Research Article

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Leptosphaeria biglobosa inhibits the production of sirodesmin PL by *L. maculans*

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Abstract

BACKGROUND: Phoma stem canker is caused by two coexisting pathogens, *Leptosphaeria maculans* and *L. biglobosa*. They coexist because of their temporal and spatial separations, which are associated with the differences in timing of their ascospore release. *L. maculans* produces sirodesmin PL, while *L. biglobosa* does not. However, their interaction/coexistence in terms of secondary metabolite production is not understood.

RESULTS: Secondary metabolites were extracted from liquid cultures, *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb) or sequentially 7 days later (Lm+Lb). Sirodesmin PL or its precursors were identified in extracts from 'Lm only' and 'Lm+Lb', but not from 'Lm&Lb'. Metabolites from 'Lb only', 'Lm&Lb' or 'Lm+Lb' caused significant reductions in *L. maculans* colony area. However, only the metabolites containing sirodesmin PL caused a significant reduction to *L. biglobosa* colony area. When oilseed rape cotyledons were inoculated with conidia of 'Lm only', 'Lb only' or 'Lm&Lb', 'Lm only' produced large gray lesions, while 'Lm&Lb' produced small dark lesions similar to lesions caused by 'Lb only'. Sirodesmin PL was found only in the plant extracts from 'Lm only'. These results suggest that *L. biglobosa* prevents the production of sirodesmin PL and its precursors by *L. maculans* when they grow simultaneously *in vitro* or *in planta*.

CONCLUSION: For the first time, *L. biglobosa* has been shown to inhibit the production of sirodesmin PL by *L. maculans* when interacting simultaneously with *L. maculans* either *in vitro* or *in planta*. This antagonistic effect of interspecific interaction may affect their coexistence and subsequent disease progression and management.

Keywords: interspecific competition; oilseed rape; secondary metabolites; phoma stem canker; *Plenodomus lingam*; *Plenodomus biglobosus*

1 INTRODUCTION

Offspring of most living organisms are provided with enough resources for initial development, but they must obtain resources for the fundamental energy required for further development, growth, survival and reproduction.[1] 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.[1–3] Interactions between pathogens and host plants do not occur in isolation; a host is likely to interact with many different species.[4] There will be resource competition between different species, called interspecific competition.[3,5] Plant pathogens that share the same host face resource competition for successful infection of the host.

Phoma stem canker is an economically important disease of oilseed rape caused by two closely related hemi-biotrophic ascomycete fungal pathogens: *Leptosphaeria maculans* (*Plenodomus lingam*) and *L. biglobosa* (*P. biglobosus*).[6–8] This disease caused annual yield losses of >£74M in the UK, despite the use of fungicides.[9,10] The distribution of *L. maculans* and *L. biglobosa* varies globally. Whereas in parts of eastern Europe and China only *L. biglobosa* is found, both pathogens can be found to coexist in northern and western Europe, Australia, and Canada.[11] These two pathogens can coexist because they occupy different ecological niches due to slight differences in their biological and epidemiological characteristics.[11] One example is the difference in their temperature optima for pseudothecial (fruiting body) maturation, which causes a temporal separation of ascospore release in Western Europe.[7–12] This enables the resource to be partitioned

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and the two *Leptosphaeria* spp. to coexist on the same plant because a later *L. biglobosa* ascospore release coincides with the elongation of the stem and emergence of later leaves to infect the upper stems, causing upper lesions. By contrast, while the earlier *L. maculans* ascospore release is associated with stem base cankers due to infection of earlier leaves.[13,14] *L. maculans* is considered economically more important than *L. biglobosa* due to its association with the more damaging stem basal cankers, while *L. biglobosa* is associated with upper stem lesions.[7,13] However, recent reports suggest that *L. biglobosa* can also be detected in stem basal cankers as well as upper stem lesions.[15–18]

There has been limited work investigating the interactions between *L. maculans* and *L. biglobosa*. *In vitro* work has shown that *L. maculans* is able to inhibit the germination of *L. biglobosa* spores due to the presence of a secondary metabolite called sirodesmin PL.[19] *L. maculans* produces sirodesmin PL, a nonhost selective epipolythiodioxopiperazine (ETP) phytotoxin, but *L. biglobosa* does not.[13,20,21] The function of sirodesmin PL is still unknown. Mutant isolates that did not produce sirodesmin PL were still able to cause normal disease symptoms on oilseed rape cotyledons; however, a mild reduction in stem canker severity and 50% reduction in fungal biomass was observed with mutant isolates.[19] This suggests that sirodesmin PL is not required for infection and disease progression. Studies have shown that mutants without sirodesmin PL had less antibacterial and antifungal activity than the wild type but did not have decreased growth or fertility,[22,23] suggesting that it may be used as an interference competition strategy. Interference competition is a strategy of interspecific competition, where a competing pathogen produces a secondary metabolite that is toxic to competing species, limiting access of other pathogens to the resource. For example, early establishment of *F. subglutinans* in maize can reduce the production of the toxic metabolite trichothecene by *F. graminearum* and temporarily protect the plant from colonization by the more toxic and damaging *F. graminearum*.[24] Similarly, previous work has shown that the pre-inoculation of oilseed rape plants with *L. biglobosa* induced resistance to *L. maculans*.[25,26] However, the effectiveness of *L. biglobosa* (weakly virulent type) in inducing resistance to *L. maculans* (highly virulent) infection was compromised if *L. biglobosa* was added at 64 h or later after *L. maculans*.[25] This may be due to the time required for production of sirodesmin PL by *L. maculans* needs up to 3 days,[27] since 64 h is about 2.5 days. This suggests that sirodesmin PL plays an important role in the interactions between *L. maculans* and *L. biglobosa*. There is a need to investigate the interactions between *L. maculans* and *L. biglobosa* in relation to secondary metabolite production, especially sirodesmin PL production by *L. maculans* and its effects on disease development, for improving phoma stem canker control strategies.

2 MATERIALS AND METHODS

2.1 Pathogen and media preparation

2.1.1 Pathogen preparation

L. maculans isolate ME24, obtained from stem canker of oilseed rape cultivar Apex in 2002,[28] and *L. biglobosa* isolate WH17-Why-1, obtained from phoma leaf lesion of oilseed rape cultivar Whisky in 2017, were used in this study. The *L. maculans* and *L. biglobosa* isolates were grown on 20% V8 agar plates for 14 days, and agar plugs were cut at the growth front of the plate for inoculation of liquid culture or agar plates.

