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Maximising the effective life of fungicides to control oilseed rape diseases through improved resistance management

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1. Abstract

Control of oilseed rape diseases depends on a combination of non-chemical practices (e.g. varietal resistance) and foliar fungicides; the latter predominately of a single chemical group – the azoles. This project aimed to understand the risk from fungicide resistance development for all oilseed rape diseases, and provide evidence to advocate effective cost-effective resistance management strategies. It was found that all modes of action should be considered at similar risk for resistance development and that resistance management should be considered across the entire fungicide programme. The polycyclic disease *Pyrenopeziza brassicae* – which causes light leaf spot – is likely to be a greater resistance risk than other pathogens (which are monocyclic) and was the focus of the experimental work.

Mutations conferring some degree of insensitivity to triazoles – G460S and S508 – have previously been identified in the UK *P. brassicae* population. Field experiments tested whether particular fungicide strategies select more or less strongly for azole insensitive mutants. The proportion of the G460S mutation exceeded 60% in most field trials, reaching 90% in 2019, meaning it was not possible to determine whether fungicide programmes were having an impact on selection. However, it did provide an opportunity to determine whether disease control by azoles was affected by presence of this mutation. The efficacy of azole and non-azole fungicides, even at sites where the proportion of the G460S mutation was high, was similar. This suggests that the G460S mutation does not confer a substantial decrease in the effectiveness of the currently available azoles in the field. This is positive news, as robust disease and fungicide-resistance management strategies require a variety of modes of action. Most light leaf spot strains now carry G460S or S508T in combination with *CYP51* promoter inserts, and azoles have been shown to be as effective as a non-azole alternative. Therefore, the use of all effective modes of action, in mixtures and in alternation, should be encouraged throughout the fungicide programme. Evidence from other countries shows it is important to not be complacent about development of resistance, including in other oilseed rape diseases, such as phoma leaf spot/stem canker and sclerotinia stem rot.

The trials were conducted across three low-disease-pressure years in light leaf spot susceptible but phoma stem canker resistant varieties. A yield uplift of between 0.17 and 0.27t/ha was required to cover the cost of the fungicide programme (i.e. to break even). As a result, applying no fungicides was often the most cost effective option, regardless of whether fungicides were applied in alternation and mixtures. Alternating modes of action and using co-formulated products are among the simplest resistance management strategies. ‘Balanced mixtures’, where the appropriate dose of two different modes of action are used to maximise disease control, yield and resistance management, are likely to be effective but require field experimentation to support their use. An integrated approach, using a range of disease management tools and strategies, such as varietal resistance, is likely to offer the most sustainable and, potentially, more cost-effective disease management approach.

2. Introduction

Economic losses attributable to light leaf spot (*Pyrenopeziza brassicae*) and phoma leaf spot/stem canker (*Leptosphaeria maculans/Leptosphaeria biglobosa*) were estimated to be over £210 million nationally in 2018 (www.cropmonitor.co.uk), with light leaf spot overtaking stem canker as the major disease affecting oilseed rape in England and Wales. Control depends on a combination of non-chemical practices (e.g. varietal resistance) and a significant contribution from foliar fungicides; predominately a single chemical group, the azoles. Two alterations (G460S and S508T) in the sterol 14α-demethylase (PbCYP51) protein conferring decreased sensitivity to triazoles have been identified recently in isolates taken from the UK *P. brassicae* population (Carter *et al.*, 2014). There is currently no evidence available to the industry to demonstrate the impact these strains are having on the effectiveness of current treatment regimes and whether the fungicide programmes used are selecting for fungicide insensitive strains.

Oilseed rape receives three fungicide applications in a typical season; one or two applications in the autumn and winter for light leaf spot/phoma control and one or two during flowering for sclerotinia control and to top up light leaf spot control. Until 2015, control of both diseases was entirely reliant on azole containing products such as Cirkon (prochloraz and propiconazole), Orius (tebuconazole) and Proline (prothioconazole). Refinzar became briefly available (penthiopyrad and picoxystrobin) but was withdrawn in 2018, leaving the industry reliant on azole chemistry prior to February once again. There is a greater range of mode of action groups to control *Sclerotinia sclerotiorum*, however, azoles are still used at flowering particularly if light leaf spot is present. According to the most recent DEFRA pesticide usage survey report, at least 71% of products applied to oilseed rape were azoles (Garthwaite *et al.*, 2019b).

The aim of fungicide resistance management is to slow selection for fungicide resistant strains in the pathogen population. In a recent peer-reviewed worldwide analysis of evidence on the effectiveness of fungicide resistance management strategies (van den Bosch *et al.*, 2014), the authors identified ‘governing’ or first principles which can be used to predict whether specific changes to fungicide programmes will increase or decrease selection for fungicide resistance. These principles are generic and have been tested in a wide range of pathosystems, however, experimentation is required to determine whether specific anti-resistance tactics are practical and cost effective for particular pathogen : crop combinations.

Insensitivity to triazoles has been identified in isolates taken from the UK *P. brassicae* population (Carter *et al.*, 2014). Two mutations in the azole target encoding sterol 14α-demethylase gene *PbCYP51*, resulting in amino acid substitutions G460S and S508T, have been associated with azole insensitivity. These alterations affect the CYP51 azole binding site and are similar to those reported

to confer resistance to triazoles in other plant pathogens including *Zymoseptoria tritici* (previously *Mycosphaerella graminicola*). For example, PbCYP51 G460S and S508T are identical to ZtCYP51 G476S, found in an azole insensitive lab mutant, and S524T, found in many *Z. tritici* field strains, respectively. From yeast expression (Cools *et al.*, 2011; Carter *et al.*, 2014) and protein modelling studies (Mullins *et al*., 2011), there is evidence to suggest that both G460S and S508T are likely to confer insensitivity to all triazoles and also explain the high levels of cross-resistance measured for *P. brassicae* field strains (Carter *et al*., 2014). Other *CYP51* mutations that have evolved in plant pathogens, for example V136A, Y137F and I381V in *Zymoseptoria tritici*, can differentially affect the binding of azoles and cross resistance between different azoles does not appear to be complete (Cools & Fraaije, 2013; Dooley *et al.*, 2015).

Despite reports of azole resistance in UK light leaf spot populations, there is currently no specific evidence-based resistance management guidance for the industry on best practice when using this mode of action group for disease control in oilseed rape. Similarly, current Fungicide Resistance Action Committee (FRAC) guidelines advise the application of general fungicide resistance management guidelines for use of SDHIs in oilseed rape and there are no specific recommendations for best practice (Anon, 2015). Any findings relating to oilseed rape will also have significance for horticultural crops. In vegetable brassicas, the majority of fungicides applied are azoles, although a proportion of the crop is treated with QoI fungicides (Garthwaite *et al.*, 2014). Many diseases affecting oilseed rape can infect vegetable brassicas including *Alternaria brassicae* and *Alternaria brassicicola*. These are considered minor pathogens on oilseed rape, but can downgrade vegetable produce. Any change in fungicide programme on oilseed rape must consider the impact on these pathogens.

In the work presented here, field experiments compared selection for insensitive azole strains of *P. brassicae* under different treatment strategies. The experiments aim to demonstrate the distribution of azole resistance in oilseed rape crops as well as provide direct, practical evidence which can be used to advocate effective strategies: through AHDB knowledge exchange with levy payers/agronomists and revised guidance from the Fungicide Resistance Action Group (FRAG).

2.1. Aims and objectives

The aim of this project was to identify fungicide strategies, through field experimentation, to decrease the selection for fungicide resistance in oilseed rape pathogens, without compromising disease control or yield.

The specific objectives were:

Objective 1. Determine the risk of fungicide resistance affecting fungicides used to control oilseed rape diseases;

Objective 2. Test which resistance management strategies are most effective at slowing fungicide resistance selection in *P. brassicae* comparing application of solo products against mixtures and alternation;

Objective 3. Conduct an economic analysis of fungicide anti-resistance management strategies for the industry.

Objectives 1 and 2 were funded by AHDB Cereals and Oilseeds. Industry contributions, as cash and in-kind, were used to fund objective 3.

3. Materials and methods

3.1. Determine the risk of fungicide resistance affecting fungicides used to control oilseed rape diseases

The risk assessment method developed by Grimmer *et al*., (2014) was used to assess the risk of resistance occurrence for modes of action deployed to control the major oilseed rape pathogens. The aim was to help prioritise resistance management where it is most important and identify whether pathogens considered minor on oilseed rape, but major on vegetable brassicas, such as *A. brassicae* and *A. brassicicola*, are also potentially at risk. The method was based on determining the first detection of resistance (FDR) time (defined as the time in years from introduction of a mode of action for use against a specific pathogen and the first detection of resistance in that pathogen) using specific traits associated with fast or slow occurrence of fungicide resistance. These include the number of latent periods of a pathogen per year, fungicide molecular complexity, the number of host species the pathogen has and the agronomic system in which the crop is grown. The assessment was done for all modes of action groups, past and present, used to control the four main diseases affecting oilseed rape including the casual pathogens of phoma leaf spot/stem canker (*Leptosphaeria maculans* and *Leptosphaeria biglobosa*), light leaf spot (*Pyrenopeziza brassicae*),

sclerotinia stem rot (*Sclerotinia sclerotiorum)* and dark leaf and pod spot (*Alternaria brassicae* and *Alternaria brassicicola*). The following model was used from Grimmer *et al*., (2014):

$$\sqrt{FDR} = 4.322 - 0.02666l - 0.002357m + c + s$$

Where FDR = first detection of resistance (in years), l = number of latent periods per year, m = molecular complexity of the molecule, c = number of host species (1 to 9 host species = 0, 10+ host species = 0.887) and s = agronomic system (outdoor = 0, protected = -0.649). The R^2 for this model was 60.7% with a standard error of 0.615, the latter of which was used to calculate the standard error for calculated values. The parameters used to calculate the first detection time to resistance for the pathogens mentioned previously are shown in Table 40 in the appendix. The latent period information (number of days at 15°C) was taken from Grimmer *et al.* (2015) for *Alternaria brassisicola* (7 days) and *Pyrenopeziza brassicae* (20 days). Monocyclic pathogens, *Sclerotinia sclerotiorum* and *Leptosphaeria maculans/Leptosphaeria biglobosa* were considered to have 1 disease cycle per year. For *Alternaria brassicae* the information on the number of days for the latent period at 15°C (8 days) was derived from Hong *et al.*, (1996). This, coupled with the likely availability of hosts to infect, was used to derive the number of latent periods per year. For simplicity, the number of latent periods per year for the two *Leptosphaeria* spp*.* were considered to be the same. The values for the latent period for each plant pathogen is shown in Table 40 and molecular complexity of each active ingredient in Table 41 in the Appendix.

3.2. Test which resistance management strategy is most effective at slowing fungicide resistance selection in *P. brassicae*: comparing the application of solo products against mixtures and alternation

Two field experiments were conducted by ADAS in each of two years (harvest years 2017 and 2018), with four experiments completed in the final year (harvest year 2019). In harvest years 2017 and 2018, these were conducted in Yorkshire and Herefordshire on cv. Fencer (2017/18 Recommended List light leaf spot rating of 5 and phoma stem canker rating of 8). In harvest year 2019, two trials were conducted in west Wales on cvs. INV1155 and Phoenix and in Herefordshire on cv. Fencer. In all experiments, fungicides were applied as two spray programmes, with the first in November/December when light leaf spot symptoms were visible in the crop (dependent on weather) and the second in January/February (when reinfection was visible in fungicide treated plots). Products were not chosen to be completely representative of commercial practice, they were designed to test whether resistance management strategies influence the selection for fungicide insensitivity. Similarly, first fungicide applications were done slightly later than would be done commercially to ensure there was disease in the crop for sampling. The preference at the beginning of the project was to use a solo product therefore Vertisan was applied [as full rate equivalent of

Refinzar (160g/L penthiopyrad)] as the alternation and mixture partner in year 1. This strategy has been used successfully in similar experiments, however, given that the product was not formulated for use on oilseed rape, in 2018 and 2019 it was replaced with Refinzar. The products and doses applied to all experiments in harvest year 2017 are outlined in Table 1 and harvest years 2018 and 2019 in Table 2.

Table 1. Treatments as products and rates applied in harvest year 2017.

Trt	Product and fungicide application timing	
	Timing 1 - November/December	Timing 2 - January/February
1	Untreated	Untreated
2	Proline 275 0.63 L/ha	-
3	Proline 275 0.315 L/ha	Proline 275 0.315 L/ha
4	Proline 275 0.315 L/ha	Vertisan 0.4 L/ha
5	Vertisan 0.8 L/ha	Proline 275 0.63 L/ha
6	Proline 275 0.63 L/ha	Vertisan 1.0 L/ha
7	Proline 275 0.315 L/ha + Vertisan 0.8 L/ha	Proline 275 0.315 L/ha + Vertisan 0.8 L/ha
8	Proline 275 0.315 L/ha + Vertisan 0.4 L/ha	Proline 275 0.315 L/ha + Vertisan 0.4 L/ha
9	Proline 275 0.63 L/ha	Vertisan 0.4 L/ha
10	Vertisan 0.4 L/ha	Proline 275 0.63 L/ha

Table 2. Treatments as products and rates applied in harvest years 2018 and 2019.

Trt	Product and fungicide application timing	
	Timing 1 - November/December	Timing 2 - January/February
1	Untreated	Untreated
2	Proline 275 0.63 L/ha	-
3	Proline 275 0.315 L/ha	Proline 275 0.315 L/ha
4	Proline 275 0.315 L/ha	Refinzar 0.5 L/ha
5	Refinzar 1.0 L/ha	Proline 275 0.63 L/ha
6	Proline 275 0.63 L/ha	Refinzar 1.0 L/ha
7	Proline 275 0.315 L/ha + Refinzar 1.0 L/ha	Proline 275 0.315 L/ha + Refinzar 1.0 L/ha
8	Proline 275 0.315 L/ha + Refinzar 0.5 L/ha	Proline 275 0.315 L/ha + Refinzar 0.5 L/ha
9	Proline 275 0.63 L/ha	Refinzar 0.5 L/ha
10	Refinzar 0.5 L/ha	Proline 275 0.63 L/ha
11	Refinzar 0.5 L/ha	Refinzar 0.5 L/ha

The treatments were designed to test and compare tactics to slow resistance selection including mixtures and alternation. Specific treatments and their rationale are described below:

Treatment 1: determine the frequency of *P. brassicae* strains in the local population without fungicide treatment;
Treatments 2 and 3: quantify the effect of splitting the dose on resistance selection;
Treatments 4 to 11: test whether dose, mixture or alternation of different modes of action is the best strategy to slow fungicide resistance selection.

