Research Article

Received: 21 July 2023 Revised: 28 August 2023 Accepted article published: 28 September 2023 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.7799

Co-inoculation timing affects the interspecific interactions between phoma stem canker pathogens *Leptosphaeria maculans* and *Leptosphaeria biglobosa*

Evren Bingol,[a] Aiming Qi,[a] Chinthani Karandeni-Dewage,[a] Faye Ritchie,[b] Bruce D. L. Fitt[a] and Yong-Ju Huang[a*]

Abstract

BACKGROUND: Phoma stem canker is an economically important disease of oilseed rape, caused by two co-existing fungal pathogen species, *Leptosphaeria maculans* (*Plenodomus lingam*) and *Leptosphaeria biglobosa* (*Plenodomus biglobosus*). *Leptosphaeria maculans* produces a phytotoxin called sirodesmin PL. Our previous work showed that *L. biglobosa* has an antagonistic effect on the production of sirodesmin PL if it is simultaneously co-inoculated with *L. maculans*. However, the effects of sequential co-inoculation on interspecific interactions between the two pathogens are not understood.

RESULTS: The interactions between *L. maculans* and *L. biglobosa* were investigated in liquid culture by inoculation with *L. maculans* first, followed by *L. biglobosa* sequentially at 1, 3, 5 or 7 days later and vice versa; the controls were inoculated with *L. maculans* only, *L. biglobosa* only, or *L. maculans* and *L. biglobosa* simultaneously. The results showed that *L. biglobosa* inhibited the growth of *L. maculans*, the production of both sirodesmin PL and its precursors if *L. biglobosa* was inoculated before, or simultaneously with, *L. maculans*. However, the antagonistic effects of *L. biglobosa* were lost if it was co-inoculated 5 or 7 days after *L. maculans*.

CONCLUSION: For the first time, the results of this study provided evidence that the timing when *L. maculans* and *L. biglobosa* meet significantly influences the outcome of the interspecific competition between them. *Leptosphaeria biglobosa* can inhibit the production of sirodesmin PL and the growth of *L. maculans* if it is inoculated before *L. maculans* or less than 3 days after *L. maculans* in liquid culture. There is a need to further investigate the timing of co-inoculation on interactions between *L. maculans* and *L. biglobosa* in their host plants for improving the control of phoma stem canker.

Keywords: interspecific interactions; *Leptosphaeria maculans*; *Leptosphaeria biglobosa*; phoma stem canker; secondary metabolites; sirodesmin PL

1 INTRODUCTION

A plant host in nature is subjected to various pest and disease stresses throughout its life cycle, often caused by plant-pathogenic fungi.[1] Oilseed rape, also known as canola (*Brassica napus*) is affected by at least 16 different pathogens worldwide; they infect different parts of the plant in different stages of its life cycle.[2] Phoma stem canker (blackleg) is one of the most economically damaging diseases of oilseed rape globally.[3] In the UK alone, it causes annual yield losses worth > £80M, despite the disease control strategies using fungicides and resistant cultivars.[4] Phoma stem canker is caused by two closely related fungal pathogen species, *Leptosphaeria maculans* (*Plenodomus lingam*) and *Leptosphaeria biglobosa* (*Plenodomus biglobosus*).[5–7] In Europe, phoma stem canker epidemics are initiated by ascospores released from mature pseudothecia developed on previous crop debris in autumn.[8,9] These ascospores get dispersed by wind, land on leaf surfaces and upon successful infection, cause phoma leaf spots.[10,11] Hyphae of *L. maculans* and *L. biglobosa* then grow through the leaf, along the petiole and down to the stem, causing stem cankers at the end of the cropping season.[12,13] Fungicides (most commonly prothioconazole and tebuconazole) are applied at the phoma leaf spotting stage in efforts to stop further pathogen growth into the stem.[14,15] Furthermore, the global distribution of these pathogens varies; only *L. biglobosa* is found in

* *Correspondence to: Y-J Huang, Centre for Agriculture, Food and Environmental Management Research, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, UK. E-mail: y.huang8@herts.ac.uk*

a *Centre for Agriculture, Food and Environmental Management Research, School of Life and Medical Sciences, University of Hertfordshire, Hatfield, UK*

b *Disease and Pest Management, ADAS Boxworth, Cambridge, UK*

China, whereas both of the pathogens are found to co-infect oilseed rape in other parts of the world, such as Europe, Canada and Australia.[16,17]

When co-infection of a single host by multiple pathogens occurs, the interactions between the pathogens can be classified into three groups: competition, co-operation or co-existence.[1,18] Competition can lead to co-existence through niche specialisation, if permitted by the biology of the pathogens. In the case of *L. maculans* and *L. biglobosa*, co-existence in oilseed rape is facilitated by the small differences in the ecological niches they occupy.[6] The optimal temperatures required for pseudothecial maturation of these pathogens are different, with pseudothecia (sexual fruiting bodies) of *L. maculans* maturing faster than those of *L. biglobosa* at low temperatures (e.g., at 5–10 °C), leading to differences in the timing of ascospore release.[19] This produces a temporo-spatial separation in the infection of oilseed rape by these pathogens, and therefore allows their co-existence.[6] Previous studies showed that *L. maculans* has mainly been associated with severe stem basal cankers, whereas *L. biglobosa* has mainly been associated with superficial upper stem lesions; therefore *L. biglobosa* has been considered less damaging than *L. maculans*.[11,16] However, recent studies have found that in the UK, ascospores of *L. maculans* and *L. biglobosa* can be released at similar times, and that *L. biglobosa* can cause stem basal cankers, especially on cultivars with effective resistance genes against *L. maculans*.[20]

When interspecific competition occurs, it can manifest in three different ways.[1] Resource-mediated (exploitation) competition arises when a resource becomes limited, whereby the species that can use the resource most efficiently outcompetes the others and survives, sometimes even leading to competitive exclusion of other species.[21,22] Host-mediated (apparent) competition is when the development of one of the competitors primes the plant immune system against other competing pathogens, reducing their chance of successfully infecting the host.[23] An example of host-mediated competition strategy has been observed between *L. maculans* and *L. biglobosa*. It was reported that pre-inoculation of oilseed rape leaves with *L. biglobosa* primes the plant immune system and induces systemic acquired resistance against *L. maculans*.[24–26] However, if *L. biglobosa* was inoculated 64 h or later after *L. maculans*, the induction of systemic acquired resistance was lost,[26] suggesting that the timing when *L. maculans* and *L. biglobosa* meet and interact affects the outcome of their competition related to disease severity.

The third interspecific competition is interference competition; it occurs when one of the competitors directly interferes with another competitor's access to a limited resource, often by producing toxins.[1] This interference competition strategy has been observed between *L. maculans* and *L. biglobosa*. *Leptosphaeria maculans* produces a non-host selective epipolythiodioxopiperazine (ETP) phytotoxin called sirodesmin PL, while *L. biglobosa* does not.[27–29] Sirodesmin PL has antimicrobial and antifungal properties.[27,28] The sirodesmin PL produced by *L. maculans* was shown to inhibit the growth of *L. biglobosa in vitro*.[29,30] The *in vitro* growth of *L. biglobosa* was inhibited when it was inoculated with liquid culture containing sirodesmin PL.[29] Previous work showed that a mutant isolate of *L. maculans* that did not produce sirodesmin PL was still able to cause disease symptoms on oilseed rape, only with reduced antimicrobial and antifungal activity, suggesting that sirodesmin PL is produced as an interference competition strategy, but not required for disease development.[30] Similar studies were done for another ETP, gliotoxin, which is produced by *Aspergillus fumigatus*, the causative agent of pulmonary aspergillosis.[31] It was shown that mutant isolates of *A. fumigatus* that do not produce gliotoxin were still able to cause invasive aspergillosis, only with reduced cytotoxic effects, suggesting that gliotoxin is not indispensable for disease development.[32]

Recent work showed that *L. biglobosa* can inhibit the production of sirodesmin PL by *L. maculans* both *in vitro* and *in planta*, when it is co-inoculated simultaneously with *L. maculans*.[29] However, when *L. biglobosa* was co-inoculated sequentially 7 days after *L. maculans* in liquid culture, the production of sirodesmin PL by *L. maculans* was not inhibited.[29] Previous work showed that it took approximately 3 days for *L. maculans* to produce sirodesmin PL.[28–30] We hypothesised that the timing when *L. maculans* and *L. biglobosa* meet influences the outcome of the interspecific competition related to sirodesmin PL production and pathogen growth. The aim of this research was therefore to investigate the effects of sequential co-inoculation on interspecific interactions between *L. maculans* and *L. biglobosa* in terms of production of sirodesmin PL and relative growth of the two pathogens *in vitro*, and the impact of the interspecific interactions on phoma stem canker management.

