Ramularia leaf spot of barley

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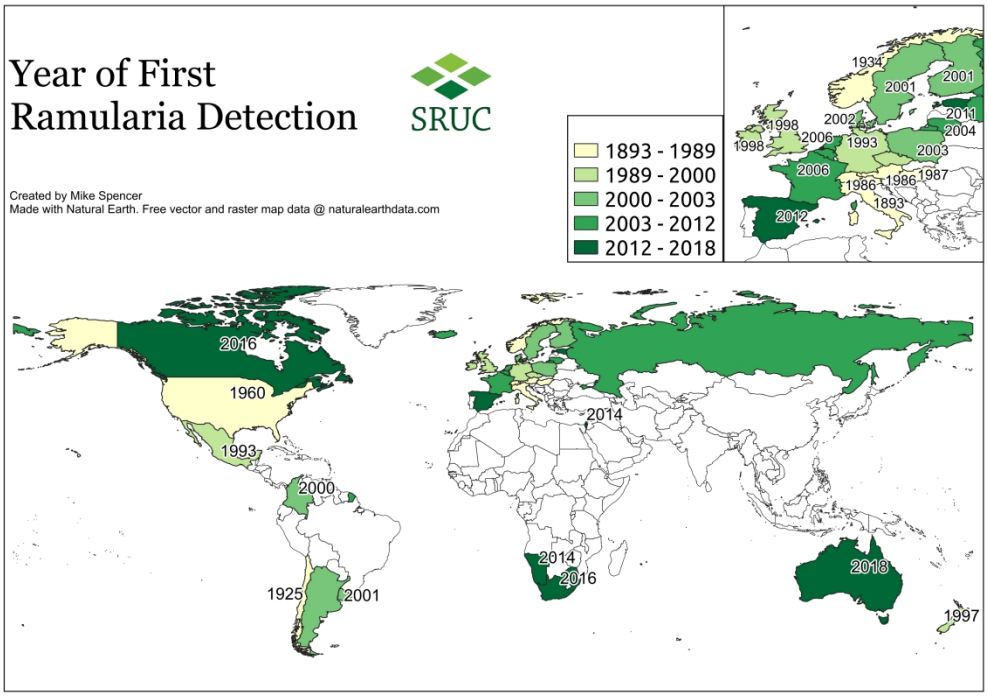


Figure 1 Global distribution of Ramularia leaf spot and the year of first report (Spencer et al, 2019)

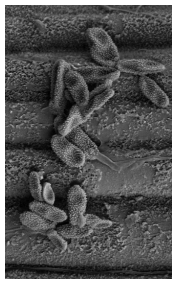
R.collo-cygni located within seed pre-sowing

R. collo-cygni present in asymptomatic seedlings

Necrosis on lower sensescing leaves and occasional sporulation

Secondary spore structure on straw and stubble

Airborne spores land on leaf surfaces and invade via stomata

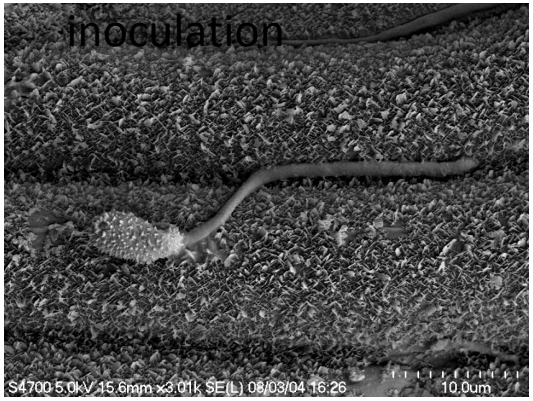


Spores from alternative hosts

Crop reaches flowering

Post flowering RLS symptoms – initial pepper spots enlarge and coalesce, spread to stem and awns

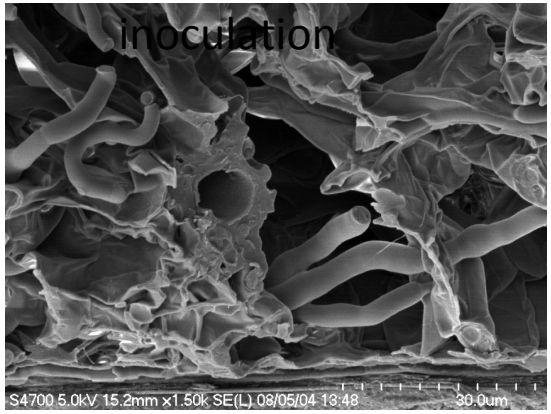
3 days post inoculation



Barley -upregulated
Pathogenesis response genes
SM Transport
Flavenoids
PP pathway
Barley-downregulated
Photosynthesis

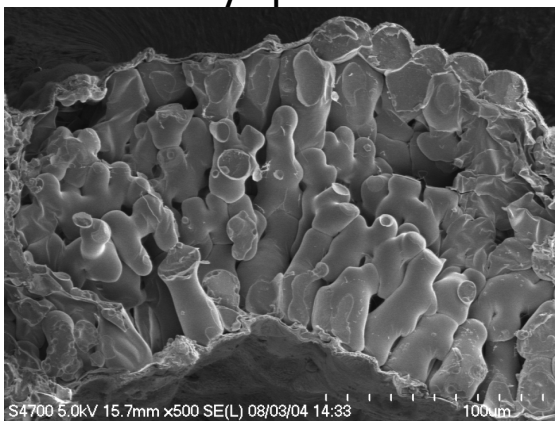
R. collo-cygni – upregulated
Effectors
Cell Wall degrading

7 days post inoculation



Barley-upregulated
Ethylene and jasmonic acid signalling
Lignification
Flavonoids
PP pathway
Barley-downregulated
Photosynthesis
R. collo-cygni – upregulated
Effectors
Transport

12 days post



Barley-upregulated
Ethylene and jasmonic acid
Senescence
Tyrosine decarboxylase

R. collo-cygni – upregulated
Effectors
Transport

Ramularia leaf spot in barley – The danger within

One of the major issues challenging barley (*Hordeum vulgare*) growers in many countries is the control of Ramularia leaf spot (RLS), caused by the fungal pathogen, *Ramularia collo-cygni.* This small, seemingly inconsequential fungus has moved from the realms of minor status in cereal growing countries in the early to mid-20th century to a major economic barley disease across many continents with only limited options in terms of effective control. Within this story lie emerging challenges and questions which are still in the process of being investigated by researchers to provide effective advice to farmers and growers

