Project Report No. 420

October 2007

Price: £4.50

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

by

Caroline Young[1], Peter Werner[2] and Jon West[3]

[1] ADAS Drayton, Alcester Road, Stratford on Avon, Warwickshire CV37 6XG
[2] CPB Twyford Ltd., Church Street, Thriplow, Herts SG8 7RE
[3] Rothamsted Research, Harpenden, Herts AL5 2JQ

This is the final report of a 36 month project, which started in April 2004. The project was funded by a contract of £15,000 (plus £5,000 in-kind) from HGCA (Project no. 2949), £118,007 from Defra (LK 0957) and an in-kind contribution of £98,069 from CPB Twyford making a total of £236,076.

CONTENTS

ABSTRACT

Sclerotinia stem rot in oilseed rape, caused by *Sclerotinia sclerotiorum*, occurs in the UK at an average incidence of 2%, but there are occasional major outbreaks with high yield loss, which are very difficult to predict. The aims of this Defra LINK – HGCA project were: [1] to improve the management of *Sclerotinia* and reduce fungicide use, [2] to assess the potential of polymerase chain reaction (PCR)-based inoculum detection for risk assessment, and [3] to assess the potential of apetally as a disease avoidance mechanism, using near-isogenic lines of oilseed rape. The apetalous (AP) and full petal (FP) lines used in this project were developed by CPB-Twyford.

The numbers of petals and stamens sticking to oilseed rape leaves are a guide to risk of stem rot developing. In this project, petals, stamens and sepals stuck to leaves and in axils were counted daily on randomly selected plants in each plot. However, the numbers counted did not relate directly to the final levels of stem rot. This was most likely because of large differences in stem rot on lines that had similar petal and stamen stick. The difference in stem rot could be a resistance mechanism, or because sclerotinia is avoided in some way during its life cycle.

The results of testing flower parts for sclerotinia infection showed that AP lines could not avoid infection completely through lack of petals. The percentage of petals, stamens and sepals infected with *Sclerotinia* was determined at early, mid and late flower by agar plating tests (ADAS) and also by polymerase chain reaction (PCR) tests (Rothamsted Research). According to agar plate tests, in general, where there was a moderate to high level of FP petals testing positive for *S. sclerotiorum*, there was also stamen infection on both AP and FP lines, but at lower levels than for petals on the FP lines. However, more stamens tested positive on the AP lines than the FP lines, e.g. in 2004, at early flower there were 37.5% of stamens testing positive on the AP lines and 24.2 % on the FP lines. The likelihood is that petals on the FP plants shield FP stamens from airborne spores which are circulating in the crop from the source on the ground (fungal fruiting bodies called apothecia). The same infection pattern was true for sepals (not tested in 2006), but in general lower levels of sepals tested positive than for stamens.

PCR tests found much higher levels of infection in all flower parts than agar tests but did not detect differences between levels of petal and stamen infection. A quantitative PCR (qPCR) test developed recently shows good potential for assessing stem rot risk from airborne spore inoculum. The qPCR test gives an indication of the peaks of spore numbers in the air, which correspond to periods of highly infective flower parts. The qPCR test could in future be based upon a simple protocol requiring one or two small spore traps to be operated during flowering and a small number of DNA assays.

Without petals, AP plants are still at risk from infection via stamens. Flower parts infected with *S. sclerotiorum* need to stick to leaves for at least a couple of days to cause leaf infection, and in each year of the project, there were similar numbers of stamens stuck on leaves of the AP or FP lines as petals on the FP lines. However, petals were found to be approximately six times as infective as stamens, with about 10% of petals on leaves leading to a lesion, whereas less than 2% of stamens initiated a leaf lesion.

Our results indicate that there is potential for sclerotinia-resistant cultivars, and that apetally does lead to some avoidance of stem rots. In 2006 the near-isogenic lines AP5 and FP5 differed in stem rot incidence, with 2.2 and 12.6%, respectively. These lines have a background derived from the cultivar Winner which also had relatively high level of stem rot, 8.8%. However, the near-isogenic lines AP1

and FP1 were derived from Nickel which had low stem rot (2.6%), and both AP1 and FP1 also had low stem rot. Apetally in winter OSR is a multiple recessive gene trait and is difficult to breed. It is unlikely to be commercially viable unless further work is done to develop stable material in a good genetic background.

In summary, the key messages are:

1. Petals sticking to leaves indicate a risk of infection, but the numbers of petals sticking may not relate directly to the final stem rot incidence.
2. Petals and stamens can both cause infection, but petals are approximately six times more infective than stamens.
3. A quantitative PCR test shows promise for detecting airborne sclerotinia inoculum within a crop, and could be used in an inoculum based disease forecasting scheme.
4. There is evidence that some winter oilseed rape cvs may be more resistant than others to stem rot, and this needs further investigation. Different cvs may have different resistance mechanisms.
5. Apetalous lines derived from OSR varieties susceptible to sclerotinia have lower stem rot incidence than full petal lines. However, it is currently difficult to breed a stable line.

SUMMARY

Introduction

Since 1994 *Sclerotinia* stem rot has occurred in England and Wale in winter oilseed rape (OSR) crops at an average incidence each year of 2% (http://cropmonitor.csl.gov.uk/wosr/surveys/wosr.cfm), with a mean yield loss of 1%. However, there is occasional high incidence (20% or more of the crop affected) on individual crops that causes significant yield loss. Fungicides can be effective but the timing of sprays is critical and it is difficult to predict this timing. Inoculum (sclerotia) can build up in the soil if the disease is not controlled, increasing the risk of infection later in the rotation to OSR and other crops such as potatoes, peas, carrots and beans. There is likely to be pressure for shorter crop rotations with the demand for more biofuel crops, which will increase the risk of sclerotinia infection, because a larger proportion of sclerotia will remain viable in soil with shorter time intervals between susceptible crops.

Since 1990, research has confirmed that infection of oilseed rape leaves with *Sclerotinia* occurs via infected petals. Other flower parts have been shown to also carry *Sclerotinia*, e.g., pollen, anthers, filaments & stamens. It is probable that these would be a route for infection by *Sclerotinia* in apetalous cultivars. There is a relationship between the percentage of petals that carry *Sclerotinia* and stem rot levels prior to harvest, as assessed by agar plating, but the relationship is variable and may not be a reliable predictor of stem rot. The development of PCR methods for detection of *Sclerotinia* inoculum gives the potential to process a large number of samples, with a high level of sensitivity. Culturing petals onto agar has been a consistently reliable method and has been used widely in Canada, but is slow to give results (8 – 10 days in the UK) and may lead to underestimation of *Sclerotinia.*

The primary route of infection of oilseed rape by *Sclerotinia* when petals are not present has not been previously investigated. There is evidence that apetalous oilseed rape develops less stem rot than full petal cultivars, under the same inoculum conditions and host genetic background. Until now, apetalous lines of winter oilseed rape have not been stable and showed significant reduction in vigour. Near isogenic lines, which enable direct physiological comparison of apetalous and full petal lines, were not available, but current apetalous lines developed by CPB-Twyford show potential for commercially acceptable yields. There is interest in apetalous oilseed rape primarily because of the potential for higher seed yields from canopies that are not shaded by petals. Apetalous spring oilseed rape is commercially available in Canada but apetalous winter cultivars are not yet commercially available.

A better understanding of the process of infection of oilseed rape by *S. sclerotiorum* and quantification of inoculum would improve forecasting of disease risk and lead to more effective targeting of fungicide applications. Such information may also be useful for incorporation into a UK decision support system, such as the HGCA sclerotinia risk assessment scheme (developed from previous work, HGCA Project Report no. OS56). Knowledge of infection routes would help plant breeders target disease escape traits in oilseed rape.

This project had five main work objectives, achieved though a combination of field work at ADAS Rosemaund, field and polytunnel work at CPB-Twyford, and laboratory work at Rothamsted Research:
Objective 1: Determine the distribution of inoculum on plants
Objective 2: Quantify the importance of petals, stamens and sepals in the infection process
Objective 3: Compare stem rot in full petal and apetalous NILs
Objective 4: Assess PCR-based sclerotinia detection

Objective 5: Confirm infection pathway in different oilseed rape lines

The overall aims of this project were:
[i] Improve the overall management of sclerotinia stem rot in OSR, through improved assessment of disease risk and more effective targeting of fungicides;
[ii] Develop and assess the potential of PCR testing for inoculum detection on flower parts;
[iii] Assess the potential of apetally as a disease avoidance mechanism.

The project results fall into three main sections (see below): chapter [1] field infection and stem rot, chapter [2] quantification of the relative importance of flower parts in the infection process, and chapter [3] the development of the PCR test for sclerotinia.

[1] Field infection of apetalous and fully petalled winter oilseed rape with Sclerotinia sclerotiorum

Near-isogenic lines (NILs) of apetalous oilseed rape were developed at CPB-Twyford, UK following two backcrosses to the winter oilseed rape variety Nickel and 4 selfing generations. Two of these NILs, FP1 (fully petalled) and AP1 (apetalous), differing only with respect to the presence or absence of petals were sown in September 2003, 2004 and 2005, on fields at high risk for *S. sclerotiorum* at ADAS Rosemaund, Herefordshire. In 2003 and 2004, each line had 10 replicate plots. For the crop sown on 1 September 2003, the mean spring plant counts for each NIL were: AP1, 13.0 and FP1, 12.6 (plants/m^2). For the crop sown on 7 September 2004, the average spring plant counts for each NIL 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, and the average 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 FP5.

Plots were inoculated with *S. sclerotiorum* at sowing in 2005 by scattering 40g sclerotia per plot (approximately 25 sclerotia/m^2). 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 separately, on the main stem (except in 2006 when sepals were not counted). The number of racemes in flower on the same plant was noted. 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. In 2004 and 2005, a pair of leaf discs (1cm diameter) was 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 just before 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. In the Pinchbeck trial, stem rot incidence was assessed, but flower sticking counts were not made. Plot yields for the ADAS and CPB-Twyford trials were measured in 2006 only.

Table 1. Percentage of flower parts testing positive for *Sclerotinia sclerotiorum* at early, mid and late flowering, ADAS Rosemaund 2006, from agar plate and PCR tests on full petal (FP) and apetalous (AP) near-isogenic oilseed rape lines.

				Agar plate results				PCR results		
2006	**GS**	**[1]NIL**		**Petal**	**Stamen**	**Leaf**		**Petal**	**Stamen**	**Leaf**
11-May	**4.2**	**FP**		72.2	55.6	2.8		94.5	91.7	83.3
		AP		-	58.3	13.9		-	88.9	91.7
19-May	**4.5**	**FP**		97.2	42.6	14.6		97.2	83.3	61.1
		AP		-	72.2	11.1		-	86.1	55.6
29-May	**4.9**	**FP**		8.4	2.8	0.0		97.2	80.6	83.3
		AP		-	8.3	0.0		-	47.2	66.7

[1]NIL = near-isogenic line

The agar plate test results for percentage of flower parts testing positive for *S. sclerotiorum* showed that in almost all cases where there was moderate or higher levels of FP petals testing positive for *S. sclerotiorum*, there was also stamen infection, but at lower levels than petals (Table 1). For stamen tests on AP and FP lines, at any one sample time there were more stamens testing positive on the AP lines than the FP lines, e.g., in 2004, at early flower there were 37.5 and 24.2 % of stamens testing positive on the AP and FP lines, respectively. The likelihood is that petals on the FP plants shield FP stamens from airborne spores which are circulating in the crop from apothecia on the ground. The same infection pattern on stamens in 2004 and 2005 was true for sepals (not tested in 2006), but in general lower levels of sepals tested positive than for stamens. The percentage of leaf discs testing positive was similar for any one sample time for AP and FP lines, suggesting no difference in spore circulation among leaves in either near isogenic line (NIL). Where there was very low petal infection such as in 2004, very few sepals, stamens or discs were infected.

The PCR test results are presented in Table 1 to give a concise comparison with the agar plate test results, but will be discussed in the PCR section below. The PCR test detects dead as well as living spores and mycelium, and the results may therefore not give a good indication as to the levels of flower parts that are actually infective. In general, PCR test results indicated a much higher % of flower parts testing positive for sclerotinia than the agar plate test results.

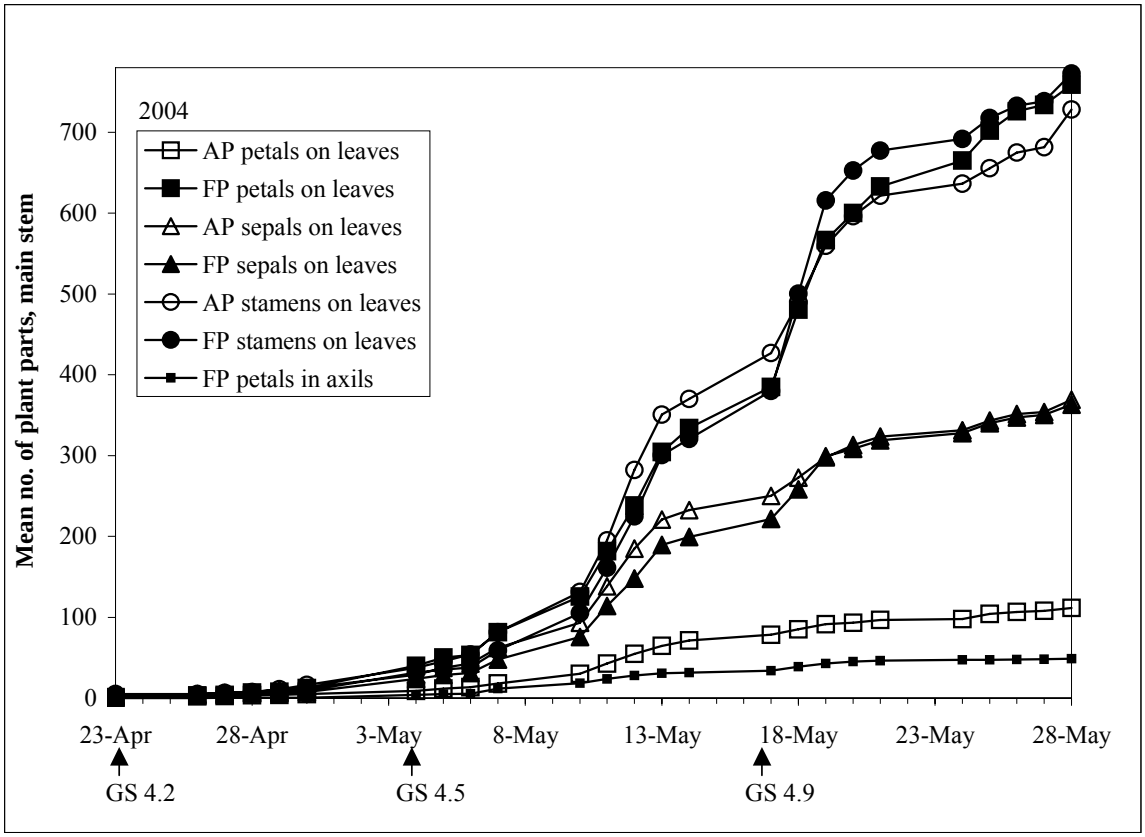


Figure 1. Flower parts sticking on apetalous (AP) and full petal (FP) oilseed rape leaves and axils, ADAS Rosemaund 2004.

The pattern and duration of flower part stick on oilseed rape leaves is important for the development of stem rot, because for stem rot to develop, flower parts infected with *S. sclerotiorum* need to stick to leaves for at least a couple of days. In general, in each year the numbers of petals from FP plants sticking to leaves were similar to the number of stamens from AP or FP plants on leaves, as expected, and these were the most numerous flower parts counted on leaves (Figure 1). The number of sepals sticking to leaves was relatively low compared to stamens or FP petals, but was similar for AP and FP plants. Sclerotinia infection can also occur via infected flower parts caught in leaf axils. Fewer flower parts in total were counted in axils (e.g., FP petals in axils shown in Figure 1), as illustrated by final cumulative axil counts in 2004: FP petals, 49; AP stamens, 72, FP stamens, 65, AP sepals, 43 and FP sepals 39. In each of the three years, the counts of flower parts in axils were similar and always much lower than on leaves. In a dry year with little or no sticking on leaves, this might not always be the case, as axils often remain wet with dew and tend to retain flower parts caught in them.

Table 2. Sclerotinia stem rot incidence in oilseed rape lines, ADAS Rosemaund 2004-6.

	2004*	2005	2006*
AP 1	4.9	3.6	5
FP 1	7	4.3	3.9
AP 3			1.9
AP 4			3.1
AP 5			2.2
FP 5			12.6
Nickel			2.6
Winner		10.7	8.8
SED	0.8		1.6

* significant difference in stem rot between entries for that year, $p \leq 0.01$; winner was not included in the analysis for 2005 because it was the surrounding field crop, not part of the trial design.

In the first two years of the project when the NILs AP1 and FP1 were tested, there was more stem rot in FP1 plots (Table 2), but the difference between the two lines was similar, given the differences in numbers of flower parts sticking to leaves. FP1 had significantly more stem rot than AP1 only in 2004. In 2005 the crop of cv Winner surrounding the trial plots had noticeable stem rot, and an assessments showed an average of 10.7% incidence. Therefore, in 2006, the commercial lines Winner and Nickel were included in the trial plan along with the additional AP and FP lines. In 2006 the NILs AP1 and FP1 both had low stem rot, as did Nickel (Figures 2 and 3). AP1 and FP1 were derived from Nickel. However, the NILs AP5 and FP5 differed in stem rot incidence, with 2.2 and 12.6%, respectively. Winner also had relatively high % stem rot, 8.8% (Figures 2 and 3). AP5 and FP5 were derived from Winner. AP3 and AP4 both had low % stem rot, consistent with their backgrounds (AP3 was a sister line to AP1 and AP4 had an independent source from an Express background).

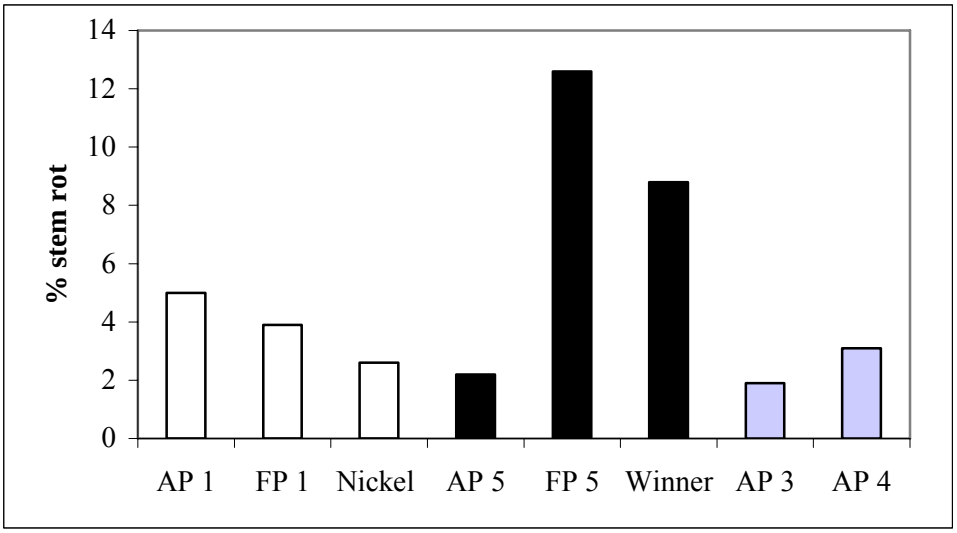


Figure 2. Sclerotinia stem rot incidence in oilseed rape lines, ADAS Rosemaund 2006. (LSD = 2.2, F prob <0.001).

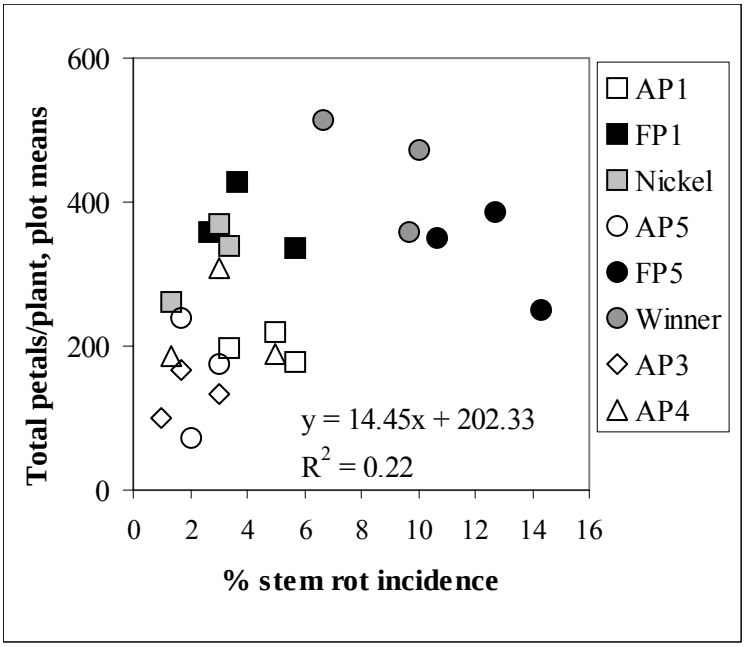


Figure 3. Petal sticking (cumulative counts) and Sclerotinia stem rot incidence on winter oilseed rape lines: full petal (FP), apetalous (AP), and two commercial varieties, ADAS Rosemaund, 2006.