2 MATERIALS AND METHODS

2.1 Pathogen and media preparation

The isolates used in this study were *L. maculans* isolate ME24, which was obtained from phoma stem canker on oilseed rape cultivar Apex in 2002,[12] and *L. biglobosa* isolate WH17-Why-1, obtained from phoma leaf spot of cultivar Whisky in 2017.[29] The *L. maculans* isolate ME24 carries three avirulence (*Avr*) effector genes (*AvrLm1*, *AvrLm6*, *AvrLm7*). These isolates of *L. maculans* and *L. biglobosa* were grown on 20% clarified V8 (CV8) agar plates for 7 days, followed by cutting of agar plugs from growth fronts of the colonies for inoculation of 20% CV8 liquid media.

CV8 media were prepared by the addition of 15 g L^{-1} calcium carbonate ($CaCO_3$) to V8 juice, agitation for 15 min using a magnetic stirrer, then centrifugation at 1400 × *g* for 5 min. The pellet was discarded, and the supernatant was used as the CV8 media. For CV8 agar plates, 12 g L^{-1} agar was added and 15 mL of autoclaved CV8 agar media was poured into each 9 cm diameter Petri dish. For CV8 liquid media, 75 mL of autoclaved CV8 broth was poured into each 250 mL conical flask.

2.2 Inoculation of liquid media

For sequential co-inoculations, the treatments were as follows: *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneous co-inoculation (Lm&Lb), initial inoculation with *L. maculans* followed by sequential co-inoculation with *L. biglobosa* at 1, 3, 5 and 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5, Lm + Lb-7), and initial inoculation with *L. biglobosa* followed by sequential co-inoculation with *L. maculans* at 1, 3, 5 and 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5, Lb + Lm-7). Three agar plugs (8 mm diameter) of each pathogen were used to inoculate 75 mL of 20% CV8 liquid media in each 250 mL conical flask according to the treatments. Flasks for 'Lm only' and 'Lb only' were inoculated with three agar plugs as sole cultures. Inoculated flasks were placed in a shaking incubator at 80 rpm and 18 °C in continual darkness. Mycelia were harvested and liquid culture filtrates were collected at 14 days from initial inoculation. Three independent experiments were done. There were two replicated flasks for each treatment for two

experiments and three replicated flasks per treatment for one experiment.

To compare the mycelial growth rates of *L. maculans* and *L. biglobosa* in liquid culture, the same inoculation method was used, and the treatments were *L. maculans* only (Lm only), *L. biglobosa* only (Lb only) and *L. maculans* and *L. biglobosa* simultaneous co-inoculation (Lm&Lb). Mycelia were harvested at 1, 3, 5, 7, 10 and 14 days post inoculation (dpi). Two independent experiments were done. There were two replicated flasks for each treatment/time point in each experiment.

2.3 Filtration of mycelia and secondary metabolite extractions from culture filtrates

Mycelia were harvested by filtering the liquid media using a Büchner funnel, pat-dried, placed in a sterile 15 mL Falcon tube and stored at −20 °C overnight before freeze-drying. The necks of the sterile 15 mL Falcon tubes were covered with parafilm and five small holes were pierced before the tubes were placed in a freeze-dryer for 48 h. After freeze-drying, the tubes were weighed using an analytical balance, and the weight of an empty 15 mL Falcon tube was subtracted to obtain the weight of freeze-dried mycelia.

The secondary metabolites were extracted from culture filtrates as previously described.[29] Culture filtrates were aliquoted into sterile tubes in duplicate (35 mL each) and 15 mL of ethyl acetate was added to 35 mL of culture filtrate, inverted 30 times, left in a fume hood for 10 min for the phases to settle, then centrifuged at 1000 × *g* for 5 min. For each replicate, a total of 20 mL of the upper (organic) phase was pipetted into a sterile 50 mL Falcon tube. Ethyl acetate was evaporated under a stream of nitrogen using a sample concentrator. Secondary metabolites were then re-suspended in 500 µL of ethyl acetate.

Using 1 mL syringes, re-suspended secondary metabolites were passed through 0.45 µm syringe filters into high-performance liquid chromatography (HPLC) vials. This was followed by pipetting 150 µL of sample into a new HPLC vial with a 200 µL glass insert and it was stored at 4 °C until required for HPLC analysis.

2.4 Analysis of the composition of secondary metabolites

To identify the composition of secondary metabolites in culture filtrates across different treatments, analysis of samples was done using a Shimadzu Prominence HPLC machine with a diode array detector (SPD-20A; Shimadzu Corporation, Kyoto, Japan). The HPLC column used was a C_{18} column (Varian pursuit 5, 150 mm × 4.6 mm), with 10 µL of sample injection volume and a flow rate of 1 mL min^{-1}.

HPLC-grade water and acetonitrile were used in the HPLC mobile phase. The method started as a linear gradient, going from 85% water and 15% acetonitrile to 100% acetonitrile over 40 min, maintaining there for 3 min, then starting a linear gradient back to 85% water and 15% acetonitrile over 5 min, leading to a total run time of 53 min per sample. The HPLC method was adapted from Pedras and Biesenthal.[33] HPLC analytes were measured by ultraviolet (UV) light absorption in the range of 190–400 nm, and the results were visualised at 254 nm for analysis, using the Lab solution program (version 5.92) by Shimadzu Corporation. The concentrations of sirodesmin PL and its precursors were measured using a gliotoxin standard curve; limit of detection (LOD) was 70 mg L^{-1} and limit of quantification (LOQ) was 200 mg L^{-1}, as described by Fortune *et al.*[29]

2.5 DNA extraction from mycelia

Freeze-dried mycelial samples were ground using a pestle and mortar and approximately 20 mg of each ground sample was transferred into each sterile 2 mL screw-cap tube. DNA extractions from mycelia were done using the DNAmite Plant DNA Extraction Kit (Microzone Ltd, King William St, Amblecote, Stourbridge, UK), according to the manufacturer's guidelines, with minor modifications, described later.

Three sterile metal beads were added to each tube containing a ground mycelial sample and 1 mL of lysis solution was added. The samples were processed using a FastPrep machine at 4.0 m s^{-1} for 40 s. This was followed by the addition of 100 µL of protein precipitant solution into each tube, vortexing for 10 s, and centrifugation at 8000 × *g* for 5 min at 4 °C. Following centrifugation, 500 µL of the supernatant from each tube was transferred into a new sterile 1.5 mL tube containing 500 µL of capture solution. These tubes were inverted ten times and incubated on the bench for 5 min before centrifugation at 11 300 × *g* for 7 min at 4 °C to pellet the DNA. The supernatant was discarded, and any remnants were removed using a pipette. DNA pellets were re-suspended in 60 µL of nuclease-free water after drying in the fume hood for 30 min, then placed at −20°C for 16 h. Extracted DNA samples were then thawed at 20 °C and centrifuged at 7000 × *g* for 10 min at 4 °C, and 50 µL of the supernatants containing the DNA were transferred into a new set of sterile 0.5 mL tubes. DNA concentration of each sample was measured using Qubit DNA Broad Range Assay Kit (Invitrogen, Carlsbad, CA, USA) and diluted to 20 ng μL^{-1} ready for quantitative polymerase chain reaction (qPCR) analyses.

2.6 Relative growth of *L. maculans* and *L. biglobosa* in different treatments

To investigate the relative growth of *L. maculans* and *L. biglobosa* in different treatments, the amounts of *L. maculans* and *L. biglobosa* DNA were quantified using SYBR Green (Agilent, Santa Clara, CA, USA) qPCR, with the primers LmacF (5′- CTTGCCCACCAATTGGATCCCCTA-3′)/LmacR (5′-GCAAAATGTGCTGCGCTCCAGG-3′) (for *L. maculans*) and LbigF (5′-CCTTCTATCAGAGGATTGGT-3′)/LmacR (5′-CGTTCTTCATCGATGCCAGA-3′) (for *L. biglobosa*).[13] The qPCR reaction mixtures were prepared at a total volume of 20 µL, containing 10 µL of SYBR Green (containing ROX as reference dye), 6.3 µL of nuclease-free water, 0.6 µL of forward primer (10 µM), 0.6 µL of reverse primer (10 µM) and 2.5 µL of DNA sample in duplicate in 96-well plates. To produce standard curves, ten-fold dilutions ranging from 10^4 to 10^{-1} pg of *L. maculans* or *L. biglobosa* DNA obtained from pure cultures were used. The amounts of *L. maculans* or *L. biglobosa* DNA in samples were calculated using the standard curves with the Stratagene MxPro-Mx3000P v3.20 software.

