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Components of resistance to diseases in winter oilseed rape cultivars: CORDISOR

by

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Abstract

The project had two aims: (i) to test whether measuring components of resistance to stem canker (caused by *Leptosphaeria maculans*) and light leaf spot (caused by *Pyrenopeziza brassicae*), especially symptomless growth, will improve selection of resistant cultivars; and (ii) to produce new methods to rate cultivar resistance to stem canker and light leaf spot in winter oilseed rape cultivar selection and evaluation trials.

The quantity of *L. maculans* DNA in a crown (stem base) sample from an individual winter oilseed rape plot before harvest was very closely related to the mean severity of canker symptoms recorded on the same plants. However, plant to plant variation (sample error) in both symptom severity and amount of pathogen DNA reduced the closeness of the relationship between amount of DNA in one plant sample and canker severity in a separate plant sample from the same plot at the same time. Sample sizes were ≤10 plants per plot, so increasing sample size to 30 plants per plot would reduce this variation but would increase collection and processing time and costs of DNA extraction and quantitative PCR (qPCR) analysis. The relationship between amount of *L. maculans* DNA and pre-harvest canker severity in the same plot became less good as *L. maculans* DNA was quantified in crowns at progressively earlier time-points. Amounts of *L. maculans* DNA in crowns were small in the winter but increased greatly from flowering onwards when canker symptoms became visible. *L. maculans* DNA in petioles of leaves with visible phoma leaf spots sampled in the autumn or winter could not be used as a good predictor of final canker severity on cultivars although there were clear reductions in pathogen DNA caused by fungicide use. In some cases, amounts of *L. maculans* DNA were large for some cvs that subsequently had relatively low canker severity, suggesting that a potential mechanism of resistance may operate in the stem or at the boundary between petiole and stem. Cultivars were ranked for amount of *L. maculans* DNA in stems in winter. The more susceptible cvs like Bristol and Winner tended to have more *L. maculans* DNA in stems than the more resistant cvs like Hearty and ES Astrid.

Difficulties in identifying the most appropriate time to sample meant that the relationship between amount of *L. maculans* DNA in petioles and final canker severity at harvest was not a reliable method to predict cultivar "field" resistance. However, the method can provide other useful information, such as which leaf layers contribute most to stem disease and therefore should be sprayed with a fungicide. There was much more DNA of *L. maculans* in petioles of senescing leaves than in those of the lowest green leaf.

Symptom development and production of *L. maculans* DNA were investigated in controlled conditions using an extended set of varieties (six European cvs used in the CORDISOR field experiments and 30-40 Chinese cvs thought to be more susceptible to *L. maculans*) that were inoculated at single positions on leaves using a small drop of ascospore suspension. Large differences between cultivars in visible symptoms and in amounts of *L. maculans* DNA were measured at different times and distances from the inoculation point and these are thought to be potentially good indicators of 'field' resistance to *L. maculans*. Large differences in severity of visible symptoms also occurred following inoculation of wounded cotyledons with conidia (asexually-produced spores) of a set of different isolates (races) of *L. maculans*. More *L. maculans* DNA was produced in leaf 2 than leaf 1, when both were inoculated at the same time (i.e. leaf 1 slightly older).

The project showed for the first time that *Pyrenopeziza brassicae* infection of the main shoot tip (meristem) of oilseed rape plants in winter was a common and widespread occurrence. Amounts of DNA of *P. brassicae* in leaves or main shoots (meristems) related well to subsequent severity of light leaf spot (LLS) symptoms at some dates (particularly when visible symptoms were severe in late winter/early spring, when visual assessments are normally made for Recommended List evaluation). It is probable that the early samples taken in December and January, particularly in Scotland, had not had sufficient thermal time to allow the pathogen to grow and spread on plants (increasing biomass) through secondary infection, which limited the ability to discriminate between resistant and susceptible cvs. Sampling in late winter/spring appears to provide the best material for qPCR studies to discriminate cultivars for resistance to *P. brassicae*. There was always much more *P. brassicae* DNA in samples of leaf blades (lamellae) of recently expanded leaves than in the main shoot tip (meristem). The relationship between the amount of *P. brassicae* DNA and LLS severity in a range of cvs was not dependent on the total amount of DNA at the particular site or time of sampling. Measurements of LLS severity by visual assessment of plots (1-9 score) often did not relate well to mean disease severity measured by close inspection of ten plants. Incubation of ten newly expanded leaves per plot in plastic bags in a cool room for a few days provided a good visualisation of LLS symptoms to discriminate differences in cultivar resistance to *P. brassicae*. There were significant differences in amounts of pathogen DNA for both *L. maculans* and *P. brassicae* between sites that had high or low severity of visible disease and between untreated and fungicide-treated plots.

In these experiments, yield loss due to canker occurred only if mean canker severity was > 2.5 (over half stem cankered) by mid-June proceeding to reduce yield by 15% per additional unit of canker severity (0-6 scale). The occurrence of stem splitting, caused by very warm spring weather during stem extension, did not increase canker severity. There were few differences in number and time of leaf production between cvs and little difference in leaf or petiole length between cvs but these factors did vary considerably between sites. Canker severity assessed as cross-sectional area of canker at the stem base (at maximum severity) was very closely related to canker severity assessed as percentage of stem girdled by canker. Discrimination between different cvs could be improved by recording the percentage of canker (either as area or girdling) as a continuous variable, rather than on the 0-6 scale currently used in HGCA RL assessments, although data recording would be more time-consuming.

With respect to the first aim of the project, it is clear that measurement of components of resistance can help in the breeding of more resistant cvs. The project showed new information on the development of stem canker and pathogen DNA in the stem in thermal time – pathogen growth was not linear but increased from the onset of flowering at different rates for different cvs. This was particularly important as the project also demonstrated that yield losses due to stem canker were insignificant if canker severity could be managed to be less than 2.5 on the 0-6 scale.

Regarding the second aim, to produce new methods to rate cultivar resistance to stem canker and light leaf spot in winter oilseed rape cultivar selection and evaluation trials, the project showed that exisiting, relatively 'low-tech' methods were best but that, particularly with inoculated controlled environment studies, there appears to be great potential in measuring pathogen growth from its DNA in order to screen genotypes for resistance to *L. maculans* at early growth stages.

The project also showed that infection of meristems by *P. brassicae* was widespread before Christmas, and as this is though to lead to symptoms of plant stunting, indicates that late-autumn foliar fungicide applications should be used against light leaf spot. The amount of DNA of *P. brassicae* on leaves sampled from field plots in late-winter was a reasonable indicator of cultivar resistance to LLS but this has not been tested in controlled conditions.

Summary Report

Introduction

The project aimed to produce new methods to improve measurement of disease resistance in winter oilseed rape cultivars. The project focussed on resistance to the two main diseases of winter oilseed rape in the UK, stem canker and light leaf spot. Disease resistance ratings are currently calculated from the visual assessment of disease and do not explain the relative importance of resistance expressed in different tissues (leaf, stem, pods) at different times or stages of disease development – information which is valuable for breeding new cultivars with enhanced disease resistance and reduced reliance on fungicides for disease control.

The approach used in the project was to evaluate 20 different oilseed rape cultivars for their growth and amounts of disease at a range of field sites in England and Scotland. The 20 cvs (Table S1) were chosen to represent a wide range of resistances and susceptibilities to canker and light leaf spot, using predominantly cvs from the HGCA recommended list at the start of the project (cv. ES Astrid replaced Aragon after first year). Additionally at some sites that included untreated and fungicide-treated plots (disease severity reduced greatly), yield data were collected and used to assess the effect of stem canker on yield. The visible disease symptoms recorded in different ways were compared with measurements of amount of DNA of the causal pathogens using quantitative PCR on selected plant tissues sampled at key growth stages.

Table S1. OSR cvs used in CORDISOR experiments in harvest years 2005-2007

Cultivar	Type	Breeder	UK Agent
Disco	Restored hybrid	Raps GbR, GER	Saaten Union
Fortis	Conventional	Novartis, Ger	Syngenta
Recital	Conventional	Novartis Gmbh, GER	Syngenta
Winner	Conventional	Raps GbR	Saaten Union
Expert	Conventional	Moment, Fr	Advanta
Courage	Conventional	Van der Have, NE	Advanta
Canberra	Conventional	Cargill, Fr/ DSV,Ger	Monsanto
Escort	Conventional	Limagrain, Fr	Nickersons
Royal	Restored hybrid	Novartis Gmbh, GER	Syngenta
Elan	Restored hybrid	NPZ, GER	KWS-UK
Lioness	Conventional	DSV, Ger	Cebeco SI
NK Bravour	Conventional	Syngenta, Fr	Syngenta
Castille	Conventional	Monsanto, Fr	Monsanto
NK Victory	Conventional	Syngenta, Fr	Syngenta
Aragon *	Conventional	Lembke, Ger	KWS-UK
Apex	Conventional	Lembke, Gr	Advanta
Bristol	Conventional	Cargill. Fr	
Shannon	Conventional	Nickersons	Nickersons
Hearty	Conventional (HEAR)	Monsanto	Monsanto
Ontario	Conventional	Nickerson GEIE	Nickersons
ES Astrid	Conventional	Euralis, Fr	Grainseed Ltd.

* replaced by ES Astrid for 2006 & 2007

Field experiment methods

Visual plant and disease data were recorded on a set of 20 cvs of winter oilseed rape at 12 different sites in each of three seasons (harvest years 2005, 2006 and 2007). At Rothamsted, over the first two seasons of the project, one cv (NK Bravour) was grown in additional plots next to the main experiment and was sampled for quantification of pathogen DNA in plant tissues more frequently than the main experiment. The same set of cold-stored seed was used each year for field experiments. Disease and plant assessments were made according to an established protocol (see Appendix 1 for details; summarised in Table S2) at specific times (Table S3). Additionally growth stage was recorded [Sylvester-Bradley & Makepeace (1985)] and presence of other diseases on leaves, stems or pods (e.g. downy mildew, powdery mildew, Alternaria, botrytis, white leaf spot, etc.) noted.

Table S2. Summary of CORDISOR plant and disease assessments (10 plants per plot unless stated)

Stem canker	Light leaf spot	Plant growth
SC1 Phoma leaf spot (individual plant visual assessments of N° leaves affected and % total leaf area affected, plus 1-9 plot score according to RL protocol)	**LLS1** Light leaf spot (incubate plants in plastic bags in cool (10°C) room for 5 days then score N° leaves affected and % total leaf area affected on individual plants, plus 1-9 plot score at time of sampling according to RL protocol)	**P1** For each plant sampled, record N° unfolded leaves present & N° lost.
SC2 Petiole infection (dispatched for PCR: 10 whole plants separated with blue-roll/tissue in a labelled plastic bag per plot)	**LLS2** Apical meristem infection(whole plants wrapped in blue-roll in a plastic bag and dispatched for PCR)	**P2** Plant height (mm to apex of main stem)
SC3 Stem canker score (individual plants scored on 0-6 scale where: 0 = uninfected, 1 = < 25% stem circumference girdled, 2 = 26-50% girdled, 3 = > 51-75% girdled, 4 = > 76-100% girdled, 5= 100% girdled + stem weak, 6 = plant dead or lodged)	**LLS3** LLS symptoms on leaves of individual plants (without incubation score N° leaves affected and % total leaf area affected), plus 1-9 plot score at time of sampling according to RL protocol.	**P3** Stem diameter (mm) individual plant measurement **P3*** 1-4 Score of average plot stem diameter (+ measure best and worst). Optional if felt that this is quicker than P3.
SC4 Stem canker score (RL) (0-6 scale: as above)	**LLS4** (now deleted as part of LLS1 and LLS3)	**P4** Plant density (N° plants in 4 x 0.5 m rows and note distance between rows) **P4***as P4 in best and worst plots with other plots scored on a relative 1-9 scale.
	LLS5 LLS stems (score % area affected on main stem)	
	LLS6 LLS pods (score % pods affected and % total area of pods affected)	

Table S3. Summary of assessment times for CORDISOR project

Exp.	Treatments	Main disease assessments (10 plants per plot/line) (other diseases assessed if present)							
		Oct	**Nov/ Dec**	**Jan**	**mid-March**	**April/ May**	**June**	**July**	notes
1. RRes (inoculated for LLS)	+/- fungicide (weekly phoma checks in autumn)	LLS1, SC1, P1	LLS1, SC1, 2 P1, 4	LLS1, 2 SC1 P1	LLS3, SC1, 3, P1, 2	LLS3, 5, SC 3, P 2	LLS5, 6, SC4 P3	LLS5, 6, SC3, P4 YIELD	
2. SC plot, Boxworth	+/- fungicide (weekly phoma checks in autumn)	SC1 P1	SC1, 2 P1, 4	SC1 P1	SC1, 3 P1, 2	SC 3 P 2	SC4 P3	SC3 P4 YIELD	
3. Breeder nursery, England	Inoculated/ uninoculated	SC1 P1	SC1, 2* P1	SC1, LLS1, 2* P1	SC1, 3 P1, 2	SC 3 P 2	SC3 P3*	SC3	SC2*= PCR on selected sites LLS1, 2* PCR on selected sites
4. Breeder nursery Scotland	Different sowing rates	LLS1 P1	LLS1 P1	LLS1, 2* P1	LLS3 P1, 2	LLS3, 5 P 2	LLS5, 6 P3*	LLS5,6	
5. LLS expt. RRes (inoculated field expt)	-	LLS1 P1	LLS1, 2 P1	LLS1, 2 P1	LLS3 P1, 2	LLS3, 5 P 2	LLS 5, 6 P3	LLS5, 6	
6. SC expt. RRes (inoculated field expt)	-	SC1 P1	SC1, 2 P1	SC1 P1	SC1, 3 P1, 2	SC 3 P 2	SC4 P3	SC3	
7. RL trial, England	+/- fungicide (yield)	SC1 P1	SC1, 2 P1, 4*	SC1 P1	SC1, 3 P1, 2	SC 3 P 2	SC3 or 4 P3*	SC3 P4* YIELD	
8. RL trial, Scotland	+/- fungicide (yield)	LLS1 P1	LLS1 P1, 4*	LLS1, 2 P1	LLS3 P1, 2	LLS 3, 5 P 2	LLS5, 6 P3*	LLS5, 6 P4* YIELD	

Data were collated at Rothamsted, and analysed by ANOVA using a statistical software package (Genstat) for each site x sample date. Cultivar means for each variable recorded were copied into Excel databases, for each season. These databases were used for further analysis of relationships between different variables and especially the relationships between severity of visual symptoms (e.g. canker or light leaf spot) and the amount of pathogen DNA (*L. maculans* or *P. brassicae*) in the same samples or samples from the same plots taken at earlier time-points.

Field experiment visual assessment results

Plant growth

There was little difference in stem diameter between cvs but there were differences between sites. Stem diameter and splitting of the stem (caused by hot weather during stem extension particularly in Apr/May 2006) did not affect canker severity. Petiole length (lowest green leaf sampled) varied only slightly between cvs, but varied greatly between sites and times of year. There was little difference in mean height between cultivars. By mid-November, on average six leaves were present and 4-6 leaves had been shed, with little difference in leaf turn-over between cvs but clear differences between sites. By January, on average 8-10 leaves were present with 7-8 leaves having been shed. The rosette leaf scars (from leaves that contribute to stem base canker) were produced by the first 10-13 leaves. There were large differences in petiole lengths between sites or assessment dates but usually no significant differences between cultivars. Similarly the number of leaves present, shed or in total differed more between sites and dates than between cultivars at a particular site on a given date.

Disease development and measurement

- Generally canker severity was low in 2005 but high in 2006 and 2007. Light leaf spot severity varied greatly between sites and was greatest in March and April.
- There was little difference between canker severity recorded by extent of girdling or by cross-sectional area affected for discriminating cultivar resistance.
- The 1-9 plot score used for phoma leaf spot (*Leptosphaeria maculans*) appeared to vary substantially between observers, occasionally under-estimating disease present on lower leaves. Furthermore, there was little relation between phoma leaf spot severity and final canker severity. The timing of onset of phoma leaf spot and winter temperature affected canker severity greatest. Fungicide sprayed in mid and late autumn gave good control of canker.
- Incubation of ten newly expanded leaves per plot in plastic bags in a cool room for 4-5 days allowed good visualisation of LLS symptoms to assess differences in cultivar resistance to *Pyrenopeziza brassicae*. Substantially more light leaf spot symptoms were visible after a 4-5 day incubation than a 2-day incubation or no incubation, especially in early to mid-winter. Generally there were large differences in LLS severity on leaves between the 20 cvs tested at each site with greatest severity on cv. Hearty and least on cv. Elan.

- There was no clear relationship between light leaf spot (LLS, *Pyrenopeziza brassicae*) severity on leaves in late winter and LLS severity on pods in June, indicating that either the second release of ascospores in spring is influential and/or that a different resistance mechanism occurs on pods and/or that avoidance of meristem infection is critical (PCR data confirmed that at some sites meristems in untreated plots became infected before winter; this could result in stunting of plants and ensure that subsequent growth stages would be infected. Similarly, fungicide applied in autumn decreased LLS severity on pods the following June for most cultivars).
- There was a good relationship (R^2 = 0.55 in 2005) between LLS severity on pods (1-9 scale in June-July, - means of four sites) and the HGCA LLS resistance score for different cultivars.
- There was a good relationship (R^2 = 0.53 to 0.56 in 2005) between canker severity (mean score, HGCA index or French G2 index in June-July, - means of five sites) and the HGCA canker resistance score for different cultivars.
- The incidence and severity of phoma leaf spotting was significantly less on cv. Hearty in some data-sets (eight sites x sample dates) but not all. Hearty had a significantly lower incidence of phoma leaf spot at five sites at the start of the season but at most of these sites, incidence on cv. Hearty increased to be the same as on other cvs later in the season (e.g. February). In contrast, at ADAS Boxworth there was no significant difference in incidence of phoma leaf spot between cvs early in the season, but cv. Hearty had a significantly lower incidence in the spring. Occasionally, cvs Escort, ES Astrid and Fortis had lower incidences of phoma leaf spot than other cvs. In general, comparing all sites, as Canberra, ES Astrid and Hearty had the smallest canker indices, while cv. Bristol had the largest canker index.
- Thermal time from first leaf spotting to canker formation was investigated at Rothamsted. Thermal time from the onset of 10% phoma leaf spot (time A) to first canker appearance (B) was 958, 1117 (estimated) and 1275 degree-days in 2005, 2006 and 2007, respectively (mean of all cvs).
- Rates of canker development from first appearance (time 'B') in 2005-2007 for cv. Bristol were; 0.0026, 0.0046 and 0.0040; for cv. Expert were 0.0008, 0.0017 and 0.0011 and for cv. ES Astrid (2006-2007 only) were 0.0019 and 0.0006 canker severity units per degree day (Figure S1).

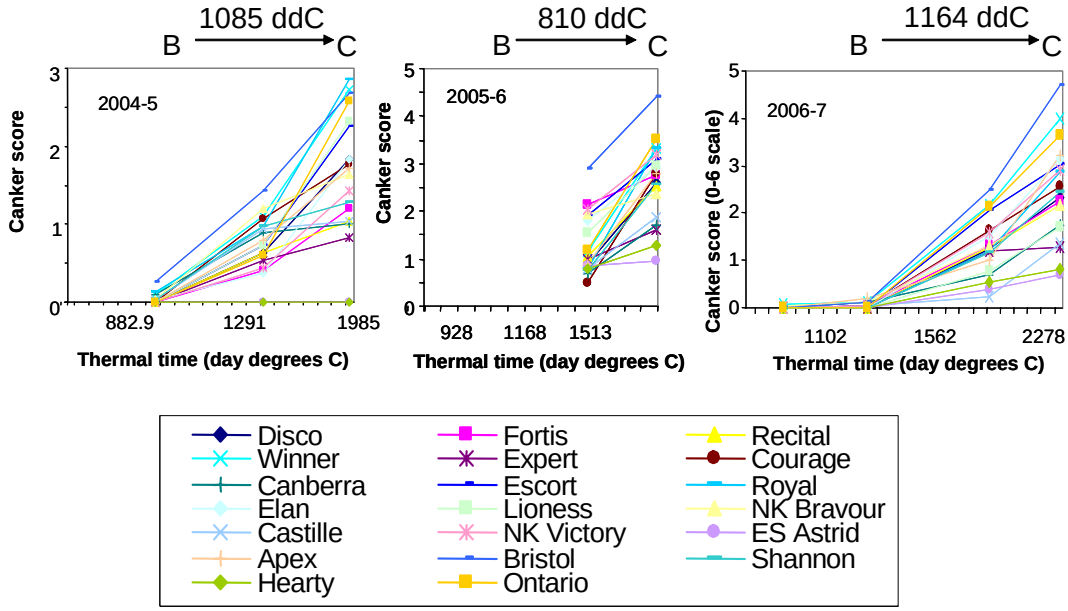

Figure S1. Increase in canker severity (0-6 scale) against thermal time (B= first appearance of canker; C = final canker measurement) at Rothamsted 2005-2007. NB different y-axis scales

• Cultivars with the highest rate of canker development at Rothamsted were: (2005) Royal, Winner and Bristol; (2006) Bristol, Ontario and Winner; (2007) Bristol, Winner and Ontario (Fig S1). Cultivars with the lowest rate of canker development at Rothamsted were: (2005) Expert, Canberra and Castille (no ES Astrid nor Hearty at Rothamsted in 2005); (2006) ES Astrid, Hearty, Expert, Canberra and Castille; (2007) ES Astrid, Hearty, Expert and Castille. The rate of canker development varied each year but the ranking of cultivars appeared to be very consistent each year at this site (Fig S1). The lower incidence of phoma leaf spot on ES Astrid and Hearty than other cvs could explain the subsequent low severity of canker on them, but the reduced rate of canker development in Expert, Canberra and Castille was probably due to operation of quantitative resistance as they had a similar incidence of phoma leaf spot to the other cvs. In contrast, cultivars Bristol, Winner, Ontario and possibly Royal appear to have increased rates of canker development, suggesting that they have less effective quantitative resistance.

- For light leaf spot (LLS), cvs Hearty, Shannon and Recital were most susceptible, while cv. Elan alone had less LLS than all other cultivars.
- There was very little or sporadic development of alternaria leaf and pod spot, powdery mildew, downy mildew and sclerotinia stem rot (SSR) with SSR more severe than normal in 2007 (but with no significant differences between cvs).

