fitt, B. D. L. (1999). Differentiating a and b groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. Plant Pathology, 48, 161-175.

Willis, C. E., Foster, S. P., Zimmer, C. T., Elias, J., Chang, X., Field, L. M., Williamson, M. S. and Emyr Davis, T. G. (2020) Investigating the status of pyrethroid resistance in UK populations of the cabbage stem flea beetle (*Psylliodes chrysocephala*). Crop Protection. 138. 105316.

Yang, Y., Marcroft, S. J., Forsyth, L., Zhao, J., Li, Z., Van de Wouw, A. and Idnurm, A. (2020). Sterol demethylation inhibitor fungicide resistance in *Leptosphaeria maculans* is caused by modifications in the regulatory region of ERG11. *Plant Disease.* 104, 1280-1290.

Yunus-Khan, T., Atabani, A. E., Badruddin, I. A., Badarudin, A., Khayoon, M. S. and Triwahyono, S. (2014). Recent scenario and technologies to utilize non-edible oils for biodiesel production. In Renewable and Sustainable Energy Reviews. 37, 840-851.

Zamani-Noor, N. and Knüfer, J. (2018). Effects of host plant resistance and fungicide application on phoma stem canker, growth parameters and yield of winter oilseed rape. Crop Protection, 112, 313–321.

Zhang, X., White, R. P., Demir, E., Jedryczka, M., Lange, R. M., Islam, M. *et al.* (2014). *Leptosphaeria spp.,* phoma stem canker and potential spread of *L. maculans* on oilseed rape crops in China. Plant Pathology, 63, 598-612.

Zheng, X., Koopmann, B. U., and von Tiedemann (2020) A global survey on diseases and pests in oilseed rape – current challenges and innovative strategies of control. Frontiers in Agronomy, 2, 590908

Zhou, Y., Fitt, B. D. L., Welham, S. J., Gladders, P., Sansford, C. E. and West, J. S. (1999). Effects of severity and timing of stem canker (*Leptosphaeria maculans*) symptoms on yield of winter oilseed rape (*Brassica napus*) in the UK. European Journal of Plant Pathology, 105, 715–728.

Appendices

APPENDICES 1 – Poster Presentations

A) University of Hertfordshire Research Conference – April 2018 & International Congress of Plant Pathology – Boston, USA – August 2018

Development of inoculation methods to understand interactions of *Pyrenopeziza brassicae* (Light leaf spot) and *Leptosphaeria maculans* (Phoma stem canker) during leaf infection on oilseed rape.

Fortune, J.A.[1], Mitrousia, G.[1], Ritchie, F.[2], Fitt, BDL[1] & Huang, Y[1]
[1] School of Life and Medical Sciences, University of Hertfordshire, UK [2] ADAS Boxworth, Cambridge, UK

Interspecific interactions between fungal pathogens causing light leaf spot (*Pyrenopeziza brassicae*) and phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) *in vitro*.

Fortune, J.A.[1], Mitrousia, G.[1], Ritchie, F.[2], Fitt, BDL.[1] & Huang, Y[1]

[1] School of Life and Medical Sciences, University of Hertfordshire, UK
[2] ADAS Boxworth, Cambridge, UK

Introduction

- UK oilseed rape (*Brassica napus*) yields have not increased in the last 10 years, in part due to yield losses from the pathogens *Leptosphaeria maculans* and *Pyrenopeziza brassicae* responsible for the phoma stem canker and light leaf spot diseases, respectively.
- These plant pathogens do occur independently; however, they are often observed together on leaves or stem tissues of the same individual plant, yet their interactions is not clear.
- Since they often share the same resources, these pathogens must interact.
- This work aims to understand the interspecific interactions between these pathogens *in vitro* on different media and at different temperatures.

Methods

For each treatment (Table 1), a total of 4 plugs (co-cultures 2 plugs of each fungus) were cultured in 75 ml of clarified V8 juice broth in a rotary shaker set at 80RPM and 18°C for 14 days. Each treatment was done in replicate and placed in a randomised position in the rotary shaker.

Using miracloth, the liquid broth was separated away from the fungal colonies and each treatment was pooled into individual Duran bottles. 75 mL of ethyl acetate (EtOH was poured into each Duran bottle and then shaken for 45 seconds. The two phases were allowed to settle for 30 minutes. For each treatment, the top phase was pipetted into two 50ml Falcon tubes and centrifuged at 6000 RPM for 3 minutes. 40 mL of clear supernatant from centrifuged Falcon tubes were pooled into two new skirted 50ml Falcon tube (20ml in each). This was repeated for each treatment. The excess EtOH was evaporated using a sample concentrator. Then re-suspended in 1 mL of EtOH.

Plates were inoculated with fungal plug following a randomisation onto clarified V8 juice agar. 8mm, 8mm and 4 mm diameter fungal plugs of *L. maculans*, *L. biglobosa* and *P. brassicae*, respectively. 20 μL of extracted secondary metabolites (SM) from each treatment (Table 1) were pipetted onto a fungal plug in triplicate. Samples were incubated at 18°C. Assessments were made at 2, 7 and 10 days for *L. maculans* and *L. biglobosa*, and weekly for *P. brassicae* by measuring the average colony diameter.

Table 1. Treatment list for production of secondary metabolites in Liquid culture and for plug bioassay.

Results

- For Lm, colony growth was significantly reduced when plugs were inoculated with SM from liquid cultures that did not contain Lm, with the exception of Lm vs Lb SM.
- SM derived from Pb only caused the greatest reduction in average colony diameter after 7 days.

Figure 1. Average colony diameter of *L. maculans* and *L. biglobosa* after 7 days when inoculated with different secondary metabolites. Bars that do not share a letter are significantly different according to Tukey.

Figure 2. Average colony diameter of *P. brassicae* after 7 days when inoculated with different secondary metabolites. Bars that do not share a letter are significantly different according to Tukey.

- For both Lb and Pb, SM deriving from liquid cultures with Lm only or Lm vs Pb caused the only significant reduction in colony diameter.
- For Pb, SM from Lb vs Pb caused a small yet significant reduction in colony growth compared to EtOH control.
- SM from Lm vs Pb did not cause a significant reduction.

Conclusions

- *L. maculans* releases antagonistic secondary metabolite/s that reduce the colony diameter of both *L. biglobosa* and *P. brassicae*.
- *L. biglobosa* can detoxify the antagonistic secondary metabolite/s.
- Secondary metabolites from *L. biglobosa* and *P. brassicae* cause small, but significant reductions in *L. maculans* colony diameter.
- *P. brassicae* release antagonistic metabolites that reduce *L. maculans* colony diameter, however *L. maculans* has a mechanism to detoxify these secondary metabolite/s.

University of Hertfordshire UH | Chadacre Agricultural Trust | COBBOLD TRUST | European Union European Regional Development Fund | Hertfordshire Local Enterprise Partnership PERFECTLY PLACED FOR BUSINESS | ADAS

Interspecific interactions between fungal pathogens causing phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) *in vitro*.

Fortune, J.A.[1], Karendeni-Dewage, C.S.[1], Ritchie, F.[2], Fitt, BDL.[1] & Huang, Y[1]

[1] School of Life and Medical Sciences, University of Hertfordshire, UK
[2] ADAS Boxworth, Cambridge, UK

j.fortune@herts.ac.uk or Twitter: @James_Fortune11

Introduction

- Phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) and light leaf spot (*Pyrenopeziza brassicae*) are the two most economically damaging diseases to UK oilseed rape; together causing > £150M losses annually (Fig. 1).
- These plant pathogens do occur independently; however, they are also observed together on leaves or stem tissues of the same plant (Fig. 2); their interactions are not clear.
- This work aims to understand the interspecific interactions between these pathogens *in vitro* on different media when inoculated with different extracted secondary metabolites.
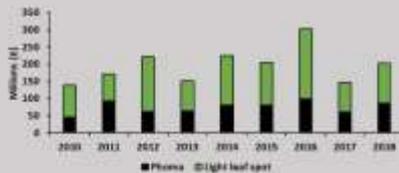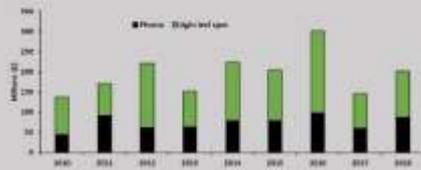
Figure 1. England and Wales oilseed rape yield losses due to phoma stem canker and light leaf spot (CropMonitor, 2018).

Figure 2. Symptoms of phoma leaf spot (PLS) and light leaf spot (LLS) on the same oilseed rape leaf.

Methods

For each treatment (Table 1), plugs were cultured in clarified V8 juice broth in a rotary shaker for 14 days. Each treatment was done in three replicates and placed in a randomised position in the rotary shaker. Liquid broth and fungal mass were separated. Secondary metabolites were extracted using Ethyl acetate (EtOAc). Dried metabolites were re-suspended in 1 mL of EtOAc.

Table 1. Treatment list for production of secondary metabolites in Liquid culture and for plug bioassay from combinations of *L. maculans* (Lm), *L. biglobosa* (Lb) and *P. brassicae* (Pb).

Treatment	Description	Replicates	Liquid Culture	Plug Bioassay
1	Lm Only	3	✓	✓
2	Lb Only	3	✓	✓
3	Pb Only	3	✓	✓
4	Lm vs Pb	3	✓	✓
5	Lb vs Pb	3	✓	✓
6	Lm vs Lb	3	✓	✓
7	EtOAc	3	✗	✓

Clarified V8 agar plates were inoculated with fungal plugs of *L. maculans* (Lm) or *L. biglobosa* (Lb) or *P. brassicae* (Pb). Extracted secondary metabolites from each treatment (Table 1) were pipetted onto the respective fungal plug in triplicate. Average colony diameter for *L. maculans*, *L. biglobosa* and *P. brassicae* was calculated for each treatment. Results are presented as the decrease in the colony diameter for individual treatments and isolates relative to the EtOH control.

Preliminary studies to identify differences between the samples were tested using HPLC with the 'EtOAc' as the baseline.

Results

- The colony diameter of *L. maculans* was not significantly reduced by any of the secondary metabolites tested after 7 days post inoculation (dpi) (Fig. 3).
- The colony diameters of *L. biglobosa* and *P. brassicae* were significantly reduced by secondary metabolites derived from *L. maculans*, with the exception of 'Lm vs Lb', when assessed after 7 days post inoculation (Fig. 3).
- There are five peaks on the chromatogram from the 'Lm only' sample that are greater than those found from the 'Lm vs Lb' sample (Fig. 4).
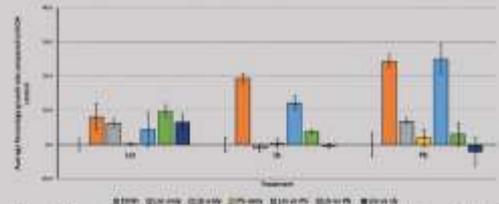
Figure 3. Diameter reduction (%) compared to EtOH control after 7 pdi when inoculated with different secondary metabolites from *L. maculans* (Lm), *L. biglobosa* (Lb) or *P. brassicae* (Pb). Bars that do not share a letter are significantly different according to Tukey tests.
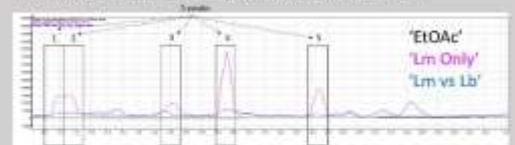
Figure 4. HPLC chromatogram when EtOAc, 'Lm only' and 'Lm vs Lb' sample were tested.

Conclusions

- *L. maculans* produces antagonistic secondary metabolites that decrease the colony diameter of *L. biglobosa* and *P. brassicae*.
- *L. biglobosa* appears to detoxify the antagonistic secondary metabolites produced by *L. maculans*.
- The secondary metabolites derived from *L. maculans* have a greater inhibitory effect on growth of *P. brassicae* than on *L. biglobosa*.
- There are 5 areas of interest between these two samples that require further investigations.

APPENDICES 2 – Events

A) Hertfordshire Science Partnership launch - March 2018

B) International Congress of Plant Pathology – Boston, USA – August 2018

C) Agritech Week 2018: Solving the challenges of crop protection – November 2018

D) Doctoral Training Alliance – Autumn School 2018

E) Agrifood Charities Partnership conference – April 2019
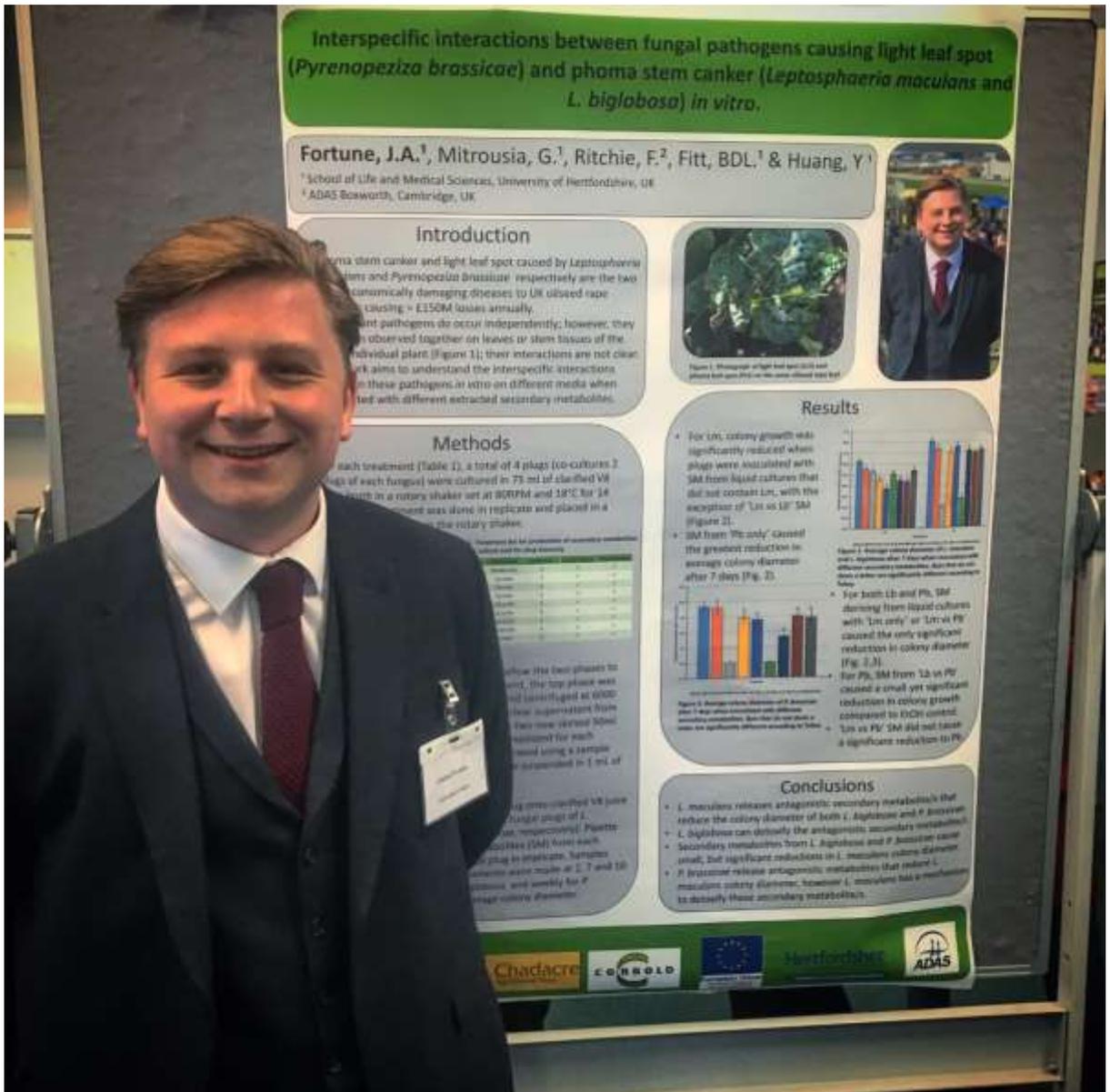
F) International Rapeseed Congress – Berlin, Germany – June 2019

G) European Plant Science Retreat – Nottingham, UK – July 2019

H) Royal Society of Biology – Parliamentary Networking Event – October 2019

I) **Agrifood Charities Partnership – Management of Diseases and Pests of Oilseed Rape – June 16th 2021**

J) Cereals 2021 – June 2021

K) 7th Virtual BCPC Disease Review – October 2021

L) Agritech East – REAP Conference 2021 – Changing Time(s) for Agriculture – November 2021

APPENDICES 3 – Awards

A) University of Hertfordshire Life and Medical School Deans Awards 2019 – Student Engagement with Research

APPENDICES 4 - Taxonomy of common oilseed rape disease causal pathogens. Phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*), Light leaf spot (*Pyrenopezizia brassicae*), Verticillium stem stripe (*Verticillium longisporum*), Sclerotinia stem rot (*Sclerotinia sclerotiorum*) and Dark leaf spot (*Alternaria brassicicola*) (CABI 2022a-f).

	Leptosphearia maculans	*Leptosphaeria biglobosa*	*Pyrenopeziza brassicae*
KINGDOM	*Fungi*	*Fungi*	*Fungi*
PHYLUM	*Ascomycota*	*Ascomycota*	*Ascomycota*
CLASS	*Dothideomycetes*	*Dothideomycetes*	*Leotiomycetes*
ORDER	*Pleosporales*	*Pleosporales*	*Helotiales*
FAMILY	*Leptospheariaceae*	*Leptospheariaceae*	*Dermateacea*
GENUS	*Leptosphaeria*	*Leptosphaeria*	*Pyrenopeziza*
SPECIES	*Maculans*	Biglobosa	*Brassicae*

	Verticillium longisporum	*Sclerotinia sclerotiorum*	*Alternaria brassicicola*
KINGDOM	*Fungi*	*Fungi*	*Fungi*
PHYLUM	*Ascomycota*	*Ascomycota*	*Ascomycota*
CLASS	*Sordariomycetes*	*Leotiomyctes*	*Dothidiomycetes*
ORDER	*Hypocreales*	*Helotiales*	*Pleosporales*
FAMILY	*Incertaesedis*	*Sclereotiniaceae*	*Pleosporaceae*
GENUS	*Verticillium*	*Sclerotinia*	*Alternaria*
SPECIES	*Longisporum*	*Sclerotiorum*	*Brassicicola*

APPENDICES 5 – Media Recipes

1.   POTATO-DEXTROSE AGAR (PDA)

For 500 mL,

19.5 g	Potato-dextrose agar
500 mL	Deionised water

2.   DEIONISED WATER AGAR (WA)

For 500 mL

7.5 g	Bacto-agar
500 mL	Deionised water

3.   MALT EXTRACT AGAR (MEA)

For 500 mL

15 g	Malt extract
10 g	Bacto-agar
500 ml	Deionised water

4.   V8 AGAR (V8)

For 500 mL

100 mL	V8 juice
0.5 g	Sucrose
7.5 g	Bacto-agar
400 mL	De-ionised water

5. CLARIFIED V8 AGAR (V8*)

For 500 mL

100 mL	Clarified* V8 juice
0.5 g	Sucrose
7.5 g	Bacto-agar
400 mL	De-ionised water

To Clarify V8 juice

a. 5 g $CaCO_3$ was added to 300 mL V8 juice
b. Stirred continuously for 15 min using an automatic stirrer
c. Poured into sterile 50 mL Falcon tubes
d. Centrifuged for 10 min at 4000 RPM
e. Supernatant poured into a sterile 50 mL Falcon tube and stored at -20°C until required
f. Pellet discarded

6. CLARIFIED V8 BROTH (V8*B)

For 500 mL

100 mL	Clarified* V8 juice
0.5 g	Sucrose
400 mL	De-ionised water

All media were prepared in 1 L Duran bottles and sterilized using an autoclave. If the volume of media required exceeded 1 L, then the media were pooled into a larger conical flask (2 or 5 L), mixed using an automatic stirrer then decanted into individual 1 L Duran bottles before sterilization.

APPENDICES 6 – Effects of secondary metabolites derived from simultaneously co-inoculated liquid culture on the growth of *L. maculans, L. biglobosa and P. brassicae in vitro*

This experiment was repeated independently three times. There were significant differences between the experiments for the effect of the secondary metabolites on colony area at either 7- or 10 days post inoculation when applied to *L. maculans* ($F_{2,71}$ = 105.19, P <0.001, LSD = 0.46; $F_{2,71}$ = 204.04, P <0.001, LSD = 1.16, respectively), *L. biglobosa* ($F_{2,71}$ = 12.83, P <0.001, LSD = 0.91; $F_{2,71}$ = 75.31, P <0.001, LSD = 1.89, respectively), or *P. brassicae* ($F_{2,69}$ = 61.83, P <0.001, LSD = 0.05; $F_{2,69}$ = 2.18, P <0.122, LSD = 0.11, respectively). Therefore, experiments were analysed as experiments rather than across experiments.

Experiment 1

For *L. maculans*, there was a small yet significant difference in the colony area (cm^2) when different secondary metabolites were applied at 7-and 10-days post inoculation. ($F_{7,23}$ = 10.27, P <0.001, LSD = 1.14; $F_{7,22}$ = 11.35, P <0.001, LSD = 2.746) (Fig. A6.1 & A6.2). At 7dpi, the only extracts that caused significant differences from the two control extracts, 'EtOAc' (10 cm^2) and 'Media Only' (9.57 cm^2), were those extracts that contained *L. biglobosa,* 'Lb only' (7.28 cm^2), 'Lb&Pb' (7.238 cm^2) and 'Lm&Lb' (6.916 cm^2); they were not significantly different from each other (Fig. A6.1).

At 10 dpi all pathogen extracts a similar pattern was seen; the extracts that contained *L. biglobosa* were significantly smaller than the control extracts 'EtOAc' (21.98 cm^2) and 'Media Only' (22.26 cm^2). However, the 'Pb only' (17.29 cm^2) extract was also significantly different from both control extracts. The effect of these extracts on colony area were not significantly different from each other. The extracts that contained *L. maculans*, with the exception of 'Lm&Lb' (14.68 cm^2), were not significantly different from the controls. The only extract that caused a significantly smaller colony area than 'Lm only' (18.75 cm^2) was the 'Lm&Lb' extract.
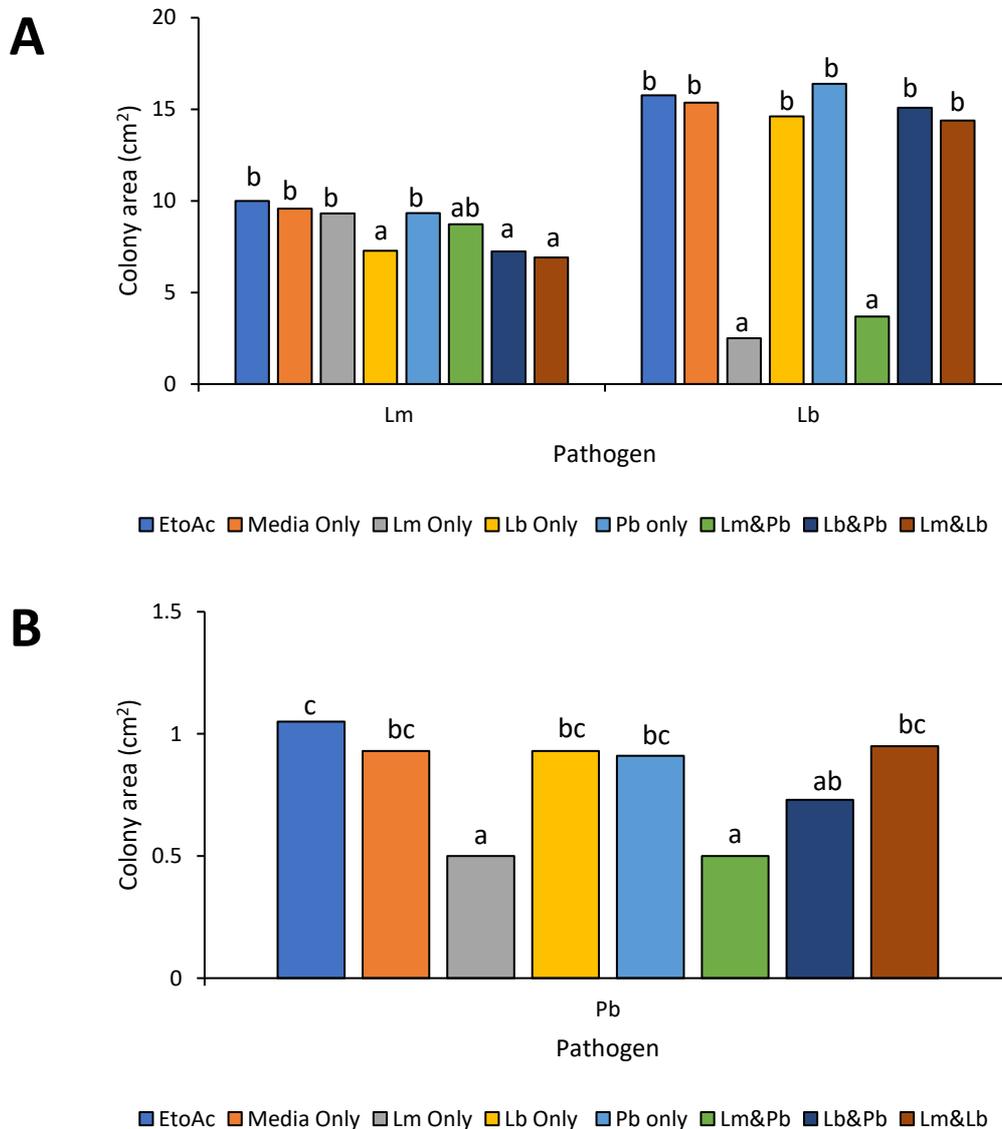
Figure A6.1: Colony area (cm^2) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 7 dpi for experiment 1. A post-hoc Tukey LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm and Lb = 23 d.f., Pb = 22 d.f.).
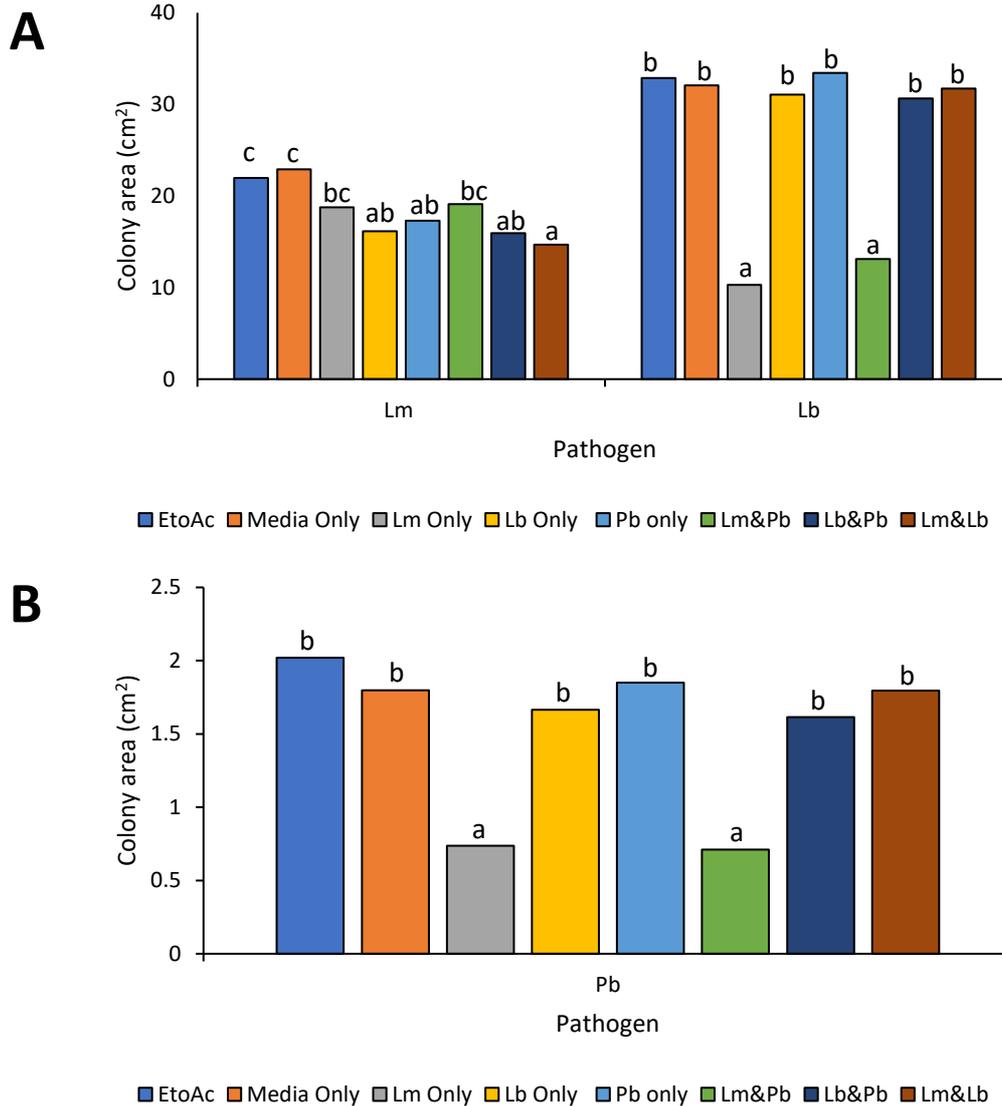
Figure A6.2: Colony area (cm²) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with _L. maculans_ (Lm), _L. biglobosa_ (Lb) (A) or _P. brassicae_ (Pb) (B) or simultaneous co-cultures of these pathogens at 10 dpi for experiment 1. A post-hoc Tukey test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lb = 23d.f., Lm and Pb = 22 d.f.).

For *L. biglobosa* and *P. brassicae,* there was a large significant difference in the colony area (cm^2) when different secondary metabolites were applied at 7- and 10- days post inoculation (Day 7, *L. biglobosa* = $F_{7,23}$ = 99.67, *P* < 0.001, LSD = 1.70; *P. brassicae* = $F_{7,22}$ = 12.98, *P* <0.001, LSD = 0.1775 and Day 10, *L. biglobosa* = $F_{7,23}$ = 99.67, *P* < 0.001, LSD = 2.65, $F_{7,22}$ = 12.13, *P* < 0.001, LSD = 0.4399) (Fig. A6.1 and A6.2). At 7- and 10 days post inoculation, the effects of applications of different secondary metabolite extracts had the same pattern on both pathogens. For both *L. biglobosa* and *P. brassicae,* only two extracts caused significant reductions in colony area compared to the two control extracts, EtOAc (32.87 cm^2 and 2.016 cm^2, respectively) and Media only (32.07 cm^2 and 1.797 cm^2, respectively); they were 'Lm only' (10.31 cm^2 and 0.74 cm^2, respectively) and 'Lm&Pb' (13.13 cm^2 and 0.71 cm^2 respectively). For *L. biglobosa*, the colony area when 'Lm only' was applied was significantly smaller than when the 'Lm&Pb' extract was applied at 10 dpi but not at 7 dpi, whereas for *P. brassicae* there was no significant difference between colony diameter for both 7 and 10 dpi.

Experiment 2

For *L. maculans*, there was a small yet significant difference in the colony area (cm^2) when different secondary metabolites were applied at 7 dpi but there was not at 10 dpi. ($F_{7,23}$ = 4.15, *P* =0.009, LSD = 1.49; $F_{7,23}$ = 0.116, *P* = 0.116, LSD = 3.885) (Fig. A6.3 and A6.4) At 7dpi, none of the extracts caused a significant difference when compared to the two control extracts, 'EtOAc' (12.02 cm^2) and 'Media only' (9.08 cm^2). For *L. biglobosa* and *P. brassicae* there was a large significant difference in the colony area (cm^2) when different secondary metabolites were applied at 7- and 10- days post inoculation (Day 7, *L. biglobosa* = $F_{7,23}$ = 38.53, *P* < 0.001, LSD = 1.53; *P. brassicae* = $F_{7,22}$ = 12.43, *P* <0.001, LSD = 0.1534 and Day 10, *L. biglobosa* = $F_{7,23}$ = 27.29, *P* < 0.001, LSD = 2.86, $F_{7,22}$ = 11, *P* < 0.001, LSD = 0.240) (Fig. A6.3 and A6.4).

At 7- and 10 days post inoculation, the effects of applications of different secondary metabolite extracts had the same pattern on both pathogens. For both *L. biglobosa* and *P. brassicae,* only two extracts caused significant reductions in colony area compared to the two control extracts, EtOAc and Media only; they were 'Lm only' and 'Lm&Pb'.
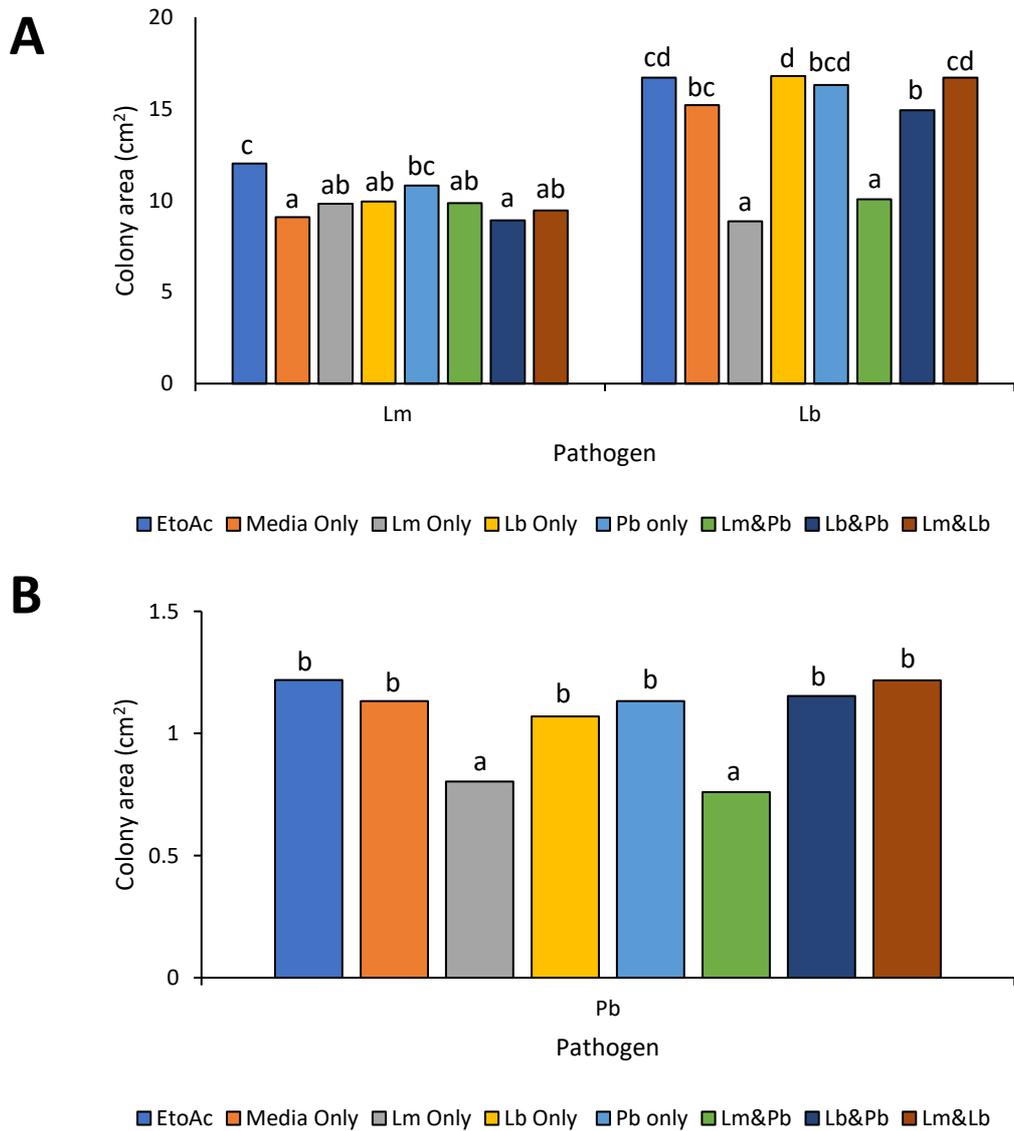
Figure A6.3: Colony area (cm^2) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 7 dpi for experiment 2. A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm and Lb = 23d.f., Pb = 22 d.f.).
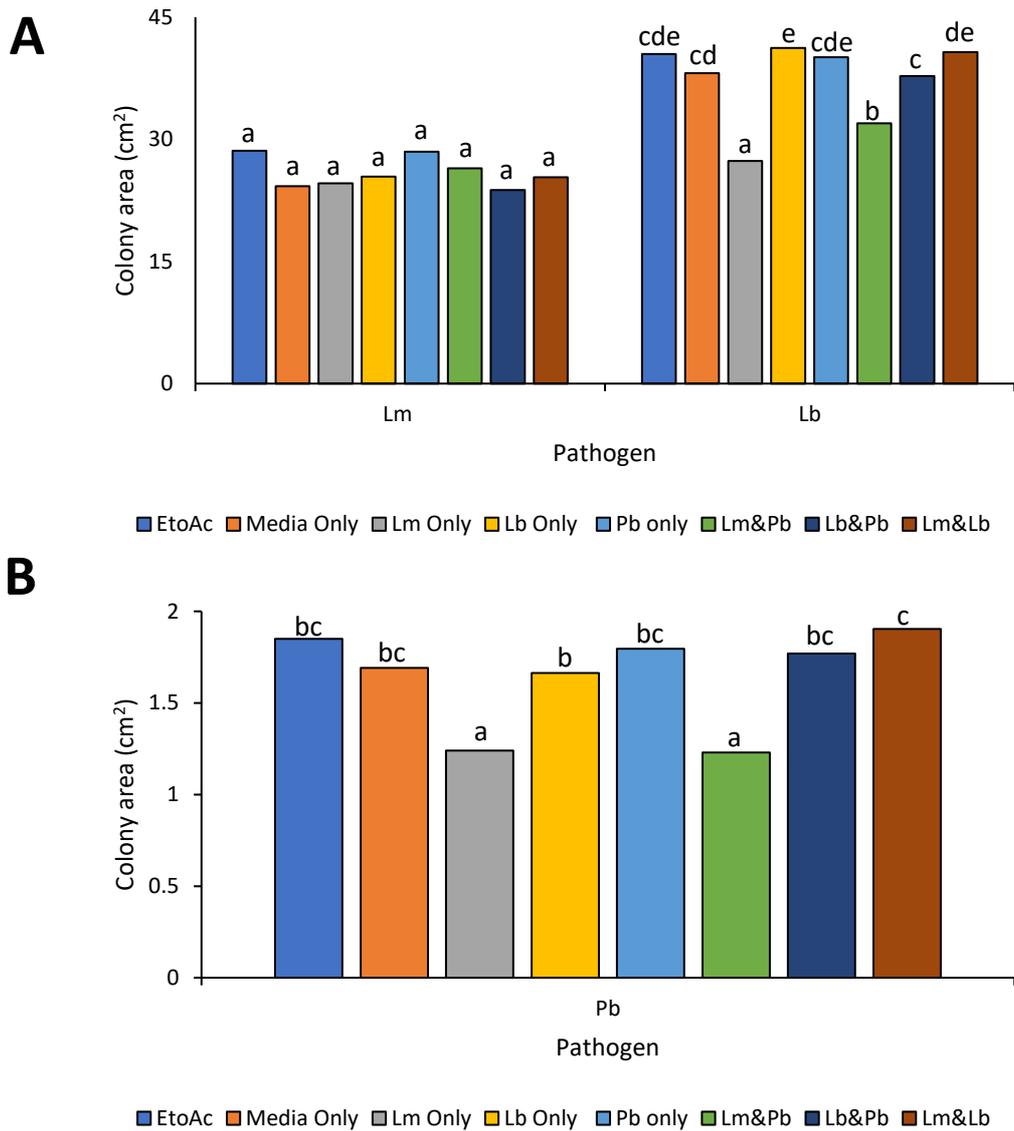
Figure A6.4: Colony area (cm²) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 10 dpi for experiment 2. A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm and Lb = 23d.f., Pb = 22 d.f.).

