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Kaczmarek, M; Piotrowska, MJ; Fountaine, JM; Gorniak, K; McGrann, GRD; Armstrong, A; Wright, KM; Newton, AC; Havis, ND

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Infection strategy of *Ramularia collo-cygni* and development of Ramularia leaf spot on barley and alternative graminaceous hosts

M. Kaczmarek[a,b], M.J. Piotrowska[a], J.M. Fountaine[a, c], K. Gorniak[a], G.R.D. McGrann[a], A. Armstrong [d] , K.M. Wright [e] , A.C. Newton[e] and N.D. Havis[a*]

[a] Crop and Soil Research Department, Scotland's Rural College (SRUC), King's Buildings, Edinburgh EH9 3JG, UK

[b] Present address: Centre for Ecosystems, Society and Biosecurity,Forest Research,Alice Holt Lodge,Farnham, GU10 4LH

[c] Present address: Syngenta, Jealott's Hill International Research Centre, Bracknell, RG42 6EY, UK

[d] Nothern Research Station (Forest Research), Bush Estate, Roslin, EH25 9SY

[e] Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

[*]Corresponding Author (e-mail): neil.havis@sruc.ac.uk

[Abstract]

Ramularia leaf spot (RLS) is a newly important disease of barley across temperate regions worldwide. Despite this recent change in importance the infection biology of the causal agent, *Ramularia collo-cygni* (*Rcc*) remains poorly understood. Confocal microscopy of the infection process of two transgenic *Rcc* isolates, expressing either GFP or dsRed reporter markers, was combined with light microscopy during field infection to track the progression of *R. collo-cygni in planta*. Infection of stomata, including the development of a previously unreported stomatopodium structure, results in symptomless development and intercellular colonisation of the mesophyll tissue. Transition to necrotrophy is associated with breakdown of host chloroplast and the formation of aggregates of conidiophores. In addition to barley, *Rcc* forms a compatible interaction with winter wheat and a number of perennial grass species. An incompatible reaction was observed with two dicotyledonous species. These

results provide further insights into the host interactions of this fungus and suggest that RLS could be a potential threat to other agriculturally important crops.

Introduction

Infection by *Ramularia collo-cygni* (*Rcc)* (Sutton & Waller, 1988) can result in Ramularia leaf spot disease on barley (RLS) leading to loss of green leaf area in infected plants (Havis *et al*., 2015; Walters *et al*., 2008). RLS can lead to yield losses of up to 20 per cent in barley, with an average loss in Scotland at 0.4 tonnes per hectare (Oxley & Havis., 2004). The development of PCR-based methods for detection of the fungus in barley tissue have expanded our understanding of pathogen's life cycle particularly the importance of seed-borne infection in disease etiology (Havis *et al*., 2014; Havis *et al.,* 2006a; Frei *et al.,* 2007; Taylor *et al.,* 2009). Recent evidence has suggested that *Rcc* is likely to undergo sexual reproduction (Piotrowska et al., 2016) however, there are many unknowns still surrounding the infection process and biology of this organism. Studies using scanning electron microscopy of naturally infected leaves (Stabentheiner *et al.,* 2009) and fluorescently labelled transgenic *Rcc* isolates (Thirugnanasambandam *et al.,* 2011) have provided valuable insights to the infection process of *Rcc*. The development of GFP- and dsRED tagged *Rcc* isolates in particular has great potential to further characterise the biology of this disease through non-invasive *in planta* live-cell imaging techniques. Using tagged fungal isolates in-depth spatio-temporal analysis of the infection cycle beginning with conidia germinating on the leaf surface under moist conditions can be performed. The fungus enters through open stomata within 24 hours after spore germination on the leaf surface (Sutton & Waller, 1988; Walters *et al.,* 2008) as observed in the related plant pathogen *Zymoseptoria tritici* (Goodwin *et al.,* 2011). Although the apparently directional growth of young *Rcc* hyphae towards stomata has been observed *in planta*, it remains unclear how the pathogen detects the presence of stomatal pores (Stabentheiner *et al.*, 2009). Following stomatal penetration, *Rcc* establishes an

epiphytic hyphal network (Thirugnanasambandam *et al.*, 2011) typically extending above the infection site interconnecting colonised stomata on the leaf surfaces. This initial development of *Rcc* is asymptomatic and the fungus can complete its life cycle without producing any symptoms during the entire barley growing season (Nyman *et al.*, 2009) reminiscent of an endophytic lifestyle rather than necrotrophy.