Foliar disease was assessed on 10 plants per plot at 3 different stages: 4 to 8 weeks after Timing 1 application, immediately prior to the Timing 2 application and 4 to 8 weeks after the Timing 2 fungicide application. Disease was monitored weekly from November onwards in Treatments 1 and 3 at each trial site to identify when first symptoms were observed and on which leaf layer. Once clear differences in light leaf spot incidence and severity were observed, all plots were assessed. Additionally, stem disease was assessed on 25 plants per plot prior to harvest and the incidence of stem canker checked on 20 plants from across untreated plots. Twenty infected leaves were taken from plots when symptoms were observed prior to the Timing 2 application. This leaf layer was tagged and subsequent leaf samples were removed 6 to 8 weeks later when new lesions were visible on the newly emerged leaves. Pyrosequencing assays were used as described by Carter et al., (2013) to quantify the change in the percentage of G460S in the resistant pathogen in the population (and hence quantify resistance selection) after treatment with each strategy.

Individual plots were harvested and moistures determined to report yield at 91% dry matter. Refinzar was not registered for use after 30 November 2018. Use after this deadline was covered by an experimental permit supplied by ADAS and seed from all plots receiving an application after this deadline was sent to landfill.

3.3. Isolation and *PbCYP51* gene analysis of *Pyrenopeziza brassicae* strains

Fungal isolates were obtained from diseased oilseed rape leaves with characteristic white conidiomata. In brief, a single pustule was picked into a drop of sterile water using a sterile needle, and the suspension was streaked onto 3% malt extract agar (MEA) and incubated at 15°C for 10 days. Single colonies were then used to establish single-spore cultures and these strains were further tested for azole sensitivity according to Carter *et al*. (2014). DNA was extracted from lyophilized mycelium using a MasterPure™ Yeast DNA kit (Epicentre, USA). PCR reactions to amplify the coding and upstream regulatory (promoter) region of the *PbCYP51* gene were done in 40 µl volumes using Easy A cloning Enzyme (Agilent Technologies, UK). PCR reactions were carried out with 4 µl Easy A cloning buffer (10 x stock), 0.8 µl dNTPs (10mM stock), 32.4 µl PCR grade water, 0.2 µl each of primers LLSCYP51UPF1 (5'-tgtaagtgggatggcgaaagaa-3') and Pb CYP51 R (5'-cgatgatacagagcagcaattcagaa-3') (100µM stock), 0.4 µl Easy A cloning enzyme and 2 µl genomic DNA (20 ng total). PCR was done using a Biometra T3 thermocycler with reaction conditions of: 95°C for 2 mins; 40 cycles of 95°C for 10 secs, 62°C for 20 secs, 72°C for 1 min; 72°C for 5 mins; with a final hold at 4°C. PCR products were visualized by agarose gel electrophoresis to ensure a single PCR amplicon. PCR products were sent to MWG Eurofins (UK) for purification and sequencing using primers LLSCYP51UPF1, Pb CYP51 R, LLSCR1 (5'-acgaatttggttcctgcta-3') and LLSCF1 (5'- caaccctctccttgactcaac-3'). Sequences were analysed using Geneious software and checked for presence of new *PbCYP51* genotypes.

3.4. Quantification of PbCYP51 G460S and S508T in light leaf spot populations

Samples consisting of 20 leaves per plot were taken 6-8 weeks after the first and second fungicide application. Occasionally fewer leaves per sample were processed due to low disease pressure in the field. Upon arrival, lesion rich areas (~ 4 cm^2) covered by white conidiomata were sampled from each leaf. Leaf cuttings were pooled according to plots and frozen at -20°C until further use. Samples, fresh or from freezer, were ground under liquid nitrogen, and genomic DNA was extracted from 50–100 mg of powdered plant tissue sample using a MasterPure™ Yeast DNA kit. DNA was quantified using nanodrop measurements and diluted to 20 ng μl^{-1}. SNP detection Pyrosequencing assays were carried out according to Carter (2013) with some modifications.

First round PCR reactions were done in 10 µl volumes containing: 1 µl Easy A cloning buffer (10 x stock), 0.2 µl dNTPs (10 mM stock), 6.7 µl PCR grade water, 0.05 µl each of primers LLSSHORTF2 (5'-ttatttccctgatccgatgaagt-3') and LSSR2 (5'-cccgccagactatgcacat-3'), each (100 µM stocks), 0.1 µl Easy A cloning enzyme, and 2 µl genomic DNA (40 ng total for field DNA samples, 20 ng for reference *P. brassicae* isolates). PCR was done using a Biometra T3 thermocycler with reaction conditions of: 95°C for 2 mins; 40 cycles of 95°C for 10 secs, 62°C for 20 secs, 72°C for 1 min; 72°C for 5 mins; with a final hold at 4°C. PCR products (2 µl) were subsequently visualized by agarose gel electrophoresis to ensure a simple PCR amplicon.

The second PCR round (nested PCR) was carried out in 50 ul reactions using GoTaq Flexi (Promega). Reactions contained 10 µl GoTaq Flexi buffer, 4 µl $MgCl_2$ solution (25 mM stock), 1 µl dNTPs (10 mM stock), 0.25 µl each of forward primer and biotinylated reverse primer (100 µM stocks), 0.25 µl GoTaq G2 Flexi DNA polymerase (5 U ul^{-1} stock), 31.75 µl PCR grade water and 2.5 µl (1:50 diluted) PCR product template from 1st round PCR. PCR was done using a Biometra T3 thermocycler with reaction conditions of: 94°C for 2 mins; 40 cycles of 94°C for 30 secs, 63°C for 30 secs, 72°C for 45 secs; 72°C for 5 mins; with a final hold at 4°C. PCR products (2 µl) were subsequently visualized by agarose gel electrophoresis to ensure the correct PCR amplicon was present.

Pyrosequencing runs were done using a Pyromark ® Gold 96 reaction kit (Qiagen) on a Pyromark PSQ MA96 instrument (Biotage) according to the method described by Carter (2013). All samples were screened in duplicate using a sequencing primer and the mean values taken. Reference *P. brassicae* isolates of known wild type, G460S and S508T genotype were included in each run as controls, along with a no-template water control. Assays were used to quantify the change in the percentage of mutations (G460S and S508T) in the pathogen population (and hence quantify resistance selection) before and after treatment with each strategy and compared with untreated populations.

3.5. Economic analysis of fungicide anti-resistance management strategies for the industry

Eighteen industry-funded oilseed rape field trials were conducted in each year in Herefordshire, Ceredigion and North Yorkshire over 3 years to evaluate the economics of current fungicide programmes compared with fungicide programmes designed around generic resistance management principles. Fungicide treatments targeting light leaf spot as a two-spray programme were applied; the first application (Timing 1) was applied in November and a second application (Timing 2) when symptoms were first seen from January onwards. There were four core treatments which were included in all the industry partner trials to allow direct comparisons across sites and seasons (Table 2). Only data derived from the core treatments are presented in this report. An economic analysis of the core treatments, using yields from all trials, was carried out to determine whether these strategies, which were considered to be effective resistance management strategies, were cost effective for growers. Products tested included Orius (tebuconazole), Proline (prothioconazole), Folicur (tebuconazole), Refinzar (penthiopyrad and picoxystrobin), Filan (boscalid) and Propulse (fluopyram and prothioconazole). These were grouped for the analysis into the four treatment strategies and analysed together. All were applied at 50% of the recommended label dose. The prices per application for spraying (per ha: £12.85) and product (price dependent on the product applied) was taken from Nix 2019 or another supplier as required. There was no adjustment in the cost per ha for spraying for multiple crop protection products at the same time e.g. if the fungicide was applied at the same time as a herbicide.

Table 3. Core treatments to test for efficacy of programmes to slow resistance selection.

Trt	Product and fungicide application timing	
	Timing 1	Timing 2
1	Untreated	Untreated
2	Azole	Azole
3	Alternative mode of action group (e.g. SDHI)	Azole
4	Azole + alternative mode of action group	Azole + alternative mode of action group

4. Results

4.1. Determine the risk of fungicide resistance affecting fungicides used to control oilseed rape diseases

Out of the four pathogens investigated *Sclerotinia sclerotiorum* was predicted, on average, to take the longest (~18 years) to become resistant to the modes of action investigated (Table 4). The first detection of resistance (FDR), on average, for all modes of action for *Leptosphaeria* spp. and *Pyrenopeziza brassicae* was similar and predicted to take approximately 7 and 9 years respectively. Although not a widespread problem in oilseed rape, *Alternaria* spp., was expected to develop resistance against a range of modes of action after ~7 years. There were similarities in the predicted FDR for SDHI and DMI chemistry (~11 years), with QoI fungicides predicted to develop resistance in the shortest time (9.6 years) although this difference over all was small.

Note that these FDR values are predictions, based on associations between traits and speed of resistance development. Resistance evolution includes random elements, which can substantially affect the actual time taken for resistance to develop. The predicted values should not be over-interpreted, but give an indication of relative risk.

Table 4. Time to the first detection of resistance (FDR) in years for four oilseed rape pathogens and the four main modes of action applied to oilseed rape in the UK.

	Pathogen				
Mode of action	*Alternaria* spp.	*Leptosphaeria* spp.	*Sclerotinia sclerotiorum*	*Pyrenopeziza brassicae*	*Average FDR (mode of action)*
SDHI	6.9	10.9	17.5	9.0	*11.1*
DMI	7.4	11.2	18.3	9.6	*11.6*
QoI	5.7	9.4	15.6	7.7	*9.6*
MBC	8.4	12.7	19.8	10.7	*12.9*
Average FDR (pathogen)	*7.1*	*11.1*	*17.8*	*9.3*	

4.2. Azole sensitivity phenotyping and genotyping of *P. brassicae* strains

Strains from three isolate collections were further characterised during 2018 using *PbCYP51* gene analysis which included both the regulatory region (promoter) and the coding sequence. The first collection of 27 strains came from different locations in the UK during the period 2003-2011. The second collection contained 34 strains that originated from a single winter oilseed rape field in Northumberland in 2016. *PbCYP51* gene sequence analysis showed that wild-type strains were present during 2003-2007, G460S was detected in a 2003 strain and was found at a higher frequency (30%) then S508T (19%) which was first detected in 2007. *PbCYP51* promoter inserts were only detected in strains isolated in 2011 and one strain carried a combination of a 151 base pair (bp) promoter insert and S508T (Figure 1).

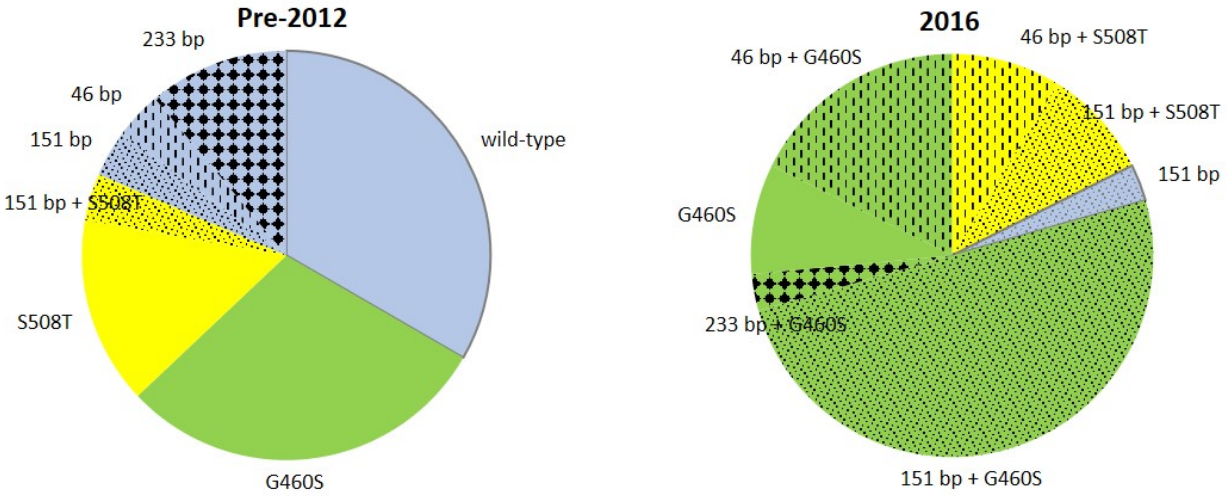

Figure 1. *PbCYP51* gene sequence analysis, including promoter region, of *P. brassicae* strains isolated pre-2012 (n = 27) and in 2016 (n = 34).

Only 3 out of the 34 strains tested in 2016 carried no *PbCYP51* promoter inserts. Thirty out of the 34 strains carried a PbCYP51 target alteration, G460S or S508T, in combination with a promoter insert of 46, 151 or 233 bp. Six strains (18%) carried S508T in combination with a 46 or 151 bp insert, 71% carried G460S with promoter inserts of 46, 151 or 233 bp, 9% carried G460S without promoter inserts and 3% (1 strain) had no target site mutation but a 151 bp promoter insert. Strains with G460S and a 151 bp promoter insert were most common at 50% (17 out of 34 strains). Carter *et al*. (2014) showed that the different promoter inserts are based on duplications of the *PbCYP51* promoter region, all having a duplicated stretch of 46 bp in common. Under exposure to azoles, the expression of *PbCYP51* is 4 to 50-fold higher in strains carrying promoter inserts.

Additional *PbCYP51* characterisation of a selection of strains isolated in 2018 showed that strains carrying G460S in combination with promoter inserts of 44, 46, 151 or 210 bp (Figure 2) have become most common.

