March 2014

Project Report No. 553

Control of ramularia leaf spot in a changing climate

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CONTENTS

1. Abstract

The LINK project on Control of Ramularia Leaf Spot in a Changing Climate (CORACLE) made substantial advances in understanding and controlling this disease of barley. Ramularia leaf spot, caused by the fungus *Ramularia collo-cygni*, has become important in temperate regions throughout the world. It has been important in Scotland and Ireland since 1998 and has become widespread in England since 2009. While it is most significant on spring barley in the UK, it is increasingly found on winter barley.

CORACLE showed that Ramularia has a negative effect on barley yields and must be controlled. Fungal spores were shown to disperse mainly at the end of the growing season, in July and August, following prolonged periods of leaf are wetness. It also showed that Ramularia epidemics can be predicted from leaf surface wetness during the period of stem extension, normally in May or June for spring barley. Using these insights into the epidemiology of Ramularia, CORACLE improved a disease forecasting system to help farmers apply appropriate fungicide sprays.

In research on disease control, CORACLE showed that seed treatment can reduce levels of *R. collo-cygni* in seed and thus reduce epidemics in susceptible varieties. The most effective treatment for established Ramularia remains a late-season spray of a foliar fungicide but the *R. collo-cygni* fungus is genetically diverse and has evolved resistance to several important groups of fungicides. It is regarded as a medium-to-high risk for further development of fungicide resistance. This means that, in future, control will increasingly need to focus on choosing a resistant variety and high standards of seed hygiene.

CORACLE found good genetic resistance to Ramularia in diverse varieties of winter and spring barley. An important discovery was that the *mlo* gene, which controls powdery mildew in many spring barley varieties, substantially increases susceptibility to Ramularia. The strength of the effect of *mlo* depends on environmental conditions. However it is possible to select for improved Ramularia-resistance in *mlo* varieties. This is reflected in the current Recommended List of Spring Barley, which includes several varieties, which combine excellent resistance to mildew with improved resistance to Ramularia.

A critical stage in the life-cycle of the *R. collo-cygni* fungus is the transition from growing harmlessly within the barley plant to becoming an aggressive parasite. Research in CORACLE showed that physical stress plays a central role in this transition and that plants which are better able to tolerate physical stress are generally less susceptible to Ramularia. A key goal for barley breeding is to produce varieties which combine Ramularia resistance, resistance to other diseases and tolerance of stress.

2. Key messages

2.1. Significance of Ramularia leaf spot on barley

- Ramularia leaf spot has become an important disease of barley and has now been detected in many barley growing countries worldwide.
- Ramularia has a negative effect on the yield of both spring and winter barley. While it is still more significant on spring barley in the UK, it is increasingly found in winter barley too.
- Most spore dispersal of the pathogen occurs in July and August after prolonged surface wetness in the crop.
- The severity of Ramularia epidemics can be predicted on the basis of leaf surface wetness in the crop at the time of stem extension in May or June.

2.2. Control of Ramularia leaf spot in barley crops

- The fungus can move from infected seed into the plant without displaying symptoms. The majority of seed stocks in the UK are now contaminated by Ramularia.
- Seed treatments can reduce epidemics in susceptible varieties but the most effective treatments are late season foliar sprays.
- The fungus is genetically diverse and should be regarded as a medium-to-high risk for development of fungicide resistance.

2.3. Breeding barley varieties resistant to Ramularia leaf spot

- There is wide genetic diversity for susceptibility to Ramularia among varieties of both spring and winter barley.
- The *mlo* gene for mildew resistance tends to increase susceptibility of barley to Ramularia, although the extent to which it does so is affected by the environment.
- Plant breeders can select for improved resistance to Ramularia, even in the presence of *mlo*, by selecting progeny lines from genetically diverse parents.

3.   Infection and pathology of Ramularia leaf spot

3.1.   Infection, growth and development of *Ramularia collo-cygni*

Also see Section 7.1

The movement of *Ramularia collo-cygni* (*Rcc*) within barley was examined in controlled condition experiments. A previously modified isolate of *Rcc* with an incorporated green fluorescent protein (GFP) was used in the study (Thirugnanasambandam *et al.,* 2011). The colonisation of leaves was studied using drop inoculations of a mycelial suspension of the GFP fungus. Colonisation of the leaves consisted of the growth of epiphytic hyphae although entry to the leaf was observed via stomata. (Figure 1A). This point of entry was previously observed for hyphal tubes from germinating spores (Stabentheiner *et al.*, 2009). During this phase the leaf was asymptomatic. During lesion formation a brick-like network of mycelia developed in the mesophyll layer of the leaf. Conidiophores were seen emerging from the stomata 14 days post inoculation (Figure 1B).
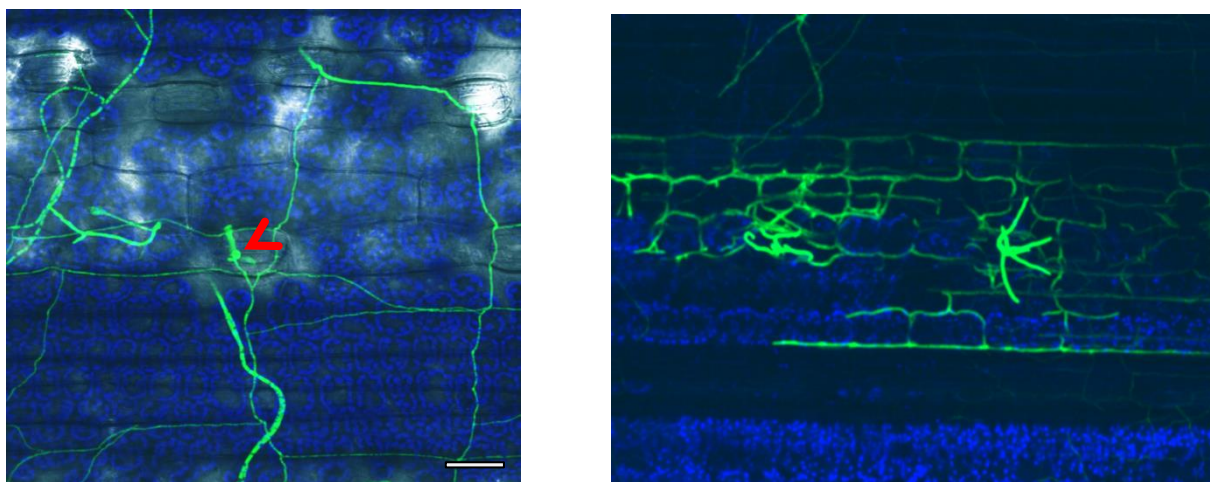
Figure 1. (A, left) Epiphytic growth of *Ramularia collo-cygni* on leaf surface. (B, right) Mesophyll colonisation and spore development.

Endophytic hyphae were physically different from epiphytic hyphae. They were thicker and more closely resembled the structure formed in the sub-stomatal cavity. Inoculation with the GFP isolate after barley heading was also carried out. During kernel development a thick layer of hyphae was observed in the layer between the pericarp and the aleurone layer (Figure 2A). The fungus was also observed in the embryo of seed. The deep seated nature of the fungus means that treatment of infection by seed treatments may be difficult.

Seed infected by GFP-*Rcc* was germinated and the movement of the fungus into developing tissue was observed. The fungus continued to move into the embryo post germination and grew intercellularly in the embryo and developing leaf tissue. Colonisation of the vascular bundles also took place and this could explain the movement of the fungus in barley in the absence of symptoms and spore movement (Figure 2B).
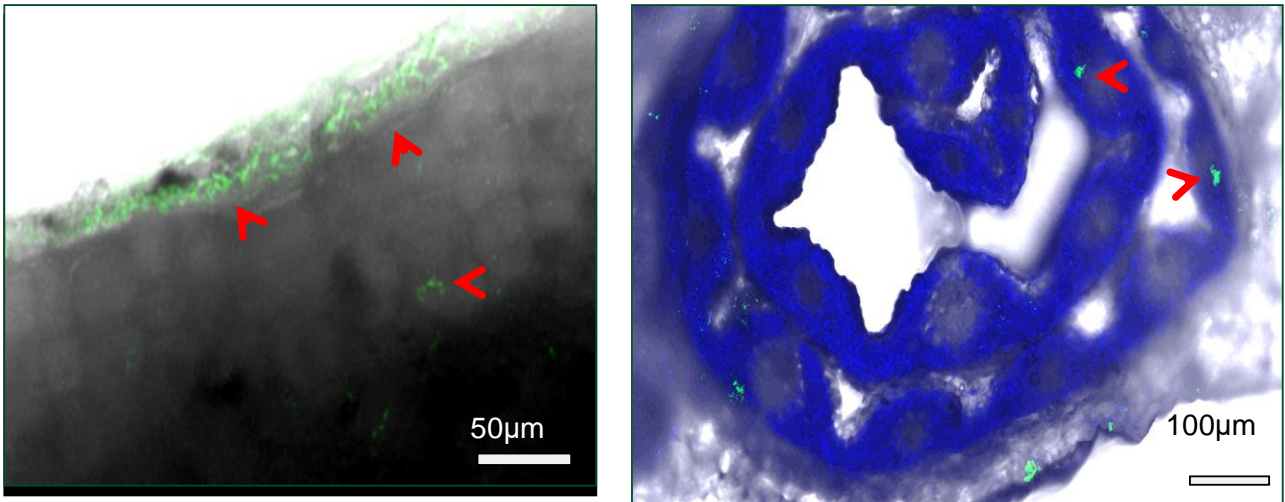
Figure 2. (A, left) Infection of seed by GFP-*Rcc*. (B, right) Colonisation of vascular bundles by GFP-*Rcc*.

The nature of the colonisation of seed by *Rcc* and the asymptomatic growth of the fungus closely resembles the growth of the mutualistic fungal endophytes of grasses *Epichloe* sp and *Neotyphodium* sp. The interaction between some endophytes and graminaceous hosts has been related to the protection of the host plant from herbivory. However, this has not been tested for *Rcc* and barley. Glasshouse experiments examining the growth of barley infected with *Rcc* indicated that high levels of *Rcc* may lead to increased height of plants in unstressed controlled conditions (Figure 3).
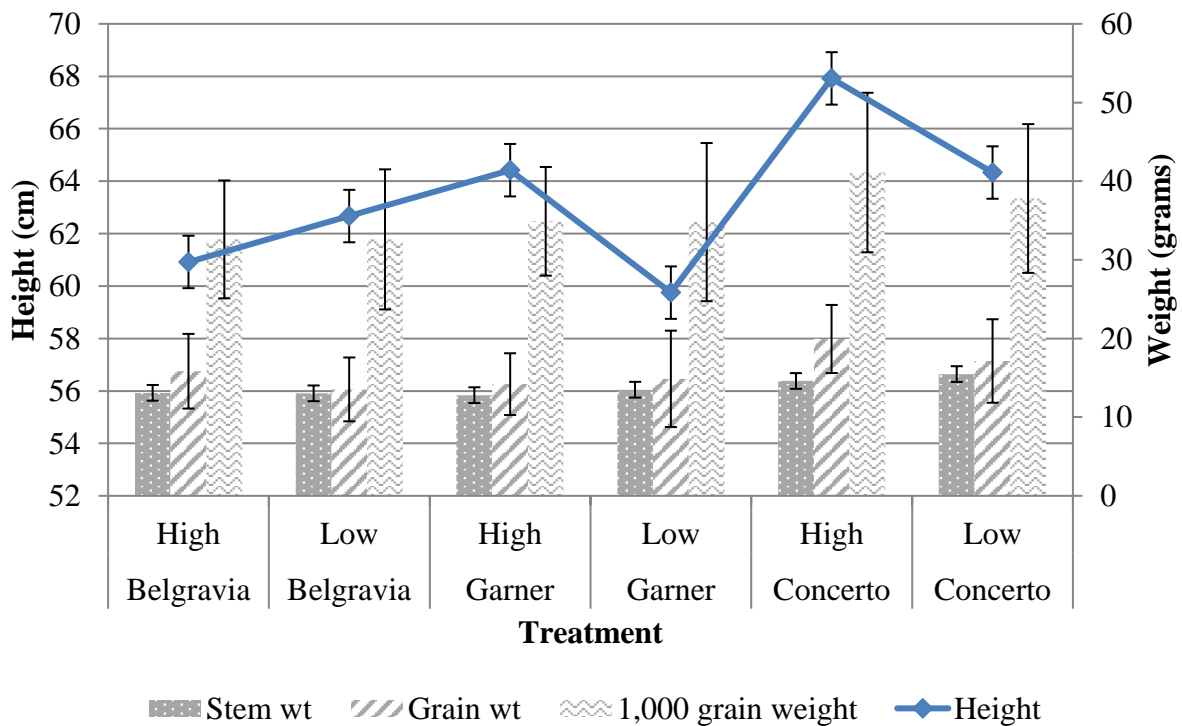
Figure 3. Effect of high or low levels of infection by *Rcc* on plant growth and biomass of three barley varieties in controlled conditions. Bars indicate ±1 standard error. Plant heights varied significantly (P<0.05) but other traits did not.

3.2. Role of seed infection in barley crops

Also see Section 7.2

The movement of the fungus in crops in the field was studied in three field trials. Two spring barley trials and one winter barley trial were carried out at Drumalbin Farm, Lanark. Plants were harvested at fortnightly intervals for the spring barley and monthly intervals for the winter trial. Plants were separated into leaf layers and DNA extracted and Rcc levels quantified using a qPCR assay (Taylor et al, 2011). Symptoms were recorded later in the season.