There was a trend towards increasing numbers of petals stuck to plants (counts were cumulative over time during the flowering period) with higher % sclerotinia stem rot incidence, but overall the relationship was not significant (R^2= 0.22, Figure 3). The AP lines all had relatively low petal stick and low stem rot. However, although all the FP lines (including Winner and Nickel) had broadly similar numbers of petals sticking, there were big differences in stem rot incidence, e.g., FP5 had 12.6% stem rot and Winner had 8.8 %, but FP1 had 3.9% stem rot and Nickel had 2.6%. FP5 was derived from Winner and FP1 was derived from Nickel. Therefore, there may be a genetic resistance to sclerotinia in Nickel and related lines that should be investigated further.

Further work should focus on looking for resistance among existing OSR varieties. Given that it is currently difficult to breed a stable and high yielding apetalous line, it would be worth doing field trials designed to compare stem rot in winter OSR FP lines. In addition, the mechanisms of resistance should be investigated for different varieties, as this would enable breeders to focus on traits that would minimise yield loss.

To improve current risk-assessment schemes it would be helpful to have better predictions of petal stick and infection with weather. Good progress has been made in a previous projects on sclerotinia in lettuce using a modelling approach with weather and infection data (Defra project HH3215TFV, Forecasting Sclerotinia in field grown lettuce, and current HDC project FV294, Outdoor lettuce: forecasting and control of sclerotinia). There is detailed weather, petal stick and stem rot field data from previous and current OSR projects that could be used to develop a field model for use in stem rot prediction.

[2] Quantifying the relative importance of petals, stamens and sepals in the infection process for Sclerotinia sclerotiorum

Near-isogenic lines (NILs) of apetalous oilseed rape were developed at CPB-Twyford, UK following two backcrosses to the winter oilseed rape variety Nickel and sister line segregation at F_4. Two of

these NILs, FP1 (fully petalled) and AP1 (apetalous), differing only with respect to the presence or absence of petals were sown in seed trays and transferred to soil in a polythene tunnel in the October of 2003, repeated in autumn 2004. The 10m x 4m polythene tunnel had previously been used annually during the previous 10 years for the production of seed crops of oilseed rape and had developed a very high risk of *Sclerotinia sclerotiorum* stem rots. In addition 5g of sclerotia harvested from infected stems of the previous crop were incorporated into the soil. The lines AP1 and FP1 were planted at the 3-4 true leaf stage. A two x two blocked lattice design was adopted with a 3 x 10 arrangement of individual plants within each block. The front 10 plants of each block were labelled and used for the more detailed assessments in each year. Watering was delivered by overhead mist spray manually applied as required during the autumn, winter and spring. The quantity of water delivered was adjusted according to prevailing weather conditions in order to maintain very high humidity (in excess of 90%) without saturating the soil. In both seasons it was found that by late May/early June the temperatures within the polytunnel were often above 40^{o}C and fungal *S. sclerotiorum* rots ceased developing.

In 2004 flowering commenced on 14 April. By 4 May, disease progress was judged to be well advanced. The numbers of leaves (green entire leaves, not senescent), petals adhering to leaves, sepals on leaves, stamens on leaves, petals per flower (10 flowers sampled) and *S. sclerotiorum* leaf rots were counted on each of the 10 labelled plants in each block. On 16 July all main stem and branch-stem *S. sclerotiorum* rots were counted, together with an estimate of the total % of each plant affected.

In 2004-5 the polytunnel was set up as for 2003/4 but more detailed observations were made in order to determine the cause and developmental course of each *S. sclerotiorum* rot. At the onset of first petal drop (11 April) four leaves were marked with a tag on each of the 10 individually labelled plants within each replicate. Every two or three days the upper surface of each of these tagged leaves was photographed in situ using a digital camera, care being taken not to disturb leaves. In addition, two sets of visual records which were made on 3 May and 16 May 2005. On these two dates each tagged leaf was inspected and the number and position of any *S. sclerotiorum* leaf rots noted. Additional counts of leaf, stem and pod rots on the whole of the plant were also made on these dates. The photographic records were used to make complete counts of petals, sepals and stamens sticking to each leaf and particularly to identify sclerotinia leaf rots. For each leaf rot identified it was possible to work back through the photographic record to follow the course of development of the rot and identify the initiating tissue.

In 2005, flowering commenced on 7 April. Counts from the 20 key plants from each NIL showed a mean petal number of 3.99 and 4.00 for the FP NIL and 0.82 and 0.79 for the AP NIL in 2004 and 2005 respectively. Table 3 shows the numbers of petals sticking to the leaves in 2004 and 2005, an additional 120 narrow petals were counted on the AP leaves in 2005. In both years, more stamens were counted on the AP leaves than on the FP leaves, it is likely that this is a consequence of a 'masking' effect since stamens overlaid by petals would be hidden and not counted. In 2004, sepals were counted and found to be nearly equal in numbers on the FP and AP NILs (213 and 219) but in 2005 no rots were attributable to sepals (using the photographic analysis) and counts were not made. The totals of number of leaf rots initiated show about four times as many in the FP material compared to the AP material (Table 3). The data was analysed for homogeneity of the FPvAP effect over years and for the overall FPvAP effect by contingency chi-squared. The differences between the FP and AP NILs were highly significant for the number of both petals and stamens sticking to the leaves and for the number of leaf rots and final stem rots.

Table 3. 2004 and 2005 polytunnel assessments. Tabulated data give counts for debris on leaves and initial and final *S. sclerotiorum* rots (total over two replicates). Homogeneity of distribution over years and between fully-petalled and apetalous (FP v AP) lines is tested by contingency chi-squared.

		2004	2005	**Total flower parts**	year effect $\chi^2_{(1)}$	*p*	FP v AP $\chi^2_{(1)}$	*p*
PETALS ON LEAVES [+]	FP	508	296	**804**				
	AP	142	49	**191**	*6.44*	*0.011*	*344.5*	*0.000*
Stamens ON LEAVES	FP	342	199	**541**				
	AP	455	293	**748**	*0.79*	*0.375*	*41.5*	*0.000*
TOTAL LEAF ROTS INITIATED	FP	43	39	**82**				
	AP	7	14	**21**	*1.79*	*0.181*	*30.3*	*0.000*
FINAL ROTS ON STEM	FP	26	43	**69**				
	AP	7	4	**11**	*2.03*	*0.154*	*38.7*	*0.000*
ROTS CAUSED BY PETALS *	FP		25	**25**				
	AP		8	**8**			*7.5*	*0.006*
ROTS CAUSED BY STAMENS *	FP		5	**5**				
	AP		3	**3**			*1.1*	*0.285*

[+] excludes counts of narrow petals produced by apetalous line

* not recorded in 2004. χ^2 based on test of homogeneity of rots over reps in 2005

In 2005, the majority of sclerotinia lesions on leaves were initiated from petals, as determined from the photographic records, on both the FP and AP NILs (Table 4).

Table 4. 2005, numbers of leaf rots initiated in the full petal and apetalous near-isogenic lines, categorised by the initial infection source

Source	FP	AP	Total
petal	18	7	**25**
petal clump	6	1	**7**
petal narrow		1	**1**
petal+stamen	1		**1**
stamen	5	2	**7**
stamen clump		1	**1**
droplet	3		**3**
ground contact	1		**1**
can't ID	3	2	**5**
Grand Total	**37**	**14**	**51**

It is clear from these studies that *B. napus* leaves can be infected through routes other than petals acting as the initial nutrient source for the fungal growth. This study notably eliminates sepals as a significant intermediary infection tissue, this may be owing to the shape of the sepals which prevents good adhesion to the leaf. In contrast, petals are flexible and soft, presenting an ideal nutrient source for *S. sclerotiorum*, the ability to create a humid micro-climate and a good contact between the petal and the leaf surface. Stamens fall somewhere between these two providing a flexible soft tissue for initial spore germination but less often result in infection into the leaf. This may be a consequence of the reduced adhesion and smaller contact area leading to a less than ideal micro-climate for the early stages of the fungal growth.

Further work is needed to establish the link between airborne inoculum levels and field environmental conditions which lead to infections of leaves by *S. sclerotiorum*. In addition, the relationship between leaf infections and main stem lesions needs to be investigated in the field, in locations where *S. sclerotiorum* risk is high.

[3] Development of a PCR test for Sclerotinia sclerotiorum

Frozen plant tissue samples were received at Rothamsted Research (RRES) from field experiments at ADAS Rosemaund in 2004, 2005 and 2006. These were petal, stamen, and leaf discs taken from plots at GS 4.2, 4.5 and 4.9 (early to late flowering) from 20 plots in the first two seasons but only from six plots in 2006. Additionally in 2006, 12 petals and 12 stamens were taken from three FP plots twice per week from 25 April to 2 June 2006. Sepals were also sampled in 2004.

All petal and stamen samples were tested by agar plating at ADAS Rosemaund and by PCR at RRES. In the first season, sepal infection was found to be zero in a sub-set tested by PCR. Other evidence suggested that sepals were not a source of infection and so sepals were not tested in 2005 or 2006. Twelve leaf discs from each of 2 AP and 2 FP plots were tested by PCR at each sample date. Of the additional twice-weekly samples taken in 2006 ten petals and ten stamens per plot on each sample date were tested by PCR. Air samples were taken each week from two Burkard (Hirst-type) spore traps operating at each end of the plots in the field at ADAS Rosemaund sampling air at 10 L/minute. Unexposed prepared drums of wax-coated tape were supplied by RRES and sampled air over a week-long period. Each daily (48mm) spore tape section was cut longitudinally to give two sub-sections, one for PCR tests and the other for traditional spore counting by microscopy.

To make DNA available for PCR, plant and air samples were processed using a commercial detergent-based product, MicroLYSIS (Microzone, http://www.microzone.co.uk/), used in combination with thermal cycling in a PCR block according to the manufacturer's protocol, followed by a step in which polysaccharides were removed. The PCR reaction used for the first two seasons was based on 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. It produced relatively weak bands when the PCR product was run on an electrophoresis gel. A new *S. sclerotiorum*-specific primer set was developed and DNA from the 'negatives' from 2005 were retested with the new PCR. Air samples from two Burkard spore traps used in the field experiment at ADAS Rosemaund in 2006 were tested using the new set of *S. sclerotiorum*-specific primers in a SYBR-green quantitative PCR. This allows an estimation of the number of *Sclerotinia* spores sampled in air each day. The technique was tested by spraying suspensions of *Sclerotinia* ascospores, from a dilution series, onto prepared wax-coated tapes, as used in the Burkard air sampler. Air samples, petal, stamen and a sub-set of leaf samples from 2004, 2005 and 2006 were tested by PCR by the end of February 2007.

There was a poor correlation between the incidence of Sclerotinia presence on petals measured by two different techniques – agar plating and PCR (Figure 4). This is mainly due to four occasions when the PCR method indicated a much higher infection incidence than the agar method. It is thought that the agar method will only indicate the presence of viable spores and mycelium of *S. sclerotiorum*, while the PCR technique would detect dead spores.

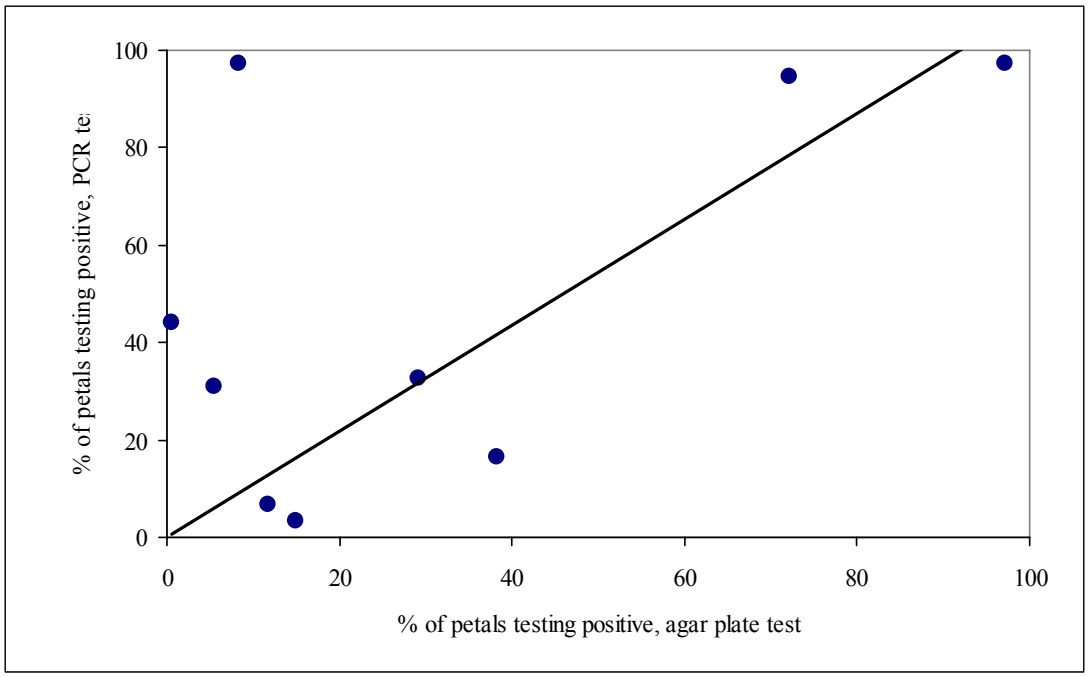


Figure 4. Incidence of sclerotinia in petals as detected by PCR and agar plating.

For leaves in particular (Figure 5), the PCR method consistently indicated a higher incidence of *Sclerotinia* infection. As with the petal samples, this could be due to the detection of unviable (dead) spores present on leaves, or under-estimation by agar plating because growth of *S. sclerotiorum* was inhibited by other fungal species growing on the agar.

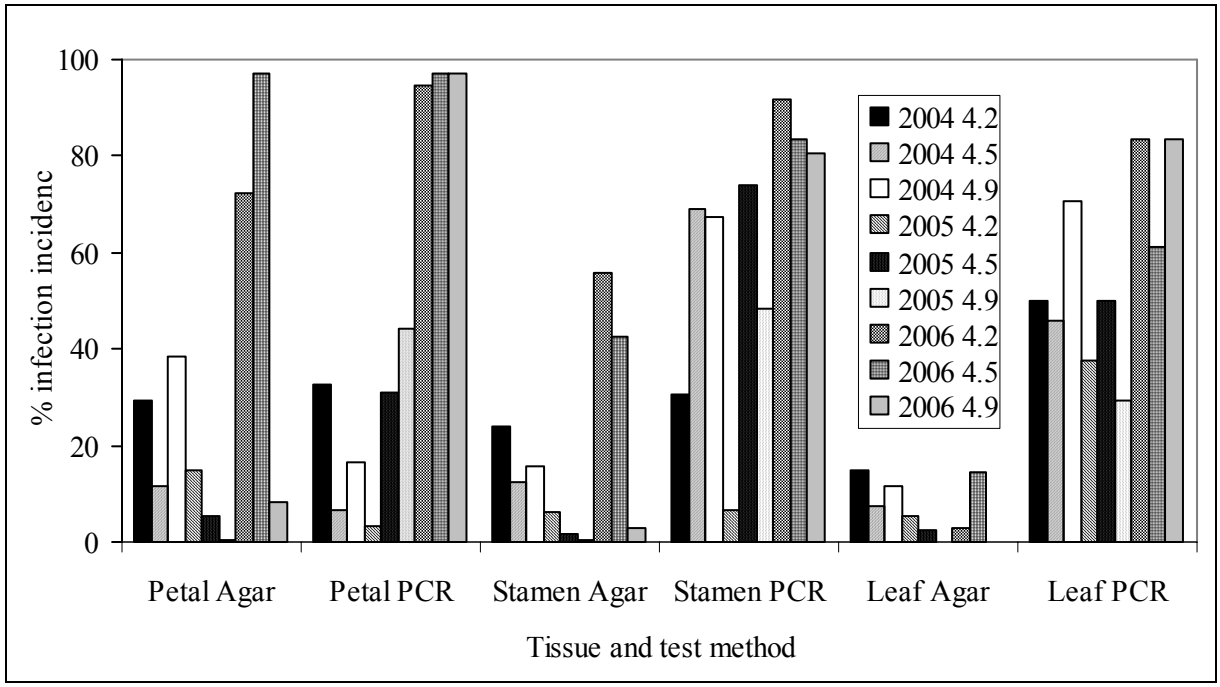


Figure 5. Incidence of sclerotinia infection on different plant tissues at three growth stages, 2004, 2005 and 2006, measured by agar plating or PCR.

PCR tests in the first two seasons (2004 and 2005), 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

semi-quantitative, numbers of *S. sclerotiorum* ascospores could only be estimated by a laborious microscopy method, counting spores within a field width of 200µm. However, such identification was subjective as Sclerotinia and Botrytis species have very similar spores. The patterns of quantified levels of *S. sclerotiorum* DNA in air samples from traps 1 and 2 reflect the numbers of spores present in air at each end of the experiment over the flowering period of 2006 (Figure 6). The spore pattern found in the two traps varies slightly due to wind direction but trap one has a large peak in spore numbers from 11 to the 15 May 2006, while trap two had a large peak at the end of May (25-29 May).

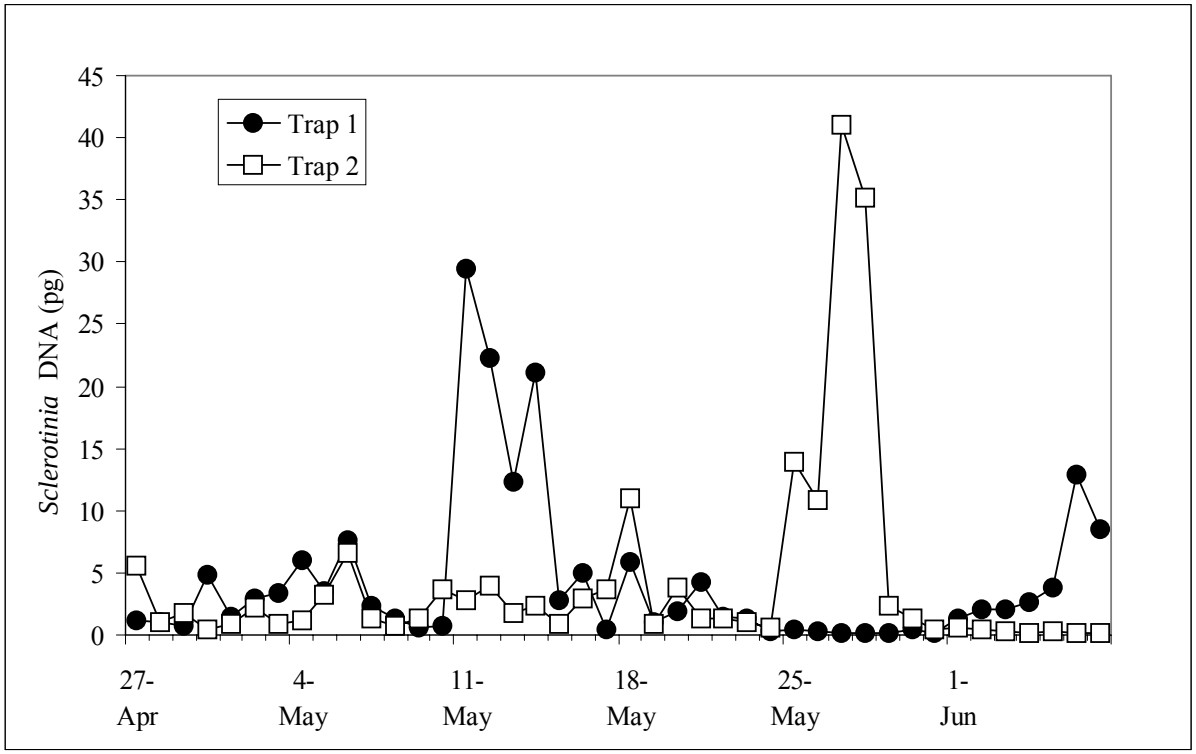


Figure 6. Sclerotinia DNA in air samples from spore traps in a winter oilseed rape crop, from PCR tests, at ADAS Rosemaund, 2006.

