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Effects of geographical location on phoma stem canker and yield of oilseed rape crops in the UK

by

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SUMMARY

Phoma stem canker, caused by *Leptosphaeria maculans* and *L. biglobosa,* is the most important disease of winter oilseed rape in the UK. Experiments at different sites in England examining the effects of fungicide timing on severity of phoma stem canker epidemics have produced conflicting results. The effects of geographic location may have contributed to these differences.

Ascospore release was monitored in three growing seasons (2005/06-2007/08) at Rothamsted. The onset of ascospore release was affected by temperature and rainfall and subsequent release by rainfall. Using quantitative PCR (qPCR), maximum *L. maculans* ascospore release was observed earlier in the season than that of *L. biglobosa*.

The national winter oilseed rape disease survey (1990 - 2006) showed regional differences in cultivar choice and patterns of fungicide application. Field experiments, using 42-45 different oilseed rape cvs/breeding lines, were done at Rothamsted during four growing seasons (2003/04, 2005/06, 2006/07, 2007/08). The severity of phoma stem cankers differed between cvs.

Regional variation in the distribution of *L. maculans* and *L. biglobosa* in stem cankers on commercial crops was determined in June/July 2001-2003 and 2006. DNA of both *L. maculans* and *L. biglobosa* was identified in 65% of samples. Geostatistical mapping and qPCR revealed the greatest amounts of *L. maculans* DNA in basal stem cankers in southern England (where epidemics are most severe) whilst that of *L. biglobosa* was greatest northern England. Similar patterns were observed in geostatistically mapped predictions of the time between onset of phoma leaf spot (autumn) and of phoma stem canker (spring). Basal stem cankers were associated with *L. maculans;* upper stem lesions were associated with *L. biglobosa.*

Geographic variation in *L. maculans* population structure was surveyed in the 2006/07 growing season. In total 120 isolates were analysed (collected from sites in Hertfordshire, Bedfordshire, Norfolk, Lincolnshire and Gloucestershire) using a plant set with known resistance (*Rlm*) genes. All isolates were virulent on cvs with *Rlm2, Rlm3* and *Rlm9* whilst 88% were not virulent on the cv. with *Rlm7*. Nineteen races were identified; the populations at Hertfordshire and Bedfordshire sites were closely related. Genetic variation in these populations was assessed using minisatellites; all isolates were unique.

1 INTRODUCTION

1.1 Phoma stem canker

The most economically important disease of winter oilseed rape in the UK is phoma stem canker, caused by *L. maculans* and *L. biglobosa*, which caused an average annual loss of €56M in 2000-2002 (Fitt *et al.*, 2006a). Ascospores released from pseudothecia (fruiting bodies) that develop on the stubble of the previous season's crop are the main inoculum initiating phoma stem canker epidemics each season (Aubertot *et al.*, 2006; West *et al.*, 2001). Once released, ascospores may be wind-dispersed up to 5 km from their source (Hall, 1992). However, the majority of ascospores are deposited within 500 m (Aubertot *et al.*, 2006; West & Fitt, 2005). Both *L. maculans* and *L. biglobosa* penetrate the leaf through stomata or wounds (Hammond *et al.*, 1985). Lesions caused by *L. maculans* are grey in colour and contain many dark spots (pycnidia) whilst lesions caused by *L. biglobosa* are smaller, have a dark margin with a light brown centre and contain few, if any, pycnidia (Toscano-Underwood *et al*., 2001). Pycnidia, the asexual fruiting bodies, produce spores (conidia) that may serve as a secondary inoculum in parts of the world such as Australia (West *et al*., 2001).

Both *L. maculans* and *L. biglobosa* spread, without causing symptoms, from the leaf through the petiole to the stem where they invade and kill host cell tissue resulting in the formation of stem cankers or stem lesions (Hammond & Lewis, 1987; Hammond *et al.*, 1985). In the UK, basal stem cankers are associated with *L. maculans* leaf lesions and upper stem lesions are often associated with *L. biglobosa* (Fitt *et al.*, 2006b; West *et al.*, 2002a). Basal stem lesions may spread and crack open during pod development and seed ripening to form cankers that may completely girdle the stem (West *et al.*, 2001). Stem cankers reduce yield by restricting water transport through the stem, resulting in premature pod ripening, and pods and seeds can become shrivelled. In severe cases, cankers may sever plants from their root systems, resulting in lodging or plant death (Gugel & Petrie, 1992; West *et al.*, 2001). After harvest, both *L. maculans* and *L. biglobosa* are able to survive on stem debris (Salam *et al.*, 2003).

Both *L. maculans* and *L. biglobosa* have a worldwide distribution. It is likely that these pathogens spread through seed transmission of *B. oleracea*, *B. napus*, *B. rapa* and other brassica seed (Fitt *et al.*, 2006a). A mixed population of *L. maculans* and *L. biglobosa* occurs in the western European countries including the UK (Fitt *et al.,* 2006a; Humpherson-Jones, 1983), although regional variation in the frequencies of the two species have been reported. In the UK, there is regional variation in the incidence and severity of phoma stem canker epidemics (cropmonitor.co.uk) and in the date of first leaf spotting (www.rothamsted.bbsrc.ac.uk/ppi/phoma/).

1.2 Disease control

The use of resistant cultivars is a key method of disease control. There are two types of resistance against *L. maculans*, namely race-specific (monogenic) and race-non specific (polygenic). Race-specific resistance is expressed in the leaves of seedlings and adult plants whilst race non-specific resistance is assessed either just before or at harvest. In oilseed rape, race-specific resistance genes (*Rlm* and *LepR*) recognise *L. maculans* avirulence alleles (*AvrLm*) thus preventing leaf lesion formation and subsequent spread and production of stem cankers (Delourme *et al.*, 2006; Huang *et al.*, 2006a; Huang *et al.*, 2006b). *Leptosphaeria maculans* has a high evolutionary potential and rapid 'breakdown' of race-specific resistance has occurred when there has been large scale cropping of cultivars with a single *Rlm* gene (Gout *et al.*, 2006). Race-non specific resistance to *L. maculans* is considered to be more durable than race-specific resistance. Race non-specific resistance, involving quantitative trait loci (QTL), acts in the petiole and stem some time after initial infection of leaves by *L. maculans*. It may be possible to breed cultivars with durable resistance to *L. maculans* by selecting lines which combine both race-specific and race-non specific resistance mechanisms. However, little is known about the mechanisms of oilseed rape resistance to *L. biglobosa* and it is believed that race-specific *Rlm* genes that operate against *L. maculans* are not effective against *L. biglobosa* (Brun *et al.*, 1997; Fitt *et al.*, 2006a; Somda *et al.*, 1998).

Fungicides can be applied as seed treatments, soil treatments, coated fertiliser granules, or as foliar sprays, depending upon phoma stem canker epidemiology and economics of oilseed rape production in different countries (Aubertot *et al.*, 2006; West *et al.*, 2001). Foliar sprays of fungicides, including triazoles, benzimidazoles and strobilurins are used where fungicide applications are cost effective because high yields are attainable (Aubertot *et al.*, 2006; Kruse & Verreet, 2005). A yield response is achieved only when fungicides are applied at the correct time. This is because these fungicides are generally protectant not curative compounds and have only a limited period of action due to degradation, leaf expansion and the production of new untreated leaves (Aubertot *et al.*, 2006; Gladders *et al.*, 1999; West *et al.*, 1999; West *et al.*, 2001). In Europe, fungicide sprays are more effective in controlling phoma stem cankers when they are applied in the autumn rather than in the spring (Kruse & Verreet, 2005; Steed *et al.*, 2007). It is also important that fungicides are applied at only the correct dose and at the right time to prevent the emergence and build up of fungicide resistance, soil erosion through compaction of tram-lines following autumn application and increased costs for the growers. In addition, *L. maculans* ascospore discharge has been shown to be reduced by chemical treatment of stubble after harvest (Wherrett *et al.* 2003).

In the management of phoma stem canker, it is important to minimise the exposure of oilseed rape crops to ascospores, the inoculum, in the autumn. Stubble management, through crop rotation and tillage, is an important way to achieve this. Huang *et al.* (2003a) showed that, although *L. maculans* and *L. biglobosa* cannot survive burial on upper stem debris, *L. maculans* can survive on stem base debris buried in sand for 12 months and then produce pseudothecia upon return to the surface. Ploughing affects the availability of the inoculum by preventing the dispersal of air-borne ascospores (Thurwachter *et al.*, 1999). The burial of crop residues also accelerates their decomposition. For example, a burial period of three years results in low amounts of residues; therefore a four year rotation is advisable (Turkington *et al.*, 2000; West *et al.*, 2001). Other methods of disease control include altering sowing date to prevent the coincidence of young plants and maximum ascospore release (needs to be balanced against the need to optimise the physical conditions that influence crop emergence) and by increasing sowing density and/or crop size (Aubertot *et al.*, 2004; Aubertot *et al.,* 2006).

The use of accurate, weather-based models to predict the onset of ascospore release allows for effective deployment of disease control measures, such as altered sowing date and use of fungicide applications, to be made on a regional or farm scale (Salam *et al.*, 2007). For example, a weather-based model fitted to data from 40 oilseed rape field experiments done under different weather conditions in growing seasons between 1992-1993 and 2001-2002 at a wide range of UK sites has been developed (Evans *et al.*, 2008). The model predicts disease development in three stages; stage 1 predicts the onset of phoma leaf spotting; stage 2 predicts the onset of phoma stem canker; stage 3 predicts the severity of phoma stem cankers at harvest. To help growers with fungicide application decisions, this model is used to predict the date of 10% incidence of phoma leaf spotting (recommended threshold for fungicide applications) at different locations in the UK and is available online (http://www.rothamsted.bbsrc.ac.uk/ppi/phoma/). In addition, predictions of the incidence of phoma stem canker, on a regional, cultivar, cultivar resistance rating and autumn fungicide application basis are available online (http://www.cropmonitor.co.uk/wosr/tools/disease/phoma_reg/forecast.cfm).

1.3 Regional variation in fungicide response

There is concern in the UK that fungicides are being used unnecessarily as well as inappropriately. Experiments done at Rothamsted Research and by ADAS in 2000/01 to 2002/03 growing seasons suggest that application of fungicides after a threshold incidence of plants with phoma leaf spot has been reached in the autumn results in a significant reduction in stem canker severity with a concomitant economic yield response. However, similar experiments done by The Arable Group (TAG), at the same time, at locations throughout

England gave different results. TAG found that application of fungicide once a threshold incidence of plants with phoma leaf spot had been reached resulted in a decrease in stem canker severity but rarely gave an economic yield response. The differences in the results between these studies could have been due to differences in the cultivars used, in the local pathogen population as well as to differences between UK regions in pathogen ascospore release patterns.

1.4 Aim and objectives

AIM: To examine geographical variation in phoma stem canker in the UK with the overall aim of reducing current levels of fungicide application. This will be done through the following objectives:

Objective 1: To investigate the effects of regional differences in agronomic practice, such as cultivar choice and fungicide application, on severity of phoma stem canker epidemics.

Objective 2: To determine both regional and seasonal differences in the distribution of *L. maculans* and *L. biglobosa* in phoma stem cankers on oilseed rape in England.

Objective 3: To study the differences in seasonal patterns of ascospores release between *L. maculans* and *L. biglobosa*.

Objective 4: To determine regional genetic variation within *L. maculans* populations in England using neutral and selective markers.

2 MATERIALS AND METHODS

2.1 Effects of cultivar on phoma stem canker

To investigate the effect of cultivar on phoma stem canker severity field experiments were done at Rothamsted, Hertfordshire, UK over four winter oilseed rape growing seasons (2003/04[1], 2005/06, 2006/07[2] and 2007/08[3]). In these experiments, 42 (2003/04, 2005/06 and 2007/08) or 45 (2006/07) current or historical winter oilseed rape cultivars/breeding lines were grown in a randomised complete block design with three replicates. Experiments were hand sown at a sowing density of 100 seeds/m^2. To reduce edge effects and inter-plot interference, each cultivar/line had a border row of a phoma stem canker susceptible cultivar, namely cv. Shannon (2003/04 and 2005/06) or cv. Recital (2006/07 and 2007/08). The field

[1] Assessments done by Elizabeth Pirie.
[2] Assessments done with Emily Boys.
[3] Assessments done with Emily Boys and Neal Evans.

experiments received no fungicide application or additional artificial inoculum. In the 2005/06, 2006/07 and 2007/08 growing seasons, the development of phoma leaf lesions was regularly assessed in the autumn/winter and phoma stem cankers were assessed in the spring/summer of each season. Assessments were done on 10 plants randomly selected from each plot of each cultivar (30 plants per cultivar in total). Disease incidence was recorded as the proportion of assessed plants with disease symptoms. Assessment of external stem disease began at the first sign of disease and continued until harvest. Stem canker severity was judged on a 0-4 scale: 0 = no canker; 4 = 100% stem discoloured and girdled (Figure 2.1). At harvest in all four seasons, 10 whole plants per replicate of each cultivar/breeding line were removed; the internal severity of phoma stem cankers was assessed by cutting through the base of the stem at the root collar and scoring the extent of necrotic tissue on a 0-4 scale (Zhou *et al*., 1999; Figure 2.1).

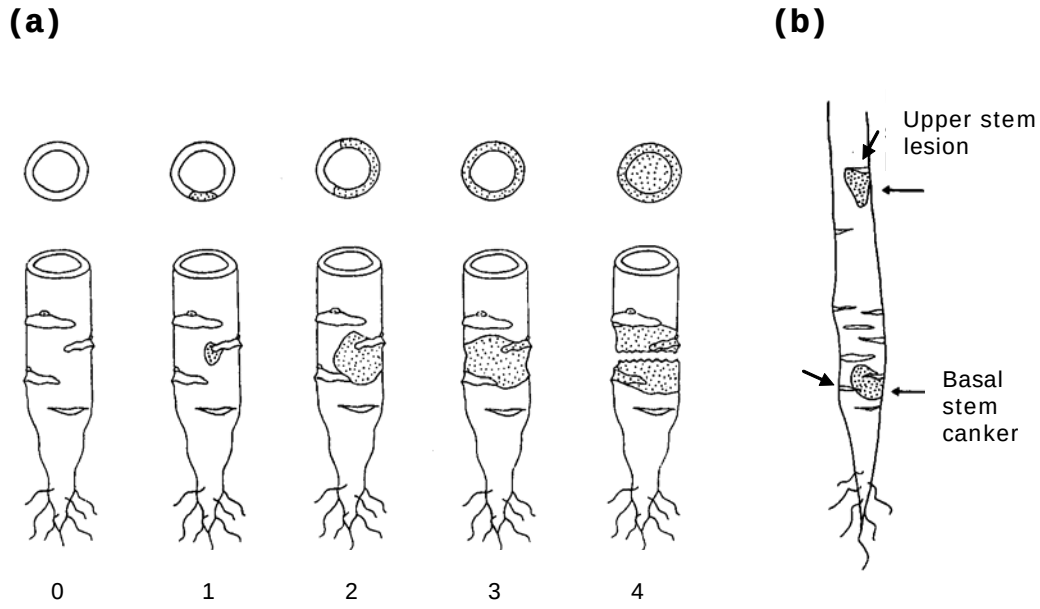

Figure 2.1. Schematic diagrams of winter oilseed rape stem bases to illustrate stem cankers with different severity scores (0-4 scale), showing (a) external and internal symptoms and (b) position and the symptoms of basal stem cankers and upper stem lesions. External symptoms scored as; 0 - no disease, 1 - less than half the stem girdled by lesions, 2 - more than half the stem girdled by lesions, 3 - whole stem girdled and weakened by lesions, 4 - plant dead. Internal symptoms scored as; 0 - no disease, 1 < 50 % of the cross section with symptoms, 2 = 50 – 75 % of the cross section with symptoms, 3 > 75 % of the cross section with symptoms, 4 = 100 % of the cross section with symptoms. Adapted from Zhou *et al.* (1999).

2.2 Regional differences in agronomic practice

To investigate regional differences in agronomic practices, data from the national winter oilseed rape surveys (1990-2006; www.cropmonitor.co.uk) were collated and provided by

Judith Turner and Sharon Elcock, FERA, York, UK. Information was provided on cultivar choice and fungicide use (chemical group, rate of application and the number and the timing of applications). Data were organised into six regions (Northern, Lincolnshire, Midlands and north Wales, South-central, South-east and South-west and south Wales) shown in Figure 3.2 and summarised accordingly.

Figure 2.2. Regional division of England and Wales. Regional division was based upon phoma risk regions identified by the PASSWORD project (Gladders *et al.*, 2004). Sampling

sites from assessment of *L. maculans* genetic diversity (Objective 4) are shown: (A) Skirbeck Farm, Benniworth, Lincolnshire, (B) Morley, Wymondham, Norfolk, (C) The Manor, Wyboston, Bedfordshire, (D) Rothamsted, Harpenden, Hertfordshire and (E) Manor Farm, Daglingworth, Gloucestershire.

2.3 Regional differences in distribution of *L. maculans* and *L. biglobosa* on oilseed rape in England

A survey of *Leptosphaeria* populations in England and Wales was done by sampling field-sown winter oilseed rape plants with visible basal stem cankers 1-2 weeks before harvest (typically late June or early July) in 2001, 2002, 2003 and 2006[4]. Samples were collected by ADAS (2001-2003) and The Arable Group (TAG; 2006) growers and farm consultants from commercial crops (approximately 10 per site). The oilseed rape cultivar and ordnance survey grid reference of each site was recorded. In total, 962 lesions from 117 sites were received (Table 2.1). Cankers/lesions occurring <5 cm from the stem base were classified as basal stem cankers. Lesions occurring >5 cm from the stem base were classified as upper stem lesions. The internal severity, assessed by cutting a cross section, of basal stem cankers was scored on a 0-5 scale according to the percentage of the stem circumference affected (0 = no symptoms visible, 1 = 0-25% affected, 2 = 25-50%, 3 = 50-75%, 4 = 75-100% and 5 = plant dead) (West *et al.*, 2002a). A piece, approximately 10 mm in diameter, was excised from a canker lesion on each stem.

Table 4.2. Numbers of oilseed rape stem samples* received in June/July in 2001, 2002, 2003 and 2006.

Year	Number of sampling sites	Number of basal stem cankers[c]	Number of upper stem lesions[d]	Total number of lesions
2001[a]	27	182	60	242
2002[a]	20	87	12	99
2003[a]	36	178	61	239
2006[b]	32	364	18	382
Total	115	811	151	962

* Selected plants were cut 30 cm above the stem base to give samples.
[a] Twenty five plants from each site were assessed for disease severity, the 10 plants with the most severe symptoms were selected (at a minority of sites <10 stems had visible basal stem cankers). Stems were collected by ADAS.
[b] A minimum of 10 plants with visible basal stem cankers was selected. Stems were collected by The Arable Group.
[c] Lesions occurring <5 cm from the stem base.
[d] Lesions occurring >5 cm from the stem base.

DNA was extracted from the pieces of basal stem canker and upper stem lesion according to the method of Graham *et al.* (1994)[5]. Uniplex PCR was done on extracted DNA to identify *L. maculans* and *L. biglobosa* DNA using species specific primers (Mahuku *et al.,*

[4] 2001, 2002 and 2003 surveys were coordinated and disease assessments done by Maria Eckert and Ze Liu.
[5] 2001-2003 samples done by Maria Eckert and Ze Liu.

1996)[6]. To determine the relative amounts of *L. maculans* and *L. biglobosa* DNA in samples, quantitative PCR (qPCR) was done[7] using the Sigma SYBR Green qPCR kit.

For each survey year (2001, 2002, 2003 and 2006), the severity of phoma stem canker epidemics was predicted at the sites of weather stations within England and Wales using the model of Evans *et al*. (2008). The model uses climatic variables to predict two key dates in the development of a phoma stem canker epidemic. These dates are *DI,* the date in autumn when 10% of plants in a crop are affected by phoma leaf spotting, and *Dc*, the date in the following spring when 10% of plants are affected by stoma stem canker. Having predicted these dates, the severity of a phoma stem canker epidemic can be predicted by summing the thermal time between Dc and the harvest date Dh. The abundance of *L. maculans* and *L. biglobosa* DNA in stem cankers and the outputs from the weather-based model of Evans *et al.* (2008) were mapped across England and Wales by geostatistical methods[8].

2.4 Variation in *L. maculans* and *L. biglobosa* ascospores release

Ascospore release was monitored at Rothamsted during the 2005/06, 2006/07 and 2007/08 winter oilseed rape growing seasons using a Burkard 7-day volumetric spore sampler (Burkard Manufacturing Company Ltd, Rickmansworth, UK) surrounded by oilseed rape stubble (including tap root; collected 2-3 weeks after harvest) from the previous season's crop (Figure 2.3).

[6] 2003 done by Ze Liu.
[7] 2003 done by Ze Liu.
[8] Predictions made using model and geostatistics done by Ben Marchant.

