Sequential infections by 32 isolates of Phoma medicaginis increase production of phytoestrogens in Medicago polymorpha var. brevispina

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Sequential infections by 32 isolates of *Phoma medicaginis* increase production of phytoestrogens in *Medicago polymorpha* var. *brevispina*

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ABSTRACT

Context. Studies of Phoma black stem and leaf spot disease (caused by *Phoma medicaginis*) in annual medics (*Medicago* spp.) normally involve a 'once-only' inoculation not reflecting multiple pathogen infection and phytoestrogen production cycles in the field. Phytoestrogen production by plants can result in lower ovulation rates in grazing animals. **Aims.** We aimed to determine whether sequential infections by *P. medicaginis* increase production of phytoestrogens in annual medics, and to measure the genetic diversity of isolates. **Methods.** In a greenhouse experiment, pathogenicity and virulence were investigated across 32 isolates of *P. medicaginis* following one, two or three rounds of inoculation of *M. polymorpha* var. *brevispina*. Production of the phytoestrogens coumestrol and 4′-*O*-methyl coumestrol was measured, and correlation with disease parameters assessed. DNA sequencing using *ITS*, *β-tubulin*, *calmodulin* and *P. medicaginis*-specific *EFNI-1α* was applied for phylogenetic analysis of isolates from Western Australia and elsewhere. **Key results.** Across isolates, highest leaf disease incidence was 76%, petiole disease incidence 61%, leaf disease severity 52% and petiole disease severity 53%. Stem coumestrol content range was 45–1247 mg kg^{-1}, and 4′-*O*-methyl coumestrol 0–344 mg kg^{-1}. All measures were highest after three rounds of inoculation. Overall, there was a positive correlation of leaf disease incidence with coumestrol content ($P < 0.05$) and of both leaf and petiole disease incidence with 4′-*O*-methylcoumestrol content ($P < 0.01$, $P < 0.05$, respectively). Phylogenetic analysis revealed a high degree of genetic similarity among Western Australian isolates, generally grouping into a single separate cluster across the four markers, and genetically distinct from isolates sourced outside Australia. **Conclusions.** Leaf disease incidence was the best discriminating disease parameter for coumestrol and 4′-*O*-methylcoumestrol content. Western Australian isolates of *P. medicaginis* were genetically similar and unique, possibly due to geographic separation. **Implications.** The study emphasised the importance of sequential inoculations when screening annual *Medicago* genotypes towards developing cultivars with superior disease resistance and enhanced animal reproductive outcomes.

Keywords: 4′-*O*-methylcoumestrol, annual medic, annual *Medicago* spp, coumestrol, genetic variation, phoma black stem and leaf spot, *Phoma medicaginis*, phytoestrogen.

Introduction

For decades there has been strong interest in annual *Medicago* species as high-quality forage legumes (Chatterton and Chatterton 1996). They are highly valued for animal production (Lüscher *et al.* 2014) and play a critical role in dryland farming systems (Barbetti *et al.* 2006). Phoma black stem and leaf spot (caused by *Phoma medicaginis*) is a damaging disease on both annual and perennial *Medicago* species in temperate and Mediterranean regions (Lamprecht and Knox-Davies 1984). It greatly reduces seed and herbage yield and, where severe, can cause incomplete defoliation and premature death in highly susceptible annual *Medicago* cultivars (Barbetti 1989*b*, 1995*a*; Barbetti and Fang 1991; Barbetti and Nichols 1991; Ellwood *et al.* 2006*b*). *Phoma medicaginis* has a wide host

range (e.g. Lamprecht and Knox-Davies 1984), also infecting *Pisum* and *Trifolium* species (Barbetti and Khan 1987). Classification of *Phoma* species can be challenging (Fatehi *et al.* 2003), particularly when based on morphology, where the major distinction from *Ascochyta* species has long been conidial size and aseptate conidia (Boerema and Bollen 1975); however, this has been resolved with the use of molecular sequencing (e.g. Kamphuis *et al.* 2008, 2012).

First disease symptoms are brown to black spots on leaves, stems and petioles, and as disease progresses, these coalesce causing chlorosis and finally abscission of leaflets and stem collapse (Barbetti 1987, 1989*b*). *Phoma medicaginis* can also cause significant root disease on annual *Medicago* species (Barbetti 1989*a*), and a rot complex in both crowns (Rodriguez and Leath 1992) and roots (Rodriguez 2005) of lucerne (*Medicago sativa*). In Western Australia, surveys showed it to be widely associated with root disease in annual *Medicago* species (You *et al.* 2000). The disease is particularly severe during wet weather conditions in spring when conidia from pycnidia within lesions are readily spread by rain splash (Barbetti 1987, 1989*b*; Barbetti *et al.* 2006; Ellwood *et al.* 2006*a*). Rising spring temperatures also foster the disease (Barbetti 1987, 1991), and in annual *Medicago* species, disease is most severe at day/night temperatures of 21°C/16°C, followed by 18°C/13°C, and least severe at 15°C/10°C (Barbetti 1987).

Phoma medicaginis infection stimulates production of phytoestrogens in annual *Medicago* species (Barbetti 1991, 1995*a*, 2007; Barbetti and Nichols 1991; Barbetti *et al.* 2006, 2020). Fields *et al.* (2018) reported that lucerne inoculated with *Stemphylium vesicarium* contained 169 ± 25.1 mg kg^{-1} of coumestrol compared with 3.4 ± 0.84 mg kg^{-1} in control plants. Phytoestrogens can provide some advantages to plants, such as protection against some fungal and bacterial pathogens (Wasserman *et al.* 2013) and against attack by pests (He and Dixon 2000; Deavours and Dixon 2005). However, they can result in lower ovulation rates in grazing animals, with consequent reduced reproduction or even infertility, particularly with sheep (Croker *et al.* 1994*a*, 1994*b*, 1999, 2005; Smith *et al.* 1979) but also with cattle (Barbetti *et al.* 2020).

These phytoestrogens are similar to the endogenous estrogens of mammals in terms of structure and function (Pierson and Ferkin 2015). The majority of phytoestrogens are phenolic compounds including isoflavones and coumestans, for which extensive research has been conducted. (Patisaul and Jefferson 2010). Phytoestrogen content in annual *Medicago* species can also increase in absence of plant disease (Francis and Millington 1965), for instance, in response to soil type (Francis and Millington 1971), superphosphate availability (Marshall and Parkin 1970) and stage of plant growth (Francis and Millington 1965). However, it is *P. medicaginis* that particularly stimulates elevated levels of phytoestrogens such as coumestrol (Barbetti and Fang 1991; Barbetti and Nichols 1991; Barbetti 2007) in annual *Medicago* species.

Although various management strategies such as close grazing (Barbetti 1989*b*; Barbetti *et al.* 2006, 2020) and application of fungicides (Barbetti 1983, 1989*b*) offer reduction of Phoma black stem and leaf spot, locating host resistance remains the most promising approach (Barbetti 1989*b*, 1990; Barbetti *et al.* 2020). In addition, our recent study (Omidvari *et al* 2021) highlighted that the intrinsic ability of annual *Medicago* cultivars to produce phytoestrogens in the absence of the pathogen, and their comparative ability to produce phytoestrogens in the presence of *P. medicaginis*, were together critical in developing more resistant cultivars with improved reproductive outcomes for grazing animals. However, in that study, we utilised a 'once-only' pathogen inoculation, which contrasts with what happens naturally in the field. There is a need for studies that better epitomise the in-field, multiple infection cycles in relation to final disease incidence/severity and production cycles of coumestrol and 4′-*O*-methyl coumestrol.

We hypothesised that levels of both Phoma black stem and leaf spot and phytoestrogens would be higher after multiple infection cycles. Therefore, in the present study, we investigated differences across leaf and stem disease incidence and severity during and after multiple infection cycles of 32 isolates of *P. medicaginis*, and the relationship of these disease parameters with production of coumestrol and 4′-*O*-methyl coumestrol. We also examined phylogenetically informative gene regions to determine the diversity of local isolates of *P. medicaginis* and how these compared with isolates from elsewhere. The local isolates had previously been investigated for their virulence and effect on phytoestrogen production across three annual *Medicago* cultivars (*M. truncatula* cv. Cyprus, *M. polymorpha* var. *brevispina* cv. Serena, and *M. murex* cv. Zodiac) (Omidvari *et al.* 2021). The relative virulence of these isolates varied across different assessment parameters and their effects on phytoestrogen production were also different.

Materials and methods

Isolates of *P. medicaginis*

Thirty-two isolates of *P. medicaginis* collected from a range of annual *Medicago* species and cultivars in Western Australia were used. These were obtained from the Culture Collection at the Department of Primary Industries and Regional Development, Western Australia, as lyophilised cultures in glass ampules. Full isolate details are provided in Omidvari *et al.* (2021).

