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The role of seeds and airborne inoculum in the initiation of leaf blotch (*Rhynchosporium secalis*) epidemics in winter barley

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Both airborne spores of *Rhynchosporium secalis* and seed infection have been implied as major sources of primary inoculum for barley leaf blotch (scald) epidemics in fields without previous history of barley cropping. However, little is known about their relative importance in the onset of disease. Results from both quantitative real-time PCR and visual assessments indicated that seed infection was the main source of inoculum in the field trial conducted in this study. Glasshouse studies established that the pathogen can be transmitted from infected seeds into roots, shoots and leaves without causing symptoms. Plants in the field trial remained symptomless for approximately four months before symptoms were observed in the crop. Covering the crop during part of the growing season was shown to prevent pathogen growth, despite the use of infected seed, indicating that changes in the physiological condition of the plant and/or environmental conditions may trigger disease development. However, once the disease appeared in the field it quickly became uniform throughout the cropping area. Only small amounts of *R. secalis* DNA were measured in 24 h spore-trap tape samples using PCR. Inoculum levels equivalent to spore concentrations between 30 and 60 spores per m^3 of air were only detected on three occasions during the growing season. The temporal pattern and level of detection of *R. secalis* DNA in spore tape samples indicated that airborne inoculum was limited and most likely represented rain-splashed conidia rather than putative ascospores.

Keywords: barley leaf scald, real-time PCR, seed infection, spore trap

Introduction

Leaf blotch infections caused by *Rhynchosporium secalis* are common in winter and spring barley grown in the whole of the UK (Zhan *et al*., 2008), but disease is especially severe in the west of England and in Scotland due to increased humidity. However, the levels of disease in crops can alter from year to year, with the cost of losses in winter barley estimated at £2·57 million due to *R. secalis* infection alone in fungicide-treated crops (Hardwick *et al.,* 2002). Initial infection can result from infected barley stubble and/or crop debris on the soil (Stedman, 1977; Fitt *et al.,* 1986) and most commonly occurs when successive barley crops have been grown in the same field. Symptom development in fields with no previous history of barley cropping can arise from the sowing of infected seed or by infection from air-dispersed putative ascospores. Once infection is established in a crop, conidia and/or putative ascospores can then be easily spread throughout the crop by rain splash or wind.

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It was suggested by Hauptfleisch (1929) that *R. secalis* infection was seed-borne, and it is thought that seed infection could be extremely important for the introduction of *R. secalis* into new areas with no history of barley cultivation (Habgood, 1971; Lee *et al.,* 2001). Skoropad (1959) demonstrated that the efficacy of disease transmission on seeds was dependent on seed germination rates and temperature. Habgood (1971) and Kay & Owen (1973) showed that leaf blotch symptoms could develop from symptomless seeds. At present, barley seeds are examined for *R. secalis* infection by visual assessments. The symptoms of leaf blotch generally occur on the lemmas and paleas producing characteristic irregular or elliptical lesions with dark brown edges and pale centres (Lee *et al.,* 2001). Lee *et al.* (2002) using competitive PCR demonstrated that visual assessments correlate poorly with *R. secalis* DNA levels from the same seed. Recent studies by Brunner *et al.* (2007) demonstrated that UK *R. secalis* populations are genetically very similar to those found in Australia and that the initial establishment of this population was probably due to the transportation of seed from old British cultivars during the early part of the last century.

Another possible source of inoculum is ascospores, although no teleomorph of *R. secalis* has yet been

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discovered. Recent work using DNA-based markers has confirmed that *R. secalis* populations are highly genetically variable, with the majority of variation distributed within individual fields (McDonald *et al.*, 1999; Salamati *et al.*, 2000). Therefore, it was suggested that sexual recombination events are common in populations of *R. secalis*, and this is of major importance for its epidemiology and adaptation to the environment (Salamati *et al.*, 2000). However, somatic recombination in *R. secalis* has also been demonstrated in the laboratory (Newton, 1989), although these events are not thought to occur in field conditions. Two opposite mating type genes have been shown to be present in the UK *R. secalis* population, but it is not known if these genes are functional (Foster & Fitt, 2003). Linde *et al.* (2003) demonstrated that in many locations the two mating type genes are found in equal frequencies, indicating that *R. secalis* is undergoing regular sexual recombination. However, despite the evidence indicating that sexual recombination can occur, sexually produced spores have not, so far, been observed.

