

Ramularia collo-cygni: the biology of an emerging pathogen of barley

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Introduction

Ramularia collo-cygni is a pathogen of increasing importance on barley crops in Northern Europe (Sachs *et al.*, 1998; Pinnschmidt & Hovmøller, 2003; Oxley & Havis, 2004) and has also been reported recently on oats and wheat (Huss *et al.*, 2005). It is the cause of Ramularia leaf spot (RLS), a disease which occurs late in the season and is characterized by small necrotic lesions, usually with a yellow halo (Salamat & Reitan, 2006). Although RLS was first reported more than 100 years ago, with Cavara (1893) describing the pathogen associated with it as *Ophiocladium hordei*, it was not until the early 1980s that RLS began to attract serious scientific attention. However, it was often confused with necrotic spotting caused by abiotic factors and pathogens such as *Pyrenophora teres* (Sachs *et al.*, 1998). Nevertheless, RLS has been reported from crops across Europe, including Denmark (Pinnschmidt & Hovmøller, 2004), the Czech Republic (Minaříková *et al.*, 2004), Germany (Sachs, 2000), Austria (Huss *et al.*, 1987), Scotland (Oxley *et al.*, 2002), as well as New Zealand (Sheridan, 2000; Harvey, 2002). The rapid leaf senescence induced by RLS results in premature loss of green leaf area in crops and can lead to substantial yield losses (Huss *et al.*, 1992; Pinnschmidt & Hovmøller,

Abstract

Ramularia collo-cygni is now recognized as an important pathogen of barley in Northern Europe and New Zealand. It induces necrotic spotting and premature leaf senescence, leading to loss of green leaf area in crops, and can result in substantial yield losses. The fungus produces a number of anthraquinone toxins called rubellins, which act as host nonspecific toxins with photodynamic activity. These toxins induce lipid peroxidation and are possibly the cause of the chlorosis and necrosis observed in leaves infected with *R. collo-cygni*. The fact that the fungus can remain latent in barley plants until flowering, coupled with its very slow growth *in vitro*, makes it difficult to detect in crops. As a result, the epidemiology of this pathogen remains poorly understood. However, the recent development of rapid and reliable PCR methods for specific detection of *R. collo-cygni* offers the prospect of increased understanding of its epidemiology and improved disease control.

2004). For example, in the UK in 2004, average yield losses were 0.4 tonnes ha⁻¹ and these were frequently associated with a loss in grain quality (Oxley, 2007).

The fact that *R. collo-cygni* has been recognized as a major pathogen of barley only relatively recently is due in large part to difficulties in isolating and identifying the fungus (Sutton & Waller, 1988; Frei & Gindrat, 2000; Sachs, 2004). However, the recent development of molecular probes for detection of *R. collo-cygni* (Havis *et al.*, 2006a; Frei *et al.*, 2007) is likely to have a significant impact on studies on the epidemiology and population biology of this pathogen. This review will examine the taxonomy, biology and life cycle of *R. collo-cygni* and look at what is known about the mechanisms by which it causes leaf spotting and premature senescence in leaves.

Taxonomy

Ramularia collo-cygni was not considered to be a 'typical' *Ramularia* species because of its curled conidiophores (like a swan's neck; Fig. 1) and conidia with eccentrically positioned scars (Braun, 1998; Crous *et al.*, 2000). Initially, therefore, it was considered distinct from *Ramularia* and thought to typify the genus *Ophiocladium* Cavara (Braun,

1998). The fungus was described as *Ophiocladium hordei*, but was transferred to the genus *Ramularia* by Sutton & Waller (1988) as *R. collo-cygni*. Its taxonomy and nomenclature can be summarized as follows:

Ramularia collo-cygni B. Sutton and J.M. Waller, *Trans Brit Mycol Soc* **90** (1): 57 (1988).

≡ *Ophiocladium hordei* Cavara, 1893.

≡ *Ovularia hordei* (Cavara) Sprague, 1946.

≡ *Ramularia hordeicola* Braun, 1988.

In 2000, Crous *et al.* reported the results of phylogenetic analyses carried out on ITS-1, ITS-2 and 5.8S DNA sequence data from 46 species of *Mycosphaerella*, including three species with *Ramularia* anamorphs. This work showed that not only does *R. collo-cygni* cluster with other typical *Ramularia* species, but the teleomorph of *R. collo-cygni*, if it exists, is likely to be a species of *Mycosphaerella* (Crous *et al.*, 2000). Interestingly, Braun (cited in Salamati & Reitan, 2006) reported the existence of *Asteromella*-like structures on barley leaves in close association with *R. collo-cygni* and suggested that these structures might represent the microsporidial state of the fungus. More recently, Salamati & Reitan (2006) obtained the *Asteromella* stage of *R. collo-cygni* from *in vitro* cultures of the fungus. These observations support the work of Crous *et al.* (2000) and the suggestions of Braun (2004) that the teleomorph stage of *R. collo-cygni* is likely to be a species of *Mycosphaerella*.

