Plant Pathology (2006) **55**, 387–400 Doi: 10.1111/j.1365-3059.2006.01383.x

Visual and PCR assessment of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) cultivars

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Methods to assess light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape cultivars were compared in laboratory, controlled-environment and field experiments. In controlled-environment experiments with seedling leaves inoculated at GS 1,4, the greatest differences in percentage area affected by *P. brassicae* sporulation were observed with inoculum concentrations of 4×10^3 or 4×10^4 spores mL^{-1}, rather than 4×10^2 or 4×10^5 spores mL^{-1}, but older leaves had begun to senesce before assessment, particularly where they were severely affected by *P. brassicae*. In winter oilseed rape field experiments, a severe light leaf spot epidemic developed in 2002/03 (inoculated, September/October rainfall 127·2 mm) but not in 2003/04 (uninoculated, September/October rainfall 40·7 mm). In-plot assessments discriminated between cultivars best in February/March in 2003 and June in 2004, but sometimes failed to detect plots with many infected plants (e.g. March/April 2004). Ranking of cultivar resistance differed between seedling experiments done under controlled-environment conditions and field experiments. The sensitivity of detection of *P. brassicae* DNA extracted from culture was greater using the PCR primer pair PbITSF/PbITSR than using primers Pb1/Pb2. *P. brassicae* was detected by PCR (PbITS primers) in leaves from controlled-environment experiments immediately and up to 14 days after inoculation, and in leaves sampled from field experiments 2 months before detection by visual assessment.

Keywords: cultivar resistance, disease assessment, PCR diagnostics, plant disease symptomatology, rapeseed, sporulation

Introduction

Since the 1970s light leaf spot, caused by *Pyrenopeziza brassicae* (anamorph *Cylindrosporium concentricum*), has been a major disease of winter (autumn-sown) oilseed rape (*Brassica napus* spp. *oleifera*) in the UK, particularly in the high-rainfall regions of Scotland and northern England (Figueroa *et al*., 1995; Fitt *et al*., 1999; Sutherland *et al*., 1998). In France, the disease was first recognized in 1977 (Brun *et al*., 1979), was a problem in the 1980s when epidemics occurred annually (Pilet *et al*., 1998), became less severe in the 1990s but has recently caused serious yield losses (> 0·8 t ha^{-1}) (J-C Pruvot, personal communication). Light leaf spot also occurs in Germany (Amelung & Daebeler, 1991) but is not considered a serious problem at present. In Poland, light leaf spot can be a serious disease after wet, mild winters (Karolewski, 1999). There is a need to introduce cultivars with good field resistance to *P. brassicae* in these countries.

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To breed for field resistance to *P. brassicae*, there is a need for accurate methods to determine severity of light leaf spot on different cultivars in field trials. In the UK, levels of field resistance to light leaf spot, assessed on the UK recommended list rating (0–9) scale (e.g. Anonymous, 1998; www.hgca.com for recommended lists since 2002), differ between winter oilseed rape cultivars and change from season to season. The resistance rating of cv. Apex decreased from 7 in 1996 to 5 in 2001; the rating for Bristol was 5 in 1994, 3 in 1995 and 2 in 1997. There is evidence that quantitative trait loci (QTL) are involved in polygenic field resistance to *P. brassicae* (Pilet *et al*., 1998), although some major genes for seedling resistance have been identified in wild brassicas (Bradburne *et al*., 1999). However, these changes in field resistance suggest that *P. brassicae* populations change rapidly, possibly through sexual reproduction, since both *P. brassicae* mating types occur in the UK (Majer *et al*., 1998) and Poland (Karolewski *et al*., 2004). UK recommended list cultivar resistance ratings are currently based on several assessments of light leaf spot damage made *in situ* in field plots (www.hgca.com) (Fig. 1). However, these assessments do not currently account for some important phases of the light leaf spot epidemics. For example, stem disease, which is thought to be important in the carry-over of light

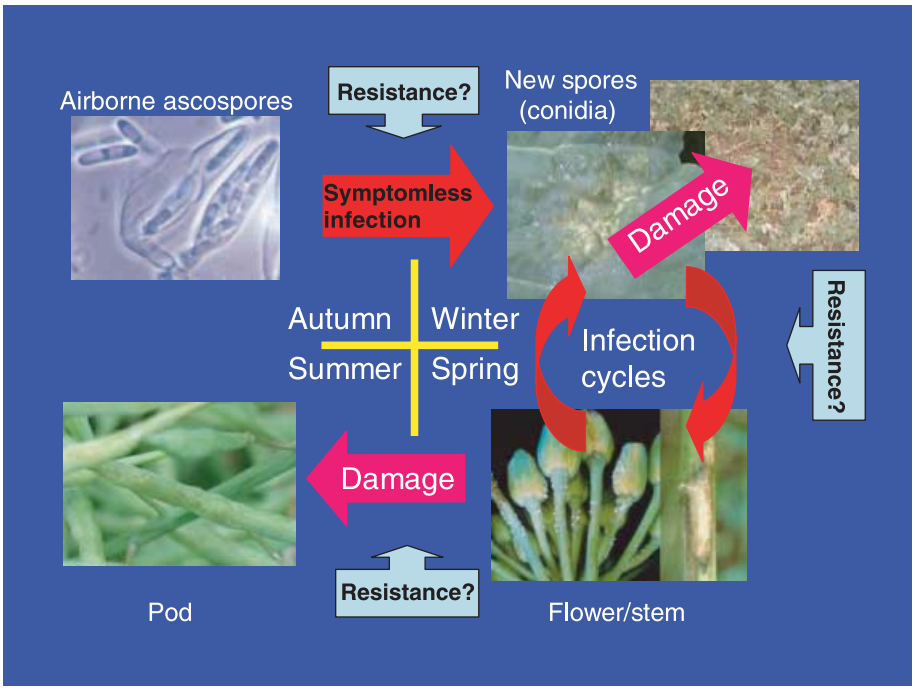

Figure 1 Seasonal cycle of light leaf spot epidemics in Europe in relation to potential components of oilseed rape resistance to *Pyrenopeziza brassicae*. Epidemics are initiated in autumn by airborne ascospores, with secondary spread of this polycyclic disease by splash-dispersed conidia. Damage to crops is associated with symptoms on leaves in winter and on pods in spring/summer. It is not known whether resistance operates at the leaf, stem or pod phases of epidemics.

leaf spot from one growing season to the next, is not assessed. Therefore, there is a need to test whether different methods can be used to assess components of cultivar field resistance at different plant growth stages to improve the robustness of the resistance ratings. Bradburne *et al.* (1999) found that resistance assessments carried out on detached seedling cotyledons did not correlate well with those on adult plants. However, new methods for artificial inoculation of seedling leaves using *P. brassicae* conidia or ascospores have been developed (Figueroa *et al.*, 1995; Gilles *et al.*, 2000a, 2001; Karolewski *et al.*, 2002). Whether there is a good correlation between the resistance response of young plants grown under controlled environment conditions and the resistance response of plants of the same cultivars under field conditions has not been reported. Therefore, we examine whether field resistance of cultivars can be discriminated in inoculated seedling tests by comparison with results observed in winter oilseed rape plots.

Currently, calculations of UK recommended list ratings, based on visual assessment of light leaf spot in winter oilseed rape plots, are based on an estimate of the percentage area of plants with light leaf spot *in situ*, without sampling or incubation. After initial infection of winter oilseed rape in autumn by airborne ascospores (Gilles *et al.*, 2001) (Fig. 1), *P. brassicae* has a long symptomless phase until the first visible necrotic lesions appear in January/February (Figueroa *et al.*, 1995; Fitt *et al.*, 1998a, 1998b; Gilles *et al.*, 2000b). Visual assessment of *P. brassicae* infection is not reliable before necrotic lesions appear, unless plants are first incubated for several days at high humidity in polyethylene bags to encourage sporulation (Fitt *et al.*, 1998a). From results of winter oilseed rape fungicide experiments in Scotland, Su *et al.* (1998) suggested that it is possible to predict potential yield loss caused by light leaf spot from assessments made at GS 3,3 (flower buds visible) (Sylvester-Bradley & Makepeace, 1985), when disease incidence (% plants affected) is assessed after incubation of plants in polyethylene bags. However, in field experiments, yield response to fungicides in field experiments does not always relate well to the recommended list resistance rating of a particular cultivar for light leaf spot, when light leaf spot is the predominant disease (J. Thomas, NIAB Cambridge UK, personal communication). This paper describes work comparing the *in situ* in-plot method of assessment with methods involving sampling and incubation of plants.