A better understanding of the importance of different inoculum sources will enable more effective control strategies to be developed. The objective of this study was to investigate the role of seeds and airborne inoculum in the initiation of barley leaf blotch epidemics in a field experiment where crop debris was excluded as a potential inoculum source. The rationale for covering the crop until disease symptoms appeared in the remaining plots was to test the hypothesis that conidia and ascospore release from plant material in autumn and winter could initiate epidemics of leaf blotch in barley crops. This had previously been indicated in research by Shaw & Royle (1989). The study used conventional visual plant pathological techniques, in combination with spore trapping and quantitative real-time PCR developed previously by Fountaine *et al.* (2007).

Materials and methods

Field trial at Rothamsted

The area chosen had not been cropped with barley for the last five years, having been fallow for the last two years and prior to that cropped with spring oilseed rape. The nearest barley stubble field was at least 0·8 km away and the plots were about 500 m from grassland (Rothamsted sports field). The whole field measured 33 × 14 m and contained 12 small plots (3 × 3 m) of winter barley cv. Siberia, which showed moderate to high resistance to barley leaf blotch (rating = 7) during the HGCA recommended list trials of 2004/5. A path of at least 3 m wide sown by seed drill with winter wheat cv. Hereward surrounded each plot (Fig. 1). The barley plots were either sown with 'clean' seed from Dalgety Agriculture (Bury St Edmunds, UK) or farm-saved 'infected' seed harvested in the previous year from untreated plots at Rothamsted. The certified barley seeds supplied by Dalgety Agriculture had been visually assessed and were found to be free of disease symptoms, whereas most of the farm-saved infected seeds showed symptoms of *R. secalis* on the surface of the seeds. All seeds were sown without the use of seed treatments on 23 September 2003 at a drill rate of 350 seed m^{-2}. Immediately after sowing a pre-emergence herbicide (flufenacet and pendimethalin (Crystal), BASF Agricultural Products) was applied at a rate of 4 L ha^{-1}. Tents manufactured from aluminium tubing and acetate clear plastic (Fig. 2) were placed in the centre of selected plots (Fig. 1) to exclude airborne inoculum (Shaw & Royle, 1989). By covering the crop, the effect of excluding conidia and airborne spores as an inoculum source, which is an issue in other closely related species such as light leaf spot of oilseed rape (*Pyrenopeziza brassicae*), could be tested in the period of the growing season when ascospore release is anticipated. The covers were later removed in order to determine whether the clean barley plants which had been excluded from airborne inoculum could subsequently become infected. The use of wheat plants between the plots was to help minimise the spread of inoculum from plot to plot via rain splash. The mini tents were secured in the soil to a depth of 250 mm, covering all the plants in the centre square metre of each plot in a 'miniature greenhouse'. All tents were regularly checked and any holes repaired using parcel tape until the development of disease symptoms, after which all the tents were removed from the trial plots. None of the plots in the field experiment received any fungicide applications throughout the growing season, but all other inputs were made according to standard farm practice with a spring

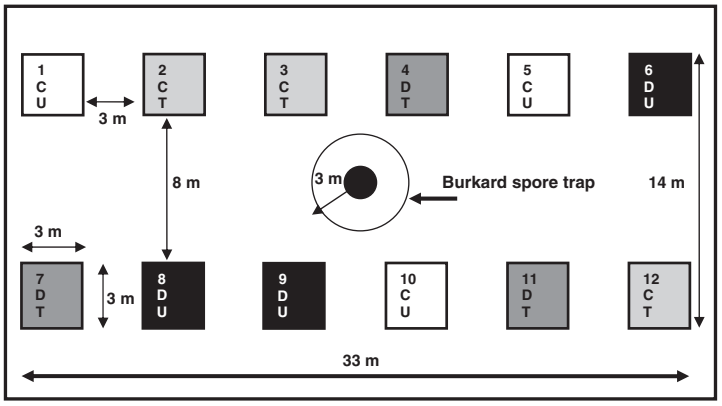

Figure 1 Layout of the contiguous block field experiment. Twelve 3 × 3 m plots of winter barley, separated by 3 m gaps of winter wheat. Plots designated with C and D were drilled with symptomless clean and farm-saved infected seed, respectively. Plots designated with T and U were covered with tents or left uncovered, respectively. All plants within 3 m of the spore traps were removed.