The qPCR reactions were done using a Stratagene 3005P qPCR machine (Agilent). Thermal cycling profiles consisted of one cycle of 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, 60 °C (for *L. maculans*) or 55 °C (for *L. biglobosa*) for 30 s, 72 °C for 45 s, and a reading step of 83 °C for 15 s, then one cycle of 95 °C for 1 min, (60 °C for *L. maculans*/55 °C for *L. biglobosa*) for 1 min, and 95 °C for 15 s to produce a dissociation curve.

2.7 Statistical analysis

To compare the growth rates of *L. maculans* and *L. biglobosa*, a logistic equation was fitted to measurements of mycelial dry weight from each individual replicate against days post inoculation. The data were analysed using the non-linear model directive

in the GenStat program (22nd edition).[34] The fitted logistic equation was:

$$Y = \frac{A}{1 + e^{(-k \times (t - t_0)}}$$

In this logistic equation, Y is the measurement of mycelial dry weight (in grams) at different time points, t is the time in dpi, A is the upper asymptotic mycelial dry weight, k is the slope of the logistic curve at inflection measuring the relative growth rate in mycelial dry weight (i.e., the growth-rate coefficient), and t_0 is the time at which the mycelial dry weight reaches half of the value of A.

To compare the differences between different treatments in mycelial dry weight, production of sirodesmin PL and its precursors, and relative pathogen DNA in homogenised mycelia, analysis of variance was done using the GenStat program (22nd edition).[34] Since three independent experiments were done for the same 11 treatments, experiment was assigned as a factor when analysing the data to assess whether the experiment had a significant effect. For measurements of sirodesmin PL and its precursors, the data were transformed using a common logarithm to homogenise the variance between treatments before they were subjected to analysis of variance. The F-values that were significant at $P < 0.05$ indicated significant effects of the factors. The Tukey *post hoc* test was used to separate the means of the treatments at 5% significant probability.

3 RESULTS

3.1 Morphology of mycelia in liquid cultures and mycelial dry masses of *L. maculans* and *L. biglobosa* in different treatments

The morphology of mycelia in liquid cultures was different between different treatments (Fig. 1). The mycelia of the 'Lm only' treatment were light brown, round and had an intact structure whereas the mycelia of the 'Lb only' treatment were red and spread across the liquid media, without an intact structure. The morphology of mycelia in treatments with initial inoculation of *L. maculans*, followed by sequential co-inoculation by *L. biglobosa* at 1, 3, 5 or 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5 and Lm + Lb-7) were more similar to that of the 'Lm only' treatment. The morphology of mycelia in treatments with initial inoculation of *L. biglobosa* followed by sequential co-inoculation by *L. maculans* at 1, 3, 5 or 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5 and Lb + Lm-7) and *L. maculans* and *L. biglobosa* simultaneous co-inoculation (Lm&Lb) were similar to that of the 'Lb only' treatment.

At 14 dpi, mycelia were harvested and freeze-dried. There were no significant differences between treatments in mycelial dry weight, except for the treatment 'Lm only' (0.33 g) being significantly greater than all other treatments ($F_{(10,44)} = 4.89$, $P < 0.001$) (Fig. 2). The mycelial dry weights of other treatments ranged from 0.21 to 0.27 g. The interactions between experiments and treatments were not significant ($F_{(20,44)} = 1.60$, $P = 0.095$).

3.2 Effects of sequential co-inoculation of *L. maculans* and *L. biglobosa* on production of secondary metabolites by *L. maculans in vitro*

The colour of culture filtrates from liquid cultures was different between different treatments (Fig. 3). Culture filtrate of the 'Lm only' treatment had a light yellow colour, whereas culture filtrate of the 'Lb only' treatment had a dark yellow/orange colour. This difference was due to production of a pigment by *L. biglobosa*, which was not produced by *L. maculans*.[35] The colours of culture filtrates from liquid cultures where *L. maculans* was inoculated before *L. biglobosa* (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5 and Lm + Lb-7) were similar to that of the 'Lm only' control treatment. However, a slight production of pigment was observed in the Lm + Lb-1 treatment. The colours of culture filtrates from liquid cultures where *L. biglobosa* was inoculated before *L. maculans* (Lb

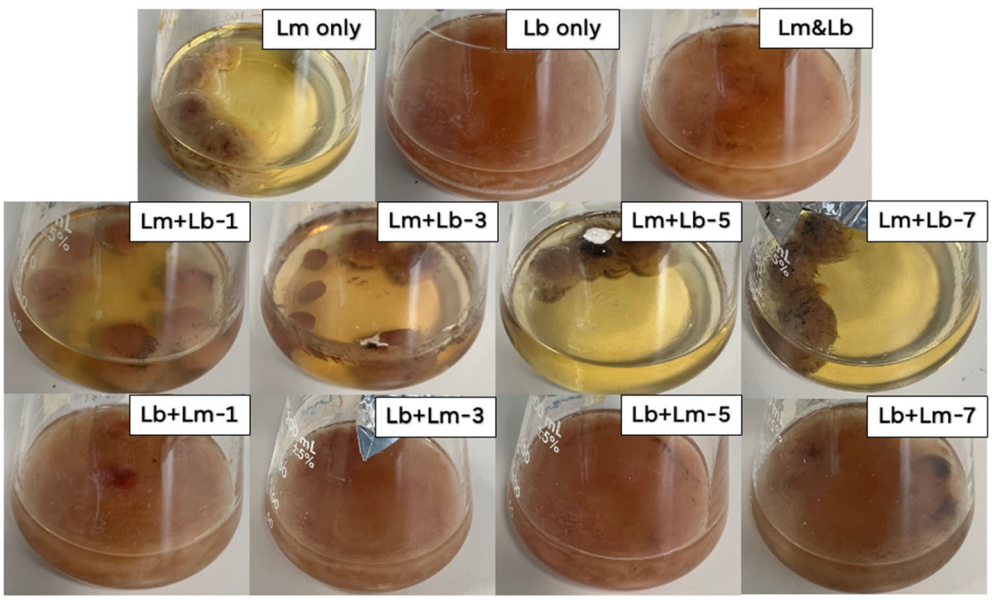

Figure 1. Morphology of mycelia in liquid cultures inoculated with *Leptosphaeria maculans* only (Lm only), *Leptosphaeria biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* co-inoculated simultaneously (Lm&Lb), first inoculation with *L. maculans* followed by co-inoculation with *L. biglobosa* sequentially at 1, 3, 5 or 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5, Lm + Lb-7), and first inoculation with *L. biglobosa* followed by co-inoculation with *L. maculans* sequentially at 1, 3, 5 or 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5, Lb + Lm-7) at 14 days after the first inoculation.

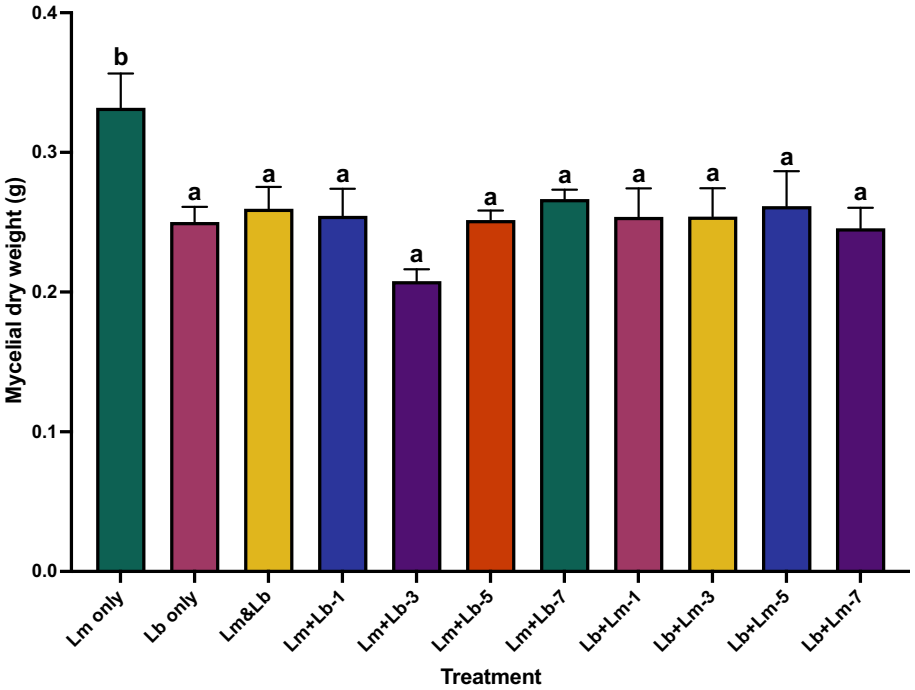

Figure 2. Average dry weight (g) of mycelia obtained from three independent experiments consisting of liquid cultures inoculated with *Leptosphaeria maculans* only (Lm only), *Leptosphaeria biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* co-inoculated simultaneously (Lm&Lb), first inoculation with *L. maculans* followed by co-inoculation with *L. biglobosa* sequentially at 1, 3, 5 or 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5, Lm + Lb-7), and first inoculation with *L. biglobosa* followed by co-inoculation with *L. maculans* sequentially at 1, 3, 5 or 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5, Lb + Lm-7). Tukey's HSD tests were used to separate the mean dry mycelial weight values across different treatments. Columns that do not share a common letter were considered significantly different ($P < 0.05$). Error bars show standard errors of the mean (SEM).

