Understanding *Sclerotinia* infection in oilseed rape to improve risk assessment and disease escape

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Abstract

It is difficult to predict when major outbreaks of *sclerotinia* stem rot will occur on oilseed rape (OSR) in the UK. An improved risk assessment scheme would help to time fungicide applications more effectively. This work aims to improve risk assessment of OSR stem rot, by investigating the role of flower parts in the infection process and assessing the potential of PCR based detection methods for *Sclerotinia*. Near isogenic lines (NILs) of full petal and apetalous OSR were established in field plots at ADAS Rosemaund in 2004,'05 &'06. Air samples analysed by PCR and microscopy indicated that ascospores were frequently present during flowering although numbers varied each season. Each year, flower parts were sampled at early, mid and late flowering and tested for *Sclerotinia* by agar plating and PCR. In general, in all years more stamens of the apetalous NIL tested positive for *Sclerotinia* than for the full petal NIL, based on agar plate tests. For example, in 2004 at late flowering when 38 % of petals from the full petal NIL tested positive, 16% of full petal stamens tested positive compared to 33% of apetalous stamens. Therefore, petals may shield stamens from airborne *Sclerotinia* ascospores. Counts of flower parts on individual leaf layers and in axils, showed that overall, more flower parts stuck to leaves of the full petal NIL than to the apetalous NIL. Conditions causing flower parts to stick to the leaves and stems appear to be important for epidemics. For one pair of NILs, in all three years there was no significant difference in % stem rot between full petal and apetalous lines. For another pair of sister DH-lines, tested only in 2006, there was 12.6 % stem rot in the full petal line compared to 2.2% in the apetalous line.

Key words: *Sclerotinia sclerotiorum*, oilseed rape, apetalous, petal stick, disease forecasting

Introduction

Sclerotinia stem rot in oilseed rape (OSR), caused by *S. sclerotiorum*, is a sporadic disease in the UK, but occasional high incidence infections of individual crops cause significant yield loss. Inoculum (sclerotia) can build up in the soil if the disease is not controlled, increasing the risk of infection to subsequent OSR and other crops in the rotation. Despite the low average UK production loss, the high impact of severe crop infection has resulted in the widespread use of fungicides. In surveys across England and Wales from 1993-1998 (by MAFF; now Defra), 49% of OSR crops were sprayed to protect against *Sclerotinia*, and although this had declined to 32% by 2000, at least half of these applications were considered unnecessary. Resistance to MBC fungicides is now widespread in *S. sclerotiorum* in France and UK farmers may soon be faced with similar problems. New fungicides are being developed but are likely to be more expensive and subject to future fungicide resistance problems, unless they are used with greater precision than is currently practised. The challenge in the UK is, therefore, to reduce the unnecessary fungicide applications, and identify those high-risk OSR crops which justify a fungicide treatment. Reliable forecasting methods have not yet been developed successfully for the UK and for progress to be made, two key elements are required: i) knowledge of the infection process of *S. sclerotiorum* and ii) a quick and accurate method of assaying the inoculum load, and hence infection risk. This work aims to improve risk assessment of OSR stem rot, by investigating the role of flower parts in the infection process, and assessing the potential of PCR-based methods for detecting *Sclerotinia* in air samples and plant tissues.

Methods

Plant material and field experiments: Near-isogenic lines (NILs) of apetalous oilseed rape were developed at CPB-Twyford, UK following two backcrosses to the winter oilseed rape variety Nickel. Two lines, FP1 (fully petalled) and AP1 (apetalous), differing only with respect to the presence or absence of petals were sown in 2003, 2004 and 2005, on fields at high risk for *S. sclerotiorum* at ADAS Rosemaund, Herefordshire. Each line had 10 replicate plots, each 6.5×12 m. For the crop sown on 1 September 03, the spring plant counts were: AP1, 13.0 and FP1, 12.6 (plants/m^2). For the crop sown on 7 September 04, the spring plant counts were: AP1, 67.2 and FP1, 58.9 (plants/m^2). For the crop sown on 7 September 05, each line had 3 replicate plots, each 6.5×12m, and the spring plant counts for FP1 and AP1 respectively, were 27.9 and 31.8 plants/m^2. Other lines sown in 2005 included apetalous lines AP3, AP4 and AP5 and fully petalled FP5. Lines AP3, AP4 and AP5 were breeding lines with differing genetic backgrounds. AP5 and FP5 are sister doubled haploid lines from the AP1×Winner cross. Plots were inoculated with *S. sclerotiorum* at sowing in 2005 by scattering 40g sclerotia per plot (approximately 25 sclerotia/m^2; sclerotia were collected from the previously infected crop at Rosemaund). Every week-day

