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Effectiveness of varietal resistance and risk prediction for the control of ramularia leaf spot of barley under Irish growing conditions

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Abstract

Ramularia leaf spot (RLS) of barley, caused by the fungal pathogen *Ramularia collo-cygni*, is a significant threat to the viability of spring barley production in Ireland. As a relatively new disease of barley, limited information is available on the development and impact of the disease under Irish conditions. RLS symptoms often only develop after anthesis and the final fungicide application, therefore some decision support is required for growers to be able to make sound integrated pest management (IPM) decisions. In the present study field trials were conducted on spring barley in 2016-2018 to determine if environmental conditions during stem extension, specifically leaf wetness, could be used to aid decisions relating to the intensity of fungicide control required later in the season for the control of RLS. The trials were conducted on four spring barley varieties subjected to one of five fungicide treatments at awn emergence 1) untreated control 2) pyraclostrobin 3) prothioconazole and chlorothalonil 4) decreased/increased rates of prothioconazole and chlrothalonil depending on risk of RLS development and 5) exclusion of prothioconazole or addition of bixafen depending on risk of RLS development. In 2018, although moderate-high levels of disease were predicted, a prolonged dry period post-stem extension resulted in no disease development. In 2016 and 2017 moderate levels of disease developed in the trials, with various significant ($P < 0.05$) interactions recorded between site, year, variety and fungicide treatment, depending on specific variable assessed: visual leaf symptoms, pathogen load in the leaf or grain as determined by qPCR or final grain yield. This was further evident in the relationships between visual symptoms and detectable *R. collo-cygni* biomass in the leaf with yield, these contrasted between seasons with weak relationships detected in 2016 (R^2 = -0.019 and R^2 = 0.176 for visual and biomass respectively) and strong relationships detected in 2017 (R^2 = -0.748 and R^2 = -0.5 for visual and biomass respectively). The variability in responses to the variety and fungicide treatments and the relationships between visual disease symptoms and biomass further highlight the unpredictability of RLS.

Keywords

spring barley; disease control; decision support system; leaf wetness; integrated pest management, *Ramularia collo-cygni*.

1.0 Introduction

Ramularia Leaf Spot (RLS) is a foliar disease of both winter and spring barley (*Hordeum vulgare*), caused by the ascomycete fungal pathogen, *Ramularia collo-cygni*. Since the mid-1990s RLS has rapidly become a serious global threat to barley production (Havis et al., 2015). The fungus can induce early senescence, with lesions prematurely reducing the green leaf area available for photosynthesis during grain filling, which can result in grain yield losses of up to 1.0 t/ha in North-Western Europe (Havis et al., 2018). In addition to loss of yield, RLS is often associated with reduced grain quality, which can dramatically impact the value of the crop if destined for distilling or malt production. It is for these economic reasons that strategies to effectively manage RLS must be developed.

As a newly established disease of barley, gaps in knowledge exist as to how the disease develops and spreads (Havis et al., 2015). Through the development of molecular diagnostics it has been possible to readily detect *R. collo-cygni* in seed stocks and, subsequently, its movement from seed to seedlings, indicating the role of seed transmission in the initiation of epidemics (Zamani-Noor et al., 2009; Havis et al. 2014). As the pathogen also produces an abundance of wind dispersed conidia in the necrotic lesions, seed transmission is unlikely to be the sole cause of the initial infection. Secondary structures have also been detected in stubble, possibly providing an additional means of the pathogen to survive between growing seasons (Salamati & Reitan, 2006). Furthermore, although primarily a disease of barley *R. collo-cygni* is also able to infect numerous gramineous hosts creating potential refuges for the pathogen. Although detectable throughout the life of the barley crop, induction of the typical rectangular lesions often only occurs post-anthesis and in response to stresses imposed upon the plant.

The current impetus to adopt integrated pest management (IPM) strategies in crop production systems must be supported by the continual development of pest control strategies that minimise the need for pesticides (Lamichhane et al., 2016). Key to such IPM based strategies is the initial prevention or suppression of the pest. Within cereal production systems this can be achieved through a number of means including; use of varietal resistance; manipulation of the crop environment through altering sowing date or planting method and subsequent agronomic practises; and, if required, the timely intervention with carefully selected pesticides determined through monitoring of pest activity (Barzman et al., 2015; Creissen et al., 2019). Unfortunately, as with most other aspects of the *R. collo-cygni*

pathosystem, limited information is available on the effectiveness of varietal resistance and agronomic practices to prevent or even suppress RLS epidemics. For instance, to date no major resistance genes have been identified against R. *collo-cygni* in barley (McGrann et al., 2014). Where significant quantitative effects have been identified, large genotype x environment interactions exist further demonstrating the difficulties of utilising quantitative resistance to control RLS (Pinnschmidt et al. 2006b; Pinnschmidt and Sindberg 2009; Hjortshøj 2012).

In the absence of effective non-fungicidal control measures, increased reliance has been placed on fungicides for RLS control. However, the long asymptomatic development period of *R. collo-cygni* creates difficulties in developing tailored fungicide-based control strategies. As the development of symptoms post anthesis can be rapid, any fungicide application must be made on the assumption that the disease will develop later in the season. As such, control of RLS in geographical regions where significant RLS related yield losses can occur has become reliant upon the routine, prophylactic application of fungicides during booting/awns emerging growth stage (GS) 45-49 (Zadoks et al., 1974), irrespective of the presence of the pathogen or risk of disease development (Havis et al., 2015). The combination of increasingly restrictive regulations relating to the authorisation of fungicides (Jess et al. 2014) and the development of fungicide resistance amongst *R. collo-cygni* populations to those currently available (Rehfus et al. 2019) seriously undermine the viability of fungicide based control strategies

To ensure the longevity of fungicide actives and/or varietal resistances it is essential to minimise their exposure to the target pathogen as much as is feasibly possible. For fungicides, this can be achieved through dose reduction, and/or mixing or alternating different fungicide chemistries (van den Bosch et al., 2014). Limiting the development of epidemics will undoubtedly reduce exposure of varietal resistances to the pathogen and thereby prolong the effective lifespan of the varietal resistance. However, in a pathosystem where pathogen levels and potential impacts on production are often only known post symptom expression, and past the timepoint where intervention can be effective, limiting the development of epidemics is difficult to achieve without the means to predict risk of disease development.

Although research into *R. collo-cygni* and the development of RLS is challenging due to the nature of the disease development, specifically the large influence external

environmental factors can have on its progress, recent studies have suggested that high humidity/leaf wetness around stem extension maybe an important determinant of the disease development (Salamati and Reitan 2006; Havis et al. 2013). Havis et al. (2013) developed a Decision Support System (DSS) based on this relationship, which allows the user to target RLS with appropriate fungicides at GS45-49 in a site-specific manner. However, following analysis of the DSS over multiple seasons Havis et al. (2018) have since questioned the strength of this relationship. Although the authors found no environmental factor significantly related to RLS across seasons, in individual seasons relationships with leaf surface wetness were identified, both at stem extension, but also cumulatively up until GS59. Unfortunately, under Irish growing conditions the benefit of a fungicide application beyond GS49 is questionable as it has been highlighted that applications delayed to GS59 are less effective at protecting yields than those administered at GS49 (Glynn & Grace, 2017). Though the duration of leaf wetness at stem extension may not provide the high level of confidence required to omit a RLS specific fungicide application, in situations where predicted disease levels are low it may be possible to omit or reduce the rate of application of the fungicide most at risk of resistance development without adversely impacting control. The aim of the study was to i) identify whether environmental conditions, such as duration of leaf wetness during stem extension, can be used to aid decisions relating to intensity of fungicide application later in the season for RLS control, and ii) determine whether varieties believed to differ in levels of resistance to RLS can provide a non-chemical control measure which can be incorporated into an IPM strategy. To address these aims spring barley field trials were conducted in Ireland across 3 growing seasons (2016-2018) in which RLS severity, *R. collo-cygni* biomass in plant tissues (DNA quantification), and grain yields were assessed.

