Temporal and Spatial Changes in the Pea Black Spot Disease Complex in Western Australia

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Abstract

Tran, H. S., Li, Y. P., You, M. P., Khan, T. N., Pritchard, I., and Barbetti, M. J. 2014. Temporal and spatial changes in the pea black spot disease complex in Western Australia. *Plant Dis.* 98:790-796.

Black spot (also referred to as Ascochyta blight, Ascochyta foot rot and black stem, and Ascochyta leaf and pod spot) is a devastating disease of pea (*Pisum sativum*) caused by one or more pathogenic fungi, including *Didymella pinodes*, *Ascochyta pisi*, and *Phoma pinodella*. Surveys were conducted across pea-growing regions of Western Australia in 1984, 1987, 1989, 1996, 2010, and 2012. In total, 1,872 fungal isolates were collected in association with pea black spot disease symptoms. Internal transcribed spacer regions from representative isolates, chosen based on morphology, were sequenced to aid in identification. In most years and locations, *D. pinodes* was the predominant pathogen in the black spot complex. From 1984 to 2012, four new pathogens associated with black spot symptoms on leaves or stems (*P. koolunga*, *P. herbarum*, *Boeremia exigua* var. *exigua*, and *P. glomerata*) were confirmed. This study is the first to confirm *P. koolunga* in association with pea black spot symptoms in field pea in Western Australia and show that, by 2012, it was widely present in new regions. In 2012, *P. koolunga* was more prevalent than *D. pinodes* in Northam and *P.*

pinodella in Esperance. *P. herbarum* and *B. exigua* var. *exigua* were only recorded in 2010. Although *A. pisi* was reported in Western Australia in 1912 and again in 1968 and is commonly associated with pea black spot in other states of Australia and elsewhere, it was not recorded in Western Australia from 1984 to 2012. It is clear that the pathogen population associated with the pea black spot complex in Western Australia has been dynamic across time and geographic location. This poses a particular challenge to development of effective resistance against the black spot complex, because breeding programs are focused almost exclusively on resistance to *D. pinodes*, largely ignoring other major pathogens in the disease complex. Furthermore, development and deployment of effective host resistance or fungicides against just one or two of the pathogens in the disease complex could radically shift the make-up of the population toward pathogen species that are least challenged by the host resistance or fungicides, creating an evolving black spot complex that remains ahead of breeding and other management efforts.

Field pea, *Pisum sativum*, is a valuable food and feed crop (5) and, when rotated with the other crops such as cereals, it can provide an effective break in the disease cycles of some pathogens (18). Black spot is a major constraint to pea production in Australia (10,20) and elsewhere (8). For example, yield loss caused by black spot ranges from 30 to 75% in Australia, France, and Canada (25,45). In most pea crops in Australia, the minimum yield loss is >10% (8,34). In Western Australia, Salam et al. (47) estimated yield loss due to black spot at up to 50%. Various control strategies for black spot are well documented (8,18,54,56,57). In general, the most effective approaches employ a combination of removal or avoidance of inoculum sources, seed treatment, utilizing available host resistance, sowing pathogen-free seed, manipulating sowing date to avoid the major ascospore showers, and application of fungicides (18).

Black spot historically is caused by one or more pathogenic fungi belonging to the phylum Ascomycota, including *Didymella pinodes* (also known as *Ascochyta pinodes*), *A. pisi*, *Phoma pinodella* (syn. *P. medicaginis* var. *pinodella* and formerly known as *A. pinodella*) (5,18,20,34), and, recently, *P. koolunga* (17). Among the pathogens in the complex, *D. pinodes* is believed to be the most important (37,50). For example, *D. pinodes* is the dominant and most important pathogen in the black spot complex in Canada (23), southern Australia (9), and France (51), even

though *A. pisi* also occurs in southern France (50). Black spot is a polycyclic disease (18). Primary infection of pea black spot pathogens usually starts with very small “pin-prick” lesions or flecks on the leaf surface after rain events of as little as 0.2 mm or in humid conditions (5,8,46). Uninterrupted wet conditions can bring about coalescence of expanding lesions and, eventually, senescence of the leaf (5).