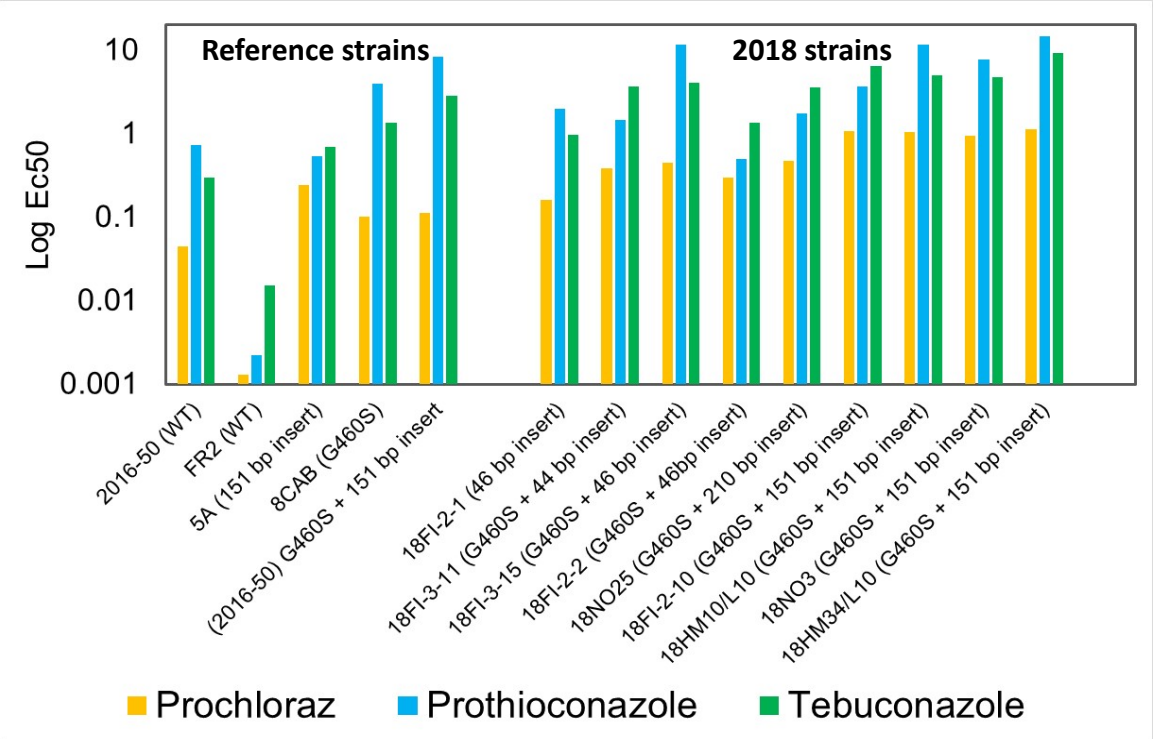

Figure 2. Genotype-to-phenotype relationship of isolates carrying different PbCYP51 variants. Strains marked with FI, NO and HM originated from Fife, Northumberland and Yorkshire, respectively.

The combination of *PbCYP51* mutations and promoter inserts resulted in the most azole insensitive phenotypes. There was a high level of cross-resistance between the three azoles (prochloraz, prothioconazole and tebuconazole) tested.

4.3. Detection of PbCYP51 G460S and S508T using SNP detection pyrosequencing assays

Pyrosequencing was carried out using a nested PCR approach according Carter (2013) with some modifications to improve the sensitivity and specificity. The final assay for detection for G460S was carried out with forward primer 460F1 (5'-gagtccatatcttccattc-3'), biotinylated reverse primer 460BIOR1 (5'-cgaactgctctccgatacacctat-3') and sequencing primer 460S1 (5'-gagtccatatcttccattc-3'). Sequence to analyse was G/AGTGCCGGCAGACATAGGTGTATCGG and assays were performed on the PSQ MA96 (Biotage) using the nucleotide dispensation order CAGCTGCGC. The allele frequencies were determined using the PyroMark ID SNP run software. Possible theoretical outcomes of the Pyrograms for homogeneous (100%) and heterogeneous populations (50% of each allele) of *P. brassicae strains* carrying G460S (ggt into agt) are shown in Figure 3.

G/G

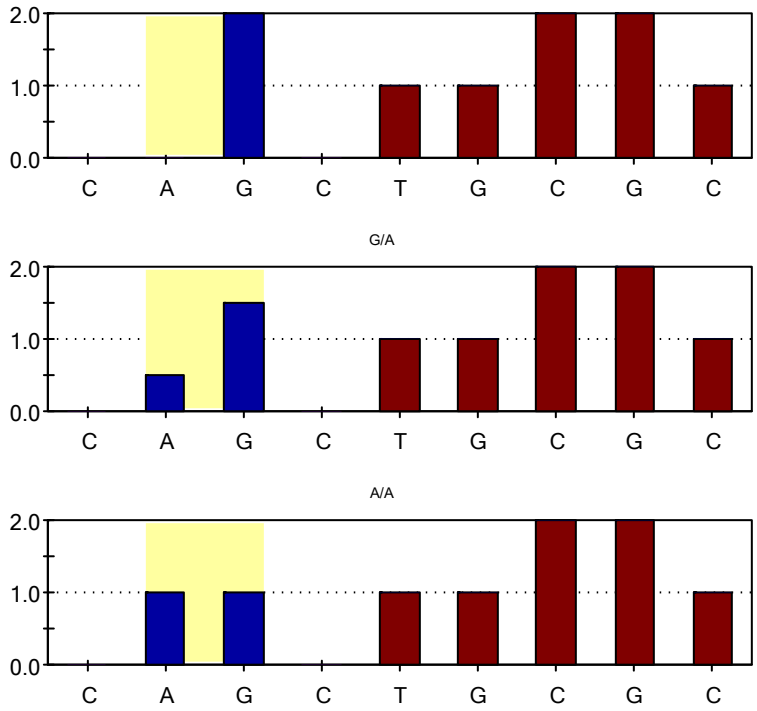

Figure 3. Selected theoretical outcomes for detection of PbCYP51 G460S (ggt to agt) in *Pyrenopeziza brassicae* field populations based on homogeneous or heterogeneous (50% mixtures) presence of mutations in test samples. G/AGTGCCGGCAGACATAGGTGTATCGG is the sequence to analyse in the Pyromark ID SNP run software.

Unfortunately, the assay to detect PbCYP51 S508T using SNP detection pyrosequencing was not reliable or didn't work with several primer sets that we tested, including the set reported by Carter (2013) (data not shown). In addition, we also attempted one allele-specific real-time PCR design without success. Because strains carrying PbCYP51 S508T are outcompeted in current field populations by G460S strains (see Figures 1 and 2), which are generally less azole sensitive, no further attempts were undertaken and we focused on quantitative G460S detection in leaf samples.

4.4. Test which resistance management strategy is most effective at slowing fungicide resistance selection in *P. brassicae*: comparing the application of solo products against mixtures and alternation

Eight experiments were conducted to determine whether different fungicide resistance management strategies had an impact on the proportion of strains with the G460S mutation over harvest years 2017, 2018 and 2019. Drilling dates, fungicide application dates and leaf sampling dates are shown in Table 5 and Table 6. Disease data as well as the proportion of strains detected with the G460S mutation are presented alongside light leaf spot incidence and severity results for individual sites. Pod disease was present (between 5.1 and 14.2% pod area affected; average of 8.5% pod area affected) with no statistically significant differences between treatments for most experiments (data not shown).

Table 5. Drilling date and fungicide application dates for all eight experiments.

Location and harvest year	Drilling date	Fungicides applied	
		Timing 1	Timing 2
Yorkshire 2017	28 August 2016	15 December 2016	3 March 2017
Herefordshire 2017	2 September 2016	28 November 2016	15 March 2017
Yorkshire 2018	2 September 2017	21 December 2017	9 April 2018
Herefordshire 2018	4 September 2017	21 December 2017	23 March 2018
Pembrokeshire 2019	7 September 2018	10 January 2019	26 March 2019
Ceredigion 2019	30 August 2018	9 January 2019	20 March 2019
Herefordshire A 2019	28 August 2018	10 December 2019	18 February 2019
Herefordshire B 2019	30 August 2018	12 October 2019	18 February 2019

Table 6. Leaf sampling dates for all eight experiments.

Location and harvest year	Leaves sampled	
	Timing 1	Timing 2
Yorkshire 2017	No sample*	2 May 2017
Herefordshire 2017	16 February 2017**	18 April 2017**
Yorkshire 2018	19 March 2018	23 May 2018**
Herefordshire 2018	23 March 2018	8 May 2018
Pembrokeshire 2019	15 March 2019	13 May 2019
Ceredigion 2019	9 March 2019	14 May 2019
Herefordshire A 2019	15 February 2019	3 April 2019
Herefordshire B 2019	15 February 2019	3 April 2019

*no or low disease present. **pyrosequencing data not available.

At the Herefordshire site in 2017, fungicides were applied on 28 November 2016 and 15 March 2017, with leaf samples taken on 16 February and 18 April 2017. Light leaf spot was assessed for the first time in the trial on 16 February 2017 and, although there was a trend for lower levels of light leaf spot incidence and severity in some treatments (from the Timing 1 application only), these differences were not statistically significantly different (Table 7). At the second disease assessment

on 15 March, both the incidence and severity of light leaf spot were significantly decreased by all Timing 1 applications of Proline regardless of the dose applied compared to the untreated control. Four weeks later on the 18 April, there were no statistically significant differences between the untreated control and any other treatment for light leaf spot incidence (between 68 and 98% plants affected in treated plots; untreated control 93%) and severity (between 0.25 and 0.55% leaf area affected; untreated control 0.82% leaf area affected).

Table 7. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Herefordshire site in 2017. Fungicides were applied on 28 November 2016 and 15 March 2017.

Treatment	Prior to sampling* 16 February 2017		First sample* 15 March 2017		Second sample 18 April 2017	
	inc	sev	inc	sev	inc	sev
Untreated	48	0.39	73	1.43	93	0.82
Proline 275 0.63 L/ha Timing 1 only	30	0.13	35	0.61	70	0.49
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	50	0.29	38	0.24	68	0.25
Proline 275 0.315 L/ha fb Vertisan 0.4 L/ha	43	0.20	43	0.42	80	0.60
Vertisan 0.8 L/ha fb Proline 275 0.63 L/ha	43	0.50	68	0.69	93	0.63
Proline 275 0.63 L/ha fb Vertisan 0.8 L/ha	5	0.21	13	0.06	68	0.31
Proline 275 0.315 L/ha + Vertisan 0.8 L/ha x 2	30	0.15	43	0.41	75	0.46
Proline 275 0.315 L/ha + Vertisan 0.4 L/ha x 2	25	0.20	38	0.48	73	0.44
Proline 275 0.63 L/ha fb Vertisan 0.4 L/ha	33	0.23	23	0.20	88	0.40
Vertisan 0.4 L/ha fb Proline 275 0.63 L/ha	10	0.52	73	1.13	98	0.55
Fpr.	ns	ns	<0.001	<0.001	ns	ns
SED (27df)			8.9	0.221		
LSD (*P*=0.05)			18.2	0.453		

*effects of Timing 1 treatments only.

Due to the low levels of light leaf spot infection in the Herefordshire trial, only a few samples tested positive in the first PCR round for pyrosequencing (samples taken on 16 February). Because of this, we decided to further focus on the molecular characterisation of strains instead. At this site, there were statistically significant differences for yield, with responses ranging from 0.15 to 0.69 t/ha (Table 8). Treatments that significantly improved yield relative to the untreated control included Proline applied at the full recommended rate in the autumn only (5.81 t/ha), Proline applied as a two spray programme at 50% of the recommended label dose at both timings (5.77 t/ha), Proline applied in the autumn at 50% of the recommended label dose followed by Vertisan applied at 50% of the recommended label dose (equivalent dose of penthiopyrad in Refinzar) in the spring (5.65 t/ha), the full recommended label dose of Proline in the autumn followed by the full dose of Vertisan (as the equivalent dose of penthiopyrad in Refinzar) in the spring (5.68 t/ha) and a tank mixture of Proline and Vertisan of 50% of the recommended label rates for both products and applied at both the autumn and spring timing (5.98 t/ha).

Table 8. Yield (t/ha) from the Herefordshire trial in 2017.

Treatment	Yield 91% Dry Matter 19/07/2017
Untreated	5.29
Proline 275 0.63 L/ha Timing 1 only	5.81
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	5.77
Proline 275 0.315 L/ha fb Vertisan 0.4 L/ha	5.65
Vertisan 0.8 L/ha fb Proline 275 0.63 L/ha	5.48
Proline 275 0.63 L/ha fb Vertisan 0.8 L/ha	5.68
Proline 275 0.315 L/ha + Vertisan 0.8 L/ha x 2	5.98
Proline 275 0.315 L/ha + Vertisan 0.4 L/ha x 2	5.37
Proline 275 0.63 L/ha fb Vertisan 0.4 L/ha	5.49
Vertisan 0.4 L/ha fb Proline 275 0.63 L/ha	5.44
Fpr.	0.010
SED (27df)	0.172
LSD (*P*=0.05)	0.353

At the Yorkshire site in 2017, fungicides were applied on 15 December 2016 and 3 March 2017, with leaf samples taken on 23 February 2017 and 2 May 2017. Light leaf spot was assessed for the first time in the trial on 16 February 2017 and fungicide treatment (Timing 1 only at this point) significantly decreased disease incidence for the Proline and some Proline + Vertisan treatments compared to the untreated control, but not the Vertisan only treatments (Table 9). Differences in light leaf spot severity were also observed and followed a similar pattern, however, these results were marginally not statistically significantly different. At the second disease assessment, which was done immediately prior to the second fungicide application, there was no effect of fungicide treatment on light leaf spot incidence, with 88% plants affected in the untreated control and a range of 60 to 73% plants affected depending on the fungicides applied. Despite an earlier difference between the two products, all Timing 1 fungicide applications of Proline and Vertisan were providing significant reductions in light leaf spot severity compared to the untreated control. At the final disease assessment on 12 April, there were no statistically significant effects of fungicide treatment on light leaf spot incidence and severity compared to the untreated control. Pod disease was present (between 5.1 and 14.2% pod area affected; average 8.5% pod area affected) with no statistically significant differences between treatments (data not shown).

Table 9. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Yorkshire site in 2017. Fungicides were applied on 15 December 2016 and 3 March 2017.

Treatment	Prior to sampling*		First sample*		Second sample	
	16 February 2017		3 March 2017		12 April 2017	
	inc	sev	inc	sev	inc	sev
Untreated	90	2.47	88	2.21	100	6.43
Proline 275 0.63 L/ha Timing 1 only	45	1.28	68	0.74	98	5.53
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	68	1.41	63	0.63	78	3.46
Proline 275 0.315 L/ha fb Vertisan 0.4 L/ha	68	1.28	58	1.18	100	5.63
Vertisan 0.8 L/ha fb Proline 275 0.63 L/ha	93	2.54	73	0.47	100	5.91
Proline 275 0.63 L/ha fb Vertisan 0.8 L/ha	40	0.40	58	0.61	98	5.89
Proline 275 0.315 L/ha + Vertisan 0.8 L/ha x 2	75	1.65	60	0.64	88	2.18
Proline 275 0.315 L/ha + Vertisan 0.4 L/ha x 2	58	1.18	60	0.66	85	3.75
Proline 275 0.63 L/ha fb Vertisan 0.4 L/ha	60	1.40	60	0.28	90	4.44
Vertisan 0.4 L/ha fb Proline 275 0.63 L/ha	85	1.87	80	0.95	98	7.29
Fpr.	<0.001	0.052	ns	0.024	ns	ns
SED (27df)	9.467	0.598		0.470		
LSD (*P*=0.05)	19.425	1.232		0.965		

*effects of Timing 1 treatment only.

