Distribution and Survival of Ascochyta Blight Pathogens in Field-Pea-Cropping Soils of Australia

J. A. Davidson, South Australian Research and Development Institute (SARDI), GPO Box 397, Adelaide, South Australia, 5001, Australia, and School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, South Australia 5064, Australia; M. Kryszyska-Kaczmarek, C. J. Wilmshurst, A. McKay, and Herdina, SARDI, Adelaide, Australia; and E. S. Scott, School of Agriculture, Food and Wine, The University of Adelaide, Australia

Abstract

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Phoma koolunga, *Didymella pinodes*, and *P. medicaginis* var. *pinodella* were detected in DNA extracted from soil following field pea crops across four states in the southeastern and western regions of Australia. *P. koolunga* was commonly detected in soil from South Australia but rarely in other states whereas *D. pinodes* plus *P. medicaginis* var. *pinodella* were widespread in all regions tested. The quantity of DNA of these pathogens detected in soils prior to growing field pea was positively correlated with ascochyta blight lesions on field pea subsequently grown in infested soil in a pot bioassay and also on field pea in

naturally infected field trials. The quantity of DNA of the soilborne pathogens was greatest following a field pea crop and gradually decreased in the following 3 years. The DNA tests were used to quantify the DNA of the pathogens in field pea plants sampled from naturally infected field trials in South Australia over two seasons. The combined results of DNA tests and pathogen isolation from the plants indicated that *P. koolunga* and *D. pinodes* were equally responsible for the ascochyta blight symptoms in the diseased trials, while *P. medicaginis* var. *pinodella* had a minor role in the disease complex.

Ascochyta blight is the most common disease of South Australian field pea crops, with individual crop losses as high as 35% (3,10,14). This disease is caused by a complex of fungi: *Didymella pinodes* (Berk. & A. Bloxam) Petr. (syn. *Mycosphaerella pinodes* (Berk. & A. Bloxam) Vestergr.) and *Phoma medicaginis* var. *pinodella* (L.K. Jones) Boerema and *D. pisi* Lib. (4,17). *D. pinodes*, considered the primary pathogen in this complex, releases large numbers of airborne ascospores during rainfall in fall and early winter (April to June), when field pea crops are sown or are emerging in southern Australia (5,6). No major gene resistance is available (3) and fungicides are often uneconomical (14); therefore, the main disease control strategy has been to delay sowing of field pea until 4 to 6 weeks after the first rains in fall to minimize infection from these ascospores (2,10,11).

P. koolunga J.A. Davidson, D. Hartley, Priest, S. Kaczmarek, Herdina, A. McKay & E.S. Scott has recently been recognized as an important component of this disease complex in the state of South Australia, and causes severe symptoms on field pea indistinguishable from those caused by *D. pinodes* (8). No teleomorph of this pathogen has been recognized and rain splash dispersal of in situ inoculum is likely to be the predominant form of dispersal. Preliminary studies using DNA assays based on the highly conserved internal transcribed spacer (ITS) region of the genome (15) have detected *P. koolunga*, *D. pinodes*, and *P. medicaginis* var. *pinodella* in field-pea-cropping soils of South Australia (9).

D. pinodes and *P. medicaginis* var. *pinodella* have been isolated from soil up to 20 and 5 years, respectively, after field pea plants

were grown (16,21,22). They survive as chlamydospores, mycelium, or sclerotia. Chlamydospores are able to withstand temperatures of at least 100°C for 12 to 15 h (22) and can survive for over 12 months in inoculated sterile soil maintained at -20 to 25°C, with specimens incubated below 15°C remaining infectious. High soil temperatures are detrimental to the pathogens and none survived for more than 1 month at 35°C or 4 months at 30°C (21). Inoculum is splashed from the soil onto the leaves of new crops, or the pathogens may infect the epicotyl and the base of the stem, causing foot rot (7,19). Chlamydospores have not been described in association with *P. koolunga* but this pathogen forms pseudosclerotia in culture (8). These structures may play a role in the survival and importance of *P. koolunga* in soil. Alternatively, the pathogen may survive as mycelium in or on organic matter.

