Seedborne Infection of Pyrethrum by *Phoma ligulicola*

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ABSTRACT

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The incidence of *Phoma ligulicola* in Tasmanian pyrethrum seed and methods of managing seedborne mycoflora were determined. Fourteen different fungi were regularly isolated from seed, including *Alternaria tenuissima*, *Stemphylium botryosum*, and *P. ligulicola*, which have been documented as pathogens of pyrethrum. Comparisons between the incidence of these fungi from seed surface-sterilized with sodium hypochlorite and nontreated seed indicated they occurred both within and on the outside of the seed. A polymerase chain reaction (PCR) test for the detection of *P. ligulicola* was also developed, with a detection limit of 800 fg of fungal DNA. The assay detected infested seed lots down to an incidence of 0.5%. Reliable amplification of the target DNA was achieved with the addition of bovine serum albumin to reduce the influence of inhibitors from pyrethrum seed. Agar plate tests and PCR demonstrated variability with pyrethrum cultivars in the presence and viability of *P. ligulicola* in seed. Effective management strategies for the reduction of seedborne *P. ligulicola* included the regular use of fungicides for reducing foliar disease intensity in the seed fields prior to harvest. Seed treatments with fludioxonil and thiabendazole/thiram also significantly reduced the incidence of seedborne *P. ligulicola* and increased seed germination and seedling survival.

Additional keywords: seedborne pathogen, *Tanacetum cinerariaefolium*

Ray blight, caused by *Phoma ligulicola*, is a major threat to production of pyrethrum (25) and chrysanthemum (3,4). The disease is found in pyrethrum-producing regions such as East Africa (30), Papua New Guinea (31), and Tasmania, Australia (22,27). Epidemics in Tasmanian pyrethrum fields occur annually in early spring. Symptoms range from necrotic spots on leaves and lesions on stems (23), to flower and bud blight (23,27). If infection of the developing flowering stem is not managed by the strategic use of fungicides in spring, the disease can lead to complete loss of yield and early termination of fields (23,25).

In chrysanthemum, inoculum sources can include alternative Compositae hosts (9), pseudosclerotia and conidia within the soil (6), infected cuttings (8), and airborne ascospores (3). Soilborne inoculum of *P. ligulicola* causing disease in chrysanthemum is mildly pathogenic to leaves or stems of globe artichoke (*Cynara scolymus* L.), rudbeckia (*Rudbeckia hirta* L.), zinnia

(*Zinnia elegans* L.), sunflower (*Helianthus annuus* L.), and dahlia (*Dahlia variabilis* Desf.), but severely pathogenic to lettuce (*Lactuca sativa* var. *crispa* L.), suggesting chrysanthemum crops should be geographically separated from these crops and avoided in their rotations (9). In rotations with nonsusceptible hosts, the inoculum contribution of pseudosclerotia is probably minimal, as their survival in natural soil (<30 weeks) and compost (<8 weeks) is limited (6). Numbers and viability of conidia also decline rapidly in natural soil (6). However, the pathogen-free status of the planting material is of paramount importance in reducing the probability of disease within commercial chrysanthemum crops. For example, symptomless infected cuttings were considered the major means of spread of this disease throughout the United States (4). Symptomless surface colonization of cuttings and roots by *P. ligulicola* were demonstrated from non-sterile soil where the fungus remained viable as an epiphyte for at least 12 weeks. These studies also demonstrated epiphytic colonization of 12 other ornamental species, including pyrethrum, but for no more than 8 weeks (8).

In contrast to chrysanthemum, little knowledge exists regarding the inoculum sources for ray blight disease in pyrethrum. The contribution of soilborne inoculum such as pseudosclerotia to the

epidemic is unknown. However, rotations between pyrethrum in the same field are generally at least 3 years, suggesting that soilborne inoculum contributes to the epidemic only minimally. Pyrethrum fields in Tasmania are not generally within the same geographic region as ornamental production, and therefore are not as likely to act as alternative hosts. The presence of the teleomorph, *Didymella ligulicola*, has not yet been confirmed in Tasmania, suggesting minimal involvement of ascospores which could contribute to long-distance dispersal (29,35). Vegetative propagation by splitting mother plants into several propagules, which may contain infected cuttings, has been used for field establishment in the past. Seed has become the primary means of propagation since 1999, and anecdotal evidence has suggested that seed may act as an inoculum source.

The objectives of this study were to: (i) characterize the mycoflora associated with pyrethrum seed; (ii) develop a polymerase chain reaction (PCR) test for the detection of seedborne *P. ligulicola*; (iii) quantify the incidence and viability of *P. ligulicola* in pyrethrum seed; (iv) examine the efficacy of different fungicide seed treatments for the reduction of seedborne *P. ligulicola*; and (v) quantify the relationship between foliar disease intensity and seedborne infection by *P. ligulicola*.