For *L. biglobosa*, the colony area when 'Lm only' extract was applied was significantly smaller than when the 'Lm&Pb' extract was applied at 10 dpi but not at 7 dpi, whereas for *P. brassicae* there was no significant difference in colony area for both 7 and 10 dpi.

Experiment 3

For *L. maculans*, there was a small yet significant difference in the colony area (cm^2) when different secondary metabolites were applied at 7 dpi but there was not at 10 dpi. ($F_{7,23}$ = 28.46, *P* < 0.001, LSD = 0.60; $F_{7,23}$ = 27.03, *P* < 0.001, LSD = 1.199) (Fig. A6.5 & A6.6). At 7dpi, only one of the extracts, 'Lm&Lb' (9.87cm^2) caused a significant difference when compared to the two control extracts, 'EtOAc' (12.88 cm^2) and 'Media only' (11. 71cm^2). However, at 10dpi the only extract that caused a significantly smaller colony diameter than the two control extracts, 'EtOAc' (31.50 cm^2) and 'Media only' (29.76 cm^2) was the 'Lb&Pb' (25.72 cm^2) extract. For *L. biglobosa* and *P. brassicae,* there was a large significant difference in the colony area (cm^2) when different secondary metabolites were applied at 7- and 10- days post inoculation (Day 7, *L. biglobosa* = $F_{7,23}$ = 167.07, *P* < 0.001, LSD = 1.27; *P. brassicae* = $F_{7,23}$ = 16.51, *P* <0.001, LSD = 0.1281 and Day 10, *L. biglobosa* = $F_{7,23}$ = 126.08, *P* < 0.001, LSD = 2.826, $F_{7,23}$ = 16.33, *P* < 0.001, LSD = 0.252) (Fig. A6.5 & A6.6).

At 7- and 10 dpi, the applications of different secondary metabolite extracts had the same effect on *L. biglobosa* (Fig. A6.5 & A6.6). For both assessment time points for *L. biglobosa,* only two extracts caused significant reductions in colony area compared to the two control extracts, EtOAc (14.12 cm^2 and 36.92 cm^2, respectively) and Media only (14.04 cm^2 and 38.61 cm^2, respectively); they were 'Lm only' (3.72 cm^2 and 18.57 cm^2, respectively) and 'Lm&Pb' (4.53 cm^2 and 22.02 cm^2, respectively).

For *L. biglobosa*, the colony area when 'Lm only' was applied was significantly smaller than when the 'Lm&Pb' extract was applied at 10 dpi but not at 7 dpi.
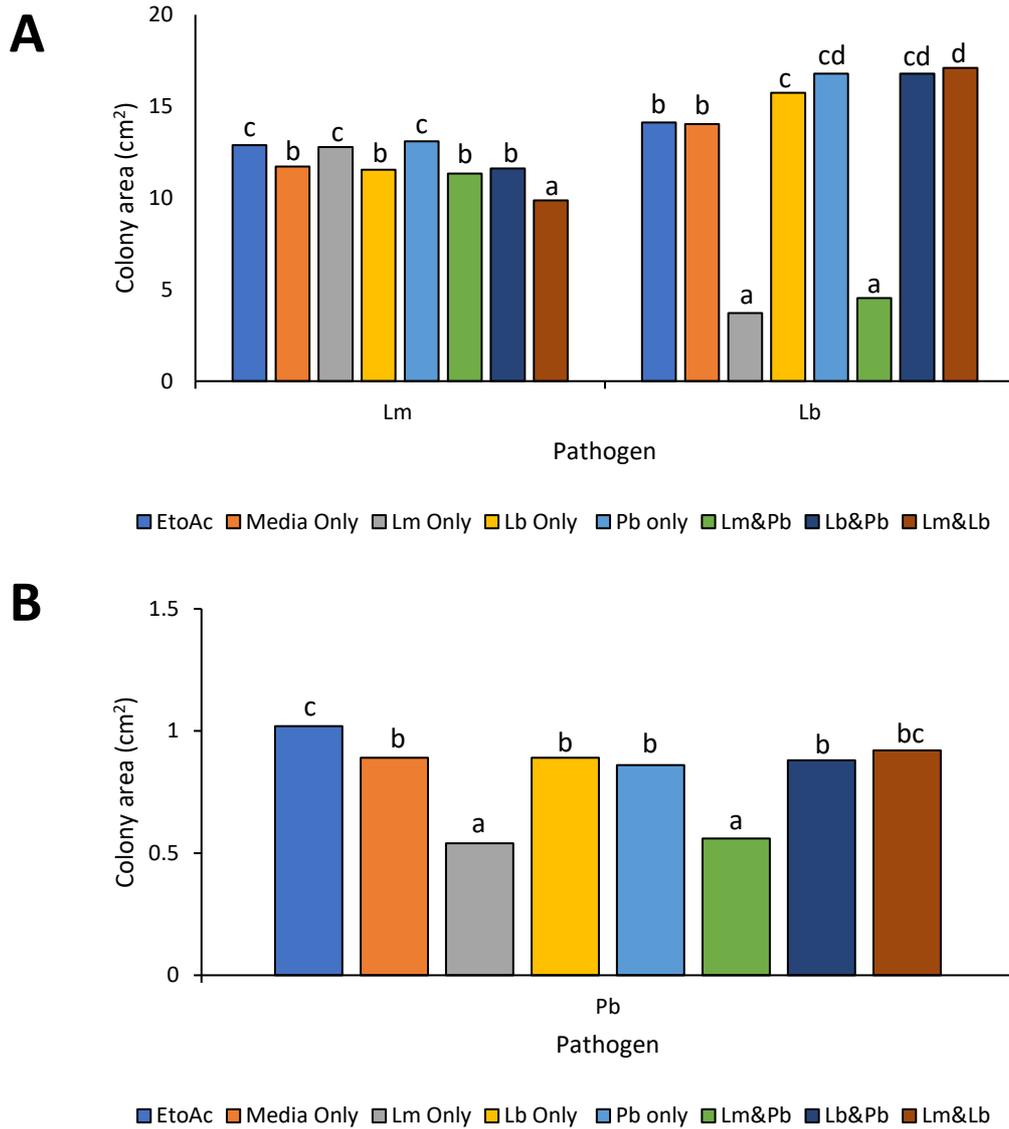
Figure A6.5: Colony area (cm²) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) (B) or simultaneous co-cultures of these pathogens at 7 dpi for experiment 3. A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm, Lb or Pb = 23 d.f.).
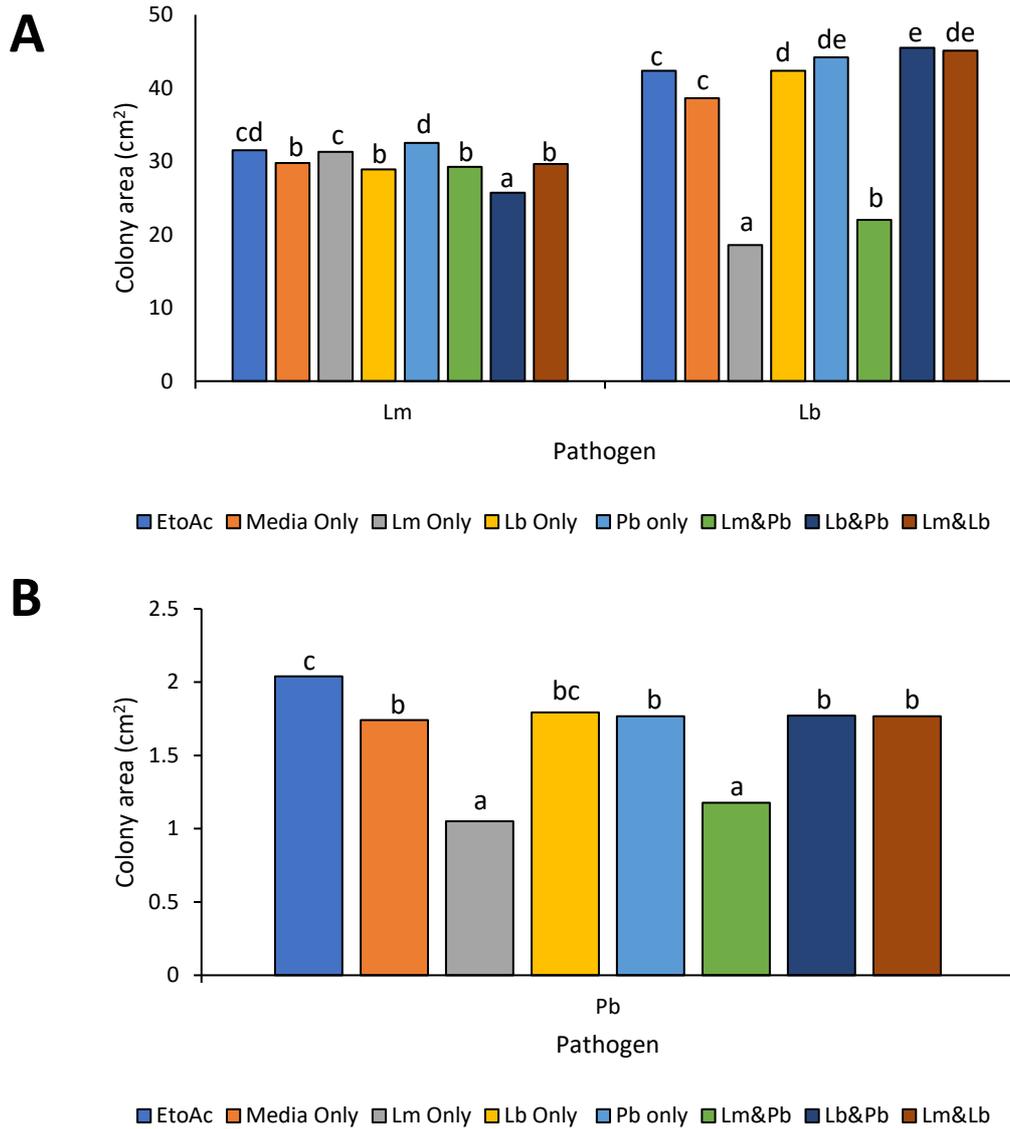
Figure A6.6: Colony area (cm²) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) (A) or *P. brassicae* (Pb) or simultaneous co-cultures of these pathogens at 7 dpi for experiment 3. A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm, Lb or Pb = 23 d.f.).

For the 7dpi assessments when extract 'Lb only' (15.75 cm^2) and 'Lm&Lb' (17.10 cm^2) were applied, the colony areas of *L. biglobosa* were significantly different from each other but were not significantly different from when extracts 'Lb&Pb' (16.79 cm^2) or 'Pb only' (16.46 cm^2) were applied. However, at the 10dpi assessment when extract 'Lb only' (42.37cm^2) and 'Lb&Pb' (45.50 cm^2) were applied, the colony areas of *L. biglobosa* were significantly different from each other but were not significantly different from when extracts 'Pb only' (44.19 cm^2) and 'Lm&Lb' (45.11 cm^2) were applied.

For *P. brassicae* at both 7 and 10 dpi, there were only two extracts that were significantly different from the two control extracts, 'EtOAc' (1.02 cm^2 and 2.04 cm^2, respectively) and 'Media only' (0.89 cm^2 and 1.742 cm^2, respectively); they were 'Lm only' (0.54 cm^2 and 1.05 cm^2, respectively) and 'Lm&Pb' (0.56 cm^2 and 1.18 cm^2, respectively).

APPENDICES 7 – Effects of secondary metabolites derived from sequentially co-inoculated liquid culture on the growth of *L. maculans* or *L. biglobosa in vitro*

Experiment 1

For *L. maculans*, there was a small yet significant difference in the colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,24}$ = 20.34, *P* < 0.001, LSD = 2.019 (Fig. A7.1). There was no significant difference in colony area when the 'Lm Only' (23.42 cm^2) extract was applied to *L. maculans* compared to the 'Ethyl acetate' control (25.43 cm^2). The colony areas when these treatments were applied were significantly greater than when a treatment that contained *L. biglobosa* was applied. The colony area when the 'Lb only' (21.06 cm^2) extract was applied was significantly greater than when the 'Lm+Lb' (17.81 cm^2) extract was applied. However, the 'Lm&Lb' extract (19.21 cm^2) was not significantly different from the other two extracts that contained *L. biglobosa*.

For *L. biglobosa*, there were large significant differences in colony area (cm^2) when different secondary metabolites were applied when assessed 7 dpi ($F_{4,21}$ = 79.49, *P* < 0.001, LSD = 2.444) (Fig. A7.1). There was no significant difference in colony area when the Ethyl acetate (22.11 cm^2) or the 'Lm&Lb' (21.35 cm^2) secondary metabolites were applied. However, the colony areas when all other treatments were applied were significantly different from those with these two extracts. The colony area of *L. biglobosa* was the smallest when the 'Lm+Lb' (5.68 cm^2) was applied. This was significantly smaller in area than when the 'Lm only' (9.85cm^2) or 'Lb only' (15.29 cm^2) extracts were applied; 'Lb only' extract resulting in a significantly larger *L. biglobosa* colony area then when 'Lm only' was applied.

A

L. maculans

B
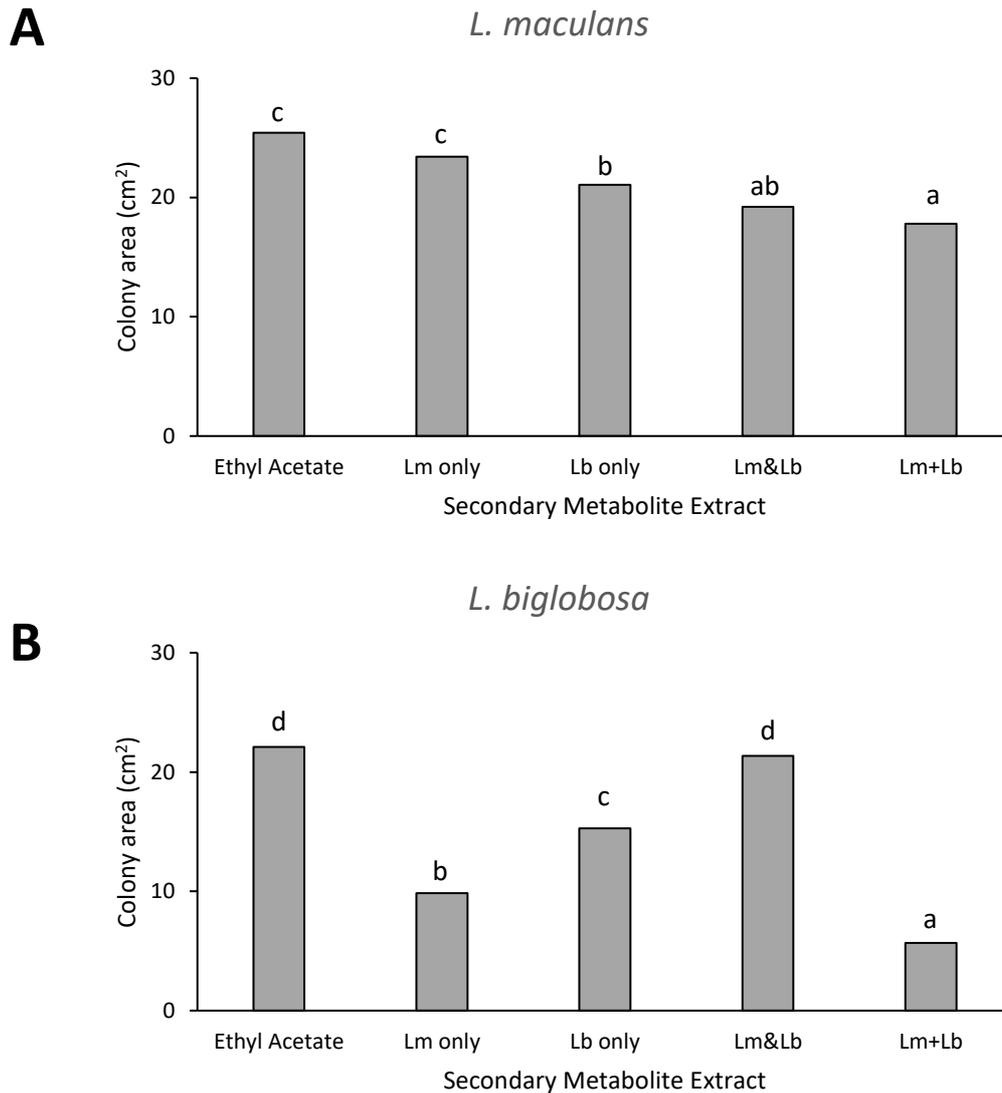
L. biglobosa

Figure A7.1: Colony area (cm²) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm) (A), *L. biglobosa* (Lb) (B) simultaneous and sequentially inoculated co-cultures of these pathogens at 7 dpi for experiment 1. Treatments included an Ethyl acetate control (Ethyl acetate), and four metabolite extracts *L. maculans* only (Lm), *L. biglobosa* (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially after 7 days (Lm+Lb). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm = 23 d.f., Lb = 21 d.f.).

<u>Experiment 2</u>

For *L. maculans*, there was a small yet significant difference in the colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,23}$ = 8.40, P < 0.001, LSD = 1.222 (Fig. A7.2). There was no significant difference in colony area when the 'Lm Only' (25.82 cm^2) extract was applied to *L. maculans* compared to the 'Ethyl Acetate' control (26.93 cm^2). The colony areas when these treatments were applied were significantly greater than when the 'Lm&Lb' (23.95 cm^2) or 'Lm+Lb' (24.62 cm^2) secondary metabolites were applied. However, the colony area when 'Lb only' (24.72 cm^2) extract was applied was only significantly different from the ethyl acetate control (Fig. A7.2). For *L. biglobosa*, there were large significant differences in colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,24}$ = 143.04, P < 0.001, LSD = 1.561). There was no significant difference in colony area when the Ethyl acetate (23.97 cm^2), 'Lb only' (25.75 cm^2) or the 'Lm&Lb' (24.77 cm^2) secondary metabolites were applied. However, the colony area when 'Lb only' extract was applied was significantly greater than that of the Ethyl acetate control. The colony areas when the 'Lm only' (13.18 cm^2) and 'Lm+Lb' (13.49 cm^2) extract were applied were not significantly different from each other.

Experiment 3

For *L. maculans*, there was significant difference in the colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,24}$ = 28.96, P < 0.001, LSD = 2.626 (Fig. A7.3). There was no significant difference in colony area when the 'Lm Only' (23.11 cm^2) extract was applied to *L. maculans* compared to the 'Ethyl Acetate' control (25.05 cm^2). The colony areas when these treatments were applied were significantly greater than when a treatment that contained *L. biglobosa* was applied. The colony areas when the 'Lb only' (16.18 cm^2), 'Lm+Lb' (15.26 cm^2) or 'Lm&Lb' (14.96 cm^2) extract were applied were not significantly different from each other. For *L. biglobosa*, there were large significant differences in colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,23}$ = 158.31, P < 0.001, LSD = 1.944).
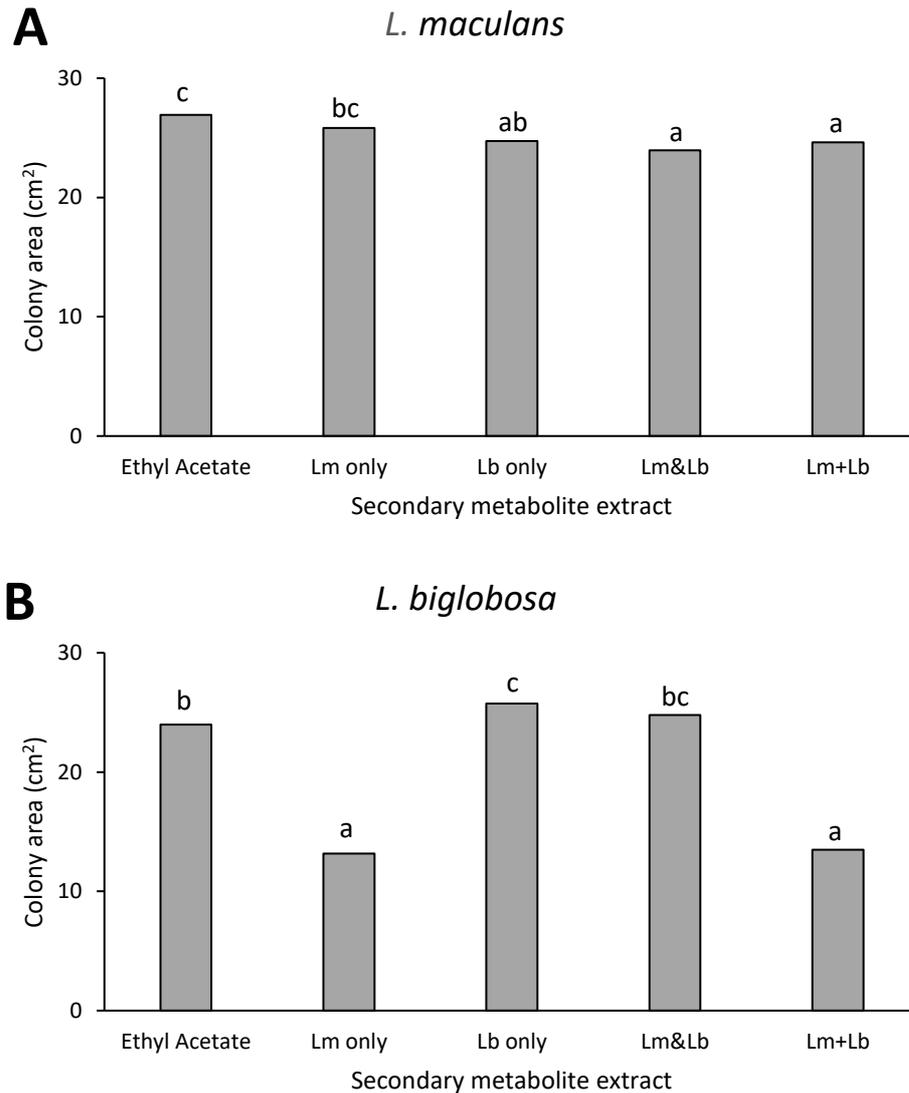
Figure A7.2: Colony area (cm^2) when agar plates with different secondary metabolites extracted from liquid cultures were inoculated with *L. maculans* (Lm)(A), *L. biglobosa* (Lb) (B) simultaneous and sequentially inoculated co-cultures of these pathogens at 7 dpi for Experiment 2. Treatments included an Ethyl acetate control (Ethyl acetate), and four metabolite extracts *L. maculans* only (Lm), *L. biglobosa* (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially after 7 days (Lm+Lb). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm = 23 d.f., Lb = 24 d.f.).
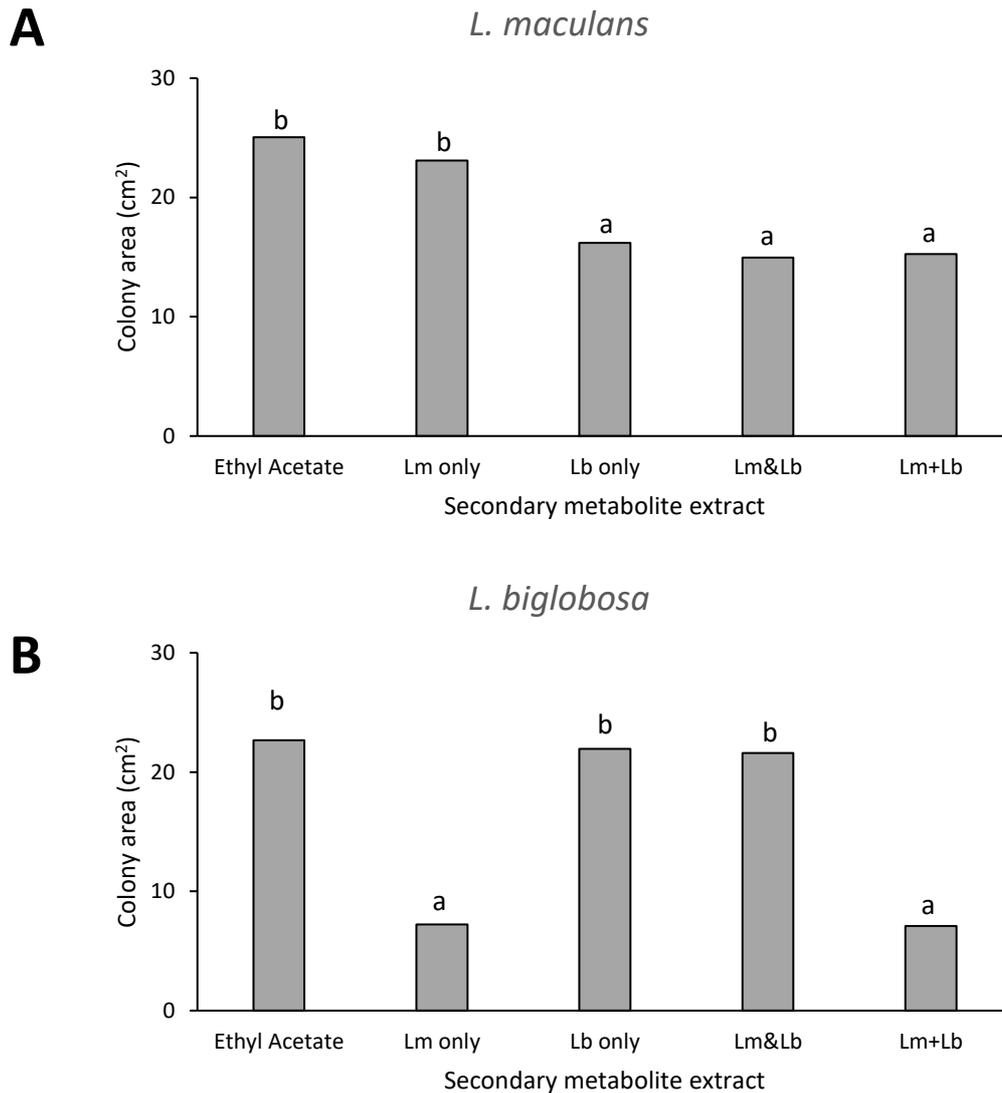
Figure A7.3: Colony area (cm²) when inoculated with different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm) (A), *L. biglobosa* (Lb) (B) simultaneous and sequentially inoculated co-cultures of these pathogens at 7 dpi for experiment 3. Treatments included an Ethyl acetate control (Ethyl Acetate), and four metabolite extracts *L. maculans* only (Lm), *L. biglobosa* (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially after 7 days (Lm+Lb). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Lm = 24 d.f., Lb = 23 d.f.).

There was no significant difference in colony area when the Ethyl acetate (22.68 cm^2), 'Lb only' (21.94 cm^2) or the 'Lm&Lb' (21.6 cm^2) secondary metabolites were applied. The colony areas when the 'Lm only' (7.22 cm^2) and 'Lm+Lb' (7.1 cm^2) extract were applied were not significantly different from each other.

APPENDICES 8 - Effects of secondary metabolites derived from sequentially co-inoculated liquid culture on the growth of *S. sclerotiorum in vitro.*

For Experiment 1, there were large significant differences in *S. sclerotiorum* colony area (cm^2) when different secondary metabolites were applied when assessed at 2 dpi ($F_{4,24}$ = 13.73, *P* < 0.001, LSD = 0.875) (Fig. A8.1). The only extract that resulted in a significantly different colony area from when the 'Ethyl Acetate' (28.65 cm^2) extract was applied was 'Lm+Lb' (11.49 cm^2); this resulted in a colony area that was significantly smaller than when all other treatments were applied. However, when the 'Lm only' (20.66 cm$^{2)}$) extract was applied the colony area was significantly smaller than when the 'Lb Only' (34.36 cm^2) or 'Lm&Lb' (32.75 cm^2) extracts were applied; these extracts were not significantly different from each other.

For Experiment 2, there were large significant differences in *S. sclerotiorum* colony area (cm^2) when different secondary metabolites were applied when assessed at 2 dpi ($F_{4,24}$ = 13.73, *P* < 0.001, LSD = 0.507) (Fig. A8.1). The only extracts that resulted in a significantly different colony area from when the 'Ethyl Acetate' (19.08 cm^2) extract was applied were 'Lm only' (2.97 cm^2) and 'Lm+Lb' (1.31 cm^2); the *S. sclerotiorum* colony area was significantly larger when 'Lm only' extract was applied than when the 'Lm+Lb' extract was applied. There was no significant difference in colony area when the other extracts were applied.

For Experiment 3, there were large significant differences in *S. sclerotiorum* colony area (cm^2) when different secondary metabolites were applied when assessed at 2 dpi ($F_{4,24}$ = 35.58, *P* < 0.001, LSD = 0.898) (Fig. A8.1). The only extracts that resulted in a significantly different colony area from when the 'Ethyl Acetate' (61.38 cm^2) extract was applied were 'Lm Only' (29.53 cm^2) and 'Lm+Lb' (17.24 cm^2); the *S. sclerotiorum* colony area was significantly larger when 'Lm Only' extract was applied than when the 'Lm+Lb' extract was applied. There was no significant difference in colony area when the other extracts were applied.
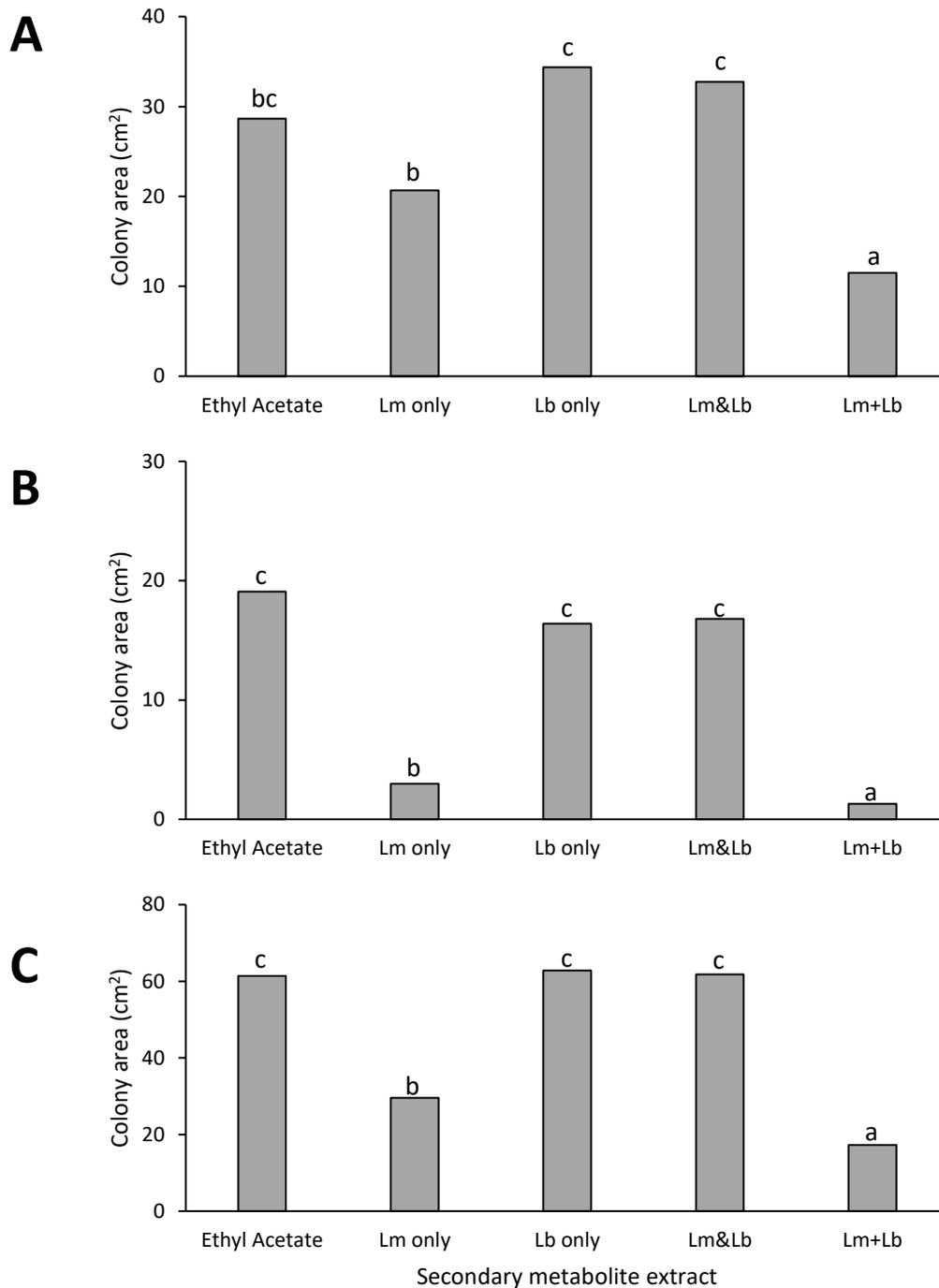
Figure A8.1. Colony area (cm^2) when _S. sclerotiorum_ was inoculated on agar plates and different secondary metabolites extracted from liquid cultures inoculated with _L. maculans_ (Lm), _L. biglobosa_ (Lb) simultaneous or sequentially inoculated co-cultures of these pathogens were applied. Assessments were made at 2 dpi for experiments 1 (A), 2 (B) and 3 (C). Treatments included an Ethyl acetate control (Ethyl acetate), and four metabolite extracts _L. maculans_ only (Lm), _L. biglobosa_ (Lb), _L. maculans_ and _L. biglobosa_ inoculated simultaneously (Lm&Lb), _L. maculans_ and _L. biglobosa_ inoculated sequentially after 7 days (Lm+Lb). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (24 d.f.).

Figure A8.2. Colony area (cm^2) when *S. sclerotiorum* was inoculated onto agar plates and different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) simultaneous and sequentially inoculated co-cultures of these pathogens were applied. Assessments were made at 2 dpi for experiments 1, 2 and 3 combined. Treatments included an Ethyl acetate control (Ethyl Acetate), and four metabolite extracts (*L. maculans* only (Lm), *L. biglobosa* only (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa inoculated* sequentially after 2 days (Lm+Lb)). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (74 d.f.).