+ Lm-1, Lb + Lm-3, Lb + Lm-5 and Lb + Lm-7) or simultaneous co-inoculation of both pathogens (Lm&Lb) were similar to that of the 'Lb only' control (Fig. 3).

The composition of secondary metabolites of the culture filtrates was analysed by HPLC. There were significant differences between different treatments in the average concentrations of sirodesmin PL ($F_{(10,44)} = 1129.98$, $P < 0.001$) (Fig. 4(a)) and its precursors ($F_{(10,44)} = 1294.62$, $P < 0.001$) (Fig. 4(b)). For the treatments 'Lb only', 'Lm&Lb', 'Lm + Lb-1', 'Lb + Lm-1', 'Lb + Lm-3', 'Lb + Lm-5' and 'Lb + Lm-7', there were no significant differences between them since no unique maxima corresponding to sirodesmin PL or its precursors were identified in the HPLC chromatograms in those treatments. For all other treatments, unique maxima corresponding to sirodesmin PL were identified at 16.3 min retention time. For the treatments 'Lm only' (892.8 mg L^{-1}), 'Lm + Lb-5' (832.2 mg L^{-1}) and 'Lm + Lb-7' (971.4 mg L^{-1}), there were no significant differences between them in the concentration of sirodesmin PL. However, 'Lm + Lb-3' (334.5 mg L^{-1}) was significantly different from all other treatments (Fig. 4(a)). Although the retention time was similar for the unique maxima corresponding to sirodesmin PL, the 'Lm + Lb-3' treatment had a 62.6% reduction in concentration when compared to the 'Lm only' control treatment. Furthermore, unique maxima corresponding to the precursors of sirodesmin PL were identified at 11.2 min retention time. For the treatments 'Lm only' (381.4 mg L^{-1}), 'Lm + Lb-5' (358.2 mg L^{-1}) and 'Lm + Lb-7' (368.7 mg L^{-1}), there were no significant differences between them in the concentrations of the precursors of sirodesmin PL. Corresponding to the toxin itself, 'Lm + Lb-3' (185.4 mg L^{-1}) was significantly different from all other treatments (Fig. 4(b)). Likewise, even though the retention time was similar for all the unique maxima corresponding to the precursors of sirodesmin PL, the 'Lm + Lb-3' treatment had a 51.4% reduction in concentration when compared to the 'Lm only' control. The interactions between experiments and treatments were not significant, neither for sirodesmin PL ($F_{(20,44)} = 0.46$, $P < 0.969$) nor for its precursors ($F_{(20,44)} = 0.88$, $P = 0.607$).

3.3 Comparison of relative pathogen growth in liquid culture co-inoculated with *L. maculans* and *L. biglobosa*

At 14 days post initial inoculation, mycelia were harvested and freeze dried for DNA extraction and qPCR. The relative growth of *L. maculans* and *L. biglobosa* in liquid cultures with different co-inoculation treatments was assessed by measuring the amounts of *L. maculans* or *L. biglobosa* DNA using qPCR. The amounts of *L. maculans* or *L. biglobosa* DNA in 50 ng DNA extracted from cultures containing both *L. maculans* and *L. biglobosa* were expressed as percentage. There were significant differences between different treatments in percentage of *L. maculans* DNA ($F_{(10,44)} = 545.86$, $P < 0.001$; Fig. 5). For the treatments 'Lb only', 'Lm&Lb', 'Lm + Lb-1', 'Lb + Lm-1', 'Lb + Lm-3', 'Lb + Lm-5' and 'Lb + Lm-7', there were no significant differences between them in percentages of *L. maculans* DNA, with all containing 0 to 7% of *L. maculans* DNA. For the treatments with *L. biglobosa* inoculated first, followed by *L. maculans* at 1, 3, 5 or 7 days later ('Lb + Lm-1', 'Lb + Lm-3', 'Lb + Lm-5' and 'Lb + Lm-7'), the percentage of *L. maculans* DNA was consistently less than that of 'Lm&Lb' (3.10%), with 1.11%, 1.41%, 1.29% and 1.40%, respectively. Moreover, the treatments 'Lm only' (100%), 'Lm + Lb-5' (97.4%) and 'Lm + Lb-7' (97.4%) were not significantly different

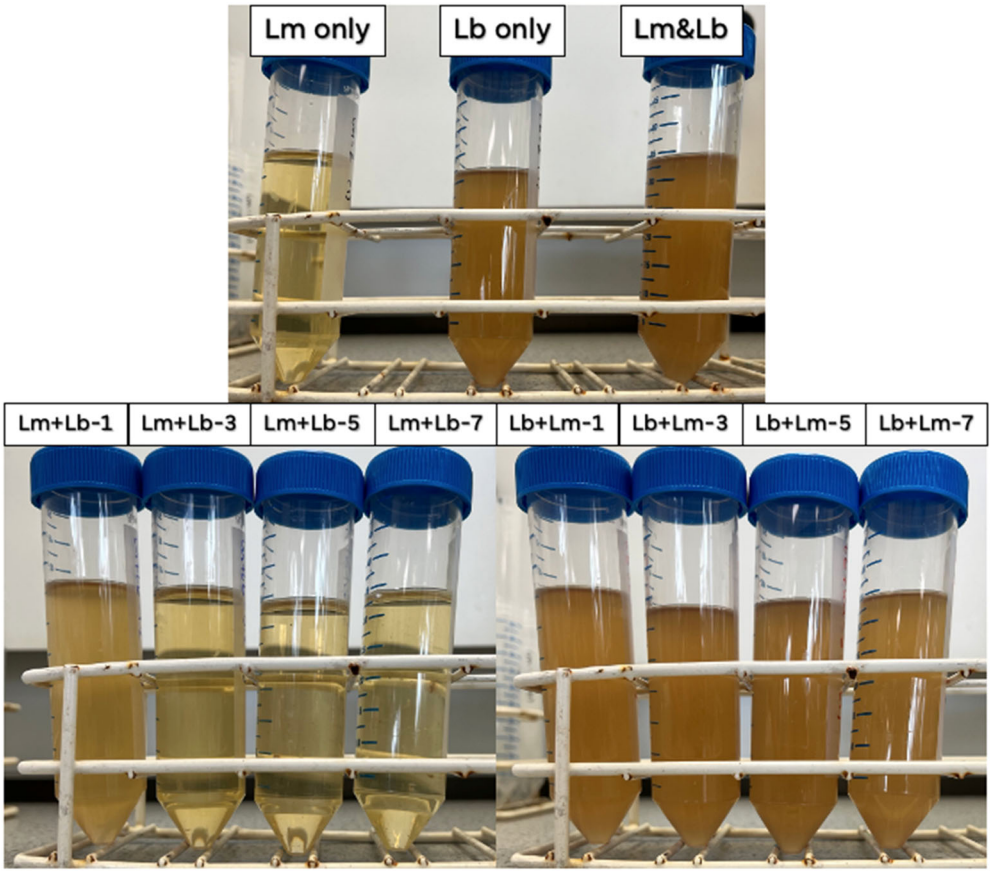

Figure 3. Colour of culture filtrates from liquid cultures inoculated with *Leptosphaeria maculans* only (Lm only), *Leptosphaeria biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* co-inoculated simultaneously (Lm&Lb), first inoculation with *L. maculans* followed by co-inoculation with *L. biglobosa* sequentially at 1, 3, 5 or 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5, Lm + Lb-7), and first inoculation with *L. biglobosa* followed by co-inoculation with *L. maculans* sequentially at 1, 3, 5 or 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5, Lb + Lm-7), at 14 days after the first inoculation.

