Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*)

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Controlled environment and field experiments were done to investigate effects of the fungicide Punch C (flusilazole plus carbendazim) on growth of *Leptosphaeria maculans* and *L. biglobosa* in oilseed rape. In controlled environment experiments, for plants inoculated with *L. maculans*, fungicide treatment decreased lesion size and amount of *L. maculans* DNA in leaves; for plants inoculated with *L. biglobosa*, fungicide did not affect lesion size or amount of pathogen DNA. When release of ascospores was monitored using a Burkard spore sampler, the timing and pattern of ascospore release differed between the four seasons. In 2006/07, the majority of ascospores released were *L. maculans,* while in 2007/08 the majority were *L. biglobosa;* in both seasons *L. maculans* ascospores were released before *L. biglobosa* ascospores. In field experiments in 2002/03 and 2003/04, fungicide treatment decreased severity of stem canker on cv. Apex but gave no significant yield response. In 2006/07 and 2007/08, fungicide treatment decreased phoma leaf spot incidence in autumn and stem canker severity at harvest, and increased yield. Fungicide treatment decreased stem canker severity more on cv. Courage, with a good yield response, than on cv. Canberra. In 2002/03 and 2003/04, fungicide treatment decreased the frequency of spread of *L. maculans* into stem pith tissues and in 2006/07 fungicide decreased the amount of *L. maculans* DNA in stem tissues (measured by quantitative PCR). These results are used to suggest how effects of fungicides on

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interactions between *L. maculans* and *L. biglobosa* might affect severity of phoma stem canker and yield response.

Keywords: ascospores, blackleg, carbendazim, disease control, flusilazole, *Phoma lingam*

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Introduction

Many damaging crop diseases are associated with two or more related co-existing fungal pathogens (Gudelj *et al*., 2004; Fitt *et al*., 2006a). For example, wheat leaf blotch diseases are caused by two related species *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) and *S. nodorum* (teleomorph *Phaeosphaeria nodorum*) (Bearchell *et al*., 2005; Shaw *et al*., 2008); cereal eyespot disease on stem bases is caused by *Oculimacula yallundae* and *O. acuformis* (Bierman *et al*., 2002); two related species *Mycosphaerella brassicicola* and *M. capsellae* cause co-existing leaf spots on oilseed rape leaves (Gudelj *et al*., 2004). Effectiveness of control of such diseases by fungicides may be associated with their effects on proportions of the two related species, especially if one of them is more damaging. It has been demonstrated that fungicides can select for one of the two eyespot pathogen species on winter wheat; sprays with the fungicide prochloraz increased proportions of *O. acuformis* and decreased proportions of *O. yallundae* compared to proportions in untreated plots (Bierman *et al*., 2002). Phoma stem canker, a disease of worldwide economic importance on oilseed rape/canola (*Brassica napus*) and brassica vegetables, and responsible for losses worth more than £500M each growing season (West *et al*., 2001; Fitt *et al*., 2008), is caused by the two closely related species *Leptosphaeria maculans* and *L. biglobosa* (Shoemaker & Brun, 2001), which co-exist on their host (Fitt *et al*., 2006a). *L. maculans* is more damaging, causing stem base canker; *L. biglobosa* is generally less damaging, causing upper stem lesions (West *et al*., 2002a; Huang *et al*., 2005). Therefore, the proportion of the two species in local populations has been shown to affect the severity of stem canker epidemics (Stonard *et al*., 2010a).

In the UK, control of severe phoma stem canker epidemics relies on the application of foliar fungicides (West *et al*., 2002b). However, the effectiveness of fungicide application varies from season to season and from region to region (West *et al*., 2002b; Steed *et al*., 2007; Stonard *et al*., 2010a). To optimise the use of fungicides for successful management of phoma stem canker in

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winter oilseed rape, previous studies investigating timing of autumn fungicide sprays have assessed stem canker severity in the following summer at harvest (Gladders *et al*., 2006). However, there has been little work on how effects of fungicides on the two co-existing phoma stem canker pathogens *L. maculans* and *L. biglobosa* might influence the severity of stem canker epidemics. In the UK, commercial fungicides used on oilseed rape, such as Punch C (flusilazole plus carbendazim) must provide effective control of both phoma stem canker and light leaf spot (*Pyrenopeziza brassicae*); the latter is particularly damaging in northern England and Scotland (Fitt *et al*., 1998; Gilles *et al*., 2000; Boys *et al*., 2007).

Previous *in vitro* studies indicate that *L. maculans* and *L. biglobosa* differ in their sensitivity to triazole fungicides. In terms of *in vitro* mycelial growth, isolates of *L. maculans* were more sensitive to the triazole fungicides flusilazole and tebuconazole than isolates of *L. biglobosa* (Eckert *et al*., 2010). Whether triazole fungicides have the same effects on growth of the two species *in planta* as *in vitro* is not clear. Although differences in sensitivity to flusilazole amongst *L. maculans* isolates have been observed (Eckert *et al*., 2010), it is not known whether the differences are caused by alterations in the *CYP51* gene of *L. maculans* (*LmCYP51*), which have led to development of fungicide resistance in other pathogens (Cools & Fraaije, 2008). Sprays with azole (triazole and imidazole) fungicides for control of septoria leaf blotch of wheat can lead to development of fungicide resistance in *M. graminicola* populations, which has been correlated with alteration of the target-encoding sterol 14α-demethylase protein (CYP51). Isolates highly resistant to azole fungicides commonly carry several mutations in the *CYP51* gene (Cools & Fraaije, 2008). Comparison of β-tubulin gene sequences of *L. maculans* and *L. biglobosa* isolates with β-tubulin sequences of known MBC fungicide-resistant isolates showed that no mutations commonly known to confer resistance to carbendazim had been observed in *L. maculans* and *L. biglobosa* (Eckert et al., 2010). To improve the effectiveness of fungicide applications, there is a need to investigate whether foliar application of fungicides to winter oilseed rape crops affects the proportions of the two *Leptosphaeria* species or

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selects for fungicide resistance alleles in pathogen populations, both of which would affect severity of phoma stem canker epidemics.

In the same way that effects of fungicide are assessed at the end of the season, in the UK, where oilseed rape is sown in autumn (August/September) and harvested the following summer (July), cultivar rating for resistance is based on assessment of phoma stem canker symptoms before harvest (Fitt *et al*., 2006b; www.hgca.com). However, such assessments provide no evidence whether cultivar interactions with the two co-existing pathogen species affect the severity of pre-harvest symptoms. There is evidence that pre-inoculation with *L. biglobosa* can activate local and systemic defence responses in oilseed rape to decrease the *in planta* development of *L. maculans*. In controlled environment experiments, oilseed rape plants pre-inoculated with *L. biglobosa* conidia before a challenge inoculation with *L. maculans* conidia developed smaller leaf lesions than plants without *L. biglobosa* pre-treatment (Mahuku *et al*., 1996). Furthermore, it has been shown that this activation of host defence responses by *L. biglobosa* occurs in winter oilseed rape crops; treatment of winter oilseed rape plants with ascospores of *L. biglobosa* in the autumn resulted in decreased stem canker severity before harvest (Liu *et al*., 2006, 2007). However, it is not clear how important these interactions between *L. maculans* and *L. biglobosa* are in determining the severity of stem canker on different cultivars.

In Europe, epidemics of phoma stem canker on winter oilseed rape are initiated in autumn by air-borne ascospores (West *et al*., 1999; Huang *et al*., 2005). Germinated ascospores infect leaves to produce phoma leaf spots from which the pathogen grows along petioles into stems to initiate stem base cankers or upper stem lesions (Huang *et al*., 2006). As epidemics of phoma leaf spot early in autumn are associated with more severe basal cankers and greater yield loss the following summer, foliar fungicides must be applied in time to prevent the pathogens from growing to stems to cause stem cankers (Zhou *et al*., 1999; Gladders *et al.,* 2006). Therefore, optimal timing of fungicide applications depends on timing of ascospore release. Weather-based models for forecasting the

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timing of ascospore release can be used to guide the timing of fungicide application (Salam *et al*., 2007; Huang *et al*., 2007). Since *L. maculans* and *L. biglobosa* differ in aggressiveness and *in vitro* fungicide sensitivity, the relative proportions of *L. maculans* and *L. biglobosa* in populations may affect the efficacy of fungicide applications. However, it has been difficult to distinguish ascospores of *L. maculans* and *L. biglobosa* in samples collected from air by visual methods. Although ascospores of *L. maculans* and *L. biglobosa* can be distinguished after they germinate (Huang *et al*., 2001), this technique is demanding and time-consuming. Furthermore, it is not practical to use it to distinguish ascospores from spore sampler tapes because the density of ascospores on them is usually great; once the ascospores germinate, the germ tubes from different ascospores overlap and it is difficult to distinguish one germinated ascospore from another. Recently, the development of molecular biological techniques based on polymerase chain reaction (PCR) and species-specific primers for *L. maculans* and *L. biglobosa* have made it possible to investigate the relative proportions of the two species in the air samples using quantitative PCR (qPCR) (Kaczmarek *et al*., 2009). This paper describes work to investigate (1) effects of fungicide on growth of *L. maculans* and *L. biglobosa in planta*; (2) seasonal differences in the release of ascospores between *L. maculans* and *L. biglobosa* in relation to effects of fungicide on development of phoma leaf spots and phoma stem canker on winter oilseed rape.