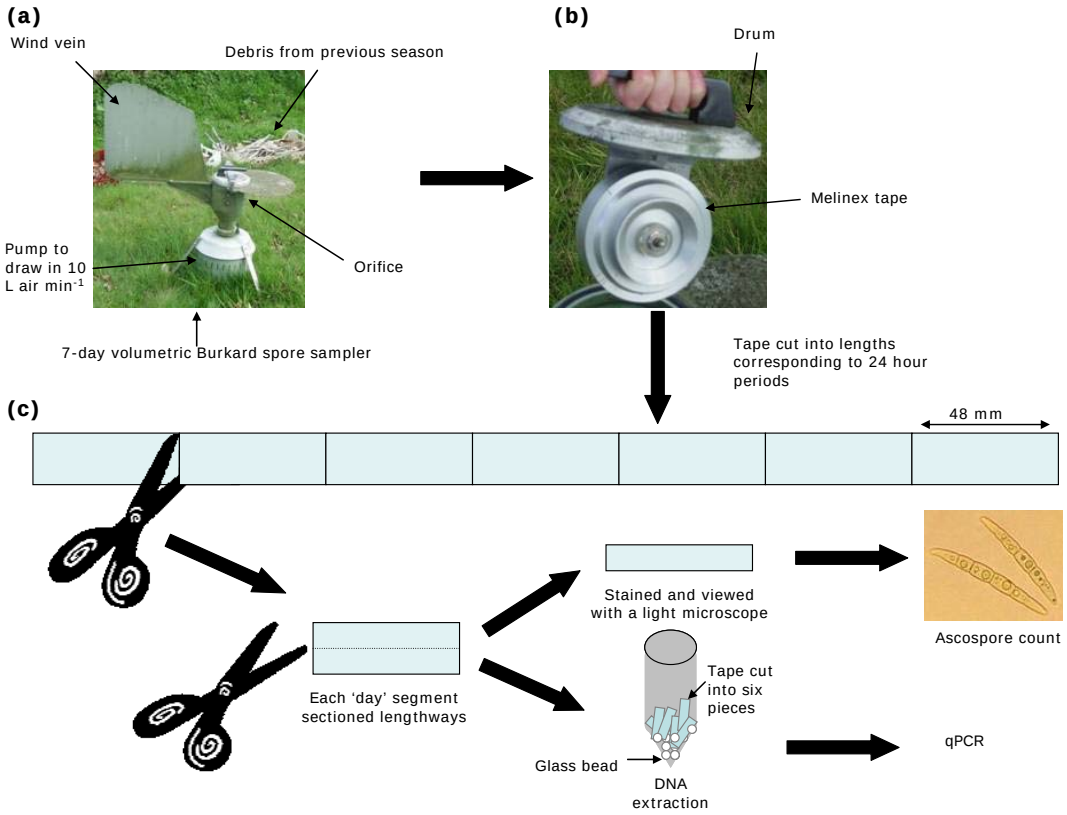

Figure 2.3. Burkard air-sampling. (a) The Burkard spore sampler was surrounded by oilseed rape debris of the previous season, the pump draws in air at a rate of 10 L min^{-1}. Ascospores in the air spora entered the Burkard through the orifice and impacted upon (b) a Vaseline-coated melinex tape attached to a rotating drum. The drum rotated once every seven days after which it was replaced. The tape was removed from the drum and (c) cut into pieces corresponding to 24 hr periods. Each of these 'day' segments was cut lengthways to give two pieces: one piece was stained with trypan blue and *L. maculans/L. biglobosa* ascospores were counted on the tape by microscopy. The second piece of tape was cut into six pieces and placed in a sterile 1.5 mL microcentrifuge tube with 50 µL volume of acid washed glass beads; DNA was extracted from these pieces and the amounts of *L. maculans* and *L. biglobosa* DNA on the tape determined by qPCR using species specific primers and SYBR green chemistry.

Monitoring of release of ascospores began 24 August in 2005, 6 September in 2006 and 22 August in 2007. Once removed from the spore sampler, tapes were divided into pieces 48 mm in length corresponding to 24 h sampling periods. Pieces corresponding to each day's sample were then divided lengthways, along the centreline in the direction of rotation, into two halves. The number of *Leptosphaeria maculans* and *L. biglobosa* ascospores on one half was immediately counted by light microscopy after staining. The number of spores present per unit volume of air (m^3) was calculated by multiplication of the mean number of spores per microscope traverse by a conversion factor of 2.09 (McCartney *et al.*, 1997). The remaining half was stored at -20°C in a 1.5 mL microcentrifuge (Figure 2.3). In the 2006/07 and 2007/08 growing seasons relative amounts of *L. maculans* and *L. biglobosa* DNA on spore tapes (tape halves stored at -20°C) was assessed. DNA was

extracted from one half of each piece of tape corresponding to one day from August/September until 31 December; on pieces of tape collected from 1 January onwards DNA was extracted from pieces for alternate days. The amounts of *L. maculans* and *L. biglobosa* DNA were quantified by qPCR.

2.5 Regional differences in genetic diversity of *L. maculans* populations in England

Spring oilseed rape cv. Drakkar (with no known race-specific genes for resistance to *L. maculans*) was sown, as a trap cultivar, at TAG sites in Hertfordshire, Lincolnshire, Norfolk, Bedfordshire and Gloucestershire, in August 2006 (Figure 2.2). The plots were infected by the local, natural *L. maculans* inoculum; no additional inoculum such as infected debris was applied. When phoma leaf spotting was first observed (October/November), leaves with characteristic phoma leaf spot symptoms were sent to Rothamsted from each site. Samples were washed, dried and processed on the day of arrival at Rothamsted. Lesions were cut from leaves and placed in humidity chambers (55 mm Petri dishes containing two layers of 1.5 cm^3 no. 1 filter paper moistened with 200 µl sterile deionised water). Humidity chambers were then sealed and placed at 20°Cfor 24 – 48 h to induce exudation of cirri (visible as pink knobs with a light microscope) from pycnidia (asexual fruiting bodies) from which conidia (asexual spores) could be collected, using a sterile mounted needle, and transferred to V8 agar medium. Isolates were purified to obtain single spore cultures, DNA extracted and confirmed as *L. maculans* using PCR. A sub-set of 24 isolates as selected from each site and these isolates were further characterised.

A differential plant set (seed supplied by M Balesdent, INRA Versailles) comprising genetically fixed cultivars/lines of *Brassica* species that possess one or two *Rlm* genes was used. These were Darmor-MX (*Rlm6*), 190-1-1 (*Rlm9*), 150-2-1 (*Rlm5*), 23-2-1 (*Rlm7*), 189-1-1 (*Rlm4*), Columbus (*Rlm1* + *Rlm3*), Bristol (*Rlm2* + *Rlm9*) and 22-2-1 (*Rlm2*); cv. Westar was used as susceptible control. Genotyping at the Avr 8 locus was not done due to unavailability of seed of this differential cultivar. Seeds were sown directly into compost in compartmentalised trays (5 replicates). Plants were grown in a glasshouse (20 – 23°C (day)/16 – 20°C (night)) for 14 days until inoculation. Ten-14 days after sowing, a plastic stake was placed next to each plant and a small piece of electrical wire was used to attach the plant to the stake for extra support and uprightness (Figure 2.4). Inoculation was done with 10µl suspension with 10^6 conidia ml^{-1}. Trays were covered with a transparent plastic lid and then transferred to a growth cabinet with a 16 h photoperiod and 20°C day/16°C night. Immediately after inoculation, trays were kept in the dark for 48 h (lids were covered with black polyethylene bags).

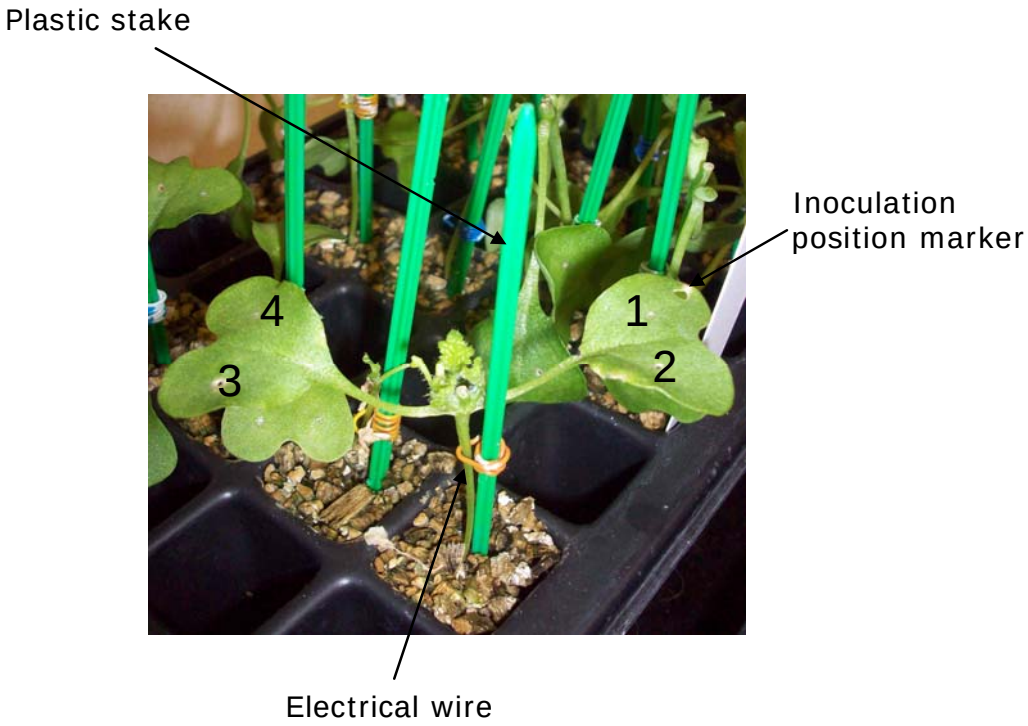

Figure 2.4. Inoculation of oilseed rape cotyledons. Each seedling was supported by a plastic stake to which it was attached using coated electrical wire. A hole was created in one cotyledon half per seedling to serve as an inoculation position marker: each cotyledon half was wounded and inoculated with 10 µL conidial (10^6 ml^{-1}) suspension in a clockwise direction from the inoculation marker at positions 1,2,3 and 4. Emerging leaves were removed by pruning.

Lesion phenotypes displayed on infected cotyledons of seedlings in the differential plant set were scored on a 1-6 scale at 14 and at 18/19 days after inoculation according to the IMASCORE system (Balesdent *et al.*, 2006). Plants giving scores of 1-3 were rated as resistant and isolates were termed avirulent, whereas plants giving scores 4-6 were classed as susceptible and isolates were termed virulent (Figure 2.5).

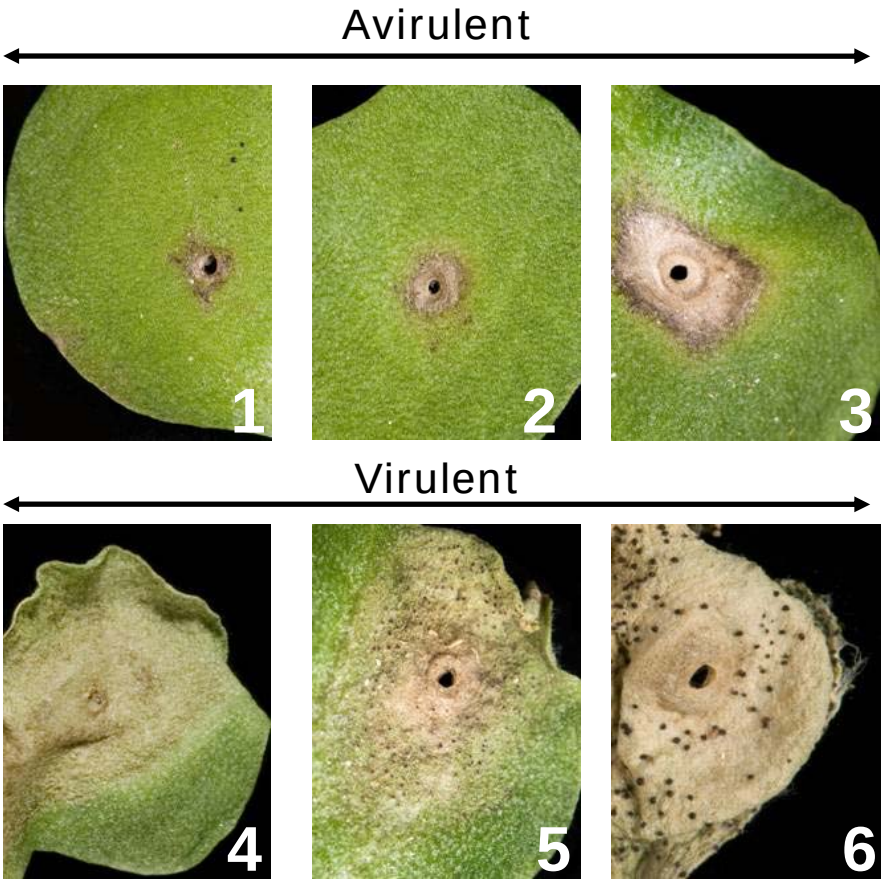

Figure 2.5. Lesion phenotypes occurring on cotyledons of differential plant set. Lesions were scored at 14 and 18 or 19 days after inoculation. Isolates with scores of 1-3 (dark, necrotic lesions) showed a resistant phenotype and were rated avirulent; isolates with scores of 4 (pale lesion), 5 (pale lesion with pycnidia) or 6 (senescent with pycnidia) showed a susceptible phenotype and were classed virulent.

Avirulence (Avr) alleles for each isolate were identified through interaction phenotypes across the differential set; for example an isolate avirulent on Darmor-MX (*Rlm6*) possessed the Avr allele *AvrLm6*. Isolates were classified into races based upon Avr alleles (Avr8 for which genotyping was not possible was indicated in brackets). For example, the race *Av4-5-6-7-(8)* is comprised of isolates possessing the Avr alleles *AvrLm4*, *AvrLm5*, *AvrLm6* and *AvrLm7.*

To examine genetic variation between isolates, fourteen minisatellite markers (*MinLm3*, *MinLm4, MinLm5, MinLm6*, *MinLm8*, *555, 632, 1377, 1721, 2451, 935.2, 967, 1838* and *1368*) were used to characterise isolates. The relatedness of isolates collected across the TAG sites was examined using dendrograms produced to reflect Nei's genetic distances using data from minisatellite markers or those from avirulence alleles.

2.6 Rothamsted weather data

A synoptic weather station collected daily meteorological data (temperature and rainfall) at Rothamsted, from 0900 h GMT to 0900 h GMT on the following day. Temperature was recorded by a 107 thermistor probe (Campbell Scientific, Loughborough, UK) and rainfall was measured using a 0.2 mm ARG100 tipping bucket rain gauge (Campbell Scientific, Loughborough, UK). Data were obtained from the Electronic Rothamsted Archive.

2.7 Statistical analysis

Data were saved as Microsoft Excel files. Preliminary analysis, calculation of means etc., was done by Microsoft Excel. Graphs were produced using SigmaPlot version 10.0. Analysis of variance (ANOVA) and regression was done using Genstat version 9 (Payne *et al.*, 2007).

3 RESULTS

3.1 Effect of cultivar on phoma stem canker

The incidence of phoma leaf spot and stem canker (external symptoms) in relation to degree days from sowing is shown in Figure 3.1. The earliest onset of both phoma leaf spot and phoma stem canker was in the 2005/06 growing season, onset in the 2006/07 and 2007/08 growing seasons were similar. The maximal proportion of plants with phoma leaf spot symptoms was greatest in the 2005/06 growing season (100%) and least in the 2006/07 growing season (51%). There were approximately 1340°C-days between the onset of leaf spotting and that of stem cankers in all seasons. The proportion of plants with phoma stem canker (external symptoms) reached 93%, 96% and 21% in the 2005/06, 2006/07 and 2007/08 growing seasons, respectively. Stem canker severity (based on internal symptoms) was greater in the 2005/06 and 2006/07 growing seasons than in 2003/04 and 2007/08 and varied between cultivars (Table 3.1; Figure 3.2). Statistical analysis showed significant differences (P <0.001) between different growing seasons and between cultivars in stem canker severity. There was a significant difference (P <0.001) between cultivars in stem canker severity under high (2005/06 and 2006/07) or low (2003/04 and 2007/08) inoculum concentration which accounted for some, but not all, of the differences between seasons observed in the severity of stem canker on the same cultivars.

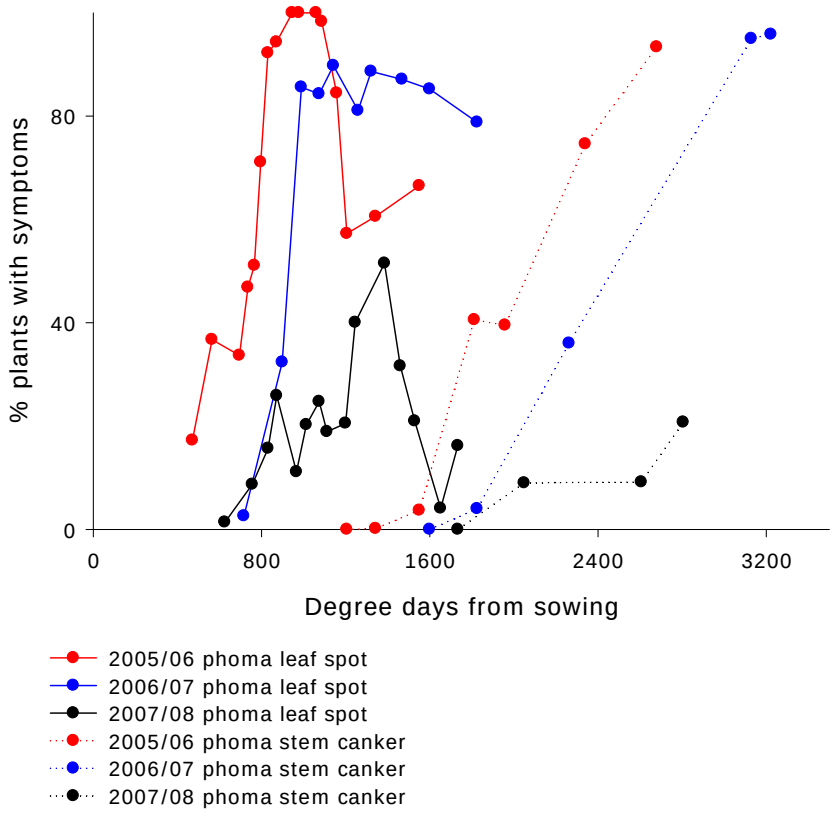

Figure 3.1. Incidence (% plants with symptoms) of phoma leaf spot and phoma stem canker in oilseed rape cvs in the 2005/06, 2006/07 and 2007/08 winter oilseed rape growing seasons at Rothamsted in relation to accumulated degree days from sowing (13/09/2005, 31/08/2006 and 28/08/2007; temperature data collected at a weather station 0.5 to 1.5 km from field experiment).

Table 3.1. Mean severity of internal stem canker severity across 42-46 different oilseed rape cultivars/breeding lines in the 2003/04, 2005/06, 2006/07 and 2007/08 winter oilseed rape growing seasons at Rothamsted.

Growing season	Mean canker severity (0-4 scale)[a]
2003/2004	0.75
2005/2006	2.56
2006/2007	3.15
2007/2008	0.38

[a] The internal severity of phoma stem canker was assessed on a 0-4 scale (Zhou *et al.*, 1999) in late June/early July in each growing season.

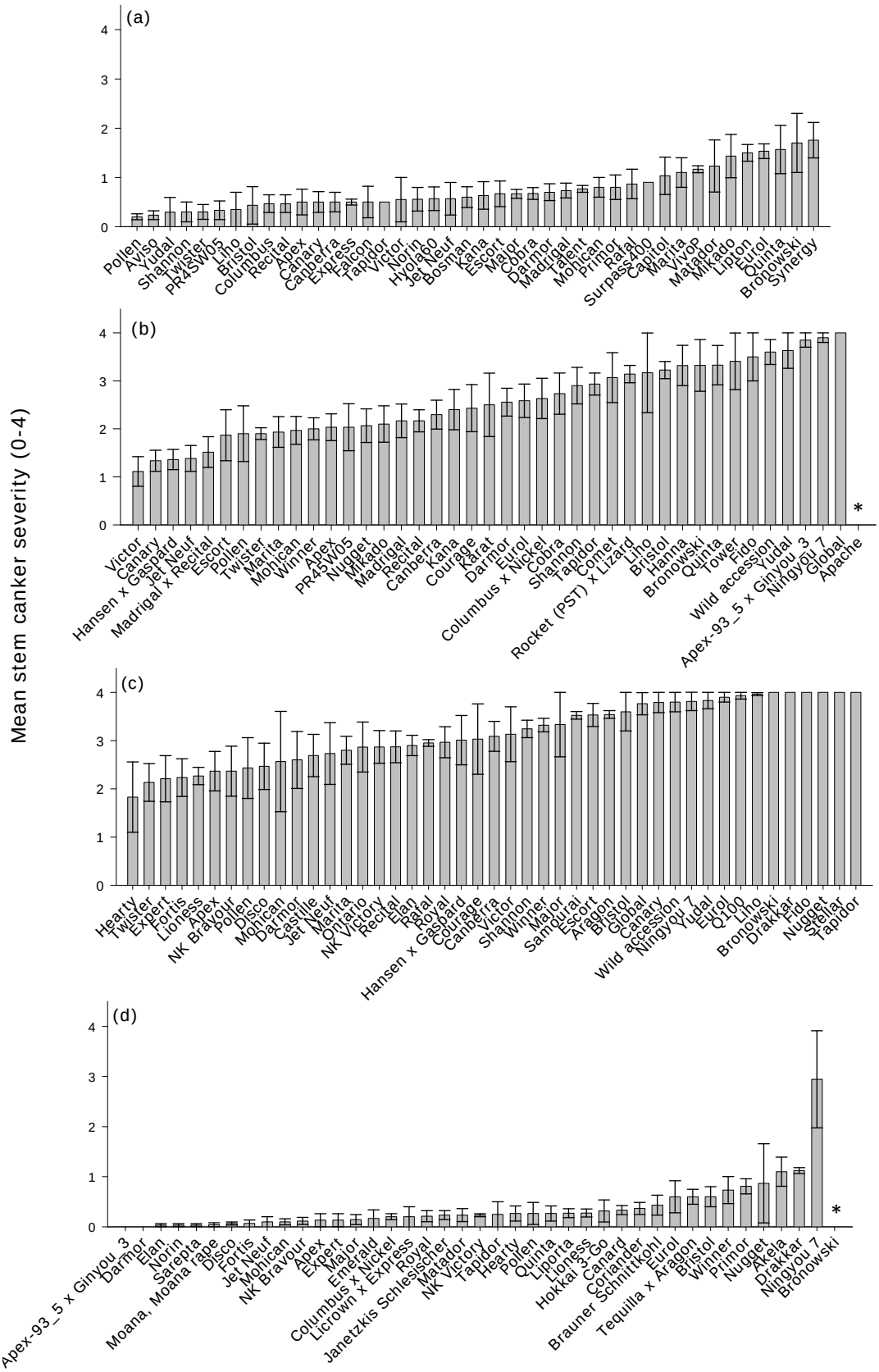

Figure 3.2. Internal severity of phoma stem cankers (0-4 scale) on 42-45 field sown oilseed rape cultivars/breeding lines in the (a) 2003/04,(b) 2005/06, (c) 2006/07 and (d) 2007/08 winter oilseed rape growing seasons at Rothamsted. (*) cv. Apache did not survive in the 2005/06 growing season, cv. Bronowski did not survive in the 2007/08 growing season. Error bars show the standard error of the individual means; approximately 30 plants assessed per cultivar in each year.