2.0 Materials and Methods

2.1 Field trial design

Spring barley field trials were conducted over three consecutive growing seasons (2016, 2017 and 2018) at two sites differing in disease pressure;, Oak Park, Co. Carlow (52.8655, -6.9095), considered a medium disease pressure environment; and Kildalton, Pilltown, Co. Kilkenny (52.3450, -7.3064), considered a high disease pressure environment (Table 1). Each trial consisted of a randomised split–plot design with four replications. Barley variety (n=4;

Table 2) was designated as sub-plot and fungicide treatment (n=5; Table 3) as whole plot, giving 20 plots (2.5 m x 10 m) per replicate and 80 plots in total per site season. Barley varieties were selected based on their RLS resistance ratings according to the UK's Agriculture and Horticulture Development Board (AHDB), recommended list for 2015-16. On a scale of 1-9 with 9 indicating resistance, the variety KWS Irina was the most resistant of the chosen cultivars (7), Propino and RGT Planet were moderately resistant (6) while Olympus was the most susceptible (4) (https://projectblue.blob.core.windows.net/media/Default/Imported%20Publication%20Docs/AHDB%20Cereals%20&%20Oilseeds/Varieties/RL2015-16/Recommended%20Lists%20for%20cereals%20and%20oilseeds%202015-16.pdf).

2.2 Disease risk assessment and fungicide application

At GS<30 (late tillering) all plots, except the plots destined to be the fungicide untreated treatment, received a cover spray of prothioconazole (Proline, Bayer) and pyraclostrobin (Modem, BASF) at 50% recommended label rates (Table 3). At GS49 (awns emerging) plots received one of five fungicide treatments (Table 3), two of which were altered to reflect the risk of RLS development. That level of risk was determined by the minutes of leaf wetness (MLW) accumulated during the two week period during stem extension. In accordance with Havis et al. (2013) disease risk deemed low for MLW < 4500, medium for MLW 4500 – 7500, and high for MLW > 7500. Relatively humidity >90% was used as a proxy for leaf wetness and was recorded using a mobile weather station located in the field trial (Kildalton), or a stationary weather station located within 200m of the field trial (Oak Park).

The fungicide treatments applied at GS49 were as follows: 1) fungicide untreated to determine levels of disease across the trials; 2) a 'QoI' treatment of pyraclostrobin (Modem, BASF) applied at 50% of the recommended label rate to provide broad spectrum disease control without impacting RLS development due to prevalence of QoI resistance in the Irish *R. collo-cygni* population; 3) a 'reference' treatment combination of prothioconazole (Proline, Bayer CropScience) and chlorothalonil (Bravo 500, Syngenta), both applied at 50% of their recommended label rate and used as referencefor the final two treatments which were modified depending on the calculated RLS risk based on MLW as described above; 4) The 'DSS rate' treatment in which rates of both prothioconazole and chlorothalonil depended on RLS risk. Where risk was deemed low (<4000 MLW) both products were applied at 25% of their respective recommended label rates; where risk was deemed medium (4000-7500

MLW) both products were applied at 50% of their respective recommended label rate; where risk was deemed high (>7500 MLW) both products were applied at 75% of their respective recommended label rates; 5) The 'DSS product' treatment in which the components of the 'reference' treatment changed depending on RLS risk as described above. Where risk was deemed low prothioconazole was omitted and only chlorothalonil was applied; where risk was deemed high an SDHI bixafen was included with the prothioconazole (in the form of the co-formulated product Siltra, Bayer CropScience) and chlorothalonil, and each applied at 50% of their respective recommended label rates.

2.3 Disease and yield assessment

Disease was assessed visually on leaf 2 (flag-1) of 10 main stems selected from throughout each plot (outer rows were not scored to avoid edge effects) at GS75, approximately 2-4 weeks post final fungicide application. All plots were combine harvested and yields recorded as t/ha (15% moisture).

2.4 Sampling for quantitative PCR analysis

Concurrent with the visual assessment at GS75 10 leaves (leaf 2) were also sampled per plot for total *R. collo-cygni* genomic DNA quantification. All leaves were air dried for 7 days, freeze dried for an additional 48 hours, and subsequently ground into a fine powder using a mixer mill (MM400 Retsch). At harvest a 1kg sample of grain was collected from each plot from which approximately 100 grains were randomly sampled and ground into a fine powder. DNA was then extracted by adding 5ml of extraction buffer (Tris-HCL 400nM, NaCL 5M, EDTA 500nM, pH 8.0 containing 2% Sodium Dodecyl Sulfate, 1% β-mercaptoethanol, 2% Polyvinylpyrrolidone 40 and Phenanthroline 5mM) to 1g of ground powder (leaf or grain), incubating for 30 minutes at 65°c, initially washing with ice cold ammonium acetate 7.5M and precipitating with isopropanol overnight before a finally washing with 70% ethanol and suspending in molecular grade water (Taylor et al. 2010). The extracted DNA was quantified using a Nanodrop (Nanodrop 2000^{TM}Thermofisher), and each sample brought to a final DNA concentration of 20ng/μl^{-1}.

2.5 Quantitative PCR assessment of pathogen DNA in leaves and grain

As the accurate identification of RLS in infected plants has historically been problematic due to the confusion with physiological leaf spots and other diseases (Havis et al., 2015) quantities of *R. collo-cygni* biomass in both the leaves and grain were also determined using a

198 qPCR assay as described by Taylor et al. (2010), with some minor modifications as described
199 below. Due to the large number of reactions to be completed, and the need for standard
200 controls a plasmid containing the target amplicon was generated. Briefly, DNA was
201 extracted from *R. collo-cygni* cultures (isolate *DK05 Rcc 001* kindly provided by Neil Havis,
202 Scotland's Rural College) using a GenElute Plant Genomic DNA Miniprep Kit (Merck
203 KGaA, Darmstadt, Germany) in accordance to the manufacturer's instructions. A 466bp of
204 the internal spacer regions (ITS), encompassing the 115bp fragment used in the qPCR was
205 generated using the primers (Ram466F 5'-ACTGAGTGAGGGAGCAATCC-3' and
206 Ram466R 5'-CCTACCTGATCCGAGGTCAA-3') and subsequently cloned using the
207 pGem®-T Easy Vector System (Promega, Madison, WI 53711 USA) into cells of *E. coli*
208 strain JM109 as per manufacturer's instructions. Subsequent plasmid extraction was
209 performed using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, PLN70, USA)
210 following manufacturer's instructions. PCR was performed to validate the ligation of required
211 DNA fragment into the plasmid, using Ram466F and Ram466R primers, followed by gel
212 electrophoresis.