1) Diagnosis of plant disease
2) Global distribution
3) Life cycle of the fungus
4) Environmental effects on disease expression
5) Genetics of disease resistance
6) Pathogen variability
7) Control of RLS by fungicides
8) Future prospects/further information

Diagnosis of plant disease

Although the early reports of *R. collo-cygni* focussed on descriptions of the fungal structures (Sutton and Waller, 1988) the fungus was reported to produce small elongated brown lesions with pinkish white sporulating hyphae on the leaf underside of Triticale (*Triticum secalum*) (Sutton and Waller, 1988). A detailed description of symptoms appearing barley crops was provided by Huss et al, 1987. Symptoms of necrosis were recorded on the upper leaves of the barley crop. Salamati and Reitan (2006) summarised disease development in the following way. The appearance of small paper spots (2.5 x 0.5mm) on the upper canopy which spread and become abundant on leaves, sheaths and awns. The spots were described as having a yellow chlorotic halo. A guide to identifying RLS was produced in 2010 (BASF, 2010). More recently the distinguishing characteristics of RLS have been collated into a published guide (AHDB, 2018). The guide recommends that growers use the 5R's to successfully diagnose the symptoms of the disease. 1) Ring of chlorosis around the lesion 2) Rectangular shape 3) Restricted by the leaf veins 4) Reddish-brown colouration 5) Right through the leaf.

Perhaps the major challenge facing growers across many countries is successfully identifying the disease Ramularia leaf spot in barley. Physiological stresses are known to cause the production of necrotic areas on barley leaves e.g. damage due to excessive light levels (Wu & Von Tiedemann, 2002), lack of manganese in the crop (TEAGASC, 2020). In the case of abiotic stresses, many nutritional deficiencies produce symptoms which appear throughout the development of the crop but physiological leaf spots (PLS) tend to appear on the upper canopy at the same time as RLS (Wu and von Tiedemann, 2002). A major difference between PLS and RLS is that the browning and necrosis is generally only seen on the upper leaf surface and symptoms are not mirrored on the underside of the leaf.

Barley (*Hordeum vulgare*) as a host is susceptible to many biotic agents as well as *R. collo-cygni.* Several them can cause symptoms which may be confused with RLS and during initial studies a

degree of confusion occurred. In particular, the early symptoms of *Pyrenophora teres* (Sachs et al, 1998) caused problems for researchers. The spot form of the fungus *P. teres* f. *maculata* can form small rectangular lesion which can be mistaken for RLS but they do not have chlorotic halo of RLS and tend to elongate along the length of the leaf, unlike RLS (BASF, 2010; AHDB, 2018).

Diagnosis of the RLS by visual means has been supplemented by additional tests. Sachs incubated leaves on acidified agar and the resultant reddish colour indicated the presence of the fungus. (Sachs, 2006). This simple lab test was soon surpassed by the development of molecular diagnostics assays (Havis et al, 2006; Frei et al ,2007; Taylor et al 2010). These assays allowed the confirmation of important parts of the fungal life cycle and advances in epidemiology studies. (Walters et al, 2008; Havis et al, 2015).

Global Distribution of Disease

The initial description of Ramularia leaf spot symptoms was on barley in the North of Italy (Cavara, 1893). The next description of the fungus was not for a further 32 years when the fungus was identified in Chile and there were only sporadic reports in other countries. In the middle of the 1980's the fungus was identified on barley crops in the Lambach region of Austria by Herbert Huss (Huss et al, 1987). From this initial report the disease spread rapidly in Central Europe before moving into all of the major barley growing regions of Europe at the start of the 21st century (Walters et al, 2008; Havis et , 2015) see insert box in Figure 1.

Although the fungus was described in South America in the early part of the twentieth century (Sutton and Waller, 1988) it was only reported as a serious economic issue at the star of this century. The disease was reported in the Buenos Aries province of Argentina in 2001 (Khier et al, 2001). Since that initial report, RLS has also been reported in other parts of Argentina and Uruguay (Carmona et al, 2013; Pereyra, 2013).

In North America, the fungus was identified in many plant species in the 1970's (Sutton and Waller, 1988) and has also now been identified in barley crops in Canada (Kelly Turkington, personal communication).

The disease was identified in New Zealand at the end of the last century (Harvey, 2002), around the same time as its first appearance in the United Kingdom. Disease epidemics have been common in the last twenty years (Soonie Chng, personal communication). In recent years, after an initial report in Tasmania RLS has been reported in other regions of Australia (Tasmanian Government, 2020). *R. collo-cygni* has also been detected in seed samples from Tanzania and Israel (Michael Hess, personal communication) and is known to be present in barley crops in South Africa (Beukes et al, 2016).

Major epidemics continue to be reported in New Zealand and across Europe, with 2017 a particularly devastating year in the UK. The current distribution reflects the investigations so far carried out. More widespread testing may reveal the fungus is present in other South American, North-African and possibly Asian countries.