Results from the field experiments show that Rcc moves from seed into developing leaves (Figure 4). The highest levels of Rcc DNA appear in the F-1 and F-2 layers at the end of the season. Analysis of spore sampler results for the same site during these trials indicated no significant spore release until mid-July. This suggests that the fungus is moving systemically within the host rather than relying on infection via external spore production. Highest Ramularia symptoms were recorded on the F-1 leaf layer in both spring and winter barley.
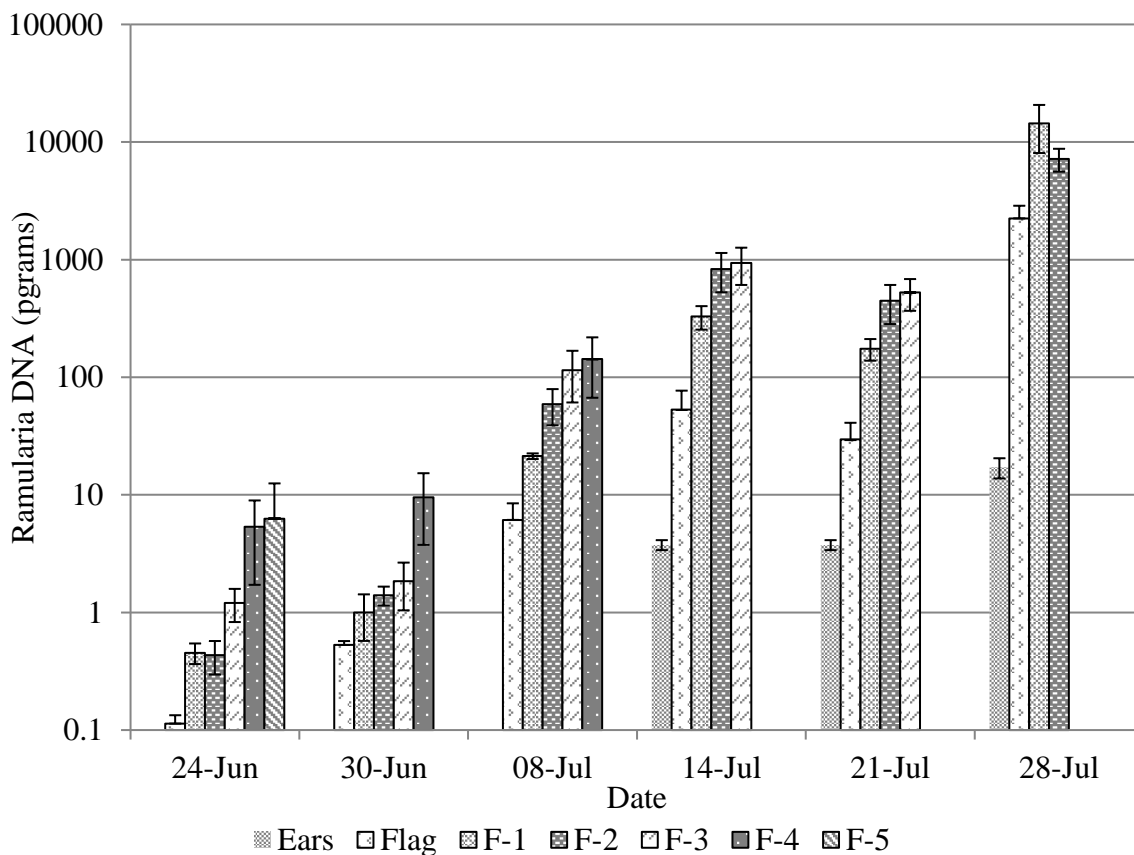
Figure 4. *Rcc* DNA levels in spring barley trial 2009.

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Annual seed testing of seed samples from commercial crops around the UK showed more Rcc in Scotland in general but levels varied within Scotland (Figure 5).

Analysis of Ramularia leaf spot epidemics and Rcc DNA levels in seed have shown that the environment plays a significant factor in disease severity as it affects the rate at which the fungus can colonise barley (Havis et al., 2013).
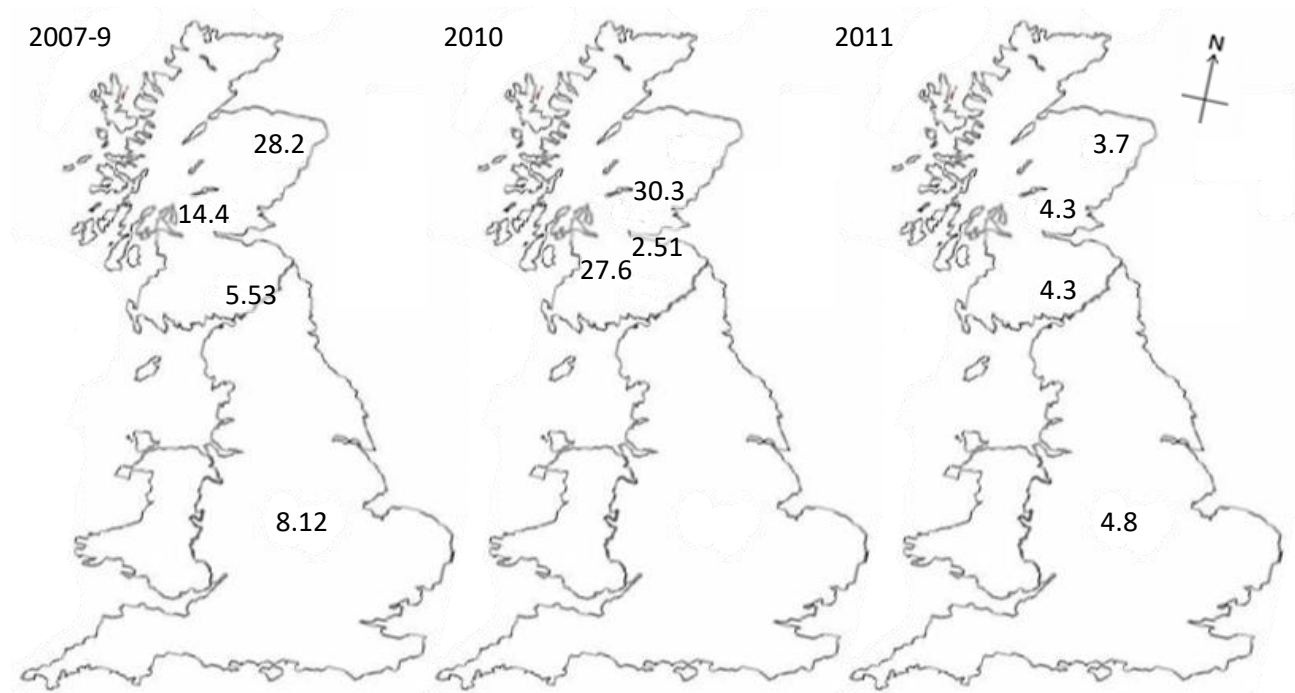
Figure 5. *Rcc* DNA levels in barley from commercial crops. Levels were influenced by site and year.

3.3. Relationship of Ramularia leaf spot to senescence and oxidative stress

Also see Sections 7.3, 7.4 and 7.5

As a late season disease, the development of Ramularia leaf spot symptoms has been associated with an overall decline in the host antioxidant system, which protects plants against stress (Schützendübel et al., 2008). The relationship between Ramularia and the control of the level of chemical oxidation within the plant (redox homeostasis) is not yet understood. Histochemical staining for two reactive oxygen species (ROS), peroxide and superoxide ions, was studied in the varieties Power, which is moderately resistant to Ramularia, and Braemar, which is highly susceptible. Hydrogen peroxide levels increased in both varieties (Figure 6A&B) as disease development progressed (Figure 6C) and the amount of *R. collo-cygni* fungus in the plant built up (Figure 6D). Levels of hydrogen peroxide were higher in the susceptible variety Braemar (Figure 6A&B) whereas superoxide did not appear to accumulate in either variety during Ramularia symptom development.
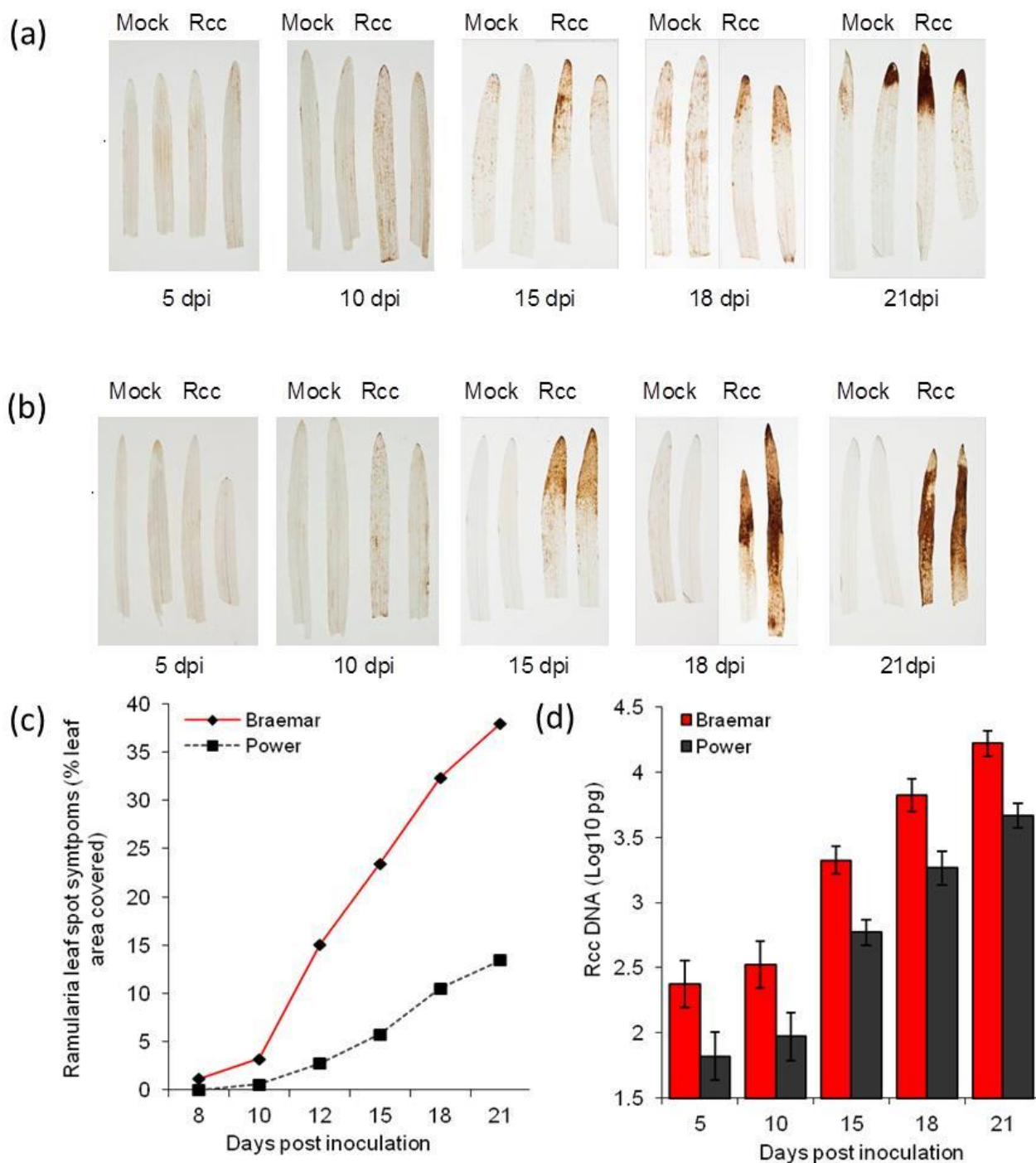
Figure 6. Hydrogen peroxide accumulation during Ramularia leaf spot development. Accumulation of 3,3'-diaminobenzidine (DAB) indicates a low level of hydrogen peroxide, revealed by the reddish-brown stain in Power (a) and a higher level in the more susceptible variety Braemar (b). Disease symptoms progressed more rapidly in Braemar than Power (c) while a larger amount of the *R. collo-cygni* fungus, indicated by the amount of DNA, was present in Braemar (d).

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Transcription of the major genes involved in scavenging hydrogen peroxide was studied because it indicates the extent to which the plant is able to adapt to oxidative stress. Increased transcription of scavenger genes was observed in leaves of Braemar inoculated with *R. collo-cygni* at the same time as increased hydrogen peroxide levels. No accumulation of peroxide scavenger transcripts was seen in the resistant variety Power. The increased level of hydrogen peroxide led us to test the role of this ROS in the disease transition of *R. collo-cygni*. Mutant plants unable to scavenge excess peroxide owing to reduced activity of a major peroxide scavenger, catalase, showed no difference in Ramularia symptoms or fungal biomass compared to wild-type plants with normal expression of peroxide-scavenging genes.

Next we examined whether imbalances to plant ROS status affected disease development using a series of barley lesion mimic mutants. These plants are affected in the metabolism of ROS owing to defects in genes involved in cellular metabolism, signalling and defence and consequently express symptoms similar to disease lesions even in the absence of disease. Mutations in two genes, both involved cation signalling, led to plants with less visible disease development which was not associated with a reduction in fungal biomass in five out six mutants tested. ROS imbalance as a factor in Ramularia leaf spot development was further analysed using an *in planta* infiltration assay with reagents that will manipulate hydrogen peroxide levels. We found that infiltration with catalase or hydrogen peroxide at 5, 7 or 10 days post inoculation (dpi) had no effect on disease development in Power and two other varieties resistant to Ramularia leaf spot. However, infiltration of catalase 5 dpi resulted in an increase in disease symptoms in Braemar and three other susceptible varieties. These results indicate that an imbalance to *in planta* ROS status may be involved in triggering the transition of *R. collo-cygni* to its disease causing phase.

In field situations, redox imbalance resulting in transient changes in ROS levels typically occur during periods of plant stress. The expression of Ramularia leaf spot symptoms has been shown to be increased when the host has been exposed to high light stress prior to fungal inoculation (Makepeace et al., 2008). Plants that had been waterlogged prior to inoculation also show increase disease expression, but not all varieties which were susceptible to abiotic stress induced Ramularia leaf spot.

To further study the link between abiotic stress and this disease, the disease response of a transgenic barley line that over-expressed a barley transcription factor gene, *Stress-induced NAC1* (*HvSNAC1*), in a Golden Promise background was examined. *HvSNAC1* overexpression has been shown to enhance drought tolerance in rice and barley (Hu et al., 2006; Al Abdallat et al., 2014). Both independent transgenic lines, OE#03 and OE#11, have reduced disease development (Figure 7A) and lower levels of fungal DNA compared to wild type Golden Promise plants (Figure 7B). Experiments examining the potential mechanism behind this resistance suggested

that delayed leaf senescence in the transgenic lines, as indicated by extended retention of chlorophyll measured in dark-induced senescence assays, may be involved (Figure 7C).