Further work on inexpensive spore traps for use within crops and quick processing methods for PCR on spore trap samples would enable farmers to assess inoculum levels, which are an important indicator of infection risk.

Detection of infection on individual petals or other flower structures by conventional PCR may be also be useful because the incidence of petal infection (rather than severity or extent of mycelial colonisation) combined with the right conditions for petals to stick to leaves and stems appears to be a major factor in infections leading to stem rot epidemics. However, for a conventional PCR test on individual petal samples to be of use, further work will be needed to develop sample protocols and test procedures that produce quick results for farmers.

Overall key messages:

- Petals sticking to leaves indicate a risk of infection, but the numbers of petals sticking may not relate directly to the final stem rot incidence.

- Petals and stamens can both cause infection, but petals are approximately six times more infective than stamens.
- A quantitative PCR test shows promise for detecting airborne sclerotinia inoculum within a crop, and could be used in an inoculum based disease forecasting scheme..
- There is evidence that some winter oilseed rape cvs may be more resistant than others to stem rot, and this needs further investigation. Different cvs may have different resistance mechanisms.
- Apetalous lines derived from OSR varieties susceptible to sclerotinia have lower stem rot incidence than full petal lines. However, it is currently difficult to breed a stable line.

Key messages from overall project aims:

Project aim [1] Improved management of *Sclerotinia* and reduced fungicide use

- No major changes to current advice.
- Spray at early flowering is essential if risk is high.
- Assess risk variables as on HGCA sclerotinia decision guide.
- Additional assessment by grower: extent of petal stick.
- No sticking on leaves could be a clear guide to omit or delay a spray (taking weather factors into account).
- There are differences in susceptibility to stem rot between OSR cvs.

Project aim [2] Assess potential of PCR-based inoculum detection

- Petal tests are still a good way to assess disease risk (petal infection is related to stem rot), but agar plate tests may be better than PCR for petal tests, because they detect viable inoculum.
- Quantitative PCR has potential for use in an inoculum-based disease risk scheme using air samples (spore samplers can be inexpensive, and only one or two sample points per field needed).

Project aim [3] Assess the potential of apetally as a disease avoidance mechanism

- Fewer flower parts are produced and stick to AP leaves, therefore the risk of infection by sclerotinia is reduced, but not avoided.
- In two out of three field trials, AP1 had less stem rot than FP1 - (but was significantly less only in the first year).
- AP winter oilseed rape is very difficult to breed (recessive multiple gene trait) and unlikely to be commercially viable unless more resources are invested in solving the breeding difficulties.

CHAPTER 1

INFECTION OF APETALOUS AND FULLY PETALLED WINTER OILSEED RAPE WITH *SCLEROTINIA SCLEROTIORUM*

Introduction

Since 1994 *Sclerotinia* stem rot in oilseed rape (OSR) has occurred in the UK at an average incidence each year of 2%, (http://cropmonitor.csl.gov.uk/wosr/surveys/wosr.cfm) with a mean yield loss of 1%. However, there is occasional high incidence (20% or more of the crop affected) on individual crops that causes significant yield loss, e.g., there were very high losses in 1992, and again in 2007 across the UK. Inoculum (sclerotia) can build up in the soil if the disease is not controlled, increasing the risk of infection later in the rotation to OSR and other crops such as potatoes, peas, carrots and beans. There is likely to be pressure for shorter crop rotations with the demand for more biofuel crops, which will increase the risk of sclerotinia infection, because a larger proportion of sclerotia will remain viable in soil with shorter time intervals between susceptible crops. Despite the low average effect on UK production, the high impact consequences of severe crop infection have resulted in the widespread use of fungicides as prophylactic treatments and there is scope to reduce their use. In MAFF surveys from 1993-1998, 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 the causal pathogen *Sclerotinia sclerotiorum* in France (Penaud *et al*, 2001), 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.

Since 1990, research has confirmed that infection of oilseed rape leaves with *Sclerotinia* occurs via infected petals. Germination of *Sclerotinia* ascospores on oilseed rape petals can occur within 3 hrs, and subsequent invasion into a leaf from an infected nutrient base such as a petal within 70 h (Jamaux *et al*., 1995). Other flower parts have been shown to also carry *Sclerotinia*, e.g., pollen (Huang *et al*., 1997; Huang & Kokko 1992), anthers, filaments & stamens (Huang & Kokko, 1992). It is probable that these would be a route for infection by *Sclerotinia* in apetalous cultivars. There is a relationship between the percentage of petals that carry *Sclerotinia* and stem rot levels prior to harvest, as assessed by agar plating (Gugel & Morrall, 1986; Turkington & Morrall, 1993), but the relationship is variable and may not be a reliable predictor of stem rot (Turkington *et al*., 1991; Davies *et al*., 1999).

The development of polymerase chain reaction (PCR) methods for detection of *Sclerotinia* inoculum give the potential to process a large number of samples, with a high level of sensitivity (Freeman *et al*, 2002). Culturing petals onto agar has been a consistently reliable method and has been used widely in Canada (Turkington & Morrall, 1993), but is slow to give results (8 – 10 days in the UK, Davies *et al*, 1999) and may lead to underestimation of *Sclerotinia* (Lefol & Morrall, 1996). In the UK, contamination of plates with *Botrytis cinerea* is a problem (Davies *et al*, 1999) and *Sclerotinia* can be underestimated. Immunodetection methods for *Sclerotinia* have been developed (Lefol & Morrall, 1996; Jamaux & Spire, 1994) but rely on microscopic examination, which is time consuming for multiple samples, and do not have the sensitivity of PCR methods. An antibody test developed in a previous LINK-HGCA project was successful at detecting very low numbers of ascospores on petals by microscopic examination (Turner *et al*., 2002, i.e., HGCA Project Report no. OS56). However, the test needs further development to enable practical use in the field or as a laboratory service.

The primary route of infection of oilseed rape by *Sclerotinia* when petals are not present has not been investigated. Evidence suggests that apetalous oilseed rape develops less stem rot than full petal cultivars, under the same inoculum conditions and host genetic background (Jiang, 2001), but so far there are no refereed publications to demonstrate that apetalous cultivars develop significantly less stem rot than the equivalent full petal cultivars. Until now, apetalous lines of winter oilseed rape have not been stable and showed significant reduction in vigour. Near isogenic lines, which enable direct physiological comparison of apetalous and full petal lines, were not available, but current apetalous lines developed by CPB-Twyford show potential for commercially acceptable yields (Gemmill *et al.*, 2003, Evans *et al.*, 2003). There is interest in apetalous oilseed rape primarily because of the potential for higher seed yields from canopies that are not shaded by petals. Apetalous spring oilseed rape is commercially available in Canada but apetalous winter cultivars are not yet commercially available. If apetalous cultivars were shown to reduce the incidence of *Sclerotinia* stem rot, then further cost savings would also result through the decrease in the amount of fungicides needed to control the disease in the UK crop. This would also result in more flexible rotations and reduced risks to horticultural and other break crops owing to the reduction of *S. sclerotiorum* inoculum in the soil.

A better understanding of the process of infection of oilseed rape by *S. sclerotiorum* and quantification of inoculum would improve forecasting of disease risk and lead to more effective targeting of fungicide applications. Such information may also be useful for incorporation into a decision support system, such as the sclerotinia risk assessment tool available on the HGCA website (www.HGCA.com). Risk assessment schemes which rely mostly of historical and husbandry factors have been devised in other countries (e.g., Twengstrom *et al*, 1998; Koch *et al*, 2006) but have not been tested in the UK. There is potential for cost savings and an increase in gross margins. For example, assuming a reduction from 40% to 20% of crops treated, and an area of 100,000 ha of treated crop (assuming a total area of crop of 500,000 ha), cost savings from the reduction in fungicides would be about £2.25m per year (average fungicide costs £8 ha^{-1}, application costs £10 ha^{-1}, and wheeling losses 1%, £4.50 ha^{-1}). Detailed knowledge of infection routes will help plant breeders target disease escape traits in oilseed rape. This will establish if the use of apetalous cultivars would lead to an effective avoidance mechanism and a reduction in fungicides used for *Sclerotinia*.

The overall aim of this project was to improve the management of sclerotinia stem rot in OSR, through improved assessment of disease risk and quantification of inoculum, and more effective targeting of fungicides. A further objective was to develop and assess the potential of PCR testing for inoculum detection on flower parts and in air samples, and compare inoculum assay results against final stem rot levels. Finally, the potential of apetally as a disease avoidance mechanism was assessed, and at the same time the apetalous lines were used as a tool to investigate the infection pathway.

Methods

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 September 2003, 2004 and 2005, on fields at high risk for *S. sclerotiorum* at ADAS Rosemaund, Herefordshire. In 2003 and 2004, each line had 10 replicate plots, each 6.5 x 12 m. For the crop sown on 1 September 2003, the mean spring plant counts for each NIL were: AP1, 13.0 and FP1, 12.6 (plants/m^2). For the crop sown on 7 September 2004, the average spring plant counts for each NIL were: AP1, 67.2 and FP1, 58.9 (plants/m^2). For the crop sown on 7 September 2005, each line had 3 replicate plots, each 6.5 x 12m, and the average 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 (average spring plant counts/m^2 were 33.3, 25.3, 28.0 and 25.3, respectively). Line AP3 was a sister line to AP1 with one further backcross to Nickel. Line AP4 was from an independent source in an Express background. Lines AP5 and FP5 were sister doubled haploid lines from an AP1 x Winner cross.

Plots were inoculated with *S. sclerotiorum* at sowing in 2005 by scattering 40g sclerotia per plot (approximately 25 sclerotia/m^2; the sclerotia were collected from the previously infected crop at ADAS 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 separately, on the main stem (except in 2006 when sepals were not counted). The number of racemes in flower on the same plant was noted. Height of the plants was measured at early flower (GS 4.2) for 10 plants/plot selected at random. 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 50µg/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. In 2004 and 2005, a pair of leaf discs (1cm diameter) was 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 2005-2006 was also established by CPB-Twyford at Pinchbeck, Norfolk, with the same oilseed rape lines, but with two replicate plots for each line. In the Pinchbeck trial, stem rot incidence was assessed, but flower sticking counts were not made. Plot yields for the ADAS and CPB-Twyford trials were measured in 2006 only.

Results

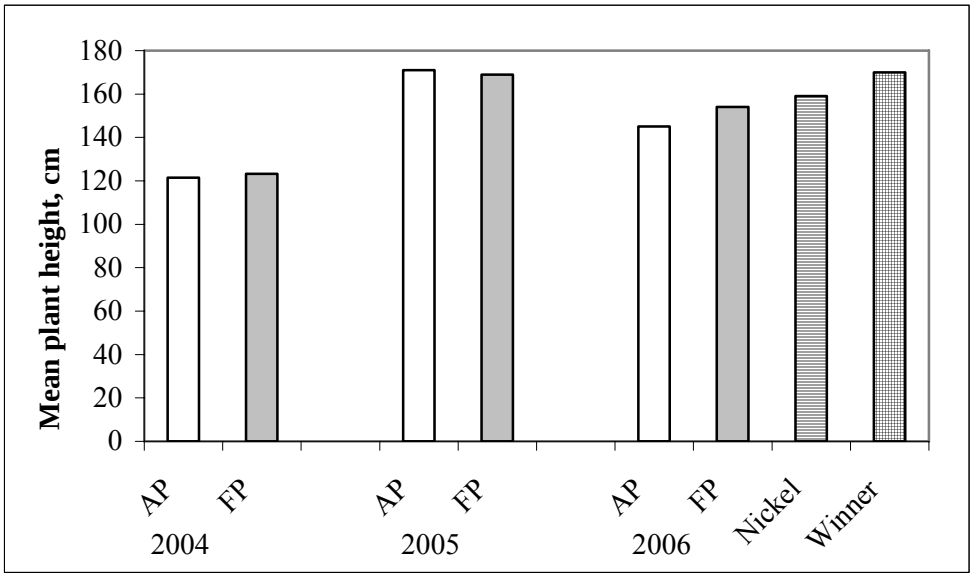


Figure 7. Height of apetalous (AP1), full petal (FP1) and commercial oilseed rape lines, ADAS Rosemaund 2004-2006.

There were no significant differences between the height of the apetalous (AP) and full petal (FP) lines at early flower in any one year, but there were differences between years. For example, in 2004 the crop was initially thin (mean spring count of 13 plants m^2), averaging 120 cm in height during flowering, but in 2005 the spring plant counts were high (63 plants m^2) and the crop was taller than in 2004 during flowering (170 cm) (Figure 7). In 2006 both Nickel and Winner were slightly taller than the FP or AP lines.

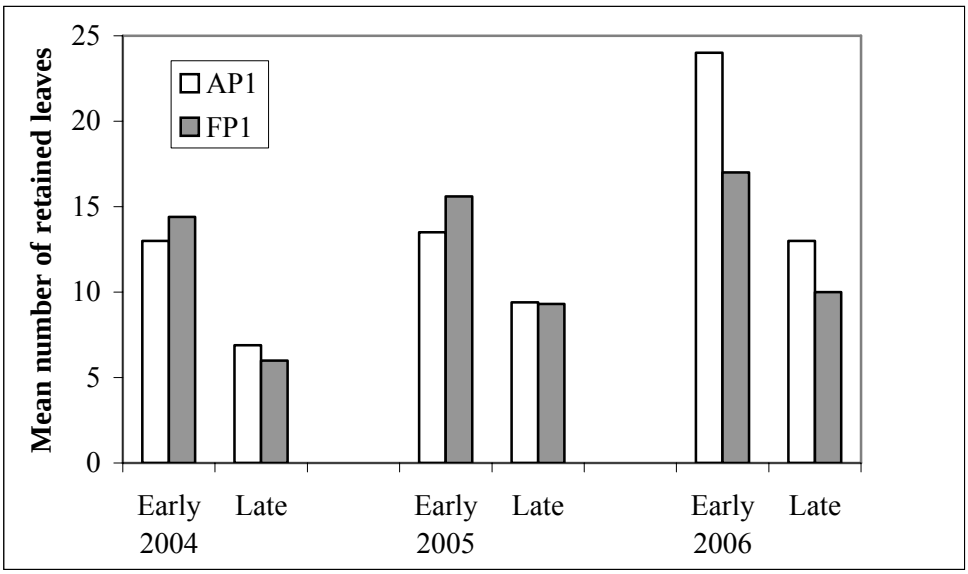


Figure 8. Number of retained leaves at early (GS 4.2) and late (GS 4.9) flower, for apetalous (AP1) and full petal (FP1) lines, ADAS Rosemaund, 2004-6.

In each year, there were more leaves retained early in flowering than late flowering (Figure 8). The least number of retained leaves by late flowering was in 2004, with an average of just 6 leaves per plant (upper leaves), compared to an average of 11 leaves per plant at late flowering in 2006. Where lower leaves are retained on plants, the risk of infection with sclerotinia may be higher, because the extra leaf area gives additional opportunity for petal and stamen stick.

The agar plate test results for percentage of petals testing positive for *S. sclerotiorum* showed that, in general, the airborne spore inoculum over the trials was low to moderate in 2004, low in 2005, and high in 2006 (Table 5). In almost all cases where there was moderate to high levels of FP petals testing positive for *S. sclerotiorum*, there was also stamen infection, but at lower levels than for petals. For stamen tests on AP and FP lines, at any one sample time there were more stamens testing positive on the AP lines than the FP lines, e.g., in 2004, at early flower there were 37.5 and 24.2 % of stamens testing positive on the AP and FP lines, respectively. The likelihood is that petals on the FP plants shield FP stamens from airborne spores which are dispersed in the crop from apothecia on the ground. The infection pattern on stamens in 2004 and 2005 was similar for sepals (not tested in 2006), but, in general, lower levels of sepals tested positive than for stamens. The percentage of leaf discs testing positive was similar for any one sample time for AP and FP lines, suggesting no difference in spore circulation among leaves in either near isogenic line (NIL). Where there was very low petal infection such as in 2004, very few sepals, stamens or leaf discs were infected.

The PCR test results (Table 5) give a concise comparison with the agar plate test results, but will be discussed in the PCR section later in this report. These PCR results are semi-quantitative tests which detect dead as well as living spores and mycelium, and may therefore not give a good indication as to

the levels of flower parts that are actually infective. PCR test results generally indicated a much higher % of flower parts testing positive for sclerotinia than the agar plate test results.

Table 5. Percentage of flower parts testing positive for *Sclerotinia sclerotiorum* at early, mid and late flower, 2004 – 2006, from agar plate and PCR tests on full petal (FP1) and apetalous (AP1) near-isogenic oilseed rape lines.

				AGAR PLATE TEST RESULTS					PCR TEST RESULTS		
2004	**GS**	**[1]NIL**		**Petal**	**Sepal**	**Stamen**	**Leaf**		**Petal**	**Stamen**	**Leaf**
23-Apr	4.2	[2]FP		29.2	6.7	24.2	15.0		32.5	30.6	50.0
		[3]AP		*	26.7	37.5	14.2		*	31.9	45.8
06-May	4.5	FP		11.66	12.5	12.5	7.5		6.7	69.2	45.8
		AP		*	34.4	40.5	6.7		*	78.3	50.0
20-May	4.9	FP		38.3	14.2	15.8	11.7		16.7	67.5	70.8
		AP		*	18.3	33.3	9.2		1.1	71.7	75.0
2005	**GS**	**NIL**		**Petal**	**Sepal**	**Stamen**	**Leaf**		**Petal**	**Stamen**	**Leaf**
29-Apr	4.2	FP		15.0	4.0	6.0	5.5		3.3	6.7	37.5
		AP		*	10.5	5.5	5.5		*	4.2	62.5
06-May	4.5	FP		5.5	3	1.5	2.5		30.8	74.1	50.0
		AP		*	3.5	2.5	1.0		*	70.0	45.8
16-May	4.9	FP		0.5	0.0	0.5	0.0		44.2	48.3	29.2
		AP		*	0.0	0.0	0.0		*	58.3	29.2
2006	**GS**	**NIL**		**Petal**		**Stamen**	**Leaf**		**Petal**	**Stamen**	**Leaf**
11-May	4.2	FP		72.2		55.6	2.8		94.5	91.7	83.3
		AP		*		58.3	13.9		*	88.9	91.7
19-May	4.5	FP		97.2		42.6	14.6		97.2	83.3	61.1
		AP		*		72.2	11.1		*	86.1	55.6
29-May	4.9	FP		8.4		2.8	0.0		97.2	80.6	83.3
		AP		*		8.3	0.0		*	47.2	66.7
[1] NIL = near-isogenic line											
[2] FP = fully petalled											
[3] AP = apetalous (no petals)											

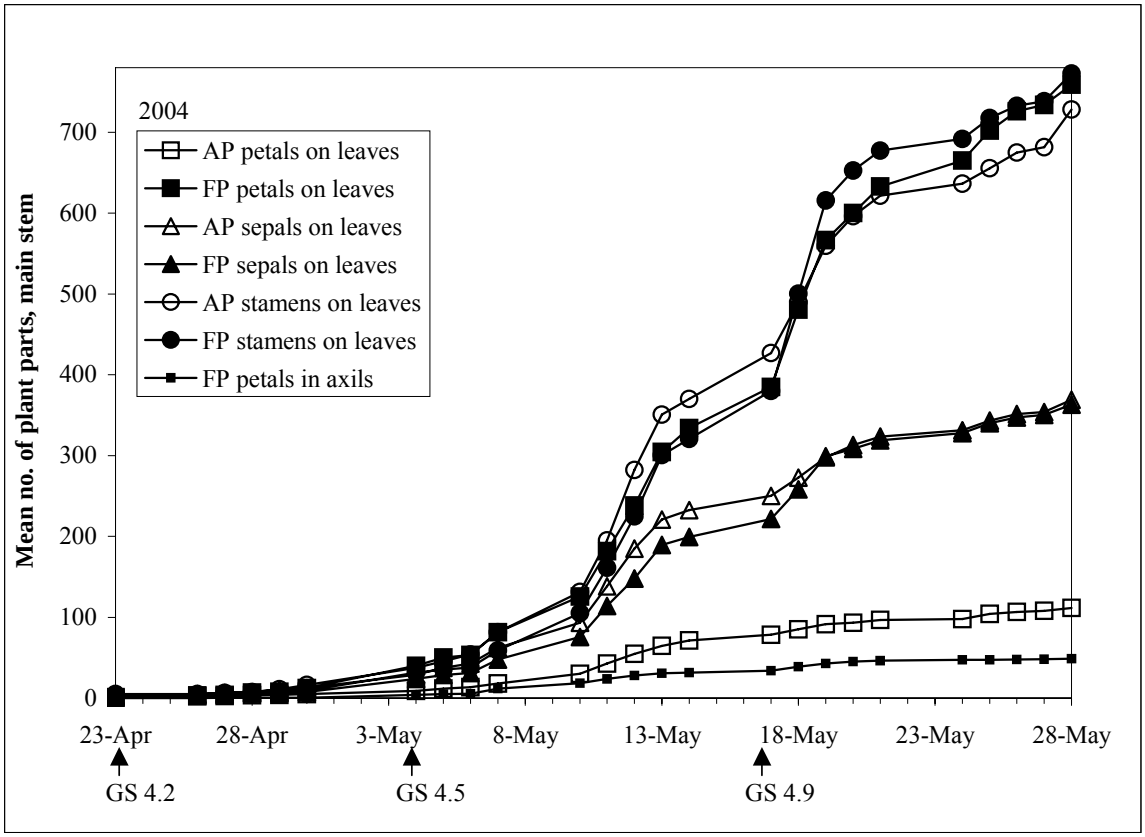


Figure 9. Flower parts sticking on apetalous (AP) and full petal (FP) oilseed rape leaves and axils, ADAS Rosemaund 2004 (cumulative counts).