Materials and methods

Selection of cultivars and fungicide

For field experiments in 2002/03 and 2003/04, the cultivar (cv.) Apex was used since this was a major cultivar grown in the UK at that time. To investigate interactions between effects of cultivar resistance and fungicide, cvs Courage and Canberra differing in their rating for resistance against *L. maculans* were used in controlled environment and field experiments in 2006/07 and 2007/08. Courage was susceptible to *L. maculans* with a rating of 3; Canberra and Apex were more resistant

against *L. maculans* with a rating of 6 on a 1-9 scale (www.hgca.com). The fungicide used in all experiments was the commercial product Punch C (containing 250 g L^{-1} flusilazole, 125 g L^{-1} carbendazim), the main fungicide used then against phoma stem canker and light leaf spot in the UK. The flusilazole component of Punch C was effective for control of phoma stem canker and the carbendazim was for control of light leaf spot (Ashworth, unpublished).

Effects of fungicide treatment on growth of *L. maculans* and *L. biglobosa* in leaf tissues in controlled environment experiments

Plants of cvs Courage and Canberra were grown in pots (9 cm diameter) containing peat-based compost and a soluble fertiliser with one plant per pot. Plants were initially grown in a glasshouse until they had three fully expanded leaves, then transferred to a growth cabinet at 20°C with a 12 hour photoperiod (210 µmol m^{-2} sec^{-1}). The second and third leaves of each plant were inoculated by placing a 15 µL drop of ascospore suspension (10^4 ascospores mL^{-1}) on the lower part of the leaf lamina close to the main vein. Before inoculation, the lower part of the leaf was gently rubbed with a wet tissue so that drops of spore suspension remained on the leaf. There were three inoculation treatments: (1) *L. maculans* only; (2) *L. biglobosa* only; (3) mixture of *L. maculans* and *L. biglobosa* (suspensions of *L. maculans* and *L. biglobosa* ascospores were mixed in a 1:1 ratio). Ascospores of *L. maculans* were collected from pseudothecia produced on UK oilseed rape stem base debris; ascospores of *L. biglobosa* were collected from pseudothecia on stem debris from Poznan, Poland (Huang *et al*., 2003). Stem pieces with mature pseudothecia producing ascospores of only *L. maculans* or only *L. biglobosa*, confirmed by isolation or PCR (Liu *et al*., 2006), were stored at -20°C until required.

There were three fungicide treatments: (1) untreated (control, sprayed with distilled water); (2) sprayed with the commercial fungicide product Punch C (DuPont Limited, Stevenage, UK) at 6 days post inoculation (dpi) (early spray); (3) sprayed at 11 dpi (late spray). A preliminary experiment

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using the field application rate recommended by the manufacturer (0·8 L Punch C in 200 L of water ha^{-1}) showed that plants were damaged when sprayed at this concentration in glasshouse conditions. When plants were sprayed at a 1:10 dilution of the recommended field application rate, no physiological changes (or symptoms of damage) were observed and there were differences in the development of phoma leaf spots between sprayed and unsprayed plants. Therefore, a 1:10 dilution was chosen for the controlled environment experiments and plants were sprayed until run-off. The experiment was arranged in a randomised complete block design with five replicate blocks (two plants per block). Development of phoma leaf spot lesions was assessed by measuring lesion diameter across two diagonals per lesion at 20 dpi. To investigate whether fungicide treatment affects symptomless growth of *L. maculans* or *L. biglobosa* along the leaf petiole from a leaf lesion to the stem, the inoculated leaves were detached at 22 dpi. The leaf lesion and a 10 cm length of the leaf petiole (measured from the inoculation site) were cut from each leaf and placed in a 50 mL tube to be freeze dried for DNA extraction (see below). The symptomless growth of *L. maculans* or *L. biglobosa* in the leaf petiole was then measured by qPCR (Huang *et al*., 2009).

Monitoring the release of *L. maculans* and *L. biglobosa* ascospores

Release of ascospores of *L. maculans* and *L. biglobosa* in the air was monitored using a Burkard 7-day recording spore sampler (Burkard Manufacturing Company Ltd., Rickmansworth, UK). Each growing season, the Burkard sampler was surrounded radially by oilseed rape stems affected by phoma stem canker from previous growing season as described by Huang *et al.* (2005). The spore sampler was operated from August to April in 2002/03 and 2003/04, and from August to February in 2006/07 and 2007/08. At 7-day intervals, the exposed tape was removed from the sampler drum and cut into pieces 48 mm long (each representing 24 h). Each 48 mm long piece of tape was cut in half length-wise. One half was mounted onto a microscope slide for counting ascospores (Huang *et al*., 2005; Lacey & West, 2006); the other half was placed in a 1·5 mL Eppendorf tube and stored at

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-20°C for DNA extraction and qPCR (see below). The amounts of *L. maculans* and *L. biglobosa* DNA on each spore tape quantified by qPCR were converted to number of ascospores (total *L. maculans* or *L. biglobosa* DNA divided by DNA per ascospore). To estimate the amount of DNA per ascospore (the size and shape of ascospores of *L. maculans* and *L. biglobosa* are similar; it was assumed that the mean amount of DNA per ascospore was the same for these two species), 40 days (each day when more than 200 ascospores were counted on the tape) were selected; for each day, all the ascospores on half of the tape had been counted under a microscope and the amounts of *L. maculans* and *L. biglobosa* DNA on the other half of the tape had been quantified using qPCR. The mean amount of DNA per ascospore was then estimated (total pathogen DNA divided by total number of ascospores).

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Effects of fungicide on development of phoma leaf spots and stem canker in winter oilseed rape

Four field experiments were done at Rothamsted (Table 1). In each growing season, the winter oilseed rape cvs were sown in late August at 80 seeds m^{-2}. Plots were arranged in a randomised block design, with three replicates in 2002 (20 x 3 m plots), four replicates in 2003 (10 x 3 m plots) and three replicates in 2006 and 2007 (15 x 3 m plots). The timing of early fungicide sprays was adjusted from season to season according to the first date when 10% of plants were affected with phoma leaf spot and the timing of late sprays was 1-2 months after the early spray, when weather and ground conditions permitted. The fungicide Punch C was applied at the recommended rate (0·8L in 200L water ha^{-1}) on two occasions (early spray and late spray) during each growing season (Table 1). At the end of each season, the experiments were combine harvested and the yields of individual plots were determined at 90% dry matter.

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Phoma leaf spotting (% plants affected) was assessed by randomly sampling ten plants from each plot regularly between September and February or September and April. In 2002/03 and

2003/04, phoma leaf spotting was assessed only on untreated plots; in 2006/07 and 2007/8, it was assessed on both treated and untreated plots. Each plant was assessed for the presence of phoma leaf spots. Phoma stem canker was assessed each season before harvest (2 July 2003, 19 July 2004, 2 July 2007 and 1 July 2008) by randomly sampling 30 (2002/03), 25 (2003/04) or 20 (2006/07 and 2007/08) plants from each plot. Phoma stem canker severity was assessed by cutting the stem base of each plant and scoring the area of necrotic tissue in the cross-section using a 0–6 scale (0, healthy stem with no affected tissue; 1, 1– 25% of the cross-section affected; 2, 26 – 50% of the cross-section affected; 3, 51– 75% of the cross-section affected; 4, 76 – 99% of the cross-section affected; 5, 100% of the cross-section affected, plant still alive; 6, 100% affected, dead stem with a hollow or severely necrotic pith), modified from the 1-6 scale of Lô-Pelzer *et al*. (2009).

Effects of fungicide treatment on growth of *L. maculans* and *L. biglobosa* in stem tissues

To assess the effects of fungicide application on growth of *L. maculans* or *L. biglobosa* in stems, before harvest in 2002/03 (2 July 2003) and 2003/04 (19 July 2004), 30 plants of each treatment were chosen at random and small pieces (5 × 5 mm) were cut from the stem base cortex and stem base pith tissue of each plant. These pieces were cut in half. One half was used for fungal isolation (West *et al*., 2002a) and the other half used for DNA extraction and end-point PCR (Liu *et al*. 2006). In 2006/07, 10 ten plants were randomly sampled from each plot on 2 July 2007. Stem canker severity on each plant was scored on the 0–6 scale, then a 6 cm long piece of stem base was sampled. Each of the sampled pieces of stem base was cut up and placed in a 50 mL tube to be freeze-dried. Each freeze-dried stem base piece was ground into powder for DNA extraction and qPCR (see below).

Sensitivity of *L. maculans* isolates to the fungicide flusilazole

A total of 123 isolates of *L. maculans* from the UK and other countries (www.oregin.info) were initially tested *in vitro* for sensitivity to flusilazole using the method described by Eckert et al.

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(2010). From these 123 isolates, a subset of 13 isolates differing in their sensitivity to flusilazole as measured by ED_{50} value (effective dose at which 50% of growth inhibited) was selected for further study. To investigate whether differences in sensitivity to flusilazole amongst *L. maculans* isolates are related to alterations in the *CYP51* gene of *L. maculans* (*LmCYP51*), this gene was amplified from the subset of *L. maculans* isolates.