The process that triggers the transition of *Rcc* from asymptomatic to the symptom causing phase remains poorly understood. Host genetic factors (McGrann *et al*., 2014; 2015a; 2015b) and environmental stimuli (Brown & Makepeace, 2009; Makepeace *et al*., 2008; Peraldi *et al*., 2014) appear to play important roles in the expression of RLS. The appearance of RLS symptoms is typically observed on plants late in the growing season, usually after the ear emergence (Schützendübel *et al*., 2008; Walters *et al.*, 2008). Once the necrotic lesions appear, the remainder of the leaf becomes chlorotic and then necrotic, usually starting from the tip and leaf margins (Huss, 2004). These small, pale to medium brown pepper spots are usually surrounded by a yellow halo (Salamati & Reitan, 2006). The numerous local infections of the leaf tissue that usually occur during mass sporulation can often coalesce to form larger necrotic areas. Periods of high leaf surface wetness are a key environmental factor that induces the rapid sporulation of the pathogen (Sutton & Waller, 1988; Huss, 2004; Havis *et al*., 2012). Detailed descriptions of *Rcc* colonisation during the transition to disease have not been described. Although Sutton and Waller (1988) first suggested that once inside the leaf, *Rcc* grows intercellularly, forming branched hyphae which colonise the mesophyll tissue, no evidence was presented to support this statement. Stabentheiner *et al.* (2009) showed the presence of fungal hyphae in the mesophyll layer of naturally infected samples from the field. However, it was not confirmed that these hyphae were specifically from *Rcc*. As such the biological events resulting in the change from endophytic to necrotrophic growth remains undetermined. Besides barley, *Rcc* has been isolated from other

cereal crops including wheat, oat, rye and maize (Huss, 2004). RLS symptoms may appear regularly on rye whereas on wheat they developed only under favourable conditions. Huss *et al.* (2004) also noted that infection of maize was mainly asymptomatic although certain cultivars may develop characteristic disease symptoms. Wild grass species such as such as common couch grass (*Elymus repens*), annual wild barley (*Hordeum murinum*), annual grass *Echinochloa crus-galli* (Huss, 2004) and silky bent-grass, *Apera spica-venti* (Frei, 2004) have also been suggested as potentially important sources of inoculum during later crop development. However, recent evidence has suggested the primary source of infection in barley crops is infected seed (Havis *et al*., 2014). In New Zealand, *Rcc* has also been recorded on several grass species such as *Agrostis* spp., *Bromus cartharticus* and *Glyceria fluitans* (Cromey *et al.*, 2004). These data combined with the recent demonstration that *Rcc* can infect and cause RLS disease on the model grass species, *Brachypodium distachyon* (Peraldi *et al.* 2014) suggests a potentially broad host range for this pathogen.

The aim of this study was to characterise the foliar infection biology of *Rcc* on barley and other potential host- and non-host plant species through live-tissue imaging of fluorescent tagged *Rcc* isolates. Improved understanding of *Rcc* development during host- and non-host interactions will provide insights into the host range of *Rcc* and offer new perspectives on the potential evolution of the fungus and any associated host specialisation.

Materials and methods

Fungal isolates and inoculum preparation

Two *Rcc* field isolates collected from naturally infected leaves of the spring barley cv. Braemar and two transgenic isolates were used in this study. The field isolates originated fromScotland, isolate B1, and Denmark, isolate DK05Rcc001. Transgenic *Rcc* isolates 8B9 (Rcc-8B9-GFP) and Stratego (Rcc-ST-DsRed) expressing GFP and DsRed fluorescent proteins, respectively, have been previously described (Thirugnanasambandam *et al.*, 2011). Fungal cultures were maintained on clarified V8 juice agar (10 mM $CaCO_3$ in 20 % (v/v) V8 juice, 1.5 % agar) at 15^{o} C in the dark. Inoculum was prepared from mycelial fragments of Rcc isolates from two-week old spread-plates by scraping the colony surface with a sterile spatula, and then filtering through sterile glass wool in the neck of a sterile glass funnel. The mycelium harvested from a single spread plate was diluted in 5 mL sterile distilled water prior to inoculation.

Plant material

Barley seeds (*Hordeum vulgare*) cvs. Optic, Belgravia, Garner and Cocktail were germinated in pots and maintained in a glasshouse under16 h light at 18^{o}C and 8 h dark at 16^{o}C day/night regime. RLS resistance ratings are available for Optic, Belgravia and Garner (http://cereals.ahdb.org.uk/varieties/ahdb-recommended-lists/spring-barley-2015-16.aspx). Belgravia has the highest resistance rating (7) whereas both Optic (5) and Garner (4) are more susceptible. There is no official rating available for Cocktail although in Scottish trials this cultivar was as susceptible as Optic (Oxley & Havis, 2009).

In the early infection comparative study between barley and possible alternative hosts, naked barley (*Hordeum vulgare var nudum*), winter wheat (*Triticum aestivum*), cv Alchemy, oats (*Avena fatua*), the perennial grasses, cocks-foot (*Dactylis glomerata*), Italian ryegrass (*Lolium multiflorum*), black grass (*Alopecurus myosuroides*) were germinated and grown under the same conditions as described for barley.