The pattern of flower part stick on OSR leaves is important for the development of stem rot, because for stem rot to develop, flower parts infected with *S. sclerotiorum* need to stick to leaves for at least a couple of days. In each year the numbers of petals from FP plants sticking to leaves were similar to the number of stamens from AP or FP plants on leaves, as expected, and these were the most numerous flower parts counted on leaves (Figure 9). Relatively low numbers of petals were counted on leaves in AP plots, indicating low numbers of petals on AP flowers (the flowers were not completely apetalous) and also low cross contamination with petals from any adjacent FP plots. The number of sepals sticking to leaves was relatively low compared to stamens or FP petals, but was similar for AP and FP plants. OSR leaves become larger further towards the base of each plant, and therefore it was not surprising that more flower parts per leaf were counted on the lowest leaves, and that most of the stem rot lesions developed at the sites of petioles of these lower leaves.

Sclerotinia infection can also occur via infected flower parts caught in leaf axils. Fewer flower parts in total were counted in axils (e.g., FP petals in axils shown in Figure 9), as illustrated by final cumulative axil counts in 2004: FP petals, 49; AP stamens, 72, FP stamens, 65, AP sepals, 43 and FP sepals 39. In each of the three years, the counts of flower parts in axils were similar and always much lower than on leaves. In a dry year with little or no sticking on leaves, this might not always be the case, as axils often remain wet with dew and tend to retain flower parts caught in them, which can lead to direct infection of stems.

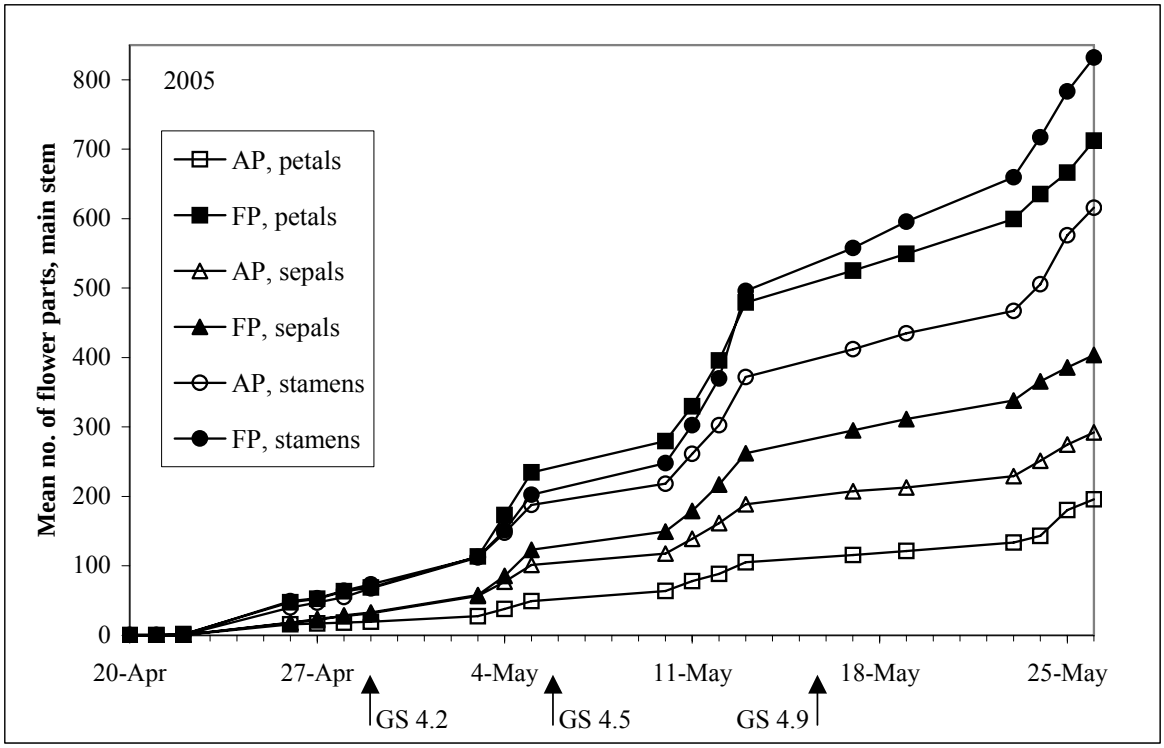


Figure 10. Flower parts sticking on apetalous (AP) and full petal (FP) oilseed rape leaves, ADAS Rosemaund 2005 (cumulative counts).

In 2005, the counts of FP petals and FP and AP stamens were different, unlike 2004, but as in 2004 these counts were higher than the AP or FP sepal counts (Figure 10). As in 2004, counts of AP petals were low.

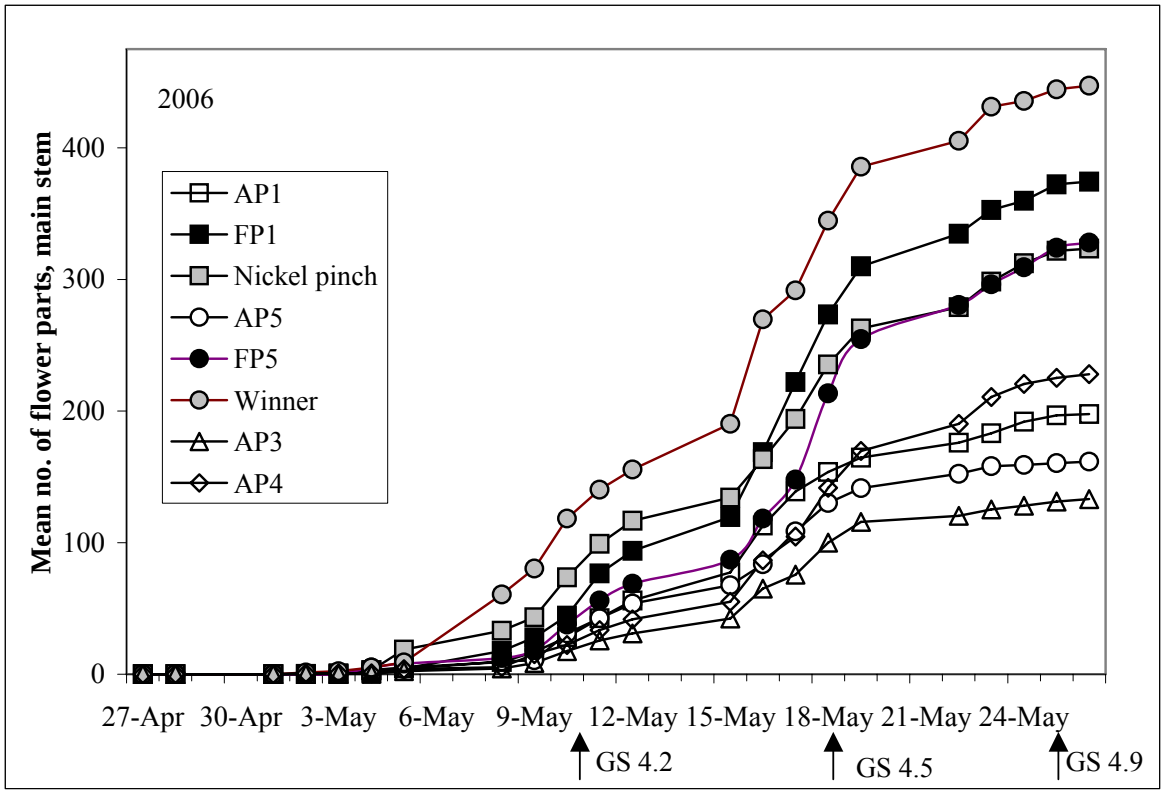


Figure 11. Petals sticking on apetalous (AP) and full petal (FP) oilseed rape leaves, ADAS Rosemaund 2006 (cumulative counts).

In 2006, petal stick counts on additional FP (closed black symbols in Figure 11) and commercial lines (Winner and Nickel) (grey symbols in Figure 11) were highest compared to counts of petals from the AP lines (open symbols in Figure 11). ANOVA of the final cumulative petal count showed significant differences between the various OSR lines overall. However, final numbers of petals stuck were not directly related to the incidence of stem rot across OSR lines, e.g., Nickel and Winner both had relatively high petal stick counts, but Winner developed 12.6% stem rot compared to Nickel with 2.6% (see Figure 13 below).

As in previous years, but not shown in Figure 11, counts of stamens on all lines were similar to FP petal counts. Sepals were not counted in 2006, because 2004 and 2005 results had confirmed observations from other previous OSR trials that sepals rarely stick to leaves. The AP lines were not completely pure, with an average of 1.4 petals per flower appearing in 2006, compared to <1 petal per flower on average in 2004. This was attributed to about 15% outcrossing of the stocks in the previous year which, owing to the recessive nature of the apetally trait, led to the production of petals in about 15% of the individuals.

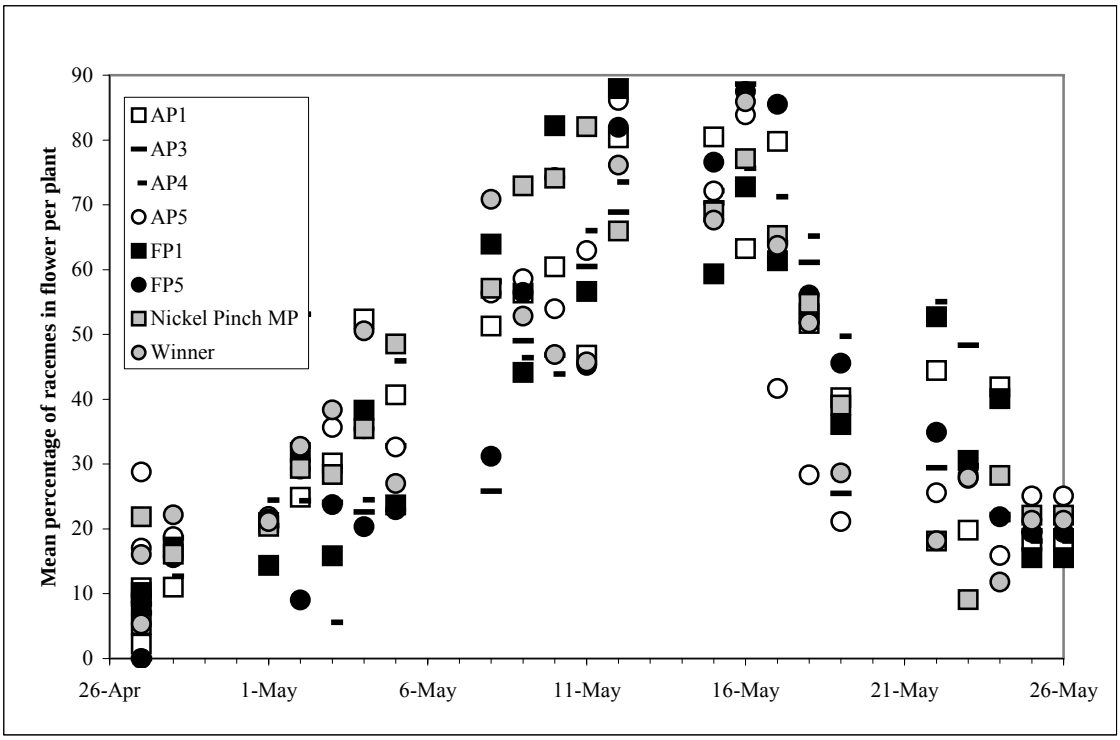


Figure 12. Percentage of racemes in flower for winter oilseed rape lines, ADAS Rosemaund 2006.

There were no obvious differences in flowering time between the OSR lines used in 2006, as shown by the percentage of racemes in flower at any one time during flowering (Figure 12). Differences in flowering time might have been one explanation for differences in % stem rot, e.g., a very early flowering cv might have had had a higher % of infected petals sticking to leaves than a late flowering cv during a peak of airborne spore infection.

Table 5. Sclerotinia stem rot incidence in oilseed rape lines, ADAS Rosemaund 2004-6.

	2004*	2005	2006*
AP 1	4.9	3.6	5
FP 1	7	4.3	3.9
AP 3			1.9
AP 4			3.1
AP 5			2.2
FP 5			12.6
Nickel			2.6
Winner		10.7	8.8
SED	0.8		.6

* significant difference in stem rot between entries for that year, $p \leq 0.01$; winner was not included in the analysis for 2005 because it was the surrounding field crop, not part of the trial design.

In the first two years of the project when the NILs AP1 and FP1 were tested, there was more stem rot in FP1 plots (Table 5), but the difference between the two lines was not as large as expected, given the differences in numbers of flower parts sticking to leaves. FP1 had significantly more stem rot than AP1 only in 2004. In 2005, the crop of cv Winner surrounding the trial plots was noted to have higher stem rot incidence than in the trial plots of AP1 and FP1, and 100 plants of Winner were assessed for stem rot at 25 random locations across the field. The range was 0 – 25% stem rot per location, with a mean of 10.7%. Therefore, in 2006, the commercial lines Winner and Nickel were included in the trial plan along with the additional AP and FP lines. In 2006, the NILs AP1 and FP1 both had low stem rot, as did Nickel (Figure 13). AP1 and FP1 were derived from Nickel. However, the NILs AP5 and FP5 differed in stem rot incidence, with 2.2 and 12.6%, respectively. Winner also had relatively high % stem rot, 8.8% (Figure 13). AP5 and FP5 were derived from Winner, reflected in the high % stem rot found in AP5. AP3 and AP4 both had low % stem rot, consistent with their backgrounds (AP3 was a sister line to AP1 and AP4 had an independent source from an Express background).

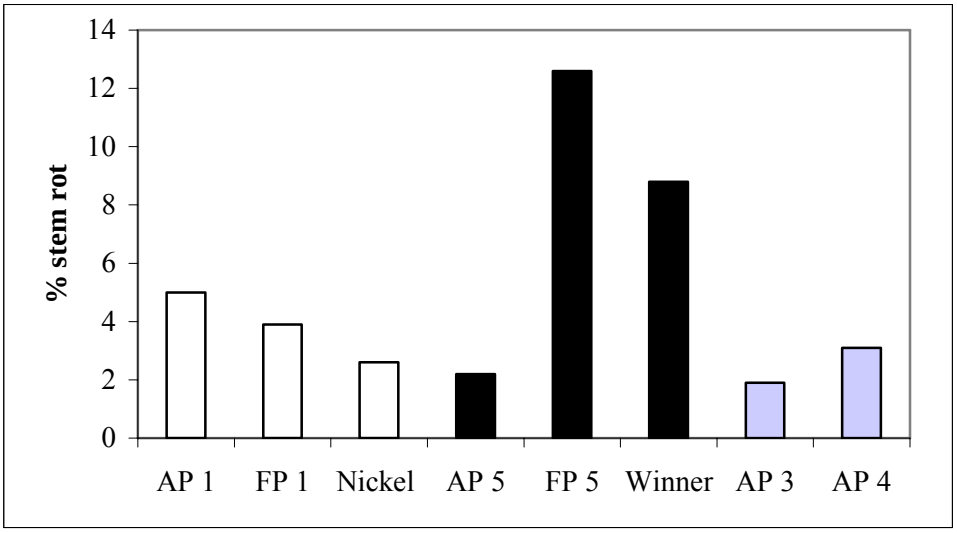


Figure 13. Sclerotinia stem rot incidence in oilseed rape lines, ADAS Rosemaund 2006. (LSD = 2.2, F prob <0.001).

Table 6. Stem rot incidence and location in apetalous (AP) and full petal (FP) near isogenic lines (NILs) of winter oilseed rape, ADAS Rosemaund, 2004 and 2005.

	NIL	Upper	Mid	Low	Secondary racemes	% Stem Rot
2004	**AP**	0.20	*1.2	3.50	0.80	*4.9
	FP	0.10	2.70	4.10	1.30	7.00
	AP	0.03	0.57	2.97	0.00	3.57
2005	**FP**	0.03	0.43	3.83	0.00	4.30

* Significant difference, $p < 0.005$

Nearly all stem rot lesions developed on the main stem, with fewer or no lesions on secondary racemes (Table 6). Most lesions were located mid or low plant. Therefore, sclerotinia infection on an individual plant is most likely to cut off the main stem, below most of the secondary raceme stems and can therefore cause significant loss on that plant.

Table 7. Oilseed rape yields, ADAS Rosemaund and CPB- Twyford 2006.

Yields, t/ha, 2006		
OSR line	**ADAS Rosemaund**	**CPB-Twyford, Pinchbeck**
AP1	3.60	4.26
AP3	3.82	4.52
AP4	3.82	4.71
AP5	3.12	3.20
FP1	3.27	3.96
FP5	3.57	5.27
Nickel Pinch MP	4.10	4.28
Winner	3.84	4.40

ANOVA, yields 2006, ADAS Rosemaund and CPB-Twyford Pinchbeck					
	df	ss	ms	vr	F pr
Line	7	5.79	0.83	20.61	<.001
Site	1	5.62	5.62	140.15	<.001
Line.Site	7	2.04	0.29	7.25	<.001
Residual	34	1.36	0.04		
Total	49	14.81			

The AP lines in general yielded more than the FP lines at each individual site (Table 7). The yields at CPB-Twyford at Pinchbeck were higher than the Rosemaund yields, mainly because the CPB site had no stem rot, but could also be due to site and weather factors. FP5 had relatively high yields at Pinchbeck compared to other AP and FP lines at the same sight, but at Rosemaund FP5 yields were similar to FP1 yields, most probably due to high stem rot % on FP5 at Rosemaund.

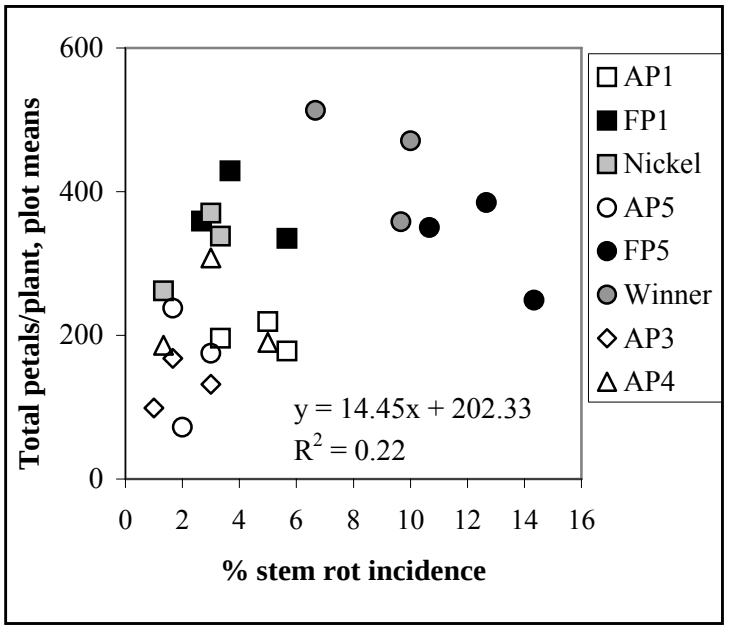


Figure 14. Petal sticking and Sclerotinia stem rot incidence on winter oilseed rape lines: full petal (FP), apetalous (AP), and two commercial varieties, ADAS Rosemaund, 2006.