(A)

(B)

(C)
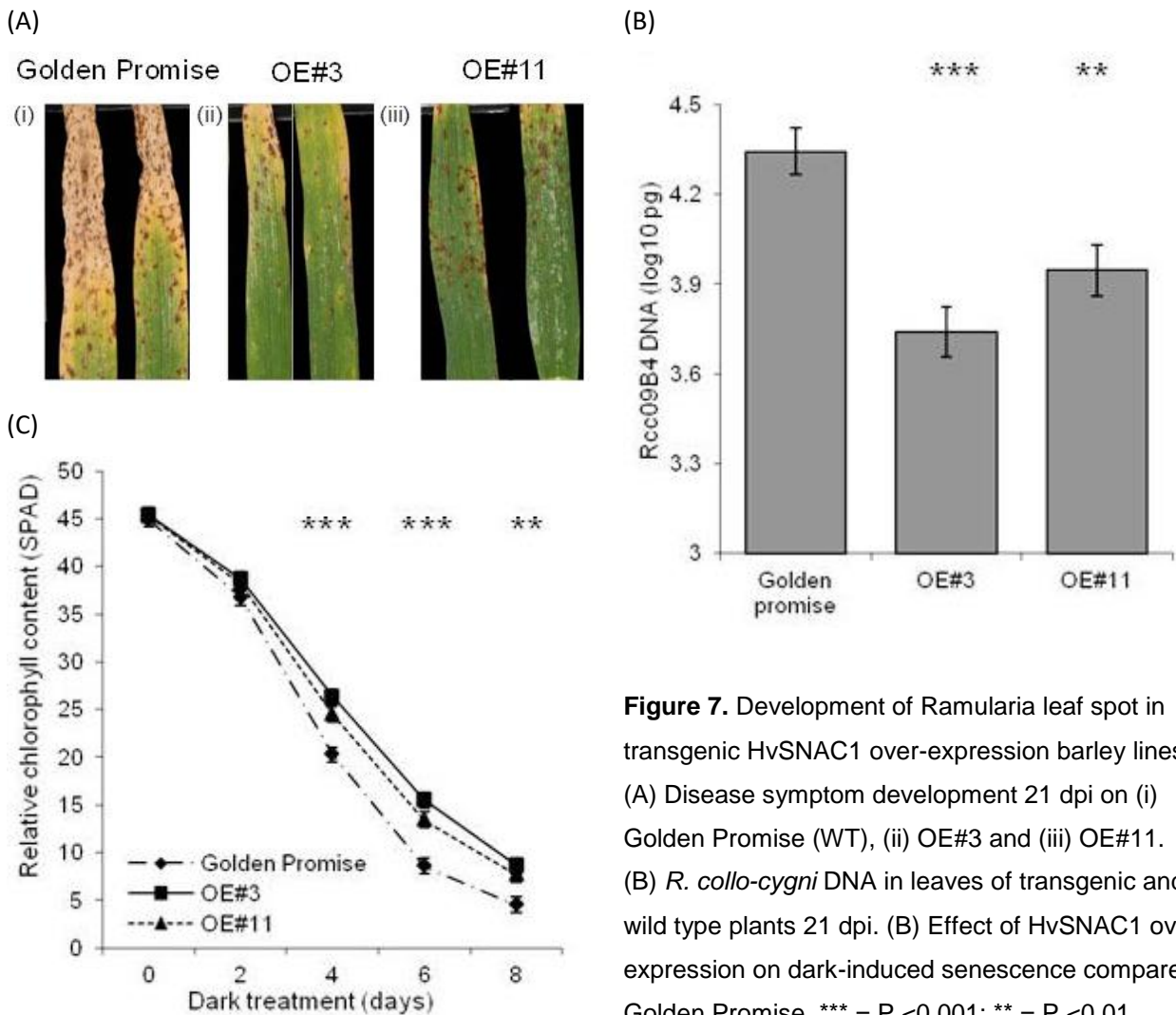
Figure 7. Development of Ramularia leaf spot in transgenic HvSNAC1 over-expression barley lines. (A) Disease symptom development 21 dpi on (i) Golden Promise (WT), (ii) OE#3 and (iii) OE#11. (B) *R. collo-cygni* DNA in leaves of transgenic and wild type plants 21 dpi. (B) Effect of HvSNAC1 over-expression on dark-induced senescence compared to Golden Promise. *** = P <0.001; ** = P <0.01.

Leaf senescence can be an important factor in endophytic fungi becoming pathogenic (Schulz and Boyle, 2005) and the senescence process results in ROS imbalances due to degradation of the chloroplast, a major source of ROS in plants (Mittler et al., 2004). Therefore we tested the hypothesis that chloroplast breakdown is important for disease transition using the barley *albostrians* mutants which are defective in a plastid differentiation, resulting in plants with green leaves, green and white striped leaves and white leaves (Hess et al., 1994). Reduced levels of Ramularia symptoms were observed on plants with green and white striped leaves compared to green leaf mutants (see Figure 8A.i-ii & 8B). Disease lesions were rarely observed on leaves from the albino mutants (Figure 8A.iii & 8B). However, despite the reduced disease symptom expression on these plants, there were no significant differences in the levels of *R. collo-cygni* DNA from plants with green, striped or white leaves (Figure 8C).
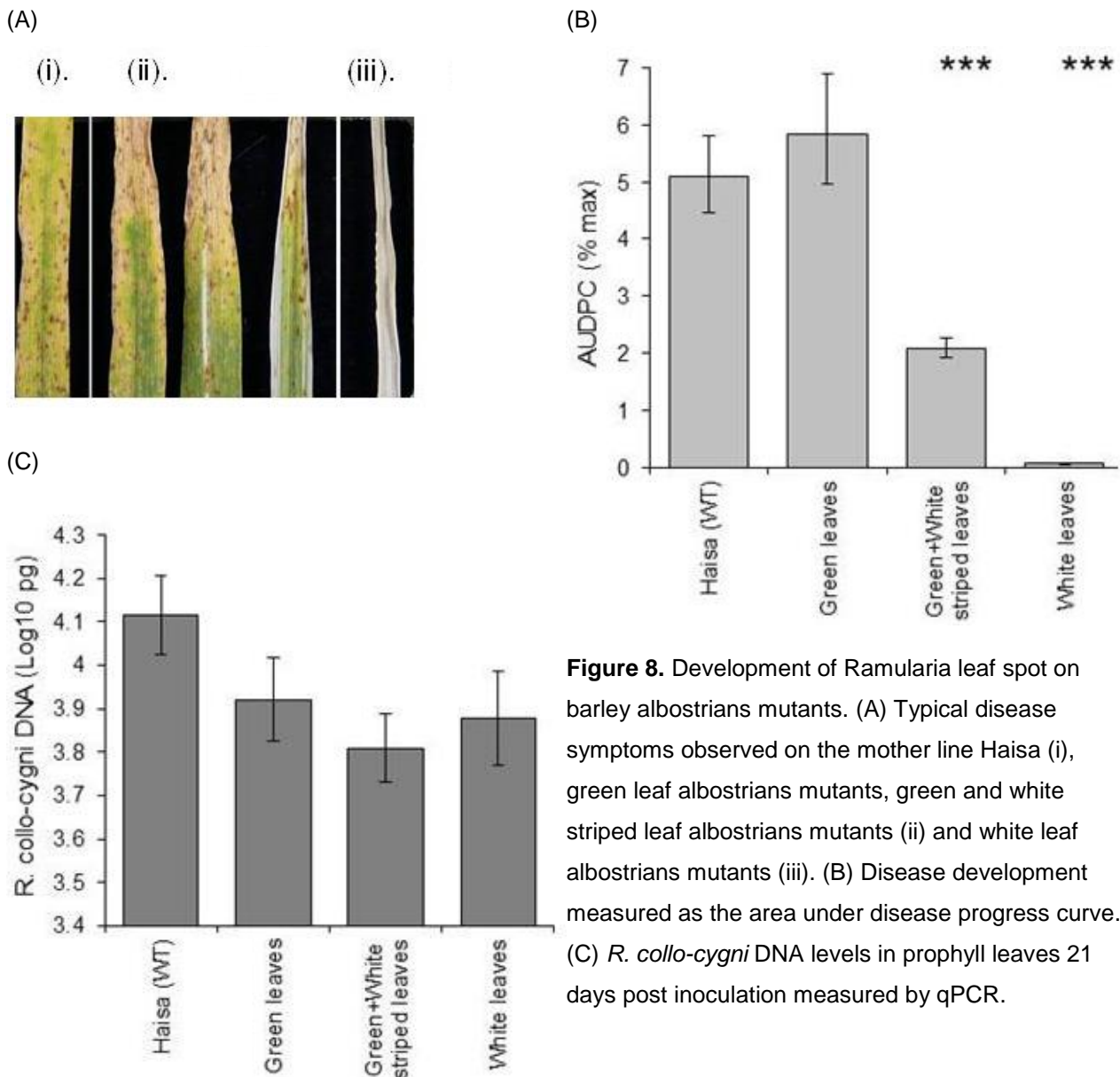
Figure 8. Development of Ramularia leaf spot on barley albostrians mutants. (A) Typical disease symptoms observed on the mother line Haisa (i), green leaf albostrians mutants, green and white striped leaf albostrians mutants (ii) and white leaf albostrians mutants (iii). (B) Disease development measured as the area under disease progress curve. (C) *R. collo-cygni* DNA levels in prophyll leaves 21 days post inoculation measured by qPCR.

White leaved albostrians mutants have been previously reported to show enhanced resistance against the biotrophic powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*, and symptomless supersusceptibility to the hemibiotrophic spot blotch fungus *Bipolaris sorokiniana* (Jain et al., 2004; Schafer et al., 2004). It remains be proved whether or not chloroplast degradation acts as a signal critical for the transition to disease in pathogens with a late necrotrophic stage of development such as *R. collo-cygni*.

4. Epidemiology and control of Ramularia leaf spot

4.1. Insensitivity to fungicides

Also see Sections 7.3, 7.4 and 7.5

Ramularia leaf spot was initially controlled by QoI fungicides, but during 2002 there was a marked decline in activity of QoI fungicides in comparison with previous years. This was due to the development of QoI resistance among populations of *R. collo-cygni* in the UK that developed between 2001 and 2002. A single amino acid substitution, changing glycine (G) to alanine (A) at position 143 in the cytochrome b gene, was responsible for this situation. It was only possible to detect that this mutation had occurred recently because of the presence of the G143 allele in *R. collo-cygni* in the Rothamsted Hoosfield archive. This archive also showed that the pathogen had exisited in low levels in barley plants during the 19th century. However, the levels of *R. collo-cygni* dramatically increased from 1996 (Figure 9).
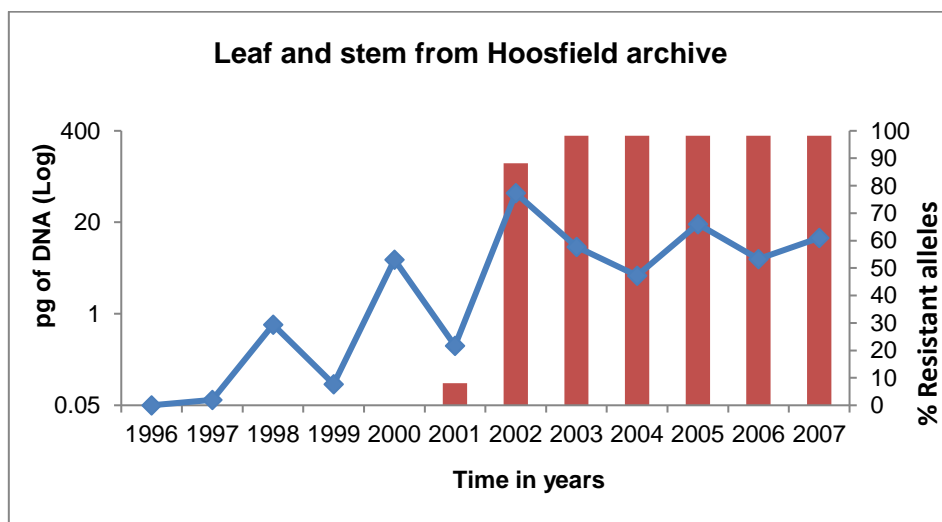
Figure 9. *Ramularia collo-cygni* DNA levels and percentage of A143 alleles (QoI-resistant) present in archived samples using allele-specific real-time PCR.

Currently, control of Ramularia leaf spot is achieved using a combination of fungicide treatments. As the pathogen *R. collo-cygni* has a history of resistance development it is important to understand the potential for resistance to develop. During the CORACLE project, we have developed a highly accurate fungicide testing system to assess the efficacy of a range of fungicides. This is particularly important for the Succinate Dehydrogenase Inhibitor (SDHI) and Demethylation Inhibitor (DMI) groups because it will enable monitoring of pathogen populations for insensitivity to these important fungicides and allow establishment of baseline sensitivity values against which to detect changes in sensitivity. Currently, no resistance has been detected to the SDHI fungicide group and the EC50 values obtained during the project were within the expected range for field control of the disease.

The DMI fungicide group achieves levels of control expected using field rates of fungicide. However, there has been a slight decline in the efficacy of this group in recent years and further work is needed to test if these declines in fungicide performance are linked to the specific mutations found in the CYP51 gene encoding the target site protein. This is challenging, as the DMI fungicides have been used in barley crops for a long period of time and single mutations can be also be detected in the oldest isolates in the SRUC culture collection. Therefore, this suggests that selection due to DMI fungicide treatments has been occuring for a number of years; a similar situation occurs in the related wheat pathogen *Zymoseptoria tritici* (Septoria tritici blotch) and this suggests a further study using the Hoosfield archive is required to understand the changes over time.

For the newer SDHI fungicides, it is possible to predict the development of resistance before it occurs in the field using laboratory generated mutants under high selection by an SDHI fungicide. The generation of stable laboratory mutants of *R. collo-cygni* has been achieved. These mutants have specific point mutations in the genes encoding subunits of the fungicide target protein. The mutants have also been shown in inoculation assays to have little or no negative effects on the growth of the pathogen compared to the wild type isolate. However, as these mutants have been generated in the laboratory, they or combinations of them might never appear in the field.

4.2.   Field control of Ramularia leaf spot

Also see Section 7.9

Control of Ramularia leaf spot has relied almost entirely on the application of effective fungicides. Initial treatments in Europe were based on triazole fungicides (Huss & Sachs, 1998). However, the introduction of the strobilurins in the late 1990s heralded a change in fungicide choice as a strobilurin in conjunction with chlorothalonil (Bravo®) at booting stage was identified as the optimum control programme (Havis et al., 2002). The appearance of a widespread mutation in the fungus in the early 21st century rendered the strobilurins ineffective against Ramularia. Control programmes were again dominated by the triazoles, especially prothioconazole (Proline®), until the early part of the decade when the second generation of carboximide fungicides (succinate dehydrogenase inhibitors; SDHIs) became available for growers. A number of these are available now to growers and initially they were partnered with other fungicides to promote an anti-resistance strategy.