Detached leaf assay

Seeds of barley (*Hordeum vulgare*) cv. Optic were germinated and maintained in a glasshouse under 16 h light at 20°C and 8 h dark at 16°C until plants reached the boots swollen stage (GSZ 45-49; Zadocks *et al.*, 1971). Detached-leaf assays were performed as described in Thirugnanasambandam *et al.* (2011) and Newton *et al.* (2001) with some modifications. Briefly, leaf sections approximately 3-5 cm in length were taken from the second and fifth – sixth leaf, gently abraded near the centre of the adaxial surface with a soft paintbrush to disrupt the surface wax structure, and placed abaxial surface down on 0.5 % distilled water agar containing 150 mg L^{-1} benzimidazole (Sigma-Aldrich, UK) in sealed polystyrene boxes (79 x 47 x 22 mm; Stewart Plastics Ltd, Surrey, UK). The abraded area of each leaf was inoculated with 10 µL of the *Rcc* mycelial fragment suspension and the boxes incubated in a controlled environment cabinet (Model LT1201, Leec Ltd, Nottingham, UK) at 17°C, light intensity 200 $\mu molm^{-2}s^{-1}$.

Whole plant inoculation assay

Spot-inoculation of whole barley leaves was performed as described for detached leaf assays (Thirugnanasambandam *et al.*, 2011; Newton *et al.*, 2001). Up to ten inoculation sites per leaf were drop-inoculated with 10 µL of mycelial fragments. For second leaves, inoculum was placed in the central region of a leaf blade whilst later leaves e.g. F-1 and flag, were inoculated on opposite sides of a midrib. Leaf segments 2 - 3cm long with the inoculation zone in the centre were then mounted and analysed microscopically on subsequent days throughout the life span of each infected barley plant. At least five inoculated leaves were studied for each time point. The experiment was repeated three times.

Confocal laser scanning microscopy (CLSM) conditions

Plant material inoculated with transgenic *Rcc* isolates was examined using, a Leica SP2 CLSM (Leica Ltd, London, UK) on a DM6000 microscope fitted with a FI/RH filter block (excitation filter BP 490/15, dichroic mirror 500, emission filter BP 525/20; excitation filter BP 560/25, dichroic mirror 580, emission filter BP 605/30) and Leica water-dipping lenses (HCX APO L10x /0.30 W U-V-1, L20x /0.50 W U-V-1, L40x /0.80 W U-V-1 or L63x / 0.90 W U-V-1). GFP fluorescence was imaged at the excitation wavelength of 488 nm and emission was collected at 500–530 nm.

Plant cell wall autofluorescence signal was detected by sequential imaging using HeNe laser for GFP detection as described above, and a lime laser at the excitation wavelength of 541 nm and peak emission was collected at 550-580 nm light wavelengths that were emitted by plant cell walls.

The autofluorescence signal from chlorophyll was collected simultaneously at light wavelengths between 650 and 700 nm. Transmission images were captured using the microscope transmission detector of the microscopes to collect 488-nm light passing through the leaf. Unless otherwise stated, images are overlay projections of z-stacks presented as maximum intensity projections and were assembled and edited using image editing software MacBiophotonics® ImageJ or Adobe Photoshop® CS5 Extended Edition.

Light microscopy conditions

Light microscopy was performed either using a Reichert-Jung Polyvar Photomicroscope (Reichert Technologies, Ney York, USA) with brightfield or differential interference contrast (DIC) optics, and 40x (1.0 NA) plan apochromat objective, or using a Nikon Eclipse TE2000 inverted microscope with DIC optics and a 40x (1.0 NA) plan fluor objective (Nikon Corporation, Tokyo, Japan). Images from the Polyvar microscope were acquired by Canon

EOS 600d SLR camera whilst images from the Eclipse microscope were captured with a DXM1200F camera and ACT-1 software.

Aniline blue staining

Leaf material from field samples exhibiting typical RLS symptoms were cleared and fixed with 1:1 v/v solution of glacial acetic acid and absolute ethanol until chlorophyll was completely removed. Fixed leaf samples were submerged twice for 30 minutes in sterile distilled water to remove excess acetic acid/ ethanol solution, and subsequently dehydrated with a series of increasing concentration of ethanol (25, 50, 75, 85, 95 and 100 %). Samples were stained with aniline blue stain (aniline blue/ ethanol 1:1 v/v) for 15, 30 and 60 minutes. To remove excess of aniline blue, leaves were briefly destained with absolute ethanol prior to mounting on a microscope slide.

***R. collo-cygni* detection in seeds**

Rcc levels were monitored in barley seeds used in this study by quantitative PCR (qPCR analysis (Taylor *et al.,* 2009). Genomic DNA was extracted from 100 seeds by milling samples in a mixer mill Retsch MM200 in to a fine powder. DNA was extracted from 1 g of finely ground material using the method of Fraaije *et al.* (1999). All batches of barley seeds used in inoculation experiments were confirmed free of *Rcc* DNA. Seed samples from the winter wheat trials in 2009 and 2010 were tested for the presence of *Rcc* DNA. DNA was extracted as for barley except a 200 seed sample was used for milling. *Rcc* DNA was detected and quantified using qPCR as previously described (Taylor *et al*., 2010).