In Western Australia, *D. pinodes* was first recorded in 1923 (12) and later in 1977 (48). *A. pisi* was first reported in 1912 (49) and later in 1927 and 1968 (48), and *P. pinodella* was first reported in 1954 (13). Recently, *P. herbarum*, *P. glomerata*, and *Boeremia exigua* var. *exigua* have been reported to be associated with pea black spot in Western Australia (29,30,52). Furthermore, Davidson et al. (17) reported *P. koolunga* associated widely with pea black spot on field pea in South Australia and reported the results of DNA soil analyses, suggesting its presence at two locations in Western Australia (19).

Because *P. herbarum* (30), *P. glomerata* (48), and *B. exigua* var. *exigua* (29) had only recently been found on field pea in Western Australia and because the symptoms on pea caused by *P. koolunga* are indistinguishable from those caused by *D. pinodes* (17), this study was undertaken to determine the full spectrum of pathogens associated with the pea black spot complex in Western Australia. This article reports the composition and relative incidences of pathogens involved with the pea black spot disease complex in Western Australia and how the composition of the pathogen complex has changed over time and with geographic location.

Materials and Methods

Fungal isolation. Pea plants with typical pea black spot lesions on leaves or stems were collected from various locations across Western Australia from August to September, when the plants were

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Accepted for publication 12 December 2013.

at the flowering stage, in 2010 and 2012. Location and numbers of collection sites varied by year, and included Dalwallinu, Northam, Wickepin, Nyabing, Esperance, and Medina (Fig. 1; Table 1). Isolates from diseased leaves or stems collected in 1984, 1987, 1989, and 1996 that had been stored in lyophilized glass ampules since isolation in the Western Australian Culture Collection at the Department of Agriculture and Food Western Australia, South Perth, Western Australia, were revived. Geographical origins of these isolates included Latham, Coomberdale, Konnongorring, Meckering, Katanning, Needilup, Avondale, Medina, Merredin, Beverley, and Mount Barker in Western Australia (Fig. 1).

Segments of approximately 3 mm² of diseased leaves and stems were surface sterilized by soaking sequentially in 70% ethanol (30 s) and 10% sodium hypochlorite (1 min), then rinsing three times in sterilized deionized water. Segments were then placed onto potato dextrose agar or Coon's medium (3), both amended with aureomycin hydrochloride (20 ppm), and incubated at 25°C for 28 days. Isolates obtained were grouped based on colony morphology of *D. pinodes*, *P. pinodella*, or other *Phoma* spp. The morphological traits to identify species associated with the disease complex were based on the Commonwealth Mycological Institute (CMI) and International Mycological Institute (IMI) descriptions of each pathogen (27,36,43,44), except for *P. koolunga* (17) and *B. exigua* var. *exigua* (7). Representative isolates (20% of each group) were subjected to single-spore isolation on Coon's agar and, subsequently, DNA extraction and sequencing were conducted.

DNA extraction, amplification, sequencing, and analysis. DNA was extracted from mycelia collected from single-spore cultures of 217 representative isolates based on the protocol described by Ceniz (14), but with some modification. In brief, mycelia from 2-week-old cultures growing on Coon's medium were subcultured into 20-ml McCartney bottles containing 10 ml of potato dextrose broth and incubated at 25°C for 2 weeks without agitation. The mycelial mat was removed, blotted dry with filter paper, washed with Tris-EDTA (TE) buffer, and pelleted by centrifugation (5 min at 13,000 rpm), and the supernatant was decanted.

Mycelia were crushed in 300 µl of extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, and 0.5% [wt/vol] sodium dodecyl sulfate) using a conical grinder (catalog number Z359947; Sigma-Aldrich). Tubes were maintained at -20°C for 10 min in 150 µl of 3 M sodium acetate (pH 5.2) and then centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a fresh tube, an equal volume of isopropanol was added and maintained at 25°C for 5 min, and subsequently tubes were centrifuged for 5 min at 13,000 rpm to collect precipitated DNA. The pellet was washed with 70% (wt/vol) ethanol, vacuum dried for 10 min, and resuspended in 50 µl of TE buffer. The concentration and quality of the extracted DNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). All DNA samples were stored at 4°C until use. A polymerase chain reaction (PCR)-based assay using primers TW81 (5'-GTTTCC GTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGT TCAGCGGGT-3') (16,17) was used to amplify from the 3' end of 16S rDNA, across internal transcribed spacer (ITS)1, 5.8S ribosomal (r)DNA, and ITS2 to the 5' end of the 28S rDNA. Amplification was performed in a volume of 50 µl with 0.4 µM each primer, 200 µM dNTPs, 2.5 mM MgCl₂, 2 µl of DNA, 1× PCR reaction buffer, and 1 unit of Taq polymerase. Conditions for amplification included a denaturation step of 2 min at 94°C; followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C; and a final elongation step of 72°C for 10 min. The PCR products were separated by electrophoresis on a 1% (wt/vol) agarose gel containing ethidium bromide at 10 µg/ml and viewed under UV light. PCR products of 500 bp (20 µl of each) were sequenced by the Australian Genome Research Facility Ltd. or Macrogen Inc. GENEIOUS software (ver. 4.8.5; Biomatters Ltd.) was used for sequence manipulation and alignment (cost matrix of 65% similarity = 5.0/-4.0, gap open penalty = 12, gap extension penalty = 3, and alignment type = global alignment with free end gaps). Sequences from all isolates were compared with those in GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) function incorporated in GENEIOUS.