The Fungicide Performance Trial results from 2012 showed a similar trend with the SDHIs controlling symptoms and protecting yield (Figure 10). Proline continued to perform well. A new multisite fungicide (Phoenix®; folpet) showed little activity against Ramularia. The single SDHI product (Imtrex®; fluxpyroxad) was slightly superior to the co-formulated product Adexar, although it is not recommended as an unpartnered spray. Siltra X Pro® is bixafen + prothioconazole,

Adexar® is fluxopyroxad + epoxyconazole, Bontima® is izopyrazam + cyprodinil, and Ignite® is the new formulation of epoxyconazole. Trials have shown that the SDHIs could give very effective control of Ramularia as a late season foliar spray and there is interest in utilising them as seed treatments. However they are considered high risk for the appearance of resistance and require careful management.
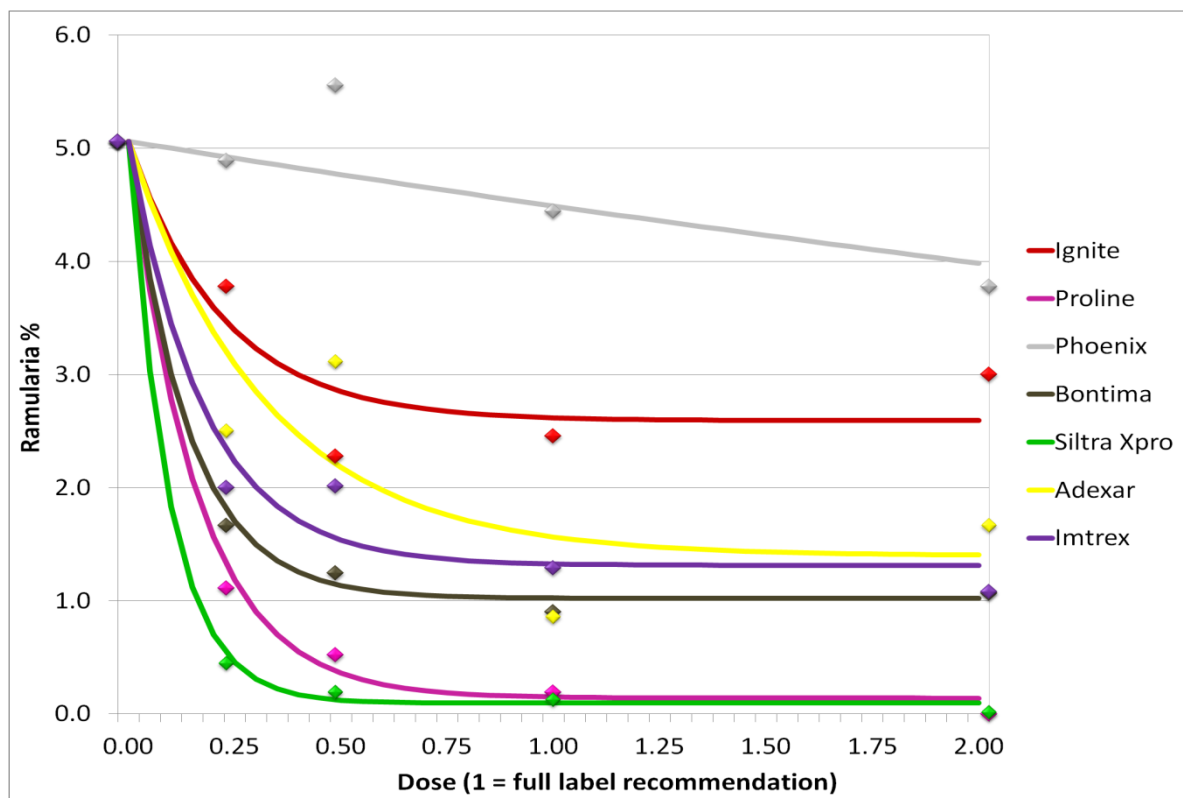
Figure 10. Activity of protectant fungicides against Ramularia leaf spot in 2012.

Reducing Ramularia leaf spot by trying to reduce or eliminate seed-borne *R. collo-cygni* was examined in a number of field trials. A range of biological and physical seed treatments were tested for their activity against Ramularia (Table 1). A standard seed treatment (Raxil Pro ®; prothioconazole, tebuconazole and triazoxide) was also included in the tests, which used varieties susceptible, intermediate and resistant to Ramularia.

Table 1. Treatments used in experiments on reducing or eliminating seed-borne *R. collo-cygni.*

Physical Treatments	Biological Treatments
Hot water 1 (2h at 52°C then 72 h at 25°C)	Cedomon® - Pseudomonas chlororaphis
Hot water 2 (1h at 21°C then 10 min at 52°C	Subtilex™ - Bacillus subtilis
Dry heat (60°C for 3 days)	
Steam (using Thermoseed™ conditions)	
Microwaving (25 secs in an 800W oven)	

R. collo-cygni DNA levels were quantified in seed pre- and post-treatment and also in leaf tissue through the course of the trials (Table 2). The only treatment which consistently reduced *R. collo-cygni* DNA levels in seed was the hot water treatment. However, testing through the season showed that *R. collo-cygni* DNA levels could recover in the hot water-treated seeds.

Table 2. Trials in which there was a significant reduction in Ramularia leaf spot by seed treatment alone.

Treatment \ Year	2009 B	2009 L	2010 B	2010 L	2011 B	2011 L	2012 B	2012 L
Hot water 1	-9%, 4%, 10%	-4%, 0%, -15%	2%, 5%, 22%*	-22%, - 4%, 14%	-11%, -25%, 47%	-22%, 5%, -33%	34%*, -14%, 6%	-1%, -29%, 21%
Hot water 2	n/a	n/a	n/a	n/a	n/a	n/a	32%*, 0%, -21%	-28%, -7%, 4%
Dry Heat	n/a	n/a	n/a	n/a	n/a	n/a	28%*, -20%, -11%	-27%, 18%, 51%
Steam	22%, -9%, 10%	n/a	24%*, 13%, 3%	-39%, -20%, 7%	2%, 2%, 13%	-11%, 13%, 0%	32%*, 23%, 24%*	-1%, 39%, 29%
Microwave	n/a	n/a	n/a	n/a	5%, 15%, -20%	0%, 18%, 38%	5%*, -16%, 12%	-11%, 12%, 16%
Fungicide	2%, 6%, 10%	2%, 6%, 4%	-9%, -6%, 27%*	-22%, -4%, 14%	7%, 22%, 26%*	11%, 18%, 8%	21%*, -28%, -22%	-2%, 36%, 30%
Cedomon	-26%, -9%, 7%	n/a	6%, 10%, 26%*	-26%, 12%, 21%	n/a	n/a	n/a	n/a
Subtilex	-12%, -12%, 25%*	n/a	-10%, -8%, 16%*	-39%, -20%, 0%	n/a	n/a	n/a	n/a

B= Bush Estate, L = Lanark. Colours indicate varieties: O= Optic, C = Cocktail, D =Decanter

* Significant reduction in RLS (P<0.05) by seed treatment alone

4.3. Spore tape analysis

Also see Section 7.10.

A series of Burkard spore samplers were set up in Scotland and in England to monitor the spread and distribution of Rcc spores over the course of a growing season. Automated meteorological stations were sited next to the samplers in order to identify the environmental conditions associated with spore release.

A general pattern of spore release was observed with the major events taking place late in the season (after flowering in the crop) with occasional events in spring and autumn (Figure 11). Analysis of the meteorological data indicated that the only parameter consistently associated with spore release was surface wetness in the crop. Analysis of the results from Bush (Scotland)

indicated that spore release reached a maximum in July and August and that spore counts increased with surface wetness in the crop in the same and preceding days. Over a longer time period, using four years' worth of data from the Bush site, temperature was shown to have a minor influence on spore release. An increase in spore numbers was observed as temperatures rose from 5°C and 15°C but there was no significant further rise in spore release as temperatures increased above 15°C. The early peaks observed at sites in March 2012 could have been a response to the unusually warm spring, when temperatures rose above 25°C. In that year, however, spore numbers declined within a number of days at Lanark and Bush and remained very low until late in the season. The unusually high spore counts in March 2012 could have resulted from spore release from senescent leaves on winter crops or from crop debris.
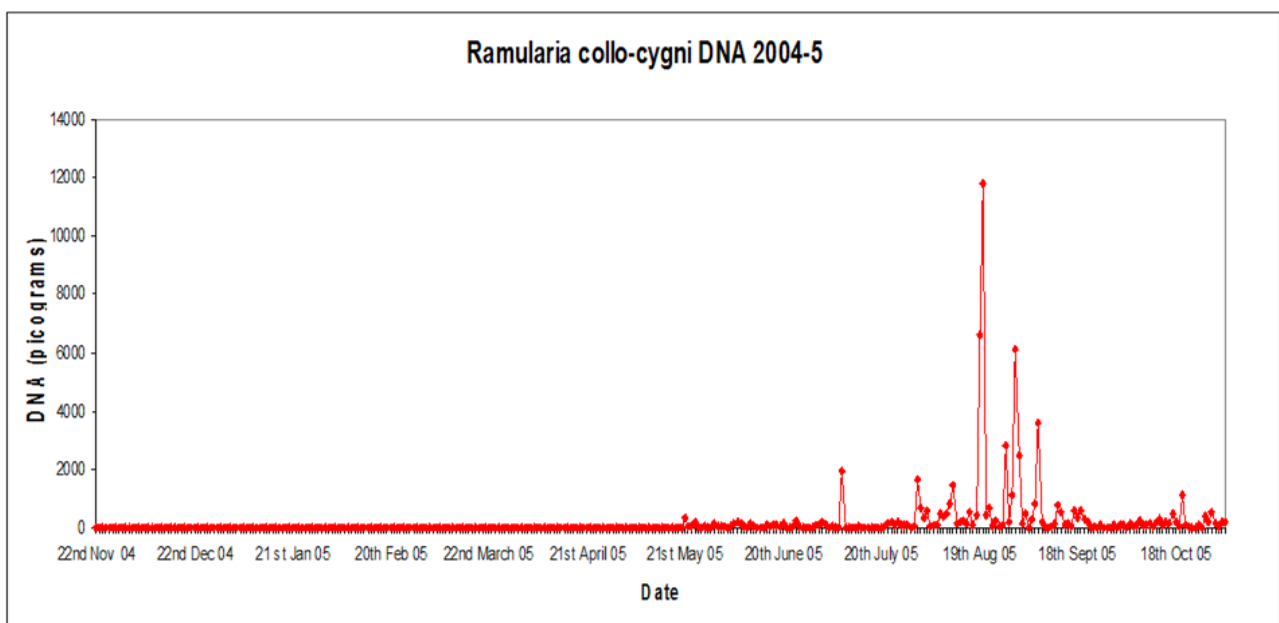
Figure 11. Sampling spores of *R. collo-cygni*. Top: A graph of *R. collo-cygni* DNA in the airborne spore population, showing a peak of spore release in August. Bottom: A Burkhard spore trap *in situ*.

4.4. Risk assessment and economic impact

Also see Section 7.11.

Based on observations in Norway of a link between humidity conditions in early June and Ramularia leaf spot epidemics in spring barley (Salamati & Reitan, 2006), a risk forecasting scheme was constructed based on equivalent dates and growth stages for spring barley in Scotland. The dates at both sites coincided with the appearance of the first node in the crop and the beginning of stem extension (GS31). An analysis of historic meteorological data and disease observations at the Bush site indicated a correlation between surface wetness in the crop and high disease levels, expressed as AUDPC (area under disease progress curve; Figure 12a). A similar correlation was observed in spring barley crops at 4 different sites around Scotland in 2008 (Figure 12b). Based on the same crop growth stage, a risk forecast was designed for winter barley crops (first 2 weeks in April). A similar relationship was observed (Figure 13).
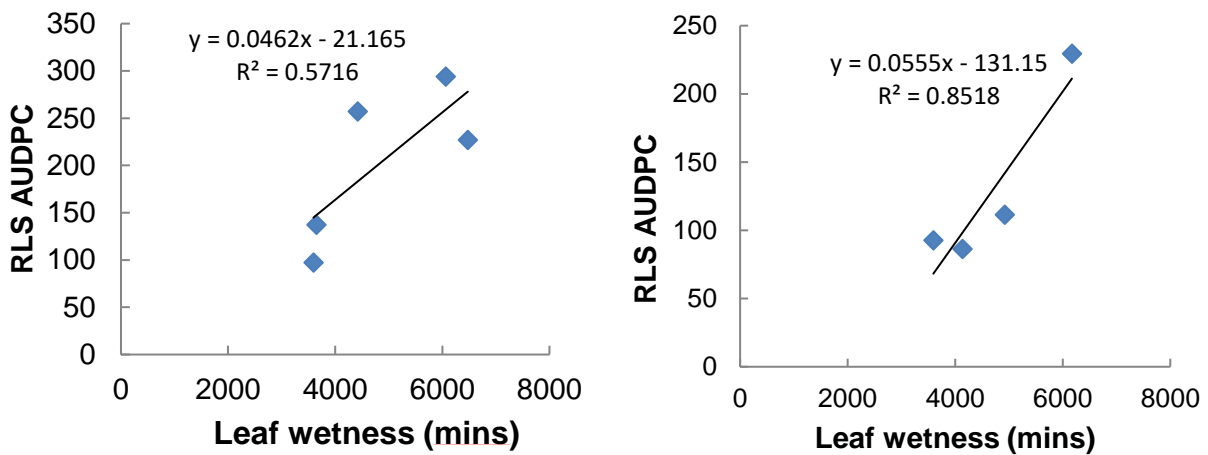
Figure 12. The relationship between leaf surface wetness and severity of Ramularia leaf spot. a (left): Five years of results from a trial site at Bush, Midlothian, from 2005 to 2009. b (right): Data from four sites in 2008.
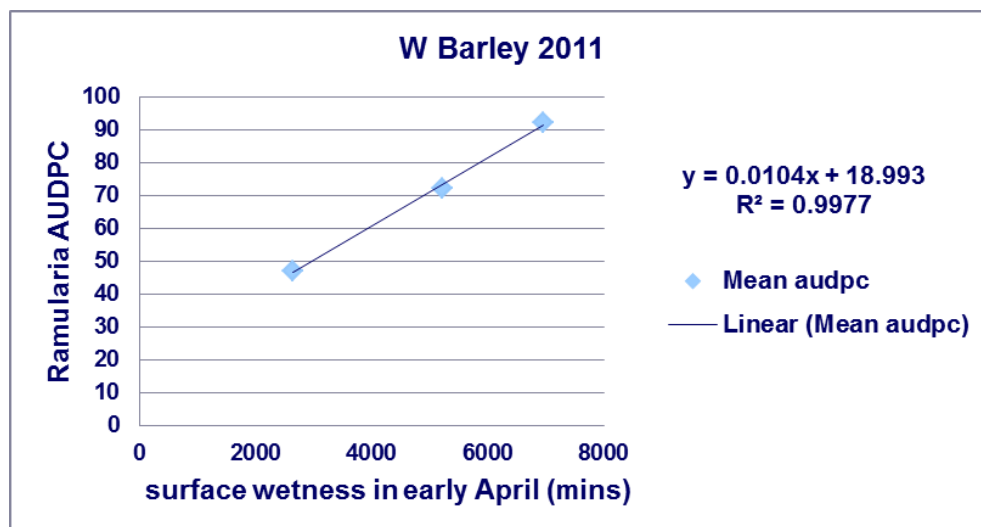
Figure 13. Relationship between leaf surface wetness and Ramularia leaf spot in winter barley at three sites in 2011.