Life cycle of *R. collo-cygni*

Conidia of *R. collo-cygni*, in common with spores of most fungal pathogens of plants, require moisture for germination and early development on the leaf surface. Indeed, Huss (2004) found that dew on leaf surfaces was sufficient to trigger conidial germination and subsequent development. On leaves of a suitable host, germination and mycelial growth is rapid, with entry into the leaf through open stomata within 24 h (Sutton & Waller, 1988). Once inside the leaf, the fungus grows intercellularly, forming branched hyphae which colonize the mesophyll tissue (Sutton & Waller, 1988). Under controlled conditions, the first symptoms of RLS appear after 7 days (Huss & Sachs, 1998): brown to blackish brown spots, 1–2 mm long and usually sharply delineated by leaf veins (Fig. 2). Neighbouring leaf spots can coalesce to form larger dark areas. Once leaf spots appear, the remainder of the leaf becomes chlorotic and then necrotic, usually starting at the leaf tip and margins (Huss, 2004). These are typical symptoms of RLS and appear on spring and winter barley under field conditions, usually after heading. Fungal sporulation only occurs in necrotic tissue, with conidiophores emerging through stomata in caespituli of up to 15, from underlying stomata. As indicated above, the terminal part of each conidiophore is strongly curved (Fig. 1) (Sutton & Waller, 1988), with the apical region

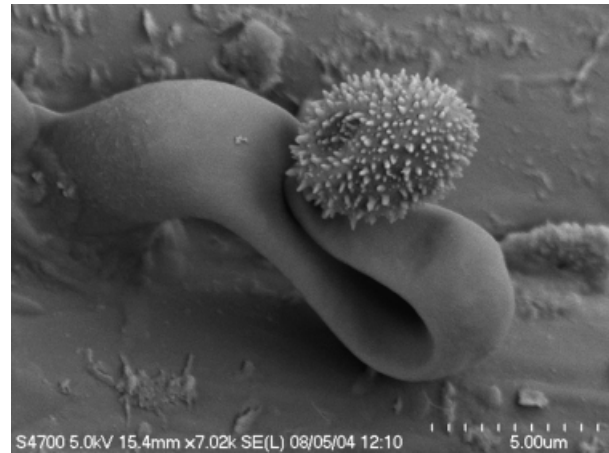


Fig. 1. Photomicrograph of the curled conidiophore of *Ramularia collo-cygni*. Note that it resembles a swan's neck or shepherd's crook.

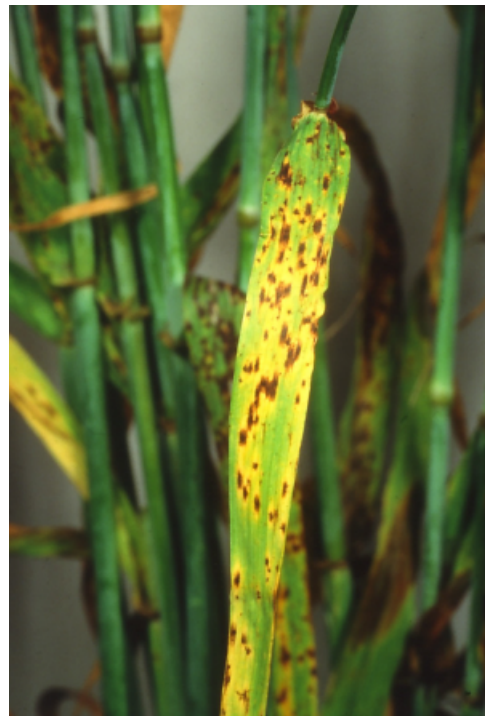


Fig. 2. Typical symptoms of *Ramularia* leaf spot (RLS) on barley leaves.

producing up to five conidia. *Ramularia 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.

