Identification of Sources of Resistance to *Phoma medicaginis* Isolates in *Medicago truncatula* SARDI Core Collection Accessions, and Multigene Differentiation of Isolates

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ABSTRACT

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Phoma medicaginis is a necrotrophic fungal pathogen, commonly found infecting the annual medic *Medicago truncatula*. To differentiate eight *P. medicaginis* isolates, five gene regions were examined: actin, β -tubulin, calmodulin, translation elongation factor 1- α (EF-1 α), and the internal transcribed spacer ribosomal DNA. Sequence comparisons showed that specimens isolated from *M. truncatula* in Western Australia formed a group that was consistently different from, but allied to, a *P. medicaginis* var. *medicaginis* type specimen. EF-1 α contained a hyper-

variable 55-bp repeat unit, which forms the basis of a rapid polymerase chain reaction-based method of reliably distinguishing isolates. Characterization of three isolates showed that all exhibited a narrow host range, causing disease only in *M. sativa* and *M. truncatula* among eight commonly cultivated legume species sampled. Infection of 86 *M. truncatula* single-seeded accessions showed a continuous distribution in disease phenotypes, with the majority of accessions susceptible. On a 1-to-5 disease reaction scale increasing in severity, individual fungal isolates showed means of 2.6 to 3.2, and scores ranged from 1 to 4.8 among accessions. The results presented here suggest that *M. truncatula* harbors specific and diverse sources of resistance to individual *P. medicaginis* genotypes.

Phoma medicaginis is one of the most serious necrotrophic pathogens on *Medicago sativa* (alfalfa) in temperate and Mediterranean regions (20,28,39). On this crop, the disease is known as spring black stem and leaf spot. Disease symptoms typically include seedling blight, stem canker, and leaf spot. Infected leaves undergo chlorosis and defoliate, while stem and petiole lesions may progress, causing crown and root rot. The disease spreads rapidly in cool and wet conditions because pycnidia within lesions disperse conidia by rain splash.

P. medicaginis is also a significant pathogen on *M. truncatula* in Australia, where *M. truncatula* is one of the most widely grown annual medics, often in legume pasture-cereal rotations (11,34). Reductions in seed and herbage yield occur, with almost complete defoliation and premature death in the most susceptible cultivars (1,3). *M. truncatula* is widely recognized as a model for legume genomics and as a reference species to identify important genes in crop species through comparative mapping (10,44,45). Increasingly, this species also is being used to dissect the genetic basis of resistance to necrotrophic diseases and to identify new sources of resistance that may not be available in crops; for example, against *Aphanomyces euteiches* (32), *Ascochyta rabiei*, and *A. lentis* (T. Pfaff, *personal communication*), *Colletotrichum trifolii* (41),

Fusarium oxysporum (J. Lichtenzveig, *personal communication*), and *Phytophthora medicaginis* (N. D'Souza, *personal communication*).

Previous studies in annual *Medicago* spp. have shown genetic variability in *Phoma medicaginis* resistance (31), although no extensive screens have been carried out in *M. truncatula* itself. This study uses accessions from the South Australian Research and Development Institute (SARDI) core collection. The core collection was created by Skinner et al. (37) using over 4,000 *M. truncatula* accessions collected from the Mediterranean basin since the 1960s, and contains three subspecies as defined by Heyn on the basis of pod characteristics (23): *M. truncatula* subsp. *truncatula*, *longeaculeata*, and *tricycla*. The core is highly diverse, with an average of 25 microsatellite alleles per locus among 192 different accession individuals sampled (12).

Phoma isolates causing disease on *Medicago* spp. normally are ascribed to *P. medicaginis* Malbr. & Roum. in Roum. var. *medicaginis* Boerema (syn. *P. herbarum* Westend. var. *medicaginis* Fckl. and *Ascochyta imperfecta* Peck), an ascomycete with no known teleomorph. The CABI Bioscience world database of fungal names lists *P. medicaginis* as occupying the group *Incertae sedis* (of uncertain placement) within the class *Dothideomycetes* and subclass *Pleosporales*. *Phoma* is a complex genus that has been described as the anamorph phase of a number of different species, such as *Didymella rabiei* and *Mycosphaerella pinodes*. The *Phoma* isolates in this study originally were identified on the basis of morphology, the major distinction with *Ascochyta* spp. being conidial size and aseptate conidia (5,8). However, these features are poorly defined and inconsistent, making classification of *Phoma* spp. difficult (15). Genetic studies have helped to resolve relatedness, particularly among *Ascochyta* species (7,14,33).