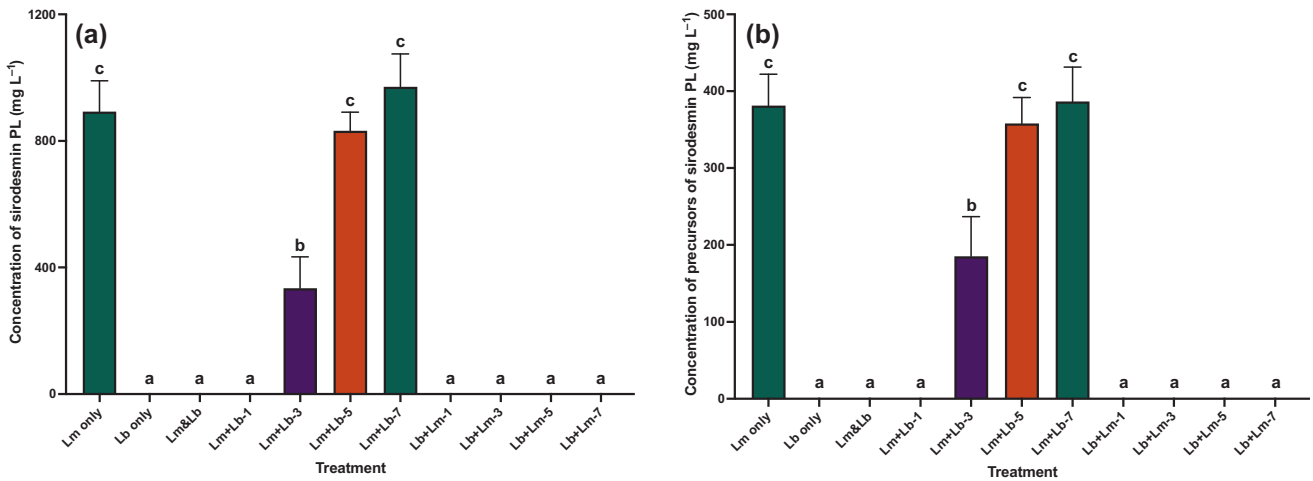

Figure 4. Average concentration of (a) sirodesmin PL (mg L^{-1}) and (b) the precursors of sirodesmin PL (mg L^{-1}) in secondary metabolite extracts obtained from three independent experiments with treatments consisting of liquid cultures inoculated with *Leptosphaeria maculans* only (Lm only), *Leptosphaeria biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* co-inoculated simultaneously (Lm&Lb), first inoculation with *L. maculans* followed by co-inoculation with *L. biglobosa* sequentially at 1, 3, 5 or 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5, Lm + Lb-7), and first inoculation with *L. biglobosa* followed by co-inoculation with *L. maculans* sequentially at 1, 3, 5 or 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5, Lb + Lm-7). Tukey's HSD tests were used to separate the mean concentration of the precursors of sirodesmin PL across different treatments. Columns that do not share a common letter were considered significantly different ($P < 0.05$). Error bars show standard errors of the mean (SEM).

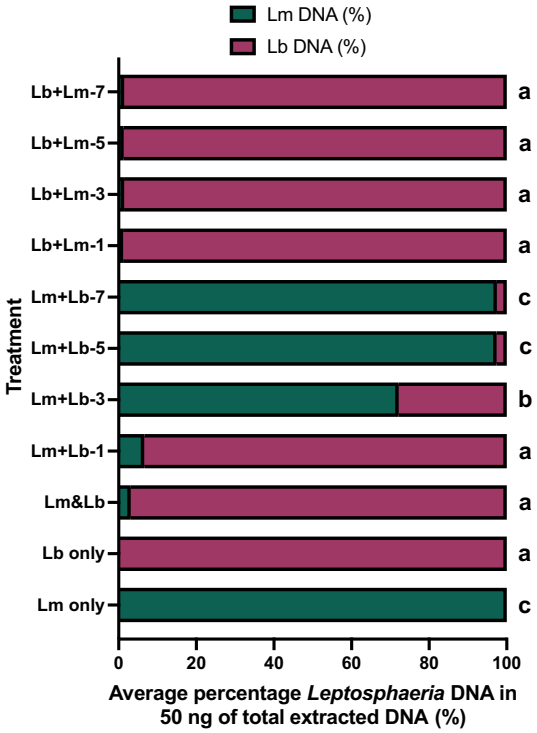

Figure 5. Average percentage of *Leptosphaeria* DNA (%) in homogenised mycelia obtained from three independent experiments with treatments consisting of liquid cultures inoculated with *Leptosphaeria maculans* only (Lm only), *Leptosphaeria biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* co-inoculated simultaneously (Lm&Lb), first inoculation with *L. maculans* followed by co-inoculation with *L. biglobosa* sequentially at 1, 3, 5 or 7 days later (Lm + Lb-1, Lm + Lb-3, Lm + Lb-5, Lm + Lb-7), and first inoculation with *L. biglobosa* followed by co-inoculation with *L. maculans* sequentially at 1, 3, 5 or 7 days later (Lb + Lm-1, Lb + Lm-3, Lb + Lm-5, Lb + Lm-7). Tukey's HSD tests were used to separate the mean percentage of *L. maculans* DNA in total extracted DNA across different treatments. Columns that do not share a common letter were considered significantly different ($P < 0.05$).

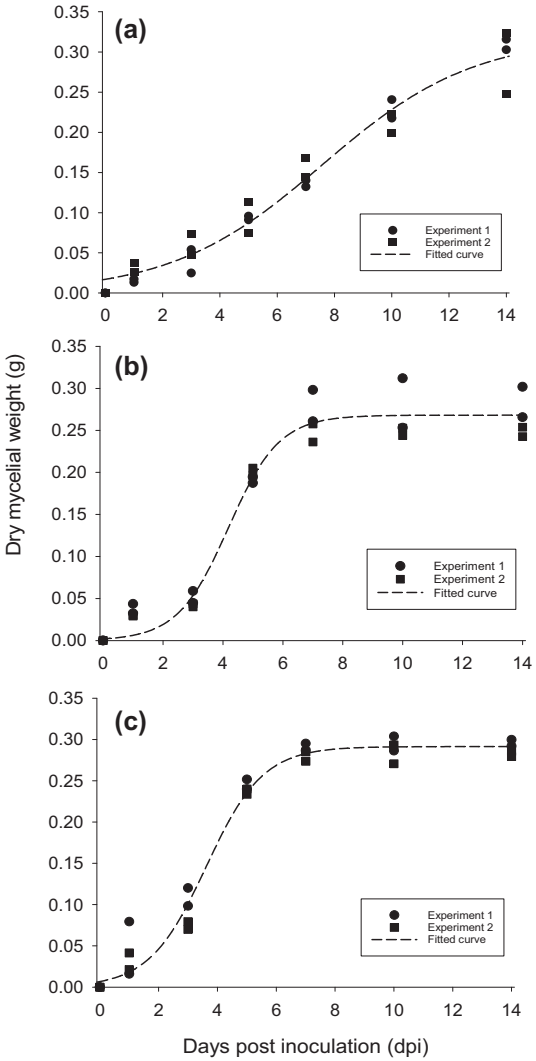

Figure 6. Fitted logistic curves for dry mycelial weight (g) of (a) *Leptosphaeria maculans* only (Lm only), (b) *Leptosphaeria biglobosa* only (Lb only) and C) *L. maculans* and *L. biglobosa* co-inoculated simultaneously (Lm&Lb) treatments from two independent experiments at 1, 3, 5, 7, 10 and 14 days post inoculation (dpi) in liquid culture. Dry mycelial weight in 'Lm only' treatment increased continuously from day 0 to 14 with the growth rate coefficient *k* value of 0.38 and the coefficient of determination (R^2) of 96.6%. Dry mycelial weight in 'Lb only' and 'Lm&Lb' treatments increased from day 0 to 7 to reach an asymptote and maintained the plateau thereafter until the experiment ended in 14 days, with the growth rate coefficient *k* values of 1.18 and 1.03, the coefficients of determination (R^2) of 96.8% and 97.9%, respectively.

from each other. However, 'Lm + Lb-3' (72.2%) was significantly different to all other treatments (Fig. 5). The reduction in *L. maculans* DNA between the 'Lm only' control treatment and 'Lm + Lb-3' was 27.8%; the concentrations of sirodesmin PL and its precursors for 'Lm + Lb-3' were also less than that of the 'Lm only' treatment but the percentage decreases were greater, at 62.6% and 51.4%, respectively. The interactions between experiments and treatments were not significant ($F_{(20,44)} = 0.94$, $P = 0.546$).