during the course of flowering, petals, sepals and stamens, that were stuck to each leaf on one randomly selected plant/plot, were counted on each leaf and leaf axil, on the main stem (except in 2006 when sepals were not counted). For the purposes of this study, stuck petals were those remaining on each leaf after a light tap. On three occasions (early, mid and late flowering), 1 petal, 1 stamen and 1 sepal (but no sepals in 2006) were sampled from one fully open flower from 12 plants per plot, and placed (petals face down) onto agar (PDA amended with 50ug/l streptomycin sulphate). Agar plates were assessed for *Sclerotinia* after 8-10 days, which allowed *S. sclerotiorum* to be distinguished from *Botrytis*. The opposite petal/stamen/sepal from each sampled flower was placed in the labelled well of an ELISA plate, frozen, and sent for PCR testing at Rothamsted Research. A pair of leaf discs (1cm diameter) were sampled from 1 leaf at mid height from areas with no adhered flower parts obvious, from 12 plants per plot, and one of the pair tested for *Sclerotinia* by agar plating and the other by PCR. Stem rot was assessed immediately prior to harvest on 300 plants per plot.

A trial similar to the ADAS Rosemaund trial in 2005-6 was also established by CPB-Twyford at Pinchbeck, Norfolk, with the same oilseed rape lines, but with two replicate plots for each line. Plot yields for the ADAS and CPB-Twyford trials were measured in 2006 only.

Detection of Sclerotinia in plant tissues (petals, stamens and leaves) and air

Individual petals, stamens or leaf discs were placed into 0.2ml PCR tubes and heated for 5mins at 95ºC. DNA was released using a commercial detergent product, MicroLYSIS (Microzone, http://www.microzone.co.uk/), used in combination with thermal cycling in a PCR block according to the maunfacturer's protocol followed by a step in which 2mg PVPP (Polyvinylpyrrolidone;Sigma) and 40µl of TE buffer (pH8.0) is added and vortexed and spun at 12,000rpm for 15 minutes to remove polysaccharides. The supernatant was removed to a new 0.2 ml tube and 2.5x ethanol & 10µl ammonium acetate added, vortexed and spun at 13,000rpm for 15 minutes. The supernatant was discarded and the remaining pellet air dried and resuspended in 10µl water. This was kept frozen at –20ºC and 1 µl used per PCR reaction. The PCR reaction used the primers and method reported in Freeman et al. (2002) with the modification of 30 cycles at the final minimum annealing temperature rather than 16 cycles.

In all three seasons, Burkard Hirst-type air samplers (Lacey & West, 2006) were operated in the field, sampling air at 10l/minute. Each daily (48mm) spore tape section was cut longitudinally along the centre-line of the spore trace using scissors to give two sub-sections (each 7mm × 48 mm). One sub-section was placed into a 1.5 ml Eppendorf tube (waxed surface facing the inside of the tube), labelled and stored at -20ºC until further processing by disruption of the air sample by shaking in a fast-prep machine with acid-washed Ballotini beads, followed by DNA extraction and PCR. The duplicate sub-section of tape was mounted on a microscope slide for traditional spore counting by microscopy.

Results

Incidence of infection of flower parts with airborne ascospores of *S. sclerotiorum* was moderate in 2004 and low in 2005, with a maximum of 38% and 15%, respectively of petals testing positive for *Sclerotinia* at early flowering. Infection was high in 2006, with a maximum of 97% of petals testing positive at mid flowering (Table 1).

Table 1 % flower parts testing positive for *Sclerotinia*, ADAS Rosemaund 2006 (agar plate tests).