Fig. 1. Locations in southwest Western Australia where diseased field pea (*Pisum sativum*) samples were collected and isolations of causal fungi associated with pea black spot disease made from 1984 to 2012. Numbers in parentheses after each location indicates the year or years of sample collection.

Neighbor-joining trees were produced using the Geneious Tree Builder function of GENEIOUS. The Jukes-Cantor genetic distance model and the bootstrap resampling method (random seed = 978.393, number of replications = 1,500, and support threshold higher than 70%) were used. All sequences were submitted to the GenBank database (accession numbers KF424317 to KF424535). An isolate of *Alternaria infectoria* H2012-242 was selected as the out-group (bootstrap value = 100%). The multi-alignment of the sequences was deposited in TreeBASE (submission 14544; <http://purl.org/phylo/treebase/phylows/study/TB2:S14544>).

Results

Spatial and temporal pathogen composition. For 1984 and 1987, *D. pinodes* in Meckering and *P. pinodella* in Needilup were the only pea black spot pathogens isolated. *D. pinodes* accounted for 40 and 100% of isolates in 1984 and 1987, respectively, whereas *P. pinodella* was only isolated in 1984, where it accounted for 60% of the isolates (Table 1).

In 1989, diseased samples were collected from six different locations. In total, 1,040 fungal isolates associated with the black

Table 1. Incidence of black spot pathogens isolated from typical diseased stem or leaf lesions on field pea (*Pisum sativum*) in 1984, 1987, 1989, 1996, 2010, and 2012 at various locations across the grain belt of southwest Western Australia