Another potential method for assessing cultivar resistance to light leaf spot is the detection of symptomless infection by PCR, which has proved to be a useful method for assessing resistance of oilseed rape cultivars to *Leptosphaeria maculans* (phoma stem canker) (Kenyon *et al.*, 2004). *Pyrenopeziza brassicae* DNA was detected by PCR assay in oilseed rape leaves (cv. Bristol), inoculated with conidia 13 days after inoculation in a controlled environment (at a constant temperature of 16°C), using primers Pb1 and Pb2 when assessment was done without incubation (Foster *et al.*, 2002). Using a nested PCR assay, detection was possible at 6 days after inoculation (Foster

et al., 2002). The use of a nested PCR protocol involves additional sample handling steps, which increase both the time taken to do the assay and the possibility of sample mix-up or contamination. There is therefore a need to develop a more sensitive PCR assay that does not rely on the use of a nested PCR step, and to test PCR diagnosis on leaves sampled from winter oilseed rape plots. This paper compares different methods of assessment of light leaf spot (*P. brassicae*) in winter oilseed rape cultivar field plots and controlled environments, including PCR detection of symptomless infection.

Materials and methods

Development of PbITSF and PbITSR PCR primers

PCR primers ITS4 and ITS5 (White *et al*., 1990) were used to amplify the ribosomal RNA region incorporating the internal transcribed spacer (ITS) regions and the 5·8S rRNA gene from *P. brassicae* isolates JH26 (*MAT1-1*) and CRB (*MAT1-2*) (Foster *et al*., 1999) and from isolates 22-433 (*MAT1-1*) and 22-432 (*MAT1-2*) of the closely related discomycete *Oculimacula* (= *Tapesia*) *yallundae* (Dyer *et al*., 2001). The resulting PCR products were sequenced using the ABI Prism Big Dye™ terminators cycle sequencing kit version 3·0 (Applied Biosystems, USA) and the sequences deposited in GenBank under accession numbers AJ305235 and AJ305236 (*P. brassicae*) and AY713293 and AY713294 (*O. yallundae*). Homology searches were done using the BLAST algorithm (Altschul *et al*., 1997) and fungal ITS sequences with the closest homology to the *P. brassicae* and *O. yallundae* sequences were used to create a multiple sequence alignment using CLUSTALX (Thompson *et al*., 1997; data not shown). From the multiple sequence alignment, the PCR primers PbITSF (5′-TTGAACCTCTCGAAGAAGTTCAGTCT-3′) and PbITSR (5′-AGATTTGGGGGTTGTTGGCTAA-3′) were designed to amplify a 461 bp diagnostic PCR product specifically from *P. brassicae* isolates. The primers were tested against DNA from *O. yallundae* and the known oilseed rape pathogens *Leptosphaeria maculans*, *Alternaria brassicae*, *A. brassicicola*, *Peronospora parasitica*, *Plasmodiophora brassicae*, *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Verticillium longisporum*. The *P. brassicae*-specific PCR fragment was not amplified from any of these DNA samples, showing that the primers are specific for *P. brassicae* (data not shown).

Genomic DNA was extracted from mycelium of *P. brassicae* isolate NH10 grown in potato dextrose broth (PDB) at 16°C in the dark for 14 days in a Petri dish (Foster *et al*., 1999). The sensitivity of primers Pb1TSF and Pb1TSR was compared with that of primers Pb1 (5′-CAA CAT TGC CTG GTA TTG AGA AAC-3′) and Pb2 (5′-ATC TGA TAC GCC TAC ACC GTC C-3′), which amplify a region flanking the *P. brassicae* mating type (*MAT*) loci (Foster *et al*., 1999). To determine the sensitivity of both diagnostic assays, *P. brassicae* genomic DNA from isolate NH10 was serially diluted in water (range from 10 ng to 1 fg per reaction) and used in PCR reactions with each set of primers. The PCR reactions and cycling parameters (Foster *et al*., 1999, 2002) were the same for both pairs of primers. However, in the current work, a different polymerase (Red-Hot Taq, ABGene, UK) and a different thermocycler (GeneAmp PCR System 2700, Applied Biosystems) were used. PCR products were analysed by running 6 μL of each reaction on 1·5% agarose gels containing 0·5 μg mL^{-1} ethidium bromide in 1 × TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) buffer and visualized on an ultraviolet transilluminator.

P. brassicae inoculum for field and controlled-environment experiments

In October 2002, field experiment plots were inoculated with *P. brassicae*-infected oilseed rape debris in order to compare development of light leaf spot on winter oilseed rape cultivars in response to different amounts of primary inoculum. Inoculum consisted of 20-cm-long pieces of upper stems (*c.* 10 kg of stems per plot) collected after harvest at the end of the 2001/02 season. In 2003/04, plots were not inoculated and received only natural background *P. brassicae* ascospore inoculum. Conidial inoculum of *P. brassicae* populations for controlled environment experiments was obtained by washing conidia from *P. brassicae*-infected winter oilseed rape leaves [cvs Apex, Bosman, Canberra, Kana, Marita, Recital with pustules (acervuli) of *P. brassicae*] sampled from Rothamsted field experiments in March 2003 or April 2004. Immediately after collecting samples, *P. brassicae* conidial suspensions from different cultivars were mixed and appropriate concentrations for experiments made by dilution after haemocytometer counts.

Controlled-environment experiments on *Brassica napus* seedlings

Oilseed rape plants for controlled-environment experiments (Table 1) were grown in compost (75% peat, 12% loam, 10% grit, 3% vermiculite; Petersfield Products, UK) in controlled-environment cabinets (80% relative humidity, 12 h light/12 h dark, 190 E m^{-2} s^{-1} light intensity at canopy level, supplied by fluorescent and tungsten lighting) at 16°C. These plants were inoculated using an aerosol sprayer (Chrom Atomiser, Camlab, UK) with the fresh conidial suspensions of *P. brassicae*. Directly after spraying with fresh conidial suspensions, plants were covered with polyethylene bags for 48 h and maintained in the controlled-environment cabinet. In experiments 1, 2 and 3, leaves were again covered with polyethylene bags 14 days after inoculation.

Experiment 1: visual assessment of light leaf spot on cultivars inoculated at GS 1,4

Oilseed rape seeds (28 cultivars, Table 2) were sown in 2·5-cm-diameter jiffy pots. One pot of each cultivar was randomly placed in one of four 35 × 21 cm plastic seed trays. Twenty days after germination, when plants were at the four-leaf stage, each tray of plants was sprayed with

Table 1 Methods for assessment of light leaf spot (*Pyrenopeziza brassicae*) severity on oilseed rape (*Brassica napus*) in controlled-environment experiments

	Oilseed rape cultivars	Growth stage[a] at inoculation with conidia	Leaves assessed[b]	Sample times	Assessment method
Expt 1	28 cultivars (Table 2)	GS 1,4	3, 4	21 days after inoculation	Visual[c]
Expt 2	37 cultivars (Table 2)	GS 1,6–1,7	5, 6	21 days after inoculation	Visual[c]
Expt 3	Apex, Bosman, Canberra, Kana, Marita, Recital	GS 1,4	3, 4	21 days after inoculation	Visual[c]
Expt 4	Apex, Canberra	GS 1,6–1,7	5 or 6	1–14 days after inoculation	PCR[d]

[a]Sylvester-Bradley & Makepeace (1985).
[b]True leaves were numbered in order of appearance.
[c]Assessement was done by estimation of percentage leaf area with *P. brassicae* sporulation (acervuli) (Gilles *et al.*, 2000a).
[d]PCR reactions done using PbITSF and PbITSR primers on individual whole leaves sampled.

a different concentration of *P. brassicae* conidia (4×10^5, 4×10^4, 4×10^3 or 4×10^2 mL^{-1}) until droplets ran off the leaf surfaces. In this experiment there were three replicates, making a total of 12 trays. To minimize interplot interference (dispersal of inoculum from those plants inoculated at high concentration to those inoculated at low concentration), trays were arranged in treatment blocks and placed on separate benches. Seed trays were placed in larger gravel trays (80 × 50 cm) filled with tap water to maintain soil wetness. At 21 days after inoculation, leaves 3 and 4 were assessed by estimating the percentage of the leaf surface area with *P. brassicae* sporulation (Karolewski *et al*., 2002).