Inoculum preparation

Inoculum preparation was as described by Omidvari *et al.* (2021). Briefly, preserved isolates in lyophilised ampoules were subcultured onto Sanderson and Srb medium

(Dhingra and Sinclair 1995) for 21 days at 25°C (±2°C) under 16 h/8 h light/dark. After 3 weeks, 5 mL sterile water was added to each plate and then conidia were harvested by rubbing the colony surface with a bent glass rod. Conidial concentration of each isolate was adjusted to 5×10^6 spores mL^{-1} by using a haemocytometer counting chamber, and the inoculum produced was always applied immediately in virulence tests (Chihaoui *et al.* 2015).

Virulence tests

The highly susceptible *M. polymorpha* var. *brevispina* (Barbetti 1990, 1995*a*, 1995*b*) was used to evaluate the virulence of different isolates across multiple inoculations. A single cultivar was chosen in order to avoid cultivar × isolate interactions as found in our earlier study (Omidvari *et al.* 2021). To remove any potential fungal seed contamination and to ensure high germination rates, seeds were surface-sterilised with 70% ethanol for 30 s, washed with sterile distilled water, and then scarified to break hardseed dormancy (when freshly ripened they also contain embryo dormancy) using sandpaper. Seeds were then transferred to Petri dishes lined with moist, sterile blotting paper. Imbibed seeds were transferred to water agar plates and incubated at 25°C in darkness until radicles emerged (Mhadhbi *et al.* 2005). Seeds were then transplanted into pots 6 cm by 6 cm by 14 cm, containing a portion of steam-pasteurised potting mix that consisted of 2.5 m^3 fine composted pine bark, 1 m^3 coco peat, 5 m^3 brown river sand, 10 kg slow-release fertiliser Osmoform NXT (22 N:2.2 P_2O_5:9.1 K_2O:1.2 Mg + trace elements; Everris International, Geldermalsen, the Netherlands), 10 kg dolomite (CalMag), 5 kg gypsum clay-breaker, 5 kg extra fine limestone, 4 kg iron heptasulfate and 1 kg iron chelate. Potting mix was pasteurised at 63°C for 45 min. Each pot was sown with four seeds at 5 mm depth and pots were maintained in a naturally lit greenhouse at 18°C (±4°C), watered daily with deionised water and allowed to drain to field capacity. Plants were fertilised weekly, according to manufacturer's recommendation, with Thrive (Yates Australia, Sydney, NSW, Australia), containing macronutrients (25 N:5 P:8.8 K) and the full range of micronutrients needed for plant growth.

Plants were spray-inoculated with the conidial suspension of each isolate until run-off, using a hand-held and operated aerosol sprayer. Tween 20 (0.001%) was added to conidial suspensions to ensure that inoculation droplets remained attached to the plant. Control plants were also treated with 0.001% Tween 20®, but in sterile distilled water. Inoculated plants were then placed into clear plastic containers with lids, measuring 770 mm long, 570 mm wide and 475 mm high, used as inoculation/incubation chambers to maintain high humidity (40 pots per container). Walls and lids of incubation boxes were internally misted with sterile distilled water and box lids closed for 72 h post-inoculation to maintain high humidity (Ellwood *et al.* 2007). The whole experiment was fully repeated once.

Multiple inoculation timings and disease assessments

There were three inoculation timings. The first inoculation was when plants were 6 weeks of age, with assessments made *in situ* for the different disease parameters at 8 weeks of age (14 days post-inoculation (dpi)), and four plants from each isolate randomly picked for harvest a further 2 weeks later at 10 weeks of age (i.e. 28 dpi) for phytoestrogen assessments. The second and third inoculations were conducted when plants were 10 and 18 weeks old, respectively, with similar time intervals for disease assessments (14 dpi) and harvesting of plants for phytoestrogen analyses (28 dpi).

Four disease assessment parameters were used, as reported previously (Omidvari *et al.* 2021): (1) leaf disease incidence (%LDI), calculated as the percentage of leaves per pot showing disease symptoms; (2) leaf disease severity (%LDS), visually estimated as the leaf area diseased per pot as a percentage of the total leaf area; (3) petiole/stem disease incidence (%PDI), calculated as the percentage of petioles/stems per pot showing disease symptoms; (4) petiole disease severity (%PDS), visually estimated as the petiole/stem area diseased per pot as a percentage of the total petiole/stem area. All disease assessments were made by the same person throughout.

Phytoestrogen assessment

Solvents

Details of solvent use for phytoestrogen assessments are as provided previously (Omidvari *et al.* 2021). Coumestrol and 4′-*O*-methylcoumestrol standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ALB Technology (Henderson, NV, USA), respectively, and used to prepare a calibration curve from 1 to 100 mg L^{-1} with nine concentrations for each standard. The relationship between peak area and concentration was linear, with correlation coefficient (r) >0.9997 for both coumestrol and 4′-*O*-methylcoumestrol. High-pressure liquid chromatography (HPLC)-grade methanol, HPLC-grade acetonitrile and HPLC-grade trifluoroacetic acid were purchased from Sigma-Aldrich. Water was obtained from a Milli-Q water purification unit (Millipore Australia). The stock standard solutions (100 μg mL^{-1}) of coumestrol and 4′-*O*-methyl coumestrol were prepared in aqueous methanol (2:8 v/v) and stored in the dark at 4°C. A reference standard (10 μg mL^{-1}) for HPLC retention times was prepared daily by dilution of the stock solutions with aqueous methanol (2:8 v/v).

Quantitation

For phytoestrogen assessment, plants sampled for each isolate or control treatment were harvested and bulked together into a single composite sample. Details of quantification of phytoestrogens used in phytoestrogen assessments are as provided by Omidvari *et al.* (2021). Three weeks after the final disease assessment, stem samples were harvested from all treatments for analysis of contents coumestrol and 4′-*O*-methyl coumestrol. Stem samples were used because diseased leaves and petioles had dehisced and because stems are a reliable indicator or phytoestrogen levels (Barbetti and Fang 1991). Samples were oven-dried at 70°C, and finely ground in two steps: first with a mechanical grinder that coarsely ground the stem material, and second by freezing with liquid nitrogen and finely grinding the material in a mortar and pestle. Ground samples (0.2 g) were extracted via methodology adapted from Fields *et al.* (2018) with 2 mL aqueous methanol (2:8 v/v) in glass sample vials (8 mL). All stem samples were hand-mixed for 30 s and put on an end-over-end mixer for 16 h at room temperature. Samples were filtered through glass fibre syringe filters, and supernatant (1 mL) was transferred into 2-mL glass auto-sampler vials. Extracted samples were analysed for coumestrol and 4′-*O*-methyl coumestrol content by HPLC. For comparative analysis, the extracts were spiked with 4-hydroxycoumarin (50 μg mL^{-1}) as an internal standard and relative quantifications determined based on HPLC–diode array detector (DAD) peak areas that were normalised based on the area of the recovered internal standard peak. Retention times for coumestrol and 4′-*O*-methylcoumestrol were at 28.5 and 43.8 min, respectively. These two compounds were identified in the samples based on their relative retention times and absorbance spectra. The HPLC analyses were performed with an Agilent 1260 series instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, auto injector and DAD. Separation was performed using an Apollo reversed phase C18 column (250 mm × 4.6 mm ID, 5 μm particle size; Grace Discovery Sciences, Bannockburn, IL, USA) at 25°C. The mobile phase consisted of solvent A 0.1% (v/v) trifluoroacetic acid in water, and solvent B acetonitrile. The elution gradient was as follows: 0–40 min, 20–50% B; 40–41 min, 50–50% B; 41–50 min, 50–100% B; and held at 100% B for 10 min. The flow rate set at 1 mL min^{-1} with the compounds detected at 254 nm and injection volume 20 μL. The column was re-equilibrated for 5 min between samples. Amounts of coumestrol and 4′-*O*-methylcoumestrol were quantified as mg kg^{-1} dry weight of plant material extracted.

In addition to the above, three separate subsamples were taken from each of the dried harvest materials from each isolate × timing of inoculation treatment (and similarly for control treatment), and separately assessed for amounts of coumestrol and 4′-*O*-methylcoumestrol. The mean of the values for three subsamples is presented as the coumestrol or 4′-*O*-methylcoumestrol value for each isolate, or for the control. Preliminary studies (data not presented) highlighted that three subsamples ensured a reliable and repeatable measure of the level of coumestrol or 4′-*O*-methylcoumestrol present.