3.4 Comparison of mycelial growth rates of *L. maculans* and *L. biglobosa*

Results for the mycelial dry weight showed that there were differences between *L. maculans* and *L. biglobosa* in growth pattern and growth rate (Fig. 6). The mycelial growth of the 'Lm only' treatment increased steadily from day 0 to 14, reaching an average mycelial dry weight of 0.30 g by 14 dpi. The growth rate coefficient '*k*' value, estimated from the fitted logistic equation, was 0.38 (Fig. 6(a)). For the 'Lb only' treatment, mycelial growth rate coefficient was more than three times faster ($k = 1.18$) than for the 'Lm only' treatment. The mycelial growth for the 'Lb only' treatment reached a plateau (i.e., asymptote) at 0.26 g by 7 dpi and did not increase any further from 7 to 14 dpi (Fig. 6(b)). Interestingly, mycelial growth in the 'Lm&Lb' treatment showed a similar growth rate coefficient ($k = 1.03$) to that of the 'Lb only' treatment. Average mycelial dry weight for 'Lm&Lb' reached an asymptote at 0.29 g by 7 dpi and did not increase any further from 7 to 14 dpi (Fig. 6(c)).

4 DISCUSSION

The results of this study provide the first evidence that the timing of co-inoculation (i.e., the timing when *L. maculans* and *L. biglobosa* meet) strongly affects the interspecific interactions between *L. maculans* and *L. biglobosa* in terms of sirodesmin PL production and relative pathogen growth. Under sequential co-inoculation, for treatments where *L. biglobosa* was inoculated first, followed with *L. maculans* at 1, 3, 5 or 7 days later, the production

of sirodesmin PL and its precursors was inhibited in all the treatments (Fig. 4). This research also confirmed previous work that simultaneous co-inoculation of *L. maculans* and *L. biglobosa* (Lm&Lb) inhibited the production of sirodesmin PL and its precursors.[29] However, when *L. maculans* was inoculated first, followed by *L. biglobosa* at 3, 5 or 7 days later, the production of sirodesmin PL and its precursors was not inhibited. These findings indicate that if there was any production of sirodesmin PL, *L. maculans* must be the first pathogen to be inoculated at least 3 days before *L. biglobosa*. This is supported by results from previous studies that it took up to 3 days for *L. maculans* to produce sirodesmin PL.[28,29] This study showed that *L. biglobosa* was still able to inhibit the production of sirodesmin PL and its precursors and retain its competitive advantage even when it was co-inoculated sequentially 1 day after *L. maculans* (Lm + Lb-1). This was because *L. biglobosa* had an antagonistic effect on the growth of *L. maculans*, confirmed by the proportion of the amount of *L. maculans* DNA by species-specific qPCR, with only 6.6% of *L. maculans* DNA detected in 'Lm + Lb-1' (Fig. 5). The antagonistic effect of *L. biglobosa* on the growth of *L. maculans* was also confirmed by both morphology of mycelia and pigment production (Figs 1 and 3). It was previously reported that *L. maculans* mycelia grew more intactly compared to *L. biglobosa* in liquid culture[3] and *L. biglobosa* produced pigmentation whereas *L. maculans* did not.[35] Both morphology of mycelia and pigmentation in culture filtrates suggest that the growth of *L. maculans* was inhibited in liquid cultures where *L. biglobosa* was inoculated before *L. maculans* or the two pathogens were co-inoculated simultaneously (Figs 1 and 3). This is further supported by the results of species-specific qPCR analyses, which showed that those treatments consistently had less than 3.0% *L. maculans* DNA, confirming that the growth of *L. maculans* was inhibited by *L. biglobosa* (Fig. 5). Although the 'Lm + Lb-1' treatment had more *L. maculans* DNA compared to the 'Lm&Lb' treatment, this difference was not significant.

Results showed that *L. biglobosa* started to lose its inhibitory effects when it was co-inoculated sequentially 3 days after *L. maculans*; this was mainly due to some sirodesmin PL and its precursors being produced, even though it was significantly less compared to that of the 'Lm only' control treatment. These results suggest that *L. biglobosa* has a partial antagonistic effect on *L. maculans* at the early stages of sirodesmin PL production since it takes approximately 3 days to produce sirodesmin PL.[28,29] However, *L. biglobosa* completely lost its ability to exert antagonistic effects on *L. maculans* growth and sirodesmin PL production if it was co-inoculated sequentially 5 or 7 days after *L. maculans*. This confirms previous work that *L. biglobosa* could not inhibit sirodesmin PL production by *L. maculans* if it is co-inoculated 7 days after *L. maculans*.[29] This was mainly due to sirodesmin PL already produced by *L. maculans* by 5–7 days, and the competitive advantage 'shifted' in favour of *L. maculans*. Therefore, *L. maculans* produces sirodesmin PL as an interference competition strategy against *L. biglobosa*. In order to out-compete *L. maculans*, *L. biglobosa* needs to act before the production of sirodesmin PL.

Although different hypotheses can be formulated about how these interactions occur, the exact mechanisms of the antagonistic effects of *L. biglobosa* on *L. maculans* remain unknown. Recent investigations have reported differences between *L. maculans* and *L. biglobosa* in terms of metabolic capacities. *Leptosphaeria biglobosa* is more efficient compared to *L. maculans* in terms of nutrient acquisition and utilisation of natural resources,[29,36] which is congruent with past reports indicating tissue colonisation by *L. biglobosa* occurs more rapidly compared to *L. maculans*.[10,37] The findings of this study further support this hypothesis since the mycelial growth rate coefficient of *L. biglobosa* was more than three times greater than that of *L. maculans* (Fig. 6). Furthermore, mycelial weight of the 'Lb only' treatment reached its maximum by 7 dpi and did not increase any further, suggesting that *L. biglobosa* has already utilised all the nutrients in the limited liquid media by 7 dpi. However, the mycelial growth of the 'Lm only' treatment increased steadily and reached its maximum by 14 dpi (Fig. 6). This can explain the reason why the 'Lm only' treatment was significantly greater in mycelial dry weight than all other treatments in the sequential co-inoculation experiments (Fig. 2). Moreover, mycelial growth rate and pattern of 'Lm&Lb' treatment were both similar to those of the 'Lb only' treatment, suggesting that the inhibition of growth of *L. maculans* is because *L. biglobosa* has utilised the nutrients in the condition with limited food source (e.g., limited volume of liquid culture). Previous studies on comparison of *L. maculans* and *L. biglobosa* growth were done by measuring radial growth of mycelia in agar plates.[25,29] However, this work was done in liquid cultures in shaking incubators so that nutrients in the media were equally distributed. Therefore, the differences in metabolic capacity between *L. maculans* and *L. biglobosa* may have been due to differences in their nutritional strategies. Although both *Leptosphaeria* species were characterised to have hemi-biotrophic lifestyles, differences between these two pathogens in terms of nutritional strategies were reported, with some even referring to *L. biglobosa* as a 'necrotroph'.[38,39] Additionally, one of the recent investigations of differences in metabolic capacity between *Leptosphaeria* species reported *L. biglobosa* to be less specialised, with *L. maculans* co-evolving more strictly with the plant host.[36] Therefore, one of the hypotheses to explain the mechanisms of these interactions is that *L. biglobosa* utilised the resources more efficiently and potentially caused competitive exclusion of *L. maculans* by employing a resource-mediated (exploitative) competition strategy.[1] In return, *L. maculans* inhibited the growth of *L. biglobosa* dependent upon successful production of sirodesmin PL by employing an interference competition strategy.[1,27–30] Results of this study supported this hypothesis and highlighted the significant influence of co-inoculation timing on the interspecific interactions between *L. maculans* and *L. biglobosa*.

An additional hypothesis to explain the mechanisms of these interactions between *L. maculans* and *L. biglobosa* is that *L. biglobosa* interferes with the gene expression related to production of sirodesmin PL by *L. maculans*. It was reported that 20 co-regulated genes are involved in the sirodesmin PL biosynthetic gene cluster (BGC) in *L. maculans*.[28,40] Our results showed that *L. biglobosa* inhibits the precursors of sirodesmin PL; this suggests that it would interfere with the expression of genes that are active in early stages of sirodesmin PL biosynthesis. It is known that sirodesmin PL biosynthesis starts with two amino-acids, tyrosine and serine.[28,41] The first step is the O-prenylation of L-tyrosine residue by dimethylallyl pyrophosphate, a reaction catalysed by a 4-*O*-dimethylallyl-L-tyrosine synthase, encoded by *sirD*.[42] The second step is the condensation of dimethylallyl-L-tyrosine and serine, catalysed by a two-module non-ribosomal peptide synthetase known to be indispensable for sirodesmin PL biosynthesis, encoded by *sirP*.[28,30,40] Furthermore, this biosynthetic cluster is regulated by a cross-control pathway gene, encoding for a Zn(II)2Cys6 transcription factor through *sirZ*.[28,30,43,44] There is a need to investigate if expression of one or more of these genes is disrupted, whether sirodesmin PL can be produced or not.