2006	GS	Near isogenic lines	Petals	Stamens	Leaves
11-May	4.2	Full petal	72.2	55.6	2.8
		Apetalous	*	58.3	13.9
19-May	4.5	Full petal	97.2	42.6	14.6
		Apetalous	*	72.2	11.1
29-May	4.9	Full petal	8.4	2.8	0.0
		Apetalous	*	8.3	0.0

In general, for each individual sample time, a higher % of stamens tested positive for the AP line than the FP line. In 2004 when moderate infection levels were detected, a higher % of sepals also tested positive for the AP line than the FP line. Usually, the number of leaf discs testing positive for *Sclerotinia* was similar for AP and FP lines. In all years, few petals were recorded stuck to the AP1 line, suggesting little cross plot petal spread between AP and FP plots. Generally, the highest number of flower parts stuck to leaves were stamens from AP and FP lines, and petals from the FP1 line (Fig 1.). Sepals from either line stuck in relatively low numbers. Most flower parts stuck to the lower leaves, with maximum flower fall occurring at mid-late flowering. The overall pattern of stick in leaf axils was the same as on leaves, but at much lower numbers, usually 1 – 2 petals, sepals or stamens per axil.

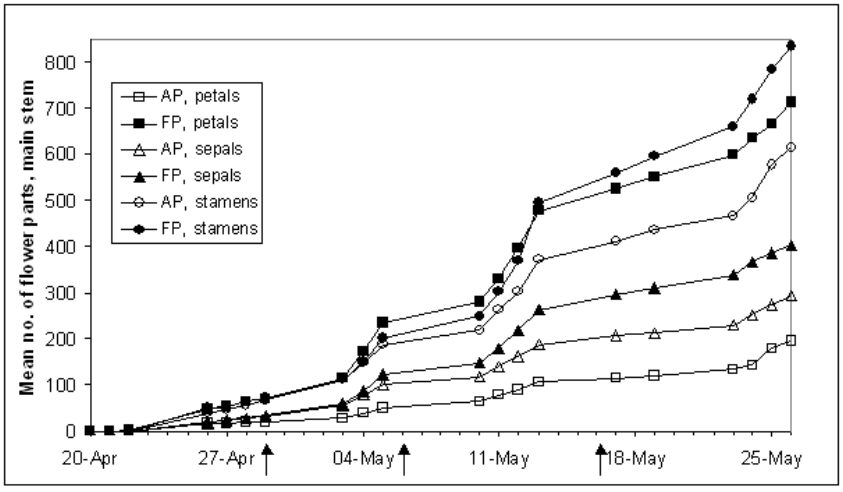

Fig.1. Cumulative flower part stick on oilseed rape leaves, ADAS Rosemaund 2005

Incidence of stem rot was low in 2004 and 2005, with 4.9 and 3.6% plants with stem rot on AP1, respectively, and 3.6 and 4.3% stem rot on FP1, respectively. In 2006, stem rot incidence was again low on AP1 and FP1, but significantly higher on FP5 (Fig 2.). AP1 and FP1 have a background from variety Nickel, and AP5 and FP5 have a background from variety Winner.

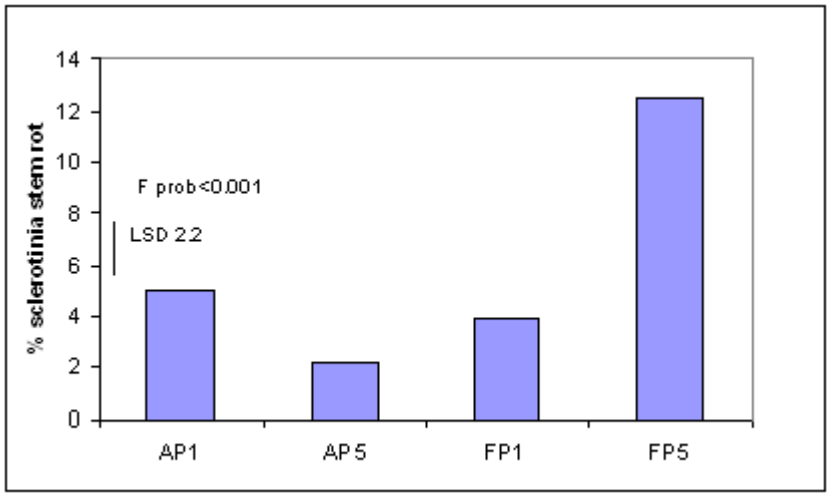

Fig 2. *Sclerotinia* stem rot on apetalous (AP) and full petal (FP) lines, ADAS Rosemaund 2006