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Primers LmCYP51F (5' ATGGCTGTTCTTGCTACCGTTG 3') and LmCYP51R (5' CTCGACCTTCTCCCTCCTCTCC 3'), designed using the *LmCYP51* gene sequence (accession number: AY142146), were used to amplify the whole *LmCYP51* gene (Griffiths & Howlett, 2002). DNA was extracted from mycelium of *L. maculans* isolates as described by Eckert *et al*. (2010). PCR products were sent to Eurofins MWG Operon (London, UK) for direct sequencing. The sequences of the *LmCYP51* gene from different isolates were compared. The *LmCYP51* genes were translated and the predicted LmCYP51 proteins were aligned with the CYP51 proteins of organisms from different phyla, including human (HsCYP51A1, NP_000777), *Mycobacterium tuberculosis* (MtCYP51B1, ZP_03419138), *Saccharomyces cerevisiae* (ScCYP51F1, NP_011871), *Candida albicans* (CaCYP51F1, AAF00597), *Arabidopsis thaliana* (AtCYP51G1, NP_172633) and *Mycosphaerella graminicola* (MgrCYP51F1, AY730587) using ClustaW.

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DNA extraction and quantitative PCR (qPCR)

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*Samples from affected leaves or stems***.** Affected leaves or stems were freeze-dried and ground into powder using a mortar and pestle. DNA was extracted from a 20 mg sub-sample (from each ground sample) using a DNA extraction kit (DNAMITE Plant Kit, Microzone Ltd, UK) and quantified on a Nanodrop ND-1000 spectrophotometer (Labtech International, UK). The amounts of *L. maculans* or *L. biglobosa* DNA in each leaf or stem samples were quantified using a Sigma SYBR Green qPCR kit (Sigma, Gillingham, UK) with specific primers LmacF/LmacR (for *L. maculans*) and LbigF/LmacR (for *L. biglobosa*) (Liu *et al*., 2006; Huang *et al*., 2009). Standard curves were

generated by using known amounts of *L. maculans* or *L. biglobosa* DNA (from 1 to 10^4 pg μL^{-1}) from pure cultures. Results were expressed as amount (pg) of *L. maculans* or *L. biglobosa* DNA in 50 ng total DNA from affected plant tissue.

Samples from spore sampler. Each half tape piece stored in a 1·5 mL tube was cut into six equal sized pieces and then placed in a sterile 2 mL screw-topped tube with acid-washed glass beads (0·15 g; particle size 425 – 600 µm; Sigma, UK). DNA was extracted from the tape pieces using the CTAB protocol (Kaczmarek *et al*., 2009). The amounts of *L. maculans* and *L. biglobosa* DNA were quantified by SYBR green qPCR as described by Kaczmarek *et al*. (2009), using *L. maculans* and *L. biglobosa* specific primers developed by Mahuku *et al*. (1996). The results obtained by qPCR were expressed as amount of *Leptosphaeria* species DNA mL^{-1}. These data were then converted to absolute amounts of DNA and numbers of ascospores of *L. maculans* or *L. biglobosa* deposited on half tape pieces each day.

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Statistical analysis

For data from controlled environment experiments, residual diagnostic plots for analysis on a natural scale indicated that transformation was needed to stabilize the variance. Therefore, the data for size of lesions and amounts of DNA of *L. maculans* or *L. biglobosa* were ln-transformed (natural logarithm) before ANOVA. Amounts of *L. maculans* or *L. biglobosa* DNA on tapes were also ln-transformed. A generalized linear model with a Poisson distribution and logarithmic-link function was fitted to describe the relationship between numbers of ascospores (light microscopy) and the ln-transformed amounts of DNA of *L. maculans, L. biglobosa* or *L. maculans* plus *L. biglobosa* detected by qPCR. To examine differences between *L. maculans* and *L. biglobosa* in the amounts of DNA quantified on tapes in months (August to February) of the 2006/07 and 2007/08 growing

seasons, ANOVA analyses were done. Data from field experiments were analysed using ANOVA to determine whether there were significant differences between different fungicide treatments in severity of phoma leaf spots, growth of *L. maculans* or *L. biglobosa* in stem tissues, severity of stem canker or yield. All the analyses were done using GENSTAT statistical software (Payne *et al*., 2007).

Results

Effects of fungicide treatment on growth of *L. maculans* and *L. biglobosa* in leaf tissues in controlled environment experiments

There was a significant difference in size of leaf lesions between fungicide treatments (P<0·001, SED 0·12, 68 df); spraying with fungicide Punch C significantly decreased the size of leaf lesions of *L. maculans* that developed on cvs Courage and Canberra (Fig. 1). There was also a significant difference in size of leaf lesions between inoculum treatments (P<0·001, SED 0·13, 68 df) but no difference between cultivars. There was an interaction between spray timing and inoculum treatment (P<0·001, SED 0·21, 68 df). For plants inoculated with *L. biglobosa* alone or with a mixture of *L. maculans* and *L. biglobosa*, there was no significant difference in lesion development between cultivars or between fungicide treatments.

There were significant differences in amount of pathogen DNA between fungicide treatments (P<0·001, SED 0·10, 68 df; Fig. 2), inoculum (P<0·001, SED 0·12, 68 df) and cultivars (P<0·001, SED 0·08, 68 df). When the data for *L. maculans* and *L. biglobosa* DNA were analysed separately, sprays of Punch C decreased the amounts of DNA of *L. maculans* (P=0·025, SED 0·27, 5 df) and *L. biglobosa* (P=0·047, SED 0·18, 5 df) in the leaf tissues. There was an interaction between cultivar and inoculum treatment (P=0·007, SED 0·20, 68 df). There was more *L. maculans* DNA in leaves of Courage than leaves of Canberra but there was little difference between the two cultivars in amount of *L. biglobosa* DNA.

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Monitoring the release of *L. maculans* and *L. biglobosa* ascospores

The numbers and patterns of ascospores released varied between growing seasons (Figs 3a,c; 4a,c). In all four growing seasons, few or no ascospores were observed before mid-September. The dates of the first major ascospore release (the date when ≥ 10 ascospores m^{-3} air were collected by the Burkard spore sampler; Huang *et al*., 2005) differed between seasons (Table 1). After the first major release of ascospores was observed, ascospores continued to be released until spring in each season. In 2003/04, when there was little rainfall in September/October, ascospore release started about 2 months later than in the other three growing seasons and there were fewer ascospores released than in the other seasons.

In 2006/07 and 2007/08, periodic changes in amounts of *L. maculans* and *L. biglobosa* DNA (determined by qPCR) showed that there were differences between the two seasons in patterns of *L. maculans* and *L. biglobosa* ascospore release (Fig. 5). In 2006/07, the daily amount of *L. maculans* DNA was greater than that of *L. biglobosa* DNA on most days (Fig. 5a). The mean amount of *L. maculans* DNA was significantly greater than that of *L. biglobosa* DNA (P = 0·001; SED 0·10; 304 df). This suggests that there were significantly more *L. maculans* ascospores than *L. biglobosa* ascospores. There were significant differences between different months in the amount of DNA of *L. maculans* (P <0·001; SED 0·21; 152 df) or *L. biglobosa* (P <0·001; SED 0·20; 152 df). In 2007/08, the amount of *L. maculans* DNA was less than that of *L. biglobosa* DNA on most days (Fig. 5b). The mean amount of *L. maculans* DNA was significantly less than that of *L. biglobosa* DNA (P < 0·001; SED 0·12; 294 df). There were significant monthly differences in amount of *L. maculans* DNA (P <0·001; SED 0·34; 160 df) or *L. biglobosa* DNA (P <0·001; SED 0·25; 160 df).

The estimated amount of DNA per ascospore was 5 pg. The daily amounts of *L. maculans* or *L. biglobosa* DNA were then converted to give the daily numbers of *L. maculans* or *L. biglobosa* ascospores. In both seasons, *L. maculans* ascospores were observed earlier than *L. biglobosa*

ascospores (Fig. 5c, d). After the first major release, ascospores of both species were released on most days in autumn. Regression analysis demonstrated significant (P <0·001) relationships between the number of ascospores counted by light microscopy and the amount of pathogen DNA detected by qPCR. Poisson distributions with logarithmic link functions were fitted to describe relationships between the number of ascospores (counted on half of the spore tape by light microscopy) and amount of *Leptosphaeria* spp. DNA (detected by qPCR on the other half of the spore tape):

$\ln N = 0{\cdot}87\ (0{\cdot}31) + 1{\cdot}33\ (0{\cdot}06)D$ for *L. maculans*

$\ln N = 2{\cdot}31(0{\cdot}26) + 0{\cdot}47(0{\cdot}06)D$ for *L. biglobosa*

$\ln N = 0{\cdot}71(0{\cdot}35) + 0{\cdot}80(0{\cdot}06)D$ for *L. maculans* plus *L. biglobosa*

where N is the number of ascospores, D is ln-transformed amount of pathogen DNA and data in parentheses are standard errors of co-efficients.