MATERIALS AND METHODS

Mycoflora of pyrethrum seeds. The mycoflora from commercial pyrethrum seed fields harvested from 1999 to 2004 were assessed by plate tests at a maximum of 30 days after harvest. Samples of harvested seed were stored in a paper bag at 4°C. Two hundred arbitrarily selected seeds from each field were surface-sterilized in 1% sodium hypochlorite for 2 min, rinsed in sterile distilled water three times, and placed on 2% water agar (WA) in petri dishes. Ten seeds were placed on each petri dish and incubated in the dark at 20°C. A further 10 non-surface-sterilized seeds were placed directly on WA in each of 20 petri dishes. Fungi were identified based on morphological characteristics after 21 days incubation using standard mycological keys (5). The presence of *P. ligulicola* was confirmed by subculturing hyphal tips to potato dextrose (Amyl Laboratories, Melbourne, Australia), malt extract (malt extract-oxid L39 and oxid

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agar no. 1), and oatmeal agars for identification (7,29,34).

Detection of *P. ligulicola* in pyrethrum seed by PCR. The efficiency of three methods of DNA extraction from pyrethrum seed for the detection of *P. ligulicola* using a PCR test previously described for detection from mycelium were tested (26). Five seed lots were created by mixing 190, 95, 45, 20, and 10 seeds from a commercially harvested cultivar containing 21% of seeds infected with *P. ligulicola*, determined by repeated plate tests, and made up to 380 seeds with a commercially harvested seed lot of the same cultivar from which *P. ligulicola* was not detected. These gave estimated final incidences of 10.5, 5.25, 2.5, 1.1, and 0.5%, respectively. Four replicates of each seed lot were prepared. In the first extraction method (method 1), approximately 100 mg of seed was ground to a powder using a mortar and pestle with liquid nitrogen, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. In the second extraction method (method 2), the entire seed sample was ground to a fine powder in a seed mill (Glen Creston Ltd., UK) and 100 mg used for DNA extraction using the DNeasy Plant Mini Kit (Qiagen). Final elutions of DNA from the columns using both these methods were conducted with 100 µl of standard elution buffer. In the third and fourth methods, each seed lot was shaken in 50 ml of CTAB buffer for 2.5 h at room temperature. Two milliliters of the resultant solution was then removed for DNA extraction using the DNeasy Plant Mini Kit (Qiagen) (method 3) or purification with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (method 4). For the latter method, an ethanol precipitation was then conducted and resultant DNA collected in 10 µl of TE buffer. The presence of DNA extracted from all methods was verified by separation on a 1% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) stained with ethidium bromide (0.5 mg/liter) and visualized under UV light. The concentrations of extracted DNA were assessed by comparison to molecular mass standards (Bio-Rad Laboratories, Hercu-

les, CA). Purity was judged by the extraction of DNA in one or multiple bands.

Detection of *P. ligulicola* from DNA extractions from pyrethrum seed used the primer pairs (PL1/PL2) previously developed to amplify a 230-bp product within the ITS region (26). In brief, the PCR was performed in 50-µl reactions containing 2.5 mM MgCl₂, 1 mmol deoxynucleoside triphosphate, 0.5 µmol of each oligonucleotide primer, and 1 unit *Taq* polymerase (Qiagen) in 1× PCR Buffer (Qiagen). Optimization of the PCR conditions to minimize PCR inhibition by plant extracts was conducted with different concentrations (1, 3, 5, 10, 15, and 20%) of bovine serum albumin (BSA) (Roche Diagnostics, Alameda, CA). One microliter containing 20 to 25 ng of DNA was used as the template for each reaction. PCR reactions were conducted with a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) thermocycler using the following conditions: 5 min at 94°C for initial denaturation followed by 35 cycles of 30 s of denaturation at 94°C, 1 min of annealing at 56°C, and 2 min extension at 72°C. Reactions concluded with a final extension at 72°C for 10 min. PCR products were visualized in a 2% agarose gel (ICN Biochemicals, Ohio) and 1× TBE (40 mM Tris-borate, 1 mM EDTA, pH 8.0) buffer containing ethidium bromide under UV light.