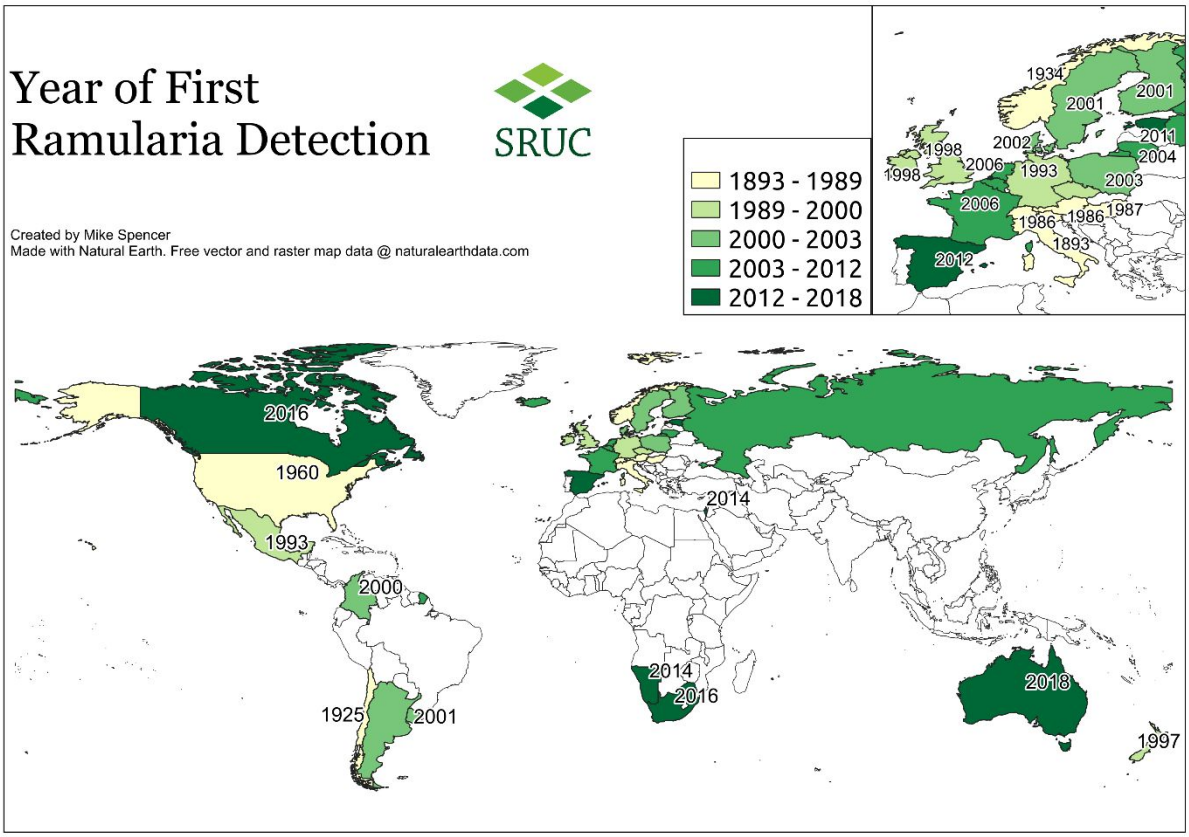


Figure 1 Global distribution of Ramularia leaf spot and the year of first report (Spencer et al, 2019)

Life cycle of the fungus

The fungus is known to infect grass and other hosts and exhibits a non-specific life cycle, which makes control a challenge. Host range is summarised in Table 1

Host	Common name	Source
Hordeum vulgare	Barley	Cavara, 1893
Triticale secalum	Triticale	Sutton & Waller, 1988
Elytrygia repens	Scutch grass	Huss et al, 2004
Elymus caninus	Bearded couch grass	Huss et al 2004
Echinochloa crus-galli	Annual grass	Huss, 2004
Avena sativa	Oat	Huss, 2004
Triticum aestivum	Wheat	Huss, 2004
Secale cereale	Rye	Huss, 2004
Quercus robur	Oak trees	Heuser and Zimmer, 2002
Triticum durum	Durum wheat	Frei and Gindrat, 2000
Poa pratensis	Common meadow grass	Frei and Gindrat, 2000
Lolium perenne	Perennial ryegrass	Frei and Gindrat, 2000
Agropyron repens	Couch grass	Frei and Gindrat, 2000
Brachypodium distachyon	Stiff brome grass	Peraldi et al, 2014
Hordeum murinum	Wall or false barley	Frei, 2004
Apera spica-venti	Silky bent grass	Frei, 2004

Agrostis spp	Bent grass species	Cromey et al, 2004
Bromus cartharticus	Prairie grass	Cromey et al, 2004
Glyceria fluitans	Floating sweet grass	Cromey et al, 2004
Lollium multiflorum	Italian Ryegrass	Kaczmarek et al, 2016

Table 1. Reported host range for the fungus *Ramularia collo-cygni.*

Infection of plants with *R. collo-cygni* occurs in a few ways. The fungus has been shown to be seed borne (Havis et al, 2006; Frei et al, 2007) and in barley the fungus has been shown to grow internally within barley plant (Frei et al, 2007; Havis et al, 2014) without causing any symptoms. Although fungal sporulation has been reported on many of the species in Table 1 the characteristic Ramularia leaf spot symptoms have only been observed on barley.
Several studies have sought to determine the exact location of *R. collo-cygni*. Detailed dissection of barley seed by Matusinsky indicated the presence of fungal DNA in the covering layers of the seed and the lemma, and in lower amounts in the pericarp and embryo (Matusinsky et al. 2011). In other studies, with higher fungal infection the fungus was identified in the endosperm (Havis et al. 2009a; Oxley and Havis 2010; Havis et al. 2014a; Clemente et al. 2014; Hess et al. 2014). Indeed ,later whole plant inoculation studies with a green fluorescing protein (GFP)-transformed isolate developed by Thirugnanasambandam et al. (2011) showed *R. collo-cygni* accumulation under the seed coat outside the aleurone layer and the GFP signal in all seed component parts, including the endosperm (Kaczmarek et al. 2013).