The aims of this study were to (i) quantify the distribution of *P. koolunga* in relation to the known field pea pathogens *D. pinodes* and *P. medicaginis* var. *pinodella* in Australia; (ii) investigate the relative survival of soilborne inoculum of *P. koolunga*, *D. pinodes*, and *P. medicaginis* var. *pinodella* in the years following ascochyta blight-affected field pea crops; (iii) determine the relative contribution of each of these pathogens to the disease complex in naturally infected field pea plants; and (iv) determine the infectivity of the soilborne inoculum in terms of disease resulting from planting into ascochyta blight-infested soils.

Materials and Methods

Presence and quantity of pathogen DNA in field-pea-cropping soils. The South Australian Research and Development Institute (SARDI) in Adelaide provides a routine DNA-based testing service, PreDicta B, to assist Australian grain growers to assess the risk from soilborne diseases before planting a crop (15). This service is delivered through accredited advisors distributed across the nation. An optimized sampling strategy has been developed for collection of soil samples from fields, involving a composite sample from 45 soils cores (10 mm in diameter, 100 mm in depth) in individual fields or management zones (12,13). Composite soil

Corresponding author: J. A. Davidson,
E-mail: jenny.davidson@sa.gov.au

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samples, 500 g per individual field, from the field-pea-cropping zones in the four states across the southeastern and western regions of Australia were submitted in fall 2008 and 2009 for commercial testing to detect a range of soilborne pathogens, including *D. pinodes*, *P. medicaginis* var. *pinodella*, and *P. koolunga*, using quantitative real-time polymerase chain reaction (qPCR) assays. The primers and probes for these assays were designed from the highly conserved internal transcribed spacer (ITS) region of the ribosomal genome (8,15) (Table 1). *D. pinodes* and *P. medicaginis* var. *pinodella* are identical in this region (17) and the same DNA test was used to quantify the combined DNA of these two pathogens, designated “*DpPmp*”. A separate DNA test was used to quantify DNA of *P. koolunga*. Both assays were designed using sequence information from local isolates and from GenBank, and specificity was evaluated using a range of target and closely related nontarget isolates (8). DNA standards were prepared by separate 10-fold dilutions of DNA from 200 pg/μl to 2 fg/μl, using DNA extracted from *P. koolunga* isolate 147/03 and *D. pinodes* isolate 11/01.

Soil samples were oven dried and DNA was extracted by the commercial service provided by SARDI (15). PCR conditions used by the SARDI laboratory have been described elsewhere (13,18,20). The quantity of DNA (picograms per gram of soil) of *D. pinodes*, *P. medicaginis* var. *pinodella*, and *P. koolunga* was estimated in these samples and designated as high (>316 pg/g of soil), medium (101 to 316 pg/g of soil), low (13 to 100 pg/g of soil), or below detection limit (<13 pg/g of soil). These categories were based on earlier experiments on infectivity of soilborne inoculum of the ascochyta blight pathogens (9). The results were mapped, using ArcGIS version 9.3.1, to the nearest town to show the total number of tests per locality and the percentage of tests in each category of high, medium, low, or below detection limit.

Soil from yield evaluation trial sites for field pea in the states of South Australia and Western Australia established and managed by other researchers was tested for the presence of DNA of *DpPmp* and *P. koolunga* prior to sowing. Soil cores were taken in a W pattern, to a depth of 10 cm (15), at 48 field pea trial sites in April 2009 in South Australia and at 30 sites in April 2010 in Western Australia. Cores from each site were bulked (500 g per site) and subjected to the same DNA tests described above. The cropping history of field pea and other pulses (i.e., faba bean, chickpea, and lentil) as well as legume pastures was collated for these trials sites where possible and compared with the quantity of pathogen DNA using unbalanced analysis of variance.