Molecular identification

DNA extraction

Sample DNA was extracted from 2-week-old fungal cultures of 28 isolates previously identified as *P. medicaginis* growing on Sanderson and Srb medium. The remaining four isolates were referenced according to their already available molecular identification details in GenBank. A modified procedure of Cenis (1992) was used for DNA extraction. Briefly, fresh mycelial mat, ~2 mm by 2 mm, was put into a 2-mL tube containing two ceramic beads (2 mm) and 300 μL extraction buffer (200 mM Tris–HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS). The mycelial mat was homogenised in a Precellys Evolution homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) at 2490*g* in two cycles of 60 s. Then, 3 M sodium acetate (150 μL, pH 5.2) was added and maintained at room temperature for 10 min. Tubes were then centrifuged at 16 816*g* for 10 min and the supernatant was transferred to a fresh 2-mL Eppendorf tube. An equal volume of isopropanol (~450 μL) was added to the tube and maintained for at least 15 min at 22°C to precipitate the DNA. Precipitated DNA was pelleted by centrifuging at 16 816*g* for 15 min, then washed with 70% w/v ethanol, vacuum-dried for 10 min and resuspended in 50 μL Tris-EDTA buffer. DNA samples were held at 4°C until needed.

Polymerase chain reaction amplification and DNA sequencing

The internal transcribed spacer (*ITS*) ribosomal DNA (rDNA) region can be of limited value for resolution of closely related species or strains (Ellwood *et al.* 2006*a*); therefore, three additional phylogenetically informative regions including *β-tubulin*, *calmodulin*, and *P. medicaginis*-specific elongation factor nested intron (*EFNI*)-*1α* were examined for distinguishing isolates. A PCR-based assay was conducted using each primer set to amplify a DNA product of predicted size from *P. medicaginis* isolates (Table 1) (Ellwood *et al.* 2006*a*). Amplification was performed in a volume of 25 μL with 1 μL each primer (final concentration of each primer 10 pmol $μL^{-1}$), 1 μL 10 ng DNA template, 12.5 μL DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA), and 9.5 μL nuclease-free water. DNA amplifications were carried out in a 9600 Thermocycler (PerkinElmer, Waltham, MA, USA) programmed at 95°C for 8 min, followed by 35 cycles at 95°C for 15 s, then 55°C (58°C for *β-tubulin*; Table 1) for 20 s, then 72°C for 60 s, then a final elongation step of 72°C for 5 min. The DNA products of predicted size from *P. medicaginis* were visualised by electrophoresis on a 1% (w/v) agarose gel

Table 1. Primer sets for PCR assay of target loci within *Phoma medicaginis* isolates used in this study.

Primer	Locus	Definition	Primer DNA sequence (5′→3′)	Annealing temp. (°C)	Product size (bp)
Bt1a	*β-Tub*	β-Tubulin	TTCCCCCGTCTCCACTTCTTCATG	58	350
Bt1b			GACGAGATCGTTCATGTTGAACTC		
CAL-228F	*CAL*	Calmodulin	GAGTTCAAGGAGGCCTTCTCCC	55	500
CAL-737R			CATCTTTCTGGCCATCATGG		
Forward	*EFNI-1α*	Elongation factor nested intron	CACACCACCATGCCCACA	55	400
Reverse			TTAGCATCTTGTCTTGAAACCT		
TW81	*ITS*	Internal transcribed spacer region	GTTTCC GTAGGTGAACCTGC	55	530
AB28			ATATGCTTAAGT TCAGCGGGT		

containing GelRed stain (diluted to 1/10 000) and viewing under UV light. PCR products were then sequenced by Macrogen, Seoul, South Korea.

Sequence alignment and phylogenetic analysis

The *P. medicaginis* isolates in this study were initially compared with closely related species using *ITS* sequence data alone because those data were available in GenBank. Then additional phylogenetic trees were constructed including *β-tubulin*, *calmodulin*, *P. medicaginis*-specific *EFNI-1α* fragments. The 32 isolates used in the phylogenetic tree included 28 sequenced isolates from this study with the addition of another four isolates (WAC4736, WAC4738, WAC4741 and WAC7980) previously sequenced by Ellwood *et al.* (2006*a*) (see accession numbers in Table 2).

For *ITS*, we selected the most closely related GenBank *ITS* sequences from five *P. medicaginis* reference isolates in GenBank (AY504634, CBS316.90, DQ092494, KF181254 and MK765001) and, with our 32 *ITS* isolates' sequence data, assembled a tree rooted to *Phaeosphaeria nodorum* (U77362). For the second tree, *P. medicaginis* isolate CBS316.90 from GenBank was included, with accession nos AY831518, AY831541, AY831548 and AY831563 (Table 2) relating to *β-tubulin*, *calmodulin*, *P. medicaginis*-specific *EFNI-1α* and *ITS* sequences, respectively. Sequence alignment and phylogenetic tree building were performed using Geneious v. 9.1.4 (Biomatters, San Diego, CA, USA). Sequence consensuses were used for BLAST in NCBI, and multiple alignment (alignment type, global alignment with free end gaps; cost matrix, 65% similarity (5.0/−4.0); gap open penalty, 12; gap extension penalty, 3) was used for phylogenetic tree building (genetic distance model, Tamura–Nei; tree building method, neighbour-joining tree; resampling method, bootstrap; random seed, 534 442; no. of replications, 100; support threshold, >50%).

Experimental design and statistical analyses

The whole greenhouse experiment was fully repeated once. There was one annual *Medicago* species (*M. polymorpha* var. *brevispina*), 32 *P. medicaginis* isolates and one control (mock inoculation), and three inoculation times and corresponding harvest times. The total number of pots in each experiment was 396, comprising cultivar (1) × isolates (33) × inoculation times (3) × replications (4), where 132 pots were inoculated once (at 6 weeks, harvested at 10 weeks), 132 pots were inoculated twice (at 6 and 10 weeks, and harvested at 14 weeks) and 132 pots were inoculated three times (at 6, 10 and 18 weeks and harvested at 22 weeks). Each experiment was arranged as a randomised block design using the 'Generate a Standard Design' function in GenStat 18.1 (18th Edition; VSNi, Hemel Hempstead, UK). Normality of data and homogeneity of the original and repeat experimental datasets were tested before conducting analyses. Data from the original and the repeat experimental datasets were not significantly different ($P > 0.05$) by *t*-test. Therefore, data from the original and repeat experiments were combined, and analysed as a single dataset. %LDI, %PDI, %LDS and %PDS were assessed. Fisher's least significant differences (l.s.d.s) were used to indicate significant differences between treatments. Correlations among all of the disease parameters and between all of the disease parameters and phytoestrogens were computed by using the linear regression function of GenStat.

Results

Disease expression across *P. medicaginis* isolates

There were significant effects of *P. medicaginis* isolate on %LDI, %PDI, %LDS and %PDS (all $P \leq 0.001$) in every round of inoculation, confirming variation for virulence across isolates of *P. medicaginis* as assessed across the different disease parameters. Control plants in all inoculation rounds remained disease-free (Table 3).

%LDI. For the first inoculation, the three most virulent isolates in terms of %LDI were WAC3704 (41%), WAC4744 (39%) and WAC4260 (38%) and three least virulent isolates were WAC3653, WAC4254 and WAC4741 (all 0%).

Table 2. *Phoma medicaginis* isolates and GenBank accession numbers for gene regions used in this study.

Isolate/source	GenBank accession number			
	β-Tubulin	*Calmodulin*	*EFNI-1α*	*ITS*
WAC3653	OK381631	OK381658	OK381605	MW342151
WAC3654	OK381632	OK381659	–	MW342152
WAC3655	OK381633	OK381660	OK381606	MW342153
WAC3656	OK381634	OK381661	OK381607	MW342154
WAC3657	OK381635	OK381662	OK381608	MW342155
WAC3658	OK381636	OK381663	OK381609	MW342156
WAC3659	OK381637	OK381664	OK381610	MW342157
WAC3704	OK381638	OK381665	OK381611	MW342158
WAC3705	OK381639	OK381666	–	MW342159
WAC3706	OK381640	OK381667	OK381612	MW342160
WAC4251	OK381641	OK381668	OK381613	MW342161
WAC4252	OK381642	OK381669	OK381614	MW342162
WAC4254	OK381643	OK381670	OK381615	MW342163
WAC4255	OK381644	OK381671	OK381616	MW342164
WAC4256	OK381645	OK381672	OK381617	MW342165
WAC4257	OK381646	OK381673	OK381618	MW342166
WAC4258	OK381647	–	OK381619	MW342167
WAC4259	OK381648	OK381674	OK381620	MW342168
WAC4260	–	OK381675	OK381621	MW342169
WAC4261	OK381649	OK381676	OK381622	MW342170
WAC4732	OK381650	OK381677	OK381623	MW342173
WAC4733	OK381651	OK381678	OK381624	MW342174
WAC4734	OK381652	OK381679	OK381625	MW342175
WAC4736	AY831516	AY831539	AY831553	AY831561
WAC4738	AY831515	AY831538	AY831552	AY831560
WAC4739	OK381653	OK381680	OK381626	MW342176
WAC4741	AY831514	AY831537	AY831551	AY831559
WAC4742	OK381654	OK381681	OK381627	MW342177
WAC4744	OK381655	OK381682	OK381628	MW342178
WAC4745	OK381656	OK381683	OK381629	MW342179
WAC6729	OK381657	OK381684	OK381630	MW342181
WAC7980	AY831510	AY831534	AY831549	AY831555
CBS316.90	AY831518	AY831541	AY831548	AY831563

Isolates prefixed WAC obtained from Department of Agriculture and Food, Western Australia (full isolate details provided in Omidvari *et al.* 2021); isolate prefixed CBS obtained from GenBank. *EFNI*, elongation factor nested intron; *ITS*, internal transcribed spacer region.