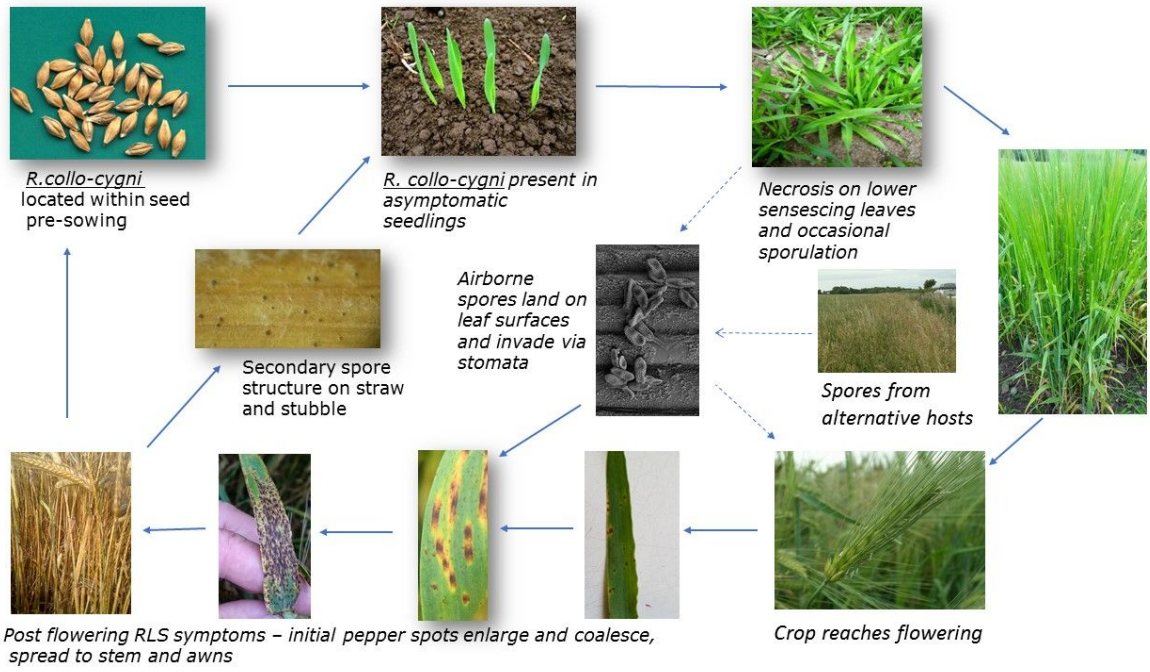


Figure 2 *Ramularia collo-cygni* lifecycle in barley

After the seed germinates and the plant develops the movement of the fungus has been tracked using a combination of microscopy and molecular diagnostics. The fungus has been shown to move in the plant asymptomatically (Havis *et al.,* 2004; Salamati & Reitan, 2006). Huss reported that the fungus can survive as a saprophyte on senescing leaves in winter barley crops (Huss, 2004). The movement of the fungus can be tracked throughout the growing season as successive leaf layers emerge (Frei et al., 2007; Havis et al., 2014; Kaczmarek *et al.,* 2017). During the vegetative growth stage of the plant the crop appears asymptomatic. The role of spore movement from senescent

material or alternative hosts is still being studied and remains contentious. Although many studies have pointed to the seed borne stage (Walters et al., 2008; Havis et al 2015), a recent study in Estonia could not detect movement from seed (Mäe et al., 2018). The studies with the fluorescent isolates indicated that the fungus grows intercellularly, forming branched hyphae in the leaf mesophyll layer (Kaczmarek et al. 2013). A brick like pattern is formed as hyphae surround the leaf cells but do not penetrate any of the cell walls. This study confirmed the initial growth pattern suggested by Sutton and Waller (1988).

Interestingly, there have been reports that in some southern hemisphere countries the pathogen displays symptoms earlier in the development of the crop i.e. pre flowering (e.g. Harvey, 2002, Ignacio Erreguena, personal communication). The causes of this observation remain unclear.

After flowering in barley crops, disease symptoms begin to appear on the upper leaves in the canopy. The initial pepper spots formed on the leaf enlarge but remain bound by leaf veins. Detailed studies indicate that the hyphae always stop at leaf veins and make no attempt the penetrate a leaf vein (Kaczmarek et al. 2013). Neighbouring leaf spots eventually coalesce to form larger dark areas. As symptoms expand, the remainder of the leaf becomes chlorotic and then necrotic, usually starting at the leaf tip and margins (Huss, 2004). Fungal sporulation generally only occurs in necrotic tissue, with conidiophores emerging through stomata in caespituli of up to 15, from underlying stromata. The terminal part of each conidiophore is strongly curved (Sutton & Waller, 1988), with the apical region producing up to five conidia. *R. collo-cygni* grows preferably, although not exclusively, on abaxial leaf surfaces and Huss (2004) estimates that a heavily infected leaf can produce up to 50, 000 conidia.

The necrosis which appears in the leaf is due to the production of secondary metabolites by the fungus. Fungal mycelia were found to have a purple pigmentation and Heiser et al 2003 speculated that these could be a range of photoactive polycyclic aromatic toxins, similar to cercosporin, produced by several species of *Cercospora.* They identified some of the coloured metabolites, including an anthraquinoid, identified as rubellin D. When applied to barley leaves, rubellin D induced light- and concentration-dependent necrosis, and in a model system was shown to exhibit photodynamic activity, triggering the light-dependent production of reactive oxygen species (ROS), and ultimately the peroxidation of α-linolenic acid (Heiser *et al.,* 2003). Later work by Heiser et al (2004) indicated these toxins are not host specific. Several isomers of rubellin have been shown to be produced by the fungus and rapid conversion of types appears to take place during the expression of disease symptoms (Miethbauer *et al.,* 2003; Heiser *et al.,* 2004; Miethbauer *et al.,* 2006. Rubellin B has been shown to be the dominant form found in leaves (Miethbauer *et al.,* 2003). A role for rubellins in pathogenicity was suggested by Heiser et al, 2004. However more recent work by Dussart et al (2018a) suggested no correlation between susceptibility to rubellin D and disease susceptibility. At the same time research indicated a few gene clusters in the fungal genome relating to secondary metabolites (Dussart et al, 2018b). The interaction between the fungal secondary metabolism and the host remains under researched.

Environmental effects on disease expression

Understanding the interaction between the fungus, host and environment has presented researchers with significant challenges. The late season production of symptoms has challenged researchers attempting to define the factors which influence disease epidemics.

Case study 1) One of the early important studies was carried out by Huss et al, 2003 at the Lambach field station in Austria. In this study pots of two winter barley varieties (Virgo and Dido) were moved into a wind exposed polytunnel after first leaf emergence. One set of pots were placed into dry conditions overnight to remove the possibility of dew forming on the leaves. In the other set dew formed naturally. During the daytime, the plants were kept in identical conditions in a polytunnel. By the time the plants reached milk ripeness (approx. GS 75-80; Zadoks, 1974), the plants which has

experienced dew on the leaves showed typical symptom formation and conidiophore formation on senescing leaves. The plants which had been kept dry stayed green and symptomless. This was the first experimental evidence of the importance of dew in symptom formation.
Studies following this identified relative humidity in crops at GS 31 as an indicator of RLS severity in Central Norway (Salamati and Reitan, 2006). Here, the authors investigated the links between weather conditions and disease severity. They found a correlation between long leaf wetness duration and days with precipitation at GS15-30 and final RLS severity. Havis et al 2012 investigated the potential for leaf wetness for 14days at GS30/31 to act as a risk warning for RLS in barley crops. Within season analysis of disease levels and meteorological data showed some correlation between leaf wetness and final disease levels but analysis over multiple years showed that differences in disease levels could not be explained solely by one environmental parameter (Havis et al., 2018).
Other environmental factors have been associated with the appearance of disease in barley crops. Heiser et a*l.,* 2003 reported on the unpublished work from E Sachs, which suggested light intensity was important in determining RLS symptom levels. However subsequent work by Makepeace (2006) suggested light intensity prior to inoculation exerts a significant effect on RLS. Plants grown under low light conditions before inoculation exhibited fewer RLS symptoms than those grown under high light. Increasing light intensity post inoculation led to fewer RLS symptoms, suggesting that although light is required for the toxins produced by *R. collo-cygni* (see above) to exert their effects, excessively high light intensities might have a negative effect on pathogenicity (Makepeace, 2006).
Formayer et al. (2004) reported that high humidity levels were crucial for the outbreak of RLS epidemics in Austria, while radiation intensity was only of minor importance. Marik et al. (2011) found that stronger symptom expression was positively affected by a higher number of rainy days in the three weeks post heading. These authors also reported that higher temperatures and lower rainfall post flowering reduced disease levels in the Czech Republic. Havis et al (2018) found a correlation between RLS levels in spring barley and temperature and rainfall experienced by the crop from sowing up the ear emergence (GS59). However, this observation was not seen across all years of the study.
Understanding the impact of the environment and other biotic factors on RLS epidemics will require many more years of study.