Experiment 2: visual assessment of light leaf spot on cultivars inoculated at GS 1,6–1,7

The resistance of 37 oilseed rape cultivars to *P. brassicae* was compared by inoculating plants at GS 1,6–1,7 (Sylvester-Bradley & Makepeace, 1985) (two replicates) (Table 2). Leaves 5 and 6 of four plants of every cultivar were inoculated with a conidial suspension (5×10^5 conidia mL^{-1}) until droplets ran off the leaf surfaces. Plants growing in separate pots (diameter 9 cm) were placed in a randomized design. At 21 days after inoculation, leaves 5 and 6 were assessed by estimating the percentage of the leaf surface area affected by *P. brassicae* sporulation (Karolewski *et al*., 2002).

Experiment 3: visual assessment of light leaf spot on six cultivars inoculated at GS 1,4

Six oilseed rape cultivars (Apex, Bosman, Canberra, Kana, Recital, Marita) were sown individually in 2·5-cm-diameter Jiffy pots. Twenty days after germination, when plants were at the four-leaf stage, three replicate plants of each cultivar were sprayed with a different concentration of *P. brassicae* conidia (4×10^5, 4×10^4, 4×10^3 or 4×10^2 mL^{-1}) until droplets ran off the leaf surfaces. Inoculated plants were randomly placed in nine large 80 × 50 cm plastic seed trays. Each seed tray was filled with tap water to maintain soil wetness. At 21 days after inoculation, leaves 3 and 4 were assessed by estimating the percentage of the leaf surface area with *P. brassicae* sporulation (Karolewski *et al*., 2002).

Experiment 4: detection of symptomless P. brassicae *infection using PCR primers PbITSF and PbITSR on oilseed rape leaves 1–14 days after inoculation*

Fifty plants each of oilseed rape cultivars Apex (UK resistance rating 5) and Canberra (7) were inoculated at GS 1,6–1,7 (Sylvester-Bradley & Makepeace, 1985) by spraying with *P. brassicae* conidia (10^5 conidia mL^{-1}). Just before and immediately after inoculation, one leaf was removed from each of three replicate plants of each cultivar and stored at –20°C. Thereafter, plants were arranged in a completely randomized design and maintained in controlled-environment cabinets for 14 days after inoculation. Every day, one leaf (leaf 5 or 6) from each of three replicate plants of each cultivar was removed at random and stored at –20°C. DNA was extracted from leaves collected, using a protocol modified from that of Graham *et al*. (1994). Leaves (*c.* 3 g fresh weight) were freeze-dried and ground separately using a pestle and mortar and approximately 300 mg of ground leaf tissue suspended in 600 μL of extraction buffer (2% hexadecyltrimethylammonium bromide, 100 mM Tris HCl, 1·4 M NaCl, 20 mM EDTA) plus 20 μL β-mercaptoethanol and incubated at 70°C for 30 min. Samples were extracted twice with an equal volume of a 24:1 chloroform and isoamyl alcohol. DNA was precipitated from the aqueous phase by addition of 0·1 volume of 6 M ammonium acetate and 2 volumes of ethanol, then incubated at –20°C for 1 h. After centrifugation, DNA pellets were washed with 1·0 mL 70% ethanol, air-dried for 25 min and resuspended in 100 μL TE (10 mM Tris HCl pH 7·5 at 25°C, 1 mM EDTA) buffer. DNA concentration was determined spectrophotometrically at 260 nm. For PCR reactions, DNA samples were adjusted to concentrations 5, 50, 100, 250, 500 or 1000 ng μL^{-1}.

Table 2 Severity of light leaf spot (percentage leaf area with *Pyrenopeziza brassicae* sporulation) on oilseed rape cultivars 21 days after inoculation with conidia at GS 1,4 (experiment 1) or GS 1,6–1,7 (experiment 2)

Cultivar (resistance rating)	Percentage leaf area affected[a]				
	Experiment 1 (conidia mL^{-1})				Experiment 2 (conidia mL^{-1})
	400	4000	4×10^5	4×10^4	5×10^5
Apex (5)[b]	6·7	6·7	25·0	13·3	89·4
Aviso (5)[c]	1·7	16·7	21·7	11·7	90·3
Batory[d]	–[f]	–	–	–	88·1
Bazyl[d]	–	–	–	–	90·0
Bermuda[d]	–	–	–	–	87·2
Bolko[d]	–	–	–	–	87·2
Bor[d]	–	–	–	–	92·8
Bosman[d]	0	11·7	16·7	10·0	89·4
Bristol (3)[b]	3·3	10·0	10·0	13·3	90·6
Canary (7)[c]	1·7	13·3	15·0	10·0	87·5
Canberra (8)[b]	1·7	21·5	31·7	15·0	88·4
Capitol (7)[b]	18·3	18·3	25·0	10·0	88·8
Cobra (5)[b]	0	5·0	16·7	15·0	89·4
Columbus (5)[b]	0	6·7	13·3	8·3	89·4
Escort (7)[b]	1·7	18·3	13·3	8·3	75·0
Eurol (7)[b]	0	5·0	18·3	21·7	90·0
Express (8)[b]	0	6·7	21·7	8·3	87·5
Falcon (7)[b]	10·0	21·7	31·7	23·3	88·8
Gara	–	–	–	–	87·5
Jet Neuf (3)[b]	0	10·0	21·7	13·3	87·8
Kana[d]	0	11·7	6·7	11·7	93·1
Leo[d]	–	–	–	–	86·9
Lipton (3)[b]	6·7	11·7	21·7	21·7	90·0
Madrigal (6)[b]	5·0	13·3	23·3	13·3	88·4
Mar	–	–	–	–	88·1
Marita[d]	0	6·7	6·7	20·0	90·3
Mohican (8)[c]	0	21·7	23·3	23·3	89·7
Norin[e]	0	1·5	16·7	29·1	90·3
Pollen (8)[c]	0	13·3	18·3	3·3	91·2
Polo	–	–	–	–	88·1
PR4SW05 (6)[b]	1·7	5·0	28·3	10·0	87·5
Recital (6)[b]	3·3	3·3	21·7	16·7	92·5
Shannon (3)[b]	0	15·0	18·3	18·3	85·9
Synergy (6)[b]	–	–	–	–	88·1
Talent (8)[c]	3·3	11·7	10·0	26·7	91·9
Twister (6)[c]	0	16·7	15·0	20·0	88·8
Vivol (6)[c]	0	10·0	18·3	21·7	88·1
Zenith[e]	0	1·7	26·7	20·0	–
SED (d.f.)	5·08 (52)	6·18 (51)	10·76 (54)	8·16 (53)	2·70 (36)

[a]Plants were incubated at 16°C for 21 days after inoculation of leaves 3 and 4 (experiment 1) or leaves 5 and 6 (experiment 2). Plants were covered with polyethylene bags for 48 h after inoculation, and then again between 14 and 21 days after inoculation.
[b]Resistance rating used in UK recommended list (Anonymous, 1998; www.hgca.com).
[c]Resistance rating provided by Procolza (France).
[d]Resistance of Polish cultivars has not been tested.
[e]Resistance of cultivar not known.
[f]–, not tested in this experiment.

