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Advanced article

Abstract:

Ramularia collo-cygni, the agent responsible for Ramularia leaf spot disease of barley, possesses many mechanisms for avoiding detection by the host, which enables it to survive as an endophyte for much of the plant's life cycle before eventually turning pathogenic. The fungus has also eluded farmers and scientists for decades as it is a late season disease that often displays symptoms after the last possible fungicide intervention when crop scouting for disease has been completed. Visual symptoms are also hard to distinguish from other pathogens which has resulted in misdiagnoses. The ability of the fungus to spread not only via infected seed, but also on infected straw and wind dispersal, coupled with its ability to rapidly evolve resistance to fungicides, has resulted in an escalation in the perceived threat it presents to barley production worldwide. A greater understanding of the plant-fungus interaction is required if future control strategies are to be successful.

Key words:

Ramularia leaf spot, barley, integrated pest management, plant pathogen, fungal pathogen, endophyte.

Key Concepts:

- Plant-fungi interactions can be complex and highly variable, including mutualism, parasitism, and commensalism.
- Global movement of seed can significantly influence the spread of seed-borne diseases.
- Plant breeding for disease control requires identification of a suitable breeding target, which may be challenging.
- Breeding for resistance to one pathogen may lead to control issues for another pathogen.
- Integrated pest management practices must consider non-chemical control measures and, when using pesticides, anti-resistance strategies.
- Single site fungicides are vulnerable to losses in efficacy due to the evolution of resistance in the pathogen population.

Introduction:

Barley (*Hordeum vulgare*) is one of the most widely grown crops in the world, occupying approximately 10% of the crop growing area worldwide (Leff et al., 2004). Pests and pathogens represent a serious threat to crop production worldwide and barley is no

exception (Oerke and Dehne, 2004; Newton et al., 2011; Savary et al., 2019). Of the main diseases that affect barley, Ramularia leaf spot (RLS), caused by the fungus *Ramularia collo-cygni*, has recently attracted significant attention from barley producers because if uncontrolled the fungus can have a significant impact upon both grain yield (yield penalty ranges from 10% to 70%; S Pereyra pers. comms) and quality (Huss et al., 1992; Oxley and Havis 2004; Oxley, 2007).

Previously considered an issue only in northern Europe and wetter parts of western Europe, RLS is now considered a significant threat to the barley production worldwide (Oxley and Havis, 2004). Since its formal identification in Italy over 100 years ago (Cavara 1893), RLS has received little attention from farmers, agronomists and researchers. This is primarily due to its similarity in appearance to other diseases, such as net blotch (*Pyrenophora teres*), and physiological leaf spotting, a common condition of barley caused by abiotic stress which also produces similar small necrotic spots (Sachs et al., 1998; Walters et al., 2008). Another reason *R. collo-cygni* has received little attention is that the fungus has, until recently, been effectively controlled with foliar fungicide applications. However, the ability of *R. collo-cygni* to evolve resistance to single site fungicides continues to concern the industry (Piotrowska et al., 2017). In a number of countries, effective control is now only achieved with multisite chemistry, which is unlikely to remain in the crop protection toolkit due to regulatory changes, particularly within Europe.

Little is known about the potential for integrated pest management (IPM) solutions to control RLS. There are currently no commercially available varieties displaying resistance to RLS though some may appear to be more tolerant of infection than others (S. Kildea and N. Havis pers. comms). In order for the effect of non-chemical control techniques to be determined we must better understand the biology and epidemiology of the fungus, which despite being able to infect many hosts, including ryegrass, oats and wheat, only produces symptoms on barley (Kaczmarek et al., 2016). Typical RLS symptoms (Fig. 1) display as a rectangular shape and a reddish-brown colour often surrounded by a chlorotic halo, and are visible on both sides of the leaf (Havis et al., 2018). A greater understanding of the interaction between the fungus and its barley host will likely be key in the development of future IPM solutions to RLS.