Over the four seasons of this study, 49 cultivars were grown in more than one season. The mean internal stem canker severity for each cultivars across all seasons is shown in Figure 3.3. Cultivar Ningyou7 had the greatest mean canker severity, cv. Twister the smallest canker severity. All the cultivars on the current HGCA Recommended List that were included in these experiments had a mean stem canker severity of less than 2. The ranking of cultivars, according to severity of internal stem canker pre-harvest, is shown in Table 3.2. The best ranked cultivars with the smallest mean stem canker severity across all four seasons were cvs Twister and Fortis. By contrast, cvs Fido and Global had the lowest mean ranks as they consistently had high stem canker severity scores. Several cultivars had large variances; for example cv. Shannon was ranked 3rd in the 2003/04 growing season but ranked 21st in 2005/06 and 22nd in 2006/07 and cv. Canary was ranked 12th in 2003/04, 4th in 2005/06 and 27th in the 2006/07 growing season. In contrast, some cultivars had variances of just 0.5; for example cv. Expert was ranked 5th in 2006/07 and 6th in the 2007/08 growing season and Wild accession was ranked 30th in 2005/06 and 31st in the 2006/07 growing season. The HGCA resistance rating of cultivars generally decreased with decreasing rank.

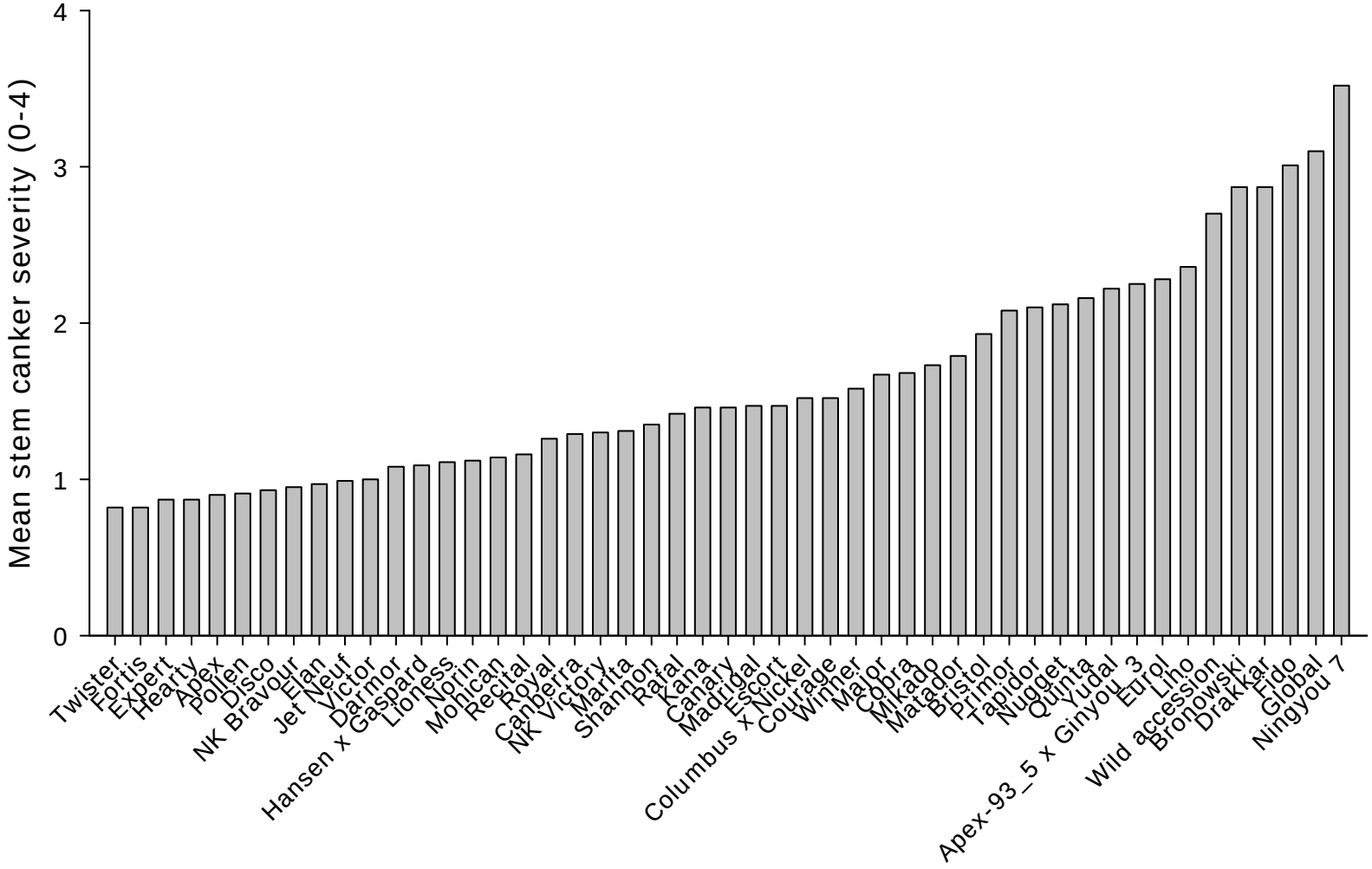

Figure 3.3. Mean stem canker severity of cultivars/breeding lines grown in at least two growing seasons (2003/04, 2005/06, 2006/07 and 2007/08) at Rothamsted. Internal stem canker severity was assessed pre-harvest (late June/early July) in each season on a 0-4 scale (Zhou *et al.*, 1999). Data presented is back transformed from a logit (x+0.1) transformation, SED 0.28; approximately 30 plants were assessed per cultivar in each season.

Table 3.2. Ranking of cultivars for internal stem canker severity scores in field experiments done at Rothamsted in the 2003/04, 2005/06, 2006/07 and 2007/08 winter oilseed rape growing seasons.

	Harvest year of field experiment[a]					
Cultivar[b]	**2004**	**2006**	**2007**	**2008**	**Mean**	**Variance of rank**
Twister	2	5	2	*	3	3
Fortis	*	*	4	2	3	2
Apex	4	7	3	7	5.25	4.25
Expert	*	*	5	6	5.5	0.5
Hearty	*	*	1	12	6.5	60.5
Pollen	1	6	8	11	6.5	17.67
Disco	*	*	9	5	7	8
Elan	*	*	11	3	7	32
Norin	10	*	*	4	7	18
NK Bravour	*	*	7	9	8	2
Victor	5	1	19	*	8.33	89.33
Jet Neuf	7	2	17	8	8.5	39
Darmor	13	15	10	1	9.75	38.25
Lioness	*	*	6	15	10.5	40.5
Recital	8	11	13	*	10.67	6.33
Mohican	17	8	12	10	11.75	14.92
Hansen x Gaspard	*	3	21	*	12	162
Canary	12	4	27	*	14.33	136.33
Royal	*	*	16	13	14.5	4.5
Canberra	11	13	20	*	14.67	22.33
Marita	22	9	14	*	15	43
Shannon	3	21	22	*	15.33	114.33
NK Victory	*	*	15	16	15.5	0.5
Kana	14	17	*	*	15.5	4.5
Madrigal	18	14	*	*	16	8
Escort	16	10	25	*	17	57
Columbus x Nickel	*	20	*	17	18.5	4.5
Rafal	20	*	18	*	19	2
Winner	*	12	23	23	19.33	40.33
Major	19	*	26	14	19.67	36.33
Mikado	26	16	*	*	21	50
Courage	*	19	24	*	21.5	12.5
Cobra	21	22	*	*	21.5	0.5
Matador	25	*	*	18	21.5	24.5
Bristol	9	25	28	25	21.75	74.25
Yudal	6	31	29	*	22	193
Apex-93_5 x Ginyou_3	*	29	*	19	24	50
Nugget	*	18	34	22	24.67	69.33
Quinta	27	26	*	21	24.67	10.33
Liho	15	27	32	*	24.67	76.33
Primor	24	*	*	26	25	2
Tapidor	23	24	33	20	25	31.33
Eurol	28	23	30	24	26.25	10.92
Bronowski	29	28	36	27	30	16.67
Wild accession	*	30	31	*	30.5	0.5
Drakkar	*	*	37	28	32.5	40.5
Ningyou 7	*	34	38	29	33.67	20.33
Global	*	33	35	*	34	2
Fido	*	32	39	*	35.5	24.5

* indicates seasons when individual cultivars were not grown in experiment.
[a] Assessments of internal stem canker severity were done in late June/early July in each season.
[b] Only cultivars that were grown in more than one season are included. Cultivars are ordered according to their mean rank.

3.2 Regional differences in agronomic practice

Over the period between 1990 and 2006, cv. Apex formed the greatest proportion of crops in all the winter oilseed rape growing regions of England and Wales (Figure 3.4). In addition to Apex, cvs Bristol, Falcon and Winner were also grown in all regions. Cultivars Cobra and Royal were recorded only in the Northern region, Fortis only in Lincolnshire, Caracas in the south-west and south Wales and cvs Castille and Lipton only in the Midlands and north Wales. However, by 2007 cv. Castille had become the most popular cultivar (31% of crops) in England (www.cropmonitor.co.uk). These 'most-used' cultivars vary in their phoma stem canker and light leaf spot resistance ratings (www.HGCA.com).

In autumn and early winter, 1990-2006 (Figure 3.5. (a)), benzimidazoles (methyl benzimidazole carbamate), imidazoles (demethylation inhibitor) and triazoles (demethylation inhibitor) were the main groups of fungicides applied to winter oilseed rape crops in all regions of England and Wales. Winter oilseed rape crops received up to four fungicide applications each season (Figure 3.6). However, the proportion of crops receiving four applications was much lower than those receiving one, two or three applications. In addition, a large proportion of crops in all regions received no fungicide application; this proportion was greatest in the northern and Midlands and north Wales regions. However, by 1998, >90% of crops were sprayed (Sharon Elcock, pers. com.). The mean number of fungicide applications in each region ranged from 1.17 (i.e. 1) in the northern region to 1.76 (i.e. 2) in the south-west and south Wales region (Table 3.3).

Under all fungicide regimes both the incidence (Figure 3.7 (a)) and severity (Figure 3.7 (b)) of phoma stem canker were greatest in the south-central and south-east regions. Differences between the regions in the proportion of plants affected within crops following different fungicide regimes were not significant. Surprisingly, both the incidence and severity of phoma stem canker were lower in unsprayed crops than in sprayed crops surveyed.

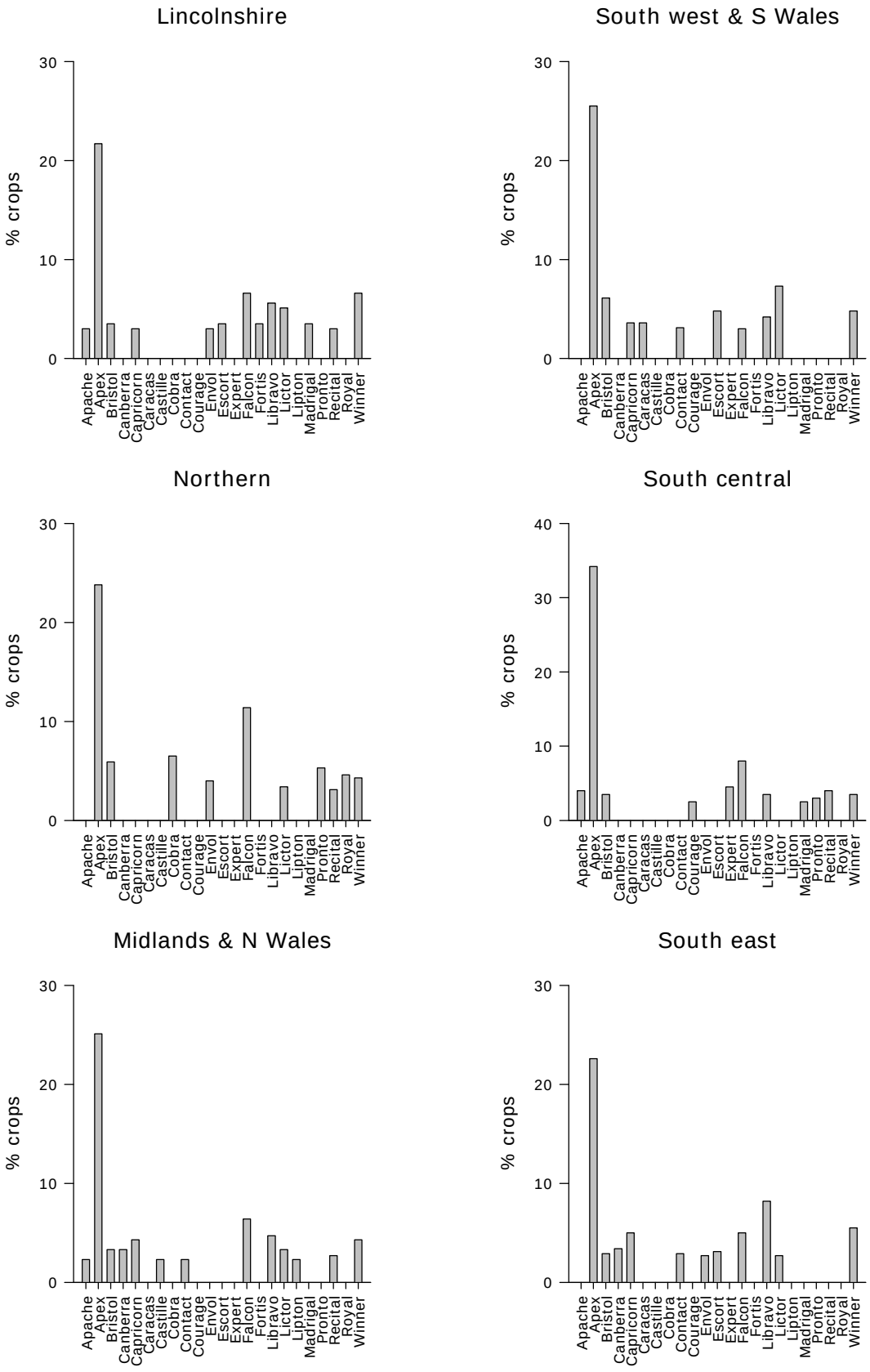

Figure 3.4. Cultivar choice in England and Wales, 1990-2006. Figures shows the percentage of commercial crops sown with different cultivars (this may be as a component of a mixture). Remaining cultivars are unknown or have a small sample size (<5 crops). Data were collected in the annual winter oilseed rape nation disease survey in England (www.cropmonitor.co.uk). The data were collated by Judith Turner and Sharon Elcock, FERA. Regions are shown in Fig 2.2.

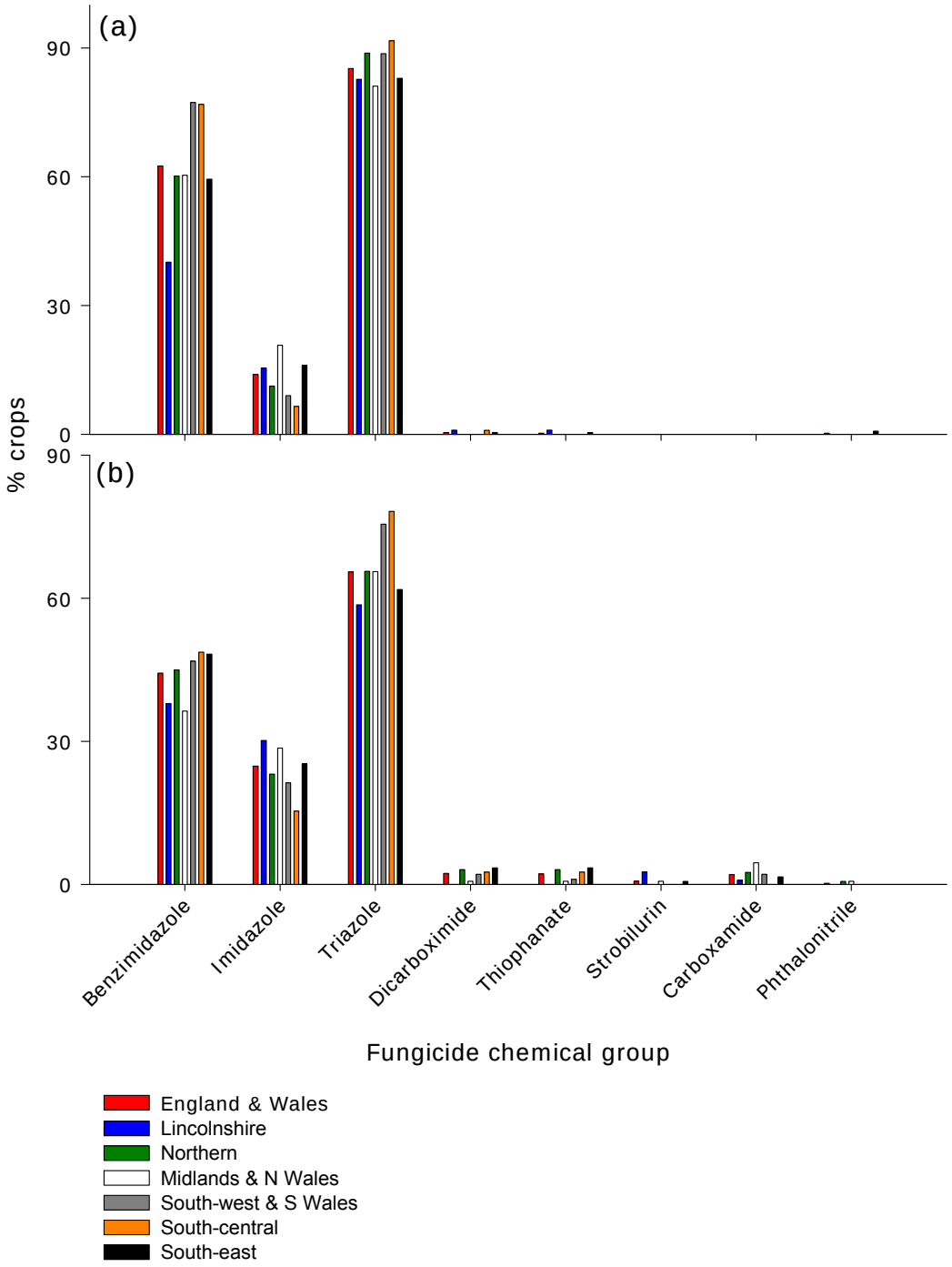

Figure 3.5. Proportions of commercial winter oilseed rape crops treated with a range of fungicide groups in England and Wales (1990-2006) (a) in autumn and early winter and (b) in late winter and spring. Fungicides may have been applied alone or as a component of a mixture with other fungicides with the same or different chemical group. Data were collected in the annual winter oilseed rape national survey in England (www.cropmonitor.co.uk). The data were collated by Judith Turner and Sharon Elcock. Regions are shown in Fig 2.2.