For the second inoculation, isolates WAC4736 (38%), WAC4251 (37%) and WAC4254 (36%) were most virulent and WAC4741 (12%) was least virulent. For the third inoculation, most virulent isolates were WAC4255 (76%) and WAC4734 (75%) and least virulent was WAC4741 (43%) (Table 3).

%PDI. The most virulent isolates in the first inoculation as assessed by %PDI were WAC4744 (27%) and WAC4732 (26%) and the least were WAC3653, WAC3656, WAC4254, WAC4736, WAC4738 and WAC4741 (all 0%) and WAC3706 (2%). For the second inoculation, the most virulent isolates were WAC4736 (22.5%), WAC4259 (21.1%), WAC4260 (20%) and WAC4739 (19.9%), whereas least virulent were WAC4255 (2%), WAC4741 (2%) and WAC3653 (3%). In relation to the third inoculation, most virulent were WAC4732 (61%) and WAC4260 (58%) and least was WAC7980 (13%) (Table 3).

Table 3. Phoma black stem and leaf spot disease parameters, namely leaf disease incidence (%LDI), petiole/stem disease incidence (%PDI), leaf disease severity (%LDS) and petiole/stem disease severity (%PDS) at 14 days post-inoculation, and production of coumestrol and 4′-*O*-methylcoumestrol in plants at 28 days post-inoculation, with 32 *P. medicaginis* isolates from Western Australia onto *M. polymorpha* var. *brevispina*.

Isolate	Inoculation round	%LDI	%PDI	%LDS	%PDS	Total coumestrol	Total 4'-*O*-methyl coumestrol
						(mg kg^{-1} stem dry weight)	
Control	First	0.0	0.0	0.0	0.0	45	0
	Second	0.0	0.0	0.0	0.0	90	0
	Third	0.0	0.0	0.0	0.0	142	19
	Mean	0.0	0.0	0.0	0.0	*92*	*6*
WAC3653	First	0	0	0	0	182	17
	Second	16.0	3.0	5.0	2.0	590	70
	Third	59.0	42.0	32.0	22.0	640	169
	Mean	25.0	15.0	12.3	8.0	471	85
WAC3654	First	18.0	7.0	16.0	6.0	316	15
	Second	30.0	7.0	32.0	2.0	356	60
	Third	48.0	45.0	46.0	29.0	358	149
	Mean	32.0	19.7	31.3	12.3	343	75
WAC3655	First	16.0	12.0	6.0	5.0	199	27
	Second	28.0	16.0	32.0	20.0	288	59
	Third	60.0	24.0	25.0	17.0	552	163
	Mean	34.7	17.3	21.0	14.0	346	83
WAC3656	First	6.0	0	6.0	0.0	282	38
	Second	29.0	12.0	20.0	11.0	332	45
	Third	54.0	32.0	21.0	20.0	442	181
	Mean	29.7	14.7	15.7	10.3	*352*	*88*
WAC3657	First	26.0	16.0	7.0	6.0	339	82
	Second	25.0	12.0	14.0	17.0	517	87
	Third	61.0	51.0	25.0	37.0	563	150
	Mean	37.3	26.3	15.3	20.0	473	106
WAC3658	First	15.0	9.0	6.0	5.0	265	20
	Second	26.0	14.0	7.0	10.0	663	112
	Third	66.0	41.0	32.0	22.0	817	284
	Mean	35.7	21.3	15.0	12.3	582	139
WAC3659	First	34.0	16.0	20.0	20.0	195	11
	Second	31.0	15.0	32.0	25.0	280	46
	Third	48.0	41.0	25.0	25.0	554	174
	Mean	37.7	24.0	25.7	23.3	343	77
WAC3704	First	41.0	14.0	30.0	19.0	229	11
	Second	30.0	8.0	27.0	7.0	424	69
	Third	57.0	46.0	20.0	21.0	741	268
	Mean	42.7	22.7	25.7	15.7	465	116
WAC3705	First	29.0	12.0	20.0	5.0	299	19
	Second	24.0	11.0	28.0	9.0	294	46
	Third	59.0	51.0	29.0	31.0	491	344
	Mean	37.3	24.7	25.7	15.0	361	136

(*Continued on next page*)

Table 3. (*Continued*).

Isolate	Inoculation round	%LDI	%PDI	%LDS	%PDS	Total coumestrol	Total 4'-O-methyl coumestrol
						(mg kg^{-1} stem dry weight)	
WAC3706	First	24.0	2.0	5.0	0.0	104	0
	Second	29.0	10.0	15.0	5.0	401	99
	Third	49.0	34.0	16.0	22.0	502	197
	Mean	34.0	15.3	12.0	9.0	336	99
WAC4251	First	27.0	16.0	10.0	11.0	264	29
	Second	37.0	19.0	36.0	14.0	282	70
	Third	60.0	42.0	21.0	16.0	796	194
	Mean	41.3	25.7	22.3	13.7	447	98
WAC4252	First	35.0	12.0	36.0	10.0	391	19
	Second	24.0	17.0	45.0	12.0	489	70
	Third	60.0	53.0	31.0	25.0	537	134
	Mean	39.7	27.3	37.3	15.7	472	74
WAC4254	First	0.0	0.0	0.0	0.0	215	17
	Second	36.0	19.0	42.0	14.0	486	56
	Third	62.0	55.0	38.0	35.0	573	160
	Mean	32.7	24.7	26.7	16.3	425	78
WAC4255	First	20.0	7.0	6.0	6.0	352	35
	Second	19.0	2.0	8.0	0.0	748	98
	Third	76.0	48.0	29.0	34.0	847	226
	Mean	38.3	19.0	14.3	13.3	649	120
WAC4256	First	28.0	13.0	5.0	5.0	223	11
	Second	31.0	15.0	22.0	6.0	790	120
	Third	56.0	46.0	16.0	18.0	934	184
	Mean	38.3	24.7	14.3	9.7	649	105
WAC4257	First	35.0	9.0	29.0	11.0	267	17
	Second	31.0	19.0	26.0	14.0	286	36
	Third	56.0	41.0	41.0	31.0	522	166
	Mean	40.7	23.0	32.0	18.7	358	73
WAC4258	First	29.0	15.0	16.0	11.0	447	54
	Second	27.0	17.0	31.0	21.0	526	119
	Third	69.0	54.0	42.0	52.0	631	247
	Mean	41.7	28.7	29.7	28.0	535	140
WAC4259	First	23.0	6.0	16.0	14.0	221	25
	Second	25.0	21.0	20.0	12.0	260	26
	Third	66.0	54.0	32.0	26.0	502	128
	Mean	38.0	27.0	22.7	17.3	328	60
WAC4260	First	38.0	20.0	29.0	21.0	332	35
	Second	34.0	20.0	24.0	17.0	347	43
	Third	64.0	58.0	45.0	42.0	559	181
	Mean	45.3	32.7	32.7	26.7	413	86
WAC4261	First	31.0	19.0	21.0	22.0	116	0
	Second	26.0	16.0	21.0	15.0	166	19

(*Continued on next page*)

Table 3. (*Continued*).