Results

Symptomless infection characteristics

During asymptomatic development, infection was clearly restricted to the leaf surface and substomatal cavities. A thin spider web-like network of hyphae, driven by regular hyphal fusion, radiated from the inoculation site and colonised leaf surface. This epiphytic hyphal network appeared well organised as the pathogen used epidermis cell junctions and topography of the leaf for colony establishment (Fig 1a)..

Similarly to the related plant pathogens, *Z. tritici* and *Pseudocercospora fijiensis* (syn. *Mycosphaerella fijiensis*), *Rcc* gained entry into the host tissue by direct penetration of open stomatal pores. Development of a morphologically distinct structure, a stomatopodium, was observed at the hyphal tip prior to stomatal penetration (Fig 1a). Similar stomatopodium, known to occur in *P. fijiensis,* has not been reported previously in *Rcc* and appeared spherical or cylindrical in shape and somewhat swollen with the diameter of approximately 4 - 5 µm which was much thicker than leaf surface colonising hyphae. Stomatopodia could also develop as side branches of the epiphytic hyphae which facilitated penetration of stomatal pores (Fig 1b). Following entry to the substomatal cavity, stomatopodia started branching becoming multibranched, thick conidiogenous basal aggregates by 7 dpi (Fig 1c). As the fungus developed, an increasing number of stomata with coniodiogenous aggregates were observed (Fig 1d). Characteristic swan necked *Rcc* conidiophores rising from mycelial aggregates developed from 14 dpi onwards (Fig 1e). The mesophyll layer is then colonised by thick hyphal extensions. At the edge of the colonised area the fungus was able to colonise more of the mesophyll layer after entering the leaf via stomata (Fig 1f). Throughout this initial development leaves remained asymptomatic with typical RLS not observed until approximately 4 weeks post inoculation.

Transition of the fungal life style and symptomatic phase

A transition in fungal growth was observed from 20 dpi as endophytic colonisation progressed into necrotrophy. At this stage *Rcc* exhibited an invasive growth into mesophyll layer of the leafh. Mesophyll colonisation appeared intercellular, developing thick endophytic hyphae radiating outwards from stomatal cavities (Fig 1g). Diameter of the intercellular hyphae had a range of approximately 3 - 5 µm, compared to epiphytic hyphae with an average diameter of 1.2 µm. The intercellular growth of *Rcc* within the mesophyll layer had a 'brickwork-like' pattern. This pattern appeared highly regulated (Fig 1h), with long hyphae typically extending parallel to leaf axis connected by side branches every –two to three rows of mesophyll cells. No invasion of plant cells was observed and the hyphae did not cross the leaf veins. Intercellular hyphae were much thicker than those growing on the surface and substomatal cavity and were usually highly vacuolated (Fig 1i).

The development of a lesion around the infected stomata usually occurred 5 - 7 days after first observation of the aggressive colonisation of palisade mesophyll, around 25-27dpi. Lesion formation was associated with a loss of the chlorophyll fluorescence signal suggesting collapse of the cells in the affected areas (Fig 2 a1). The newly formed, small lesions, called pepper spots were clearly visible from 25 dpi and were associated with red discolouration of the surrounding tissue, presumably related to production of the rubellin toxins (Fig 2 a2). Mesophyll cells that collapsed due to the infection by intercellular hyphae emitted autofluorescence (Fig 2b).

As the lesion expanded encompassing the branched endophytic mycelium, the fungus appeared to develop long, but less-branched hyphae and actively grew away from the necrotic area (Fig 2c). This fungal growth habit was observed within leaf tissue presenting as a chlorotic halo surrounding the developing lesion. No penetration of vascular bundles was observed at any stage of infection progression (Fig 2c, 2d). Long chains of conidiophores

emerged through the collapsed epidermis (Fig 2e) which caused necrotic symptoms on the leaves (Fig 2f)

Simultaneous infection of barley by GFP- and DsRed- tagged *Rcc* isolates

In a whole plant inoculation assay, spring barley cv. Optic was challenged with two transgenic isolates *Rcc*-8B9-GFP and *Rcc*-ST-DsRed to observe whether these isolates could coexist and simultaneously establish infection on the same leaf. Prior to the co-inoculation experiment, the colonisation of barley by the transgenic isolate *Rcc*-ST-DsRed was verified *in planta*. *Rcc*-ST- DsRed colonisation was identical to the infection of barley by the isolate *Rcc*-8B9-GFP (Fig 2g). Co-inoculation experiments revealed that both isolates were able to coexist within a small area of the leaf. However, both isolates during the establishment of their epiphytic networks appeared to avoid exploring the same grooves between epidermis cells (Fig 2h). Although, the sporulation of both fungal strains developed at 15 dpi, no instance of simultaneous formation of spores of both genotypes at one stoma was noted, possibly suggesting competition for the ecological niche (data not shown).