The objectives of this study were to distinguish *P. medicaginis* genotypes isolated from *Medicago truncatula* by examining phylo-

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genetically informative gene regions, to evaluate SARDI core accessions for sources of resistance to *P. medicaginis*, and examine the interaction between pathogen genotype and host resistance.

MATERIALS AND METHODS

Fungal isolates. All available *P. medicaginis* isolates recovered from *M. truncatula* in Western Australia (WAC4736, 4738, 4741, 7977, and 7980) were obtained from the Department of Agriculture and Food, Western Australia (DAFWA, South Perth, Western Australia). Isolates OMT1 and OMT5 were isolated from naturally infected *M. truncatula* in Perth, Western Australia. *P. medicaginis* var. *medicaginis* CBS316.90 and *P. pinodella* CBS318.90 standard reference cultures were acquired from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). Other species sequenced in this study from DAFWA included *Leptosphaerulina trifolii* WAC6693, *P. pinodella* WAC7978, *P. exigua* WAC7988, and *Ascochyta lentis* SATAL (Table 1). Representative cultures of OMT1 and OMT5 have been deposited with DAFWA.

Fungal isolation and culture. Isolates OMT1 and OMT5 were isolated by surface sterilization of diseased material (5% ethanol and 1% sodium hypochlorite for 1 min), followed by rinsing with distilled water and plating onto tap water agar with benzimidazole (50 ppm). The plates were incubated at 22°C for 24 to 48 h, and the leading edge of colonies subcultured onto wheat meal agar (WMA; 12 g of ground wheat meal, 12 g of agar, and 1 liter of distilled water). Conidia were harvested at 14 days and single spore cultures produced to ensure homogeneity.

DNA isolation and sequencing. Fungal isolates were grown in 10 ml of half-strength potato dextrose broth (19.5 g of potato dextrose and 1 liter of distilled water) (Becton Dickinson, Franklin Lakes, NJ) at 22°C for 48 h. Mycelial mats were blotted to remove excess medium. Genomic DNA was obtained by modifying a protocol described by Matallana et al. (30). Fungal material (0.1 to 0.2 g) was ground in liquid nitrogen, homogenized in 500 µl of extraction buffer (50 mM Tris, pH 8, 25 mM EDTA, pH 8, 100 mM NaCl, and 1% sodium dodecyl sulfate), combined with 20 µl of proteinase K (Sigma) at 2.5 mg/ml, and incubated at 65°C for 1 h. The samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol). DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 6) and 0.7 volume of isopropanol and pelleted at 20,000 × g for 15 min. The samples were washed with 80% ethanol, resuspended in 100 µl of Tris-EDTA (10 mM Tris and 1 mM EDTA, pH 8), and treated with 1 µl of RNAase (10 mg/ml, 1 h at 37°C).

Polymerase chain reaction amplification and DNA sequencing. The internal transcribed spacer (ITS) ribosomal DNA (rDNA) region is of limited value in resolving closely related species or strains, as shown for example in the genera *Botryosphaeria* (38) and *Cladosporium* (43). Therefore, four additional phylogenetically informative regions—actin, β-tubulin, calmodulin, and translation elongation factor 1α (EF-1α)—were examined for their effectiveness in distinguishing isolates.

Primers ITS1 and ITS4 (42) were used to amplify ITS1, the 5.8S subunit, and ITS2 of rDNA. Primers bt2a and Bt2b were used to amplify β-tubulin (19). β-Tubulin, calmodulin, and EF-1α gene regions were amplified using primer sets described by Carbone and Kohn (9). We designed *P. medicaginis*-specific elongation factor nested intron (EFNI)-1α primers to sequence beyond G/C-rich stretches. Primer sequences were forward, 5'-CACACCACCATGCCCCACA-3' and reverse, 5'-TTAGCATCTTGCT-TGAAACCT-3'.