Most stem rot lesions were on the lower third of the plant, which is where most of the flower parts stuck. There was no stem rot recorded at the CPB-Twyford site at Pinchbeck in 2006 and yields were significantly different between the two sites, for the various lines, and also for the site by line interaction.

PCR tests showed that ascospores of S. sclerotiorum were present in the air almost continuously during the flowering period of the crop. As the PCR test was not quantitative, numbers of S. sclerotiorum ascospores could only be estimated by a laborious microscopy method using two transverse traverses of the daily spore tape, counting spores within a field width of 200µm. However, such identification was subjective as other *Sclerotinia* and Botrytis species have very similar spores.

PCR detection of S. sclerotiorum in plant tissues was not always consistent with the results of agar plate tests. Initial results indicate occasional rare underestimation of infection thought to be caused by poor DNA release and extraction or inhibition of the PCR reaction by other chemicals present. Control methods such as quantifying DNA extracted, were implemented to detect this. More importantly the PCR method was found occasionally to indicate a higher incidence of *Sclerotinia* on plant tissues than the agar plate tests. This could be because DNA in non-viable spores would lead to a positive result.

Discussion

There were large differences in crop establishment between the three years of the project, with plant counts very low in 2004 (dry conditions at sowing), high in 2005, and intermediate in 2006. Despite this, there were similarities in 2004 to 2006 in the levels of flower stick and stem rot incidence in the AP1 line, and for the FP1 line. The higher % of stamens and sepals testing positive for *Sclerotinia* in the AP1 NIL compared to the FP1 NIL suggest that petals shield the stamens and sepals from

airborne spores. More flower parts stuck to the lower leaves, and this was where stem rot lesions almost always occurred, as expected. Few or no stem rot lesions were recorded on secondary racemes; almost all were recorded on the main raceme in all years. The AP NIL had similar numbers of stamens and sepals sticking to leaves as for the FP line. Therefore, although the AP NIL may avoid infection by petals there is an opportunity for infection via other flower parts, particularly as the AP stamens and sepals usually had a higher % testing positive for *Sclerotinia* than those of the FP NIL. For the purposes of this study, the agar plate test for detecting *Sclerotinia* was an easy and useful method. However, it takes too long to be useful as an aid to making fungicide application decisions.

In each year, the AP1 and FP1 line had disappointingly low incidences of stem rot, with only the 2004 crop showing a significantly higher incidence of stem rot in FP1 than AP1. However, when additional lines with a wider genetic background were tested in 2006, the doubled haploid line FP5 showed a relatively high stem rot incidence (12.6%), significantly more than the sister line AP5, FP1 or AP1. The difference in the background of the lines could be a source of resistance to *Sclerotinia*, as there are no big differences in flower stick that could explain the difference in stem rot incidence. This will be investigated in further work. Whilst the differences in stem rot between the lines were partly explained by levels of petal stick there was no detectable relationship between stamen stick and stem rot. The use of PCR-based diagnostics applied to air samples would be enhanced greatly if a quantitative method could estimate numbers of *S. sclerotiorum* ascospores. A purely qualitative method applied to air samples gave little information as there were nearly always some spores present in air at the time of flowering. A quantitative PCR (qPCR) method may also provide new information on the rate of colonisation of leaf and stem tissues by *S. sclerotiorum*, as a method of testing for quantitative host resistance, as reported by Latunde-Dada et al. (2007) for *Leptosphaeria maculans* and *Pyrenopeziza brassicae*. Detection of infection on individual petals or other flower structures by conventional PCR seems preferable to the use of qPCR since the incidence of petal infection (rather than severity) combined with the right conditions for petals to stick to leaves and stems appears to be the main factor driving stem rot epidemics. However, simpler approaches, such as the use of agar plate tests may provide sufficient information for risk assessment schemes.

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