2.1.2 Identifying the optimal media for culturing of the pathogens

Agar plugs (10 mm) of *L. maculans* isolate ME24 and *L. biglobosa* isolate WH17-Why-1 were inoculated in the middle of plates (9 cm) containing five different growth media: malt extract agar (MEA), water agar (WA), potato dextrose agar (PDA), 20% V8 juice agar (V8) or 20% clarified V8 agar (V8 Clar). V8 juice was clarified by adding 15 g L^{-1} of calcium carbonate to the V8 juice agitated using a magnetic stirrer for 15 min before being centrifuged at 1400 *g* for 5 min. The pH of the clarified V8 media was 5.86, whereas the pH of the V8 only media was 6.15. The pellet was discarded. The supernatant was stored at −20 °C until required. The supernatant was classed as clarified V8 juice. The inoculated plates were incubated for 7 days at 21 °C in continuous darkness before the colony diameter was measured. Each treatment was replicated three times. The colony diameter was converted to colony area (cm^2).

2.2 Concentration of sirodesmin PL production by *L. maculans*

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 80 rpm and 18 °C for 14 days in continual darkness. Secondary metabolite extractions were taken at six time points. There were three replicates for each time point. The experiment was replicated two times (experiments 1 and 2). The time points for experiment 1 were 1, 3, 7, 8, 10, and 14 days post inoculation (dpi) and for experiment 2 were 1, 3, 6, 8, 10, and 14 dpi. The secondary metabolites were extracted from each sample and analyzed using high-performance liquid chromatography (HPLC).

The secondary metabolites from liquid culture were extracted using ethyl acetate. The culture filtrate from each replicate was split equally into two 50-mL Falcon tubes (~37.5 mL in each). Each Falcon tube was then topped up to 50 mL using ethyl acetate (EtOAc) (~12.5 mL), then gently inverted 30 times to increase secondary metabolite uptake, then left for 1 h so that the phases could settle before being centrifuged at 3200 *g* for 5 min. For each replicate, 20 mL of the organic phase was pipetted into a clean 50-mL Falcon tube and the EtOAc was evaporated under a constant stream of nitrogen using a sample concentrator. The dried metabolites were resuspended in 0.5 mL of EtOAc. Falcon tubes containing the same treatment were combined into sterile plastic sample bottles. Using a syringe, the resuspended metabolites were passed through a 0.45-µm syringe filter into a clean HPLC sample vial. A different HPLC vial was prepared with a glass insert and 200 µL of the resuspended metabolites was pipetted into the insert. The samples were stored at 20 °C until required for HPLC analysis.

To measure the concentration of sirodesmin PL, a gliotoxin standard curve ($R^2 = 0.999$, $y = 2^6x - 56\ 900$) was created using maximal areas of gliotoxin samples visualized at 254 nm at concentrations of 0, 10, 50, 100, 250, 500, and 1000 mg L^{-1} using HPLC analysis. This gliotoxin standard curve was used to calculate the concentration of sirodesmin PL at each time point. The level of detection (LOD) and level of quantification (LOQ) were 70 and 200 mg L^{-1}, respectively. The mean concentration at each time point was calculated. Gliotoxin was used for the standard curve because it belongs to the same class of fungal secondary metabolites as sirodesmin PL, ETP, has been used in previous work as a standard curve,[23] and is commercially available.

2.3 Effects of *L. biglobosa* on secondary metabolite production by *L. maculans in vitro*

To investigate the effects of *L. biglobosa* on secondary metabolite production by *L. maculans*, *L. biglobosa* was simultaneously or sequentially inoculated with *L. maculans*. Clarified 20% V8 juice broth was inoculated with four different treatments: *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb), and *L. maculans* and *L. biglobosa* sequentially (Lm+Lb). For the 'Lm+Lb' treatment, the liquid media was inoculated with *L. maculans* followed by *L. biglobosa* inoculation after 7 days. Three plugs of each pathogen were used per corresponding treatment, therefore cultures containing both the pathogens had six fungal plugs. Each inoculation treatment was done in triplicate. The experiment was repeated three times. To identify the secondary metabolite composition of the different treatments, secondary metabolites were extracted from liquid media using ethyl acetate after 14 dpi. The samples were prepared and analyzed using HPLC. To identify the compound responsible for each unique HPLC maximum, individual 1-mL fractions were taken for liquid chromatography–mass spectrometry (LC–MS) analysis.

To identify the secondary metabolite composition, the samples were analyzed using a Shimadzu Prominence HPLC machine (Kyoto, Japan) with a diode array detector (SPD-M20A) using 20-µL injections. Separations were performed on a C_{18} column (Varian pursuit 5, 150 × 4.6 mm). A linear gradient 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 was used. 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^{-1} was used. Results were visualized at 254 nm using Lab solution version 5.92 (Shimadzu Corporation) but wavelengths were detected from 190 to 400 nm. The LOD and LOQ were calculated to be 70 and 200 mg L^{-1}, respectively. All samples and chemicals used were passed through a 0.45-µm nylon microfilter before being analyzed, although all chemicals used were of HPLC analytical grade and water was double distilled. The expected pattern of maxima for deacetylsirodesmin PL, phomamide, and sirodesmin PL were identified using information from Pedras *et al.*[27]

2.4 Identification of secondary metabolites using LC–MS

A Waters I-Class UPLC system coupled to a Xevo Micro TQ-S mass spectrometer was used for analysis. Chromatographic separation was performed on a Waters BEH C18 column (2.1 × 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^{-1} and a gradient separation performed 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 performed in positive ion mode over the range 50–650 *m/z* using a scan time of 0.2 s. The probe capillary voltage was 3 kV, the cone voltage was fixed at 20 V, and the collision energy was fixed at 3 V. Desolvation gas flow was set at 1000 L h^{-1}, with a temperature of 500 °C, and a cone gas flow of 150 L h^{-1} was used, with source temperature set at 150 °C.

2.5 Effects of secondary metabolite extracts on growth of *L. maculans* and *L. biglobosa* on agar plates

Fungal plugs of *L. maculans* or *L. biglobosa* (8 mm in diameter) were inoculated onto the middle of clarified V8 juice (V8 Clar) agar plates. Each fungal plug was then inoculated with 20 µL of the corresponding secondary metabolite extract from each treatment ('Lm only', 'Lb only', 'Lm&Lb', 'Lm+Lb') or ethyl acetate (control). Each treatment had five replicate plates. Plates were sealed using Parafilm and incubated at 18 °C in continual darkness. Colony diameters for *L. maculans* and *L. biglobosa* were recorded at 7 dpi. The colony diameter was converted to colony area (cm^2). The experiment was repeated three times.