Subheading 1: Distribution of *R. collo-cygni*

Since the first report of *R. collo-cygni* in 1893 in Italy, the fungus has been detected in all the temperate regions worldwide and RLS outbreaks have been reported on all continents except Antarctica (Fig. 2; Spencer et al., 2019). The existence of a seed-borne stage in the lifecycle of *R. collo-cygni* (Matusinsky et al., 2011) is thought to be responsible for the sporadic distribution of the pathogen worldwide, as the trading of barley seeds across countries and even continents is common practice. Despite a few countries such as Norway or Chile having detected *R. collo-cygni* early in the last century, the majority of RLS outbreaks have been reported in the last 20 years, which can partly be explained by the difficulty to identify RLS symptoms and correctly diagnose the disease. The use of quantitative PCR to screen historic barley tissue samples from the mid-19th century revealed that *R. collo-cygni* DNA was present in the UK as early as 1854, suggesting that despite the first official detection of RLS in the UK in 1998, previous epidemics must have occurred but remained unnoticed or were misdiagnosed (Fountaine and Fraaije, 2009). As RLS is becoming more prevalent and more difficult to

control, and as diagnostic techniques such as molecular detection become affordable, there is little doubt that more countries will report RLS epidemics in the future.

Subheading 2: Ramularia leaf spot epidemiology

The classification of *R. collo-cygni* has been subject to debate for several decades, but a recent study of its genome has confirmed the classification of this ascomycete fungus within the class of the Dothideomycetes, in the Capnodiales order, in the Mycosphaerellaceae family (McGrann et al., 2016). Unlike other closely related fungi, such as *Zymoseptoria tritici* or *Dothistroma septosporum*, the agents responsible for Septoria tritici blotch of wheat and red band needle blight of pine trees, respectively (Kabir et al., 2015; Tiley et al., 2018), *R. collo-cygni* exhibits several characteristics of endophytic fungi. Several studies showed that *R. collo-cygni* is often present in barley seeds (Fig.3a) and is predominant in the lemma, pericarp and embryo (Matusinsky et al., 2011). Fungal DNA movement can be followed from seeds to seedling as the fungus develops asymptomatically within its host (Fig. 3b to e; Havis et al., 2014). Ramularia leaf spots may, under specific conditions such as waterlogging, be seen on dying leaves early in the growing season (Fig. 3c; McGrann and Havis 2017), but RLS symptoms generally appear post anthesis (Fig 3f; Walters et al., 2008). Late in the growing season RLS symptoms can be seen developing on all parts of the plant including stems, awns and grains (Fig. 3g; Sachs 2002). Secondary spore structures have been observed on straw and decaying tissue but their role as secondary sources of inoculum for seedlings is still unclear (Fig. 3h; Salamati and Reitan 2006).

The understanding of the life cycle of *R. collo-cygni* has improved in the past 15 years, however, the relative influence of the different stages in the fungal life cycle on disease epidemiology is still contentious. If the seed-borne stage has helped explain the movement of the fungus on a global scale, the movement of the fungus among and within crops is still being actively investigated. Many studies have now shown that *R. collo-cygni* DNA is present in many barley crops and in some countries such as in the UK, the fungus seems to be ubiquitous in barley seed further highlighting the importance of the seed-borne stage in disease epidemiology (Havis et al, 2015). The importance of air-borne spores in the biology of *R. collo-cygni* is still subject to debate, but this mode of transmission may play a role in the infection of upper leaf layers (Fig. 3d). Several studies on spore movement have given conflicting findings. Schützendübel et al. (2008) found a close relationship between spore movement and disease epidemics in trials in Germany. These findings were corroborated by Zamani-Noor (2011), who showed that spore release from winter crops contributed to disease levels in spring crops. Indeed, *R. collo-cygni* spores have been detected in countries where disease epidemics have been infrequent (Havis et al, 2015). However, there are also a number of studies demonstrating that *R. collo-cygni* moves from infected seed into developing plant tissue both in controlled environments and in the field, confirming the vertical transmission of the fungus (Nyman et al., 2009; Zamani-Noor et al., 2009). Havis et al. (2014) studied the growth of *R. collo-cygni* from infected seed in field conditions. The presence or absence of external inoculum was measured by the use of continuous spore trapping and quantitative PCR. *R. collo-cygni* DNA was tracked moving up the crop canopy in the absence of external spore movement indicating that epidemics in the UK develop from seed-borne infection and that late season spore movement does not influence disease