Based on these observations, a risk forecast is produced based on figures from meteorological stations across Scotland on an annual basis. The early timing of the forecast allows growers to adjust their fungicide programmes to reduce Ramularia leaf spot. The parameters which affect Ramularia epidemics are being elucidated and have been combined into a risk assessment for the crop (Table 3). Analysis of trials data will continue to weigh up the relative importance of the factors and produce a risk algorithm similar to those for eyespot and Fusarium.

Table 3. Parameters included in risk forecast for Ramularia leaf spot in spring and winter barley.

| Risk Factor	Winter Barley	Spring Barley
Previous season epidemic		
High disease levels and spore release	+	+
Low disease levels and spore release	_	_
Varietal choice		
Tolerant	Neutral	Neutral
Susceptible	+	+
Cultivation system		
Minimum Tillage	+	+
Ploughed	_	_
Sowing date		
Early (pre spring barley harvest)	+	N/A
Late (post spring barley harvest)	-	N/A
Surface wetness at GS 31		
Prolonged periods of 100% wetness in the crop	+	+
Few periods of crop wetness	-	-
Winter barley epidemics		
High disease levels and spore release	N/A	+
Low disease levels and spore release	N/A	-

Ramularia AUDPC values from winter and spring barley trials run by SRUC were plotted on a graph against yields and a regression analysis carried out. The analysis showed a similar yield response in winter and spring crops to increasing AUDPC (Figure 14). The gradient of the lines was very similar for both crops.

The yield loss can be plotted against AUDPC. Assuming a price a £128t/ha for feed barley, a reduction in AUDPC of 96 will give an economic benefit (Figure 15). Assuming a price of £147t/ha for malting barley a reduction in AUDPC of 74 would be economically viable. The yield loss figure on the y-axis can be calculated from a contour plot which establishes the relationship between the value of barley (V, £/t) and the cost of treatment (C, £/ha) (Figure 16).

Ramularia forecasts for Scotland are disseminated through SRUC's Crop Clinic web page (http://www.sruc.ac.uk/info/120118/crop_clinic). In April and June, risk forecasts are posted for winter and spring barely, respectively. Forecasts are also publicised through SRUC's Twitter feed.
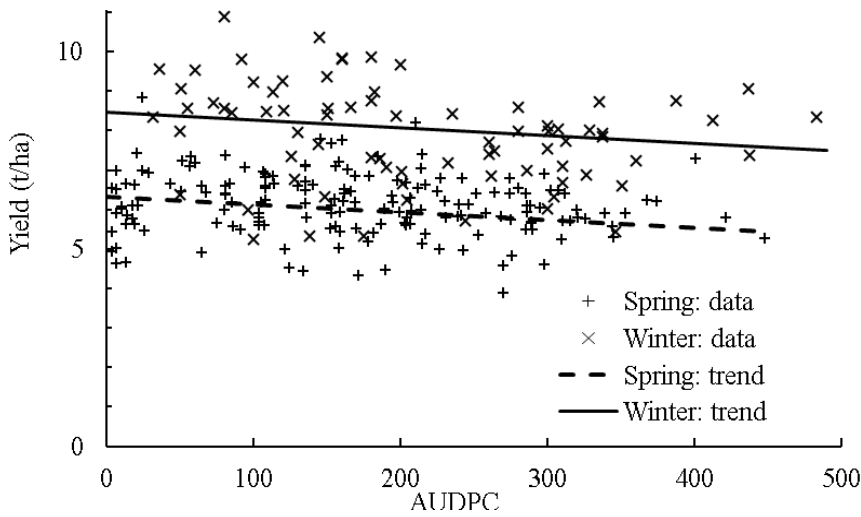
Figure 14. Relationship of yield to area under the disease progress curve (AUDPC) of Ramularia leaf spot in spring and winter barley.
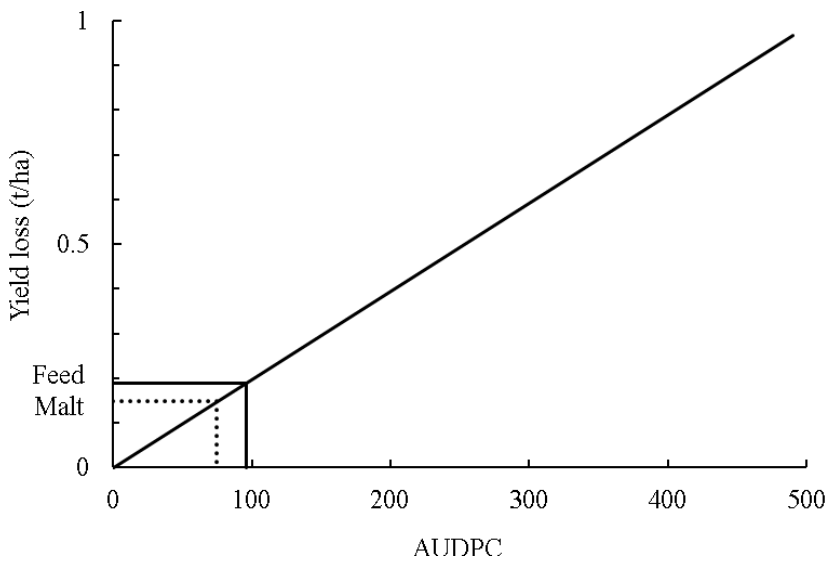
Figure 15. Relationship between yield loss and area under the disease progress curve (AUDPC) of Ramularia leaf spot in barley.
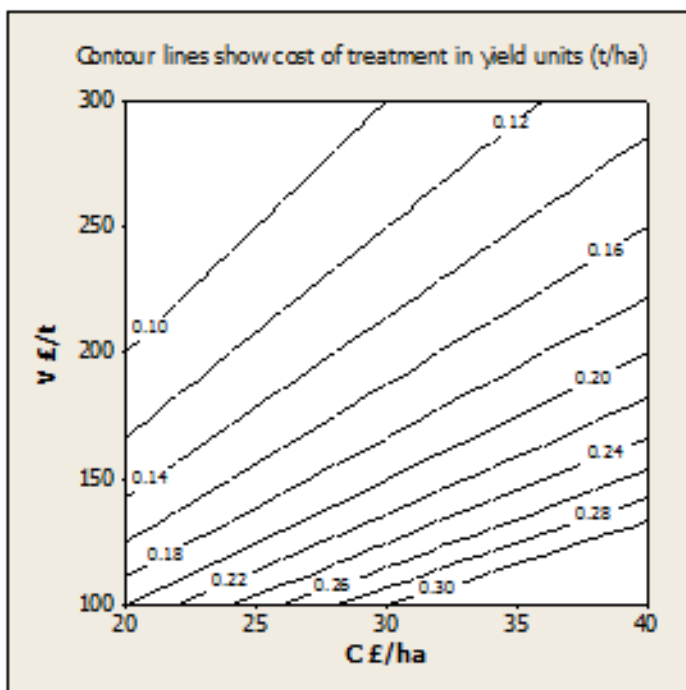
Figure 16. Value of barley (V) against the cost of treatment, allowing calculation of the economic benefit of controlling Ramularia leaf spot.

5. Genetics and breeding for resistance to Ramularia leaf spot

5.1. The effect of *mlo* mildew resistance on Ramularia leaf spot

Also see Section 7.12.

mlo genes have been very important in breeding spring barley over the last 25 years because they have provided almost complete, durable resistance to powdery mildew, formerly the most severe disease of barley in most areas. About 15 years ago, however, it became apparent that *mlo* was also associated with increased susceptibility to facultative fungal diseases including blast, net blotch and *Fusarium* head blight (reviewed by Brown & Rant 2013). These studies were done in laboratory conditions and could not easily be extrapolated to field situations because the environment can modify the effect of *mlo* on facultative pathogens.

The greater susceptibility of *mlo* plants to other facultative diseases, the widespread use of spring barley varieties with *mlo* mildew-resistance and the emergence of Ramularia as an important new disease of barley led to the hypothesis that *mlo* genes may be implicated in the increased prevalence and severity of Ramularia. We tested this hypothesis in field trials and seedling tests, in which the presence of an *mlo* gene increased the severity of Ramularia symptoms in barley lines bred from a cross of Braemar, an *mlo* variety which is very susceptible to Ramularia, with Power, a non-*mlo* variety with moderate resistance to Ramularia. Much the strongest genetic effect on Ramularia severity was that of the *mlo* gene, although the extent to which *mlo* was associated with greater susceptibility to Ramularia varied between experiments on seedlings and between field trial sites (Figure 17).
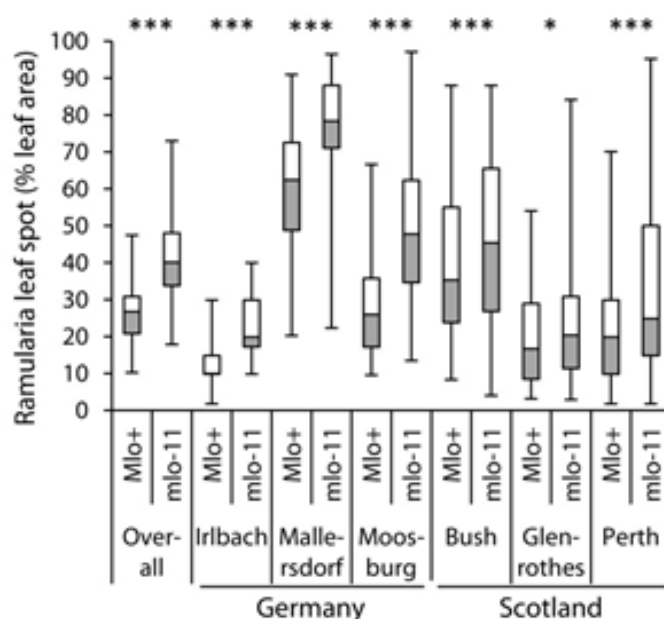
Figure 17. Boxplot showing the effects of *mlo* on Ramularia leaf spot development in field trials of progeny of Power x Braemar. Note that in all trials, there was greater severity of Ramularia on plant with the *mlo-11* mildew-resistance gene than in those with the form of the gene conferring susceptibility to mildew (*Mlo+*), although the size of the difference varied between trials.

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Further experiments were done on plants carrying mutations in *ROR* genes, which reduce the resistance of *mlo* barley plants to mildew. For *mlo* mildew-resistance to be fully effective, both the *ROR1* and *ROR2* genes are required. Ramularia symptoms were reduced in plants carrying both *mlo* and *ROR* mutations but the amount of fungal DNA was not affected (Figure 18). This implies that there is genetic variation in barley in the extent to which the plant permits the fungus to progress from the endophytic phase of its life cycle to becoming a necrotrophic parasite.
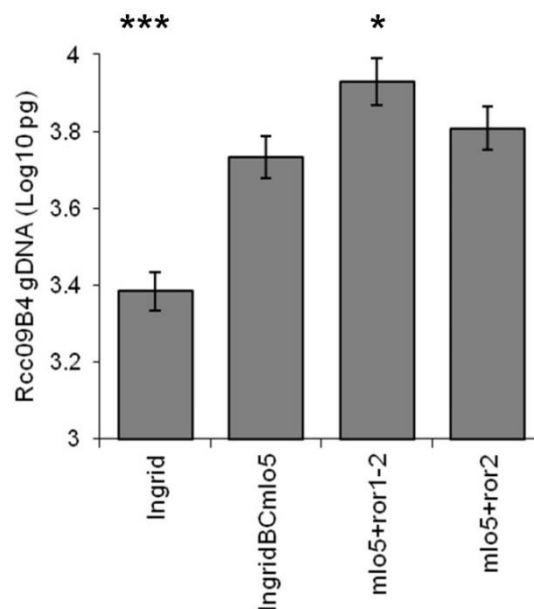
Figure 18. Mutations in the *ROR1* and *ROR2* genes reduce the extent to which *mlo* enhances the symptoms of Ramularia leaf spot susceptibility (top) but do not reduce the amount of *R. collo-cygni* DNA in barley leaves (bottom). Left to right: Ingrid, Ingrid + *mlo-5*, Ingrid + *mlo-5* + *ror1-2*, Ingrid + *mlo-5* + *ror2*.

These results indicate that the widespread use of *mlo* to control mildew may inadvertently have stimulated the emergence of Ramularia leaf spot as a major disease of barley. Other agricultural and climatic changes during the last 15–20 years may also be implicated in the rise of Ramularia, or may interact with *mlo*. It should be possible, however, for breeders to restore the value of *mlo* in barley breeding by selecting lines which have both an *mlo* gene for resistance to mildew and enhanced resistance to Ramularia. The presence of a combination of resistance to Ramularia and strong resistance to mildew in several spring barley varieties on the current Recommended List indicates that this can be achieved. Further experiments, including field trials, are required to test whether or not *mlo* plants are also more susceptible to net blotch and *Fusarium* in field conditions.

5.2. Association genetics of resistance to Ramularia leaf spot

Also see Sections 7.13 and 7.14.

The companies participating in CORACLE and SRUC ran a series of trials, coordinated by JIC, to investigate variation between barley varieties in susceptibility to Ramularia leaf spot. The aims of this research were to improve methods of trialling and scoring Ramularia; to identify varieties which were good sources of resistance to Ramularia, particularly in diverse environments; and to conduct a genetic analysis in order to detect portions of the barley genome which carry genes for resistance to Ramularia and to identify varieties which carry different resistance genes. The latter goal is especially significant because if varieties with different genes are inter-crossed, they may produce progeny lines which have better resistance than either parent.