Quantification of pathogen DNA *in planta* by qPCR

The pathogen DNA data was produced from plant samples taken at selected times from field experiments and processed directly at Rothamsted or NIAB or posted from other sites (SAC sites and breeders' nurseries in Scotland and England) to Rothamsted. In some cases, posted samples were assessed for visible disease symptoms in addition to processing of selected plant tissues for pathogen DNA measurement. At Rothamsted, all selected tissue samples (upper or lower petioles, meristems or recently expanded leaves) were frozen, freeze-dried, and powdered and DNA was extracted using a commercial kit (DNAmite, Microzone, Haywards Heath) (see Appendix 2 for details). Different quantitative PCR (qPCR) protocols were used according to equipment available at each site (Rothamsted and NIAB). Although various methods with different primers were initially investigated at Rothamsted, both NIAB and Rothamsted sites used SYBR green systems to quantify *L. maculans* in plant tissues and a similar method was used at Rothamsted to assay *P. brassicae*. This method measures fluorescence of a dye that binds to double-stranded DNA. Non-target DNA (e.g. from the plant) that is not replicated in the PCR reaction produces a background low level of fluorescence but this is below a set detection threshold. Therefore, only the DNA of the target (plant pathogen in this case) is measured, the amount produced after a set number of replication cycles depending on the initial amount of target DNA in the sample. Samples were also collected from selected lines from the 'TN' population (a mapping population from a cross between a European cv Tapidor and a Chinese cv NingYou), which appears to be segregating for a number of genetic traits including resistance to *L. maculans*), which provided samples that were more susceptible than the 20 cvs used in CORDISOR field experiments (this TN-population was being grown in the UK as part of the NOVORB LINK project).

Results

The optimal sample timing to quantify DNA of *L. maculans* in leaf petioles was taken as the time when maximal pathogen DNA was present in the petiole of the lowest green leaf. This was found to be 300-400 degree-days (ddC) after the onset of leaf spotting (1 month in autumn). This occurred in early November 2004 (maximal pathogen DNA at ≈300 ddC), and in late November 2005 (leaves 7 and 8; 400 ddC). This conclusion was based on detailed measurements on a single cv. (NK Bravour). However, it is not clear whether this time with maximum DNA represents the earliest time that pathogen is likely to reach the stem. Additionally as it was not always known at what date a particular leaf

was first infected, it was often difficult to identify the most appropriate time to sample. The considerable variation due to the position of lesions on leaves and other sources of variation meant that the relationship between amounts of *L. maculans* DNA in petioles and final canker severity at harvest was not a reliable method to assess cultivar "field" resistance. However, these data provide other useful information, such as which leaf layers contribute most to stem cankers and therefore should be sprayed with a fungicide. There was nearly always more *L. maculans* DNA in petioles of senescing leaves than in petioles of adjacent green leaves (the lowest green leaf) sampled at the same time. Amounts of pathogen DNA present in upper and lower petiole pieces from different cvs at different times were also compared and showed no clear relationship in most samples (Table S4). There was sometimes more pathogen DNA in lower petioles than in upper petioles. In only two samples (Rothamsted Feb 06 and Nov 06) was the pathogen DNA in one half of the petiole a good predictor of that in the other half.

Table S4 Relative amounts and relationship between *L. maculans* DNA in upper (UP) and lower petioles (LP) at different sites and times

Site x date	R^2	P	Ratio of *L. maculans* DNA present, UP:LP
RRES Dec 04	0.25	0.0004	0.27
RRES Dec 05	0.005	0.6	0.54
RRES Dec 05 (outlier removed)	0.08	NS	0.54
RRES Jan 05	0.39	0.007	0.24
RRES Feb 06	0.98	<0.001	0.72
RRES Feb 06 (outlier removed)	0.07	NS	1.25
RRES Oct 06 (lowest green leaf)	0.006	0.25	1.12
RRES Oct 06 (senescing leaf)	0	0.99	2.04
RRES Oct 06 (cultivar means, green leaf)	0.38	0.019	3.8
RRES 17 Nov 06 (3 reps x 7 cvs)	0.2	0.003	7.1
RRES 23 Nov 06 (single rep x 20 cvs)	0.77	<0.001	2.2
Boxworth Nov/Dec 06	0.01	0.73	0.9
Boxworth Jan 07	0.06	0.43	1.8
Monsanto Nov 05	0.03	0.5	3.15
Monsanto Feb 06	0.08	0.54	2.57
Syngenta Dec 04	0	0.96	1.75
Syngenta Dec 05	0.01	0.71	2.4
Syngenta Feb 05	0.01	0.53	1.93
Teversham (NIAB site) Jan 05	0	0.81	0.95

- There was no clear relationship between the amount of *L. maculans* DNA in petioles in the autumn or winter and canker severity in the same individual plots the following June.
- Quantities of *L. maculans* DNA in stems were very closely related to severity of canker symptoms recorded on the same plant but frequently plant to plant variation in both symptom severity and amount of pathogen DNA meant that plot sample sizes should be more than 10 plants per plot for comparison of DNA at one time-point against canker at a different time-point (i.e. on different plants from the same plot). Amounts of pathogen DNA increased in stems gradually throughout the winter and spring but increased greatly from around flowering onwards (when visible canker symptoms began to appear). General trends in the ranking of cultivars according to cumulative amounts of *L. maculans* DNA in stems in spring show consistent differences between more susceptible cvs like Bristol and Winner and resistant cvs like Hearty.
- Amounts of *P. brassicae* DNA were well related to subsequent severity of LLS symptoms at some sites but not at others (particularly not if samples were taken early in the season in Scotland). Perhaps there was insufficient time to allow pathogen biomass to increase through secondary infection, which limited discrimination between resistant and susceptible cvs. Sampling in late winter and spring appears to be the best timing to provide material for qPCR studies to discriminate cultivars for resistance to *P. brassicae*.
- There was always much more *P. brassicae* DNA in samples of lamellae of newly expanded leaves than in the main shoot tip (meristem).
- Goodness of fit of regression of *P. brassicae* DNA against LLS severity was not related to the total amount of pathogen DNA (i.e. the ability to predict LLS severity from amount of pathogen DNA did not rely on there being a large amount of pathogen DNA).

Controlled environment experiments to assess quantitative resistance to *L. maculans*

A sub-set of six CORDISOR cultivars, together with 30-40 commercial Chinese oilseed rape cvs were tested using two methods in controlled environments: (i) point inoculation with *L. maculans* ascospore suspension followed by assessment of visual symptoms at two times (9 and 20 days post inoculation) and measurement of pathogen DNA by qPCR; (ii) point inoculation of wounded leaves using a conidial suspension of each of six different *L. maculans* isolates, followed by assessment of visual symptoms after a period of incubation.

Both methods demonstrated large differences in symptom development or pathogen DNA between the Chinese and the CORDISOR (i.e. European) cvs. European cvs were the most resistant and many of the Chinese cvs were very susceptible (i.e. had much more pathogen DNA present). There was more pathogen DNA on leaf 2 than leaf 1, when leaves were inoculated with *L. maculans* ascospores at the same time (i.e. leaf 1 was slightly older).

Canker-yield loss relationships

Yield loss due to stem canker was investigated by expressing the yield of each cultivar grown in untreated (diseased) plots as a percentage of the yield in fungicide-treated (relatively healthy) plots. The percentage yield loss of cultivars was compared against different measurements of canker severity; (a) mean canker score of the plot before harvest (usually assessed in mid-late June), (b) HGCA canker index, (c) 'G2' canker index. The latter two indices take into account the number of plants in each class of the 0-6 canker severity scale used to assess this disease. However the 'G2' method progressively weights the effect of canker severity as the severity increases. The equations are based on canker data collected using the following scale:

0	no symptoms observable
1	<25% girdling of the stem
2	26-50% girdling
3	51 -75% girdling
4	76 -100% girdling
5	100% girdling + stem weakness
6	100% girdling + stem death

The HGCA canker index is calculated as:

$$\frac{(0a + 1b + 2c + 3d + 4e + 5f + 6g)}{(a+b+c+d+e+f+g)} \times \frac{100}{6}$$

where a, b, c, d, e, f, g are the number of plants in each disease category

The 'G2' index is calculated as:

$$\frac{(0a + 1b + 3c + 5d + 7e + 9f)}{(a+b+c+d+e+f)}$$

where a-f are the numbers of plants in each disease category (0-5 scale in which category f (stem 100% girdled) comprises f and g in the HGCA 0-6 score; 0, 1,3, 5 ,7 and 9 are coefficients).

At Rothamsted in 2005, 2006 and 2007, yield was reduced only if the mean canker severity was >2.5 (over half stem cankered) by mid-June, with increasing yield loss as mean canker severity increased further. Canker severity was <2.5 in June 2005 so there was no significant yield loss at Rothamsted. In 2006 and 2007, a 'broken-stick' approach that regressed yield as a percentage of the fungicide-treated yield per cv (y) against mean canker score (x) (only for cvs with canker severity >2.5) gave significant regressions (2006: R^2 = 0.35, P=0.02: y=-14.1x +120; 2007: R^2 = 0.74, P=0.0007: y=-15.9x +126). When Rothamsted mean canker score or canker G2 index (a combination of canker severity and incidence) was plotted against % yield (yield as a percentage of the respective cv's fungicide-treated plot yield), regressions of all points including those for low disease severities produced lower goodness of fit with either linear or polynomial functions fitted than the linear broken-stick approach. Generally, yield was reduced when the G2 index was >4 at Rothamsted (2006 regression of points greater than G2 index of 4; 2007 regression of points greater than G2 index of 4; R^2 = 0.44, P=0.013: y=-10.4x + 128.4; 2007: R^2 = 0.87, P=0.00072: y=-13.3x + 144). In contrast to these results from Rothamsted, there was no effect of canker severity on yield at ADAS Boxworth in 2006 or 2007 comparing yields of untreated and fungicide-treated plots. This was partially because canker severity was moderately high even in the fungicide-treated plots, but nevertheless some cvs had mean canker scores of 3.7 (cv. Ontario) and 3.8 (cv. Winner) in 2007 in ADAS untreated plots (or G2 indices up to 6).

Implications and knowledge transfer

Knowledge transfer activities, such as presentations at Cereals events, talks to crop consultants, oral and poster presentations at international conferences, press articles and a review of *P. brassicae* biology have been made. Material to be published as research papers in refereed scientific journals has been prepared. A list of publications to date using information from this project is given below:

Papers and reviews

Evans N, Baierl A, Semenov MA, Gladders P, Fitt BDL. 2008. Range and severity of a plant disease increased by global warming. Journal of the Royal Society Interface 5: 525-531.

Boys EF, Roques SE, Ashby AM, Evans N, Latunde-Dada AO, Thomas JE, West JS, Fitt BDL. 2007. Resistance to infection by stealth: *Brassica napus* (winter oilseed rape) and *Pyrenopeziza brassicae* (light leaf spot) in Europe. *European Journal of Plant Pathology* 118: 307-321.

Aubertot JN, West JS, Bousset-Vaslin L, Salam MU, Barbetti MJ, Diggle AJ. 2006. Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 114: 91-106.

West JS, Fitt BDL. 2005. Population dynamics and dispersal of *Leptosphaeria maculans* (blackleg of canola). *Australasian Plant Pathology* 34: 457-461.

Popular articles

West JS, Thomas J, Gladders P, Booth E, Latunde-Dada AO, Evans N, Fitt BDL, 2005. Components of Resistance to Diseases in Winter Oilseed Rape: CORDISOR *RRA Newsletter 16. January 2005*.

West JS, Thomas J, Gladders P, Booth E, Latunde-Dada AO, Evans N, Fitt BDL, 2004. Components of Resistance to Diseases in Winter Oilseed Rape: CORDISOR. *GCIRC Bulletin 21, XII 2004*, pp.79-81.

Conference proceedings

BDL. Fitt, N Evans, P Gladders, Y-J Huang and JS West (2008) Phoma stem canker and light leaf spot on oilseed rape in a changing climate. *Aspects of Applied Biology* **88**; Effects of Climate Change on Plants: Implications for Agriculture. pp 143-145.

West JS, Rogers SL, Latunde-Dada AO, Pirie EJ, Stonard JF, Huang YJ, Atkins SD, Fitt BDL. 2008. Quantifying DNA of Leptosphaeria maculans and Pyrenopeziza brassicae in oilseed rape tissues using qPCR. 9th International Congress of Plant Pathology, Healthy and safe food for everybody, August 24-29, 2008, Torino, Italy.

West JS, Gladders P, Booth E, Thomas J, Jennaway R, Werner P, Bowman J, Nightingale M, Padbury N, Clarke M, Jellis GJ, Foster V, Rogers SL, Kenyon D, Atkins SD, Boys EF, Huang Y-J, Evans N, Latunde-Dada AO, Li Q, Fitt BDL. 2008. Factors affecting canker and light leaf spot severity in oilseed rape. BSPP Break Crops Workshop, Peterborough, 24 June 2008, p16.

West JS, Rogers SL, Gladders P, Fitt BDL 2008. Climate change set to worsen severity of stem canker. HGCA R&D Conference 'Arable Cropping in a Changing Climate', 23-24 January 2008, Belton Woods, Lincs. Abstract p135.

Latunde-Dada AO, West JS, Rogers SL, Downes K, Roques SE, Pirie E, Stonard JF, Huang Y-J, Fitt BDL 2007. New methods to understand quantitative resistance to *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape. Proceedings of the 12th International Rapeseed Congress: sustainable development in cruciferous oilseed crop production. Wuhan, China, March 26-30, 2007. Abstracts p310.

West JS, Latunde-Dada AO, Rogers SL, Huang Y-J, Evans N, Fitt BDL 2007. *Leptosphaeria maculans* avirulence management for durable control of phoma stem canker on oilseed rape. Proceedings of the 12th International Rapeseed Congress: sustainable development in cruciferous oilseed crops production. Wuhan, China, March 26-30, 2007. Vol. IV. p2-4.

West JS, Latunde-Dada AO, Huang Y-J, Evans N, Fitt BDL 2006. Avirulence management for durable control of stem canker of oilseed rape in Europe. *Aspects of Applied Biology* 80, *Delivering sustainability within profitable farming systems – is it possible?*. pp 171-176, The Association of Applied Biologists, Wellesbourne.

West JS, Latunde-Dada AO, Huang Y-J, Evans N, Fitt BDL 2005. Enhanced avirulence management for durable control of blackleg in Europe. In: Potter, T. (Ed), 14th Australian Research Assembly on Brassicas, Port Lincoln, 2005. Proceedings. SARDI, Primary Industries and Resources South Australia, pp 76-81

Other KT Activities

A) Events:

The project featured at both the Defra and Rothamsted Research displays at Cereals 2005 and a Defra SA LINK leaflet was distributed. Jon West made a presentation on durability of resistance to phoma stem canker at the 14th Australian Research Assembly on Brassicas, Port Lincoln, South Australia, 6th October 2005; and a similar presentation

at the Rothamsted Research Association meeting on 24th November 2005 at Rothamsted. Jon West presented an overview of the project's objectives, methods and results at the UK-Brassica Research Community Annual Meeting, John Innes Centre, 24th May 2006. A simplified version of this presentation is available on the UK-Brassica Research Community website: http://www.brassica.info/ukbrc/meet06.htm.

The project featured at Cereals 2006 (14-15 June) as part of the HGCA display and the Defra leaflet was distributed at the Rothamsted display. Additionally, the project was publicised at ADAS open days at Rosemaund (6 June), Boxworth (8 June) and High Mowthorpe (29 June) and in the HGCA oilseed rape disease and pest management workshops in different parts of England (autumn 2008).

B) Press articles

An article by Mark Sanderson that featured the project was published in Syngenta Farmer's Guardian in October 2005. An article entitled, 'Research In Focus: stem canker in rape', by Sarah Henly, appeared in 'Crops', issue date: 15 October 2005.

Jon West attended a press briefing organised by Bayer, and reported recent research findings, leading to an article in Farmers Weekly on 13 October 2006 'Phoma control crucial as OSR area expands' by Andrew Blake.

Scientific Report

Abstract

The project had two aims: (i) to test whether measuring components of resistance to stem canker (caused by *Leptosphaeria maculans*) and light leaf spot (caused by *Pyrenopeziza brassicae*), especially symptomless growth, will improve selection of resistant cultivars; and (ii) to produce new methods to rate cultivar resistance to stem canker and light leaf spot in winter oilseed rape cultivar selection and evaluation trials.

The quantity of *L. maculans* DNA in a crown (stem base) sample from an individual winter oilseed rape plot was very closely related to the mean severity of canker symptoms recorded on the same plants before harvest. However, plant to plant variation (sample error) in both symptom severity and amount of pathogen DNA reduced the closeness of the relationship between amount of DNA in one plant sample and canker severity in a separate plant sample from the same plot and same time, when plot sample sizes were ≤10 plants. The relationship between amount of *L. maculans* DNA in crowns and pre-harvest canker severity became less good as samples were taken at progressively earlier time-points in the same field-plot. Amount of *L. maculans* DNA in crowns remained small during the winter but increased greatly from flowering onwards when symptoms became visible. General trends in the ranking of cultivars for amount of *L. maculans* DNA in stems in winter show differences between more susceptible cvs like Bristol and Winner and more resistant cvs like Hearty and ES Astrid. *L. maculans* DNA in petioles of leaves with visible phoma leaf spots sampled in the autumn or winter could not be used as a predictor of final canker severity. Difficulties in identifying the most appropriate time to sample meant that the relationship between amount of *L. maculans* DNA in petioles and final canker severity at harvest was not a reliable method to predict cultivar "field" resistance. However, the method can provide other useful information, such as which leaf layers contribute most to stem disease and therefore should be sprayed with a fungicide. There was much more DNA of *L. maculans* in petioles of senescing leaves than in those of the lowest green leaf.

The project showed for the first time that *Pyrenopeziza brassicae* infection of the main shoot tip (meristem) of OSR plants in winter was a common and widespread occurrence. Amounts of DNA of *P. brassicae* in leaves or main shoots (meristems) were related well to subsequent severity of light leaf spot (LLS) symptoms at some locations on some dates (particularly when visible symptoms were severe in late winter/early

spring, when visual assessments are normally made for Recommended List evaluation). It is probable that early samples taken in December and January, particularly in Scotland, had not had sufficient thermal time to allow pathogen biomass to increase through secondary infection, which limited discrimination between resistant and susceptible cvs. Sampling in late winter and spring appears to provide the best material for qPCR studies to discriminate cultivars for resistance to *P. brassicae*. There was always much more *P. brassicae* DNA in samples of lamellae of recently expanded leaves than in the main shoot tip (meristem). Goodness of fit of regressions of *P. brassicae* DNA against LLS severity was not related to the general amount of DNA. Measurements of LLS severity by visual assessment of plots (1-9 score) often did not relate well to mean disease severity measured by close inspection of ten plants. Incubation of ten newly expanded leaves per plot in plastic bags in a cool room for a few days provided a good visualisation of LLS symptoms to discriminate differences in cultivar resistance to *P. brassicae*. The large differences between cultivars in visible symptoms and amounts of *L. maculans* DNA measured at different times and distances from the ascospore-inoculation point in tests using European and Chinese cultivars grown in controlled conditions may be good indicators of resistance to *L. maculans*. Large differences in severity of visible symptoms occurred following inoculation of wounded cotyledons with conidia of a set of different races of *L. maculans*.

In these experiments, yield loss due to canker occurred only if mean canker severity was >2.5 (over half stem cankered) by mid-June and reduced yield by 15% for each additional unit of canker severity (0-6 scale). The occurrence of stem splitting, caused by very warm spring weather during stem extension, did not increase canker severity. There were few differences in leaf production and turnover between cvs and little difference between leaf or petiole length between cvs but these factors did vary considerably between sites. Canker severity assessed as cross-sectional area of canker at the stem base (at maximum severity) was very closely related to canker severity assessed as the percentage of stem girdled by canker. Discrimination between different cvs could be improved by recording the percentage of canker (either as area or girdling) as a continuous variable, rather than on the 0-6 scale currently used in HGCA RL assessments, although data recording would be more time-consuming.

Abbreviations: cv(s) – cultivar(s), qPCR – quantitative PCR,

Chapter 1. General introduction

The project aimed to produce new methods to improve measurement of disease resistance in winter oilseed rape cultivars. The project focussed on resistance to the two main diseases of winter oilseed rape in the UK, stem canker and light leaf spot. Disease resistance ratings are currently calculated from the visual assessment of disease and do not explain the relative importance of resistance expressed in different tissues (leaf, stem, pods) at different times or stages of disease development – information which is valuable for breeding new cultivars with enhanced disease resistance and reduced reliance on fungicides for disease control.

The approach used in the project was to evaluate 20 different oilseed rape cultivars for their growth and amounts of disease at a range of field sites in England and Scotland. The 20 cvs (Table 1) were chosen to represent a wide range of resistances and susceptibilities to canker and light leaf spot, using predominantly cvs from the HGCA recommended list at the start of the project (cv. ES Astrid replaced Aragon after first year). Additionally at some sites that included untreated and fungicide-treated plots (disease severity reduced greatly), yield data were collected and used to assess the effect of stem canker on yield. The visible disease symptoms recorded in different ways were compared with measurements of amounts of DNA of the causal pathogens using quantitative PCR at on selected plant tissues sampled key growth stages.

Table 1. OSR cvs used in CORDISOR experiments in harvest years 2005-2007

Cultivar	Type	Breeder	UK Agent
Disco	Restored hybrid	Raps GbR, GER	Saaten Union
Fortis	Conventional	Novartis, Ger	Syngenta
Recital	Conventional	Novartis Gmbh, GER	Syngenta
Winner	Conventional	Raps GbR	Saaten Union
Expert	Conventional	Moment, Fr	Advanta
Courage	Conventional	Van der Have, NE	Advanta
Canberra	Conventional	Cargill, Fr/ DSV,Ger	Monsanto
Escort	Conventional	Limagrain, Fr	Nickersons
Royal	Restored hybrid	Novartis Gmbh, GER	Syngenta
Elan	Restored hybrid	NPZ, GER	KWS-UK
Lioness	Conventional	DSV, Ger	Cebeco SI
NK Bravour	Conventional	Syngenta, Fr	Syngenta
Castille	Conventional	Monsanto, Fr	Monsanto
NK Victory	Conventional	Syngenta, Fr	Syngenta
Aragon*	Conventional	Lembke, Ger	KWS-UK
Apex	Conventional	Lembke, Gr	Advanta
Bristol	Conventional	Cargill. Fr	
Shannon	Conventional	Nickersons	Nickersons
Hearty	Conventional (HEAR)	Monsanto	Monsanto
Ontario	Conventional	Nickerson GEIE	Nickersons
ES Astrid	Conventional	Euralis, Fr	Grainseed Ltd.

* replaced by ES Astrid for 2006 & 2007

Chapter 2. Visual plant and disease assessments

2.1 Methods

Visual plant and disease da+ta were recorded on a set of 20 cvs of oilseed rape at 12 different sites in each of three seasons (harvest years 2005, 2006 and 2007). Additionally, at Rothamsted, over the first two seasons of the project, one cv (NK Bravour) was grown in additional plots next to the main experiment and was sampled for quantification of pathogen DNA in plant tissues more frequently than the main experiment. The same set of cold-stored seed was used each year for field experiments. Disease and plant assessments were made according to an established protocol (see Appendix 1 for details; summarised in Table 2) at specified times (Table 3). Growth stage [Sylvester-Bradley & Makepeace (1985)] & presence of other diseases on tissues studied (leaves, stems or pods) e.g. downy mildew, powdery mildew, alternaria, botrytis, white leaf spot, etc.) were recorded.