213 Quantification reactions were performed in volumes of 20µl as per Taylor et al.,
214 (2010), containing 8.7µl PCR grade water, 4µl LightCycler® Multiplex DNA Master (Roche
215 Molecular Systems, Inc. 4300 Hacienda Dr, Pleasanton, CA 94588, United States), 1 µl each
216 of RamF6 forward and RamR6 reverse primer (400nmol), 0.3µl Ram6 FAM probe and 5ng
217 of the extracted leaf or grain DNA (normalized to 20ng/µl). The reaction was performed
218 using a LightCycler® 96 Instrument (Roche Molecular Systems), and analysed with
219 LightCycler® 96 SW 1.1 software. The qPCR was conducted using the following conditions:
220 pre-incubation for 30s at 95°C, followed by 45 cycles of 5s at 95°C and 30s at 58°C. Each
221 dilution for the standard curve was run in triplicate and uncharacterised samples were run in
222 duplicate.

223 **2.6 Statistical Analysis**

224 Analysis of variance (ANOVA) was used to evaluate differences between treatments for
225 visual symptoms, *R. collo-cygni* biomass in the leaf, grain yield and *R. collo-cygni* biomass in
226 the grain. Separate models were used to analyse these datasets for differences between the
227 main effects (site, year, variety, fungicide treatment) and all interactions between them. Non-
228 significant interactions terms ($P > 0.05$) were then removed from each respective model. Data
229 from visual leaf assessments, *R. collo-cygni* biomass in the leaf and grain required $\log_{(10)}$

transformation to normalize the distribution of residuals, whilst grain yield residuals were normally distributed and therefore did not require transformation. Due to the distribution of residuals, when investigating agreement between the datasets and raw plot scores, a spearman rank correlation was used with a $P > 0.05$ significance. All statistical analysis was performed in Genstat V14 (VSN International Ltd. 2011).

3.0 Results

3.1 Visual disease on leaf 2

Based on the total minutes of leaf wetness (MLW) during stem extension the trials at Kildalton were consistently deemed to be at high risk of RLS development, whilst those at Oak Park were deemed at a low, high and moderate risk in 2016, 2017 and 2018, respectively)(Table 1). Due to an extended period of drought post stem extension in 2018 no visual RLS developed and therefore was absent from the analysis. Significantly higher levels of disease were recorded at Kildalton than Oak Park, reflective of the longer periods of MLW experienced at Kildalton (Figure 1). Higher levels of disease were recorded in 2017 than 2016 (P <0.001). Whilst significant effects of fungicide treatment on disease levels were recorded, these were dependent on barley variety, or the year, or the site (Table 4). In the case of the interaction of fungicide treatment with variety, no differences between the 'reference' treatment and either decision support system (DSS) treatment were observed irrespective of variety, demonstrating that additional fungicide input, whether through increased rates of application or the addition of the SDHI, was not required even when increased risk of disease was predicted. In the single site (Oak Park, 2016) where low risk of disease was predicted the reduction in fungicide input in both the 'DSS rate' and 'DDS product' was equally as effective at maintaining disease control as the 'reference' treatment. Disease levels in the untreated and QoI treatment plots depended on variety. In the untreated plots, cv. Propino displayed the highest levels of disease, whilst cv. RGT Planet had the lowest. Although the QoI fungicide treatment had significantly lower levels of disease compared to the untreated, levels depended on variety, with Irina displaying higher levels of disease compared to the others (Figure 2).

3.2 Quantification of R. collo-cygni biomass in leaf 2

Similar to the visual disease data, extremely low or no amounts of *R. collo-cygni* biomass were detected in the 2018 collection and so were excluded from the analysis. When restricted

to both 2016 and 2017 an overall moderately positive relationship between visual disease and quantifiable *R. collo-cygni* biomass was detected for leaf 2 (R^2=0.58; d.f. = 298, P<0.001) (Figure 3). As such, factors effecting visual disease severity (year, site and treatment and their interactions) also effected levels of *R. collo-cygni* biomass. However, barley variety had a significant effect on *R. collo-cygni* biomass levels, but not visual disease. Additionally, three way interactions between treatment x variety x site and treatment x site x year were also observed for *R. collo-cygni* biomass (Table 5). Comparable to the visual disease data no differences in *R. collo-cygni* biomass were observed between the 'DSS' treatments and the 'reference' treatment. However unlike the visual symptoms, cv. RGT Planet had the highest levels of detectable *R. collo-cygni* in the untreated plots. Similar to the visual symptoms, levels of *R. collo-cygni* were greatest in cv. Irina following the QoI treatment, most likely contributing to the significant effect of variety (Figure 4).

3.3 Effect of Ramularia on grain yield

As significant differences in disease levels were observed between 2016 and 2017 the relationship between levels of disease and grain yield was determined for both the combined dataset (2016 and 2017) and for each year individually. For the combined dataset a weak negative relationship between grain yield and both visual symptoms (R^2 = -0.45, P < 0.001), detectable biomass (R^2 = -0.42, P < 0.001) was observed (Figure 5). When assessed individually no relationships were observed in 2016 for either visual symptoms or biomass levels with grain yield (R^2 = -0.019, P = 0.811 and R^2 = 0.176, P = 0.036, respectively) (Supplementary Figure 1). However, in 2017 strong negative relationships were detected for both visual symptoms and biomass quantities with grain yield (R^2= -0.748, P < 0.001 and R^2= -0.5, P <0.001 respectively) (Supplementary Figure 2). Accordingly, treatment, year, site, variety, and various interactions between these factors had significant effects on grain yield (Table 6). These differences were generally due to differences in the responses to the QoI treatment between Kildalton and Oak Park and between the two years, with yield responses following the QoI fungicide treatment lower under higher disease pressures experienced at Kildalton and in 2017. Again no differences were apparent between the 'reference' treatment and 'DSS' treatments irrespective of the risk of disease predicted. Additionally, varietal yields differed depending on site and year, although cv. RGT Planet tended to provide amongst the highest yields irrespective of both (Figure 6).

3.4 Quantification of R. collo-cygni biomass in harvested grain

A weak positive relationship between quantities of *R. collo-cygni* in leaf 2 and the harvested grain was detected (R^2=0.33, d.f. = 281, P<0.001) (Supplementary Figure 3). Levels of DNA detected in the grain were considerably lower when compared to those detected in the leaves, with a maximum mean value of 2.9pg in grain compared to max of 26977pg in leaves. Fungicide treatment, site and year, and the interactions between site x year, and treatment x site had significant effects on the level of *R. collo-cygni* biomass detected in the grain (Table 7). Overall, higher levels were detected in 2017 when compared to 2016 and at Kildalton when compared to Oak Park. Significant differences between the treatments in 2017 were largely due to lower *R. collo-cygni* biomass levels being detected in the DSS rate treatment, which due to the high risk predicted at both sites that year was a treatment of increased rates of prothioconazole and chlorothalonil (Figure 7).

4.0 Discussion

This study investigated whether it is possible to predict risk of RLS by examining the effects of various risk modifiers on fungal development, disease expression and grain yield. Risk modifiers under investigation were barley varietal resistance and environmental conditions (MLW) at stem extension. By imposing levels of risk (low, medium, high) based on the total number of MLW for the 14 day period after the start of stem extension it was possible to demonstrate that, despite some years being deemed high risk, the 'reference' fungicide treatment of a mixture of prothioconazole and chlorothalonil, each applied at half their respective recommended label rates, was sufficient to control RLS. Unfortunately, as only one of the sites was deemed low risk, it is difficult to determine if either omitting the azole or reducing the dose of either fungicides would provide adequate control under a low risk scenario.