Year	Location	Species isolated	Number of isolates	Total number of isolates		Isolates (%)
				By location	By year	
1984	Needilup	<i>Didymella pinodes</i>	2	5	5	40.00
		<i>Phoma pinodella</i>	3	...	...	60.00
	Mean	...	2.5	...	...	...
1987	Meckering	<i>D. pinodes</i>	5	5	5	100.00
	Mean	...	5	...	...	...
1989	Avondale	<i>D. pinodes</i>	184	209	1,040	88.00
		<i>P. pinodella</i>	23	...	...	11.00
		<i>Phoma</i> sp.	2	...	...	1.00
	Mean	...	69.7	...	...	...
	Beverly	<i>D. pinodes</i>	228	249	...	91.60
		<i>P. pinodella</i>	21	...	...	8.40
	Mean	...	124.5	...	...	...
	Medina	<i>D. pinodes</i>	128	146	...	87.70
		<i>P. pinodella</i>	16	...	...	11.00
		<i>Boeremia exigua</i> var. <i>exigua</i>	2	...	...	1.40
	Mean	...	48.7	...	...	...
	Coomberdale	<i>D. pinodes</i>	165	187	...	88.20
		<i>P. pinodella</i>	18	...	...	9.60
		<i>B. exigua</i> var. <i>exigua</i>	4	...	...	2.10
	Mean	...	62.3	...	...	...
	Merredin	<i>D. pinodes</i>	66	74	...	89.20
		<i>P. pinodella</i>	8	...	...	10.80
	Mean	...	37	...	...	...
	Mount Baker	<i>D. pinodes</i>	74	175	...	42.30
		<i>P. pinodella</i>	92	...	...	52.60
		<i>B. exigua</i> var. <i>exigua</i>	9	...	...	5.10
	Mean	...	58.3	...	...	...
1996	Katanning	<i>D. pinodes</i>	2	8	8	100.00
	Konnongorring	<i>D. pinodes</i>	2	...	...	100.00
	Latham	<i>D. pinodes</i>	2	...	...	100.00
	Merredin	<i>D. pinodes</i>	2	...	...	100.00
	Mean	...	2	...	...	...
2010	Medina	<i>D. pinodes</i>	51	150	150	34.00
		<i>P. pinodella</i>	78	...	...	52.00
		<i>P. herbarum</i>	21	...	...	14.00
2012	Mean	...	50	...	...	...
	Medina	<i>D. pinodes</i>	127	165	664	77.00
		<i>P. pinodella</i>	36	...	...	21.80
		<i>P. glomerata</i>	2	...	...	1.20
	Mean	...	55	...	...	...
	Esperance	<i>D. pinodes</i>	41	85	...	48.10
		<i>P. pinodella</i>	16	...	...	19.00
		<i>P. koolunga</i>	28	...	...	32.90
	Mean	...	28.6	...	...	...
	Wickepin	<i>D. pinodes</i>	84	118	...	71.50
		<i>P. pinodella</i>	34	...	...	28.50
	Mean	...	59	...	...	...
	Northam	<i>D. pinodes</i>	19	100	...	19.00
		<i>P. koolunga</i>	81	...	...	81.00
	Mean	...	50	...	...	...
	Nyabing	<i>D. pinodes</i>	74	102	...	72.50
		<i>P. pinodella</i>	25	...	...	24.50
		<i>P. koolunga</i>	3	...	...	2.90
	Mean	...	34	...	...	...
	Dalwallinu	<i>D. pinodes</i>	80	94	...	85.20
		<i>P. pinodella</i>	7	...	...	7.00
		<i>P. koolunga</i>	3	...	...	3.50
		<i>Phoma</i> sp.	4	...	...	4.30
	Mean	...	23.4	...	...	...
Total	...	...	...	1,872	...	...

spot complex in pea were collected; at each location, the number of fungal isolates varied from 74 to 249 (Table 1). At some locations, including Avondale, Beverly, Medina, Coomberdale, and Merredin, *D. pinodes* was the predominant species, ranging from 87.7% (128 isolates) to 91.6% (228 isolates) of the isolates obtained. *P. pinodella* was also recovered from the six locations but at a lower incidence of 8.4 to 52.6%. At Mount Baker, *P. pinodella* predominated at 52.6%, while *D. pinodes* and *B. exigua* var. *exigua* accounted for 42.3, and 5.1% of 175 isolates, respectively. *B. exigua* var. *exigua* was identified at three of the six locations: Medina (two isolates, 1.4%), Coomberdale (four isolates, 2.1%), and Mount Baker (nine isolates, 5.1%).

In 2012, the composition of black spot pathogen complex in Western Australia radically changed, with the widespread appearance of *P. koolunga*, identified at four of the six locations: Esperance, Northam, Nyabing, and Dalwallinu. The proportion of *P. koolunga* isolated in comparison with *D. pinodes* and *P. pinodella* was different across these locations. For example, at Esperance, the percentage of *P. koolunga* isolates (32.9%) was lower than that of *D. pinodes* (48.1%) but nearly double that of *P. pinodella* (19%). However, at Northam, *P. koolunga* (81%) dominated *D. pinodes* (19%) while there were no *P. pinodella* isolates. At Nyabing and Dalwallinu, *P. koolunga* constituted only a small percentage (2.9 and 3.5%, respectively) while *D. pinodes* was the predominant species (72.5 and 85.2%, respectively).

In general, the composition of pathogens associated with pea black spot recorded in 1996, 2010, and 2012 changed radically compared with that recorded in the 1980s. In 1996, *D. pinodes* was the only species identified at four locations across the Western Australian grain belt (Katanning, Konnongorring, Latham, and Merredin); however, only eight isolates were recovered that year. In 2010, there were three species identified at Medina, including *D. pinodes*, *P. pinodella*, and *P. herbarum*, the latter constituting 14% of all isolates at that site. Furthermore, the composition of *D. pinodes* and *P. pinodella* was substantially different from that recorded in the 1980s. For example, in 2010, *P. pinodella* was the predominant species, accounting for 52%, while *D. pinodes* made up 34%. In 2012, at the same location (Medina), although neither *B. exigua* var. *exigua* nor *P. herbarum* were isolated, *P. glomerata*, a new species in terms of the black spot complex for Western Australia (52), was isolated at an incidence of 1.2% compared with *D. pinodes* (77%) and *P. pinodella* (21.8%). In 2012, at Medina, *D. pinodes* was the predominant species rather than *P. pinodella*, as had been the case in 2010.