Cultivar ES Astrid (phoma canker RL resistance rating 8) was selected to replace cv. Aragon (rating 2) after the 2004-05 season to provide data for a more resistant cultivar extending the range of disease resistance available. Plants were also assessed (June 2006, Spalding site) orsampled and assessed (May 2007, Cowlinge site) from the T-N population of the NOVORB LINK project as this material included some commercial European winter OSR cvs (common to the CORDISOR project) and also very canker-susceptible lines.

Table 2. Summary of CORDISOR Plant and Disease assessments (10 plants per plot unless stated)

Stem canker	Light leaf spot	Plant growth
SC1 Phoma leaf spot (individual plant visual assessments of N° leaves affected and % total leaf area affected, plus 1-9 plot score according to RL protocol)	**LLS1** Light leaf spot (incubate plants in plastic bags in cool (10°C) room for 5 days then score N° leaves affected and % total leaf area affected on individual plants, plus 1-9 plot score at time of sampling according to RL protocol)	**P1** For each plant sampled, record N° unfolded leaves present & N° lost.
SC2 Petiole infection (dispatched for PCR: 10 whole plants separated with blue-roll/tissue in a labelled plastic bag per plot)	**LLS2** Apical meristem infection (whole plants wrapped in blue-roll in a plastic bag and dispatched for PCR)	**P2** Plant height (mm to apex of main stem)
SC3 Stem canker score (individual plants scored on 0-6 scale where: 0 = uninfected, 1 = < 25% stem circumference girdled, 2 = 26-50% girdled, 3 = > 51-75% girdled, 4 = > 76-100% girdled, 5= 100% girdled + stem weak, 6 = plant dead or lodged)	**LLS3** LLS symptoms on leaves of individual plants (without incubation score N° leaves affected and % total leaf area affected), plus 1-9 plot score at time of sampling according to RL protocol.	**P3** Stem diameter (mm) individual plant measurement **P3*** 1-4 Score of average plot stem diameter (+ measure best and worst). Optional if felt that this is quicker than P3.
SC4 Stem canker score (RL) (0-6 scale: as above)	**LLS4** (now deleted as part of LLS1 and LLS3)	**P4** Plant density (N° plants in 4 x 0.5 m rows and note distance between rows) **P4***as P4 in best and worst plots with other plots scored on a relative 1-9 scale.
	LLS5 LLS stems (score % area affected on main stem)	
	LLS6 LLS pods (score % pods affected and % total area of pods affected)	

Table 3. Summary of assessment times for CORDISOR project

Exp.	Treatments	Main disease assessments (10 plants per plot/line) (other diseases assessed if present)							
		Oct	Nov/ Dec	Jan	mid-March	April/ May	June	July	notes
1. RRes (inoculated for LLS)	+/- fungicide (weekly phoma checks in autumn)	LLS1, SC1, P1	LLS1, SC1, 2 P1, 4	LLS1, 2 SC1 P1	LLS3, SC1, 3, P1, 2	LLS3, 5, SC 3, P 2	LLS5, 6, SC4 P3	LLS5, 6, SC3, P4 YIELD	
2. SC plot, Boxworth	+/- fungicide (weekly phoma checks in autumn)	SC1 P1	SC1, 2 P1, 4	SC1 P1	SC1, 3 P1, 2	SC 3 P 2	SC4 P3	SC3 P4 YIELD	
3. Breeder nursery, England	Inoculated/ uninoculated	SC1 P1	SC1, 2* P1	SC1, LLS1, 2* P1	SC1, 3 P1, 2	SC 3 P 2	SC3 P3*	SC3	SC2* PCR on selected sites only
4. Breeder nursery Scotland	Different sowing rates	LLS1 P1	LLS1 P1	LLS1, 2* P1	LLS3 P1, 2	LLS3, 5 P 2	LLS5, 6 P3*	LLS5, 6	LLS1, 2* PCR on selected sites
5. LLS expt. RRes (inoculated field expt)	-	LLS1 P1	LLS1, 2 P1	LLS1, 2 P1	LLS3 P1, 2	LLS3, 5 P 2	LLS 5, 6 P3	LLS5, 6	
6. SCt expt. RRes (inoculated field expt)	-	SC1 P1	SC1, 2 P1	SC1 P1	SC1, 3 P1, 2	SC 3 P 2	SC4 P3	SC3	
7. RL trial, England	+/- fungicide (yield)	SC1 P1	SC1, 2 P1, 4*	SC1 P1	SC1, 3 P1, 2	SC 3 P 2	SC3 or 4 P3*	SC3 P4* YIELD	
8. RL trial, Scotland	+/- fungicide (yield)	LLS1 P1	LLS1 P1, 4*	LLS1, 2 P1	LLS3 P1, 2	LLS 3, 5 P 2	LLS5, 6 P3*	LLS5, 6 P4* YIELD	

Data were collated at Rothamsted, and analysed by ANOVA using a statistical software package (Genstat) for each site x sample date. Cultivar means for each variable recorded were copied into three Excel databases, one for each season. These databases were used for further analysis of trends, relationships between different variables and extensively to assess the relationship between visual symptoms such as canker or light leaf spot severity and the amount of respective pathogen DNA found in the same samples or samples from the same plots but taken at an earlier time-point.

2.2 Results

Plant growth

There was little difference in stem diameter between cvs but there were differences between sites. Stem diameter and presence of splitting on the stem (caused by hot weather during stem extension, particularly in Apr/May 2006) did not affect canker severity. Petiole length (lowest green leaf sampled) varied only slightly between cvs but varied greatly between site and time of year. There was little difference in mean height between cultivars. By mid-November on average six leaves were present and 4-6 leaves shed, with little difference in leaf turn-over between cvs but clear differences between sites. By January, on average, 8-10 leaves were present with 7-8 leaves already shed. The rosette leaf scars (from leaves that contributed to stem canker) were produced by the first 10-13 leaves. There were large differences in petiole lengths between sites or assessment dates but usually no significant differences between cultivars. Similarly the number of leaves present, shed or in total varied more between sites and dates than between different cultivars at a particular site on a given date.

Disease development and measurement

- Generally canker severity was low in 2005 but high in 2006 and 2007. Light leaf spot severity varied greatly between sites and was greatest in March and April.
- There was little difference between canker severity recorded by extent of girdling or by cross-sectional area affected for discriminating cultivar resistance.
- The 1-9 plot score used for phoma leaf spot (*Leptosphaeria maculans*) appeared to vary substantially between observers, occasionally under estimating disease present on lower leaves. Furthermore, there was little relation between phoma leaf spot severity and final canker severity. The timing of onset of phoma leaf spot and winter temperature affected canker severity greatest. Fungicide sprayed in mid and late autumn gave good control of canker.
- Incubation of ten newly expanded leaves per plot in plastic bags in a cool room for 4-5 days allowed good visualisation of LLS symptoms to assess differences in cultivar resistance to *Pyrenopeziza brassicae*. Substantially more light leaf spot symptoms were visible after 4-5 day incubation than a 2-day or no incubation, especially in early-mid-winter. Generally there were large differences in LLS severity on leaves between the 20 cvs tested at each site, with greatest severity on cv. Hearty and least on cv. Elan.

- There was no clear relationship between light leaf spot (LLS, *Pyrenopeziza brassicae*) severity on leaves in late winter and LLS severity on pods in June, indicating that either the second release of ascospores in spring is influential and/or that a different resistance mechanism occurs on pods and/or that avoidance of meristem infection is critical (PCR data confirmed that at some sites meristems in untreated plots became infected before winter and this could result in stunting of plants and ensure that subsequent growth stages would be infected. Similarly, fungicide applied in autumn decreased LLS severity on pods the following June for most cultivars).
- There was a reasonable relationship (R^2 = 0.55 in 2005) between LLS severity on pod (1-9 scale in June-July, - means of four sites) and the HGCA LLS resistance score for different cultivars.
- There was a good relationship (R^2 = 0.757; P<0.0001) between visible LLS data for selected sites x dates x cvs (sites sampled for pathogen DNA) expressed as a proportion of the sum of LLS severity on all the cvs assessed at the individual site x time and the average proportion per cv compared against the 2006-7 HGCA RL LLS resistance rating (see Figure 6).
- There was a good relationship (R^2 = 0.53 to 0.56 in 2005) between canker severity (mean score, HGCA index or French G2 index in June-July, - means of five sites) and the HGCA canker resistance score for different cultivars.
- The incidence and severity of phoma leaf spotting was significantly less on cv Hearty in some data-sets (eight sites x sample dates) but not all. Hearty had significantly lower incidence of phoma leaf spot at five sites at the start of the season but at most of these sites, incidence on cv. Hearty increased to be the same as on other cvs later in the season (e.g. February). In contrast, at ADAS Boxworth there was no significant difference in incidence of phoma leaf spot between cvs early in the season, but cv. Hearty had a significantly lower incidence in the spring. Occasionally, cvs Escort, ES Astrid and Fortis had lower incidence of phoma leaf spot than other cvs. In general, comparing all sites, cvs Canberra, ES Astrid and Hearty had the smallest canker indices, while cv. Bristol had the largest canker index.
- Thermal time from first leaf spotting to canker formation was investigated at Rothamsted. Thermal time from the onset of phoma leaf spot (time A) (10% plants of Hearty) to first canker appearance (B) was 958, 1117 (estimated) and 1275 degree-days in 2005, 2006 and 2007 respectively (mean of all cvs).
- Rates of canker development from first appearance (time 'B') in 2005-2007 for cv. Bristol were; 0.0026, 0.0046 and 0.0040; for cv. Expert were 0.0008, 0.0017 and 0.0011 and for cv. ES Astrid (2006-2007 only) were 0.0019 and 0.0006 canker severity units per degree day (Figure 1).

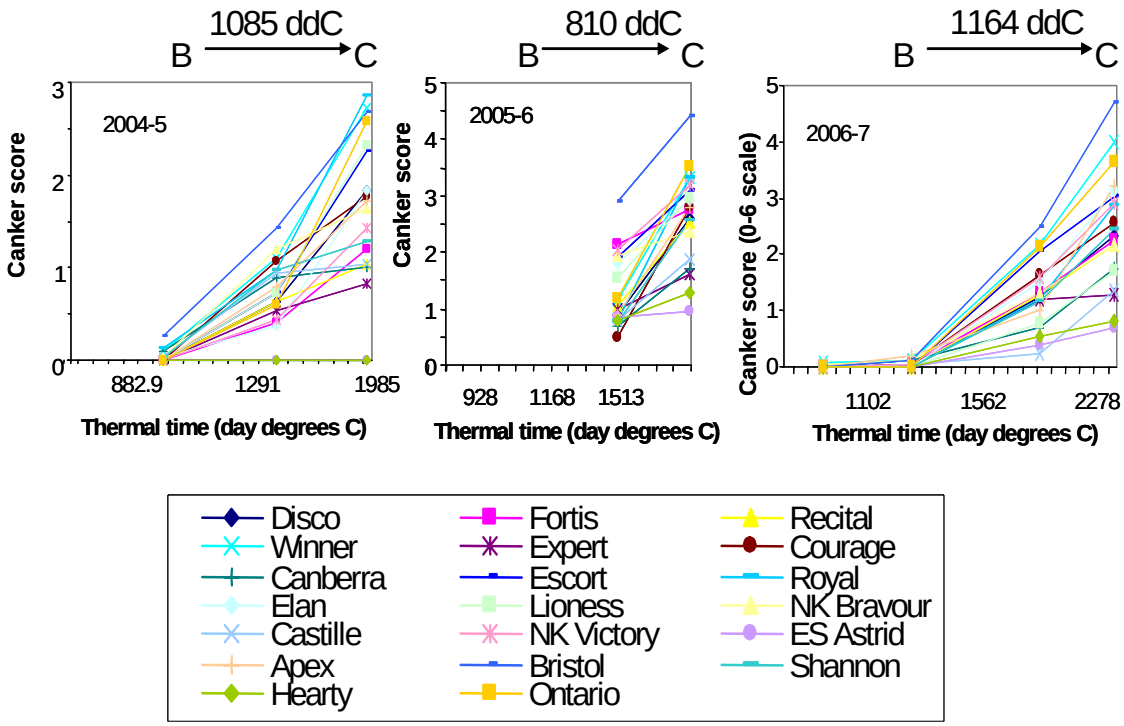

Figure 1. Increase in canker severity (0-6 scale) against thermal time (B= first appearance of canker; C = final canker measurement) at Rothamsted 2005-2007. NB different y-axis scales

Cultivars with the highest rate of canker development at Rothamsted were: (2005) Royal, Winner and Bristol; (2006) Bristol, Ontario and Winner: (2007) Bristol, Winner and Ontario (Figure 1). Cultivars with the lowest rate of canker development at Rothamsted were: (2005) Expert, Canberra and Castille (no ES Astrid nor Hearty at Rothamsted in 2005); (2006) ES Astrid, Hearty, Expert, Canberra and Castille; (2007) ES Astrid, Hearty, Expert and Castille. The rate of canker development varied each year but the ranking of cultivars appeared to be very consistent each year at this site (Figure 1). The lower incidence of phoma leaf spot on ES Astrid and Hearty than other cvs could explain the subsequent lower severity of canker on them, but the reduced rate of canker development in Expert, Canberra and Castille was probably due to operation of quantitative resistance as they had a similar incidence of phoma leaf spot to the other cvs. In contrast, cultivars Bristol, Winner, Ontario and possibly Royal appear to have increased rates of canker development, suggesting that they have less effective quantitative resistance.

- For light leaf spot (LLS), cvs Hearty, Shannon and Recital were most susceptible, while cv. Elan alone had less LLS than all other cultivars.
- There was very light or sporadic development of alternaria leaf and pod spot, powdery mildew, downy mildew and sclerotinia stem rot (SSR) with SSR more severe than normal in 2007 (but with no significant differences between cvs).

Chapter 3. Quantification of pathogen DNA *in planta* by qPCR

3.1. Methods

Sample collection and processing for DNA extraction

The pathogen DNA data was produced from plant samples taken at selected times from field experiments and processed directly at Rothamsted or NIAB or posted from other sites (SAC sites and breeders' nurseries in Scotland and England) to Rothamsted. In some cases, posted samples were assessed for visible disease symptoms in addition to processing of selected plant tissues for pathogen DNA measurement. Samples were also collected from selected lines from the 'T-N' population (a mapping population from a cross between a European cv. Tapidor and a Chinese cv. NingYou, which appears to be segregating for a number of genetic traits including resistance to *L. maculans*), which provided samples that were more susceptible than the 20 cvs used in CORDISOR field experiments (this TN population was grown as part of the NOVORB LINK project). At Rothamsted, selected tissue samples (upper or lower petioles, meristems or recently expanded leaves) were frozen, freeze-dried, and powdered (see Appendix 2 for details). Freeze-dried powdered samples were stored at 5°C until DNA had been extracted and were kept throughout the project in case re-extraction was necessary (it was not).

DNA extraction (Rothamsted protocol)

At Rothamsted, selected tissue samples (upper or lower petioles, meristems or recently expanded leaves), which had been washed and blotted dry if dirty, were frozen, and freeze-dried. DNA was extracted from tissue samples using a commercial kit (DNAmite, Microzone, Haywards Heath) according to the manufacturer's directions using a sub-sample of 20 mg of freeze-dried powdered plant tissue in a 2 ml tube with three stainless steel ball bearings (ø 4 mm) and 1 ml of solution 'LA' and shaken for 20 seconds at 4.0 ms^{-1} in a fastprep machine, solution 'PA' added, centrifuged for 5 minutes at 10,000 rpm, the supernatant transferred to solution 'CA' and centrifuged for 7 minutes a 13,000 rpm to pellet the DNA. The DNA pellet was re-suspended in 100 µl of water and the quality of DNA was assessed using a spectrophotometer (nanodrop ND-1000). The DNA was then archived at -20°C. DNA standards of *B. napus* were initially produced using a DNeasy mini plant kit (Qiagen).

DNA extraction (NIAB protocol)

Frozen petiole material was cut into appropriate lengths and freeze dried. Dried material was ground in a coffee grinder, mixed carefully, weighed and a 20 mg sub-sample taken for DNA extraction. Extraction was performed with Nucleospin kits (Quiagen).

Quantitative PCR-introduction

Different quantitative PCR (qPCR) protocols were used according to equipment available at each site (Rothamsted or NIAB). Although various methods (Sybr Green and Taq-man) with different primers were initially investigated at Rothamsted, eventually both NIAB and Rothamsted sites used Sybr green systems to quantify *L. maculans* in plant tissues and a similar method was used at Rothamsted to assay *P. brassicae*. This method measures fluorescence of a dye that binds to double-stranded DNA. Non-target DNA (e.g. from the plant), which is not replicated in the PCR reaction produces a background low level of fluorescence but this is below a set detection threshold, so only the DNA of the target (plant pathogen) is measured, the amount produced after a set number of replication cycles depending on the initial amount of target DNA in the sample.

Quantitative PCR (Rothamsted protocol)

The *L. maculans* Taq-man quantitative PCR, initially proposed to be used at Rothamsted, was found to have insufficient specificity. As an alternative, the SYBR green system used successfully at NIAB was used (but with a different real-time PCR machine). A new Taq-man q-PCR method was successfully developed with alternative primers and a probe. This method was used for quantification *of L. maculans* in approximately half of the sample sites x dates sampled in seasons 2004/05 and 2005/06 but was discontinued in favour of using a SYBR green method, which was easier and enabled many of the same reagents to be used for both *L. maculans*- and *P. brassicae*-specific qPCRs. A selection of samples tested in different qPCR runs using the Taq-man method were retested with the new SYBR green method to confirm that the results were consistent. The final SYBR-green based methods, used for the most *L. maculans* assays (including all the 2006/07 season) and all *P. brassicae* assays over the entire project are described.
Quantitative PCRs were done in Bioplastic (EU) 96 x 0.2 ml PCR plates capped with Bioplastic EU optical thin wall 8 cap strip low background (Bioplastics, Landgraaf, The Netherlands). SYBR green mix (Sigma-Aldrich, Gillingham, Dorset) was used for qPCR reactions.

L. maculans primers used in the final protocol had the sequences:

LmF 5'-GTGGCGGCAGTCTACTTTGA-3' and

LmR 5'-GAGTCCCAAGTGGAACAAACA-3'.

P. brassicae primers used were:

PbITSF 5'-TTGAACCTCTCGAAGAAGTTCAGTCT-3' and

PbITSR 5'-AGATTTGGGGGTTGTTGGCTAA-3'

For either *L. maculans* or *P. brassicae* specific qPCR, a total of 2.5 µl of the extracted DNA was used with 17.5 µl per well of qPCR mix made up from a total mix of 1000µl SYBR Green mix, 800 µl ultra pure dH20, 10 µl rox dye and 8 µl of each primer (LmF and LmR, or PbITSF and PbITSR) in a qPCR machine Mx3000P (Stratagene, Amsterdam, The Netherlands) (in the first year, the machine used was a 7500 Real Time PCR machine (Applied Biosystems, USA). PCR conditions for *L. maculans* comprised 95°C for 2 min followed by 50 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 45 s and 83°C for 15 s. There followed a dissociation cycle of 95°C for 1 min, 60°C for 30 s and 95°C for 30 s.

PCR conditions for *P. brassicae* were 95°C for 2 min followed by 35 cycles of 95°C for 15 s, 58°C for 30 s, 72°C for 45 s and 82°C for 36 s. There followed a dissociation cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

The dissociation curve was produced to confirm that target DNA had been amplified. Three replicates per run of each DNA sample were used with standard non-template controls of water and *Brassica napus* DNA. A calibration curve was produced from 3 replicates of pure target pathogen DNA on a dilution series ranging from 10 ng to $1x10^{-4}$ ng in 10-fold increments with *B. napus* DNA. The calibration curve standards were made up of *B. napus* DNA and pathogen DNA to make up a total of 50 ng, i.e. *B. napus* : *L. maculans* or *B. napus, P. brassicae* 40 ng:10 ng, 49 :1, 49.9: 0.1, 50: 0.01, 50: 0.001 and 50: 0.0001. The run was repeated if the calibration curve had a goodness of fit (R^2 value) less than 0.98. Additionally as independent standards, 3 replicates of pure pathogen DNA used at amounts of 1 ng and 0.01 ng per reaction were used in each run and a conversion factor was calculated from the mean of the differences between the values calculated automatically from the calibration curve and the actual amounts used. The conversion factor was applied to produce an adjusted value for all unknown samples. Results were expressed as pg pathogen DNA per sample (i.e. per 2.5 µl sub-sample of 100 µl extracted from 20 mg plant material). Occasionally the results for one of the three replicates were not included in calculating the average amount of DNA for the sample they differed substantially from the results for the other two replicates. For *L. maculans*, values less than 1 pg were considered as zero when regressed against disease data, but most *P. brassicae* DNA amounts were less than 1 so these were used directly when related to factors such as disease symptom severity.

Quantitative PCR (NIAB protocol)

Quantitative PCR was carried out successfully for *L. maculans* at NIAB using a previously established LightCycler-based q PCR method with the same *L. maculans* primers as described above (Primers HVF1 and HV26c; Defra CSG 15 report, 2004). The primers amplify a 377 base pair product specific to *L. maculans* and do not cross react with the closely related species *L. biglobosa*. All experiments were carried out using a Lightcycler™ with the standard reaction mixture of 10 µl absolute QPCR SYBR Green capillary Mix (ABGene) which contains 3mM MgCl2, 2 µl of primer (5 µM stock), 2 µl DNA template and 6 µl water. Reaction mixtures were loaded into chilled glass capillary tubes by centrifugation at 700 g for 5 s. PCR cycling conditions were as follows: initial denaturation at 95ºC for 15 min (to activate the 'hot start' TaqDNA polymerase) followed by 30 cycles of: 95ºC for 0 s, 62ºC for 5 s and 72ºC for 15 s. PCR product accumulation was measured once during each cycle following the end of the extension step.