Survival of pathogen DNA in soil from field pea disease management trial sites over time. Soil cores were sampled from field pea disease management trial sites in fall (April to May) each year from 2006 to 2010. These field experiments, described elsewhere (14), were conducted from 2007 to 2009 in South Australia to assess fungicide efficacy against ascochyta blight in field pea, each with two or three times of sowing. The sites represented medium to high (MH), medium (M), and low (L) rainfall cropping regions in South Australia, with annual average rainfall of 468, 375, and 327 mm, respectively. The soil types at the MH and M rainfall sites were calcareous clay-loams with a surface soil (0 to 10 cm) and alkaline with pH (water) ranging from 7.5 to 8.2 at MH (Turret-

field) and 8.3 to 8.5 at M (Hart). The soil type at Minnipa, the L rainfall site, was a calcareous sandy loam with a surface soil (0 to 10 cm) and alkaline with pH (water) ranging from 8.4 to 8.8. Soil cores were collected as above to 500 g/site before each management trial was planted and were collected again in the years following the field pea trial until 2010. All soil samples were subjected to the DNA tests described above. Field pea and pulse cropping history was collated for these trials sites where possible and compared with the quantity of pathogen DNA using unbalanced analysis of variance.

Quantification of DNA of the fungal pathogens in naturally infected field pea plants. Field pea plants (‘Kaspa’) were sampled from the disease management trials described above at regular intervals during the growing seasons of 2007, 2008, and 2009. Six plants were collected at random from buffer (nonexperimental) plots at each time of sowing every 2 weeks at the M and MH rainfall sites and every 4 weeks at the L rainfall site and were assessed for percent disease severity. In 2007 and 2008, fungi were isolated from a representative lesion on the stem and a leaf of each plant and were identified based on morphological characteristics of conidia and cultures to confirm the presence of *D. pinodes*, *P. medicaginis* var. *pinodella*, and *P. koolunga*. A total of 697 isolates was collected across the six trials.

The plants were placed into plastic bags with pinprick holes for ventilation and stored at -20°C until lyophilized using a Cuddon FD80 (Blenheim, New Zealand). The plants were milled and DNA was extracted from 1 g of the homogenized sample by the PreDicta B service, and target DNA was quantified using qPCR as described above. The results were expressed as picograms of target DNA per g dry plant. The concentration of target DNA in the field pea plants was regressed against percent disease severity on plants and the resulting slopes were compared using simple linear regression, with *DpPmp* and *P. koolunga* as the groups.

In this experiment, more DNA of *P. koolunga* than *DpPmp* was always detected in the naturally infected field-grown pea plants even when the isolation frequency for *P. koolunga* was lower and the quantity of soilborne DNA was less than that of *DpPmp*. This may have been due to a greater density of *P. koolunga* in the plant material. The following procedure was carried out to estimate the amount of DNA per unit weight of mycelium for *D. pinodes*, *P. koolunga*, and *P. medicaginis* var. *pinodella*. Mycelial broth cultures of each pathogen were produced as follows. Single conidium-derived isolates of the three pathogens had been collected from typical ascochyta blight leaf and stem lesions on naturally infected field pea plants and stored as mycelial plugs in sterile water at 4°C (8). Ten isolates from the three regions described above were selected for this study: *D. pinodes* (isolates FT07005, FT07009, and FT07015), *P. koolunga* (isolates DAR78535, FT07006, FT07010, and FT07013), and *P. medicaginis* var. *pinodella* (FT06009, FT07007, and FT07011). Mycelial plugs were removed from storage vials and placed onto potato dextrose agar (PDA). These were incubated for 10 to 14 days under fluorescent light and blacklight for 12 h per day and night at 22°C. Conidial suspensions of individual isolates were prepared by flooding the plates with sterile reverse osmosis (RO) water and gently rubbing the culture surface with a sterile glass rod to suspend the conidia. Sterilized yeast extract broth (10 g of Bacto yeast extract plus 10 g of sucrose made up to 1 liter with sterile RO water) was poured into 20 Schott bottles (100 ml/bottle) and cooled to room temperature, after which 1 ml of conidial suspension was added to each bottle. Each of the 10 isolates was added to four bottles. The bottles were incubated at room temperature for 7 days on a platform shaker at 100 rpm. Mycelium was harvested by passing the resulting cultures through sterile filter paper (Filtach, grade 1803, 90 mm in diameter) in a ceramic Buchner funnel (90 mm in diameter) using vacuum suction. The funnel and vacuum bottle were cleaned after each bottle of broth by washing in sterile RO water, soaking in 0.95% hypochlorite (Milton) for 3 min, and rinsing in sterile RO water. The mycelium from each bottle was patted dry, weighed, wrapped in aluminum foil, and freeze dried. DNA was extracted from 0.1 g of