(Figs 3, 4, 5 near here)

Effects of fungicide on development of phoma leaf spots and stem canker in winter oilseed rape

In 2003/04, the late release of ascospores was associated with a late start of the phoma leaf spot epidemic (Table 1, Fig. 3). In the 2002/03 season, phoma leaf spots were first observed on 31 October but in the 2003/04 season phoma leaf spots were not observed until 23 December. In both seasons, on untreated plants incidence (% plants affected) of leaf spot reached >90% but the period with an incidence of leaf spot > 80% was longer in the 2002/03 than the 2003/04 season. After the late leaf spotting epidemic in 2003/04, stem canker development started later; phoma stem canker was not observed until 23 May in 2004 while stem canker was observed on 7 May in 2003. Before harvest, the severity of stem canker in untreated plots was less in 2004 than in 2003 (Table 2). Fungicide treatments decreased the severity of stem canker in 2002/03 (P = 0·02; SED 0·22; 6df) and 2003/04 (P <0·001; SED 0·11; 9df), with the late spray decreasing severity more than the early

spray in both seasons. However, there was no significant difference in yield between treated and untreated plots in both seasons (Table 3).

(Table 2 near here)

In 2006/07 and 2007/08, early fungicide treatment decreased the incidence of phoma leaf spot in autumn (Fig. 4b,d). There were differences in control of the epidemics between spray timings and between seasons. There was no significant difference between the two cultivars in incidence of phoma leaf spot, so the data are presented as the means of both cultivars. The development of phoma leaf spots in untreated plots differed between seasons. In 2006/07, incidence of phoma leaf spotting reached 40% in late October with a maximum of 78% plants affected in late November in untreated plots (Fig. 4b). In 2007/08, although leaf lesions were observed in early October, the incidence of leaf spot did not reach 60% until mid-December with a maximum of 70% plants affected in early January in untreated plots (Fig. 4d). In 2006/07, there were significant differences between fungicide treatments in incidence of phoma leaf spot for assessments between 2 November and 14 December 2006 (Fig. 4b). In 2007/08, the epidemic was not severe; there was no significant difference in incidence of phoma leaf spot between fungicide treatments except for the assessment on 11 December 2007 (P <0·05, SED 9·4, 10 df) (Fig. 4d). In both seasons, there were no differences between late-sprayed and untreated plots in incidence of phoma leaf spot (Fig. 4b, d).

Fungicide treatment decreased the severity of phoma stem canker in 2006/07 and 2007/08 (Table 2). In both growing seasons, the severity of stem canker was greatest in untreated plots. In 2006/07, fungicide treatment significantly decreased the final stem canker severity (P <0·001, SED 0·21, 10 df). However, there was no difference in stem canker severity between early sprayed and late sprayed plots. There was a significant difference in stem canker severity between cultivars (P <0·001, SED 0·17, 10 df), with stem canker severity greater on Courage than on Canberra. There was no interaction between fungicide spray timing and cultivar. In 2007/08, after the smaller incidence of phoma leaf spot in autumn, the stem canker severity was less than in 2006/07. There was a

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significant difference in stem canker severity at harvest between cultivars (P<0·01, SED 0·22, 10 df) but there was no difference between fungicide treatments. For cv. Courage, fungicide treatment generally decreased the severity of stem canker by harvest; for cv. Canberra, there was no significant difference in stem canker severity between treated and untreated plots (Table 2).

In 2006/07, there was a significant difference in yield between treated and untreated plots (P <0·005, SED 0·11, 10 df) and between cultivars (P <0·01, SED 0·09, 10 df) (Table 2). Overall, treated plots (4·05 t ha^{-1}) yielded more than untreated plots (3·60 t ha^{-1}); cv. Courage (4·05 t ha^{-1}) yielded more than cv. Canberra (3·74 t ha^{-1}). There was an interaction between cultivar and fungicide treatment (P <0·05, SED 0·16, 10 df). Treated plots of cv. Courage yielded more than untreated plots. However, for cv. Canberra, there was no difference in yield between treated and untreated plots. In 2007/08, there was a significant difference in yield between treated and untreated plots (P <0·01, SED 0·15, 10 df). Overall, treated plots (3·98 t ha^{-1}) yielded more than untreated plots (3·50 t ha^{-1}). Treated plots of cv. Courage yielded more than untreated plots but there was no significant difference in yield between treated and untreated plots of cv. Canberra (Table 2).

Effects of fungicide treatment on growth of *L. maculans* and *L. biglobosa* in winter oilseed rape stem tissues

In 2003 and 2004, effects of fungicide on growth of *L. maculans* and *L. biglobosa* in stem tissues were investigated by isolation of *L. maculans* or *L. biglobosa* cultures from stem base cankers before harvest and by end-point PCR. In 2003, the frequency of detection of *L. maculans* or *L. biglobosa* in stem pith tissue was less in plots treated with either early or late fungicide sprays than in untreated plots (Table3). For example, frequency of detection of *L. maculans* in stem pith tissue was 13% for plants from plots with the late fungicide spray and 47% for plants from untreated plots. Compared with untreated plots, the late spray treatment decreased the frequency of detection of *L. maculans* but increased the frequency of detection of *L. biglobosa* in cortex tissues. In 2004, fungicide treatment

decreased the frequency of detection of *L. maculans* in stem pith tissue but did not affect frequency of detection of *L. biglobosa* (Table 3). Both early and late fungicide treatments decreased the frequency of detection of *L. biglobosa* but did not affect the frequency of detection of *L. maculans* in stem base cortex tissue.

In 2007, when effects of fungicide on growth of *L. maculans* and *L. biglobosa* in stem tissues were investigated by quantification of pathogen DNA in stem tissues using qPCR, fungicide treatment significantly decreased the amount of *L. maculans* DNA in the stem base tissue ($P<0{\cdot}001$, SED 0·24, 72 df) (Fig. 6a). There was no significant difference between cultivars in amount of *L. maculans* DNA and no interaction between cultivar and spray timing. Fungicide treatment significantly decreased the amount of *L. biglobosa* DNA in cv. Canberra ($P<0{\cdot}001$, SED 0·16, 74 df) but not in cv. Courage (Fig. 6b). When *L. maculans* and *L. biglobosa* DNA were analysed together, there was no difference between cultivars but there was a difference between spray treatments ($P = 0{\cdot}017$, SED 2·86, 5 df). The amount of *L. maculans* DNA in the stem base was significantly greater than the amount of *L. biglobosa* DNA ($P<0{\cdot}001$, SED 2·03, 6 df). There was an interaction between fungicide treatment and *Leptosphaeria sp.* ($P = 0{\cdot}009$, SED 3·79, 10 df). Fungicide treatments decreased the amount of *L. maculans* DNA more than amount of *L. biglobosa* DNA. There was a linear relationship between the amount of *L. maculans* DNA in stems and the stem canker severity score at harvest:

$$D = 3{\cdot}77S - 4{\cdot}42,\ R^2 = 0{\cdot}77,$$

where D is amount of pathogen DNA and S is the stem canker score. However, there was no clear relationship between the amount of *L. biglobosa* DNA in stems and the stem canker severity score at harvest.

(Fig. 6, Table 3 near here)

Sensitivity of *L. maculans* isolates to the fungicide flusilazole

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The 123 *L. maculans* isolates showed large differences in sensitivity to flusilazole as measured by ED_{50} value (Hood, unpublished). From these 123 isolates, a subset of 13 isolates with a wide range of sensitivity to flusilazole was selected (Table 4). Using primers LmCYP51F and LmCYP51R, a single PCR product was amplified from *L. maculans* genomic DNA with the expected size of 1632bp. The *LmCYP51* gene was amplified and sequenced from all 13 isolates. Alignment of *LmCYP51* gene sequences of the 13 isolates showed that one isolate (LmMX007) had nucleotide changes at two positions (position 489 changed from T to C; position 901 changed from G to A). Translation into the predicted LmCYP51 protein revealed one nucleotide change to code for amino acid substitution D336G. Alignment with other CYP51 proteins showed that the altered residue in isolate LmMX007 is not conserved. Therefore, this variation in *LmCYP51* probably reflects the unique geographic origin and host source of this individual isolate compared to the other isolates tested. The results indicate that the differences in sensitivity to flusilazole between *L. maculans* isolates were not due to alterations in the *LmCYP51* gene.