epidemics. *R. collo-cygni* can also infect many other plant species, including major crops such as wheat and oats as well as many species of weeds. A recent study suggested that wild grasses could act as sources of inoculum (Mäe et al., 2018), however, the role of these alternative hosts in RLS epidemiology remains to be fully established. Changes in environmental conditions are also known to play a major role in RLS epidemiology. Formayer et al. (2004) reported that a high humidity level was crucial for the outbreak of RLS epidemics, whereas light radiation intensity was of minor importance. A study by Makepeace et al. (2008) showed that light intensity plays an important role in mediating RLS severity. A later study by Marik et al. (2011) showed that strong symptomatic expression was positively affected by a higher number of rainy days in the three weeks post heading in the crop and also reported that higher temperatures and lower rainfall post flowering reduced disease levels in the Czech Republic. More recent work in the UK has highlighted a correlation between high rainfall and temperatures during the growing season and increased RLS levels in UK spring barley crops (Havis et al., 2018). Further highlighting the importance of environmental conditions on RLS development, McGrann and Brown (2018) showed that waterlogging plants resulted in increased RLS severity.

Subheading 3: Plant-fungus interactions

The asymptomatic growth of *R. collo-cygni* is marked by the colonisation of the intracellular spaces of the host by the fungus. During this phase of development, *R. collo-cygni* derives its energy from nutrients available in the apoplastic medium which is marked by an increase in the expression of fungal genes involved in sugar metabolism (Sjokvist et al., 2019). Concomitantly, the fungus also secretes effectors including Lysine motif (LysM)-containing effectors, to avoid recognition by the host which would lead to the host mounting an immune response against *R. collo-cygni* (Sjokvist et al., 2019). LysM-containing effectors are small secreted proteins that highly specifically bind chitin, a known pathogen-associated molecular pattern (PAMP), to prevent recognition of fungal chitin by the host receptors, therefore avoiding PAMP-triggered immunity (PTI) in the host (Godfrey and Rathjen, 2012). Proteins containing LysM domains have been identified in several fungal species closely related to *R. collo-cygni,* including the wheat pathogen *Z. tritici* as well as *Cladosporium fulvum,* the agent responsible of tomato leaf mould (Bolton et al., 2008; Lee et al., 2014). During the asymptomatic phase, the host responses include an increased expression of genes involved in lignification and cell wall reinforcement as well as a downregulation of the expression of photosynthesis-related genes (Sjokvist et al., 2019). However, at the physiological level no differences in photosynthesis efficiency were observed between infected barley plants and non-infected ones (C. Burrell unpublished data).

The appearance of RLS disease symptoms reflects the transition in lifestyle of the fungus as it changes from endophytic to necrotrophic growth. The reason for the change in lifestyle remains unclear but this transition has been linked with stresses in the plant such as rapid changes in conditions, high light intensity, waterlogging as well as flowering (Makepeace et al., 2008; McGrann and Brown, 2018; Schützendübel et al., 2008). Changes occurring in the plants during flowering, such as the degradation of the anti-oxidant system, are thought to act as a signal triggering necrotrophic growth of *R. collo-cygni* (Schützendübel et al., 2008). The host genotype is also known to mediate RLS severity as highlighted by the role of the *Mildew Locus O* (*MLO*) mutation in