Winter oilseed rape field experiments

Visual assessment of light leaf spot in field experiments (before or after incubation)

Field experiments were done in the 2002/03 and 2003/04 growing seasons on the Rothamsted farm. Seed of six cultivars was sown on 21 August 2002 or 22 August 2003 (80 seeds m^2) in plots (20 × 3 m) arranged in three replicate blocks. Three cultivars (Bosman, Kana, Marita) were bred for Polish climatic conditions and three were from the UK recommended list, with different resistance ratings [Apex (5), Canberra (8), Recital (6)]. In between plots with test

Table 3 Methods for assessment of light leaf spot (*Pyrenopeziza brassicae*) severity in winter oilseed rape experiments at Rothamsted (six cultivars) in 2002/03 and 2003/04

			Visual						
			In situ (plot)	Plants sampled[b]					
				Before incubation	After incubation[e]				
Sample date	Growth stage[i] (GS)	PCR[a]	Plot area affected (%)[h]	Leaves affected (%)	Leaves affected (%)	Plant area affected (%)	Leaf area affected (%)	Stem area affected (%)	Pod area affected (%)
2002/03[f]									
January	1,9–1,13	No	No	Yes	Yes	No	No	No[d]	No[d]
February	1,12–1,18	No	Yes	No	Yes	Yes	Yes	No[d]	No[d]
March	2,1–2,7	No	Yes	No	Yes	Yes	Yes	Yes	No[d]
April	3,5–3,7	No	Yes	No	Yes	Yes	Yes	Yes	Yes
May	4,7–5,5	No	Yes	No	Yes	Yes	Yes	Yes	Yes
June	5,9–6,5	No	Yes	No	No[c]	Yes	No[c]	Yes	No[d]
2003/04[g]									No[d]
November	1,5–1,6	No	Yes	No	Yes	Yes	Yes	No[d]	No[d]
December	1,7–1,8	Yes	Yes	No	Yes	Yes	Yes	No[d]	No[d]
January	1,7–1,10	Yes	Yes	No	Yes	Yes	Yes	No[d]	No[d]
February	1,10–1,15	Yes	Yes	No	Yes	Yes	Yes	No[d]	No[d]
March	2,0–2,3	Yes	Yes	No	Yes	Yes	Yes	No[d]	No[d]
April	3,1–3,5	No	Yes	No	Yes	Yes	Yes	Yes	Yes
May	4,5–5,3	No	Yes	No	Yes	Yes	Yes	Yes	Yes
June	5,9–6,3	No	Yes	No	No[c]	Yes	No[c]	Yes	Yes

[a]To detect *P. brassicae*, primers PbITSF and PbITSR were used on individual leaves; from each of 10 plants from one plot of each cultivar, the oldest available leaf was sampled.
[b]Assessement was done by estimation of percentage area of organ or whole plant with *P. brassicae* acervuli (Gilles *et al.*, 2000a) on 10 plants per plot sampled from three plots (in three blocks) of each cultivar.
[c]All leaves had abscissed.
[d]Stem/pod had not yet developed.
[e]Before assessment plants were incubated for 5 days in polyethylene bags at 8°C; for all samples it was also possible to assess percentage of plants affected.
[f]Plots were inoculated with infected oilseed rape stem debris after sowing.
[g]Plots were not inoculated.
[h]Assessment done in the plot using UK recommended list protocol for assessing light leaf spot on leaves or pods (no incubation of plants).
[i]Sylvester-Bradley & Makepeace (1985).

cultivars, plots of the same size were sown with the susceptible cultivar Shannon (3). *In situ* and laboratory assessments of light leaf spot were done at monthly intervals, starting from January in 2002/03 and November in 2003/04 (Table 3). Assessment of light leaf spot *in situ* was done using the UK recommended list protocol (www.hgca.com): visual examination of the crop canopy in three areas of each plot; ignore all naturally senescent tissue; include all necrosis and chlorosis attributable to light leaf spot; estimate percentage area affected and record the average percentage area of crop affected with light leaf spot in the three areas. For the laboratory assessments, 10 plants (with roots) were sampled from each of the 18 plots and incubated for 5 days at 8°C in polyethylene bags (Fitt *et al.*, 1998a). The severity of light leaf spot was measured by estimation of percentage area of the whole plant affected by light leaf spot (with *P. brassicae* sporulation); counting the number of leaves on each plant and noting how many were affected; estimation of percentage area of each leaf affected; estimation of percentage area of each stem affected (late in season); estimation of percentage area of all pods affected (late in season).

PCR detection of P. brassicae *DNA in oilseed rape leaves collected from the 2003/04 field experiment*

During the 2003/04 field experiment, 10 leaves from one plot of each cultivar were sampled regularly and PCR diagnosis was done using the primer pair PbITSF/PbITSR (Table 3). The oldest leaves were taken from 10 plants at random each month from 9 December 2003 until 15 March 2004 (when light leaf spot symptoms were first visible). Directly after sampling, leaves were washed in water, dried for 30 min at 20°C and frozen at −20°C. DNA was extracted from leaves collected, using a protocol modified from that of Lee & Taylor (1990). For PCR reactions, DNA samples from leaves sampled in December, January and February were adjusted to concentrations of 200, 500 or 1000 ng μL^{-1}, and DNA samples from samples taken in March were adjusted to 100 ng μL^{-1}.

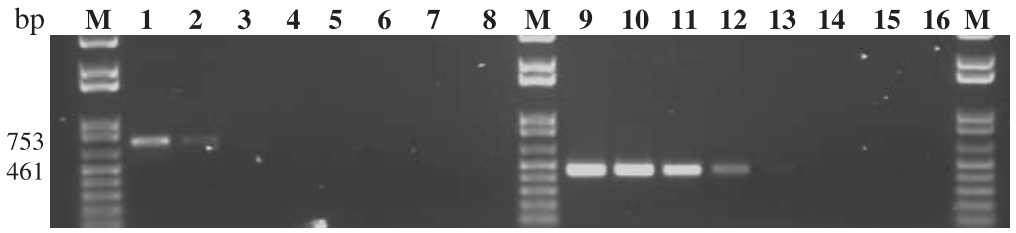

Figure 2 PCR amplification of genomic DNA from *Pyrenopeziza brassicae* isolate NH10 (grown on potato dextrose broth, Foster *et al.*, 1999) serially diluted in water, using primers Pb1 and Pb2 (lanes 1–8) or PbITSF and PbITSR (lanes 9–16). M, 1 kb plus DNA ladder (Invitrogen Life Technologies, UK); lanes 1 and 9, 10 ng DNA; 2 and 10, 1 ng DNA; 3 and 11, 100 pg DNA; 4 and 12, 10 pg DNA; 5 and 13, 1 pg DNA; 6 and 14, 100 fg DNA; 7 and 15, 10 fg DNA; 8 and 16, negative (water) control.

Data analysis

Data from the experiments were analysed with the statistical software Genstat 5 (Payne *et al.*, 1993). Results from controlled-environment and field experiments were analysed by analysis of variance (ANOVA). Correlations were then used to compare the different methods for assessment of light leaf spot in field experiments, including *in situ* assessments used in UK recommended list trials, and correlation coefficients were calculated.

Results

Comparative sensitivity of primer pairs Pb1/Pb2 and PbITSF/PbITSR for detection of *P. brassicae* DNA from culture

PCR amplification of *P. brassicae* DNA of isolate NH10 using primers Pb1/Pb2 produced a 753 bp amplicon, while a product of 461 bp was amplified using the PbITSF and PbITSR ITS primers. It was possible to detect down to 1 ng of *P. brassicae* DNA with primers Pb1 and Pb2 (Fig. 2) and as little as 1 pg when primers PbITSF and PbITSR were used.

Controlled-environment experiments

Experiment 1: visual assessment of light leaf spot on cultivars inoculated at GS 1,4

For 16 out of 28 cultivars tested, the maximum percentage leaf area with sporulation was observed when the *P. brassicae* conidial concentration was 4×10^4 mL^{-1}. The mean percentage leaf area affected (average for all cultivars) generally increased with increasing concentration of conidia from 4×10^2 to 4×10^4, but decreased when the concentration increased further to 4×10^5 mL^{-1}. Significant differences in severity of light leaf spot symptoms were found between cultivars at all four concentrations of conidia (4×10^5, 4×10^4, 4×10^3 and 4×10^2 mL^{-1}) (Table 2, Fig. 3a). However, there were interactions between cultivar and inoculum concentration used. Generally, the percentage of sporulating leaf area was greatest at the two intermediate concentrations (e.g. cv. Pollen 18·3 and 13·3% at 4×10^4 and 4×10^3, respectively, and 3·3% at 4×10^5 mL^{-1}). However, for some cultivars the severity of symptoms increased with increasing inoculum concentration (e.g. cvs Bristol, Envol, Marita, Norin, Talent). These interactions were complicated by the effects of light leaf spot on leaf senescence, especially at high inoculum