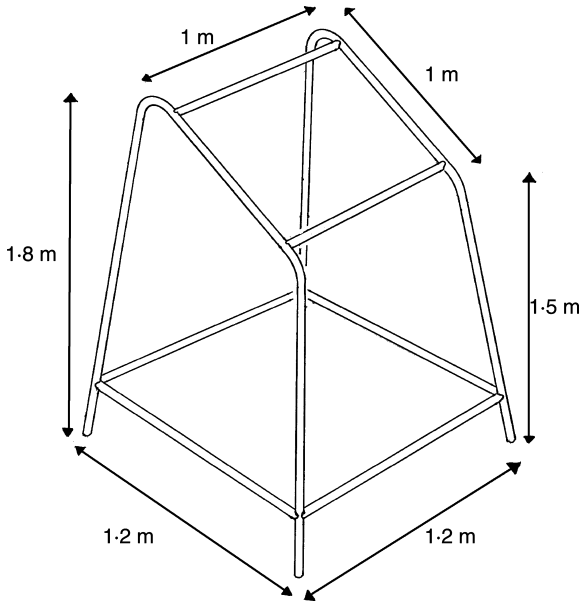

Figure 2 The basic aluminium frame of the tent manufactured from 2·5 cm diameter tubing.

application of 167 kg ha^{-1} of ammoniacal nitrogen 18·4%, nitric N 11·6% and water-soluble sulphur (SO_3) 19·0% (Sulphur Gold, TERRA Nitrogen UK Ltd). The crop was harvested on 7 September 2004.

Field trial sampling and visual assessments

Field assessments were carried out every week to check for the development of disease symptoms, until the time that they first appeared in multiple plots (symptoms were visible in all uncovered plots sown with farm saved seeds on 17 February). All tents were then removed and plants were sampled from each of the nine, 1 m^2 subplots, contained in all 12 of the main plots. The visual assessments for *R. secalis* leaf blotch were carried out by counting the number of lesions on whole plants, and the plant growth stages were also recorded for each subset of 10 plants sampled. Following a disease assessment, all plants were discarded except for the 10 plant samples from the central 1 m^2 subplot. Plants from this subplot were further dissected, with each plant being separated into roots, white stems (colourless and located below the surface of the soil), green stems (found above the surface of the soil) and leaves (10 of each for each plant section, from three replicates). These were then frozen for future DNA extraction and PCR testing. The same procedures were used for samples taken on 24 April 2004. Visual assessments were also performed on 10 plants per plot during each month of the growing season.

Trapping of airborne inoculum

A Burkard 7-day recording volumetric spore trap (Burkard Manufacturing Co. Ltd.) was set up in the central strip of the field immediately after the crop was sown with wheat (Fig. 1). A 3 m radius directly surrounding the spore trap was kept clear to decrease rain splash-dispersed spores being sucked into the trap. The spores were collected on Melinex polyester film tape (Burkard Manufacturing) coated with a mixture of paraffin wax and petroleum jelly. The tape was replaced every seven days. For analysis, the tape was cut into 48 mm sections, each representing a 24 h period.

DNA extraction and quantification of *R. secalis* DNA using real-time PCR

DNA was extracted from all sampled plant and fungal material using the method of Fraaije *et al.* (1999) except that the DNA extraction buffer was amended with 5 mM 1,10-phenanthroline monohydrate and 2% (wt/vol) polyvinylpyrrolidone K30 (Sigma-Aldrich Chemie GmbH) to clean the DNA (Zhang & Stewart, 2000). The DNA was quantified using the fluorescent dye thiazole orange (Sigma-Aldrich) as described previously (Fraaije *et al.*, 2005). Spore tape DNA extractions were done using Ballotini beads (Jencons Ltd.) in a FastPrep machine (Savant Instruments) as described by Calderon *et al.* (2002). DNA was extracted from half of each 24-h tape sample and 1 μL from 200 μL final DNA solution was used in each PCR assay. Quantitative real-time PCR measurements were carried out in a Stratagene Mx3000P real-time PCR machine (Stratagene, La Jolla, California, USA) with the cytochrome *b* Locked Nucleic Acid (LNA) probe assay recently developed by Fountaine *et al.* (2007).

Determination of *R. secalis* seed infection and its transmission in controlled environment studies

Seeds from the two seed batches used in the field experiment were visually assessed and tested by real-time PCR to establish differences in seed infection levels and to determine whether symptomless seeds were infected with *R. secalis*. Five randomly selected seeds with, and five seeds without symptoms on the grain surface of each seed batch were analysed.