symptom development. The wild type *MLO* gene acts as a negative regulator of plant defence therefore allowing biotrophic fungi such as *Blumeria graminis*, the agent responsible for powdery mildew, to infect the plant (Jørgensen and Mortensen, 1977). Plants carrying the loss of function mutation, *mlo,* show complete resistance to powdery mildew but increased susceptibility to other pathogens including *R. collo-cygni* (Makepeace et al., 2007; McGrann et al., 2014). Barley breeding programs have been extensively relying on the *mlo*-mediated resistance to control powdery mildew resulting in more than 70 % of barley varieties used in Europe carrying the *mlo* mutation (Dreiseitl, 2012). Although the mechanism responsible for symptom formation is still unclear, it was long hypothesised to be the result of the release of toxic secondary metabolites in the host by the fungus; this hypothesis was strengthened by the discovery of the rubellin toxins in infected barley leaves (Miethbauer et al., 2003). Rubellins are non-host specific toxins that induce cell death in a light- and concentration-dependent manner (Heiser et al., 2003). The exact mode of action of the toxin is currently unknown but an *in vitro* study showed the production of reactive oxygen species (ROS) by light-activated rubellins (Heiser et al., 2003), which is a common cause of cellular damage and disease symptoms. Furthermore, the ROS hydrogen peroxide (H_2O_2) was detected in leaves infected by *R. collo-cygni* (McGrann and Brown, 2018), supporting the link between RLS symptom development and the release of rubellins in the host. However, in a recent study of the secondary metabolism of *R. collo-cygni*, Dussart et al. (2018a) showed that the fungal genome contains several putative gene clusters involved in the biosynthesis of other secondary metabolites, including betaenones, a family of phytotoxins produced by *Phoma betae* known to induce chlorosis on beet leaves (Ichihara et al., 1983), suggesting that other metabolites may be involved in symptom development. Casting further doubts on the relationship between rubellins and symptom development, the expression of the putative core gene responsible for rubellin biosynthesis is highest prior to symptom development, and declines over time when symptoms appear (Dussart et al., 2018a). A preliminary study of the role of the rubellin D toxin produced by *R. collo-cygni* suggests that no direct correlation exists between rubellin sensitivity and RLS resistance, as high sensitivity to the toxin does not always result in high susceptibility to the disease (Dussart et al., 2018b). No causal link between RLS symptom development and rubellin release and action in the host has yet been found. Despite the presence of several secondary metabolite biosynthetic clusters encoded in the genome of *R. collo-cygni*, no other compounds have been identified to date. However, the observation of pink exudates, which are particularly evident when *R. collo-cygni* is grown *in vitro* in competition with other fungal pathogens of barley such as *Pyrenophora teres*, suggests that *R. collo-cygni* may produce other secondary metabolites that may play diverse roles in fungal growth and/or host colonisation (Fig. 4; Dussart et al., 2018a). Although transformants and mutants of *R. collo-cygni* have been generated previously using *Agrobacterium*-mediated transformation and UV mutagenesis (Thirugnanasambandam et al., 2011; Piotrowska et al., 2017), developing a method to target specific genes in the rubellin biosynthesis pathway will be required to assess the role of this metabolite in *R. collo-cygni* biology. Similarly, identifying other secondary metabolites and their respective role in the biology of *R. collo-cygni* may help better understand the interactions between *R. collo-cygni* and its host.

Subheading 4: Crop Disease Management

Ramularia leaf spot infection leads to a reduction in green leaf area resulting in grain yield losses. To combat these losses we must explore whether certain components of IPM, as defined by the European Commission (European Union 2009), offer a viable solution for the control of RLS. These principles include prevention and suppression, monitoring, decision-making, non-chemical methods, pesticide selection, reduced pesticide use and anti-resistance strategies. This section will address each eight of these principles.

At the time of writing there are no known sources of plant genetic resistance to RLS. A greater understanding of the molecular mechanisms responsible for triggering the fungus to become pathogenic may lead to the identification of breeding targets. Some varieties may show fewer symptoms than others in trials, however these results are rarely consistent due to the significant effect of the environment on disease symptom formation. Plant tolerance to RLS must also be further explored as trial data suggests that some varieties may be able to suppress the formation of necrotic lesions due to *R. collo-cygni,* despite having relatively high amounts of fungal DNA within the leaf and seed tissue (Mulhare et al. unpublished data). The ability of such varieties to cope with abiotic stress could maintain *R. collo-cygni* in the endophytic stage by suppressing the signal triggering necrotrophic growth. Selection for such varieties in RLS resistance breeding programmes may therefore have additional benefits in terms of tolerance to abiotic stresses.

Knowledge of the potential impact some agronomic practices may have on RLS is also lacking, although practices that reduce potentially infected crop residues may lead to a reduction in RLS in the following crop. Such practices include rotation of crops, rather than growing consecutive barley crops, and removal of infected crop residues (stubble, trash) from the soil surface after harvest by ploughing, both of which could effectively remove secondary spore structures (S. Kildea and N. Havis pers. comms.). The significance of the seed-borne phase for the dispersal of the fungus suggests that sourcing seed free from *R. collo-cygni* would be a viable solution to the issue. However, the global trade of barley seed and high frequency of *R. collo-cygni* infection detected in European seed stocks severely restricts this as a viable option (Havis et al., 2014; J. Mulhare et al. unpublished data). As in-field monitoring of the crop prior to disease symptoms being visible requires a molecular assay, efforts to establish a decision support system to predict risk from RLS have been attempted. However, these attempts have largely failed due to the predominant effect various combinations of environmental stresses have on disease development (Havis et al., 2018).