2.6 Effects of *L. biglobosa* on disease development and sirodesmin PL production by *L. maculans in planta*

2.6.1 Preparation of L. maculans *and* L. biglobosa *inoculum and plant material*

L. maculans and *L. biglobosa* conidial inoculum was obtained from 2-week-old cultures grown on 20% V8 juice agar as described by Huang *et al.*[28] The suspensions were measured using a hemocytometer slide under a light microscope and suspensions were adjusted to 1×10^7 conidia mL^{-1}. The 'Lm&Lb' inoculum was prepared by combining a sample of each conidial spore suspension in a 1:1 ratio. Cultivar Charger, which has a moderate disease resistance rating score of 4 according to the AHDB Recommended List resistance ratings (0–9 scale, with 9 being good resistance), was used for this study (https://ahdb.org.uk/knowledge-library/recommended-lists-archive). Cotyledons were prepared by sowing seeds of winter oilseed rape cv. Charger into 40-well plug trays placed in a controlled environment cabinet set at 20/18 °C and 12 h/12 h day/night. Plug trays were filled with John Innes (No. 3) soil and Miracle Grow multipurpose compost mixed in a 1:1 ratio. Plants were regularly watered to ensure soil remained moist. True leaves were removed to prolong the lifespan of cotyledons.

2.6.2 Experimental design and plant inoculation

There were four treatments, 'Lm only', 'Lb only', 'Lm&Lb' and a sterilized distilled water control, 'SDW'. There were 30 plants per treatment. The experiment was designed using a randomized block design with three replicates. Cotyledons of 14-day-old seedlings were inoculated. The inoculation sites were prepared by wounding the cotyledon using a sterilized needle. Each cotyledon was wounded in two locations, therefore there were four inoculation sites per plant. For inoculation, 10 µL of conidial suspension was pipetted onto each inoculation site according to the treatment and experimental design. Inoculated plants were covered with tray covers to maintain high humidity for 48 h. The inoculated plants were kept in a controlled environment cabinet at 18/16 °C and 12 h/12 h day/night.

2.6.3 Assessment of disease development

To measure the size of lesions, cotyledons were excised from each plant at 17 dpi, placed inside a 9 × 9 cm square, and a standardized photograph was taken. The lesion area was calculated by processing the standardized photographs using ImageJ software.[29] The total number of pixels were converted into cm^2. The experiment was repeated three times.

2.6.4 Assessment of sirodesmin PL production in planta

Due to the *in planta* secondary metabolite detection requiring very severely diseased plant tissue, a separate experiment with the same design as those used for disease development experiments was done and the cotyledons were excised from each plant at 26 dpi, rather than at 17 dpi. The standardized photograph was also taken for assessing the lesion area using ImageJ software. After lesion phenotype photographs were taken,

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lesions from 30 plants of each treatment were excised. The lesions were excised according to their size. For small (e.g. plants inoculated with Lb only) or no lesions (e.g. control), an 8 mm diameter corer centered over each inoculation site was used. For larger lesions, a scalpel was used to excise the lesion. Lesions from five plants were grouped together and placed into 2-mL screw cap tubes. There was a total of six 2-mL screw cap tubes per treatment. After 48 h of freeze-drying, three small metal balls (3 mm) 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^{-1}; this step was repeated three times, samples were placed on ice in between runs of the FastPrep. Lysed samples were centrifuged at 15 700 *g* for 5 min and 500 µL of ethyl acetate supernatant was pipetted into a sterile 50 mL Falcon tube. Supernatants for each treatment were combined and evaporated to residue. Each treatment sample was resuspended in 500 µL of ethyl acetate and passed through a 0.45 µm Polytetrafluoroethylene (PTFE) syringe filter into a 2 mL HPLC vial for HPLC analysis. For identification of the compound responsible for each unique HPLC maximum, individual 1.5 mL fractions were taken for between 11–12.5 min and 16–17.5 min for each sample. Fractions were evaporated to residue before being resuspended in 110 µL of ethyl acetate before being analyzed by LC–MS.

2.7 Statistical analysis

The statistical analysis of the data was done using GenStat 22nd edition.[29] To analyse the effects of different media types on colony area of different pathogens, the effect of co-inoculation of *L. maculans* and *L. biglobosa* on the concentration of sirodesmin PL and its precursors, a factorial analysis of variance (ANOVA) was done to determine significant differences between treatments on each pathogen. For the effect of secondary metabolite extracts on colony area, unbalanced ANOVA was used. For data at 17 dpi, a factorial ANOVA was done using 'treatment' and 'experiment number' as factors. For data at 26 dpi, an ANOVA was done using only 'treatment' as the factor. Variance ratio values (i.e., *F*-test) that were significant at $P < 0.05$ were used to provide evidence for significant effects of factors. *Post hoc* Fisher least significant difference tests were applied to separate means between different media and different secondary metabolite extracts for each pathogen.

3 RESULTS

3.1 Optimal media for colony growth

There were significant differences in the colony area on different media for *L. maculans* ($F_{4,13} = 169.37$, $P < 0.001$, LSD = 1.51) and *L. biglobosa* ($F_{4,13} = 21.31$, $P < 0.001$, LSD = 3.55). Both pathogens had significantly larger colony areas when cultured on 20% clarified V8 juice agar than on all other media tested (Fig. 1). For *L. maculans*, the colony area was not significantly different between water agar (5.6 cm^2) and MEA (6.4 cm^2), but was significantly smaller than that on PDA (10.4 cm^2). The largest colony area was on clarified V8 agar, which was significantly larger than that on V8 agar (Fig.1). For *L. biglobosa*, there were no significant differences between MEA (13.6 cm^2), WA (15.4 cm^2) and PDA (15.7 cm^2) or between MEA and V8 (11.6 cm^2). However, the colony area was significantly larger on clarified V8 agar (23.3 cm^2) than those on other media (Fig.1). The clarified V8 was the optimum medium for both *L. maculans* and *L. biglobosa*, therefore clarified V8 was used for liquid culture and agar plates in this study.

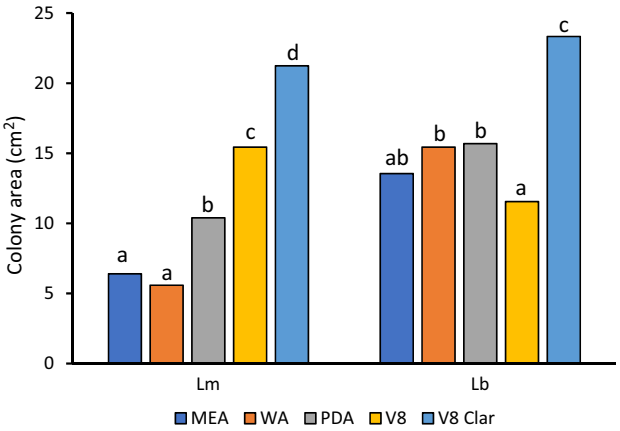

Figure 1. Colony area (cm^2) of *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) when cultured on different media types at 7 days post inoculation. Malt extract agar (MEA), water agar (WA), potato dextrose agar (PDA), 20% V8 juice agar (V8) and 20% clarified V8 agar (V8 Clar) (df = 13, LSD = 1.51 and 3.55 cm^2 for Lm and Lb, respectively). Letters represent Fisher's protected least significant difference test between different media tested on each pathogen. Fisher LSD tests were used to separate the mean values of colony areas for each pathogen. Columns that do not share a letter are significantly different at $P = 0.05$.