Table 1. Quantitative polymerase chain reaction primer and probe sequences used to quantify DNA of *Phoma koolunga* and *Didymella pinodes* plus *P. medicaginis* var. *pinodella* (*DpPmp*) in soil, plant, and fungal assays

Species, primers, probes	Sequence 5'–3'
<i>P. koolunga</i>	
Forward primer	CGCAGACTCGCCTCACAG
Reverse primer	AATGTACTTTTGGACGCCGCT
Probe	6FAM-CGCTTTGCACTCATAA-MGB
<i>DpPmp</i>	
Forward primer	AGAGACCGATAGCGCACAAG
Reverse primer	AGTCCAGGCTGGTGCAGGA
Probe	6FAM-CATGTACCTCTCTCCGGG-MGB

² FAM = carboxyfluorescein and MGB = minor groove binder.

lyophilized hyphae and tested using qPCR as above, and the results were expressed as micrograms of target DNA per gram of lyophilized hyphae.

Infectivity of the soilborne inoculum tested in a pot bioassay.

The relationship between soilborne inoculum of the ascochyta blight pathogens and subsequent disease severity on seedlings of field pea was investigated in a pot bioassay using a serial dilution of naturally infested soil. Soil (2 kg) was collected in May 2006 from a site in the MH rainfall region following an ascochyta blight-affected field pea crop in 2005. The soil was serially diluted by mixing 1:4 11 times with steam-sterilized sand (Waikerie Sand from Berri) supplied by Plant Growth Services at SARDI. These 11 dilutions, as well as the original soil sample and the sterile sand, were subjected to the DNA tests described above. Soil from each of the dilution series was placed into 12-cm pots, two pots per sample. Each pot was sown with 4 seeds of field pea 'Parafield' and watered by overhead irrigation twice daily for 5 min. The length of ascochyta blight lesions on stems and percent leaf area diseased (%LAD) were measured weekly for 6 weeks, beginning 6 weeks after sowing. The quantity of DNA in the soil dilutions was regressed against the length of stem lesions (in millimeters) and the %LAD on plants. The resulting slopes were compared using simple linear regression with *DpPmp* and *P. koolunga* as the groups.

Fungi were isolated from representative lesions on the stems and leaves of the plants and identified based on morphological characteristics of conidia and cultures to confirm the presence of *D. pinodes*, *P. medicaginis* var. *pinodella*, and *P. koolunga* (8). Single conidium-derived cultures were subjected to the DNA tests to corroborate identification by cultural methods. DNA was extracted from mycelia scraped from the cultures and subjected to the DNA assays described above (Table 1).

Statistical analysis. Statistical analyses (Student's *t* tests, unbalanced analyses of variance, and simple linear regressions) were conducted using Genstat version 12.1. Data for the picograms of DNA were transformed to logarithm (log DNA + 1) for statistical analyses. Untransformed data are presented in text and tables, while transformed data are presented in the figures. When *F* values indicated significant differences, mean separation was based on least significant differences at 5% level of probability.

Results

Presence and quantity of pathogen DNA in field-pea-cropping soils. Over the 2-year period of 2008 to 2009, 1,042 commercial soil samples from the southeastern and western cropping regions across four Australian states were tested for the presence of *P. koolunga* and *DpPmp* DNA (Table 2). The DNA of *DpPmp* was detected in 44% of these soil samples, varying between 40 and 52% for each state. Conversely, *P. koolunga* was detected in only 6% of all the samples, with the highest incidence in South Australia at 6%, while the incidence was 3% or less in the other states. *P. koolunga* DNA was widely distributed throughout South Australian field-pea-cropping soils but limited in distribution in Western Australia and the eastern states of Victoria and New South Wales (Fig. 1).