Research into integrated solutions to RLS control have recently been limited in part due to a lack of knowledge of the potential for non-chemical control and, until recently, high efficacy of many fungicide groups. Recent work has shown the potential for plant resistance elicitors to be used in conjunction with synthetic fungicides in order to achieve adequate RLS control (Walters et al., 2011; Havis et al. unpublished). These findings are particularly timely as in 2017 a shift affecting the sensitivity of the pathogen population to two of the major fungicide classes, the succinate dehydrogenase inhibitors (SDHIs) and the demethylation inhibitors (DMIs) in sterol biosynthesis was observed. As a result, these chemicals are now regarded as being totally ineffective against the fungus in the UK and Germany (N. Havis and M. Hess pers. comms.). The multisite chlorothalonil currently remains effective in these countries, however it will no longer be approved for use in Europe from 20th May 2020 as it is deemed to be of high risk to amphibians and fish (European Food Safety Authority 2018). This will leave barley growers without an

effective tool to manage RLS and due to the ability of the fungus to adapt and evolve resistance to fungicides it is unlikely that any new fungicide groups in the pipeline will provide a long term solution on their own.

Subheading 5: Concluding remarks

R. collo-cygni is often described as "an endophyte gone wrong" as it develops asymptomatically, colonising its host apoplast for most of the growing season before becoming necrotrophic and causing losses in grain yield and quality. RLS has now become an important threat to barley production in all the temperate regions of the world, and isolates of *R. collo-cygni* insensitive to several major fungicides have recently been identified in the fields in several European countries. Furthermore, the lack of resistant varieties renders the control of this disease even more difficult. Despite recent advances, the biology of this fungus and its interactions with barley remain poorly understood. Future areas of research include improving the understanding the interaction between the pathogen and its host, assessing the structure of the *R. collo-cygni* population globally, developing IPM solutions to RLS and identifying potential reasons why this pathogen has become an important threat to barley production in recent years.

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Glossary:

Effectors: Small proteins secreted by pathogens to help colonise their hosts.

Endophyte: A micro-organism that lives inside a plant without causing damage to its host.

Integrated Pest Management: Holistic approach to achieve economic- and environmental-friendly plant protection.

Biotrophic: Biotrophic pathogens keep their host alive and extract nutrient from live cells.

Necrotrophic: Necrotrophic pathogens induce host cell death and feed on dead cell content.

Secondary metabolite: Organic compounds produced by living organisms that are not involved in growth, reproduction or development but confer an advantage to the organisms under specific conditions.

Apoplast: The space outside the plasma membrane of a plant cell including the extracellular space and cell wall.