3.2 Concentration of sirodesmin PL production by *L. maculans* over 14 days

When the concentration of sirodesmin PL was measured at regular time intervals over 14 days in culture filtrates of *L. maculans*, sirodesmin PL was not detected until 3 dpi. The concentration of sirodesmin PL increased from 3 dpi until 10 dpi and then decreased from 10 dpi (Fig. 2).

3.3 Effects of *L. biglobosa* on sirodesmin PL production by *L. maculans in vitro*

3.3.1 Comparison of secondary metabolite chromatograms

For the secondary metabolites extracted from liquid culture, there were three unique maxima that were present only in the 'Lm only' and 'Lm+Lb' 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. When *L. maculans* was cultured simultaneously with *L. biglobosa* ('Lm&Lb') the three maxima unique to 'Lm only' and 'Lm+Lb' were not present and its chromatogram was like that of 'Lb only'.

3.3.2 Identification of maxima found on the chromatograms from extracted secondary metabolites using LC–MS

Maximum 1 contained ions in its LC–MS positive ion spectra (Fig. 3(a)) at *m/z* 445.20, which corresponds to a monocharged molecule with a molecular weight 444.5 Da; deacetylsirodesmin is a known secondary metabolite produced by *L. maculans* and has a molecular weight of 444.5 Da.[30,31] Additionally, ions were observed at *m/z* 381.24 $[M - S_2 + H]^+$, corresponding to deacetylsirodesmin lacking two sulfur atoms; *m/z* 403.23 $[M - S_2 + Na]^+$, corresponding to the sodium adduct of sirodesmin lacking two sulfur atoms; and *m/z* 467.19 $[M + Na]^+$, corresponding to the sodium adduct of deacetylsirodesmin (Fig. 3(a)). In addition to these, ions were observed at *m/z* 319.26, which corresponds to 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.[30,31]

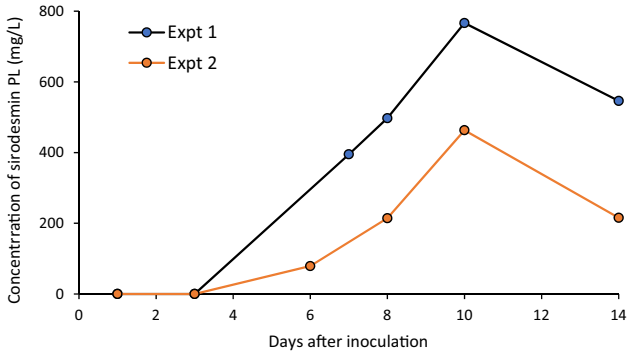

Figure 2. Concentration of sirodesmin PL (mg L^{-1}) produced in clarified V8 liquid media inoculated with *Leptosphaeria maculans* over 14 days post inoculation in experiment 1 (Expt 1) and experiment 2 (Expt 2). Concentrations were calculated using a gliotoxin standard curve ($y = 2^6x - 56900$; $R^2 = 0.999$).

Maximum 2 from the chromatogram contained ions in its LC–MS positive ion spectra (Fig. 3(b)) at *m/z* 487.19 (97%), which corresponds to a monocharged molecule with a molecular weight 486.6 Da. Sirodesmin PL is a known secondary metabolite produced by *L. maculans* with a molecular weight of 486.6 Da.[30,31] Additionally, the following ions were found: *m/z* 423.27 $[M - S_2 + H]^+$, corresponding to sirodesmin PL lacking two sulfur 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.

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

3.3.3 *Secondary metabolite composition and concentration*

There were significant differences between different treatments in concentrations of sirodesmin PL 'precursors' (deacetylsirodesmin and phomamide) ($F_{3.11} = 23.87$, $P < 0.001$, LSD = 125.11) (Fig. 3(c)). The extracts that had concentrations of 'precursors' greater than the level of detection (LOD = 70 mg L^{-1}) and level of quantification (LOQ = 200 mg L^{-1}) were 'Lm only' (283.3 mg L^{-1}) and 'Lm +Lb' (357.5 mg L^{-1}) (Fig. 3(c)), and they were not significantly different from each other. The maxima for precursors were not detected in the 'Lm&Lb' or 'Lb only' treatments.

There were significant differences between treatments in the concentrations of sirodesmin PL ($F_{3.11} = 134.00$, $P < 0.001$, LSD = 128.77) (Fig. 3(c)). The extracts that had concentrations greater than the level of detection (LOD = 70 mg L^{-1}) and level of quantification (LOQ = 200 mg L^{-1}) were 'Lm only' and 'Lm+Lb' (Fig. 3(c)). There was no significant difference in the concentration of sirodesmin PL between 'Lm only' (747.4 mg L^{-1}) and 'Lm+Lb' (831.5 mg L^{-1}). The maxima for sirodesmin PL were not detected in the 'Lm&Lb' or 'Lb only' treatments.

3.3.4 *Effects of secondary metabolite extracts on growth of* L. maculans *and* L. biglobosa *on agar plates*

The secondary metabolites had a significant effect on the colony areas of *L. maculans* ($F_{4.72} = 24.62$, $P < 0.001$, LSD = 2.029) and *L. biglobosa* ($F_{4.73} = 87.10$, $P < 0.001$, LSD = 1.972) (Figs 4 and 5). The secondary metabolite extracts had greater effects on growth of *L. biglobosa* than on growth of *L. maculans* (Fig. 4). For *L. biglobosa*, the extracts that caused significant differences in colony area compared with the 'EtoAc' control (21.3 cm^2) were 'Lm only' (10.1 cm^2) and 'Lm+Lb' (8.7 cm^2) (i.e. 52.7% and 59.3% reduction compared to the EtOAc control in 'Lm only' and 'Lm+Lb', respectively), but they were not significantly different from each other. The Lb only (20.3 cm^2) and Lm&Lb (22.0 cm^2) were not significantly different from each other, nor from the EtOAc control (Fig. 4(b)). For *L. maculans*, the three treatments that differed significantly from the EtOAc control (25.8 cm^2) were 'Lb only' (19.4 cm^2), 'Lm&Lb' (18.2 cm^2) and 'Lm+Lb' (18.2 cm^2) (i.e. 24.8%, 29.5%, and 29.5% reduction, respectively), but they were not significantly different from each other. There was no significant difference between 'Lm only' (24.1 cm^2) and the ethyl acetate control in colony area of *L. maculans* (Fig. 4(a)).