## APPENDICES 9 - Effects of secondary metabolites derived from sequentially co-inoculated liquid culture on the growth of *V. longisporium in vitro.*

For Experiment 1, there were large significant differences in *V. longisporum* colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,23}$ = 138.37, *P* < 0.001, LSD = 0.337) (Fig. A9.1). The only extracts that resulted in a significantly different colony area from when the 'Ethyl acetate' (5.35 cm^2) extract was applied were 'Lm+Lb' (3.37 cm^2) and 'Lm only' (2.39 cm^2). The colony area when 'Lm only' secondary metabolite extract was applied was significantly smaller than when the 'Lm+Lb' extract was applied. The colony areas when 'Lb only' (5.13 cm^2) or 'Lm&Lb' (5.15 cm^2) extracts were applied were not significantly different from each other, or the 'Ethyl Acetate' control.

For Experiment 2, there were large significant differences in *V. longisporum* colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,24}$ = 52.69, *P* < 0.001, LSD = 0.357) (Fig. A9.1). The only extracts that resulted in a significantly different colony area from when the 'Ethyl Acetate' (5.45 cm^2) extract was applied were 'Lm+Lb' (3.97cm^2) and 'Lm only' (3.76 cm^2); the colony areas when 'Lm only' and 'Lm+Lb' secondary metabolite extracts were applied were not significantly different from each other. The colony areas when 'Lb only' (5.47 cm^2) or 'Lm&Lb' (5.47 cm^2) extracts were applied were not significantly different from each other, or the 'Ethyl Acetate' control.

For Experiment 3, there were large significant differences in *V. longisporum* colony area (cm^2) when different secondary metabolites were applied when assessed at 7 dpi ($F_{4,24}$ = 54.34, *P* < 0.001, LSD = 0.583) (Fig. A9.1). The only extracts that resulted in a significantly different colony area from when the 'Ethyl Acetate' (5.93 cm^2) extract was applied were 'Lm+Lb' (2.88 cm^2) and 'Lm only' (3.56 cm^2); the colony area when 'Lm only' secondary metabolite extract was applied was significantly larger than when the 'Lm+Lb' extract was applied.
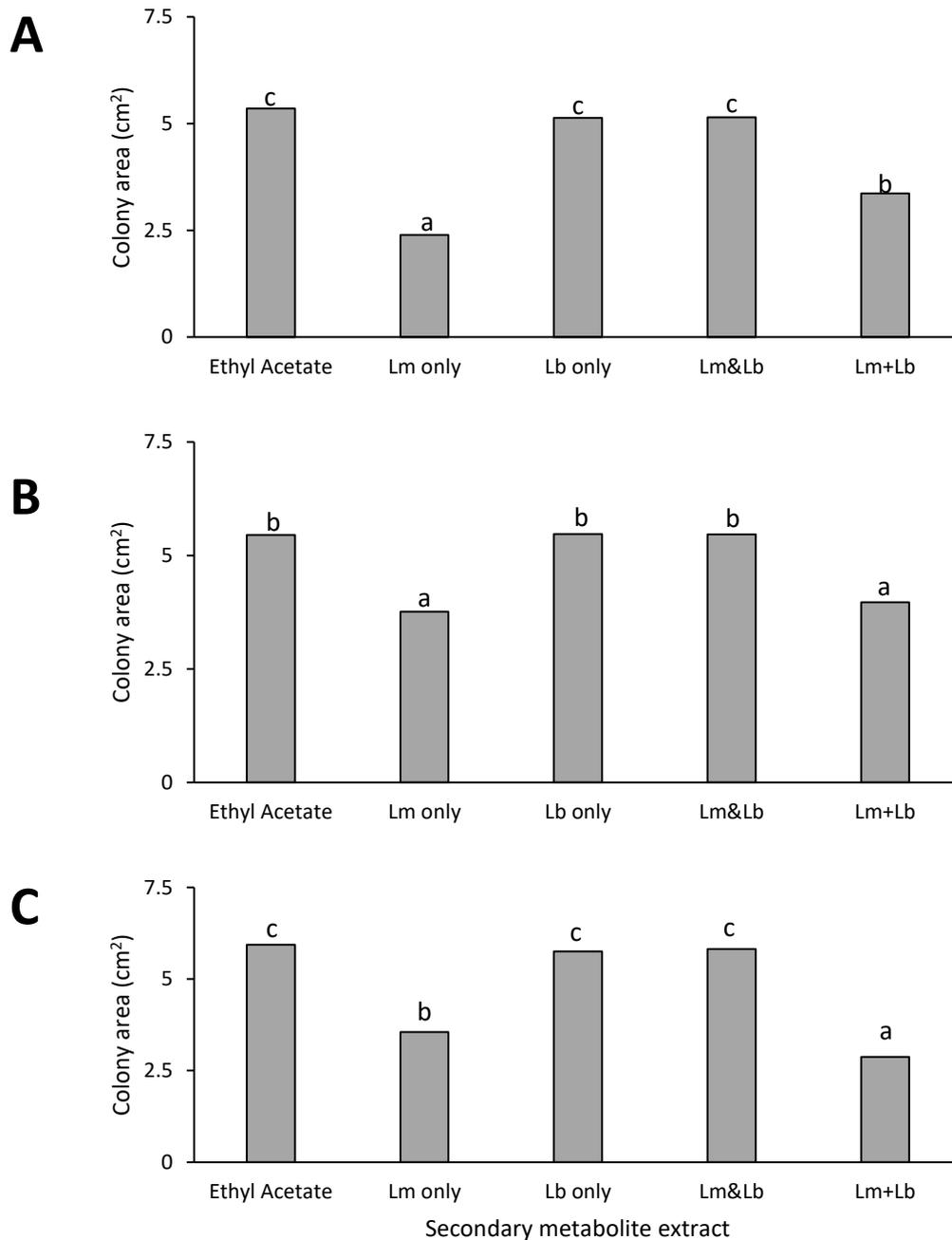
Figure A9.1. Colony area (cm^2) when *V. longisporum* were inoculated onto agar plates and different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb), simultaneous and sequentially inoculated co-cultures of these pathogens were applied. Assessments were made at 7 dpi for experiments 1 (A), 2 (B) and 3 (C). Treatments included an Ethyl acetate control (Ethyl Acetate), and four metabolite extracts (*L. maculans* only (Lm), *L. biglobosa* only (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially after 7 days (Lm+Lb)). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (Expt 1 = 23 d.f., Expt 2 and 3 = 24 d.f.).

The colony areas when 'Lb only' (5.75 cm^2) or 'Lm&Lb' (5.82 cm^2) extracts were applied were not significantly different from each other, or the 'Ethyl Acetate' control.
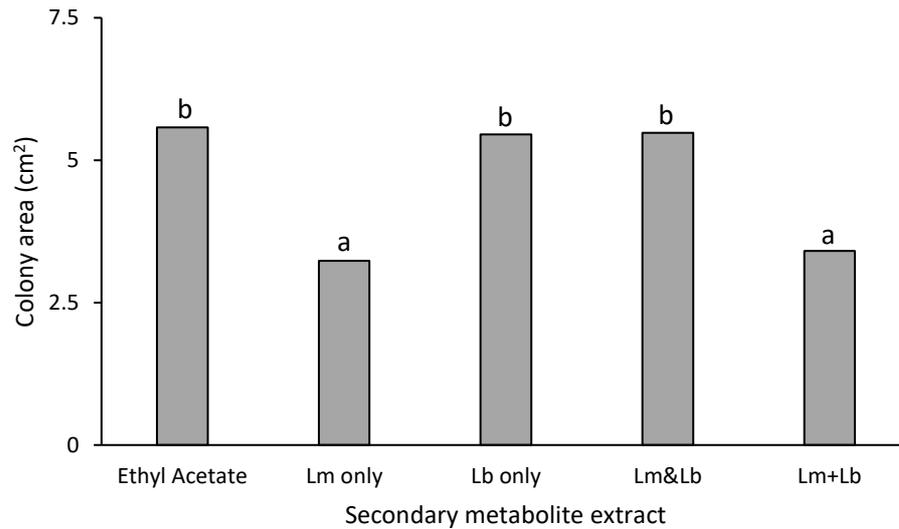
Figure A9.2. Colony area (cm^2) when agar plugs of *V. longisporum* were inoculated onto agar plates and different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* (Lm), *L. biglobosa* (Lb) simultaneous or sequentially inoculated co-cultures of these pathogens were applied. Assessments were made at 7 dpi for Experiments 1, 2 and 3 combined. Treatments included an Ethyl acetate control (Ethyl acetate), and four metabolite extracts (*L. maculans* only (Lm), *L. biglobosa* only (Lb), *L. maculans* and *L. biglobosa* inoculated simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially after 2 days (Lm+Lb)). A post-hoc Fisher's protected LSD test was done for each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen (73 d.f.).

APPENDICES 10 - qPCR thermal profiles for *L. maculans* (a), *L. biglobosa* (b) or *P. brassicae* (c).
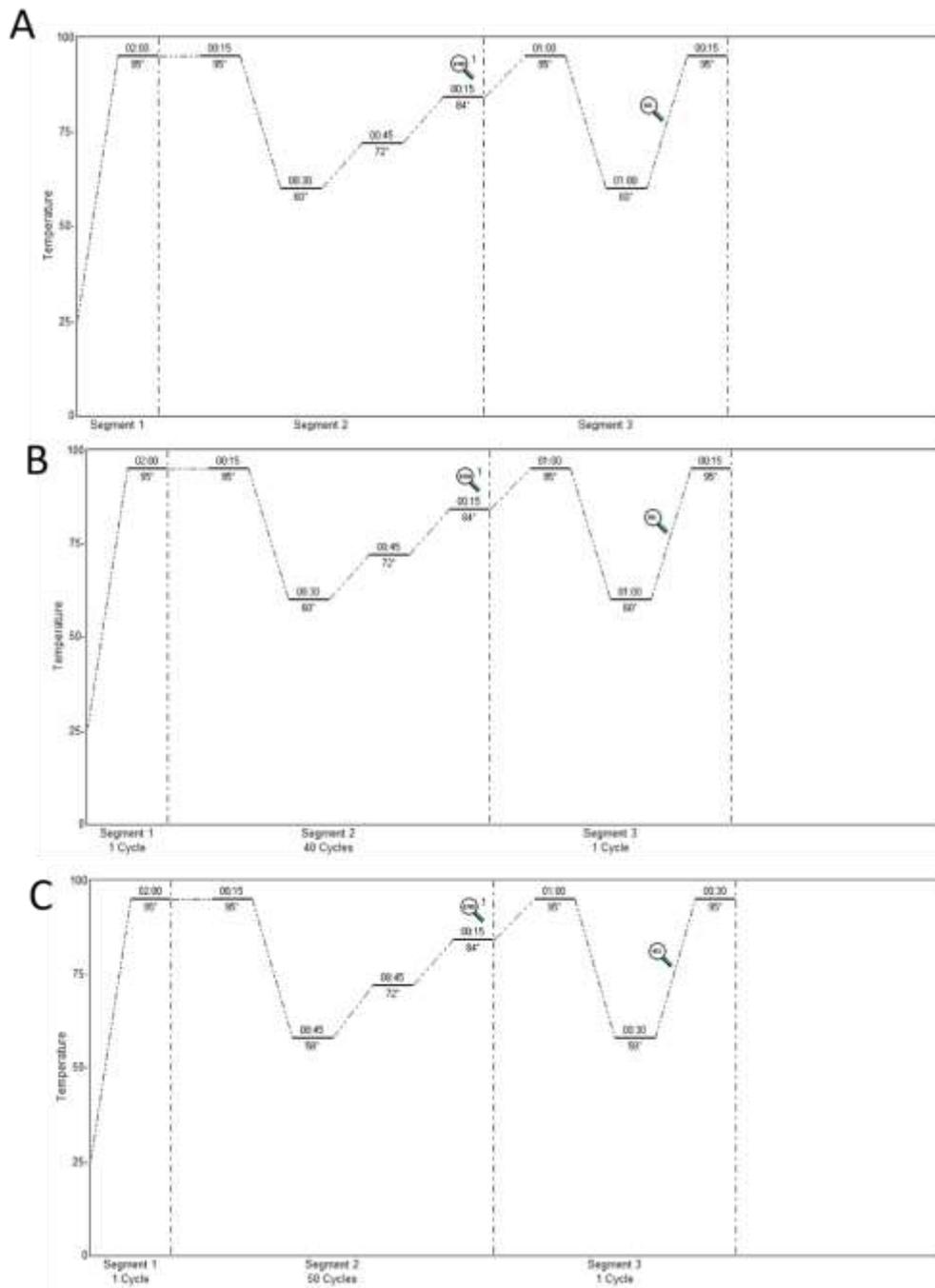
APPENDICES 11. qPCR standard curves and dissociation curves for *L. maculans* (a), *L. biglobosa* (b) or *P. brassicae* (c), and an example of range of samples fitting within the standard curve range (d).

Standard Curve

B

Log fit values

SYBR Standards, RSq:0.994
SYBR, Y = -4.179*LOG(X) + 31.31, Eff. = 73.5%
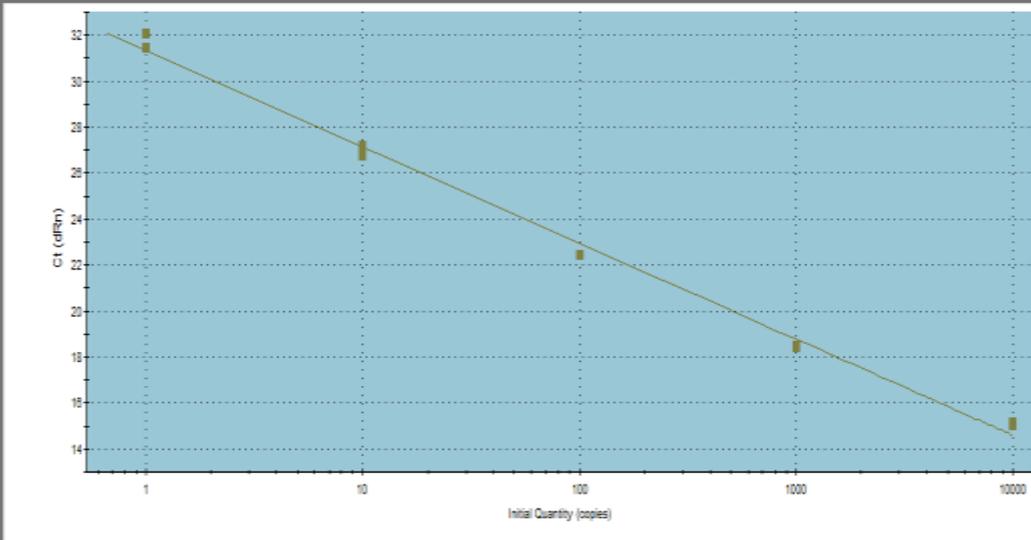
Dissociation Curve
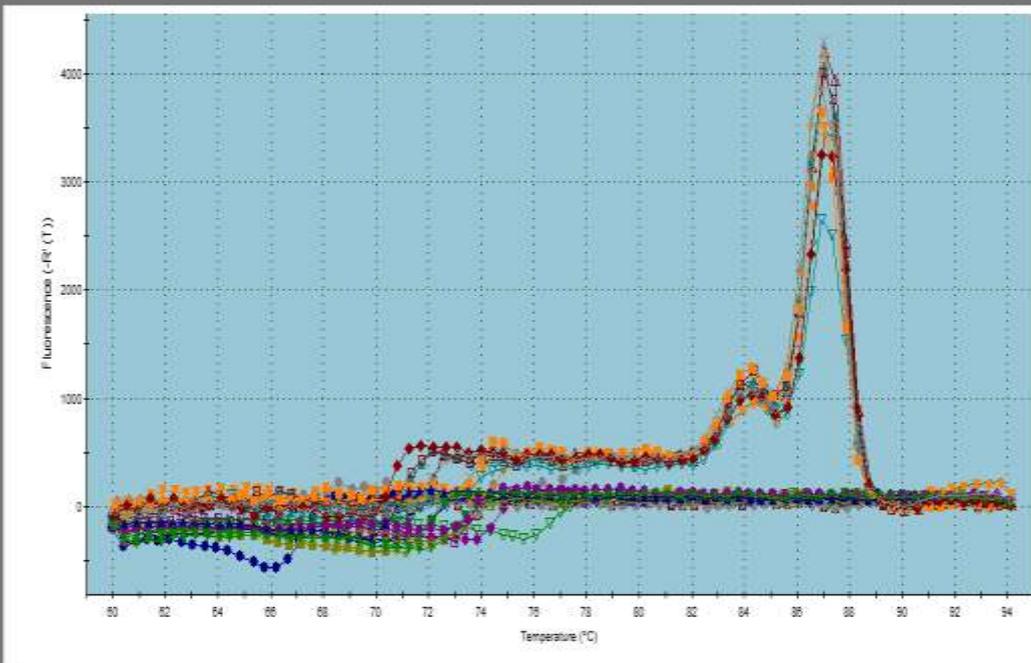
Standard Curve

C

Log fit values
- SYBR Standards, RSq:0.996
- SYBR, Y = -4.165*LOG(X) + 14.29, Eff. = 73.8%

Dissociation Curve

Standard Curve

Log fit values

■ SYBR Standards, RSq:0.997
▲ SYBR Unknowns
── SYBR, Y = -4.292*LOG(X) + 32.78, Eff. = 71.0%
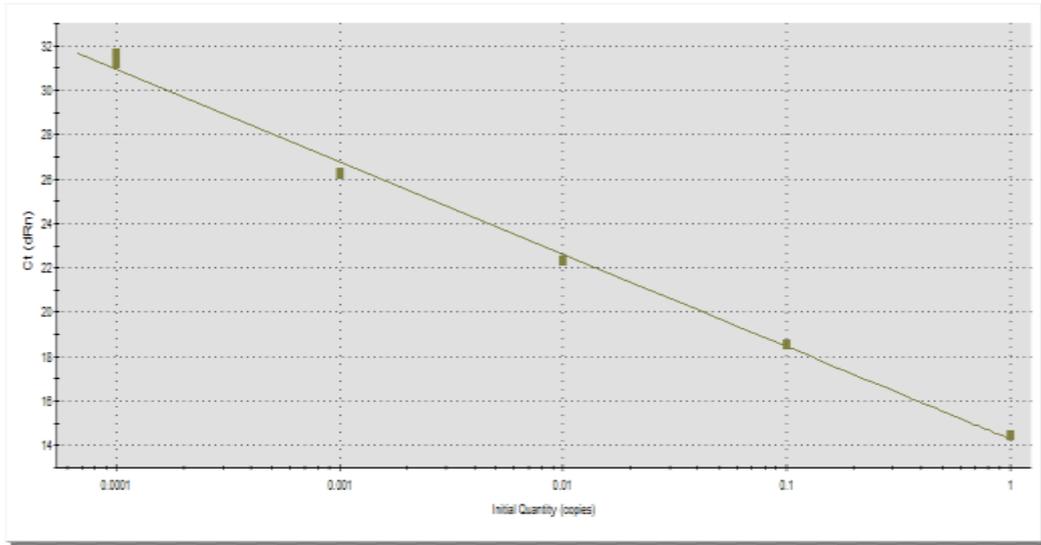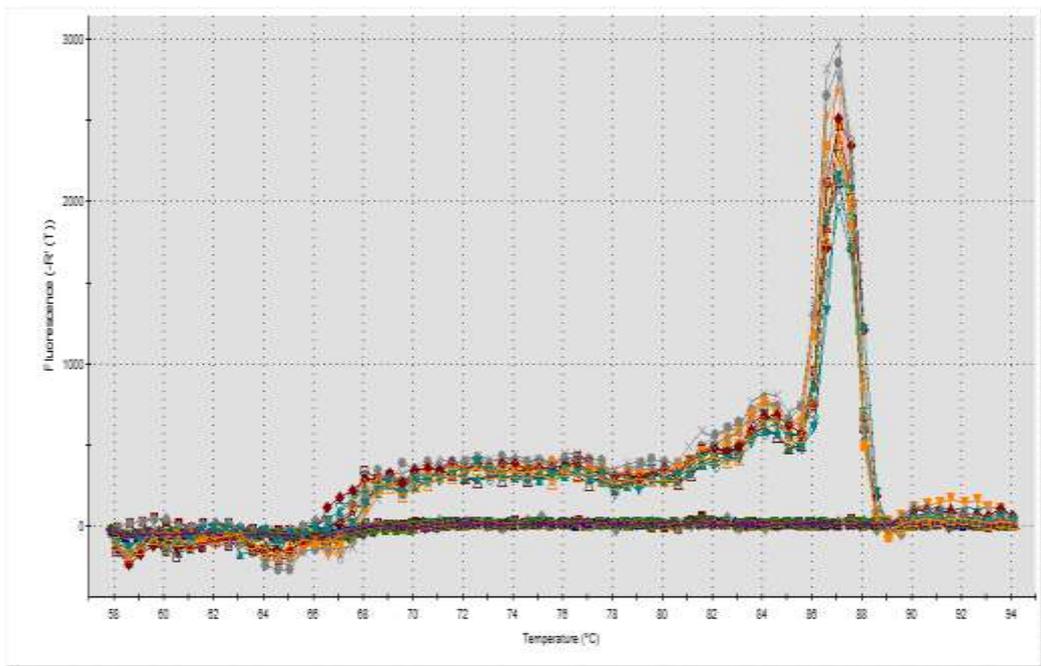
D
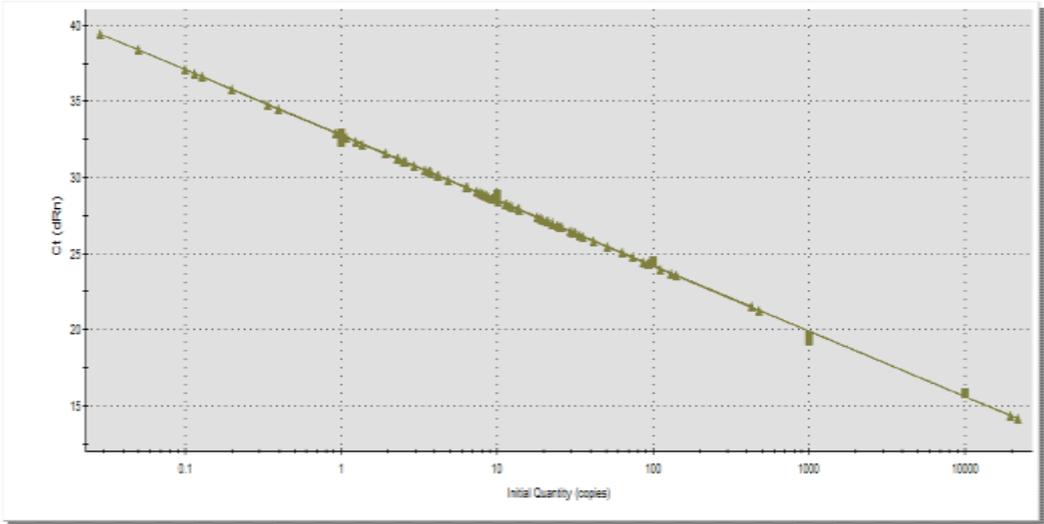
APPENDICES 12 – Lesion area when solo of co-inoculated with *L. maculans* or *L. biglobosa*.
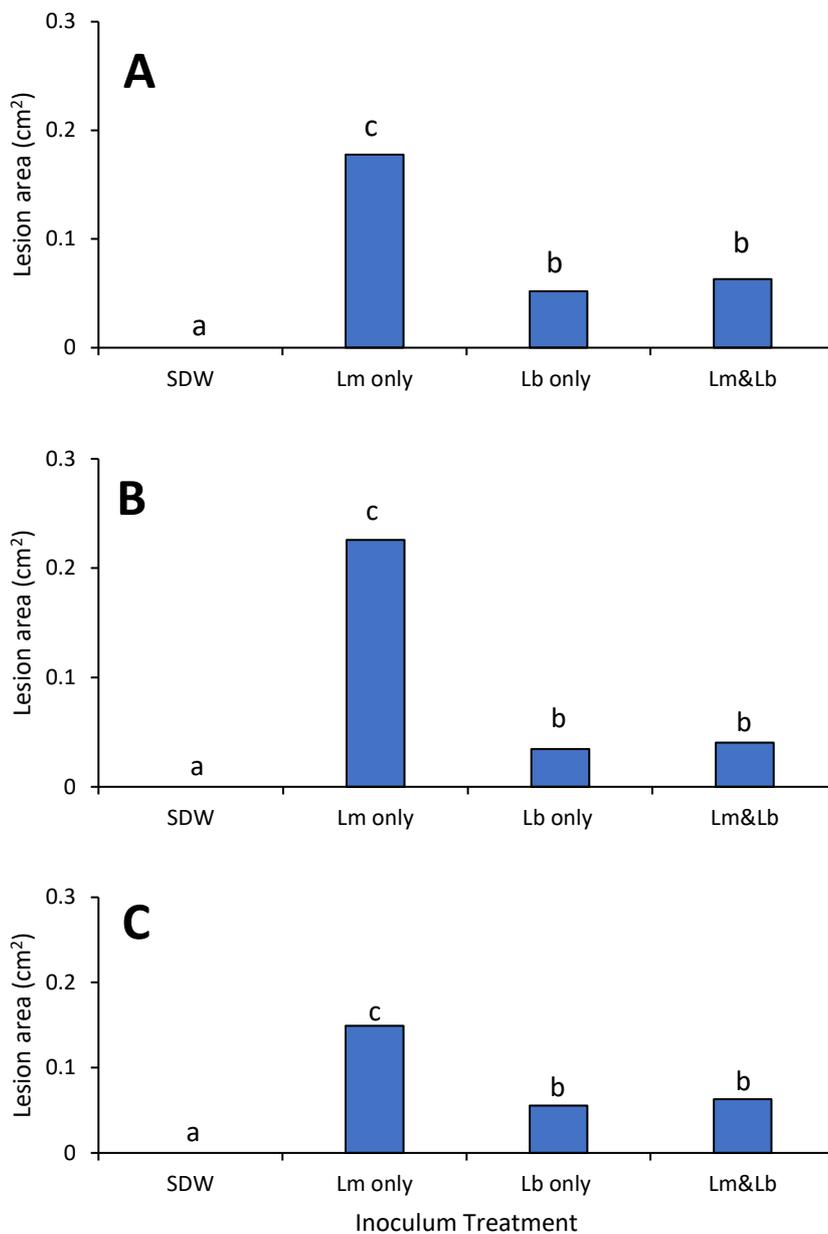
Figure A12.1: Lesion area (cm²) on cv. Charger cotyledons inoculated with *L. maculans* only (Lm only) or *L. biglobosa* only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control for Experiment 1 (A), 2 (B) or 3 (C) at 17 days post inoculation. Data were square root -transformed for statistical analysis. Unprotected Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (*P* < 0.05).

APPENDICES 13 –Effect of solo or co-inoculation of *L. maculans* or *L. biglobosa* on the growth of *L. maculans* and *L. biglobosa* in cotyledons at 26 dpi.

The growth of *L. maculans* and *L. biglobosa* in cotyledons was assessed by quantifying the pathogen DNA. At 26 dpi, there was a significant difference in the quantity of *L. maculans* DNA between treatments ($F_{3.15}$ = 13.84, *P* < 0.001, LSD = 26.68) (Fig. A13.1). The 'Lm only' treatments had a significantly greater quantity of *L. maculans* DNA (104 pg/ng) than all other treatments. There was no significant difference in the quantity of *L. maculans* DNA between the other samples, even though the mean quantity of *L. maculans* DNA in the 'Lm&Lb' was 23 pg/ 50 ng of sample DNA and the mean *L. maculans* DNA in the 'SDW' and 'Lb only' was 0pg. There was a 99.5% less *L. maculans* DNA in the 'Lm&Lb' treatment than in the 'Lm only' treatment.

There was a significant difference in the quantity of *L. biglobosa* DNA between treatments ($F_{3.15}$ = 10.80, *P* < 0.001, LSD = 0.61) (Fig. A13.1). The 'Lb only' treatments had a significantly greater quantity of *L. biglobosa* DNA (1.65 pg/ 50 ng of sample DNA) than all other treatments. The quantity of *L. biglobosa* DNA was not significantly greater in the 'Lb only' treatment than in the 'Lm&Lb' (1.67 pg/ 50 ng of sample DNA) treatment; both samples were significantly greater than the 'SDW' (0pg/ 50 ng of sample DNA) and 'Lm only' (0 pg/ 50 ng of sample DNA) treatments. There was 18% less *L. biglobosa* DNA in the 'Lm&Lb' treatment than in the 'Lb only' treatment.
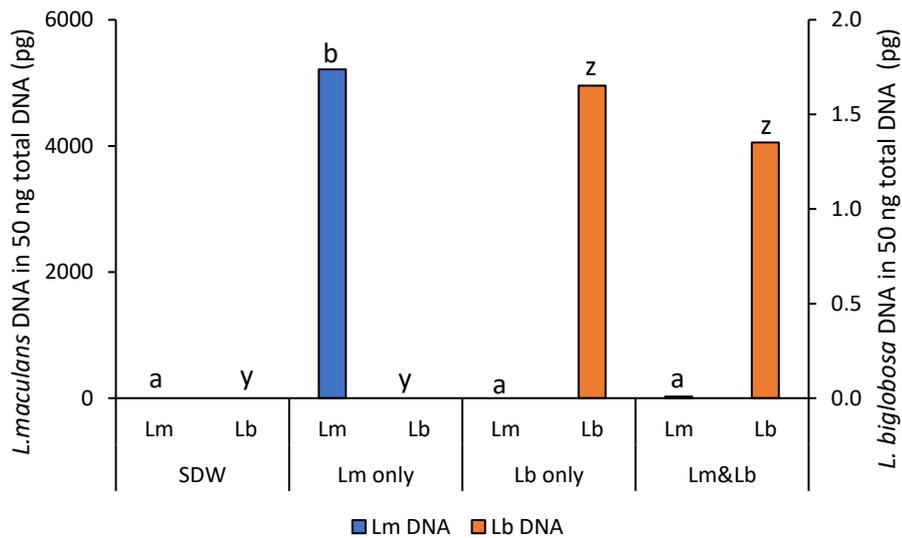
Figure A13.1: Quantity of *L. maculans* (blue) and *L. biglobosa* (orange) (pg) DNA in 50 ng of extracted DNA from plant samples with *L. maculans* only (Lm only) or *L. biglobosa* only (Lb only) or both (Lm&Lb) or with sterilised distilled water (SDW) as a control for all replicates combined at 26 days post inoculation. Data were square root-transformed for statistical analysis. Fisher LSD tests were used to separate the mean values of colony areas. Columns that do not share a letter are significantly different (*P* < 0.05) (a-b for *L. maculans* and y-z for *L. biglobosa*).

APPENDICES 14 – Field Experiment Plans

A) 2017/18 Cropping Season

Plots drilled on 3.5m centres to give adeq

PLOT	DISCARD	DISCARD	1	2	3	4	5	6
BLOCK	DISCARD	DISCARD	1	1	1	1	1	1
TREATMENT	DISCARD	DISCARD	11	1	10	6	8	5
PLOT	DISCARD	DISCARD	7	8	9	10	11	12
BLOCK	DISCARD	DISCARD	1	1	1	1	1	1
TREATMENT	DISCARD	DISCARD	4	3	12	9	2	7
PLOT	DISCARD	DISCARD	13	14	15	16	17	18
BLOCK	DISCARD	DISCARD	2	2	2	2	2	2
TREATMENT	DISCARD	DISCARD	1	6	2	8	10	7
PLOT	DISCARD	DISCARD	19	20	21	22	23	24
BLOCK	DISCARD	DISCARD	2	2	2	2	2	2
TREATMENT	DISCARD	DISCARD	9	4	12	5	11	3
PLOT	DISCARD	DISCARD	25	26	27	28	29	30
BLOCK	DISCARD	DISCARD	3	3	3	3	3	3
TREATMENT	DISCARD	DISCARD	10	4	3	1	6	12
PLOT	DISCARD	DISCARD	31	32	33	34	35	36
BLOCK	DISCARD	DISCARD	3	3	3	3	3	3
TREATMENT	DISCARD	DISCARD	11	2	9	7	5	8

		25/10/2017	28/11/2017
Trt	Variety	T1	T2
1	Quartz	untreated	untreated
2	Quartz	Proline 0.315	Proline 0.315
3	Flamingo	untreated	untreated
4	Flamingo	Proline 0.315	Proline 0.315
5	Barbados	untreated	untreated
6	Barbados	Proline 0.315	Proline 0.315
7	Charger	untreated	untreated
8	Charger	Proline 0.315	Proline 0.315
9	Django	untreated	untreated
10	Django	Proline 0.315	Proline 0.315
11	MH13JO76	untreated	untreated
12	MH13JO76	Proline 0.315	Proline 0.315

B) 2018/ 19 Cropping Season

PLOT	DISCARD	1	2	3	4	5	6	DISCARD
BLOCK		1	1	1	1	1	1	
TREATMENT		9	6	2	5	7	8	
PLOT	DISCARD	7	8	9	10	11	12	DISCARD
BLOCK		1	1	1	1	1	1	
TREATMENT		3	10	1	11	4	12	
PLOT	DISCARD	13	14	15	16	17	18	DISCARD
BLOCK		2	2	2	2	2	2	
TREATMENT		1	9	11	6	8	7	
PLOT	DISCARD	19	20	21	22	23	24	DISCARD
BLOCK		2	2	2	2	2	2	
TREATMENT		5	12	3	10	2	4	
PLOT	DISCARD	25	26	27	28	29	30	DISCARD
BLOCK		3	3	3	3	3	3	
TREATMENT		10	11	5	6	12	7	
PLOT	DISCARD	31	32	33	34	35	36	DISCARD
BLOCK		3	3	3	3	3	3	
TREATMENT		4	8	3	2	1	9	

		16/11/2018	11/12/2018
Trt	Variety	T1	T2
1	Quartz	untreated	untreated
2	Quartz	Proline 0.315	Proline 0.315
3	Flamingo	untreated	untreated
4	Flamingo	Proline 0.315	Proline 0.315
5	Barbados	untreated	untreated
6	Barbados	Proline 0.315	Proline 0.315
7	Charger	untreated	untreated
8	Charger	Proline 0.315	Proline 0.315
9	Django	untreated	untreated
10	Django	Proline 0.315	Proline 0.315
11	MH13JO76	untreated	untreated
12	MH13JO76	Proline 0.315	Proline 0.315

C) 2019/20 Cropping Season

PLOT	DISCARD	307	308	309	310	311	312	DISCARD
BLOCK		3	3	3	3	3	3	
TREATMENT		9	10	5	4	11	8	
PLOT	DISCARD	301	302	303	304	305	306	DISCARD
BLOCK		3	3	3	3	3	3	
TREATMENT		3	6	7	12	1	2	
PLOT	DISCARD	207	208	209	210	211	212	DISCARD
BLOCK		2	2	2	2	2	2	
TREATMENT		4	12	9	7	8	1	
PLOT	DISCARD	201	202	203	204	205	206	DISCARD
BLOCK		2	2	2	2	2	2	
TREATMENT		3	10	6	5	11	2	
PLOT	DISCARD	107	108	109	110	111	112	DISCARD
BLOCK		1	1	1	1	1	1	
TREATMENT		6	1	9	7	11	8	
PLOT	DISCARD	101	102	103	104	105	106	DISCARD
BLOCK		1	1	1	1	1	1	
TREATMENT		4	5	12	3	2	10	

		05/11/2019	**16/12/2019**
Treatment	**Variety**	**T1**	**T2**
1	Quartz	untreated	untreated
2	Quartz	Proline 0.315	Proline 0.315
3	Flamingo	untreated	untreated
4	Flamingo	Proline 0.315	Proline 0.315
5	Barbados	untreated	untreated
6	Barbados	Proline 0.315	Proline 0.315
7	Charger	untreated	untreated
8	Charger	Proline 0.315	Proline 0.315
9	Django	untreated	untreated
10	Django	Proline 0.315	Proline 0.315
11	MH13JO76	untreated	untreated
12	MH13JO76	Proline 0.315	Proline 0.315

APPENDICES 15 – Incidence and severity of Phoma leaf spotting at the post T1 and T2 assessments.

The 2017/18 cropping season

During the 2017/18 cropping season, the average severity of phoma leaf spotting from all cultivars was similar at T1 + 5 weeks (5 weeks after the early fungicide spray) to that at T2 + 6 weeks (6 weeks after the later fungicide spray). There were no significant differences between cultivars in the severity of *L. maculans* leaf spotting at both post-fungicide application assessments between cultivars ($F_{5,35}$ = 1.68, *P* = 0.182, LSD = 5.33; $F_{5,35}$ = 2.10, *P* = 0.104, LSD = 1.60). However, there were significant decreases in average severity of *L. maculans* phoma leaf spotting for untreated plots (5.8 and 3.2, respectively; $F_{1,35}$ = 6.30, *P* = 0.029, LSD = 3.3) and plots treated with prothioconazole (2.1 and 1.4, respectively; $F_{1,35}$ = 15.98, *P* <0.001, LSD = 0.92) between T1 + 5 weeks and T2 + 6 weeks.