There is considerable debate about the aetiology of the disease. Nevertheless, although RLS symptoms can start appearing in barley under field conditions early in the growing season (e.g. Harvey, 2002; Fig. 3), *R. collo-cygni* is known as a late season pathogen of barley, with typical RLS

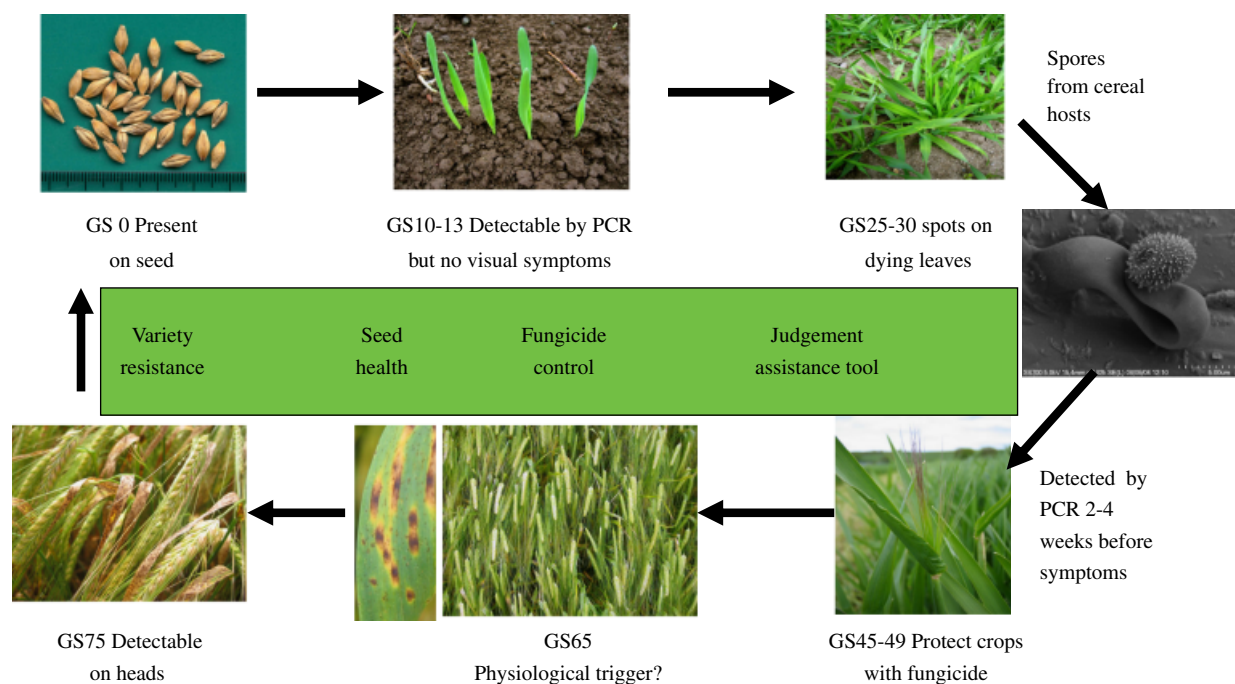


Fig. 3. Proposed life cycle of *Ramularia collo-cygni*. Inoculum sources include contaminated/infected seed and spores from other graminaceous hosts. Although symptoms of infection can be observed on leaves of young plants, the crop often appears symptomless until heading, when typical symptoms appear and the fungus sporulates profusely. The fungus can be detected on heads of barley, giving rise to seed contamination/infection. Understanding the life cycle fully is crucial for developing effective control measures. GS0 to GS75 represent stages on the Zadoks growth guide for barley.

symptoms appearing on foliage after emergence of the ear (Sachs *et al.*, 1998; Pinnschmidt & Hovmøller, 2003; Oxley & Havis, 2004; Fig. 3). During stem extension in barley, *R. collo-cygni* can survive as a saprophyte on the dead lower leaves (Huss, 2004). However, there is also evidence that it grows systemically and asymptotically in the plant (Havis *et al.*, 2004; Salamati & Reitan, 2006), a conclusion confirmed by PCR-based detection methods (Havis *et al.*, 2006a,b). In these circumstances, symptom development and fungal sporulation appear to be triggered by the transition of the plant from vegetative to reproductive growth (Salamati & Reitan, 2006; Fig. 3). Whether environmental conditions have a role to play in the appearance of symptoms and fungal sporulation late in the season is not known.

It has been suggested that the symptoms on barley leaves induced by *R. collo-cygni* are dependent on light intensity (E. Sachs, unpublished results, quoted in Heiser *et al.*, 2003). Interestingly, recent work by Makepeace (2006) has shown that light intensity prior to inoculation exerts a significant effect on RLS. Plants grown under low light conditions before inoculation exhibited fewer RLS symptoms than did those grown under high light. However, increasing light intensity after plants were inoculated led to fewer RLS symptoms, suggesting that although light is required for the toxins produced by *R. collo-cygni* (rubellins; see below)

to exert their effects, excessively high light intensities might have a negative effect on pathogenicity (Makepeace, 2006).