3.4 Effects of *L. biglobosa* on lesion development and sirodesmin PL production by *L. maculans*

3.4.1 *Disease development* in planta

There were differences in lesion phenotypes between the four different treatments (Fig. 6). When cotyledons were inoculated with *L. maculans* conidia only (Lm only), the lesions were large, gray, sunken, and not defined. However, when cotyledons were co-inoculated simultaneously with *L. maculans* and *L. biglobosa* (Lm&Lb), the lesions were small, dark, and well-defined; these were similar to the lesions developed from inoculation with *L. biglobosa* conidia only (Lb only). By 26 dpi, the lesions of 'Lm only' had spread to the whole cotyledons while the lesions of 'Lm&Lb' did not spread and remained similar as lesions of 'Lb only' (Fig. 6). 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. 7(a)). 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. 7(a)). Similarly, at 26 dpi ($F_{3.458} = 690.08$, $P < 0.001$, LSD = 7.35), the lesion area was significantly greater for 'Lm only' (153.3 mm^2) than that for 'Lb only' (24.6 mm^2) or 'Lm&Lb' (27.1 mm^2) (Fig. 7(b)). There was no significant difference between experiments ($F_{2.1106} = 0.81$, $P = 0.444$, LSD = 1.603).

3.4.2 *Production of sirodesmin PL* in planta

There was a difference in the presence or absence of sirodesmin PL and its precursors between treatments *in planta*. Sirodesmin PL and its precursors were present only in the 'Lm only' treatment, and were absent from the 'SDW', 'Lb only' and 'Lm&Lb' treatments (Fig. 8). The LC–MS chromatograms showed that there were three unique maxima at retention times 4.95, 5.03, and 5.11 min in the 'Lm only' sample. At retention time 4.95 min, the positive ion spectra contained ions with *m/z* 242.29 (30%) and 243.35 (5%). These ions remain unidentified.

At retention time 5.03 min (Fig. 8(a)), the positive ion spectra (Fig. 8(b)) contained ions at *m/z* 319.26 (100%) and 320.29 (15%) with a mean *m/z* of 319.4. This 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.[30,31]

At retention time 5.11 min (Fig. 8(c)), the positive ion spectra (Fig. 8(d)) contained an ion at *m/z* 487.18, which corresponds to a monocharged molecule with a molecular weight of 486.57 Da. Sirodesmin PL has a molecular weight of 486.6 Da.[30,31] The positive ion spectra (Fig. 8(d)) also contained an ion at *m/z* 445.24, which corresponds to a monocharged molecule with a molecular weight of 444.24 Da. Deacetylsirodesmin PL is a known secondary

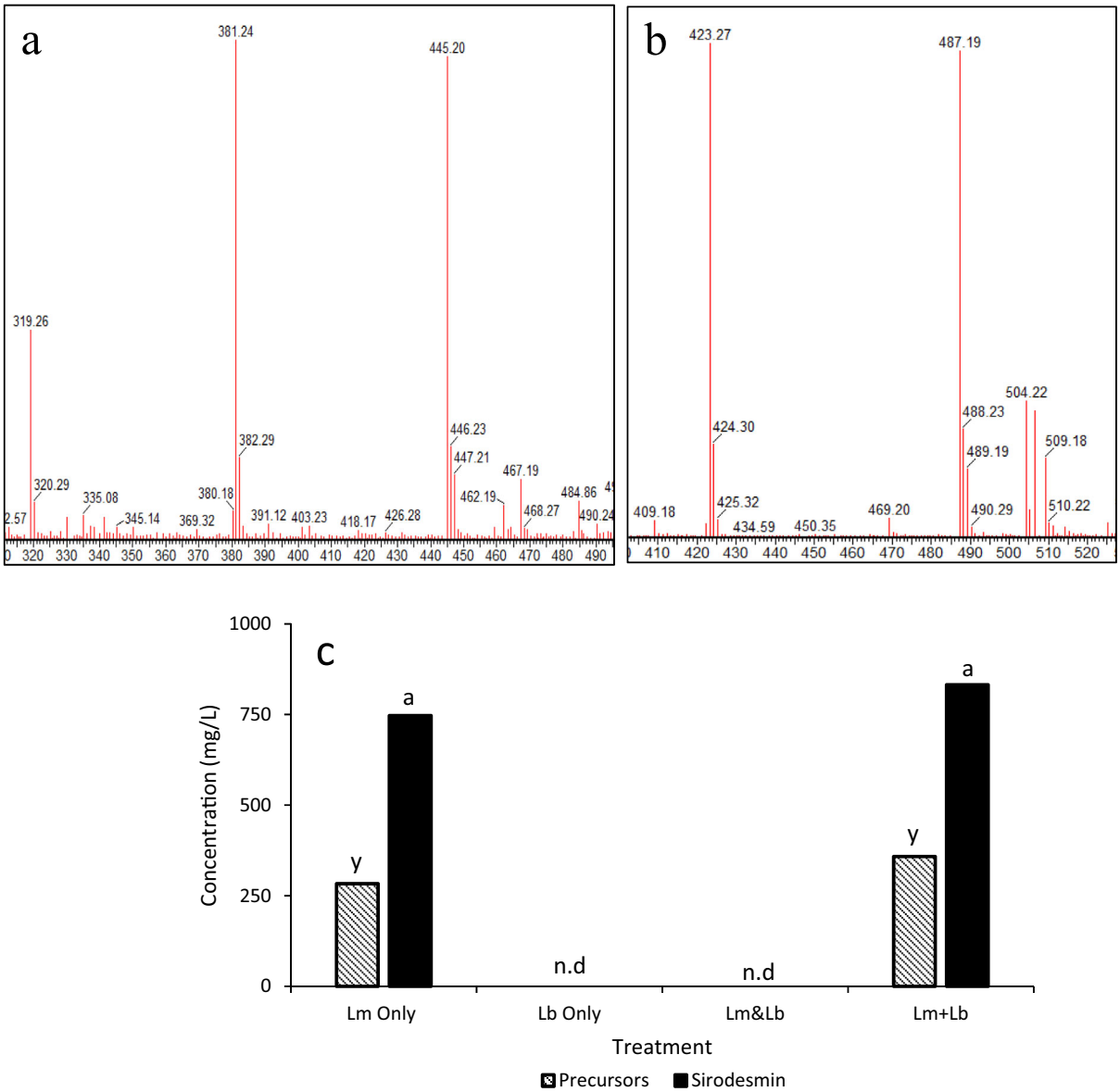

Figure 3. LC–MS positive ion spectra for the 'precursors' HPLC maximum at retention time 11–12 min (a) and 'sirodesmin' HPLC maximum at retention time 16–17 min (b). Concentration of sirodesmin PL and its precursors (mg L^{-1}) (c) extracted from clarified V8 liquid culture inoculated with *Leptosphaeria maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb) or *L. maculans* and *L. biglobosa* sequentially (Lm +Lb). For the Lm+Lb treatment, the liquid media was inoculated with *L. maculans* first then 7 days later with *L. biglobosa*. Fisher LSD tests were used to separate the mean values of concentrations. Columns that do not share a letter are significantly different ($P = 0.05$). Samples where a maxima corresponding to 'precursors' or 'sirodemsin PL' were not detected are indicated (n.d).