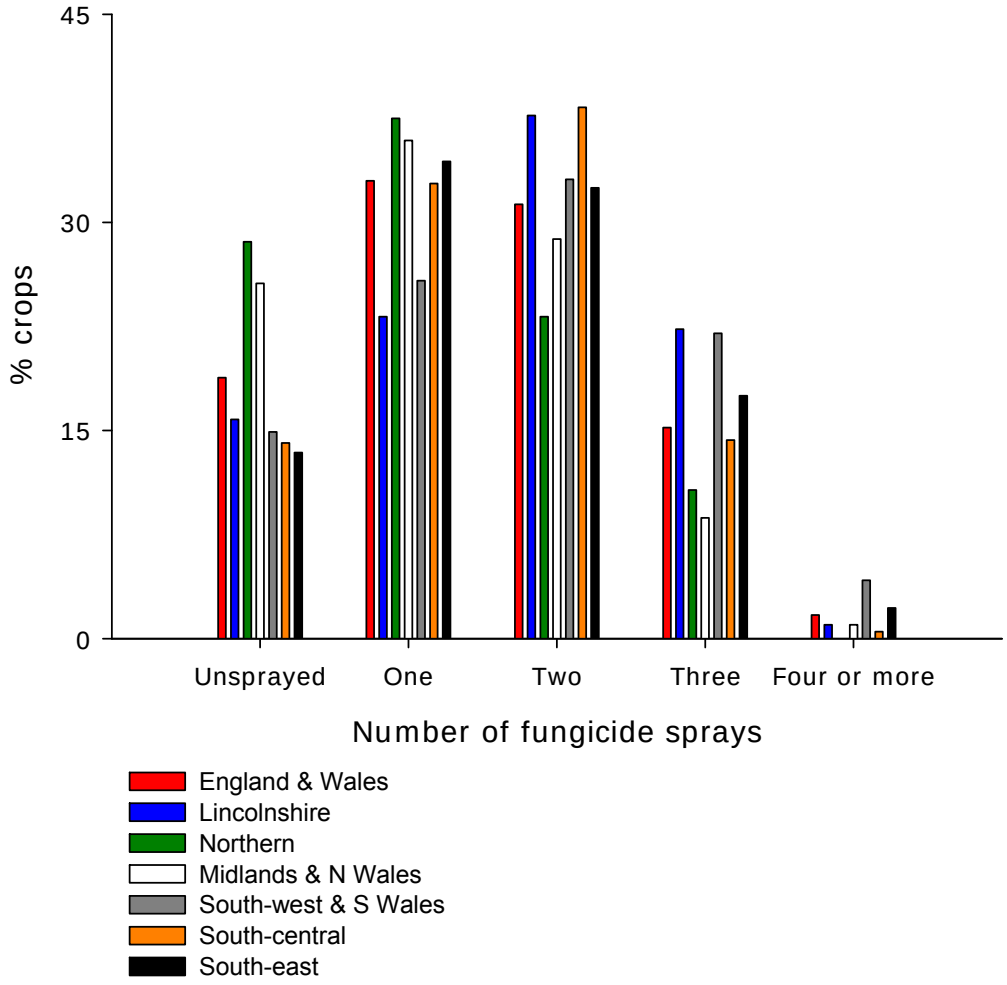

Figure 3.6. Proportion of commercial winter oilseed rape crops receiving no, one, two, three or four fungicide applications in England 1990-2006**.** Data were collected in the national winter oilseed rape survey in England (www.cropmonitor.co.uk) and were collated by Judith Turner and Sharon Elcock. Regions are shown in Fig 2.2.

Table 3.3. Mean number of fungicide applications in English and Welsh regions between 1990 and 2006.

Region[a]	**Mean number of fungicide applications**
Lincolnshire	1.69
Midlands & North Wales	1.23
Northern	1.17
South West & South Wales	1.76
South-central	1.54
South East	1.59

[a]Regions are shown in Fig 2.2.

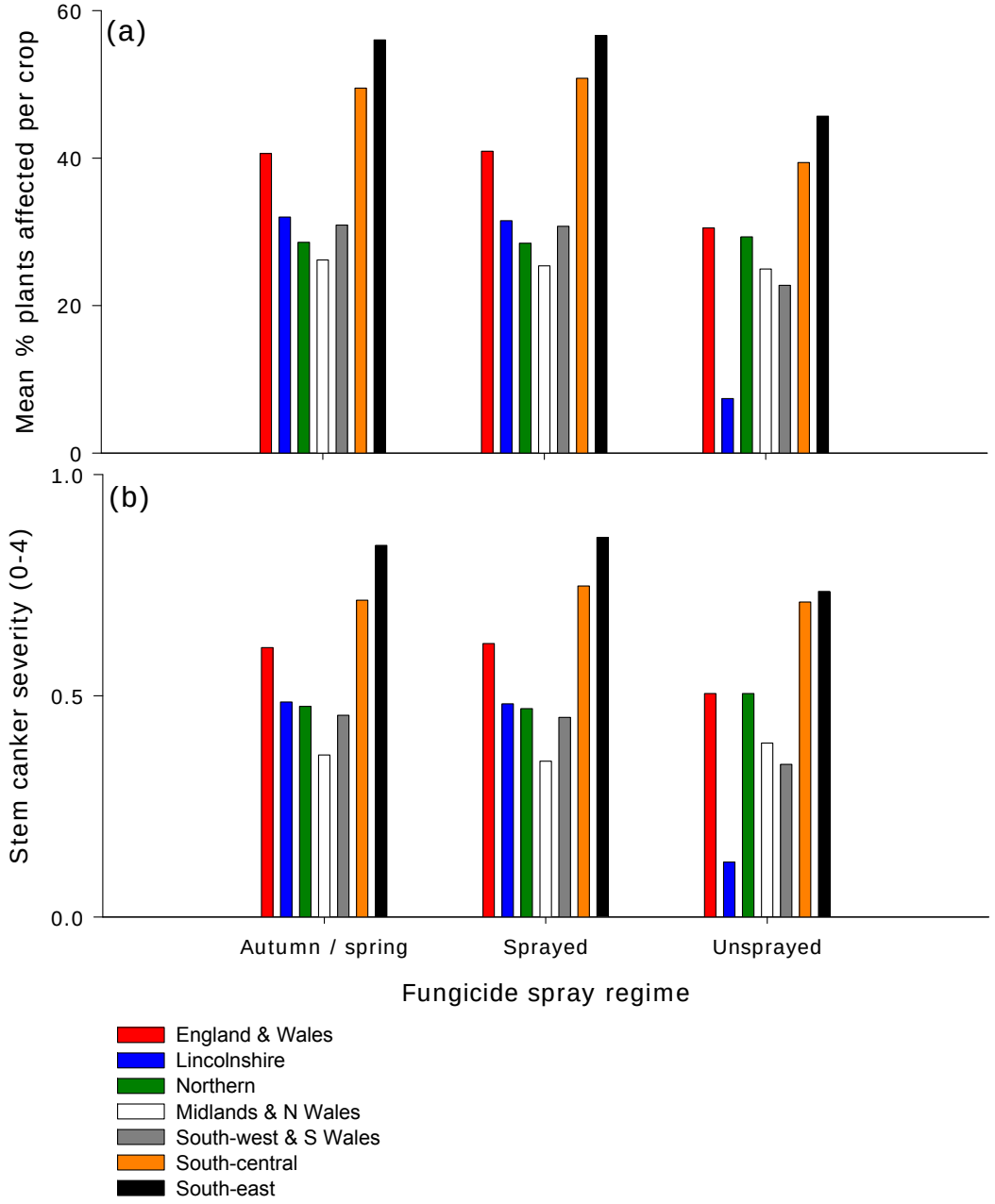

Figure 3.7. Incidence and severity of phoma stem canker severity following different fungicide regimes (1990-2006). Stem canker severity was assessed on a 0-4 scale (Zhou *et al.*, 1999). Data were collected in the national winter oilseed rape survey in England (www.cropmonitor.co.uk) and were collated by Judith Turner and Sharon Elcock. Regions are shown in Fig 2.2.

3.3 Regional differences in the distribution of *L. maculans* and *L. biglobosa* in England

Of the 962 lesion samples analysed, *Leptosphaeria* DNA was identified in 912 (775 basal stem canker samples and 136 upper stem lesion samples) of the samples. In most samples (65%), both *L. maculans* DNA and *L. biglobosa* DNA were identified. However, those with only *L. maculans* DNA (29%) formed a greater proportion than samples with *L. biglobosa*

DNA (5%) alone. In total, *L. maculans* DNA was identified in 95% (863) of samples whilst *L. biglobosa* DNA was identified in 70% (640). Likewise, in the majority of basal (66%) and upper stem (57%) samples, DNA from both *L. maculans* and *L. biglobosa* was identified jointly (Figure 3.8). Differences in the proportion of samples in which only *L. maculans* DNA or only *L. biglobosa* DNA or both *L. maculans* DNA and *L. biglobosa* DNA was identified between basal stem canker samples and upper stem lesion samples were significant (*P* <0.001).

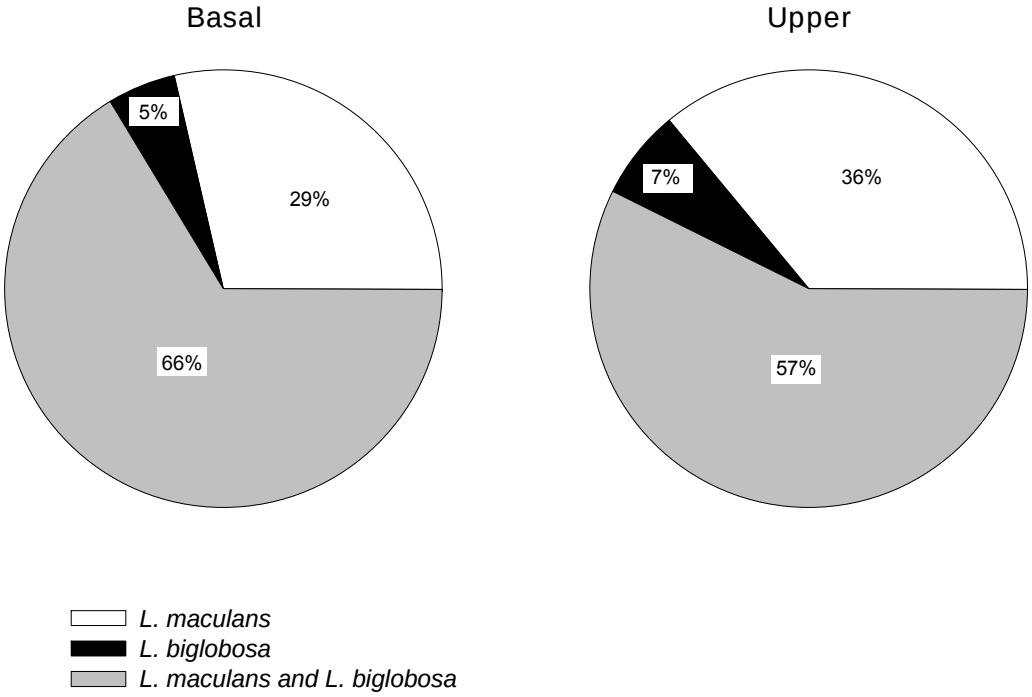

Figure 3.8. Proportions of basal stem canker or upper stem lesion samples in which *L. maculans*, *L. biglobosa* or both *L. maculans* and *L. biglobosa* DNA were identified by uniplex traditional PCR. Samples were collected from England and Wales in the 2001-2003 and 2006 stem surveys.

The proportions of samples in which *L. maculans, L. biglobosa* or both *L. maculans* and *L. biglobosa* DNA were identified are shown in Figures 3.9 – 3.12. For basal stem cankers, the proportion of samples with *L. maculans* DNA alone was greater than that with *L. biglobosa* DNA alone in all regions in all survey years, with the exception of the south-west region in the 2001 survey. DNA from both *L. maculans* and *L. biglobosa* DNA was identified jointly in a greater proportion of samples than either *L. maculans* or *L. biglobosa* DNA singly in all years except 2003. In 2003, *L. maculans* DNA alone was identified in the greatest proportion of samples. In all survey years, *L. maculans* DNA (alone or in combination with *L. biglobosa* DNA) was identified in a high proportion of basal stem cankers collected from the south-east (2001 = 88.5%, 2002 = 100%, 2003 = 100% and 2006 = 100%). By contrast, in the northern region of England *L. biglobosa* DNA (alone or in combination with *L. maculans* DNA) was found in a relatively larger proportion of basal stem canker samples (2001 = 82%,

2002 = 72.5%, 2003 = 28.6% and 2006 81.5%) as compared to the amount of *L. biglobosa* DNA identified in basal stem cankers from other regions. The results of the chi-squared test established highly significant (P<0.001) differences between the 2001, 2002, 2003 and 2006 surveys in the proportion of basal stem cankers in which *L. maculans* or *L. biglobosa* DNA was identified.

In upper stem lesions DNA from both *L. maculans* and *L. biglobosa* were identified jointly in the greatest proportion of stems in the 2001 and 2006 surveys; in the 2002 survey, the proportion of samples in which both *L. maculans* and *L. biglobosa* DNA was identified equalled that of those samples in which only *L. maculans* DNA was identified; in 2003 *L. maculans* DNA alone was identified in the greatest proportion of samples. *Leptosphaeria biglobosa* DNA alone was not identified in samples in 2002 and was identified only in upper stem lesions collected from the south-central and south-east regions in 2001, the north in 2003 and from the south central region in 2006.

Using qPCR, *L. maculans* DNA was identified in all of the basal stem canker samples tested whilst *L. biglobosa* DNA was found in 96%. Co-occurance of *L. maculans* and *L. biglobosa* DNA was found in 75% of canker samples. For 77% of all samples, the amount of detected *L. maculans* DNA was greater than that of *L. biglobosa*. A significantly (P<0.001) greater quantity of *L. maculans* DNA than *L. biglobosa* DNA was detected in basal stem cankers; a greater quantity of *L. biglobosa* DNA than *L. maculans* DNA was found in upper stem lesions, although this difference was not significant (Figure 3.11).

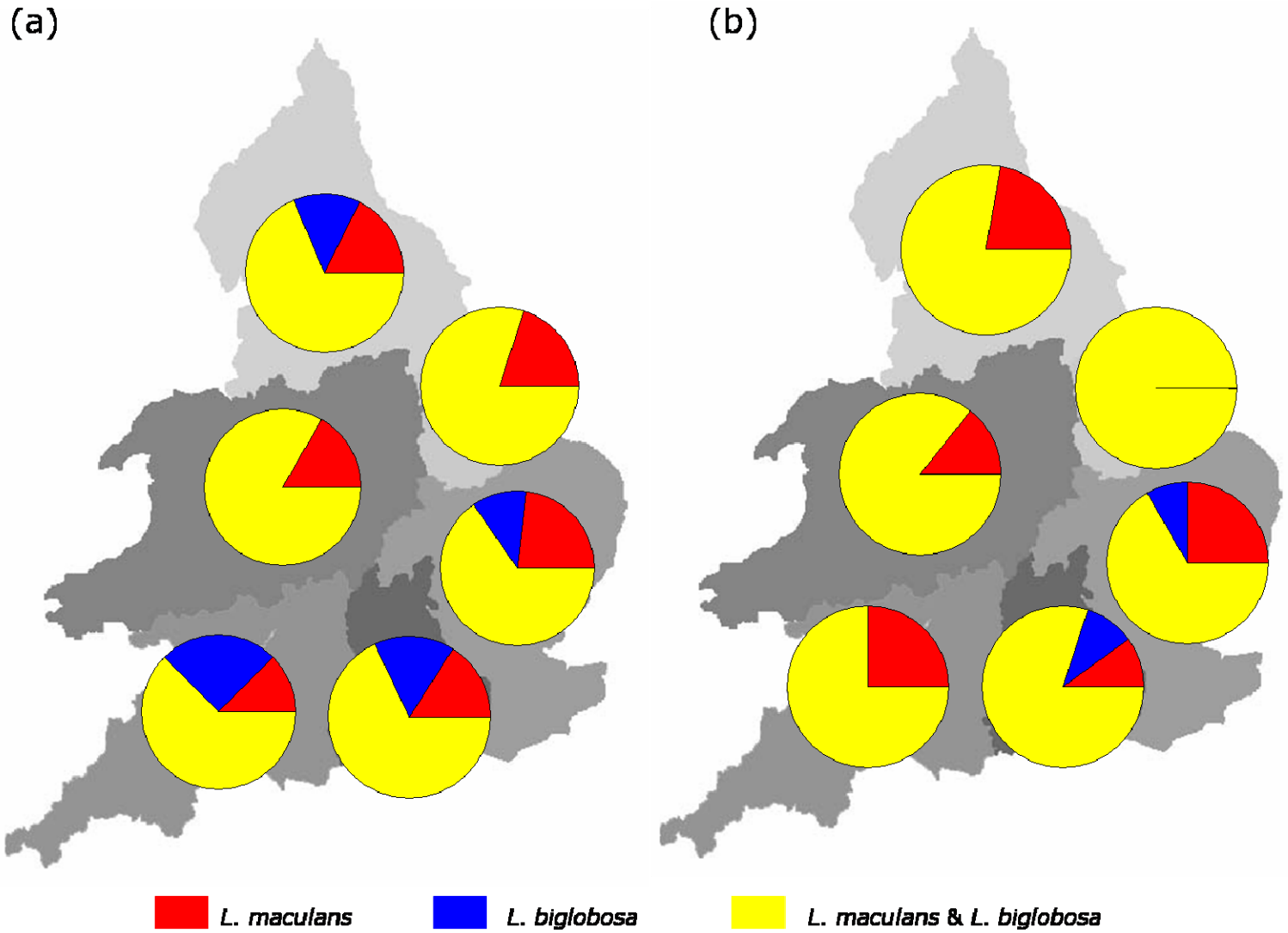

Figure 3.9. Proportions of (a) basal stem cankers and (b) upper stem lesions in which *L. maculans*, *L. biglobosa* or both *L. maculans* and *L. biglobosa* DNA was identified in using uniplex traditional PCR in winter oilseed rape samples collected from commercial crops in June and July 2001 for different regions (Fig 2.2). Lesions occurring <5 cm from the stem base were classed as basal stem cankers whilst those occurring >5 cm from the stem base were termed upper lesions. Samples were supplied by ADAS and piecharts are placed atop the regions of England and Wales where samples were sourced.

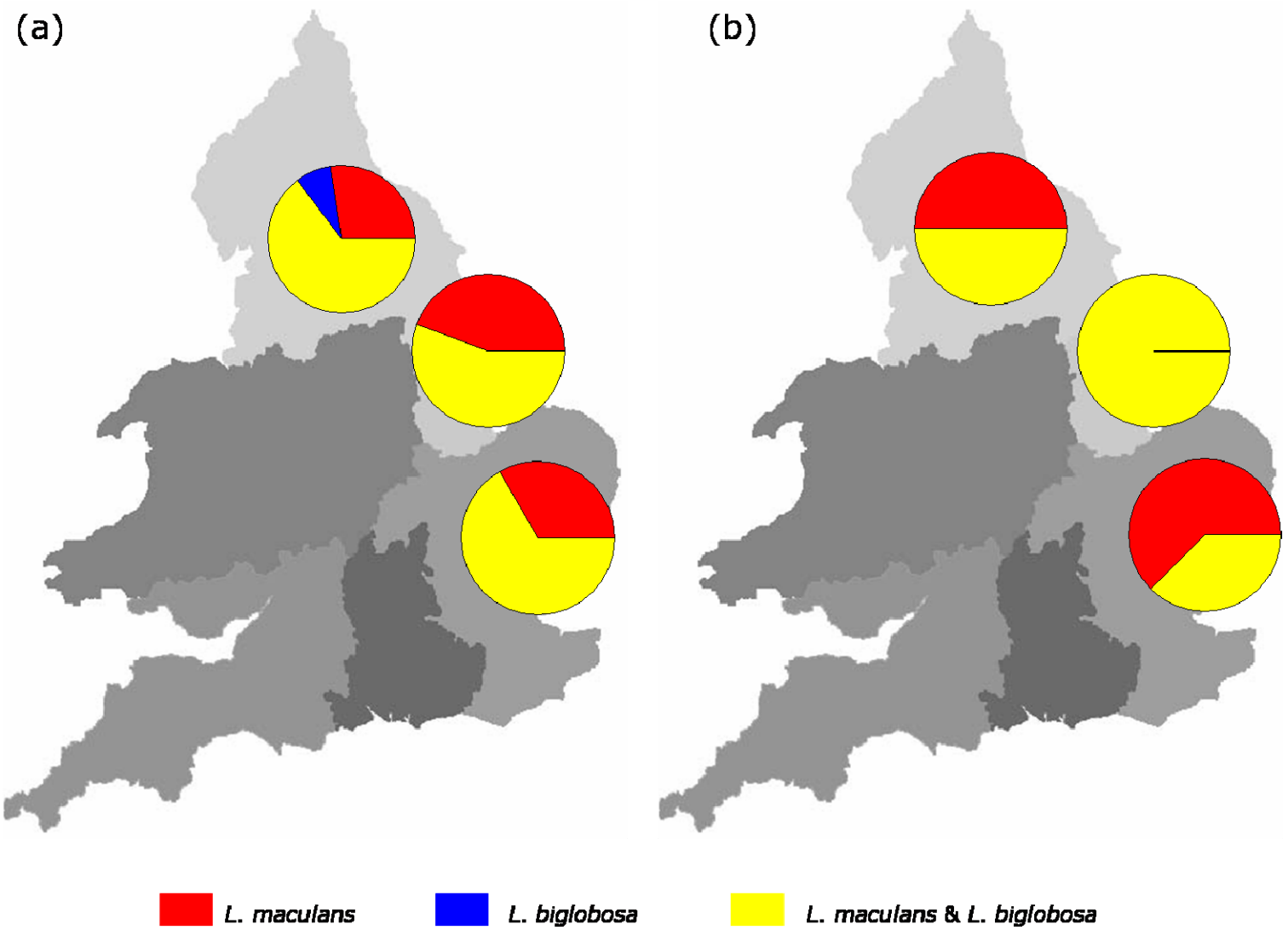

Figure 3.10. Proportions of (a) basal stem cankers and (b) upper stem lesions in which *L. maculans*, *L. biglobosa* or both *L. maculans* and *L. biglobosa* DNA was identified in using uniplex traditional PCR in winter oilseed rape samples collected from commercial crops in June and July 2002 for different regions (Fig 2.2). Lesions occurring <5 cm from the stem base were classed as basal stem cankers whilst those occurring >5 cm from the stem base were termed upper lesions. Samples were supplied by ADAS. and piecharts are placed atop the regions of England and Wales where samples were sourced.