Actin primers were designed to anneal to conserved regions of the *Act1* gene in *Septoria tritici* (GenBank AJ300310), *Cercospora beticola* (AF443281), *Botryotinia fuckeliana* (AJ000335), *Cladosporium herbarum* (AJ300320), and *Colletotrichum gloeosporioides* (AF112537). The primers amplify an approximately 400-bp intron-spanning fragment. Primer sequences were forward, 5'-TTGACCGACTACCTCATGAAGT-3' and reverse, 5'-AGATACCTGGGTACATGGTGGT-3'.

Polymerase chain reaction (PCR) products were purified with UltraClean PCR Cleanup columns (MolBio, Solana Beach, CA). Amplicons were direct sequenced with BigDye v. 3.1 Terminator Cycle Sequencing Ready Reaction Mix (ABI, Foster City, CA) and run on an ABI Prism 377 DNA sequencer.

Phylogenetic analyses. To compare *P. medicaginis* isolates from *M. truncatula* with related species, ITS sequence data from the six additional species sequenced in this study (Table 1) and ITS sequences from eight GenBank ITS species were assembled. The most closely related GenBank sequences were *D. applanata* DAP428170, *D. bryoniae* AF297228, *D. pinodes* AF362064, *Epicoccum nigrum* AF455455, *P. glomerata* PGL428533, and *P. herbarum* AY293803. *Leptosphaeria maculans* L07735 and *Phaeosphaeria nodorum* PNU77362 were selected as distant Dothideomycetes.

Alignments were generated using the ClustalW algorithm in BioEdit v.5.0.9 (21). Gaps were treated as a fifth character, and all ambiguous characters and parsimony uninformative characters were excluded prior to analysis. Partition homogeneity tests (25) were conducted using PAUP* version 4.0b10 (40) to determine

TABLE 1. Fungal isolates and GenBank accession numbers for gene regions used in this study

Species	Isolate/source ^b	Host	Date, location	GenBank accession numbers ^a				
				Actin	β-Tubulin	Calmodulin	EFNI-1α	ITS ^c
<i>Ascochyta lentis</i>	WAC SATAL	<i>Lens culinaris</i>	1998, Merredin, WA	...	...	...	...	AY131201
<i>Leptosphaerulina trifolii</i>	WAC6693	<i>Medicago polymorpha</i>	1992, Avondale, WA	...	...	...	...	AY831558
<i>Phoma exigua</i>	WAC7988	<i>M. sativa</i>	1981, Harvey, WA	...	...	...	...	AY131200
<i>P. pinodella</i>	CBS318.90	<i>Pisum sativum</i>	1990, Netherlands	...	...	...	...	AY831562
<i>P. pinodella</i>	WAC7978	<i>M. truncatula</i>	1981, Perth, WA	...	...	...	...	AY831556
<i>P. medicaginis</i>	OMT1, TS	<i>M. truncatula</i>	2000, Perth, WA	...	...	...	...	AY131199
<i>P. medicaginis</i>	OMT5, TS	<i>M. truncatula</i>	2000, Perth, WA	AY831519	AY831507	AY831531	AY831547	AY831554
<i>P. medicaginis</i>	WAC4736	<i>M. truncatula</i>	1986, Gnowangerup, WA	AY831528	AY831516	AY831539	AY831553	AY831561
<i>P. medicaginis</i>	WAC4738	<i>M. truncatula</i>	1986, Gnowangerup, WA	AY831527	AY831515	AY831538	AY831552	AY831560
<i>P. medicaginis</i>	WAC4741	<i>M. truncatula</i>	1986, Gnowangerup, WA	AY831526	AY831514	AY831537	AY831551	AY831559
<i>P. medicaginis</i>	WAC7977	<i>M. truncatula</i>	1981, Perth, WA	AY831524	AY831512	AY831536	AY831550	AY831557
<i>P. medicaginis</i>	WAC7980	<i>M. truncatula</i>	1981, Perth, WA	AY831522	AY831510	AY831534	AY831549	AY831555
<i>P. medicaginis</i> var. <i>medicaginis</i>	CBS316.90	<i>M. sativa</i>	1990, Czechoslovakia	AY831530	AY831518	AY831541	AY831548	AY831563

^a EFNI = elongation factor nested intron.