metabolite produced by *L. maculans* and has a molecular weight of 444.5 Da.[30,31] The following ions were also found in the positive ion spectra (Fig. 8(d)): *m/z* 424.32 $[M - S_2 + H]^+$, corresponding to sirodesmin PL lacking two sulfur atoms, *m/z* 460.24 $[M - S_2 + K]^+$, corresponding to the potassium adduct of sirodesmin PL lacking two sulfur atoms, 465.20 $[M - S_2 + CH_3CN + H]^+$ corresponding to the acetonitrile adduct of sirodesmin PL lacking two sulfur atoms, and *m/z* 504.25 $[M + NH_4]^+$, corresponding to the ammonium adduct of sirodesmin PL.

4 DISCUSSION

The results of this study for the first time provide evidence that *L. biglobosa* can inhibit *L. maculans* production of sirodesmin PL and its precursors to increase its competitiveness over *L. maculans* both *in vitro* and *in planta*. No sirodesmin PL nor its precursors were produced when *L. maculans* and *L. biglobosa* were grown together simultaneously *in vitro* (liquid culture) or *in planta* (cotyledons of oilseed rape), suggesting that the production of sirodesmin PL and its precursors by *L. maculans* had been inhibited by *L. biglobosa*. The inhibition of sirodesmin PL production has advantages for *L. biglobosa* growth because sirodesmin PL has antibacterial and antifungal properties.[19] There was an inhibition of *L. biglobosa in vitro* growth when secondary metabolite extracts containing sirodesmin PL were applied; this suggests that *L. maculans* uses sirodesmin PL as a form of interference interspecific competition strategy to outcompete *L. biglobosa*. This supports previous findings that *L. maculans*

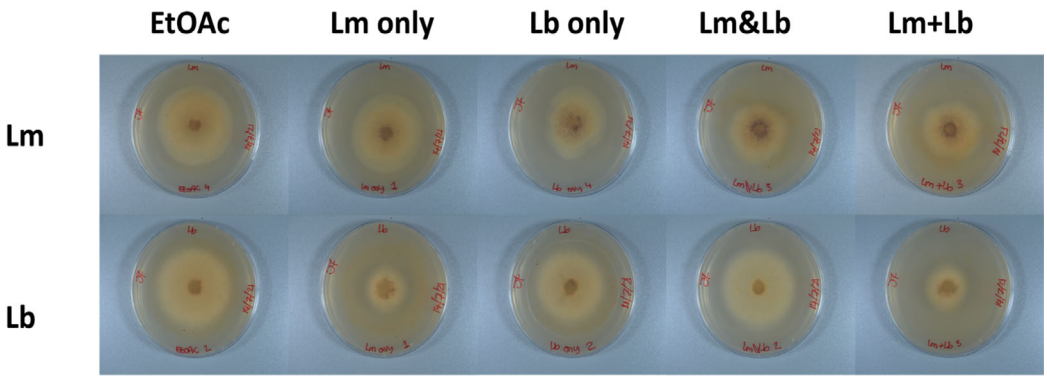

Figure 4. Colony phenotypes of *Leptosphaeria maculans* or *L. biglobosa* on clarified V8 agar plates at 7 days post inoculation (dpi). Fungal plugs of *L. maculans* or *L. biglobosa* were inoculated with different secondary metabolites extracted from liquid cultures inoculated with *L. maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* inoculated sequentially 7 days later (Lm+Lb) or ethyl acetate control (EtOAc).

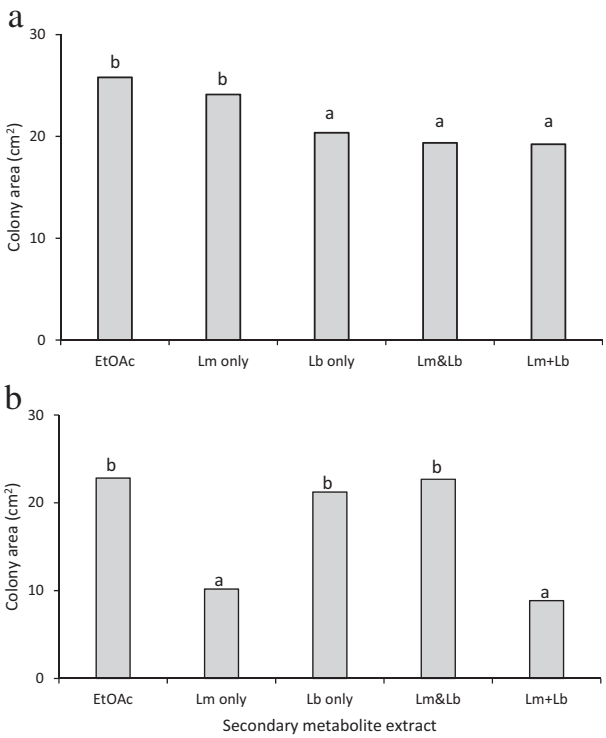

Figure 5. Colony areas (cm^2) of *Leptosphaeria maculans* (a) or *L. biglobosa* (b) on clarified V8 agar plates at 7 days post inoculation. Fungal plugs of *L. maculans* or *L. biglobosa* were inoculated with different secondary metabolites extracted from clarified V8 liquid culture inoculated with *L. maculans* only (Lm only), *L. biglobosa* (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb), *L. maculans* and *L. biglobosa* sequentially (Lm+Lb) or ethyl acetate control (EtOAc). For the 'Lm+Lb' treatment, the liquid media was inoculated with *L. maculans* first then 7 days later with *L. biglobosa*. Data presented are means of three experiments. The *post hoc* Fisher's least significant difference (LSD) tests were done within each pathogen. Columns that share the same letter are not significantly different from each other within each pathogen at $P = 0.05$.

has an inhibitory effect on *L. biglobosa*.[19] Additionally, this study provides evidence that *L. biglobosa* may use an interspecific exploitation competition strategy. *L. biglobosa* had a greater colony area on clarified V8 agar than that of *L. maculans*, suggesting that *L. biglobosa* colonizes the resource more efficiently than

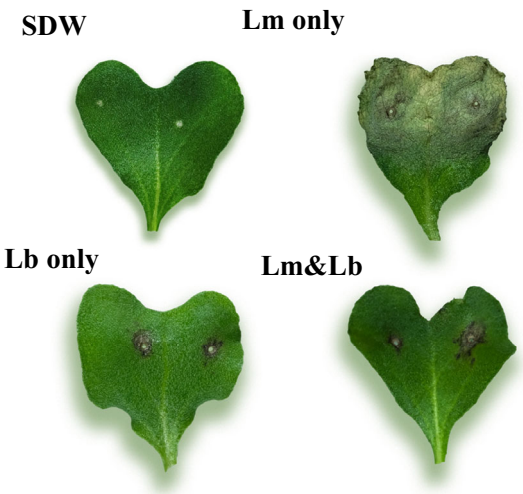

Figure 6. Lesion phenotypes on cotyledons of oilseed rape cultivar Charger at 26 days post inoculation. Cotyledons were inoculated with conidial of *Leptosphaeria maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb) or sterilized distilled water (SDW) as a control.