The DNA of *DpPmp* was detected in 77% of the soil samples from the field pea yield evaluation sites in South Australia and Western Australia, while *P. koolunga* DNA was detected in 35% of the soils from the South Australian trial sites across the three main field-pea-cropping regions and in 22% of the soils from the Western Australian trial sites, in the Esperance and Katanning regions (Table 3). A variable amount of DNA was detected in each year, ranging from zero to a maximum of 6,457 pg/g of soil for *DpPmp* and a maximum of 9772 pg/g of soil for *P. koolunga*. *P. koolunga* was coincidental with *DpPmp* in all but three of the South Australian samples but the latter two pathogens were regularly detected in soils in which *P. koolunga* was not detected (Fig. 2). The mean quantity of *DpPmp* DNA (73 pg/g of soil) was significantly ($t = 6.04$, $df = 146$, $P < 0.001$) higher than the mean quantity of *P. koolunga* DNA (8 pg/g of soil) averaged across all the samples. The mean quantity of *DpPmp* DNA detected in the soils decreased significantly ($P < 0.05$) with increasing number of years since a pulse crop or legume pasture had been grown (Fig. 3) but, in some cases, pathogen DNA was not detected in soils within 3 months of the field pea crop being harvested. The mean quantity of *P. koolunga* DNA did not vary with number of years since a pulse crop or legume pasture was grown.

Survival of pathogen DNA in soil from field pea disease management trial sites over time. The mean quantity of *DpPmp* DNA (219 pg/g of soil) was significantly ($P < 0.001$) higher than the mean quantity of *P. koolunga* DNA (46 pg/g of soil) averaged for all the soils from the disease management trial sites tested over the 4 years of this study. When each site was analyzed separately (Table 4), the quantity of *DpPmp* DNA was significantly ($P < 0.001$)

Table 3. Quantity of DNA of *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (*DpPmp*) and *P. koolunga* in soils of field pea yield evaluation trials sampled presowing in fall (April to May) in the states of South Australia (SA) in 2009 and Western Australia (WA) in 2010

State, region	Samples ^y	Interval ^z	Number of positive samples	
			<i>DpPmp</i>	<i>P. koolunga</i>
SA				
Eyre Peninsula	10	1	10	6
Mallee	1	1	0	0
Mid and Upper North	25	1–5	17	6
South East	3	ND	3	0
Yorke Peninsula	8	1–20	6	4
Total	47	...	36	16
WA				
BindiBindi	1	2	1	0
Esperance	3	3–6	2	3
Geraldton	8	1–4	6	0
Katanning	9	1–4	8	3
Merredin	5	1–3	5	0
Total	26	...	22	6

^y Number of samples.

^z Cropping interval = range of years since a pulse crop or legume pasture. ND = not data.

Table 2. Results of commercial soil samples tested for DNA of *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (*DpPmp*) and *P. koolunga* in fall (April to May) 2008 and 2009 from four states across the southeastern and western cropping regions of Australia

State ^y	Total tests ^z	Number of tests per category ^x							
		<i>DpPmp</i>				<i>P. koolunga</i>			
		BDL	Low	Medium	High	BDL	Low	Medium	High
NSW	352	212	81	33	26	351	0	1	0
SA	258	151	53	29	25	217	25	15	1
Vic	272	148	65	30	29	256	12	3	1
WA	160	77	45	21	17	151	4	4	1
Total	1,042	588	244	113	97	975	41	23	3

^x BDL = Below Detection Limit (< 13 pg/g soil); Low = (13–100 pg/g soil); Medium = (101–316 pg/g soil); High = (316 pg/g soil).

^y NSW = New South Wales, SA = South Australia, Vic = Victoria, and WA = Western Australia.

^z Total number of tests.

higher than that of *P. koolunga* at the MH and M rainfall sites but not at the L rainfall site. There was significantly ($P < 0.01$) more *DpPmp* DNA at the MH rainfall site than at the M rainfall site, whereas the quantity of *P. koolunga* DNA was statistically equivalent at these two sites. There was only one instance when the quantity of *P. koolunga* DNA was higher than the quantity of *DpPmp* DNA: the 2007 M rainfall site sampled in fall 2008 following the ascochyta blight infected trial (*data not shown*). The quantity of pathogen DNA detected at the L rainfall site was significantly ($P < 0.01$) lower than at the MH and M rainfall sites for both *DpPmp* and *P. koolunga*.