^b Isolates prefixed CBS were obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). Isolates prefixed WAC were obtained from the Department of Agriculture and Food, Western Australia (South Perth, Western Australia). The DNA sequences for OMT1 were identical to OMT5 where GenBank accession numbers are not provided. TS = this study.

^c ITS = internal transcribed spacer region.

whether the sequence data from different gene regions were congruent. Phylogenetic analysis based on parsimony was performed on separate and combined data sets in PAUP. The most parsimonious trees were obtained by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple equally most parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (24). In the initial analyses, all characters were unweighted and unordered; thereafter, characters were reweighted according to the consistency index. Branch and branch node supports were determined using 1,000 bootstrap replicates (16) and characters were sampled with equal probability (however, weights were applied).

Plant cultivation. The *M. truncatula* core collection accessions were acquired from the Genetic Resource Centre, SARDI (GPO Box 397, Adelaide, South Australia 5001). Accessions DZ315 and DZ045 were obtained from the Institut National de la Recherche Agronomique (INRA), Montpellier, France. To ensure homogeneity, a single randomly sampled individual from each accession was single-seeded for two generations. Experiments were conducted in two stages. Isolate pathogenicity on an initial set of 10 *M. truncatula* accessions and the host range among seven other legume species was examined. Virulent isolates then were screened on 86 *M. truncatula* accessions. The initial set of *M. truncatula* accessions was composed of A17, Borung, DZ315, DZ045, Sa8618, Sa11734, Sa15951, Sa24968, Sa27063, and Sa30199. *M. sativa* accessions Sa35043, Sa36325, and Sa38082

were obtained from SARDI. *Lupinus albus* cv. Kiev Mutant was obtained from the DAFWA. Seed for other cultivars were sourced commercially. These included: *Cicer arietinum* cv. Sona; *Lens culinaris* cv. Digger; *Lupinus angustifolius* cvs. Unicrop, Belara, and Quinlock; *Pisum sativum* cv. Parafield; *M. sativa* cv. Sceptre; and *Vicia faba* cv. Fiord.

To ensure even germination, seed were scarified with sand using a mortar and pestle, transferred to a petri dish lined with blotting paper, and irrigated with sterile water. The seed were held at 4°C for 48 h, and germinated at room temperature overnight. Seedlings were sown in pasteurized composted pine bark fines (Richgro Garden Products, Jandakot, Western Australia), and grown under natural light in a temperature-controlled greenhouse (22 and 18°C, day and night, respectively).

Plant inoculations. Inoculum was prepared by growing fungal isolates on WMA at 22°C for 21 to 28 days under UV. Conidia were harvested by incubating plates with 10 ml of distilled water for 20 min. The suspension was filtered through glasswool, adjusted to 1×10^6 spores/ml, and Tween 20 added to 0.05%. Inoculation of *Medicago* accessions was performed at the fourth trifoliate stage, or between 3 and 4 weeks of age for species of other legume genera. Two methods of inoculation were used. Initial pathogenicity and host range tests were performed by spraying with an artist's airbrush to runoff, using a rotating platform to ensure an even distribution over plant surfaces. Screens of isolates on 86 *M. truncatula* accessions used spot inoculation with 10- μ l droplets of spore suspension on five leaves per plant. These were the unifoliate leaf, the middle and one lateral leaflet of the first trifoliate leaf, and the middle and one lateral leaflet of the second trifoliate leaf. This method was developed because *M. truncatula* accessions of the SARDI core are highly variable in their growth habit, leaf architecture, and surface characteristics (i.e., presence or absence of leaf hairs), which affects the volume of inoculum adhering to the leaf surface. To ensure high humidity to stimulate conidia germination, plants were placed in sealed propagator trays for 48 h.

A minimum of three plants per replicate pot and three replicates per accession were inoculated. Plants were evaluated macroscopically for disease phenotype at 7 days postinoculation (dpi), and rescored at 10 dpi to confirm more resistant disease reactions. Disease scores of accessions with extreme phenotypes were verified independently. Spray-inoculated plants were evaluated according to the 1-to-5 scale devised by Salter and Leath (36), with an additional class, 0, for complete absence of symptoms. Spot-inoculated plants were rated on a related scale as follows: 1 = small brown or black flecks at the inoculation site (typically 3 mm in diameter); 2 = large lesions spreading beyond the inoculation site, chlorosis limited to a 1- to 2-mm margin around the lesion; 3 = necrotic lesion and chlorosis covering up to one-third of the leaf area; 4 = necrotic lesion and chlorosis covering up to four-fifths of the leaf area; and 5 = necrotic lesion, entire leaf chlorotic or dead (Fig. 1). Individual accession disease scores were averaged for data comparisons. The statistical software package JMP IN (version 5.1; SAS Institute, Cary, NC) was used to compare means using the Tukey-Kramer honestly significant difference test at $P \leq 0.05$.