A series of eight trials of 76 spring barleys was run to optimise trialling methods. Data were obtained from six of them, from two sites in Bavaria, Germany in both 2010 and 2011, a further site in Bavaria in 2011 and one in Scotland in 2011. These trials established that there is a very wide range of variation in susceptibility to Ramularia in spring barley, with a ten-fold range of susceptibility from an average of 3.6% across all trials for the most resistant variety to 37% for the most susceptible. There was extensive variety-by-site interaction, however, so that the ranking of varieties differed substantially between trials. This is a common issue in crop variety trials for many traits. Even so, the most resistant varieties tended to be resistant in all trials while the most susceptible varieties were susceptible in most trials, implying it is unlikely that breeders will make grossly erroneous decisions about selecting varieties for Ramularia resistance.

Having established a trialling methodology, a further series of eight trials was run on 263 spring barley varieties in 2013 and data was collected from six of them, four in Bavaria, one in Scotland and one in Norway. These varieties were chosen from the set established by the Association Genetics of UK Elite Barley (AGOUEB) project. The aims were to study the resistance of a wider range of varieties and to produce data for association genetic analysis. There was an even wider range of responses than in the first series of trials, from 3% to 72% Ramularia disease on flag

leaves on average over the complete series of trials. Although there was some variation between the relative scores of different varieties at the six sites, the correlation between the sites was moderately strong and the varieties' scores therefore moderately consistent. There was a strong effect of heading data on Ramularia scores, with earlier-heading varieties having higher scores, possibly because there was more time for the disease to develop in the upper leaves. There was also a weak effect of height, with taller varieties tending to have somewhat lower scores; this effect is commonly seen in splash-borne diseases. Resistant and susceptible varieties were identified for use in future research and plant breeding programmes.

The association genetic analysis of Ramularia resistance in spring barley used 4,398 single-nucleotide polymorphism (SNP) markers in 4,067 mapped positions in the barley genome. Genes affecting susceptibility to Ramularia were detected on chromosomes 4H, 5H and 6H, as well as an unmapped locus. The quantitative trait loci (QTLs) on chromosomes 5H and 6H were expressed consistently at all the trial sites. The one on 4H had a statistically significant effect in three trials and the unmapped locus in only one trial. The 4H gene maps to the opposite end of the chromosome to the *mlo* mildew-resistance gene, so in this association genetics analysis, there was no correlation between the presence of *mlo-11* and Ramularia severity. The lack of such an effect may have been due to population sub-division, because plant breeders tend to intercross *mlo-11* varieties or, alternatively, varieties with the wild-type *mlo*⁺ allele. This is because selection of a coadapted gene complex helps to reduce the yield penalty associated with an *mlo* allele (Kjær *et al.* 1990). The resulting sub-division of the population makes it difficult to detect a phenotypic effect associated with the *mlo* locus. Further analysis is continuing to finalise the positions of the association QTLs and to determine effect of the *mlo* locus on Ramularia susceptibility in these varieties.

Field trials of winter barley were less successful. A total of seven trials of 80 varieties were sown at sites in Germany in 2010/11 and 2011/12 but data could only be obtained from one trial and even that was not considered adequate. Two trials of 252 winter varieties from the AGOUEB material were grown in polytunnels in 2012/13 and inoculated artificially. Mean Ramularia scores ranged from 4% to 48% across the two trials, implying a wide range of genetic variation in winter barley bred for UK conditions. Although there were substantial differences between the two trials, the most resistant varieties had low scores in both of them.

Association genetic analysis of the winter barleys used 4,585 SNP markers at 4,067 mapped positions. Ramularia severity was associated with markers on chromosomes 1H, 3H, 5H and 6H. Those on chromosomes 1H and 5H showed statistically significant variation in their effects between the two trials while those on 3H and 6H did not. These results should be treated with caution, owing to differences between the two trials. A further polytunnel trial is in progress at JIC

in 2013/14, with the aim of obtaining an additional replicate of Ramularia scores on this set of winter barley varieties.

In Septoria tritici blotch, caused by *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*), a fungus closely related to *R. collo-cygni*, some fungal isolates have low virulence on some wheat varieties. The question of whether or not similar variety-by-isolate interaction exists in Ramularia leaf spot was investigated. The experiments used 12 isolates of *R. collo-cygni* and 19 spring barley varieties, including parents of crosses used for genetic analysis, sources of resistance to Ramularia and some susceptible varieties for comparison. There was statistical evidence of a weak variety-by-isolate interaction effect but in general, the results imply that resistance to Ramularia is likely to have a stable effect against the whole population of the *R. collo-cygni* fungus.

5.3.　Genetics of resistance to Ramularia leaf spot in winter barley

Also see Section 7.15.

In most of continental Europe and Scandinavia, Ramularia leaf spot is at least as serious a disease of winter barley as of spring barley. In Scotland, while it is common on winter barley it is of greater economic significant on spring barley. In England, however, most of the outbreaks observed so far have been on winter barley crops.

Sejet Plant Breeding I/S conducted a study of the genetics of Ramularia leaf spot and physiological leaf spotting as a contribution to CORACLE. Three winter barley genotypes differing in levels of Ramularia under field conditions were evaluated and genes for resistance to Ramularia were mapped. The variety Stratego was highly susceptible whereas Hobart and a progeny line of Vilna x Labea (VL) were either resistant or tolerant. These lines were crossed in all three possible combinations and the resulting populations grown in six field trials in Denmark and Scotland between 2008 and 2010.

There was substantial segregation of susceptibility to Ramularia in crosses of Stratego with both the more resistant lines. A considerable proportion of progeny were more resistant than either parent (Figure 19), implying firstly that, despite its susceptibility, Stratego carries genes for resistance to Ramularia and secondly, that plant breeders can combine genes from different sources to make gradual increases in the resistance of new varieties.
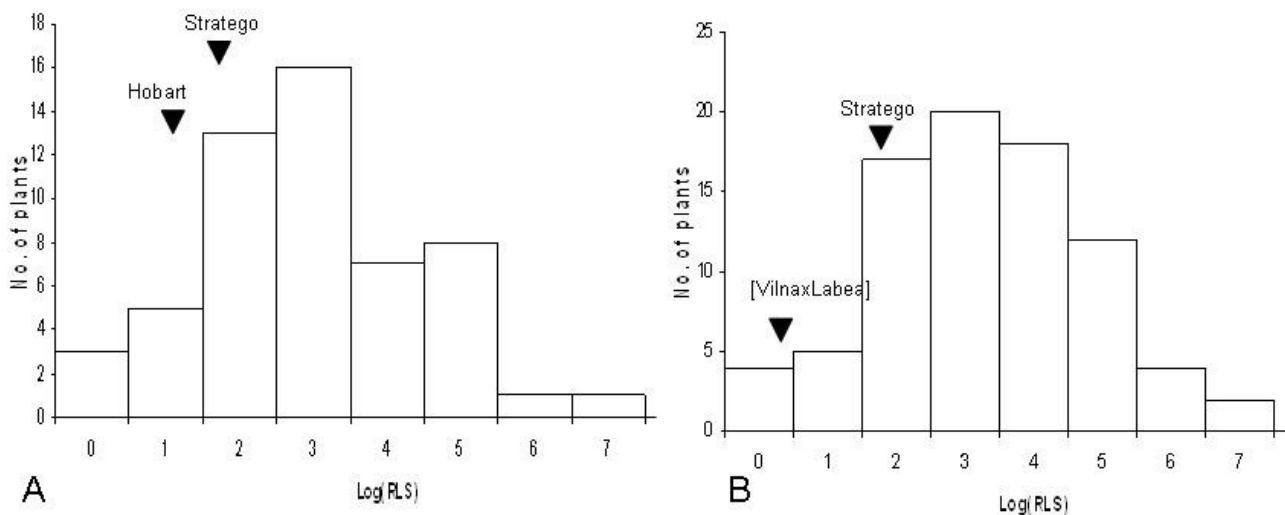
Figure 19. Distribution of Ramularia leaf spot scores from trials in Scotland in 2010. A: Stratego x Hobart, B: Stratego x (Vilna x Labea). Note that numerous progeny exceeded the scores for parents in each direction.

Genetic mapping was used to identify genes controlling substantial proportions of resistance to Ramularia. Depending on the trial, between 26% and 50% of genetic variation could be assigned to defined positions in the barley genome. Three such positions were identified in VL x Stratego and one in Hobart x Stratego. In one case, resistance was increased by the gene in Stratego whereas in the other three cases, Stratego contributed susceptibility. The fact that a large proportion of genetic variation could not be explained by these genes implies that an important contribution to Ramularia resistance is made by the collective effect of several, possibly many genes with small effects, probably distributed throughout the barley genome.

An important technical point in running field trials of Ramularia is that it is crucial to keep them free of other diseases. As Ramularia symptoms are mainly expressed late in the growing season, earlier attacks by other pathogens might interfere with scoring Ramularia. The environment clearly affected the trials and in some cases, genes increasing Ramularia leaf spot symptoms decreased physiological leaf spotting and *vice-versa*.

5.4. Improved methods of trialling barley lines and varieties for Ramularia resistance

See Section 7.16.

The research in CORACLE resulted in improvements to methods of trialling large numbers of barley varieties for resistance to Ramularia leaf spot. This is an important goal for plant breeding because much of the advance made in breeding for resistance to any disease is achieved by creating new combinations of many genes which individually have small effects on the disease. While the use of DNA markers is beginning to support selection for disease resistance, most selection is still done by identifying lines which show lower levels of disease in field or other trials,

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combined with other desirable traits such as yield and quality. This will continue to be the main way in which new cereal varieties are bred for the foreseeable future.

In field trials, the principal aim should be to use a site which gets high levels of Ramularia infestation and where other facultative foliar diseases are unimportant. Currently, rust can be controlled with a strobilurin (QoI) fungicide while powdery mildew can be controlled with specialist fungicides. High levels of environmental stress, such as waterlogging or drought, should be avoided if possible because it can alter the varieties' rankings. In particular, spring barleys which have *mlo* mildew resistance and normally have good resistance to Ramularia can become heavily diseased by Ramularia under stressful conditions; the effect of stress on such a variety, Decanter, in laboratory conditions was reported by Makepeace *et al.* (2008). If the trial site is uneven, for instance if there is a slope or a gradient in soil type, these factors can influence Ramularia levels and should be included as covariates in statistical analysis. An example of an uneven site was one in Germany on chalky soil which sloped downhill towards a stream running through a wood; higher humidity at the bottom of the hill appeared to stimulate stronger Ramularia symptoms than at the top.

Small plots down to $1m^2$ can produce good Ramularia data provided there is no large variation in environmental factors at the trial site, but smaller plots may produce inconsistent results. If small plots are used, guard rows should be sown or the trial should be surrounded by other plots. Wide tracks running through the trial or bare ground around the trial can reduce humidity levels and thus lower the severity of Ramularia. These issues are less serious for larger plots.

A method of inoculating adult barley plants was devised, based on the method of Makepeace *et al.* (2008), modified by Peraldi *et al.* (2014). This is suitable for inoculating individual plants, for example in a polytunnel, or very small tussocks in a field situation. If tussocks are used, they should be sown very close together and steps should be taken to ensure a high level of humidity for several days before and two weeks after inoculation. The inoculation method is not recommended for plots as large as $1m^2$ because of the excessive amount of fungal material required. A detailed protocol for inoculation in polytunnels has been distributed to the member companies of CORACLE.

A field guide to scoring Ramularia will be produced for open-access publication in summer 2015.

Figure 20. Inoculation of adult plants of winter barley in a polytunnel at KWS UK Ltd.

Figure 21. Symptoms of Ramularia leaf spot in a polytunnel trial of winter barley.

5.5.   Integrated research on control of Ramularia

Also see Section 7.17.

A paper which reviews the full range of recent research on Ramularia in Europe and South America, including key results from the CORACLE LINK project, has been accepted for publication.

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7. Appendix A: Summaries of papers resulting from CORACLE

Authors' affiliations given here are those at which they did the relevant work, in most cases during the CORACLE project.

7.1 Mechanism of infection of barley by the ascomycete fungus, *Ramularia collo-cygni*

See Section 3.1 of report

Maciej Kaczmarek[1], James M Fountaine[1], Adrian C Newton[2], Nick D Read[3], Neil D Havis[1]
[1] Scotland's Rural College, [2] James Hutton Institute, [3] University of Edinburgh

Paper in preparation

The filamentous fungus *Ramularia collo-cygni* causes a late season disease on spring and winter barley called Ramularia Leaf Spot (RLS). It has become an increasingly important problem for EU farmers in the past decade and has recently been recognised as a major disease of barley in the UK. The lack of apparent varietal resistance to the disease has led to significant amounts of fungicide being applied to crops in north western and central Europe in order to maintain green leaf area and prevent significant yield loss. These factors have contributed to an increasing focus on achieving a better understanding of the fundamental biology of this elusive pathogen in order to develop more successful strategies of RLS management. Disease development throughout the life cycle of the host plant barley has been analysed by the employment of transgenic *R. collo-cygni* isolate expressing GFP reporter molecule and confocal laser scanning microscopy. We have examined the seed-borne stage and the mode of fungal transmission into barley seedlings. In addition, we have analysed the potential for sexual reproduction of this important fungal pathogen by the range of correlative microscopy techniques such as Cryo-SEM, CLSM and light microscopy. Here we describe for the first time the nature of speculated spermogonial stage called *Asteromella*. We also present preliminary evidence that suggests the existence of a perfect stage in this fungus that, if functional, could resemble closely related *Mycosphaerella* species.

7.2 Evidence for seed transmission and asymptomatic growth of *Ramularia collo-cygni* in barley (*Hordeum vulgare*)

See Section 3.2 of report

Neil D Havis[1], Marika Nyman[2], Simon J P Oxley[3]
[1] Scotland's Rural College, [2] Technical University of Munich, [3] AHDB

Plant Pathology (2014) 63: 929–936

Ramularia collo-cygni (*Rcc*) is becoming an increasing problem for barley growers across Europe. However, the life cycle of the pathogen is only slowly being elucidated. *Rcc* DNA was detected in a number of harvested seed samples from 1999 to 2010, with mean levels peaking in winter barley samples in 2009. A number of experiments were carried out to determine if the pathogen can move from barley seed to seedlings, and also from seed through the developing plant and into the subsequent generation of seed, both in controlled experiments and in field trials. Results from testing of seed indicated that the fungus is widespread at the end of the growing season in harvested grain samples and can be transmitted to developing plants from infected seed stock. Examination of infected seedlings did not reveal the presence of spores but fungal structures were found within the leaf. The location of the fungus within seed was examined, with Rcc DNA found in

both embryo and non-embryo tissue. The implications for barley production of the pathogen being seed-borne are discussed.