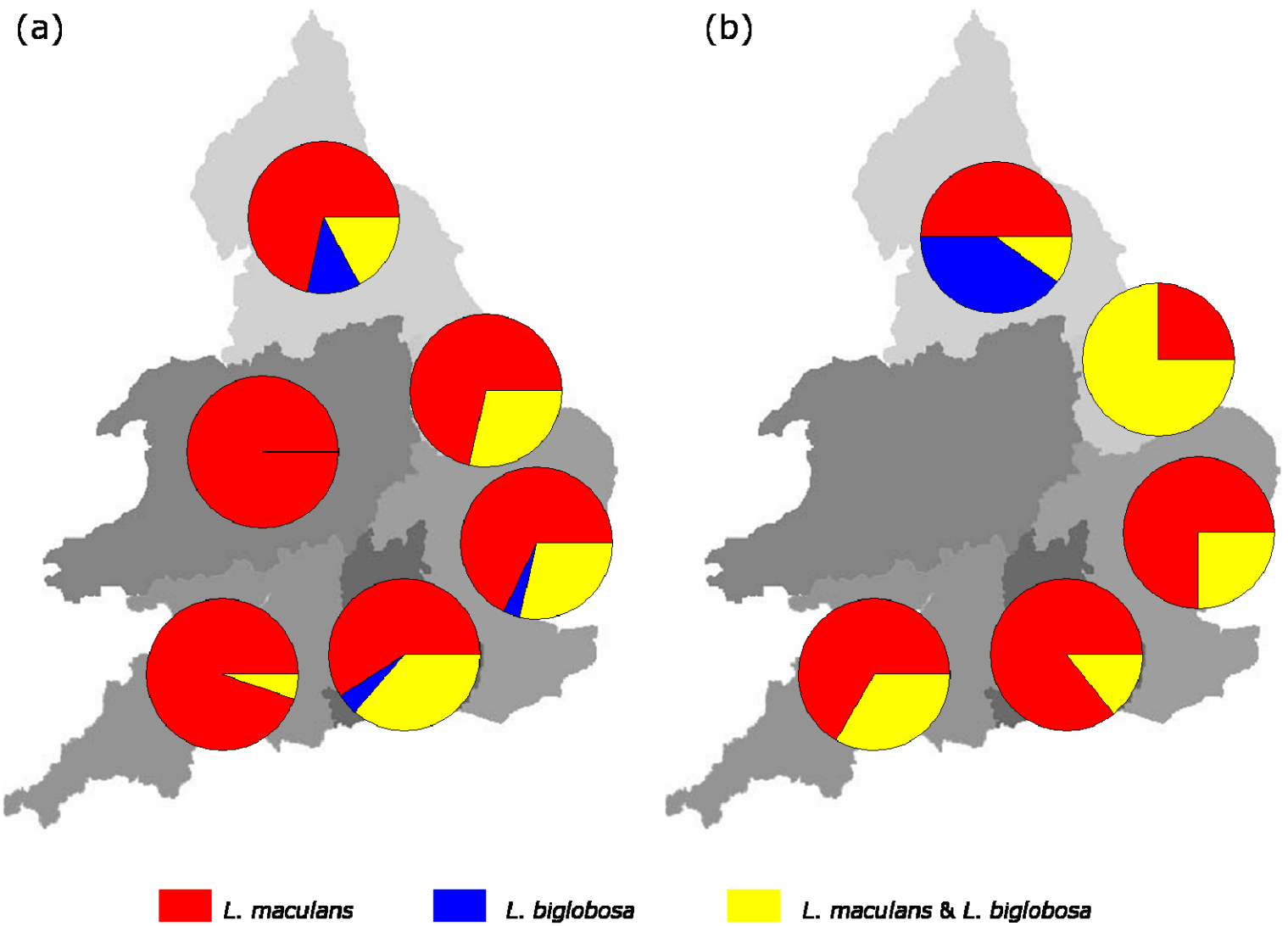

Figure 3.11. Proportions of (a) basal stem cankers and (b) upper stem lesions in which *L. maculans*, *L. biglobosa* or both *L. maculans* and *L. biglobosa* DNA was identified in using uniplex traditional PCR in winter oilseed rape samples collected from commercial crops in June and July 2003 for different regions (Fig 2.2). Lesions occurring <5 cm from the stem base were classed as basal stem cankers whilst those occurring >5 cm from the stem base were termed upper lesions. Samples were supplied by ADAS and piecharts are placed atop the regions of England and Wales where samples were sourced.

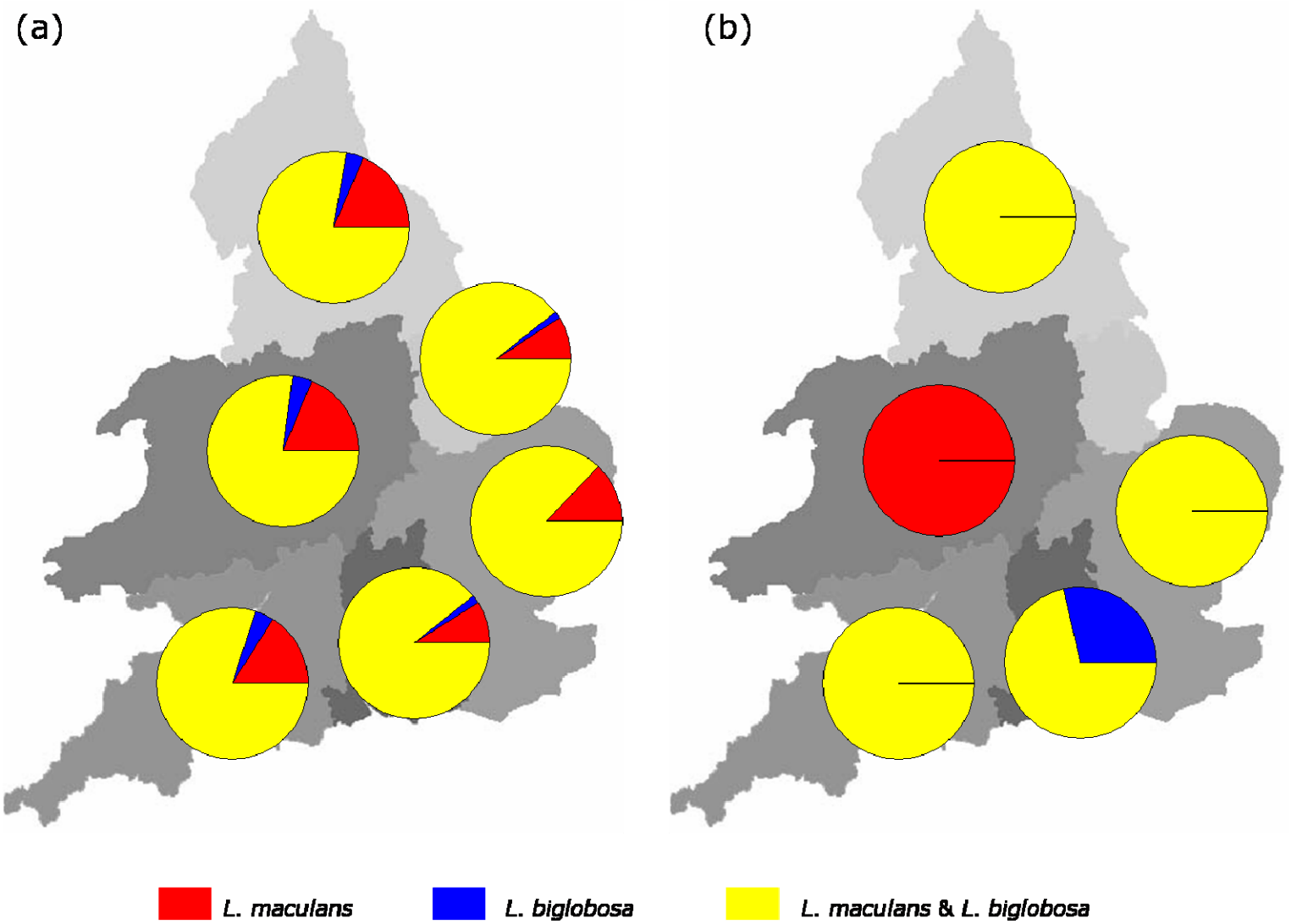

Figure 3.12. Proportions of (a) basal stem cankers and (b) upper stem lesions in which *L. maculans*, *L. biglobosa* or both *L. maculans* and *L. biglobosa* DNA was identified in using uniplex traditional PCR in winter oilseed rape samples collected from commercial crops in June and July 2006 for different regions (Fig 2.2). Lesions occurring <5 cm from the stem base were classed as basal stem cankers whilst those occurring >5 cm from the stem base were termed upper lesions. Samples were supplied by TAG and piecharts are placed atop the regions of England and Wales where samples were sourced.

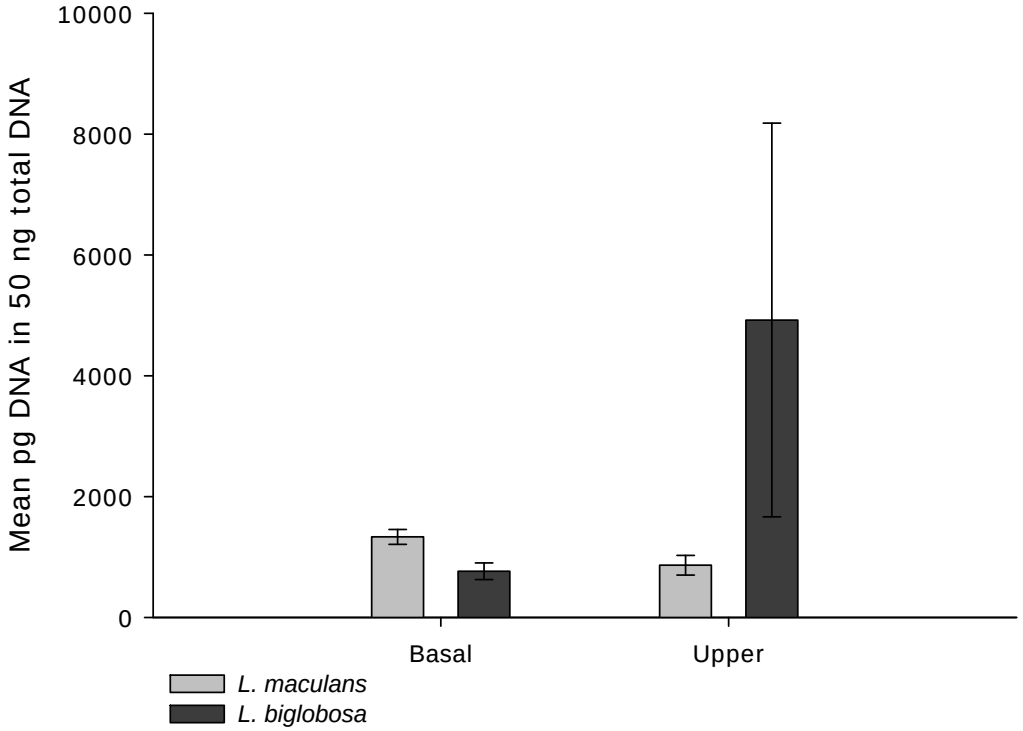

Figure 3.11. Amounts of *L. maculans* and *L. biglobosa* DNA in basal stem cankers and upper stem lesion as quantified by qPCR. The error bars show the standard error of the individual means: basal (805 d.f.) and upper (150 d.f.).

Samples were received from 21 oilseed rape cultivars. The mean amount of *L. maculans* DNA in basal cankers and upper lesions was significantly greater than that quantified for *L. biglobosa* in all but five cultivars namely Catalina, Cohort, Fortress, Gemini and Recital (Figure 3.12).

Geostatistics was used to map spatial variation in the amount of *L. maculans* and *L. biglobosa* DNA quantified in basal stem cankers (Figure 3.13). In each year, the smallest amounts of *L. maculans* were observed in north east of England. Conversely in 2001 and 2002, this region had the greatest amounts of *L. biglobosa*. Geostatistical maps of predicted stem canker severity show the lowest stem canker severity in the north east of England (Figure 3.13, (g)-(j)). The maps (Figure 3.14) show that there was no obvious persistent pattern in the predicted date of the appearance of leaf spotting. However, the time taken between leaf spotting and stem canker emergence was greatest in the north of England in all years. Thus the time between stem canker emergence and harvest was shortest in the north and, consequently, stem canker development was less than in the south.

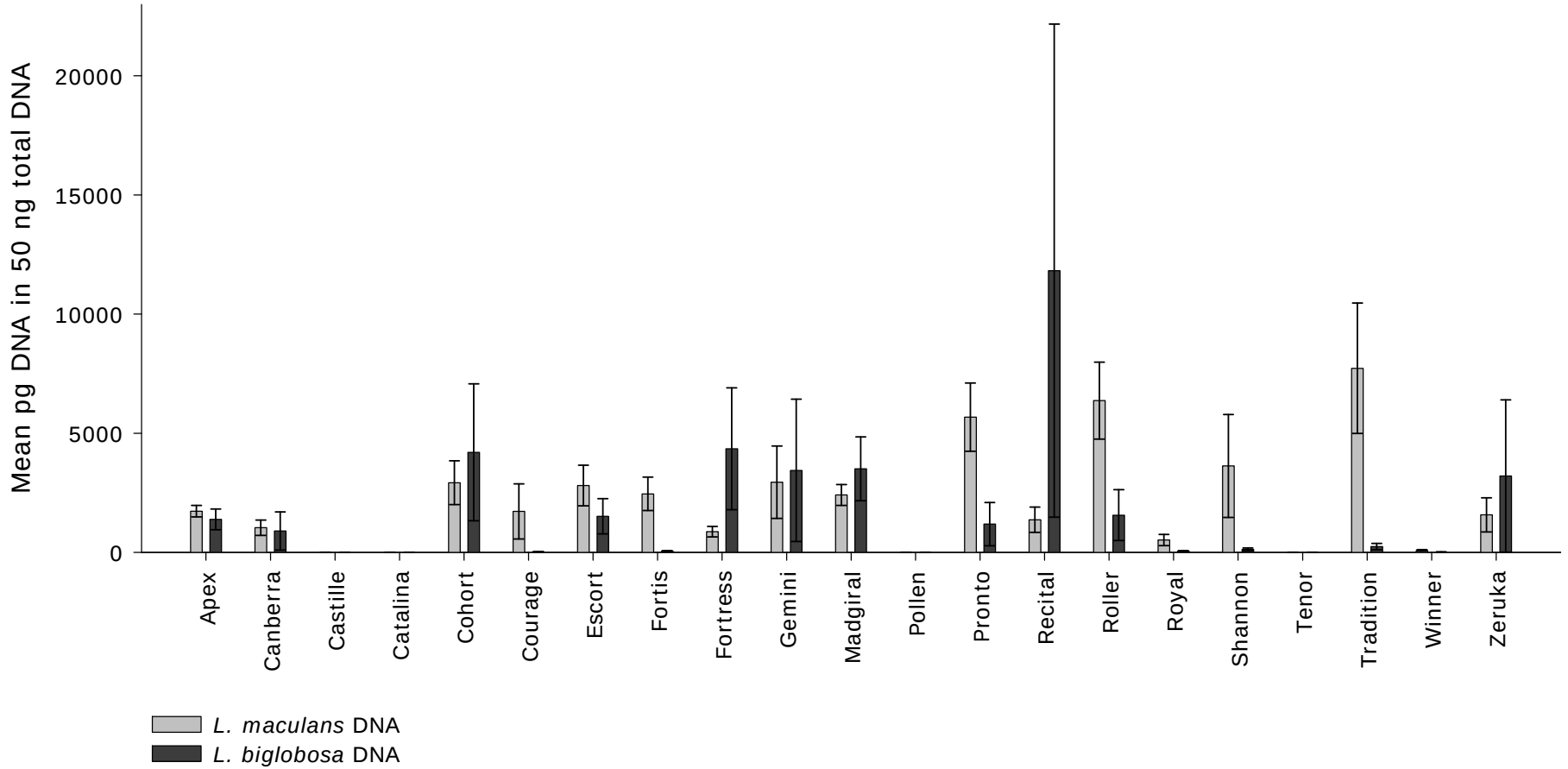

Figure 3.12. Amounts of *L. maculans* and *L. biglobosa* DNA in stem samples obtained from a range of winter oilseed rape cultivars. The amounts of DNA were determined by qPCR. The vertical bars show the standard errors of the individual means: Apex 139 d.f., Canberra 30 d.f., Castille 47 d.f., Catalina 7 d.f., Cohort 11 d.f., Courage 6 d.f., Escort 56 d.f., Fortis 19 d.f., Fortress 20 d.f., Gemini 8 d.f., Madrigal 40 d.f., Pollen 8 d.f., Pronto 27 d.f., Recital 47 d.f., Roller 8 d.f., Royal 47 d.f., Shannon 13 d.f., Tenor 11 d.f., Tradition 4 d.f., Winner 201 d.f. and Zeruka 8 d.f.

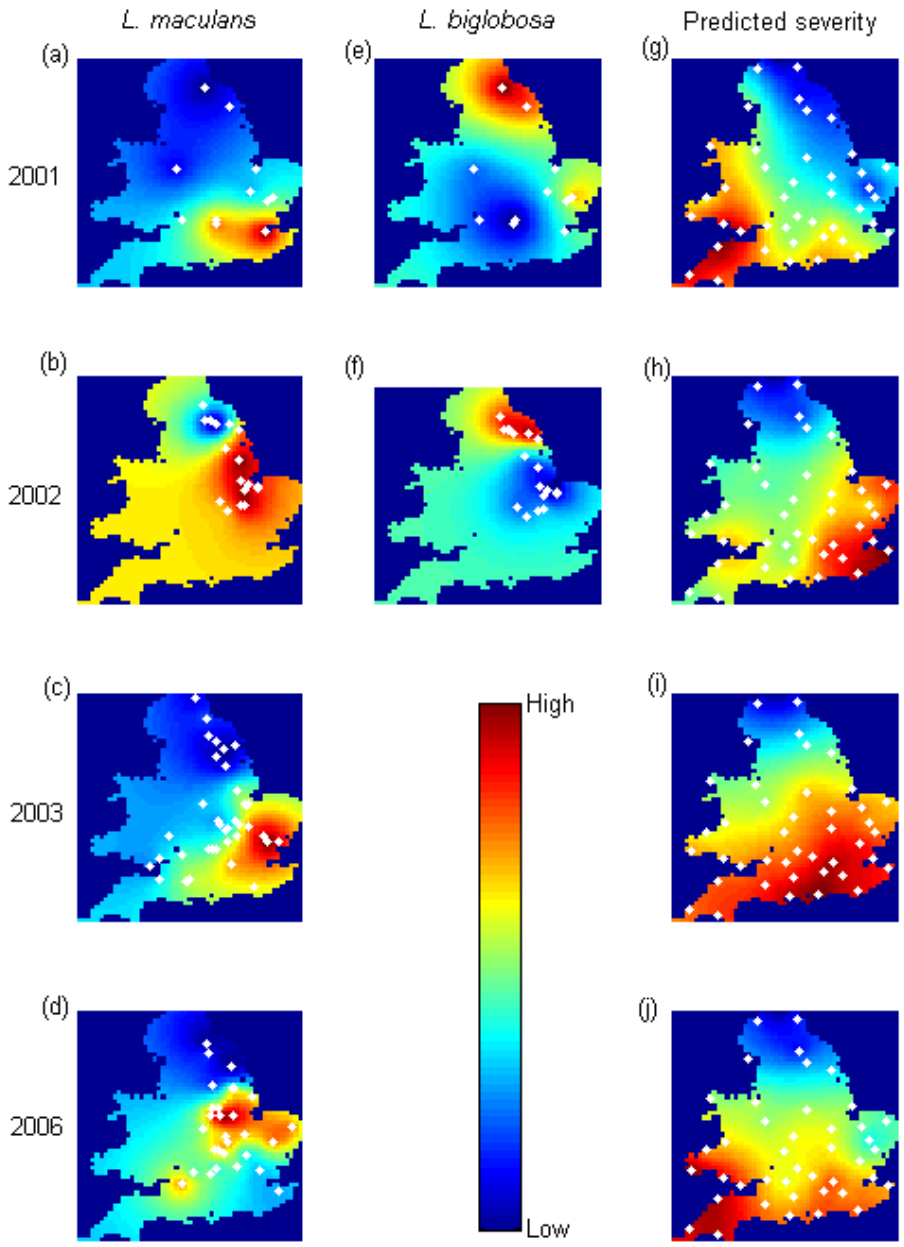

Figure 3.13. Geostatistical maps showing the amounts of *L. maculans* and *L. biglobosa* DNA in basal stem cankers and the predicted severity of phoma stem canker. Maps of the spatially varying component of (i) log transformed amount of *L. maculans* DNA (plots A-D), (ii) log transformed amount of *L. biglobosa* DNA (plots E-F) and (iii) phoma stem canker severity as predicted by the model of Evans *et al*. (2008) (plots G-J) at harvest in 2001, 2002, 2003 and 2006. The location of sample sites/weather station sites are marked. Maps were produced by Ben Marchant (Stonard *et al.* 2009).