RESULTS

DNA sequencing. Primers designed to amplify each of the five gene regions (ITS rDNA region, actin, β -tubulin, calmodulin, and EF-1 α) gave a PCR product in each *P. medicaginis* isolate. However, the EF-1 α universal primers described by Carbone and Kohn (9) gave poor sequencing chromatograms, due to the existence of G-C-rich intron stretches resulting in polymerase slippage and abrupt sequencing signal reduction. The EF-1 α amplicons ranged in size from 600 to 800 bp (not shown), twice the expected length found among other Dothideomycetes. Due to the apparent

Fig. 1. *Phoma medicaginis* disease symptoms. **A to E**, Spot inoculation scoring categories and **F**, a susceptible *Medicago sativa* spray-inoculated accession at 10 days postinoculation. Spot inoculation disease reaction (DR) scores: **A**, DR = 1, small brown or black flecks at the inoculation site (typically 3 mm in diameter); **B**, DR = 2, large lesions spreading beyond the inoculation site, chlorosis limited to a 1- to 2-mm margin around the lesion; **C**, DR = 3, necrotic lesion and chlorosis covering up to one-third of the leaf area (middle leaf); **D**, DR = 4, necrotic lesion and chlorosis covering up to four-fifths of the leaf area; **E**, DR = 5, necrotic lesion, entire leaf chlorotic or dead.

polymorphism, nested *P. medicaginis*-specific intron-targeted primers (EFNI-1 α) were designed to characterize the intervening sequence. GenBank accession numbers for each gene region are given in Table 1.

WAC4736, 4738, 4741, 7977, 7980, OMT1, and OMT5 exhibited a 97.2 to 97.4% identity in nucleotide composition compared with *P. medicaginis* var. *medicaginis* CBS316.90 across the five gene regions. This suggests that these isolates are closely allied to, but different from, this reference culture. The majority of nucleotide differences between isolates were accounted for by high polymorphism in EF-1 α . Excluding *P. medicaginis* var. *medicaginis* CBS316.90, calmodulin and EFNI-1 α together resolved four genotypes. Examination of the intron sequences showed the existence of a 55-bp unit repeated five consecutive times in each isolate.

Phylogenetic analyses. The *P. medicaginis* isolates in this study initially were compared with closely related species using ITS sequence data alone because, for most species, only this data was available in GenBank. The aligned data set consisted of 601 characters, of which 80 were parsimony informative. The data set contained significant phylogenetic signal compared with 1,000 random trees ($P < 0.01$, $g1 = -0.91$), and initial heuristic searches resulted in 47 most parsimonious trees.

Fig. 2. Phylogram of one of 47 most parsimonious trees based on nuclear DNA internal transcribed spacer region sequence data. *Phoma medicaginis* isolates obtained from *Medicago truncatula* in Western Australia are in bold. Tree length is 165 steps (CI = 0.66, RI = 0.77), and branch support (bootstrap values) is given above branches based on 1,000 bootstrap replicates. The tree is rooted to *Phaeosphaeria nodorum*.

Of these 47 most parsimonious trees, one (Fig. 2) shows that *P. medicaginis* var. *medicaginis* CBS316.90 is more similar to *P. medicaginis* isolates from *M. truncatula* than to other fungal species. The tree also shows no clear association between species of the same genus designation and clade, with strong bootstrap support (>80) for *D. pinodes* grouping with *E. nigrum*, *D. bryoniae* with *P. exigua*, and moderate support (>50) for *A. lentis* grouping with *P. herbarum* and *P. medicaginis*.