The sensitivity of the PCR test was examined using three different methods. The first method used a dilution series of *P. ligulicola* genomic DNA from isolate DZ 62547 ranging from 8 ng/µl to 8 fg/µl, diluted in sterile distilled water. Concentrations were determined by comparison to a molecular mass standard (Bio-Rad Laboratories) on a 2% agarose gel (described above). Each dilution was replicated and the PCR was conducted twice. The two subsequent methods examined the sensitivity of the PCR to detect *P. ligulicola* when pyrethrum seed DNA was also present in the extractions. To test whether the PCR could detect one infected seed in a 100-mg sample of healthy seeds, 20 replicates of seed lot mixtures were produced containing one seed randomly selected from a highly infected seed lot (65%) and the remaining from a seed lot where *P. liguli-*

cola was not detected by repeated plate tests or PCR. DNA was extracted from these seed lots using method 1. The detection limit was further examined with the DNA extracted by method 1 from the range of replicate incidence batches (10.5 to 0.5%), described above to examine the reliability of DNA extraction techniques.

Incidence of *P. ligulicola* in pyrethrum seed. The incidence of *P. ligulicola* in commercial seed harvested from fields in 1995 to 2003 was assessed by plate tests and PCR in 2003. Plate tests used the techniques described above to characterize the spectrum of mycoflora associated with pyrethrum seed and hence determined if the *P. ligulicola* detected by PCR was viable. Incidence of *P. ligulicola* in seed from fields harvested in 1999 to 2004, in which previous information was available on the incidence of *P. ligulicola* directly following harvest, was compared with the incidence following testing in 2004.

DNA was extracted from 10 replicates of 100 mg of seed following method 1, and PCR detection of *P. ligulicola* was undertaken using a final concentration of 10% BSA within the reaction mixture described above. The incidence of *P. ligulicola* within the seed (%) was estimated from the number of bulk samples in which *P. ligulicola* was detected (e.g., 1/10 bulk samples testing positive = 0.14% estimated incidence; 2/10 = 0.29%; 3/10 = 0.47%; 4/10 = 0.68%; 5/10 = 0.92%; 6/10 = 1.21%; 7/10 = 1.59%; 8/10 = 2.12%; 9/10 = 3.02%; 10/10 = 100%) (11).

Relationship between foliar disease intensity and incidence of seedborne *P. ligulicola*. The relationship between foliar disease intensity prior to harvest and the incidence of seedborne *P. ligulicola* was described. Epidemics of different intensities were generated by the application of fungicides of varying efficacy within a commercial field approaching its first harvest at Table Cape (UTM: 55 G 394130 5658008). Fungicide treatments were: chlorothalonil (1,440 g a.i./ha), azoxystrobin (250 g a.i./ha), kresoxim-methyl (150 g a.i./ha), boscalid and pyraclostrobin (200 g a.i./ha and 400 g a.i./ha, respectively), chlorothalonil and pyrimethanil (750 g a.i./ha and 300 g a.i./ha), and a nontreated control. Fungicide details are listed (Table 1). Fungicides were applied three times from early spring on 24 August, 12 September, and 3 October 2004. Plots were 19.2 m² (3.2 × 6 m) and separated by 1-m buffers to minimize spray drift. Fungicides were applied with a gasoline-driven Solo 432 backpack sprayer with a 1.6-m boom fitted with hollow cone nozzles spaced at 40-cm intervals, delivering 300 liters/ha.

Foliar disease intensity was measured on 20 August (prior to fungicide applications), 13 October, and 18 November. Within each plot, 20 flowering stems were systematically selected along a diagonal

Table 1. List of fungicides and active ingredients used in trials

Product	Active ingredient	Supplier
Amistar WG	500 g/kg azoxystrobin	Syngenta Australia
Bravo 720	720 g/liter chlorothalonil	Syngenta Australia
Sporekill	120 g/liter didecyltrimethyl-ammonium chloride	Lefroy Valley Australia
Thiram	800 g/kg thiram	Barmac Industries Pty. Ltd., Queensland, Australia
Tecto SC	500 g/liter thiabendazole	Syngenta Australia
Maxim 100 FS	100 g/liter fludioxonil	Syngenta Australia
Walabi SC	375 g/liter chlorothalonil and 150 g/liter pyrimethanil	Bayer Crop Science Australia
BAS516	100 g/liter pyraclostrobin and 200 g/liter boscalid	NuFarm Australia
Strobry WG	500 g/kg kresoxim-methyl	BASF Australia Limited

transect. Each stem was removed from the base of the plant stored at 4°C until assessment within 48 h. Defoliation severity was defined as the height to defoliation (leaves either completely necrotic or abscised) divided by the total stem height × 100. On each flowering stem, the number of diseased and healthy flowers and/or buds was also counted. This enabled the calculation of the “incidence of flowering stems with ray blight” defined as the number of flowering stems with any diseased flowers and/or buds divided by the total number of flowering stems × 100, and the “incidence of flowers with ray blight” defined as the number of diseased flowers and/or buds divided by the total number of flowers on each stem × 100.