The quantity of pathogen DNA in the soils increased following field pea in all cases except for the M rainfall site in 2008 and then declined each subsequent year. The MH and M rainfall sites were similar in terms of quantity of pathogen DNA per year while the L site was significantly ($P = 0.05$) different from the other sites. At the MH and M rainfall sites, the decline in pathogen DNA was significant ($P < 0.001$) for *P. koolunga* but not for *DpPmp* (Fig. 4). Extremely low levels of pathogen DNA (<20 pg/g of soil) were consistently detected at the L site, although this increased to above 100 pg/g of soil following a trial with severe ascochyta blight in 2009.

Quantification of DNA of the fungal pathogens in naturally infected field pea plants. In all, 54% of the total of 697 isolates obtained from the field pea plants sampled from the disease management trials in 2007 and 2008 were *D. pinodes*, whereas 41% were *P. koolunga* and only 5% were *P. medicaginis* var. *pinodella* (Table 5). *P. koolunga* was the most common pathogen (87%) isolated from plants at the M rainfall site in 2007 and made up 50% of isolates in 2008 from the same site, whereas it made up only 9% of the isolates from the L rainfall site.

There was a significant ($P < 0.001$) linear relationship between the percent disease severity on plants and the quantity of pathogen DNA in the plants (log picograms of DNA + 1 per gram of plant

tissue) collected from each trial each season (Fig. 5). The only nonsignificant relationship was at the L rainfall trial in 2008, where very little disease developed. The linear relationship between percent disease and log (picograms of DNA + 1) of *DpPmp* ($R^2 = 0.39$) and of *P. koolunga* ($R^2 = 0.59$) combined for all trials was significant ($P < 0.001$) and accounted for 39 and 58% of variation, respectively. These slopes were significantly different ($P < 0.001$), in that consistently more *P. koolunga* DNA (log [picograms of DNA + 1] = $3.47 + 0.0582 \times$ percent disease) than of *DpPmp* (log [picograms of DNA + 1] = $2.87 + 0.0402 \times$ percent disease) was detected at a given disease severity.

The quantity of fungal DNA per gram of freeze-dried mycelia was not significantly different among the three pathogens, with an

Fig. 2. Relationship between log (picograms of DNA + 1) of *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (*DpPmp*) and log (picograms of DNA + 1) of *Phoma koolunga* in the soils sampled prior to sowing from the field pea yield evaluation sites in the states of South Australia in 2009 and Western Australia in 2010.

Fig. 1. Distribution of **A**, *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* and **B**, *Phoma koolunga* in field-pea-cropping soils in southeastern and western regions of Australia sampled for commercial testing in fall (March to May) 2008 and 2009. Each pie (circle) on the map represents a sample location and the pie size increases with the number of samples from that site. Percentage of risk categories detected at each site is shaded in each pie according to the legend.

average of 285 µg of DNA per gram of freeze-dried mycelium. Hence, the ratio of DNA per gram of plant tissue in field pea plants sampled from the field trials was not altered for these pathogens.

Infectivity of the soilborne inoculum tested in a pot bioassay. The quantity of pathogen DNA in the soil dilution series decreased exponentially with soil:sand dilution factor (*DpPmp* $R^2 = 0.87$, $P < 0.001$; *P. koolunga* $R^2 = 0.86$, $P < 0.001$). There was a logarithmic relationship between the summed amount of pathogen DNA in the soil and the disease measured at each assessment (Fig. 6). The highest regression coefficient was attained in the fifth week of assessment: length of stem $R^2 = 0.54$ ($P < 0.001$) and %LAD $R^2 = 0.25$ ($P < 0.001$). The regression between stem lesions and log

Fig. 3. Relationship between the number of years since a pulse crop or legume pasture was grown and the mean quantity of pathogen DNA (log [picograms of DNA + 1] per gram of soil) in the soil sampled prior to planting field pea in the states of South Australia in 2009 and Western Australia in 2010. For DNA of *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (*DpPmp*): minimum least significant difference = 0.564, average least significant difference = 1.356, maximum least significant difference = 2.175; for DNA of *P. koolunga*: not significant, $P = 0.05$. Gray columns = *DpPmp*, black columns = *P. koolunga*.