EF-1 α showed substantial differences in length and sequence between *P. medicaginis* isolates and other species. The dissimilarity prevented sequence alignments with other species, and therefore phylogenetic analyses using combined gene regions were conducted among *P. medicaginis* isolates only. ITS and calmodulin sequence data were excluded from the analyses because the ITS had no informative regions, and a partition homogeneity test was significant ($P = 0.001$) that EFNI-1 α and calmodulin data were in conflict. The aligned data set of actin, β -tubulin, and EFNI-1 α consisted of 1,056 characters, of which 46 were parsimony informative. The data set contained significant phylogenetic signal compared with 1,000 random trees ($P < 0.01$, $g1 = -1.21$), and heuristic searches resulted in a single most parsimonious tree (Fig. 3). Isolates WAC4736, WAC4741, and OMT5 were selected as representatives of each of the three genotypic groups of *P. medicaginis* isolates from *M. truncatula* for infection experiments.

Disease evaluations. *P. medicaginis* isolates WAC4736, WAC4741, and OMT5 caused disease only on *M. sativa* and *M. truncatula* and not on any of the other legume species tested. *M. sativa* accessions showed levels of disease equivalent to *M. truncatula* (data not shown, Fig. 1F, spray inoculation symptoms). Disease reaction (DR) scores ranged between 2 and 4 at 7 dpi, with clear lesions and pycnidia forming by 10 dpi.

Most of the 86 accessions in the *M. truncatula* core collection were susceptible to *P. medicaginis* (Table 2). Mean DR scores ranged from 2.6 to 3.2 between the isolates, with WAC4741 the most virulent. DR scores among accessions ranged from 1 to 4.8. Sixteen of the accessions (Sa2193, Sa2715, Sa2729, Sa3054, Sa8618, Sa9062, Sa9357, Sa9596, Sa10964, Sa11734, Sa14829, Sa17562, Sa27774, Sa27784, Sa28089, and Sa28645) were susceptible to all three isolates, with DR scores ≥ 3 . Only accessions Sa1489 and Sa8623, with DR scores ≤ 2 against each isolate, were considered partially resistant. However, genotype-specific resistance in accessions exhibiting a score ≤ 1.5 was observed against

Fig. 3. Single most parsimonious phylogram of *Phoma medicaginis* isolates based on actin, β -tubulin, and elongation factor nested intron (EFNI)-1 α sequence data. Tree length is 52 steps (CI = 0.98, RI = 0.99), and branch support (bootstrap values) is given above branches based on 1,000 bootstrap replicates.

individual isolates. These included Sa396, Sa3047, Sa1502, Sa9119, Sa9456, Sa9707, Sa10481, Sa11753, Sa11954, Sa12451, Sa17498, Sa18346, Sa18543, Sa24576, Sa27063, and Sa28064.

DISCUSSION

A prerequisite to understanding isolate specificity and host resistance lies in resolving relationships with allied fungal species and between isolates. In the first step, we compared *P. medicaginis* to the most genetically similar species in GenBank. Twenty predominantly North American *P. medicaginis* ITS sequences available in GenBank and the *P. medicaginis* var. *medicaginis* CBS316.90 reference culture examined here share the same single nucleotide difference with the isolates in this study. This suggests that the *P. medicaginis* isolates from *M. truncatula* form a group closely related to all other isolates sequenced to date, but that isolates other than those from *M. truncatula* in Western Australia are distinct, as depicted in the ITS tree and supported in the combined analysis tree.

Maximum parsimony of the ITS regions showed no support for monophyletic genera, but rather strongly supports polyphyly among *Phoma* and *Didymella* spp. The number of nucleotide differences indicates that *Phoma* spp. could be assigned to different genera or, conversely, the genus *Leptosphaerulina* expanded to incorporate *Ascochyta*, *Epicoccum*, and *Phoma*, assuming GenBank accessions are from correctly identified fungi. Faris-Mokaiesh et al. (14) and Reddy et al. (35) found similar phylogenetic conflicts among *Phoma* and *Didymella* spp., whereby two organisms from different genera were more similar than two organisms of the same genus. The ITS tree is not in total agreement with the sections proposed by Boerema et al. (6), based on morphology and generic synonyms. The majority of the species examined here, including *P. medicaginis*, were placed into section *Phyllostictoides*. However, *P. herbarum* and *E. nigrum* were assigned to sections *Phoma* and *Peyronellaea*, respectively, and *L. trifolii* was designated as miscellaneous. Giraud et al. (18) have suggested that the phylogenetic species concept may be more appropriate than the biological species concept in organisms with life cycles