For the controlled environment study, five hundred seeds from each seed batch were sampled. Each seed was thoroughly examined with a hand lens and were separated into seed lots with and without symptoms. Symptomless seeds from the 'clean' seed batch and seeds with symptoms from the farm-saved 'infected' seed batch were sown into autoclaved compost (John Innes, No 2) in propagator trays (50 × 20 cm) and incubated at 5, 10, 16, 20 and 25°C in separate controlled environment cabinets (Fisons 600H). The light intensity used was 200 μ Einstein m^{-2} s^{-1} PAR. After germination, the seedlings in the trays were watered daily, so that the soil always remained damp regardless of the incubation temperature. Seedlings were sampled at growth stage (GS) 8–9 (coleoptiles breaking through soil surface) and at GS 14–15 (4 or 5 leaves unfolded). These samples were taken on different sampling dates, as the growth of the seedlings varied,

depending on the incubation temperature. Ten seedlings were sampled at each growth stage using ethanol-cleaned forceps. Seedlings were dissected into lower root, seed, upper root and above soil stem/leaf sections and *R. secalis*-infection levels determined using real-time PCR. Microscopy was carried according to the methods developed by Davis & Fitt (1990) to examine the plant material for fungal structures of *R. secalis*.

Data analysis

The visual assessment and quantitative real-time PCR data sets were transformed using natural logarithms, ln(x + 0·5) for visual data and ln(x + 0·1) for PCR data, to normalize the residuals from fitted models. Results were examined with a 2-way analysis of variance (ANOVA) using Genstat, 8th edition, (VSN International, Ltd), with plant, plot and block as random factors. The weather data for the 2003–2004 growing season were obtained from the Electronic Rothamsted

Archive Meteorological Database (http://www.era.iacr.ac.uk).

Results

Effect of seed source and covering of plants on leaf scald epidemics

The first symptom of leaf blotch, a single lesion in one of the plots drilled with farm-saved infected seed, was seen on 3 February 2004. The tents were removed two weeks later when all uncovered plots sown with farm-saved infected seed showed symptoms. Plants from farm-saved seed had an average of 5·6 lesions per plant compared to the symptomless clean seed which had only 0·18 lesions per plant (Table 1). However, covering the crop significantly reduced the formation of symptoms; at the time the tents were removed, there were no symptoms of blotch in any of the covered areas, whether drilled with symptomless clean or farm-saved infected seed. Disease was recorded in seedlings surrounding the tents in the same plots (data not shown). Following removal of the tents, disease levels in these previously covered areas remained lower than in plots that had not been covered (Fig. 3). In the covered plots, disease levels in plots drilled with clean seed remained much lower than in those drilled with farm-saved infected seed, and the same effect was also seen for the plots that were not covered. Results obtained from the ANOVA on all plots showed that tenting greatly reduced symptoms of *R. secalis* ($P < 0·001$) and plots sown with clean seed had less disease than plots sown with farm-saved infected seed ($P < 0·001$). The interaction was significant ($P < 0·001$) because the covered plants were free of symptoms regardless of seed source. The difference between the seed types and the effect of covering the crop with tents both remained highly significant ($P < 0·001$) for up to two months after the removal of the tents. However, four months after removal of the tents disease severity levels were relatively closer, the main effect differences were highly significant ($P = 0·006$ for seed source, $P < 0·001$ for tenting) and the interaction was no longer significant ($P = 0·4$).

Table 1 Visual assessment of barley leaf blotch, recorded at GS 23–34 (17 Feb 2004) and GS 77 (2 Jun 2004). Samples were taken from the centre 1 m^2 subplot of plots of winter barley grown from clean (symptomless) or farm-saved (infected) seed sources and covered or left open until then. Ten plants per subplot were assessed. Back transformed mean values per plant are given

Seed	Tent cover	Leaf blotch[a] GS 23–34	Leaf blotch[b] GS 77
Clean	Open	0·18	5·3
	Tented	0	0·95
Farm-saved	Open	5·60	11·2
	Tented	0	3·6
cv of mean[c]		12%	21%

[a]Disease expressed as number of lesions per plant. Analysed on log scale: main effects and interaction significant ($P < 0·001$, df 1,6). Block and plot variances not significantly larger than plant-plant variance.
[b]Disease expressed as number of lesions per plant. Analysed on log scale: main effects of seed source, $P < 0·006$ (1,6 df); tenting $P < 0·001$. Interaction $P = 0·4$. Blocks did not differ more than expected from plot-plot variation, but this was significantly more than plant-plant variation ($P < 0·001$; 6108 df).
[c]Coefficient of variation from sem on log scale; this variability applies to any mean in the column.