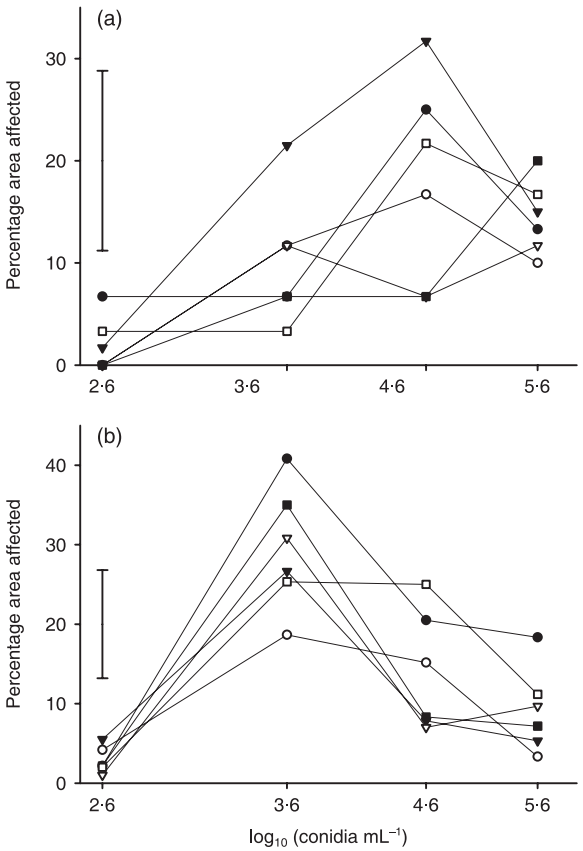

Figure 3 Severity of light leaf spot (% leaf area with *Pyrenopeziza brassicae* sporulation) on oilseed rape cultivars Apex (●), Bosman (○), Canberra (▼), Kana (▽), Marita (■) and Recital (□) 21 days after inoculation of leaves 3 and 4 at GS 1,4 with *P. brassicae* conidial concentrations: 4×10^5, 4×10^4, 4×10^3 or 4×10^2 mL^{-1} in experiment 1 (a) and experiment 3 (b). Plants were covered with polyethylene bags for 48 h after inoculation, and then again between 14 and 21 days after inoculation. The horizontal axis was $\log_{10}$-transformed. Vertical bars indicate SEDs (46 d.f.).

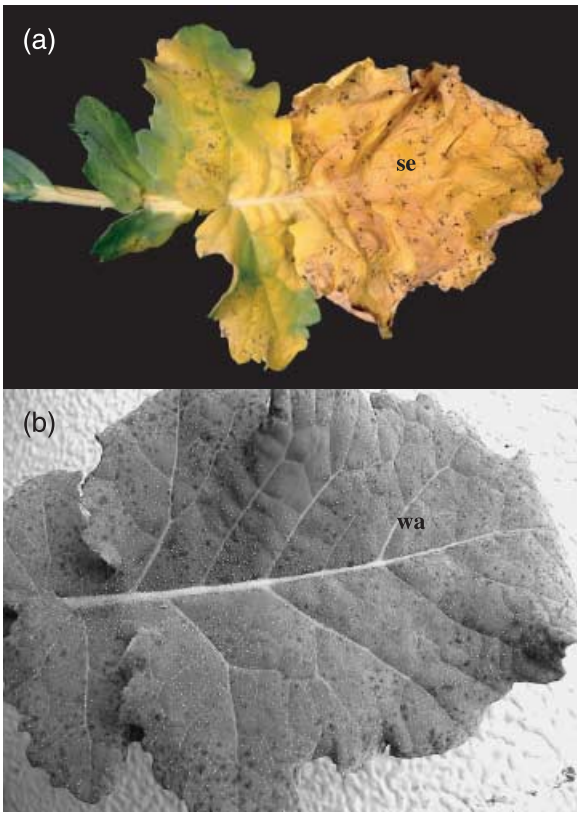

Figure 4 Symptoms of light leaf spot on leaves of oilseed rape cv. Apex 21 days after inoculation with *Pyrenopeziza brassicae* conidia at GS 1,4 (leaf 4, experiment 1, a) or GS 1,7 (leaf 6, experiment 2, b). Large numbers of white acervuli were visible on green leaf tissue of leaf 6 (wa) whereas leaf 4 had senesced (se) and light leaf spot could not easily be assessed.

concentrations. For some cultivars, leaves of plants inoculated at GS 1,4 had started to become senescent 21 days after inoculation, and sporulation could not be assessed easily (Fig. 4a).

Experiment 2: visual assessment of light leaf spot on cultivars inoculated at GS 1,6–1,7

All cultivars were severely affected by *P. brassicae* (Table 2, mean = 89% leaf area with sporulation). The cultivar Escort was significantly less affected ($P = 0{\cdot}008$) than other cultivars. In this experiment infected leaves were still green, so that large numbers of white *P. brassicae* acervuli could easily be seen when plants were assessed 21 days after inoculation (Fig. 4b).

Experiment 3: visual assessment of light leaf spot on six cultivars inoculated at GS 1,4

The maximum percentage leaf area with sporulation was observed when the *P. brassicae* conidial concentration used was 4×10^3 mL^{-1} (Fig. 3b; 3·6 on $\log_{10}$ scale). The mean percentage leaf area affected (average for all cultivars) generally increased with increasing concentration of conidia from 4×10^2 to 4×10^3 and then decreased when concentration increased to 4×10^4 and 4×10^5 mL^{-1}. There were significant differences between cultivars in severity of light leaf spot symptoms at the three higher concentrations (4×10^5, 4×10^4 and 4×10^3 mL^{-1}) (Fig. 3b). However, there were interactions between cultivar and inoculum concentration used. Although the concentration at which percentage leaf area with sporulation was greatest in experiment 3 differed from that in experiment 1, the overall pattern was similar, with percentage leaf area affected with sporulation greatest at the two intermediate concentrations.

Experiment 4: detection of symptomless P. brassicae *infection on oilseed rape leaves 1–14 days after inoculation using PCR primers PbITSF and PbITSR*

PCR reactions using primers PbITSF/PbITSR produced a 461 bp amplicon which was detected in all DNA samples extracted from *B. napus* (cvs Apex and Canberra) leaves harvested over the 14-day time course (Table 4). It is significant that this diagnostic PCR product was also

Table 4 *Pyrenopeziza brassicae* infection of individual leaves (5 or 6) of oilseed rape cvs Apex and Canberra, inoculated with conidia (10^6 mL^{-1}) at GS 1,6–1,7, assessed by PCR[a] on inoculated leaves (experiment 4)

	Days after inoculation														
Cultivar/DNA (ng μL^{-1})	0[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Apex															
5	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
100	–	–	–	+	+	–	–	+	+	+	+	+	+	+	+
500	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Canberra															
5	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
100	–	+	+	–	+	+	+	+	+	–	–	+	+	+	+
500	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+, presence of *P. brassicae* detected.

[a]PCR detection of *P. brassicae* DNA done using primers PbITSF and PbITSR on individual leaves (5 or 6) sampled at random. A leaf was harvested from each of three replicate plants at each time point.

[b]PCR was also done on inoculated leaves harvested immediately after inoculation with *P. brassicae* conidia.

amplified in DNA extracted from leaf samples harvested immediately after inoculation with *P. brassicae* conidia. The pathogen was detected reproducibly in all template DNA samples adjusted to concentrations higher than 100 ng μL^{-1}. However, with diminishing sensitivity of the assay at higher dilutions (e.g. 5 ng μL^{-1}), detection of the diagnostic PCR product was possible only in the leaf samples that were harvested at later times during the incubation period. No PCR product was amplified from healthy *B. napus* DNA.

Winter oilseed rape field experiments

Visual assessment of light leaf spot in field experiments (before or after incubation)

In the 2002/03 field experiment with inoculated plots, symptoms of light leaf spot were observed on oilseed rape when the first assessment was made in January (Fig. 5), whilst in the uninoculated 2003/04 experiment they were not observed until March, although assessments were started in November (Fig. 6). The autumn was wet in 2002/03 (September/October rainfall 127·2 mm), whereas in 2003/04 it was dry (September/October rainfall 40·7 mm). In the January 2003 assessment, immediately after sampling, severity of light leaf spot on different cultivars ranged from 64 to 81% of leaves affected (data not presented). After incubation for 5 days, the proportion of leaves affected increased to 79–85% (Fig. 5b). All assessment methods showed severe light leaf spot symptoms in February 2003. In April, light leaf spot severity was very low in *in situ* assessments on plots (Fig. 5a), although it was high when samples were assessed after incubation (Fig. 5b–e). Light leaf spot severity had increased in May, in both *in situ* and incubated assessments. The percentage of the whole plant affected declined in June (Fig. 5c), after abscission of the remaining leaves, although light leaf spot severity increased on the developing pods (Fig. 5f). Analysis of variance showed that differences between cultivars in severity of light leaf spot were significant ($P < 0{\cdot}01$, 10 d.f.) in January/February for all except one (whole plant assessment after incubation, Fig. 5c) assessment method used. However, the ranking of cultivars by different assessment methods was not consistent. Subsequently, all assessment methods, with the exception of assessments *in situ* in March (Fig. 5a, $P = 0{\cdot}001$, 10 d.f.) and in April on stems (Fig. 5e, $P = 0{\cdot}022$, 10 d.f.) showed no differences between cultivars.