Isolate	Inoculation round	%LDI	%PDI	%LDS	%PDS	Total coumestrol	Total 4'-O-methyl coumestrol
						(mg kg^{-1} stem dry weight)	
	Third	55.0	48.0	34.0	27.0	351	91
	Mean	37.3	27.7	25.3	21.3	211	37
WAC4732	First	28.0	26.0	31.0	28.0	96	0
	Second	28.0	13.0	31.0	16.0	287	22
	Third	66.0	61.0	52.0	52.0	858	249
	Mean	40.7	33.3	38.0	32.0	414	90
WAC4733	First	26.0	11.0	22.0	22.0	212	20
	Second	29.0	19.0	16.0	19.0	341	53
	Third	47.0	42.0	38.0	34.0	505	207
	Mean	34.0	24.0	25.3	25.0	353	93
WAC4734	First	31.0	18.0	41.0	20.0	258	29
	Second	29.0	9.0	23.0	8.0	539	55
	Third	75.0	56.0	36.0	35.0	785	218
	Mean	45.0	27.7	33.3	21.0	527	101
WAC4736	First	7.0	0.0	6.0	0.0	390	30
	Second	38.0	23.0	37.0	34.0	415	29
	Third	58.0	50.0	51.0	53.0	451	146
	Mean	34.3	24.3	31.3	29.0	419	68
WAC4738	First	9.0	0.0	5.0	0.0	358	49
	Second	30.0	17.0	11.0	10.0	521	73
	Third	54.0	21.0	36.0	31.0	571	124
	Mean	31.0	12.7	17.3	13.7	483	82
WAC4739	First	24.0	13.0	5.0	5.0	207	9
	Second	31.0	20.0	31.0	16.0	679	135
	Third	55.0	32.0	19.0	17.0	1247	166
	Mean	36.7	21.7	18.3	12.7	711	103
WAC4741	First	0.0	0.0	0.0	0.0	99	0
	Second	12.0	2.0	5.0	1.0	781	41
	Third	43.0	19.0	16.0	8.0	1166	174
	Mean	18.3	7.0	7.0	3.0	682	72
WAC4742	First	26.0	12.0	20.0	11.0	349	32
	Second	21.0	12.0	7.0	10.0	828	80
	Third	67.0	38.0	31.0	28.0	968	180
	Mean	38.0	20.7	19.3	16.3	715	97
WAC4744	First	39.0	27.0	30.0	21.0	401	65
	Second	25.0	17.0	21.0	14.0	874	95
	Third	63.0	51.0	35.0	33.0	878	190
	Mean	42.3	31.7	28.7	22.7	718	117
WAC4745	First	34.0	20.0	38.0	20.0	182	19
	Second	25.0	17.0	24.0	22.0	567	60
	Third	61.0	33.0	24.0	22.0	682	193
	Mean	40.0	23.3	28.7	21.3	477	91

(*Continued on next page*)

Table 3. (*Continued*).

Isolate	Inoculation round	%LDI	%PDI	%LDS	%PDS	Total coumestrol	Total 4'-O-methyl coumestrol
						(mg kg^{-1} stem dry weight)	
WAC6729	First	34.0	22.0	39.0	29.0	401	51
	Second	31.0	17.0	23.0	7.0	598	56
	Third	54.0	21.0	12.0	10.0	607	197
	Mean	39.7	20.0	24.7	15.3	535	101
WAC7980	First	31.0	16.0	30.0	30.0	428	13
	Second	23.0	9.0	16.0	3.0	909	95
	Third	52.0	13.0	13.0	12.0	949	195
	Mean	35.3	12.7	19.7	15.0	762	101
First inoculation overall mean		23.2	11.2	16.7	11.0	263	*24*
Second inoculation overall mean		26.7	13.6	22.2	11.9	483	*65*
Third inoculation overall mean		57.0	40.7	29.2	26.9	658	*183*
Overall mean		35.6	21.8	22.7	16.6	468	*91*
Significance of isolates		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$		
l.s.d. ($P = 0.05$) isolates		2.8	2.2	2.3	2.0		
Significance of inoculation order		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$		
l.s.d. ($P = 0.05$) inoculation order		0.8	0.7	0.7	0.6		
Significance isolates × inoculation order		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$		
l.s.d. ($P = 0.05$) isolates × inoculation order		4.8	3.9	4.0	3.5		

Full isolate details are provided in Omidvari *et al.* (2021).

%LDS. For the first inoculation, the most virulent isolates with regard to %LDS were WAC4734 (41%), WAC6729 (39%) and WAC4745 (38%), whereas WAC3653, WAC4254 and WAC4741 rated nil LDS. For the second inoculation, the most virulent isolates were WAC4252 (45%) and WAC4254 (42.5%) and the least virulent were WAC3653 (5%), WAC4741 (5%), WAC3658 (7%) and WAC4742 (7%). For the third inoculation, most virulent were WAC4732 (52%) and WAC4736 (51%) and least virulent WAC6729 (12%) and WAC7980 (13%) (Table 3).

%PDS. For the first inoculation, most virulent isolates in terms of %PDS were WAC7980 (30%), WAC6729 (29%) and WAC4732 (28%), whereas WAC3653, WAC3656, WAC3706, WAC4254, WAC4736, WAC4738 and WAC4741 all rated no nil PDS. In the second inoculation, most virulent was WAC4736 (34%) and least virulent were WAC4255 (0%), WAC4741 (1%), WAC3653 (2%) and WAC3654 (2%), but these last four did not differ from the control plants. For the third inoculation, most virulent isolates were WAC4736 (53%), WAC4732 (52%) and WAC4258 (52%) and least virulent WAC4741 (8%) and WAC6729 (10%) (Table 3).

Disease expression across different rounds of inoculation

There were significant main effects of inoculation rounds on all disease parameters %LDI, %PDI, %LDS and %PDS (all $P \leq 0.001$) (Table 3). The highest %LDI was recorded for the third inoculation (57.6%) and the lowest for the first inoculation (23.5%), with the second inoculation intermediate (26.6%). The highest, intermediate and lowest %PDI occurred for the third (41.6%), second (13.8%) and first (11.6%) inoculations, respectively. With regard to %LDS, the third inoculation had the highest rating (30.1%), followed by the second (22.2%) and first (17.5%) inoculations. Likewise, for %PDS, the third inoculation had the highest rating (27.3%), the second inoculation was intermediate (12.3%), and the first inoculation had the lowest (11.3%) (Table 3).

Disease expression interaction effects

There was a significant *P. medicaginis* isolate × inoculation round interaction effect on all disease parameters %LDI, %PDI, %LDS and %PDS (all $P \leq 0.001$) (Table 3).

Leaf disease incidence (%LDI) was highest with isolates WAC4255 and WAC4734 in the third round of inoculation,

whereas isolates WAC3653, WAC4254 and WAC4741 showed the lowest %LDI in the first round of inoculation (Table 3).

Petiole disease incidence (%PDI), was highest for isolates WAC4732 and WAC4260 in the third round of inoculation, whereas it was lowest for isolates WAC4736, WAC3653, WAC3656, WAC4254, WAC4738, WAC4741 and WAC3706 in the first round of inoculation and for isolates WAC4741, WAC4255 and WAC3653 in the second round of inoculation (Table 3).

Leaf disease severity (%LDS) was highest for isolates WAC4732, WAC4736 in the third round of inoculation, whereas it was lowest for isolates WAC3653, WAC4741 and WAC4254 in the first round of inoculation (Table 3).

Petiole/stem disease severity (%PDS) was highest for isolates WAC4736, WAC4732 and WAC4258 in the third round of inoculation, whereas WAC4736 was one of six isolates with the lowest %PDS in the first round of inoculation (Table 3).

Phytoestrogen expression across different rounds of inoculation

There were highly significant overall effects of inoculation round in terms of phytoestrogen expression ($P \leq 0.001$) (Table 3). The highest level of coumestrol was for the third inoculation (658.3 mg kg^{-1} stem dry weight), and the lowest level for the first inoculation (262.5 mg kg^{-1}), and the level for the second inoculation was intermediate (483.4 mg kg^{-1}) (Fig. 1). Likewise, the highest level of 4′-*O*-methylcoumestrol was for third inoculation (183.2 mg kg^{-1}), the lowest for the first inoculation (24.2 mg kg^{-1}), and the level for the second inoculation was intermediate (65 mg kg^{-1}) (Fig. 1).

Phytoestrogen expression across *P. medicaginis* isolates

Overall, Phoma black stem and leaf spot resulted in very high levels of both coumestrol and 4′-*O*-methyl coumestrol compared with levels in uninoculated control plants (Table 3).

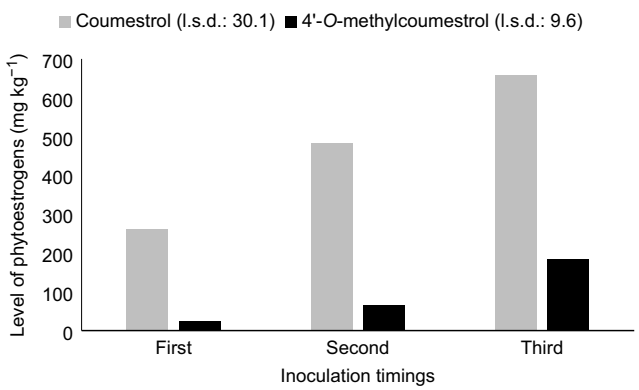

Fig. 1. Production of coumestrol and 4′-*O*-methylcoumestrol at 28 days post-inoculation for three inoculation timings: first at 6 weeks only; second repeated at 10 weeks; third repeated at 18 weeks after germination.