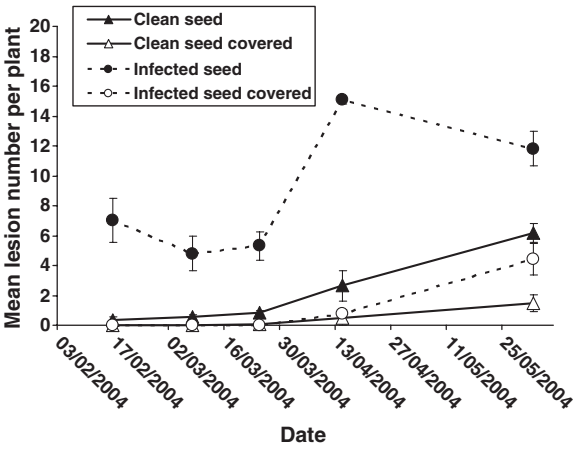

Figure 3 The average number of leaf blotch (*Rhynchosporium secalis*) lesions per plant for barley sown from symptomless clean seed or farm-saved infected seed based on analysis of 10 plants sampled from the centre 1 m^2 subplot. Each of the treatment combinations were replicated three times in the contiguous block design. The plastic covers were removed for the crop on 3 Feb 2004. Bars indicate standard error of the mean.

Detection of *R. secalis* in plant samples using real-time PCR

All plant materials (roots, seeds, white stems, green stems and leaves) sampled on 17 February from plots which never had covers, had detectable amounts of *R. secalis*

DNA in real-time PCR throughout the trial. However, plant samples from uncovered plots sown with farm-saved infected seeds (plots 6, 8 and 9) had higher levels of *R. secalis* than those collected from uncovered plots sown with clean seeds (plots 1, 5 and 10). The roots sampled from uncovered plots sown with farm-saved seeds contained around 6 pg *R. secalis* DNA, compared to around 0·6 pg for the clean seed roots (Fig. 4). The PCR results from the 17 February samples showed that no *R. secalis* DNA could be detected in samples from plots sown with either the farm-saved seed (plots 4, 7 and 11) or symptomless clean seeds (plots 2, 3 and 12) when grown under-cover. Nearly all plant parts sampled from all plots contained *R. secalis* DNA on 24 April 2004. The highest levels of *R. secalis* DNA were always found in the leaves and decreased towards the roots. *Rhynchosporium secalis* DNA levels were lower in plots sown with clean seed ($P < 0{\cdot}001$) and in covered plots ($P < 0{\cdot}001$).

The interaction between seed and cover was also significant, indicating that covering prevented disease development even when the seed was heavily infected with *R. secalis* ($P < 0{\cdot}001$). Differences in *R. secalis* DNA levels between the different plant parts (roots, seeds, stems and leaves) were also significant ($P < 0{\cdot}001$) as was the interaction between plant parts and cover ($P < 0{\cdot}001$). Patterns and levels of significance were similar on both sampling dates (Table 1).

Detection of airborne inoculum of *R. secalis*

DNA of *R. secalis* was not detected in spore tape samples collected between 4 October and 30 December (Fig. 5). Small daily amounts of *R. secalis* DNA, < 2 pg, were detected between 20 January and 24 July during the period of the growing season when leaf blotch symptoms were visible. Detection of airborne inoculum was not linked with rainfall events (data not shown). Assuming a detection threshold of 0·1 pg of pathogen DNA in a 24 h air sample is equivalent to approximately 3 spores m^{-3} (Fraaije *et al.*, 2005), *R. secalis* spore concentrations between 30 and 60 spores m^{-3} were only detected on three occasions. Anything detected below the threshold of 0·1 pg of pathogen is regarded as zero and not within the scope of this assay.