Genetics of disease resistance

From the earliest reports of the appearance of RLS in barley crops it became apparent that the genetics of the host influenced the development of symptoms. (Walters et al, 2008) The study from Harvey (2002) in New Zealand suggested that even low disease levels in the crop can exert a disproportionately negative effect on yield. Yield losses due the RLS were reported by both Huss et al, 1992 and Pinnschmidt & Hovmøller, 2004 indicating a susceptibility of the plant to the disease. In Europe the average yield losses attributed to RLS in barley have been reported to be in the region of 0.4 t/ha in Europe (Hess et al. 2007; Walters et al. 2008). However varietal and regional effects have been reported on disease severity (Hess et al. 2011; Oxley and Havis 2009). More recently epidemics in South American barley crops have produced yield losses as high as 70% in susceptible cultivars (Havis et al, 2014). In both Uruguay and Argentina losses were associated with a significant reduction in grain size, with less than 10% of grains reaching 2.5mm in size (Pereyra 2013; Clemente et al. 2014). In addition to reducing yield and grain size in crops, RLS has been implicated in a reduction in malt quality (Pinnschmidt and Jørgensen 2009; Hess et al. 2011). Pinnschmidt and Jørgensen (2009) attributed the yield loss from RLS to a reduction in thousand grain weight in infected plots. The impact of the fungus on the plant during its long asymptomatic phase has recently garnered interest and a project was initiated in Scotland to examine the interaction between the fungus and photosynthesis in the plant prior heading. The interaction was examined using a combination of controlled conditions and field experiments (Burrell et al, 2020). The authors of this work observed no change in photosynthesis, measured as maximum quantum yield, in either

system prior to symptom expression. They also noted that there was no difference in CO_2 uptake per unit leaf area in the same leaves and concluded that the yield response from *R. collo-cygni* was primarily related to the loss of photosynthetic area in the symptomatic phase (Burrell et al. 2020).
The effect of variety on RLS symptom severity has been studied for the last 20 years. Sheridan (2000) was one of the first studies in the Southern hemisphere to identify that differences in varietal susceptibility to RLS existed. The need for new varieties in the southern hemisphere was highlighted by Pereyra et al (2014). The authors reported that of all the varieties used in Uruguay in 2013 the best options were still only moderately susceptible, and none were resistant.
Work in Denmark by Hans Pinnschmidt and colleagues on a large panel of spring and winter cultivars demonstrated a wide range of susceptibility but importantly also identified some moderately resistant lines (Pinnschmidt and Hovmøller 2003; 2004; Pinnschmidt et al. 2006). This work suggested that varietal resistance could be utilised effectively to control RLS in Denmark. Improved resistance was also reported in Germany in the 6-row winter barley, Duet, by Hess et al (2006). The authors also pointed out that the maturity of the varieties should be considered, when comparing disease scores.
This variation in varietal susceptibility has also been reported in a number of European countries e.g. Lithuania (Leistrumaitė and Liatukas 2006), Norway (Reitan and Salamati, 2006), Slovakia (Gubiš et al. 2008) and also in the UK (Oxley et al, 2008) . Significant differences in the RLS-susceptibility of winter barley cultivars was found in the Czech Republic (Mařík et al. 2011) but only limited variation between spring cultivars (Matušinsky et al. 2013). In all these cases, there was quantitative variation in resistance with no clear division between resistant and susceptible cultivars suggesting that RLS-resistance is generally a quantitative trait.
An early project on breeding for RLS resistance identified a correlation between the presence of *mlo* mildew resistance and susceptibility of barley to RLS (Bistrich et al, 2006). The authors had examined a wide range of varieties, advanced breeding lines and Genebank accessions over several seasons and sites. The *Mlo* gene in barley is required to confer susceptibility to powdery mildew (*Blumeria graminis*) therefore non-functional (*mlo*) alleles confer recessively inherited resistance to this disease. Breeders have used the *mlo-11* allele especially, and *mlo-9* in spring barley breeding programmes across Europe (Jørgensen 1992). Pinnschmidt et al (2006) reported on trials of 75 barley cultivars in Denmark. Of the 13 most susceptible to RLS only one did not have *mlo* while all the 18 least susceptible lines lacked *mlo*. One of the difficulties of effectively trialling RLS in field experiments is the presence of other factors including disease. This was highlighted by an analysis of RLS scores from many naturally infected trials in Denmark. Here, two-thirds of variation between trial plots was found to be attributable to extraneous factors, including cultivars' susceptibility to other diseases. Only the remaining one third of variation was related to cultivar susceptibility to RLS. However, removing the other factors revealed a strong association between the presence of *mlo* and high susceptibility to RLS. A series of inoculated trials in Denmark showed the same association (Pinnschmidt and Sindberg 2009).
Several studies were carried out to investigate the relationship between *Mlo.* These experiments utilised seed lines, which varied in alleles of the *Mlo* gene. The results largely supported the conclusion of the field trials undertaken in Denmark but also raised other questions about the interaction between *mlo* mildew resistance and RLS. Makepeace et al (2007) used near isogenic lines (NILs) to breed different *mlo* alleles, replacing Mlo^+ wild type allele, into a susceptible background. In field trials with low RLS disease levels *mlo* alleles were associated with a moderate decrease in RLS. These results contradicted the results of the Danish trials.
Case study 2) A separate experiment by Makepeace et al (2008) had indicated that high light levels before inoculation could increase symptom formation in controlled conditions. In order to investigate the influence of light and *mlo* on RLS susceptibility, a further set of experiments was conducted on NILs with either Mlo^+ (mildew-susceptible wild-type) or *mlo-5* (mildew-resistant) in controlled environment chambers. When light levels were increased before inoculation RLS symptoms on both wild and modified *mlo* lines were increased but the disease levels were

significantly greater in the *mlo* lines. Conversely lowering light levels before inoculation lowered RLDS symptoms but there were no significant differences between *Mlo*$^{+}$ and *mlo-5* lines (Brown and Makepeace 2009). McGrann repeated this work and found that *R. collo-cygni* DNA levels were much higher in the *mlo-5* lines in field trials and controlled environment experiments (McGrann et al, 2014a).