In all examined plant material, the number of substomatal aggregates appeared higher for *Rcc*-8B9-GFP than for *Rcc*-ST-DsRed. Numbers of stomatal aggregates were counted as an indication of successful infection for both isolates across ten previously collected low magnification images of infection development at 7 dpi. T-test analysis showed that there was significant difference between the numbers of the observed basal aggregates per leaf analysed with *Rcc*-8B9-GFP producing significantly more aggregates than *Rcc*-ST-DsRed (P = 0.004721; mean values 5.3 and 2.8, for *Rcc*-8B9-GFP and *Rcc*-ST-DsRed infected samples, respectively).

Analysis of naturally infected leaf samples by light microscopy

To validate the results obtained from inoculation experiments an additional analysis of the latter stages of *Rcc* development following leaf senescence was examined in naturally heavily infected barley field samples from two UK sites (West Sussex cv Optic and Bush Estate, Midlothian cv Cocktail). The aniline blue method proved reliable for staining of fungal structures present on the leaf surface. However, intercellular hyphae colonising the mesophyll layer of leaves, observed with the confocal microscopy of the transgenic isolates, remained unstained and could not be readily visualised by conventional light microscopy.

Aniline blue staining of naturally infected field sampled leaves with RLS symptoms revealed massive sporulation within the necrotic lesions (Fig 3a). The majority of sporulating conidiophores were observed as fungal aggregates erupting from stomata (Fig 3b). Towards the edge of the necrotic lesion, instances of sporulation associated with the infection of stomata became much less frequent. Instead, conidiophores were observed erupting through the epidermis anticlinal walls (Fig 3b, 3c). Furthermore, the long continuous chains of conidiophores also developed in large numbers in grooves between epidermal cells directly adjacent to vascular bundles (Fig 3d). We also observed such chains of conidiophores following inoculation with hyphal fragments of the transgenic isolate *Rcc*-8B9-GFP (Fig 2e) where they were linked to intercellular mycelium in the mesophyll that was clearly restricted by vascular bundles (Fig 1h; Fig 2c). At the edge of the lesion observed on the inoculated detached leaves, within a chlorotic area, sporulation was rarely associated with substomatal cavities (Fig 3d). Here sporulation was observed, where chains of conidiophores burst through the anticlinal grooves of adjacent epidermis cells (Fig 2e). However, autofluorescence was also detectable around dead inoculum. In the region of leaf where dead hyphae were prevalent, the development of a lesion has occurred. Lesion formation was indicated by gradual fading and subsequent loss of detectable chlorophyll autofluorescence signal (Fig 2d). The similar infection stages observed between naturally infected field

samples and detached leaves inoculated with hyphal fragments confirms the suitability of the inoculation technique for studying this pathogen.

Effect of varietal variation on *Rcc* colonisation of spring barley

Four cultivars of spring barley, Belgravia, Garner, Optic and Cocktail that differ in their official AHDB resistance ratings for RLS were inoculated with *Rcc*-8B9-GFP to examine whether or not different levels of fungal development are exhibited during asymptomatic infection of these different varieties.

No apparent differences in *Rcc* development were observed during early stages of colonisation in any of the cultivars. Isolate Rcc-8B9-GFP was able to infect each of the cultivars at a similar rate starting from establishing an organised epiphytic hyphal network and infecting stomata. However, first instance of a mature form of conidiogenous aggregates and sporulation was on Cocktail as early as 8 dpi and the slowest development of these structures was found on Belgravia at 12 dpi, which also had the highest AHDB resistance rating to RLS. Optic and Garner, which have the lower RLS resistance ratings, showed the first signs of conidiogenous aggregates and sporulation at 10 dpi (results not shown)

Analysis of alternative hosts of *Rcc* (supplementary data)

Similarly to development in barley, isolate *Rcc*-8B9-GFP gained entry into wheat plants via stomata without triggering any apparent resistance response, suggesting a compatible interaction had occurred (Fig 4a). The fungus developed an organised hyphal network and as infection progressed, typical hyphal aggregates were observed in stomatal cavities which subsequently gave rise to conidiophores and conidia (Fig 4b). Since the fungus was able to colonise wheat and sporulate without any obvious cell death response from the plant, this

observation confirms that wheat could be a potentially very important *Rcc* host and the fungus could survive from season to season overwintering in wheat crops. To assess the potential risk *Rcc* infection may pose to wheat, seeds of different recommended and candidate wheat varieties from Scottish field trial sites were tested for the presence of *Rcc* DNA using qPCR. *Rcc* DNA was detected in all 35 wheat varieties tested (Table 1). *Rcc* DNA levels ranged from 0.002 pg to 0.681 pg with a mean value of 0.127 pg per 100 ng of DNA. The varieties Claire and Timber had the lowest levels of *Rcc* DNA whereas Cassius was the highest (Table S1). These values are much lower than those typically observed in barley seeds (Havis *et al*., 2014).