3.2 Results

3.2.1 *L. maculans*

The optimal sample time to quantify DNA of *L. maculans* in leaf petioles was taken as the time when maximal pathogen DNA was present in the petiole of the lowest green leaf. This was found to be 300-400 degree-days (ddC) after the onset of leaf spotting (≈1 month in autumn). This occurred in early November 2004 (maximal pathogen DNA at ≈300 ddC), and in late November 2005 (leaves 7 and 8; 400 ddC). This conclusion was based on detailed measurements on a single cv. (NK Bravour). However it is not known whether this time with maximum DNA represents the earliest time that pathogen is likely to reach the stem. Additionally as it was not clear at what date a particular leaf was first infected, it was often difficult to identify the most appropriate time to sample. The considerable variation due to the position of lesions on leaves and other sources of variation mean that the relationship between amounts of *L. maculans* DNA in petioles and final canker severity at harvest was not a reliable method to assess cultivar "field" resistance. However, these data provide other useful information, such as which leaf layers contribute most to stem cankers and therefore should be sprayed with a fungicide. There was nearly always considerably more *L. maculans* DNA in petioles of senescing leaves than in petioles of adjacent green leaves (the lowest green leaf) sampled at the same time (data not shown). Amounts of pathogen DNA present in upper

and lower petiole pieces from different cvs at different times were also compared and showed no clear relationship in most samples (Table 4). There was sometimes more pathogen DNA in lower petioles than in upper petioles. In only two samples (Rothamsted Feb 06 and Nov 06) was the pathogen DNA in one half of the petiole a good predictor of that in the other half.

Table 4. Relative amounts and relationship between *L. maculans* DNA in upper (UP) and lower petioles (LP) at different sites and times

Site x date	R^2	P	Ratio of *L. maculans* DNA present, UP:LP
RRES Dec 04	0.25	0.0004	0.27
RRES Jan 05	0.39	0.007	0.24
RRES Dec 05 (outlier removed)	0.08	NS	0.54
RRES Dec 05	0.005	0.6	0.54
RRES Feb 06	0.98	<0.001	0.72
RRES Feb 06 (outlier removed)	0.07	NS	1.25
RRES Oct 06 (lowest green leaf)	0.006	0.25	1.12
RRES Oct 06 (senescing leaf)	0	0.99	2.04
RRES Oct 06 (cv. mean, green leaf)	0.38	0.019	3.8
RRES 17 Nov 06 (3 reps x 7 cvs)	0.2	0.003	7.1
RRES 23 Nov 06 (single rep x 20 cvs)	0.77	<0.001	2.2
Boxworth Jan 07	0.06	0.43	1.8
Boxworth Nov/Dec 06	0.01	0.73	0.9
Monsanto Nov 05	0.03	0.5	3.15
Monsanto Feb 06	0.08	0.54	2.57
Syngenta Dec 05	0.01	0.71	2.4
Syngenta Feb 05	0.01	0.53	1.93
Syngenta Dec 04	0	0.96	1.75
Teversham (NIAB site) Jan 05	0	0.81	0.95

There was no clear relationship between amounts of *L. maculans* DNA in petioles in the autumn or winter and canker severity in the same individual plots the following June (Table 5a), nor if data were combined per cv (Table 5b)

Similarly, there was a poor relationship between mean amount of *L. maculans* DNA in petiole tissues of different leaves or leaves sampled at different times, different sites or in different cultivar replicates of the same site (i.e. testing consistency of method) (Table 5c); and a poor relationship between cumulative pathogen DNA from many sites compared against RL ratings (Table 5d). There was a poor relationship between

pathogen DNA in petioles and subsequently in crowns (Table 5e). There was a poor relationship between the amounts of pathogen DNA in petioles of respective cvs taken at one time-point (e.g. November) and that on leaves from the same plots at a later time-point (e.g. January), between different replicates of the same cvs, and also between leaves of the same cvs sampled at the same time but from different sites (Table 5c).

Table 5a: Regressions of amount of *L. maculans* DNA (pg) present in upper petiole (UP), lower petiole (LP) or whole petioles (WP) against canker severity (or log of severity) in respective

Comparison	R^2	P
RRes Nov / Dec '04 LP v. plot canker score (June)	0.00006	0.913
RRes Nov / Dec '04 UP v. plot canker score (June)	0.0251	0.438
RRes Jan '05 LP v. plot canker score (June)	0.0163	0.685
RRes Jan '05 UP v. plot canker score (June)	0.2309	0.048
Syngenta Feb '05 LP v. plot canker score (June)	0.0148	0.480
Syngenta Feb '05 UP v. plot canker score (June)	0.0182	0.419
Syngenta Dec '04 LP v. plot canker score (June)	0.0032	0.629
RRes Dec '05 LP v. plot canker score (June)	0.0072	0.532
RRes Dec '05 LP v. plot canker score (June) (results above 1pg only)	0.0387	0.289
RRes Dec '05 UP v. plot canker score (June)	0.009	0.482
RRes Dec '05 UP(results above 1pg only)	0.0156	0.489
RRes Feb '06 LP (not 96) v. plot canker score (June)	0.0139	0.481
RRes Feb '06 UP (not 96) v. plot canker score (June)	0.0763	0.098
RRes Feb '06 UP (not 96 + 88) v. plot canker score (June)	0.0098	0.567
RRes Oct 06 UP v. plot canker score (June) girdling	0.0154	0.422
RRes Oct 06 UP v. plot canker score (June) internal	0.0113	0.492
RRes Oct 06 LP v. plot canker score (June) girdling	0.0266	0.290
RRes log of plot canker score (June 07) v. *Lm* DNA in UP Nov06	0.000	NS
RRes Oct 06 v. log plot canker score (girdling) June 07	0.03	nd
RRes Oct 06 LP v. plot canker score (June) internal	0.0315	0.249
RRes Nov '06 WP v. plot canker score (June) girdling	0.0122	0.590
RRes Nov '06 WP v. plot canker score (June) internal	0.0054	0.722
Boxworth Nov/Dec '06 LP v. plot canker score (June)	0.0041	0.844
Boxworth Nov/Dec '06 UP v. plot canker score (June)	0.0976	0.349
Boxworth WP (Nov/Dec) v. plot canker score (June)	0.1053	0.330
RRes WP Nov/Dec 04 v. plot canker score Jun-05	0.0974	0.193
RRes WP Jan 05 v. plot canker score Jun-05	0.0899	0.227
NIAB Dec 04 UP V canker score (June 2005)	0.006	NS
NIAB Dec 04 LP V canker score (June 2005)	0.158	NS
NIAB Jan 05 LP V canker score (June 2005)	0.105	NS
NIAB Lmac DNA LP nov05 V canker score 2006	0.0	NS
NIAB Lmac DNA LP Jan06 V canker score 2006	0.017	NS
NIAB LP Dec06 V canker score June 07	0.015	NS
NIAB LP Jan07 V canker score June 07	0.037	NS

[1] = nd not determined

Table 5b. Regressions of mean amount of *L. maculans* DNA (pg) in petiole tissues per cv against cv canker score (i.e. biological reps per cv pooled to reduce sample variability

Comparison	R^2	P
Boxworth Jan '07 WP mean cv v. mean cv canker score (June)	0.033	0.697
RRes cv mean LP (nov06) v. cv mean canker score (June 07)	0.049	[1]nd
RRes cv mean LP (Nov 06) v. log mean cv canker score (June 07)	0.068	nd

[1] = nd not determined

Table 5c. Regressions of mean amount of *L. maculans* DNA (pg) in petiole tissues of different leaves or at different times, different sites or in different biological replicates of the same cv (i.e. testing consistency of method)

Comparison	R^2	P
RRes Oct 06 LP lowest green leaf v. LP first senescing leaf	0.003	nd
Monsanto Nov 05 LP 7 cvs in 1 replicate v. LP in 2 other replicates	0.5 (r1 v r2); 0.002(R1 v R3)	nd
Monsanto Nov 05 UP of 7 cvs in 1 replicate v. LP in 2 other replicates	0.44 (R1vR2); 0.002 R1 v R3	nd
Boxworth Nov 05 LP of 1 rep of 7 cvs v. in a second rep	0.45	nd
Boxworth Nov 05 UP of one replicate plot of each of 7 cvs v. UP in a second replicate	0.02	
LP of 7 cvs in Nov 05 at Monsanto v. Boxworth	0	
UP of 7 cvs in Nov 05 at Monsanto v. UP Boxworth	0.266	
NIAB Dec 04 LPV Jan 05 LP	0.032	NS
NIAB Nov 05 LPV Jan 06 LP	0.086	NS
NIAB LP Dec06 V Jan 07 LP	0.079	NS
NIAB LP Jan06 V LP Jan 07	0 (if forced to origin)	NS

[1] = nd not determined

Table 5d. Regressions of cumulative amounts[2] of *L. maculans* DNA in petioles per cv against cv RL resistance ratings (both integer and decimal scores)

Comparison	R^2	P
RL canker resistance rating <2007 v. LP *L. maculans* DNA[2]	0.2	**0.05**
Sum of *L maculans* DNA in lower petioles (12 sites X dates) against 2008-9 RL canker rating (decimal)	1.6	0.288

[2] (pathogen DNA per cultivar as a proportion of total pathogen DNA per site, summed over many sites. This does not include two Boxworth datasets, which only had a subset of 7 cvs tested).

Table 5e. Regressions of amount of pathogen DNA in petioles (individually or cumulative over many sites) against pathogen DNA in crowns (section 5).

Comparison	R^2	P
RRes LP Nov 06 v. DNA in crowns in Nov 06 or Feb 07	<0.1	NS
RRes LP v. DNA in crown Jan 2005	<0.1	NS
sum of proportional *Lm* DNA in crowns (12 site x dates) v.sum of proportional *Lm* DNA in lower petioles (12 site x dates)	**0.46**	0.09
sum of proportional *Lm* DNA in crowns (RRES Jan/Feb05,06,07) that in lower petioles (RRES Nov/Dec04,05,06)	0.02	NS

Quantities of *L. maculans* DNA in crowns (stem base) were closely related to severity of canker symptoms recorded on the same plant but frequently, plant to plant variation in both symptom severity and amount of pathogen DNA meant that plot sample sizes should be more than 10 plants per plot. For comparison of pathogen DNA at one time-point (biological source) against canker at a different time-point (i.e. on different plants of the same plot), there was often not a good relationship, particularly for DNA from crowns sampled early in the season (e.g. winter) (Table 6).

Table 6: Regressions of *L.maculans* DNA in stem base material against visual stem canker scores

Comparison	R^2	Probability
RRes Feb '06 Crown v. plot canker score (June)	0.2367	0.029
TN population - crown v. plot canker score May '07	0.8147	<0.001
Rothamsted May '07 5 pooled stems per cv v. plot canker score (girdling) June	0.4524	0.003
RRes May '07 5 pooled stems per cv v. plot canker score (internal) June	0.573	0.002
RRes May '07 5 individual stems per cv v. plot canker score (girdling) June	0.595	0.042
RRes May '07 5 individual stems per cv v. plot canker score (internal) June	0.615	0.038
RRes May '07 5 individual stems per cv *L.maculans* DNA v. plot canker score - Girdling (June)	0.292	<0.001
RRes May '07 5 individual stems per cv *L.maculans* DNA v. plot canker score - Internal (June)	0.2971	<0.001
RRes May '07 *L.maculans* DNA pg in stems v. plot canker score June (girdling)	0.3231	<0.0001
RRes May '07 *L.maculans* DNA pg in stems v. plot canker score June (internal)	0.3052	0.0001
RRes March '07 crown v. plot canker score (June) girdling	0.0602	0.398
RRes March '07 crown v. log plot canker score (June) girdling	0.0852	not done
RRes March '07 crown v. plot canker score (June) internal	0.0704	0.359
ADAS Boxworth Nov/Dec '06 Crowns v. plot canker score (June)	0.0114	0.755
Boxworth Crown *L.maculans* DNA (Nov / Dec '06) v.plot canker score (June)	0.0114	0.755
Boxworth mean cv crown *L.maculans* DNA (Nov / Dec '06) v.mean plot canker score (June)	0.0029	0.919
Boxworth Crown *L.maculans* DNA (Jan '07) v. plot canker score (June)	0.3886	0.017
Boxworth mean cv crown *L.maculans* DNA (Jan '07) v. mean plot canker score (June)	0.7188	0.016
Boxworth crown *L.maculans* DNA (Jan 07) (mean of cv) v.log of cv mean canker score (June)	0.6183	not done
Boxworth Crown *L.maculans* DNA (Feb '07) v. plot canker score (June)	0.0587	0.404
Boxworth Crown *L.maculans* DNA (Feb '07) v. plot canker score (June) (2 points removed)	0.3744	0.034
Boxworth mean cv crown *L.maculans* DNA (Feb '07) v. mean plot canker score (June)	0.176	0.349
L.maculans DNA in crowns of 20 diff cvs (Nov 06) and in crowns from the same plots (Feb 07) (RRes)	0.0239	not done
L. maculans DNA in crowns (Nov 2006) at Rothamsted v. that at Boxworth at the same time	0.92	0.003
L. maculans DNA in crowns (Feb 2007) at RRes v. ADAS	0.02	not done
L. maculans DNA in 5 individual and 5 pooled crowns (22 May 07) at RRes	0.84 (% area)	P<0.001
v. plot canker score in June (as % area and % girdling)	0.73 (%girdling)	P<0.001
RRes (Feb '07) cv mean crown *L.mac* DNA v. cv mean plot canker score – Girdling (June)	0.107	0.172
RRes (Feb '07) cv mean crown *Lm* DNA v. **log** cv mean plot canker score (girdling) in June 07	0.0601	not done
RRes (Feb '07) cv mean crown *L.mac* DNA v. cv mean plot canker score - Internal (June)	0.0977	0.175
RRes (March '07) mean cv crown *L.mac* DNA v. mean cultivar canker score - Girdling (June)	0.0184	0.749
RRes (March '07) mean crown *L.mac* DNA v. mean cultivar canker score - Internal (June)	0.015	0.681
RRes (May '07) mean cv stem *L.mac* DNA v. cv mean plot canker score - Girdling (June)	0.5548	0.054
RRes (May 07) mean cv stem *L.mac* DNA v. cv mean plot canker score - Internal (June)	0.5152	0.069

Amounts of DNA of *L. maculans* in crowns (stem bases) increased gradually throughout the winter but increased greatly from around 900 degree-days (early-mid March) and particularly from flowering onwards (when visible canker symptoms begin to appear) (Figure 2). In the winter, the relative amounts of pathogen DNA in samples of each cultivar (Figure 2 inset box) did not reflect the final levels in June, so early samples were a poor predictor of canker severity. This suggests that factors operating after flowering have the greatest affect on canker development.

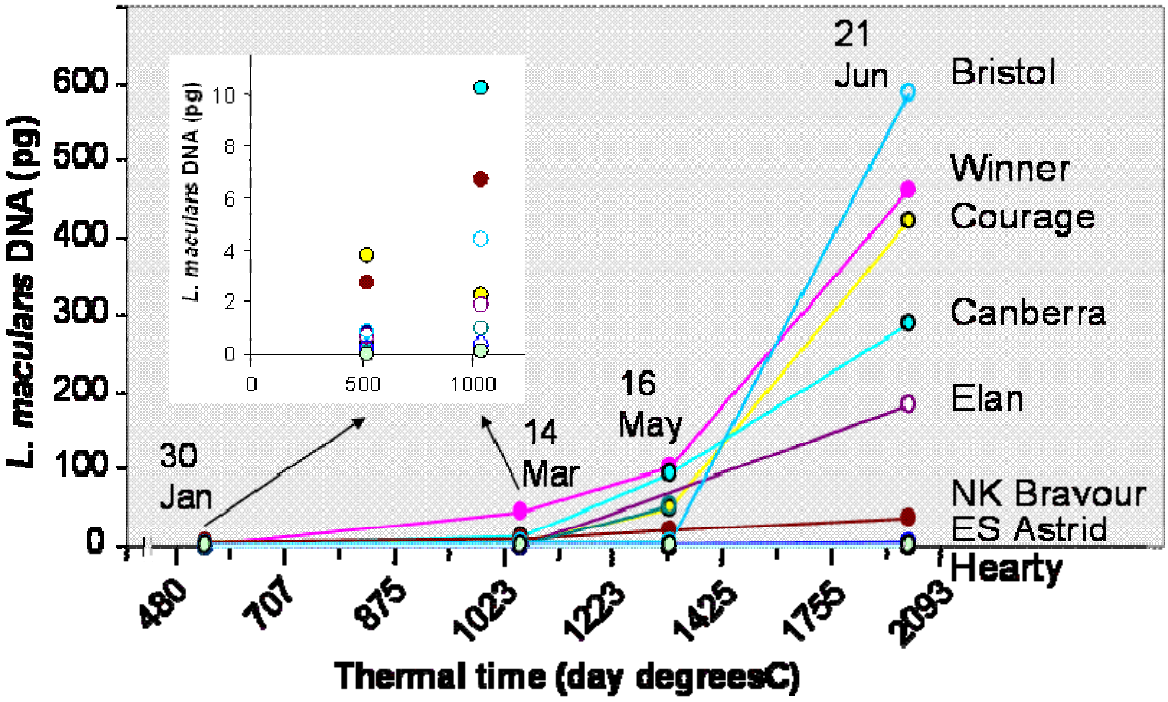

Figure 2. Change in amount of *Leptosphaeria maculans* DNA in crowns at Rothamsted in 2006-7 against thermal time

General trends in *L. maculans* DNA in stems in spring show consistent differences between more susceptible cvs like Bristol and Winner and more resistant cvs like Hearty and ES Astrid (e.g. Figure 3). The cluster of points near to the origin of Figure 3 represent amounts of pathogen DNA in samples collected in February and March.

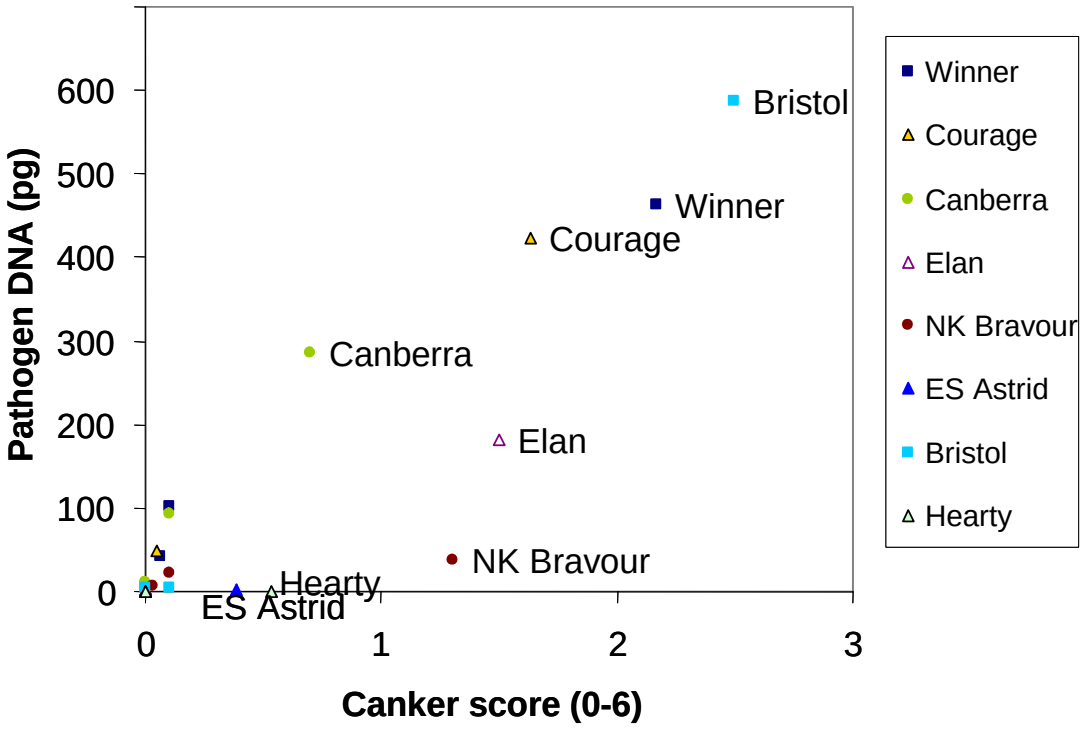

Figure 3. *L.maculans* DNA against canker severity in Feb, March and May 2007 at Rothamsted for 8 different cvs

Table 7 shows that there was no effect of petiole length on the amount of pathogen DNA in petioles nor on the ratio of pathogen DNA in upper to lower petioles.

Table 7. Relationship between amount of *L. maculans* DNA (pg) and petiole length (cm)

RRes Dec '05 DNA whole petiole v. Dec '05 petiole length	R^2 = 0.0475	P=0.356
RRes Feb '06 DNA whole petiole v. Jan '06 petiole length (cm)	R^2 = 0.0366	P=0.419
RRes Feb DNA in whole petiole v. April petiole length	R^2 = 0.0474	P=0.357
RRES Dec 05 petiole length (cm) v. ratio of *L.maculans* DNA upper petiole: whole petiole	0	NS
RRES Dec05 petiole length (cm) v. ratio of *L. maculans* DNA upper petiole: lower petiole (Hearty and ES Astrid omitted as very high DNA ratios)	0	NS

3.2.2 *P. brassicae*

DNA of *P. brassicae* was detected in meristem tissue at all sites (data not shown). Amounts of *P. brassicae* DNA were generally low (defined as < 1pg detected per reaction in >90% of samples tested) at most sites, particularly those sampled early in the season (November to January) (Table 8). However, the goodness of fit of regressions of *P. brassicae* DNA against LLS severity was not related to the total amount of pathogen DNA (i.e. the ability to predict LLS severity from amount of pathogen DNA did not rely on there being large amounts of pathogen DNA) (Table 16). One factor that accounts for the lack of a relationship between pathogen DNA in meristem samples and visible LLS is that occasionally a replicate may be found to have 10 or 100 times more pathogen DNA in the meristem sample than other replicates of the same cv. and this then skews the data point for that cv. This could be due to contamination by spores during sample handling or inclusion of partially expanded leaves in the shoot-tip sample that have much more pathogen DNA present than the unexpanded leaf primordial and meristem (see results from leaf samples below, Figure 4).

The amount of *P. brassicae* DNA in meristem tissue was significantly related to the current and subsequent severity of visual LLS symptoms at Rothamsted in February 2006 and 2007 and at SAC Angus in Jan '06 but not at other sites/dates (Table 9). Most samples were taken early in the season (November-January, particularly in Scotland. Perhaps the early sampling regimes did not give sufficient time to allow pathogen biomass to increase through secondary infection, which limited discrimination between resistant and susceptible cvs. Sampling in late winter and spring appears to be the best time to provide material for qPCR studies to discriminate cultivars for resistance to *P. brassicae*. There was little improvement on the relationship between pathogen DNA in meristems and visible LLS symptoms when individual replicate plot data were pooled per cv (Table 11). e amount of *P. brassicae* DNA in meristem tissue was not significantly related to visual LLS symptoms on stems or pods except for stems at Rothamsted in June 2007 (Table 10).