Moreover, competition under limited food source conditions (e.g., liquid culture), the rates of nutrient uptake by *L. maculans* and *L. biglobosa* may also affect the production of sirodesmin PL. Only one isolate of each pathogen was used in this study. There are seven subclades in *L. biglobosa*[17,45]; there is a need to investigate whether other subclades have similar antagonistic effects against *L. maculans*.

The results of this study have potential practical importance for phoma stem canker management. Firstly, this study confirmed the antagonistic effects of *L. biglobosa* on *L. maculans* if it is inoculated before *L. maculans* suggesting that it is possible to identify a weakly pathogenic isolate of *L. biglobosa* and use it as a biocontrol agent against *L. maculans*, which will potentially provide a sustainable and environmentally friendly way to control phoma stem canker by reducing fungicide sprays. Previous work showed that *L. biglobosa* inhibited *L. maculans* growth through inhibiting the production of sirodesmin PL both *in vitro* (liquid culture) and *in planta* (host) when they were co-inoculated simultaneously,[29] which suggests that the antagonistic effects of *L. biglobosa* on *L. maculans* observed in the liquid cultures in sequential co-inoculation, are likely to occur in their host plants. To test this hypothesis, there is a need to do experiments with sequential co-inoculation on host plants. Secondly, results of this study suggest that the difference in timing of *L. maculans* and *L. biglobosa* ascospore release in natural conditions may lead to differences in incidence of simultaneous and/or sequential co-inoculations, which may affect the severity of phoma leaf spotting and subsequently the severity of phoma stem canker. Because phoma stem canker epidemics are initiated by ascospores,[8,19,20,46] if ascospores of *L. biglobosa* are released earlier with a larger amount than ascospores of *L. maculans*, this will be analogous to the sequential co-inoculation with *L. biglobosa* before *L. maculans*, leading to *L. biglobosa* inhibiting the growth of *L. maculans*, subsequently leading to less severe phoma stem canker. This hypothesis is indirectly supported by previous results on timing/abundance of *L. maculans* and *L. biglobosa* ascospore release and the severity of phoma stem canker in field experiments.[20,47] For example, for the field experiments with nine cultivars, the mean severity of stem canker in the summer of 2012 (i.e., 2.28) was less than that in the summer of 2011 (i.e., 4.03). This may have been due to ascospores of *L. biglobosa* being released earlier with a larger amount than ascospores of *L. maculans* in the autumn 2011[20] so that *L. biglobosa* could have inhibited the growth of *L. maculans* leading to less severe canker in the summer of 2012. However, disease severity in field conditions can be affected by many other factors, such as rainfall, temperature, cultivar resistance and composition of pathogen races. Furthermore, the timing of ascospore release is also affected by weather conditions. Although weather-based models have been developed to predict the timing of ascospore release,[8,19,46] those models do not distinguish *L. maculans* from *L. biglobosa* ascospore release. There is a need to develop models specifically for the prediction of *L. maculans* or *L. biglobosa* ascospore release and use data from field experiments with on-site ascospore release monitoring to validate the models for guiding fungicide applications.

ACKNOWLEDGEMENTS

This research work was supported by the University of Hertfordshire, Chadacre Agricultural Trust, Felix Thornley Cobbold Agricultural Trust, the Perry Foundation, the UK Biotechnology and Biological Sciences Research Council (BBSRC, P00489X/1), the Innovate UK (102641), AHDB Cereals & Oilseeds (RD-2140021105), and the Department for Environment, Food and Rural Affairs (Defra, CH0110). The authors thank James Stanley and Christine Gigou for technical support and assistance.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1 Dutt A, Andrivon D and Le May C, Multi-infections, competitive interactions, and pathogen coexistence. *Plant Pathol* **71**:5–22 (2021). https://doi.org/10.1111/ppa.13469.
2 Zheng X, Koopmann B, Ulber B and von Tiedemann A, A global survey on diseases and pests in oilseed rape—current challenges and innovative strategies of control. *Front Agron* **2**:1-15 (2020). https://doi.org/10.3389/fagro.2020.590908.
3 Zhang X, White RP, Demir E, Jedryczka M, Lange RM, Islam M *et al*., *Leptosphaeria* spp., phoma stem canker and potential spread of *L. maculans* on oilseed rape crops in China. *Plant Pathol* **63**:598–612 (2014). https://doi.org/10.1111/ppa.12146.
4 CropMonitor. Secure.fera.defra.gov.uk. (2022). Retrieved 24 February 2022, from https://secure.fera.defra.gov.uk/cropmonitor/.
5 Shoemaker R and Brun H, The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Can J Bot* **79**:412–419 (2001). https://doi.org/10.1139/b01-019.
6 Fitt BDL, Huang Y, Bosch F and West J, Coexistence of related pathogen species on arable crops in space and time. *Annu Rev Phytopathol* **44**:163–182 (2006). https://doi.org/10.1146/annurev.phyto.44.070505.143417.
7 de Gruyter J, Woudenberg JHC, Aveskamp MM, Verkley GJM, Groenewald JZ and Crous PW, Redisposition of phoma-like anamorphs in *Pleosporales*. *Stud Mycol* **75**:1–36 (2013). https://doi.org/10.3114/sim0004.
8 Huang Y, Fitt BDL, Jedryczka M, Dakowska S, West J, Gladders P *et al*., Patterns of ascospore release in relation to phoma stem canker epidemiology in England (*Leptosphaeria maculans*) and Poland (*Leptosphaeria biglobosa*). *Eur J Plant Pathol* **111**:263–277 (2005). https://doi.org/10.1007/s10658-004-4421-0.
9 Bousset L, Jumel S, Garreta V, Picault H and Soubeyrand S, Transmission of *Leptosphaeria maculans* from a cropping season to the following one. *Ann Appl Biol* **166**:530–543 (2015). https://doi.org/10.1111/aab.12205.
10 Huang Y, Toscano-Underwood C, Fitt BDL, Hu X and Hall A, 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 Pathol* **52**:245–255 (2003). https://doi.org/10.1046/j.1365-3059.2003.00813.x.
11 West J, Balesdent M, Rouxel T, Narcy J, Huang Y, Roux J *et al*., Colonization of winter oilseed rape tissues by A/Tox+ and B/Tox0 *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathol* **51**:311–321 (2002). https://doi.org/10.1046/j.1365-3059.2002.00689.x.
12 Huang Y, Li Z, Evans N, Rouxel T, Fitt BDL and Balesdent M, Fitness cost associated with loss of the AvrLm4 avirulence function in *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *Eur J Plant Pathol* **114**:77–89 (2006). https://doi.org/10.1007/s10658-005-2643-4.
13 Huang YJ, Pirie EJ, Evans N, Delourme R, King GJ and Fitt BDL, Quantitative resistance to symptomless growth of *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape). *Plant Pathol* **58**:314–323 (2009).
14 Sewell TR, Hawkins NJ, Stotz HU, Huang YJ, Kelly SL, Kelly DE *et al*., Azole sensitivity in *Leptosphaeria* pathogens of oilseed rape: the role of lanosterol 14α-demethylase. *Sci Rep* **7**:15849 (2017). https://doi.org/10.1038/s41598-017-15545-9.

15 Fortune JA, Qi A, Ritchie F, Karandeni Dewage CS, Fitt BDL and Huang YJ, Effects of cultivar resistance and fungicide application on stem canker of oilseed rape (*Brassica napus*) and potential interseasonal transmission of *Leptosphaeria* spp. inoculum. *Plant Pathol* **70**:2115–2124 (2021). https://doi.org/10.1111/ppa.13453.

16 Fitt BDL, Brun H, Barbetti M and Rimmer S, World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *Eur J Plant Pathol* **114**:3–15 (2006). https://doi.org/10.1007/s10658-005-2233-5.

17 Dilmaghani A, Balesdent MH, Didier JP, Wu C, Davey J, Barbetti MJ *et al.*, The *Leptosphaeria maculans-Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathol* **58**:1044–1058 (2009). https://doi.org/10.1111/j.1365-3059.2009.02149.x.