(Table 4 near here)

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Discussion

The results of both controlled environment and field experiments indicate that the fungicide Punch C (flusilazole plus carbendazim) decreased *in planta* growth of *L. maculans* in oilseed rape more than that of *L. biglobosa*. For example on cv. Courage, in controlled environment experiments in Punch C treated plants there was a 27-fold decrease in amount of *L. maculans* DNA associated with a decrease in size of leaf lesions by comparison with untreated plants inoculated with *L. maculans*; by contrast there was no decrease in *L. biglobosa* DNA or lesion size in the plants inoculated with *L. biglobosa.* This suggests that Punch C is more effective in controlling the growth of *L. maculans* than that of *L. biglobosa* in oilseed rape leaf tissues. Thus these *in planta* results confirm the conclusions from previous *in vitro* experiments which showed that the triazole fungicides flusilazole

and tebuconazole were more effective against *L. maculans* than against *L. biglobosa* (Eckert *et al*., 2010). This conclusion is further supported by results from the field experiments. Although fungicide treatment decreased the frequency of both *L. maculans* and *L. biglobosa* detected in stem pith in the 2002/03 and 2003/04 seasons, the frequency of *L. maculans* was decreased more than that of *L. biglobosa*. Similarly, although fungicide treatment decreased the amount of DNA of both *L. maculans* and *L. biglobosa* in stem tissues by harvest in 2007/08, the amount of *L. maculans* DNA was decreased more than that of *L. biglobosa* DNA. These results suggest that use of fungicides influences the proportions of these two co-existing *Leptosphaeria* species on oilseed rape, as it influences proportions of the two co-existing *Oculimacula* species that cause eyespot on cereals (Bierman *et al.,* 2002). The results also suggest that control of stem canker by fungicides applied in autumn may reflect these effects of fungicide on the two species, since there was far more *L. maculans* DNA than *L. biglobosa* DNA detected in stem bases of untreated plants, suggesting that phoma stem canker is mainly caused by *L. maculans,* as indicated by previous studies using different methods (West *et al*., 2002a). Furthermore, there was a good relationship between stem canker severity and amount of *L. maculans* DNA in stem tissues but no clear relationship with amount of *L. biglobosa* DNA in stem tissues. There was no evidence of resistance to triazole fungicides in *L. maculans* populations that decreased their effectiveness, unlike for *M. graminicola* populations on wheat in the UK (Cools & Fraaije, 2008; Cools *et al*., 2010). However, there were only 13 isolates analysed and these isolates were from different parts of the world. There is a need to test and sequence the *LmCYP51* gene of more isolates, especially for isolates recovered from regions where fungicides are used regularly for control of phoma stem canker.

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The evidence that ascospores of *L. maculans* are often released before those of *L. biglobosa* in the UK, obtained by combining qPCR with air sampling in 2006/07 and 2007/08, may explain why the early fungicide treatment decreased the amount of *L. maculans* DNA in stem tissues in 2006/07 more than did the late treatment, whereas the late treatment decreased the amount of *L.*

biglobosa DNA more. This direct evidence for differences between the two species in timing of ascospore release is supported by previous indirect evidence through assessing leaf spotting (West *et al.,* 2002a) and explains how the two species co-exist on oilseed rape in the UK through separation in time (Fitt *et al.,* 2006a). Given the differences between the two species in their contribution to the severity of phoma stem canker epidemics, these results provide evidence for the benefits of early fungicide sprays timed to coincide with the first appearance of leaf spots in autumn (West *et al*., 2002b; Gladders *et al.,* 2006; Steed *et al.,* 2007). The major release of ascospores in 2003/04 that started 2 months later and was in smaller numbers than in 2002/03 led to a less severe stem canker epidemic, confirming previous work over different seasons and regions suggesting that the timing and number of ascospores released in autumn affects the severity of stem canker epidemics the following summer (West *et al.,* 2002b; Huang *et al*., 2005; Evans *et al.,* 2008). Quantitative PCR has also been used to demonstrate seasonal differences in production of ascospores of the two species in Poland (Kaczmarek *et al.*, 2009). It is likely that effects of fungicide on relative amounts of *L. maculans* and *L. biglobosa* DNA in stems from the previous season contribute to differences in production of ascospores on stem debris between seasons, since the conditions for production and maturation of these two species are similar (Toscano-Underwood *et al*., 2003). Differences in type and timing of fungicide use may also contribute to the regional differences in proportions of the two species in the UK (Stonard *et al.,* 2010a, 2010b).

These results demonstrate how use of qPCR on air samples to distinguish the two species can improve disease forecasting to optimise fungicide use (West *et al.,* 2008). Results of field experiments in 2006/07 and 2007/08 suggest that the time and relative number of ascospores of *L. maculans* and *L. biglobosa* affect the severity of phoma stem canker epidemics. In 2006/07, ascospores of *L. maculans* were released earlier and in larger numbers than those of *L. biglobosa*. By contrast, in 2007/08 ascospores of *L. biglobosa* were released in larger numbers than those of *L. maculans.* This difference between seasons in composition of airborne inoculum may have

contributed to the difference in severity of stem canker epidemics, with the stem canker epidemic less severe in 2007/08 than 2006/07. The qPCR technique is also being used in diagnostic estimation of spores and mycelium of other fungal pathogens (Fountaine *et al*., 2007; Luo *et al*., 2007) and in detection of avirulence alleles in *L. maculans* airborne inoculum (Van de Wouw *et al*., 2010). A function fitting analysis using the amounts of DNA of the *Leptosphaeria* spp. on tapes and ascospore counts suggests that the qPCR technique is more accurate for estimation of relative number of ascospores of *L. maculans* and *L. biglobosa* when larger numbers of ascospores are observed on the tapes, as it is for estimation of frequencies of avirulence alleles of *L. maculans* from airborne inoculum (Van de Wouw *et al*., 2010). Deployment of a regional network of spore samplers in the oilseed rape growing areas of the UK and DNA-based analysis of the samples to determine the composition of pathogen populations during the autumn period when spray decisions are made (West *et al*., 2008) could greatly benefit the industry by improving effectiveness of control and decreasing national losses from phoma stem canker.

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These results suggest that effects of fungicide on interactions between *L. maculans* and *L. biglobosa* in autumn play an important role in determining the relationship between autumn fungicide timing and severity of phoma stem canker the following summer. In controlled environment experiments, in untreated leaves inoculated with *L. maculans*, the lesions were larger and contained more *L. maculans* DNA than in untreated leaves inoculated with a mixture of *L. maculans* and *L. biglobosa*, providing further evidence that there are interactions between *L. maculans* and *L. biglobosa*. Previous studies have shown that *L. biglobosa* can activate local and systemic defence against *L. maculans* in oilseed rape (Mahuku *et al*., 1996; Chen & Fernando, 2006; Liu *et al*., 2006). Treatment with fungicide decreased the size of lesions in leaves inoculated with *L. maculans* but did not affect the size of lesions in leaves inoculated with a mixture of *L. maculans* and *L. biglobosa*, suggesting that interactions between *L. maculans* and *L. biglobosa* can affect the effectiveness of fungicide treatments. In the UK, ascospore release lasts for 4-5 months (Huang *et*

al., 2005); thus the timing and relative numbers of *L. biglobosa* and *L. maculans* ascospores released will affect the interactions between *L. maculans* and *L. biglobosa* and effectiveness of fungicide treatments. Timing of fungicide application in autumn is crucial to prevent the spread of *L. maculans* from leaf lesions to stems, since in autumn plants are generally smaller and temperatures greater than later in the season, so that the pathogen can grow rapidly along the petiole to the stem (West *et al.*, 2002a; Huang *et al*., 2006). To control stem canker epidemics, it is recommended that fungicide is applied when a threshold of 10% plants with phoma leaf spots is reached (Gladders *et al.,* 2006; http://www.rothamsted.ac.uk/leafspot). Results of this work demonstrate forecasting to optimise control of phoma stem canker epidemics and increase yield can be improved if the spray timing is accurately based on information about timing and abundance of ascospores of *L. maculans* and *L. biglobosa*, combined with effective deployment of quantitative (Huang *et al.,* 2009) and major gene resistance in oilseed rape cultivars (Brun *et al.,* 2010).

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Table 1 Dates of fungicide Punch C (flusilazole plus carbendazim) applications, cultural procedures and dates of stages in phoma leaf spot/stem canker epidemic development in field experiments with winter oilseed rape at Rothamsted in four growing seasons. Release of ascospores was monitored using a Burkard spore sampler; observations of first phoma leaf spot lesions (in autumn) and first stem cankers (in spring) were made on untreated plots.

		2002/03	2003/04	2006/07	2007/08
Fungicide	Untreated	none	none	none	none
regime	Early spray[a]	4 Nov	24 Nov	13 Oct	24 Oct
	Late spray[b]	16 Jan	9 Feb	13 Nov	22 Dec
Cultural	Cultivar	Apex	Apex	Courage,	Courage,
procedure				Canberra	Canberra
	Sowing date	20 Aug	26 Aug	24 Aug	22 Aug
	Harvest date	14 July	2 Aug	20 July	15 July
Epidemic	First ascospores[c]	20 Oct	12 Dec	22 Sept	17 Sept
development	First leaf spotting[d]	31 Oct	23 Dec	28 Sept	3 Oct
	First stem canker[e]	7 May	23 May	2 May	12 Jun

[a]Date of early spray was adjusted according to the date when 10% plants were affected with phoma leaf spot.

[b]Date of late spray was 1-2 months after the first spray when weather and ground conditions permitted.

[c]First date when ≥ 10 ascospores m^{-3} air were collected by a Burkard spore sampler.

[d]First date when ≥ 5% plants were affected in untreated plots.

[e]First date when ≥ 5% plants had stem base canker in untreated plots.

Table 4 Isolates of *Leptosphaeria maculans* used for amplification and sequencing of the *LmCYP51* gene. These isolates differ in sensitivity to flusilazole *in vitro*, as measured by ED_{50} (effective dose of 50% of growth inhibited).