Interactions between cultivar and fungicide treatment were not detected at either assessment timing ($F_{5,35}$ = 1.56, *P* = 0.212, LSD = 7.53; $F_{2.35}$ = 1.15, *P* = 0.365, LSD = 2.26). There was no significant difference between treatments at T1 + 5 weeks ($F_{11,35}$ = 1.74, *P* = 0.124, LSD = 8.13) but there was a difference at T2 + 6 weeks ($F_{11,35}$ = 3.08, *P* = 0.010, LSD = 2.19) (Fig. A15.1). A post-hoc Fisher's protected LSD test identified a difference at the T2 + 6 weeks post-fungicide assessment.
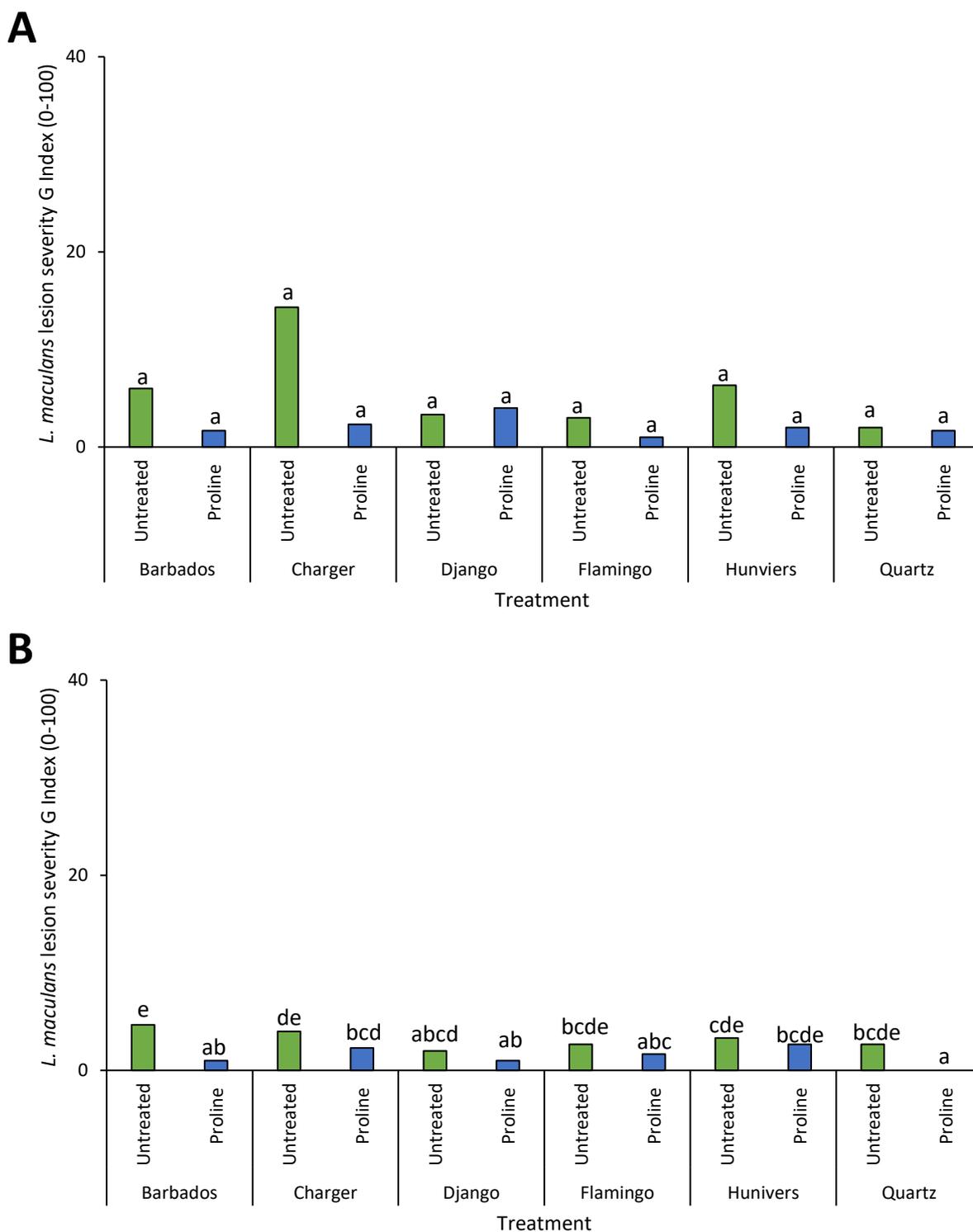
Figure A15.1: Severity of *L. maculans* leaf spots (% plants affected) at A) T1 + 5 weeks (27 November 2017) and B) T2 + 6 weeks (09 January 2018) at Terrington St Clement during the 2017/2018 cropping season. Fisher's protected LSD tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05. (35 d.f.).

At T2 + 6 weeks, the treatment with the smallest severity score was treated cv. Quartz (0.0), but this was not significantly different from treated cvs Barbados (1.0), Django (1.0) and Flamingo (1.7) or untreated cv. Django (2.0). The only treatment that was significantly different from untreated cv. Django was untreated cv. Barbados (4.7); this severity score was not significantly greater than those of untreated cvs Quartz (2.7), Flamingo (2.7), Charger (4.0) or Hunivers (3.3) or treated cv. Hunivers (2.7).

During the 2017/18 cropping season, the mean incidences of phoma leaf spotting on untreated plots (38.3 and 32.2%, respectively) were greater than on treated plots (18.9 and 14.4%, respectively) at both T1 + 5 weeks and at T2 + 6 weeks ($F_{1,35}$ = 16.06, P < 0.001, LSD = 10.06; $F_{1,35}$ = 16.79, P < 0.001, LSD = 8.96). There were no significant differences in the incidence of *L. maculans* leaf spotting between cultivars at either T1 + 5 weeks or T2 + 6 weeks post-fungicide assessment ($F_{5,35}$ = 2.3, P = 0.077, LSD = 18.31; $F_{5,35}$ = 2.10, P = 0.104, LSD = 15.98), respectively. There was no significant interaction between cultivar and fungicide application for *L. maculans* leaf spotting incidence at both T1 and T2 ($F_{5,35}$ = 1.37, P = 0.275, LSD = 24.65; $F_{5,35}$ = 1.15, P = 0.365, LSD = 22.59) (Fig. A15.2). Despite this, there was a significant difference between treatments observed at both post-fungicide assessments ($F_{11,35}$ = 2.91, P = 0.014, LSD = 25.89; $F_{11,35}$ = 3.08, P = 0.010, LSD = 21.94, respectively). At T1 + 5 weeks, the treatment that had the smallest mean incidence of plants with *L. maculans* lesions was treated cv. Flamingo (10%). However, this score was only significantly smaller than the incidence scores for untreated cvs Hunivers (50%) and Charger (63%); the incidence score for untreated cv. Charger was significantly greater than that for all other treatments except for untreated cv. Hunivers. However, the incidence score for untreated cv. Hunivers was not significantly greater than those of untreated cvs Flamingo (30%), Barbados (33%) or Django (33%) and treated cv. Django (27%).

At T2 + 6 weeks, the treatment that had the smallest incidence was prothioconazole treated cv. Quartz (0%); this was not significantly different from treated cvs Barbados (10%), Django (10%), Flamingo (17%) and untreated cv. Django (20%).
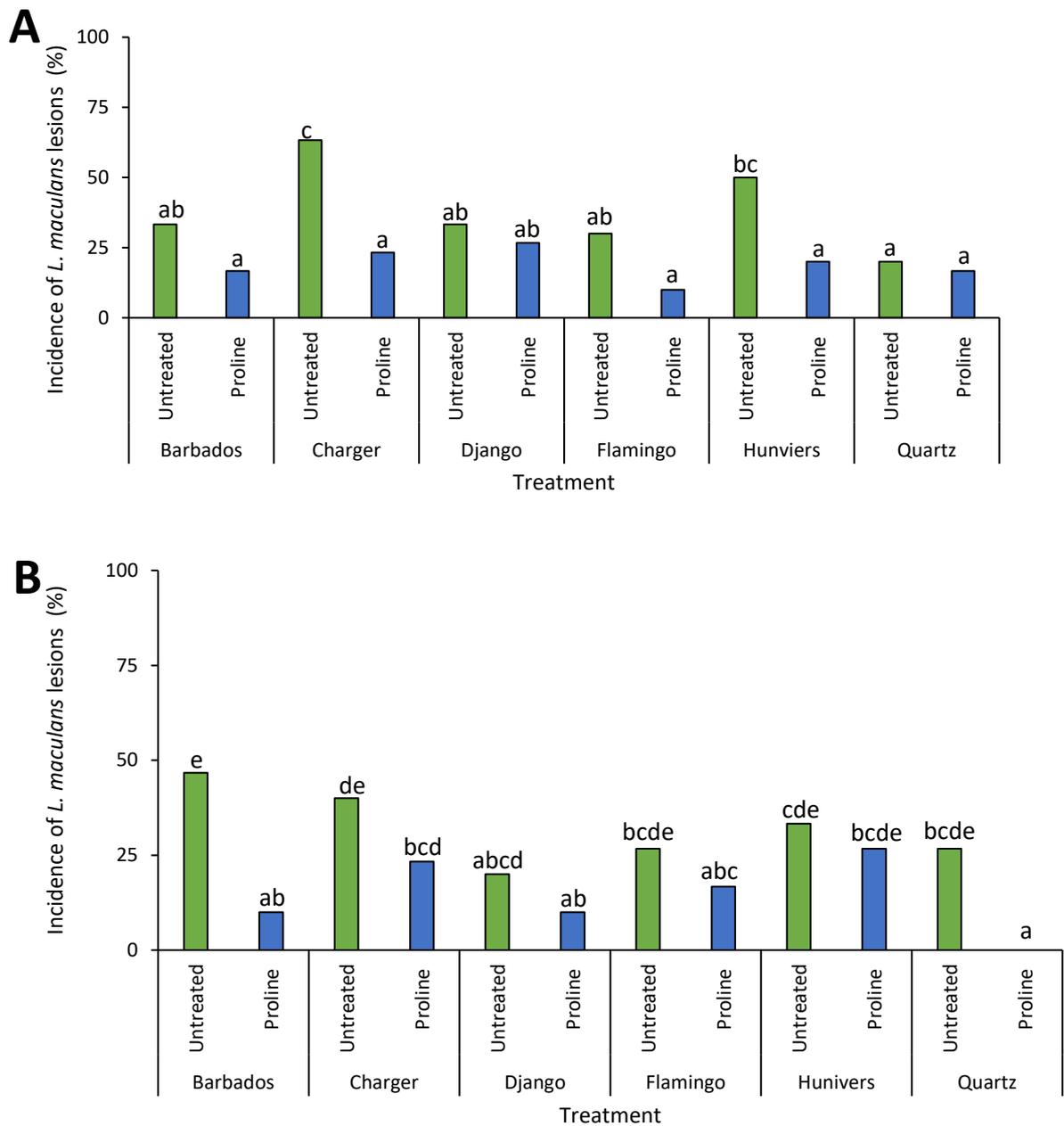
Figure A15.2: Incidence of *L. maculans* leaf spots (% plants affected) at A) T1 + 5 weeks (27 November 2017) and B) T2 + 6 weeks (09 January 2018) at Terrington St Clement during the 2017/2018 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05. (35 d.f.).

The only treatment that untreated cv. Django was significantly different from was untreated cv. Barbados (47%); this treatment was not significantly different from untreated cvs Quartz (27%), Flamingo (27%), Hunivers (33%), Charger (40%) and treated cv. Hunivers (27%). Treated cv. Charger was only significantly different from the treatments with the smallest and greatest severity scores (treated cv. Quartz and untreated cv. Barbados).

The 2018/19 cropping season

During the 2018/19 cropping season, the average severity of *L. maculans* lesions differed between T1 + 4 weeks and T2 + 5 weeks assessments (Fig. A15.3). The severity of *L. maculans* lesions on untreated plots was greater at T2 + 5 than at T1 + 4 weeks. There was no significant difference in average severity score of *L. maculans* lesions between untreated and treated plots at T1 + 4 weeks, whereas there was a significant difference at the T2 + 5 weeks assessment ($F_{1,35}$ = 0.82, *P* = 0.376, LSD = 2.65; $F_{1,35}$ = 37.76, *P* < 0.001, LSD = 6.00). At T1 + 4 weeks, there was no significant difference between untreated (1.56) and treated (2.22) plots. However, at T2 + 5 weeks in plots that received an application of prothioconazole (1.4), the lesions were significantly smaller than those in plots that remained untreated (19.2).

At T1 + 4 weeks, there were no significant differences in the severity of *L. maculans* lesions between cultivars nor was there an interaction between the two treatments ($F_{5,35}$ = 1.14, *P* = 0.370, LSD = 2.65; $F_{5,35}$ = 0.67, *P* = 0.653, LSD = 3.75). There was no significant difference between treatments at the post-T1 assessment, reaffirmed by a Fisher's protected LSD post hoc test ($F_{11,35}$ = 0.88, *P* = 0.569, LSD = 3.76). In contrast, at the post-T2 assessment, there was an overall difference in severity of *L. maculans* lesions between cultivars ($F_{,35}$ = 2.75, *P* = 0.045, LSD = 10.39). Cv. Charger (21.2) had significantly more severe *L. maculans* lesions than all other cultivars. A significant interaction between cultivar and application of fungicide was not found ($F_{5,35}$ = 1.81, *P* = 0.151, LSD = 14.70).

Figure A15.3: Severity of *L. maculans* leaf spots at A) T1 + 4 weeks (11 December 2018) and B) T2 + 5 weeks (10 January 2019) at Terrington St Clement during the 2018/2019 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05. (35 d.f.).

There were significant differences between treatments found at the post-T2 assessment ($F_{11,35}$ = 5.14, *P* < 0.001, LSD = 15.13). Treated cv. Quartz had the smallest *L. maculans* lesion severity (0.033); this value was significantly different only from untreated cvs Flamingo (15.7), Django (19.7), Hunivers (20.3) and Charger (38.7). The severity scores on all other treatments were significantly smaller than on untreated cv. Charger. However, the only cultivars that were significantly different from untreated cv. Hunivers were treated cultivars. The only treatment for which the severity score was significantly different from untreated cvs Quartz (7.3) and Barbados (13.7) was untreated cv. Charger.

During the 2018/19 cropping season, the mean incidence of *L. maculans* lesions on untreated plots was greater at T2 + 5 weeks (10.3) than at T1 + 4 weeks (1.89) (Fig. A15.4). There were no overall significant differences in the incidence of plants with *L. maculans* lesions between cultivars or treatments with/without application of fungicides, nor was there an interaction between the two treatments found at T1 + 4 weeks ($F_{5,35}$ = 0.84, *P* = 0.533, LSD = 16.37; $F_{1,35}$ = 0.95, *P* = 0.340, LSD = 9.45; $F_{5,35}$ = 1.13, *P* = 0.374, LSD = 23.15). There was no significant difference in *L. maculans* lesion incidence between treatments at the post T1 assessment ($F_{1.35}$ = 0.99, *P* = 0.484, LSD = 22.99). However, for the T2 + 5 weeks assessment, there were overall significant differences between cultivars ($F_{5.35}$ = 3.11, *P* = 0.028, LSD = 20.50) with cv. Quartz (31.7%) having a significantly smaller overall incidence of plants with *L. maculans* lesions than cv. Charger (66.7%). All other cultivars were not significantly different from either cv. Quartz or Charger. Overall, there was also a significantly smaller incidence of *L. maculans* lesions when a spray of prothioconazole was applied (14.4%), in comparison to untreated plots (73.9%) ($F_{1.35}$ = 108.47, *P* = <0.001, LSD = 11.84). All cultivars that had received a spray of prothioconazole had a significantly smaller incidence of plants with *L. maculans* phoma lesions than the equivalent untreated plots. There was a significant difference in *L. maculans* lesion incidence between treatments at the post-T2 assessment ($F_{11.35}$ = 11.36, *P* = <0.001, LSD = 28.92). A post hoc Fisher's protected LSD test showed that the treatments that had the smallest mean incidence of *L. maculans* lesions were treated cv. Quartz and treated cv. Django (3.3%); however, these were not significantly smaller than those for any other treated cultivars, except cv. Charger (37%).

Figure A15.4: Incidence of *L. maculans* leaf spots (% plants affected) at A) T1 + 4 weeks (11 December 2018) and B) T2 + 5 weeks (10 January 2019) at Terrington St Clement during the 2018/2019 cropping season. Fisher's protected LSD tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (35 d.f.).

The treatment that had the greatest mean incidence of plants with *L. maculans* lesions was untreated cv. Charger (96.67%); this was significantly different from all other cultivars except for untreated cvs Flamingo (77%) and Django (77%). However, all other untreated cultivars were not significantly different from the incidence scores on untreated cvs Flamingo or Django. All treated plots had significantly smaller *L. maculans* lesion incidences than untreated cvs Flamingo or Django.

The 2019/20 cropping season

During the 2019/20 cropping season, the average severity of phoma leaf spots differed between T1 + 6 weeks and T2 + 5 weeks assessments. The severity of *L. maculans* phoma leaf spotting on untreated plots was greater at T2 + 5 than at T1 + 6 weeks. At the post T1 assessment, there was no overall significant difference between cultivars ($F_{5,35}$ = 1.42, P = 0.258, LSD = 6.23). However, there was an overall significant difference due to the application of prothioconazole, with treated plots (0.11) having a smaller phoma leaf spotting severity score than untreated plots (7.56) ($F_{1,35}$ = 18.42, P < 0.001, LSD = 3.60). A significant interaction between cultivar and fungicide application was not found at T1+ 6 weeks ($F_{5,35}$ = 1.37, P = 0.274, LSD = 8.81). There were significant differences between treatments ($F_{11,35}$ = 2.94, P = 0.02) (Fig. A15.5). The *L. maculans* leaf spotting severity score for untreated cv. Charger (15.0) was significantly greater than the severity scores on all treated plots and untreated cvs Quartz (0.7) or Barbados (3.3). The treatments that had the smallest *L. maculans* leaf spotting severity score were treated cvs Quartz (0.0), Flamingo (0.0), Django (0.0) and Hunivers (0.0); these were only significantly smaller than those of untreated cvs Flamingo (9.0), Hunivers (9.0) and Charger. In contrast, overall significant differences at T2 + 5 weeks assessment were found between cultivar ($F_{5,35}$ = 4.76, P = 0.004, LSD = 5.54). Overall, cv. Hunivers had the greatest severity score (13.7), which was significantly greater than all other treatments, except Charger (10.1) and Django (10.3).

The cultivar that had the smallest mean severity score was cv. Quartz (2.1), which was significantly smaller than all over cultivars except Barbados (6.2) and Flamingo (5.9).
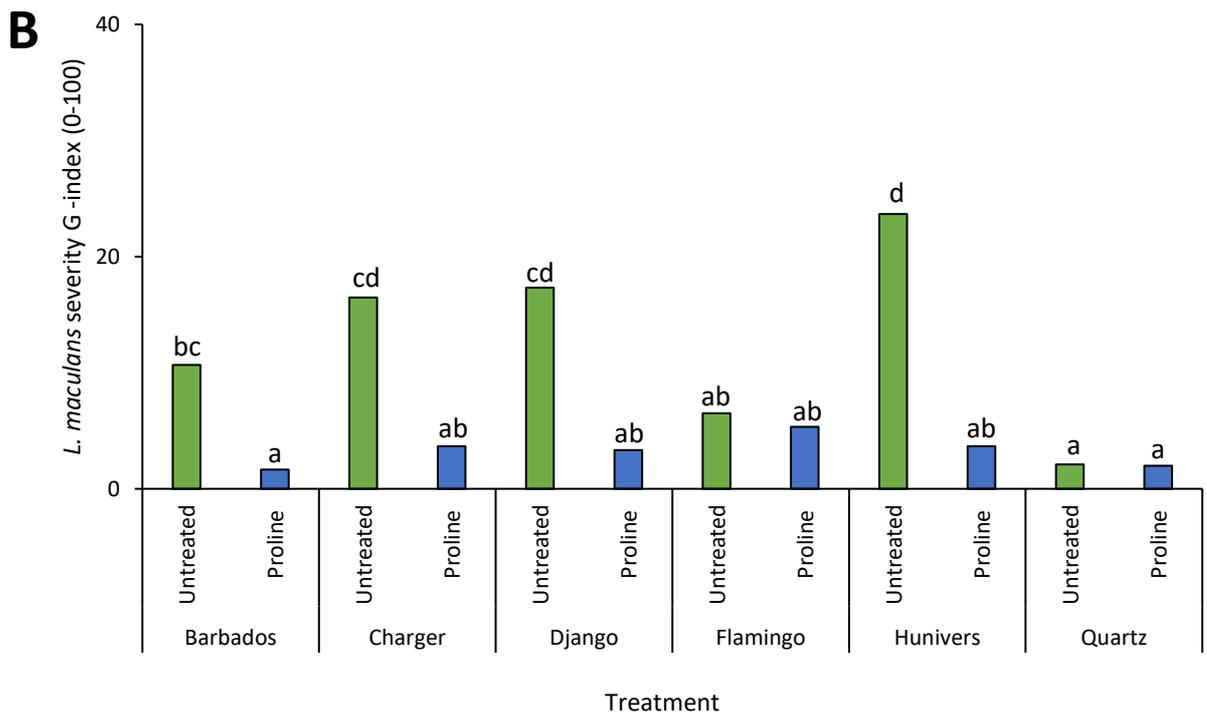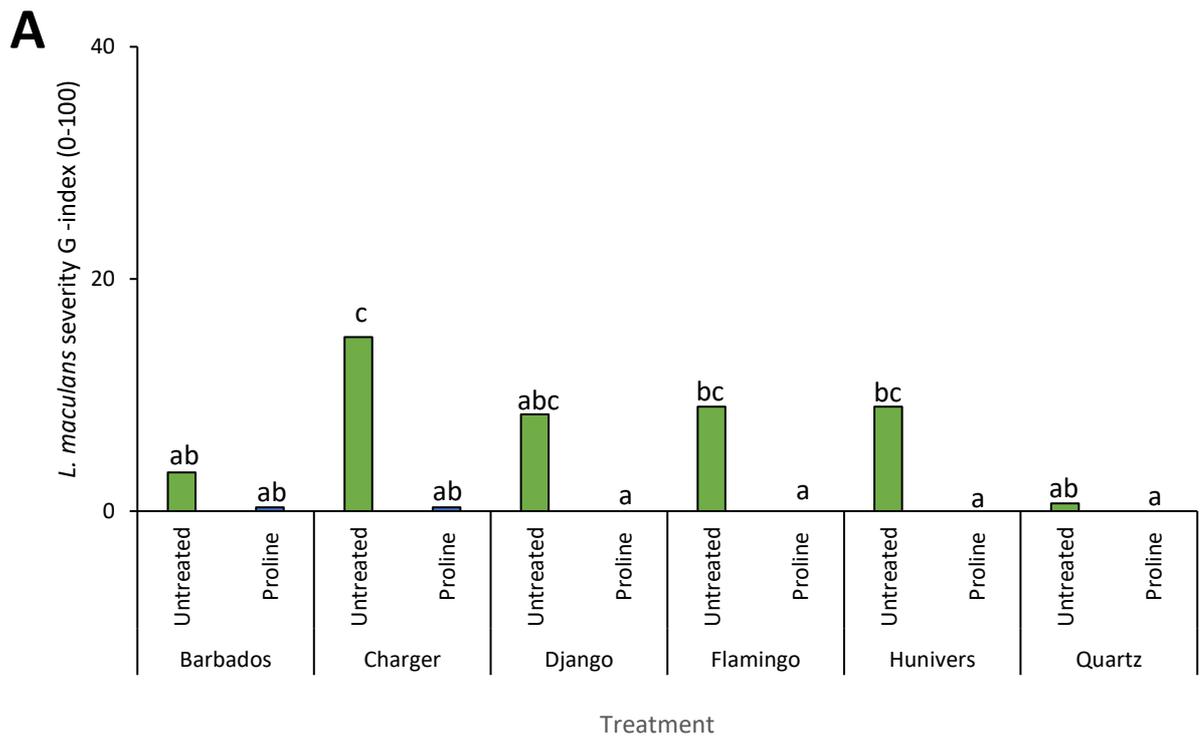
Figure A15.5: Severity of *L. maculans* leaf spots at A) T1 + 4 weeks (12 December 2019) and B) T2 + 5 weeks (20 January 2020) at Terrington St Clement during the 2019/2020 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (35 d.f.).

There was also a significant difference between plots treated with prothioconazole and untreated plots ($F_{1,35}$ = 38.07, P < 0.001, LSD = 3.20), with the untreated plots (12.8) having a greater *L. maculans* leaf spotting severity score than those that received an application of prothioconazole (3.3). A significant interaction between cultivar and treatment was found ($F_{5,35}$ = 4.20, P = 0.008, LSD = 7.83). The only cultivars whose untreated severity score was not significantly different from the treated score were Quartz (2.1, 2.00 respectively) and Flamingo (6.50, 5.33 respectively). There was a significant difference in severity score after the application of prothioconazole on all other cultivars. There was a significant difference between treatments ($F_{11,35}$ = 7.54, P < 0.001, LSD= 7.83). The treatment that had the smallest *L. maculans* leaf spotting severity score was treated Barbados (1.7), which was significantly different only from untreated cvs Barbados (10.7), Charger (16.5), Django (17.3) and untreated Hunivers (23.7). Untreated Hunivers had the greatest severity score and that was significantly greater than those for all treatments that received an application of prothioconazole and untreated cvs Flamingo, Quartz and Barbados.

During the 2019/20 cropping season, the mean incidence of phoma leaf spots on untreated plots was greater at T2 + 5 weeks (19.4%) than at T1 + 4 weeks (38.1%). At both post fungicide assessments, there were significant differences in incidence of plants with *L. maculans* phoma leaf spots between cultivars and between treatments with/without fungicide application (Cultivar - $F_{5,35}$ = 3.17, P = 0.027, LSD = 17.86; $F_{5,35}$ = 4.38, P = 0.006, LSD = 16.07, Fungicide – $F_{1,35}$ = 54.39, P < 0.001, LSD = 10.31; $F_{1,35}$ = 53.70, P <0.001, LSD = 22.72). At both post T1 and T2 assessments, the cultivar with the smallest incidence was cv. Quartz and cv. Hunivers had the greatest incidence. At the post T1 and T2 assessment, the incidence of plants with *L. maculans* lesions on cv. Hunivers was significantly greater only than on cvs Quartz and Barbados. At the post T1 assessment, the incidence of leaf spotting on cv. Quartz (3.3%) was not significantly different from on cvs Barbados (11.7%) and Flamingo (18.3%), whereas at the post T2 assessment incidence on cv. Quartz (21.7%) was not significantly different from on cvs Barbados (26.7%) and Django (38.3%). At both post T1 and T2 assessments, untreated plots had a greater incidence than treated treatments (T1 + 6 weeks- 37.8% and 1.1%, T2 + 5 weeks - 54.4% and 21.7% respectively). However, an interaction between cultivar

and fungicide application was found only at T1+ 6 weeks, not at T2 + 5 weeks ($F_{5,35}$ = 3.15, P = 0.027, LSD = 25.26; $F_{5,35}$ = 2.61, P = 0.054, LSD = 22.72) (Fig. A15.6).

At T1 + 6 weeks, most cultivars had a significantly smaller incidence of *L. maculans* leaf spotting when an application of prothioconazole was applied than when they were untreated. However, this was not the case for cvs Barbados or Quartz where the mean incidence score was smaller but not significantly smaller so the incidence of plants with *L. maculans* differed between treatments at both spray timings ($F_{11,35}$ = 7.81, P < 0.001, LSD = 25.26; $F_{11,35}$ = 8.06, P < 0.001, LSD = 22.72). At T1 + 6 weeks, the treatments that had the smallest incidence were treated cvs Quartz, Flamingo, Django and Hunivers, all of which had an incidence of 0% (Fig. A15.6). The only treatments that were not significantly different were treated cvs Barbados (3.3%), Charger (6.7%) and untreated cv. Barbados (20.0%). The treatment that had the greatest incidence was untreated cv. Hunivers (63.3%). However, this incidence score was not significantly different from untreated cvs Django (43.3%) and Charger (56.7%). At T2 + 5 weeks, treated cv. Barbados had the smallest incidence score (16.67%); the only treatments that had a significantly greater incidence score were untreated cvs Django (56.67%), Charger (66.67%), Flamingo (70%) and Hunivers (73.33%); there was no significant difference between these four untreated treatments.
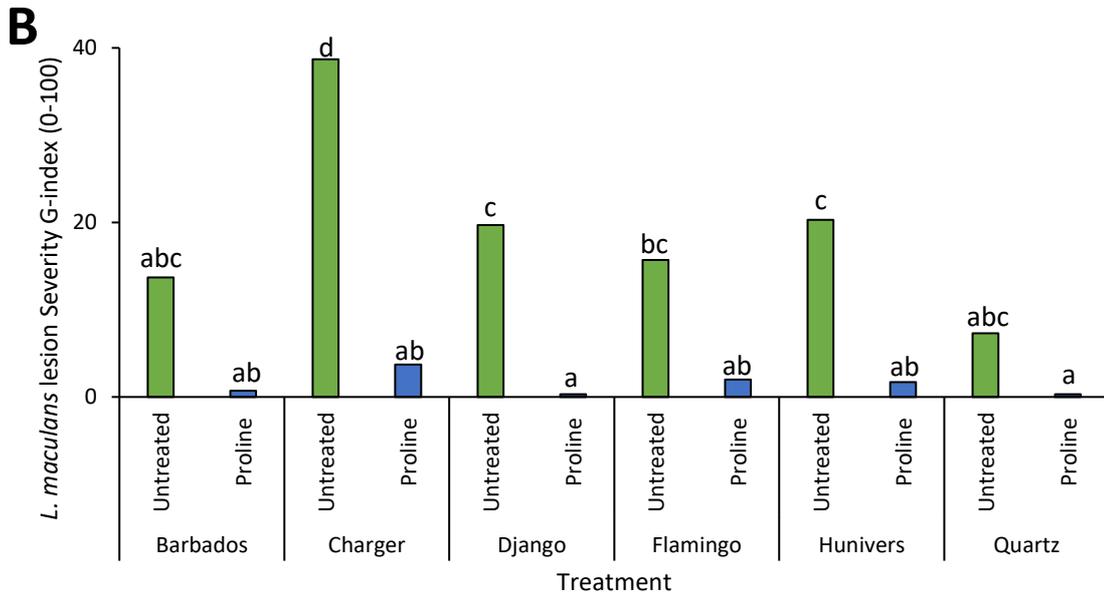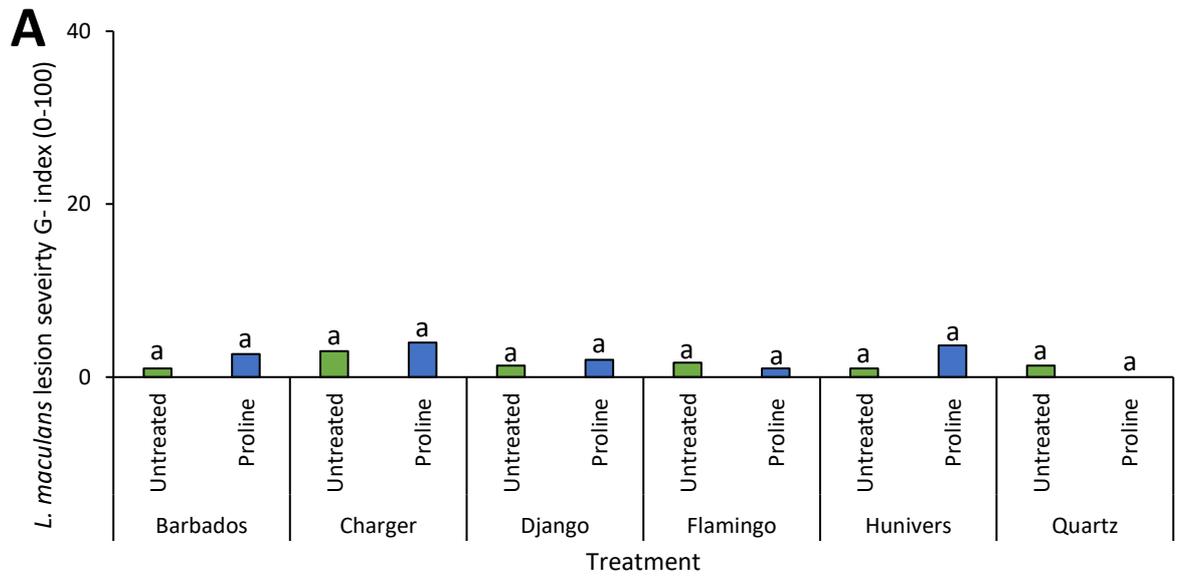
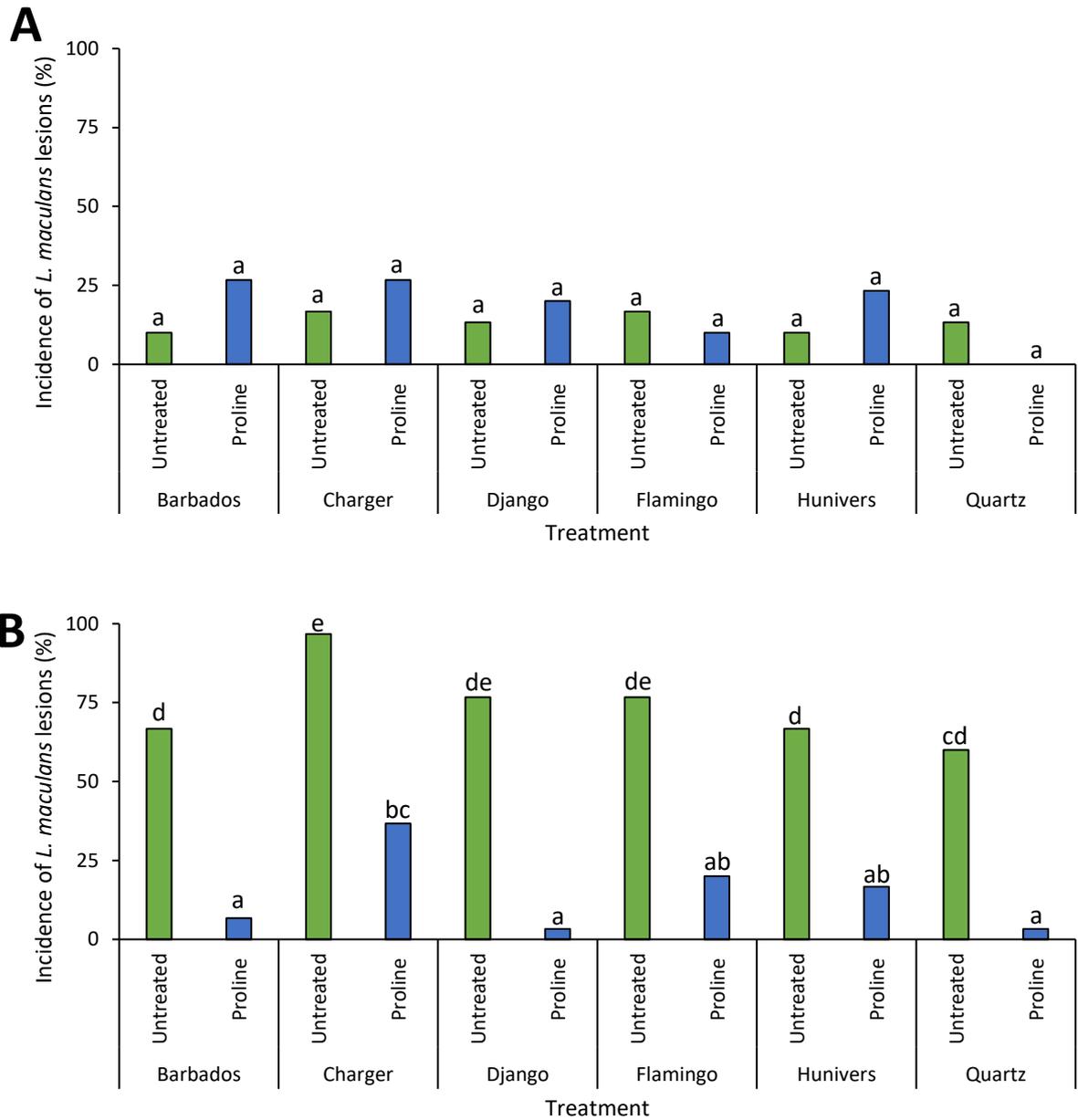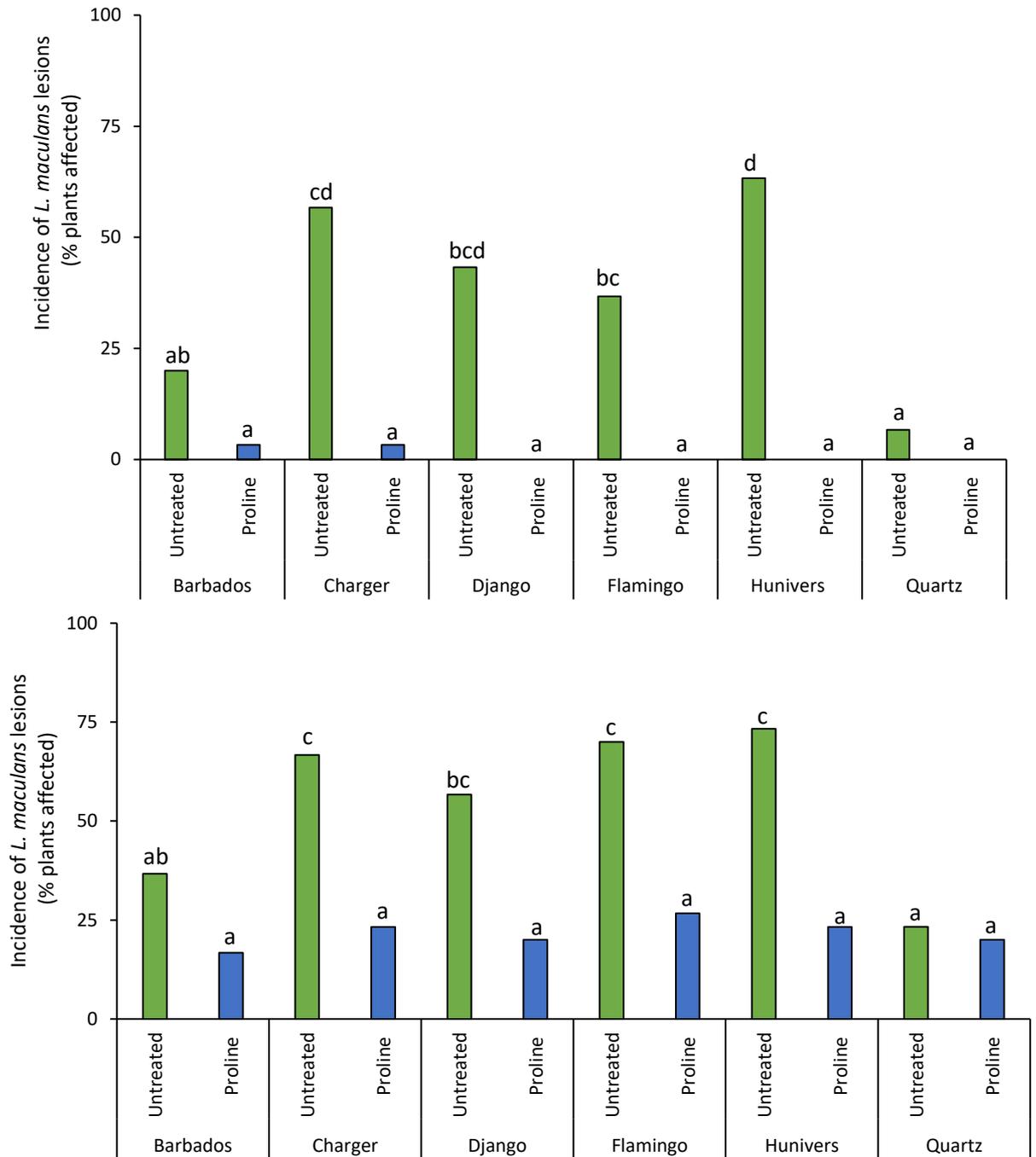**Figure A15.6: Incidence of *L. maculans* leaf spots at A) T1 + 4 weeks (12 December 2019) and B) T2 + 5 weeks (20 January 2020) at Terrington St Clement during the 2019/2020 cropping season. Post Hoc Fisher's protected LSD tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (35 d.f.).**

APPENDICES 16 – Incidence and severity of light leaf spot

The 2017/18 cropping season

During the 2017/2018 cropping season, there were no overall significant differences in the severity of light leaf spot in April between cultivars ($F_{5.35}$ = 0.51, P = 0.766, LSD = 0.24), nor were there significant differences between treatments that received an application of prothioconazole and those that had not ($F_{1.35}$ = 2.74, P = 0.111, LSD = 0.14). There was no significant overall interaction between cultivars and fungicide treatment ($F_{5.35}$ = 0.90, P = 0.498, LSD = 0.34). Additionally, there were no significant differences between treatments ($F_{11.35}$ = 0.89, P = 0.563, LSD = 0.34) (Fig. A16.1). During the 2017/2018 cropping season, there were no overall significant differences in the incidence of light leaf spot in the April assessment between cultivars ($F_{5.35}$ = 0.76, P = 0.584, LSD = 21.02) or between fungicide treatments ($F_{1.35}$ = 2.29, P = 0.144, LSD = 12.3). A significant overall interaction between cultivar and application of prothioconazole was found ($F_{5.35}$ = 0.96, P = 0.463, LSD = 29.72). Additionally, there were no significant differences in the incidence of light leaf spot between fungicide treated and untreated ($F_{11.35}$ = 0.99, P = 0.482, LSD = 29.72) plots (Fig. A16.1).