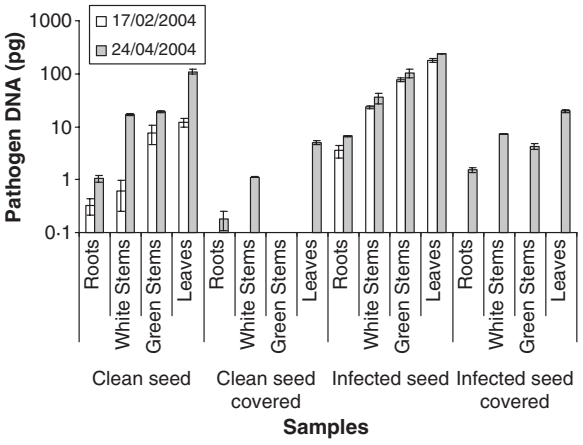

Figure 4 The average amount of *Rhynchosporium secalis* DNA detected in different plant sections sampled from barley plots sown with symptomless clean or farm-saved infected seeds. The centre 1 m^2 of each plot was left untreated, or was covered with tents until 17 Feb 2004. Ten plants per plot were sampled on 17 Feb 2004 (GS 23–24, after removal of the tents) and on 24 April 2004 (GS 31–32) and tested using quantitative real-time PCR. Bars indicate standard error of the mean.

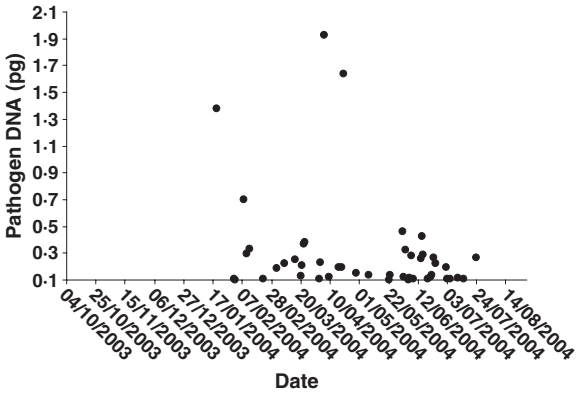

Figure 5 Temporal distribution of *Rhynchosporium secalis* DNA detected on spore trap tapes between 4 Oct 2003 and 23 Aug 2004.

Seed infection and transmission of disease in controlled environment studies

Seeds with symptoms had approximately twice as much *R. secalis* DNA as randomly-selected seeds from the same batch (Table 2). Most seeds without symptoms tested positive for presence of *R. secalis* in real-time PCR. However, the average infection level of seeds sampled from the symptomless clean seed batch was approximately 10-fold lower than seeds sampled from the farm-saved infected seed batch, irrespective of the sampling method.

Table 2 Level of *Rhynchosporium secalis* in clean (symptomless) or farm-saved (infected) barley seed of various origins, determined by visual assessments and quantitative real-time PCR

Seed lot	Sampling method[a]	Visual symptoms[b]	Pathogen DNA (pg) per sample[c]
Clean seed	At random	0	0·18
	Selective	1·2	0·26
Farm-saved	At random	0·6	1·53
	Selective	1·2	3·0
SED or proportionate error in each mean		0·26	34%

[a]Seeds were randomly selected or selected for symptoms of *R. secalis* infection.
[b]Visual symptom scores; 0, no symptoms; 1, flecking symptoms; 2, lesions visible. ANOVA: main effect of sampling method, $P < 0{\cdot}001$; seed lot and interaction $P > 0{\cdot}1$, not significant.
[c]Back-transformed mean; analysis on log scale (equivalent to the measurement scale). ANOVA: main effect of seed lot, $P < 0{\cdot}001$; sampling method and interaction $P > 0{\cdot}2$, not significant.

Most parts of plants produced from symptomless clean seed tested negative for *R. secalis* DNA at all growth stages and temperatures tested (data not shown). As expected, more samples were positive for *R. secalis* infection when farm-saved infected seeds were sown (Fig. 6). The highest levels of pathogen DNA were detected after growth at 16°C. At this temperature, DNA of *R. secalis* was detected in all plant parts sampled at GS 8–9 and 14–15. None of the plants grown in the controlled environment showed any symptoms of leaf blotch at GS 14–15. No spores or hyphal/mycelium structures resembling *R. secalis* were observed in any of the samples examined by light microscopy.