In 2016 and 2017 the prediction of risk based upon the MLW during the period of stem extension were indicative of the levels of disease experienced later during grain filling. Unfortunately, for 2018 the predicted risk (moderate-high) that was forecast for the trials failed to develop at either site. This was likely due to the fact that from late-May through to mid-July (approximately GS49-GS75) almost no rainfall was recorded at either site and this disease development was curtailed. Although 2018 was an unusual year, it demonstrates that, whilst the MLW surrounding stem extension may indicate levels of risk, the development of RLS is also highly dependent on weather conditions that follow this period. This finding is in

general agreement with Havis et al. (2018) who found that although factors such as MLW, temperature and rainfall are likely to contribute to RLS, further research is required to understand the specific conditions that lead to RLS epidemics. However, for forecasting or risk prediction systems to be effective they must predict disease development in advance so that timely interventions can be made if required. If, as the case appears to be for RLS, these conditions are environment specific and continue up until the development of visually observable disease symptoms then the value of such a prediction system would be questionable, as appropriate and timely fungicide interventions would be impossible.

Variable levels of RLS disease were experienced across the study. Although these trials provided more information on the control of this disease, for each of the disease components assessed (visual disease, *R. collo-cygni* biomass in both leaf and grain, and impacts of disease on grain yield) multiple levels of interactions between the studied factors were identified. This further demonstrates the changeable nature of the pathogen/disease and the difficulties facing growers and agronomists in deciding upon the most effective means of achieving control. Within the trials where significant levels of RLS were recorded, and where the disease was allowed to develop unchecked by fungicide, substantial reductions in yield occurred. As outlined by Kildea et al (2018), such yield losses have the potential to seriously undermine the profitability of barley production in Ireland. Unfortunately the relationship between yield loss and levels of *R. collo-cygni*, whether assessed as visual symptoms of RLS or as quantity of *R. collo-cygni* biomass in leaf 2 was not always clear, especially where different levels of fungicides were applied. As significant site and year effects on disease levels were observed, their influences on grain yield were examined separately. Whilst moderate levels of disease were detected in 2016 only a weak negative relationship with yield was observed. Conversely, in 2017 where higher levels of disease were observed a much stronger relationship was observed. Although the 2017 data demonstrates the clear impacts RLS can have on barley yields, the poor relationship in 2016 also highlights that further insights into this relationship need to be examined. A lack of relationship between disease levels and grain yield is not uncommon in cereals as there are many factors, other than disease, that influence grain yield (genetic yield potential of the variety, environmental conditions, nutritional status of the crop etc.) (Powell et al. 2012).

Across the various fungicide treatments a pre-stem extension fungicide treatment of prothioconazole and pyraclostrobin was applied and although this fungicide application was not applied for RLS control, it may have impacted the epidemic development. Since 2016

resistance to the azole fungicides, including prothioconazole has been detected throughout European populations, including Irish populations (Rehfus et al. 2019). Further analysis of the azole sensitivity status of the populations from each of the trials is required to determine if differences concerning the impacts on yield between the two years are in part due to differences in activity of this initial fungicide application. As spring barley yields are determined by what happens from early tillering onwards (Kennedy et al., 2017), any alleviation of stresses, such as those potentially imposed by RLS following the pre-stem extension fungicide, may have limited potential yield losses.

Fungicides can provide an effective strategy for control of RLS when used correctly, however, for the majority of fungicides there is a significant risk of resistance developing in the pathogen population which can have devastating consequences for fungicide efficacy (Fountaine & Fraaijie, 2009). The effects of fungicide resistance are clearly demonstrated here by the lack of control from the QoI treatment (Figure 1). Equally, varietal resistance is often viewed as key to alleviating the reliance on fungicides , thereby providing an effective IPM tool by ensuring both disease control and delaying the evolution of fungicide resistance (Lamichhane, 2016). Unfortunately, as an elusive disease that has only been considered a significant threat to barley production this century, limited information is available on varietal resistances to RLS, and where it is proposed to exist its reliability is questioned (AHDB 2019). Within the present study four modern commercial varieties were selected based on their published resistances for the UK (AHDB 2015) and whilst the reliability of the UK recommended list rating for RLS has been questioned this continues to be the perceived resistance of these varieties (Neil Havis *personal communication*). Levels of disease present in the untreated control plots of each of these varieties did not reflect their rating, with the variety proposed to be most susceptible, Olympus, exhibiting similar levels of visual disease and detectable *R. collo-cygni* biomass as the variety proposed to be most resistant, cv. KWS Irina. Furthermore, the two varieties that exhibited both the least and most visual symptoms, cv. RGT Planet and cv. Propino respectively, were regarded as having similar levels of moderate resistance. As the AHDB recommended list is based on the performance of varieties in the UK (this includes a trial conducted in Northern Ireland) it is possible that the Irish *R. collo-cygni* population is different and as such has developed to display different responses to these varieties. What this aspect of the study does confirm is that differences in resistances between varieties do exist. How best to exploit these varieties and further utilise RLS resistance in breeding programmes and on farm remains to be determined.

Unfortunately, the complexity of utilising varietal resistance is further compounded by the fact that the visual differences observed were not always reflected by the levels of *R. collo-cygni* biomass detected in the same leaf layer at the same time point. For instance, cv. RGT Planet had consistently the lowest levels of visible disease and Propino the highest (Figure 2), whilst cv. RGT Planet had the highest levels of detectable *R. collo-cygni* biomass, whilst cv. Propino had the lowest (Figure 4). Furthermore, although no differences were observed between the varieties following the 'reference' treatment or either DSS based treatments, increased visual symptoms and *R. collo-cygni* biomass levels were detected in cv. KWS Irina following the QoI treatment (Figure 2). Both these findings suggest that if varietal resistance levels are to be reliably determined a greater understanding as to the relationship between the barley plant and the pathogen will be required. In the former case of cv. RGT Planet, it may be a form of resistance, whereby high levels of the pathogen are sustained in the plant before it imposes sufficient stresses to initiate the pathogenic phase of the disease. In the case of cv. KWS Irina, the QoI fungicide may have created the environment whereby *R. collo-cygni* was allowed to proliferate possibly through direct inhibition of competing organisms within the barley microbiome, or indirectly through physiologically influencing the barley plant.

Whilst a weak relationship was detected between levels of *R. collo-cygni* biomass in leaf 2 and the harvested grain, no relationship existed between grain yield and *R. collo-cygni* biomass in the grain. Surprisingly, given the quantities of *R. collo-cygni* biomass detected in the leaves, and the levels of visual symptoms, the quantity of *R. collo-cygni* biomass detected in the grain was very low. Furthermore, levels were low when compared to those previously published by (Oxley & Havis 2010). This may simply just be due to differences in the qPCR assay setup but could also be due to differences in the Scottish and Irish *R. collo-cygni* populations. Considering the former, in the present study quantities were determined from standard curves established using plasmids containing the target fragment, whilst in the study of Oxley & Havis (2010) it was from standard curves established using genomic DNA. Undoubtedly seed borne infections contribute to RLS epidemics (Zamani-Noor et al., 2009; Havis et al., 2014). The specific role it plays remains unresolved, however, as observed in the present study, even where high levels of RLS control were achieved *R. collo-cgyni* was still detectable in the subsequent grain suggesting generating disease free seed will be a challenge. In 2016 no differences were observed between the different treatments in terms of levels of *R. collo-cygni* biomass in the grain, irrespective of the differences that existed in leaf 2. In the

higher pressure year of 2017, the only difference between treatments was between the 'DSS rate' treatment, which had increased rates of both the azole and chlorothalonil, and the untreated, potentially suggesting a relationship between persistence of foliar control and levels in the subsequent harvested grain. Even though these differences existed, it was still detectable in the 'DSS rate' treatment and as no differences existed between this treatment and the 'reference' treatment the increased spend for a marginal decrease in levels of *R. collo-cygni* in the grain would be unjustified economically but equally from an anti-resistance perspective.