There was a trend towards increasing numbers of petals stuck to plants with higher % sclerotinia stem rot incidence, but overall the relationship was not significant (R^2= 0.22, Figure 14). The AP lines all had relatively low petal stick and low stem rot. However, although all the FP lines (including Winner and Nickel) had broadly similar numbers of petals sticking, there were big differences in stem rot incidence, e.g., FP5 had 12.6% stem rot and Winner had 8.8 %, but FP1 had 3.9% stem rot and Nickel had 2.6%. FP5 was derived from Winner and FP1 was derived from Nickel. Therefore, there may be a genetic resistance to sclerotinia in Nickel and related lines that should be investigated further.

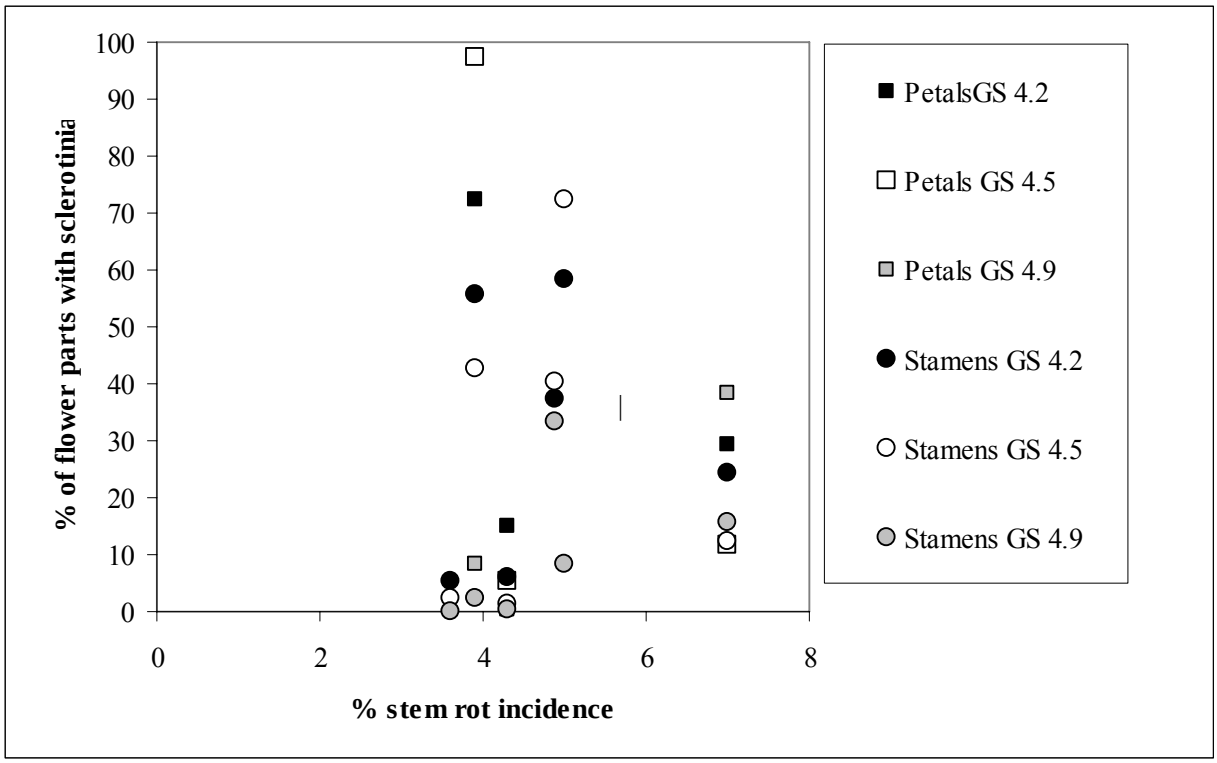


Figure 15. Percentage of petals and stamens testing positive for sclerotinia and final % stem rot incidence, for winter oilseed rape near-isogenic lines AP1(apetalous) and FP1 (full petal).

There was no clear relationship between the % of flower parts testing positive for sclerotinia by agar plating and final levels of stem rot (Figure 15, $R^2 = -0.058$), and therefore there was no clear evidence for the exact timing of infection events occurred during flowering each year. Flower testing was done only on the AP1 and FP1 lines in each year, and stem rot was generally low on both these lines, with a maximum of 7% on FP1 in 2006. When relationships between petals only or stamens only and final % stem rot were examined for each of the growth stages 4.2, 4.5 and 4.9, all were non-significant. This was most likely due to a combination of limited data for each growth stage separately, and low stem rot on both the AP and FP lines. There was no significant relationship between petal or stamen infection and final stem rot for AP plants only or for FP plants only.

Discussion

In summary, stamens were found to be a route for sclerotinia infection as well as petals, but a larger proportion of petals than stamens had sclerotinia present. On FP plants, the petals appear to shield the stamens from airborne spores. In this study, final stem rot levels were not found to be significantly related to levels of sclerotinia inoculum on flowers, most probably because flower testing was, in hindsight, only carried out on lines which were somewhat resistant to sclerotinia infection (the AP1 and FP1, Nickel-related, near-isogenic lines). When susceptible near-isogenic lines of AP5 and FP5 were compared (Winner related), the FP5 line had significantly more stem rot than the AP5 line (12.6% vs 2.2%). In no situation did the AP lines escape stem rot completely.

Infection of flowers with sclerotinia is an indicator of the spore inoculum levels over a crop, and is therefore an important factor in assessing the risk of stem rot. In previous research (Turkington & Morrall, 1993; Turkington *et al*, 1991), flower infection levels in susceptible OSR cvs have been shown

to have a direct relationship to stem rot incidence, but in this work, this was not the case. However, only AP1 and FP1 were used for flower tests, but AP1 and FP1 and Nickel appeared relatively resistant to stem rot compared to other FP lines such as FP5 and Winner. Therefore a relationship between % flower parts testing positive and final levels of stem rot was unlikely to exist for AP1 and FP1, because FP1 had more infected flower parts in total sticking to its leaves than AP1, yet had similar low levels of stem rot to AP1. However, based on other research, there is still a case for developing a quick and simple test to help growers determine times of high inoculum and therefore high infection risk of OSR leaves from infected flower parts. Agar plate testing requires sampling of flowers at many locations across a crop and is slow (up to 10 days for UK samples which always contain Botrytis) to give results. It is therefore impractical for routine testing of the levels of sclerotinia airborne spores. However, it does assay for viable sclerotinia on flowers, whereas the semi-quantitative PCR test on flowers will detect dead spores which can give a misleadingly high indication of (see later section in this report on the PCR test development). It may be better to develop simple and inexpensive spore traps to assay airborne spore numbers over a crop, which could give quick results by a quantitative PCR test, yet only require one or two sampling points per crop.

A larger proportion of stamens were found to be infective on AP1 than FP1, which suggests that petals may shield sepals from airborne spores which circulate through the crop from apothecia on the ground. Stamens stuck to leaves in roughly equal numbers to petals from the FP lines, and therefore are likely infection routes on AP lines. Sepals were also found to have sclerotinia, generally at similar levels to stamens, but sepals stuck to leaves in relatively low numbers and were therefore less likely to cause infection. The % of leaf discs from mid-plant which tested positive for sclerotinia were similar on AP and FP lines, suggesting no difference between AP or FP lines in the numbers of spores circulating within the lower part of the crop. The role of pollen in infection was not investigated in this work, but it is known to carry sclerotinia spores (Huang *et al*, 1997). While it is probable that equal amounts of pollen would stick to leaves in AP or FP lines, it is possible that a larger proportion of pollen is infected in AP lines than FP lines, as is the case for stamens. This could be another significant infection route for AP lines.

Sticking of infected flower parts on leaves is a key stage in the development of stem rot. In this work, final stem rot levels tended to be higher with more petals, stamens and sepals stuck to leaves. All the AP lines had relatively low petal stick and low stem rot, but there were exceptions with the FP lines. All the FP lines had relatively high petal stick, but FP1 and Nickel in particular developed low stem rot compared to FP5 or Winner. Therefore, petal stick may not be directly related to stem rot but it is an indicator of risk. Without flower stick, no stem rot will develop. High levels of flower stick indicate disease risk, but are not always associated with high levels of stem rot.

In each year, although there was variability, the cumulative counts of flower sticking showed that in total, FP1 always had more flower parts sticking to leaves than AP1, as expected. This was true for the additional AP and FP lines tested in 2006. AP lines are therefore expected to get less leaf infection and stem rot then FP lines. However, AP lines can still become infected by other flower parts, in particular, stamens. The counts of petals, stamens and sepals in axils were very low compared to counts on leaves, but stamen and sepal counts were similar between AP and FP lines, and could be a significant route of infection, but probably not the main route.

The lower leaves of the plants had the most flower parts counted on them, because the lower leaves are the largest in area. Most stem rot lesions developed on the lower plant in this project, at the location of the highest flower part count. This supports the idea of the main infection route being by flower parts sticking to leaves.

The results provide indications that there is potential for sclerotinia-resistant cultivars. The comparisons of apetalous material with fully petalled near-isogenic lines showed, with some qualification, that the absence of petals does lead to some avoidance of stem rots. In 2006 the near-isogenic lines AP5 and FP5 differed in stem rot incidence, with 2.2% and 12.6%, respectively. These lines have a background derived from the cultivar Winner which also had relatively high level of stem rot, 8.8%. However, the stem rot levels in the near-isogenic lines AP1 and FP1 were not as different as expected, with only the 2004 results showing significantly more stem rot in FP1 compared to AP1 (4.9% vs 7.0%). It was suggested from observations made in 2005 and confirmed in the 2006 trial that Nickel which was the donor of the background genotype for the AP1/FP1 NILs showed a significantly lower number of stem rots than Winner (2.6% vs. 8.8%), despite being exposed to an equivalent number of infective petals, suggesting that there may be an additional mechanism operating to confer some partial resistance in Nickel. Unfortunately the nature of this was not a subject of the current studies and the effect served to partially obscure the disease avoidance mechanism of the apetally in the AP1/FP1 NILs.

The differences in crop establishment each year (spring plants counts, mean of AP1 and FP1, 13, 63 and 30 plants/m^2, in 2004,'05 and '06, respectively) were not related to flower stick on leaves, eg., around 400 petals in total were counted on the high density crop in 2006 compared to over 700 in the low density crop of 2004. In low plant density crop, the plants are usually bigger with more branches. The differences in flower stick are likely a reflection of weather, particularly rain and wind. The timing of flowering appeared similar across all lines, whereas differences in timing, such as onset of early flower, could have influenced infection levels. The location of stem rot lesions was the same in all years for all crop densities, with nearly all lesions occurring in the lowest third of each plant, on the main stem, and few or no lesions on secondary racemes. This location causes the maximum yield loss in plants. Within each year, the AP1 and FP1 lines had very similar establishment and crop height, and appeared the same in every respect except for the numbers of petals and also yields. Where there was no stem rot at the CPB-Twyford site in 2006, the AP lines generally yielded more than the FP lines, as found by Gemmill *et al* (2003). At the ADAS site, where there was some stem rot on all lines, the difference in yield between the AP and FP lines was smaller. AP winter OSR is a multiple recessive gene trait and difficult to breed, and unlikely to be commercially viable unless more resources are used to develop improved stable material in a good genetic background. The AP lines in this project were not completely pure, with an average of 1.4 petals per flower appearing in 2006, compared to <1 petal per flower on average in 2004.

Sclerotinia stem rot requires the right combination of many factors to allow development of the stem lesions. It is therefore difficult to devise a robust disease prediction scheme when it is difficult to quantify the risk at every step in the sclerotinia cycle. The crop has to be flowering and weather must be suitable for apothecial germination, ascospore release, spore circulation in the air, flower parts falling in sufficient numbers, sticking of flower parts on leaves, and infection of leaves. Current advice is to check all risk factors as outlined in the HGCA sclerotinia decision guide and in absence of other guidelines, spray particularly at early flower. However, if airborne inoculum could be easily assayed, this would help in assessing disease risk and timing of fungicide sprays, if any. If the crop has no flowers sticking and the weather forecast is dry, infection is less likely even if inoculum levels are high. But petals and flower debris can stick to leaves with only a small amount of rainfall, and axils usually remain moist even when the crop is generally dry, and it is possible that in dry weather it is even more important to have a method of measuring airborne spore inoculum to order to be able assess disease risk. From previous work (Davies *et al*, 1999) there appear to be years of high infection efficiency

when not all factors such as inoculum levels appear to be a risk factor, yet stem rot levels are high. The challenge still remains in the UK to predict the years of major outbreaks.

Further work should focus on looking for resistance among existing OSR varieties. Given that it is currently difficult to breed a stable and high yielding apetalous line, it would be worth doing field trials designed to compare stem rot in winter OSR FP lines. In addition, the mechanisms of resistance should be investigated for different varieties, as this would enable breeders to focus on traits that would minimise yield loss. For example, this could include not only plants that have a reduced number of leaf infections via thick leaf wax, but plants that slow down the growth of *S. sclerotiorum* and develop small, partial stem lesions as opposed to large, stem-girdling lesions that cause complete death of the upper canopy.

To improve current risk-assessment schemes it would be helpful to have better predictions of petal stick and infection with weather. Good progress has been made in a previous projects on sclerotinia in lettuce using a modelling approach with weather and infection data (Defra project HH3215TFV, Forecasting Sclerotinia in field grown lettuce, and current HDC project FV294, Outdoor lettuce: forecasting and control of sclerotinia). There is detailed weather, petal stick and stem rot field data from previous and current OSR projects that could be used to develop a field model for use in stem rot prediction.

References

Davies J M Ll, Gladders P, Young CS, Dyer C, Hiron L, Locke T, Lockley D, Ottway C, Smith JA, Thorpe G, Watling M, 1999. Petal culturing to forecast *Sclerotinia* stem rot in winter oilseed rape: 1993-1998. *Aspects of Applied Biology* **55**. Protection and Production of Combinable Break Crops. p129-134.

Evans EJ, Gemmill JM, Werner CP and Williams E, 2003. Physiological factors contributing to yield enhancement in winter apetalous oilseed rape (*Brassica napus*). Proceedings of the 11th International Rapeseed Congress, AP1.12

Freeman, Ward, Calderon & McCartney, 2002. *Eur. J. Plant Pathology.* **108,** 877-886.

Gemmill J M. 2003. The physiology and agronomy of the apetalous character in oilseed rape (*Brassica napus* L). University of Newcastle, UK, PhD Thesis.

Gemmill J M, Evans E J, Werner CP and Williams E, 2003. Influence of the apetalous character on the distribution of pods and seeds in near-isogenic lines of winter oilseed rape (*Brassica napus*). Proceedings of the 11th International Rapeseed Congress, AP5.11

Gugel RK, Morrall RAA, 1986. Inoculum-disease relationships in *Sclerotinia* stem rot of rapeseed in Saskatchewan. *Canadian Journal of Plant Pathology* **8**, 89-96.

Huang HC, Kokko E, Erickson RS, 1997. Infection of Alfalfa pollen by *Sclerotinia sclerotiorum.* *Phytoparasitica* **25**, 17-24.

Huang HC, Kokko EG, 1992. Pod rot of dry peas due to infection by ascospores of *Sclerotinia sclerotiorum*. *Plant Disease* **76**, 597-600.

Jamaux I, Spire D, 1994. Development of a polyclonal antibody-based immunoassay for the early detection of *Sclerotinia sclerotiorum* in rapeseed petals. *Plant Pathology* **43**, 847-862.

Jamaux I, Gelie B, Lamarque C, 1995. Early stages of infection of rapeseed petals as leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. *Plant Pathology* **44**, 22-30.

Jiang L, 2001. A mutant with apetalous flowers in oilseed rape (Brassica napus): Mode of inheritance and influence on crop physiology and *Sclerotinia* infection. Georg-August University, Gottingen, PhD Thesis.

Koch, S.; Dunker, S.; Kleinhenz, B.; Rohrig, M.; Friesland, H.; Tiedemann, A. von, 2006. Development of a new disease and yield loss related forecasting model for Sclerotinia stem rot in winter oilseed rape in Germany. Conference: Working Group "Integrated Control in Oilseed Crops". Bulletin OILB/SROP **29**, No. 7, 335-341. Eds. Koopmann, B.; Cook, S.; Evans, N.; Ulber, B. Publ: International Organization for Biological and Integrated Control of Noxious Animals and Plants (OIBC/OILB), West Palaearctic Regional Section (WPRS/SROP), Dijon, France.

Lefol C, Morrall RAA, 1996. Immunofluorescent staining of *Sclerotinia* ascospores on canola petals. *Canadian Journal of Plant Pathology* **18**, 237-241.

Penaud A, Huguet B, Wilson V, Leroux P, 2001. Benzimidazole resistance of *Sclerotinia sclerotiorum* in French oilseed rape crops. In: Young CS, Hughes KJD, eds. *Proceedings of Sclerotinia 2001- The Xl International Sclerotinia Workshop,* York 8th-12th July 2001: Central Science Laboratory, York, England. 117-118.

Turkington TK, Morrall RAA, 1993. Use of petal infestation to forecast *Sclerotinia* stem rot of canola: the influence of inoculum variation over the flowering period and canopy density. *Phytopathology* **83**, 682-689

Turkington TK, Morrall RAA, Gugel RK, 1991. Use of petal infestation to forecast *Sclerotinia* stem rot of canola: evaluation of early bloom sampling. *Canadian Journal of Plant Pathology* **13**, 50-59.

Turner J, Young C, Riding A, Gladders P, 2002. *Sclerotinia* control in oilseed rape: progress with quantitative diagnosis and development of a web-based forecasting scheme. HGCA Project Report OS56, Home-Grown Cereals Authority, London.

Twengstrom E, Sigwald R, Svensson C, Yuen J, 1998. Forecasting Sclerotinia stem rot in spring sown oilseed rape. *Crop Protection* **17**, 405-411.

CHAPTER 2

QUANTIFYING THE RELATIVE IMPORTANCE OF PETALS, STAMENS AND SEPALS IN THE INFECTION PROCESS FOR *SCLEROTINIA SCLEROTIORUM* IN OILSEED RAPE

Introduction

Sclerotinia stem rot in oilseed rape (*B. napus*) is a sporadic disease in the UK, and the challenge is to reduce unnecessary fungicide applications. This could be achieved through improved agronomic technologies enabling the identification of those high-risk oilseed rape crops which justify a fungicide treatment or through the breeding of oilseed rape varieties with a genetic tolerance or avoidance mechanism. The first of these options has been considered in the first chapter of this report and by Davies *et al* (1999) and suggests that the development of methods to quantify the inoculum presence in individual fields may be a cost effective way to target the application of expensive fungicides. To date no reliable genetic resistance to *S. sclerotiorum* in *B. napus* has been identified although it has been suggested by Jiang (2001) that the use of apetalous material could provide a substantial avoidance mechanism. However, the use of apetalous varieties of oilseed rape presents breeders with a significant challenge since the trait is not easily stabilised, involves the introduction of recessive alleles at each of several loci and is associated with an inherent yield penalty. Prior to committing extensive resources to breeding advanced lines of apetalous oilseed rape it is therefore valuable to attempt to quantify the extent to which apetally might successfully avoid *S. sclerotiorum* stem rot. Current knowledge (e.g., Turkington & Morrall, 1991) clearly identifies petals as a major element in the initial infection process but previous studies have solely utilised conventional fully petalled material. There is therefore little information regarding the potential for other flower parts, such as the sepals or the stamens, or even direct infection of spores on leaf laminar, to initiate the infection process. The aim of this study was to utilise an apetalous line of *B. napus* together with a near isogenic sister line (NIL) which produced normal petals in order to quantify the relative importance of the different flower parts in the initial infection process.