Various grass species have been implicated as hosts for *Rcc* (Cromey *et al*., 2004; Frei, 2004; Huss *et al*., 2004; Peraldi *et al*., 2014). Initial infection of Italian ryegrass (*L. mulitiflorum*) occurred in identical manner as observed in barley and wheat plants, with penetration of stomata by stomatopodia (data not shown) and establishing spiderweb-like epiphytic network of hyphae. However, colonisation of subsequent stomata followed by sporulation appeared to be more rapid and abundant in Italian ryegrass with spore formation occurring as early as 5 dpi compared to 8 and 10 dpi for barley and wheat, respectively.

Development of *Rcc* on Cock's foot (*D. glomerata*) suggested an incompatible interaction. Although stomatopodia formation and attempts to infect were observed (Fig 4c), no further development, such as substomatal aggregates, was recorded. An initial epiphytic hyphal network formed, but this hyphal growth appeared to be much less organised compared to that observed on other hosts (Fig 4c). *Rcc* hyphae appeared to rapidly collapse as indicated by the loss of GFP expression (Fig 4d).

Discussion

The recent establishment of RLS as an important disease of barley has led to renewed efforts to understand the biology of this disease (Havis *et al*., 2015). The ability of the fungus to complete its life cycle asymptomatically (Havis *et al.,* 2014) has led to suggestions that is actually an endophyte (Salamati & Reitan, 2006). The results presented here indicate that *Rcc* invades and colonises barley extensively, growing inter-cellularly through the mesophyll layer in the absence of disease symptoms. The transition to disease is associated with stress in the host plant e.g. waterlogging, light stress or post anthesis and is accompanied by an apparent loss of host chlorophyll (Makepeace *et al*., 2008; Schutzendubel *et al*., 2008).

Recent scanning electron microscopy (SEM) examinations of naturally infected leaves from the field have provided an initial insight into *Rcc* development on barley (Stabentheiner *et al*., 2009) but successful transformation of the fungus with fluorescent marker tags has facilitated studies of asymptomatic infection on barley (Thirugnanasambandam *et al.,* 2011). Studies on *Rcc* are challenging due to its sparse or even lack of sporulation *in vitro* (Sutton & Waller, 1988).

Rcc infection begins with the rapid formation of a mycelial network on the surface of the inoculated leaf. Penetration of leaf tissue occurred always through the stomatal pore as previously reported (Stabentheiner *et al*., 2009; Thirugnanasambandam *et al.* 2011). This mode of entry appears common to members of the *Mycosphaerellae* fungi including *Z. tritici* and *P. fijiensis* (Palmer & Skinner, 2002; Churchill, 2011). Stomatal penetration may be less likely to trigger defence reactions caused by the damage of host tissues during infection in line with the stealth mode of pathogenesis suggested for *Z. tritici* (Goodwin *et al*., 2011). The observation that the host epidermal cells remained intact during the early stages of *Rcc* infection is consistent with this hypothesis but may also indicate endophytic development is important for *Rcc*. Both Stabentheiner *et al*. (2009) and Thirugnanasambandam *et al.* (2011) stated that no specialised penetration structures were formed by *Rcc* during penetration of

stomatal pores. Although in this study invasive hyphae were observed to enter open stomata without producing any morphologically distinct structure, penetration of a stoma was often facilitated by a structure called a stomatopodium. This structure appeared to form as a thickening of the invasive hypha that forms above the stomatal pores entering between guard cells (Fig 1b). Stomatapodia were frequently but not exclusively associated with penetration of stomata (Fig1b). Furthermore, it was observed that this structure formed on the leading tip of hypha but also could develop as side branches extending from hyphal network. Similar structures have been reported previously in the closely related fungus, *P. fijiensis* (Balint-Kurti *et al.*, 2001) but this is the first report of such a structure in *Rcc*.

The development of an apparently organised network of epiphytic hyphae confirms previous observation that invasive hyphal networks appear on leaf surface prior to penetration but the method of stomatal recognition remains unclear. It remains to be determined which mechanisms are involved in this growth habit. Once inside substomatal cavities, stomatopodia develop into thick conidial bases (Fig 1c) as observed by Thirugnanasambandam *et al.* (2011). These fungal aggregates in the substomatal cavity remain connected by the epiphytic hyphal network on the leaf surface. Within these aggregates, which comprised a group of swollen, often highly vacuolated cells the characteristic *Rcc* swan-neck conidiophores are produced. Initially, the typical sporulation rising from subsequent stomatal pores was associated with some local necrosis of tissue surrounding stomata. This could be due to mechanical damage during conidiophore emergence but RLS macroscopic symptoms were not observed until at least 25 dpi. However, we have determined that during later stages of development, from 20 dpi (Fig 1g), the substomatal aggregates begin expansion into mesophyll tissue surrounding the cavities and produced an organised endophytic network of swollen, heavily branched hyphae that colonise intercellular space between mesophyll cells. The substomatal aggregates were associated with every successful stomatal infection of plant hosts in this study.