For the first inoculation, levels of coumestrol produced ranged from 45 to 447 mg kg^{-1} and levels of 4′-*O*-methyl coumestrol ranged from 0 to 82 mg kg^{-1}. The isolate that resulted in the highest level of coumestrol was WAC4258 (447 mg kg^{-1}), and the lowest levels of coumestrol were caused by isolates WAC4732, WAC4741, WAC3706 and WAC4261 (96, 99, 104 and 116 mg kg^{-1}, respectively). For the first inoculation, isolate WAC3657 resulted in the greatest production of 4′-*O*-methyl coumestrol (82 mg kg^{-1}).

For the second inoculation, the greatest amount of coumestrol was produced in plants inoculated with isolates WAC7980 and WAC4744 (909 and 874 mg kg^{-1}, respectively), and the least in plants inoculated with WAC4261 (166 mg kg^{-1}). In relation to 4′-*O*-methylcoumestrol, the highest production resulted from isolates WAC4739, WAC4256, WAC4258, WAC3658 (135, 120, 119 and 112 mg kg^{-1}, respectively).

For the third inoculation, the greatest amount of coumestrol resulted from inoculation with isolates WAC4739 and WAC4741 (1247 and 1166 mg kg^{-1}, respectively). In terms of 4′-*O*-methylcoumestrol, isolates WAC3705, WAC3658 and WAC3704 (344, 284 and 268 mg kg^{-1}, respectively) caused greatest production.

Phytoestrogen expression interaction effects

There was a significant *P. medicaginis* isolate × round of inoculation interaction effect on both coumestrol and 4′-*O*-methylcoumestrol production ($P \leq 0.001$) (Table 3). Production of coumestrol was greatest for isolates WAC4739 and WAC4741 in the third round of inoculation, whereas nearly one-third of the isolates produced the lowest level of coumestrol in the first round of inoculation (Table 3). Production of 4′-*O*-methyl coumestrol was highest for isolates WAC3705, WAC3658 and WAC3704 in the third round of inoculation, whereas isolates WAC3706, WAC4261, WAC4732 and WAC4741 did not produce any 4′-*O*-methyl coumestrol in the first round of inoculation (Table 3).

Correlation of phytoestrogen production with disease parameters

Overall, meaned across the three inoculations, there was a significant positive correlation of leaf disease incidence with coumestrol ($P < 0.05$), and of both leaf and petiole disease incidence with 4′-*O*-methyl coumestrol contents ($P < 0.01$ and $P < 0.05$, respectively). This was most evident in the first inoculation, where %LDI was significantly and positively correlated with production of coumestrol ($P < 0.05$), and third inoculation, where %LDI was significantly and positively correlated with coumestrol and 4′-*O*-methylcoumestrol ($P < 0.05$ and $P < 0.01$, respectively) and %PDI was correlated with 4′-*O*-methylcoumestrol production ($P < 0.05$).

Table 4. Correlation coefficients (*r*) among Phoma black stem and leaf spot disease parameters, namely leaf disease incidence (%LDI), petiole/stem disease incidence (%PDI), leaf disease severity (%LDS) and petiole/stem disease severity (%PDS), and for disease parameters with phytoestrogen contents in *Medicago polymorpha* var. *brevispina* in first, second and third inoculation rounds by *Phoma medicaginis* separately and meaned across all inoculation rounds as a group.

Disease parameter	Inoculation round	%LDI	%PDI	%LDS	%PDS	Coumestrol	4′-O-methyl coumestrol
%LDI	First	–	0.73**	0.70**	0.67**	0.30*	0.15n.s.
	Second	–	0.59**	0.58**	0.41**	0.10n.s.	0.16n.s.
	Third	–	0.66**	0.50**	0.52**	0.38*	0.55**
	All round of inoculations	–	0.87**	0.62**	0.73**	0.32*	0.58**
%PDI	First	–	–	0.65*	0.74*	0.20n.s.	0.22n.s.
	Second	–	–	0.49**	0.62**	0.15n.s.	0.03n.s.
	Third	–	–	0.67*	0.71**	0.003n.s.	0.37*
	All round of inoculations	–	–	0.63*	0.80*	0.15n.s.	0.36*
%LDS	First	–	–	–	0.78*	0.20n.s.	0.08n.s.
	Second	–	–	–	0.51**	0.20n.s.	0.04n.s.
	Third	–	–	–	0.79*	0.13n.s.	0.17n.s.
	All round of inoculations	–	–	–	0.74**	0.02n.s.	0.18n.s.
%PDS	First	–	–	–	–	0.15n.s.	0.03n.s.
	Second	–	–	–	–	0.20n.s.	0.11n.s.
	Third	–	–	–	–	0.10n.s.	0.28n.s.
	All round of inoculations	–	–	–	–	0.05n.s.	0.23n.s.

For significance of *r* values: *$P < 0.05$; **$P < 0.01$; n.s., not significant ($P > 0.05$).

Correlation between disease factors

Leaf disease incidence was significantly and positively correlated with %PDI, %LDS and %PDS in first, second and third inoculation rounds (all $P < 0.001$) and for all inoculation rounds grouped and meaned together ($P < 0.001$) (Table 4). %PDI was significantly and positively correlated with leaf disease severity %LDS and petiole disease severity (%PDS) in the first ($P < 0.05$ and $P < 0.01$), second (both $P < 0.001$) and third ($P < 0.01$ and $P < 0.001$ respectively) inoculation rounds. %LDS was significantly and positively correlated with %PDS in the first ($P < 0.05$), second ($P < 0.001$) and third ($P < 0.05$) inoculation rounds and for all inoculation rounds grouped and meaned together ($P < 0.001$) (Table 4).

Sequence alignment and phylogenetic analysis

ITS region

The *P. medicaginis* isolates were initially compared by using *ITS* sequence data alone because it provided the most readily available comparative data in GenBank. The phylogenetic tree constructed with 32 isolates of *P. medicaginis* as well as five accessions from GenBank (AY504634, KF181254, CBS316.90, MK765001 and DQ092494) separated into two clades (Fig. 2). The *P. medicaginis* isolates from GenBank clustered together to form Clade I. All isolates from the present study (including four *P. medicaginis* isolates previously sequenced by Ellwood *et al.* 2006*a*; shown in bold in Fig. 2) clustered together into Clade II. Generally, there was a very high degree of similarity for all isolates from Western Australia (Fig. 2). All sequences have been deposited in the GenBank database (see details in Table 2).

Concatenated sequencing of *β-tubulin*, *calmodulin* and *EFNI-1α*

Sequencing of *EFNI-1α* highlighted significant differences in length and sequence among *P. medicaginis* isolates and compared with other species. This dissimilarity inhibited sequence alignments with other species such that the phylogenetic tree using combined gene regions was solely constructed among *P. medicaginis* isolates. The tree of concatenated sequences of *β-tubulin*, *calmodulin* and *EFNI-1α* constructed for the 32 isolates of *P. medicaginis* and CBS316.90 retrieved from GenBank separated into two clades (Fig. 3). WAC3704 and WAC3706 clustered together in Clade I. All remaining isolates (including four isolates previously sequenced by Ellwood *et al.* 2006*a*) clustered together with CBS316.90 to form Clade II.

Discussion

All *P. medicaginis* isolates tested were virulent on *M. polymorpha* var. *brevispina*; however, the expression of