Discussion

The emergence of the barley crop under the covers was quicker by about 1 week due to the warmer climate and thus the plants developed at a faster rate throughout the whole growing season. On the day that the tents were removed (GS 23–24), there were clear effects of both covering and seed batches on the epidemic development of *R. secalis* (Table 1). Plants in symptomless open plots sown with clean seeds had significantly less disease than open plots sown with farm-saved infected seed (Fig. 3). These results were supported by the real-time PCR data. All plant parts (roots, white stems, green stems and leaves) sampled from plots sown with farm-saved infected seeds showed higher levels of *R. secalis* DNA than equivalent samples taken from plots sown with clean seeds. Detection of *R. secalis* in all of the plant parts sampled indicates a systemic infection spreading from the seed. However, the greater infection levels were always found in infected uncovered seed and these caused greater disease levels throughout the growing season when compared to all the other seed types and conditions (Fig. 3).

Rhynchosporium secalis was initially not detected in plants sampled from covered subplots, irrespective of seed source for reasons that are unclear. Suppression of disease development in the covered crop may have been due to the differences in soil water potential which could have influenced rhizosphere bacterial communities involved in disease suppression. Another possible explanation is that the environmental conditions for the covered crop may not have been conducive to fungal growth as it may have been too warm and dry, but ideal for greater plant growth enabling disease escape (D.J. Lovell, Rothamsted Research, personal communication). Skoropad (1959) demonstrated that the rate of germination of a seedling could prevent the infection occurring at increased temperature (higher than the optimal 16°C) by causing the coleoptiles to grow away more rapidly from the infected area of the seed. Winter barley is now drilled as early as possible in the UK, when the soil temperature will be higher and therefore this should reduce the effective transmission of *R. secalis* from seed to seedlings . However, during this experiment no data for the environmental impact of the covers were taken so it is not possible to determine what factors caused the lack of both

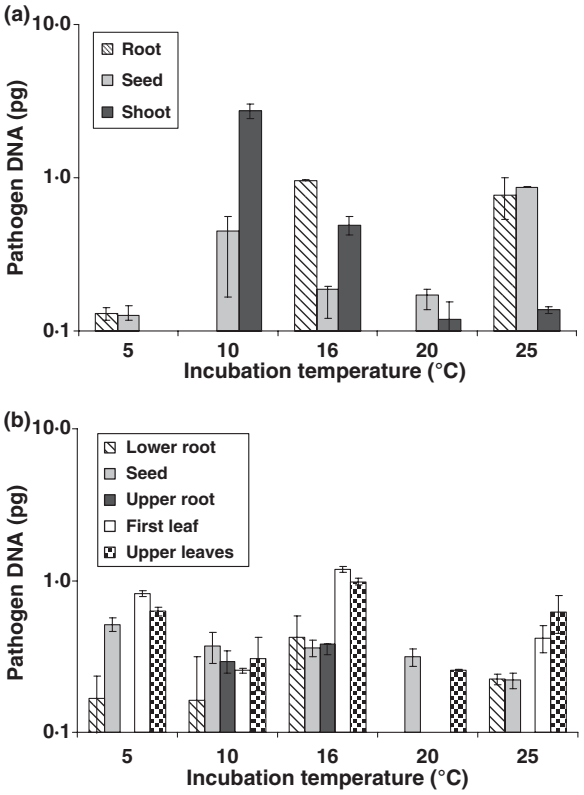

Figure 6 Detection of *Rhynchosporium secalis* (using quantitative real-time PCR) in plants originating from farm-saved infected seed germinated at different temperatures. a, samples from GS 8–9; collected 14 (5°C), 9 (10°C), 5 (16°C) and 4 (20 and 25°C) days after sowing. b, samples from GS 14–15; collected 50 (5°C), 39 (10 and 16°C) and 24 (20 and 25°C) days after sowing. Bars indicate standard error of the mean.

visual disease and positive PCR results for samples taken from under covers on 17 February from both farm-saved and clean seed.

Four weeks after the removal of the tents, *R. secalis* DNA was detected in plant samples from all plots and this was followed eventually by leaf blotch symptoms (Table 1; Fig. 3). This could be due to a delayed spread of inoculum from the seed following a change in the environment, because highest levels of pathogen DNA were still recorded for plant parts (including roots) sampled from plots sown with the farm-saved infected seed. However, it is also possible that infection could have developed from the outside plots following the removal of the covers and these plants could have been more susceptible to symptom development due to their earlier 'softer' growth environment containing elevated temperatures and humidity and reduced amounts of other outside factors such as wind and frost. The development of a uniform disease pattern following the removal of the covers is probably the results of conidia spreading across all plots as shown in Fig. 3 with the exception of the infected uncovered seed plots which had initially higher pathogen levels due the role of seed infection.