On 15 February 2005, seed was hand harvested from each plot, air-dried, and cleaned. The incidence of viable *P. ligulicola* in seed from each plot was assessed in plate tests following storage at 4°C in a paper bag for no longer than 28 days. Two

hundred arbitrarily selected seeds from each crop were placed directly on WA (10 seeds per plate) and incubated in the dark at 20°C. Fungi were identified 21 days later based on morphological characteristics using standard mycological keys

(5,29). Linear regression analyses were conducted using Genstat 8, Version 1 (Adept Scientific Inc., Bethesda, MD). Goodness-of-fit between disease intensity in October and November, and the incidence of seedborne *P. ligulicola*, was

Fig. 1. Detection limit of the *Phoma ligulicola* primer pair (PL1/PL2). The primer pair was used in the polymerase chain reaction with serial dilutions of *P. ligulicola* total genomic DNA. Lanes 1 to 7 contain 8 ng, 800 pg, 80 pg, 8 pg, 800 fg, 80 fg, and 8 fg of total genomic DNA, respectively. Lane M contains the 100-bp ladder (Geneworks, Adelaide, Australia). Uppermost band = 500 bp.

Table 2. Incidence (%) of fungi isolated from surface-sterilized or nontreated commercial pyrethrum seeds harvested between 2000 and 2005

Cultivar (line)	Sterilization	Year	Incidence (%)							
			<i>Aureobasidium pullulans</i>	<i>Alternaria spp.^y</i>	<i>Cladosporium spp.^y</i>	<i>Stemphylium botryosum</i>	<i>Ulocladium atrum</i>	<i>Phoma ligulicola</i>	<i>Microsphaeropsis sp.</i>	Others ^z
1-1	None	2000	80	45	45	6	1	22	0	3
1-1	Surface	2000	60	39	22	8	3	17	0	5
2-1	None	2000	69	33	18	4	3	19	3	7
2-1	Surface	2000	74	35	12	7	2	18	2	2
3-1	None	2000	80	27	8	1	6	12	0	6
3-1	Surface	2000	75	29	5	3	4	9	1	4
4	None	2000	74	16	6	0	1	7	0	9
4	Surface	2000	70	15	8	1	1	8	0	5
1-1	None	2001	60	90	14	4	4	4	2	0
1-1	Surface	2001	45	84	8	2	0	4	1	0
1-2	None	2001	72	54	30	0	6	2	0	4
1-2	Surface	2001	52	25	16	0	0	1	0	6
1-3	None	2001	78	84	30	0	6	2	0	4
1-3	Surface	2001	59	41	16	0	1	2	0	2
2-1	None	2001	65	74	30	0	2	4	2	0
2-1	Surface	2001	42	43	18	0	0	4	4	0
2-2	None	2001	74	64	32	0	4	1	0	1
2-2	Surface	2001	23	38	19	0	2	0	0	2
3-1	None	2001	64	92	22	0	2	1	0	2
3-1	Surface	2001	52	34	10	0	0	0	0	1
3-2	None	2001	75	68	68	0	12	2.5	0	2
3-2	Surface	2001	63	12	35	0	1	0	0	1
1-1	None	2002	64	65	25	22	16	10	7.5	10
1-1	Surface	2002	58	41	18	18	5	4	6	8
2-1	None	2002	45	91	15	14.5	17	5	0	12
2-1	Surface	2002	23	76	10	8	2	3	0	7
1-1	None	2003	100	69	16	6	20	3	3	0
1-1	Surface	2003	60	43	8	0	2	1	2	2
2-1	None	2003	85	58	13	8	12	2	1	2
2-1	Surface	2003	74	38	5	1	4	4	1	0
4	None	2003	100	24	6	0	1	0	0	0
4	Surface	2003	81	14	4	0	0	0	0	0
5	None	2003	100	10	14	0	10	0	0	0
5	Surface	2003	74	0	0	0	2	0	0	0
1-1	None	2004	63	43	32	0	10	28	0	0
1-1	Surface	2004	41	12	12	0	1	12	0	0
2-1	None	2004	58	40	18	0	5	15	0	0
2-1	Surface	2004	34	22	6	0	0	10	0	0
1-1	None	2005	75	25	23	4	5	10	4	0
1-1	Surface	2005	64	15	4	9	0	7	3	0
2-2	None	2005	68	33	13	4	5	5	3	0
2-2	Surface	2005	46	29	7	11	1	0.5	2	0

^y *Alternaria* spp. include *A. alternata* and *A. tenuissima*; *Cladosporium* spp. include *C. cladosporioides* and *C. herbarum*.