Table 8. Sites x dates with low amounts of *P.brassicae* DNA detected in meristem samples

2004-5	2005-6	2006-7
RRES Nov 04	CPBT Feb 06	SU Kinross Feb 07
RRES Jan 05	RRES Dec 05	SAC Aberdeen Jan 07
Nickerson Jan 05	Monsanto Feb 06	SAC Angus Jan 07
SAC Aberdeen Dec 04		ADAS Boxworth Jan 07
		RRES Dec 06
		RRES Feb 07

Table 9: Summary of relationships found between amount *P.brassicae* DNA (pg) in meristems v LLS severity (% leaf area affected) at different times (simultaneously or subsequently)

Comparison	R^2 (>0.5)	P value (<0.05)
Rothamsted meristem *P.brassicae* DNA Feb '07 v. 30-Jan '07 % LLS leaf area	0.5281	<0.001
Rothamsted meristem *P.brassicae* DNA Feb '07 v. 14-March '07 % LLS leaf area	0.5761	0.001
SU Kinross meristem (A) *P.brassicae* DNA Dec '06 v. 21-Dec '06 % LLS leaf area	0.0334	0.44
SU Kinross meristem (C) *P.brassicae* DNA Dec '06 v. 21-Dec '06 % LLS leaf area	0.0013	0.879
SU Kinross meristem A Feb '07 v % LLS leaf area (23 Feb 07)	0.0998	0.49
SU Kinross meristem B Feb '07 v % LLS leaf area (23 Feb 07)	0.0002	0.975
SAC Aberdeen Jan '07 meristem *P.brassicae* DNA in v % LLS leaf area (16 Jan)	0.0763	0.033
SAC Angus Jan '07 *P.brassicae* DNA in meristem v. % LLS leaf area (22 Jan)	0.0402	0.457
Boxworth Jan 07 *P.brassicae* DNA in meristem v. % LL S leaf area 3 Apr 07	0.0506	0.439
SAC Angus meristem *P.brassicae* DNA Jan '06 v. % LLS leaf area 14-Apr	0.0938	0.021
Rothamsted Dec 05 *P.brassicae* in meristem v % LLS leaf area (Dec 05)	0.0146	0.362
Rothamsted Dec 05 *P.brassicae* DNA in meristem v % LLS leaf area 4 Apr 06	0.003	0.682
Rothamsted Feb-06 meristem *P.brassicae* DNA v % LLS leaf area 31-Jan-06	0.4327	<0.001
Rothamsted Feb-06 meristem *P.brassicae* DNA v % LLS leaf area 4-Apr-06	0.1062	0.040
Rothamsted Nov 04 meristem *P.brassicae* DNA v % LLS leaf area 24 Nov 04	0.0284	0.298
Rothamsted Nov-04 meristem *P.brassicae* DNA v % LLS leaf area 14-Jan-05	0.0039	0.701
Rothamsted Nov-04 meristem *P.brassicae* DNA v % LLS leaf area 17-Mar-05	0.0351	0.247
Rothamsted Jan-05 meristem *P.brassicae* DNA v % LLS leaf area 24-Nov-04	0.1733	0.007
Rothamsted Jan-05 meristem *P.brassicae* DNA v % LLS leaf area 14-Jan-05	0.0103	0.532
Rothamsted Jan-05 meristem *P.brassicae* DNA v % LLS leaf area 17-Mar-05	0.0043	0.688
SAC Angus Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 24-Nov-04	0.0015	0.772
SAC Angus Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 21-Jan-05	0.0066	0.537
SAC Angus Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 23-Mar-05	0.0223	0.255
SAC Angus Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 11-May-05	0.0002	0.906
SU Kinross Dec-04 meristem *P.brassicae* DNA v % LLS leaf area 18-Dec-04 (assessed 3 days after sampling)	0.1584	0.091
SU Kinross Dec-04 meristem *P.brassicae* DNA v % LLS leaf area 20-Dec-04 (assessed 5 days after sampling)	0.1131	0.159
SU Kinross 3-Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 5-Feb (assessed 2 days after sampling)	0.0026	0.831
SU Kinross 3-Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 7-Feb-05 (assessed 4 days after sampling)	0.0002	0.950
SU Kinross 3-Feb-05 meristem *P.brassicae* DNA v % LLS leaf area 01-Apr-05	0.0003	0.947

Table 10. Summary of relationships found between *P.brassicae* DNA in meristems v LLS severity scored in other ways (general 1-9 plot score, or on stems or pods)

Comparison	R^2 (>0.5)	P value (<0.05)
SU Kinross Feb 06 meristem *P.brassicae* DNA v Feb plot LLS severity	0.013	0.484
SU Kinross Feb 06 meristem *P.brassicae* DNA v Mar plot LLS severity	0.0157	0.441
Rothamsted meristem *P.brassicae* DNA Feb '07 v % stem area LLS 21-July'07	0.2567	0.022
Nickerson Jan-05 meristem *P.brassicae* DNA v % stem area LLS 11-Jul	0.0142	0.365
Rothamsted meristem *P.brassicae* DNA Feb'07 v % pod incidence LLS Jun '07	0.1052	0.163

Table 11. *P.brassicae* cv mean DNA in meristem v. mean cv % LLS leaf area (i.e. individual replicate plot data pooled per cv)

Comparison	R^2	P value (<0.05)
SSU Kinross mean meristem cv *P.brassicae* DNA (pg) Dec '06 v. mean cv % LLS leaf area 21-Dec '06	0.0145	0.613
SU Kinross mean meristem cv *P.brassicae* DNA (pg) Dec '06 v. mean cv % LLS leaf area (26-Apr '07)	0.1164	0.141
SAC Aberdeen Jan '07 mean cv meristem *P.brassicae* DNA (pg) v. mean % LLS leaf area (Jan)	0.0846	0.213
SAC Angus Jan '07 mean cv meristem *P.brassicae* DNA (pg) v. mean % LLS leaf area (Jan)	0.0768	0.506
Boxworth Jan 07 mean cv meristem *P.brassicae* DNA v. mean % LLS leaf area (3-Apr 07)	0.2557	0.247
SAC Angus Jan 06 mean cv meristem *P.brassicae* DNA v. mean cv % LLS leaf area 14-Apr 06	0.2055	0.045
Rothamsted Nov 04 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 24-Nov-04	0.0728	0.249
Rothamsted Nov 04 cv mean meristem *P.brassicae* DNA v. % mean cv LLS leaf area 14-Jan-05	0.0623	0.288
Rothamsted Nov 04 cv mean meristem *P.brassicae* DNA v. % mean cv LLS leaf area 17-Mar-05	0.0088	0.694
Rothamsted Dec 05 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf are 4-Apr 06	0.0212	0.539
Rothamsted Jan-05 cv mean meristem *P.brassicae* DNA v.mean cv % LLS leaf area 24-Nov-04	0.1358	0.109
Rothamsted Jan-05 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 14-Jan-05	0.0007	0.911
Rothamsted Jan-05 cv mean meristem *P.brassicae* DNA v.mean cv % LLS leaf area 17-Mar-05	0.0406	0.394
Rothamsted Feb 06 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 31-Jan-06	0.3865	0.003
Rothamsted Feb 06 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 4-Apr-06	0.009	0.689
SAC Angus Feb-05 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 24-Nov-04	0.0117	0.650
SAC Angus Feb-05 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 21-Jan-05	0.0002	0.948
SAC Angus Feb-05 cv mean meristem *P.brassicae* DNA v. mean cv % LLS leaf area 23-Mar-05	0.0109	0.661
SAC Angus Feb-05 cv mean meristem *P.brassicae* DNA v. mean cv% LLS leaf area 11-May-05	0.0007	0.913

Amounts of *P. brassicae* DNA detected in newly expanded leaves were always much higher than that detected in shoot tips (meristems) (e.g. Figure 4) and was usually significantly related to the amount in meristems (Table 12). The amount of *P. brassicae* DNA in leaf tissue was significantly related to the current and subsequent severity of visual LLS symptoms at two out of five sites/times investigated in the 2006-7 season (Table 13). For site x dates where this relationship was not significant, pooling of data per cv still did not improve the relationship (Table 14).

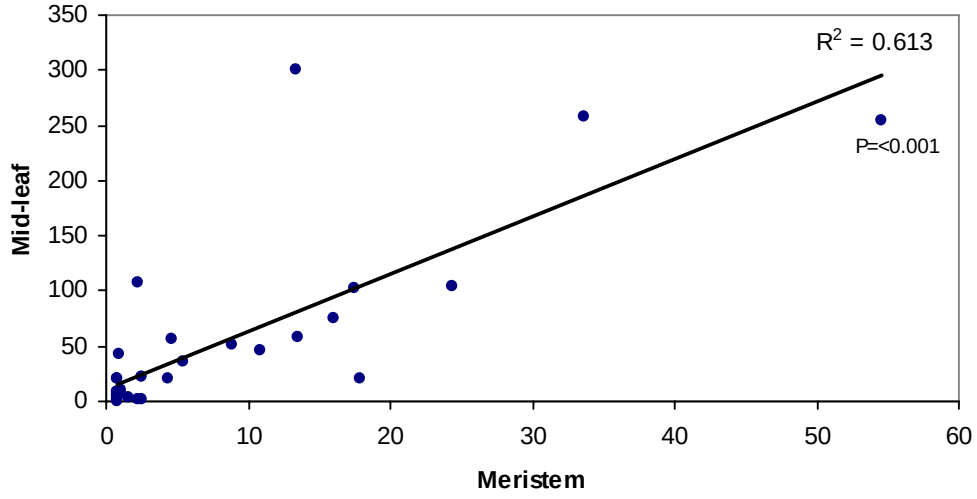

Figure 4. Example of the relative amounts and relationship between amounts of *P. brassicae* DNA found in meristem and mid-canopy leaf tissues of the same plot samples (Rothamsted Feb 2007)

Table 12. Relationship between amounts of *P. brassicae* DNA measured in leaves and meristems at the same sample site and date

Comparison	R^2 (>0.5)	P value (<0.05)
Rothamsted Feb '07 meristem v mid-leaf P.brassicae DNA	0.613	P=<0.001
Rothamsted Feb '07 meristem v leaf P.brassicae DNA (cv average)	0.6844	P=<0.001
SU Kinross Dec '06 meristem v leaves P.brassicae DNA (average of rep A & B)	0.3159	P=0.009
SU Kinross Feb '07 meristem v leaf P.brassicae DNA rep A	0.4709	P=0.088
SU Kinross Feb '07 meristem v leaf P.brassicae DNA rep B	0.7477	P=0.012
SAC Aberdeen Jan '07 meristem v leaf P.brassicae DNA	0.0781	P=0.03
SAC Aberdeen Jan '07 meristem v leaves P.brassicae DNA (cv average)	0.2558	P=0.023
SAC Angus Jan '07 meristem v leaf P.brassicae DNA	0.0633	P=0.347
SAC Angus Jan '07 meristem v leaf P.brassicae DNA (cv average)	0.0288	P=0.687
Rothamsted Nov/Dec '04 meristem v leaf P.brassicae DNA	0.1375	P=0.366

Table 13. Summary of regressions of *P.brassicae* DNA in mid-leaves against LLS severity (% leaf area affected)

Comparison	R^2 (>0.5)	P value (<0.05)
Rothamsted mid-leaf *P.brassicae* DNA (pg) Feb '07 v. 30-Jan '07 % LLS leaf area	0.5912	<0.001
Rothamsted mid-leaf *P.brassicae* DNA (pg) Feb '07 v. 14-March '07 % LLS leaf area (only data of 14 cv for %LLS)	0.7908	<0.001
SU Kinross leaves (A) *P.brassicae* DNA (pg) Dec '06 v. 21-Dec '06 % LLS leaf area	0.06	0.783
SU Kinross leaves (C) *P.brassicae* DNA (pg) Dec '06 v. 21-Dec '06 % LLS leaf area	0.0524	0.332
SU Kinross leaves A Feb '07 v. % LL S leaf area (23 Feb 07)	0.0065	0.864
SU Kinross leaves B Feb '07 v. % LL S leaf area (23 Feb 07)	0.00005	0.988
SAC Aberdeen Jan '07 *P.brassicae* DNA in leaves v. % LLS leaf area (16 Jan)	0.1543	0.001
SAC Angus Jan '07 *P.brassicae* DNA in leaves v. % LLS leaf area (22 Jan)	0.00005	0.988

Table 14. Summary of regressions of *P. brassicae* cv mean DNA in mid-canopy leaves against mean cv LLS severity (i.e. individual replicate plot data pooled per cv)

SU Kinross mean leaf cv *P.brassicae* DNA (pg) Dec '06 v. mean cv % LLS leaf area 21-Dec '06	R^2 =00023	0.841
SU Kinross mean leaf cv *P.brassicae* DNA (pg) Dec '06 v. mean cv % LLS leaf area (26-Apr ' 07)	R^2= 0.0098	0.677
SAC Angus Jan '07 mean cv leaf *P.brassicae* DNA (pg) v. mean cv % LLS leaf area (Jan)	R^2 = 0.2959	0.163
SAC Aberdeen Jan '07 mean cv leaf *P.brassicae* DNA (pg) v. mean % LLS area (June)	R^2 = 0.4663	P=0.0009

Table 15. *P. brassicae* DNA in mid-canopy leaves v pod LLS severity

Rothamsted Feb '07 mean cv *P.brassicae* DNA (mid-leaf) v. mean % pod LLS incidence (June)	R^2 = 0.098	P=0.179

Table 16. Test of relationship between the goodness of fit for meristem *P.brassicae* DNA-visible LLS comparisons and the amount of pathogen DNA present

Mid-point *P.brassicae* DNA v. R^2 value	R^2 = 0.0655	P=0.250
Average *P.brassicae* DNA v. R^2 value	R^2 = 0.0207	P=0.523

Table 17: Tests of consistency of method at different sites or dates

SU Kinross P brassicae DNA in meristems Dec 06 v. Feb 07 *P brassicae* DNA in meristems	R^2= 0.25	Not done
SU Kinross *P brassicae* DNA in meristems Dec 06 v. Feb 07 *P brassicae* DNA in leaves	R^2= 0.62	0.035
SU Kinross *P brassicae* DNA (pg) in meristems sampled in Feb06 v. Dec 05 from the same plots	R^2 = 0.0002	NS
RRES *Pb* DNA in meristems in Dec 05 v. Feb 06 based on cultivar means (means of replicate plots)	R^2 = 0	NS
P. brassicae DNA in meristems (average per cv of all reps) at SU Kinross in Dec 2006 v. Feb 2007	0	Not done (flat line)

The total amount of *P. brassicae* DNA in leaves sampled from the five sites x dates sampled in 2007 was expressed per cv as a proportion of the total *P. brassicae* DNA in the samples at each site and accumulated, then the result compared against the RL cv LLS resistance rating (Figure 5) to give a relationship with R^2 = 0.302; *P*=0.01.

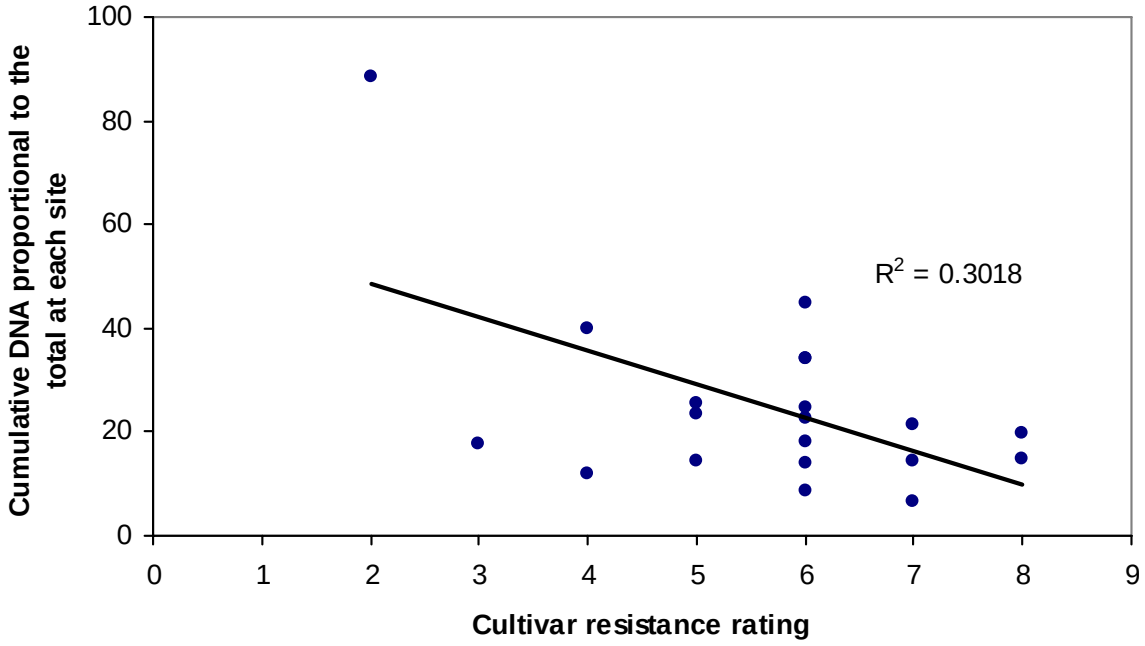

Figure 5. Cumulative proportional *P. brassicae* DNA in leaves of oilseed rape sampled from five sites in January or February 2007 against HGCA CEL recommended list cultivar resistance rating (2006-7) (R^2 = 0.302; *P*=0.01).

The same process, done for the cumulative total *P. brassicae* DNA in meristems, from 8 sites Jan / Feb V RL rating 2006/07 (totals proportional for each site based on total Pb DNA per site) gave no clear relationship (R^2 = 0.026). Therefore the pathogen DNA quantified in leaves gives a better indication of cultivar resistance than pathogen DNA in meristems. However, for comparison, the visible LLS data for the same sites, dates and cvs (when only a subset were tested) was also expressed as a proportion of the sum of

LLS severity on all the cvs assessed at the site x time and the average proportion per cv compared against the 2006-7 RL resistance rating (Figure 6), which gave a good relationship (R^2 = 0.757; P<0.0001). That is to be expected as the RL rating is calculated in a similar way but for more site x dates. Unfortunately comparisons with yield are not possible as no yield data were collected in 2007 from sites that had LLS but no canker.

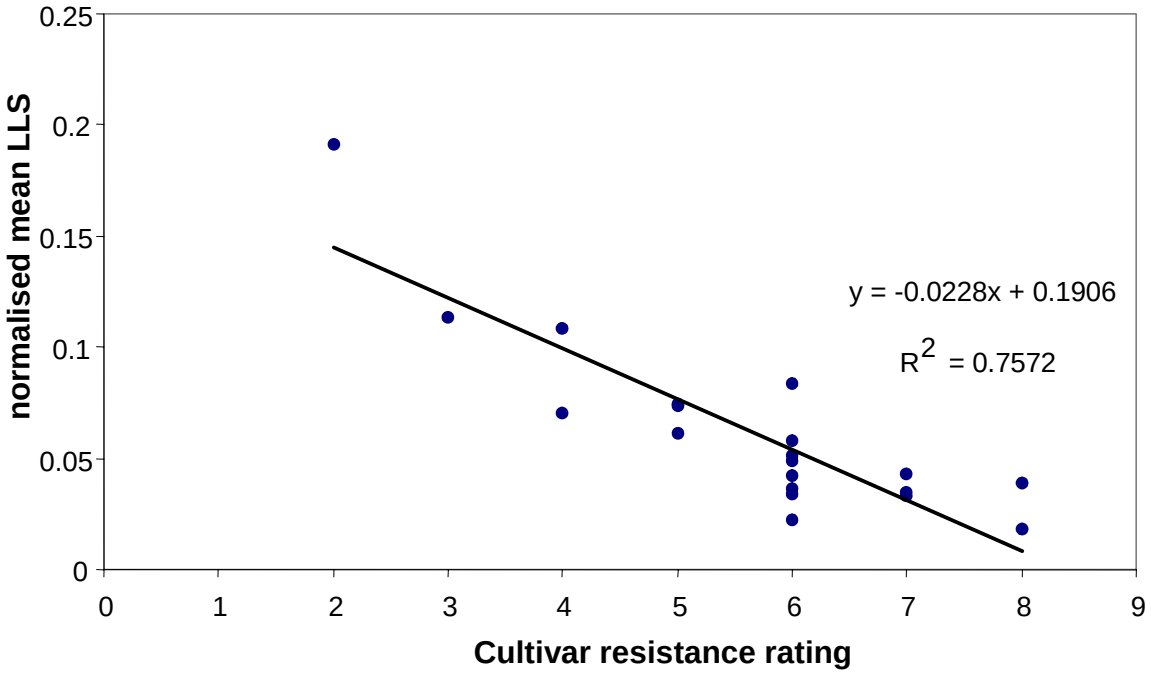

Figure 6. Cumulative proportional mean severity of LLS at the same five site x dates as the DNA data (Figure 5) in 2007 against HGCA RL cv resistance rating (2006-7). (R^2 = 0.757; P<0.0001).

Chapter 4. Controlled environment experiments to assess quantitative resistance to *L. maculans*

4.1 Methods

A sub-set of six CORDISOR cultivars, together with 30-40 commercial Chinese oilseed rape cvs, were tested using two methods in controlled environments (i: seedling study) point inoculation of seedlings with *L. maculans* ascospore suspension followed by assessment of visual symptoms at two times (9 and 20 days post inoculation) and measurement of pathogen DNA by qPCR; (ii: cotyledon study) point inoculation of wounded cotyledons using a conidial suspension of each of six different *L. maculans* isolates, followed by assessment of visual symptoms after a period of incubation. For the seedling study (i), the plant material used was 18 Chinese and 5 UK (CORDISOR) cvs, inoculated with small (20 µl) drop of asospores (2 x 10^4 /ml) at the same positions of leaf 1 & 2. These were incubated at 100% RH, 20°C day/ 18 °C night. Visible symptoms were assessed and tissue samples were taken 9 d.p.i (29 Nov 07) and 20 d.p.i (10 Dec 07). A plant of cv 16, which was inoculated but not incubated under high RH was used as the control (pathogen DNA representing the level present in the inoculum). A total of 6 plants per cv was used, 3 plants sampled on 29 Nov & 3 on 10 Dec by taking pieces of leaf 2 cm x 2 cm, initially around the inoculation point and contiguously every 2 cm along the leaf and petiole towards the stem. Pieces from the same leaf position (leaf 1 or leaf 2) were pooled for the three pseudo-reps for processing for a single pathogen DNA measurement.

For the cotyledon study (ii) nine plants each of 33 Chinese and six UK cvs (UK1:Courage, UK2:Hearty, UK3: Winner, UK4: Elan, UK5: Bristol, UK6: NK Bravour) were used, having been sown on 16 Nov 07. Each plant was tested with four isolates out of six used (isolates: A- 06 N37, B- 06 N83, C- 06 N92, D- 06 R54, E- 06 R56, F- 06 R74) by inoculating one isolate per half of a cotyledon. Inoculations were made by pricking the cotyledon position with a sterile needle, followed within 1 minute by addition of a droplet of inocuulm suspension (15 µl drop of 2 x 10^7 spores/ml). The 20 tested cvs were arranged randomly in blocks with 3 plants of the same cv per row, 5 rows per tray. Disease severity was assessed visually on 11 Dec 07 using a 0-6 score (0 = no symptoms, 1-3 = resistant - necrosis with margins of increasing sized lesion (fungus apparently contained), 4-6 = susceptible - no margin, fungus spreading with increasing sized lesions).