Intercellular growth was observed after 25 dpi, but the aggregates that developed by this time point at the edge of the infection did not immediately produce spores. Instead they directly expanded into the mesophyll layer. Leaves still appeared asymptomatic up to a week after the initial colonisation of the mesophyll suggesting *Rcc* growth was still endophytic at this stage. These endophytic mycelium eventually gave rise to mass sporulation via stomata and through the epidermis at cell junctions, inducing massive collapse of mesophyll tissue and subsequent RLS symptom expression. This could indicate a change in fungal growth from endophytic to necrotrophic. After epidermal cells collapse heavy colonisation of the intercellular space between mesophyll cells was observed. Collapse of mesophyll tissue in wheat is associated with proliferation of *Z. tritici* hyphae (Kema *et al*., 1996) potentially due to a release of intracellular nutrients into the apoplast (Keon *et al*., 2007).

It has been proposed that *Rcc* is an opportunistic saprophyte that is able to recognise and respond to a stress response in the host, be it the switch from vegetative to reproductive phase (Schutzendübel *et al.,* 2008), exposure to extreme environmental stress (Brown & Makepeace *et al*., 2009; Makepeace *et al*., 2008; Peraldi *et al*., 2014), or altered host stress and cell death regulation pathways (McGrann *et al*., 2014; 2015a; 2015b) by becoming a necrotrophic pathogen. These characteristics are typical of plant endophytes that can adapt rapidly to the growth habit and internal environment of the host that they have colonised (Schulze &Boyle, 2005). Seed-borne transmission of *Rcc* (Havis *et al*., 2014) together with asymptomatic sporulation, seen here and in previous work (Thirugnanasambandam *et al.,* 2011) supports the classification of *Rcc* as an endophyte. This suggests that *Rcc* inoculum may spread within a barley crop during the growing season without apparent symptoms, with disease only occurring under specific host and environmental conditions.

Several authors have reported the isolation of *Rcc* from many crop and perennial grass species in addition to barley (Huss, 2004; Frei, 2004; Cromey *et al.,* 2004). Alternative hosts should

therefore be considered as another important source of RLS within the growing season as they can facilitate pathogen survival through the winter period becoming a source of inoculum between the growing seasons. Winter wheat is one of the most important crops in the world and has been reported to a compatible host for *Rcc* (Huss, 2004). Asymptomatic infection of winter wheat is similar to barley suggesting that not only could wheat be a source of fungal inoculum for barley, it can potentially develop the disease on its own. The pathogen behaved in the same way and pace on wheat as in barley, and was able to sporulate therefore completing the life cycle. Furthermore, *Rcc* DNA was detected in wheat seeds suggesting the fungus can be potentially seed borne in this host (Table 1, Table Sp1). This could have serious implications for wheat production worldwide. Further study of the *Rcc* – wheat system is merited.

Infection on Italian ryegrass (*L. multiflorum*) was also akin the barley infection but more rapid indicated by much faster development of substomatal aggregates. Whether *Rcc* originated from perennial grasses and subsequently evolved to be the pathogen of the main cultivated crops is unknown. Evolutionary adaptation observed as a host jump from native grasses to crops have previously been described for of the wheat pathogen *Z. tritici* (Stukenbrock *et al.*, 2007; 2012). The findings described here suggest that ryegrass could be a major inoculum source for *Rcc* as this grass species can often be seen growing next to crop fields.

Results from the inoculation experiments with *D. glomerata* showed that this grass species is not a host for *Rcc*. The fungus was not able to establish infection despite repeated attempts in independent inoculation experiments. Interestingly, the initial development of the fungus was similar to barley and other hosts with some directional growth towards stomata and attempts to penetrate observed. However, no further development occurred suggesting that mechanisms of incompatibility could exhibit themselves only during the infection of stomata

RLS has now become a plant disease of major importance for barley growers, despite being known for over a century (Cavara, 1893). Factors that contribute to the increase in prevalence of RLS remain to be conclusively determined. It is therefore essential to employ all available tools and resources, such as the fluorescently tagged *Rcc* isolates (Thirugnanasambandam *et al.*, 2011), to increase our understanding of *Rcc* infection of barley and to study other potentially important sources of the disease, such as alternate hosts. For determination of different stages of the lifecycle of this fungus, transgenic *Rcc* isolates can be used to further investigate the spread of inoculum from seeds to plants and plants to seeds, and in addition, to address the question of whether *Rcc* is truly persisting in barley as an endophyte. Coupled with the PCR based techniques that enable the quantification of *Rcc* in infected leaf and seed material (Taylor *et al.*, 2010), visual analysis of the infection could provide knowledge on inoculum pressure required on the host before disease symptoms are seen and determine the trophic niche inhabited by this fungus.