Methods

Near-isogenic lines (NILs) of apetalous oilseed rape were developed at CPB-Twyford, UK following two backcrosses to the winter oilseed rape variety Nickel and sister line segregation at F_4. Two of these NILs, FP1 (fully petalled) and AP1 (apetalous), differing only with respect to the presence or absence of petals were sown in 30ml module seed trays and transferred to soil in a polythene tunnel in the October of 2003 and repeated in autumn 2004. The 10m x 4m polythene tunnel had previously been used annually during the previous 10 years for the production of seed crops of oilseed rape and had developed a very high risk of *Sclerotinia sclerotiorum* stem rots. Prior to planting the soil was prepared by rotavation, incorporating NPK fertiliser enhanced with trace elements and sulphur in the form of sulphite. In addition 5g of sclerotia harvested from infected stems of the previous crop were incorporated into the soil. The polytunnel was planted with a central path and 2 equal sized beds to each side of the path. The lines AP1 and FP1 were planted at the 3-4 true leaf stage. Owing to the need to block the plants (to avoid cross contamination with petals from the FP line as far as possible) replicate blocks were limited. Accordingly, a two x two blocked lattice design was adopted with a 3 x 10 arrangement of individual plants within each block. This design enabled the spatial factors of the left to right and door to far end of the tunnel to be treated as fixed effects. The front 10 plants of each block were labelled and used for the more detailed assessments in each year. Watering was delivered

by overhead mist spray manually applied as required during the autumn, winter and spring. Once flowering began, the overhead watering was applied a minimum of 3 times per week. The quantity of water delivered was adjusted according to prevailing weather conditions in order to maintain high humidity (in excess of 90%) without saturating the soil. In both seasons, it was found that by late May/early June the temperatures within the polytunnel were on many days above 40^{o}C and fungal *S. sclerotiorum* rots ceased developing. Few of the leaf rots initiated were observed to develop into a true stem rot with sclerotia.

Assessments in 2003-2004

During the first season of this study, flowering commenced on 14 April 2004. The progression of petal drop and *S. sclerotiorum* leaf rots was monitored and on the 4 May, disease progress was judged to be well advanced. The numbers of leaves (green entire leaves, not senescent), petals adhering to leaves, sepals on leaves, stamens on leaves, petals per flower (10 flowers sampled) and *S. sclerotiorum* leaf rots were counted on each of the 10 labelled plants in each block. A further assessment on the 16 July counted all main stem and branch-stem *S. sclerotiorum* rots, together with an estimate of the total percentage of each plant affected by rot.

Assessments in 2004-2005

In the second season the polytunnel was set up as for 2003-2004 but more detailed observations were made in order to determine the cause and developmental course of each *S. sclerotiorum* rot. At the onset of first petal drop (11 April), 4 leaves were marked with a tag on each of the 10 individually labelled plants within each replicate. Every two or three days the upper surface of each of these tagged leaves was photographed *in situ* using a digital camera, care was taken not to disturb the leaves or dislodge adhering flower parts. In addition to these photographic records two sets of visual records which were made on 3 May 2005 and 16 May 2005. On these two dates, each tagged leaf was inspected and the number and position of any *S. sclerotiorum* leaf rots noted. In addition other lesions such as phoma leaf spots were recorded in order to aid with the later analysis of the photographs. Additional counts of leaf, stem and pod rots on the whole of the plant were also made on these dates. The photographic records were used to make complete counts of petals, sepals and stamens sticking to each leaf and particularly to identify sclerotinia leaf rots. For each leaf rot identified, it was possible to work back through the photographic record to follow the course of development of the rot and identify the initiating tissue.

Results

Flowering commenced in the polytunnel on 14 April in 2004 and on 7 April in 2005. As expected the apetalous NIL produced some petals most of which were reduced in width (narrow) and tending to become filamentous. Counts from the 20 key plants from each NIL showed a mean petal number per flower of 3.99 and 4.00 for the FP NIL and 0.82 and 0.79 for the AP NIL in 2004 and 2005 respectively. Table 8 shows the numbers of petals sticking to the leaves in 2004 and 2005, an additional 120 narrow petals were counted on the AP leaves in 2005. In both years, more stamens were counted on the AP leaves than on the FP leaves, it is likely that this is a consequence of a ‘masking’ effect since stamens overlaid by petals would be hidden and not counted. In 2004, sepals were counted and found to be nearly equal in numbers on the FP and AP NILs (213 and 219), but in 2005, no rots were attributable to sepals (using the photographic analysis) and counts were not made. The totals of number of leaf rots initiated show about four times as many in the FP material as in the AP (Table 8). The data was analysed for homogeneity of the FP v AP effect over years and for the overall FP v AP

effect by contingency chi-squared. The FP NILs had significantly higher numbers than the AP NILs of both petals and stamens sticking to the leaves and for the number of leaf rots and final stem rots.

Table 8. 2004 and 2005 polytunnel assessments. Counts for debris on leaves and initial and final *S. sclerotiorum* rots (total over two replicates). Homogeneity of distribution over years and between fully-petalled and apetalous (FP v AP) lines is tested by contingency Chi-squared.

	2004	2005	**Total**	year effect		FP v AP	
				$\chi^2_{(1)}$	*p*	$\chi^2_{(1)}$	*p*
petals on leaves [+]							
FP	508	296	**804**				
AP	142	49	**191**	*6.44*	*0.011*	*344.5*	*0.000*
stamens on leaves							
FP	342	199	**541**				
AP	455	293	**748**	*0.79*	*0.375*	*41.5*	*0.000*
total leaf rots initiated							
FP	43	39	**82**				
AP	7	14	**21**	*1.79*	*0.181*	*30.3*	*0.000*
final rots on stem							
FP	26	43	**69**				
AP	7	4	**11**	*2.03*	*0.154*	*38.7*	*0.000*
rots caused by petals *							
FP		25	**25**				
AP		8	**8**			*7.5*	*0.006*
rots caused by stamens *							
FP		5	**5**				
AP		3	**3**			*1.1*	*0.285*

[+] excludes counts of narrow petals produced by apetalous line

* not recorded in 2004. χ^2 based on test of homogeneity of rots over reps in 2005

Table 9. 2005, Numbers of leaf rots initiated in the FP and AP NILs, categorised by the initial infection source.

Source	FP	AP	Total
petal	18	7	**25**
petal clump	6	1	**7**
petal narrow		1	**1**
petal+stamen	1		**1**
stamen	5	2	**7**
stamen clump		1	**1**
droplet	3		**3**
ground contact	1		**1**
can't ID	3	2	**5**
Grand Total	**37**	**14**	**51**

The photographs from the 2005 season were studied and 51 *S. sclerotiorum* leaf rots were identified (Table 9). For each of these the earlier photographs of the same leaf were studied and the source of the initial infection identified (with the exception of 5 rots where the photo quality was impaired). Infections attributable to petals could be traced to either single petals or multiple layers of petals (clumps). The majority of sclerotinia lesions on leaves were initiated from petals, on both the FP and AP NILs (Table 9). On one occasion, one of the narrow petals produced by the AP NIL was responsible for the rot and on another both a petal and stamen entangled together could be identified. There were 8 events where only stamens were associated with the initial site of infection and 3 where the infection developed at the site of an earlier water droplet in the absence of any occurrence of other

tissue (Table 9). On one occasion the leaf tip touching the ground developed a rot which may have been a direct consequnce of this contact or could have been a result of an additional hidden tissue infection. There was no occasion when sepals were seen to initiate an infection and no instances of an infection developing on 'clear' leaf where no visible tissue or droplet had previously occurred. The time frame for rot development was quite variable ranging from extremely fast, as in the example of a petal induced rot when the time from the petal landing to the leaf collapsing was less than 5 days. But other occasions showed up to 7 days between the initial tissue landing and rot development. Frequently, following the initial signs of rot on leaves there was a small increase in rot area but no further growth beyond that. This pattern of arrested development was observed for most of the initiated leaf rots in both 2004 and 2005. Unfortunately it is not possible from the current experimental design to attribute this effect either to the extreme environment (during early to mid May the poly-tunnel became extremely warm, up to 45^{o}C, on more than 50% of days) or to the background genotype of the NILs used. The main source of leaf rots was, perhaps as expected, the presence of one or more petals adhering to the leaf some 2 to 6 days prior to the initial leaf rot, with significantly fewer rots observed on the AP than the FP lines (Table 8). The secondary tissue leading to infections were the stamens for which there was no significant difference between the AP and FP NILs.

Discussion

It is clear from these studies that *B. napus* leaves can be infected through routes other than petals acting as the initial nutrient source for the fungal growth. Davies *et al* (1999) have shown that all the flower part tissues, petals, sepals and stamens, are infected whilst still attached to the flower and that *in vitro* all can give rise to *S. sclerotiorum* growth. This study notably eliminates sepals as a significant intermediary infection tissue, this may be owing to the shape of the sepals which prevents good adhesion to the leaf and less often promotes the ideal microclimate for fungal establishment and transfer across tissues. In contrast, petals are flexible and soft presenting an ideal nutrient source for *S. sclerotiorum*, the ability to create a humid micro-climate and a good contact between the petal and the leaf surface. Stamens fall somewhere between these two providing a flexible soft tissue for initial spore germination but less often lead to cross infection into the leaf. This may be a consequence of the reduced adhesion and smaller surface area of contact leading to a less than ideal micro-climate for the early stages of the fungal growth. Using the data from the photographic analysis it is possible to determine that 9.6% of petals recorded on the leaves led to an early stage leaf rot whereas only 1.6% of stamens initiated a rot. This gives a relative infectivity ratio of 5.9:1 for petals vs. stamens. This finding might be extended to the field situation making the prediction that petals are about 6 times more infective than stamens. For near isogenic lines (with no genetic differences between them in resistance) it would be possible to predict that:

Number of rots = $k_i\, r(p + s/6)$

Where: k_i = infectivity constant dependent upon the prevailing conditions in the field such as inoculum levels, temperature and humidity, r = resistance constant of the variety under study, p = cumulative number of petals sticking to leaf and s = cumulative number of stamens sticking to leaf

For a comparison of fully petalled and apetalous NILs the ratio

Number of $Rots_{AP}$ / Number of $Rots_{FP}$ = $(p_{AP} + s_{AP}/6) / (p_{FP} + s_{FP}/6)$
($_{AP}$ denotes apetalous parameters and $_{FP}$ fully petalled parameters)

can be calculated and used to test the goodness of fit for this simple model for the field collected data. Using this model for the 2004 and 2005 data sets gives predicted incidence of stem rots of 1.8% and 1.4% for the apetalous AP1 line in the two years respectively. This underestimates the observed incidences which were 4.9% and 3.6% for the two years. It therefore appears that the simple polytunnel system does not provide a perfect model for the field situation but may establish one element of the whole infection process. In the field, however, a higher % of stamens carry sclerotinia inoculum on the AP NILs than the FP NILS, which would tend to increase the predicted infections from stamens in field apetalous material compared to fully petalled. Pollen is also known to carry *S. sclerotiotum*, and it is possible that a higher proportion of AP pollen carries *S. sclerotiotum* than FP pollen, as is the case for stamens.

Further work is needed to establish the link between airborne inoculum levels and field environmental conditions which lead to infections of leaves by *S. sclerotiorum*. In addition, the relationship between leaf infections and main stem lesions needs to be investigated in the field, in locations where *S. sclerotiorum* risk is high.

References

Davies J M Ll, Gladders P, Young CS, Dyer C, Hiron L, Locke T, Lockley D, Ottway C, Smith JA, Thorpe G, Watling M, 1999. Petal culturing to forecast *Sclerotinia* stem rot in winter oilseed rape: 1993-1998. *Aspects of Applied Biology* **55**. Protection and Production of Combinable Break Crops. p129-134.

Jiang L, 2001. A mutant with apetalous flowers in oilseed rape (Brassica napus): Mode of inheritance and influence on crop physiology and *Sclerotinia* infection. Georg-August University, Gottingen, PhD Thesis.

Turkington TK, Morrall RAA, Gugel RK, 1991. Use of petal infestation to forecast *Sclerotinia* stem rot of canola: evaluation of early bloom sampling. *Canadian Journal of Plant Pathology* **13**, 50-59.

CHAPTER 3

DEVELOPMENT OF A PCR TEST FOR *SCLEROTINIA SCLEROTIORUM*

Introduction

Molecular diagnostics have been developed extensively in recent years to detect a number of specific plant pathogens. The principal method relied upon for this is the polymerase chain reaction or PCR, which artificially replicates a section of DNA with a specific target sequence of bases. The target sequence may be unique to a particular species, in which case, its replication to the extent that it can be visualised by gel electrophoresis, indicates the presence of that species in the sample. The target sequence may be common to a group of organisms, such as all fungi, or it could be unique at the sub-species level, for example indicating the presence of a particular gene or allele, or the presence of a mating-type gene or gene conferring resistance to a fungicide. Large numbers of samples can be processed relatively cheaply, providing an answer in only a few days or less, depending on the sample processing steps used. The technique can therefore provide a rapid and accurate indication of the presence of the target organism in a sample, which can be cheaper, easier and more accurate than traditional mycological techniques such as agar plating tests. More recently, the technique has been improved to enable quantification of the target DNA by detecting fluorescence from a dye that binds to DNA. Other quantitative PCR methods have also been developed. One key aim of the project was to develop a PCR-based diagnostic specific to *S. sclerotiorum* and evaluate its potential for use in disease forecasting schemes

Methods

Plant samples

Frozen plant tissue samples were received at Rothamsted Research (RRES) from field experiments at ADAS Rosemaund in 2004, 2005 and 2006. These were petal, stamen, and leaf discs taken from plots at GS 4.2, 4.5 and 4.9 (early to late flowering) from 20 plots in the first two seasons but only from six plots in 2006. Additionally in 2006, 12 petals and 12 stamens were taken from three FP plots twice per week from 25 April to 2 June 2006. Sepals were also sampled in 2004.

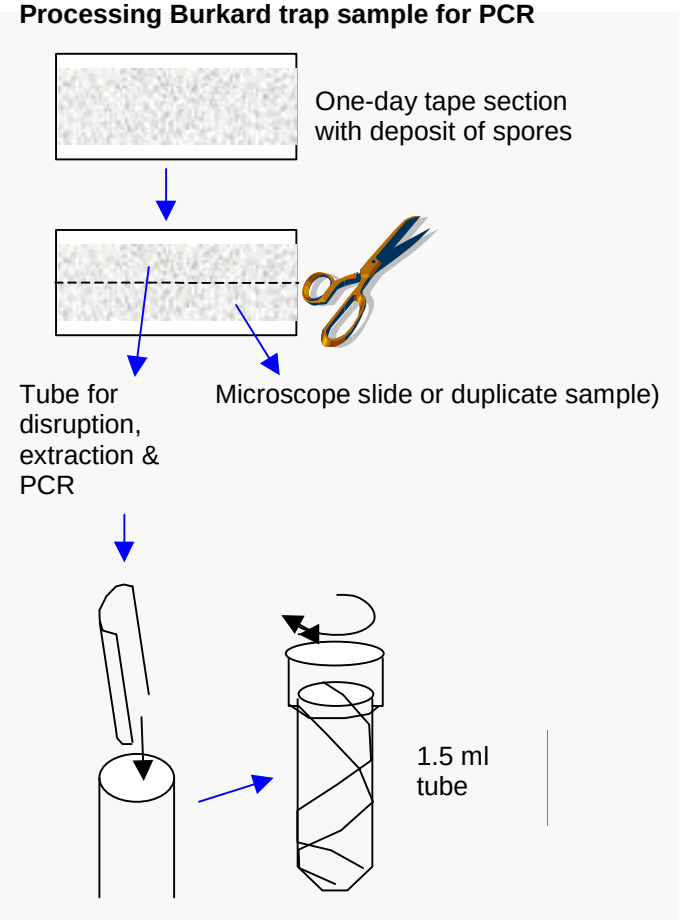
All petal and stamen samples were tested by agar plating at ADAS Rosemaund and by PCR at RRES. In the first season, sepal infection was found to be zero in a sub-set tested by PCR. Other evidence suggested that sepals were not a source of infection and so sepals were not tested in 2005 or 2006. Twelve leaf discs from each of 2 AP and 2 FP plots were tested by PCR at each sample date. Of the additional twice-weekly samples taken in 2006 ten petals and ten stamens per plot on each sample date were tested by PCR.

Air samples

Air samples were taken each week from two Burkard (Hirst-type) spore traps operating at each end of the plots in the field at ADAS Rosemaund sampling air at 10 L/minute. Unexposed prepared drums of wax-coated tape were supplied by RRES and returned by post after being used to sample air over a

week-long period. Each daily (48mm) spore tape section, held at the edge with forceps, was cut longitudinally along the centre-line of the spore trace using scissors to give two sub-sections (each 7mm x 48 mm). By slightly curving the sub-section of tape, it was placed into a 1.5 ml Eppendorf tube (waxed surface facing the inside of the tube) (see Figure 16). The tube was labelled and stored at minus 20ºC until further processing to extract DNA and PCR. The duplicate sub-section was mounted on a microscope slide, stained with trypan blue in gelvotol (Polyvinyl alcohol) for traditional spore counting by microscopy.

Figure 16.



To make DNA available for PCR, plant and air samples were processed using a commercial detergent-based product, MicroLYSIS (Microzone, http://www.microzone.co.uk/), used in combination with thermal cycling in a PCR block according to the manufacturer'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 13,000rpm 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 for the first two seasons was based on 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. It

produced relatively weak bands when the PCR product was run on an electrophoresis gel. This problem was confounded by occasional suspected false negatives, which occurred with this primer set, leading to the development, testing and optimisation of a new *S. sclerotiorum*-specific primer set. DNA from the 'negatives' from 2005 were retested with the new PCR, often giving positive reactions. It was therefore not due to an ineffective DNA extraction step as originally thought, but due to the inefficiency of the PCR. Details of the new primers will be published in a paper currently in preparation. Unfortunately there was insufficient DNA available to retest the 2004 and 2005 air samples with the new PCR primers, but air samples from two Burkard spore traps used in the field experiment at ADAS Rosemaund in 2006 were tested using the new set of *S. sclerotiorum*-specific primers in a SYBR-green quantitative PCR. This allows an estimation of the number of *Sclerotinia* spores sampled in air each day. The technique was tested by spraying suspensions of *Sclerotinia* ascospores, from a dilution series, onto prepared wax-coated tapes, as used in the Burkard air sampler. The sprayed tapes were allowed to air-dry. Unexposed control tapes were also produced. Additionally, tapes from four Burkard spore traps, which were operated simultaneously at the same location to provide a background of naturally occurring spores and pollens, were then sprayed with different amounts of *Sclerotinia* ascospores to verify that DNA from other spores and pollens would not interfere with the qPCR.

Air samples, petal, stamen and a sub-set of leaf samples from 2004, 2005 and 2006 were tested by PCR by the end of February 2007 (the milestone date for 2006 samples).

Results

Plant samples

There was a poor correlation between the incidence of *S. sclerotiorum* presence on petals measured by two different techniques: agar plating and PCR (Figure 17). This is mainly due to four occasions when the PCR method indicated a much higher infection incidence than the agar method. It is thought that the agar method will only indicate the presence of viable spores and mycelium of *S. sclerotiorum*, while the PCR technique would detect dead spores as well as viable spores and mycelium.

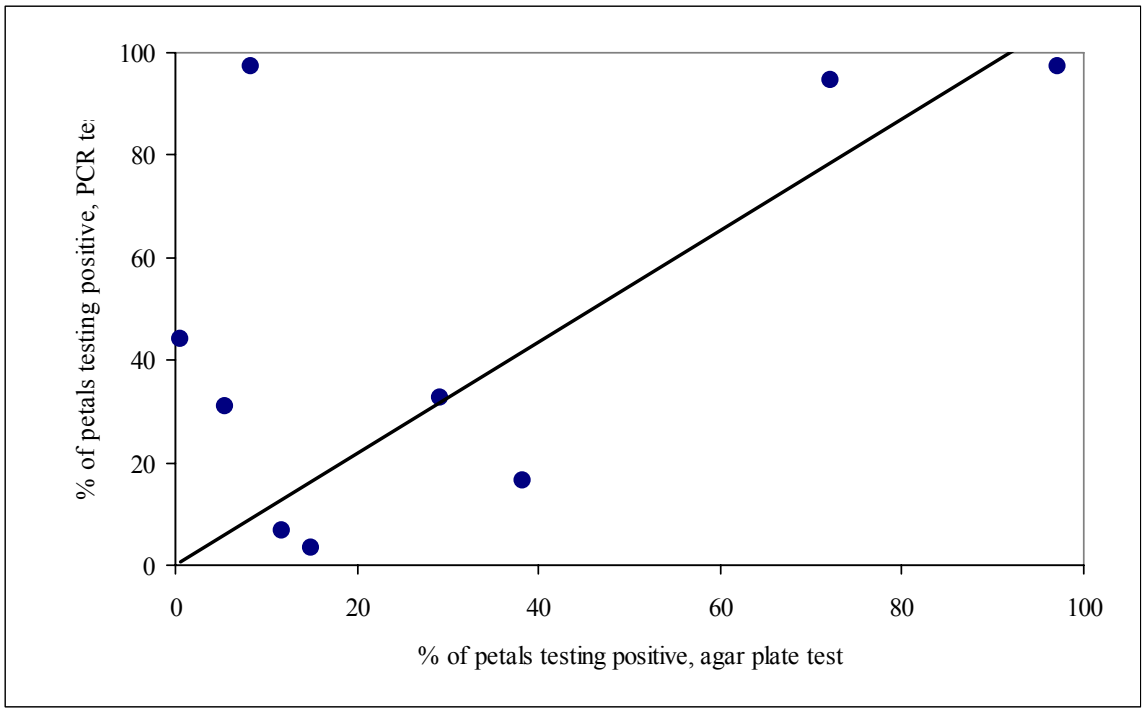


Figure 17. Incidence of *S. sclerotiorum* in petals as detected by PCR and agar plating.