Isolate	Source	Region	Year	ED_{50} (µg mL^{-1})
06R4.4	*B. napus*	Rothamsted, UK	2006	0·013
06R6.3	*B. napus*	Rothamsted, UK	2006	0·047
06R41	*B. napus*	Daglingworth, UK	2006	0·045
UK1	*B. napus*	Rothamsted, UK	1996	0·041
ME24	*B. napus*	Darrington, UK	2002	0·085
P27d	*B. napus*	Rennes, France	1996	0·003
LmCa06	*B. rapa*	Alberta, Canada	1989	0·041
IBCN51	*B. napus*	Saskatchewan, Canada	1992	0·291
99-79	*B. napus*	Canada	1999	0·033
IBCN15	*B. napus*	Victoria, Australia	1988	0·042
LmMX007	*B. oleracea*	Mexico	2002	0·281
LmAT009	*B. napus*	St. Pölten, Austria	2003	0·009
LmSE009	*B. napus*	Poznan, Poland	2006	0·035

Table 2 Effects of fungicide Punch C (flusilazole plus carbendazim) treatment on severity of basal phoma stem canker and yield of winter oilseed rape in the 2002/03, 2003/04, 2006/07, 2007/08 growing seasons at Rothamsted. Severity of basal stem cankers was assessed 2 weeks before harvest on a 0-6 scale. Plots were combine harvested and the yield determined as 90% seed dry weight.

		Cultivar	Untreated	Early[a] spray	Late[a] spray	SED[b]	df
2003	Canker severity[c]	Apex	2·3	1·9	1·6	0·22	6
	Yield (t ha^{-1})	Apex	3·1	3·1	3·2	0·13	6
2004	Canker severity	Apex	1·0	0·7	0·3	0·11	9
	Yield (t ha^{-1})	Apex	2·3	2·8	2·6	0·17	9
2007	Canker severity	Courage	2·5	1·6	2·4	0·29	10
		Canberra	2·0	1·0	1·2		
	Yield (t ha^{-1})	Courage	3·5	4·3	4·4	0·16	10
		Canberra	3·7	3·8	3·8		
2008	Canker severity	Courage	1·3	1·0	0·6	0·38	10
		Canberra	0·1	0·2	0·2		
	Yield (t ha^{-1})	Courage	3·6	4·0	4·2	0·21	10
		Canberra	3·5	3·8	4·0		

[a]Dates of fungicide sprays are given in Table 1.

[b]Approximate maximum SED.

[c]Stem canker severity was assessed on a 0-6 scale (0, healthy stem with no affected tissue; 1, 1–25% of the cross-section affected; 2, 26 – 50% of the cross-section affected; 3, 51– 75% of the cross-section affected; 4, 76 – 99% of the cross-section affected; 5, 100% of the cross-section affected, plant still alive; 6, 100% affected, dead stem with a hollow or severely necrotic pith).

Table 3 Frequency (%) of occurrence of *Leptosphaeria maculans* or *L. biglobosa* in basal phoma stem cankers on winter oilseed rape (cv. Apex) that had received different fungicide treatments in the 2002/03 and 2003/04 growing seasons. Two weeks before harvest in July 2003 and 2004, 30 plants were sampled from plots which had received no fungicide spray (untreated), an early spray or a late spray, and a small tissue sample was taken from the stem base cortex and pith tissue of each plant. These tissues were subdivided and the occurrence of *L. maculans* and *L. biglobosa* in these samples was determined by isolation of fungal cultures and end-point PCR.

		Frequency (%) of occurrence of *L. maculans* or *L. biglobosa*[a]			
		2003		2004	
		Cortex	Pith	Cortex	Pith
Untreated	Lm	64	47	77	87
	Lb	33	33	13	0
Early spray[b]	Lm	80	30	90	80
	Lb	7	0	0	3
Late spray[b]	Lm	44	13	80	37
	Lb	40	27	7	0

[a]Data represent the proportion of plants (out of 30) with the *Leptosphaeria* sp. in the relevant stem tissues.

[b]Dates of fungicide sprays are given in Table 1.

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Figure 1 Effects of the fungicide Punch C (flusilazole plus carbendazim) on development of lesions caused by *Leptosphaeria maculans* or/and *L. biglobosa* on leaves of oilseed rape cvs Courage (a) or Canberra (b) in a controlled environment experiment with five replicates. Plants were inoculated with ascospores of *L. maculans* (Lm), *L. biglobosa* (Lb) or a mixture of *L. maculans* and *L. biglobosa* (LmLb). There were three fungicide treatments; untreated, sprayed at 6 or 11 days post inoculation (dpi). The length and width of leaf lesions were measured at 20 dpi, then lesion areas were estimated by multiplying lesion length by lesion width. Vertical bars are standard deviations. Data presented are not transformed; ANOVA was done with ln-transformed (natural logarithm) data (SED 0.21, 68 df).

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Figure 2 Effects of the fungicide Punch C (flusilazole plus carbendazim) on growth of *Leptosphaeria maculans* or *L. biglobosa* in leaves of oilseed rape cvs Courage (a) and Canberra (b) in a controlled environment experiment with five replicates, measured by quantification of pathogen DNA using quantitative PCR. Plants were inoculated with ascospores of *L. maculans* (Lm), *L. biglobosa* (Lb) or a mixture of *L. maculans* and *L. biglobosa* (LmLb). LmLb-Lm represents *L. maculans* DNA quantified from leaves inoculated with LmLb; LmLb-Lb represents *L. biglobosa* DNA quantified from leaves inoculated with LmLb. There were three fungicide treatments; untreated, sprayed at 6 or 11 days post inoculation (dpi). Inoculated leaves were detached at 22 dpi for DNA extraction and quantitative PCR. Vertical bars are standard deviations. Data presented are not transformed; ANOVA was done with ln-transformed (natural logarithm) data (SED 0.28, 68 df).

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Figure 3 Changes in numbers of air-borne ascospores of *Leptosphaeria maculans* and *L. biglobosa* (a, c) in relation to incidence (% plants affected) of phoma leaf spot (b,d) on winter oilseed rape (cv. Apex) in 2002/03 (a,b) and 2003/04 (c,d) growing seasons at Rothamsted. Ascospore release was monitored using a Burkard spore sampler; the incidence of phoma leaf spots in autumn was assessed on plants sampled from plots that had not received a fungicide treatment. Dates of fungicide Punch C (flusilazole plus carbendazim) application are indicated by arrows (Table 1).

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Figure 4 Changes in numbers of air-borne ascospores of *Leptosphaeria maculans* and *L. biglobosa* (a, c) in relation to incidence (% plants affected) of phoma leaf spot (b, d) on winter oilseed rape cvs Courage and Canberra (results presented as mean of both cultivars) with different fungicide treatments in the 2006/07 (a, b) and 2007/08 (c, d) growing seasons at Rothamsted. Ascospore release was monitored using a Burkard spore sampler. Fungicide Punch C (flusilazole plus carbendazim) treatments (dates of fungicide application are indicated by arrows) were untreated (□), early spray (●) or late spray (▲) (Table 1).

Figure 5 Mean daily amounts of DNA (a, b) or number of ascospores (c,d) of *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) detected on half of a Burkard spore sampler tape during the 2006/07 (a,c) and 2007/08 (b,d) winter oilseed rape growing seasons at Rothamsted. The Burkard spore sampler was surrounded by oilseed rape debris with stem canker from the previous growing season. The amounts of *L. maculans* or *L. biglobosa* DNA extracted from half of the tapes from the spore sampler were quantified using quantitative PCR; the numbers of ascospores of each species were then estimated from the amount of DNA (the mean amount of DNA per ascospore was 5 pg).

Figure 6 Effects of fungicide Punch C (flusilazole plus carbendazim) treatments on growth of *Leptosphaeria maculans* (a) and *L. biglobosa* (b) in stem tissue of winter oilseed rape cvs Courage or Canberra in the 2006/07 growing season. Ten plants were sampled from each plot on 2 July 2007 before harvest and a 6 cm long piece of stem base was sampled from each plant for DNA extraction. The growth of the pathogens in stem tissues was measured by quantification of the pathogen DNA using quantitative PCR. Vertical bars are SED (6 df).