1 Figures and Tables:

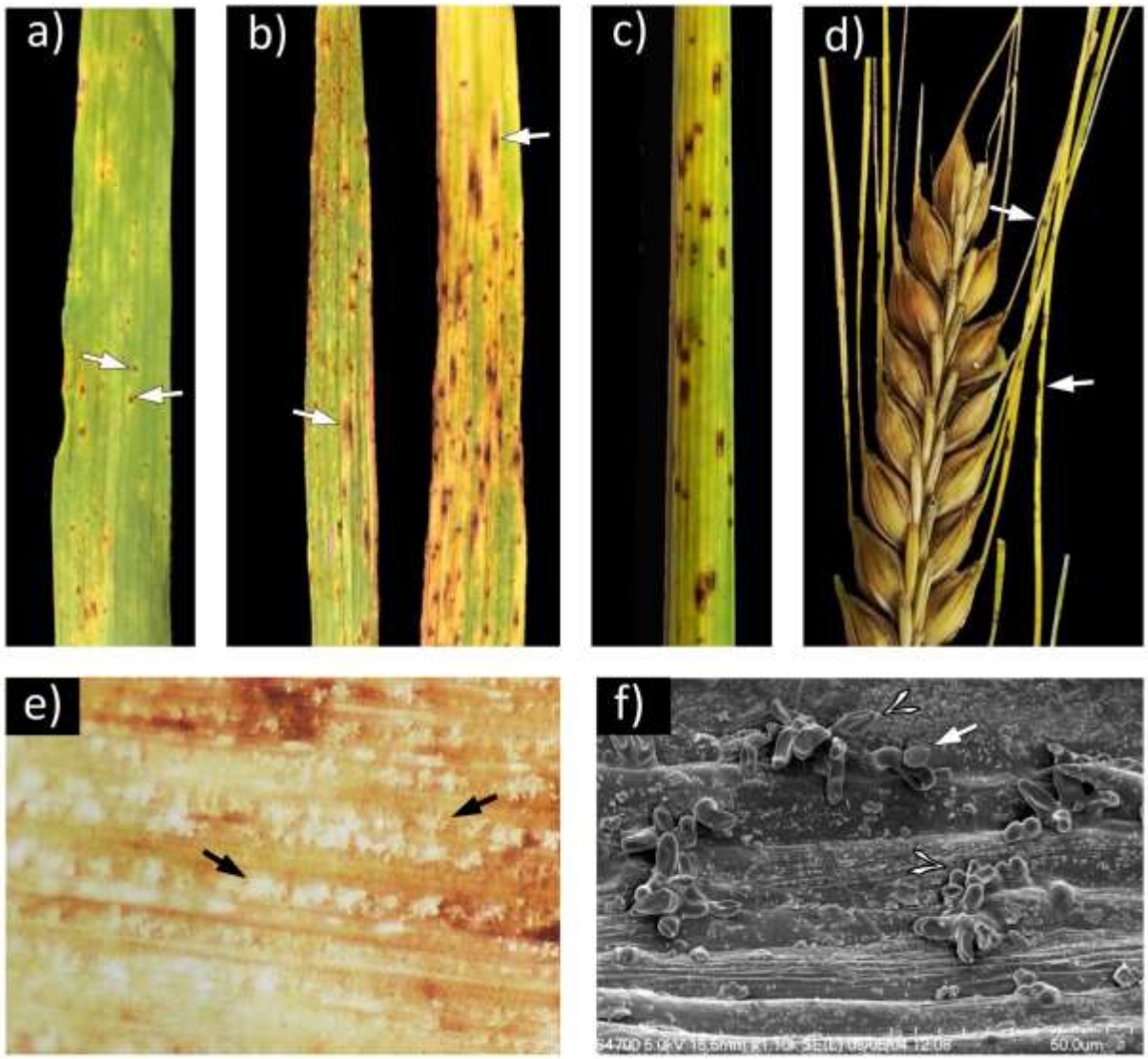

Figure 1: Ramularia leaf spot symptoms

(a) Ramularia leaf spot symptoms are first visible as "pepper spots" on barley leaves (white arrows). (b) The symptoms then turn into the typical rectangular, red-ish brown lesions surrounded by a chlorotic halo (white arrows). (c) As the infection develops the stem can become infected. (d) In severe cases, symptoms can also be seen on awns (white arrows). (e) Sporulation can be seen on the underside of the leaf forming a line of small white clusters (black arrows). (f) Conidiophores (white arrowhead) bearing single conidia (white arrow) emerge in cluster from the leaf.