^z Includes *Acremonium* sp., *Phoma epicoccina*, *Fusarium* spp., *Penicillium italicum*, *Penicillium expansum*, and other sterile fungi.

M 1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2. Detection of *Phoma ligulicola* from pyrethrum seed lots with varying incidence using polymerase chain reaction (PCR) with primer pair PL1/PL2. Lanes 1 to 12 show two independent DNA extractions from each seed lot with incidences of: lanes 1 and 2, 10.5%; lanes 3 and 4, 5.25%; lanes 5 and 6, 2.5%; lanes 7 and 8, 1.1%; and lanes 9 and 10, 0.5%. Lane 11 is a positive control showing the amplification of the approximately 230-bp PCR product from pyrethrum seed total genomic DNA. Lane 12 is a negative control containing pyrethrum seed DNA only. Lane M contains the 100-bp DNA ladder (Geneworks, Adelaide, Australia). Uppermost band = 500 bp.

Table 3. Incidence of *Phoma ligulicola* in pyrethrum seed lots harvested from 1995 to 2005 as determined by polymerase chain reaction (PCR) and plate tests either directly following harvest and/or in 2003

Cultivar (line)	Year	PCR detection		Incidence (%) by plate tests	
		Replicate extractions with <i>P. ligulicola</i> detected (<i>n</i> = 10)	Estimated incidence ^z	Directly following harvest	In 2003
1-1	1995	9	3.02	-	0
1-2	1995	9	3.02	-	0
2-1	1995	2	0.29	-	0
3-1	1995	0	0	-	0
3-2	1995	0	0	-	0
4	1995	0	0	-	0
5	1995	2	0.29	-	0
6-1	1995	0	0	-	0
6-2	1995	0	0	-	0
1-1	1996	10	100	-	0
2-1	1996	2	0.29	-	0
3-2	1996	0	0	-	0
4	1996	0	0	-	0
5-1	1996	0	0	-	0
1-1	1997	9	3.02	-	0
2-1	1997	4	0.67	-	0
3-2	1997	0	0	-	0
1-1	1998	10	100	-	0
1-2	1998	5	0.92	-	0
2-1	1998	0	0	-	0
3-1	1998	0	0	-	0
5	1998	4	0.67	-	0
1-1	1999	10	100	-	2
1-2	1999	10	100	-	0.5
2-1	1999	0	0	-	0
3-1	1999	0	0	-	0.5
4	1999	4	0.67	-	0
1-1	2000	10	100	22	1
2-1	2000	10	10	19	0
3-1	2000	10	10	12	0
4	2000	3	0.47	7	1
1-1	2001	8	2.12	4	6
1-2	2001	9	3.02	2	2
1-3	2001	2	0.29	2	1
2-1	2001	4	0.67	4	1
2-2	2001	0	0	1	0
3-1	2001	0	0	1	0
3-2	2001	2	0.29	2.5	0
1-1	2002	8	2.12	10	2.5
2-1	2002	9	3.02	5	1
1-1	2003	8	2.12	3	-
2-1	2003	0	0	2	-
4	2003	0	0	0	-
5	2003	0	0	0	-
1-1	2004	5	0.92	28	-
2-1	2004	2	0.29	15	-
1-1	2005	5	0.92	10	-
2-2	2005	2	0.29	5	-

^z Incidence estimated based on the number of seed lot replications testing positive by PCR based on the maximum likelihood estimation (11).

based on R^2 , the standard error of the estimate of Y (SE E_y), and visual inspection of residual plots. Precision of the model was assessed by the coefficient of variation (CV), defined as the square root of the mean square error, divided by the grand mean of Y (20).

Effect of fungicides on the incidence of *P. ligulicola*, seed germination, and seedling survival. The efficacy of thia-bendazole (10 ml/kg seed)/thiram (5 g/kg seed) was assessed in two replicated trials on three cultivars where the incidence of *P. ligulicola* in the nontreated seed varied between 12 and 21% as described by plate tests. Furthermore, the efficacy of dide-cyldimethyl-ammonium chloride (20 ml/kg seed) and fludioxonil (7 ml/kg seed) was assessed in another two replicated trials using one cultivar where 21% of nontreated seeds were infected with *P. ligulicola* as determined by plate tests. Fungicides were applied to approximately 3 g of seed. All fungicides and combined fungicide treatments (premixed) were applied as slurry in combination with wall-paper paste (Polycell Products, Australia). Fungicide slurries and seed were placed into a precoated sealed container and mixed for approximately 1 min to ensure total coverage then air-dried, prior to further testing within 48 h.