TABLE 2. Evaluation of 86 South Australian Research and Development Institute accessions for resistance to three genotypes of *Phoma medicaginis*

Accession	DR score ^a			Accession	DR score ^a		
	4736	4741	OMT5		4736	4741	OMT5
Sa396	1.27	2.08	2.98	Sa11954	2.9	4.12	1.21
Sa1306	1.9	3.03	1.99	Sa12451	1.23	3.22	1.23
Sa1316	2.73	3.97	2.61	Sa12455	2.1	3.29	2.08
Sa1489	1.07	1.53	1.34	Sa14829	3.42	3.69	4.13
Sa1502	1	3.11	2.69	Sa15951	1.7	3.26	2.73
Sa1516	2.57	2	3.88	Sa17498	2.91	1.5	4.14
Sa2193	3.6	3.26	3.8	Sa17562	3.99	3.64	3.36
Sa2203	1.88	4.06	2.74	Sa17590	3.37	2.64	1.36
Sa2715	3.85	3.08	3.06	Sa18346	2.7	1.31	1.18
Sa2729	3.53	3	3.85	Sa18395	1.87	4.62	2.61
Sa2841	2.43	4.11	1.69	Sa18532	3.2	3.14	1.52
Sa3047	2.37	1.26	1.84	Sa18543	3.17	4.4	1
Sa3054	4.43	3.98	4.14	Sa19957	3.14	4.57	1.81
Sa3308	2.96	3.92	2.5	Sa19995	3.1	2.98	2.19
Sa3536	2.23	3.08	2.26	Sa19998	1.92	4.54	2.66
Sa3569	1.84	1.7	2.69	Sa21302	2.19	3.17	2.16
Sa3749	1.63	3.25	3.53	Sa21362	1.93	2.75	3.42
Sa3919	4.28	2.11	4.17	Sa23859	2.06	3.83	1.26
Sa4327	1.63	2.83	3.03	Sa24576	1.48	4.22	2.17
Sa4480	1.95	2.47	3.31	Sa24968	2.5	3.75	2.32
Sa4586	2.5	3.27	2.94	Sa25654	2.25	3.92	1.93
Sa7749	1.6	4.42	3.87	Sa25915	2.8	3.06	2.95
Sa8454	2.2	2.14	1.38	Sa25941	3.33	3.2	1.32
Sa8604	1.54	2.32	4.1	Sa27063	3.2	2.33	1.64
Sa8618	3.44	3.42	3.33	Sa27192	2.03	2.64	2.63
Sa8623	1.52	2	1.48	Sa27774	4.47	4.77	3.47
Sa8871	3.25	2.03	2.65	Sa27784	3.2	3.47	3.24
Sa9062	3.63	3.58	3.82	Sa28064	1.45	2.6	1.33
Sa9119	2.6	1.32	1.93	Sa28089	3.97	4.22	3.13
Sa9141	3.73	2.14	2.57	Sa28375	3.4	3.9	1.72
Sa9295	3.44	2.35	2.82	Sa28645	4.23	4.23	4.82
Sa9357	4	3.64	3.45	Sa28889	2.27	3.93	2.79
Sa9456	1	3.25	2.43	Sa30199	2.4	3.93	3.54
Sa9596	3.93	3.33	3.1	Sa30203	3.62	4.62	2.38
Sa9670	3.06	2.9	2.24	Sa30740	3.42	2.9	2.81
Sa9707	1.2	4.32	1.14	Sa31438	2.96	4.2	3.01
Sa9710	2.48	1.24	1.88	A17	2.27	4	4.15
Sa9715	1.72	3.28	1.63	A20	2.43	2.92	3.2
Sa9856	2.22	1.58	2.12	Borung	1.97	2.58	3.28
Sa9866	2.5	2.79	1.29	DZ045	2.05	2.35	2.19
Sa9888	2.43	3.47	2.96	DZ315	1.92	3.4	2.5
Sa10481	1.38	3.14	2.94				
Sa10964	3.55	4.23	4.31				
Sa11734	3.97	4.11	3.47				
Sa11753	3.08	1.39	2.01				
				Range	1–4.47	1.24–4.77	1–4.82
				Mean	2.62	3.15	2.63
				LSD ^b	1.22	1.11	1.05