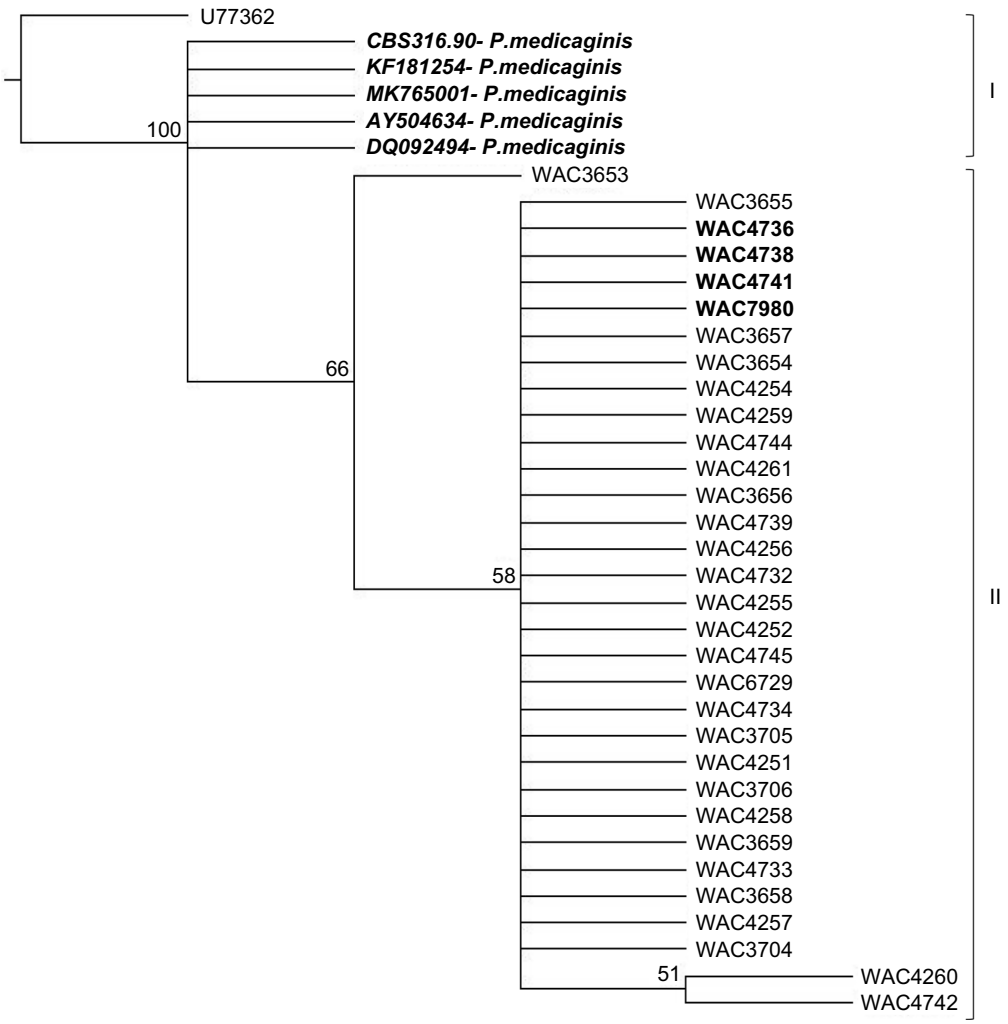

Fig. 2. Neighbour-joining tree of *ITS* generated by Geneious with the Tamura–Nei genetic distance model demonstrating the phylogenetic relationship of isolates from GenBank (in bold and italic) (Clade I) and 32 *Phoma medicaginis* isolates including four reference isolates from Western Australia previously sequenced by Ellwood *et al.* (2006*a*) (in bold). GenBank accession numbers for isolates of *P. medicaginis* submitted in the present study and as based on their *ITS* sequences are provided in Table 2. Branch support (bootstrap values) presented above branches based on 1000 bootstrap replicates. Tree is rooted to *Phaeosphaeria nodorum* (U77362).

their virulence could be similar or different in terms of the different leaf and stem/petiole disease parameters. For instance, isolates WAC4734, WAC4260 and WAC4732 showed greatest virulence on leaf and petiole/stem, whereas WAC4741 displayed low virulence on both leaves and petioles/stems. Omidvari *et al.* (2021) earlier demonstrated virulence variability among these isolates when tested on three cultivars from different annual *Medicago* species. Perhaps this is a consequence of the wide host range for *P. medicaginis* on foliage (e.g. Barbetti and Khan 1987) and roots (Rodriguez and Leath 1992; Djebali 2013) of *Medicago* species and even on other legumes (Barbetti 1984). Although *P. medicaginis* is 'a less-discriminating pathogen' as suggested by Ellwood *et al.* (2006*a*), it is a highly specialised necrotrophic fungus that can promote tissue death and also avoid or suppress host resistance responses. This contrasts with biotroph pathogens where race-specific gene-for-gene recognition is well established (Hammond-Kosack and Parker 2003). Clearly, characterisation of the genetic diversity of an even wider range of isolates of this pathogen would be beneficial.

There were effects of different timing of inoculations in the present study. %LDI, %PDI, %LDS and %PDS all increased with each subsequent inoculation applied to the plants. In-field, Phoma black stem and leaf spot disease increases during the growing season owing to 'natural exposure' to

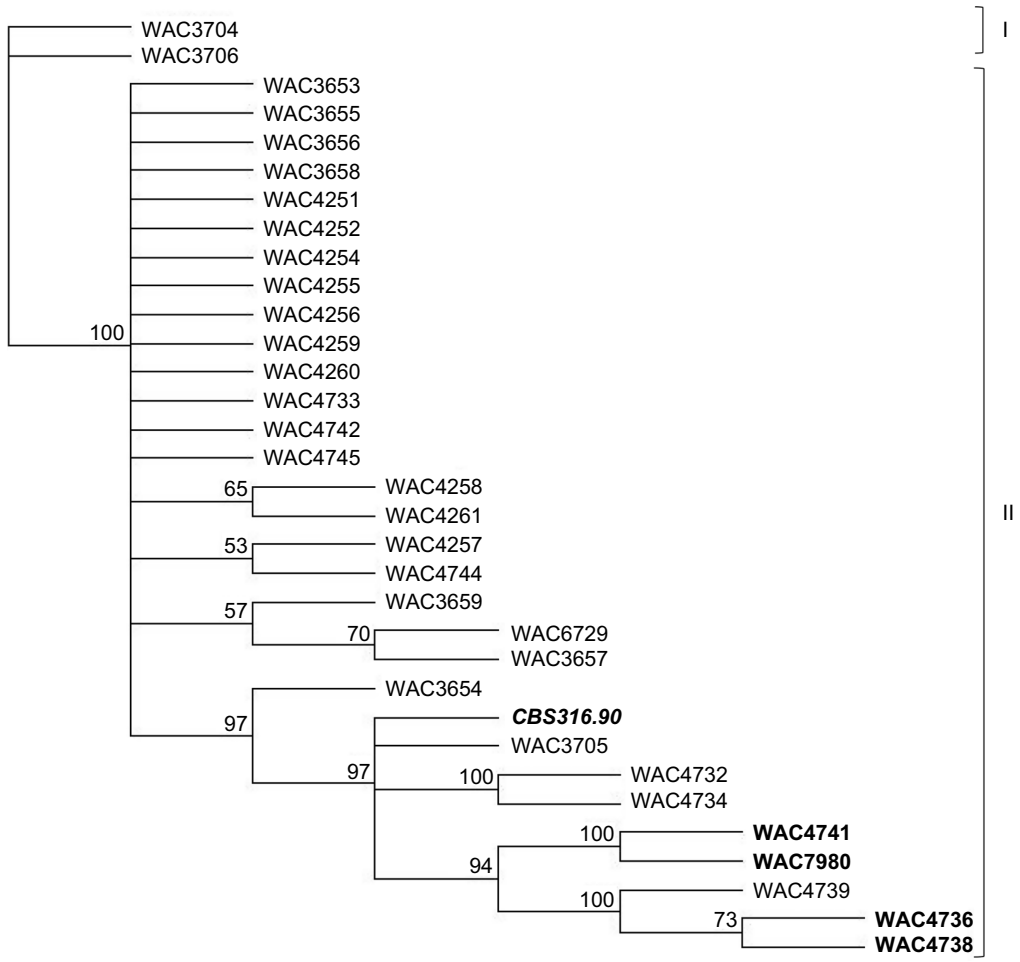

Fig. 3. Neighbour-joining tree of the concatenated sequences of *β-tubulin*, *calmodulin* and *EFNI-1α* sequence data (accession numbers are provided in Table 2) generated by Geneious with the Tamura–Nei genetic distance model. It demonstrates phylogenetic relationship of WAC3704 and WAC3706 (Clade I) and 30 other *Phoma medicaginis* isolates including four reference isolates from Western Australia previously sequenced by Ellwood *et al.* (2006*a*) (in bold) and isolate CBS316.90 from GenBank (in bold and italic). Branch support (bootstrap values) presented above branches based on 1000 bootstrap replicates.

multiple cycles of reinoculation, even to the extent that the disease causes defoliation and early plant death of very susceptible annual *Medicago* cultivars such as *M. polymorpha* var. *brevispina* cv. Sava. In lush stands, disease is most severe during conducive wet weather conditions in spring (Barbetti 1983, 1987, 1989*b*; Barbetti *et al.* 2006) that are ideal for conidial spread by rain splash (Ellwood *et al.* 2006*a*), along with rising seasonal temperatures that also help foster serious epidemics (Barbetti 1987).