The proportion of the G460S mutation was lowest in the untreated control (60.1%) and where the lowest dose of azole had been applied (64.5%), however, these differences were not statistically significantly different from other treatments tested. There were small yield responses relative to the untreated control reported in this trial ranging from 0.05 to 0.32 t/ha, however no statistically significant differences existed between the untreated control or different treatments (Table 10).

Table 10. The proportion of the G460S mutation on the second sampling date and yield from the Yorkshire trial in 2017.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
	No sample	2 May 2017	10 August 2017
Untreated	-	60.9	2.59
Proline 275 0.63 L/ha Timing 1 only	-	88.4	2.73
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	-	81.4	2.64
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	-	64.5	2.64
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	-	76.8	2.70
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	-	76.9	2.91
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	-	92.8	2.80
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	-	77.7	2.74
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	-	77.2	2.69
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	-	79.7	2.66
Fpr.	-	ns	ns
SED (27df)	-		
LSD (*P*=0.05)	-		

In 2018 and 2019, Refinzar was used as the non-azole fungicide option. At the Herefordshire site in 2018, fungicides were applied on 21 December 2017 and 23 March 2018, with leaf samples taken on 23 March 2018 and 8 May 2018. Light leaf spot was assessed for the first time on 23 March 2018 and only two treatments statistically decreased light leaf spot incidence (Refinzar 1.0 L/ha and Proline 0.315 L/ha + Refinzar 1.0 L/ha) compared to the untreated control (effects from the Timing 1 application only) (Table 11). At the second disease assessment on 12 April, effects from the Timing

2 fungicides applied in March were just starting to be visible, with the incidence of light leaf spot lower than the untreated control for most two spray programmes. Four weeks later on the 11 May, incidence of light leaf spot in all treatments was almost 100%. There were statistically significant reductions in light leaf spot severity for all fungicide programmes tested compared to the untreated control. Proline 0.315 L/ha + Refinzar 1.0 L/ha applied as a tank mixture twice was significantly more effective than all the other fungicide programmes tested (3.25% leaf area affected).

Table 11. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Herefordshire site in 2018. Fungicides were applied on 21 December 2017 and 23 March 2018.

Treatment	Prior to sampling* 23 March 2018		First sample* 12 April 2018		Second sample 11 May 2018	
	inc	sev	inc	sev	inc	sev
Untreated	95	0.81	83	1.33	100	9.83
Proline 275 0.63 L/ha Timing 1 only	85	0.84	88	0.88	100	6.05
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	83	0.82	68	0.87	100	5.60
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	90	0.90	78	0.93	100	4.63
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	73	0.60	65	0.24	100	4.90
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	88	0.69	73	0.68	100	4.85
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	50	0.30	63	0.39	95	3.25
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	85	0.65	60	0.46	100	5.19
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	83	0.50	75	1.00	100	3.98
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	90	1.02	90	0.97	100	6.80
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	83	0.52	75	0.89	100	5.10
Fpr.	0.010	ns	0.028	ns	ns	<0.001
SED (30df)	9.935		8.9			1.087
LSD (*P*=0.05)	20.291		18.3			2.219

*effects of Timing 1 treatment only.

Significant decreases in stem lesions caused by light leaf spot were observed for a range of treatments (Table 12). Only small yield responses to fungicide treatment relative to the untreated control were observed in this trial (untreated control 3.92 t/ha; yield responses from -0.06 to 0.20 t/ha). The proportion of the G460S mutation in the untreated control at both the first and second sampling date was high (63 and 79% respectively) and there were no statistically significant effects of fungicide treatment on the proportion of this mutation.

Table 12. The proportion of the G460S mutation on the first and second sampling date, stem disease severity and yield from the Herefordshire trial in 2018.

Treatment	Proportion of G460S mutation		Stem disease	Yield
	First sample	Second sample	Percentage stem area affected	91% Dry Matter
	23 March 2018	8 May 2018	18 June 2018	22 July 2019
Untreated	62.7	78.6	3.9	3.92
Proline 275 0.63 L/ha Timing 1 only	77.0	83.0	3.3	4.03
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	58.4	64.2	3.3	3.93
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	69.4	71.6	3.1	3.99
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	69.6	79.6	2.5	3.85
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	81.7	86.6	2.7	3.94
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	82.7	88.8	2.2	4.12
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	59.5	57.5	2.4	3.86
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	59.9	65.4	2.3	3.97
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	73.8	80.9	3.2	4.05
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	62.1	68.1	2.9	3.89
Fpr.	ns	ns	0.011	ns
SED (30df)			0.42	
LSD (*P*=0.05)			0.86	

At the Yorkshire site in 2018, fungicides were applied on 21 December 2017 and 9 April 2018, with leaf samples taken on 23 March and 8 May 2018. Light leaf spot was assessed for the first time in the trial on 21 February 2018 and there were statistically significant decreases in light leaf spot severity for some fungicide treatments compared to the untreated control, however, the disease severity was very low (0.08 and 0.33% leaf area affected) (Table 13). At this point in the trial, only Timing 1 fungicides had been applied and it is likely that these differences were due to variation within the trial area rather than treatment effects, as many of the treatments were the same. The final light leaf spot assessment was completed on 23 May and light leaf spot incidence was 100% in all treatments in the trial. There was no difference in disease severity between the untreated control or fungicide treatments. Light leaf spot on the pods was high, between 23 and 31% pod area affected with no statistically significant differences between treatments (Table 14).

Table 13. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Yorkshire site in 2018. Fungicides were applied on 21 December 2017 and 9 April 2018.

Treatment	First sample*		Second sample	
	21 February 2018		23 May 2018	
	inc	sev	inc	sev
Untreated	28	0.28	100	7.78
Proline 275 0.63 L/ha Timing 1 only	23	0.31	100	7.33
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	15	0.14	100	7.60
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	25	0.33	100	6.33
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	20	0.18	100	7.45
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	8	0.08	100	5.58
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	15	0.14	100	6.20
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	25	0.25	100	6.65
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	18	0.16	100	6.55
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	18	0.18	100	6.18
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	20	0.20	100	6.25
Fpr.	ns	0.039	-	ns
SED (30df)		0.074	-	
LSD (*P*=0.05)		0.151	-	

*effects of Timing 1 treatment only.

There was a significant effect of fungicide treatment on yield (Table 14). There was no statistically significant yield improvement where azoles were applied as a single autumn spray or a split programme at 50% of the recommended label dose. Most treatments where products were alternated improved yields but were not statistically significant from the untreated control. The treatments where tank mixtures were applied were the treatments with highest yields (4.12 and 4.19 t/ha). The proportion of the G460S mutation was between 89 and 96% regardless of the treatment applied.

Table 14. The proportion of the G460S mutation on the first and second sampling date, pod disease and yield from the Yorkshire trial in 2018.

Treatment	Proportion of G460S mutation		Pod disease	Yield
	First sample	Second sample	Percentage pod area affected	91% Dry Matter
	19 March 2018	23 May 2018*	11 July 2018	4 August 2018
Untreated	88.8	-	30.8	3.74
Proline 275 0.63 L/ha Timing 1 only	90.4	-	28.5	3.92
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	90.5	-	30.0	3.88
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	95.2	-	28.2	4.04
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	96.6	-	30.2	3.92
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	94.5	-	29.0	4.05
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	80.9	-	27.8	4.19
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	90.8	-	29.1	4.12
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	96.4	-	28.0	3.98
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	87.6	-	28.5	3.95
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	89.3	-	27.8	4.04
Fpr.	ns		ns	0.050
SED (29df)				0.121
LSD (*P*=0.05)				0.248

*data not available.

As pod disease was reported at a high level, a regression of disease severity against yield was carried out to determine whether this had any association with yield across the treatments (Figure 1). There was a significant regression (P=0.012: R^2=47.3) suggesting that high levels of pod disease are associated with yield loss.

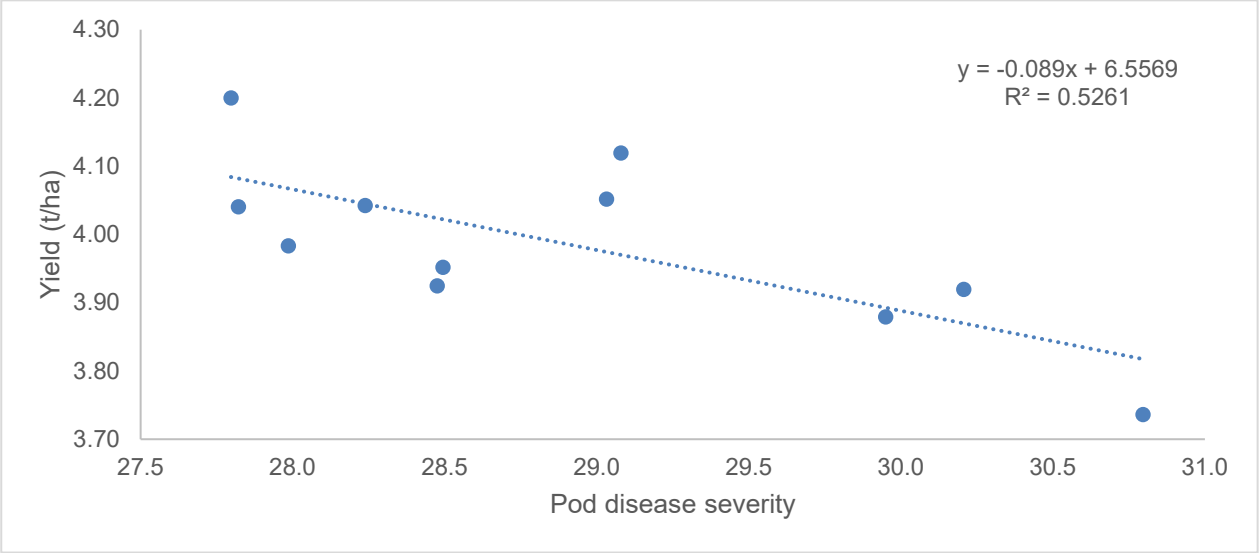

Figure 1. Regression of pod disease severity (as the percentage of pod area affected) and yield at the Yorkshire site in harvest year 2018.

In 2019, four trials were conducted; two in Herefordshire and two in Wales (Pembrokeshire and Ceredigion). The shift from Yorkshire to Wales was due to the very high levels of the G460S mutation detected in the trial in Yorkshire in 2018 (>90%).

At the Herefordshire A site in harvest year 2019, fungicides were applied on 10 December 2018 and 18 February 2019, with leaf samples taken on 5 February and 3 April. Light leaf spot was assessed for the first time in the trial on 8 February and there was no significant decrease in light leaf spot incidence and severity compared to the untreated control (Table 15). A significant effect of fungicide on light leaf spot incidence was observed at the disease assessment on 11 March for five treatments compared to the untreated control: Proline applied at 50% of the recommended label dose twice (48% plants affected), Refinzar applied at the full recommended label dose twice (43% plants affected), Proline 50% dose + Refinzar full dose applied as a tank mix twice (48% plants affected) and Refinzar applied at 50% of the recommended label dose first followed by either Refinzar (half rate) or Proline (full rate) (38 and 46% plants affected). No other significant effects of fungicides on disease incidence or severity were observed at the final assessment on 12 April.

Table 15. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Herefordshire A site in 2019.

Treatment	Prior to sampling*		First sample		Second sample	
	8 February 2019		11 March 2019		12 April 2019	
	inc	sev	inc	sev	inc	sev
Untreated	65	0.41	65	0.11	100	0.86
Proline 275 0.63 L/ha Timing 1 only	35	0.31	58	0.09	95	0.67
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	63	0.19	48	0.07	95	0.65
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	53	0.23	63	0.09	95	0.65
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	43	0.20	43	0.06	88	0.30
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	50	0.25	55	0.10	95	0.38
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	55	0.20	48	0.08	93	0.42
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	45	0.15	63	0.08	90	0.47
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	60	0.33	65	0.11	90	0.46
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	60	0.22	46	0.06	95	0.49
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	45	0.18	38	0.06	98	0.36
Fpr.	ns	ns	0.029	ns	ns	ns
SED (30df)			8.5			
LSD (*P*=0.05)			17.3			

*effects of Timing 1 treatment only.

There were yield responses to fungicide treatment of between -0.04 and 0.31 t/ha compared to the untreated control, however, these differences were not statistically significantly different (Table 16). There was a significant effect of fungicide treatment on the proportion of the G460S mutation at this site. As the sample was taken on the 15 February, the effects would relate to the Timing 1 fungicide application only and the proportion of G460S was 86% in the untreated control. Where Proline had been applied at the full recommended label dose, the proportion of the G460S mutation was between 75 and 80%. Where it was applied at half the recommended dose the proportion ranged from 55 to 93%. Where Refinzar was applied, the proportion of the mutation was between 62 and 66% regardless of the dose applied. For tank mixes, the proportion of G460S ranged from 59 to 75% of the population. No significant effects were observed at the second sample on 3 April.

Table 16. The proportion of the G460S mutation on the first and second sampling date and yield from the Herefordshire A trial in 2019.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
	15/02/2019	03/04/2019	03/08/2019
Untreated	86.4	69.6	3.71
Proline 275 0.63 L/ha Timing 1 only	80.4	69.9	3.82
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	92.8	67.7	3.80
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	55.3	74.7	4.02
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	61.8	68.5	3.81
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	80.3	68.2	3.95
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	75.0	72.4	4.02
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	59.0	71.5	3.89
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	74.8	70.2	3.67
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	65.0	70.5	3.84
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	66.0	64.2	3.82
Fpr.	0.036	ns	ns
SED (30df)	11.12		
LSD (*P*=0.05)	22.70		

At the Herefordshire B site in harvest year 2019, fungicides were applied on 12 October 2018 and 18 February 2019, with leaf samples taken on 15 February and 3 April. Light leaf spot was assessed for the first time in the trial on 13 February and there was a statistically significant decrease in light leaf spot incidence compared to the untreated control for most treatments (Table 17). At this point, only the Timing 1 fungicides had been applied and the most effective treatments were those where both products were applied in mixtures (18 to 25% plants affected), followed by Proline applied alone (23 to 43% plants affected) and then Refinzar alone (40 to 53% plants affected). Disease incidence and severity was low when the trial was assessed prior to the first leaf sample being taken on 11 March, with no statistically significant differences between treatments. At the final assessment on 2 April, light leaf spot incidence and severity had increased compared to the 11 March assessment, with significant decreases in light leaf spot incidence and severity compared to the untreated control.

There was a similar pattern for incidence compared to the first assessment on 13 February with significant reductions in light leaf spot incidence relative to the untreated control (65% plants affected). The most effective treatments were the tank mixtures of Proline and Refinzar (18% plants affected), followed by Proline at Timing 2 (30 to 43% plants affected) and then Refinzar at Timing 2 (25 to 63% plants affected). The Timing 1 only treatment was also still effective, with 33% plants affected by light leaf spot compared to 65% in the untreated control. Despite low disease levels, most treatments significantly decreased light leaf spot severity relative to the untreated control (between 0.02 and 0.08 leaf area affected), with the exception of the 50% dose of Proline only at both timings (0.12% leaf area affected) and 50% dose of Proline followed by 50% dose of Refinzar as an alternation treatment (0.20% leaf area affected).