Table 4. Mean quantity of pathogen DNA (picograms of DNA per gram of soil) in soils of field pea disease management trials in South Australia sampled in fall (April to May) each year for four consecutive seasons (2006–10)^w

Pathogen ^x	Sites ^y	DNA (pg/g) ^z
<i>DpPmp</i>	Turretfield (MH)	2,041 a
	Hart (M)	328 b
	Minnipa (L)	4 d
<i>P. koolunga</i>	Turretfield (MH)	67 c
	Hart (M)	117 c
	Minnipa (L)	4 d

^w Field pea disease management trials were described by McMurray et al. (14). First samples were taken prior to sowing field pea and sites were resampled in subsequent seasons until 2010.

^x *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (*DpPmp*) and *P. koolunga*.

^y MH, M, and L = medium-high-, medium-, and low-rainfall regions, respectively.

^z Different letters indicate significant differences at $P = 0.05$.

Table 5. Number of isolates identified as *Didymella pinodes*, *Phoma medicaginis* var. *pinodella*, and *P. koolunga* of the total number of cultures isolated from ascochyta-blight-affected field pea plants sampled from disease management trials in 2007 and 2008 in South Australia^x

Pathogen	Trial site and year (years since previous field pea crop) ^y						Isolates ^z
	Turretfield (MH)		Hart (M)		Minnipa (L)		
	2007 (5)	2008 (5)	2007 (2)	2008 (3)	2007 (10)	2008 (10)	
<i>D. pinodes</i>	36	174	11	80	37	38	376
<i>P. medicaginis</i> var. <i>pinodella</i>	16	10	3	5	2	1	37
<i>P. koolunga</i>	28	85	72	86	9	4	284
Total number of isolates	80	269	86	171	48	43	697

^x Field pea disease management trials were described by McMurray et al. (14).

^y MH, M, and L = medium-high-, medium-, and low-rainfall regions, respectively.

^z Total number of isolates per species.

(picograms of DNA + 1) of *P. koolunga* in the soil ($R^2 = 0.55$, $P < 0.001$) was statistically equivalent to the regression between stem lesions and log (picograms of DNA + 1) of *DpPmp* in the soil ($R^2 = 0.52$, $P < 0.001$). Likewise, the regressions between %LAD and log (picograms of DNA + 1) of *P. koolunga* and of *DpPmp* in the soil were statistically equivalent, even though the correlation coefficient was smaller than for stem lesions (Fig. 6B). The isolation of all three pathogens (*D. pinodes*, *P. medicaginis* var. *pinodella*, and *P. koolunga*) was confirmed by cultural methods and DNA tests.

Discussion

D. pinodes and *P. medicaginis* var. *pinodella* were widely distributed and frequently detected in the field-pea-growing soils of southeastern and western regions of Australia. Conversely, although *P. koolunga* was widely distributed across the field-pea-growing regions of the state of South Australia, it had a limited distribution in the states of New South Wales, Victoria, and Western Australia. The origin of *P. koolunga* is unknown but its wider distribution in South Australia suggests either a point of origin or reflects the intensity of field pea cropping in that state. *P. koolunga* may have spread to the other states from South Australia on field pea seed recently (1) and may become more widespread in future years. The coincidental nature of *P. koolunga* with *D. pinodes* and *P. medicaginis* var. *pinodella* also suggests that the distribution of *P. koolunga* is directly linked to field pea cropping; however, this requires substantiation.

Overall, more DNA of *D. pinodes* plus *P. medicaginis* var. *pinodella* than of *P. koolunga* was detected in soils. The smallest amounts of DNA of all three pathogens were detected in the calcareous sandy loam at the L rainfall site. The quantity of pathogen DNA detected in soil generally increased following a field pea crop

Fig. 4. Mean log (picograms of DNA + 1) of pathogen DNA in soils in the years following field pea disease management trials for the combined medium-high (MH) and medium (M) rainfall sites and for the low (L) rainfall site in South Australia from 2007 to 2010. For DNA of *Phoma koolunga* at the combined MH and M rainfall sites: minimum least significant difference = 0.6706, average least significant difference = 0.7306, maximum least significant difference = 0.7891, $P = 0.05$. All other comparisons are nonsignificant. Gray columns = *Didymella pinodes* plus *P. medicaginis* var. *pinodella* (*DpPmp*); black columns = *P. koolunga*.