The 2018/19 cropping season

During the 2018/2019 cropping season, there was an overall significant difference in the severity of light leaf spot during the April assessment between treatments that received an application of prothioconazole and those that had not ($F_{1.35}$ = 19.89, P <0.001, LSD = 0.3034). There was no overall significant difference between cultivars ($F_{5.35}$ = 2.38, P = 0.069, LSD = 0.53), nor was there a significant overall interaction between cultivar and fungicide treatment ($F_{5.35}$ = 0.70, P = 0.630, LSD = 0.74). However, there was a significant difference between individual treatments ($F_{11.35}$ = 3.21, P = 0.008, LSD = 0.74) (Fig. A16.1). The treatment that had the smallest *P. brassicae* severity score was treated cv. Charger (0.0); this was significantly smaller than for all untreated cultivars, except untreated cvs Barbados (0.6) and Django (0.6), and treated cv. Flamingo (0.8).
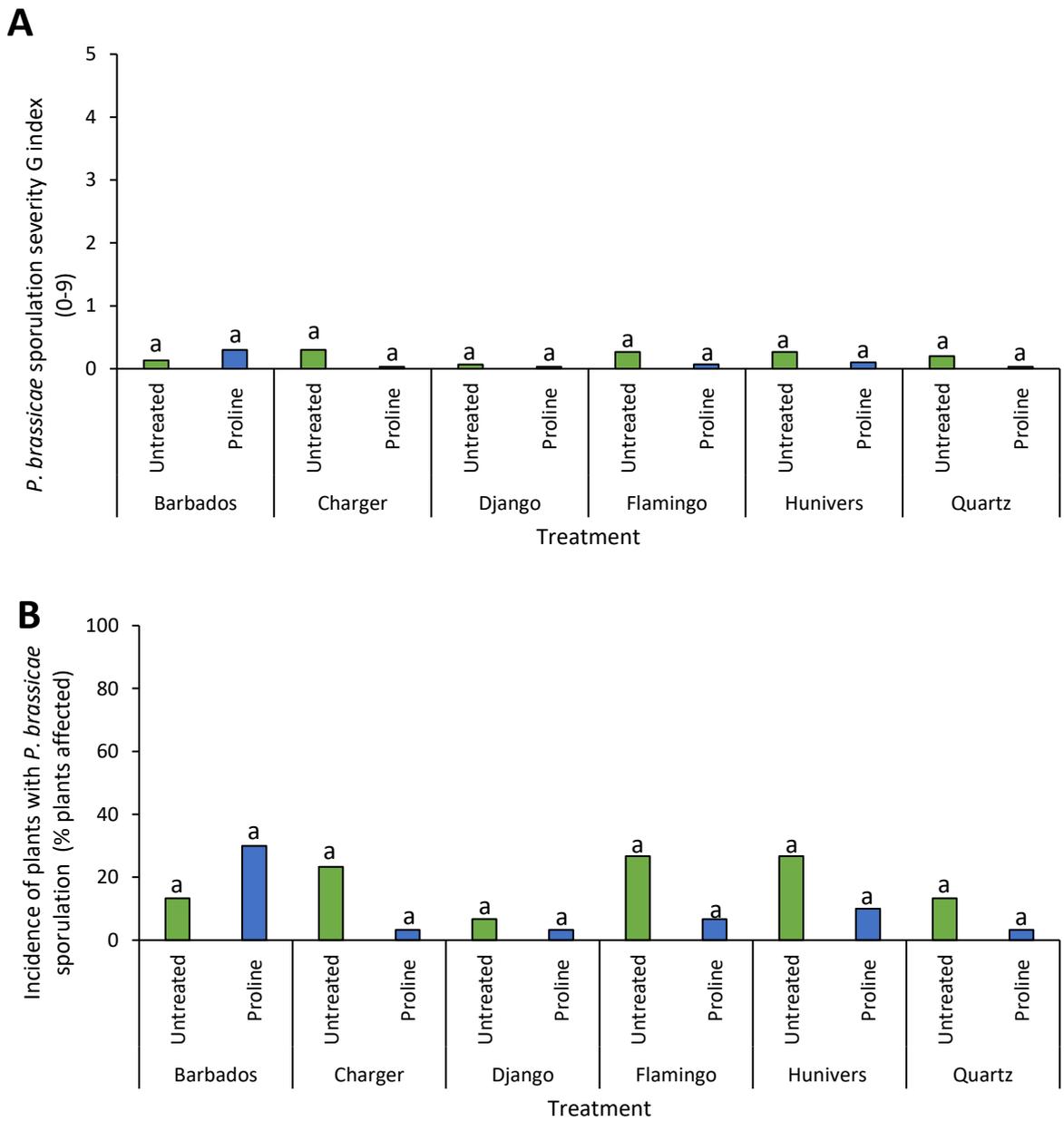
Figure A16.1: A. Average *P. brassicae* sporulation G-index severity (0-9) and B. average incidence of *P. brassicae* (% of plants with symptoms) in the field experiment (20 April 2018) at Terrington St Clement in the 2017/2018 cropping season. Fisher's Protected LSD post hoc tests are represented as letters. Bars that do not share a letter are significantly different at

P =0.05 (35 d.f.).

The treatment that had the greatest *P. brassicae* severity score was untreated cv. Flamingo (1.5); this was not significantly different from all other untreated cultivars or treated cv. Flamingo. During the 2018/19 cropping season, there were overall significant differences in the incidence of light leaf spot between cultivars during the April assessment ($F_{5.35}$ = 3.14, *P* = 0.025, LSD = 20.73) (Fig. A16.2). The cultivar that had the smallest overall mean incidence of *P. brassicae* sporulation was cv. Django (26.7%); however, it was not significantly different from all other cultivars, apart from cv. Flamingo (63.3%). Cv. Flamingo had the greatest overall severity score, that was significantly greater than that of all other cultivars. All other cultivars were not significantly different from cv. Django. There was an overall significant difference between treatments that received an application of prothioconazole (20.6%) and those that had not (61.1%) ($F_{1.35}$ = 48.89, *P* < 0.001, LSD = 11.97).

A significant overall interaction between cultivar and fungicide treatment was found ($F_{5.35}$ = 2.67, *P* = 0.047, LSD = 2.67). There was a significant difference in the incidence of *P. brassicae* sporulation between treated and untreated plots on cultivars Charger, Flamingo, Hunivers and Quartz; however, there was no significant difference between treated and untreated plots on cultivars Django and Barbados. There was a significant difference between individual treatments ($F_{11.35}$ = 7.09, *P* < 0.001, LSD = 29.32) (Fig. A16.2). The treatment that had the smallest incidence of *P. brassicae* sporulation was prothioconazole treated cv. Charger (3.3%); it was significantly different from all untreated cultivars and treated cv. Flamingo (40.0%). Untreated cv. Flamingo had the greatest mean incidence (86.7%); all other untreated cultivars apart from cvs Barbados (40.0%) and Django (33.3%) were not significantly different, and all treatments that had been treated with prothioconazole were significantly different.
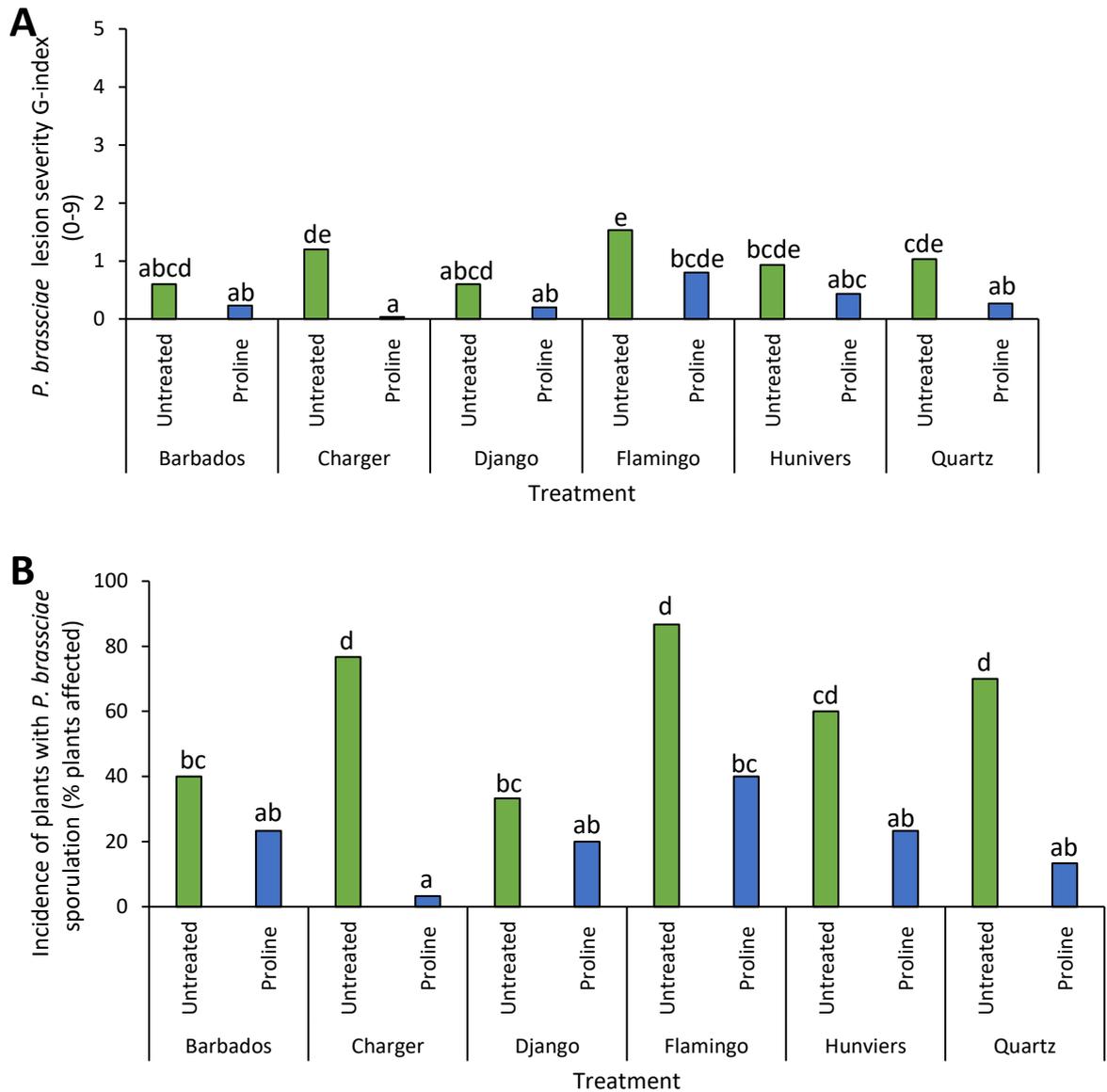
Figure A16.2: A) *P. brassicae* sporulation severity G-index (0-9) and **B)** average incidence (% of plants with symptoms) of *P. brassicae* in the field experiment (03 April 2019) at Terrington St Clement in the 2018/2019 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05. (35 d.f.).

The 2019/20 cropping season

During the 2019/2020 cropping season, there was no overall significant difference in the severity of *P. brassicae* sporulation during the April assessment between treatments that received an application of prothioconazole and those that had not ($F_{1.35}$ = 1.31, *P* = 0.264, LSD = 0.14). There was no overall significant difference between cultivars ($F_{5.35}$ = 2.04, *P* = 0.109, LSD = 0.14), or overall interaction between cultivar and fungicide treatment ($F_{5.35}$ = 0.52, *P* = 0.757, LSD = 0.197). There was no significant difference between individual treatments (Fig. A16.3). During the 2019/20 cropping season, there was no overall significant difference in the incidence of plants with *P. brassicae* sporulation during the April assessment between cultivars ($F_{5.35}$ = 2.37, *P* = 0.070, LSD = 13.47). The cultivar that had the smallest overall mean incidence of *P. brassicae* sporulation was cv. Flamingo (1.7%); however, it was not significantly different from all other cultivars. There was no overall significant difference between treatments that received an application of prothioconazole (11.7%) and those that had not (7.8%) ($F_{1.35}$ = 1.07, *P* < 0.312, LSD = 7.78). There was no significant overall interaction between cultivar and fungicide treatment ($F_{5.35}$ = 0.60, *P* = 0.704, LSD = 19.05) (Fig. A16.3). There was no significant difference between individual treatments ($F_{11.35}$ = 1.44, *P* = 0.217, LSD = 19.05).

A

B
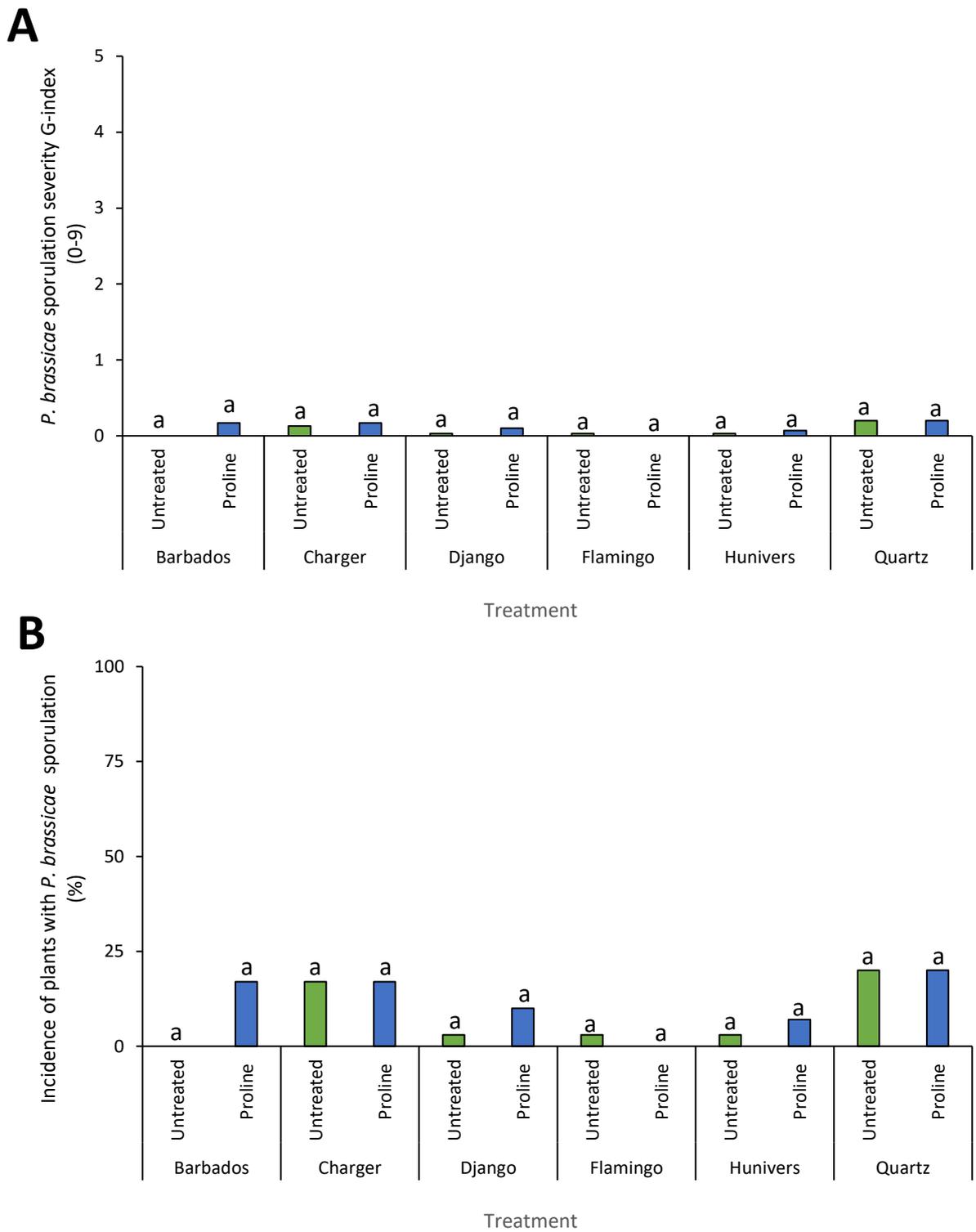
Figure A16.3: A) *P. brassicae* sporulation severity G-index (0-9) and **B)** average incidence (% of plants with symptoms) of *P. brassicae* in the field experiment (01 May 2020) at Terrington St Clement in the 2019/2020 cropping season. Fisher's tests are represented as letters. Columns that do not share a letter are significantly different at *P* =0.05 (35 d.f.).

APPENDICES 17 – Incidence and severity of phoma stem basal cankers pre-harvest

The 2017/18 cropping season

During the 2017/18 cropping season, there was a significant difference in the severity of stem basal cankers between cultivars ($F_{5.35}$ = 18.51, *P* < 0.001, LSD = 0.52). Cultivar Barbados (0.47) had the smallest severity score, but it was significantly smaller than that for all other cultivars, except for cv. Django (0.8). The cultivar that had the greatest severity score was cv. Charger (2.7); this was significantly greater than all severity scores on other cultivars. The severity score on Flamingo (1.5) was not significantly different from cvs Hunivers (1.3) and Quartz (1.1) but was significantly greater than the scores on cvs Django and Barbados. There was a significant difference between plots with or without fungicides ($F_{1.35}$ = 45.01, *P* <0.001, LSD = 0.30). The mean phoma stem canker severity score on untreated plots (1.77) was greater than on treated plots. There was a significant interaction between cultivar and fungicide treatment ($F_{5.35}$ = 3.38, *P* = 0.019, LSD = 0.73) (Fig. A17.1). There was a significant difference between untreated and treated plots in all cultivars except for cvs Barbados and Quartz. Other significant differences were observed between treatments. Untreated cv. Charger had a severity score of 3.78; this was significantly greater than the severity score for all other treatments. The treatment that had the second greatest mean phoma stem canker score was untreated cv. Flamingo (1.88); this was not significantly different from scores for all other untreated cultivars, except untreated cv. Barbados (0.68). It was significantly greater than the score for all treated cultivars, except treated cv. Charger (1.53). The treatment that had the smallest phoma stem canker severity score was treated cv. Barbados (0.25), but this was not significantly different from all other treated cultivars except treated cvs Charger and Flamingo.

Figure A17.1: A) Average severity (0-7 scale) of phoma stem canker at the stem base and B) incidence (% of plants with stem cankers) in the field experiment at Terrington St Clement, Norfolk in the 2017/2018 cropping season. Fisher's protected tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (35 d.f.).

During the 2017/18 cropping season, there was an overall significant difference in the incidence of plants with stem basal cankers between cultivars ($F_{5.35}$ = 14.49, P < 0.001, LSD = 15.32). The incidence of phoma stem canker at the stem base of cultivar Flamingo (54.2%) was significantly different only from the cultivars that had the greatest and smallest incidences. The cultivar that had the smallest incidence of phoma stem canker at the stem base was cv. Barbados (25.8%); however, this incidence was not significantly different from the phoma incidence on cv. Django (39.2%). The cultivar that had the greatest incidence of phoma stem cankers was cv. Charger (84.2%), this was significantly greater than that for all other cultivars. The incidences of stem basal canker on cvs Django, Quartz (48.3%) and Flamingo were not significantly different from each other. However, the incidence was smaller on cv. Django than on cv. Hunivers (61.7%), but not on cvs Quartz and Flamingo. There was a significant difference between plots with/without application of prothioconazole ($F_{1.35}$ = 20.59, P < 0.001, LSD = 8.84).

There was no significant interaction in incidence of stem basal cankers between cultivar and fungicide treatment ($F_{5.35}$ = 0.87, P = 0.471, LSD = 21.66). In all cultivars, the incidence of stem basal cankers was less on treated plots than on untreated plots. There were significant differences between treatments ($F_{11.35}$ = 8.89, P < 0.001, LSD = 21.7) (Fig. A17.1). The incidence of phoma stem cankers at the stem base on untreated cv. Charger (98.3%) was significantly greater than on all other cultivars. The phoma stem canker incidence on untreated cvs Hunivers (70.0%) or Charger (70.0%) was not significantly different from that on all other untreated cultivars, nor was it significantly different from treated cv. Hunivers (53.3%). %). The treatment that had the smallest incidence was treated cv. Barbados (18.3%), but this was not significantly smaller than on treated cv. Django (23.3%) or untreated cv. Barbados (33.3%).

The 2018/19 cropping season

During the 2018/19 cropping season, there was an overall significant difference in the severity of stem basal canker between cultivars ($F_{5.35}$ = 9.96, P < 0.001, LSD = 0.51). The cultivar that had the smallest mean phoma stem canker severity score was cv. Barbados

(0.27); this was significantly different from cvs Hunivers (0.85), Flamingo (1.43) and Charger (1.55). The severity score on cv. Hunivers (0.85) was not significantly different from cvs Django (0.39) or Quartz (0.48), but the severity score was significantly smaller than cvs Flamingo and Charger. There was a significant difference in phoma severity score between untreated and treated plots ($F_{1.35}$ = 7.87, P = 0.010, LSD = 0.29). The mean phoma severity score was significantly greater on untreated plots (1.03) than on those that had been treated with prothioconazole (0.63). There was no significant interaction between cultivar and fungicide treatment ($F_{5.35}$ = 0.91, P = 0.491, LSD = 0.72) (Fig. A17.2). There were significant differences in the mean phoma stem canker severity score between treatments ($F_{11.35}$ = 5.66, P < 0.001, LSD = 0.72) (Fig A17.2). Treated cv. Barbados (0.12) was the treatment that resulted in the least severe stem basal canker score, but this treatment was only significantly different from untreated and treated cvs Flamingo and Charger and treated cv. Hunivers. The treatment that had the greatest severity score was untreated cv. Charger (2.02); this was significantly different from all other treatments except for untreated cv. Flamingo. The severity scores on treatments untreated cvs Django (0.57), Quartz (0.72) and Hunivers (0.80) were not significantly different from each other, nor were they significantly different from all other treatments, except for untreated cvs Flamingo and Charger.

During the 2018/19 cropping season, there was an overall significant difference in the incidence of plants with stem basal cankers between cultivars ($F_{5.35}$ = 10.85, P < 0.001, LSD = 20.11). The cultivar with the smallest incidence of phoma stem basal canker was cv. Barbados (21.7%); this incidence was significantly smaller than that on cvs Hunivers (49.2%), Charger (71.7%) and Flamingo (72.5%); the incidences on cvs Charger and Flamingo were not significantly different from each other but both were significantly greater than on cvs Hunivers and Django; there was no significant difference between cvs Hunivers or Django. There was a significant difference between plots with/without an application of prothioconazole ($F_{1.35}$ = 12.30, P = 0.002, LSD = 11.61). The incidence of phoma stem canker was significantly greater on untreated plots (55.3%) than on treated plots (35.6 %).
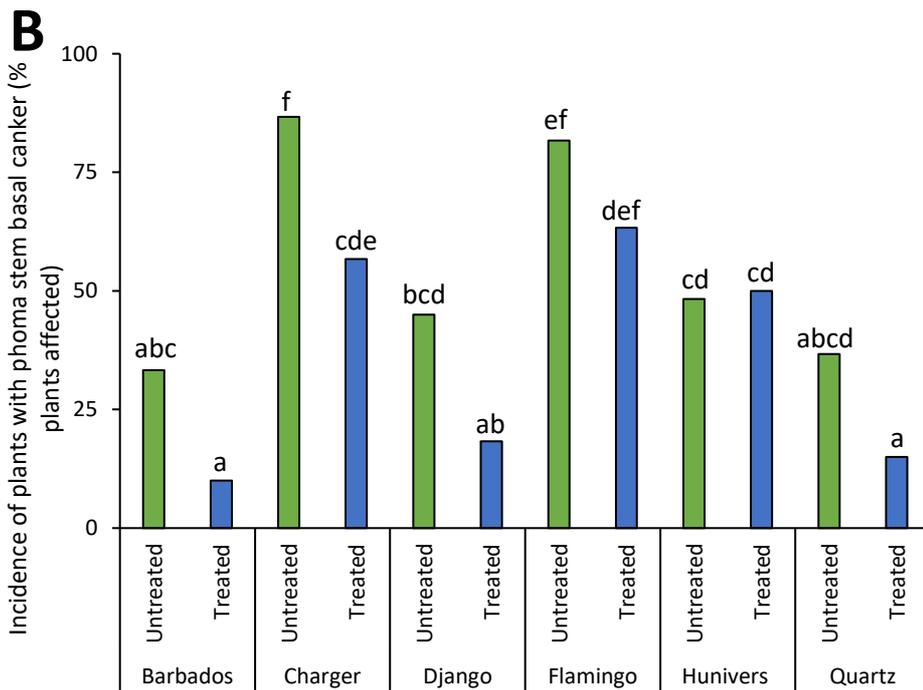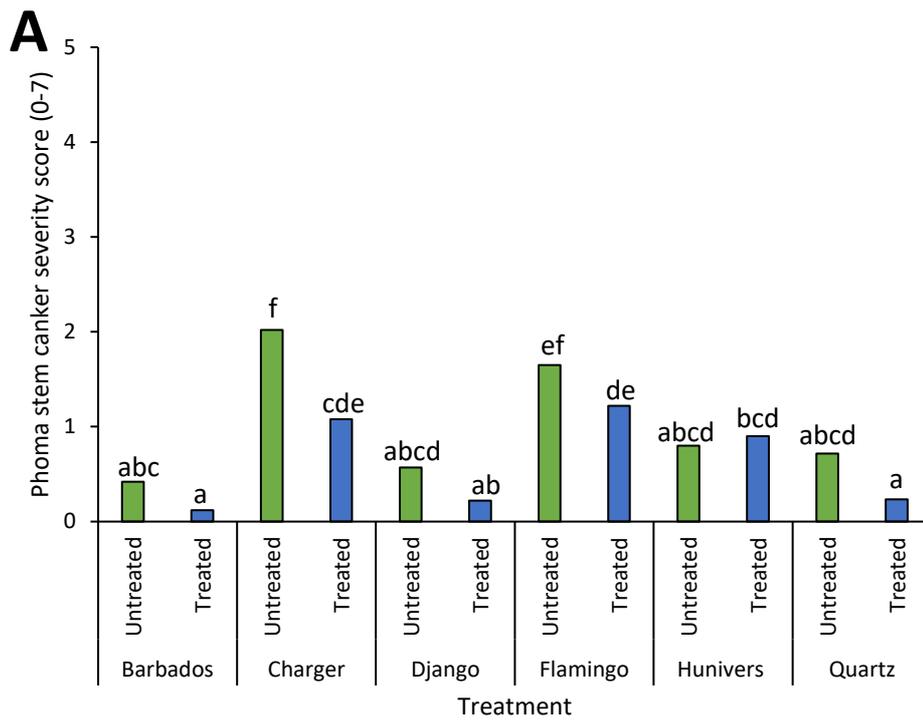
Figure A17.2: A. Average severity (0-9 scale) of phoma stem canker at the stem base and B. incidence (% of plants with stem cankers) in a field experiment at Terrington St Clement, Norfolk in the 2018/2019 cropping season. Fisher's protected post hoc tests are represented as letters. Columns that do not share a letter are significantly different P =0.05 (35 d.f.).

There was no interaction in incidence of stem basal canker between cultivar and fungicide treatment ($F_{5.35}$ =0.66, P = 0.65, LSD = 28.44). When the individual treatments were compared, there were significant differences ($F_{11.35}$ = 6.35, P < 0.001, LSD = 28.44) (Fig. A17.2). Treated cv. Barbados (10%) had the smallest incidence; however, it was significantly smaller only than those on untreated or treated cvs Hunivers, Flamingo and Charger, and untreated cv. Django (45.0%). The treatment that had the greatest incidence was untreated cv. Charger (86.7%); this was significantly greater than that on all other cultivars, except untreated (81.7%) and treated (63.3%) cv. Flamingo. Untreated cv. Quartz (36.7%) was significantly different only from untreated cvs Flamingo and Charger.

The 2019/20 cropping season

During the 2019/20 cropping season, there was an overall significant difference in the severity of stem basal cankers between cultivars ($F_{5.35}$ = 16.88, P < 0.001, LSD = 0.32). The mean phoma severity score on cv. Barbados (0.43) was not significantly different from that on cvs Quartz (0.63) and Django (0.73); these two cultivars were not significantly different from each other or from cv. Hunivers (0.94). The cultivar that had the greatest severity score was cv. Flamingo (1.59). However, this was not significantly different from cv. Charger (1.39). There was a significant difference in phoma severity score between untreated and treated plots ($F_{1.35}$ = 16.61, P < 0.001, LSD = 0.19). The mean phoma severity score was significantly greater on untreated plots (1.14) than on those that had been treated with prothioconazole (0.77).