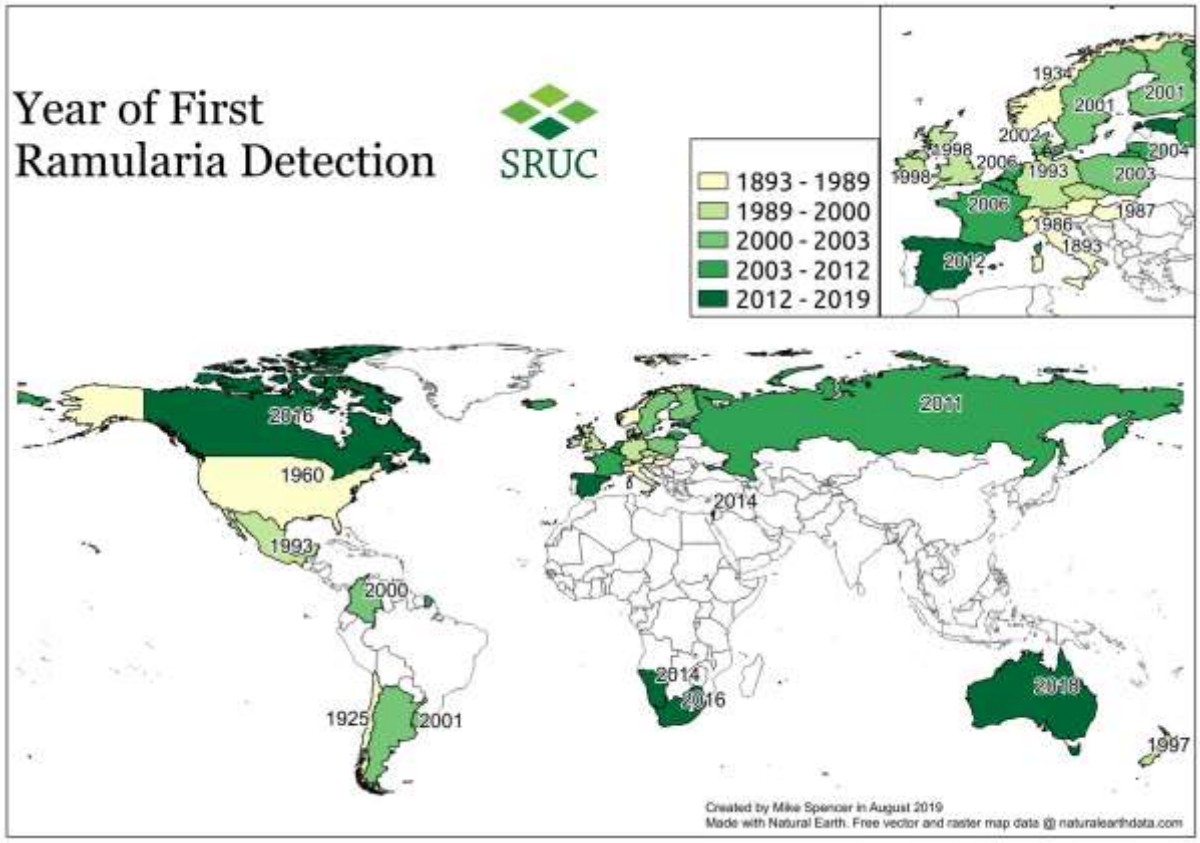

Figure 2: Distribution of *R. collo-cygni.*

The map indicates the global distribution of the fungus and the year of its first positive detection. Darker colours on the map indicate more recent reports of the fungus in crops.