7.3 Contribution of the drought tolerance-related Stress-responsive NAC1 transcription factor to resistance of barley to Ramularia leaf spot

See Section 3.3 of report

Graham RD McGrann, Andrew Steed, Christopher Burt, Rachel Goddard, Cléa Lachaux, Anuradha Bansal, Margaret M Corbitt, Paul Nicholson and James KM Brown
John Innes Centre

NAC proteins are plant transcription factors that are involved in tolerance to abiotic and biotic stresses, as well as in many developmental processes. Stress-responsive NAC1 (SNAC1) transcription factor is involved in drought tolerance in barley and rice, but has not been shown previously to have a role in disease resistance. Transgenic over-expression of *HvSNAC1* in barley cv. Golden Promise reduced the severity of Ramularia leaf spot (RLS), caused by the fungus *Ramularia collo-cygni*, but had no effect on disease symptoms caused by *Fusarium culmorum*, *Oculimacula yallundae* (eyespot), *Blumeria graminis* f. sp. *hordei* (powdery mildew) or *Magnaporthe oryzae* (blast). The *HvSNAC1* transcript was weakly induced in the RLS-susceptible cv. Golden Promise during the latter stages of *R. collo-cygni* symptom development when infected leaves were senescing. Potential mechanisms controlling HvSNAC1-mediated resistance to RLS were investigated. Gene expression analysis revealed no difference in the constitutive levels of antioxidant transcripts in either of the over-expression lines compared with cv. Golden Promise, nor was any difference in stomatal conductance or sensitivity to reactive oxygen species-induced cell death observed. Over-expression of *HvSNAC1* delayed dark-induced leaf senescence. It is proposed that mechanisms controlled by HvSNAC1 that are involved in tolerance to abiotic stress and that inhibit senescence also confer resistance to *R. collo-cygni* and suppress RLS symptoms. This provides further evidence for an association between abiotic stress and senescence in barley and the development of RLS.

7.4 Factors contributing to the transition of *Ramularia collo-cygni* from endophyte to necrotroph

See Section 3.3 of report

Graham RD McGrann and James KM Brown
John Innes Centre

Paper in preparation

Ramularia collo-cygni is an ascomycete fungus that colonises barley primarily as a benign endophyte, although this interaction can become pathogenic causing the disease Ramularia leaf spot. The factors that result in the change in fungal growth from endophytic to necrotrophic are not well understood. Exposure to abiotic stress factors caused increased disease in most susceptible and resistant host varieties examined. Symptom development is associated with an increase in hydrogen peroxide in leaves of both resistant and susceptible plants that relates well to the observed disease levels. Lowered antioxidant levels in transgenic and mutant barley plants had no effect on disease transition but imbalances to hydrogen peroxide homeostasis during asymptomatic growth of the fungus increased disease expression in most susceptible varieties but not in resistant plants. Lesion mimic mutants were used to assess the effect of altered redox

balance on disease transition and analysis of the *bst1-7* mutants' response to *R. collo-cygni* implied a role for chloroplast breakdown in disease expression. This link was investigated using the albino *albostrians* mutant. White albostrians leaves with blocked chloroplast development showed no disease symptoms whilst allowing *R. collo-cygni* biomass to build up to levels comparable to diseased green leaves. We suggest that disease transition of the endophyte *R. collo-cygni* is linked to a combination of host genetic, physiological and environmental factors that affect chloroplast breakdown causing an imbalance in redox status and releasing signals that result in this fungus switching to an aggressive necrotrophic lifestyle.

7.5 Differential effects of lesion mimic mutants in barley on disease development by facultative pathogens

See Section 3.3 of report

Graham RD McGrann, Andrew Steed, Christopher Burt, Paul Nicholson, James KM Brown
John Innes Centre

Submitted to a peer-reviewed journal

Lesion mimic mutants display spontaneous necrotic spots and chlorotic leaves as a result of mis-regulated cell death programs. Typically these mutants have increased resistance to biotrophic pathogens but their response to facultative fungi that cause necrotrophic diseases is less well studied. The effect of altered cell death regulation on the development of disease caused by *Ramularia collo-cygni*, *Fusarium culmorum* and *Oculimacula yallundae* was explored using a collection of barley necrotic (nec) lesion mimic mutants. nec8 mutants displayed lower levels of all three diseases compared to nec9 mutants which had increased *R. collo-cygni*, but decreased *F. culmorum* disease symptoms. nec1 mutants reduced disease development caused by both *R. collo-cygni* and *F. culmorum*. The severity of the nec1-induced lesion mimic phenotype and *F. culmorum* symptom development was reduced by mutation of the negative cell death regulator MLO. The significant reduction in *R. collo-cygni* symptoms caused by nec1 was completely abolished in the presence of the mlo-5 allele and both symptoms and fungal biomass were greater than in the wild type. These results indicate that physiological pathways involved in regulation of cell death interact with one another in their effects on different fungal pathogens.

7.6 Development of fungicide resistance in *Ramularia collo-cygni*

See Section 4.1 of report

James M Fountaine[1], Bart A Fraaije[2]
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Paper in preparation

The fungus *Ramularia collo-cygni* is the major biotic agent involved in Ramularia leaf spot. Although it was first described in Italy in 1893 it was only initially identified as a pathogen of spring barley in Scotland in 1998. Since then it has increased in its importance throughout the whole of the UK, as well as an increasing number of European countries. Increasing our understanding of this relatively new disease has been a challenge over the last decade, firstly for understanding the economic impact of the disease and secondly, for developing optimal disease control programmes. Advances in both areas have led to an increase in fungicide use and this remains the only reliable control method available to growers. With this increase in fungicide use, however, there is also an increased risk in the rapid development of fungicide resistance, due to the dynamic nature of this pathogen. The development of resistance in *Ramularia collo-cygni* to QoI, MBC and DMI

fungicides will be described in detail for the first time and the evolution of the specific mutations conferring resistance will be shown throughout the period of the Rothamsted Hoosfield spring barley experiment. Results will be linked to bioassay data and characterised single spore isolates. Using this information may allow us to design future spray programmes that can slow or prevent the development of future fungicide resistance problems.

7.7 Detection of barley diseases over a 150 year period of the Hoosfield spring barley archive located at Rothamsted research

See Section 4.1 of report

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Paper in preparation

The Hoosfield spring barley experiment is the only continuous spring barley experiment in the United Kingdom, running on one field site at the Rothamsted estate since 1852. Samples of leaf, straw and grain material have been collected from the field at the end of each growing season and stored in sealed glass jars. Samples from plot 4A, were selected for sampling and DNA from both the leaf/straw and then grains was extracted. The purified DNA was then used in quantitative PCR assays to allow the quantification of three economically important barley diseases *Rhynchosporium commune, Pyrenophora graminea* and *Ramularia collo-cygni* in the background of high quality barley DNA over the time period of the whole experiment in both dried leaf and grain archive material. The dynamics of each disease will be explored and this will be linked to the agronomic and climate data gathered over the 150 years of the experiment in order to evaluate the occurrence of these specific diseases over time.

7.8 Mechanism of resistance to succinate dehydrogenase inhibitors in *Ramularia collo-cygni*

See Section 4.1 of report

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Paper in preparation

Ramularia collo-cygni (Rcc) is a fungal pathogen of barley (*Hordeum vulgare*) but it can also infect other cereal crops such as wheat (*Triticum aestivum*), rye (*Secale cereale*) and oats (*Avena sativa*). Its economic impact has increased in the last two decades, as *Rcc* started to have an impact on growers' yields. *Rcc* has been present as a major barley pathogen in Scotland since 1998. Initially Quinone outside Inhibitor (QoI) fungicides were widely used to control the disease, but between 2001 and 2002 the first resistant strains appeared. Currently Succinate Dehydrogenase Inhibitors (SDHIs) are widely used and recommended as some of the most effective fungicide treatments against *Rcc* and currently all of the available data suggests that *Rcc* is still sensitive to all SDHI fungicides. However, *Rcc* has presently been exposed to SDHI fungicides in Scotland for eight growing seasons and the risk of fungicide resistance development is probably high. The aim of this study was to examine if there are already existing SDHI-resistant strains of *Rcc* on the field and if *Rcc* develops resistance to SDHIs, what mutations will form and whether resistance will cause a fitness cost. In order to answer these questions we evaluated the sensitivity of *Rcc* population to SDHIs in an *in vitro* assay. We tested isolates worldwide but mostly from Scotland from the years 2010, 2011 and 2012. To examine the possible mechanism of

resistance development to SDHIs in *Rcc*, we conducted UV mutagenesis studies that enabled us to identify the amino acid changes correlating with the resistance. We characterised mutants in terms of their fitness and pathogenicity to test if there is any fitness penalty correlating with the specific mutations.

7.9 Control of Ramularia leaf spot

See Section 4.2 of report

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Paper in preparation

Ramularia leaf spot (RLS), caused by the fungus *Ramularia collo-cygni,* is now a major disease of barley in the UK and is also reducing yield and quality in barley crops in different countries of the world. The relationship between disease severity (measured as area under disease progress curve) and yield in untreated crops has been investigated and quantified for both winter and spring barley. The levels of disease reduction at which treatment of the crop is economic can be calculated, based on the value of the harvested grain and the cost of the treatment. Control programmes have been based on late season fungicide applications as complete varietal resistance is not yet available. Fungicide timing has been investigated and recommendations have evolved as fungicides change. The rapid appearance of resistance in the fungus to the Quinone outside Inhibitor (QoI) fungicide group has led to careful management of fungicides against the disease. Triazole fungicides e.g. prothiconazole, (Proline®) and Succinate dehydrogenase inhibitors (SDHI) e.g. bixafen (Siltra XPro ®), xemium (Adexar®) and izopyrazam (Bontima®) are still highly active against the disease. The discovery of a seed borne stage in the fungal life cycle has led to interest in the use of seed treatments to reduce fungal load and decrease epidemics. Chemical, biological and physical seed treatments were tested in field conditions for their ability to control RLS. In general, foliar applications are more effective than seed treatments.

7.10 The effect of weather conditions on the release of spores of the ascomycete fungus, *Ramularia collo-cygni*

See Section 4.3 of report

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Paper in preparation

The fungus *Ramularia collo-cygni* (*Rcc*) is the main biotic cause of Ramularia leaf spot in barley. This disease is increasing in its economic importance in barley growing regions of Europe, South America and New Zealand. Symptoms generally appear late in the growing season, post flowering in the crop. The loss of photosynthetic area leads to a decrease in yield and quality of harvested grains. In order to study the influence of spore movement on disease epidemics, Hirst type spore samplers were set up at 2 sites in Scotland (Midothian and Lanarkshire). Spores were trapped on coated Mellinex tape and *Rcc* DNA levels quantified by qPCR. Automated meteorological stations were sited next to the samplers to record the weather parameters during the cropping season. Spore levels were found to reach a maximum in July and August and increase as temperatures increased from 5°C to 15 °C. However, the main parameter to influence spore release was surface wetness in the crop, either on the same or preceding days. Data from a wider area including

Germany and Poland are currently being included in the analysis. The implications of these findings on fungal biology and disease control will be discussed.

7.11 Development of a risk assessment scheme for Ramularia leaf spot epidemics

See Section 4.4 of report

Neil D Havis[1], Simon JP Oxley[2], Michael Heβ[3], Gareth Hughes[1], Fiona J Burnett[1]
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Paper in preparation

Ramularia leaf spot (RLS) epidemics have been increasing in frequency and severity in barley growing regions of the world. Losses in the UK have been estimated at 0.5 t ha^{-1} but in Uruguay they have been recorded as high at 70% of the final crop yield. The late season nature of the disease and the asymptomatic growth of the fungus *Ramularia collo-cygni* has complicated the development of an accurate forecasting scheme for the severity of disease epidemics in a growing season. Observations of a relationship between humidity in spring barley and final disease levels in Norway led to the development of a full forecasting scheme for winter and spring barley crops. The scheme is based on minutes of full surface wetness experienced by the crop at stem extension. The effect of seasonal differences on appropriate dates has been studied using data from Scotland and Germany. The accuracy of the prediction in relation to observed symptoms is discussed. There are a number of other factors which can influence the losses in a crop due to RLS and these are discussed within the paper.

7.12 A trade-off between *mlo* resistance to powdery mildew and increased susceptibility of barley to a newly important disease, Ramularia leaf spot

See Section 5.1 of report

Graham RD McGrann[1], Anna Stavrinides[1], Joanne Russell[2], Margaret M Corbitt[1], Allan Booth[2], Laetitia Chartrain[1], William TB Thomas[2], James KM Brown[1]
[1] John Innes Centre, [2] James Hutton Institute

Ramularia leaf spot (RLS), caused by the fungus *Ramularia collo-cygni*, is a serious, recently-emerged disease of barley in Europe and other temperate regions. The trade-off between strong resistance to powdery mildew conferred by *mlo* mutant alleles and increased susceptibility to RLS was investigated. In field trials and seedling tests, the presence of *mlo* alleles increased severity of RLS. Genetic analysis of a doubled-haploid population identified one QTL for susceptibility to RLS, co-localising with the *mlo-11* allele for mildew resistance. The effect of *mlo-11* on RLS severity was environmentally sensitive. Analysis of near-isogenic lines of different *mlo* mutations in various genetic backgrounds confirmed that *mlo* alleles increased RLS severity in seedlings and adult plants. For *mlo* resistance to mildew to be fully effective the genes *ROR1* and *ROR2* are required. RLS symptoms were significantly reduced on *mlo-5 ror* double mutant plants but fungal DNA levels remained as high as in *mlo-5* single mutants, implying that *ror* alleles modify the transition of the fungus from endophytism to necrotrophy. These results indicate that the widespread use of *mlo* resistance to control mildew may have inadvertently stimulated the emergence of Ramularia leaf spot as a major disease of barley.