Ramularia collo-cygni may also be transmitted via seed (Fig. 3). Indeed, there is anecdotal evidence for a seed-borne phase for this pathogen (H. Huss, Versuchsstation Lambach-Stadl-Paura, Bundesamt für Agrarbiologie, Austria, pers. comm.). Support for seed transmission comes from recent work using a PCR-based diagnostic method developed for *R. collo-cygni*. Using this diagnostic tool, the pathogen was detected in barley grain samples harvested in 1999 and 2004 in Scotland (Havis *et al.*, 2006a,b). When seed carrying the pathogen was germinated, microscopic examination of seedling leaves revealed the presence of *R. collo-cygni* within leaves, although it was not present on the leaf surface (Havis *et al.*, 2006a; Havis *et al.*, unpublished results). As highlighted by Havis *et al.* (2006a), the existence of a seed-borne stage for *R. collo-cygni* will have serious implications for barley cultivation, as the fungus could be present in the majority of crops prior to symptom expression. However, as important as seed-borne transmission would be to barley crops, it is important to remember that there are a number of other possible sources of inoculum for this pathogen, including other graminaceous hosts such as oat, wheat, rye and couch grass (*Elymus repens*) (Huss, 2004; Salamati & Reitan, 2006) (Fig. 4). Recent work by Frei *et al.* (2007) has shown that winter barley is an important source

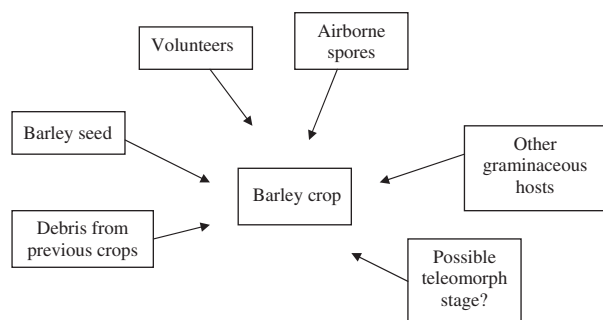


Fig. 4. Sources of inoculum of *Ramularia collo-cygni*.

of inoculum for spring barley and that the pathogen can spread from the winter to the spring crop.

It is important to note that the symptoms of RLS can easily be confused with those caused by abiotic stresses (physiological leaf spots, PLS; Wu & von Tiedemann, 2002) and by pathogens such as *Pyrenophora teres* (Sachs *et al.*, 1998). In fact, some authors have observed that *R. collo-cygni* was never the single cause of necrotic leaf spotting in experiments carried out under field conditions in Switzerland (Frei *et al.*, 2007). In this work, most necrotic leaf spotting was attributable to PLS. Reports such as these highlight the need for reliable means of detecting the fungus in plant material exhibiting symptoms of necrotic leaf spotting.

Toxin production by *R. collo-cygni*

In their work on *R. collo-cygni*, Sutton & Waller (1988) suggested that the staining reaction of mesophyll cells in the proximity of fungal hyphae might be due to production of a toxin. In fact, mycelium of *R. collo-cygni* growing on agar is coloured, the colour changing depending on the medium on which it is grown (Sutton & Waller, 1988; Salamati & Reitan, 2006). The purple colour of the mycelium led Heiser *et al.* (2003) to speculate that *R. collo-cygni* produces photoactive polycyclic aromatic toxins similar to cercosporin, produced by several species of *Cercospora*. They showed that *R. collo-cygni* produces a number of coloured metabolites, including an anthraquinoid identified as rubellin D (Fig. 5). 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 to lead to the peroxidation of α -linolenic acid (Heiser *et al.*, 2003). Subsequent work demonstrated that rubellin D also induced the peroxidation of α -linolenic acid in barley and tobacco leaves, suggesting that it is a host nonspecific toxin (Heiser *et al.*, 2004). In fact, these workers showed that, in addition to rubellin D, *R. collo-cygni* also produces rubellins A, B, C and E (Fig. 5; Miethbauer *et al.*,

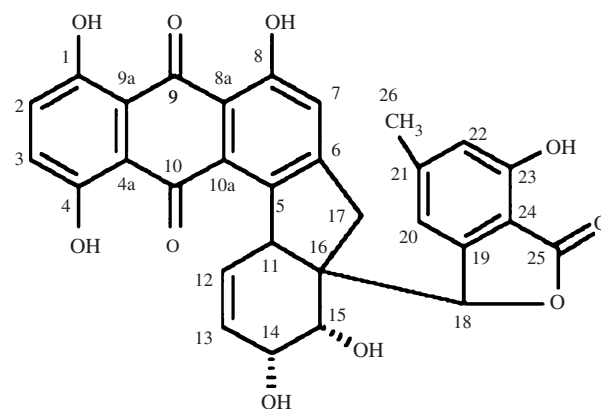


Fig. 5. Chemical structure of rubellin D.