Agar plating done at ADAS Rosemaund showed a very highly significant correlation ($P<0.001$; correlation coefficient of 0.84) between infection incidence on petals and stamens, with stamen infection consistently about 54% of the infection incidence on petals (Figure 18; $y = 0.54x + 1.39$, $R^2 = 0.84$). Two possible explanations for this may be that petals partly shield stamens from spores, which generally move upwards from ground level after gusts of wind have penetrated the crop canopy and that poorer contact with the agar medium may produce a lower apparent incidence of infection of stamens.

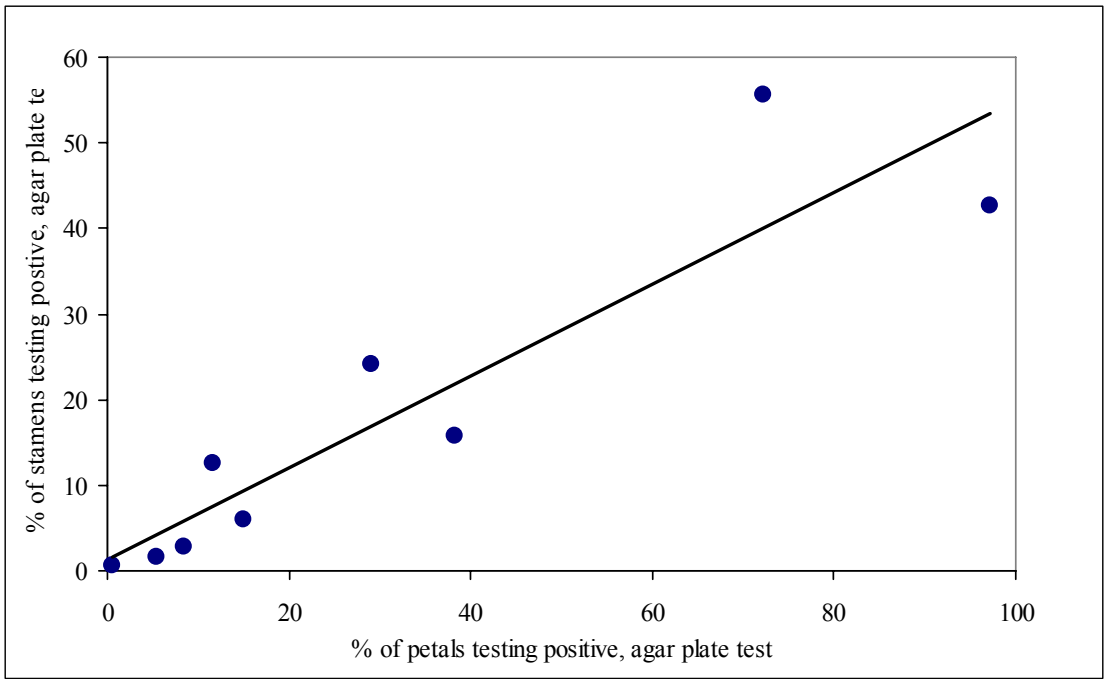


Figure 18. Incidence of *S. sclerotiorum* on petals and stamens of full petal plants, measured by agar plating, at GS 4.2, 4.5 and 4.9 in 2004, 2005 and 2006.

The correlation between petal and stamen infection measured by PCR (Figure 19) was not quite significant at the 5% level (P=0.056). This was due to three points with much higher stamen infection (60-80%) than petal infection (5-30%), contrasting with six other points where infection levels were very similar for the two flower tissue types.

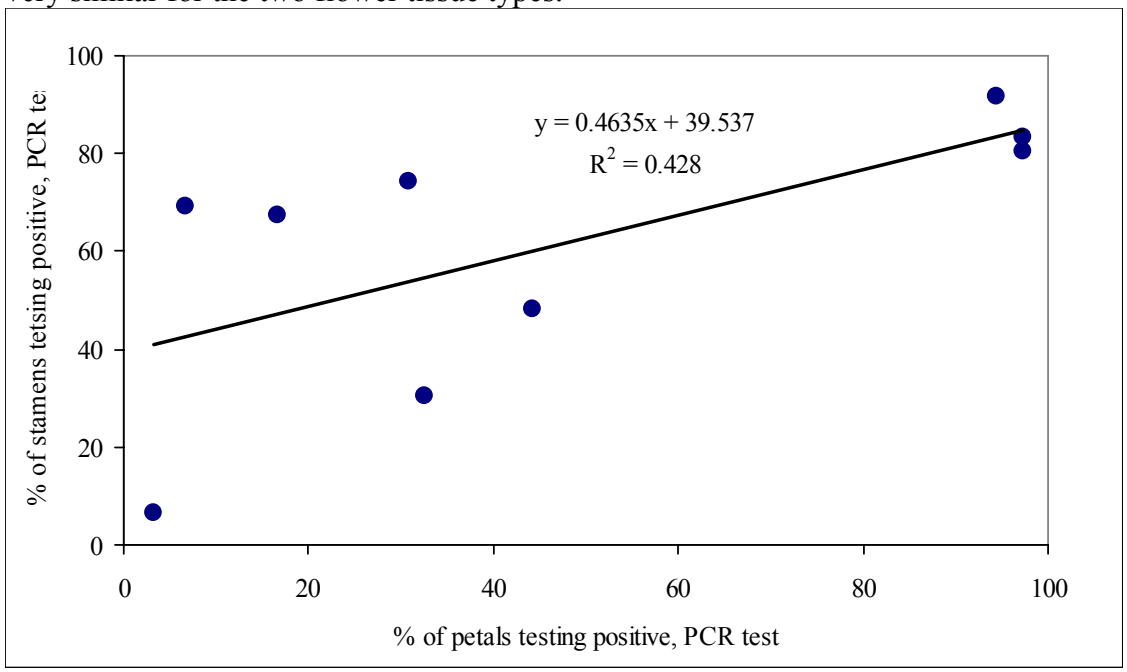


Figure 19. Incidence of *S. sclerotiorum* on petals and stamens of FP plants, measured by PCR, at GS 4.2, 4.5 and 4.9 in 2004, 2005 and 2006.

For leaves (Figure 20), the PCR method consistently indicated a higher incidence of *S. sclerotiorum* infection than with the agar plate method. As with the petal samples, this could be due to the detection of unviable (dead) spores present on leaves.

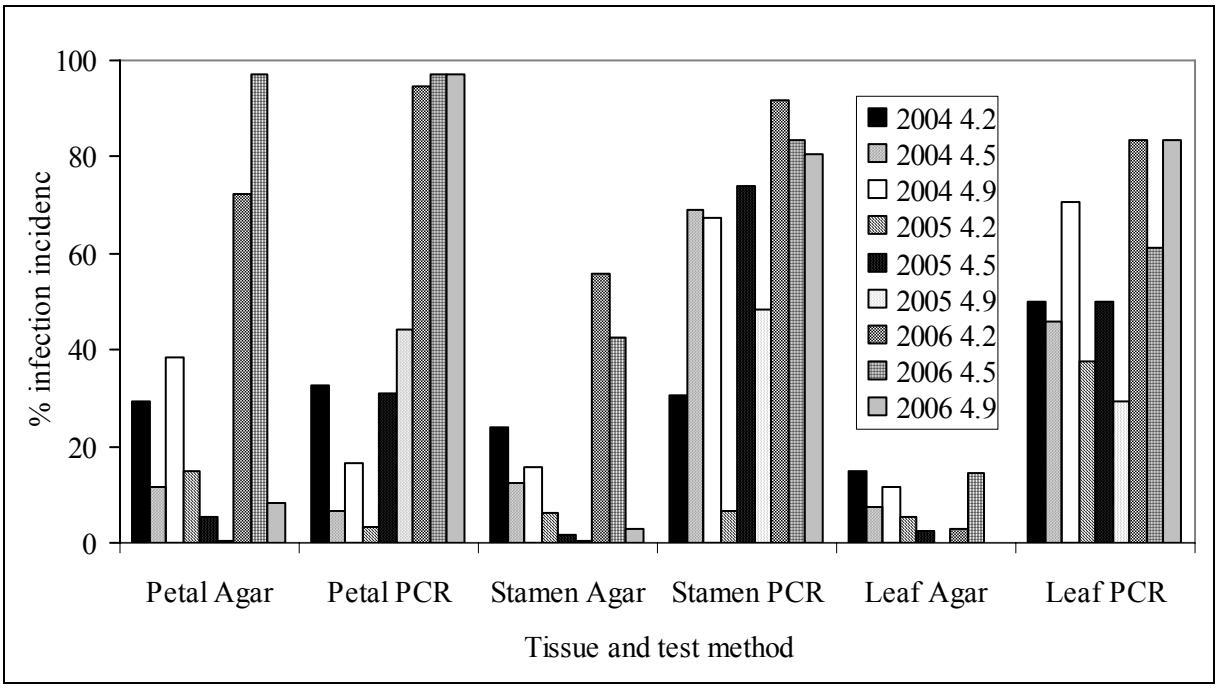


Figure 20. Incidence of *S. sclerotiorum* infection on different plant tissues at three growth stages, 2004, 2005 and 2006, measured by agar plating or PCR.

PCR data for 2004 (Figure 21) indicates a relatively low incidence of infection on petals, with some variability between plots and most petal infection occurring around early flowering (GS 4.2), a low level during mid-flowering (GS 4.5) and a moderate incidence of infection at late flowering (GS 4.9).

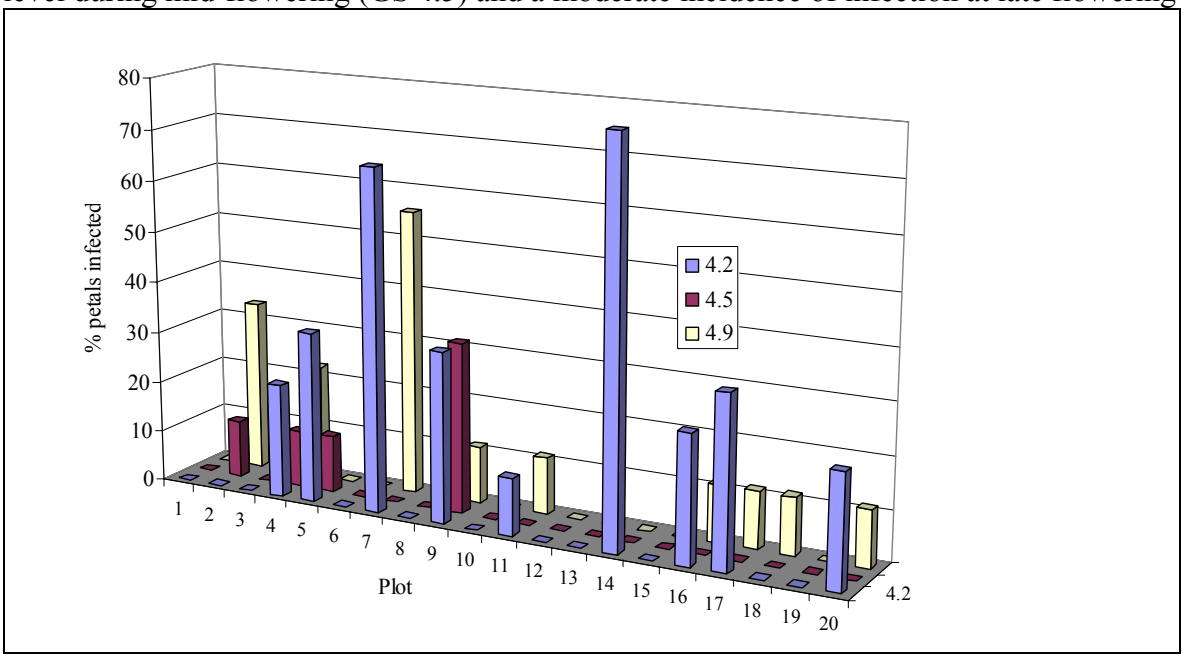


Figure 21. % Petals infected with *S. sclerotiorum*, ADAS Rosemaund 2004 (N.B. Apetalous plot numbers shown, but not tested by PCR).

PCR data for 2005 (Figure 22) indicates a high level of variability between plots (thought to be due to patchy sporulation from inoculum sources) and most petal infection occurring around mid and late flowering (GS 4.5 and 4.9).

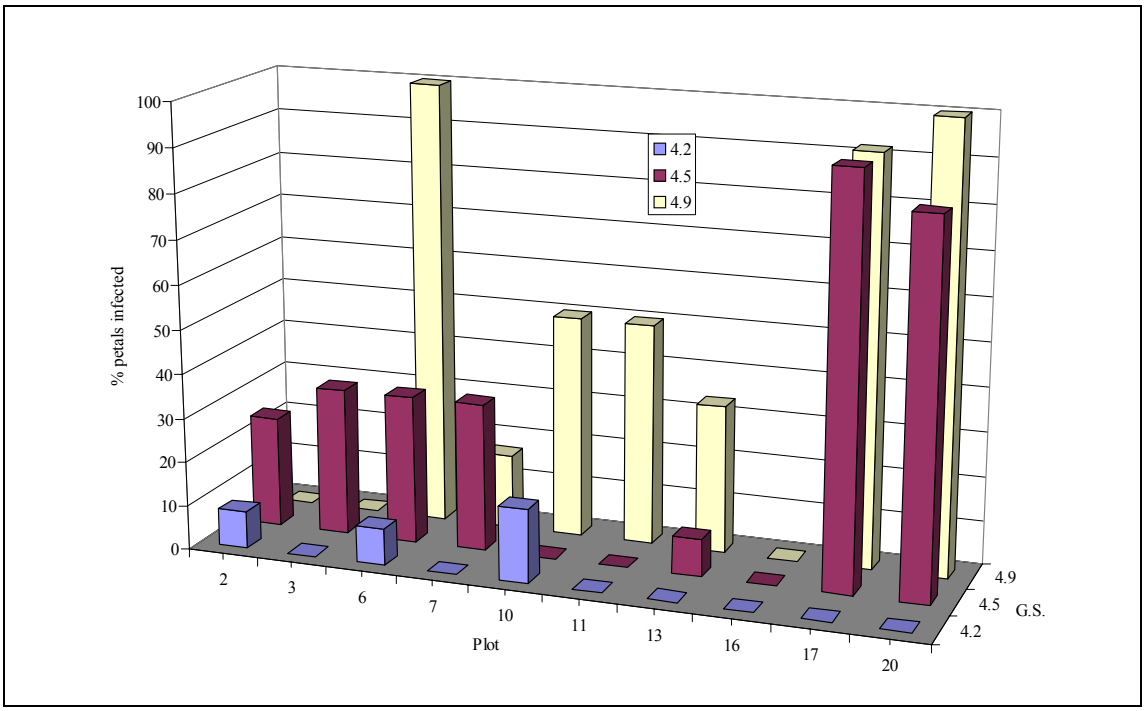


Figure 22. % Petals infected with *S. sclerotiorum*, ADAS Rosemaund 2005 (NB Apetalous plot numbers shown, but not tested by PCR).

Data of two weekly petal infection from three FP plots in 2006, measured by PCR, are shown below Figure 23). In contrast to other seasons, petal infection was relatively consistent throughout the flowering period with a moderate level initially and moderate to high incidence of infection through the mid to late flowering period. NB. For clarity, the X- and Y- axes are the reverse of the previous two graphs.

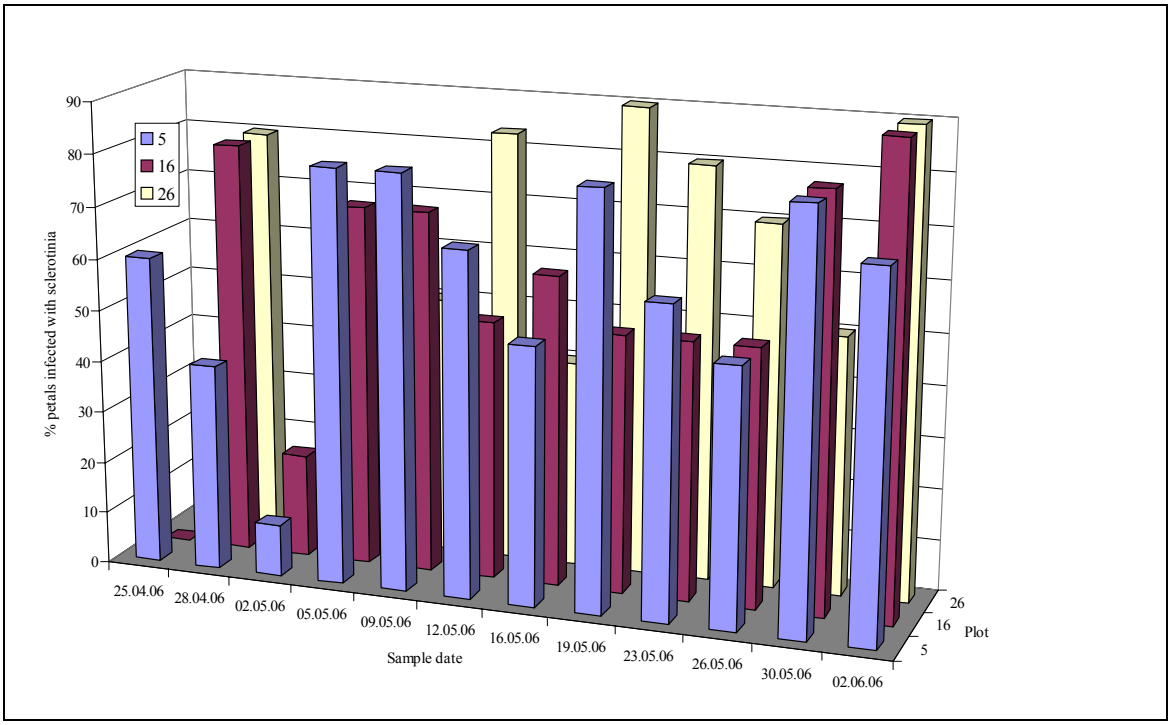


Figure 23. % Petals infected with *S. sclerotiorum*, three plots (5, 16, 26), ADAS Rosemaund 2006.

Air samples

PCR tests in the first two seasons (2004 and 2005), 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 (each 48 mm long), counting spores within a field width of 200µm. However, such identification was subjective as other *Sclerotinia* and *Botrytis* species have very similar spores. Sclerotinia-like spores were counted as having an elliptical shape, smooth surface coat and size 7-10 µm by 3.3-4.6 µm. A x40 phase contrast objective lens was used to give the best discrimination of spores.

The patterns of quantified levels of *S. sclerotiorum* DNA in air samples from traps 1 and 2 are shown in Figure 24, reflecting numbers of spores present in air at each end of the experiment over the flowering period of 2006. The spore pattern found in the two traps varies slightly due to wind direction but trap one has a large peak in spore numbers from the 11 to the 15 May 2006, while trap two had a large peak at the end of May (25-29 May). On both of these occasions there was a large amount of insect debris on the slides, followed for several days with little deposited air spora. It is likely that on these occasions a large insect had temporarily partially blocked the air intake.