The 'reference' treatment and both 'DSS' treatments were based upon the multisite fungicide chlorothalonil, which has proved very effective for the control of RLS. However since 20th May 2020 the use of chlorothalonil in European production systems is no longer permitted (Anon, 2019). The recent development of resistance to the current azoles, in particular prothioconazole and the SDHI fungicides (Rehfus et al., 2019), which until recently were extremely effective, underlines the need for alternative control strategies. Although additional azole, QoI and SDHI fungicides are being developed and currently display good efficacy against RLS, the ability of the pathogen to readily adapt is of concern for the longevity of their activity. The present study further highlights the complexities that exist in controlling RLS, and if these fungicides are to remain effective the integration of varietal resistance and risk prediction into management strategies is a must. For such IPM strategies to be effective further investigations into this complex pathosystem are immediately required.

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Figure 1 Mean ramularia leaf spot (RLS) on leaf 2 per fungicide treatment. Error bars represent least significant differences ($P < 0.05$). DSS: decision support system treatments which are based on minutes of leaf wetness (MLW), DSSprod: reference treatment modified by adding/eliminating fungicide actives, DSSrate: reference treatment modified by increasing/decreasing fungicide application rates, Ref.Prog: reference fungicide treatment, QoI: pyraclostrobin treatment.

Figure 2 Mean ramularia leaf spot (RLS) on leaf 2 per each of the four varieties studied. Error bars represent least significant differences ($P < 0.05$). DSS: decision support system treatments which are based on minutes of leaf wetness (MLW), DSSprod: reference treatment modified by adding/eliminating fungicide actives, DSSrate: reference treatment modified by increasing/decreasing fungicide application rates, Ref.Prog: reference fungicide treatment, QoI: pyraclostrobin treatment.

Figure 3 Visual ramularia leaf spot symptoms on leaf 2 versus detectable levels of *Ramularia collo-cygni* (Rcc) DNA in leaf 2. (R^2 = 0.58, d.f. = 298, $P < 0.001$, based on Log10 transformation of both values).

Figure 4 Interaction between year, variety and fungicide treatment on detectable levels of *Ramularia collo-cygni* (Rcc) DNA in leaf 2. Error bars represent least significant differences ($P < 0.05$). DSS: decision support system treatments which are based on minutes of leaf wetness (MLW), DSSprod: reference treatment modified by adding/eliminating fungicide actives, DSSrate: reference treatment modified by increasing/decreasing fungicide application rates, Ref.Prog: reference fungicide treatment, QoI: pyraclostrobin treatment.

Figure 5 A) Visual ramularia leaf spot symptoms (RLS) on leaf 2 versus grain yield (t/ha) (R^2 = -0.45, d.f. = 319, ***P*** = <.0001) and B) detectable levels of *Ramularia collo-cygni* **(Rcc)** DNA in leaf 2 versus grain yield, in both 2016 and 2017. (R^2 = -0.42, d.f. = 301, ***P*** = <.0001).

Figure 6 Effect of A) fungicide treatment and B) at both Kildalton and Oak Park on grain yield (t/ha). DSS: decision support system treatments which are based on minutes of leaf wetness (MLW), DSSprod: reference treatment modified by adding/eliminating fungicide actives, DSSrate: reference treatment modified by increasing/decreasing fungicide application rates, Ref.Prog: reference fungicide treatment, QoI: pyraclostrobin treatment.

Figure 7 Mean detectable *Ramularia collo-cygni* (Rcc) DNA levels in grain in 2016 and 2017. Error bars represent least significant differences ($P < 0.05$).

Figure 1.

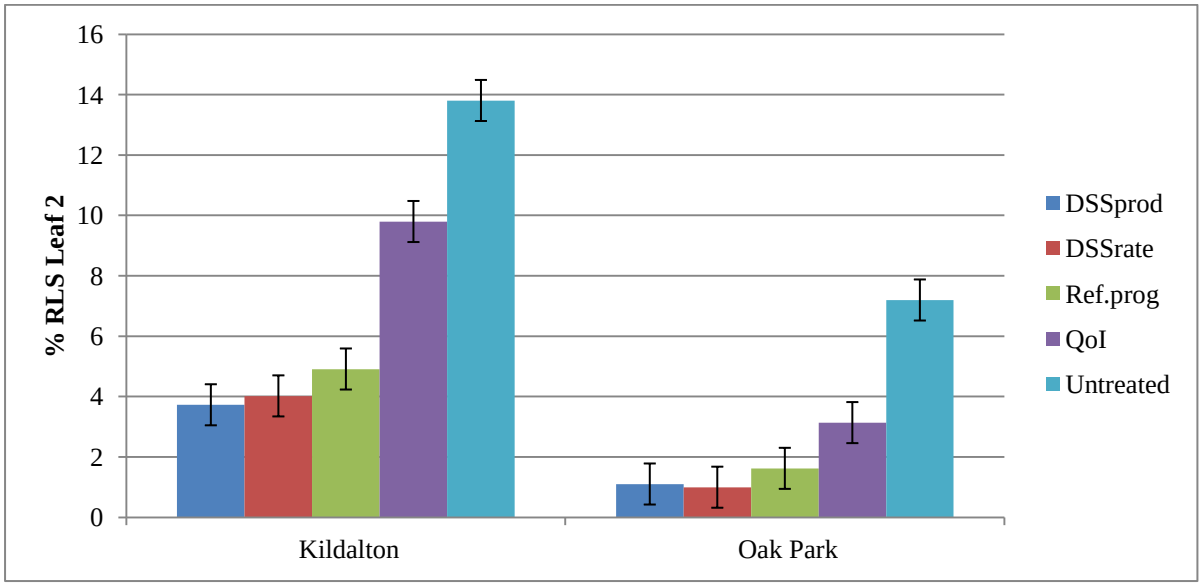

16
14
12
10
8
6
4
2
0
% RLS Leaf 2
Kildalton
Oak Park
DSSprod
DSSrate
Ref.prog
QoI
Untreated

Figure 2.