L. maculans when there is no competition. This is supported by previous studies showing that *L. biglobosa* has a faster colonization rate than *L. maculans*.[7,32,33] The reduced *L. maculans in vitro* growth when secondary metabolite extracts from liquid culture containing *L. biglobosa* were applied suggested that *L. biglobosa* also has antagonistic effects on *L. maculans* growth. Since no specific antibacterial and antifungal compounds were identified from *L. biglobosa* extracts, the effects of *L. biglobosa* on *L. maculans* growth may be due to *L. maculans* detecting *L. biglobosa* pathogen-associated molecular patterns (PAMPS) inducing defense responses, like the mechanism of PAMP-triggered immunity induced in plants,[34] increasing energy use for defense and other strategies, rather than for growth. When *L. maculans* and *L. biglobosa* were inoculated simultaneously (Lm&Lb) onto cotyledons of oilseed rape cv. Charger, the lesion phenotype and lesion size were like lesions of 'Lb only' and not similar to lesions of 'Lm only', suggesting that *L. biglobosa* inhibited the lesion development by *L. maculans*. No sirodesmin PL was detected in plants inoculated with 'Lm&Lb'.

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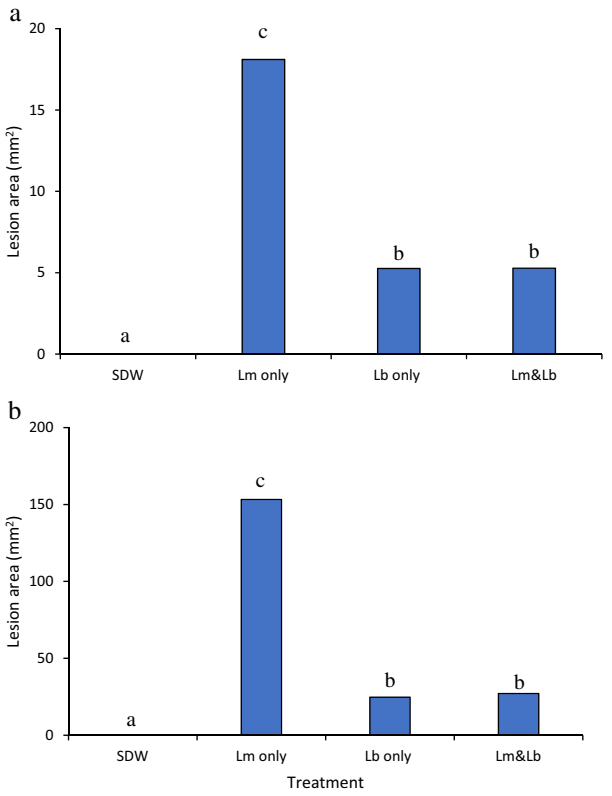

Figure 7. Phoma leaf lesion area (mm^2) on cotyledons of oilseed rape cultivar Charger at 17 (a) or 26 (b) days post inoculation (dpi). Cotyledons were inoculated with conidia suspensions of *Leptosphaeria maculans* only (Lm only), *L. biglobosa* only (Lb only), *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb) or sterilized distilled water (SDW) as a control. Data presented are means of three experiments at 17 dpi (a) or one experiment at 26 dpi (b). The *post hoc* Fisher's least significant difference (LSD) tests were used to separate the mean values of lesion areas. Columns that do not share a letter are significantly different at $P = 0.05$.

The only extracts that contained sirodesmin PL and its precursors were 'Lm only' and 'Lm+Lb' (*L. maculans* followed by *L. biglobosa* inoculation 7 days later), not the 'Lm&Lb' (*L. maculans* and *L. biglobosa* inoculation simultaneously), suggesting that *L. biglobosa* inhibits sirodesmin PL production by *L. maculans* when *L. maculans* and *L. biglobosa* grow together simultaneously. Unique detectable maxima were not identified in the 'Lm&Lb' extracts, suggesting that the production of precursors may have been 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.[23] It has been shown that *O*-prenyl-L-tyrosine is the first committed precursor for sirodesmin PL biosynthesis, a step catalyzed by prenylase (*SirP*).[30] The *sirP* mutants have been shown to inhibit the production of sirodesmin PL[23] so denaturation of *sirP* or another gene involved early in the biosynthesis pathway would probably inhibit the production of the precursors and sirodesmin PL production.[23,30,35] Also, it has been shown that the production of sirodesmin PL is regulated by the cross-pathway control gene cpaA using the transcription factor *sirZ*,[36] therefore if *L. biglobosa* disrupts the expression or products of the genes responsible for the biosynthesis of the sirodesmin PL precursors early in the pathway then downstream products and compounds will not be produced. Once the biosynthesis of sirodesmin PL precursors has started, *L. biglobosa* could not disrupt or inhibit sirodesmin PL production. This hypothesis is supported by the results showing that sirodesmin PL and its precursors were detected in extracts from 'Lm+Lb' but not 'Lm&Lb'. There is a need to investigate the expression of genes related to biosynthesis of sirodesmin PL in the presence of *L. biglobosa* to identify the potential gene inhibitors.