Reductions in the incidence of *P. ligulicola* were assessed by plate tests. Ten seeds were directly placed on WA in petri dishes and incubated in the dark at 20°C for 21 days. Seed germination was assessed by placing 400 arbitrarily selected seeds on 90 mm Whatman no. 1 filter paper moistened with sterile distilled water in each of 10 sterile petri dishes, which were then sealed and incubated in the dark at 20°C. Seeds germinated after 25 days were counted. Seedling survival was assessed by planting six replicates of 50 arbitrarily selected seeds into autoclaved (121°C for 30 min) seed raising mixture in surface-sterilized trays. Trays were placed in a heated glasshouse (20 ± 4°C) and the number of seedlings counted 28 days after planting. The efficacy of fungicides on reducing the incidence of *P. ligulicola*, improving seed germination and seedling survival, compared with a nontreated control, was compared by analysis of variance using Genstat 8, Version 1 (Adept Scientific Inc.).

RESULTS

Mycoflora of pyrethrum seeds. Fourteen fungal species were regularly associated with pyrethrum seed, and often multiple species were found on individual seeds (Table 2). The most frequently isolated in both surface-sterilized and nonsterilized seed were *Aureobasidium pullulans*, *Alternaria* spp. (*A. alternata* and *A. tenuissima*), *Cladosporium* spp. (*C. herbarum* and *C. cladosporioides*), *Stemphylium botryosum*, *Ulocladium atrum*, *P. ligulicola*, and *Mi-*

Microspheeropsis sp. with a mean incidence of 65, 43, 17, 3, 4, 6, and 1%, respectively. Seed harvested in 2000 had a higher incidence of *P. ligulicola* in most cultivars. Less *P. ligulicola* occurred in seed harvested in 2001, which coincided with the implementation of a fungicide program for seed crops. Surface sterilization of seed reduced the incidence of *A. pullulans*, *Alternaria* spp., *Cladosporium* spp., *U. atrum*, and *P. ligulicola*. The incidence of *Microspheeropsis* sp. was sporadic and not greatly affected by surface sterilization (Table 2).

Detection of *P. ligulicola* in pyrethrum seed by PCR. A 230-bp amplicon was generated by PCR from genomic DNA extracted from infected pyrethrum seed. Sensitivity tests using serial dilutions of *P. ligulicola* total genomic DNA allowed successful detection with as little as 800 fg DNA (Fig. 1). Using DNA extraction methods 1 and 2, *P. ligulicola* was detected from infected seed lots down to an incidence of 0.5% (Fig. 2). Using the third extraction method, *P. ligulicola* was not detected at incidences less than 5.25%, and at both 10.5 and 5.25%, *P. ligulicola* was only detected in 50% of replicate samples. Furthermore, *P. ligulicola* was detected in 60% of replicate extractions from mixing one seed from a highly infected seed lot with seeds from a lot with no detectable *P. ligulicola* using extraction method 1. The reliability of amplification was improved by the addition of 10% BSA (data not shown).

Incidence of *P. ligulicola* in pyrethrum seed. The incidence of seedborne *P. ligulicola* was quantified using PCR and viability assessed using plate tests (Table 3). Estimation of incidence of *P. ligulicola* based on results of PCR was useful when the incidence of *P. ligulicola* was low. However, using 10 replicates per seed lot, this test was not able to reliably estimate incidence between 3.02% (*P. ligulicola* detected in 9 of 10 replicates) and 100% (*P. ligulicola* detected in all replicates). The incidence of *P. ligulicola* as assessed by plate tests directly following harvest was on average 1.4, 2.9, 8.9, and 17.2 times greater in 2004 than in 2000, 2002 and 2005, 2001, and 2003, respectively. The incidence of seedborne *P. ligulicola* was also affected by cultivar, with cultivar 1 having at least 1.36 times more *P. ligulicola* than other cultivars.

Discrepancies were noted between the incidence of *P. ligulicola* as estimated by plate tests directly following harvest and following storage times of up to 3 years. For example, the incidence of *P. ligulicola* in "cultivar 1, seedline 1" seed declined from 22% in seed tested immediately after harvest in 2000, to 1% following retesting in 2003 (Table 3). However, in seed harvested between 2001 and 2003, the low incidence of *P. ligulicola* made conclusions regarding survival in seed difficult

(Table 3). Greatly reduced viability of *P. ligulicola* following seed storage was also noted in seed harvested from 1995 to 1999, with plate tests failing to detect *P. ligulicola* in seed lots harvested prior to 1998, despite detection by PCR (Table 3). Irrespective of year of harvest, *P. liguli-*

cola was detected by PCR in at least five of the 10 replicates of all cultivar 1 seed, while none was detected in cultivar 3 (Table 3).