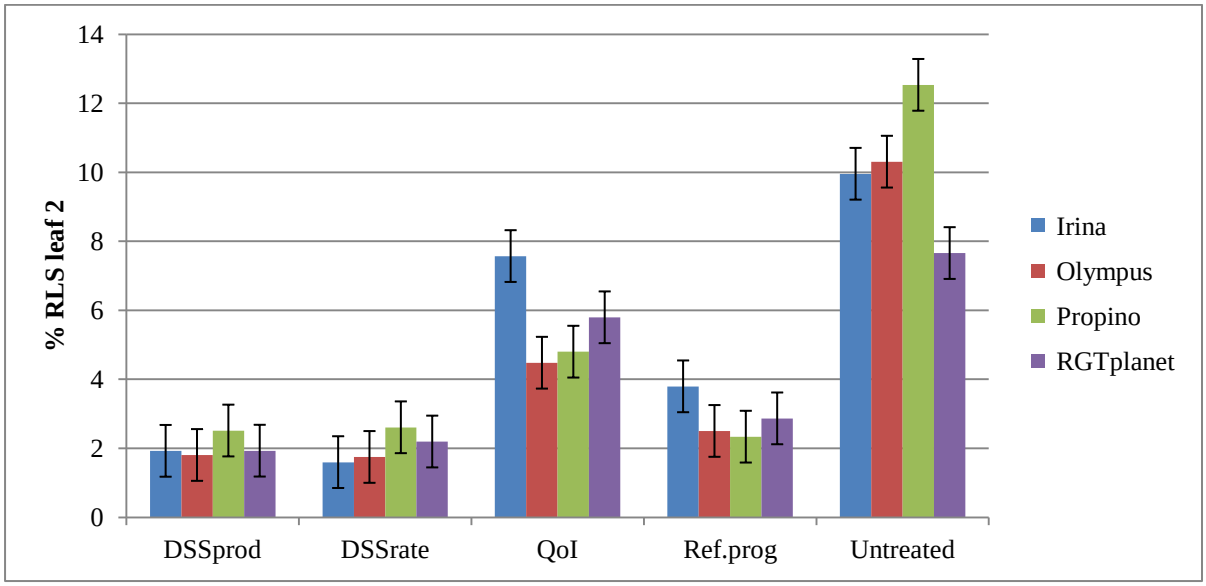

% RLS leaf 2
14
12
10
8
6
4
2
0
DSSprod
DSSrate
QoI
Ref.prog
Untreated
Irina
Olympus
Propino
RGTplanet

Figure 3.

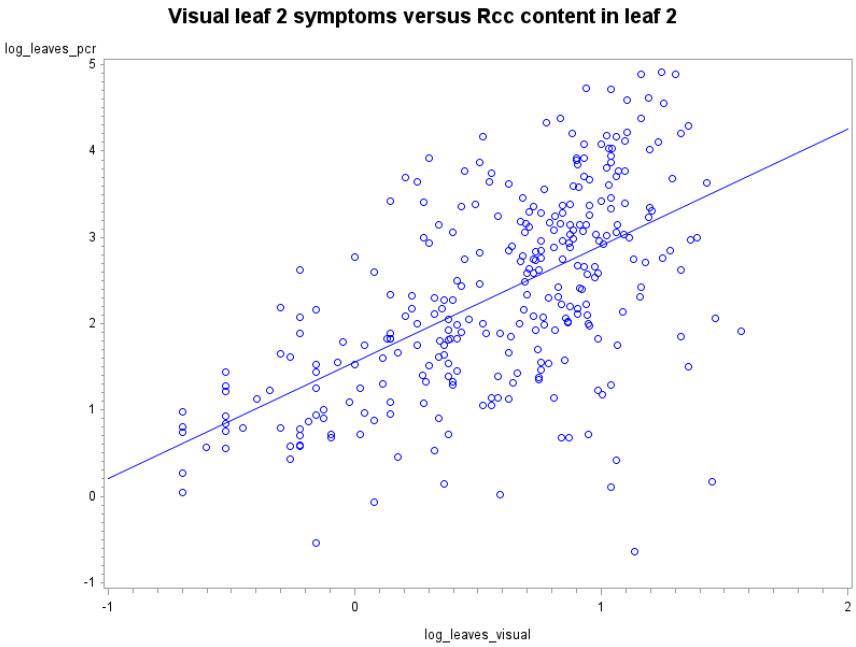

Visual leaf 2 symptoms versus Rcc content in leaf 2
log_leaves_pcr
5
4
3
2
1
0
-1
-1
0
1
2
log_leaves_visual

Figure 4.

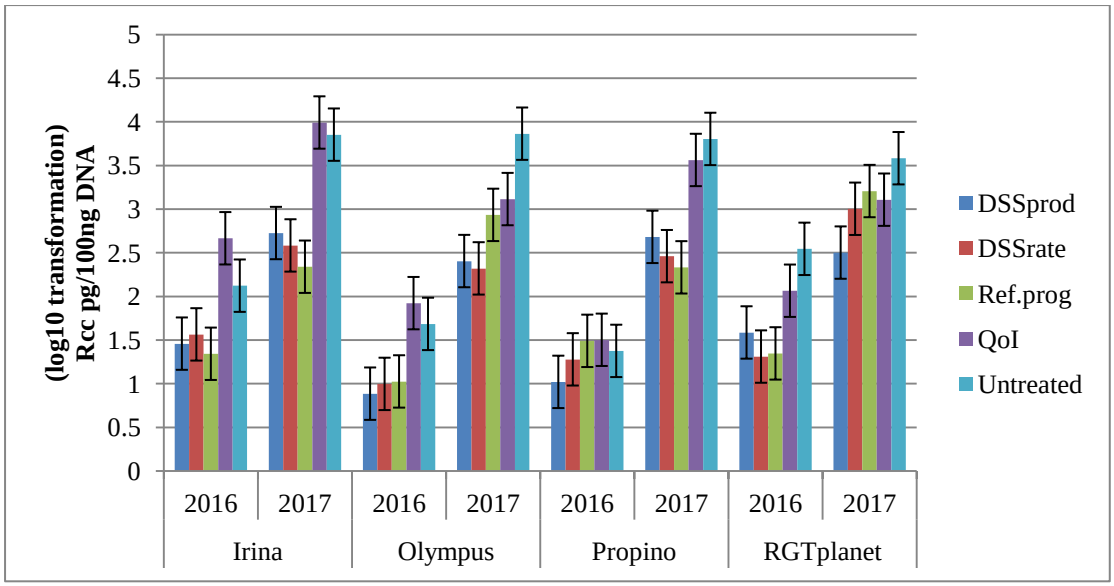

(log10 transformation)
Rcc pg/100ng DNA
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
2016
2017
2016
2017
2016
2017
2016
2017
Irina
Olympus
Propino
RGTplanet
DSSprod
DSSrate
Ref.prog
QoI
Untreated

Figure 5.