2003; Heiser *et al.*, 2004; Miethbauer *et al.*, 2006). In terms of relative ability to induce peroxidation of α -linolenic acid, rubellin A exhibited greatest activity, whereas rubellin E was ineffective (Miethbauer *et al.*, 2006). It seems likely that rubellin B, which is not soluble *in planta*, is produced first and is then converted to the more polar rubellin D, which possesses greater solubility *in planta* (Heiser *et al.*, 2004). Apparently, most of the rubellin in infected plant tissue is rubellin B (Miethbauer *et al.*, 2003).

The possibility that the rubellins might be involved in infection of host tissue by *R. collo-cygni* was ruled out by Miethbauer *et al.* (2003), as they were demonstrated to be host nonspecific toxins. Nevertheless, Heiser *et al.* (2004) proposed that they might be considered as pathogenicity factors. They suggest that rubellin production by the fungus results in oxidative stress, which in turn is involved in the formation of leaf spots. The fungus is then able to colonize the necrotic host tissue, while continued production of rubellins accelerates leaf necrosis. The early loss of green leaf area, coupled with reduced rates of photosynthesis, is likely to result in the premature ripening observed under field conditions (Heiser *et al.*, 2004).

Host resistance to *R. collo-cygni*

As indicated above, yield losses associated with infection of barley crops by *R. collo-cygni* can be substantial (Huss *et al.*, 1992; Pinnschmidt & Hovmøller, 2004). In fact, Harvey (2002) found that the correlation between disease severity and grain yield was non-linear, with low levels of infection exerting a major influence on grain yield. The disproportionate effect of infection on yield increases the importance of good disease control. Most barley varieties appear to be susceptible to the pathogen, although there is moderate resistance to *R. collo-cygni* in some varieties of both spring and winter barley (Pinnschmidt *et al.*, 2006). Some authors have reported that barley varieties carrying the *mlo* gene

conferring resistance to powdery mildew are the most susceptible to infection by *R. collo-cygni* (e.g. Reitan & Salamati, 2006). However, other work suggests that the *mlo* gene, in itself, does not increase susceptibility to *R. collo-cygni* and that the results obtained by others, e.g. Reitan & Salamati (2006), probably reflect differences in the genetic background of different varieties (Makepeace, 2006; Makepeace *et al.*, 2007). Importantly, Makepeace (2006) found that varieties most resistant to *R. collo-cygni* under controlled conditions following seedling inoculation were most susceptible as adult plants under field conditions. These data suggest that there are different genes for resistance to *R. collo-cygni* at the seedling and adult plant stages or that some resistance genes are only expressed under specific field conditions (Makepeace, 2006). The detection of possible sources of resistance to *R. collo-cygni* in screening tests carried out under field conditions (Bistrich *et al.*, 2006; Pinnschmidt *et al.*, 2006) bodes well for the breeding of resistant varieties, although, clearly, this process is in its infancy. Currently, therefore, control of this pathogen is dependent on fungicides (Oxley *et al.*, 2006).

Conclusions

Over the past 15 years, *R. collo-cygni* has become recognized as an important pathogen of barley. The reasons for this change in status are not known. However, it has been suggested that the introduction of barley varieties with increased susceptibility to abiotic stresses, coupled with decreased competition from other foliar pathogens as a result of improved control, are possible reasons for the increasing importance of *R. collo-cygni* (Salamati & Reitan, 2006). Understanding the causes of this relatively sudden appearance of *R. collo-cygni* as a major pathogen will be important in attempts to devise effective control measures. Further, it is clear from this brief review that the epidemiology of this pathogen is poorly understood. This is not surprising considering the slow growing nature of the fungus *in vitro* and the fact that it can remain undetected in plants until the reproductive phase starts. However, the recent development of rapid and reliable methods for detection of *R. collo-cygni* using PCR will make it easier to detect the pathogen in barley crops and should lead to increased information on its epidemiology. This is crucial for the development of robust systems for disease forecasting and improved disease control.

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