There was no significant interaction between cultivar and fungicide treatment ($F_{5.35}$ = 1.53, P = 0.217, LSD = 0.45). There were significant differences in the mean phoma stem canker severity score between treatments ($F_{11.35}$ = 9.88, P = <0.001, LSD = 0.45) (Fig. A17.3). The treatment with the smallest incidence was treated cv. Barbados (0.30); this was not significantly different from untreated cvs Barbados (0.35), Django (0.65) and from treated cvs Quartz (0.33), Django (0.47) and Hunivers (0.73). The treatment with the greatest severity score was untreated cv. Flamingo (2.03); this was significantly greater than all other treatments, except from untreated cv. Charger (1.2).
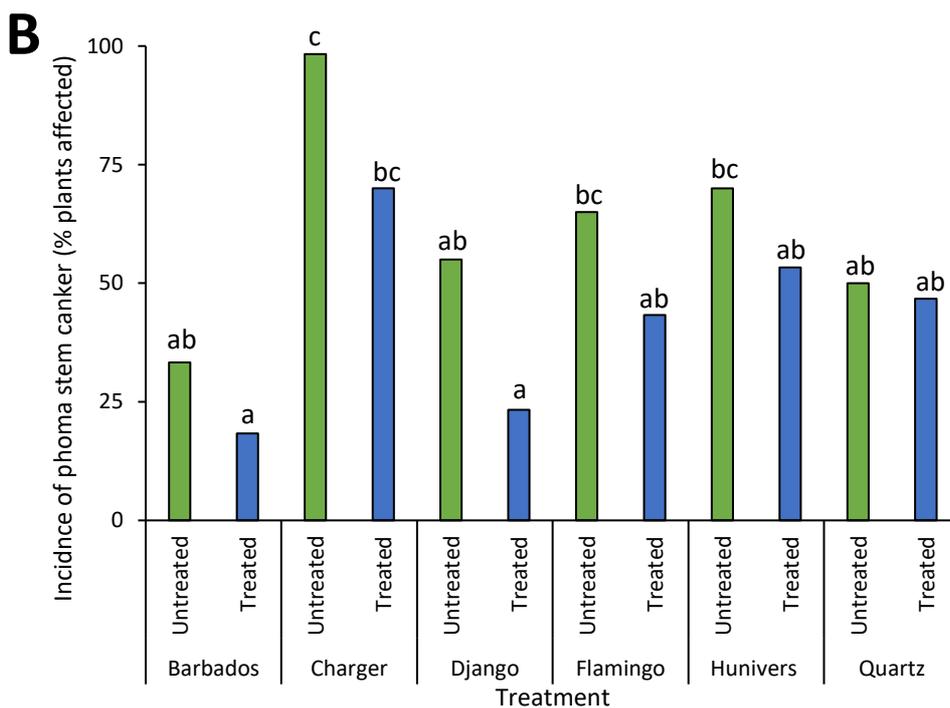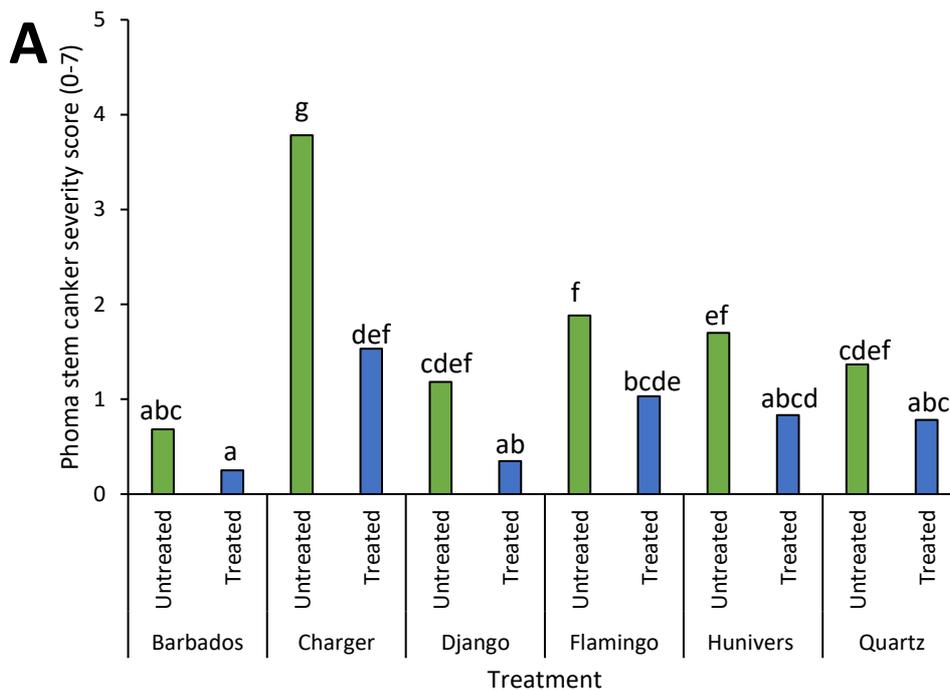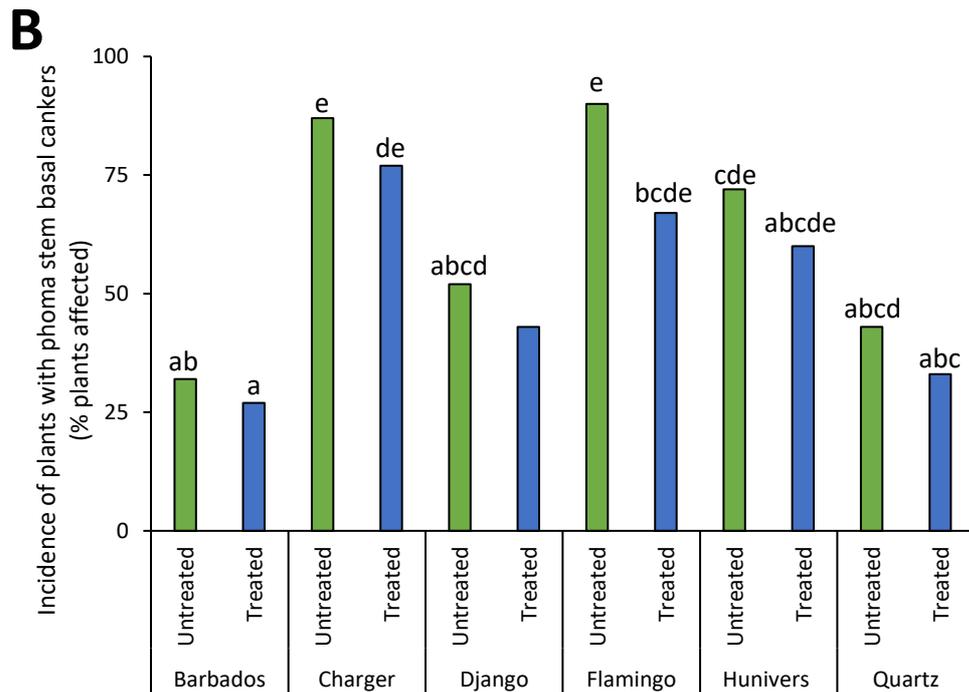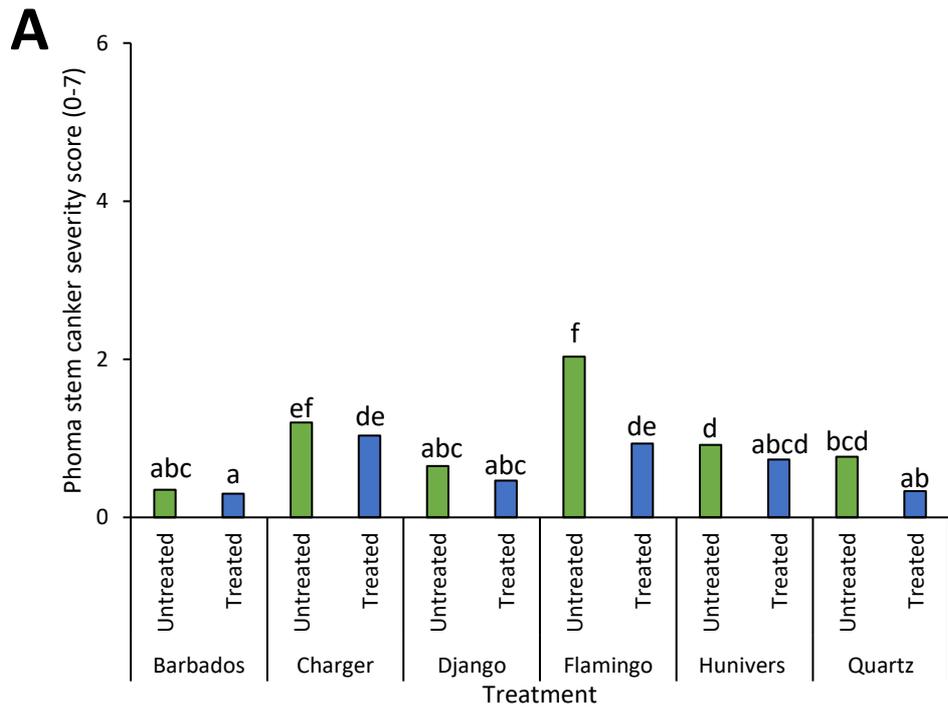
Figure A17.3: A) Average severity (0-7 scale) of phoma stem canker at the stem base and **B)** incidence (% of plants with stem cankers) in the field experiment at Terrington St Clement, Norfolk in the 2019/2020 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (35 d.f.).

However, untreated cv. Charger was not significantly different from untreated cv. Django and treated cvs Flamingo (0.93) and Charger (1.03). The severity score on treated cv. Hunivers was significantly different only from untreated cvs Flamingo and Charger.

During the 2019/20 cropping season, there was an overall significant difference in the incidence of plants with stem basal cankers between cultivars ($F_{5.35}$ = 16.47, P < 0.001, LSD = 15.61). The cultivar with the smallest incidence of phoma stem basal canker was cv. Barbados (29.2%); this incidence was significantly smaller only than on cvs Charger (81.7%), Flamingo (78.3%) and Hunivers (65.8%). They were not significantly different from each other. Cvs Flamingo and Charger were significantly different from all other cultivars except for cv. Hunivers. The incidence on cv. Hunivers was not significantly different than on cv. Django. There was a significant difference between plots with/without an application of prothioconazole ($F_{1.35}$ = 6.81, P = 0.015, LSD = 9.01). The incidence on untreated plots (62.5%) was significantly greater than that on treated plots (51.1%). There was no interaction in stem basal canker incidence between cultivar and fungicide treatment ($F_{5.35}$ = 0.34, P = 0.881, LSD = 22.07). There were significant differences in incidence between treatments ($F_{11.35}$ = 8.26, P < 0.001, LSD = 22.07) (Fig. A17.3). The treatment with the smallest incidence was treated cv. Barbados (26.7%); this was significantly smaller only than on untreated and treated cvs Flamingo, Charger and untreated cv. Hunivers. They were not significantly different from each other. The incidences on untreated cv. Django (51.7%) or treated cv. Hunivers (60.0%) were not significantly different from those on any of the other treatments.

APPENDICES 18 – Incidence and severity of Light leaf spot on stems

The 2017/18 cropping season

During the 2017/18 cropping season, there was no significant difference between cultivars in *P. brassicae* stem lesion severity ($F_{5.35}$ = 2.34, *P* = 0.073, LSD = 0.25). There was a significant difference in *P. brassicae* stem lesion severity between untreated and treated plots ($F_{1.35}$ = 10.59, *P* = 0.003, LSD = 0.14). The lesions on untreated plots (0.51) were significantly more severe than those on treated plots (0.24).

There was no overall significant interaction between cultivar and fungicide treatment ($F_{5.35}$ = 2.16, *P* = 0.092, LSD = 0.35). There was a significant difference between treatments ($F_{5.35}$ = 3.01, *P* = 0.012, LSD = 0.35). (Fig. A18.1). Treated cv. Quartz had the smallest *P. brassicae* stem lesion severity score (0.03) but this was not significantly smaller than the severity scores on untreated (0.17) or treated (0.13) cv. Flamingo and treated cv. Barbados (0.13). The treatment that had the greatest severity score was untreated cv. Quartz (0.75), but this was only significantly different from those that were not significantly different from treated cv. Quartz. The scores on these cultivars were not significantly different from each other and the severity scores of both were significantly greater only than the severity score on treated cv. Quartz. The severity scores for untreated cvs Barbados (0.42) and Django (0.53), treated cvs Hunivers (0.28) and Charger (0.32) were not significantly different from any other treatment except for treated cv. Quartz.

During the 2017/18 cropping season, there was no significant difference in *P. brassicae* stem lesion incidence between cultivars ($F_{5.35}$ = 2.27, *P* = 0.08, LSD = 1.79). There was a significant difference in *P. brassicae* stem lesion incidence between untreated and treated plots ($F_{1.35}$ = 12.85, *P* = 0.001, LSD = 1.03). The untreated plots (34.2%) had a significantly greater incidence of *P. brassicae* than treated plots (17.5%). There was no significant interaction between cultivar and fungicide treatment ($F_{5.35}$ = 2.33, *P* = 0.073, LSD = 2.53) for *P. brassicae* incidence. However, there was a significant difference between treatments ($F_{11.35}$ = 3.26, *P* = 0.007, LSD = 2.53) (Fig. A18.1).
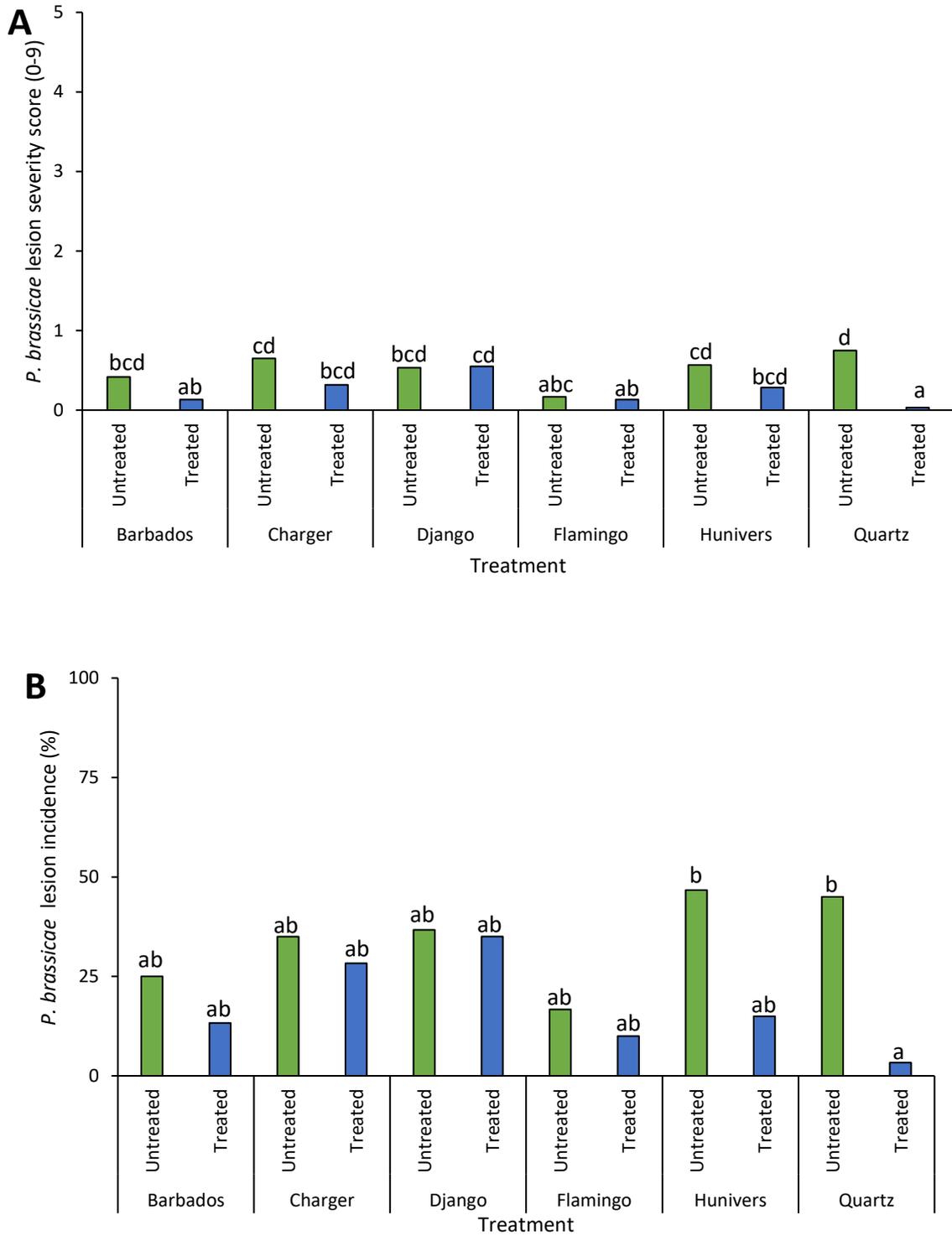
Figure A18.1: A) Average severity (0-9 scale) of *P. brassicae* stem lesions at the stem base and B) incidence (% of plants with *P. brassicae* stem lesions) in the field experiment at Terrington St Clement, Norfolk in the 2017/2018 cropping season. Statistical analysis was done on square root-transformed data. Fisher's protected LSD tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (35 d.f.).

The 2018/19 cropping season

During the 2018/19 cropping season, there was no overall significant difference in the severity of *P. brassicae* stem lesions between cultivars ($F_{5.35}$ = 0.67, *P* = 0.647, LSD = 0.35), with/without application of prothioconazole ($F_{1.35}$ = 3.03, *P* = 0.094, LSD = 0.20) nor was there a significant interaction between cultivar and fungicide application ($F_{5.35}$ = 0.52, *P* = 0.759, LSD = 0.49). There was no significant difference in severity between treatments (Fig. A18.2). In all cultivars, except for cv. Flamingo, the mean severity score was smaller for the treated plots than untreated plots, though differences were not significant. There was no overall significant difference in the incidence of *P. brassicae* stem lesions between cultivars ($F_{5.35}$ = 0.57, *P* = 0.721, LSD = 0.87). There was a significant difference in *P. brassicae* stem lesion incidence between untreated and treated plots ($F_{1.35}$ = 4.53, *P* = 0.044, LSD = 0.50). The incidence of *P. brassicae* sporulation was significantly greater on untreated (90.0%) than on treated plots (80.8%).

There was no significant interaction between cultivar and fungicide application ($F_{5.35}$ = 0.13, *P* = 0.985, LSD = 1.23). There was no significant difference in severity between treatments ($F_{11.35}$ = 0.73, *P* = 0.700, LSD = 1.23) (Fig. A18.2).

The 2019/20 cropping season

During the 2019/20 cropping season, there was a significant difference in the severity of *P. brassicae* stem lesions between cultivars ($F_{5.35}$ = 8.74, *P* < 0.001, LSD = 0.548). The cultivar that had the smallest severity score was cv. Barbados (0.8); this was significantly smaller than that of all other cultivars. The cultivar that had the greatest severity score was cv. Charger (5.6), but this was not significantly greater than the severity scores on cvs Flamingo (4.3) and Quartz (4.8). However, the severity score on cv. Charger was significantly greater than the severity score on cv. Hunivers (3.4) but the severity scores on cvs Flamingo and Quartz were not. The severity score for cv. Hunivers was not significantly different from that on cv. Django (2.2). There was no significant difference in *P. brassicae* stem lesion severity score between untreated and treated plots ($F_{1.35}$ = 0.00, *P* = 0.949, LSD = 0.3).
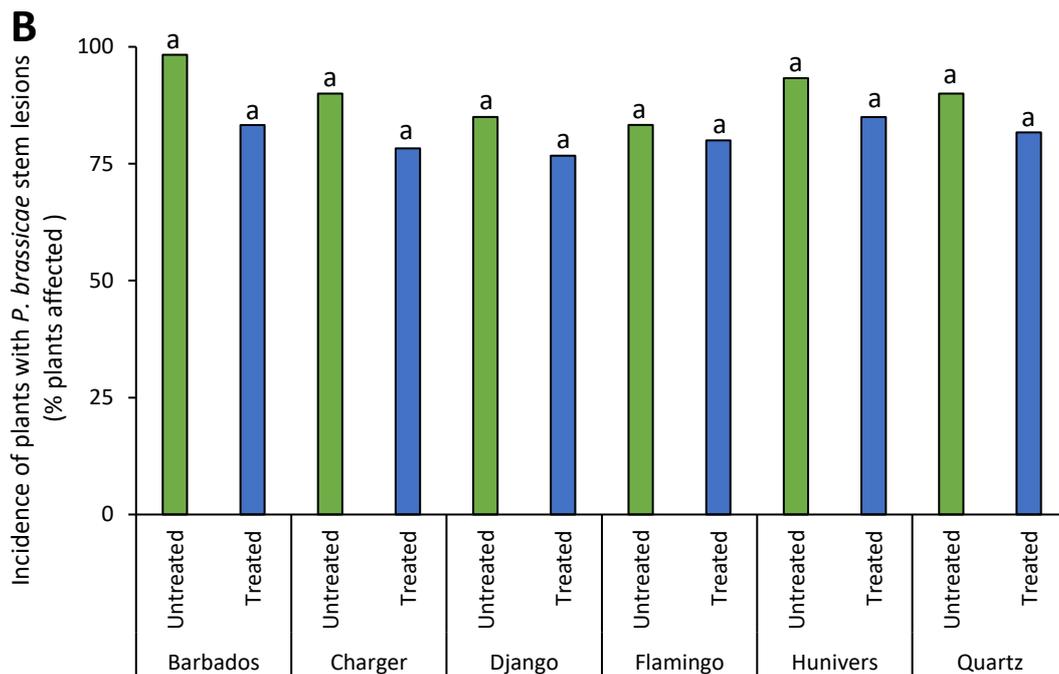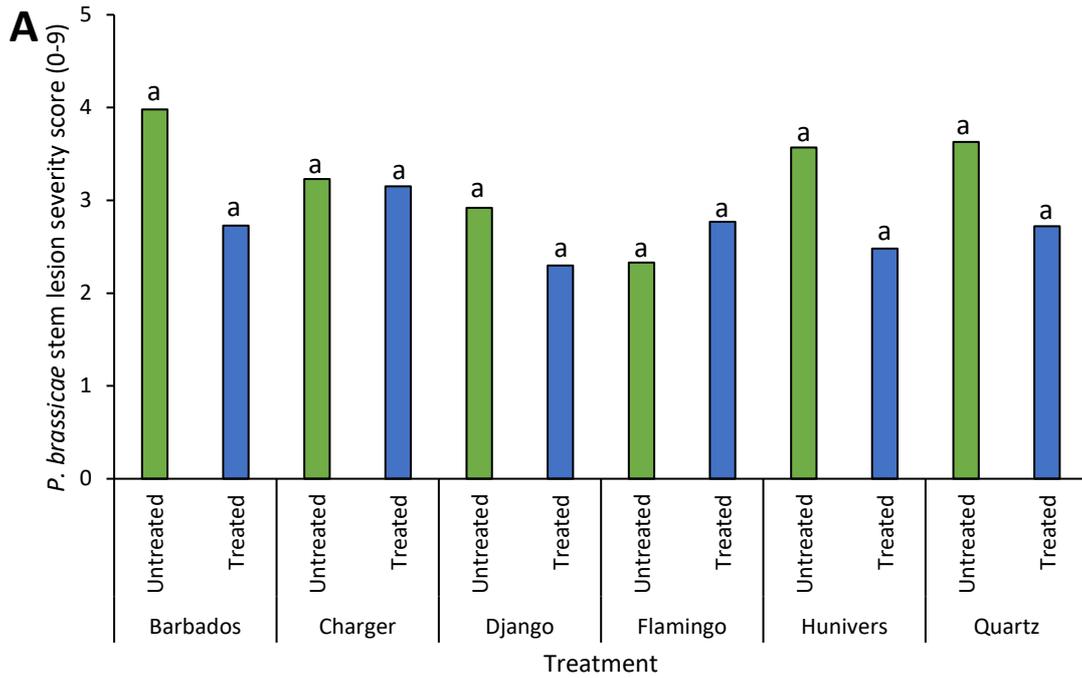
Figure A18.2: A) Average severity (0-9 scale) of *P. brassicae* stem lesions at the stem base and B) incidence (% of plants with *P. brassicae* stem lesions) in the field experiment at Terrington St Clement, Norfolk in the 2018/2019 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (35 d.f.).

There was no significant interaction between cultivar and fungicide application ($F_{5.35}$ = 0.17, P = 0.971, LSD = 0.77). There was a significant difference in *P. brassicae* stem lesion severity score between treatments ($F_{11.35}$ = 4.05, P = 0.002, LSD = 0.77). (Fig. A18.3). Untreated cv. Barbados (0.7) had the smallest *P. brassicae* stem lesion severity score, but it was not significantly different from those of untreated or treated cv. Django or treated cv. Barbados. The treatment that had the greatest severity score was untreated cv. Charger, this was not significantly different from all other treatments that were not significantly different from untreated cv. Barbados. However, untreated cv. Charger was significantly different from untreated cv. Django, but untreated or treated cvs Hunivers, Flamingo and Quartz or treated cv. Charger were not. The severity scores on both untreated or treated cv. Hunivers and untreated cv. Flamingo were not significantly different from those on treated cv. Django.

There was an overall significant difference in the incidence of *P. brassicae* stem lesions between cultivars ($F_{5.35}$ = 8.30, P < 0.001, LSD = 1.87). The cultivar with the smallest incidence was cv. Barbados (9.2%). This was significantly smaller than all other cultivars. The cultivar with the greatest incidence was cv. Charger (65.0%). However, this was significantly greater only than cvs Barbados and Django (27.5%). The incidences on cvs Django and Hunivers (41.7%) were not significantly different from each other. There was no significant difference in *P. brassicae* stem lesion incidence between untreated and treated plots ($F_{1.35}$ = 0.00, P = 0.949, LSD = 1.08). There was no significant interaction between cultivar and fungicide application ($F_{5.35}$ = 0.20, P = 0.97, LSD = 2.64). There was a significant difference in *P. brassicae* stem lesion incidence between treatments ($F_{11.35}$ = 3.86, P = 0.003, LSD = 2.64). (Fig. A18.3). Untreated cv. Barbados (8.3) had the smallest *P. brassicae* stem lesion incidence, but this was not significantly smaller than the incidences on treated cvs Barbados (10.0%) and Django (21.7%). The treatment that had the greatest incidence was untreated cv. Charger (65.0%), but it was significantly greater only than the incidence on the treatments that were not significantly different from untreated cv. Barbados. Although not significantly different from the incidence on untreated cv. Charger, the incidence on both untreated (43.3%) or treated cv. Hunivers (40.0%) or untreated cvs. Django (33.3%) and Flamingo (45.0%) were not significantly different from that of treated cv. Django (21.7%).
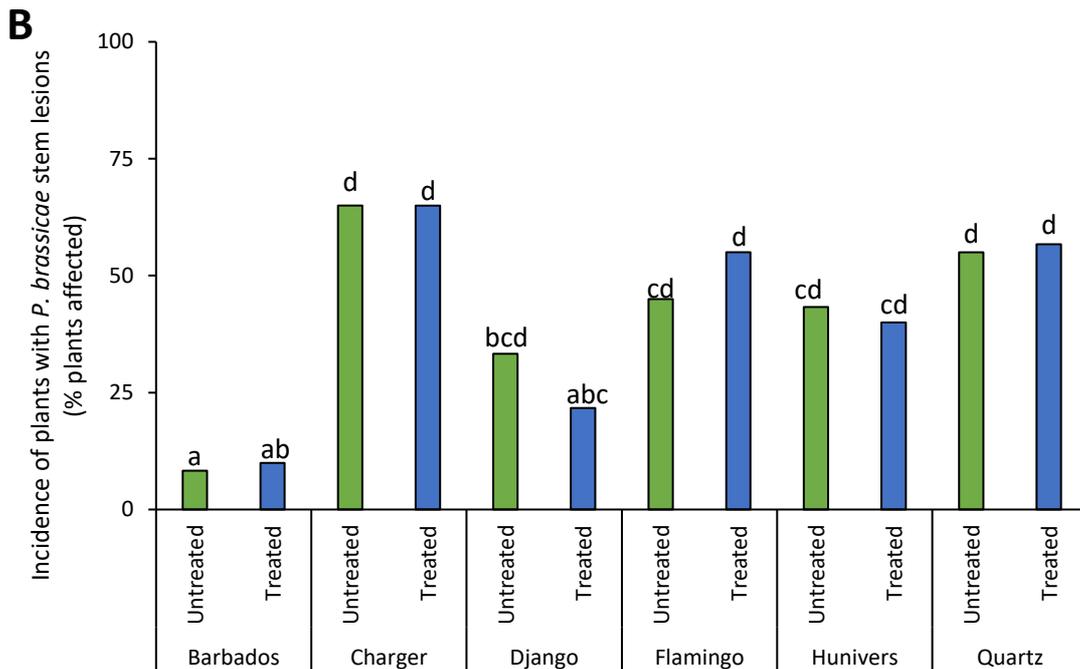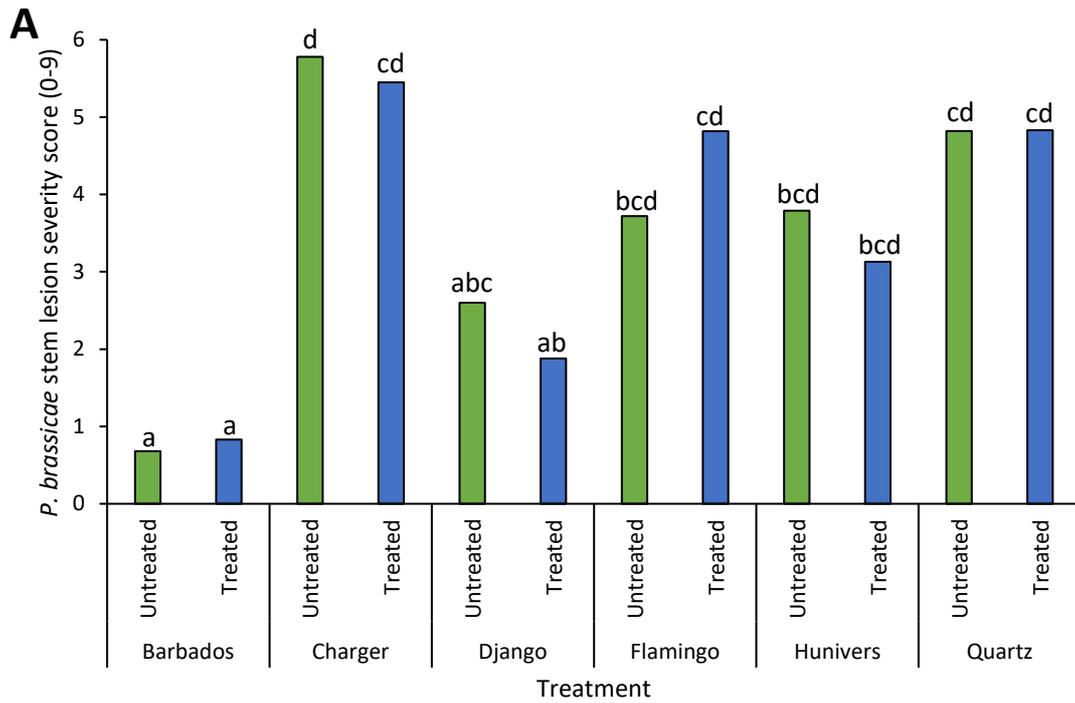
Figure A18.3: A) Average severity (0-9 scale) of *P. brassicae* stem lesions at the stem base and B) incidence (% of plants with *P. brassicae* stem lesions) in the field experiment at Terrington St Clement, Norfolk in the 2019/2020 cropping season. Statistical analysis was done on square root-transformed data. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different P =0.05 (35 d.f.)

APPENDICES 19 – Yield

The 2017/18 cropping season

In the 2017/18 cropping season, there was an overall significant difference in yield between cultivars ($F_{5.27}$= 5.12, *P* = 0.005, LSD = 0.48). The cultivar that had the smallest yield was cv. Hunivers (4.32 t/ha), but this yield was not significantly different to the yields of cvs Flamingo (4.45 t/ha), Django (4.63 t/ha) and Charger (4.78 t/ha). The cultivar that had the greatest yield was cv. Barbados (5.24 t/ha). However, it was not significantly different from those of cvs Quartz (5.09t/ha) or Charger.

There was a significant difference in yield depending on application of prothioconazole ($F_{1.27}$ = 11.81, *P* = 0.003, LSD = 0.28). The yield from treated plots (4.98t/ha) was significantly greater than on untreated plots (4.53t/ha). There was yield increase of 0.45t/ha. There was no significant interaction between cultivar and the application of prothioconazole ($F_{5.27}$ = 0.48, *P* = 0.789, LSD = 0.68). There was a significant difference in yield between treatments ($F_{5.27}$ = 3.62, *P* = 0.010, LSD = 0.68) (Fig. A19.1). The treatment that had the greatest yield was treated cv. Barbados (5.51t/ha); this was not significantly different from the yield for untreated cvs Quartz (4.84t/ha) and Barbados (4.98t/ha), and treated cvs Django (4.85t/ha), Charger (5.18t/ha) and Quartz (5.33t/ha). The treatment that had the smallest yield was untreated cv. Hunivers (4.18t/ha); this was significantly different from that for all the cultivars that were not significantly different from treated cv. Barbados, except for untreated cv. Quartz. There were no treatments that had a significantly different yield to untreated cv. Quartz.

The 2018/19 cropping season

In the 2018/19 cropping season, there was an overall significant difference in yield between cultivars ($F_{5.30}$= 9.72, *P* < 0.001, LSD = 0.26). The cultivar that had the greatest yield was cv. Django (4.12 t/ha). However, this yield was not significantly different from cv. Barbados (3.93 t/ha).
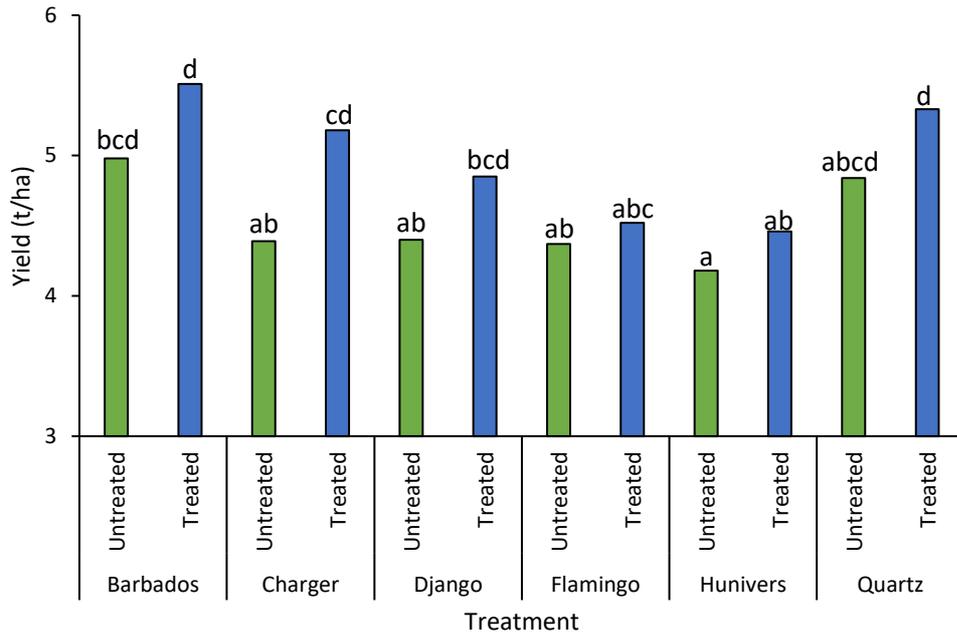
Figure A19.1: Yield from winter oilseed rape field experiment at Terrington St Clement, Norfolk in the 2017/18 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (27 d.f.).

The cultivar that had the smallest yield was cv. Quartz (3.41 t/ha); this was not significantly different from cvs Charger (3.52 t/ha), Flamingo (3.55 t/ha) or Hunivers (3.64 t/ha). There was no significant difference in yield between cvs Barbados, Hunivers or Flamingo. There was a significant difference in yield between untreated and treated plots ($F_{1.30}$ = 17.12, P < 0.001, LSD = 0.15). There was a significantly greater yield in treated plots (3.84 t/ha) than in untreated plots (3.55 t/ha); a yield increase of 0.29t/ha. There was no significant interaction between cultivar and the application of prothioconazole ($F_{5.30}$ = 1.33, P = 0.294, LSD = 0.37) (Fig. A19.2). There was a significant difference in yield between treatments ($F_{5.30}$ = 6.58, P < 0.001, LSD = 0.37) (Fig. A19.2). The treatment that had the smallest yield was untreated cv. Charger (3.28 t/ha); however, it was not significantly different from that of untreated (3.31t/ha) or treated (3.52 t/ha) cv. Quartz, or untreated cvs Flamingo (3.38 t/ha) and Hunivers (3.37 t/ha).

The treatment that had the greatest yield was treated cv. Django, but this was not significantly different from the yields for untreated cv Barbados (3.94t/ha) and Django (4.02 t/ha), and treated cvs Hunivers (3.91 t/ha) and Barbados (3.93 t/ha).

The 2019/20 cropping season

In the 2019/20 cropping season, there was an overall significant difference in yield between cultivars ($F_{5.27}$= 10.55, P < 0.001, LSD = 0.47). The cultivar that had the greatest yield was cv. Hunivers (4.03 t/ha). However, this yield was not significantly different from those of cvs Django (4.01 t/ha) and Quartz (3.72 t/ha). The cultivar that had the smallest yield was cv. Barbados (2.80 t/ha); this was not significantly different from those of cvs Flamingo (3.09 t/ha) and Charger (3.32 t/ha). The yields were not significantly different between cvs Flamingo and Charger, nor were the yields of cvs Charger and Quartz. There was a significant difference in yield of untreated and treated plots ($F_{1.27}$ = 24.60, P < 0.001, LSD = 0.27). There was a significantly greater yield in treated plots (3.81 t/ha) than in untreated plots (3.18t/ha). There was a yield increase of 0.63t/ha. There was no significant interaction between cultivar and the application of prothioconazole ($F_{5.27}$ = 2.40, P = 0.083, LSD = 0.66). There was a significant difference in yield between treatments ($F_{5.27}$ = 8.12, P < 0.001, LSD = 0.66) (Fig. A19.3).
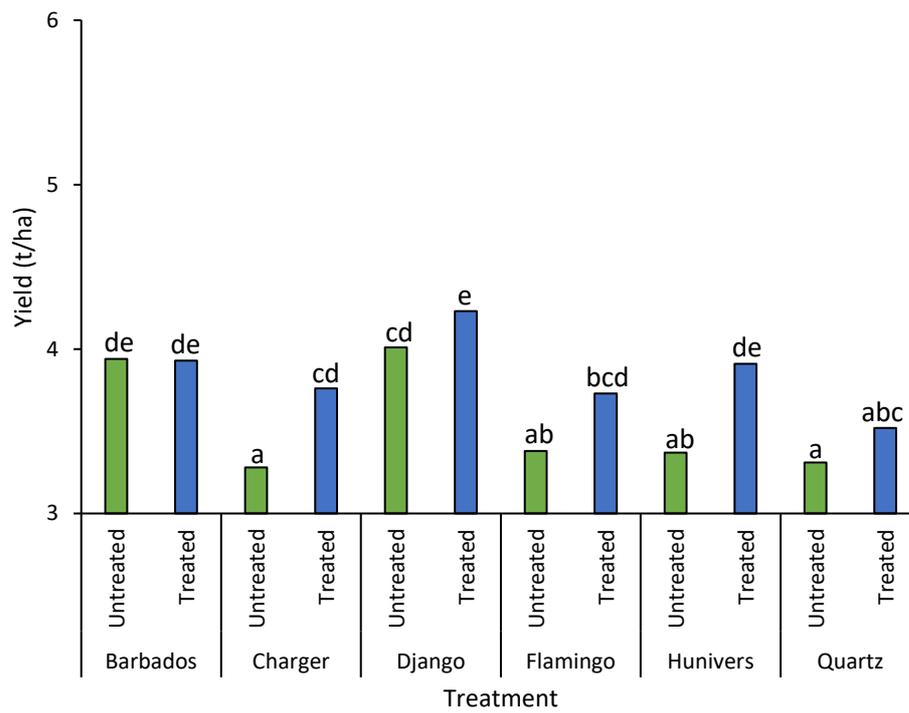
Figure A19.2: Yield from winter oilseed rape field experiment at Terrington St Clement, Norfolk in the 2018/19 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different *P* =0.05 (30 d.f.).
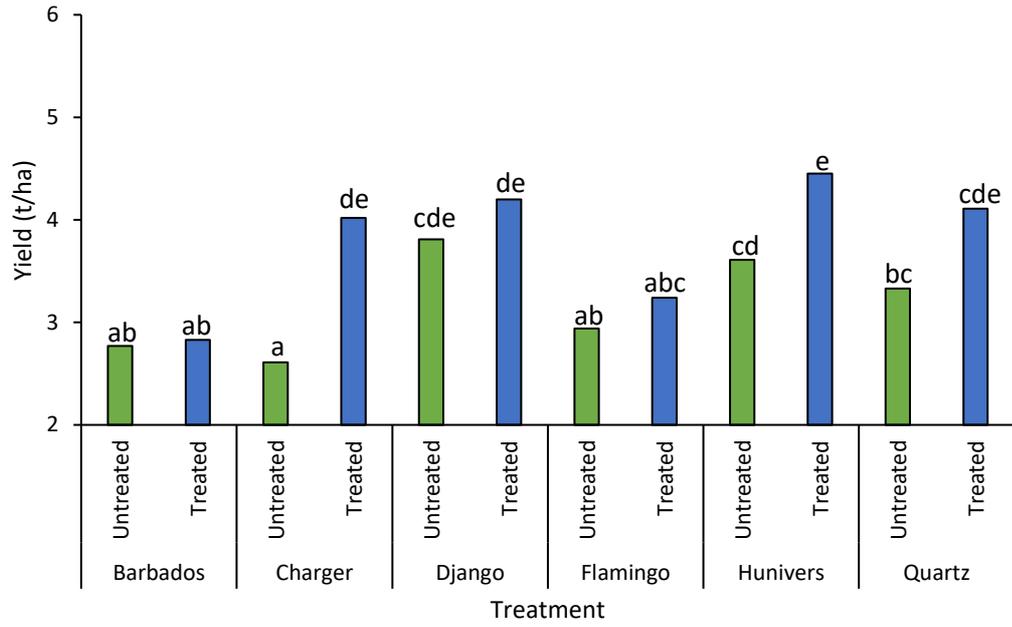
Figure A19.3: Yield from field experiment at Terrington St Clement, Norfolk in the 2019/20 cropping season. Fisher's protected LSD post hoc tests are represented as letters. Columns that do not share a letter are significantly different P =0.05 (30 d.f.).