The data were analysed by Ordinal logistic regression. This model treats the score measure as a factor which says that for a given cultivar and isolate combination, there

will be an expected number in a given category for a particular set of samples. The paucity of responses 1 to 3 made it sensible to combine them with zero score category. In this analysis, what is formed is a contingency table for each cultivar/isolate combination and set of scores. Because the scores are essentially a categorization for each leaf lobe, this means that analyses that rely on the assumption of Normality of the observations, additivity of treatment effects on this scale and constant variance are not appropriate. The analysis assumes that all measurements are independent of each other which is clearly not the case since leaf lobes on a plant are likely to be more similar than those on different plants. Results are presented as coefficients of effects relative to a reference level, in this case cultivar No.1 and isolate A. Changing the reference level changes the actual values of the effects but not the ordering of cultivars.

4.2 Results

Amounts of pathogen DNA in 2cm x 2cm leaf tissue sections at the inoculation point increased from that measured 9 days post inoculation (data not shown) to that at 20 d.p.i. (Figure 7). The ranking of cultivars in terms of amount of pathogen growth changed slightly between the two measurement dates. It is thought that the measurement at 20 d.p.i. reflects host quantitative resistance more than at 9 d.p.i as there was more time for host factors to affect the pathogen's growth.

The UK varieties were generally more resistant apart from Winner and Bristol, which the field work showed to be the more susceptible of UK varieties tested. Interestingly some Chinese lines were reasonably resistant and may form the basis of future breeding programmes to make Chinese cvs more resistant to stem canker. However, most Chinese cvs were very susceptible compared to cvs Winner and Bristol (Figure 7). This is not surprising as the pathogen is not present in China so the cultivars have not been selected for resistance to this pathogen.

A comparison of pathogen growth (DNA) on two different leaves (leaf 1 and leaf 2) inoculated at the same time (leaf 1 a few days older than leaf 2) showed consistently an increase in pathogen growth on leaf 2. Differences were proportionally large and occurred on every cv tested. This was not an artefact of the qPCR method as samples from both leaves were tested in the same qPCR runs in a random order. The reason may be due to different nutrients present in the leaves. If this finding pertained to pathogen DNA amounts in the petiole then sampling slightly different leaves from field plants may produce considerable noise in terms of the amount of pathogen DNA measured in the CORDISOR experiments.

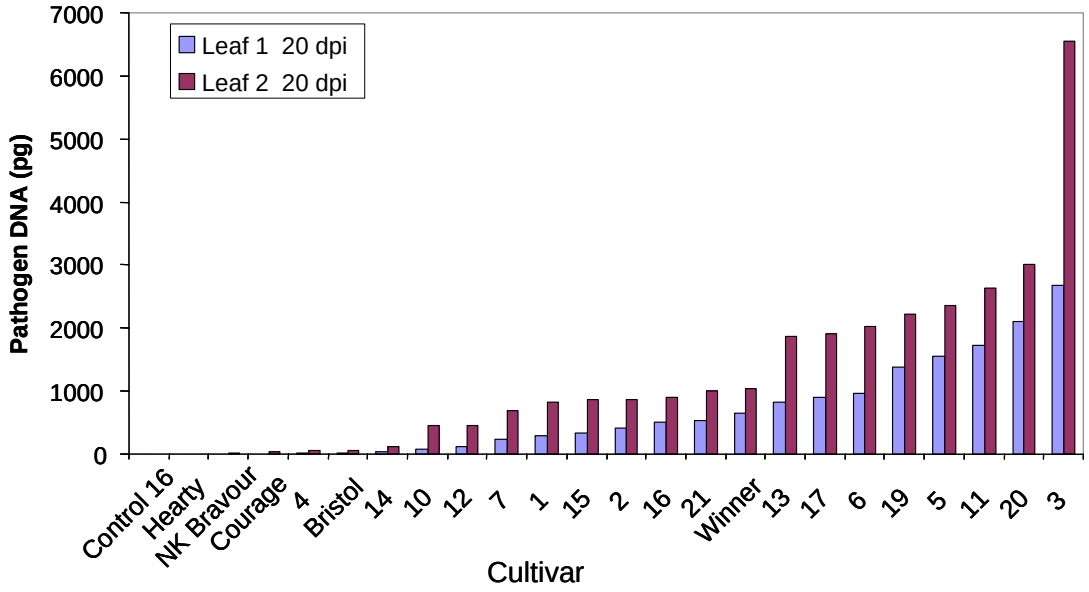

Figure 7. *L. maculans* growth (DNA) on leaf 1 and leaf 2 at inoculation point

Results of the cotyledon experiment largely supported the findings of the ascospore inoculated leaf assay. The symptom severities allowed the plants to be divided into five statistically different groups with all the UK cvs together as the most resistant cvs (Figure 8).

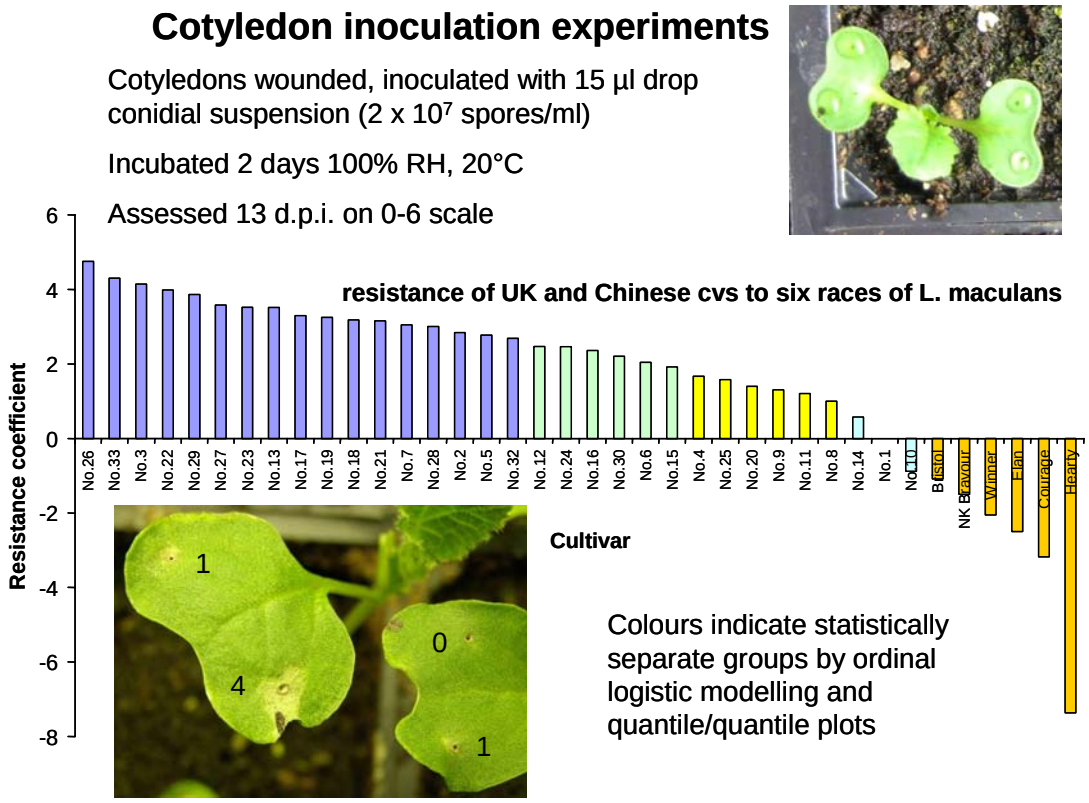

Figure 8. Groupings of Chinese and UK cvs tested by cotyledon inoculations

4.3 Discussion

Both methods demonstrated large differences in symptom development or pathogen DNA between the cvs, with the CORDISOR (i.e. European cvs) predominantly the most resistant to infection and many of the Chinese lines shown to be very susceptible (i.e. have much more pathogen DNA present). In the seedling assay, leaf 2 produced consistently more pathogen DNA than leaf 1, when inoculated with *L. maculans* ascospores at the same time (i.e. leaf 1 slightly older). Within the UK cvs, the most resistant was shown to be cv Hearty by both methods. This was the only cv known to have the resistance gene *RLm7*, which explains why it was expected to be the most resistant. However, differences in pathogen growth in cvs that became infected are thought to be due to quantitative resistance and appear to relate to the size of leaf lesion (e.g. Figure 9).

Figure 9. Examples of *L. maculans* lesions (phoma leaf spot) on cvs 6, 21, 5, Hearty, Courage and 12 (clockwise from top left).

Chapter 5. Canker-yield loss relationships

5.1 Methods

Yield loss due to stem canker was investigated by expressing the yield of each cultivar grown in untreated (diseased) plots as a percentage of the yield in fungicide-treated (relatively healthy) plots. The percentage yield loss of cultivars was compared against different measurements of canker severity (a) mean canker score of the plot before harvest (usually assessed in mid-late June), (b) HGCA canker index, (c) 'G2' canker index. The latter two indices take into account the number of plants in each class of the 0-6 canker severity scale used to assess this disease. However the 'G2' method progressively weights the canker severity as the severity increases. The equations are based on canker data collected using the following scale:

0	no symptoms observable
1	<25% girdling of the stem
2	26-50% girdling
3	51 -75% girdling
4	76 -100% girdling
5	100% girdling + stem weakness
6	100% girdling + stem death

The HGCA canker index is calculated as:

$$\frac{(0a + 1b + 2c + 3d + 4e + 5f + 6g)}{(a+b+c+d+e+f+g)} \times \frac{100}{6}$$

where a, b, c, d, e, f, g are the number of plants in each disease category

The 'G2' index is calculated as:

$$\frac{(0a + 1b + 3c + 5d + 7e + 9f)}{(a+b+c+d+e+f)}$$

where a-f are the numbers of plants in each disease category (0-5 scale in which category f (stem 100% girdled) comprises f and g in the HGCA 0-6 score; 0, 1,3, 5 ,7 and 9 are coefficients).

5.2 Results

At Rothamsted in both 2005, 2006 and 2007, yield was reduced only if the mean canker severity was >2.5 (over half stem cankered) by mid-June, with greater yield loss as mean canker severity increased further (Table 18; e.g. Fig 10, in which the data point for LLS-affected cv. Hearty causes the best regression line to appear to decline again as canker severity reduced). Canker severity was <2.5 in 2005 so there was no significant yield loss at Rothamsted. In 2006 and 2007, a 'broken-stick' approach showed that regressed yield as a percentage of the fungicide-treated yield per cv (y) against mean canker score (x) (only for cvs with canker severity >2.5) gave significant linear regressions (2006: R^2 = 0.35, P=0.02: y=-14.1x + 120; 2007 R^2 = 0.74, P=0.0007: y=-15.9x + 126, Figure 11). Expression of Rothamsted canker severity and incidence as the combined G2 index (Figure 12) improved the goodness of fit slightly for regressions of all points, including those for low disease severities, compared to regressions of mean canker score against % yield but produced a lower goodness of fit with either linear or polynomial functions applied than the linear broken-stick approach. Generally, yield was reduced when the G2 index was >4 was at Rothamsted (2006: R^2 = 0.44, P=0.013: y=-10.4x + 128.4; 2007: R^2 = 0.87, P=0.00072: y=-13.3x + 144, Figure 13), indicating that the G2 index is not directly related to yield loss as the relationship at Rothamsted in 2007 was not linear. In contrast to these results from Rothamsted, there was no effect of canker severity on yield at ADAS Boxworth in 2006 or 2007 (Figure 14) with no yield response in fungicide-treated plots. This was partially because canker severity was moderately severe even in the fungicide-treated plots, but nevertheless some cvs had mean canker scores of 3.7 (cv. Ontario) and 3.8 (cv. Winner) in 2007 in untreated plots (or G2 indices up to 6).

Put simply, the mean of 2006 and 2007 Rothamsted data indicate that yield reduces by 15% for each unit of canker severity (0-6 scale) but only once a severity of 2.5 is exceeded by mid-June.

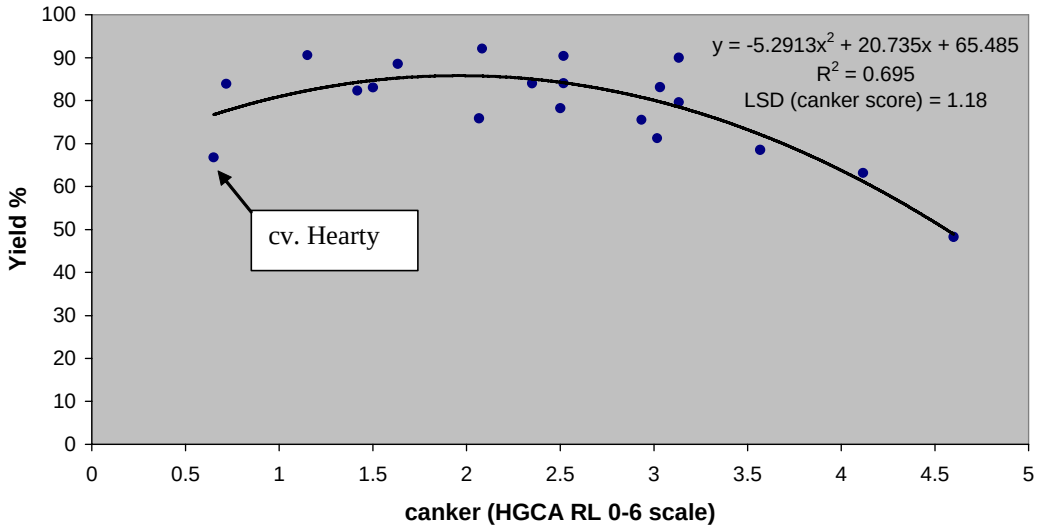

Figure 10. Untreated OSR yields at Rothamsted 2007 as a % of treated-plot yield in relation to mean canker severity per cv in mid-June

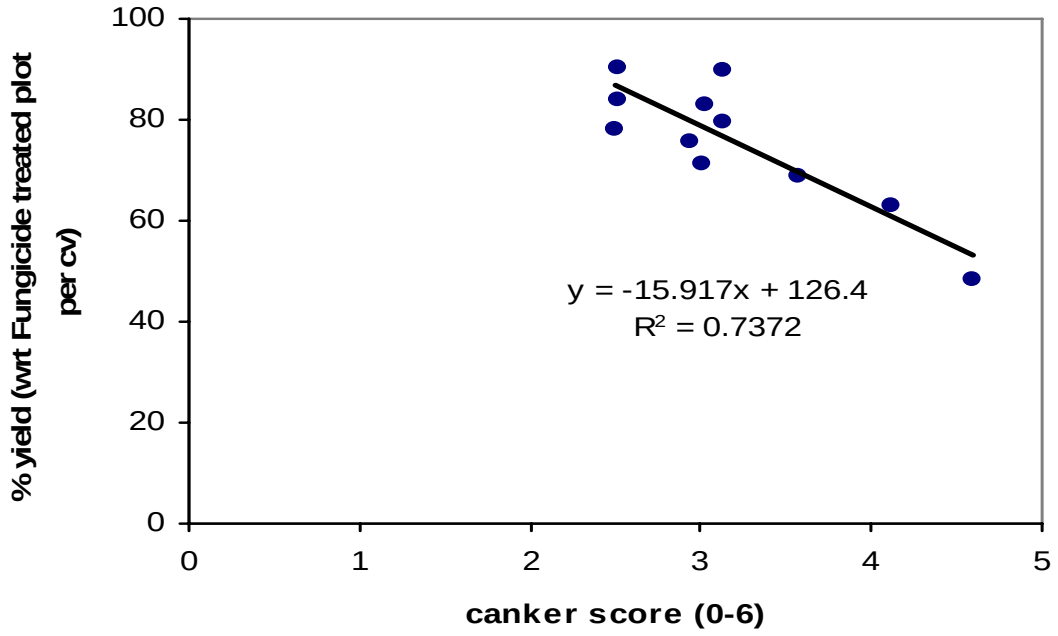

Figure 11. Regression of % yield against mean canker severity (only if over 2.5) at Rothamsted in 2007

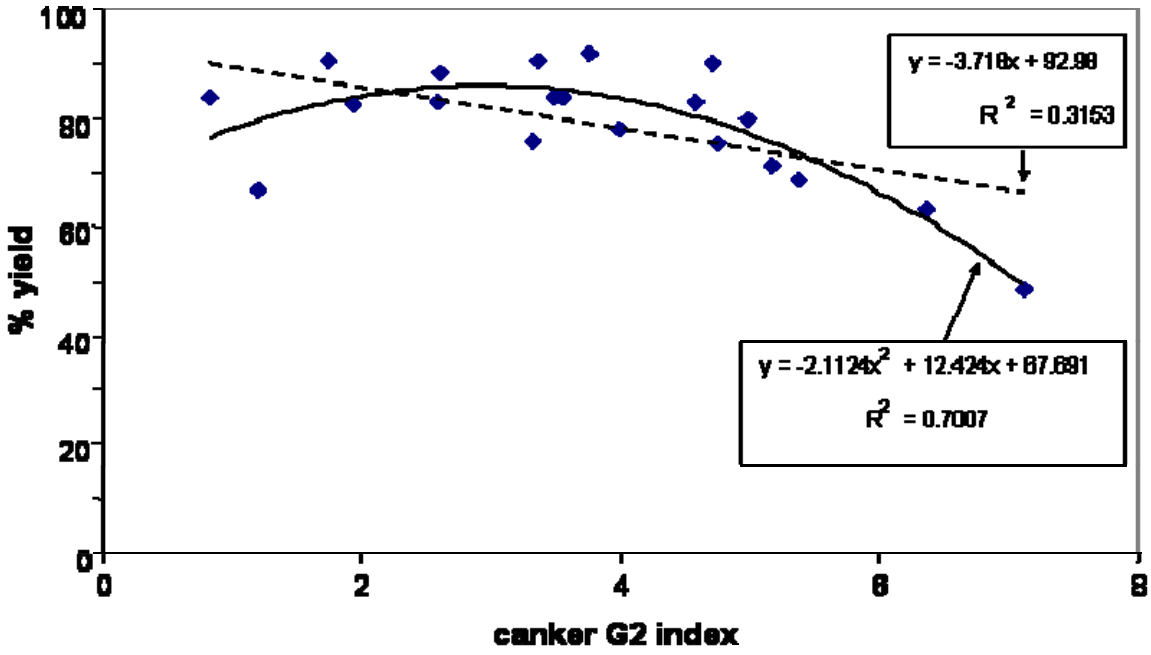

Figure 12. Rothamsted canker G2 index June 2007 data against yield as a percentage of fungicide-treated plots. Both linear (dashed line) and polynomial functions have been fitted.

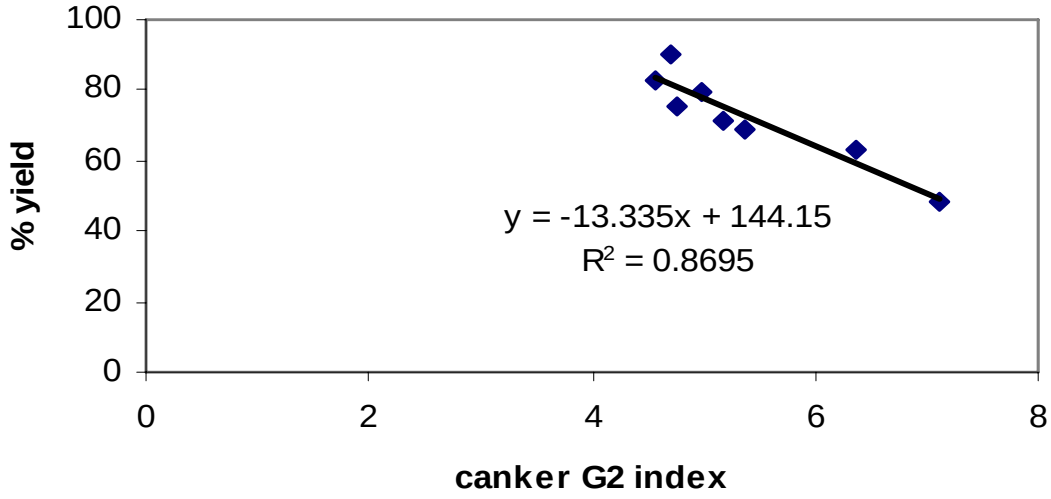

Figure 13. RRES % yield in 2007 compard to G2 canker index if over 4

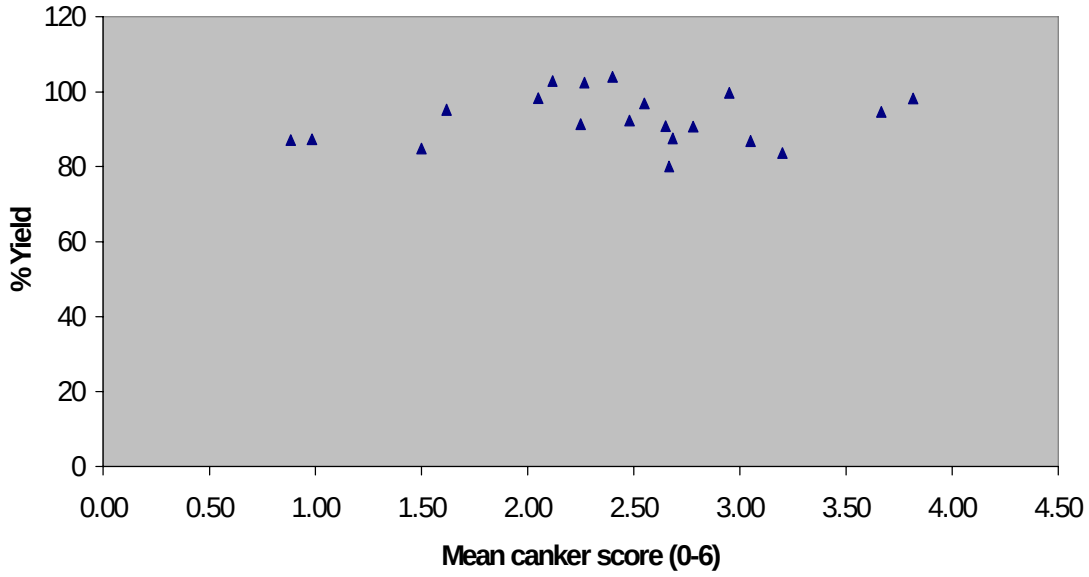

Figure 14. ADAS Untreated yield as a % of treated yield and canker severity in untreated plots 2007

Table18. Summary of canker yield loss investigation

Site	Comparison	R^2	P
RRes 2005	Canker mean score v. % yield	0	NS
RRes 2006	Canker mean score v.% yield	0.285	<0.015
RRes 2006	Canker if over 2.5 v. % yield	0.3492	0.026
RRes 2006	G2 v.% yield (linear)	0.257	0.022
RRes 2006	G2 if over 4 v % yield	0.444	0.013
RRes 2007	Canker mean score v.% yield	0.29	0.14
RRes 2007	Canker if over 2.5 v.% yield	0.737	0.0007
RRes 2007	G2 v.% yield (linear)	0.31	0.01
RRes 2007	G2 if over 4 v % yield (linear)	0.87	0.0007
Boxworth 2006	Canker mean score v. % yield	0.035	NS
Boxworth 2007	HGCA Canker index v. % yield	0.01	NS
Boxworth 2007	G2 v. % yield	0.02	NS

Chapter 6. Implications and knowledge transfer

6.1 General discussion

Canker

For measurement of *L. maculans* DNA in leaf petioles, there was a great deal of variation between plants and between different replicate plots of the same cv (sample error). This could be due to differences in the leaf position sampled (although this was standardised as much as possible), the severity of phoma leaf spots (hence amount of mycelium growing towards the stem) the location of phoma leaf spots on the leaf, or leaf size. The correct time to sample is also difficult to estimate, as for plants from sites where the sowing date, microclimate and nutrient regime resulted in very large and vigorous plants, the optimal sampling time appears to be several weeks after a particular leaf position was infected, at which time the leaf may be starting to senesce. By contrast, on small plants, sampling the lowest green leaf in the canopy (if bearing a phoma leaf spot) appears to provide better data. There was also a lot of variation, possibly caused by the small amount of fungal DNA in hyphae growing along vascular tissue being obscured by the large amount of pathogen DNA arising from mycelium growing more slowly as it colonises the parenchyma of the petiole.