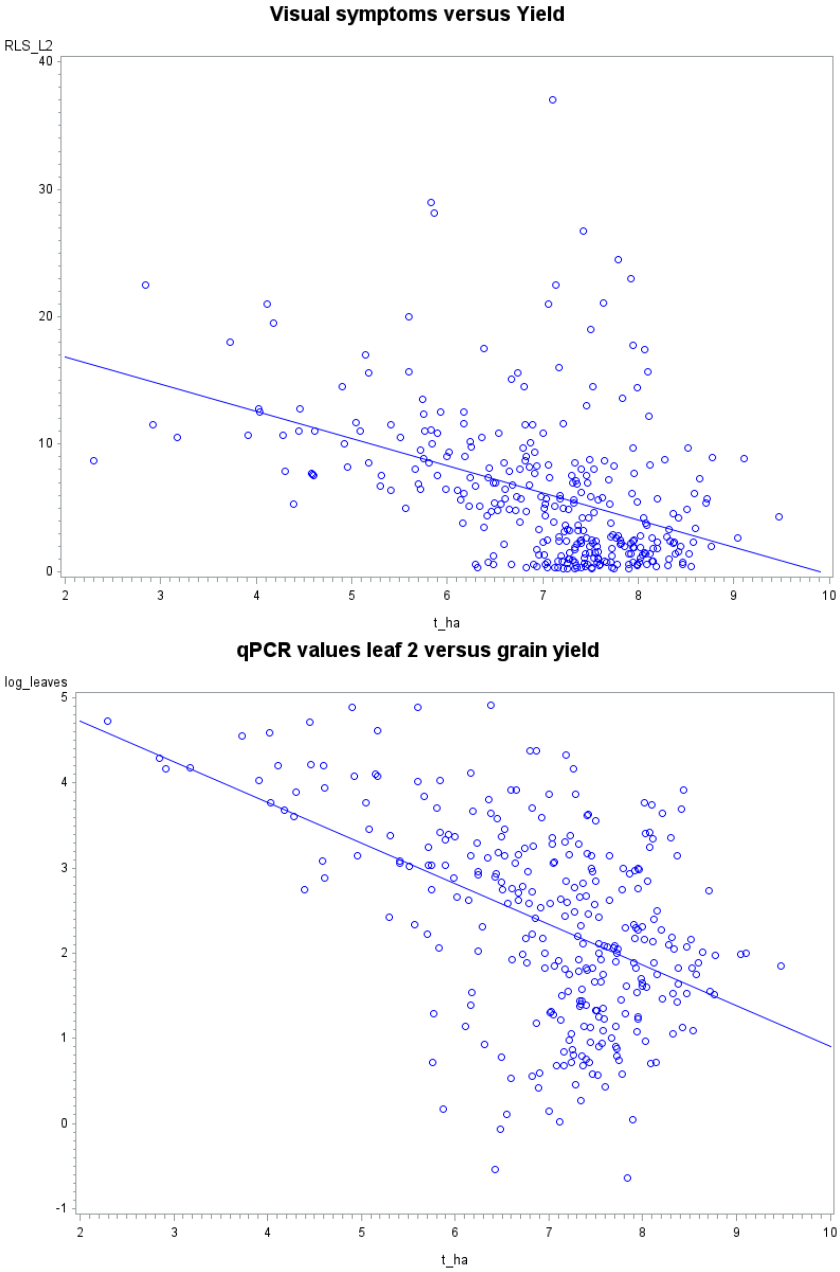
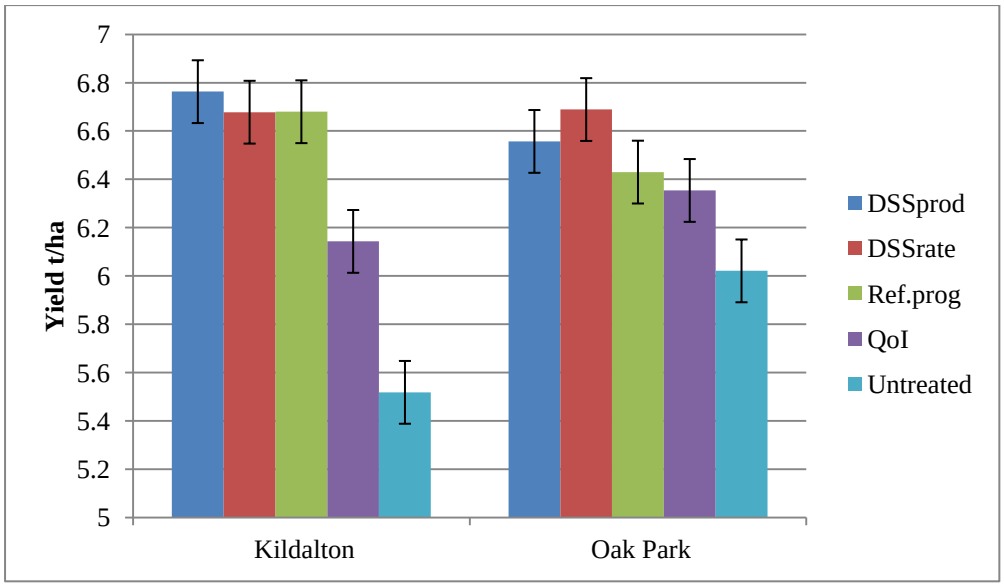

Visual symptoms versus Yield
RLS_L2
40
30
20
10
0
2
3
4
5
6
7
8
9
10
t_ha
qPCR values leaf 2 versus grain yield
log_leaves
5
4
3
2
1
0
-1
2
3
4
5
6
7
8
9
10
t_ha

Figure 6.


7
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
5.2
5
Yield t/ha
Kildalton
Oak Park
DSSprod
DSSrate
Ref.prog
QoI
Untreated

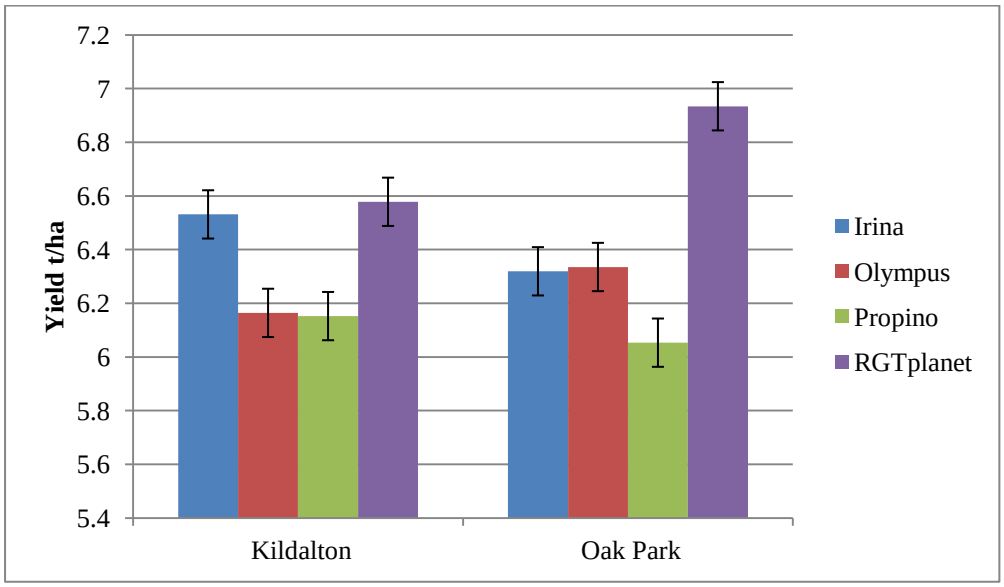

7.2
7
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
Yield t/ha
Kildalton
Oak Park
Irina
Olympus
Propino
RGTplanet

Figure 7.

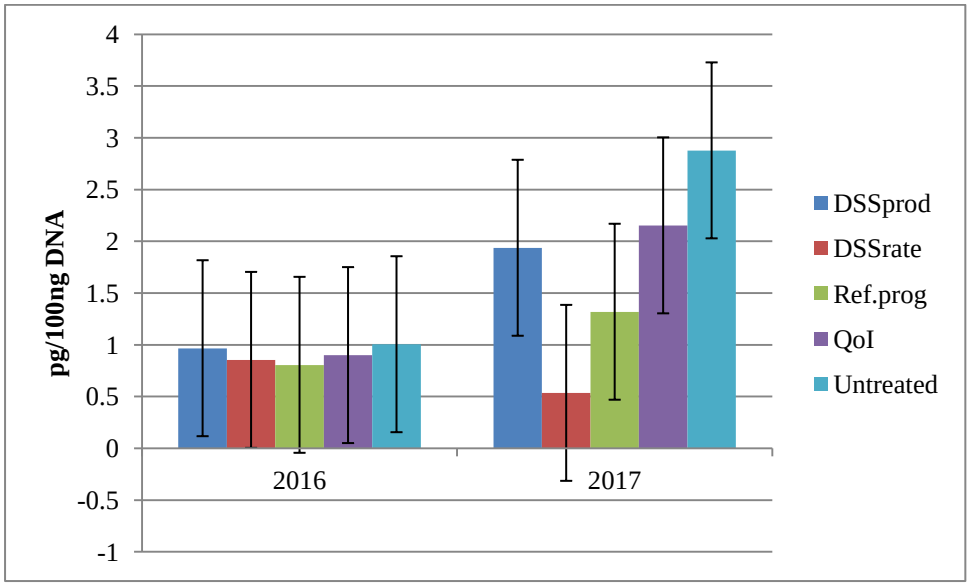

4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
pg/100ng DNA
2016
2017
DSSprod
DSSrate
Ref.prog
QoI
Untreated