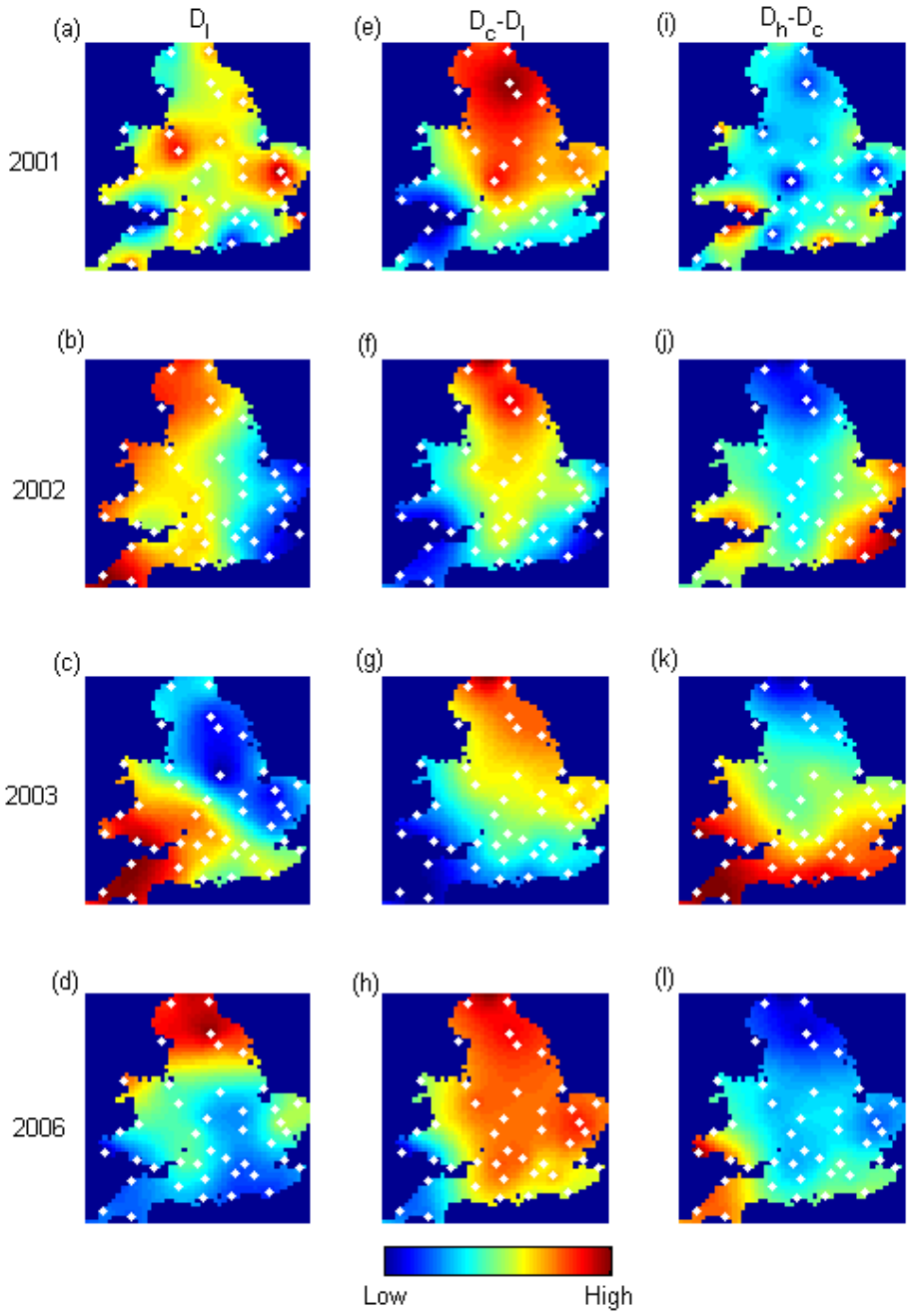

Figure 3.14. Maps of number of days between key events in stem phoma development as predicted by the model of Evans *et a*l. (2008). Plots A-D map Dl (the number of days after 15 July at which 10 % of crop is affected by leaf spotting); Plots D-G map $Dc - Dl$ (the number of days after Dl until 10 % of crop is affected by phoma stem lesions) and Plots H-K map $Dh - Dc$ (the number of days between Dc and harvest). The location of weather station sites are marked. Maps were produced by Ben Marchant (Stonard *et al.* 2009).

3.4 Variations in *L. maculans* and *L. biglobosa* ascospore release

During pseudothecial (fruiting body) maturation (1 July to 30 September), in the 2005/06 growing season the mean temperature was 16.05°C with a mean rainfall of 1.77 mm per day. In the 2006/07 growing season the mean temperature was 17.95°C with 2.17 mm rain. In the 2007/08 season 15.14°C with 1.95 mm of rain. The maximum daily temperature did not exceed 30°C during the period of pseudothecial development in the 2005/06 and 2007/08 growing seasons; the maximum daily temperature exceeded 30°C on 5 days in late July 2006. The mean rainfall and temperature for each season during the most important period for pseudothecial maturation are shown in Table 3.4.

Table 3.4. Temperature and rainfall values* during the main period of pseudothecial maturation in the 2005/06, 2006/07 and 2007/08 winter oilseed rape growing seasons at Rothamsted.

	Mean Temperature (°C)[a]			Mean rainfall (mm)[b]			Number of days with rain[c]		
Season	**July**	**Aug.**	**Sept.**	**July**	**Aug.**	**Sept.**	**July**	**Aug.**	**Sept.**
2005/06	16.73	16.14	15.29	1.26	1.89	2.17	10	9	10
2006/07	20.31	16.36	17.38	1.15	3.55	1.8	9	14	11
2007/08	15.81	15.57	14.05	2.8	2.08	0.97	17	12	8

* Temperature and rainfall data was measured by a weather station at Rothamsted, approximately 0.5 km from the Burkard spore sampler.
[a] The mean daily temperature was calculated from each day's maximum and minimum temperature (measured from 9 am to 8. 59 am GMT), this then allowed the mean temperature per month to be determined.
[b] The mean rainfall was calculated from the daily amount of rainfall, measured at 9 am GMT.
[c] Days with rain were classified as days with >0.49 mm rain.

Few *L. maculans* and *L. biglobosa* ascospores were counted on tapes before September (Figures 3.15, 3.16 and 3.17). The dates of first major release (defined by Huang *et al.* (2005) as the date when >10 ascospores m^{-3} air were counted on tapes from the Burkard spore sampler) were 15 September 2005, 28 September 2006 and 2 September 2007. In the 2007/08 season, this first major release was succeeded by 14 days with less than 10 ascospores m^{-3} air and a daily count of more than 10 ascospores m^{-3} air on consecutive days was not recorded until 23 September 2007. Overall, maximum ascospore concentrations were recorded on 31 October 2005 (1039 ascospores m^{-3} air), 20 November 2006 (797 ascospores m^{-3} air) and 16 October 2007 (821 ascospores m^{-3} air). In winter oilseed rape field experiments done 0.25 to 1 km from the Burkard spore sampler, phoma leaf lesions were observed soon after the first major ascospore release each season; stem cankers were observed from March onwards, after maximum ascospore release.

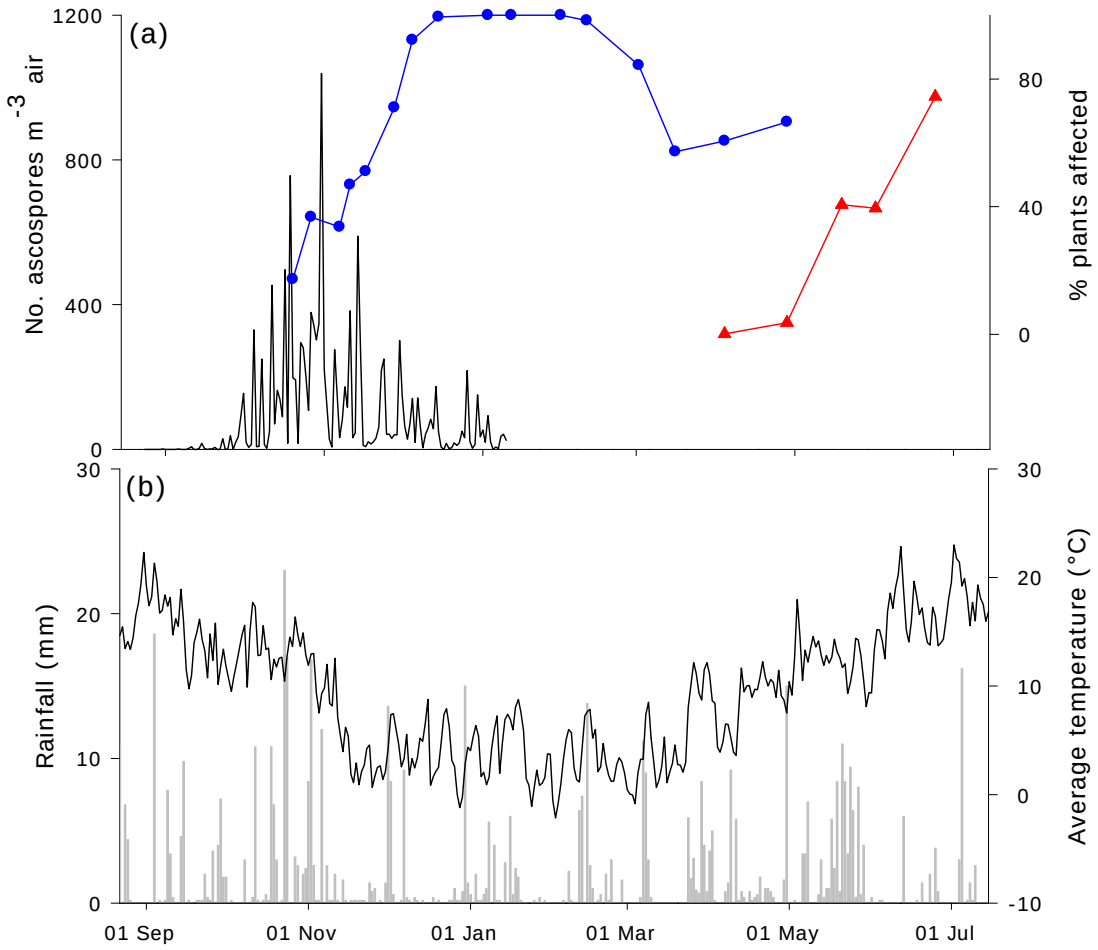

Figure 3.15. Changes in total numbers of ascospores of *L. maculans* and *L. biglobosa* during the 2005/06 winter oilseed rape growing season. Phoma leaf spot (•) and phoma stem canker (▲) incidence (% plants affected) on a field sown with winter oilseed rape cultivars, at Rothamsted, during the 2005/06 cropping season in relation to fluctuations in airborne concentrations of *L. maculans* and *L. biglobosa* ascospores captured by a Burkard spore sampler ((a) solid line) and weather data ((b) rainfall, grey histograms; temperature, solid line). The Burkard spore sampler was approximately 50 m from the field experiment. The mean disease progress was assessed on 42 oilseed rape cultivars (see section 2.1).

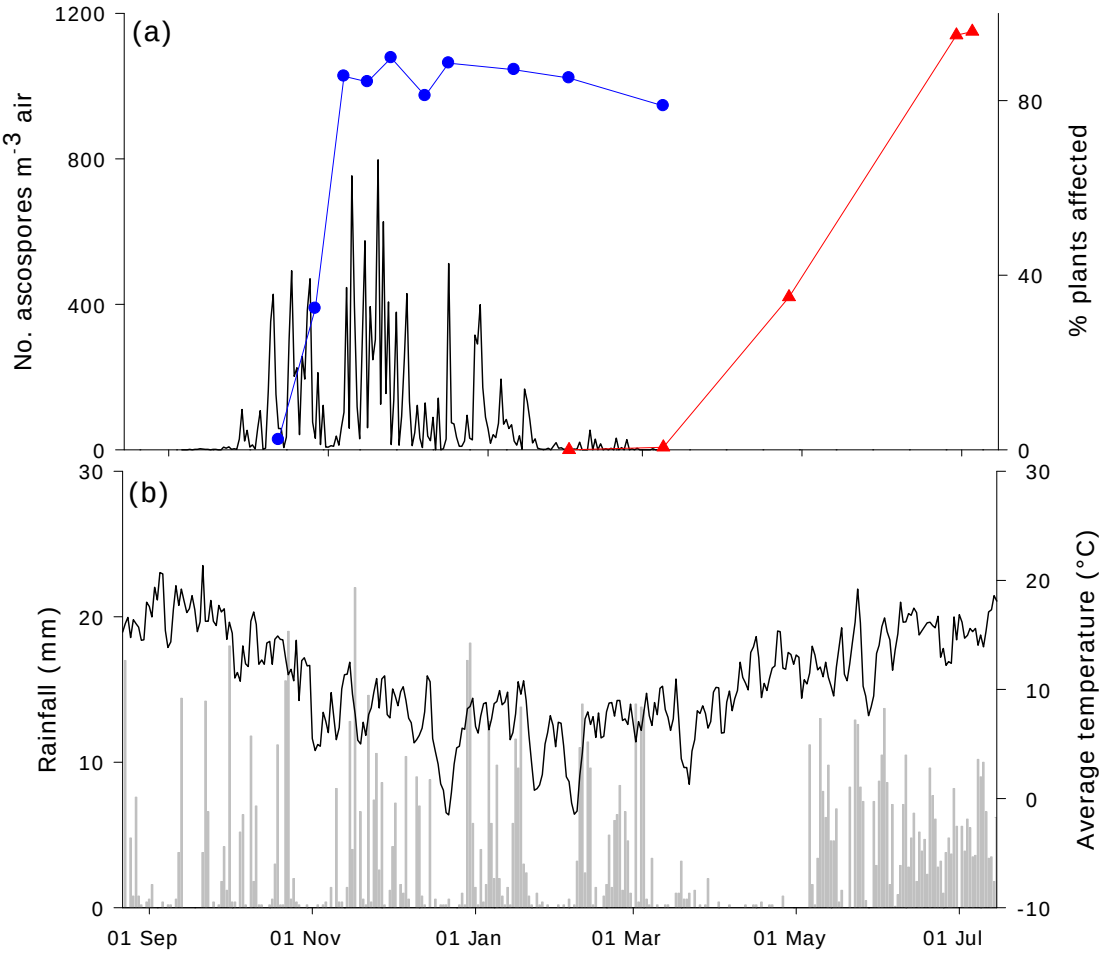

Figure 3.16. Changes in total numbers of ascospores of *L. maculans* and *L. biglobosa* during the 2006/07 winter oilseed rape growing season. Phoma leaf spot (•) and phoma stem canker (▲) incidence (% plants affected) on a field sown with winter oilseed rape cultivars, at Rothamsted, during the 2006/07 cropping season in relation to fluctuations in airborne concentrations of *L. maculans* and *L. biglobosa* ascospores captured by a Burkard spore sampler ((a) solid line) and weather data ((b) rainfall, grey histograms; temperature, solid line). The Burkard spore sampler was approximately 1.5 km from the field experiment. The mean disease progress on 42 oilseed rape cultivars is shown (see section 2.1).

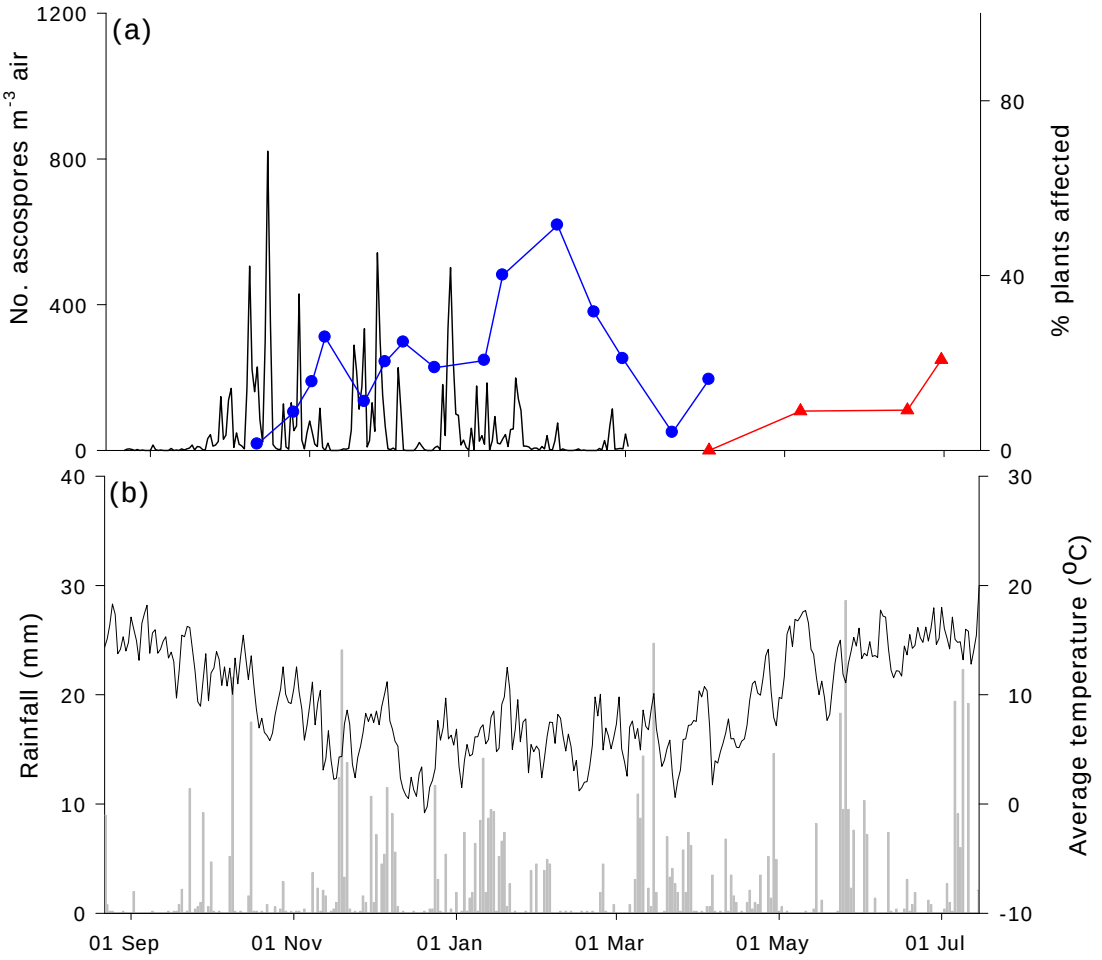

Figure 3.17. Changes in total numbers of ascospores of *L. maculans* and *L. biglobosa* during the 2007/08 winter oilseed rape growing season. Phoma leaf spot (•) and phoma stem canker (▲) incidence (% plants affected) on a field sown with winter oilseed rape cultivars, at Rothamsted, during the 2007/08 cropping season in relation to fluctuations in airborne concentrations of *L. maculans* and *L. biglobosa* ascospores captured by a Burkard spore sampler ((a) solid line) and weather data ((b) rainfall, grey histograms; temperature, solid line). The Burkard spore sampler was approximately 1 km from the field experiment. The mean disease progress on 42 oilseed rape cultivars is shown (see section 2.1).

Ascospore release consistently occurred after rainfall events in the 2005/06 (Figure 3.15), 2006/07 (Figure 3.16) and 2007/08 (Figure 3.17) growing seasons. The mean number of ascospores m^{-3} air was significantly greater (P <0.001; SED 0.087; d.f. 275) on days with at least 0.5 mm rain per day compared with those days with <0.5 mm rain (Figure 3.18). The greatest numbers of ascospores were released on days with 20 – 24.9 mm rainfall (Figure 3.18).