ITS sequencing and phylogenetic analysis. The phylogenetic tree shows that all isolates recovered from pea displaying symptoms of black spot grouped into five main clades (Fig. 2). Clade I contained more than one species, including *D. pinodes*, *P. pinodella*, and *P. glomerata* (all isolates recovered in the 1980s, 1996, and 2012). *P. herbarum* and *B. exigua* var. *exigua* from 2010 were distributed in clades II and III, with bootstrap values (BVs) of 97.9 and 97.1%, respectively. In clade IV, there were 15 isolates of *P. pinodella* derived from samples collected at Medina in 2010. All *P. koolunga* isolates were clustered in clade V (BV = 73.6%), in which an isolate from Northam was distributed into a subbranch (BV = 87.1%) that included one other isolate from Dalwallinu (BV = 77.8%) and three reference isolates from South Australia (BV = 88.2%).

Discussion

Among the pathogens in the black spot complex, *D. pinodes* is the most important and damaging (8,37,50). However, our study showed that the relative proportions of pea black spot pathogens in Western Australia changed depending upon location or year. Although *D. pinodes* predominated overall, one or more of *P. pinodella*, *P. herbarum*, *B. exigua* var. *exigua*, or *P. koolunga* were important in some years or locations, and even *P. glomerata* has some potential for importance at one specific location. These changes in the relative proportions of pea black spot pathogens between locations and years is nicely illustrated by *P. pinodella*,

being the dominant pathogen at Mount Baker in 1989 and Medina in 2010, whereas *P. koolunga* dominated at Northam in 2012.

The widespread incidence of *P. koolunga* in Western Australia is particularly noteworthy. This species was first identified by Davidson et al. (17) in South Australia and reported to be present in Western Australia at two locations between 2008 and 2009 (but only based on soil DNA analyses; 19). In South Australia, it was initially mistakenly identified as *Macrophomina phaseolina* in 1982 by Ali et al. (4). Our study is the first to show *P. koolunga* associated with pea black spot symptoms on leaves and stems in Western Australia. We identified *P. koolunga* at four different locations (Esperance, Northam, Nyabing, and Dalwallinu) in 2012. It was particularly interesting that, at Northam, the proportion of *P. koolunga* isolates (81%) quadrupled that of *D. pinodes* (19%), and it appeared to totally replace *P. pinodella*. In addition, at Esperance, although the proportion of isolates of *P. koolunga* (32.9%) was smaller than that of *D. pinodes* (48.1%), it still nearly doubled the incidence of *P. pinodella* (19%). However, at Nyabing and Dalwallinu, *P. koolunga* constituted only a small proportion (2.9 and 3.5%, respectively).

Davidson et al. (19) reported that DNA of *P. koolunga*, *D. pinodes* and *P. pinodella* was detected in soil cropped to field pea across New South Wales, South Australia, Victoria, and Western Australia. These authors concluded that it is common to detect *P. koolunga* in soil in South Australia but rarely in the other states. Of the 1,042 samples they collected, the highest incidence of *P. koolunga* was observed in South Australia at 6% but it constituted only <3% incidence in the other Australian states. In their study, Davidson et al. (19) collected 22 soil samples from across five locations in Western Australia (Bindi Bindi, Esperance, Geraldton, Katanning, and Merredin). However, of these, only six collected from Esperance and Katanning were positive for *P. koolunga* DNA. In contrast, in our study in 1989, *P. koolunga* was not associated with black spot symptoms at Katanning whereas, at the same location in 2008 to 2009, Davidson et al. (19) reported three of nine soil samples positive for *P. koolunga* in DNA. Similarly, at Esperance, Davidson et al. (16) found three soil samples positive for *P. koolunga* and two samples positive for *D. pinodes* and *P. pinodella* DNA. However, in our study at Esperance in 2012, the incidence of *P. koolunga* was higher than that of *P. pinodella* but not of *D. pinodes*. Our findings add some support to the claim of Davidson et al. (19) that *P. koolunga* “may become more widespread in future years”. Indeed, in Western Australia since the 2008–09 growing season, *P. koolunga* has spread and become widely established outside the Katanning and Esperance regions into Nyabing, Dalwallinu, and Northam.