Table 1: Minutes of leaf wetness, predicted Ramularia leaf spot risk and fungicide programme adjustment to reflect risk

Year	Location	MLW[1]	RLS Risk[2]	DSS Rate[3]	DSS Product[4]
2016	Oak Park	3,940	Low	Proline (0.2 l/ha) & Bravo (0.5 l/ha)	Bravo (1.0 l/ha)
	Kildalton	7,650	High	Proline (0.6 l/ha) & Bravo (1.5 l/ha)	Siltra (0.5 l/ha) & Bravo (1.0 l/ha)
2017	Oak Park	8,580	High	Proline (0.6 l/ha) & Bravo (1.5 l/ha)	Siltra (0.5 l/ha) & Bravo (1.0 l/ha)
	Kildalton	10,380	High	Proline (0.6 l/ha) & Bravo (1.5 l/ha)	Siltra (0.5 l/ha) & Bravo (1.0 l/ha)
2018	Oak Park	4,560	Medium	Proline (0.4 l/ha) & Bravo (1.0 l/ha)	Proline (0.4 l/ha) & Bravo (1.0 l/ha)
	Kildalton	9,240	High	Proline (0.6 l/ha) & Bravo (1.5 l/ha)	Siltra (0.5 l/ha) & Bravo (1.0 l/ha)

[1]Minutes of leaf wetness (MLW) (relative humidity >90%) were determined for a period of two weeks during stem extension.

[2]Risk of Ramularia leaf spot (RLS) development dependent on MLW; low risk = MLW <4,500; medium risk = MLW 4,500-7,500; high risk MLW >7,500

[3]Proline (Bayer CropSicence) contains 250 g/l prothioconazole, with a manufacture recommended rate of 0.8 l/ha; Bravo (Syngenta) contains 500 g/l chlorothalonil, with a manufactures recommended rate of 2.0 l/ha. Where risk was deemed low (<4000 MLW) both products were applied at 25% the manufactures recommended rate; where risk was deemed medium (4000-7500 MLW) both products were applied at 50% the manufactures recommended rate; where risk was deemed high (>7500 MLW) both products were applied at 75% the manufactures recommended rate.

[4]Siltra (Bayer CropScience) contains 60 g/l bixafen and 200 g/l prothioconazole. In all risk scenarios Bravo was applied at 1.0 l/ha. Where risk was deemed low Proline was omitted; where risk was deemed medium Proline (0.4 l/ha) was included; where risk was deemed high an SDHI was included in addition to Proline, in the form of Siltra (0.5 l/ha).

Table 2: Spring barley varieties included in the trial, their perceived resistance at the time the trials were conducted and their pedigree

Variety	Breeder	Pedigree	RLS Resistance 1 (susceptible) -9 (resistant)[1]	Year first recommended
Irina	KWS Lockow GMBH	Conchita x Quench	7	2014
RGT Planet	RAGT, UK	Tamtam x Concerto	6	2017
Propino	Syngenta Seeds Ltd.	NFC Tipple x Quench	6	2011
Olympus	Limagrain, UK	Genie x LAN 0848	4	2017

[1]Reistance rating taken from the AHDB Spring Barley recommended list for 2015/2016. In 2018 AHDB ceased publishing the Ramularia leaf spot (RLS) resistance ratings due to inconsistencies in disease assessments across the recommended list trials (AHDB, 2019). The above ratings are however the expert opinion of how varieties will react (Neil Havis personal communication)

Table 3. Fungicides applied to the different varieties in the trials 2016-2018.

	Treatment	Fungicide[1]	Product	Rate	Comment
1.	Untreated	-	-	-	To determine levels of disease in the trial
2.	QoI	Pyraclostrobin	Modem	0.625 l/ha	To provide broad spectrum disease control without impacting RLS development
3.	Standard	Prothioconazole & chlorothalonil	Proline & Bravo	0.4 l/ha & 1.0 l/ha	To provide broad spectrum disease control including RLS
4.	DSS Rate	Prothioconazole & chlorothalonil	Proline & Bravo	0.2 – 0.6 l/ha 0.5 – 1.5 l/ha	See Table 1 for further information
5.	DSS Product	Chlorothalonil +/- prothioconazole/bixafen	Bravo +/- Proline or Siltra	0.1 l/ha 0.4 l/ha (Proline) or 0.5 l/ha (Siltra)	See Table 1 for further information

[1]With the exception of the untreated control all treatments received a cover spray of Proline (0.4 l/ha) and Modem (0.625 l/ha) at late tillering (<GS30) (Zadoks et al., 1974).

Table 4: The effect of treatment on % Ramularia leaf spot on leaf 2.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Treatment	4		23.72308	5.93077	84.85	<.001
Treatment.Cv	12		1.59	0.1325	2.07	0.039
Site	1		18.34399	18.34399	215.91	<.001
year	1		11.74664	11.74664	138.26	<.001
Treatment.Site	4		0.94244	0.23561	2.77	0.029
Treatment.year	4		2.88903	0.72226	8.5	<.001
Site.year	1		0.44694	0.44694	5.26	0.023

Table 5: Factors affecting *Ramularia collo-cygni* biomass levels in leaf 2.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Treatment	4		55.701	13.9252	36.88	<.001
Cv	3		8.0296	2.6765	8.44	<.001
Treatment.Cv	12		7.6881	0.6407	2.02	0.045
Site	1		42.1909	42.1909	96.01	<.001
year	1		169.5052	169.5052	385.73	<.001
Treatment.Cv.Site	12		14.9144	1.2429	2.83	0.002
Treatment.Cv.year	12		10.8724	0.906	2.06	0.022
Treatment.Site.year	4		5.0658	1.2664	2.88	0.024

Table 6: The effect of treatment on grain yield.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Treatment	4		56.759	14.1897	31.89	<.001
Cv	3		28.5251	9.5084	54.63	<.001
Treatment.Cv	12		5.2727	0.4394	2.52	0.012
year	2		474.0053	237.0026	640.19	<.001
Treatment.Site	4		9.3216	2.3304	6.29	<.001
Cv.Site	3		5.9827	1.9942	5.39	0.001
Treatment.year	8		20.9974	2.6247	7.09	<.001
Cv.year	6		11.1105	1.8517	5	<.001
Site.year	2		172.9827	86.4913	233.63	<.001

Table 7: Significant effects for *Ramularia collo-cygni* biomass levels in grain.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Treatment	4		6.5538	1.6385	7.86	0.002
Site	1		32.2641	32.2641	132.01	<.001
year	1		4.4655	4.4655	18.27	<.001
Treatment.year	4		4.0181	1.0045	4.11	0.003
Site.year	1		9.4278	9.4278	38.57	<.001