The results of this study suggest that the timing when *L. maculans* and *L. biglobosa* meet and interact is important for the outcome of the interaction between them. In liquid culture, sirodesmin PL and its precursors were identified in the secondary metabolite extracts from 'Lm+Lb' but not from 'Lm&Lb'. This effect of timing is mainly due to the timing of *L. maculans* production of sirodesmin PL since *L. maculans* did not produce sirodesmin PL until 3 dpi, which agrees with the study by Gardiner *et al.*[23] Therefore, if *L. biglobosa* can exploit the resource before *L. maculans* can produce sirodesmin PL, *L. biglobosa* can exploit its exploitation interspecific competitive strategy to use the resource and inhibit the production of sirodesmin PL and its precursors to outcompete *L. maculans*. This is supported by the results from *in planta* experiments. Cotyledons inoculated with 'Lm&Lb' produced lesions like those of *L. biglobosa* but different from those of *L. maculans* and no sirodesmin PL was found. This finding may help to explain why the effectiveness of *L. biglobosa* in inducing resistance to *L. maculans* infection was compromised if *L. biglobosa* was added at 64 h or later after *L. maculans*[24] because 64 h is roughly 2.5 days which is required for sirodesmin PL to be produced (Fig. 2). The production of sirodesmin PL was a maximum at 10 dpi before reducing at day 14; this may be due to conservation of energy because the resource is a relatively nutrient poor by 14 days.[37] Only one cultivar of winter oilseed rape was used in this study. The antagonistic effects of interspecific competition between *L. maculans* and *L. biglobosa* may differ, with cultivars having different resistance ratings or between isolates having different pathogenicity, therefore further investigation is required to fully understand these phenomena.

The results of this study have important practical and agricultural relevance. 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 small dark lesions (*L. biglobosa* type of lesions) and affect the timing of fungicide application because the timing of fungicide application is determined based on the *L. maculans* type of lesions (large gray lesions). In the UK, as part of an integrated pest management strategy, growers are advised to accurately apply the first fungicide application when 10–20% of the plants in a crop have *L. maculans* phoma leaf spots in autumn, followed by another application if/when reinfection occurs (https://ahdb.org.uk/knowledge-library/how-to-manage-phoma-in-oilseed-rape). This study showed that when cotyledons were co-infected with *L. maculans* and *L. biglobosa* at the same time, the lesions were small and had an appearance like *L. biglobosa* lesions. Therefore, in seasons where there is simultaneous infection of plants by both *Leptosphaeria* spp., the grower may apply the fungicide later or not at all due to the advised threshold of *L. maculans* lesions not being met. In addition, *L. biglobosa* is less sensitive to azole fungicides than *L. maculans*.[14,38] In the UK, there is substantial reliance on the use of azole fungicides, particularly prothioconazole and tebuconazole.[39] Furthermore, recent studies have shown that *L. biglobosa* can also cause stem basal cankers as well as upper stem lesions[15,18,40,41] and cause yield losses.[16] Therefore, results

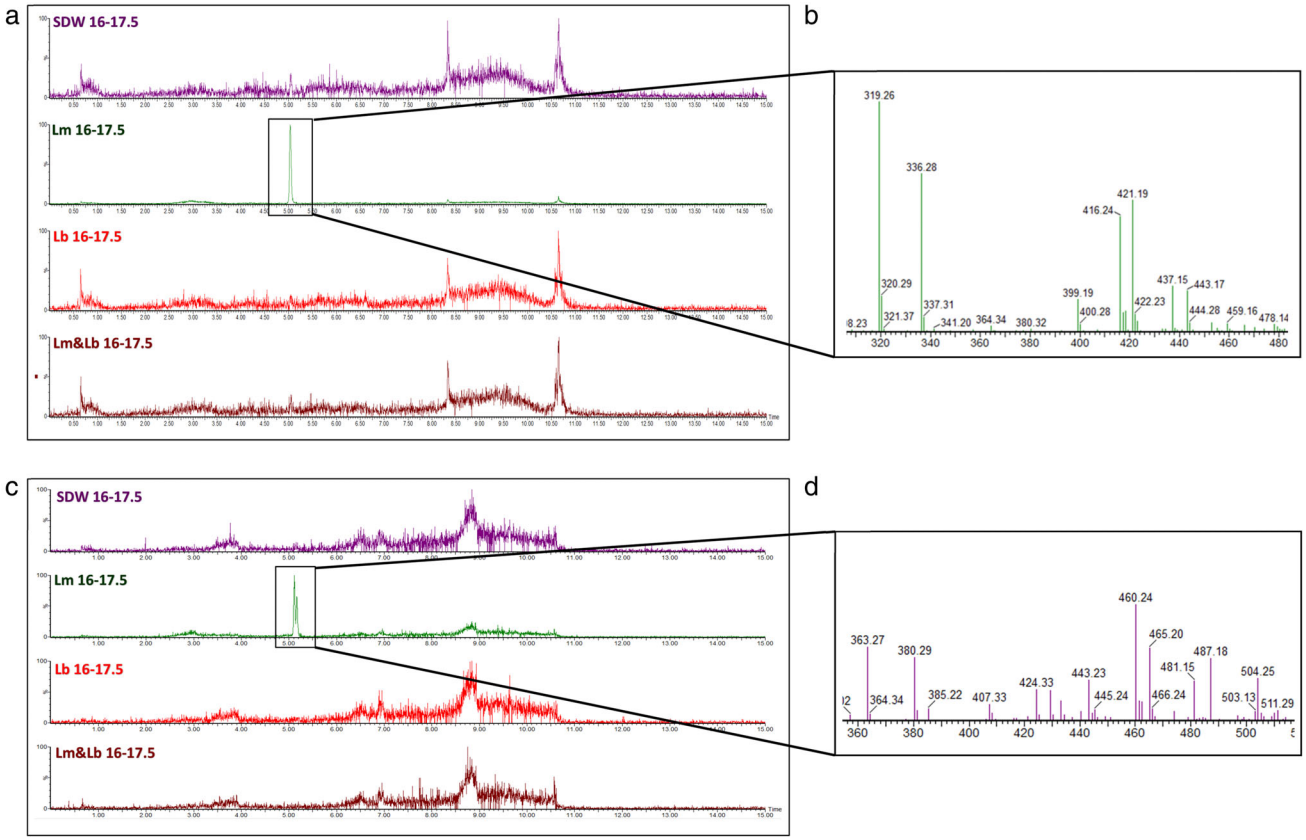

Figure 8. LC-MS chromatograms of HPLC fractions of secondary metabolite extracts from cotyledons of oilseed rape cultivar Charger at 26 days post inoculation. Cotyledons were inoculated with sterilised distilled water (SDW), *Leptosphaeria maculans* only (Lm only), *L. biglobosa* only (Lb only) or *L. maculans* and *L. biglobosa* simultaneously (Lm&Lb). HPLC fractions for 'SDW' (purple), 'Lm only' (green), 'Lb only' (red) or 'Lm&Lb' (brown) were taken from retention time 16.0–17.5 min. Unique maxima were found only in the 'Lm only' sample at retention times 5.03 min (a) and 5.11 min (c) and their ion spectrograms (b and d, respectively).

of this study suggest that the current advice on fungicide application threshold needs to be revised to integrate the appearance of *L. biglobosa* leaf spot lesions to protect the crops from both *Leptosphaeria* spp. because their interspecific interactions and inhibition of sirodesmin PL production may cause unforeseen challenges in the future.

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CONFLICT OF INTEREST

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.

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