In 2004, all methods showed that light leaf spot symptoms were absent from November to February, light leaf spot severity was low in March and then increased until June (Fig. 6), except for the whole plant assessment, which had a maximum in May (Fig. 6d). Analysis of variance showed no differences in severity of light leaf spot between cultivars for all assessment methods, except *in situ* assessments in June, when cv. Bosman had significantly less disease (Fig. 6a, $P = 0{\cdot}012$, 10 d.f.).

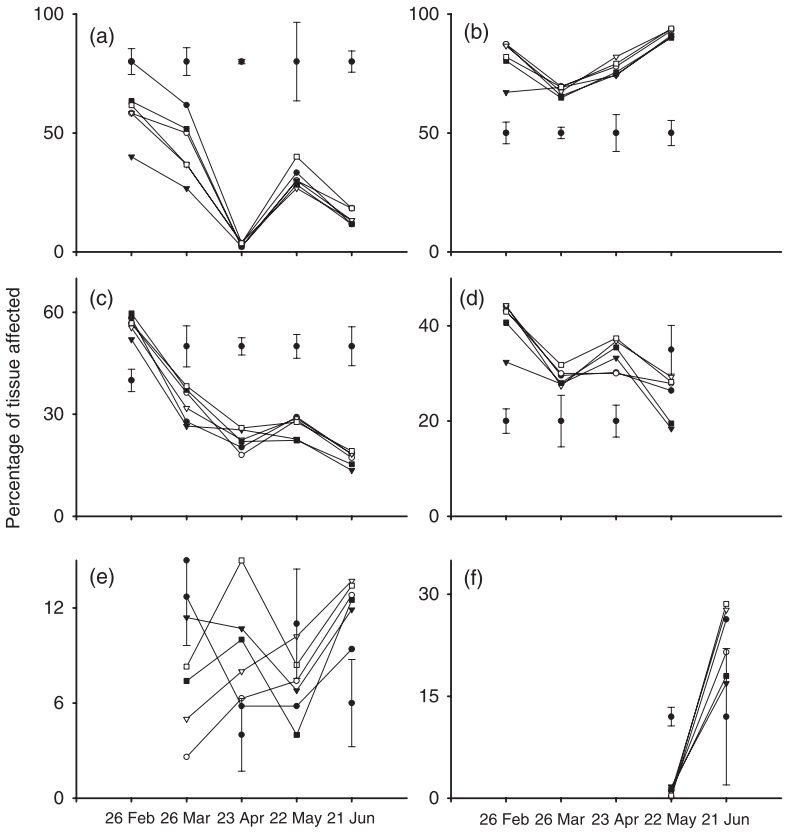

Figure 5 Development of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape cultivars Apex (●), Bosman (○), Canberra (▼), Kana (▽), Marita (■) and Recital (□) in 2002/03 assessed using six different methods: *in situ* percentage of plants affected in-plot (a); for plants sampled from plot: percentage of leaves affected (b), percentage of whole plant affected (c), percentage of leaf area affected (d), percentage of stem area affected (e), percentage of pod area affected (f). The percentage of plants affected by light leaf spot (after incubation of plants sampled from plots) was 100% throughout the sampling period. Vertical bars indicate SEDs (10 d.f.) at each date.

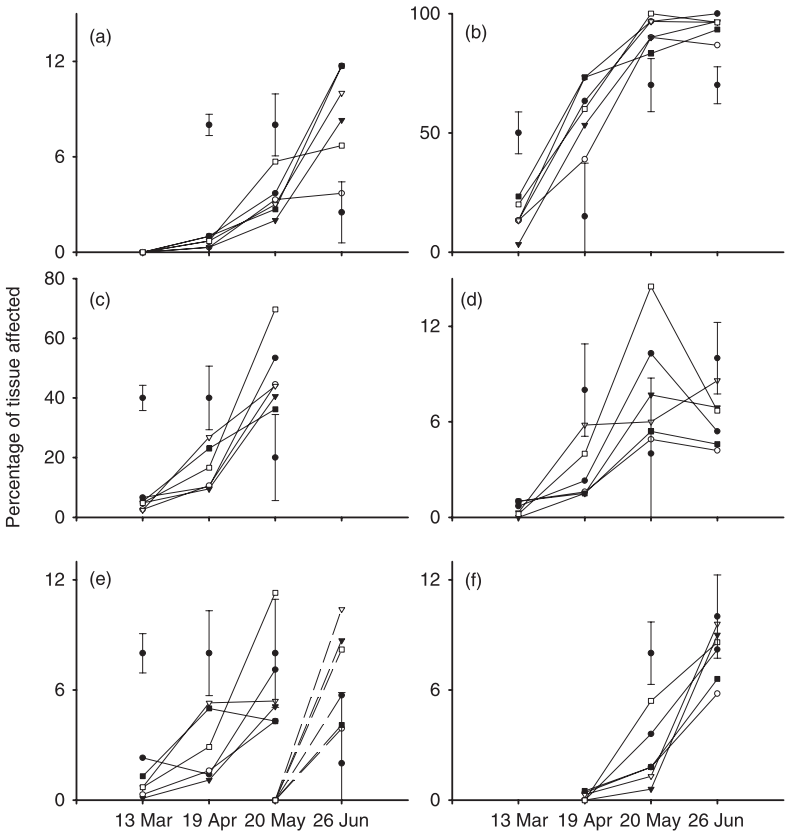

Figure 6 Development of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape cultivars Apex (●), Bosman (○), Canberra (▼), Kana (▽), Marita (■) in 2003/04 assessed using seven different methods of assessment: *in situ* percentage of plants affected in-plot (a); for plants sampled from plot: percentage of plants affected (b), percentage of leaves affected (c), percentage of whole plant affected (d), percentage of leaf area affected (solid line) and percentage of pod area affected (dashed line) (e), percentage of stem area affected (f). Vertical bars indicate SEDs (10 d.f.) at each date.

PCR detection of P. brassicae *DNA in oilseed rape leaves collected from the 2003/04 field experiment*

PCR, using PbITSF and PbITSR primers, successfully detected *P. brassicae* DNA in oilseed rape (cv. Recital) leaves in January 2004, 2 months before *P. brassicae* infection was identified using visual assessment methods (Table 5). In March, when *P. brassicae* sporulation was observed on oilseed rape leaves, the percentage of leaves in which the pathogen was detected was at least four times greater for the PCR method than for visual assessment methods.

Relationships between different methods for assessing light leaf spot on different cultivars

In 2003, when a severe light leaf spot epidemic developed, the *in situ* assessment method was correlated with assessments after sampling in February, May and June (values of the correlation coefficient > 0·5) (Table 6). However, the correlation was poor in other months in 2003, and in 2004, when the epidemic started later and was not severe. In 2004, there were good correlations between percentage of plants affected and percentage of plant area affected, percentage of leaf area affected and percentage of leaves affected, but not with the in-plot assessment, especially in April There were often good correlations between percentage leaf area affected and percentage of leaves affected, between percentage plant area affected and percentage leaf area affected in the period February to May, and between percentage plant area affected and percentage pod area affected in June. The ranking of cultivars in the seedling assays (Fig. 3) was very different from the ranking in the field experiments (Figs 5 and 6). The ranking of cultivars in the field experiments differed, depending on the plant organ being assessed (see, e.g., Fig. 6e).