and then declined in subsequent years. However, the relationship between the quantity of soilborne inoculum and the years since a host crop had been grown was extremely variable and, in some cases, pathogen DNA was not detected in the soil samples collected 3 months after a host crop was harvested. The field pea disease management trials at the L rainfall site had no disease in 2007 and 2008 (14) and no pathogen DNA was detected in the associated soils. Hence, the amount of pathogen DNA in the soil appeared to be influenced by the intensity of the ascochyta blight epidemic in the crop and also by the length of time since a host crop had been grown.

The severity of ascochyta blight symptoms on field pea seedlings in pots was correlated with the amount of soilborne inoculum in the soil dilution series. Likewise, the relative amounts of pathogen DNA detected in soil samples from disease management trial sites (14) reflected the frequency of isolation of *P. koolunga*, *D. pinodes*, and *P. medicaginis* var. *pinodella* from naturally infected field pea plants. Although the plants in these trials would have been exposed to airborne ascospores of *D. pinodes*, which are considered the primary inoculum for ascochyta blight, the results of the current study indicate that disease severity may also be influenced by the amount of soilborne inoculum.

D. pinodes and *P. medicaginis* var. *pinodella*, the recognized causal agents of ascochyta blight on field pea, can survive for up to 20 years after a field pea crop has been grown (21,22). The current study showed that DNA of *P. koolunga* is detectable in soil for at least 4 years after a field pea crop, and the potential for longer survival periods should be investigated. All the causal pathogens of ascochyta blight on field pea have the potential to be widespread in field-pea-cropping soils, and this soilborne inoculum can initiate epidemics.

Naturally infected field-grown pea plants always yielded more DNA of *P. koolunga* than *D. pinodes* plus *P. medicaginis* var. *pi-*

Fig. 6. A, Length of stem lesions (in millimeters) and, B, percent leaf area diseased (%LAD) in dilution series pot bioassay regressed against log (picograms of DNA + 1) of *Phoma koolunga* and log (picograms of DNA + 1) of *Didymella pinodes* plus *P. medicaginis* var. *pinodella* (DpPmp) in fifth week of assessment.

Fig. 5. Linear regressions between percent disease severity on plants and log (picograms of DNA + 1) of *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (◆ and bold line) and *Phoma koolunga* (* and non-bold line) per gram of plant tissue in nine field pea disease management trials in medium-high (MH), medium (M), and low (L) rainfall regions in South Australia from 2007 to 2009.

nodella, even when the isolation frequency for *P. koolunga* was lower and the quantity of soilborne DNA was less than that of *DpPmp*. The possibility that this may reflect differences in amplification efficiency was largely discounted by the finding of similar amounts of DNA per unit weight of mycelium grown in vitro for all three pathogens. Other possible reasons for the larger amount of *P. koolunga* in plant tissue include differences in density of mycelium within the plant material, or a greater number of fruiting bodies and spores of *P. koolunga* which would concentrate the DNA within the plant tissue; alternatively, the number of nuclei per conidium may vary. Further studies are required to ascertain the reason for this observation.

Both *D. pinodes* and *P. koolunga* were isolated from plant material at a higher frequency than *P. medicaginis* var. *pinodella*, indicating that the latter plays a minor role in the disease complex. The DNA tests used in this study, based on the highly conserved ITS region of the genome (8,15), do not distinguish between the closely related species *D. pinodes* and *P. medicaginis* var. *pinodella*. An assay that can distinguish between the two pathogens would facilitate interpretation of the results of soil and plant DNA tests.

This study showed *P. koolunga* to be widespread in field-pea-cropping soils of South Australia but to have limited distribution in soils of other cropping regions of Australia. It can be detected in soil for at least 4 years following an infected field pea crop and initiate epidemics of ascochyta blight on field peas. The combined results of DNA tests and pathogen isolation from the naturally infested field pea plants indicated that *P. koolunga* and *D. pinodes* were, in general, equally responsible for the ascochyta blight symptoms in field trials, whereas *P. medicaginis* var. *pinodella* appeared to play a minor role in this disease complex.

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