18 Abdullah A, Moffat C, Lopez-Ruiz F, Gibberd M, Hamblin J and Zerihun A, Host–multi-pathogen warfare: pathogen interactions in co-infected plants. *Front Plant Sci* **8**:1806 (2017). https://doi.org/10.3389/fpls.2017.01806.

19 Toscano-Underwood C, Huang YJ, Fitt BDL and Hall AM, Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. *Plant Pathol* **52**:727–736 (2003). https://doi.org/10.1111/j.1365-3059.2003.00930.x.

20 Huang YJ, Sidique SN, Karandeni Dewage CS, Gajula LH, Mitrousia GK, Qi A *et al.*, Effective control of *Leptosphaeria maculans* increases importance of L. biglobosa as a cause of phoma stem canker epidemics on oilseed rape. *Pest Manag Sci* (2022). https://doi.org/10.1002/ps.7248.

21 Tan S, Gu Y, Yang C, Dong Y, Mei X, Shen Q *et al.*, *Bacillus amyloliquefaciens* T-5 may prevent *Ralstonia solanacearum* infection through competitive exclusion. *Biol Fertil Soils* **52**:341–351 (2016). https://doi.org/10.1007/s00374-015-1079-z.

22 Dutt A, Anthony R, Andrivon D, Jumel S, Le Roy G, Baranger A *et al.*, Competition and facilitation among fungal plant parasites affect their life-history traits. *Oikos* **130**:652–667 (2021). https://doi.org/10.1111/oik.07747.

23 Aimé S, Alabouvette C, Steinberg C and Olivain C, The endophytic strain *Fusarium oxysporum* Fo47: a good candidate for priming the defense responses in tomato roots. *Mol Plant Microbe Interact* **26**:918–926 (2013). https://doi.org/10.1094/mpmi-12-12-0290-r.

24 Liu S, Liu Z, Fitt B, Evans N, Foster S, Huang Y *et al.*, 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 Pathol* **55**:401–412 (2006). https://doi.org/10.1111/j.1365-3059.2006.01354.x.

25 Shah U, Kotta-Loizou I, Fitt BDL and Coutts R, Mycovirus induced hypervirulence of *Leptosphaeria biglobosa* enhances systemic acquired resistance to *Leptosphaeria maculans* in *Brassica napus*. *Mol Plant Microbe Interact* **33**:98–107 (2020). https://doi.org/10.1094/MPMI-09-19-0254-R.

26 Mahuku G, Hall R and Goodwin P, Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oilseed rape. *Physiol Mol Plant Pathol* **49**:61–72 (1996). https://doi.org/10.1006/pmpp.1996.0039.

27 Rouxel T, Chupeau Y, Fritz R, Kollmann A and Bousquet JF, Biological effects of sirodesmin PL, a phytotoxin produced by *Leptosphaeria maculans*. *Plant Sci* **57**:45–53 (1988). https://doi.org/10.1016/0168-9452(88)90140-9.

28 Gardiner D, Cozijnsen A, Wilson L, Pedras M and Howlett B, The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. *Mol Microbiol* **53**:1307–1318 (2004). https://doi.org/10.1111/j.1365-2958.2004.04215.x.

29 Fortune JA, Bingol E, Qi A, Baker D, Ritchie F, Karandeni Dewage CS *et al.*, *Leptosphaeria biglobosa* inhibits the production of sirodesmin PL by *L. maculans*. *Pest Manag Sci* (2022). https://doi.org/10.1002/ps.7275.

30 Elliott CE, Gardiner DM, Thomas G, Cozijnsen A, van de Wouw A and Howlett BJ, Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus*. *Mol Plant Pathol* **8**:791–802 (2007). https://doi.org/10.1111/j.1364-3703.2007.00433.x.

31 Gardiner DM and Howlett BJ, Bioinformatic and expression analysis of the putative gliotoxin biosynthetic gene cluster of *Aspergillus fumigatus*. *FEMS Microbiol Lett* **248**:241–248 (2005). https://doi.org/10.1016/j.femsle.2005.05.046.

32 Cramer RA, Gamcsik MP, Brooking RM, Najvar LK, Kirkpatrick WR, Patterson TF *et al.*, Disruption of a nonribosomal peptide synthetase in *Aspergillus fumigatus* eliminates gliotoxin production. *Eukaryot Cell* **5**:972–980 (2006). https://doi.org/10.1128/ec.00049-06.

33 Pedras M and Biesenthal C, Production of the host-selective phytotoxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. *Can J Microbiol* **44**:547–553 (1998). https://doi.org/10.1139/w98-034.

34 VSN International, *Genstat for Windows*, 22nd edn. VSN International, Hemel Hempstead, UK (2022) Web page: genstat.co.uk.

35 Williams RH and Fitt BDL, Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. *Plant Pathol* **48**:161–175 (1999). https://doi.org/10.1046/j.1365-3059.1999.00333.x.

36 Frąc M, Kaczmarek J and Jędryczka M, Metabolic capacity differentiates *Plenodomus lingam* from *P. biglobosus* subclade 'brassicae', the causal agents of phoma leaf spotting and stem canker of oilseed rape (*Brassica napus*) in agricultural ecosystems. *Pathogens* **11**:50 (2022). https://doi.org/10.3390/pathogens11010050.

37 Eckert M, Maguire K, Urban M, Foster S, Fitt BDL, Lucas J *et al.*, *Agrobacterium tumefaciens*-mediated transformation of *Leptosphaeria* spp. and *Oculimacula* spp. with the reef coral gene dsred and the Jellyfish Gene GFP. *FEMS Microbiol Lett* **253**:67–74 (2005). https://doi.org/10.1016/j.femsle.2005.09.041.

38 Lowe RG, Cassin A, Grandaubert J, Clark BL, Van de Wouw AP, Rouxel T *et al.*, Genomes and transcriptomes of partners in plant-fungal-interactions between canola (*Brassica napus*) and two *Leptosphaeria* species. *PloS One* **9**:e103098 (2014). https://doi.org/10.1371/journal.pone.0103098.

39 Padmathilake K and Fernando W, Less virulent *Leptosphaeria biglobosa* immunizes the canola plant to resist highly virulent *L. maculans*, the blackleg pathogen. *Plan Theory* **11**:996 (2022). https://doi.org/10.3390/plants11070996.

40 Urquhart A, Elliott C, Zeng W and Idnurm A, Constitutive expression of transcription factor SirZ blocks pathogenicity in *Leptosphaeria maculans* independently of sirodesmin production. *PloS One* **16**:e0252333 (2021). https://doi.org/10.1371/journal.pone.0252333.

41 Gardiner DM, Waring P and Howlett BJ, The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. *Microbiology* **151**:1021–1032 (2005). https://doi.org/10.1099/mic.0.27847-0.

42 Kremer A and Li S-M, A tyrosine *O*-prenyltransferase catalyses the first pathway-specific step in the biosynthesis of Sirodesmin PL. *Microbiology* **156**:278–286 (2010). https://doi.org/10.1099/mic.0.033886-0.

43 Elliott CE, Fox EM, Jarvis RS and Howlett BJ, The cross-pathway control system regulates production of the secondary metabolite toxin, sirodesmin PL, in the Ascomycete, Leptosphaeria maculans. *BMC Microbiol* **11**:169 (2011). https://doi.org/10.1186/1471-2180-11-169.

44 Fox EM, Gardiner DM, Keller NP and Howlett BJ, A zn(ii)2cys6 DNA binding protein regulates the sirodesmin PL biosynthetic gene cluster in *Leptosphaeria maculans*. *Fungal Genet Biol* **45**:671–682 (2008). https://doi.org/10.1016/j.fgb.2007.10.005.

45 Zou Z, Zhang X, Parks P, du Toit L, Van de Wouw A and Fernando W, A new subclade of *Leptosphaeria biglobosa* identified from *Brassica rapa*. *Int J Mol Sci* **20**:1668 (2019). https://doi.org/10.3390/ijms20071668.

46 Salam MU, Fitt BDL, Aubertot J-N, Diggle AJ, Huang YJ, Barbetti MJ *et al.*, Two weather-based models for predicting the onset of seasonal release of ascospores of *Leptosphaeria maculans* or *L. biglobosa*. *Plant Pathol* **56**:412–423 (2007). https://doi.org/10.1111/j.1365-3059.2006.01551.x.

47 Huang Y, Hood J, Eckert M, Stonard J, Cools H, King G *et al.*, 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 Pathol* **60**:607–620 (2011). https://doi.org/10.1111/j.1365-3059.2010.02416.x.