Table 17. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Herefordshire B site in 2019.

Treatment	Prior to sampling*		First sample		Second sample	
	13 February 2019		11 March 2019		2 April 2019	
	inc	sev	inc	sev	inc	sev
Untreated	65	0.81	10	0.02	65	0.23
Proline 275 0.63 L/ha Timing 1 only	38	0.29	8	0.01	33	0.06
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	33	0.19	10	0.02	43	0.12
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	43	0.25	20	0.03	63	0.20
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	53	0.37	13	0.01	30	0.06
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	43	0.23	13	0.01	33	0.05
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	18	0.34	13	0.02	18	0.03
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	25	0.12	13	0.01	18	0.02
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	23	0.11	15	0.02	25	0.05
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	43	0.38	18	0.02	38	0.08
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	40	0.39	18	0.02	60	0.03
Fpr.	0.035	ns	ns	ns	0.003	0.011
SED (30df)	12.6				12.7	0.069
LSD (*P*=0.05)	25.7				26.0	0.140

*effects of Timing 1 treatment only.

There were no significant effects of fungicide treatment on yield, however, this is unsurprising given the low levels of disease reported. Yield responses ranged from -0.03 t/ha (Proline applied at Timing 1 only) to 0.38 t/ha (Proline at 50% recommended label dose applied at Timing 1 and Timing 2).

The proportion of strains with the G460S mutation was high at the first sampling date on 15 February in the untreated control (83.4%), with the lowest proportions of the mutation in treatments where no azoles were included in the programme (61.1%) and 50% dose Proline at Timing 1 followed by 50% dose Refinzar at Timing 2 (58.6%). There were, however, no statistically significant differences between the different treatments on the frequency of the G460S mutation. At the second sampling date on 3 April, the proportion of the G460S mutation in plots treated with Proline (azole only) was nearly 90%, for alternation treatments between 76 and 90% and Refinzar (non-azole only) 64% but these differences were not statistically significantly different.

Table 18. The proportion of the G460S mutation on the first and second sampling date and yield from the Herefordshire B trial in 2019.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
	15 February 2019	3 April 2019	8 August 2019
Untreated	83.2	79.8	3.70
Proline 275 0.63 L/ha Timing 1 only	84.5	89.3	3.67
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	73.7	89.6	4.08
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	58.6	79.5	3.75
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	90.2	90.3	4.07
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	73.7	78.6	3.86
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	66.7	74.7	3.89
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	85.5	74.1	4.07
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	81.4	78.3	3.89
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	83.1	75.8	3.78
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	61.1	63.8	4.03
Fpr.	ns	ns	ns
SED (30df)			
LSD (*P*=0.05)			

At the Pembrokeshire site in harvest year 2019, fungicides were applied on 10 January and 26 March 2019, with leaf samples taken on 15 March and 13 May. Light leaf spot was assessed for the first time in the trial on 12 March prior to sampling and no significant differences between treatments were observed (Table 19). At the second disease assessment on 19 April, 24 days after the Timing 2 fungicide application, significant differences in light leaf spot incidence and severity were observed despite relatively low levels reported. Light leaf spot incidence was similar to the untreated control (45% plants affected) where Proline had been applied at Timing 1 only (48% plants affected).

Treatments that decreased light leaf spot incidence significantly compared to the untreated control included Proline applied at 50% of the recommended label dose at Timing 1 and Timing 2 (25% plants affected), Proline alternated with Refinzar, regardless of dose applied (23 to 25% plants affected), Proline + Refinzar at 50% recommended label dose applied as a tank mixture at Timing 1 and Timing 2 (20% plants affected) and Refinzar applied at both Timing 1 and Timing 2 (23% plants

affected). The same pattern was observed for light leaf spot severity, with the exception of Proline applied at 50% of the recommended label dose at Timing 1 and Timing 2, which was marginally not significantly different (0.55% leaf area affected) compared to the untreated control (1.05% leaf area affected). Despite a general trend towards lower severity of light leaf spot at the final assessment on 10 May, there were no statistically significant differences between treatments.

Table 19. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Pembrokeshire site in 2019.

Treatment	Prior to sampling* 12 March 2019		First sample 19 April 2019		Second sample 10 May 2019	
	inc	sev	inc	sev	inc	sev
Untreated	54	1.03	45	1.05	100	7.00
Proline 275 0.63 L/ha Timing 1 only	33	0.28	48	0.98	100	5.53
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	48	0.69	25	0.55	88	5.63
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	52	0.73	38	0.90	100	6.28
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	33	0.39	53	1.00	85	5.83
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	54	1.10	23	0.38	100	5.98
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	44	1.06	33	0.73	100	6.48
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	29	0.28	20	0.48	83	4.50
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	40	0.68	25	0.43	100	6.28
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	40	0.68	43	0.95	95	4.35
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	42	0.96	23	0.30	93	4.93
Fpr.	ns	ns	0.024	0.034	ns	ns
SED (30df)			9.2	0.261		
LSD (*P*=0.05)			18.8	0.534		

*effects of Timing 1 treatment only.

There were no significant effects of fungicide treatment on yield (Table 20). Yield responses ranged from -0.16 t/ha (Proline applied at Timing 1 timing only) to 0.22 t/ha (Refinzar at 50% recommended label dose applied at Timing 1 and Timing 2).

The proportion of strains with the G460S mutation was high at the first sampling date on 15 March in the untreated control (75.2%). The proportion of strains in fungicide treated plots ranged from 81.4 to 93.3% and there were no significant differences between treatments (Table 20). The same was observed on the second sampling date on 13 May, with 84.0% of the population with the G460S mutation in the untreated control and treatments ranging from 73.9 to 93.9% and no significant differences between treatments.

Table 20. The proportion of the G460S mutation on the first and second sampling date and yield from the Pembrokeshire trial in 2019.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
	15 March 2019	13 May 2019	13 August 2019
Untreated	75.2	84.0	3.18
Proline 275 0.63 L/ha Timing 1 only	86.5	76.2	3.22
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	86.9	81.6	3.22
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	84.8	82.3	3.02
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	81.4	73.9	3.22
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	91.4	93.9	3.15
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	86.5	80.2	3.33
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	93.3	86.7	3.30
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	81.7	69.6	3.06
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	83.0	85.9	3.21
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	88.1	74.9	3.40
Fpr.	ns	ns	ns
SED (30df)			
LSD (*P*=0.05)			

At the Ceredigion site in harvest year 2019, fungicides were applied on 9 January and 20 March, with leaf samples taken on 9 March and 14 May. Light leaf spot was assessed for the first time in the trial on 12 March shortly after sampling and no significant differences between treatments were observed (Table 21). This was the same at both the following assessments, with no statistically significant differences for light leaf spot incidence or severity.

There were no significant effects of fungicide treatment on yield (Table 22). Yield responses ranged from 0.03 t/ha (Proline applied at Timing 1 timing only) to 0.47 t/ha (Proline 0.63 L/ha at Timing 1 followed by Refinzar 0.5 L/ha at Timing 2).The proportion of strains with the G460S mutation was high at the first sampling date on 9 March in the untreated control (74.8%). The proportion of strains in fungicide treated plots ranged from 71.0 to 90.4% and there were no significant differences between treatments (Table 22). The same was observed on the second sampling date on 13 May, with 78.4% of the population with the G460S mutation in the untreated control and treatments ranging from 64.0 to 88.9% and no significant differences between treatments.

Table 21. Light leaf spot incidence (inc) and severity (sev) on three assessment dates at the Ceredigion site in 2019.

Treatment	Prior to sampling*		First sample		Second sample	
	12 March 2019		18 April 2019		10 May 2019	
	inc	sev	inc	sev	inc	sev
Untreated	83	2.71	48	1.00	100	6.83
Proline 275 0.63 L/ha Timing 1 only	85	2.85	48	1.45	100	6.85
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	83	3.25	40	0.78	100	6.53
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	79	2.28	35	0.50	100	6.03
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	77	3.30	40	0.78	95	5.50
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	69	1.87	55	1.20	93	5.63
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	73	2.82	23	0.35	90	5.28
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	85	3.03	30	0.40	95	5.33
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	65	1.38	28	0.55	100	5.63
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	88	2.94	48	1.13	100	6.05
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	67	2.10	48	1.18	85	4.63
Fpr.	ns	ns	ns	ns	ns	ns
SED (30df)						
LSD (*P*=0.05)						

*effects of Timing 1 treatment only.

Table 22. The proportion of the G460S mutation on the first and second sampling date and yield from the Ceredigion trial in 2019.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
	9 March 2019	13 May 2019	1 August 2019
Untreated	74.8	78.4	3.73
Proline 275 0.63 L/ha Timing 1 only	72.3	88.9	3.76
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	71.0	80.8	3.90
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	76.7	67.2	3.70
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	77.8	70.8	3.91
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	81.6	82.6	3.75
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	75.4	71.4	3.94
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	87.4	64.0	3.77
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	82.1	76.5	4.20
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	90.4	85.7	3.91
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	79.7	76.8	4.00
Fpr.	ns	ns	ns
SED (30df)			
LSD (*P*=0.05)			

A set of samples were taken from field trial sites to determine if the proportion of the G460S mutation varied by site (Table 23). Most sites showed a high proportion of the G460S mutation, particularly in Fife where the proportion was 99.4%. At one of the Herefordshire sites the proportion was 50.7%, the lowest recorded in this project, however, the other sites ranged from 82.3 to 93.9%.

Table 23. Proportion of the G460S mutations reported from samples taken from a range of untreated and treated fields (samples supplied by industry partners).

Location	Number of lesions sampled	Proportion of G460S in samples (%)
East Yorkshire	18	93.9
North Yorkshire	17	82.3
Fife	15	99.4
Herefordshire	20	50.7
Kent	12	93.5

4.4.1. Cross site analyses: 2017, 2018 and 2019

Cross site analyses were conducted for individual years (harvest years 2017, 2018 and 2019) as well as across years where the same products were applied to the trials (2018 and 2019). In 2017, disease assessments were done in February (prior to sampling), March (at the first sample) and April (at the second sample) as outlined in previous sections. Only one genotype sample was obtained in 2017 due to low disease so no cross site analysis for pyrosequencing data from 2017 is included. For all assessments in 2017, there was no significant interaction between site and year (data not shown). Significant effects of fungicide treatment on disease were observed for all disease assessments, however, the effectiveness of different strategies did vary. Despite many of the autumn treatments being similar, there was variation in their performance for the control of light leaf spot. For example a full dose application of Proline ranged from 23% plants affected in one treatment to 46% plants affected in another (Table 24). The general trend was for some control of light leaf spot, however, no substantial differences between treatments existed. The percentage control of the disease varied depending on when light leaf spot was assessed in the season and when fungicides were applied. The most effective treatment gave 80% control at the first assessment and 64% at the final assessment, with averages of 40 and 26% control respectively.

Table 24. Light leaf spot incidence (inc) and severity (sev) on three assessment dates in 2017.

	Prior to sampling*		First sample		Second sample	
Treatment	inc	sev	inc	sev	inc	sev
Untreated	69	1.43	80	1.82	96	3.62
Proline 275 0.63 L/ha Timing 1 only	38	0.70	51	0.67	84	3.00
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	59	0.84	50	0.44	73	1.85
Proline 275 0.315 L/ha fb Vertisan 0.4 L/ha	55	0.74	50	0.80	90	3.11
Vertisan 0.8 L/ha fb Proline 275 0.63 L/ha	68	1.50	70	0.58	96	3.27
Proline 275 0.63 L/ha fb Vertisan 0.8 L/ha	23	0.30	35	0.33	83	3.10
Proline 275 0.315 L/ha + Vertisan 0.8 L/ha x 2	53	0.90	51	0.53	81	1.32
Proline 275 0.315 L/ha + Vertisan 0.4 L/ha x 2	41	0.69	49	0.57	79	2.10
Proline 275 0.63 L/ha fb Vertisan 0.4 L/ha	46	0.82	41	0.24	89	2.42
Vertisan 0.4 L/ha fb Proline 275 0.63 L/ha	48	1.29	76	1.04	98	3.92
Fpr.	0.001	0.005	<0.001	<0.001	0.033	0.046
SED (30df)	10.49	0.302	7.28	0.260	7.95	0.803
LSD (*P*=0.05)*	21.03	0.606	14.60	0.521	15.93	1.611

*effects of Timing 1 treatment only.

Yield responses to fungicide applications were statistically significant, with between 0.11 and 0.45 t/ha increase, with an average of 0.24 t/ha (Table 25). The single application of Proline 0.63 L/ha in the autumn (4.27 t/ha) was as effective as the two spray programmes (maximum yield achieved in other treatments 4.39 t/ha), with no statistically significant difference between this and the two spray programmes. Including Vertisan this year provided data that suggests that the relative effectiveness of a product against light leaf spot and the order (and dose) it is applied in alternation treatments and mixtures could impact on yield. For example, yield was higher where Proline 0.63 L/ha was applied as the first fungicide and followed by Vertisan (4.09 t/ha) compared to Vertisan 0.8 L/ha followed by Proline 0.63 L/ha (4.30 t/ha). This difference was marginally not statistically significant, however, yield was slightly lower where Vertisan 0.4 L/ha was applied as the first treatment suggesting that, in some years, early sprays are likely to impact disease control later in the season. The highest yield was achieved by a tank mix of Proline 0.315 L/ha + Vertisan 0.8 L/ha applied twice (4.39 t/ha).

Table 25. Yield at the Yorkshire and Herefordshire sites in 2017.

Treatment	Yield 91% Dry Matter 19/07 & 10/08
Untreated	3.94
Proline 275 0.63 L/ha Timing 1 only	4.27
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	4.20
Proline 275 0.315 L/ha fb Vertisan 0.4 L/ha	4.14
Vertisan 0.8 L/ha fb Proline 275 0.63 L/ha	4.09
Proline 275 0.63 L/ha fb Vertisan 0.8 L/ha	4.30
Proline 275 0.315 L/ha + Vertisan 0.8 L/ha x 2	4.39
Proline 275 0.315 L/ha + Vertisan 0.4 L/ha x 2	4.05
Proline 275 0.63 L/ha fb Vertisan 0.4 L/ha	4.09
Vertisan 0.4 L/ha fb Proline 275 0.63 L/ha	4.05
Fpr.	0.015
SED (28df)	0.119
LSD (*P*=0.05)	0.238

In 2018, fungicides significantly decreased light leaf spot incidence at the first two assessments and disease severity at the final assessment (Table 26). The percentage control of the disease varied depending on when light leaf spot was assessed in the season and fungicide programmes were generally less effective than they were in 2017. The most effective treatment gave 46% control at the first assessment prior to sampling and 46% control at the final assessment, with averages of 18 and 34% control respectively. The effect of fungicide dose appeared to be clearer in 2018, with higher doses in tank mixes and alternation treatments generally decreasing light leaf spot to a greater extent relative to the untreated control.