Relationship between foliar disease intensity and incidence of seedborne *P. ligulicola*. Significant relationships be-

Fig. 3. Effect of foliar disease intensity on the incidence of *Phoma ligulicola* within pyrethrum seed when significant ($P < 0.05$) regression models were detected. **A**, Incidence of *P. ligulicola* within seed (%) = $-12.19 \times$ defoliation severity in November (%) + 1.01; $R^2 = 0.98$; **B**, Incidence of *P. ligulicola* within seed (%) = $-1.95 \times$ defoliation severity in October (%) + 0.72; $R^2 = 0.89$; **C**, Incidence of *P. ligulicola* within seed (%) = $12.13 \times$ incidence of stems with ray blight in October + 0.67; $R^2 = 0.91$.

tween foliar disease intensity in October and November and the incidence of *P. ligulicola* within seed were detected (Fig. 3). Significant regressions ($P < 0.05$) were detected between the incidence of *P. ligulicola* within seed and defoliation severity in October and November and the incidence of stems with ray blight in October. The R^2 values for these significant models ranged from 0.89 and 0.98. The best predictor of the incidence of *P. ligulicola* within seed was defoliation severity in November ($R^2 = 0.98$). The slopes for these significant models ranged from 0.72 and 1.01, which indicated the incidence of *P. ligulicola* within seed increased from 0.72 to 1.01% with each 1% increase in disease intensity. SEEdy indicated that using disease intensity to predict *P. ligulicola* incidence in seed had a standard error between 5.64 and 14.78%. The coefficient of variation for the significant models ranged from 7.4 to 11.9% (Fig. 3).

Effect of fungicides on incidence of *P. ligulicola*, seed germination, and seedling survival. In all three cultivars of seed harvested in 2000, treatment of seed with thiabendazole and thiram significantly ($P < 0.001$) reduced the incidence of viable *P. ligulicola* and increased seed germination ($P = 0.021$) and seedling survival ($P = 0.001$) over nontreated seed. Application of thiabendazole and thiram reduced the incidence of viable *P. ligulicola* by 7.5 times, increased seed germination by 1.8 times, and increased seedling survival by 1.2 times (Table 4).

In cultivar 1, the effect of fludioxonil on the incidence of *P. ligulicola*, and seed germination and seedling survival, was not significantly different from that of thiabendazole and thiram (Table 4). Didecyldimethyl-ammonium chloride gave no significant reductions in the incidence of *P. ligulicola* and increases in seed germination or seedling survival compared with nontreated seed.

DISCUSSION

These experiments have provided new information on the mycoflora associated with pyrethrum seed, the incidence of *P. ligulicola* within pyrethrum seed, identified several fungicide treatments that may reduce seedborne inoculum, and demonstrated a significant relationship between foliar disease intensity during the growing season and incidence of *P. ligulicola* in seed.

Several of the fungi isolated from seed are known to be pathogenic to pyrethrum. For example, *S. botryosum* and *A. tenuissima* have been characterized as the cause of pink spot and winter blight, respectively. *Alternaria alternata*, *C. cladosporioides*, and *U. atrum* have all been confirmed as saprophytes (24). *Alternaria* spp., *Cladosporium* spp., and *Penicillium* spp., have also been associated with pyrethrum seed from India (14). The symptoms associated with disease caused by *P. ligulicola* on mature plants have been well characterized (23,27). However, *P. ligulicola* has also been associated with seedling damping-off in pyrethrum (S. J. Pethybridge, unpublished data) and damping-off and death of cuttings in chrysanthemum (4,16).

The presence of *P. ligulicola* at incidences up to 28% in commercial seed lots is of concern. Surface sterilization with sodium hypochlorite reduced the incidence of *P. ligulicola* compared with non-surface-sterilized seed, but did not eliminate it, suggesting that *P. ligulicola* may be present on both the outer pericarp tissue and within the seed. The failure of surface sterilization to eliminate *Alternaria* spp., *Cladosporium* spp., *S. botryosum*, *U. atrum*, and *Microsphaeropsis* spp. also suggests their presence both within seed and on the seed surface. Seed infested with *P. ligulicola* may contribute to primary inoculum for ray blight epidemics. This is consistent with the random distribution of

disease in early spring (21) and the occurrence of disease at isolated locations (S. J. Pethybridge, unpublished data). Reductions in seed germination and seedling survival associated with nontreated seed (S. J. Pethybridge, unpublished data) could also reduce field establishment. However, reductions in plant density would lead to a less dense canopy with microclimatic conditions less conducive to disease.