Discussion

The results of controlled-environment experiments suggest that seedling tests can potentially discriminate severity of *P. brassicae* infections between oilseed rape cultivars, providing inoculum concentration and seedling age are chosen carefully. These seedling tests are easier to do than cotyledon tests described by Bradburne *et al.* (1999). However, to discriminate best between cultivars, seedlings should be at GS 1,4–1,7 when inoculated so that leaves remain green during the whole experiment, allowing *P. brassicae* sporulation to develop. The optimum temperature for plant and disease development is 16°C, since higher temperatures (e.g. 20°C, Karolewski *et al.*, 2002) induce early senescence of leaves. The best discrimination between cultivars was at a conidial concentration of 4×10^3 or 4×10^4 mL^{-1}; lower concentrations did not produce sufficient symptoms, whilst higher concentrations provided no discrimination between cultivars. However, the poor correlation between results of seedling tests and

Table 5 PCR detection of *Pyrenopeziza brassicae* DNA in oilseed rape leaves sampled from a winter oilseed rape experiment (six cultivars) at Rothamsted in 2003/04 using PbITSF and PbITSR primers, compared with visual assessment

		Number of PCR reactions detecting *P. brassicae*[a]				Leaves affected by *P. brassicae* (%)	
		DNA concentration (ng μL^{-1})				Assessment	
Cultivar	Sampling date	100	200	500	1000	PCR[a]	Visual[b]
Apex	7 December	–[c]	0	0	0	0	0
	9 January	–	0	0	0	0	0
	12 February	–	0	0	0	0	0
	12 March	3	3	4	3	40	9
Bosman	7 December	–	0	0	0	0	0
	9 January	–	0	0	0	0	0
	12 February	–	2	1	0	20	0
	12 March	1	2	3	2	30	5
Canberra	7 December	–	0	0	0	0	0
	9 January	–	0	0	0	0	0
	12 February	–	0	2	1	20	0
	12 March	2	4	5	0	50	2
Kana	7 December	–	0	0	0	0	0
	9 January	–	1	1	1	10	0
	12 February	–	0	0	0	0	0
	12 March	1	3	2	0	40	0
Marita	7 December	–	0	0	0	0	0
	9 January	–	0	0	0	0	0
	12 February	–	1	1	1	10	0
	12 March	2	3	3	0	30	2
Recital	7 Dec	–	0	0	0	0	0
	9 Jan	–	1	1	1	10	0
	12 Feb	–	1	0	0	10	0
	12 March	1	2	2	2	40	4

[a]Detection using PbITSF and PbITSR primers was done on 10 leaves per plot sampled from one plot of each cultivar; leaves were not incubated before assessment.
[b]Assessment of *P. brassicae* sporulation done in the laboratory on 60–100 leaves after 5 days' incubation of plants in polyethylene bags at 8°C.
[c]PCR not done.

those in field plots with the same cultivars (e.g. Canberra) suggest that different components of field resistance operate at seedling and adult plant stages (Fig. 1), confirming the results of Bradburne *et al.* (1999). Therefore, seedling tests, even with new conidial or ascospore inoculation methods (Gilles *et al.*, 2000a, 2001; Karolewski *et al.*, 2002), may be more useful for studying the genetics of resistance (Bradburne *et al.*, 1999) than for assessing field resistance of cultivars.

Results of field experiments demonstrated how the optimal timing for discriminating severity of light leaf spot in plots of different winter oilseed rape cultivars differs from season to season, and is greatly influenced by the severity and timing of the light leaf spot epidemic. The seasonal differences are related to differences in availability of ascospore inoculum in autumn (Gilles *et al.*, 2000b, 2001), occurrence of autumn rainfall to initiate epidemics and winter rainfall to spread this polycyclic disease (Fitt *et al.*, 1998a; Evans *et al.*, 2003). Thus, in 2004, when the epidemic was slight, assessments done *in situ* in plots were unable to distinguish between cultivars before June, whilst in 2003, during a severe epidemic, differences between cultivars were optimal in February/March. The results demonstrate how a single in-plot assessment can fail to detect widespread infection, as in April 2003 after a very dry month, when sampling and incubation revealed that most plants were affected by *P. brassicae*. Furthermore, in April 2004 at GS 3,4, the in-plot assessments did not relate well to the percentage of plants affected (after incubation), which may provide a good prediction of yield loss (Su *et al.*, 1998). Only when there was a severe epidemic (spring 2002/03) was there a good relationship between in-plot assessments of light leaf spot on leaves and assessments after sampling and incubation (Fitt *et al.*, 1998a).

Thus, whilst in-plot measurements of light leaf spot on leaves can provide discrimination between cultivars in relation to assessment of field resistance in the UK (www.hgca.com) and for understanding resistance QTLs (Pilet *et al.*, 1998) in epidemic years, they are not always reliable when light leaf spot is not severe. To compare cultivars effectively, it may be appropriate to do in-plot assessments on several occasions each season over a number of seasons, supplemented by sampling and incubation of plants (at 8–10°C for 5 days) at the start of the growing season before visual symptoms are observed

Table 6 Correlations between different methods for assessing light leaf spot (*Pyrenopeziza brassicae*) in winter oilseed rape experiments (six cultivars) in 2002/03 (above diagonal) and 2004/05 (below diagonal)

Month/method of assessment	Plot area affected (*in situ*) (%)	Plants affected (%)	Plant area affected (%)	Leaf area affected (%)	Leaves affected (%)	Stem area affected (%)	Pod area affected (%)
February (GS 1,14)[a]							
Plot area affected (%)		[d]	0·52	0·48	0·43	[c]	
Plants affected (%)	–[b]		[d]	[d]	[d]		
Plant area affected (%)	–	–		0·06	–0·07		
Leaf area affected (%)	–	–	–		0·92		
Leaves affected (%)	–	–	–	–			
March (GS 2,3)[a]							
Plot area affected (%)		[d]	0·35	0·20	–0·33	0·14	
Plants affected (%)	–		[d]	[d]	[d]	[d]	
Plant area affected (%)	–	–		0·66	0·32	–0·04	
Leaf area affected (%)	–	–	–		0·21	0·52	
Leaves affected (%)	–	–	–	–		–0·38	
Stem area affected (%)	–	–	–	–	–		
April (GS 3,4)[a]							
Plot area affected (%)		[d]	0·07	0·22	0·38	–0·16	
Plants affected (%)	0·18		[d]	[d]	[d]	[d]	
Plant area affected (%)	0·05	0·76		0·68	0·10	0·35	
Leaf area affected (%)	0·16	0·70	0·93		0·05	0·44	
Leaves affected (%)	0·14	0·78	0·95	0·97		–0·08	
Stem area affected (%)	–0·17	0·10	0·14	0·18	0·14		
May (GS 5,1)[a]							
Plot area affected (%)		[d]	0·63	0·68	0·69	0·66	0·41
Plants affected (%)	0·43		[d]	[d]	[d]	[d]	[d]
Plant area affected (%)	0·32	0·58		0·93	0·50	0·65	0·03
Leaf area affected (%)	0·38	0·62	0·94		0·61	0·74	0·18
Leaves affected (%)	0·29	0·71	0·85	0·92		0·61	0·72
Stem area affected (%)	0·37	0·43	0·47	0·59	0·53		0·24
June (GS 6,2)[a]							
Plot area affected (%)		[d]	0·45	[c]		0·53	0·38
Plants affected (%)	0·39		[d]			[d]	[d]
Plant area affected (%)	0·32	0·41				0·28	0·93
Leaf area affected (%)							
Leaves affected (%)							
Stem area affected (%)	0·31	0·54	0·91				0·29
Pod area affected (%)	0·36	0·26	0·87			0·64	

[a]Average growth stage (Sylvester-Bradley & Makepeace, 1985).
[b]In February and March 2004, no light leaf spot was detected when plots were assessed by any visual methods.
[c]Tissue not present; either stems or pods had not developed (February–April) or most leaves had dropped off plants (June).
[d]100% of plants were affected with light leaf spot in 2003.

(Fitt *et al*., 1998b; Su *et al*., 1998). However, for assessment of light leaf spot on pods, in both seasons there was a good relationship between assessments in-plot and after sampling/incubation, suggesting that in-plot assessments are adequate for assessment of disease on pods. To overcome unreliability in arrival of ascospore inoculum in autumn, and in occurrence of rainfall to favour progress of epidemics, it is important to inoculate trials with *P. brassicae* ascospores or conidia (Fitt *et al*., 1998b; Gilles *et al*., 2000a) and provide irrigation, if necessary, or locate them in areas such as Scotland when natural inoculum and weather favourable for light leaf spot development occur reliably (Sutherland *et al*., 1998). These principles form the basis for the inoculated test protocol that is used to differentiate resistance responses for a number of oilseed rape diseases as part of the recommended list process (J. Thomas, NIAB Cambridge UK, personal communication).