Table 26. Light leaf spot incidence (inc) and severity (sev) on three assessment dates in 2018.

Treatment	Prior to sampling*		First sample		Second sample	
	inc	sev	inc	sev	inc	sev
Untreated	61	0.54	55	0.80	100	8.80
Proline 275 0.63 L/ha Timing 1 only	54	0.58	55	0.60	100	6.69
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	49	0.48	41	0.50	100	6.60
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	58	0.61	51	0.63	100	5.47
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	46	0.39	42	0.21	100	6.17
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	48	0.38	40	0.38	100	5.21
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	33	0.22	39	0.26	98	4.72
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	55	0.45	42	0.36	100	5.92
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	50	0.33	46	0.58	100	5.26
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	54	0.60	54	0.57	100	6.49
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	51	0.36	47	0.54	100	5.67
Fpr.	0.001	ns	0.008	ns	ns	<0.001
SED (30df)	5.71		5.3			0.824
LSD (*P*=0.05)	11.42		10.6			1.648

*effects of Timing 1 treatment only.

In 2018, the proportion of the G460S mutation was high across the sites, with nearly 76% of strains reported to have the mutation and no statistically significant differences between treatments (Table 27). Small yield improvements were reported compared to the untreated control of between 0.05 and

0.33 t/ha, with an average of 0.16 t/ha (Table 27). The single application of Proline in the autumn (3.98 t/ha) in the autumn was as effective as the two spray programmes (maximum yield achieved in other treatments 4.16 t/ha), with no statistically significant differences between these treatments. The order of the fungicide products in the alternation programmes and dose did not have an impact in the trials as they did in 2017, which may be due to a switch to a different non-azole co-formulation. The highest yield was achieved through a tank mix of Proline 0.315 L/ha + Refinzar 1.0 L/ha (4.16 t/ha), which is similar to the azole/non-azole combination that achieved the highest yield in 2017.

Table 27. The proportion of the G460S mutation on the first sampling date and yield in 2018.

Treatment	Proportion of G460S mutation	Yield
	First sample	91% Dry Matter
		22/07 & 04/08
Untreated	75.7	3.83
Proline 275 0.63 L/ha Timing 1 only	83.7	3.98
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	74.4	3.90
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	71.7	4.02
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	79.3	3.88
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	87.2	4.00
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	84.7	4.16
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	73.5	3.99
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	61.9	3.98
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	63.9	4.00
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	63.9	3.97
Fpr.	ns	ns
SED (29df)		
LSD (*P*=0.05)		

In 2019, control of light leaf spot was generally poorer than in 2017 and 2018 (Table 28). The percentage control of the disease achieved by the most effective treatment at the first assessment was 40%, with an average of 24%. This may be due, in part, to the Timing 1 fungicides being delayed until mid-December/early January compared to previous years. At the final assessment, the most effective treatment was giving 34% control of the disease, with an average of 20% across treatments. Fungicides were least effective against light leaf spot in this year compared to 2017 and 2018.

The G460S mutation was again high across the four sites in 2019, averaging almost 80% on the first sampling date and 78% on the second (Table 29). There was again a trend for treatments that received no azole to have a lower proportion of the mutation, however, there were no statistically significant differences between treatments. Despite relatively low disease pressure in 2019, there were still small statistically significant yield differences between treatments (Table 29). Small yield improvements were reported compared to the untreated control of between 0.03 and 0.23 t/ha, with an average of 0.12 t/ha (Table 29). The single application of Proline in the autumn (3.62 t/ha) in the autumn was as effective as many of the other two spray programmes, however, yield was significantly lower than the maximum yield achieved of 3.81 t/ha (Proline 0.315 L/ha + Refinzar 0.5 L/ha applied as a tank mixture twice).

Table 28. Light leaf spot incidence (inc) and severity (sev) on three assessment dates in 2019.

Treatment	Prior to sampling*		First sample		Second sample	
	inc	sev	inc	sev	inc	sev
Untreated	67	1.24	42	0.54	91	3.73
Proline 275 0.63 L/ha Timing 1 only	48	0.93	40	0.63	82	3.28
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	57	1.08	31	0.35	81	3.23
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	57	0.87	39	0.38	89	3.29
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	49	0.86	37	0.50	74	2.92
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	56	1.02	35	0.42	81	2.96
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	46	1.04	29	0.31	74	3.08
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	42	0.74	30	0.31	69	2.43
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	51	0.85	34	0.26	78	2.98
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	54	0.87	33	0.40	82	2.78
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	53	1.14	32	0.41	88	2.79
Fpr.	ns	ns	ns	ns	<0.001	ns
SED (30df)					4.2	
LSD (*P*=0.05)					8.2	

*effects of Timing 1 treatment only.

Table 29. The proportion of the G460S mutation on the first and second sampling date and yield in 2019.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
Untreated	79.9	77.9	3.58
Proline 275 0.63 L/ha Timing 1 only	80.9	81.1	3.62
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	81.1	79.9	3.75
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	73.1	76.5	3.62
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	77.8	75.8	3.78
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	81.7	78.3	3.66
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	76.4	74.9	3.78
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	80.9	74.3	3.81
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	80.0	73.6	3.61
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	79.6	79.7	3.78
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	73.7	69.9	3.76
Fpr.	ns	ns	0.049
SED (28df)			0.089
LSD (*P*=0.05)			0.177

Across 2018 and 2019, the first and second disease assessments determined the effectiveness of the Timing 1 fungicide application only and there were no differences between azole and non-azole treatments. Disease was slightly lower where products had been applied as tank mixtures (Table 30). The third disease assessment assessed the effectiveness of two spray programmes and Proline applied at Timing 1 only significantly decreased light leaf spot (4.4%) relative to the untreated control (5.4% leaf area affected). Two spray programmes decreased disease severity further (between 3.6 and 4.4% leaf area affected), however, these were not significantly different to the one spray programme.

Table 30. Light leaf spot incidence (inc) and severity (sev) on three assessment dates in 2018 & 2019.

Treatment	Prior to sampling*		First sample		Second sample	
	inc	sev	inc	sev	inc	sev
Untreated	65	1.01	46	0.63	94	5.42
Proline 275 0.63 L/ha Timing 1 only	50	0.81	45	0.62	88	4.41
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	54	0.88	34	0.40	88	4.35
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	57	0.79	43	0.46	93	4.02
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	48	0.70	39	0.40	83	4.01
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	53	0.81	37	0.41	87	3.71
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	41	0.77	32	0.29	82	3.63
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	46	0.64	34	0.32	79	3.60
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	51	0.68	38	0.37	85	3.74
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	54	0.78	40	0.46	88	4.02
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	52	0.88	38	0.46	92	3.75
Fpr.	0.002	ns	0.002	0.019	<0.001	<0.001
SED (30df)	5.03		3.7	0.101	2.8	0.407
LSD (*P*=0.05)	9.92		7.3	0.200	5.5	0.804

*effects of Timing 1 treatment only.

The proportion of the G460S mutation was high across both years and no differences between treatments observed (Table 31). Yields where fungicides were applied were generally statistically significantly different from the untreated control, with an average yield response of 0.13 t/ha (Table 31). Proline applied at Timing 1 (3.74 t/ha) only did not statistically improve yields relative to the untreated control (3.66 t/ha), however, the split treatment (Proline 0.315 t/ha applied twice) did (3.80 t/ha). Highest yields were achieved by tank mixes (3.87 and 3.91 t/ha) and alternation treatments of Proline and Refinzar. Where Refinzar was the first fungicide applied, yields were significantly higher compared to the untreated control (between 3.83 and 3.86 t/ha).

Table 31. The proportion of the G460S mutation on the first and second sampling date and yield in 2018 & 2019.

Treatment	Proportion of G460S mutation		Yield
	First sample	Second sample	91% Dry Matter
Untreated	78.5	78.1	3.66
Proline 275 0.63 L/ha Timing 1 only	81.8	81.4	3.74
Proline 275 0.315 L/ha fb Proline 275 0.315 L/ha	78.9	76.7	3.80
Proline 275 0.315 L/ha fb Refinzar 0.5 L/ha	73.2	75.2	3.75
Refinzar 1.0 L/ha fb Proline 275 0.63 L/ha	79.4	76.4	3.82
Proline 275 0.63 L/ha fb Refinzar 1.0 L/ha	83.7	80.0	3.77
Proline 275 0.315 L/ha + Refinzar 1.0 L/ha x 2	77.9	77.7	3.91
Proline 275 0.315 L/ha + Refinzar 0.5 L/ha x 2	79.3	71.0	3.87
Proline 275 0.63 L/ha fb Refinzar 0.5 L/ha	79.3	72.0	3.74
Refinzar 0.5 L/ha fb Proline 275 0.63 L/ha	80.2	79.6	3.86
Refinzar 0.5 L/ha fb Refinzar 0.5 L/ha	74.3	69.6	3.83
Fpr.	ns	ns	0.013
SED (28df)			0.067
LSD (*P*=0.05)			0.131

4.5. Economic analysis of fungicide anti-resistance management strategies for the industry

Eighteen trials were conducted over three years, however, disease pressure was low across all three years and this should be taken into consideration regarding the interpretation.

4.5.1. Economic analysis – commercial trials

For all comparisons, there was no significant interaction between site and the fungicide programme tested. Therefore the performance of the fungicide programmes was not influenced by the site or the individual trials included in the dataset. When comparing the performance of azole programmes and azole mixture programmes across eighteen sites, these were similar for yield (4.35 and 4.32 t/ha respectively) and statistically significantly higher than the untreated control (by 0.13 and 0.10 t/ha respectively). Yield responses in general were small and as a result, gross margin across all trials was, on average, higher for the untreated control compared with the azole only programme (-£7 per ha), although the difference was not statistically significant. The programme that included tank mixtures (-£40 per ha) was the least cost effective (Table 32). This was due, in part, to dose rates as programmes contained different products and combinations of products.

Table 32. Yield (t/ha) and gross margin (£ per ha) associated with three fungicide strategies to control light leaf spot averaged across 18 trials in 2017, 2018 and 2019.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	4.22	1032
Azole followed by azole	4.35	1025
Mixtures (different MOA applied with azole)	4.32	992
Fpr.	0.004	0.007
SED (103 df)	0.040	13.2
LSD (*P*=0.05)	0.078	26.2

Alternation, where a non-azole was applied as the first fungicide application, was tested alongside the three previous treatments in 14 out of the 18 trials (Table 33). There were no significant differences between yields, however, gross margin was significantly higher where no fungicides had been applied (£1081). There was no significant difference between the untreated control and azole only programme (-£18). Alternation was the most cost effective of the fungicide strategies based on resistance management principles, however, the gross margin was significantly lower than the untreated control (-£31). Mixtures (-£47 per ha) were also significantly less cost effective.

Table 33. Yield (t/ha) and gross margin (£ per ha) associated with four fungicide strategies to control light leaf spot averaged across 14 trials in 2017, 2018 and 2019.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	4.37	1081
Azole followed by azole	4.46	1063
Alternation (different MOA followed by azole)	4.44	1050
Mixtures (different MOA applied with azole)	4.43	1034
Fpr.	ns	0.026
SED (117 df)		15.6
LSD (*P*=0.05)		30.9

There were no significant effects of fungicide programme on yield and gross margin in the trials in North Yorkshire (Table 34). In Herefordshire, there were significant effects on gross margin, with the azole only programme performing similarly to the untreated control. All other fungicide programmes (alternation and mixtures) were significantly less cost effective (up to -£57 per ha) compared to the untreated control (Table 35).

Table 34. Yield (t/ha) and gross margin associated with the four light leaf spot disease control strategies tested in six trials in North Yorkshire in 2017, 2018 and 2019.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	4.40	1089
Azole followed by azole	4.51	1083
Alternation (different MOA followed by azole)	4.59	1104
Mixtures (different MOA applied with azole)	4.47	1046
Fpr.	ns	ns
SED (50 df)		
LSD (*P*=0.05)		

Table 35. Yield (t/ha) and gross margin associated with the four light leaf spot disease control strategies tested in six trials in Herefordshire and Ceredigion in 2017, 2018 and 2019.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	4.56	1143
Azole followed by azole	4.63	1118
Alternation (different MOA followed by azole)	4.53	1081
Mixtures (different MOA applied with azole)	4.58	1086
Fpr.	ns	0.039
SED (51 df)		23.6
LSD (*P*=0.05)		47.4

4.5.2. Economic analysis – AHDB funded trials

There were eight AHDB funded trials. These trials were primarily set up to test the effectiveness of fungicide resistance management strategies and their impact on the selection for fungicide insensitive strains of *P. brassicae*, however, they also present an opportunity to look at the economics of similar treatments tested in the commercial trials previously as a standard set of treatments. In this dataset there is also the opportunity to investigate the impact of the order of fungicide application when applied in an alternation strategy e.g. is there a difference when azoles are applied in November/December compared with a different mode of action.

Across all eight trials, there were increases in yield with fungicides of between 0.09 and 0.21 t/ha (Table 36). All of the four fungicide strategies resulted in significantly higher yields than the untreated control [azole followed by azole, alternation (azole followed by different mode of action) and mixtures (different mode of action applied with an azole)]. These differences were small, however, due to low disease pressure over all three years. Gross margin for the different strategies were between -£4 and +£2 per ha, however, there were no statistically significant differences between them.

Table 36. Yield (t/ha) and gross margin associated with the five light leaf spot disease control strategies tested in eight trials conducted in Ceredigion, Herefordshire and North Yorkshire in 2017, 2018 and 2019.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	3.73	852
Azole followed by azole	3.90	854
Alternation (different MOA followed by azole)	3.95	852
Alternation (azole followed by different MOA)	3.90	848
Mixtures (different MOA applied with azole)	4.00	853
Fpr.	<0.001	ns
SED (96 df)	0.055	
LSD (*P*=0.05)	0.108	

Looking at individual years, the highest yield responses to fungicides were 0.45 t/ha, 0.19 t/ha and 0.23 t/ha in 2017, 2018 and 2019 respectively. In 2017 and 2019, using mixtures resulted in the highest yield, however, this was usually also the most expensive fungicide programme. In 2018, all fungicide treatments performed similarly. In 2017 there were significant yield improvements, however, despite a trend for higher gross margins than the untreated control for all fungicide programmes, these differences were not statistically significantly different (Table 37). In 2018, gross margin for fungicide treatments was lower than the untreated control and these differences were not statistically significantly different (Table 38).