The PCR assay was effective for the detection of seedborne *P. ligulicola*. Optimization of this assay by the inclusion of BSA in the reaction was able to minimize PCR inhibitors associated with pyrethrum seed and provide more consistent amplification of target DNA fragments. The presence of these inhibitors has been commonly encountered in the development of such assays from a variety of plant substrates (15). The DNA extraction kit by Qiagen was the most reliable for routine detection of *P. ligulicola* in infected seed by PCR. Advantages of PCR over plate tests included reduced assay times, the ability to assess presence in larger subsamples of commercial seed, and alleviated difficulties in detecting *P. ligulicola* among other fast-growing fungi during plate tests. Similar PCR-based assays for the detection of fungal pathogens have been developed for the testing of *Leptosphaeria maculans* (33), *Alternaria brassicae* (13), *Rhynchosporium secalis* (17), *Tilletia indica* (32), and *Alternaria radicina* (28).

Several effective measures for reducing the incidence of *P. ligulicola* in seed were demonstrated. Defoliation severity in spring was highly correlated with the incidence of *P. ligulicola* within seed at harvest. Similar relationships have also been found in other pathosystems (12). This finding suggests that the incidence of *P. ligulicola* in seed can be significantly reduced if fungicides are regularly applied to seed fields prior to harvest to reduce disease intensity (25). Furthermore, sites of a higher risk of disease such as those on south facing slopes and in valleys (22) could be avoided for seed production, and sites could be established so they were geographically isolated from commercial fields. Validation of these linear regression models could be useful in the setting of a practical disease intensity threshold during the season, over which the incidence of seedborne *P. ligulicola* is unacceptable. In addition, failing the effective reduction of disease intensity within the field, fungicide seed treatments can be used to protect seeds from fungal infections after planting, or to deter the growth of seedborne fungal pathogens (1). Our trials have demonstrated significant benefits from thiabendazole/thiram and fludioxonil. Thiram has also been demonstrated to be an effective seed treatment in the control of *P. betae* (10). Fludioxonil is a broad spectrum contact fungicide with activity mostly against

Table 4. Effect of thiabendazole/thiram in three cultivars (1, 2, and 3) and didecyldimethyl-ammonium chloride, fludioxonil, and thiabendazole/thiram in one cultivar (1) on incidence of *Phoma ligulicola* detected in plate tests, seed germination, and seedling survival

Cultivar	Treatment	Incidence of <i>P. ligulicola</i> (%)	Seed germination (%)	Seedling survival (%)
1	Thiabendazole/thiram	2 b	85 a	51 a
	Nontreated	22 a ^z	45 b	43 b
	LSD (P)	1.2 (<0.001)	5.4 (0.002)	2.9 (<0.001)
2	Thiabendazole/thiram	3 b	67 a	45 a
	Nontreated	19 a	35 b	39 b
	LSD (P)	2.5 (<0.001)	5.6 (0.013)	2 (0.001)
3	Thiabendazole/thiram	2.1 b	54 a	59 a
	Nontreated	12 a	32 b	51 b
	LSD (P)	4.3 (<0.001)	4.6 (0.021)	2 (<0.001)
1	Didecyldimethyl-ammonium chloride	18 a	42 b	40 c
	Thiabendazole/thiram	3.5 b	80 a	56 ab
	Fludioxonil	2.1 b	84 a	58 a
	Nontreated	22 a	45 b	43 bc
	LSD (P)	4.7 (0.002)	7.2 (0.006)	13 (0.012)

^z Means followed by the same letter are not significantly different at $P = 0.05$.

Fusarium spp., although small amounts of this fungicide have been found in internal seed tissues and the coleoptiles of germinating corn seeds (19). Further work is needed to assess the efficiency of these fungicide treatments under a range of field conditions (18). The effectiveness of fungicide alternatives, such as thermotherapy (hot water, hot air, solar heat, aerated steam, and radiation treatment), at eliminating fungal seedborne pathogens has also been recognized, and may prove as effective as fungicides in pathogen control and preserving seed viability (1,2).

Additional studies will quantify the seed to seedling transmission efficiency and therefore the contribution of seedborne *P. ligulicola* to primary inoculum for field epidemics. Further development of treatments to reduce the incidence of *P. ligulicola* associated with pyrethrum seed will also depend upon the significance of this inoculum to field epidemics.

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