^a Disease reaction (DR) based on a minimum of three replicates composed of three plants per replicate and five leaves per plant. Scores range from 1 (resistant) to 5 (fully susceptible), where 1 = small brown or black flecks at the inoculation site (typically 3 mm in diameter); 2 = large lesions spreading beyond the inoculation site, chlorosis limited to a 1- to 2-mm margin around the lesion; 3 = necrotic lesion and chlorosis covering up to one-third of the leaf area (middle leaf); 4 = necrotic lesion and chlorosis covering up to four-fifths of the leaf area; and 5 = necrotic lesion, entire leaf chlorotic or dead.

^b Least significant difference (LSD) was calculated using the Tukey-Kramer honestly significant difference test at $P \leq 0.05$.

involving restricted dispersal between selection on the host and mating, and showed that divergence can occur in sympatry and lead to host race formation, or even speciation, by the mere process of specialization, with strong divergent adaptive selection.

The highly polymorphic *P. medicaginis* EF-1 α 55-bp repeat unit enabled isolate genotypes to be clearly distinguished. The separation of the isolates was consistent with the sum of differences from the ITS, actin, β -tubulin, and calmodulin gene regions, other features such as colony morphology (not shown), and discrimination comparable with randomly amplified polymorphic DNA analysis (not shown). The repeat was not present among the other species sequenced, and shows no homology to sequences or motifs in GenBank. The nested primers designed to EF-1 α amplify within an intron, where duplication of noncoding DNA provides an explanation for the hypervariable nature of this gene region. Therefore, the EF-1 α intron region provides a specific means of rapidly distinguishing closely related strains.

P. medicaginis isolates from *M. truncatula* failed to induce either compatible or incompatible disease phenotypes in legumes other than *M. truncatula* and the closely related *M. sativa*. This contrasts with previous reports documenting a wide host range for *P. medicaginis* (27), which detailed disease principally among the Papilionoideae, but also within the Brassicaceae and Solanaceae. Many such reports require verification but suggest that *P. medicaginis* var. *medicaginis* may be a less-discriminating pathogen, and underlines the need for characterization of the population structure, genetic diversity, and host specificity of this species.

A continuous distribution and wide range of disease phenotypes were observed among 86 accessions screened. Previous studies in the *P. medicaginis*–*M. truncatula* pathosystem have found comparably wide ranges in disease symptoms between accessions (2,31). However, this is the first study using genotyped monosporic sources of inoculum and single-seeded accessions. Our results suggest that *M. truncatula* harbors specific and diverse sources of resistance to individual pathogen genotypes, with several isolate-specific resistant accessions showing pronounced susceptibility to a second isolate with a DR score >4. In one accession, Sa2841, tissue specificity was observed, with complete resistance in the unifoliate leaf to WAC4736 but not in subsequent trifoliate leaves. Two accessions, Sa1489 and Sa8623, showed resistance to all three isolates. The genetic basis of these broad-spectrum resistance phenotypes cannot be determined until the genes responsible have been mapped or isolated.

In contrast to biotrophic pathosystems where race-specific gene-for-gene recognition is well established (22), resistance to necrotrophs usually is quantitative in nature, and necrotrophs generally are considered to have a wide host range. However, necrotrophic fungi must be highly specialized in order to promote tissue death and successfully avoid, or suppress, host resistance responses. Phylogenetic analyses recently have helped to define host-preferential clades in some species; for example, among *Ascochyta* spp. (4,15). Host plant resistance mechanisms against necrotrophs are not well understood but appear to function by interfering with the action of phytotoxins produced by the pathogen: for example, the race-specific *Hm1* gene in maize (26), the *Pyrenophora tritici-repentis* ToxA-insensitivity *tsn1* (13) and ToxB-insensitivity *tsc2* (17) loci in wheat, and resistance to the *F. graminearum* mycotoxin deoxynivalenol in wheat (29). Identification of sources of resistance in diverse accessions of *M. truncatula* offers the opportunity to characterize new forms of resistance in this genetically tractable model legume.

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