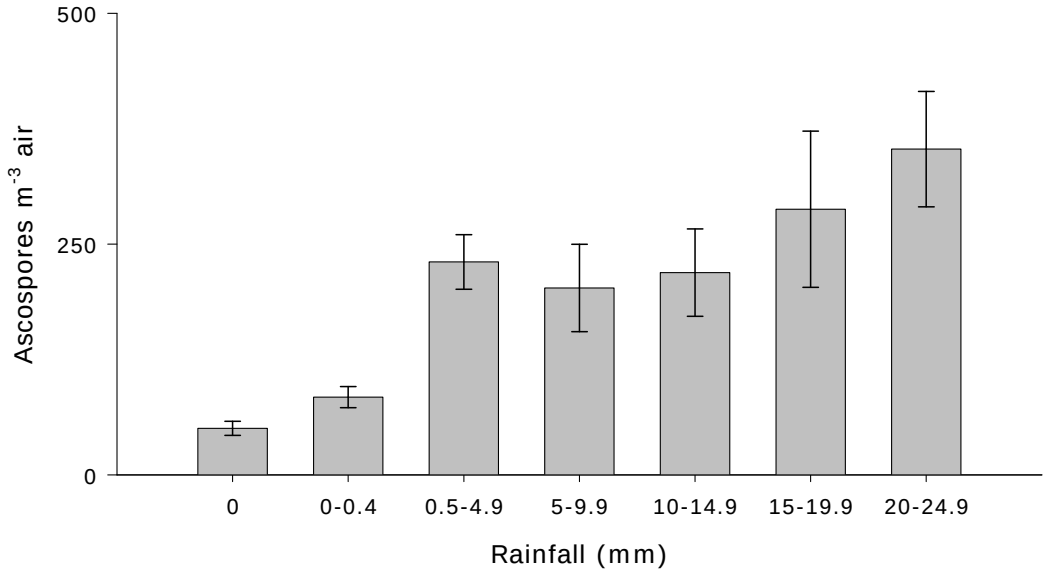

Figure 3.18. Mean total number of air-borne *L. maculans* and *L. biglobosa* ascospores on days with rainfall events in three seasons at Rothamsted from October to December. The number of ascospores per m^3 of air was calculated from counts of ascospores on tapes collected from a 7-day Burkard spore sampler surrounded by stubble from the previous season's crop in the 2005/05, 2006/07 and 2007/08 winter oilseed growing seasons at Rothamsted. Rainfall data were recorded at Rothamsted approximately 0.5 km from the Burkard spore sampler. Error bars show the standard error of the individual means: 0 mm, 87 d.f.; 0-0.4 mm 81 d.f.; 0.5-4.9 mm, 55 d.f., 5-9.9 mm, 21 d.f.; 10-14.9 mm, 15 d.f.; 15-19.9 mm 7 d.f.; 20-24.9 mm, 3 d.f.

In the 2006/07 and 2007/08 seasons, the amounts of *L. maculans* and *L. biglobosa* DNA on spore tapes were quantified using qPCR (Figure 3.19). In the 2006/07 growing season, the quantities of *L. maculans* and *L. biglobosa* DNA were maximal on 8 November 2006 (88.2 ng and 63.6 ng, respectively). In the 2007/08 growing season, *L. maculans* DNA yield was greatest on 28 October 2007 (30.8 ng); while *L. biglobosa* DNA was greatest on 16 October 2007 (45.6 ng). There were monthly differences in the mean quantities of *L. maculans* and *L. biglobosa* DNA detected (Figure 3.19). In the 2006/07 growing season significant monthly differences in the quantity of both *L. maculans* (P <0.001; SED 0.2095; d.f. 152) and *L. biglobosa* (P <0.001; SED 0.2015; d.f. 152) were observed. The mean amount of *L. maculans* DNA quantified per day was greater in all months than that of *L.*

biglobosa and was greatest in November, that of *L. biglobosa* in December (Figure 3.19). In 2007/08 growing season, there were significant monthly differences in *L. maculans* DNA (*P* <0.001; SED 0.3432; d.f. 160) and *L. biglobosa* DNA (*P* <0.001; SED 0.2488; d.f. 160) detected. The mean amount of *L. biglobosa* DNA quantified per day was greater than that of *L. maculans* in all months except August and was greatest in January whilst that of *L. maculans* was greatest in October (Figure 3.19).

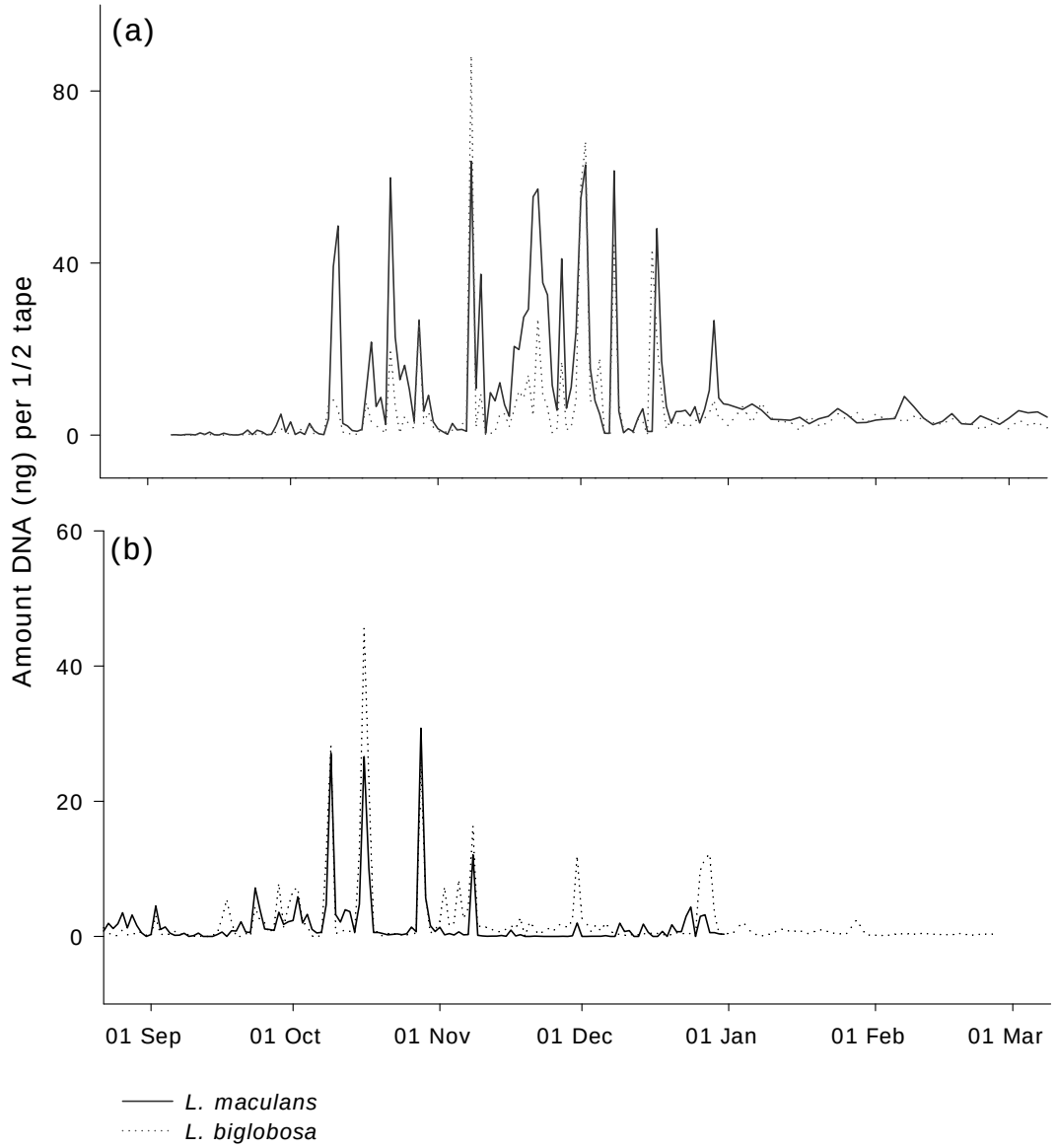

Figure 3.18. Total amounts of *L. maculans* or *L. biglobosa* DNA, quantified by qPCR, in extracts from Burkard spore sampler tapes in the (a) 2006/07 and (b) 2007/08 growing seasons at Rothamsted.

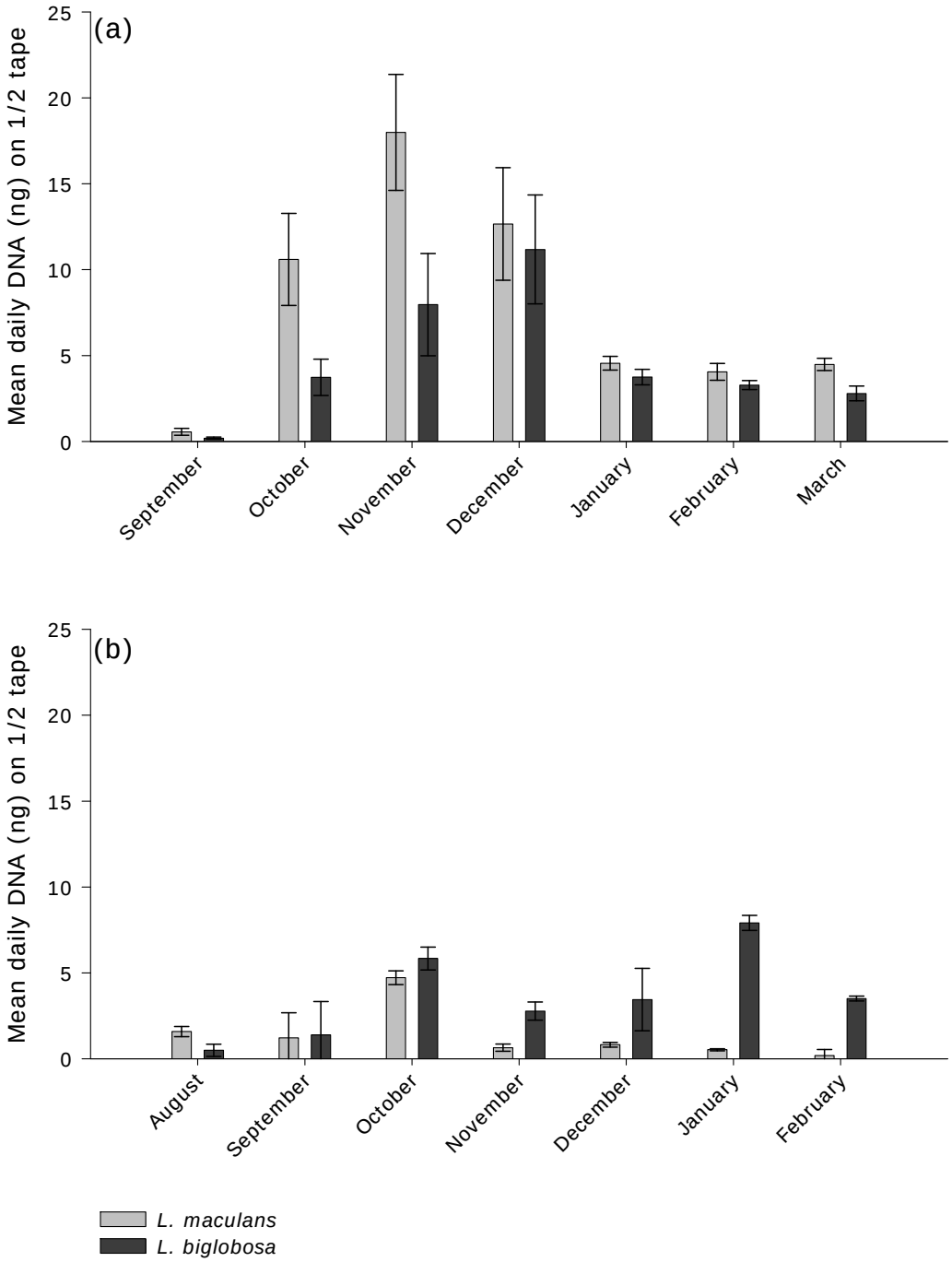

Figure 3.19. Mean daily amounts of *L. maculans* and *L. biglobosa* DNA detected on half of a Burkard spore sampler tape during the 2006/07 and 2007/08 winter oilseed rape growing season at Rothamsted. The Burkard spore sampler was surrounded by oilseed rape debris from the previous season in the (a) 2006/07 and (b) 2007/08 growing seasons. The amount *Leptosphaeria* DNA in DNA extracted from tapes removed from the spore sampler was quantified using qPCR. Error bars show the standard error of the individual means: (a) September 24 d.f., October 30 d.f., November 29 d.f., December 30 d.f., January 14 d.f., February 13 d.f., March 6 d.f. (b) August 9 d.f., September 29 d.f., October 30 d.f., November 29 d.f., December 30 d.f. , January 14 d.f., February 13 d.f.

3.5 Regional variation in the genetic diversity of *L. maculans* isolates in England

All 120 isolates were virulent on cvs Bristol (*Rlm2, Rlm9*), 02-22-1-1 (*Rlm3*) and 01-190-1-1 (*Rlm9*); therefore, no isolates possessed the *AvrLm2*, *AvrLm3* or *AvrLm9* avirulence alleles (Figure 3.20). Most isolates (84%) possessed *AvrLm7* and were avirulent on cv. 01-23-2-1 (*Rlm7*) and 58% of isolates possessed *AvrLm6* (avirulent on cv. Darmor MX (*Rlm6*)). The proportions of isolates with *AvrLm1*, *AvrLm4* and *AvrLm5* were 18%, 28% and 35% respectively.

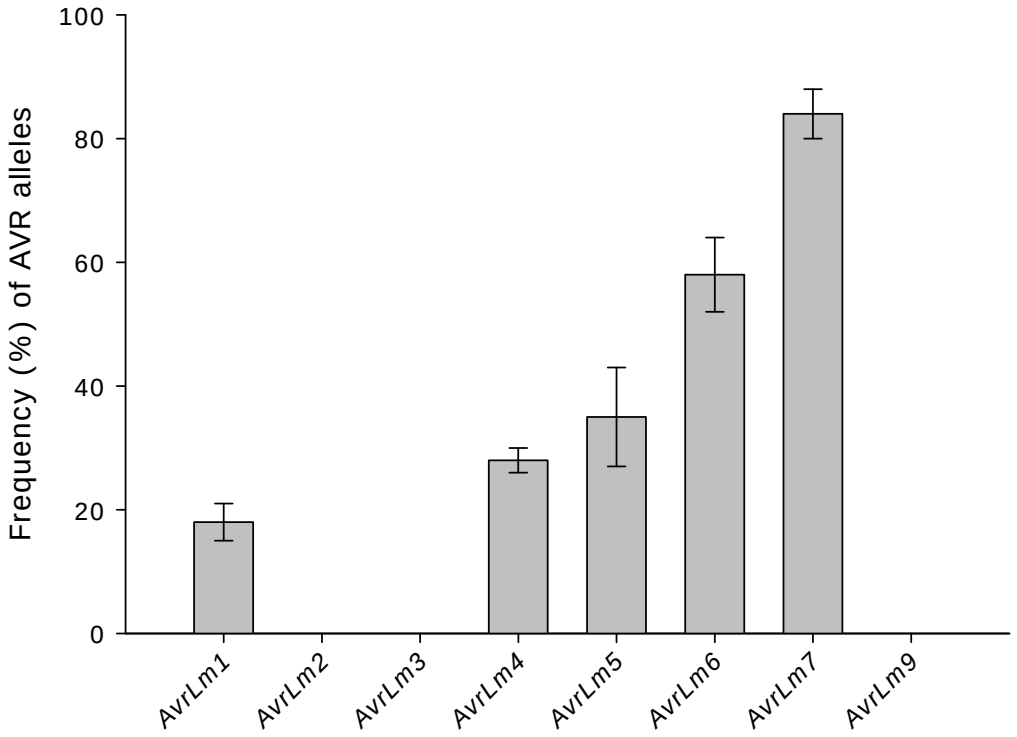

Figure 3.20. Mean frequency (%) of avirulence alleles in England. Isolates of *L. maculans* were obtained from leaf lesions occurring on cv. Drakkar in Autumn 2006. The avirulence alleles possessed by each isolate were determined using a differential set with known genes for resistance to *L. maculans*. Characterisation at the *AvrLm8* locus was not done. Error bars show the standard error of the individual means between sites, 119 d.f.

The frequency of avirulence alleles varied between sites (Table 3.5). There were significant differences between sites in the proportion of isolates possessing *AvrLm1, AvrLm5* and *AvrLm6*, respectively whilst there were no significant differences between sites in the frequency of avirulence alleles: *AvrLm4* and *AvrLm7*.

Table 3.5. Number of races and frequencies of Avr alleles in *L. maculans* isolates collected from Rothamsted and four TAG locations within England.

Sample location	No. isolates	No. races	Frequency (%) of AVR alleles[abc]							
			AvrLm1	***AvrLm2***	***AvrLm3***	***AvrLm4***	***AvrLm5***	***AvrLm6***	***AvrLm7***	***AvrLm9***
Norfolk	24	12	13	0	0	33	54	54	71	0
Bedfordshire	24	10	25	0	0	25	42	58	83	0
Lincolnshire	24	10	17	0	0	33	29	79	96	0
Hertfordshire	24	11	8	0	0	21	42	46	83	0
Gloucestershire	24	9	25	0	0	29	8	54	88	0
P[d]			0.013	-	-	0.426	<0.001	0.031	0.416	-

[a] Isolates were collected from leaf lesions on cv. Drakkar in Autumn 2006.
[b] Isolates not characterised at the *AvrLm8* loci.
[c] AVR alleles determined using differential plant set.
[d] Determined by chi-squared test.

In total, 19 uniquely individual races were identified in this study. The most diverse population, with 12 races, was Norfolk; the least diverse site was Gloucestershire with only 9 races (Table 3.5). The most common races were Av6-7-(8) (14%), Av7-(8) (13%) and Av5-6-7-(8) (11%; Table 3.6). Four races (Av1-4-6-7-(8), Av1-4-7-(8), Av1-7-(8) and (Av4-5-(8)) were represented by only a single isolate. Race Av7-(8) was identified at all sites and was represented by 25% of the isolates from the Gloucester site. In addition, a few (9 out of 120) isolates were identified at all sites that were virulent on all the differentials used (*Rlm1* to *Rlm7* and *Rlm9*), these isolates were termed Av(8) (Table 3.6). The race with the greatest number of avirulence alleles was Av1-4-5-6-7-(8) which was identified at the Norfolk, Bedfordshire and Lincolnshire sites.

Table 3.6. Races of *L. maculans* indentified on oilseed rape at Rothamsted and four TAG locations in England.

	Proportion (%) of isolates[b]					
Race[a]	**Norfolk**	**Bedfordshire**	**Lincolnshire**	**Hertfordshire**	**Gloucestershire**	**Total**
Av(8)	8.3	8.3	4.2	8.3	8.3	7.5
Av1-4-5-6-7-(8)	8.3	4.2	4.2	0	0	3.3
Av1-4-6-7-(8)	0	0	4.2	0	0	0.8
Av1-4-7-(8)	0	0	4.2	0	0	0.8
Av1-5-6-7-(8)	0	4.2	4.2	4.2	0	2.5
Av1-6-(8)	0	8.3	0	0	4.2	2.5
Av1-6-7-(8)	4.2	8.3	0	4.2	16.7	6.7
Av1-7-(8)	0.0	0	0	0	4.2	0.8
Av4-5-(8)	4.2	0	0	0	0	0.8
Av4-5-6-7-(8)	0	0	8.3	8.3	8.3	5.0
Av4-5-7-(8)	4.2	0	0	4.2	0	1.7
Av4-6-7-(8)	8.3	12.5	12.5	0	12.5	9.2
Av4-7-(8)	8.3	8.3	0	8.3	8.3	6.7
Av5-(8)	4.2	0	0	8.3	0	2.5
Av5-6-7-(8)	16.7	20.8	12.5	4.2	0	10.8
Av5-7-(8)	16.7	12.5	0	12.5	0	8.3
Av6-(8)	12.5	0	0	0	0	2.5
Av6-7-(8)	0	0	33.3	25	12.5	14.2
Av7-(8)	4.2	12.5	12.5	12.5	25	13.3

[a] Race nomenclature indicates the AVR loci for which the isolate in avirulent according to Balesdent *et al.* (2005). Numbers in brackets correspond to the AVR locus (*AvrLm8*) not characterised.
[b] Twenty four isolates from each site, isolated from cv. Drakkar in autumn 2006, were analysed.

Genetic distance analysis to examine at the relationship between locations (Figure 3.21) showed that isolates from Bedfordshire and Hertfordshire have the closest similarity. Isolates from Norfolk are closely related to those from Bedfordshire and Hertfordshire whilst

Lincolnshire and Gloucestershire isolates are in a separate branch of the dendrogram and are more similar to one another.

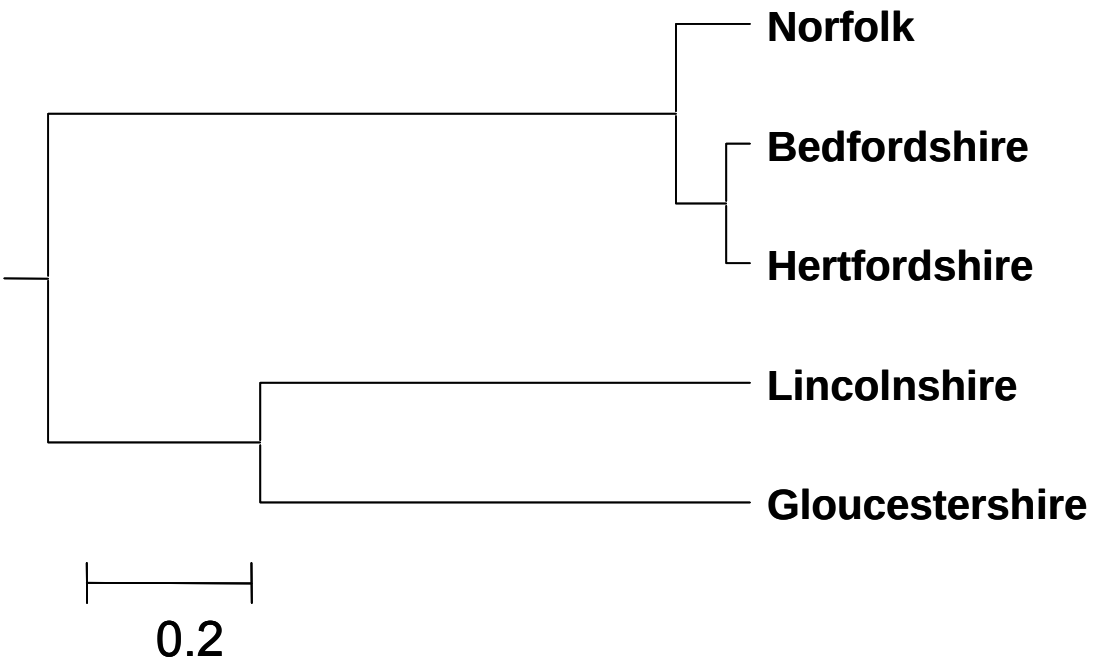

Figure 3.21. Dendrogram showing relationship between five sites in England based on *L. maculans* avirulence alleles. Dendrogram was created to reflect Nei's genetic distances. Avirulence alleles were determined using a differential plant set with known resistance genes (*Rlm1* to *7* and *Rlm9*) and scoring the interaction phenotype. Twenty four isolates were examined from each site.