These experiments provide evidence that the new, more sensitive primer pair PbITSF/PbITSR is effective for the detection of *P. brassicae* infection in symptomless oilseed rape tissue. Nevertheless, PCR diagnosis on leaves sampled from field experiments was more sensitive than visual assessments, even after incubation, with infected leaves detected 2 months earlier. However, from results of controlled-environment experiments, when *P. brassicae* was

detected immediately after inoculation, it seems that this PCR method may have detected *P. brassicae* conidia on leaf surfaces, as well as hyphae in symptomless tissue. However, in the field, inoculum concentrations are much lower than those used under controlled-environment conditions. Therefore, the highly sensitive nature of the PCR should not present a problem for field assessments. To be of value in discriminating severity of *P. brassicae* infection on different cultivars, there is a need to develop a PCR method to quantify amounts of *P. brassicae* DNA in host tissues (Fraaije *et al*., 2001). Quantitative PCR (Thomas *et al*., 2004) might be used predictively if, for example, the amount of *P. brassicae* DNA present in oilseed rape apical meristems with symptomless infection in January could be related to severity of disease later in the season (Gilles *et al*., 2000b), in the same way that symptomless *L. maculans* infection of leaf petioles sampled in November/December has been related to subsequent severity of phoma stem canker in different cultivars (Kenyon *et al*., 2004).

Acknowledgements

The research was supported by the European Commission – INCO A1 programme 'States in the phase of preaccession to the European Union' (contract ICA1-CT-2002-70005), the UK Biotechnology and Biological Sciences Research Council and Department for Environment, Food and Rural Affairs, the Home-Grown Cereals Authority and GIE ProColza. We thank D. Arkell for practical assistance.

References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–402.

Amelung D, Daebeler F, 1991. Occurrence of fertile apothecia and the epidemiology of *Pyrenopeziza brassicae* Sutton and Rawlinson (anamorph: *Cylindrosporium concentricum* Grev.) in the German Democratic Republic. *International Organisation for Biological Control Bulletin* **14**, 147–50.

Anonymous, 1998. *Oilseed Rape Variety Handbook*. Cambridge, UK: National Institute of Agricultural Botany.

Bradburne R, Majer D, Magrath R, Werner CP, Lewis B, Mithen R, 1999. Winter oilseed rape with high levels of resistance to *Pyrenopeziza brassicae* derived from wild *Brassica* species. *Plant Pathology* **48**, 550–5.

Brun H, Renard M, Jouan B, Tanguy X, Lamarque C, 1979. Observations préliminaires sur quelque maladies du colza en France. *Sclerotinia sclerotiorum*, *Cylindrosporium concentricum*, *Ramularia armoraciae*. *Sciences Agronomique de Rennes* **1979**, 7–17.

Dyer PS, Furneaux PA, Douhan G, Murray TD, 2001. A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis*. *Fungal Genetics and Biology* **33**, 173–80.

Evans N, Baeirl A, Brain P, Welham SJ, Fitt BDL, 2003. Spatial aspects of light leaf spot (*Pyrenopeziza brassicae*) epidemic development on winter oilseed rape (*Brassica napus*) in the UK. *Phytopathology* **93**, 657–65.

Figueroa L, Fitt BDL, Shaw MW, McCartney HA, Welham SJ, 1995. Effects of temperature on the development of light leaf spot (*Pyrenopeziza brassicae*) on oilseed rape (*Brassica napus*). *Plant Pathology* **44**, 51–62.

Fitt BDL, Doughty KJ, Gilles T, Gladders P, Steed JM, Su H, Sutherland KG, 1998b. Methods for assessment of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape in the UK. *Annals of Applied Biology* **133**, 329–42.

Fitt BDL, Doughty KJ, Gladders P, Steed JM, Sutherland KG, 1998a. Diagnosis of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) in the UK. *Annals of Applied Biology* **133**, 155–66.

Fitt BDL, Turner JA, Gladders P, Sutherland K, 1999. Relationships between regional weather and incidence of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape in England and Wales. *Aspects of Applied Biology* **56**, 102–6.

Foster SJ, Ashby AM, Fitt BDL, 2002. Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape. *European Journal of Plant Pathology* **108**, 379–83.

Foster SJ, Singh G, Fitt BDL, Ashby AM, 1999. Development of PCR based diagnostic techniques for the two mating types of *Pyrenopeziza brassicae* (light leaf spot) on winter oilseed rape (*Brassica napus* ssp. *oleifera*). *Physiological and Molecular Plant Pathology* **55**, 111–9.

Fraaije BA, Lovell DJ, Coelho JM, Baldwin S, Hollomon DW, 2001. PCR-based assays to assess wheat varietal resistance to blotch (*Septoria tritici* and *Stagonospora nodorum*) and rust (*Puccinia striiformis* and *Puccinia recondita*) diseases. *European Journal of Plant Pathology* **107**, 905–17.

Gilles T, Evans N, Fitt BDL, Jeger MJ, 2000b. Epidemiology in relation to methods for forecasting light leaf spot (*Pyrenopeziza brassicae*) severity on winter oilseed rape (*Brassica napus*) in the UK. *European Journal of Plant Pathology* **106**, 593–600.

Gilles T, Fitt BDL, Kennedy R, Welham SJ, Jeger MJ, 2000a. Effects of temperature and wetness duration on conidial infection, latent period and asexual sporulation of *Pyrenopeziza brassicae* on leaves of oilseed rape. *Plant Pathology* **49**, 498–508.

Gilles T, Fitt BDL, McCartney HA, Papastamati K, Steed JM, 2001. The roles of ascospores and conidia of *Pyrenopeziza brassicae* in light leaf spot epidemics on winter oilseed rape (*Brassica napus*) in the UK. *Annals of Applied Biology* **138**, 141–52.

Graham GC, Mayers P, Henry RJ, 1994. A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *Biotechniques* **16**, 48–50.

Karolewski Z, 1999. The occurrence of light leaf spot on winter oilseed rape in Western Poland in 1991–96 and the characteristics of *Pyrenopeziza brassicae* isolates. *Phytopathologia Polonica* **18**, 113–21.

Karolewski Z, Evans N, Fitt BDL, Baierl A, Todd AD, Foster SJ, 2004. Comparative epidemiology of *Pyrenopeziza brassicae* (light leaf spot) ascospores and conidia from Polish and UK populations. *Plant Pathology* **53**, 29–37.

Karolewski Z, Evans N, Fitt BDL, Todd AD, Baierl A, 2002. Sporulation of *Pyrenopeziza brassicae* (light leaf spot) on oilseed rape (*Brassica napus*) leaves inoculated with

ascospores or conidia at different temperatures and wetness durations. *Plant Pathology* **51**, 654–65.

Kenyon D, Thomas J, Handy C, 2004. Feasibility of using quantitative PCR for assessing resistance to stem canker in oilseed rape cultivars. *International Organisation for Biological Control Bulletin* **27**, 109–17.

Lee SB, Taylor JW, 1990. Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: a Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 282–7.

Majer D, Lewis BG, Mithen R, 1998. Genetic variation among field isolates of *Pyrenopeziza brassicae*. *Plant Pathology* **47**, 22–8.

Payne RW, Lane PW, Digby PGN, Harding SA, Leech PK, Morgan GW, Todd AD, Thompson R, Tunnicliffe Wilson G, Welham SJ, White RP, 1993, .*Genstat 5 Release 3 Reference Manual.* Oxford, UK: Clarendon Press.

Pilet ML, Delourme R, Foisset N, Renard M, 1998. Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.). *Theoretical and Applied Genetics* **97**, 398–406.

Su H, Fitt BDL, Welham SJ, Sansford CE, Sutherland KG, 1998. Effects of light leaf spot (*Pyrenopeziza brassicae*) on yield of winter oilseed rape (*Brassica napus*). *Annals of Applied Biology* **132**, 371–86.

Sutherland KG, Fitt BDL, Steed JM, Welham SJ, Gladders P, Turner JA, 1998. Development and control of light leaf spot (*Pyrenopeziza brassicae*) epidemics in winter oilseed rape in the UK. *Brighton Crop Protection Conference – Pests and Diseases, 1998.* Farnham, UK: BCPC, 1053–58.

Sylvester-Bradley R, Makepeace RJ, 1985. Revision of a code for stages of development in oilseed rape (*Brassica napus*). *Aspects of Applied Biology* **10**, 395–400.

Thomas J, Kenyon D, Handy C, 2004. Preliminary results on the use of quantitative PCR for assessing resistance to light leaf spot (*Pyrenopeziza brassicae*) in oilseed rape cultivars. *International Organisation for Biological Control Bulletin* **27**, 83–6.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876–82.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfland DH, Sninsky JJ, White TJ, eds. *PCR Protocols: a Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 315–22.