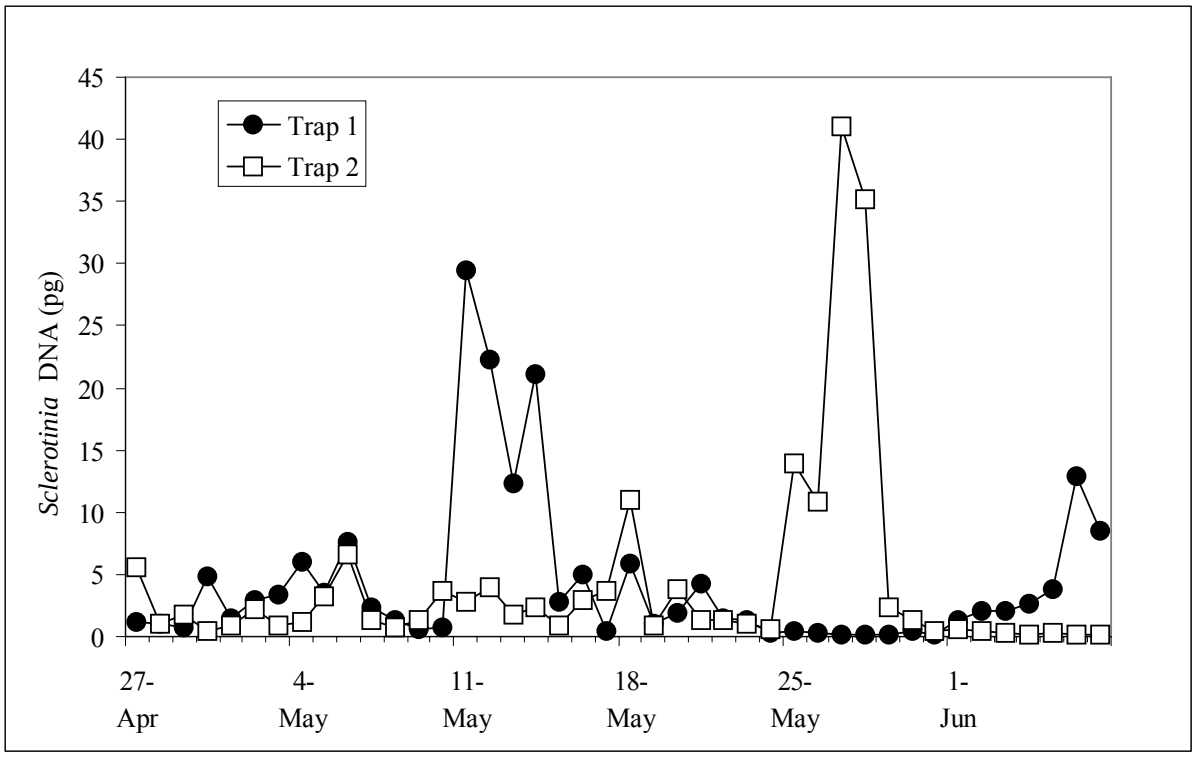


Figure 24. *Sclerotinia* DNA in air samples from spore traps in a winter oilseed rape crop, from PCR tests, at ADAS Rosemaund, 2006.

Further results of *S. sclerotiorum* infection of flower and leaf tissue samples are reported and discussed separately (chapter 1 of this report) in relation to stem infection, genotype of lines tested and epidemiology of the pathogen researched by other partners in the project.

Discussion

The value of PCR-based diagnostics applied to air samples has been greatly enhanced by the development of a quantitative method that estimates numbers of *S. sclerotiorum* ascospores. A purely qualitative method applied to air samples in 2004 and 2005 gave little information as there were nearly always some spores present in air at the time of flowering. The quantitative PCR applied to spore trap tapes clearly showed peaks of airborne inoculum. Further work on inexpensive spore traps for use within crops and quick processing methods for PCR on spore trap samples would enable farmers to assess inoculum levels, which are an important indicator of infection risk. A quantitative PCR (qPCR) method applied to plant samples 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* in oilseed rape.

Detection of infection on individual petals or other flower structures by conventional PCR may be also be useful because the incidence of petal infection (rather than severity or extent of mycelial colonisation) combined with the right conditions for petals to stick to leaves and stems appears to be a major factor in infections leading to stem rot epidemics. Agar plate test results are a good indicator of inoculum levels in a crop but the test requires an incubation time too long to be of practical use. However, for a conventional PCR test on individual petal samples to be of use, further work will be needed to develop sample protocols and test procedures that produce quick results for farmers.

References

Freeman J., Ward E., Calderon C., McCartney A. (2002) A polymerase chain reaction (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum* European Journal of Plant Pathology **108**, 877-886.

Lacey M.E., West J.S. (2006). *The Air Spora*. Pp. 156. Dordrecht, Springer.

Latunde-Dada A.O., West J.S., Roques S.E., Huang Y.-J., Pirie E., Stonard J.F., Downes K., Fitt B.D.L. (2007) New methods to understand quantitative resistance to *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape. Proceedings of the 12th International Rapeseed Congress, 26-30 March 2007, Wuhan, China, in press.

APPENDIX 1, Weather data for ADAS Rosemaund, 2004-2006

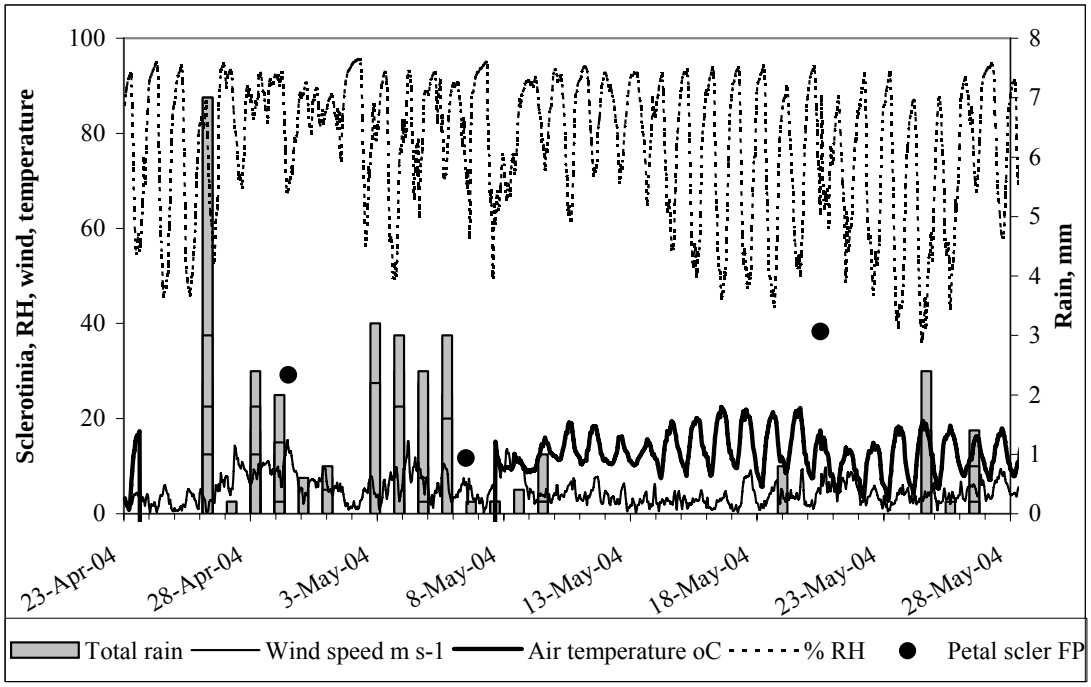


Figure 1. Weather data during oilseed rape flowering and % petals testing positive for *S. sclerotiorum* (agar plate tests), ADAS Rosemaund 2004.

Late April and early May had rain on most days in 2004 (Figure 1), which encouraged petal stick at early flower but possibly washed much of the airborne spore inoculum from the air. The late flower sample in late May had the highest % of petals testing positive for sclerotinia.

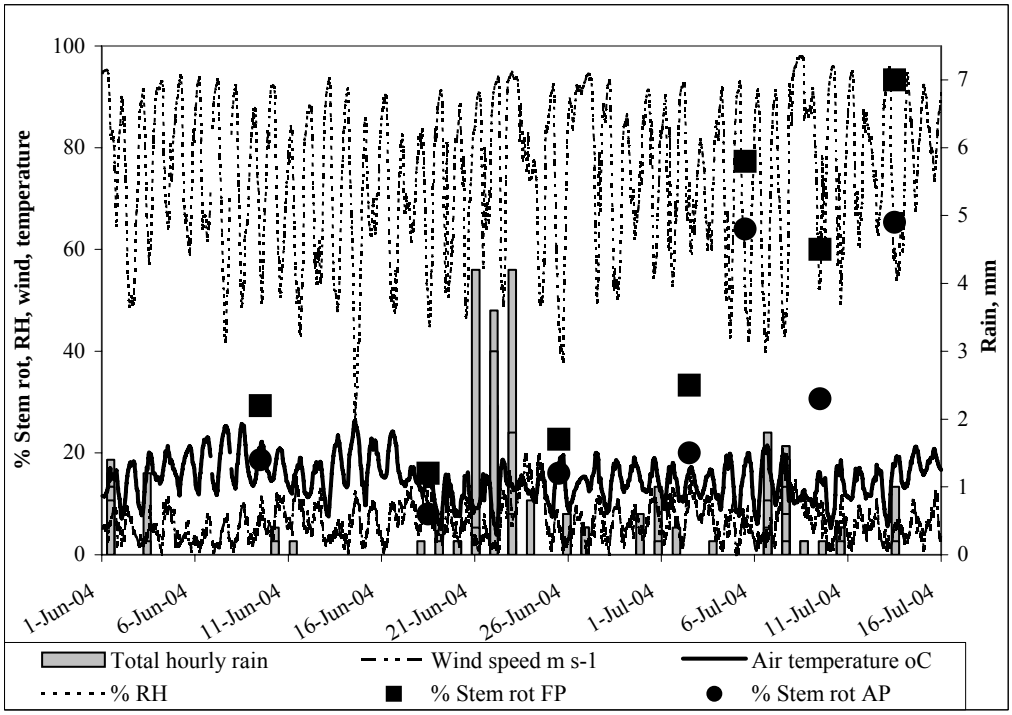


Figure 2. Weather data June-July and *S. sclerotiorum* stem rot incidence, ADAS Rosemaund 2004.

Rain at the end of June and start of July in 2004 may have promoted the increase in stem rot from initial observations of up to 2% in early June to 5% in mid July (Figure 2)

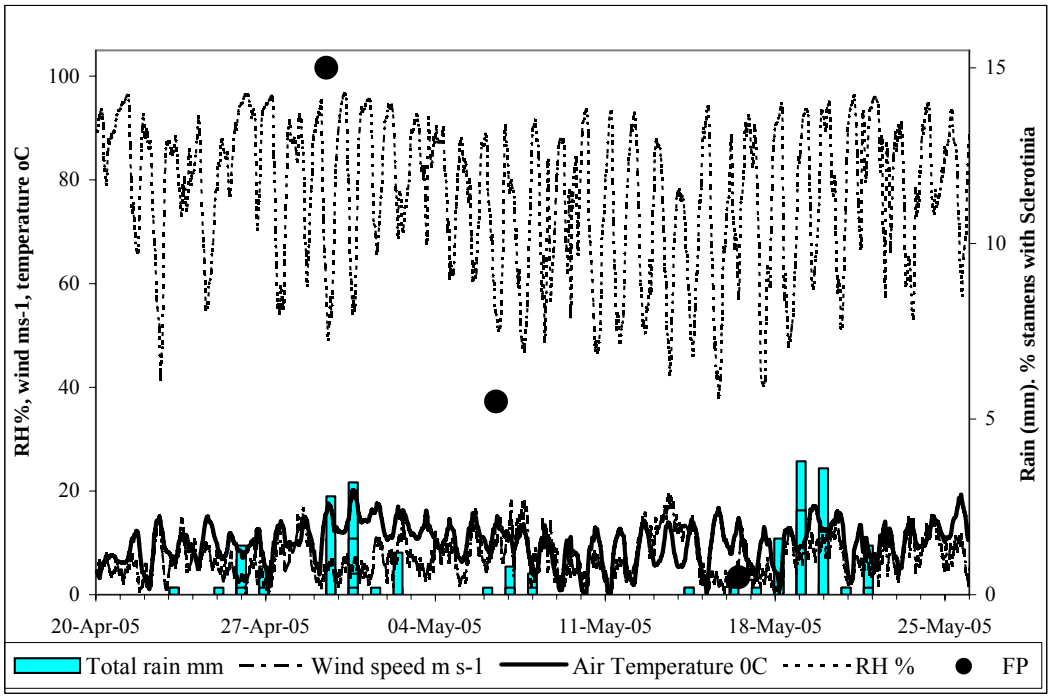


Figure 3. Weather data during oilseed rape flowering and % petals testing positive for for *S. sclerotiorum* (agar plate tests), ADAS Rosemaund 2005.

Low rainfall in mid-May 2005 may have contributed to very low inoculum levels at the late flower sample (Figure 3), due to low apothecial germination.

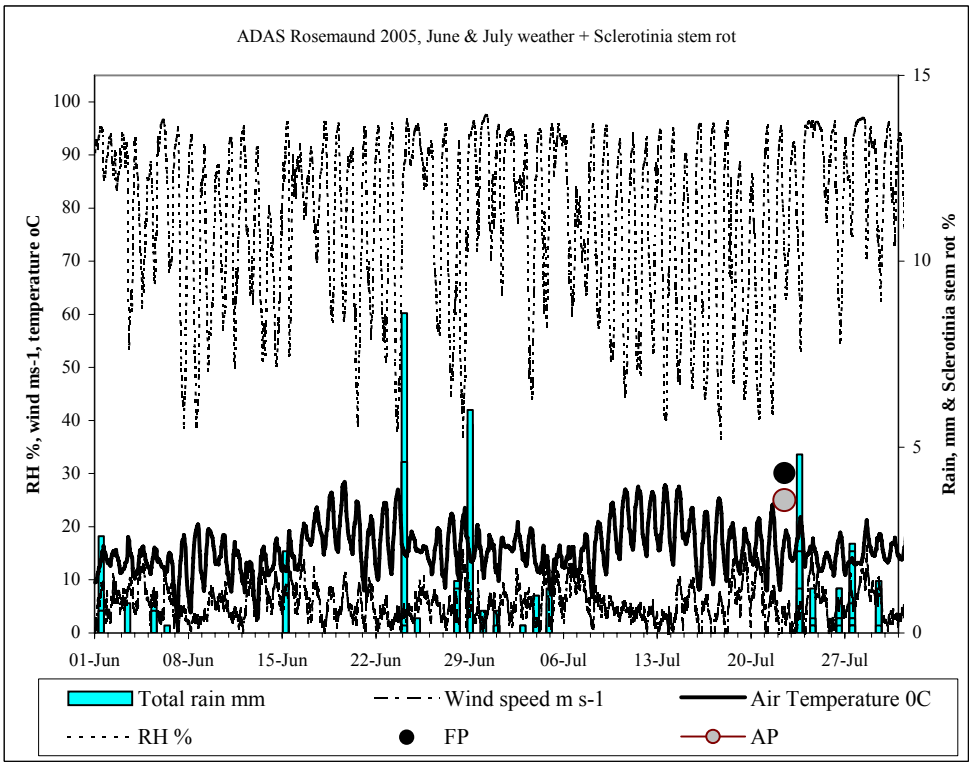


Figure 4. Weather data June-July and *S. sclerotiorum* stem rot incidence, ADAS Rosemaund 2005.

Low petal infection during flowering (Figure 4) may explain the low stem rot levels by late July 2005.

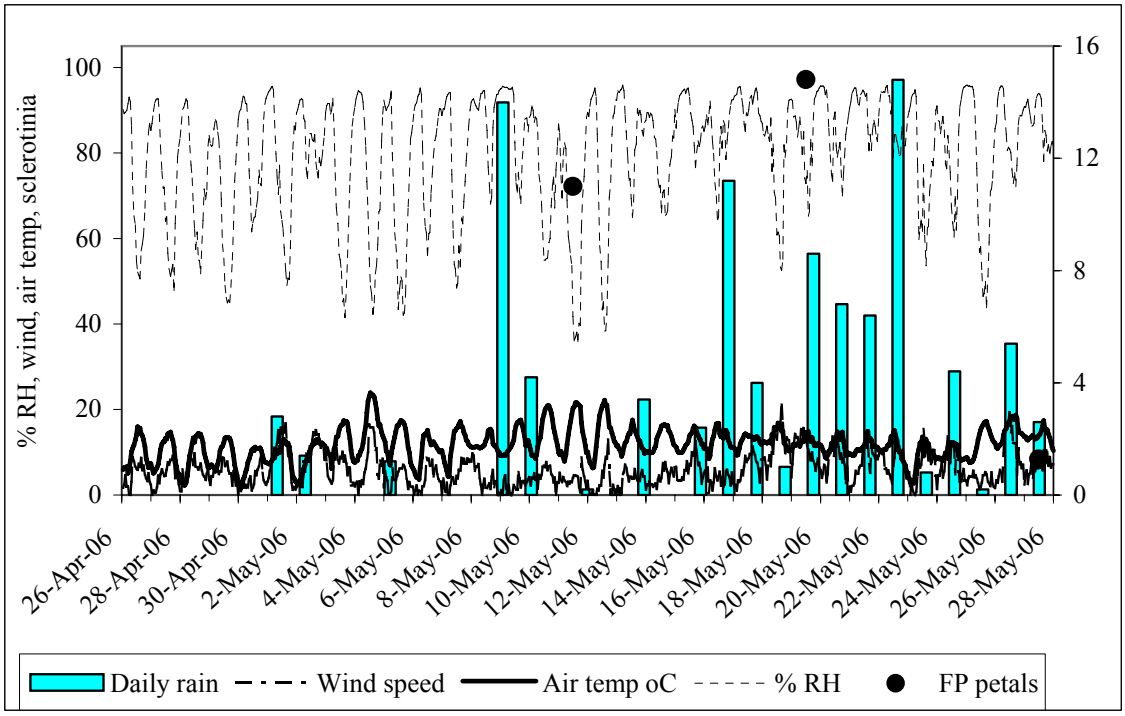


Figure 5. Weather data during oilseed rape flowering and % petals testing positive for *S. sclerotiorum* (agar plate tests), ADAS Rosemaund 2006.

Warm, wet weather during flowering would have promoted spore release and infection of petals in 2006, leading to a relatively high % of petals testing positive for sclerotinia (Figure 5).

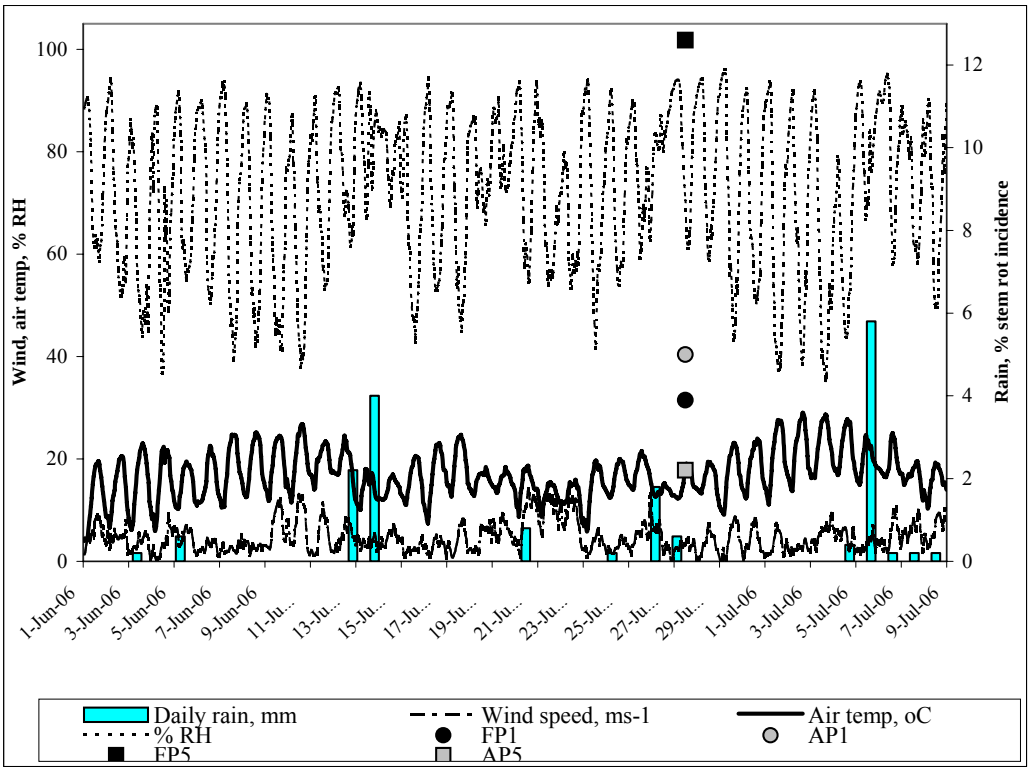


Figure 6. Weather data June-July and *S. sclerotiorum* stem rot incidence, ADAS Rosemaund 2006.

Continuously wet leaves and high petal infection during flowering in 2006 would have promoted stem rot infection (Figure 6).