Although there were reports of *Ascochyta pisi* on field pea in 1912 (49) and in 1927 and 1968 (48), there have not been any subsequent confirmed incidences of *A. pisi* in Western Australia. Additionally, we did not find it from 1984 to 2012. In view of its absence, it is possible that these early identifications were incorrect. Whatever the case, it is clear that the environmental conditions in Western Australia do not favor widespread establishment of *A. pisi*, as it would likely have been introduced multiple times on infested seed from eastern Australia. *A. pisi* still occurs in South Australia, in spite of its relatively rare appearance compared with *D. pinodes* and *P. pinodella* (2,17). Furthermore, in a survey in 1976 in South Australia, *A. pisi* was the most prevalent pathogen causing leaf and pod spot on field pea (3). In Victoria, Bretag et al. (11) reported that, of 436 seed lots tested, 94.8% of the isolates recovered were *D. pinodes*, 4.2% were *P. pinodella*, and 1.0% were *A. pisi*. In France, *A. pisi* was found at high incidence in the southern regions but *D. pinodes* remained the dominant pathogen. In Canada, 85% of seed lots infected with one or more pea black spot pathogens were infected by *A. pisi* (50). A recent pea black spot epidemic in 2005–06 in Spain was primarily attributed to *A. pisi* (24), despite it being widely considered the least damaging among the pathogens in the pea black spot complex (53). In India, *A. pisi* was recovered in 1 of 40 isolates in pea-growing areas of Himachal Pradesh (41). Although these reports highlight a clear and significant role of

Fig. 2. A, Neighbor-joining tree of internal transcribed spacer data produced with Jukes-Cantor genetic distance model. The tree was generated by GENEIOUS. Bootstrap values of 1,500 replications are shown for the major nodes. Closest BLAST results were used as references, and shown in bold; * indicates reference sequence achieved from GenBank. **B**, Enlarged images of A in accordance with the assigned clades I, II, III, IV, and V.

A. pisi as an important pathogen in the pea black spot complex, *A. pisi* is likely no longer present in Western Australia. If the reports of its presence in Western Australia in 1912, 1927, and 1968 were a case of mistaken identity, then perhaps it was never established in Western Australia on field pea. Unfortunately, these original isolates identified as *A. pisi* are no longer available for molecular comparison. The reason for its failure to establish or absence in Western Australia also warrants further investigation.

Although *B. exigua* var. *exigua* was recorded at three locations in Western Australia in 1989 (Medina, Coomberdale, and Mount Baker), there has not been any subsequent incidence. Similarly, *P. herbarum* and *P. glomerata* were only identified once at Medina, in 2010 and 2012, respectively. Such species constituted new pathogens associated with pea black spot at various times of sampling (1989, 2010, and 2012) at Medina and their first occurrence was recently documented (29,30,52). Except for the recent report of *P. glomerata* on field pea in Australia (52), this pathogen has not been reported elsewhere on pea except for a single isolation from pea rhizosphere soil in Tomaszko, Poland. However there was no molecular confirmation of identity in that study (39). It is noteworthy that *P. herbarum*, *B. exigua* var. *exigua*, and *P. glomerata* have wide host ranges (27,30,31,36). In Australia, the host range of *B. exigua* var. *exigua*, frequently classified as a weak or wound parasite (6), included 48 hosts in 14 families and another 12 species under experimental conditions (1). There are about 138 species affected by *P. herbarum* (27), and this pathogen has been reported in Australia on alfalfa (*Medicago sativa*) and soybean (*Glycine max*) in 1985, *Protea* sp. in 1991, and Arabian pea (*Bituminaria bituminosa*) in 2010 (30). According to Morgan-Jones (36), hosts of *P. glomerata* include *Vitis vinifera*, *Coniferae* spp., *Lycopersicon esculentum*, *Mallus pumila*, and *Solanum tuberosum* but, in Australia, it has been reported on other crop legumes, including *Cicer arietinum*, *Lupinus angustifolius*, *Medicago littoralis*, *M. truncatula*, and *G. max* (48).