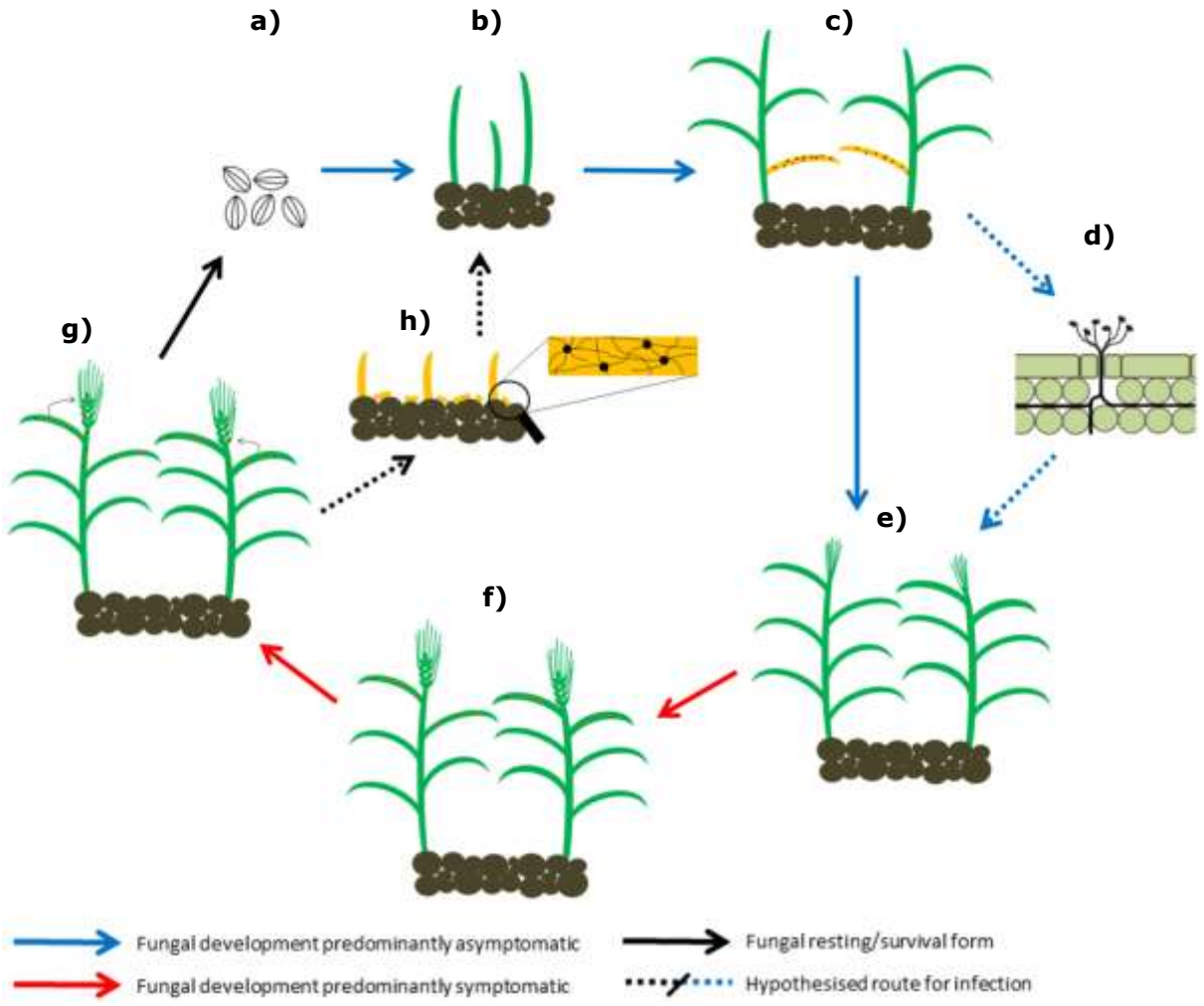

Figure 3: Life cycle of *R. collo-cygni*

R. collo-cygni DNA can be detected in the seeds (a), seedlings (b) and adult plants even in the absence of disease symptoms. RLS can be observed on senescing lower leaves (c) sometimes associated with sporulation (d) however, the role of air-borne spores in the infection remains elusive. *R. collo-cygni* colonisation is often symptomless (e) until flowering occurs, when typical RLS symptoms can be seen developing on the higher canopy (f) as well as awns and heads, therefore infecting new grains (g). Secondary spores developing on decaying straw may also act as a source of inoculum (h). Dotted lines show potential routes of infections via secondary sources of inoculum.

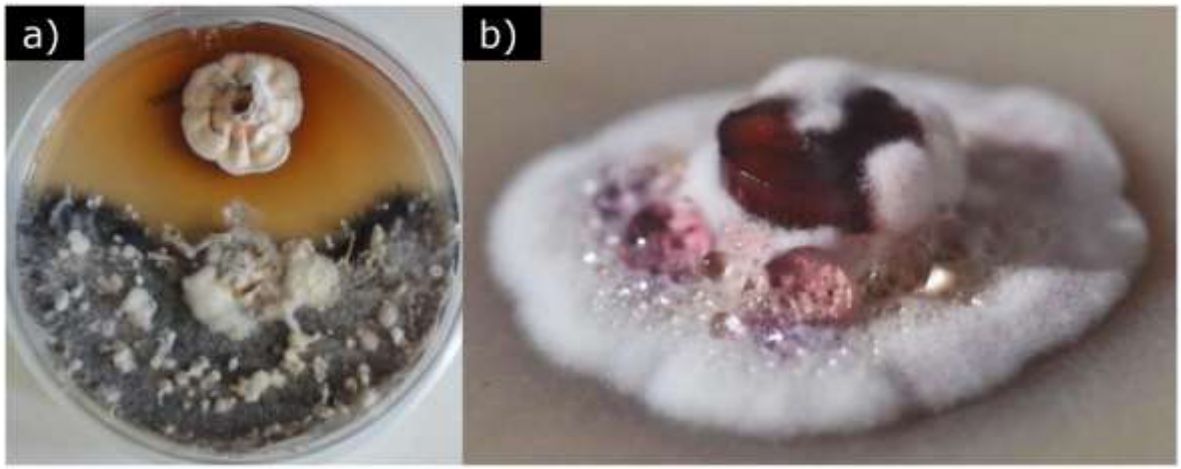

Figure 4: Red coloration observed on *Ramularia collo-cygni* cultures. (a) Red coloration of the medium surrounding the *R. collo-cygni* culture (top) after incubation in competition with *Pyrenophora teres* (bottom) for 28 days at 15°C in the dark on potato dextrose agar. (b) Exudates of a pink/red compound observed on cultures of *R. collo-cygni* grown for 14 days at 15°C in the dark on oatmeal agar.