Table 37. Yield (t/ha) and gross margin associated with the five light leaf spot disease control strategies tested in two trials conducted in North Yorkshire and Herefordshire in 2017.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	3.94	922
Azole followed by azole	4.20	953
Alternation (different MOA followed by azole)	4.22	935
Alternation (azole followed by different MOA)	4.30	960
Mixtures (different MOA applied with azole)	4.39	968
Fpr.	0.009	ns
SED (96 df)	0.114	
LSD (*P*=0.05)	0.236	

Table 38. Yield (t/ha) and gross margin associated with the five light leaf spot disease control strategies tested in six trials conducted in North Yorkshire and Herefordshire in 2018.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	3.83	884
Azole followed by azole	3.90	855
Alternation (different MOA followed by azole)	4.00	873
Alternation (azole followed by different MOA)	4.02	891
Mixtures (different MOA applied with azole)	3.99	853
Fpr.	0.038	ns
SED (96 df)	0.065	
LSD (*P*=0.05)	0.135	

In 2019, yields were increased with fungicide use, however, responses were generally low (from 0.07 to 0.23 t/ha). Gross margins were similar to the untreated control (£801) for most treatments (between £795 and £804), with the exception of alternation treatments where azoles were applied as the first spray and a non-azole at the second spray (£771), however, there were no statistically significant differences between treatments for gross margin (Table 39).

Table 39. Yield (t/ha) and gross margin associated with the five light leaf spot disease control strategies tested in four trials conducted in Herefordshire and Ceredigion in 2019.

Treatment	Yield at 91% dry matter	Gross margin
	t/ha	£
Untreated (no fungicide applied)	3.58	801
Azole followed by azole	3.75	804
Alternation (different MOA followed by azole)	3.78	800
Alternation (azole followed by different MOA)	3.65	771
Mixtures (different MOA applied with azole)	3.81	795
Fpr.	0.048	ns
SED (96 df)	0.087	
LSD (*P*=0.05)	0.175	

5. Discussion

The first objective of this project was to determine the risk of fungicide resistance for major and minor oilseed rape diseases. The trait-based risk assessment takes information on a particular active ingredient, pathogen and agronomic system and uses them to predict the risk of resistance evolution using information that is often readily available (Grimmer *et al.*, 2015). This is advantageous as it provides an indication of the risk of resistance developing before a product/mode of action is released to the market. The analysis showed that all modes of action should be considered as being at risk of resistance. At the time this project was started, a range of azoles (e.g. prothioconazole, metconazole and tebuconazole) were available plus a range of non-azole options (e.g. picoxystrobin, boscalid, azoxystrobin, dimoxystrobin) for the general and specific control of oilseed rape diseases. Across the fungicide programme, it is often the case that a particular active ingredient or active ingredients will control more than one disease meaning that the same active ingredient is used more than once during the season. For example, prothioconazole was applied an average of 1.7 times to oilseed rape in 2018 and azoxystrobin 1.08 times (Garthwaite *et al.*, 2019b). This reflects the fact that prothioconazole has activity on phoma leaf spot, light leaf spot and sclerotinia whereas azoxystrobin was only registered for use during flowering for sclerotinia control. This is relevant as a pathogen can be exposed to resistance selection from the repeated use of the same solo mode of action, even if the pathogen was not the intended target of all the applications. This does not represent a good resistance management strategy.

Alternaria spp. were predicted to be at highest risk of developing resistance. This pathogen tends to be more of an issue in the south west of England, where warm conditions can encourage disease development in the pods and in crops that have lodged. The widespread use of fungicides for control of other diseases in oilseed rape, in particular azoles, throughout the fungicide programme has been linked to the reduction in the appearance of *Alternaria* spp. and fungicides applied for sclerotinia control also have activity. Brassica vegetables, such as Brussels sprouts, purple sprouting broccoli and cabbage, were reported to receive two fungicide applications in 2017, however, this is likely to be an underestimate for long season crops (Garthwaite *et al.*, 2019a). Azoles accounted for ~56% of the total fungicides applied to vegetable brassicas, with 27% in co-formulation with another mode of action and most fungicide applications, 62%, were for ‘general disease control’. The potential effects of what is done on oilseed rape, and implications for high value vegetable brassica crops should be considered.

In Australia, an analysis of 200 populations has demonstrated that 15% of the *L. maculans* isolates retrieved were resistant to fluquinconazole which is used as a seed treatment and they found no cross resistance with prothioconazole/tebuconazole (van de Wouw *et al.*, 2017). Another recent Australian study identified *L. maculans* isolates that varied in their sensitivity to fluquinconazole, tebuconazole/prothioconazole and flutriafol, some of which are associated with modifications in the

regulatory *ERG11* regions. This can be associated with resistance to azoles in *L. maculans*, however, not all of the less azole sensitive isolates have these modifications. This suggests that other mechanisms are also likely to confer resistance. It was found that the resistance factors (RF), which are used to compare the lethal effects of the fungicide with a fully sensitive standard strain, were small so azoles were still likely to be effective in field situations. It was also suggested that, given the isolates were collected 2,000km apart and contained the same insertion, that it was possible that the modifications could appear independently in different populations.

Previous work has shown that there were no mutations on CYP51B in *L. maculans* isolates collected in the UK or that any substantial differences in fungicide sensitivity to azoles could be detected (Sewell *et al.*, 2017). But, the Australian research acts as a warning as to potential mechanisms for azole resistance that could occur in the UK. The AHDB fungicide performance project evaluates the effectiveness of azoles and new chemistry against phoma leaf spot/stem canker and light leaf spot and non-azoles perform similarly to or slightly better than azoles (Ritchie *et al.*, 2019; Walker *et al.*, 2019). The performance of the field trials suggest that there isn't substantial resistance in UK *L. maculans* populations currently, however, the Australian studies demonstrate that it is possible and resistance management should be a key consideration in fungicide programmes for this pathogen. Sclerotinia isolates with decreased sensitivity to SDHIs have been reported in the UK although these are not thought to be numerous and there have been no reports of issues with field control (FRAC, 2018). Where solo products containing SDHIs are available and are to be used for sclerotinia control, these are recommended to be mixed with an alternative mode of action (BASF, 2016). Populations with a high proportion of SDHI resistant strains have been identified in France, however, reports of problems with SDHI efficacy remains low (Anses *et al.*, 2019). Resistance management should therefore be considered across the entire fungicide programme and take into account all diseases likely to be present, whether they are the primary target of a fungicide application or not.

The second objective was to test which resistance management strategies are most effective at slowing fungicide resistance selection in *P. brassicae* comparing application of solo products against mixtures and alternation. The use of natural populations or inoculated strains of plant pathogens to determine the impact of fungicide treatment on selection for fungicide insensitivity has previously identified general principles for fungicide resistance management (van den Bosch *et al.*, 2014). These general principles can be applied to any pathosystem, however, the practicality and economic viability of resistance management strategies can differ substantially between different crop : pathogen combinations. For example, the availability and impact of cultural and chemical control methods on a pathogen can vary considerably. This means that pathosystems should be investigated separately to identify guidelines that are effective and practical for the control of pathogens of specific crops. The practicality of implementing alternation or mixture strategies can vary with the number of treatments applied and the number of modes of action available. For

example, a winter wheat crop could receive 3 fungicide applications in a season whereas for late blight control on ware potatoes, an average of 10 applications are applied (Garthwaite *et al.*, 2019b). The number of modes of action available to control diseases is likely to be substantially different for individual crops and associated diseases. *P. brassicae* isolates with decreased sensitivity to azoles were reported in Scotland in the early 2000s after reports of less effective control and it was found that a greater proportion of isolates grew on a discriminatory dose of 10 ppm flusilazole. The shift in sensitivity had not been large, however, it was considered large enough to cause issues with light leaf spot control in the field (Burnett, 2003).

CYP51 sequencing of 27 *P. brassicae* strains originating prior to 2012 (2003-2011) suggested that around 30% of the *P. brassicae* population had the G460S mutation and 19% had the S508T mutation (Figure 1). G460S and S508T strains were first detected in 2003 and 2007, respectively. CYP51 promoter inserts (46, 151 and 233 bp) were also detected in six strains, with one strain carrying a promoter insert of 151 bp in combination with S508T. Carter *et al*. (2014) showed that the *CYP51* expression is induced in strains with promoter inserts after exposure to azoles and strains with both *CYP51* mutations and promoter inserts show higher levels of azole insensitivity *in vitro*. Further characterisation of strains sampled in 2016 and 2018 showed that the frequency of G460S had increased to high levels (79% and higher) while the S508T frequency had declined (18% and lower). In addition, the majority of strains carried G460S in combination with *CYP51* promoter inserts. No new *CYP51* mutations and no combination of mutations (G460S + S508T) were detected. In laboratory studies, the expression of PbCYP51 G460S + S508T in yeast reduced the sensitivity of azoles by between 9 to 35-fold, to a greater extent than S508T alone (Carter et al., 2014). The absence of field strains carrying G460S + S508T may indicate that a fitness penalty (possibly lethal) is associated with this CYP51 variant. However, evolution of new mutations and combinations of mutations cannot be ruled out in the future as shown for *Z. tritici* (Cools and Fraaije, 2002). In addition, accumulation of additional *CYP51* promoter sequence (tandem) repeats resulting in increasing levels of azole insensitivity has also been found in a range of fungi, including banana black sigatoka pathogen *Mycosphaerella fijiensis* (Diaz-Trujillo *et al*., 2018)

In the field experiments conducted in this project, it was found that the proportion of the G460S mutation exceeded 60% in most cases, reaching 90% in samples taken in 2019. This meant that selection for G460S in experiments started from a high baseline, so the headroom for selection of this strain using fungicide treatment was small. In one experiment in 2019, there was a significant difference in the effect of fungicide treatment on the proportion of G460S mutation, however, clear conclusions were difficult to derive from this result. There was a trend for lower proportions of the G460S mutation where non-azoles had been applied compared to azoles, however, differences were small and not statistically significant.

The period of time over which the pathogen population is exposed to a fungicide is directly related to the amount of selection for resistance that occurs. And the strength of selection is largely independent of the size of the pathogen population. Hence, when we target a treatment at *L. maculans/L.biglobosa* or *Sclerotinia sclerotiorum*, we are also causing selection for insensitive *P. brassicae* strains, even if the amount of light leaf spot present is very low. When the results from the field trials are considered from an in-field efficacy point of view, there was no difference in the performance of azole and non-azole fungicides against light leaf spot, even at sites where the proportion of the G460S mutation is high. This is positive news for resistance management as a range of modes of action are required to implement a robust strategy. It also suggests that the presence of the G460S mutation does not confer a substantial decrease in the effectiveness of the currently available azoles in the field. However, novel mutations might be selected for if azoles with different CYP51 binding properties enter the market. Screening in laboratory tests in 2018 and 2019 against picoxystrobin and penthiopyrad show that *P. brassicae* remain fully sensitive to both QoI and SDHI modes of action (Kevin King, pers comm.).

Another aspect to consider at the same time as considering inherent risk is the mechanism by which resistance is conferred. For QoI, this is usually a single mutation resulting in a loss of control within a few years. For *Z. tritici* on wheat, QoI insensitivity is associated with a mutation resulting in cytochrome *b* G143A amino acid substitution (Fraaije *et al.*, 2003). It is unknown whether this mutation would be possible for *P. brassicae*. In some plant pathogens G143A cannot occur if an intron is located nearby and its splicing is prevented due to this mutation resulting in an inactive protein (Sierotzki *et al.*, 2007). Because prediction of fungicide resistance risk can only provide a likely indication of how rapidly resistance may occur, fungicide performance assessments and resistance monitoring will be important tools for future product stewardship enabling validation of optimal resistance management strategies.

In the AHDB funded trials in this project, the percentage control of light leaf spot has ranged from 26 to 68% in 2017, 19 to 42% in 2018 and 19 to 27% in 2019. The timings of fungicide applications were designed to allow some disease to develop to allow samples for pyrosequencing to be taken, however, they would represent some situations where fungicide application timing was less than optimal, particularly in 2019. Control of light leaf spot with azoles historically appears to have been more effective, although between trial and season variation is always reported. In Scotland in harvest years 2001 and 2002, the percentage control achieved by azoles in field trials ranged from 79 to 93% (Burnett, 2003). In England in harvest years 1995 to 1997, control ranged from 43 to 97% when fungicides were applied mid-November and at early stem extension (Gladders *et al.*, 1998). In AHDB funded fungicide efficacy trials over the last six years, the percentage control of light leaf spot by all modes of action in both Scotland and England has been between 30 to 85% in individual trials, with an average of 50 to 60% control being typical in individual years. No differences in the effectiveness

of azoles and other products with efficacy against light leaf spot have been observed in those trials including QoIs and SDHIs (Ritchie *et al.*, 2019). Azoles should not be actively excluded from fungicide programmes and their use in mixtures and in alternation with other fungicide modes of action should be encouraged to maintain an effective resistance management strategy.

Given that the proportion of the G460S mutation is similar across sites where control is moderate to low, questions need to be asked around other factors that could be affecting the control of the disease. There are anecdotal reports from Scotland that light leaf spot has become more difficult to control despite the use of fungicide plus light leaf spot resistant varieties. One factor could be the ability of local *P. brassicae* populations to overcome host resistance genes. It has been identified previously that there may be regional differences in *P. brassicae* populations, with differences in the extent of disease development on susceptible and resistant cultivars when exposed to light leaf spot isolates derived from different areas in England and Scotland (Klöppel *et al.*, 2015). If variety resistance can be overcome by a local population, essentially rendering it 'susceptible', and disease pressure is therefore high, there is the potential for a fungicide programme to appear to be 'less effective' especially if the application timing is suboptimal. In the current study, where first fungicides were applied later in a curative situation, the percentage control of light leaf spot achieved was substantially poorer. It is likely that a more targeted and integrated crop management approach will be the most effective way to control light leaf spot long term. Robust information on the performance of varieties, fungicides and disease risk and how to integrate these cost effectively will be essential to maintain control. Better timing and targeting of fungicide applications and reduced doses have also been shown to be effective for fungicide resistance management as it reduces the exposure of the pathogen to selective pressure. However, there is a balance between implementing these strategies and optimising disease control (van den Berg *et al.*, 2013). The current light leaf spot model is not reactive and unable to predict disease risk in 'real time' therefore limiting its use to amend fungicide programmes in season. Economic benefits from using varieties with good resistance have been demonstrated on oilseed rape in Scotland and England previously in the early 1990s (Gladders *et al.*, 1998), however, information on varieties that are commercially relevant currently is limited.