7.13 Genetics of resistance to Ramularia leaf spot in European spring barley cultivars

James KM Brown[1], Joanne Russell[2], Margaret M Corbitt[1], Graham RD McGrann[1], Allan Booth[2], William TB Thomas[2] and several staff of plant breeding and trialling companies
[1] John Innes Centre, [2] James Hutton Institute

Data analysis in progress

This paper will report genetic variation in spring barley cultivars for susceptibility to Ramularia leaf spot (RLS), caused by *Ramularia collo-cygni*. It will first report the trials of a subset of cultivars in 2010 and 2011 to establish trialling and scoring methods for a large amount of germplasm and will describe genotype-by-environment interaction in the susceptibility of varieties to RLS. It will then report the trials of the full set of cultivars in 2013 to produce data for association genetics analysis. The relationship of RLS scores to escape traits such as heading date and height will be described. Quantitative trait loci identified as affecting RLS scores will be reported, along with an analysis of the consistency of these effects across different trial sites. The effects of different ways of accounting for the effect of population structure in the lines analysed on the association genetics analysis will be described. Cultivars with particularly low average levels of RLS across the series of trials will be highlighted as potential sources of resistance, as well as susceptible lines which can be used as controls or spreaders in trials or as parents of crosses for further research on genetics of RLS. Sets of cultivars with different genes for RLS-resistance are especially important in breeding because they may give rise to transgressive segregation, leading to advances in resistance. The paper will also report experiments showing an absence of strong cultivar-by-isolate interactions in susceptibility to RLS, which implies that current breeding strategies are likely to produce varieties with durable resistance to this disease.

7.14 Resistance to Ramularia leaf spot in European winter barley cultivars

James KM Brown[1], Joanne Russell[2], Margaret M Corbitt[1], Peter Werner[3], Allan Booth[2], William TB Thomas[2]
[1] John Innes Centre, [2] James Hutton Institute, [3] KWS Seeds Ltd

Data analysis in progress

A paper reporting trials of winter barley cultivars for responses to Ramularia leaf spot (RLS), caused by the fungus *Ramularia collo-cygni* may be published separately or combined with the paper on spring barley (7.13). Methods of trialling barley lines using artificial inoculation of *R. collo-cygni*, for example in polytunnels, will be reported. Limitations on field trialling winter barley lines for RLS-resistance will be discussed briefly, emphasising the need for trials at sites with an environment to which the barley germplasm is adapted and where *R. collo-cygni* is abundant. The existence of a wide range of genetic variation in susceptibility to RLS in winter barley germplasm relevant to UK breeding will be reported. So far as possible, cultivars with consistently low RLS scores across the series of trials will be highlighted as candidate sources of resistance. Association genetics analysis will be completed on the data from all three polytunnel trials, although the depth of analysis will depend on the quality of replication of the full set of trials.

7.15 Genetics of resistance to Ramularia leaf spot in crosses of three European winter barley cultivars

See Section 5.3 of report

Rasmus L Hjorshøj, Lars B Eriksen
Sejet Plant Breeding I/S, Denmark

PhD thesis, Aarhus University, Denmark (2012): Improving Resistance to Ramularia Leaf Spot in Barley

Three winter barley genotypes differing in severity of Ramularia leaf spot under field conditions were evaluated and genes for resistance were mapped. The variety Stratego was highly susceptible whereas Hobart and a progeny line of Vilna x Labea (VL) were either resistant or tolerant. These lines were crossed in all three possible combinations and the resulting populations grown in six field trials. There was substantial genotype-by-environment interaction in this series of trials. Genes which increased RLS symptoms decreased physiological leaf spotting and *vice-versa*. The presence of transgressive segregation in levels of RLS implies that plant breeders can combine genes from different sources to make gradual increases in the resistance of new varieties. Up to half the genetic variation in RLS severity could be assigned to loci in the barley genome. Three QTLs were identified in VL x Stratego and one in Hobart x Stratego. Despite its susceptibility, Stratego contributed one QTL for resistance. The fact that a large proportion of genetic variation could not be explained by these genes implies that an important contribution to Ramularia resistance is made by the collective effect of several, possibly many genes with small effects, probably distributed throughout the barley genome.

7.16 A guide to trialling barley germplasm for resistance to Ramularia leaf spot

See Section 5.4 of report

James KM Brown[1], Neil D Havis[2]
[1] John Innes Centre, [2] Scotland's Rural College

Article in preparation

A guide to trialling barley for severity of Ramularia leaf spot (RLS) is in preparation. Topic to be covered will include: preparation of seed, noting the widespread contamination of seed by *Ramularia collo-cygni*; choice of field trial sites including physical characteristics, as well as the need for abundant *R. collo-cygni* and low levels of other facultative fungal foliar diseases; control of biotrophic fungal pathogens; the effect of abiotic stress on Ramularia trials and its potential effect on the ranking of varieties' scores; trial design and plot sizes; scoring RLS symptoms and distinguishing them from other syndromes; methods of inoculating trials where appropriate; and the potential for using DNA marker-assisted selection to supplement field trialling.

7.17 *Ramularia collo-cygni* − an emerging pathogen of barley crops (*review*)

See Section 5.5 of report

Neil D Havis[1], James KM Brown[2], Gladys Clemente[3], Peter Frei[4], Malgorzata Jedryczka[5], Joanna Kaczmarek[5], Maciej Kaczmarek[1], Pavel Matusinsky[6], GrahamRD McGrann[1], Sylvia Pereyra[7], Marta Piotrowska[1], Hind Sghyer[8], Aurelien Tellier[8], Michael Hess[8]
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Ramularia collo-cygni (Rcc) is the biotic factor responsible for the disease Ramularia leaf spot (RLS) of barley (*Hordeum vulgare*). Despite having been described over one hundred years ago and being considered a minor disease in some countries, the fungus is attracting interest in the scientific community as a result of the increasing number of recorded economically damaging disease epidemics. New reports of disease spread and fungal identification using molecular diagnostics have helped redefine RLS as a global disease. This review describes recent developments in our understanding of the biology and epidemiology of the fungus, outlines advances made in field of the genetics of both the fungus and host as well as summarising the control strategies currently available.

8. Appendix B: Knowledge exchange activities in CORACLE

Activity	Date	Organisation
Press release announcing project, with articles in Eastern Daily Press, Business Weekly, etc	June 2009	JIC
Six NIAB-TAG Winter Conferences featuring barley disease management, S. & E. England; 40-80 growers, agronomists & technical specialists per conference	Nov 2009 – Jan 2010	NIAB-TAG
Three AHDB Cereals & Oilseeds Roadshows	Jan 2010	SRUC
Seven NIAG-TAG National Agronomy Training Courses featuring barley disease management, S. England & S. Wales; 25 attending per course	Feb 2010 – Apr 2010	NIAB-TAG
5 AHDB Cereals & Oilseeds Agronomy Workshops, featuring barley disease management	Feb 2010	AHDB & NIAB-TAG
Three Maltsters' Association of Great Britain Barley events	Feb 2010	SRUC
Paper at Crop Protection in Northern Britain conference	Feb 2010	SRUC
Five AHDB Cereals & Oilseeds Disease Roadshows in England	Feb 2010	SRUC
Barley Disease Technical Note	Apr 2010	SRUC
Cereals 2010 demonstration	June 2010	JIC & SRUC
Publication of A Guide to the Recognition and Understanding of Ramularia and Other Leaf Spots of Barley	June 2010	BASF & SRUC
Three Success Through Knowledge open days	June & July 2010	SRUC
Poster at Association of Applied Biologists Biocontrol Conference	Nov 2010	SRUC
Article about Ramularia in 'Landmark Bulletin'; circulation c.3000	Jan 2011	NIAB-TAG
Three AHDB Cereals & Oilseeds/SRUC Disease Roadshows	Jan 2011	SRUC
Paper at Crop Protection in Southern Britain conference	Feb 2011	SRUC & NIAB-TAG
Two talks in Ramularia session at 4th International Barley Leaf Blight workshop	June 2011	SRUC
National Agronomy Centre Open Day, Norfolk, with field demonstration on barley disease management, including Ramularia; c.300 attending.	June 2011	NIAB-TAG
Two 'Success Through Knowledge' open days	June & July 2011	SRUC
'Cereals in Practice' event, Scotland	June 2011	SRUC
Seven NIAG-TAG National Agronomy Training Courses featuring barley disease management including Ramularia, S. & E. England; 25 attending per course Nov 2011 to April 2012	Nov 2011 – April 2012	NIAB-TAG
'Research in Focus' article in Farmers Weekly	Dec 2011	JIC
Three AHDB Cereals & Oilseeds/SRUC Disease Roadshows	Jan 2012	SRUC
Article in Farmers Weekly about control of Ramularia by SDHIs	Feb 2012	NIAB-TAG
Three papers at Crop Protection in Northern Britain conference	Feb 2012	SRUC
Article in Crop Production Magazine	Mar 2012	AHDB & SRUC
Pre-conference Ramularia workshop at EFGC Germany	Mar 2012	JIC & SRUC
Two posters at European Fungal Genetics Conference, Germany	Mar 2012	SRUC
Article about CORACLE in Farmers Weekly, April 2012	Apr 2012	SRUC
Activity	Date	Organisation
Two 'Success through Knowledge' open days	June & July 2012	SRUC
Poster at Cereals 2012	June 2012	AHDB

'Cereals in Practice' event, Scotland	June 2012	SRUC & JHI
Article about CORACLE (variety resistance and fungicides) in Farmers Weekly	July 2012	JIC, SRUC & JHI
European Aerobiology Symposium	Sept 2012	SRUC
European Foundation of Plant Pathology conference, The Netherlands	Oct 2012	SRUC
Representation to AHDB Recommended Lists about variety ratings for Ramularia	Nov 2012	JIC & SRUC
Five posters at British Society for Plant Pathology Presidential Meeting	Dec 2012	SRUC & JIC
Four SRUC/AHDB Cereals & Oilseeds Disease Roadshows	Jan 2013	SRUC
Two MAGB/AHDB Cereals & Oilseeds Malting Barley Breakfast Meetings	Jan & Feb 2013	SRUC & AHDB
Integrated Pest Management Conference, Italy	Mar 2013	SRUC
Fungal Genetics Conference, USA	Mar 2013	SRUC
20 farmers talks and agronomy meetings, Scotland	2013	SRUC
'Research in Focus' article in Farmers Weekly	Apr 2013	JIC & SRUC
Updated AHDB Cereals & Oilseeds Topic Sheet, 'Ramularia leaf spot in barley'	May 2013	AHDB, SRUC & JIC
Malting Barley Day, with field demonstration on spring barley disease management focussing on Ramularia; with Crisp Malting, H. Banham Ltd, Adams & Howling Ltd; Norfolk, c.80 attending	June 2013	NIAB-TAG
Demonstration at Cereals 2013	June 2013	AHDB, JIC & SRUC
Cereals in Practice event	June 2013	SRUC
Field Trials Open Evening for general public	June 2013	JIC
Three SRUC Trial Open Days	June & July 2013	SRUC
Two posters at Society of Experimental Biology conference, Italy	July 2013	JIC
'Fascination of Fungi' Open Day for general public, with exhibit about crop diseases including Ramularia	Oct 2013	JIC
Talk at Seed Health Symposium	Nov 2013	SRUC
Talk at JHI Barley research workshop	Nov 2013	SRUC
Recommended List meeting about disease ratings, including Ramularia	Dec 2013	JIC
Press release about effect of mlo on Ramularia with articles in Farmers Weekly, Farmers Guardian, Crop Production Magazine, Zimbabwe Star, Science Daily, Science Newsline Biology, Nigeria Sun, etc	Jan 2014	JIC
'Research in Action' article for Arable Farming	Feb 2014	JIC, SRUC, KWS & AHDB
Discussions on Ramularia control, including outputs of CORACLE, in assorted farmer, internal and external agronomist individual and group discussions, meetings and field events	Throughout	NIAB-TAG
Teacher-Scientist Network: science lessons by scientists in primary schools, including lessons on plant pathology	Throughout	JIC

9. Appendix C: Relation of Programme of Work to sections of report

Task	Description	Report sections
1.1	Develop risk algorithm for forecasting RLS as a management tool for choice of varieties and fungicides.	4.3, 4.4
1.2	Study variation in responses of UK populations of *Rcc* to triazole and carboximide fungicides and assess its impact on disease control	4.1
2.1	Develop real-time PCR to quantify *Rcc* in plants	3.2
2.2	Study extent of seed contamination by *Rcc* in samples of grain from different UK regions and different varieties, to determine current incidence of pathogen in barley seed	3.2
2.3	Study movement of *Rcc* spores into barley seeds to test if seed contamination occurs by growth of mycelium within the plant or by dispersal of spores onto the grain	3.1
2.4	Examine effectiveness of seed treatments to control RLS and produce uncontaminated grain	4.2
2.5	Examine significance of seed contamination for epidemiology of RLS and disease control	4.2
3.1	Produce *Rcc*-free seed stocks of barley varieties studied in AGOUEB as a basis for analysis of susceptibility to RLS	5.2
3.2	Run field trials of RLS-susceptibility in spring and winter varieties, comprising selections from the AGOUEB set plus other cultivars from continental Europe, as a basis for association genetics	5.2
3.3	Apply high-throughput methods of scoring RLS on the full set of AGOUEB varieties, to estimate the RLS-susceptibility of all the AGOUEB varieties and to identify new methods of estimating RLS susceptibility as alternatives to field trialling	5.2, 5.4
3.4	Obtain GoldenGate marker data on trialled varieties not included in AGOUEB	5.2
3.5	Estimate locations and effects of resistance genes by combining disease scores with marker data and estimates of relatedness, and investigate associations of resistance loci with other important phenotypes	5.2
3.6	Develop mapping populations from elite spring barley varieties with higher levels of resistance to RLS to locate resistance genes and to test the effect of the *mlo* mildew resistance gene on RLS	5.1
3.7	Conduct QTL analysis of RLS resistance in crosses of winter barley varieties	5.3
3.8	When especially important genes for resistance have been identified, develop markers suitable for use in marker-assisted selection for RLS-resistance	5.1, 5.2
3.9	Study variety-by-isolate specificity in resistance to RLS, to evaluate the risk of varieties' resistance diminishing through the emergence of virulent *Rcc* isolates	5.2
4.1	Test induction of RLS symptoms in infected plants by various abiotic stresses, to identify inducing stresses which are more tractable than high light intensity	3.3
4.2	Once stress conditions which induce RLS have been identified, develop simplified protocols to evaluate their potential for high-throughput applications such as screening for resistance in breeding nurseries	4.4
4.3	Identify effects of stress factors on host defences and fungal development will be investigated	3.3
4.4	Investigate complex effects of *mlo* mildew resistance on RLS in the field and lab, in particular to test the hypothesis that *mlo* suppresses RLS when the plant is not stressed but aggravates symptoms in stressed plants	3.3, 5.1
4.5	Investigate variation in varieties' susceptibility to toxins produced by *Rcc* and test its relationship to field resistance	3.3

10. Acknowledgements