The treatment that had the smallest yield was untreated cv. Charger (2.62 t/ha); however, it was not significantly different from those of untreated or treated cvs Flamingo and Barbados. The treatment that had the greatest yield was treated cv. Hunivers (4.45t/ha); this was not significantly different from those of untreated (3.81t/ha) or treated (4.20 t/ha) cv. Django, and treated cvs Charger (4.02 t/ha) and Quartz (4.11 t/ha).

APPENDICES 20 – qPCR analysis

2017/18

A greater amount of *L. maculans* DNA was detected than *L. biglobosa* DNA or *P. brassicae* DNA in the 2017/2018 season at Terrington St Clement (Fig. 4.12). Although small, the first *Leptosphaeria* spp. DNA maximum was detected in mid-September and both *L. maculans* and *L. biglobosa* DNA were detected at similar amounts. However, during the main period of *Leptosphaeria* spp. ascospore release events from early November to early January, both *L. maculans* DNA and *L. biglobosa* DNA were detected, but *L. maculans* DNA was detected at far greater amounts. There were no main periods of *Pyrenopeziza brassicae* ascospore release, there was a constant low level of *P. brassicae* ascospore release throughout the recording period which suggests that the ascospore release events may have occurred before sampling, similar to the results of Evans *et al.* (2017). The major ascospore release events for *L. maculans* and *L. biglobosa* were correlated with each other. The maximum amount of *L. maculans* DNA (2600 pg.) was on 7 December, *L. biglobosa* DNA (550 pg.) on 9 December and *P. brassicae* DNA (130pg) on 19 October.

2018/19

In the 2018/19 cropping season, there were sporadic, large *P. brassicae* ascospore release events that occurred throughout the recording period (Fig. 4.13). *P. brassicae* release events were more frequent and larger from September to December 2018. The largest *P. brassicae* maxima (950 pg) was on 19 December 2018. Overall, there was a very small number of *Leptosphaeria* spp. ascospores released during the 2018/19 cropping season at Terrington St Clement (Fig. 4.13). A greater amount of *L. maculans* DNA was detected than *L. biglobosa* DNA in the 2018/2019 season. The first *Leptosphaeria* spp. DNA maximum was detected in mid-October and both *L. maculans* DNA and *L. biglobosa* DNA were detected at similar amounts. On 21 October, there was a *L. biglobosa* DNA maximum that was larger than the *L. maculans* DNA maximum.

However, there was a main period of *L. maculans* ascospore release detected from early November to January with the maximum concentration of *L. maculans* DNA on 24 January. In contrast, *L. biglobosa* DNA maximum release was very limited and a consistently small amount of *L. biglobosa* DNA was detected throughout the season.

2019/20

In the 2019/20 cropping season, there were large, erratic *P. brassicae* ascospore release events that occurred throughout the recording period (Fig. 4.14). Although the release events were more frequent and larger between September and December 2019, *P. brassicae* ascospores were detected at high concentrations across the entire recording period. The largest *P. brassicae* maximum (1383 pg) was on 29 October 2019. Overall, there were large quantities of *Leptosphaeria* spp. ascospores released during the 2019/20 cropping season at Terrington St Clement (Fig. 4.14). A greater amount of *L. biglobosa* DNA was detected than *L. maculans* DNA in the 2019/2020 season. The first *Leptosphaeria* spp. DNA maximum was detected in early October and both *L. maculans* DNA and *L. biglobosa* DNA were detected at similar amounts, but a greater amount of *L. biglobosa* DNA was recorded. The *L. maculans* and *L. biglobosa* ascospore release events were well correlated, but all release events had a greater amount of *L. biglobosa* DNA than *L. maculans* DNA, except for the maximum at the end of December 2019. However, there was a main period of *Leptosphaeria* ascospore release detected from mid-October until the end of November with the maximum concentration of *L. biglobosa* DNA on 27 November (1150 pg). In contrast, *L. maculans* DNA maximum release was on 7 November (769 pg). There was a secondary smaller *Leptosphaeria* spp. ascospore release period at the end of January 2020.

APPENDICES 21 – Pseudothecial Densities

The 2017/18 season

In the 2017/18 season, there was a significant difference in $\log_e$ (pseudothecial density) on crop stubble between cultivars ($F_{5.53}$ = 13.33, P < 0.001, LSD = 0.54). Overall, cv. Charger (3.12) had a significantly greater $\log_e$ (pseudothecial density) than all other cultivars except for cv. Hunivers (2.814), but the $\log_e$ (pseudothecial density) on cv. Hunivers was significantly larger than on all other cultivars. Cv. Django (2.237), Quartz (2.232) and cv. Flamingo (2.223) were not significantly different from each other. Cv. Barbados (1.151) had the smallest mean $\log_e$ (pseudothecial density) and this was significantly smaller than that on all other cultivars. There were significant differences due to the application of fungicide ($F_{1.53}$ = 24.81, P < 0.001, LSD = 0.31) (Fig A21.1). Overall, the untreated (2.665) treatments had a greater $\log_e$ (pseudothecial density) than the fungicide treated treatments (1.887). An interaction between cultivar and fungicide treatment was found to influence the $\log_e$ (pseudothecial density)($F_{5.35}$ = 4.11, P = 0.004, LSD = 0.77). There were four cultivars, cvs Charger, Hunivers, Quartz and Flamingo, that had significantly a smaller $\log_e$ (pseudothecia density) when prothioconazole was applied compared to the untreated controls. Although the mean $\log_e$ (pseudothecial density) was greater on treated than on untreated stubble on cv. Barbados, they were not significantly different. Whereas on cv. Django the mean $\log_e$ (pseudothecia density) was greater on treated stubble than on untreated stubble, they were not significantly different.

Figure A21.1. *Leptosphaeria* spp. pseudothecial densities on stubble of different cultivars from the field experiments in the 2017/18 cropping season. Stems of the six winter oilseed rape cultivars were collected after harvest from plots that were 'Untreated' or 'Treated' with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. The pseudothecial density (number mature pseudothecia/cm^2) of each treatment was the mean of five stems. The mean values are presented with statistical analysis from natural logarithmic transformed data. Fisher's LSD tests were used to compare the log_e transformed (pseudothecial density) between different treatments. Columns that do not share a common letter are significantly different at *P* =0.05 (53 d.f.)

Untreated cv. Charger (3.684) had the greatest mean density of mature pseudothecia; this was significantly more than for all other treatments except for untreated cvs Hunivers (3.404) and Quartz (2.996). Treated cv. Barbados (0.841) had the smallest $\log_e$ (pseudothecial density); this was significantly smaller than all other densities recorded except for those on treated cvs Barbados and Quartz. Treated cv. Hunivers was only significantly different from untreated cvs Charger and Hunivers, that had significantly greater $\log_e$ (pseudothecia density), and treated cv. Barbados that had a significantly smaller $\log_e$ (pseudothecia density).

The 2018/19 season

In the 2018/19 season, there was a significant difference between cultivars in $\log_e$ (pseudothecial density) on crop stubble ($F_{5.55} = 14.44$, $P < 0.001$, LSD = 0.5136) (Fig. A21.2). Overall, cv. Charger (2.665) had a significantly greater $\log_e$ (pseudothecial density) than all other cultivars, except for cv. Flamingo (2.613). Cv. Django (1.067) had the smallest mean $\log_e$ (pseudothecial density); however, it was significantly smaller only than the $\log_e$ (pseudothecial density) on cvs Flamingo, Charger and Quartz (1.614). Cvs Quartz, Hunivers and cv. Barbados were not significantly different from each other. There was a significant difference due to the application of fungicide ($F_{1.53} = 7.65$, $P = 0.008$, LSD = 0.2956). Untreated stubble (1.956) had a significantly greater $\log_e$ (pseudothecial density) than treated stubble (1.551). An interaction between cultivar and fungicide treatment was not found to influence the $\log_e$ (pseudothecial density) ($F_{5.55} = 1.45$, $P = 0.227$, LSD = 0.7262) (Fig. A21.2). The only cultivar that had a significant difference between its untreated and treated $\log_e$ (pseudothecial density) was cv. Quartz.

Treated cv. Quartz (1.147) had a significantly smaller $\log_e$ (pseudothecial density) than untreated cv. Quartz (2.08). For all other cultivars, there was no significant difference in $\log_e$ pseudothecial density between untreated and treated samples. Untreated cv. Charger (3.022) had the greatest mean $\log_e$ (pseudothecial density); this was not significantly different from $\log_e$ (pseudothecia density) on untreated (2.69) or treated (2.535) cv. Flamingo or treated cv. Charger (2.307).
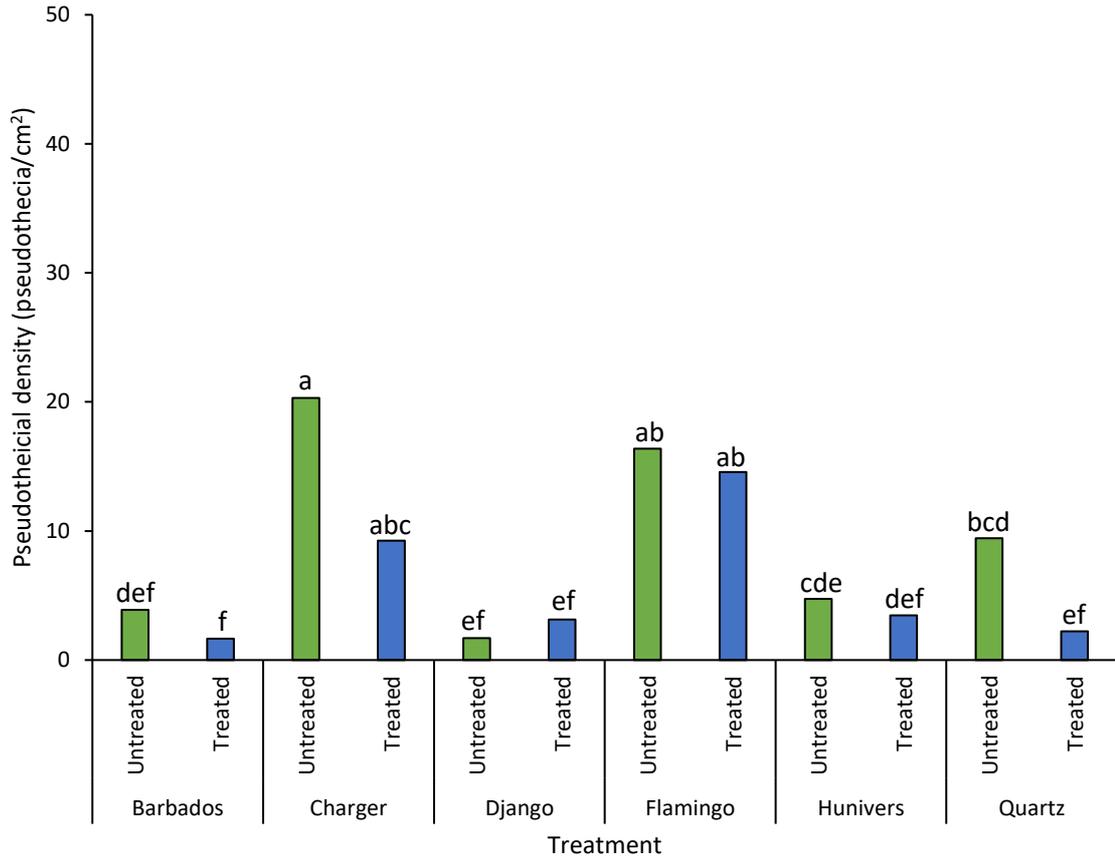
Figure A21.2. *Leptosphaeria* spp*.* pseudothecial densities on stubble of different cultivars from the field experiments in the 2018/19 cropping season. Stems of the six winter oilseed rape cultivars were collected after harvest from plots that were 'Untreated' or 'Treated' with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. The pseudothecial density (number mature pseudothecia/cm^2) of each treatment was the mean of five stems. The mean values are presented with statistical analysis from natural logarithmic-transformed data. Fisher's LSD tests were used to compare the log$_e$ transformed pseudothecial density between different treatments. Columns that do not share a common letter are significantly different at *P* =0.05 (55d.f.).

The treatment that had the smallest $\log_e$ (pseudothecia density) was treated cv. Barbados (0.917) but it was not significantly greater than untreated cvs Barbados (1.157) and Django (0.949) or treated cvs Django (1.185), Hunivers (1.449) and Quartz (1.147). However, untreated cv. Barbados and treated cv. Hunivers were not significantly different from untreated cv. Quartz that was also only significantly different from untreated cv. Charger.

The 2019/20 season

In the 2019/20 cropping season, there was a significant difference between cultivars in $\log_e$ (pseudothecial density) on crop stubble ($F_{5.53}$ = 27.15, P < 0.001, LSD = 0.5158). Overall, cv. Flamingo (2.612) had a significantly greater $\log_e$ (pseudothecial density) than all other cultivars except for cv. Charger (2.524). Cultivars Hunivers and Django were not significantly different from each other and were significantly different from all other cultivars. There was a significant difference in $\log_e$ (pseudothecial density) due to the application of fungicide ($F_{1.53}$ = 4.53, P = 0.039, LSD = 0.2975). Untreated stubble (1.678) had a significantly greater $\log_e$ (pseudothecial density) than treated stubble (1.365). An interaction between cultivar and fungicide treatment was not found to influence the $\log_e$ (pseudothecial density) ($F_{5.53}$ = 1.10, P = 0.376, LSD = 0.7296) (Fig. A21.3). The only cultivar that had a significant difference in $\log_e$ (pseudothecial density) between untreated and treated samples was cv. Charger. The $\log_e$ (pseudothecial density) was greater on untreated cv. Charger (2.961) than on treated cv. Charger (2.119). The treatment that had the greatest $\log_e$ (pseudothecial density) was untreated cv. Charger; that was significantly greater than on all other treatments except for untreated (2.518) and treated cv. Flamingo (2.699), that were not significantly different from each other, nor were they significantly different from treated cv. Charger. The $\log_e$ (pseudothecial density) of treated cv. Charger and untreated cv. Flamingo were not significantly different from untreated cv. Hunivers (1.816), but the $\log_e$ (pseudothecial density) of untreated cv. Hunivers was also not significantly different from untreated cvs Quartz (1.224) and Django (1.383) and treated cvs Quartz (1.221) and Hunivers (1.257). All these other treatments did not have a significantly greater $\log_e$ (pseudothecial density) than treated cv. Django (0.91).
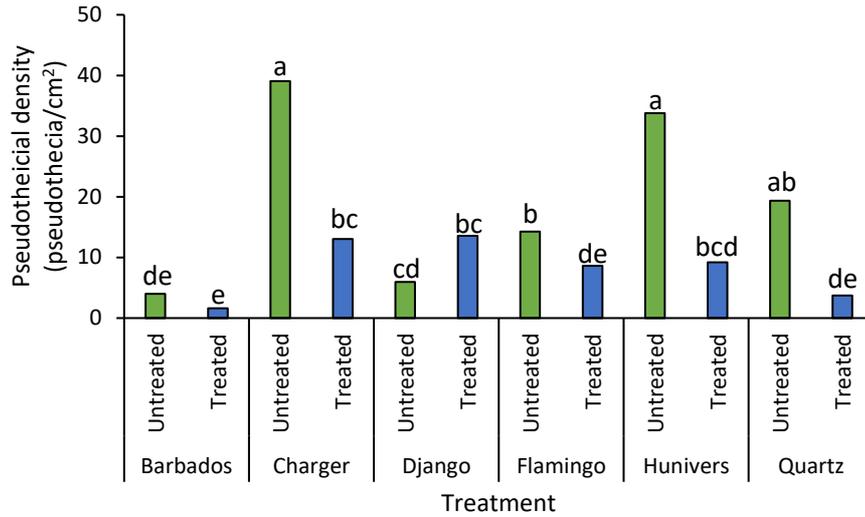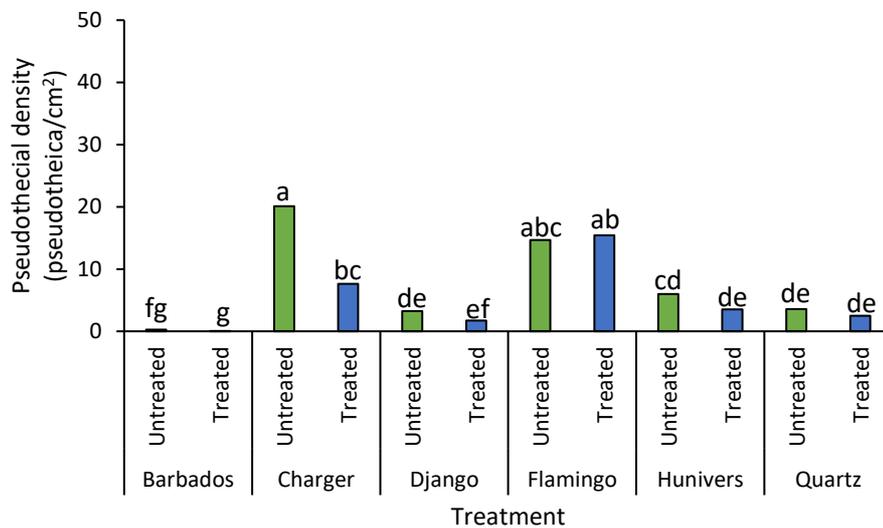
Figure A21.3. *Leptosphaeria* spp*.* pseudothecial densities on stubble of different cultivars from the field experiments in the 2019/20 cropping season. Stems of the six winter oilseed rape cultivars were collected after harvest from plots that were 'Untreated' or 'Treated' with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. The pseudothecial density (number mature pseudothecia/cm^2) of each treatment was the mean of five stems. The mean values are presented with statistical analysis from natural logarithmic transformed data. Fisher's LSD tests were used to compare the $\log_e$ transformed pseudothecial density between different treatments. Columns that do not share a common letter are significantly different at *P* =0.05 (53 d.f.).

Treated cv. Barbados had the smallest mean $\log_e$ (pseudothecia density), but it was not significantly different from the $\log_e$ (pseudothecia density) of untreated cv. Barbados but was a significantly smaller $\log_e$ (pseudothecia density) than treated cv. Django, but untreated cv. Barbados was not significantly different. Cv. Barbados (0.117) had the smallest mean $\log_e$ (pseudothecia density) and had a significantly smaller $\log_e$ (pseudothecia density) than all other cultivars.

Publications

Fortune, J.A., Qi, A., Ritchie, F., Karandeni Dewage, C.S., Fitt, B.D.L. and Huang, Y.J. (2021) Effects of cultivar resistance and fungicide application on stem canker of oilseed rape (*Brassica napus*) and potential interseasonal transmission of *Leptosphaeria* spp. inoculum. Plant Pathology. 70, 2115-2124

Effects of cultivar resistance and fungicide application on stem canker of oilseed rape (*Brassica napus*) and potential interseasonal transmission of *Leptosphaeria* spp. inoculum

James A. Fortune[1] | Aiming Qi[1] | Faye Ritchie[2] |
Chinthani S. Karandeni Dewage[1] | Bruce D. L. Fitt[1] | Yong-Ju Huang[1]

[1]Centre for Agriculture, Food and Environmental Management Research, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, UK
[2]Disease and Pest Management, ADAS Boxworth, Cambridge, UK

Correspondence
Yong-Ju Huang, Centre for Agriculture, Food and Environmental Management Research, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, AL10 9AB, UK.
Email: y.huang8@herts.ac.uk

Abstract

Phoma stem canker is a damaging disease of oilseed rape (*Brassica napus*) that causes annual yield losses to UK oilseed rape growers worth approximately £100 million, despite the use of fungicides. In the UK, oilseed rape is sown in August/September and harvested in the following July. The disease epidemics are initiated by ascospores released from *Leptosphaeria* spp. pseudothecia (ascocarps) on stem stubble in the autumn/winter. Control of this disease is reliant on the use of cultivars with "field resistance" and azole fungicides. This study investigated the effects of cultivar resistance and application of the fungicide prothioconazole on the severity of stem canker before harvest and the subsequent production of pseudothecia on the infected stubble under natural conditions in the 2017/2018, 2018/2019, and 2019/2020 cropping seasons. The application of prothioconazole and cultivar resistance decreased the severity of phoma stem canker before harvest, and the subsequent production of *Leptosphaeria* spp. pseudothecia on stubble in terms of pseudothecial density. Results showed that stems with less severe stem cankers produced fewer mature pseudothecia of *Leptosphaeria* spp. on the infected stubble. This investigation suggests that the most sustainable and effective integrated control strategy for phoma stem canker in seasons with low quantities of inoculum is to use cultivars with medium or good field resistance and apply only one spray of prothioconazole when required.

KEYWORDS
blackleg, *Brassica napus*, disease control, integrated pest management, *Leptosphaeria maculans*, oilseed rape

1 | INTRODUCTION

Phoma stem canker (also known as blackleg) is caused by two closely related coexisting ascomycete fungal pathogens, *Leptosphaeria maculans* and *L. biglobosa* (Fitt et al., 2006a; Shoemaker & Brun, 2001). This disease causes damage to oilseed rape worldwide (Fitt et al., 2006b) and accounts for annual yield losses to UK oilseed rape growers worth approximately £100 million, despite the use of fungicides and resistant cultivars (www.cropmonitor.co.uk) (Zhang et al., 2014). In the UK, phoma stem canker is a monocyclic disease that is initiated by ascospores as primary inoculum in autumn or winter. These ascospores are released from pseudothecia (sexual fruiting bodies) that mature after harvest on infected stem debris, such as stubble left in fields from the previous cropping season. Once mature, ascospores

are ejected and transported by wind-dispersal from the pseudothecia to infect suitable host plants (Gladders & Musa, 1980; Huang et al., 2005; West et al., 2001). Many ascospores travel less than 1 km, so growers are advised to isolate new crops of oilseed rape 200–500 m from previous crops (West & Fitt, 2005; www.ahdb.org.uk/frag). A successful infection is characterized by the appearance of phoma leaf spots that are usually observed in autumn. L. maculans and L. biglobosa leaf spots have different phenotypes, with L. maculans forming larger grey leaf spots with many black pycnidia (asexual fruiting bodies), while L. biglobosa forms smaller black lesions with few or no pycnidia (Fitt et al., 2006a). A recent investigation has shown that incidences of L. maculans and L. biglobosa were greater when plants were assessed using quantitative PCR than with a visual assessment (Jacques et al., 2021). The pathogens then grow from leaves along the leaf petiole towards the stem, causing stem canker by invading and killing stem cells where they continue to develop until harvest (Hammond et al., 1985; Huang et al., 2006).

It has long been accepted that an integrated approach, combining chemical control, cultivar resistance, and cultural control, is the most effective strategy for managing phoma stem canker epidemics (Gladders et al., 2006a; Huang et al., 2018; Juroszek & von Tiedemann, 2011; West et al., 2002). Growers are advised to apply fungicides only when they are needed. Accurate fungicide application timing is essential for effective control (Gladders et al., 2006a; Huang et al., 2011; West et al., 2002). In the UK, the risk of infection by Leptosphaeria spp. is greatest in autumn. Thus, the application of the first fungicide spray is recommended when 10%–20% of plants have L. maculans phoma leaf spots in autumn, followed by another fungicide application when reinfection is observed, to prevent the development of cankers in the following June/July (https://ahdb.org.uk/knowledge-library/how-to-manage-phoma-in-oilseed-rape). A range of fungicide active ingredients and modes of action are available commercially and used for protecting oilseed rape against diseases. However, there is a reliance on prothioconazole and tebuconazole, which are both azole fungicides. The use of these two active ingredients, either alone or in co-formulations, is increasing; they accounted for 34% in 2012 and 52% in 2018 of all fungicides applied on oilseed rape in the UK (Garthwaite et al., 2012, 2018). Additionally, growers are encouraged to grow cultivars with good "field resistance" as an economical and environmentally friendly method for phoma stem canker control. These methods are supplemented by cultural practices such as oilseed rape rotations and sowing UK crops by late August (Huang et al., 2018; Marcroft et al., 2004).

Previous research has shown that the greater the quantity of primary inoculum at the start of the cropping season, the more severe the stem cankers are before harvest (Bousset et al., 2019; Lô-Pelzer et al., 2009b; Marcroft et al., 2004). Therefore, there is a need to reduce the quantity of primary inoculum produced on stem debris after harvest for effective control of phoma stem canker. However, there is limited knowledge on the effect of integrated management approaches on the interseasonal transmission of pathogen inoculum. It is not known how cultivar resistance and application of fungicides affect the production of primary inoculum in the UK. The aim

of this investigation was to study the effects of fungicide application and cultivar resistance on the severity of phoma stem canker before harvest and on the subsequent development of pseudothecia on the crop stubble after harvest.

2 | MATERIALS AND METHODS

2.1 | Field experiments with different cultivars and fungicide treatments

Six cultivars with different field resistance ratings for phoma stem canker were selected for field experiments at Terrington St Clement, Norfolk. They were selected based on the AHDB Recommended List (RL) resistance ratings (0–9 scale, with 9 being good resistance) (https://ahdb.org.uk/knowledge-library/recommended-lists-for-cereals-and-oilseeds-rl-harvest-results-archive). To study the effects of cultivar resistance, the six cultivars were classified into three groups: good resistance, medium resistance, or susceptible. Cultivar Quartz (resistance rating 8) had good resistance, while cvs Barbados (resistance rating 7), Django (resistance rating 6), and Hunivers (resistance rating 7) had medium resistance, and cvs Charger and Flamingo (both resistance rating 4) were susceptible to Leptosphaeria spp. The experiments were sown in late August or early September in each of the 2017/2018, 2018/2019, and 2019/2020 cropping seasons. Each plot was 10 × 3 m. Plots were arranged in a randomized block design. Each of the six cultivars had untreated and prothioconazole-treated plots, each with three replicates: in total 36 plots. All other standard farm inputs and treatments were applied uniformly across the experiments. Proline 275 (Bayer Crop Science UK; prothioconazole-desthio 275 g/L) was used for this work because it is a triazole fungicide and representative of the main chemical group used to control oilseed rape diseases in the UK (Garthwaite et al., 2018). A half-label dose rate (0.315 L/ha) was used in two applications (early spray and late spray, respectively). The timing of the early spray was dependent on the timing of 10%–20% incidence of phoma leaf spot (% plants affected) on the susceptible cultivar Flamingo and the second spray was applied 4–6 weeks after the first spray when the soil was accessible, the wind speed was low, and there was no rain. The first sprays were applied on 25 October 2017, 16 November 2018, and 5 November 2019 and the second sprays were applied on 28 November 2017, 11 December 2018, and 16 December 2019 in the 2017/2018, 2018/2019, and 2019/2020 cropping seasons, respectively.

2.2 | Phoma stem canker assessments

In June/July each season before harvest, phoma stem canker was assessed by pulling up 20 plants randomly from each plot. The stems were cut at the crown collar and the severity of stem base canker on each plant was scored using a 0–7 scale (modified from the 1–6 scale of Lô-Pelzer et al., 2009a), whereby 0 = 0%, 1 = 1– 5%, 2 = 6%–25%,

3 = 26%–50%, 4 = 51%–75%, 5 = 76%–99%, 6 = 100% stem cross-section affected and plant still alive; 7 = 100% cross-section affected, dead stem with a hollow or severely necrotic pith. Stem cankers assessed in this work refer to stem basal cankers which were classified as cankers at the root crown or <10 cm above it (Mitrousia et al., 2018). Mean disease severity score per plot was calculated from the 20 plants per plot assessed.

2.3 | Stem incubation for pseudothecial production

Oilseed rape stems with stem cankers were sampled from the field experiments every year after harvest for pseudothecial production, as described by Huang et al. (2005). Stems from each treatment were collected together and placed in a free draining plastic tray around a Burkard spore sampler in a field plot at Bayfordbury, Hertfordshire so that pseudothecia could mature under natural conditions. The stems were collected and dried when major ascospore release events were observed using the Burkard spore sampler because the major release of ascospores indicated that most of the pseudothecia were mature (October/November). Once dried, five stems were selected randomly from each treatment. For each stem, the stem base (<10 cm above the root crown) was cut into 'stubble sticks' (5 × 0.5 cm). Four sticks from each stem were placed in a Petri dish for counting pseudothecia.

2.4 | Pseudothecial density assessment

Using a bifocal dissecting microscope at 10× magnification, the total number of mature pseudothecia on each 5 × 0.5 cm stubble stick was counted. The pseudothecial density (number of pseudothecia per cm^2) on each stubble stick was calculated. A characteristically shaped and sized *Leptosphaeria* spp. pseudothecium was considered mature when the neck was formed or the ostiole was open (i.e., ascospores were released) (Toscano-Underwood et al., 2003). The average pseudothecial density on each stem was calculated using a mean of the four stubble sticks. The average pseudothecial density per treatment was calculated using the means of the five stems per treatment (e.g., 20 stubble sticks per treatment were assessed).

2.5 | Statistical analysis

The statistical analyses of the data were done using GenStat (General Statistics) (www.vsni.co.uk). The 3-year data sets were analysed by fitting a mixed-model procedure. The fitted model consisted of the constant term and the full factorial combination of cultivar, fungicide, and year to determine the main effects of application of prothioconazole, choice of cultivar, year, and their two-way and three-way interactions on severity scores of stem canker replicated three times in each year (balanced design) and the density data of pseudothecia on crop stubble replicated between four and five times in each year (unbalanced

design). The pseudothecial density data were log$_e$-transformed to make the data more normally distributed and the variance homogenous. To analyse the effects of the three resistance groups, the fitted model consisted of the constant term and the full factorial combination of resistance, fungicide, and year. The post hoc test was done using the least significant difference (LSD) calculated at $p = 0.05$ by residual maximum likelihood (REML). The relationship between stem canker severity score and the density of pseudothecia on the sampled stems was analysed using linear regression and linear regression with groups.

3 | RESULTS

3.1 | Phoma stem canker severity

The three-way interaction of cultivar × fungicide × year was not significant, but the main effects of cultivar, fungicide, and cropping year and the effects of all two-way interactions were all significant ($p < 0.01$; Table 1). The significance of interaction between cultivar and fungicide means that the difference between fungicide treatments in stem canker severity depended on cultivars (Table 2). For example, cvs Quartz, Charger, Flamingo, and Django had significantly less severe canker in treated plants than in untreated plants, while cvs Barbados and Hunivers had no difference between the treated and untreated plants. The significance of interaction between cultivar and cropping season means that the difference among cultivars in stem canker severity depended on cropping seasons (Table 2). For example, cvs Barbados, Django, Flamingo, and Hunivers had no significant difference in canker severity scores in all three cropping seasons. However, cvs Charger and Quartz had a significantly higher canker score in 2017/2018 than in 2018/2019 and 2019/2020 cropping seasons.

When the means of cultivar × fungicide were compared, untreated cvs Charger and Flamingo had significantly greater stem canker severity scores than all other treatments tested. However, the treatment that had the most severe stem canker was untreated cv. Charger (2.46); this was significantly greater than the stem canker severity score of untreated cv. Flamingo (1.85) (Table 2). The stem canker severity score of treated cv. Charger (1.27) was significantly greater than that of untreated cv. Django (0.88), but the stem canker severity scores of untreated cvs Hunivers (1.17) and Quartz (0.97) and treated cv. Flamingo (1.14) were not significantly different from either treatment. There was no significant difference in the stem canker severity scores between untreated and treated cv. Hunivers (0.67), but the stem canker severity score of untreated cv. Barbados (0.52) was significantly less severe than that of untreated cv. Hunivers but not different from treated cv. Hunivers. There was no significant difference in stem canker severity score between treated cvs Quartz (0.48), Django (0.37), and Barbados (0.26). These treatments had significantly less severe canker scores than all other treatments, except for untreated cv. Barbados (Table 2).

When the six cultivars were divided into three resistance groups, neither the three-way interaction of resistance × fungicide × year nor the two-way interaction of resistance × year were significant

Factor	Wald statistic	df_{num}	F statistic	df_{den}	F probability
Stem canker severity score					
Cultivar	193.76	5	38.75	70.0	<0.01
Fungicide	61.62	1	61.62	70.0	<0.01
Year	27.01	2	13.50	70.0	<0.01
Cultivar × fungicide	17.62	5	3.52	70.0	<0.01
Cultivar × year	28.76	10	2.88	70.0	<0.01
Fungicide × year	14.08	2	7.04	70.0	<0.01
Cultivar × fungicide × year	13.53	10	1.35	70.0	0.22
Log_e (number of pseudothecia/cm²)					
Cultivar	220.85	5	44.17	124.4	<0.01
Fungicide	33.69	1	33.69	124.0	<0.01
Year	50.92	2	25.46	124.2	<0.01
Cultivar × fungicide	16.21	5	3.24	124.6	<0.01
Cultivar × year	49.61	10	4.96	124.8	<0.01
Fungicide × year	4.82	2	2.41	124.9	0.10
Cultivar × fungicide × year	18.58	10	1.86	124.5	0.06

TABLE 1 Testing outputs of significant probability levels by fitting a mixed model for the main effects of cultivar, fungicide, and cropping season, the two-way interactions, and the three-way interaction

Abbreviations: df_{num}, numerator degrees of freedom; df_{den}, denominator degrees of freedom.

TABLE 2 Severity of phoma stem canker from field experiments in the 2017/2018, 2018/2019, and 2019/2020 cropping seasons

Cultivar	Resistance rating[a]	Treatment	Stem canker severity (0–7 scale)			Mean of cultivar × fungicide
			2017/2018	2018/2019	2019/2020	
Barbados	7	Untreated	0.68	0.42	0.47	0.52 ef
		Treated	0.25	0.12	0.40	0.26 f
		Mean of cultivar × year	0.47 ghi	0.27 i	0.43 ghi	—
Charger	4	Untreated	3.78	2.02	1.58	2.46 a
		Treated	1.53	1.08	1.20	1.27 c
		Mean of cultivar × year	2.66 a	1.55 b	1.39 bc	—
Django	6	Untreated	1.18	0.57	0.90	0.88 d
		Treated	0.35	0.22	0.55	0.37 f
		Mean of cultivar × year	0.77 eh	0.39 hi	0.73 eh	—
Flamingo	4	Untreated	1.88	1.65	2.02	1.85 b
		Treated	1.03	1.22	1.17	1.14 cd
		Mean of cultivar × year	1.46 bc	1.43 bc	1.59 b	—
Hunivers	7	Untreated	1.70	0.80	1.02	1.17 cd
		Treated	0.83	0.90	0.87	0.87 de
		Mean of cultivar × year	1.27 bcd	0.85 dg	0.94 def	—
Quartz	8	Untreated	1.37	0.72	0.83	0.97 cd
		Treated	0.78	0.23	0.43	0.48 f
		Mean of cultivar × year	1.08 cde	0.48 ghi	0.63 fi	—

Note: The six winter oilseed rape cultivars were either untreated or treated with the fungicide prothioconazole. 'Mean of cultivar × fungicide' represents the mean phoma stem canker severity scores over three seasons of the two-way interaction between cultivar and fungicide. 'Mean of cultivar × year' represents the mean phoma stem canker severity scores across two treatments of the two-way interaction between cultivar and year. Least significant differences (LSD) were calculated at $p = 0.05$ and used to separate the mean stem canker severity score between the various treatment combinations in the two-way interactions. Values that do not share a common letter are significantly different at $p = 0.05$.
[a] Cultivar AHDB recommended list phoma resistance rating on 0–9 scale; resistance rating 8–9 was classed as good resistance, 6–7 as medium resistance, and <5 as susceptible.

($p = 0.78$ and 0.52, respectively), but the main effects of resistance, fungicide, and cropping season and the effects of the other two two-way interactions of resistance × fungicide and fungicide × year were significant ($p < 0.05$). All the three resistance groups significantly benefitted from receiving prothioconazole treatment (Figure 1). However, there was no significant difference between the 'Good' and 'Medium' resistant groups within the same fungicide treatment.