The DEFRA oilseed rape disease surveys conducted from 2017 to 2019 have shown that, in the spring in England, the percentage of plants affected by light leaf spot in individual crops ranged from 12 to 15%. This is in contrast to 23 to 44% plants affected which was typical for crops assessed from 2008 to 2015 (Anon, 2020). All trials presented here, both AHDB and industry funded, were therefore conducted in three low disease years. To cover the cost of the fungicide programme or 'break even' in the industry funded trials, a yield uplift of between 0.17 and 0.27 t/ha was required depending on the strategy implemented. Across the three years of AHDB trials in North Yorkshire, the small yield increases achieved through fungicide applications covered the costs of fungicide application and

gross margins were similar to where no fungicides were applied. In trials conducted in the West Midlands and Wales, the yield responses to fungicides were very small and the increased cost of alternation and mixture strategies meant the gross margins were significantly lower than applying no fungicides. On average, the azole only programme required a yield improvement of between 0.16 and 0.18 t/ha to cover application costs to break even and an alternation strategy, where an azole was applied in sequence with a product containing a different mode of action, or vice versa, performed similarly regardless of the order in which products were applied between 0.17 to 0.24 t/ha.

Tank mixtures were the most expensive option and required an additional yield of 0.26 and 0.29 t/ha over and above the untreated control to 'break even'. It should be noted that all products in the industry fungicide trials were applied, whether as solo products or mixtures, at 50% of the recommended label dose therefore a higher cost associated with a tank mixture would be expected. In the AHDB trials, significant yield improvements of up to 0.45 t/ha were observed with fungicide application, however, gross margins were similar regardless of whether fungicides were applied or not. As all the treatments in the AHDB trials followed similar azole/non-azole strategies with regards to dose it is likely that more 'balanced' mixtures, where the appropriate dose of individual modes of action to provide resistance management and acceptable efficacy are applied, are likely to be more cost effective. This could also be achieved with a co-formulated product where both modes of action are effective against the target disease. With the increasing pressure on margins, there is likely to be pressure to use cheaper options for disease control. Prothioconazole, the most frequently used azole on oilseed rape, has come off patent and generic options are now registered for use in the UK. This could have implications for the cost of fungicide programmes, particularly through the provision of potentially cheaper azole products at the expense of resistance management.

The sequential application of a product with the same mode of action has been proven to be a poor fungicide resistance management strategy and using a range of modes of action, either through alternating or tank mixing products, is recommended to slow resistance evolution (van den Bosch *et al.*, 2014). There is up to date information on commercially relevant fungicides through the AHDB Fungicide Performance project for phoma leaf spot/stem canker and light leaf spot, however, the economic analysis presented here highlights the importance of understanding the risk to crops in a single season in order to avoid unnecessary fungicide applications and prolong the effective life of different modes of action. Tailoring fungicide input according to risk factors e.g. drilling date, varietal resistance and disease pressure would be a more cost effective approach, however, would require additional research to implement.

Reducing the need for unnecessary fungicide applications will have an impact on costs and decrease the selection for fungicide resistance. Using varieties with better resistance has been proven to be an effective strategy to manage light leaf spot, in conjunction with appropriate fungicide use

(Gladders *et al.*, 1998). Pod disease associated with yield loss was reported in one out of the eight trials. Previous research has demonstrated that early infection from light leaf spot had the greatest potential for decreasing green leaf area and associated yield reductions (Jeffrey *et al.*, 1994). This was through a decrease in pod number rather than seed size. It also demonstrated that the need for a fungicide varies depending on when the epidemic starts to protect yield. For example, in one year a mid-December and February fungicide had a significant impact on yield, whereas in a subsequent year, a February applied fungicide was all that was required. There is still no predictive tool suitable for in-season use to help to adjust fungicide decisions and take into account drilling date or variety and guide decisions. Such a tool would help to manage costs and also be beneficial for fungicide resistance management through better strategic use of fungicides to optimise disease control.

5.1. Maintaining control of oilseed rape diseases: conclusions and practical guidelines

- Fungicide resistance management is a strategy to maintain the effectiveness of one or more modes of action for as long as possible.
- Continue to use current resistance management guidelines when planning a fungicide programme for oilseed rape so that the effectiveness of azoles and other modes of action are maintained.
- Use different modes of action either in alternation or as mixtures, across the entire fungicide programme.
- Both azoles and non-azoles are effective against light leaf spot.
- Most strains carry G460S or S508T in combination with *CYP51* promoter inserts. Strains with promoter inserts shows inducible overexpression of *CYP51* after exposure to azoles.
- Alternation, and the use of co-formulated products, is likely to be the simplest resistance management strategies to implement immediately.
- 'Balanced mixtures', where the appropriate dose of two different modes of action are used to maximise disease control and yield as well as implement a resistance management strategy, are likely to be effective and reduce fungicide inputs and costs but will require field experimentation to support use.
- Switch to using a variety with better resistance. Varieties with good light leaf spot resistance have been shown to have economic benefits, particularly where the ratings are 6 and above.
- Appropriate timing of fungicides for the control of light leaf spot, particularly in the autumn/winter, is likely to be a key factor in the success of fungicide treatments.

5.2. Maintaining control of oilseed rape diseases: future research

- **'Resistance-proof' products before they come to market.** Optimise co-formulations and tank mixture partners for efficacy and resistance management. Provide clear guidance on resistance management strategies at time of launch.

- **A reliable predictive model to help guide the need for and timing of fungicide applications for light leaf spot.** The light leaf spot model at present does not offer a 'real time' decision option. Damage from light leaf spot is associated with early introduction of the disease to the crop. Previous research has demonstrated that, in some years, the number of fungicide applications and optimal timing required to control the disease differs. A better indication of when to start the fungicide programme, taking into account variety resistance, would help fine tune fungicide use and mean that they are only used when necessary.

- **Publicly available, independent data to provide confidence in and information on the deployment of variety and fungicide dose based integrated control strategies.** AHDB generate information on variety susceptibility and fungicide efficacy through the Recommended List ® and Fungicide Performance projects, however, information is limited on how to best integrate these approaches. There is a need to demonstrate how best to use both two strategies to achieve both disease control and yield. In the long term, there are potential benefits to integrating fungicides and variety resistance to slow the selection for fungicide resistance and virulence as well as decreasing chemical inputs and associated costs to levy payers.

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8. Appendix

Table 40. Parameters used for the model to determine the risk of fungicide resistance development.

Disease	Pathogen	Latent periods per year	Number of host species	Agronomic system
Light leaf spot	*Pyrenopeziza brassicae*	11	0	0
Stem canker	*Leptosphaeria maculans*	1*	0	0
Stem canker	*Leptosphaeria biglobosa*	1*	0	0
Sclerotinia stem rot	*Sclerotinia sclerotiorum*	1	1	0
Dark leaf/pod spot	*Alternaria brassicae*	31	0	0
Dark leaf/pod spot	*Alternaria brasissicola*	27.5	0	0

**L. maculans* and *L. biglobosa* considered to be monocyclic in UK conditions (West *et al.*, 2001).

Table 41. Fungicides that were modelled to determine their risk of fungicide resistance development and their respective molecular complexities.

Fungicide Class	Active Ingredient	Molecular Complexity
carboxamide SDHI	Penthiopyrad	447
	Boscalid	399
DMI	Prothioconazole	458
	Tebuconazole	326
	Prochloraz	377
	Propiconazole	377
	Difenoconazole	495
QoI	Dimoxystrobin	438
	Picoxystrobin	495
	Azoxystrobin	646
MBC	Thiophanate methyl	407
	Carbendazim	222

High Mowthorpe, 2017

Soil texture:	Sandy Loam		
Drainage:	Good		
Previous cropping:	2014	Fodder beet	
	2015	Spring barley	
	2016	Winter barley	
Soil analysis:	P	28.20	Mg/l
	K	82.00	Mg/l
	Mg	18.00	Mg/l
	Organic matter	5.00	%
	pH	7.80	
Crop:	Winter oilseed rape		
Cultivar:	Fencer		
Sowing date:	24/08/16		
Seed rate:	3.0 kg/ha		
Fertilisers:	20/08/16	10:24:24	250 kg/ha
	21/02/17	25N 0:0:14.3 SO_3	450 L/ha
	29/03/2017	37N	220 L/ha
Herbicides:	26/08/16	Sultan	1.3 L/ha
	15/09/16	Shogun	0.5 L/ha
	29/11/16	Fusilade Max	0.7 L/ha
	05/07/17	Glyphosate	4 L/ha
Insecticides:	06/09/16	Hallmark	75ml/ha
	05/10/16	Hallmark	75ml/ha
	29/11/16	Cythrin Max	50ml/ha
Molluscicides:	N/A		

Rosemaund, 2017

Soil texture:	Silty Clay Loam		
Drainage:	Good		
Previous cropping:	2013	Winter barley	
	2014	Potatoes	
	2015	Winter wheat	
	2016	Winter barley	
Soil analysis:	P	16.40	Mg/l
	K	145.00	Mg/l
	Mg	116.00	Mg/l
	Organic matter	3.10	%
	pH	6.80	
Crop:	Winter oilseed rape		
Cultivar:	Fencer		
Sowing date:	02/09/2016		
Seed rate:	70.0 seeds/m^2		
Fertilisers:	11/03/2017	Nitrogen	47 kg/ha
	16/03/2017	Nitrogen	80 kg/ha
	16/03/2017	Sulphur	90 kg/ha
	27/03/2017	Nitrogen	92 kg/ha
	26/08/2016	Lime	3.5 t/ha
Herbicides:	19/08/2016	Falcon	0.542 l/ha
	19/08/2016	Shadow	1.986 l/ha
	24/08/2016	Samurai	2.0 l/ha
	24/08/2016	Sultan 50 SC	0.903 l/ha
	24/08/2016	Clomate	0.217 l/ha
	27/06/201	Motif	3 l/ha

Rosemaund, 2018

Soil texture:	Silty Clay Loam		
Drainage:	Good		
Previous cropping:	2015	Winter oilseed rape	
	2016	Winter beans	
	2017	Winter wheat	
Soil analysis:	P	18.6	Mg/l
	K	148.0	Mg/l
	Mg	179.0	Mg/l
	Organic matter	2.8	%
	pH	6.4	
Crop:	Winter oilseed rape		
Cultivar:	Fencer		
Sowing date:	04/09/2017		
Seed rate:	60.0 seeds/m^2		
Fertilisers:	28/08/2017	Layer Manure	6.3 t/ha
	26/03/2018	25N 14.3S	200 l/ha
	16/04/2018	25N 14.3S	280 l/ha
	07/05/2018	Efficient N28	30 l/ha
Herbicides:	05/09/2017	Shadow	2 l/ha
	28/06/2018	Samurai	4 l/ha
Insecticides:	N/A		
Molluscicides:	05/09/2017	TDS Major	5 kgs/ha

Rosemaund A, 2019

Soil texture:	Silty clay loam		
Drainage:	Good		
Previous cropping:	2014	Winter wheat	
	2015	Winter oats	
	2016	Winter wheat	
	2017	Potatoes	
	2018	Winter wheat	
Soil analysis:	P	22.0	Mg/l
	K	214.0	Mg/l
	Mg	105.0	Mg/l
	Organic matter	3.4	%
	pH	6.6	
Crop:	Winter oilseed rape		
Cultivar:	Fencer		
Sowing date:	30/08/2018		
Seed rate:	50 seeds/m^2		
Fertilisers:	23/08/2018	Layer Manure	7 t/ha
	28/02/2019	25N 14.3So3	200 l/ha
	27/03/2019	Nuram 35S	160 l/ha
	07/05/2019	Efficie-N-t 28	30 l/ha
Herbicides:	30/08/2018	Shadow	2.5 l/ha
	10/12/2018	Targa Max	0.3 l/ha
	17/07/2019	Samurai	4 l/ha
Insecticides:	24/10/2018	Hallmark	0.075 l/ha
	10/12/2018	Decis Forte	0.075 l/ha
Molluscicides:	30/08/2018	Slug pellets	

Rosemaund B, 2019

Soil texture:	Silty clay loam		
Drainage:	Good		
Previous cropping:	2015	4 year ley	
	2016	Winter wheat	
	2017	Potatoes	
	2018	Winter wheat	
Soil analysis:	P	42.6	Mg/l
	K	220.0	Mg/l
	Mg	75.0	Mg/l
	Organic matter	2.8	%
	pH	6.7	
Crop:	Winter oilseed rape		
Cultivar:	Fencer		
Sowing date:	28/08/2018		
Seed rate:	50 seeds/m^2		
Fertilisers:	22/02/2019	Gran urea 46%	150 kg/ha
	19/03/2019	26N 35So3	250 kg/ha
	22/03/2019	Gran urea 46%	125 kg/ha
Herbicides:	01/09/2018	Blanco	0.253 l/ha
	01/09/2018	Sultan 50SC	1.044 l/ha
	17/10/2018	Falcon	1.5 l/ha
Insecticides:	14/09/2018	Lambdastar	0.075 l/ha
Molluscicides:	N/A		

Ceredigion, 2019

Soil texture:	Loam		
Drainage:	Good		
Previous cropping:	2016	Winter Barley	
	2017	Winter Wheat	
	2018	Winter Wheat	
Soil analysis:	P	17.8	2
	K	224	2+
	Mg	106	3
	Organic matter	9.0	
	pH	6.7	
Crop:	Winter oilseed rape		
Cultivar:	INV 1155		
Sowing date:	07/09/2018		
Seed rate:	2.5 kg/ha		
		kg/ha	
Fertilisers:	N	232	
	P	85	
	K	85	
	S	137	
Herbicides:	09/09/2018	Sitaki CS	0.25 l/ha
	03/10/2018	Falcon	0.7 l/ha
Insecticides:	N/A		
Molluscicides:	N/A		

Cardigan, 2019

Soil texture:	Clay Loam		
Drainage:	Good		
Previous cropping:	2016	Winter barley	
	2017	Winter wheat	
	2018	Winter wheat	
Soil analysis:	P	16.2	2
	K	90	1
	Mg	77	2
	Organic matter	6.2	
	pH	6.6	
Crop:	Winter oilseed rape		
Cultivar:	Phoenix		
Sowing date:	30/08/2018		
Seed rate:	50 seeds/m^2		
		kg/ha	
Fertilisers:	N	186	
	P	56	
	K	78	
	S	74	
Herbicides:	01/09/2019	Stalwart	0.75 l/ha
	10/10/2018	Cleravo	1.0 l/ha
	10/10/2018	Dash	1.0 l/ha
	16/07/2019	Snapper	2.39 l/ha
	16/07/2019	Companion Gold	0.64 l/ha
	16/07/2019	Mesh	1.0 l/ha
Insecticides:	N/A		
Molluscicides:	N/A		