However, in several cases there was more pathogen DNA present in the lower petiole than in the upper petiole, which suggests that there may be translocation of nuclei along hyphal tubes to the hyphal front; this requires further investigation in controlled conditions. Such further work might use other methods such as the reporter genes or PCR-based detection of pathogen in different smaller sections of petioles appear to offer better ways to understand the growth of this pathogen. The original hypothesis for *L. maculans* was based on the idea that pathogen growth along the petiole was affected by quantitative resistance. Quantitative-PCR to measure pathogen growth at earlier stages in the leaf is possible – the project did not investigate this using field-collected material but interesting results were found from inoculated leaves in controlled environment studies at a late stage of the project. Results of this method (pathogen growth in leaves in CE conditions) need to be corroborated in future work against canker severity on the same cvs grown in field conditions. Although all of the cultivars tested were found to have phoma leaf spot symptoms in the field, with relatively small differences in the timing of the onset and in the incidence or severity of symptoms, the qPCR results for *L. maculans* suggest that other factors than the rate of pathogen growth in the petiole may influence final disease (canker) severity. Only cvs

Hearty and ES Astrid had significantly reduced severities of phoma leaf spot at most sites and had generally low amounts of pathogen DNA present. Not surprisingly, leaves sampled from fungicide-treated plots also produced small amounts of pathogen DNA.

In stems (crowns), there was a small amount of DNA of *L. maculans* from November through the winter and a rapid increase in pathogen DNA in stems from the onset of flowering (when visible cankers tend to first appear). Although there were occasionally good relationships between pathogen DNA in crowns collected in the winter and final canker severity, the method was not consistently accurate for all sites until used on crowns collected in May or June when there were relatively large amounts of pathogen DNA, which related very well to canker severity. The best relationships between pathogen DNA and canker severity were produced when the same stems were scored for canker as were sampled for DNA, due to the elimination of one source of sampling error. The increase in pathogen DNA in stems in spring therefore appears to mirror an increase in canker severity. Over the three seasons, this was shown consistently at Rothamsted to occur at a fast rate for cvs Bristol, Winner, Royal and Ontario and at a slow rate for ES Astrid, Hearty, Expert, Canberra and Castille, which may be an effect of quantitative resistance.

DNA of *L. maculans* was not routinely tested in leaf lamellae from CORDISOR field experiments. This decision, made at the start of the project, was based on findings from a preliminary study (Defra CSG 15 report, 2004), which suggested that products inhibitory to qPCR were produced in infected leaf lamellae particularly in senescing leaves but not in petioles, which were generally less 'green' and therefore had lower concentrations of chlorophyll and other pigments. However qPCR data were produced successfully from lamellae of field-collected green leaves tested for *P. brassicae* (section 3.2) and from green field-collected leaf lamellae sampled occasionally for *L. maculans* (data not shown) and from infected leaf lamellae tested from CE-grown plants (section 4). Amounts of *L. maculans* DNA in petioles were thought to be the product of the amount of pathogen growth in the leaf and a measure of the pathogen's spread to the stem, which is an important stage in the epidemiology of the disease. However, amounts of pathogen DNA in petioles were often very low compared to that measured directly around inoculated positions in the CE experiments. Assuming the amount of pathogen DNA in leaves is related to pathogen growth and indirectly the inverse of cultivar resistance, there is therefore, potential for CE experiments to provide a better discrimination of cultivar resistance to 'field' inoculum than obtained from petioles or leaves of field-grown plants. Further work is required to test whether data of leaf lesion size or pathogen DNA in a number of UK cvs, inoculated under controlled conditions, is well related to canker severity in field-grown plants and the UK RL resistance ratings.

Canker did not affect yield at all sites or dates. It appears that mean canker severity must exceed 2.5 (over half stem-base cankered) on the HGCA 0-6 scale by late-June before any significant yield losses occur. This is based only on data at Rothamsted (Hertfordshire) and it is possible that a high evaporation potential caused by hot and windy weather could exacerbate the impact of canker severity on yield loss so that plants in a cool climate would tolerate moderately severe stem canker without yield loss, while crops with the same symptoms but in warmer conditions would go into early senescence and suffer reduced yield. There are therefore implications for future climate change in the effect of canker on yield in addition to the increased thermal time to promote disease development in the first place.

Light leaf spot

There was no significant relationship between *P. brassicae* DNA at the meristem of cvs and RL resistance ratings for light leaf spot when plants were sampled early in the winter (December-January) but a better relationship resulted when plants were sampled later in the vegetative stage of growth (Feb-March). The same was true of the relationship pathogen DNA at the meristem and the individual plot LLS severities at the same time or subsequent times after sampling. *P. brassicae* DNA was found in greater amounts from lamellae of ten mid-canopy leaves sampled per plot than from plant apices (meristems) and this was often well related to visual LLS symptoms but leaves were only sampled at a few sites in the final season (2007). It is thought that the later sampling dates for *P. brassicae* may improve discrimination between cultivars by allowing time for initial infections on leaves (caused randomly by wind-dispersed ascospores) to sporulate and infect the plant meristem, therefore amplifying differences in disease development due to the level of host resistance/susceptibility. As expected, visible LLS data normalised according to the sum of LLS severity on all cvs per site and cumulated over many sites was very well related to the HGCA RL resistance rating.

Other diseases

There was very little alternaria leaf and pod spot, powdery mildew, downy mildew and sclerotinia stem rot in most seasons. Sclerotinia stem rot (SSR) was relatively severe in England in 2007 despite a very dry April. There was no difference in incidence of SSR between cultivars at Rothamsted. However, there was a significant difference between plots that were fungicide-treated in late-March (intentionally against LLS, which produced SSR incidence 2%) and untreated plots (SSR incidence 5.5%, SED 1.04, df 78) indicating that fungicide (boscalid) present on surfaces of leaves still had some activity several weeks later when petal fall would have favoured the pathogen. Clubroot caused

the abandonment of a trial in Scotland and there appeared to be no difference between cvs (cv Mendel, which has good resistance to clubroot, was not in the set of test cvs).

Conclusions

With respect to the first aim of the project, it is clear that measurement of components of resistance can help in the breeding of more resistant cvs. The project showed new information on the development of stem canker and pathogen DNA in the stem in thermal time – pathogen growth was not linear but increased from the onset of flowering at different rates for different cvs. This was particularly important as the project also demonstrated that yield losses due to stem canker were insignificant if canker severity could be managed to be less than 2.5 on the 0-6 scale.

Regarding the second aim, to produce new methods to rate cultivar resistance to stem canker and light leaf spot in winter oilseed rape cultivar selection and evaluation trials, the project showed that exisiting, relatively 'low-tech' methods were best but that, particularly with inoculated controlled environment studies, there appears to be great potential in measuring pathogen growth from its DNA or lesion appearance in order to screen genotypes for polygenic resistance to *L. maculans* at early growth stages.

The project also showed that infection of meristems by *P. brassicae* was widespread before Christmas, and as this is though to lead to symptoms of plant stunting, indicates that late-autumn foliar fungicide applications should be used against light leaf spot. The amounts of DNA of *P. brassicae* on leaves sampled from field plots in late-winter were reasonably related to the RL cultivar resistance ratings for LLS but it is not clear whether the assessment of visible symptoms or pathogen DNA is best related to yield loss due to LLS.

6.2 Knowledge transfer

Knowledge transfer activities, such as presentations at Cereals events, talks to crop consultants, oral and poster presentations at international conferences, press articles and a review of *P. brassicae* biology have been made. Material to be published as research papers in refereed scientific journals has been prepared. A list of publications to date using information from this project is given below:

Papers and reviews

Evans N, Baierl A, Semenov MA, Gladders P, Fitt BDL. 2008. Range and severity of a plant disease increased by global warming. Journal of the Royal Society Interface 5: 525-531.

Boys EF, Roques SE, Ashby AM, Evans N, Latunde-Dada AO, Thomas JE, West JS, Fitt BDL. 2007. Resistance to infection by stealth: *Brassica napus* (winter oilseed rape) and *Pyrenopeziza brassicae* (light leaf spot) in Europe. *European Journal of Plant Pathology* 118: 307-321.

Aubertot JN, West JS, Bousset-Vaslin L, Salam MU, Barbetti MJ, Diggle AJ. 2006. Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 114: 91-106.

West JS, Fitt BDL. 2005. Population dynamics and dispersal of *Leptosphaeria maculans* (blackleg of canola). *Australasian Plant Pathology* 34: 457-461.

Popular articles

West JS, Thomas J, Gladders P, Booth E, Latunde-Dada AO, Evans N, Fitt BDL, 2005. Components of Resistance to Diseases in Winter Oilseed Rape: CORDISOR *RRA Newsletter 16. January 2005*.

West JS, Thomas J, Gladders P, Booth E, Latunde-Dada AO, Evans N, Fitt BDL, 2004. Components of Resistance to Diseases in Winter Oilseed Rape: CORDISOR. *GCIRC Bulletin 21, XII 2004*, pp.79-81.

Conference proceedings

BDL. Fitt, N Evans, P Gladders, Y-J Huang and JS West (2008) Phoma stem canker and light leaf spot on oilseed rape in a changing climate. *Aspects of Applied Biology* **88**; Effects of Climate Change on Plants: Implications for Agriculture.pp. 143-145.

West JS, Rogers SL, Latunde-Dada AO, Pirie EJ, Stonard JF, Huang YJ, Atkins SD, Fitt BDL. 2008. Quantifying DNA of *Leptosphaeria maculans* and *Pyrenopeziza brassicae*

in oilseed rape tissues using qPCR. 9th International Congress of Plant Pathology, Healthy and safe food for everybody, August 24-29, 2008, Torino, Italy.

West JS, Gladders P, Booth E, Thomas J, Jennaway R, Werner P, Bowman J, Nightingale M, Padbury N, Clarke M, Jellis GJ, Foster V, Rogers SL, Kenyon D, Atkins SD, Boys EF, Huang Y-J, Evans N, Latunde-Dada AO, Li Q, Fitt BDL. 2008. Factors affecting canker and light leaf spot severity in oilseed rape. BSPP Break Crops Workshop, Peterborough, 24 June 2008, p. 16.

West JS, Rogers SL, Gladders P, Fitt BDL 2008. Climate change set to worsen severity of stem canker. HGCA R&D Conference 'Arable Cropping in a Changing Climate', 23-24 January 2008, Belton Woods, Lincs. Abstract p. 135.

Latunde-Dada AO, West JS, Rogers SL, Downes K, Roques SE, Pirie E, Stonard JF, Huang Y-J, Fitt BDL 2007. New methods to understand quantitative resistance to *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape. Proceedings of the 12th International Rapeseed Congress: sustainable development in cruciferous oilseed crop production. Wuhan, China, March 26-30, 2007. Abstracts p. 310.

West JS, Latunde-Dada AO, Rogers SL, Huang Y-J, Evans N, Fitt BDL 2007. *Leptosphaeria maculans* avirulence management for durable control of phoma stem canker on oilseed rape. Proceedings of the 12th International Rapeseed Congress: sustainable development in cruciferous oilseed crops production. Wuhan, China, March 26-30, 2007. Vol. IV. p. 2-4.

West JS, Latunde-Dada AO, Huang Y-J, Evans N, Fitt BDL 2006. Avirulence management for durable control of stem canker of oilseed rape in Europe. *Aspects of Applied Biology* 80, *Delivering sustainability within profitable farming systems – is it possible?*. pp. 171-176, The Association of Applied Biologists, Wellesbourne.

West JS, Latunde-Dada AO, Huang Y-J, Evans N, Fitt BDL 2005. Enhanced avirulence management for durable control of blackleg in Europe. In: Potter, T. (Ed), 14th Australian Research Assembly on Brassicas, Port Lincoln, 2005. Proceedings. SARDI, Primary Industries and Resources South Australia, pp. 76-81

Other KT Activities

A) Events:

The project featured at both the Defra and Rothamsted Research displays at Cereals 2005 and a Defra SA LINK leaflet was distributed. Jon West made a presentation on durability of resistance to phoma stem canker at the 14th Australian Research Assembly on Brassicas, Port Lincoln, South Australia, 6th October 2005; and a similar presentation at the Rothamsted Research Association meeting on 24th November 2005 at Rothamsted. Jon West presented an overview of the project's objectives, methods and results so far

at the UK-Brassica Research Community Annual Meeting, John Innes Centre, 24th May 2006. A simplified version of this presentation is available on the UK-Brassica Research Community website: http://www.brassica.info/ukbrc/meet06.htm.
The project featured at Cereals 2006 (14-15 June) as part of the HGCA display and the Defra leaflet was distributed at the Rothamsted display. Additionally, the project was publicised at ADAS open days at Rosemaund (6 June), Boxworth (8 June) and High Mowthorpe (29 June) and in the HGCA oilseed rape disease and pest management workshops in different parts of England (autumn 2008).

B) Press articles

An article by Mark Sanderson that featured the project was published in Syngenta Farmer's Guardian in October 2005. An article entitled, 'Research In Focus: stem canker in rape', by Sarah Henly, appeared in 'Crops', issue date: 15 October 2005.
Jon West attended a press briefing organised by Bayer, and reported recent research findings, leading to an article in Farmers Weekly on 13 October 2006 'Phoma control crucial as OSR area expands' by Andrew Blake.

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Appendices

Appendix 1: Field experiment plant and disease assessment methods

Disease and crop assessments

Codes used below, such as SC2, LLS3 and P1, refer to types of assessment summarised in appendix 3 and explained in more detail below.

Crop density and growth measurements

P1 At each main assessment (see dates below and in Appendix 4), in each plot, the general growth stage (P1) of the plot should be noted using the scale derived by Sylvester-Bradley & Makepeace (1985). Additionally at Rothamsted (RRES) and ADAS, plant growth should be assessed on 10 plants per plot by recording number of expanded leaves present and number shed (number of leaf scars present). Leaves are considered expanded when the petiole is visible.

P4 Crop density (P4) should be measured in experiments where yields are also taken and this should be done in November and after harvest to provide data on establishment, winter kill and to relate yield to crop density. At RRES and ADAS, this is calculated by counting the number of plants in 4 x 0.5 m rows and noting the distance between rows. At other sites (where yields are to be taken), it was agreed (minutes 8 June 04.doc) that to reduce workload, after a quick look, measure the plant density in the best and worst established plots and score other plots on a 1-9 scale which can be interpolated from the best and worst plots.

P2 Two plant height assessments (P2) should be made; Mid-March and April/May, where the height (mm) to the apex of the main stem is recorded on 10 plants per plot.

P3 Stem diameter (P3) is measured on 10 plants in untreated plots in June at RRES and ADAS, by measuring the diameter of the crown in mm. At other sites (e.g. breeders nurseries), for speed, note the smallest and largest stem diameters of plots and rate other plots on a 1-4 scale.

Disease assessments

The experiments should be inspected each week to detect the onset of phoma leaf spotting. The date and growth stage when phoma leaf spotting first appears should be noted. At RRES and ADAS, once phoma leaf spotting has been observed, all untreated plots should be assessed for disease. At all sites, assessments should be made on the dates indicated in appendix 4 (Oct, Nov/Dec, Jan, mid-March, April/May, Jun and July). In addition to the standard ‘breeders’ 1-9 assessment for Phoma and LLS (based on the HGCA protocol on page 3), a minimum of 10 plants per plot should be assessed unless otherwise stated. Plants should be sampled at random from the plot or sampling area of plots, and individual data should be collected for each plant, recording incidence (% plants affected) and severity of all diseases present on the CORDISOR assessment form (Appendices 1 and 2). Two assessment forms have been designed, the first (appendix 1a & b) for assessments up to the

onset of flowering and the second (appendix 2a & b) from flowering to harvest (this is to reduce the size of the assessment form as information to be collected changes with crop growth). For each form, two versions exist; one for RRES & ADAS and the other for all other sites.

SC1 & LLS1

Pre-flowering, for both phoma leaf spot (SC1) and light leaf spot (LLS1), two severity measurements should be recorded: (i) % areas affected per plant and (ii) number of leaves affected per plant. Other diseases should also be recorded. For LLS leaf infection (LLS1), in November and January, plants should be incubated in plastic bags at 10°C for 2-5 days prior to assessment. Breeders do a simpler assessment (LLS1*) where one fully expanded, non-senescent green leaf per plant is detached and these are incubated in a labelled plastic bag (per plot) at 10°C for 3-5 days and checked for LLS (the result can be recorded separately or in the 'notes' part of the disease assessment form).

SC2 & LLS2

PCR analysis SC2 will be in late November and January, aiming to assess petiole infection by *L. maculans*; 10 whole plants should be collected from untreated plots, plants separated with blue-roll/tissue or in individual plastic bags and placed in a single large plastic bag, labelled with the plot number. One breeder in England (TBA) should send samples to Jon West at Rothamsted by courier/next day delivery, along with samples from RRES and ADAS (RL sites send samples to NIAB). Leaves for PCR will be selected at Rothamsted according to development of phoma leaf spotting at the sample date. The petioles of these selected leaves will be measured to determine mean length prior to processing for real-time PCR.

At RRES, a more detailed regime of SC2 sampling will be made in the sub-experiment (RAW/502) (=pot experiment in original proposal – but now field-based) with samples (10 plants per plot) taken fortnightly in November and December, and petioles of two leaves of known position (senescing, and lowest green leaf) plus one stem section (approx 2 cm long at the crown) assessed from the early inoculated and a spare (naturally inoculated) plots.

Assessment LLS2 should also be in late November and January, 10 whole plants wrapped in blue-roll and placed in a labelled plastic bag per plot (from some sites these can be the same plants as for SC2). In November each LLS site should send ten plants of cv Hearty samples to Jon West at Rothamsted in the method above to enable the best site to be selected for the Nov/Dec and January LLS2 samples (leaves and apical meristems will be processed for real-time PCR). According to the proposal, LLS2 sites should be one breeder's nursery in Scotland, one in England, plus the RRES and ADAS experients, while SAC should send samples to NIAB.

At RRES, a more detailed regime of LLS2 sampling will be made in the sub-experiment (RAW/502) with samples taken fortnightly in October to January, and leaves and meristems assessed.

LLS3

From mid-March, assessment of LLS on 10 individual plants per plot is to be made directly on the plants without incubation in plastic bags (as LLS1).

LLS4

In mid-March only, in addition to the usual assessment of 10 individual plants (LLS3), an assessment to satisfy the RL protocol (LLS4) should be made w.r.t LLS only (i.e. not for downy mildew, phoma or white leaf spot as in the excerpt below):'Light leaf spot, Alternaria, downy mildew, Phoma and white leaf spot on leaves and pods

1) Examine all leaves and pods in 3 areas of each plot.

2) Ignore all naturally senescent tissue.

3) Include all necrosis and chlorosis attributable to disease.

4) Estimate % infection using the descriptions below. Record the average % infection from the 3 areas. Interpolate values if necessary.

1-9	% Infection	Leaves	Pods
1	0	No infection observable	
2	0.1	Trace of infection	
3	1	Diseased leaves with 1 small lesion; plants with a few scattered lesions	Terminal raceme with a few scattered lesions
4	5	Leaves appear 1/10 infected; diseased leaves with 2 lesions	Terminal raceme appears 1/10 infected; diseased pods with 1 or 2 lesions
5	10	Leaves appear ¼ infected; diseased leaves with few large or many small lesions	Terminal raceme appears ¼ infected; diseased pods with 2 or more lesions
6	25	Area appears ½ infected ½ green	
7	50	Area appears more infected than green	
8	75	Very little green tissue left	
9	100	Leaves/pods dead - no green tissue left	

These descriptions are guides for specific levels; interpolate between these points as necessary e.g.15%, 27%, 60% etc.'

(indented passage above taken from the HGCA recommended list winter oilseed rape trials protocol protocol 2004/05)

Post-flowering, diseases on stems and pods only should be recorded. For non-target diseases such as Sclerotinia and Botrytis, record presence on the stem as Y/N.

LLS5 For LLS stem infection in late April/ early May, June and July, score the % area affected on the main stem.

SC3 For phoma stem canker assessment (crown cankers and phoma stem lesions), score using the HGCA RL 0-6 score (0 = uninfected, 1 = < 25% stem circumference girdled, 2 = 26-50% girdled, 3 = > 51-75% girdled, 4 = > 76-100% girdled, 5= 100% girdled + stem weak, 6 = plant dead or lodged) idividually on each of 10 plants per plot.

SC4 In June, in addition to SC3, the assessment should be expanded to satisfy the RL protocol (SC4) (i.e an additional 20 stems assessed) as indicated below:

> 'Assessment is by pulling up 30 stems per plot just before swathing. Stems should be pulled at random throughout the plot, but since access is likely to be very difficult, aim to take 15 stems from the second drill row on each side of the plot, using the first 3-5m of the plot length. If sampling is not carried out prior to swathing, it must be done ***as soon as possible afterwards, within a maximum of 2 days.*** The external symptoms only should be assessed for DOPs by assigning stem base symptoms on each of the 30 stems to one of the following categories:
>
> | | |
> |---|---|
> | 0 | No infection observable |
> | 1 | <25% girdling of the stem |
> | 2 | 26-50% girdling |
> | 3 | 51 -75% girdling |
> | 4 | 76 -100% girdling |
> | 5 | 100% girdling + stem weakness |
> | 6 | 100% girdling + stem death |
>
> Any records made should show clearly the number of plants per plot in each disease category. "Five bar gate" tally systems are most appropriate. A disease index (DI) on a 0-100 scale will be calculated using the formula
>
> $$\frac{(0xa + 1xb + 2xc \text{ etc})}{(a+b+c + \text{etc})} \times 100/6$$

where a, b, c etc are the number of plants in each disease category

(indented passage above taken from the HGCA recommended list winter oilseed rape trials protocol protocol 2004/05)

LLS6 LLS disease severity on pods (LLS6) should be recorded in June and July both as % total area of pods affected and % pods affected (i.e. % incidence of pod infection per plant).