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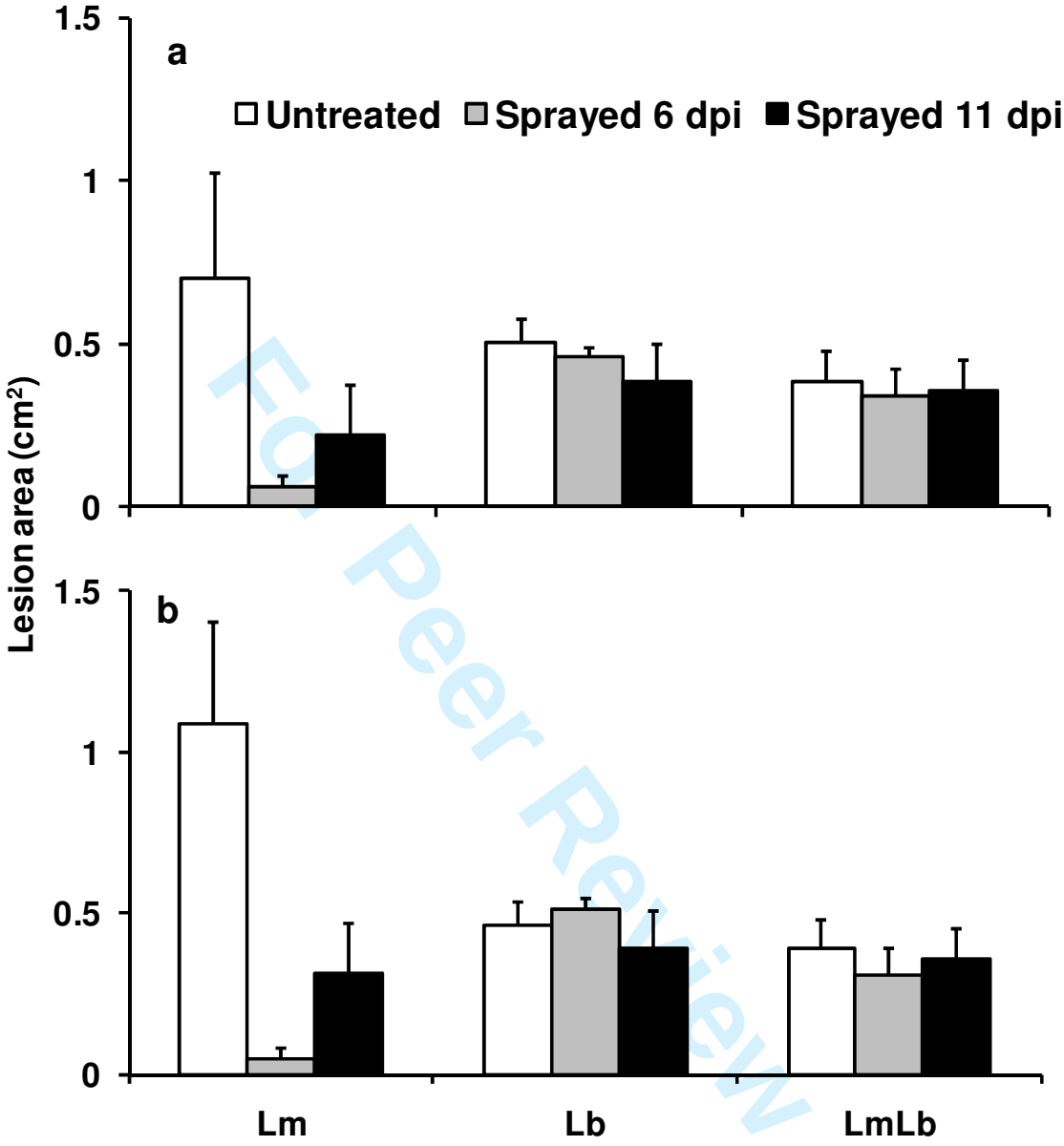
Fig. 1



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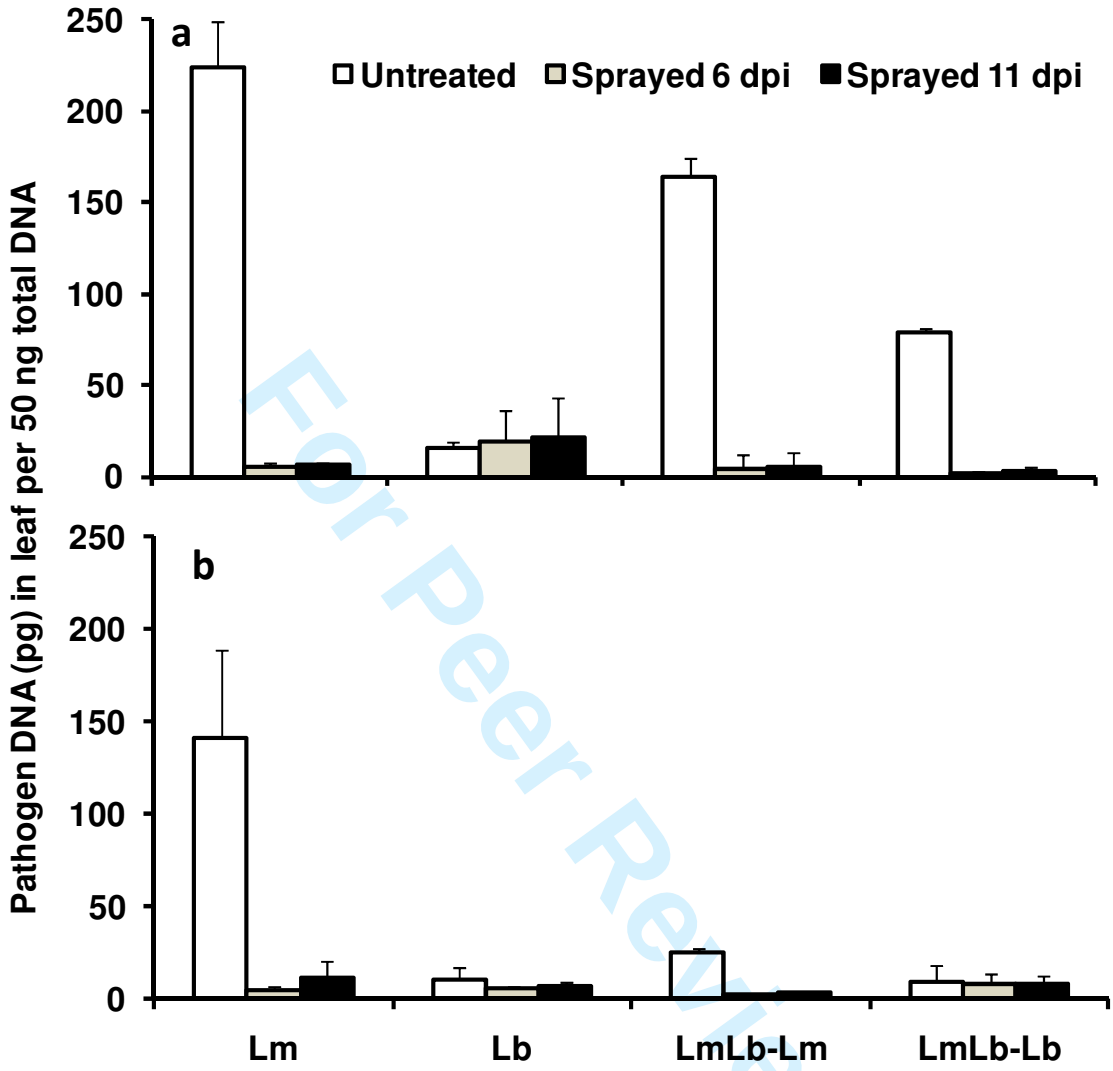
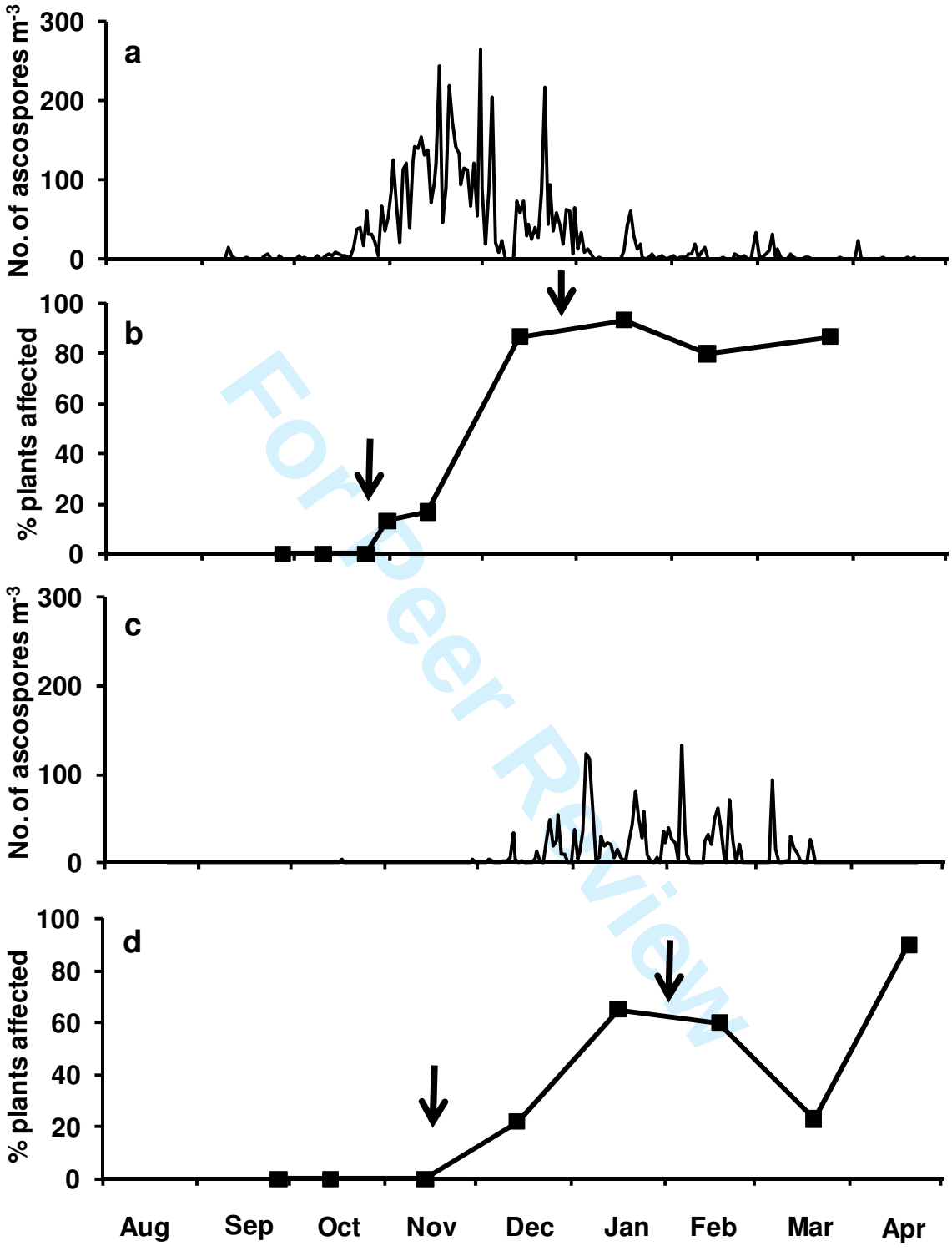