The Coracle project was established in the UK to investigate the Control of Ramularia in a Changing climate. The final project report is available online (AHDB, 2016). This project examined in more detail the effect of *mlo* alleles on RLS by carrying out a series of field trials using a double haploid combination produced from a cross between a highly RLS susceptible *mlo-11* cultivar (Braemar) and a highly RLS susceptible *Mlo*$^{+}$ cultivar (Power). The field trials were carried out in a few countries to ameliorate the environmental effect on RLS development. The results of these trials were reported by McGrann et (2014a).

The authors reported that *mlo-11* was indeed associated with increased susceptibility to RLS but that this effect was much stronger in Bavarian trials rather than in other trial sites or controlled environment experiments. The authors also carried out a quantitative trial locus (QTL)analysis on disease expression in the lines and found the only significant QTL was the *Mlo* locus. At the site with the greatest differences between lines the QTL accounted for up to 37% of the genetic variation (McGrann et al, 2014a)

Interestingly. Hofer et al (2015) reported no increase in *R. collo-cygni* DNA in grain from *mlo-5* varieties grown in Bavarian field trials over 4 seasons. The authors recommended a re-evaluation of the effect of *mlo* on the incidence and severity of necrotrophic pathogens.

However, the overall conclusion from the work on *mlo* and RLS expression in barley is that the mildew-resistance allele is very likely to contribute to the susceptibility of spring barley cultivars to RLS but the strength of this effect varies with the environment, location and genetic background (Havis et al, 2014) . This conclusion is based on the reports of significant variation in RLS-susceptibility between cultivars (Pinnschmidt et al. 2006) and other lines (McGrann et al. 2014a) with *mlo*. The future management of *mlo* in spring barley breeding programmes remains a challenge. Further work by McGrann et al (2014a) showed that the use of *ror* mutants, which partially restore mildew susceptibility in *mlo* plants, could alleviate disease symptoms without affecting *R. collo-cygni* DNA levels in leaves. This suggests that resistance to fungal infection and colonisation and symptom formation may to some extent be under separate genetic control. In order to elucidate the role of plant genetic factors on disease expression. McGrann et al (2014b) carried out a series of experiments with the stress responsive transcription factor (*SNAC1*). The NAC proteins are involved in the plant response to biotic and abiotic stress. In lines of the RLS and mildew susceptible variety, Golden Promise, over expression of this factor reduced RLS symptoms but had no effect on other disease levels. The transcription factor was found to have no effect on either stomatal conductance or reactive oxygen species induced cell death but was found to delay dark induced senescence (McGrann et al, 2014b). In an additional set of experiments the role of active oxygen species in the transition of the fungus from endophyte to necrotroph were investigated (McGrann and Brown, 2018). The authors reported that abiotic stress in seedling leaves prior to inoculation increased RLS symptoms in all susceptible and most partially resistant lines. This was attributed to accumulation of hydrogen peroxide in the leaves. This suggests that senescence in the leaf is not as important as changes in the plant reactive oxygen species.

The production of new tolerant/resistant varieties remains a challenge. One approach may be to use diverse germplasm as sources of partial resistance and select for lines with improved RLS, which still maintain broad spectrum resistance to other major economic pathogens and other desirable traits (Havis et al, 2014).

Pathogen variability

A major advance in our understanding of the host-fungus interactions has been the sequencing of the genome of the fungus and its evaluation alongside genetic information from the host. The first full *R. collo-cygni* genome sequence was published by McGrann et al (2016). This has been followed by subsequent publication of new sequences (Stam et al, 2018). These publications gave the first insight into the genetic makeup of the fungus and offered some guidance into the likely interaction between fungus and host e.g. a limited number of genes coding for cell wall degrading enzymes but many genes associated with secondary metabolism and toxin production (McGrann et al, 2016). The publication of further sequence data from several isolates from barley and other hosts offered a glimpse into the evolution and global movement of the fungus (Stam et al, 2019). The authors noted that *R. collo-cygni* had diverged significantly from other dothidiomycte fungi and that the current global population was relatively uniform with no evidence of geographical clustering or host specialisation (Stam et al, 2019). They hypothesised that the global movement of the pathogen (described in Figure 1) was due to man made movement of seed.

Case study 3) A recent project has examined for the first time the interaction between the host and the fungal transcriptome during the infection process (Sjokvist et al, 2019). A susceptible barley variety was inoculated with an aggressive isolate of *R. collo-cygni.* The infection of the host and subsequent development of symptoms were related to changes in the transcriptome of the both the fungus and the host. The infection was carried out using a mycelial suspension. After 3 days fungal growth was observed on the leaf surface and the barley cells were alive (Figure 1). After 7 days the stomata were colonised, fungal growth was observed in mesophyll layer and the barley cells were alive (Figure 2). After 12 days the fungal growth was extensive inside the leaf and the barley cells were chlorotic and collapsing (Figure 3).