A summary of all assessments for disease and PCR sampling is given in appendix 3.

Yield measurement

RL sites to follow RL protocol. Other yield sites (RRES & ADAS) will follow a protocol closely modelled on the RL protocol - at Rothamsted a plot combine, 2.1 m wide will harvest the central band of the 3m-wide plots along the 10m long yield-collection area. Samples should be taken for moisture, oil content and dry weight assessments. Yield data (adjusted to 90% dry matter) should be sent electronically to Rothamsted so that it can be analysed statistically.

Data handling

Raw data collected directly in digital format should be sent by email to Rothamsted. Alternatively, raw data written on assessment forms should be photocopied and one copy sent by post. At Rothamsted, all electronic data files should be stored in at least three locations, one of which should be the secure (read-only) web site, a backed-up CORDISOR folder on the Rothamsted main computing system, and another should be a portable format such as a CD. It is anticipated that the data will be transferred from excel documents into an Access or similar database. The paper records of raw data should be converted to electronic format as soon as possible but should be stored until at least one year after the termination of the project. At Rothamsted, all summary data of analysed raw data should be recorded in a Lab Notebook. Met data (required for any future modelling of disease progress against thermal time) should be taken from the Rothamsted daily met records on ERA. Mean daily temperature and rainfall data from ADAS Boxworth should be submitted at the end of each season.

Appendix 2: CORDISOR project processing of oilseed rape tissue samples for quantification of pathogen DNA by real-time PCR analysis

Note: Methods A, B, C and D, below, describe methods for processing different tissue samples (petiole, crown, meristem and leaf lamellae), followed by method E, a freeze drying step, and F, production of a dry powdered sample for DNA extraction.

Materials: (A, B, C, D) Oilseed rape whole plants, scalpel, Blue-roll/tissue, 50 ml centrifuge tubes with lids, forceps, latex gloves, 70% Methanol, chopping board, ruler

(E) freeze-drier, centrifuge tube lids, plastic boxes/punnets

(F) Pestles and mortars, plastic funnels, wire mesh tea strainers, RO/distilled water, 70% methanol, dissection scissors, forceps, plastic weigh-boats, gloves, blue-roll/tissue.

Methods: A) SC2 – processing of petioles for *L. maculans* analysis (main experiments sampled in Nov/Dec; plus a small number of samples fortnightly from Oct-Jan)

Ensure bench and chopping board are clean and swab with 70% methanol.

Handle one plant at a time per ten-plant plot sample. Identify leaf/leaves per plant to be processed (if leaf position data are included, leaf positions are numbered from the plant base i.e. leaf 1 is the first leaf produced; count leaf scars if present to identify number of leaves shed and continue counting leaves present from the plant base). Unless otherwise stated, for *L. maculans*, only petioles from the lowest non-senescent leaf will be used and only if phoma leaf spots are present on it. Occasionally for RAW602, other leaves may be sampled e.g. the lowest senescent leaf, or a mid-canopy leaf.

Remove selected leaf/leaves as close as possible from the plant stem using a clean scalpel (wipe scalpel blade on blue-roll moistened with 70% methanol in between each cut). Do not remove leaves by hand as vascular tissue (in which the fungus grows) can be pulled-out and left attached to the stem.

Measure and record petiole length of each leaf.

Divide the petiole into two sections – upper (nearest leaf lamellae) and lower (nearest stem base), again using a cleaned scalpel for each cut. Furthermore, for petioles larger than 0.5 cm in diameter, divide each section longitudinally to assist later freeze drying and powdering steps. For petioles longer than 18 cm, keep only the upper 9cm and the lower 9 cm.

Place upper and lower petiole sections respectively into two labelled 50 ml centrifuge tubes. Label should indicate sample site, plot number, sample date, leaf position or type (if not the standard lowest green leaf) and tissue section e.g. XXX001/15.10.04/L5/Lo (= site XXX plot 1, sampled on 15 Oct 04, leaf 5, lower petiole section). Up = upper petiole section. If leaf position is not recorded, leaf type should be recorded instead i.e. senescent, green (lowest green) or mid (mid-canopy leaf).

Continue with other plants (total of 10) from the same plot, placing tissue samples into the same respective tubes per plot and tissue section.

The tubes should be sealed with the lids supplied.

Place tubes in -20°C freezer for at least one day before the freeze drying step (method D, below)

B) Processing crown tissue for *L. maculans* analysis

As for method (A) above, but from each plant sampled and for plants pre-stem extension growth-stage, remove leaves and cut hypocotyls just below cotyledon scars. For plants post-stem extension, cut at cotyledon scars and at the top of rosette leaf scars (indicated by position at which internode length increases). Divide crown section into quarters.

Place into tubes labelled as above but with tissue identified as 'crown' instead of 'up' or 'lo'.

C) LLS2 – processing apical meristems for *P. brassicae* analysis (Main experiments in January, more frequently for RAW602).

For each of tem plants per plot sample, cut across the stem where uppermost leaves are attached using a clean knife. Trim large leaf bases and any excess stem to leave the uppermost tip of stem bearing the meristem and unfolded leaves ≈3-5 cm in length. Place in the labelled tube. The label should be as above but with the tissue identified as 'meristem', to indicate the meristem at the tip of the main stem.

D) LLS2 – processing leaf lamellae for *P. brassicae* analysis (January).

For each of ten plants per plot sample, select a mid-canopy leaf (typically the youngest fully-expanded leaf and clean of any mud), remove lamellae only (leaving petiole). Pool lamellae from selected leaves of all ten plants per plot into a single 50 ml sample tube and label with usual plot/site and date identification plus 'leaf'.

E) Freeze drying (based on using freeze drier in Bawden 212 and adjacent small chest freezer)

Samples in centrifuge tubes, prepared by methods A, B, C or D above, should have lids removed from the tubes (retain lids for later) and the tubes stacked upright in plastic boxes/punnets. The tubes in the plastic boxes should be returned to the freezer next to the freeze drier for at least 30 minutes. Ideally, the metal drying trays and wrack assembly is also placed in the freezer, loaded with the boxed open tubes and left for at least 30 minutes. The cooled drying wrack when placed into the freeze drier, cools the air in the chamber so that the samples stay frozen while the vacuum develops.

Follow the instructions indicated on the freeze drier to pre-cool the condenser (Usually, the freeze drier in Bawden 212 is used and cooling to below minus 30ºC usually takes only 10 minutes but the room should also be cool <20ºC or the freeze drier does not work well).

The lids of the tubes should be washed, rinsed several times and left to dry.

Follow the instructions indicated on the selected freeze drier to load and activate the freeze drying process.

Monitor the condenser temperature, vacuum and appearance of the samples – if any thawing is suspected, shut down and re-freeze the tubes (and wrack).

For large samples e.g. petioles it is necessary to freeze dry the samples over a period of two or three days, occasionally shutting down the drier to thaw the condenser while refreezing the samples.

Finally, remove a few tubes and if tube contents no longer feel cold and are obviously dry, remove all tubes, follow shut-down procedure for the selected freeze drier and seal tubes with clean, dry tube lids.

F) Powdering of freeze-dried samples

All equipment should be clean. Before processing each tube of freeze-dried material, the pestle, mortar should be washed, dried on blue-roll or tissue and wiped on blue-roll moistened with 70% methanol. Similarly any scissors or forceps used should be cleaned. Latex gloves should be worn. Ideally several sets of equipment should be available to allow a single break for washing and drying prior to handling more samples. Finally all equipment should be wiped with clean tissue to confirm that it is dry.

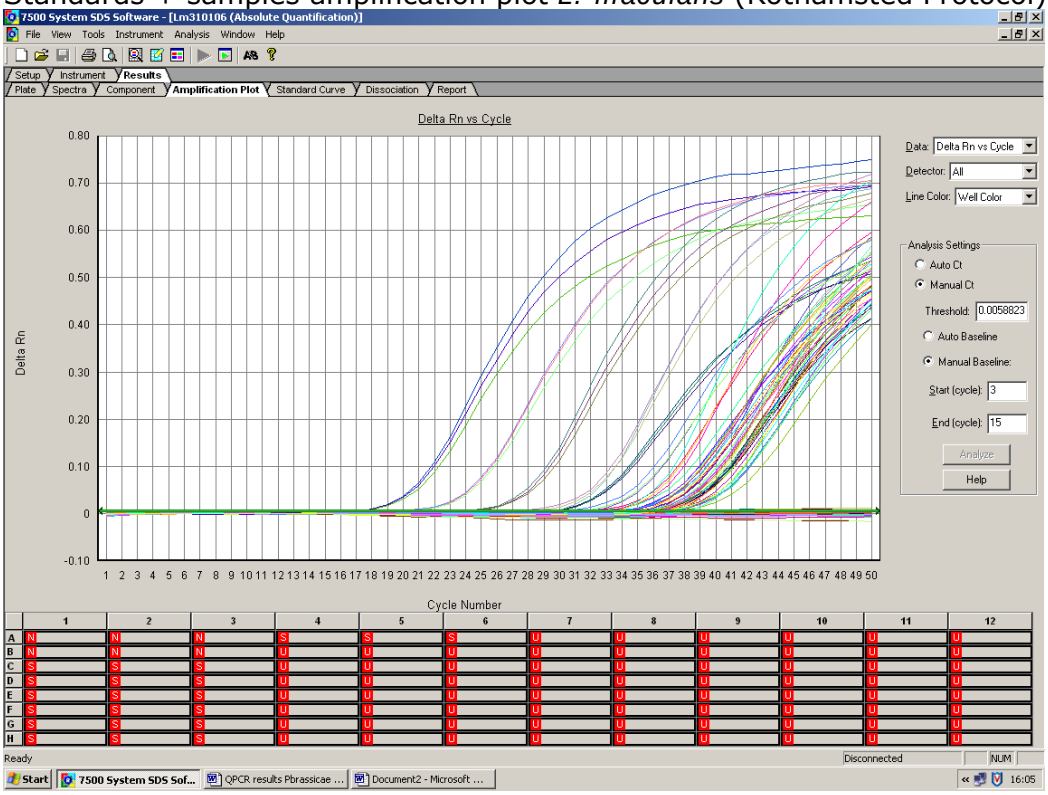
For leaf lamellae and meristem samples, it is possible to crush the sample to a fine powder in the sample tube using a metal or glass rod as a pestle. For tougher tissues, such as crown or petiole samples, all or a portion (for large tissue samples) of freeze-dried material should be transferred to a mortar and crushed with a pestle. It may be necessary either to cut it into smaller fragments with clean dissecting scissors or seccateurs, or to use a clean wire mesh tea strainer inside the mortar, grinding the plant material against the mesh of the tea strainer on the wall of the mortar for best effect. Fine powdered debris should collect at the bottom of the mortar. Continue until all material in the tube is powdered. If any material appears to be moist, it should be returned to the tube, refrozen and freeze-dried. (it appears that rarely, layers of waxy leaves of oilseed rape effectively seal the tube like parafilm and prevents the freeze drying process to leave some material moist. If this occurs frequently, suspect that the freeze drying step has not worked – was not done for long enough, or equipment was faulty. Samples should be re-frozen and freeze-dried again).

Funnel the powder from the mortar back into its original, labelled tube.

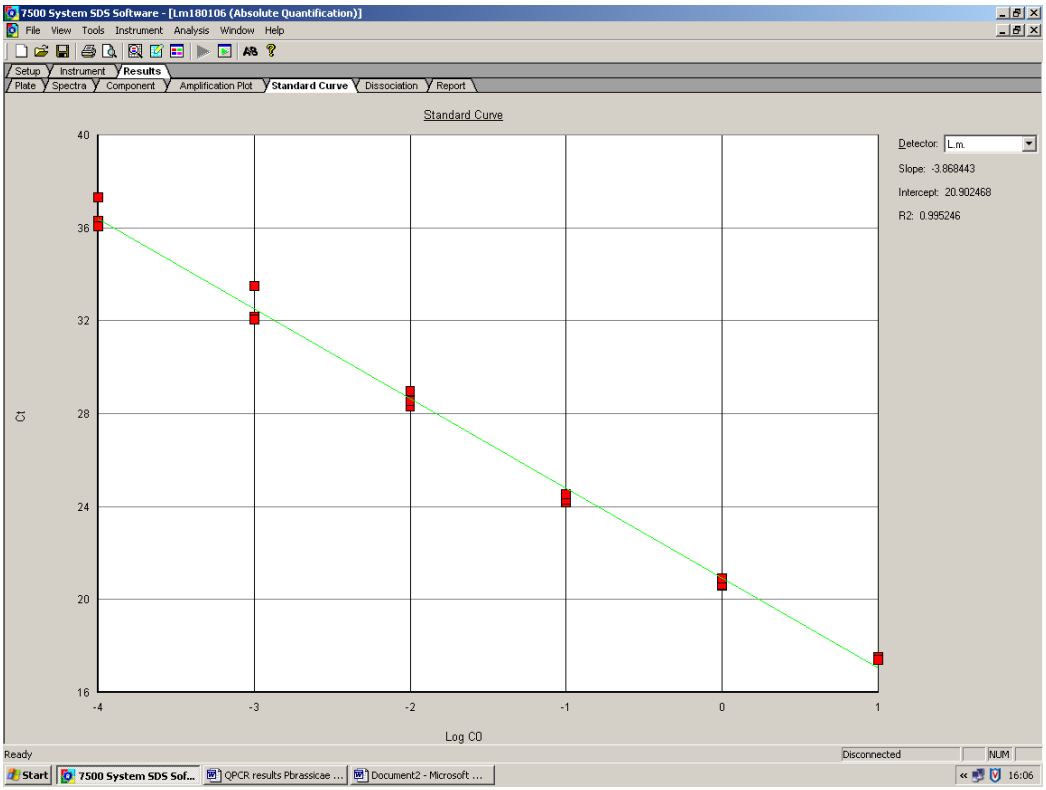
Cap the tube and store at 4-5°C for subsequent DNA extraction.

Appendix 3 Example qPCR outputs

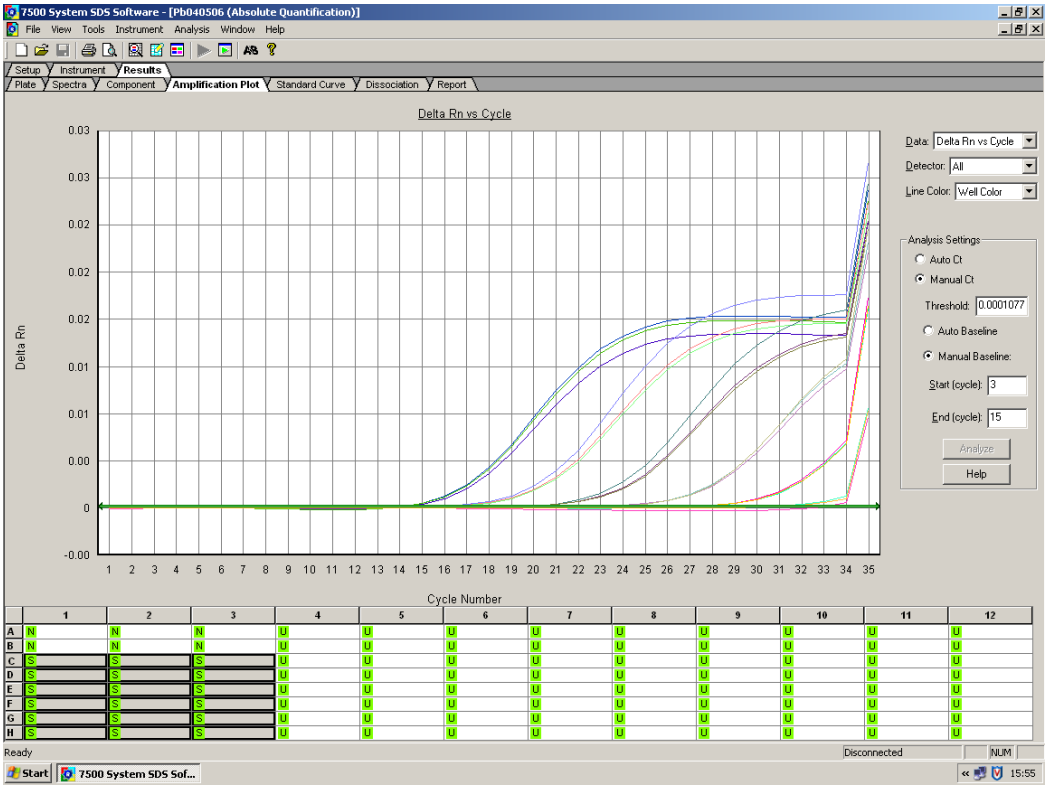
Standards + samples amplification plot *L. maculans* (Rothamsted Protocol)



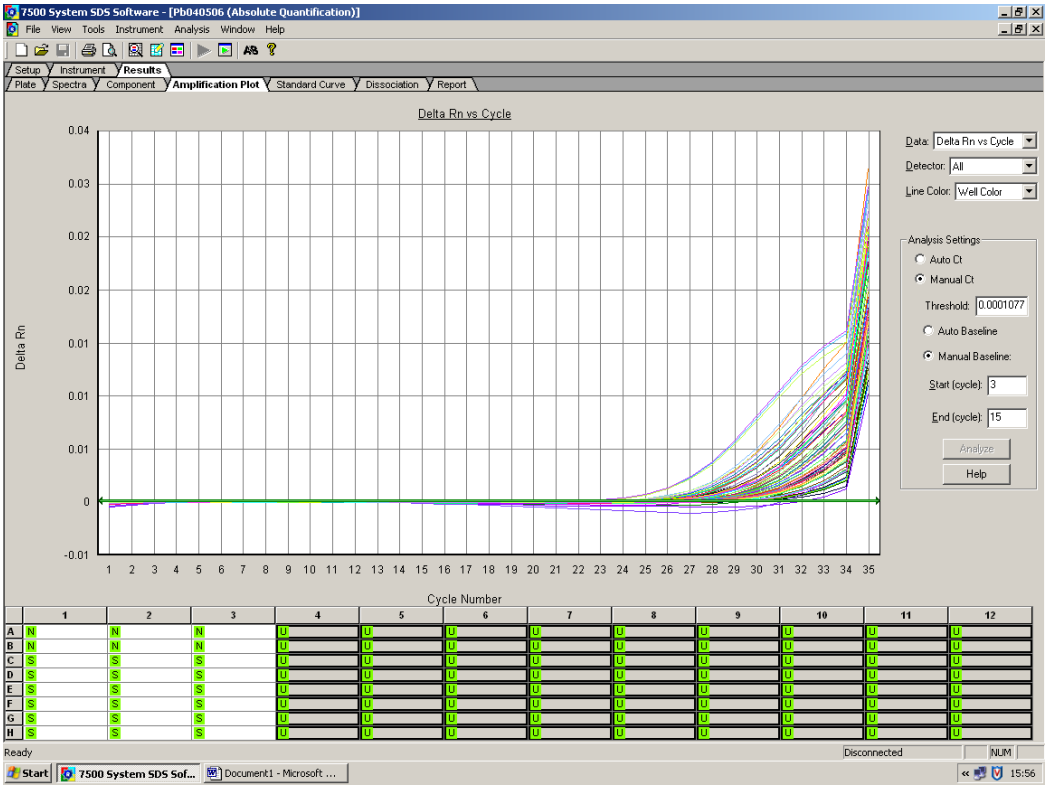
Standard curve *L. maculans*



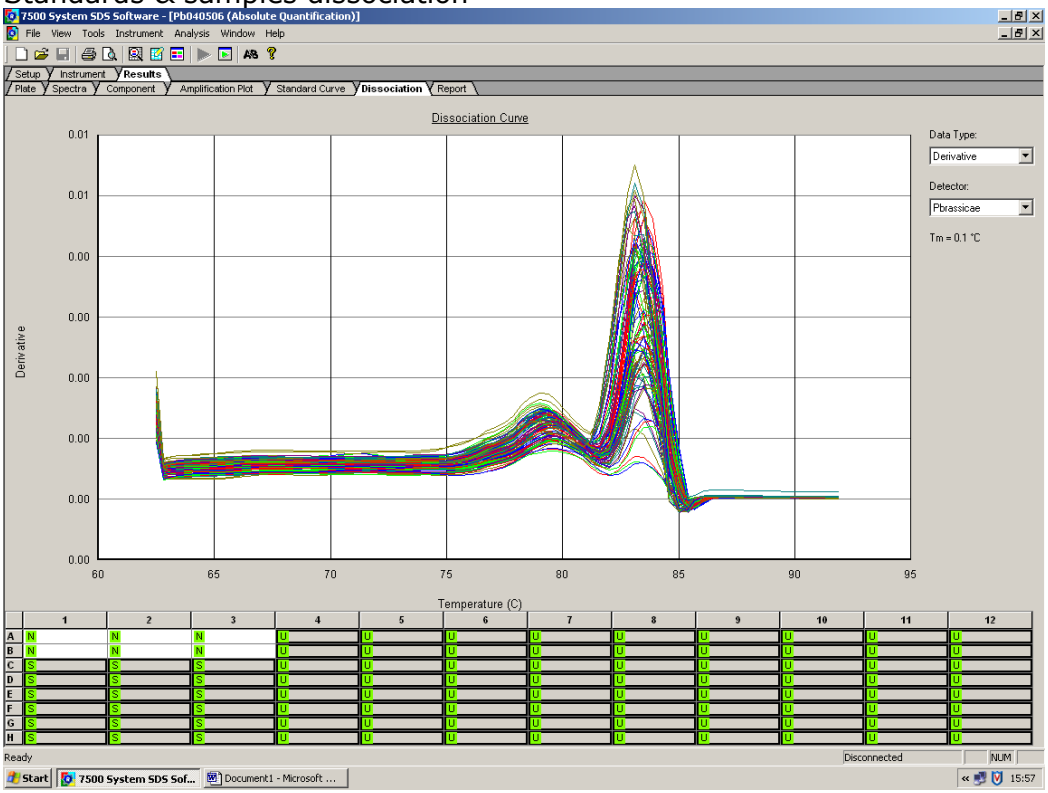
P.brassicae standards 04.05.06- amplification plot (Rothamsted Protocol)



Samples- amplification plot



Standards & samples dissociation



Standard curve