3.2 | Pseudothecial density on stem stubble

The three-way interaction of cultivar × fungicide × year and the two-way interaction of fungicide × year were not significant ($p = 0.06$ and 0.10, respectively), but the main effects of cultivar, fungicide, and cropping season and the effects of the other two two-way interactions of cultivar × fungicide and cultivar × year were significant ($p < 0.01$; Table 1). The significance of the interaction between cultivar and fungicide means that the difference between fungicide treatments in pseudothecial density depended on cultivars (Table 3). For example, cvs Barbados, Charger, Hunivers, and Quartz had significantly fewer pseudothecia per cm^2 in treated than in untreated plants, while cvs Django and Flamingo had no difference between the treated and untreated plants.

The significance of interaction between cultivar and cropping season means that the difference among cultivars in pseudothecial density was dependent on cropping season (Table 3). For example,
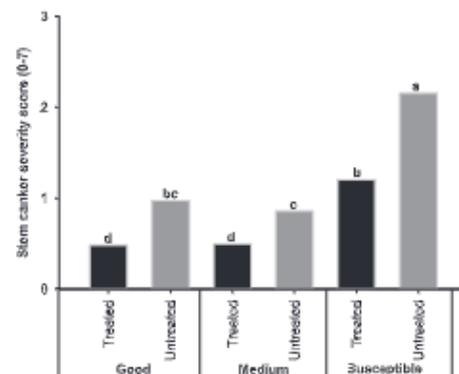

FIGURE 1 Mean severity of phoma stem canker of different fungicide treatments within resistance groups from winter oilseed rape field experiments in the 2017/2018, 2018/2019, and 2019/2020 cropping seasons. The resistance group 'Good' contained cv. Quartz, 'Medium' contained cvs Barbados, Django, and Hunivers, and 'Susceptible' contained cvs Charger and Flamingo. They were either untreated or treated with the fungicide prothioconazole. Least significant differences (LSD) were calculated at $p = 0.05$ and used to separate the mean stem canker score between different treatment combinations in the two-way interaction of resistance × fungicide. Bars that do not share a common letter are significantly different at $p = 0.05$

cvs Barbados and Charger had significantly greater pseudothecial density in 2017/2018 and 2018/2019 than in 2019/2020 while cvs Django, Hunivers, and Quartz had no significantly greater pseudothecial density in 2017/2018 than in 2018/2019 and 2019/2020. There was no significant difference in pseudothecial density for cv. Flamingo between all three cropping seasons.

When the means of cultivar × fungicide were compared, untreated cv. Charger (26.02) had the greatest pseudothecial density that was significantly greater than all other treatments (Table 3). There was no significant difference between untreated cvs Hunivers (15.47) and Flamingo (15.04) or treated cv. Flamingo (13.07). The pseudothecial density on treated cv. Flamingo was significantly different from treated cv. Hunivers (5.24) but untreated cv. Quartz (10.17) or treated cv. Charger (10.01) were not significantly different from either treatment. There were significantly more pseudothecia on stubble of untreated than treated cvs Charger and Quartz (Figure 2). There was a significantly smaller pseudothecial density on untreated cv. Barbados (2.89) than treated cv. Hunivers but the pseudothecial density on untreated cv. Django (3.59) and treated cvs Django (5.13) and Quartz (2.83) were not significantly different from that on untreated Barbados. The treatment that had the smallest pseudothecial density was treated Barbados (1.10) (Table 3).

When the six cultivars were divided into three resistance groups, the three-way interaction of resistance × fungicide × year and the two two-way interactions of resistance × fungicide and resistance × year were not significant ($p = 0.36$, 0.39, and 0.25, respectively), but the main effects of resistance, fungicide, and cropping season and the effect of the two-way interaction of resistance × year were significant ($p < 0.05$). Although pseudothecial density did not differ significantly between 'Good' and 'Medium' groups, they had a significantly smaller pseudothecial density than the 'Susceptible' group (Figure 3).

3.3 | Relationship between stem canker severity and pseudothecial density

The relationship was analysed by regressing stem canker severity score against *Leptosphaeria* spp. pseudothecial density (i.e., number of pseudothecia per cm^2; Figure 4). The correlation coefficient (r) between stem canker severity and pseudothecial density was $r = 0.85$, which supported a good simple linear relationship between the two traits ($p < 0.01$). For example, untreated cv. Charger had the greatest stem canker severity score and produced the greatest pseudothecial density (Tables 2 and 3). Stem canker severity scores and pseudothecial densities were greater on untreated stems than on prothioconazole-treated stems of the same cultivar. The stem canker severity scores and pseudothecial densities on susceptible cultivars were greater than those on untreated or treated cultivars with medium or good field resistance. Cultivars with either medium or good resistance ratings after fungicide treatment had the smallest phoma stem canker severity score and subsequently the smallest *Leptosphaeria* spp. pseudothecial density. However, comparison of the positions of lines at the intercept and/or line parallelisms of

TABLE 3 *Leptosphaeria* spp. pseudothecial densities on stubble of six cultivars from the field experiments in the 2017/2018, 2018/2019, and 2019/2020 oilseed rape cropping seasons

Cultivar	Resistance rating[a]	Treatment	Pseudothecial density (pseudothecia/cm²)			Mean of cultivar × fungicide
			2017/2018	2018/2019	2019/2020	
Barbados	7	Untreated	4.00	3.89	0.27	2.89 f
		Treated	1.62	1.65	0.03	1.10 g
		Mean of cultivar × year	2.81 ef	2.77 ef	0.14	–
Charger	4	Untreated	39.07	20.31	20.10	26.02 a
		Treated	13.06	9.24	7.60	10.01 bc
		Mean of cultivar × year	24.62 a	15.39 abc	13.15 bcd	–
Django	6	Untreated	5.93	1.70	3.25	3.59 ef
		Treated	13.59	3.14	1.74	5.13 ef
		Mean of cultivar × year	9.76 cd	2.43 f	2.49 f	–
Flamingo	4	Untreated	14.26	16.38	14.67	15.04 b
		Treated	8.62	14.57	15.43	13.07 bc
		Mean of cultivar × year	11.75 cd	15.47 ad	15.09 ad	–
Hunivers	7	Untreated	33.78	4.74	5.99	15.47 bc
		Treated	9.20	3.46	3.51	5.24 de
		Mean of cultivar × year	22.85 ab	4.10 ef	4.75 ef	–
Quartz	8	Untreated	19.35	9.44	3.57	10.17 cd
		Treated	3.73	2.22	2.48	2.83 ef
		Mean of cultivar × year	10.67 d	5.83 e	3.09 ef	–

Note: Stems of the six cultivars were collected after harvest from plots that were untreated or treated with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. 'Mean of cultivar × fungicide' represents the pseudothecial densities across three seasons of the two-way interaction between cultivar and fungicide. 'Mean of cultivar × year' represents the mean pseudothecial densities across two treatments of the two-way interaction between cultivar and year. Least significant differences (LSD) were calculated at $p = 0.05$ and used to separate the pseudothecial densities between the various treatment combinations in the two-way interactions. Values that do not share a common letter are significantly different at $p = 0.05$.

[a]Cultivar AHDB recommended list phoma resistance rating on 0–9 scale; resistance rating 8–9 was classed as good resistance, 6–7 as medium resistance, and <5 as susceptible.

FIGURE 2 *Leptosphaeria* spp. pseudothecia on oilseed rape stem stubble of two cultivars with different levels of 'field resistance'. Stem stubble from susceptible cv. Charger (a, b) and resistant cv. Quartz (c, d) from plots that were untreated (a, c) or treated with fungicide prothioconazole (b, d), from the 2019/2020 cropping season. Stubble was collected after harvest and placed in free draining trays to allow pseudothecia to mature under natural conditions. Scale bars represent 0.5 cm. White arrows indicate the pseudothecia [Colour figure can be viewed at wileyonlinelibrary.com]
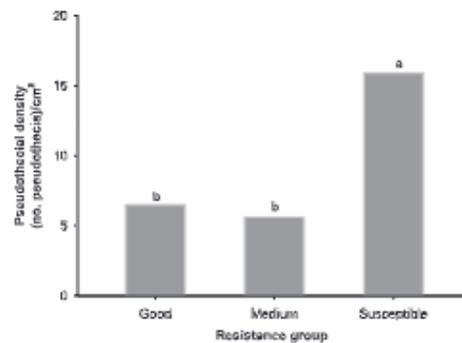
FIGURE 3 Mean *Leptosphaeria* spp. pseudothecial densities on stubble of different resistance groups from winter oilseed rape field experiments in the 2017/2018, 2018/2019, and 2019/2020 cropping seasons. The resistance group 'Good' contained cv. Quartz, 'Medium' contained cvs Barbados, Django, and Hunivers, and 'Susceptible' contained cvs Charger and Flamingo. Stems of these six winter oilseed rape cultivars were collected after harvest from plots that were untreated or treated with prothioconazole and placed in free draining trays to allow pseudothecia to mature under natural conditions. Least significant differences (LSD) were calculated at $p = 0.05$ and used to separate the mean *Leptosphaeria* spp. pseudothecial densities between resistance groups because the two-way interaction of resistance × fungicide was not significant ($p = 0.39$). Bars that do not share a common letter are significantly different at $p = 0.05$
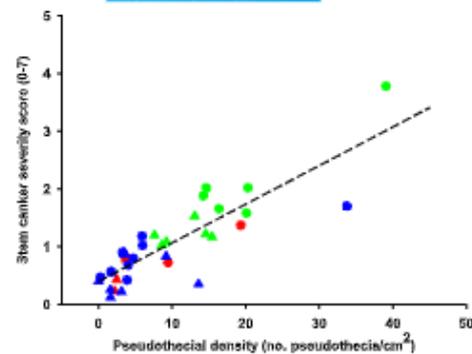

FIGURE 4 Relationship between phoma stem canker severity score before harvest and pseudothecial density of *Leptosphaeria* spp. on stems of six different cultivars from the field experiments in 2017/2018, 2018/2019, and 2019/2020 cropping seasons. The stem samples from untreated (●) or fungicide treated (▲) plots were collected after harvest and placed in free draining trays to allow pseudothecia to mature under natural conditions. The colours for groups represent field resistance rating against *Leptosphaeria* spp.: red = Good (cv. Quartz), blue = Medium (cvs Barbados, Django, and Hunivers), and green = Susceptible (cvs Charger and Flamingo). The fitted relation is $y = 0.40 + 0.067x$ ($R^2 = 71.9\%$, $df = 34$) in which y is the stem canker severity score (0–7 scale) and x the pseudothecial density (no. pseudothecia/cm^2)

the slope showed that separate lines for treated and untreated samples did not significantly improve the common fitted simple line. Fitting separate lines for grouping cultivars into 'Good', 'Medium', and 'Susceptible' field resistance levels did not significantly improve the common fitted simple line. As a result, a common simple linear relation was fitted to the combined data and it accounted for 71.9% of the variance in the observed phoma stem canker severity score by the pseudothecial density (i.e., the coefficient of determination $R^2 = 0.719$, $df = 34$; Figure 4).

4 | DISCUSSION

The application of the fungicide prothioconazole and use of cultivar resistance directly affected the severity of phoma stem canker before harvest, which indirectly affected the subsequent pseudothecial density of *Leptosphaeria* spp. on the stubble after harvest. Cultivars with medium or good resistance had less severe stem canker and subsequently fewer pseudothecia than susceptible cultivars, suggesting that using cultivar resistance will not only control phoma stem canker in the current season but also reduce the inoculum for the following cropping season. Previous studies also showed that greater stem canker severities result in greater pseudothecial densities (Lô-Pelzer et al., 2009b; Marcroft et al., 2004). These previous studies also found that quantitative cultivar resistance reduced the

severity of stem canker in crops, but stubble of crops with similar disease severity scores resulted in similar quantities of primary inoculum regardless of the level of quantitative resistance (Lô-Pelzer et al., 2009b; Marcroft et al., 2004). Surprisingly, cv. Quartz with a good resistance rating produced more pseudothecia than cv. Barbados with a medium resistance rating. This may have been due to different types of resistance in these two cultivars, because cv. Quartz has *Rlm7* gene-mediated resistance, whereas cv. Barbados has quantitative resistance. Therefore, the pseudothecia produced on stubble of cv. Quartz may be predominantly *L. biglobosa* because resistance genes like *Rlm7* are effective in controlling phoma stem canker only when caused by *L. maculans* (Huang et al., 2018; Mitrousia et al., 2018). This is supported by previous work showing that more *L. biglobosa* ascospores than *L. maculans* ascospores were discharged from mature pseudothecia on the stems of cvs Roxet and Excel with the resistance gene *Rlm7* in the 2010/2011, 2011/2012, and 2012/2013 cropping seasons (Sidique, 2015). A recent study showed that cultivars with resistance genes against *L. maculans* promote colonization by *L. biglobosa* (Kerdraon et al., 2020). This suggests that use of cultivars with good resistance against *L. maculans* may result in a shift in the ratio of *L. maculans* to *L. biglobosa* in the local *Leptosphaeria* spp. populations.

The application of fungicide prothioconazole effectively reduced the severity of stem cankers and subsequently reduced pseudothecial density at the start of the following cropping

season. The application of prothioconazole reduced the severity of stem cankers on all cultivars, suggesting that prothioconazole is still effective for the control of phoma stem canker in the UK. This study showed that when there was little *Leptosphaeria* spp. pathogen inoculum (e.g., 2019/2020 season), using a cultivar with medium field resistance without fungicide application (e.g., cv. Barbados) was more effective in reducing stem canker severity than applying fungicide to a susceptible cultivar (e.g., cv. Charger). This suggests that the use of fungicide may not be necessary or economically viable if there is little *Leptosphaeria* spp. inoculum when using a cultivar with medium or good quantitative resistance. However, the application of fungicide did significantly reduce the subsequent pseudothecial density, suggesting that use of fungicide may not be economically necessary in the current cropping season, but it will help to reduce inoculum for the next season. This study did not distinguish pseudothecia as *L. maculans* or *L. biglobosa*; even if most of the pseudothecia on the stems are *L. biglobosa*, reduction in number of pseudothecia on the crop debris is still important for control of stem canker on the next season's crop, because recent work showed that *L. biglobosa* can cause significant yield losses (Cai et al., 2018). Previous studies in oilseed rape also showed that increases in yield due to fungicide application were not cost-effective when there was little *Leptosphaeria* spp. inoculum (Gladders et al., 2006b). Studies in other crops support this observation. For the control of potato late blight (*Phytophthora infestans*), applying a low fungicide dose to a potato cultivar with good resistance was more effective in reducing disease severity than applying a greater fungicide dose to a susceptible cultivar (Ritchie et al., 2018). Results of this study showed that more cultivars had a significantly greater reduction in stem canker severity after spraying with prothioconazole under high *Leptosphaeria* spp. inoculum concentration than under low pathogen inoculum situations. This suggests that the effectiveness of fungicide application is dependent not only on the choice of cultivar but also on the pathogen inoculum concentration.

Because epidemics of phoma stem canker are initiated by ascospores released from mature pseudothecia in the previous autumn, reducing the density of pseudothecia is crucial for effective control of the disease in the following season. However, development and maturation of pseudothecia are affected by many factors. Differences in the pseudothecial densities between the three cropping seasons suggest that weather conditions affect the development of pseudothecia on crop stubble. Previous studies showed that precipitation and temperature are two major factors affecting the maturation of *L. maculans* and *L. biglobosa* pseudothecia (Huang et al., 2007; Toscano-Underwood et al., 2003). Studies on fungicide sensitivity showed that *L. biglobosa* is less sensitive to triazole fungicides than *L. maculans* (Eckert et al., 2010; Huang et al., 2011), suggesting that application of azole fungicides may favour *L. biglobosa* over *L. maculans* in production of pseudothecia on crop stubble. However, in this study the pseudothecia produced on stubble were not distinguished as *L. biglobosa* or *L. maculans*. Recent work showed that *L. biglobosa* is a key species in microbial communities on oilseed rape stubble, whereas *L. maculans* plays a minor role (Kerdraon et al., 2020). Additionally, *L. biglobosa* is more competitive than *L. maculans* at the necrotrophic growth stage on stem stubble (West et al., 1999). This suggests that the pseudothecial development and speed of maturation on oilseed rape stubble may differ between *L. biglobosa* and *L. maculans* under different environmental conditions and may lead to the changes in predominance of the two species in a local pathogen population.

Results of this study showed that the greater phoma stem canker severity before harvest, the greater the pseudothecial density of *Leptosphaeria* spp. after harvest, suggesting that cultivar resistance of the previous crop had a direct effect on inoculum production for the following cropping season; this agrees with other studies (Bousset et al., 2021; Lô-Pelzer et al., 2009b; Marcroft et al., 2004). However, we also noted that ascospore-bearing pseudothecia were present on stubble even if it was from cultivars with good resistance or treated with fungicide. This suggests that using resistant cultivars and fungicides may not prevent phoma stem canker epidemics but could help to reduce the disease severity and its impact on the yield of the crops. Brun et al. (2010) showed that stem canker severity increased with an increased number of phoma leaf lesions. This suggests that the reduction in initial inoculum concentration (i.e., fewer pseudothecia) could reduce the number of phoma leaf lesions at the beginning of the following cropping season. However, the threshold (i.e., the baseline) values of the initial inoculum that can cause a significant canker severity, or the relationship between the initial inoculum concentration and the final stem canker severity, still needs to be investigated. Increased understanding about initial inoculum concentrations at the beginning of the cropping season could help modellers to model inoculum spread to surrounding oilseed rape fields, because the level of phoma leaf spot in oilseed rape fields was found to be a function of the distance to emitting ascospore sources (Bousset et al., 2021; Zhang et al., 2014).

This investigation suggests that the most sustainable and effective integrated control strategy for phoma stem canker in seasons with little *Leptosphaeria* spp. inoculum is to use cultivars with medium or good resistance, combined with one application of fungicide. These methods could be even more effective if geographical screening work were routinely done to identify *L. maculans* population structures in different regions for effective deployment of resistance (R) genes to control phoma stem canker. Deployment of cultivars with different R genes will not only reduce phoma stem canker severity before harvest and decrease inoculum concentration for the next cropping season, but can also prolong the lifespan of cultivar resistance (van den Bosch & Gilligan, 2003; Gladders et al., 2006a; Marcroft et al., 2004; Mitrousia et al., 2018). Effective control of severe phoma stem canker epidemics relies on use of fungicides. However, it is important to acknowledge that in the UK fungicides are not applied solely for the control of phoma stem canker; they are also applied for the control of other autumn diseases such as light leaf spot, so it is important for growers to be aware of other diseases to ensure they can be controlled when necessary.

ORCID

James A. Fortune https://orcid.org/0000-0002-9980-7941
Aiming Qi https://orcid.org/0000-0002-0784-9520
Faye Ritchie https://orcid.org/0000-0001-9642-1712
Chinthani S. Karandeni Dewage https://orcid.org/0000-0001-6621-8203
Bruce D. L. Fitt https://orcid.org/0000-0003-3981-6456
Yong-Ju Huang https://orcid.org/0000-0001-6537-5792

REFERENCES

van den Bosch, F. & Gilligan, C.A. (2003) Measures of durability of resistance. *Phytopathology*, 93, 616–625.

Bousset, L., Palerme, M., Leclerc, M. & Parisey, N. (2019) Automated image processing framework for analysis of the density of fruiting bodies of *Leptosphaeria maculans* on oilseed rape stems. *Plant Pathology*, 68, 1749–1760.

Bousset, L., Vallée, P., Delourme, R., Parisey, N., Palerme, M. & Leclerc, M. (2021) Besides stem canker severity, oilseed rape host genotype matters for the production of *Leptosphaeria maculans* fruit bodies. *Fungal Ecology*, 52, 101076.

Brun, H., Chèvre, A., Fitt, B.D.L., Powers, S., Besnard, A., Ermel, M. et al (2010) Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist*, 185, 285–299.

Cai, X., Huang, Y.J., Jiang, D., Fitt, B.D.L., Li, G. & Yang, L. (2018) Evaluation of oilseed rape seed yield loss caused by *Leptosphaeria biglobosa* in central China. *European Journal of Plant Pathology*, 150, 179–190.

Eckert, M.R., Rossall, S., Selley, A. & Fitt, B.D.L. (2010) Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker of oilseed rape). *Pest Management Science*, 66, 396–405.

Fitt, B.D.L., Brun, H., Barbetti, M.J. & Rimmer, S.R. (2006b) World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, 114, 3–15.

Fitt, B.D.L., Huang, Y.J., van den Bosch, F. & West, J.S. (2006a) Coexistence of related pathogen species on arable crops in space and time. *Annual Review of Phytopathology*, 44, 163–182.

Garthwaite, D.G., Barker, I., Parrish, G., Smith, C., Chippindale, C. & Pietravalle, S. (2018) Arable crops in the United Kingdom 2018. Pesticide usage survey report 284. York: FERA. Available at: https://secure.fera.defra.gov.uk/pusstats/surveys/documents/arable2018.pdf [Accessed 1st April 2021].

Garthwaite, D.G., Hudson, S., Barker, I., Parrish, G., Smith, L. & Pietravalle, S. (2012) Arable crops in the United Kingdom 2012 (including aerial applications 2012). Pesticide Usage Survey Report Number 250. York: FERA. Available at: https://secure.fera.defra.gov.uk/pusstats/surveys/documents/arable2012v3.pdf [Accessed 1st April 2021].

Gladders, P., Dyer, C., Fitt, B.D.L., Evans, N., van den Bosch, F. & Baierl, A. et al (2006b) Pest and disease management system for supporting winter oilseed rape decisions (PASSWORD)—validation phase. Project Report No. 390. London: HGCA. Available at: https://projectblue.blob.core.windows.net/media/Default/Research%20Papers/Cereals%20and%20Oilseed/pr390.pdf [Accessed 1st April 2021].

Gladders, P., Evans, N., Marcroft, S. & Pinochet, X. (2006a) Dissemination of information about management strategies and changes in farming practices for the exploitation of resistance to *Leptosphaeria maculans* (phoma stem canker) in oilseed rape cultivars. *European Journal of Plant Pathology*, 114, 117–126.

Gladders, P. & Musa, T.M. (1980) Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. *Plant Pathology*, 29, 28–37.

Hammond, K.E., Lewis, B.G. & Musa, T.M. (1985) A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathology*, 34, 557–565.

Huang, Y.J., Evans, N., Li, Z-Q., Eckert, M., Chevre, A.-M., Renard, M. et al (2006) Temperature and leaf wetness duration affect phenotypic expression of Rlm6-mediated resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist*, 170, 129–141.

Huang, Y.J., Fitt, B.D.L., Jedryczka, M., Dakowska, S., West, J.S., Gladders, P. et al (2005) Patterns of ascospore release in relation to phoma stem canker epidemiology in England (*Leptosphaeria maculans*) and Poland (*Leptosphaeria biglobosa*). *European Journal of Plant Pathology*, 111, 263–277.

Huang, Y.J., Hood, J.R., Eckert, M.R., Stonard, J.F., Cools, H.J., King, G.J. et al (2011) Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). *Plant Pathology*, 60, 607–620.

Huang, Y.J., Liu, Z., West, J.S., Todd, A.D., Hall, A.M. & Fitt, B.D.L. (2007) Effects of temperature and rainfall on date of release of ascospores of *Leptosphaeria maculans* (phoma stem canker) from winter oilseed rape (*Brassica napus*) debris in the UK. *Annals of Applied Biology*, 151, 99–111.

Huang, Y.J., Mitrousia, G.K., Sidique, S.N.M., Qi, A. & Fitt, B.D.L. (2018) Combining R gene and quantitative resistance increases effectiveness of cultivar resistance against *Leptosphaeria maculans* in *Brassica napus* in different environments. *PLoS One*, 13, e0197752.

Jacques, N., Balesdent, M.H., Rouxel, T. & Laval, V. (2021) New specific quantitative real time PCR assays shed light on the epidemiology of two species of the *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex. *Plant Pathology*, 70, 643–654.

Juroszek, P. & Von Tiedemann, A. (2011) Potential strategies and future requirements for plant disease management under a changing climate. *Plant Pathology*, 60, 100–112.

Kerdraon, L., Barret, M., Balesdent, M.-H., Suffert, F. & Laval, V. (2020) Impact of a resistance gene against a fungal pathogen on the plant host residue microbiome: the case of the *Leptosphaeria maculans–Brassica napus* pathosystem. *Molecular Plant Pathology*, 21, 1545–1558.

Lô-Pelzer, E., Aubertot, J.N., Bousset, L., Pinochet, X. & Jeuffroy, M.H. (2009a) Phoma stem canker (*Leptosphaeria maculans*/*L. biglobosa*) of oilseed rape (*Brassica napus*): Is the G2 disease index a good indicator of the distribution of observed canker severities? *European Journal of Plant Pathology*, 125, 515–522.

Lô-Pelzer, E., Aubertot, J., David, O. & Jeuffroy, M.H. (2009b) Relationship between severity of blackleg (*Leptosphaeria maculans*/*L. biglobosa* species complex) and subsequent primary inoculum production on oilseed rape stubble. *Plant Pathology*, 58, 61–70.

Marcroft, S.J., Sprague, S.J., Pymer, S.J., Salisbury, P.A. & Howlett, B.J. (2004) Crop isolation, not extended rotation length, reduces blackleg (*Leptosphaeria maculans*) severity of canola (*Brassica napus*) in south-eastern Australia. *Australian Journal of Experimental Agriculture*, 44, 601–606.

Mitrousia, G.K., Huang, Y.J., Qi, A., Sidique, S.N.M. & Fitt, B.D.L. (2018) Effectiveness of Rlm7 resistance against *Leptosphaeria maculans* (phoma stem canker) in UK winter oilseed rape cultivars. *Plant Pathology*, 67, 1339–1353.

Ritchie, F., Bain, R.A., Lees, A.K., Boor, T.R.W. & Paveley, N.D. (2018) Integrated control of potato late blight: predicting the combined efficacy of host resistance and fungicides. *Plant Pathology*, 67, 1784–1791.

Shoemaker, R.A. & Brun, H. (2001) The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Canadian Journal of Botany*, 79, 412–419.

Sidique, S.N.M. (2015) Effects of host resistance on colonisation of *Brassica napus* (oilseed rape) by *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker). PhD thesis, Hatfield, University of Hertfordshire.

Toscano-Underwood, C., Huang, Y.J., Fitt, B.D.L. & Hall, A.M. (2003) Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. *Plant Pathology*, 52, 726–736.

West, J.S., Biddulph, J.E., Fitt, B.D.L. & Gladders, P. (1999) Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. *Annals of Applied Biology*, 135, 535–546.

West, J.S. & Fitt, B.D.L. (2005) Population dynamics and dispersal of *Leptosphaeria maculans* (blackleg of canola). *Australasian Plant Pathology*, 34, 457–461.

West, J.S., Fitt, B.D.L., Leech, P.K., Biddulph, J.E., Huang, Y.J. & Balesdent, M.H. (2002) Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on phoma leaf spot and phoma stem canker development on winter oilseed rape (*Brassica napus*) in southern England. *Plant Pathology*, 51, 454–463.

West, J.S., Kharbanda, P.D., Barbetti, M.J. & Fitt, B.D.L. (2001) Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology*, 50, 10–27.

Zhang, X., White, R.P., Demir, E., Jedryczka, M., Lange, R.M., Islam, M. et al. (2014) *Leptosphaeria* spp., phoma stem canker and potential spread of *L. maculans* on oilseed rape crops in China. *Plant Pathology*, 63, 598–612.

Chemical warfare: the fungal quest to conquer oilseed rape

By JAMES FORTUNE, DANIEL BAKER, JAMES STANLEY,
CHINTHANI KARANDENI DEWAGE, FAYE RITCHIE, BRUCE D L FITT
and YONG-JU HUANG

Centre for Agriculture, Food and Environmental Management, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Hertfordshire, AL10 9AB, UK
Corresponding Author Email: y.huang8@herts.ac.uk

Key words: Phoma stem canker, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, sirodesmin PL, interspecific interactions, phomamide

Introduction

Phoma stem canker, caused by *Leptosphaeria maculans* and *L. biglobosa*, causes an average yield loss of > £70M annually in UK oilseed rape (www.cropmonitor.co.uk) (Zhang *et al.*, 2014). Previous studies had shown that *L. biglobosa* ascospores were released later than those of *L. maculans* (Huang *et al.*, 2011). However, more recent investigations that have used qPCR analysis have reported that ascospores of both species are more frequently released at similar times (Javaid *et al.*, 2018). *L. maculans* produces sirodesmin PL, a non-host selective epipolythiodioxopiperazine; *L. biglobosa* does not (Pedras & Yu, 2009). Sirodesmin PL has an inhibitory effect on *L. biglobosa* (Elliott *et al.*, 2007). There has been limited work investigating the interaction between *L. maculans* and *L. biglobosa* at key stages of their life cycles. Therefore, this study aims to provide a better understanding of the unknown interactions between *L. maculans* and *L. biglobosa* and investigate the changes in phytotoxin production as a result of increased interspecific competition.

Materials and methods

L. maculans and *L. biglobosa* were cultured in liquid culture, either individually or dual cultured with a competing pathogen. After 14 days, a secondary metabolites ethyl acetate extraction was done for each treatment, to investigate the effect of secondary metabolites on the colony growth of *L. maculans* and *L. biglobosa*. Fungal plugs (8mm diameter) of *L. maculans* or *L. biglobosa* were inoculated onto clarified V8 juice agar plates. Each fungal plug was inoculated with the corresponding secondary metabolite extract from each treatment or ethyl acetate. Each treatment was replicated five times; the ethyl acetate control was replicated three times. Colony diameters for *L. maculans* and *L. biglobosa* were recorded at 7 days post inoculation and converted to colony areas. To investigate the changes in phytotoxin production, the secondary metabolites extracted from each treatment were analysed to identify differences in composition using HPLC and LC-MS.

Results

Analysis of interspecific interactions between the pathogens *in vitro* confirmed that different mechanisms of interspecific competition were used to out-compete each other. The secondary metabolites produced by *L. maculans* inhibited *L. biglobosa* colony growth. This inhibition was not observed when *L. biglobosa* was inoculated with secondary metabolites extracted from

the co-culture of *L. maculans* and *L. biglobosa*. There were three unique maxima found only in the secondary metabolite extracts that inhibited *L. biglobosa* colony growth. Using HPLC and LC-MS, these maxima were identified as sirodesmin PL precursors deacetylsirodesmin PL and phomamide, sirodesmin PL and an unknown compound. When *L. maculans* and *L. biglobosa* were co-inoculated, sirodesmin PL and its precursors were not produced. Additional maxima on the HPLC chromatograph were not found. Results of this study suggest that *L. biglobosa* must inhibit the formation of sirodesmin-precursor. Due to sirodesmin having an antagonistic effect on *L. biglobosa*, it is thought that this interference must happen very early in *L. maculans*-*L. biglobosa* interactions, before the production of sirodesmin. Considering application of the results for control of phoma stem canker in field conditions, if *L. maculans* and *L. biglobosa* ascospores are released at the same time, phoma leaf spot lesions may appear later or be smaller, allowing fungicides to be applied later.

Acknowledgements

References

Elliott CE, Gardiner DM, Thomas G, Cozijnsen A., Van De Wouw A, Howlett BJ. 2007. Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus*. *Molecular Plant Pathology* **8**:791-802

Huang YJ, Hood JR, Eckert MR, Stonard JF, Cools HJ, King GJ *et al.* **2011.** Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). *Plant Pathology* **60**:607-620.

Javaid A, Gajula LH, Fitt BDL, Huang YJ. 2018. Investigating regional differences in proportions of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* in southern England. *International Congress of Plant Pathology 2018, Boston, Massachusetts, U.S.A.* August 1st 2018. (Poster presentation. Abstract, Group 2: Pathogen Dispersal and Survival. 1043-P, p96).

Pedras MSC, Yu Y. 2009. Mapping the sirodesmin PL biosynthetic pathway - A remarkable intrinsic steric deuterium isotope effect on a ^{1}H NMR chemical shift determines beta-proton exchange in tyrosine. *Canadian Journal of Chemistry* **87**:564–570.

Zhang X, White RP, Demir E, Jedryczka M, Lange RM, Islam M *et al.* **2014.** *Leptosphaeria spp.*, phoma stem canker and potential spread of *L. maculans* on oilseed rape crops in China. *Plant Pathology* **63**:598-612.

26

TECHNICAL OSR DISEASE

The work being undertaken by teams of researchers to enable more disease-resistant to be bred was highlighted at an AgriFood Charities Partnership seminar. **Alice Dyer**

Why phoma is getting more difficult to control

Since the 2009/10 season, phoma stem canker has caused annual yield losses worth more than £74 million in England and Wales. This is despite growers employing resistant varieties and fungicide programmes.

Phoma stem canker is caused by two similar, coexisting pathogens – leptosphaeria maculans and L.biglobosa. These form different types of lesions on the leaf, with L. biglobosa creating a smaller, more defined lesion, whereas L. maculans forms a much larger, paler lesion. L.biglobosa tends to form up the stem causing upper stem lesions.

Ascospores

Previous studies of the pathogens found that typically L. biglobosa ascospores are released later than L.maculans, but more recent investigations using qPCR testing have revealed that ascospores of both

James Fortune is exploring how the two phoma pathogens coincide.

species are being more frequently released at similar times.

This was also seen in field trials near Kings Lynn conducted by James Fortune, who is studying for a PhD at the University of Hertfordshire to develop new strategies for better control of phoma stem canker to reduce crop losses and improve disease modelling.

He said: "The spores were released at the same time in November, which is very different to what previous studies have shown."

Mr Fortune investigated the impact this would have on disease levels because the L.maculans pathogen produces a non-host selective toxic secondary

> ## 66 The spores were released at the same time in November, which is very different to what previous studies have shown
>
> JAMES FORTUNE

metabolite called sirodesmin PL, which has been shown to have an inhibitory effect on the L.biglobosa pathogen.

In a lab he examined the effects of secondary metabolites produced from the two pathogens grown in isolation and together on the growth of L.maculans and L.biglobosa. He found that the growth of L. maculans was not affected by the introduction of different secondary metabolite extracts from either L.maculans or L.biglobosa in isolation or when grown together after seven days. However, for L.biglobosa there was a significant inhibition of growth when the L.maculans extract was applied.

"This showed the secondary metabolites produced by L. maculans inhibits the L.biglobosa colony growth," he said.

Interestingly, when the extract from L.maculans and L.biglobosa grown together was applied, this inhibition was not observed. Using analytical chemistry techniques, it was shown that sirodesmin was present in the L.maculans only extract but not in the L. biglobosa only extract or the extract when both pathogens were grown together.

The same study was then undertaken on oilseed rape cotyledons inoculated with the two pathogens either in isolation or together.

Mr Fortune said: "L.maculans formed very large, pale lesions, like what is seen in the field, while L.biglobosa formed much smaller and more concise lesions. However, when L. biglobosa and L.maculans were inoculated at the same time, they formed very similar lesions to those observed when inoculated with L.biglobosa only, suggesting that L.maculans had been outcompeted."

DNA

Preliminary DNA extractions from the lesions found that the amount of L.maculans and L. biglobosa DNA was reduced when simultaneously co-inoculated.

"When we combined the two, there was a reduction in DNA concentration for both pathogens, but the reduction was disproportionate; L.maculans

oilseed rape varieties reports.

DNA was reduced much more than L.biglobosa DNA.

"There was a 95% reduction in L.maculans compared to the L.maculans only inoculated plants, and a 40% reduction in L.biglobosa compared to the L. biglobosa only plants. There have been many suggestions that L.biglobosa can prime the plant to L. maculans which would infer a defence response. However, this may not explain the disproportionate reductions in DNA concentration.

"Guidance for applications of fungicides tends be for when there is 10-20% incidence of L.maculans leaf spots in the crop.

"If L.maculans and L.biglobosa ascospores are released at the same time, phoma leaf spot may appear smaller or later, resulting in a later application of fungicide, affecting the control of the disease."

Exploring genetic resistance to rhizoctonia

» Although there is no identified resistance to rhizoctonia, it appears that a plant's defence response to the disease can vary between varieties of commercial OSR.

Isabelle Sims, who is studying for a PhD at the University of Nottingham, examined five varieties under inoculation for seven days using a symptom scale of 0: healthy – 4: dead.

She said: "The varieties Anastasia and Savio showed around 3.5-4, so pretty much dead. Whereas varieties like Campus showed a lot of symptoms, but nowhere near as much."

When roots were measured, the variety Anastasia had the shortest roots among the varieties and showed a 95% reduction in root length under inoculation. Skye had the

❝In Anastasia, at eight hours, sclerotia had germinated and produced infection structures on the plant. This is really fast.

ISABELLE SIMS

longest roots both under inoculation and not.

Ms Sims said: "It lost 85% [of root length] under inoculation but still had good root length after seven days. Campus showed the smallest

reduction in root length post-inoculation at 79%."

Ms Sims then examined sclerotia and roots under the microscope to see how the pathogen behaved at the early stages next to different varieties.

"In Anastasia, at eight hours, sclerotia had already germinated and produced infection structures like plaques on the plant. This is really fast. Skye had a similar response with infection plaques after 48 hours but not so much at eight and 24 [hours]."

Meanwhile, Campus showed no infection plaques or sclerotia germinating within 48 hours.

"There definitely seems to be some differences in OSR varieties both in the symptoms they show, how root growth is affected and how sclerotia germinates at the roots."