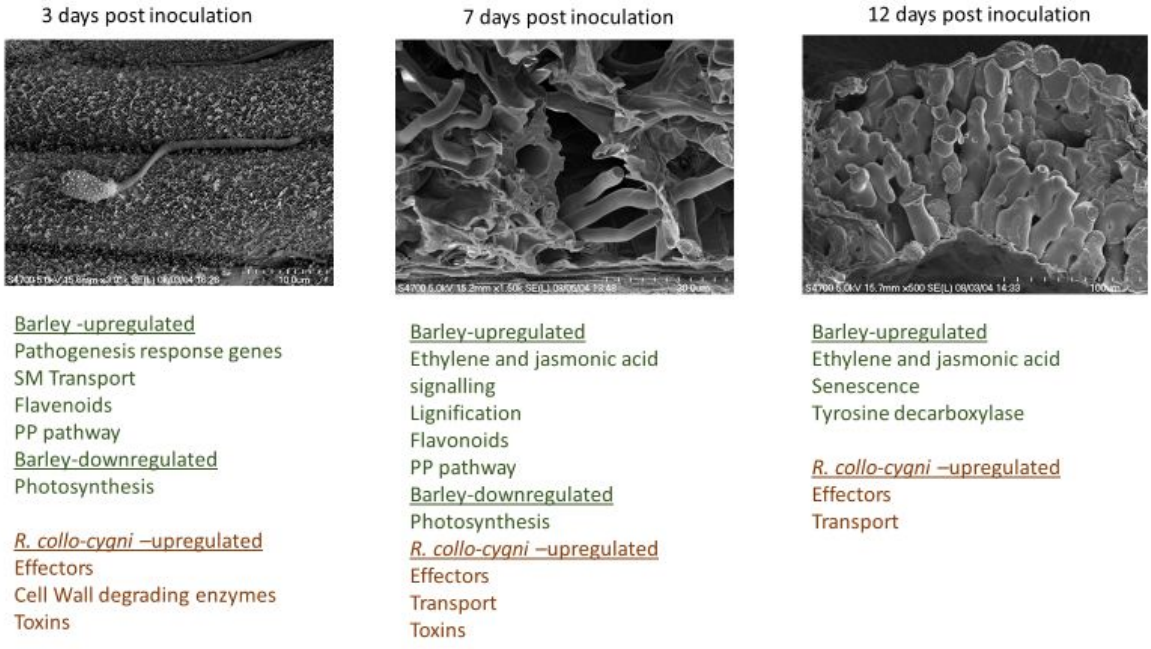


Figure 3. A pictorial representation of the infection process of barley by *R. collo-cygni* and the associated main molecular components identified to be induced or downregulated. SM= secondary metabolites, PP=phenylpropanoid.

A few changes were observed in the plant and fungal transcriptome over the course of the experiment. In the fungus after only 3 days nearly 70% of the fungal genes were expressed early on. In particular, pathogenicity factors including genes coding for secreted effectors were detected. One of these potential effectors was a chitin binding LysM domain protein. In response the host upregulated many of the classical chitin elicitor receptor kinases. As the fungus upregulated cell wall degrading enzymes, the plant responded by upregulating chitinases and glucan-endo 1,3 β-

glucosidases. The upregulation of Pathogenesis related proteins in barley suggests that the plant is detecting the pathogenesis associated molecular patterns of *R. collo-cygni.* The induction of secondary metabolite transport in the plant also suggests host defence is based the production and secretion of plant phytotoxic compounds. (Sjokvist et al, 2018). Photosynthesis in the host was strongly down regulated after 3 days with genes for light induced proteins affected. Photosynthesis was more subtly influenced through the rest of the colonisation experiment. After 7 days with apoplastic infection taking place the fungus reduced production of cell wall degrading enzyme and glycosyl hydrolases. This could a response to inhibitors produced by the host e.g. xylanase inhibitors. The host also appears to be increasing the enzymes involved in lignifying the cell walls and boosts the production of flavonoids which are acting as phytoalexins. The colonisation of the apoplast appears to induce water surface tension on the hyphae and increase stress on the host plant. The strong increase in expression of genes involved in the uptake and metabolism of hexoses after 7days suggest the fungus is deriving nutrition from the apoplast. In response to this the plant seems to be activating many of the calcium signalling pathways. The variety in the experiment had the wild type *Mlo* gene. It was found to be upregulated significantly after 7days post inoculation. The authors hypothesised that *Mlo* could mediate defence against *R. collo-cygni* but that the fungus produces effectors to supress *Mlo.* Plant defence responses are generally controlled by either the jasmonic/ ethylene pathways or salicyclic pathways depending on the fungal lifestyle. The jasmonic/ethylene pathway is associated with necrotrophic pathogens and over the course of the experiment these pathways were observed to be upregulated. The salicylic acid pathway is associated with biotrophic pathogens and no changes in gene activity was recorded in this pathosystem.
After 12 days necrosis was appearing on the leaves and the apoplast was extensively colonised. The genes proposed to be involved in rubellin production in the fungus were not found to be upregulated after 12 days, but other genes involved in secondary metabolism were found to be upregulated (Sjokvist et al, 2018). The *R. collo-cygni* transcriptome appeared to be moving towards producing acidic conditions *in planta* which could facilitate toxin production. Genes encoding for senescence factors in the infected leaves were upregulated suggesting that the natural senescence process is underway however the pathways involved in remobilisation of nutrients in the plant were not activated. The authors proposed that nutrients were being relocated into the fungus and contributed to lesion formation (Sjokvist et al, 2018). This interaction has been observed in the *Zymoseptoria tritici*/wheat pathosystem (Ma et al. 2018). This project is due shortly to reveal more details of changes within the transcriptome of the barley host and the fungus. The information will be ideally placed to identify the key molecular factors in the barley/*R. collo-cygni* interaction and will help in designing not only future breeding programmes but also potentially new control measures for the fungus (S. Radutiou, personal communication)

Control of RLS by fungicides

Given the lack of effective genetic control of RLS, control of RLS has generally been based on the timely use of fungicides (Hess et al. 2007; Havis et al, 2104). At the time of the first reports of RLS in Europe the strobilurin-based fungicides (quinone outside inhibitors; QoI) were highly effective against the disease. The QoIs, which inhibit the cytochrome bc1 complex of the respiratory chain, are an important group of chemical fungicides, active against a broad spectrum of fungal diseases (Bartlett et al. 2002). These fungicides were used extensively to control cereal pathogens. Resistance to the major wheat pathogen, *Zymoseptoria trictici* appeared in the early years of the century (Fraaije et al. 2005). At the same the strobilurin fungicides showed reduced efficacy against RLS in UK field trials. This decline in activity developed rapidly and spread to many countries (Oxley et al*.,* 2006; McCabe 2009). This rapid decline was attributed to the G143A point mutation in cytochrome b gene and was found to be prevalent in *R. collo-cygni* populations both in Scotland and Denmark (Fountaine and Fraaije, 2009). When 302 isolates of *R.collo-cygni* collected in 12 locations in the Czech Republic in 2009 were tested for the mutation the resistant allele was detected in nearly 50%

of the isolates (Matusinsky et al, 2010). In countries where strobilurin use was more regulated, the proportion of resistant:sensitive alleles remains closer to 50:50 (Jan-Eivind Kam Anderson, personal communication). The strobilurins are still used extensively in South America suggesting they are still active. Field trials in Uruguay showed improved control of RLS when azoxystrobin and chlorothalonil were used together rather than chlorothalonil on its own (Pereyra et al, 2014).