Small amounts of *R. secalis* DNA (just above the detection threshold) were detected in spore tape samples from the end of January until the end of July 2004 with the occasional higher level of up to 1·9 pg of pathogen DNA. This pattern is not typical of other ascospore-producing fungi present in other arable crops such as *Mycosphaerella graminicola*, *Pyrenopeziza brassicae* and *Oculimacula yallundae,* which all tend to produce ascospores more abundantly from senesced plants and/or stubble during late summer to early winter. However these pathogens also produce some spores during late spring to summer (Calderon *et al.,* 2002; Fraaije *et al.,* 2005). The period in which positive tape samples were detected coincided largely with the period that symptoms were visible on the leaves and not during late summer to early winter. Because of the low level of detection in comparison with other ascospore producing pathogens, it is likely that the *R. secalis* DNA detected originated from asexual spores, rather than from ascospores. Asexual spores of *R. secalis* are relatively small in comparison with asexual spores of other fungi and therefore during humid conditions, water droplets containing spores could have been sucked directly into the spore trap. It was not possible to identify putative ascospores of *R. secalis* by examining the spore tapes by microscopy since the morphology of the teleomorph is unknown. In order to identify ascospores in positive samples, work now needs to be carried out to develop an immunofluorescence microscopy assay using specific antibodies or *in situ* PCR to positively identify the source of fungal material that is causing a positive result for spore tape DNA samples.

In the controlled environment study, no leaf blotch symptoms were seen throughout the experiment. However, *R. secalis* DNA was detected in seedlings from both clean and farm-saved infected seeds at all temperatures used. Of the temperatures tested, 16°C was optimal for *R. secalis*, confirming earlier work (Skoropad, 1959). Plant roots were colonized by *R. secalis* in both the controlled environment and the field experiments. There is only one previous report of *R. secalis* infection on the root system, and affected plants had reduced root length (Martin, 1980). However, Barnes & Shaw (2003) found *Botrytis cinerea* in the roots of *Primula,* and recent work by Sesma & Osbourn (2004) has suggested that soilborne inoculum and root infection could be important in the development of rice blast disease, caused by *Magnaporthe grisea*. This fungus had previously only been considered to be a foliar pathogen but Sesma & Osbourn (2004) were able to show that root colonization can lead to systemic invasion and development of disease on aerial parts of the plant. Unfortunately, no infection structures of *R. secalis* were visualized by light microscopy. Understanding which stages/structures of *R. secalis* are important for the infection process and its life cycle could be investigated by green fluorescent protein technology to visualize by confocal microscopy, fungal development *in planta* (Rohel *et al.,* 2001).

The role of seed infection as primary inoculum for leaf blotch in winter barley crops in the UK has probably been overlooked because of the long latency of the disease. It appears that *R. secalis* can be transmitted from seeds, but may produce no symptoms in the plant for several months, and currently almost all of the commercial seed treatments used by growers are not active against *R. secalis* infection contained in the seed. Changes in the physiological condition of the plant and/or environmental conditions may trigger disease development. Seedborne infection may also contribute to the genetic variation of *R. secalis* populations as batches of seed are often combined and travel long distances before being sown. In addition to using resistant barley varieties and foliar fungicide applications, the testing of seed batches for *R. secalis* by PCR, with rejection of severely contaminated batches, or additional seed treatments with fungicides might improve disease control. Further work is now needed to establish what levels of seed infection can be tolerated before rejection should occur as many of the clean symptomless seeds used in this study were also found to be infected. Seed batches can also be tested for the presence of alleles conferring resistance to fungicides. Mutations in cytochrome *b* and β-tubulin encoding genes, targets for the quinone outside inhibitors (QoI) and methyl benzimidazole carbamate (MBC) fungicides, respectively, have been identified in populations of *R. secalis* (Wheeler *et al.*, 1995). Seed infection could also be a factor responsible for the introduction of populations of *R. secalis*, resistant to compounds such as QoI fungicides. The QoI fungicide resistance that developed in France during the summer of 2008 (FRAC, 2008) for example, could pose a risk to UK growers if seed is imported from an infected area. Therefore, routine monitoring of seed and other source of *R. secalis* inoculum using PCR may become vital for the long term viability of barley cropping in the UK.

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