Currently, breeding programs in Australia and elsewhere have focused almost exclusively on resistance to *D. pinodes* (9,22,26,38,42,59) and attempts to locate resistance to other pathogens of the pea black spot complex are rare. The one exception is Ali et al. (3), who examined resistance of pea lines to *D. pinodes*, *A. pisi*, and *P. pinodella*. However, because several different pathogens are known to be highly virulent on pea, particularly *D. pinodes* (8), *P. pinodella* (40), and *P. koolunga* (17,19,35), along with several other less virulent pathogens such as *P. herbarum* (30), *P. glomerata* (52), and *Boeremia exigua* var. *exigua* (29), it remains a significant challenge to effectively manage the black spot complex in Western Australia or where similar complexes exist. It is widely accepted that only partial resistance is available in the pea germplasm and that the inheritance of resistance is largely polygenic (26). Hence, pea genotypes with some resistance to one pathogen will not necessarily have resistance to other pathogens of this complex (H. S. Tran, unpublished data). Although thousands of pea accessions have been screened for resistance to an individual black spot complex pathogen such as *D. pinodes* (28,58), high-level resistance has not been found (55). For this reason, it is unlikely that effective resistance to all major pathogens can be developed. Our study demonstrated that the pathogens of the pea black spot complex change between years and between locations, adding to the difficulty of developing effective management strategies. Development and deployment of effective host resistance against just one or two pathogens of the complex could radically shift the make-up of the population toward species least challenged by the particular host resistance. It is also likely that the efficacy of fungicides currently applied to manage pea black spot in Australia (8,35) and elsewhere (15,32,33,56) will not be equally effective across the spectrum of pathogens. If that was the case, then it is possible that regular applications of some chemicals could change the relative proportions of individual pathogens within the complex at particular locations or over time.

DNA sequencing analysis of the ITS regions of the representative isolates showed that the pathogens *P. herbarum*, *P. glomerata*,

B. exigua var. *exigua*, and *P. koolunga*, associated with the pea black spot complex in Western Australia (17,29,30), were distinct from the two pathogens historically accepted as the main causal agents, *D. pinodes* and *P. pinodella*. In the case of *P. koolunga*, all isolates grouped into clade V, similar to the grouping of *P. koolunga* in the study by Davidson et al. (17). However, *P. koolunga* isolates found in Western Australia are likely to be different from those found in South Australia, because the three reference sequences of *P. koolunga* from South Australia were grouped in a subclade of clade V with a relatively high bootstrap value (88.2%). Although the majority of *D. pinodes* and *P. pinodella* isolates clustered into clade I, suggesting strong similarities between these two species, 15 isolates of *P. pinodella* isolated in 2010 at Medina grouped into a separate clade (clade IV). These isolates could constitute a subgroup of *P. pinodella* that warrants further investigation. An attempt by Faris-Mokaiesh et al. (21) to differentiate *D. pinodes* and *P. pinodella* by biochemical and molecular markers resulted in a conclusion that there was no clear difference between these two fungi. However, according to Onfroy et al. (40), *D. pinodes* and *P. pinodella* can readily be distinguished based on morphological characteristics (e.g., presence of pseudothecia, proportion of bicellular conidia, and size of unicellular conidia), growth rate, and random amplification of polymorphic DNA analysis. Clearly, this warrants further investigation.

In conclusion, the pathogen population associated with the pea black spot complex in Western Australia is dynamic across time and geographic location. This poses a challenge to developing effective resistance against the black spot complex, because breeding programs remain focused on resistance to *D. pinodes*, largely ignoring other pathogens. Further, development and deployment of effective host resistance or fungicides against just one or a few of the pathogens within the disease complex could radically shift the composition of the population toward pathogen species that are less sensitive to the host resistance or fungicides. This would create a black spot complex that remains ahead of any breeding efforts. Hence, it is not surprising that this disease causes severe losses worldwide, despite attempts to develop genotypes with effective resistance and the widespread utilization of fungicides.

Acknowledgments

We thank the Australian Government for providing financial assistance to H. S. Tran through a Postgraduate AUSAID Scholarship, the School of Plant Biology at The University of Western Australia for operational funding support for this research, X. L. Fang for assistance with dendrogram formulation and interpretation, and the Department of Agriculture and Food Western Australia for providing half the salary of M. Barbetti during the early period of these studies.

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