Control in more recent years has relied on the use of demethylation inhibitors (DMI) e.g. prothioconazole and succinate dehydrogenase inhibitors (SDHI) e.g. boscalid. The SDHI fungicides have been used for many years but the development of a second generation of SDHI compounds increased the control of RLS in many countries (Havis et al, 2014). The rapid development of resistance in *R. collo-cygni* to single site fungicides observed previously suggested careful stewarding of the DMI and SDHI fungicides had to be adopted. A study at the start of the decade suggested *R. collo-cygni* was medium to high risk for development of resistance to SDHI fungicides (Piotrowska et al. 2017). Many control programmes incorporated the highly effective multisite chlorothalonil to protect the single site chemicals. In some countries this multi-site was not approved for use e.g. Denmark (Jørgensen and Christiansen, 2006). However, other multi-sites have been used in combination with other fungicides to control RLS in barley (e.g. folpet) (AHDB, 2020).

Despite the stewardship programmes in 2017 across Europe a rapid decline in activity of single site fungicides towards RLS was observed (Havis et al, 2018b). The first mutations conferring reduced efficacy to SDHI fungicides were detected in 2014 (Rehfus et al, 2019). Isolates from European countries were tested for the frequency of mutations from 2014 to 2017. The sdh mutations B-H266Y/R, B-T267I, B-I268V, C-N87S, C-H146R, C-H153R and some others) were detected with increasing frequencies since 2014. DMI adapted isolates of *R. collo-cygni* were also found at a high frequency in the countries under investigation. In total fifteen different *Cyp51* haplotypes were detected in the set of isolates from 2009 to 2017. The most frequent haplotype in 2017 was *C1* haplotype. In New Zealand 33 isolates were collected from both North and South island (20 of them with either C-H142R, C-H149R, or C-N83S mutations in sdhi sub unit), so field efficacy is reducing but DMI's still remain effective (Soonie Chng, personal communication). Testing of isolates from South America is ongoing. The removal of chlorothalonil from the EU approved list in May 2020 presents a further challenge to barley growers (Dussart et al, 2020). New chemistry is slowly being released onto the global market e.g mefentrifluconazole from BASF. However, the challenge of managing new single site fungicides sustainably remains.

Timing of fungicide inputs has been studied extensively and found to have a significant effect on spray efficacy. Pereyra et al (2014) showed that in Uruguay greatest RLS control could be achieved by a three-spray programme (applications at GS33,38 and 47). This may not be practical or economic in many countries. Most studies indicate that the best effects from late treatments come from sprays at awns peeping (GS49). In Argentina, a protection window of barley against RLS by the application of an azoxystrobin + isopyrazam mixture between barley stem elongation (GS30) and first awns visible (GS49) was determined by Erreguerena et al (2014). The GS 49 treatment has also been recommended for RLS control in Switzerland (Peter Frei, *personal communication*), Germany (Hess et al. 2007) and UK (Havis et al. 2012). However, the choice and timing of fungicides in a growing season will depend on disease incidence and severity in the crop (Hess et al. 2014).

Alternatives to conventional fungicides are attracting increased attention. Plant defence elicitors which prime plants to provide broad spectrum disease control have been tested in field trials (Walters et al, 2008). These compounds are not recommended as solo treatments but did give significant reductions in RLS when applied at GS 24 followed by reduced rate fungicides at GS31 and GS 39. (Havis et al. 2009b) However, the use of elicitors needs to be managed carefully as other trials, which used a combination of defence inducing compounds, showed a negative effect on RLS (Walters et al, 2012), Therefore, more research is required before they can be used effectively (Havis et al, 2014).

The control of *R. collo-cygni* by seed treatments has been examined in many studies. Previously, good control of RLS was observed in field trials by fungicide seed treatments (Havis et al, 2010; Clemente et al. 2014). The control observed was not found to be related to restricting fungal movement in barley (Havis et al, 2010). However, the appearance of resistance to many of the fungicides has reduced the efficacy of these treatments. Non-chemical treatments have also been studied and hot water treatment has been shown to reduce fungal DNA levels (Havis et al, 2010; Zamani-Noor 2011). The control from these treatments is not always consistent in reducing RLS in the barley crop. More studies are needed to evaluate the reasons behind this observation.

Future prospects and further information

The global distribution of the fungus has been steadily expanding over the last twenty years and economic damage has been recorded on many continents. The future of Ramularia control presents many challenges. A sustainable approach will necessitate the adoption of Integrated Pest Management schemes. There are many factors to be considered in an IPM approach and some areas are under researched. A study from Croatia suggested that removing infected volunteer barley plants could an effective cultural control technique (Koric et al. 2009) and work is ongoing to establish the possibility of transmission from barley stubble to following barley crops (Havis et al, unpublished). A successful IPM programme will need to incorporate more information on rotation, varietal tolerance, seed health, risk forecasting, host-pathogen interactions, alternative control measures and fungicide efficacy.

A number of useful reviews of research in Ramularia have been published
Walters D R, Havis N D, Oxley S J P (2008). *Ramularia collo-cygni*: the biology of an emerging pathogen of barley. FEMS Microbiology Letters. 279:1–7.
Havis ND, Brown JKM, Clemente G, Frei P, Jedryczka M, Kaczmarek J, Kaczmarek M, Matusinsky P, McGrann GRD, Pereyra S, Piotrowska M, Sghyer H, Tellier A, Hess M (2015). *Ramularia collo-cygni* - an emerging pathogen of barley crops. Phytopathology 105: 895-904.
Dussart F; Creissen HE; Havis ND (2020) *Ramularia collo-cygni* – An Enemy in Waiting. In: eLS. John Wiley & Sons, Ltd: Chichester. DOI:10.1002/9780470015902.a0028896

The proceedings from a couple of meetings are available online and these can provide extra information and background.
First European Ramularia workshop 2006
http://webdoc.sub.gwdg.de/pub/mon/2007/koopmann.pdf
AHDB sponsored Ramularia workshop 2018
https://projectblue.blob.core.windows.net/media/Default/Imported%20Publication%20Docs/AHDB%20Cereals%20&%20Oilseeds/Disease/Ramularia%20leaf%20spot/Ramularia%20workshop%202018%20(presentations,%20biographies%20and%20abstracts)%20041018.pdf

Ramularia also featured in a number of barley meetings

Fourth international workshop on barley leaf blights 2011
https://www.hutton.ac.uk/events/fourth-international-workshop-barley-leaf-blights
Second international barley leaf disease Rabat. Morocco, 2017
https://repo.mel.cgiar.org/handle/20.500.11766/8020

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