Genetic differences between isolates were examined using minisatellites. The results showed that each isolate had a unique genotype (Figure 3.22). Distance analysis based on minisatellite allele profiles of the 120 isolates split the isolates into two clusters which are approximately 60% similar (Figure 3.22). Isolates from each of the five sites are included in each of these clusters. There is a cluster of 11 isolates from Hertfordshire, split by two Lincolnshire isolates, and several small clusters of Bedfordshire isolates.

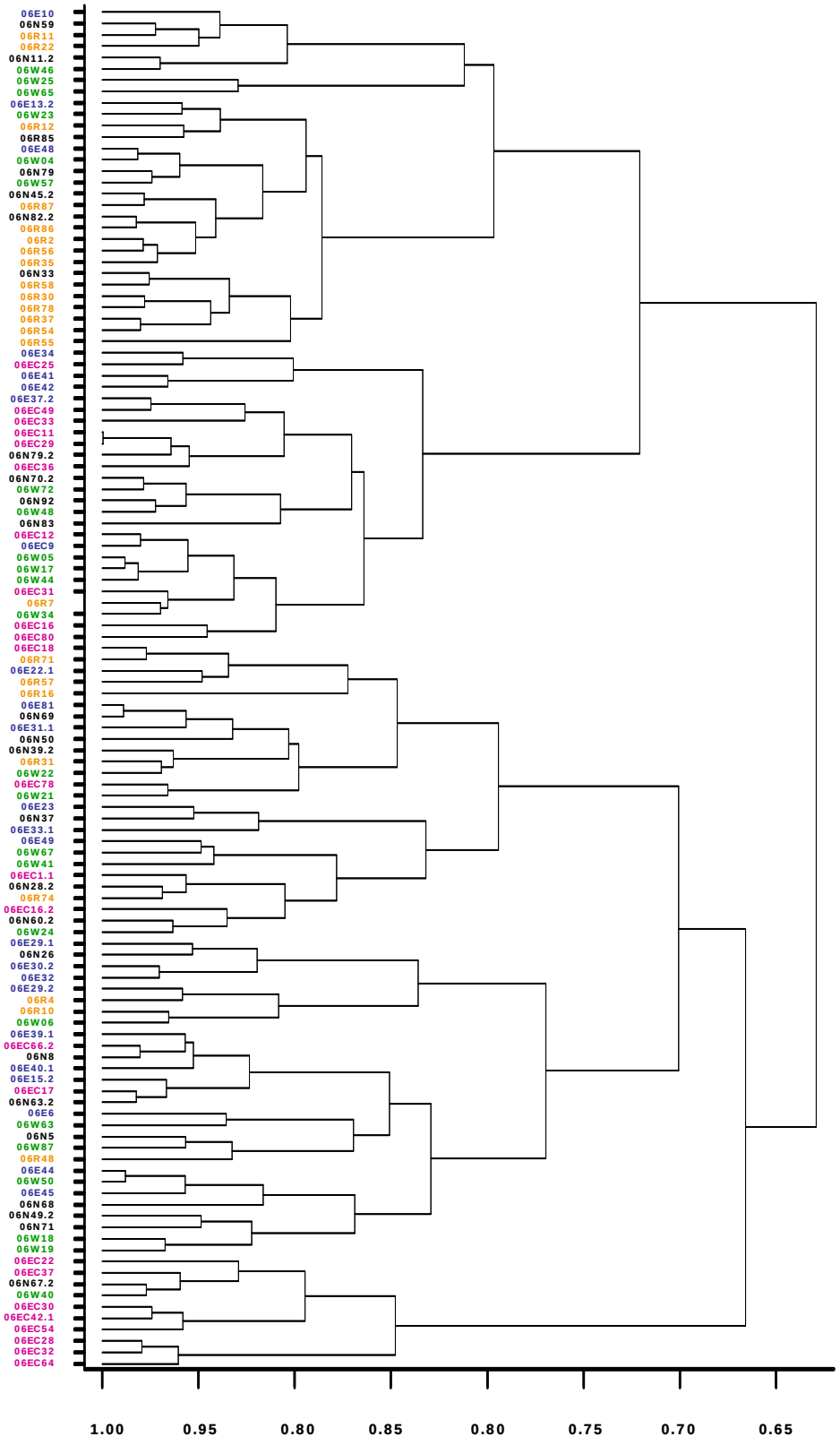

Figure 3.22. Cluster dendogram showing the similarities in minisatellite allele polymorphisms among 120 *L. maculans* isolates from five sites in England. Isolates from Norfolk are shown in blue, Bedfordshire in pink, Lincolnshire in black, Hertfordshire in orange and Gloucestershire in green. The scale shows degree of similarity (1 = 100%).

4 DISCUSSION

4.1 Factors affecting regional variation in populations of *L. maculans* and *L. biglobosa* and severity in phoma stem canker epidemics

The results of this work (summarised in Figure 4.1) demonstrate how elements of differences in geographical location, cultivar choice and fungicide application regime influence genetic variation in populations of *Leptosphaeria maculans* and the severity of phoma stem canker epidemics in England. These regional differences may help to explain the regional variation in fungicide responses observed between field experiments done at different locations in England. The main conclusions from this work are:

1. The seasonal timing of ascospore release differs between *L. maculans* and *L. biglobosa* because they have different responses to weather factors. In both the 2006/07 and 2007/08 growing seasons maximum *L. maculans* ascospore release was earlier in the season than maximum *L. biglobosa* ascospore release. In the survey of winter oilseed rape stems with basal stem cankers and/or upper stem lesions and other studies (Toscano-Underwood *et al.*, 2001; West *et al.*, 2002b), *L. maculans* was generally associated with basal stem cankers and *L. biglobosa* with upper stem lesions. Differences in patterns of ascospore release may explain this separation in space; as *L. maculans* ascospores are released early in the growing season, when temperatures are greater and plant leaves are smaller, the pathogen can rapidly grow along the petiole to the plant stem before stem elongation and therefore cause a basal stem canker. However, as *L. biglobosa* ascospores are released later in the growing season when temperatures are lower and leaves are larger the pathogen is less able to reach lower parts of the plant stem before the start of stem elongation and therefore forms an upper stem lesion. Differences between *L. maculans* and *L. biglobosa* in timing of ascospore release may result from differences in their response to temperature and rainfall and/or differences in crop residue management. Regional variation in these weather and agronomic factors may result in regional differences in ascospore release and therefore influence the regional differences in proportions of *L. maculans* and *L. biglobosa* in basal stem cankers.

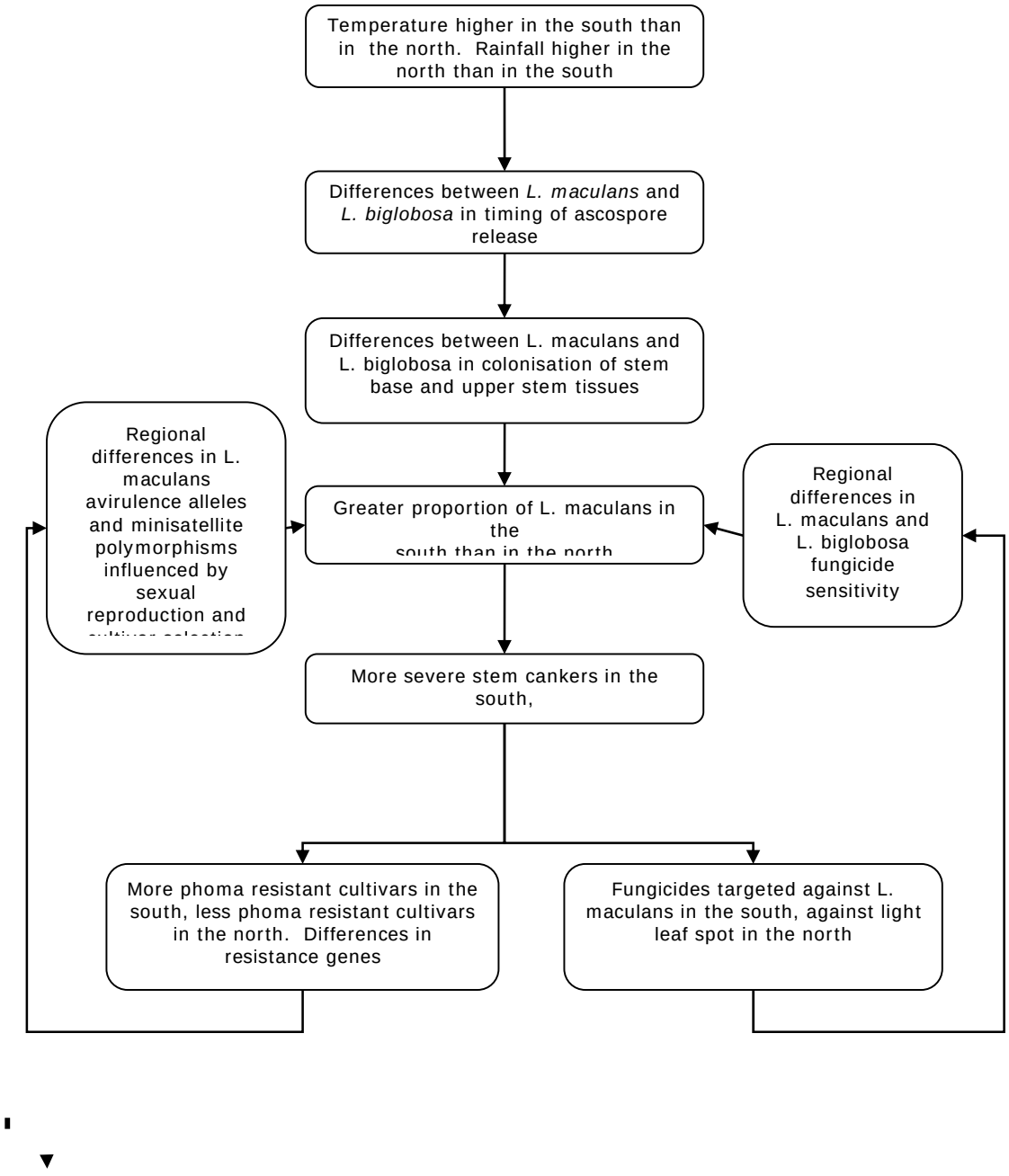

Figure 4.1. Summary of factors affecting regional variation in populations of *L. maculans* and *L. biglobosa* and in the severity of phoma stem canker epidemics in England.

2. There are regional and seasonal differences in the distribution of *L. maculans* and *L. biglobosa* in phoma stem cankers which affect the severity of epidemics on winter oilseed rape in the UK. A survey of phoma-affected oilseed rape stems from commercial crops was done (2001-2003 & 2006) in which DNA from each pathogen was quantified in basal stem cankers showed regional variation within England and Wales. The proportion of *L. maculans* DNA was greatest in basal stem cankers collected from southern England, where epidemics are most severe (Figure 1.7; www.cropmonitor.co.uk), whilst the proportion of *L. biglobosa* DNA was greatest in those from northern England. The observed pattern of regional variation matched patterns of predictions, made using a combined disease prediction and climate change model, of the time between phoma leaf spot onset (autumn) and phoma stem canker onset (spring) (Evans *et al*., 2008). This indicates that temperature affects the proportions of *L. maculans* and *L. biglobosa* in basal stem cankers. However, in the survey of plants from commercial crops with phoma stem canker done in June/July 2001-2003 and 2006, there were significant differences between cultivars in the amount of *L. maculans* and *L. biglobosa* DNA quantified in basal stem cankers and upper stem lesions, showing that cultivar choice affects the proportions of *L. maculans* and *L. biglobosa* in stem cankers and lesions. In addition, fungicide application regime may affect proportions of the two species, since *L. maculans* and *L. biglobosa* differ in their sensitivity to azole fungicides (Eckert, 2005; Hood, 2008).

3. Regional differences in choice of oilseed rape cultivars influence the race structure of *L. maculans* populations and thus the severity of phoma stem canker epidemics. The results of the English national winter oilseed rape survey showed that there is regional variation in cultivar choice. Variation in cultivar choice between northern and southern England is likely to be due to yield expectations and the high risk of phoma stem canker epidemics and low risk of light leaf spot in southern England compared to the low risk of severe phoma stem canker epidemics and high risk of light leaf spot in the north. Therefore, cultivars with a good phoma stem canker resistance rating are selected in southern England whilst those with a good light leaf spot resistance rating are selected in northern England; for example cv. Cobra (HGCA light leaf spot resistance rating of 7 and phoma stem canker resistance rating of 4) was a popular choice only in northern England.

The results of field experiments done in four growing seasons (2003/04, 2005/06, 2006/07 and 2007/08), using a range of current and historical cultivars/breeding lines at Rothamsted, showed significant differences between cultivars in the severity of phoma stem canker. The observed differences are likely to have been due to differences between cultivars in

resistance types: monogenic resistance operating in the leaves of seedlings and juvenile plants polygenic, or adult plant resistance. The cultivars Jet Neuf and Darmor, with both monogenic and polygenic resistance (Balesdent *et al.*, 2001; Delourme *et al.*, 2006; Pilet *et al.*, 2001) had low stem canker severity in all seasons, showing how combining the two types of resistance can be efficacious in producing more effective resistance to *L. maculans*.

These results showed that the race-specific resistance genes possessed by different cultivars affected the race structure of *L. maculans* populations. The most closely related sampling sites, in terms of race structure, were in Hertfordshire and Bedfordshire which were also geographically the closest. Isolates from the Hertfordshire and Bedfordshire sites were closely related to those from the Norfolk site but only distantly related to those from Lincolnshire and Gloucestershire. The close genetic relationship between isolates from sites that were geographically close together supports the theory that *L. maculans* race structure is affected by the history of oilseed rape cropping and cultivar choice (Balesdent *et al.*, 2005), since oilseed rape cropping history in England is likely to be more similar between sites close together than those far apart.

All 120 *L. maculans* isolates that were examined were found to possess to be virulent on *Rlm2, Rlm3* and *Rlm9,* these resistance genes have been used widely in commercial cultivars, for example cv. Apex (*Rlm9*) was used extensively in England between 1990 and 2006. The results of this work show that *Rlm6* and *Rlm7* would currently provide effective protection against *L. maculans*. However, it is important that the use of *Rlm6* and *Rlm7* in commercial cultivars is carefully monitored and is combined with other resistance genes and race-non specific resistance alleles, since *L. maculans,* according to the criteria of McDonald and Linde (2002), has a high evolutionary potential and may rapidly render monogenic resistance ineffective. For example, the wide scale use of Surpass 400 (*LepR3*) in Australia and of *Rlm1* resistance in France resulted in rapid changes in the local *L. maculans* populations so that these sources of resistance became ineffective very rapidly (Li & Cowling, 2003; Rouxel *et al.*, 2003).

All 120 *L. maculans* isolates had a uniquely different genotype as determined by the minisatellite analysis. Therefore, it is reasonable to conclude that the phoma leaf spots examined were initiated by ascospores rather than by asexual conidia.

4. Regional variation in choice of fungicides influences the composition of *L. maculans* and *L. biglobosa* in populations and thus the severity of phoma stem canker epidemics.

The results of the national winter oilseed rape survey show regional differences in fungicide application regime. Differences between the north and the south are likely to be due to the

increased incidence of light leaf spot and decreased incidence of phoma stem canker in the north as compared to the south. The proportions of *L. maculans* and *L. biglobosa* in phoma stem cankers may be affected by regional variation in fungicide application regime as *L. maculans* and *L. biglobosa* differ in their sensitivity to azole fungicides (Eckert, 2005; J Hood, 2009). In addition, there is regional variation in sensitivity to azole fungicides in *L. maculans* Populations: isolates from Lincolnshire are more sensitive than those from more southern sampling sites (J Hood, 2009).

4.2 Implications for improving control of phoma epidemics

1. Climate change

There is a consensus that global warming is occurring and that it is linked to human activity (Hansen *et al.*, 2005). Of concern is the prediction of an increase in severity and a northwards spread, into Scotland, of phoma stem canker due to climate change (Evans *et al.*, 2008). The results of the current study suggest that an increase in temperature would result not only in an increase in phoma stem canker severity but also a spread of *L. maculans* northwards into areas currently occupied predominately by *L. biglobosa*. A spread of *L. maculans* into areas previously dominated by *L. biglobosa* has occurred in Poland (Karolewski *et al.*, 2002, cited in Fitt et al. 2006a) and Canada (Gugel & Petrie, 1992). It is therefore important that improvements are made in control of *L. maculans* as a part of a strategy for adaptation to climate change.

The results of this work show that the *Brassica* genes, *Rlm6* and *Rlm7*, for resistance to *L. maculans* have the potential to provide effective control of phoma stem canker epidemics. However, controlled environment experiments, using Darmor-MX (*Rlm6*), Darmor (without *Rlm6*) and *AvrLm6 L. maculans* isolates, have shown that the resistance conferred by *Rlm6* (effective at 15°C) is ineffective at 25°C (Huang *et al.*, 2006). Therefore, it is possible that other genes for resistance to *L. maculans* are affected by temperature. As temperatures increase through climate change, it is important that durable sources of resistance to *L. maculans* are identified and bred into commercial winter oilseed rape cultivars.

2. Agronomy

Stubble management techniques affect pathogen survival and the production and maturation of pseudothecia of *L. maculans* and *L. biglobosa* on crop debris. This in turn affects the timing of ascospore release of each species. In this study *L. maculans* ascospore release was generally earlier than that of *L. biglobosa*. Early ascospore release results in the most severe basal stem

cankers. The results of both field and controlled environment experiments have shown that pre- or co-inoculation with *L. biglobosa* induces systemic acquired resistance to subsequent *L. maculans* infection (Liu *et al.*, 2007; Liu *et al.*, 2006). Therefore, stubble management techniques could be altered to favour the survival of *L. biglobosa*. For example, stubble should not be left standing as this results in a higher water potential at the stem base than in upper parts of the stem; this favours maturation of pseudothecia of the pathogen colonising the stem base (*L. maculans*) over that colonising upper parts of the stem (*L. biglobosa*) (Fitt *et al.*, 2006b). However, tillage followed by the recommended 4 year rotation should ensure that both *L. maculans* and *L. biglobosa* are unable to survive on any debris returned to the soil surface (Turkington *et al.*, 2000; West *et al.*, 2001).

Winter oilseed rape cultivars with qualitative resistance to *L. maculans* do not provide resistance to *L. biglobosa* (Brun *et al.*, 1997; Fitt *et al.*, 2006a; Somda *et al.*, 1998). Therefore, cultivar choice may affect the proportions of *L. maculans* and *L. biglobosa* in local populations. The results of this work found differences in the quantities of *L. maculans* and *L. biglobosa* DNA in basal stem cankers and upper stem lesions on different winter oilseed rape cultivars. These differences may affect the timing of *L. maculans* and *L. biglobosa* ascospore release and, therefore, position of stem colonisation in the following season. The use of cultivars susceptible to *L. biglobosa* infections may inhibit *L. maculans* through induced resistance.

As phoma stem canker epidemics become more severe, it will become increasingly important to apply fungicides at the correct time and dose. *Leptosphaeria maculans* and *L. biglobosa* differ in fungicide sensitivity (Eckert, 2005) and in alignment with the results of the current study it is possible to improve disease prediction models, for advice upon fungicide timing, through the ability to distinguish between *L. maculans* and *L. biglobosa* ascospore release. In addition, recommendations on application rate could be improved by knowledge of the proportions of *L. maculans* and *L. biglobosa* in local populations. By improving fungicide targeting it may be possible to reduce the current rates of fungicide applications in the UK, thus limiting the risk of the evolution of resistance to azole fungicides.

The results of this work have shown that geographic variation in climate, cultivar choice and fungicide application regime affects the severity of phoma stem canker epidemics in England. However, other factors not assessed in this study, such as land preparation and crop residue management techniques, may also affect severity of epidemics. It is likely that regional variation in these factors resulted in variation in fungicide response between sites in England. These results can be used to prepare the UK winter oilseed industry for the major challenges, including

climate change, associated with the status of oilseed rape as the choice break crop in the predominantly cereals-based arable agriculture of the United Kingdom.

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