Fig. 3



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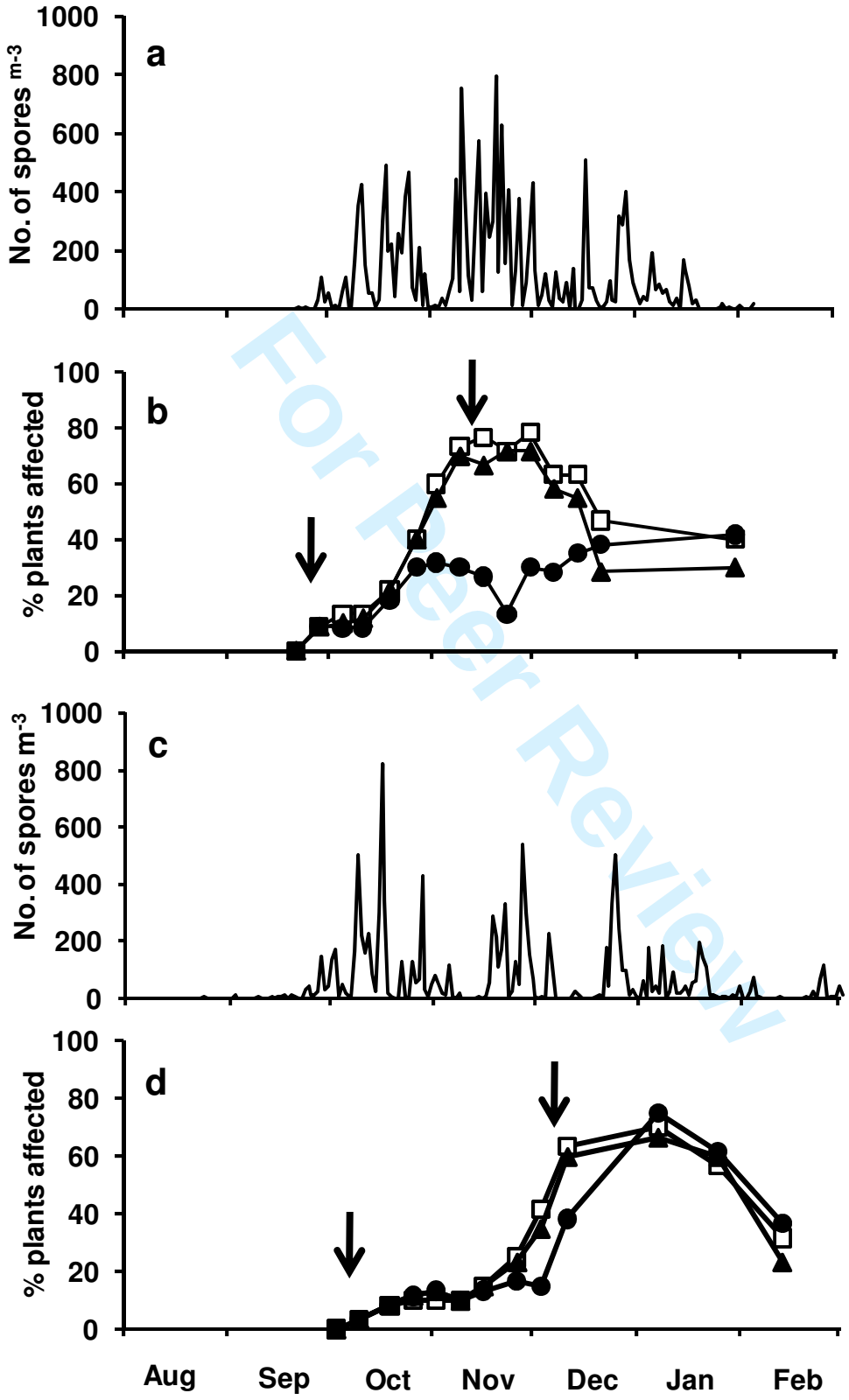

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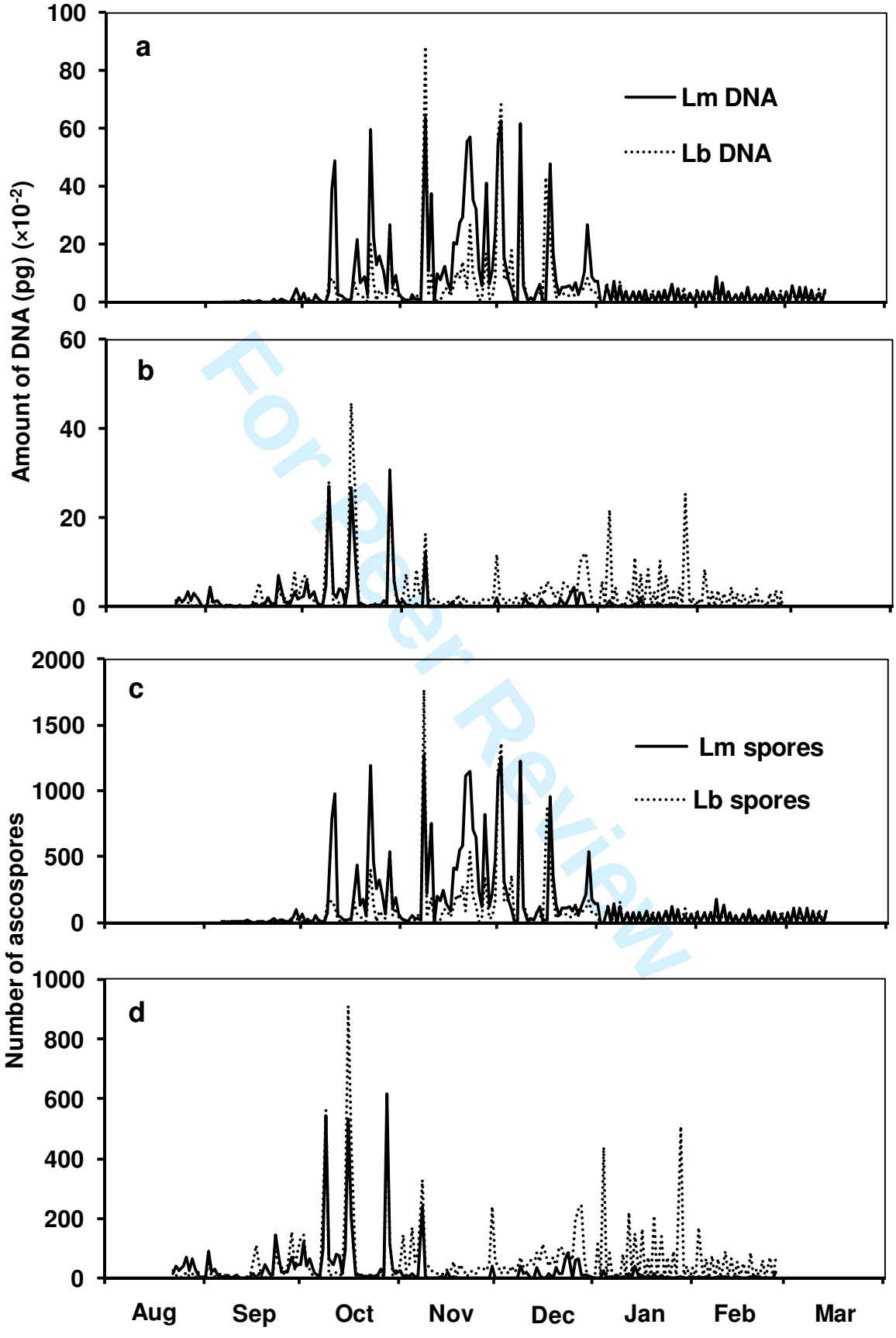

Fig.6