This study highlights how the levels of both coumestrol and 4′-*O*-methylcoumestrol can increase in plants with each additional inoculation with *P. medicaginis*. This is an important consideration when screening potential new annual *Medicago* cultivars for resistance to this disease, because single-inoculated screenings under controlled environment conditions almost certainly underestimate both the maximum levels of phytoestrogens that can be produced and the adverse consequences for animals grazing medics in fields where there are multiple infection cycles over the life of the plant. It is plausible that the production of phytoestrogens may provide some advantage to the host, such as providing protection against attack by pests (He and Dixon 2000; Deavours and Dixon 2005); however, the adverse effects of phytoestrogens on animal productivity eclipse any such benefits (Smith *et al.* 1979; Barbetti *et al.* 2006; Barbetti 2007; Reed 2016). Importantly, there are some annual *Medicago* cultivars and accessions that show moderate disease resistance and produce lower levels of coumestans in the presence of pathogens (Barbetti 1995*b*, 2007). Such genotypes would be high value not only for

the animal feedbase industry directly, but also for annual *Medicago* species pre-breeding and breeding programs. Omidvari *et al.* (2022) indicated that disease-resistant accessions of annual *Medicago* species are available in germplasm collections, which will strengthen the utilisation of these species across wider ranging agroecosystems where they are one of the main plant types in forage systems. Most positive correlations of Phoma leaf disease incidence with coumestrol and 4′-*O*-methylcoumestrol content and of Phoma stem disease incidence with 4′-*O*-methylcoumestrol were when plants had been inoculated three times, perhaps due to multiple rather than single inoculations, and this perhaps reflects the multiple infections in the field with fungal foliar pathogens, which are well known to produce significant increases in coumestrol in annual *Medicago* species (Barbetti and Fang 1991; Barbetti and Nichols 1991; Croker *et al.* 1994*a*, 1994*b*; Tivoli *et al.* 2006; Reed 2016; Fields *et al.* 2018). It is clear that disease screening programs conducted in the greenhouse and aiming to develop future cultivars are best done using multiple infection cycles.

One concern is that *M. polymorpha* var. *brevispina*, the commercial cultivar used in the present study, is not as susceptible to *P. medicaginis* and consequent production of phytoestrogens as some highly susceptible cultivars such as *M. polymorpha* var. *brevispina* cv. Serena. In very susceptible cultivars, Barbetti (1995*b*) found that coumestrol was elevated to 1420 mg kg^{-1} in stems of severely diseased plants. Even a low incidence of Phoma black stem and leaf spot is sufficient for stimulation of coumestrol production (Fuchs *et al.* 1983). For example, although Barbetti (1995*a*) found levels of coumestrol of 230–500 mg kg^{-1} in pods of diseased plants without fungicide application, they still found 30–130 mg kg^{-1} following significant disease reduction from fungicides. This is not surprising, because accumulation of phytoestrogens can positively relate to resistance to pathogens in *Medicago* species (Barbetti 1995*a*, 2007; He and Dixon 2000; Dixon *et al.* 2002; Deavours and Dixon 2005; Deavours *et al.* 2006). Further, Croker *et al.* (1994*a*) highlighted that such correlation of incidence/severity of Phoma black stem and leaf spot with coumestrol content occurs with both green and dry annual medic plant material. Importantly, in the present study, the best discriminating disease parameter for coumestrol + 4′-*O*-methylcoumestrol production was %LDI, highlighting its usefulness as an indicator in annual *Medicago* breeding programs seeking combined disease resistance and low phytoestrogen production. Significant benefits of this method are that it can be utilised out of season, involves greater control over the screening process, offers a simpler method, and requires a smaller amount of precious breeder's seed than field screening. Although low disease incidence may not result in substantial losses of herbage and seed yields, low disease incidence can still stimulate production of phytoestrogen compounds produced as phytoalexins in plants (Fuchs *et al.* 1983).

Control plants produced coumestrol in the absence of Phoma black stem and leaf spot disease; however, they produced 4′-*O*-methylcoumestrol only following the third round of inoculation. Phenolic compounds such as flavonoids and isoflavonoids, including coumestans, are an important aspect in legume plant defence (Dixon 2001), and there is evidence for their accumulation over time in the absence of obvious disease (Barbetti *et al.* 2020). For example, Reid (1990) reported that some healthy, vegetative annual *Medicago* cultivars such as Jemalong and Cyprus may contain substantial concentrations of coumestans. Although phytoestrogen levels in the absence of disease were lower than in the presence of disease, dietary coumestrol concentrations as low as 25 mg kg^{-1} can still significantly affect ovulation rates in sheep (Smith *et al.* 1979). However, it is evident that most of the production of these compounds occurs in response to fungal pathogens in *Medicago* species (e.g. Deavours *et al.* 2006; Barbetti 2007; Kamphuis *et al.* 2008; Fields *et al.* 2018). Kamphuis *et al.* (2012) reported, for both resistant and susceptible plants inoculated with *P. medicaginis*, induction of genes involved in the synthesis of flavonoids, particularly of the phenylpropanoid pathway in susceptible interactions. They suggested that this may be due to a greater number of cells successfully penetrated by *P. medicaginis* in susceptible than resistant interactions, resulting in higher isoflavonoid transcript levels. Clearly, there is a need to monitor the intrinsic levels of phytoestrogen for all potential new annual *Medicago* varieties, and especially for those targeted for disease-prone areas.

Phylogenetic analysis based on *ITS* sequence data showed that all *P. medicaginis* isolates from this study, including the four previously sequenced by Ellwood *et al.* (2006*a*), clustered together into a single clade (Clade II). Available reference strains obtained from GenBank from various locations outside Australia including Germany, Czechoslovakia, United States and China separated together into a single cluster (Clade I), and this cluster did not contain any of the isolates from Western Australia. Accordingly, whereas there is a very high degree of similarity for all isolates from Western Australia used in the present study, these isolates show a clear overall genetic distinctiveness from those originating outside Australia. Furthermore, the majority of the studied isolates clustered together in the tree of the concatenated sequences of *β-tubulin*, *calmodulin* and *EFNI-1α*. Similarly, Ellwood *et al.* (2006*a*) reported that Australian isolates were distinct from isolates obtained from other countries. Possible reasons for the phylogenetic separation of Western Australian isolates from those sourced from other countries may be genetic isolation combined with the absence of any sexual reproduction in Australia. Although the sexual stage of this fungus was reported for the first time in Russia in 2017 (Jayasiri *et al.* 2017), it remains an anamorphic ascomycete in Australia with no known sexual stage. Considering that *P. medicaginis* readily disseminates conidia by rain splash, wind and insects (Leath 1990) and is spread on seed as

hyphae on or inside the seed surface (Kernkamp and Hemerick 1953), highly related clonal lineages should be common within a location, such as in the present study for Western Australian isolates. Similarly, Castell-Miller *et al.* (2007) demonstrated that clustering of isolates from northern or southern areas of Minnesota did not reveal any clear relationship to 'adjacent' geographical locations, suggesting that the effective population size *P. medicaginis* is geographically large.

In the present study, molecular phylogenetic analysis grouped most of the isolates from different *Medicago* cultivars together and suggested a close genetic relationship among the majority of isolates recovered from different hosts and/or cultivars in Western Australia; however, there were clear isolate differences in terms of their virulence and disease patterns. Similarly, Ellwood *et al.* (2006*a*) showed that even though isolates WAC4736, WAC4741 and OMT5 exhibited various disease levels they were still clustered together based on *ITS* sequence data. However, our study highlighted that these isolates clustered separately based on *calmodulin*, *β-tubulin* and *P. medicaginis*-specific *EFNI-1α*. Australia is separated by oceans from other continents, and Western Australia is separated from other states of Australia by an arid inland region, perhaps limiting gene flow to the extent of maintaining uniqueness of Western Australian isolate genetic diversity. Finally, the fact that isolates from different *Medicago* species in this study clustered together suggests that isolates lack host specialisation, and as noted above, perhaps in part due to *P. medicaginis* being a less-discriminating pathogen (Ellwood *et al.* 2006*a*). This again underlines the need for further characterisation of the wider *P. medicaginis* population structure, genetic diversity and host specificity.

In conclusion, the study highlights variation in virulence levels in *P. medicaginis* populations, and consequent variation in disease incidence and severity in *M. polymorpha* var. *brevispina*. These variations in virulence will assist in explaining the differing levels of Phoma black stem and leaf spot in different annual *Medicago* forages and across different locations and seasons. The correlation of disease incidence with stimulation of production of phytoestrogenic compounds to levels that could adversely affect ovulation rates of grazing animals, particularly sheep, is important for prediction of which annual *Medicago* pastures are most likely to be problematic for grazing animals. That the level of phytoestrogens increased with multiple inoculation rounds is of concern for animal producers in disease-prone regions. It is critical that pre-breeding and breeding programs consider and accommodate the need for sequential inoculations when developing annual *Medicago* cultivars with superior disease resistance in combination with enhanced animal reproductive outcomes. Until such improved cultivars are available, the safest option for farmers grazing annual *Medicago* species in disease-prone regions is to consider carefully the potential for high phytoestrogen levels that could be induced following multiple cycles of *P. medicaginis* reinfection in infested stands.

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

Conflicts of interest. The authors declare that they have no conflicts of interests with regard to the conduct or publication of these studies.

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