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Table1 Detection of Rcc in wheat samples from 2009 and 2010 trials in Central Scotland

Year	Region	Crop	No of varieties	Mean Rcc DNA (pgrams) (±S.E.)	Range Rcc DNA range (pgrams)
2009	Central Scotland	Winter wheat (untreated)	35	0.49 (± 0.33)	Cassius (0.68 pg) – Claire (0.002pg)
2010	Central Scotland	Winter wheat (Full fungicide programme)	35	5.14 (± 0.078)	Viscount (14.32 pg) – Einstein (0.38 pg)
LSD (P=0.05)				0.66	

Table 2 Inoculation of transformed Rcc into various plant species in controlled experiments

Species name	Reason for use	Description of growth	1	2	3	4	5	6
Hordeum vulgare	*H. vulgare*, or barley is *Rcc* known host. This is a control to compare the extent of infection of other species against.	Hyphae grow on leaf surface, following leaf grooves, prior to entry via stomata.	+	+	+	+	+	+
Hordeum vulgare var. nudum	Naked Barley is a variant of barley that is easily detachable from its seed coat or hull and provides a second variant of barley.	Similar colonisation and infection to barley but no sporulation observed.	+	+	+	+	+	+
Triticum aestivum cv. emerald	A reported host of *Rcc* (Huss, 2004) and a major crop.	Colonisation progressed in a very similar manner to *H. vulgare.*	+	+	+	+	+	+
Lolium multiflorum	Common name annual ryegrass. Previously identified as a host of *Rcc* (Sprague, 1950).	Hyphal growth on the surface disorganised but some infection of stomata observed.	-	-	-	-	-	-
Alopecurus myosurides	A major weed of crops found in Europe.	Growth of hyphae towards stomata and evidence of potential sporulation.	+	-	+	+	+	+
Dactylis glomerata	A perennial grass sown in temperate pastures and also a common wild grass in Britain.	Colonisation of leaf surface and unsuccessful attempts to infect observed.	+	+	+	-	-	-

1. Directed growth (similar to *H. vulgare*)
2. Stomatapodium formation
3. Hyphal thickening
4. Stomatal infection
5. Sporulation on leaf surface
6. Conidiophore formation

Supplementary Table 1 – Rcc DNA levels in winter wheat from Central Scotland

Variety	2009 Rcc DNA (pgrams)	2009 S.E.	Variety	2010 Rcc DNA (pgrams)	2010 S.E.
Alchemy	0.018	0.017	Alchemy	9.745	0.215
Battalion	0.228	0.201	Beluga	4.328	0.215
Beluga	0.202	0.048	Cassius	6.193	0.215
Cassius	0.681	0.459	Chilton	4.204	0.215
Claire	0.002	0.002	Cocoon	1.768	0.215
Conqueror	0.016	0.009	Conqueror	3.480	0.215
Cordiale	0.292	0.229	Cordiale	3.660	0.215
CPBT W 144	0.089	0.043	Delphi	4.452	0.215
CPBT W 148	0.190	0.034	Denman	3.133	0.215
CPBT W 150	0.33	0.206	Duxford	1.351	0.215
CPBT W 152	0.334	0.167	Einstein	0.377	0.215
Duxford	0.066	0.007	Gallant	13.950	0.215
Edmunds	0.053	0.016	Grafton	1.780	0.215
Einstein	0.042	0.042	Gravitas	5.676	0.215
Gallant	0.103	0.026	Horatio	5.292	0.215
Gladiator	0.013	0.006	Invicta	4.703	0.215
Glasgow	0.171	0.056	JB Diego	5.613	0.215
Grafton	0.063	0.049	KWS Gator	5.818	0.215
Humber	0.025	0.019	KWS Podium	5.548	0.278
Invicta	0.118	0.013	KWS Santiago	13.470	0.215
Istabraq	0.022	0.012	KWS Saxtead	9.713	0.215
JB-Diego	0.023	0.014	KWS Solo	1.108	0.215
Ketchum	0.311	0.133	KWS Sterling	2.188	0.277
Kingdom	0.067	0.042	KWS Target	2.578	0.215
Marksman	0.015	0.015	Monterey	4.282	0.215
Oakley	0.067	0.001	Oakley	1.462	0.215
Panorama	0.07	0.07	Relay	5.304	0.373
PBI-40636	0.051	0.039	Scout	3.780	0.215
Qplus	0.207	0.179	Solstice	6.095	0.215
Robigus	0.036	0.003	Stigg	4.798	0.215
Scout	0.100	0.059	Torch	5.549	0.373
Solstice	0.081	0.042	Trident	7.443	0.215
Timber	0.004	0.002	Tuxedo	5.927	0.215
Viscount	0.239	0.120	Viscount	14.320	0.215
Walpole	0.107	0.079	Warrior	0.838	0.215