

Scotland's Rural College

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

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

*Published in:*  
Plant Pathology

*DOI:*  
[10.1111/ppa.12552](https://doi.org/10.1111/ppa.12552)

First published: 06/06/2016

*Document Version*  
Peer reviewed version

[Link to publication](#)

### *Citation for published version (APA):*

Kaczmarek, M., Piotrowska, MJ., Fountaine, JM., Gorniak, K., McGrann, GRD., Armstrong, A., Wright, KM., Newton, AC., & Havis, ND. (2016). Infection strategy of *Ramularia collo-cygni* and development of *Ramularia* leaf spot on barley and alternative graminaceous hosts. *Plant Pathology*, 66(1), 45 - 55.  
<https://doi.org/10.1111/ppa.12552>

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

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

3 M. Kaczmarek<sup>a,b</sup>, M.J. Piotrowska<sup>a</sup>, J.M. Fountaine<sup>a, c</sup>, K. Gorniak<sup>a</sup>, G.R.D. McGrann<sup>a</sup>, A.  
4 Armstrong<sup>d</sup>, K.M. Wright<sup>e</sup>, A.C. Newton<sup>e</sup> and N.D. Havis<sup>a\*</sup>

5 <sup>a</sup> Crop and Soil Research Department, Scotland's Rural College (SRUC), King's Buildings,  
6 Edinburgh EH9 3JG, UK

7 <sup>b</sup> Present address: Centre for Ecosystems, Society and Biosecurity, Forest Research, Alice Holt  
8 Lodge, Farnham, GU10 4LH

9 <sup>c</sup> Present address: Syngenta, Jealott's Hill International Research Centre, Bracknell, RG42  
10 6EY, UK

11 <sup>d</sup> Nothern Research Station (Forest Research), Bush Estate, Roslin, EH25 9SY

12 <sup>e</sup> Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee DD2 5DA,  
13 UK

14

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

16

17 **[Abstract]**

18 *Ramularia* leaf spot (RLS) is a newly important disease of barley across temperate regions

19 worldwide. Despite this recent change in importance the infection biology of the causal

20 agent, *Ramularia collo-cygni* (*Rcc*) remains poorly understood. Confocal microscopy of the

21 infection process of two transgenic *Rcc* isolates, expressing either GFP or dsRed reporter

22 markers, was combined with light microscopy during field infection to track the progression

23 of *R. collo-cygni* *in planta*. Infection of stomata, including the development of a previously

24 unreported stomatopodium structure, results in symptomless development and intercellular

25 colonisation of the mesophyll tissue. Transition to necrotrophy is associated with breakdown

26 of host chloroplast and the formation of aggregates of conidiophores. In addition to barley,

27 *Rcc* forms a compatible interaction with winter wheat and a number of perennial grass

28 species. An incompatible reaction was observed with two dicotyledonous species. These

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

## 31 **Introduction**

32 Infection by *Ramularia collo-cygni* (*Rcc*) (Sutton & Waller, 1988) can result in *Ramularia*  
33 leaf spot disease on barley (RLS) leading to loss of green leaf area in infected plants (Havis  
34 *et al.*, 2015; Walters *et al.*, 2008). RLS can lead to yield losses of up to 20 per cent in barley,  
35 with an average loss in Scotland at 0.4 tonnes per hectare (Oxley & Havis., 2004). The  
36 development of PCR-based methods for detection of the fungus in barley tissue have  
37 expanded our understanding of pathogen's life cycle particularly the importance of seed-  
38 borne infection in disease etiology (Havis *et al.*, 2014; Havis *et al.*, 2006a; Frei *et al.*, 2007;  
39 Taylor *et al.*, 2009). Recent evidence has suggested that *Rcc* is likely to undergo sexual  
40 reproduction (Piotrowska *et al.*, 2016) however, there are many unknowns still surrounding  
41 the infection process and biology of this organism. Studies using scanning electron  
42 microscopy of naturally infected leaves (Stabentheiner *et al.*, 2009) and fluorescently labelled  
43 transgenic *Rcc* isolates (Thirugnanasambandam *et al.*, 2011) have provided valuable insights  
44 to the infection process of *Rcc*. The development of GFP- and dsRED tagged *Rcc* isolates in  
45 particular has great potential to further characterise the biology of this disease through non-  
46 invasive *in planta* live-cell imaging techniques. Using tagged fungal isolates in-depth spatio-  
47 temporal analysis of the infection cycle beginning with conidia germinating on the leaf  
48 surface under moist conditions can be performed. The fungus enters through open stomata  
49 within 24 hours after spore germination on the leaf surface (Sutton & Waller, 1988; Walters  
50 *et al.*, 2008) as observed in the related plant pathogen *Zymoseptoria tritici* (Goodwin *et al.*,  
51 2011). Although the apparently directional growth of young *Rcc* hyphae towards stomata has  
52 been observed *in planta*, it remains unclear how the pathogen detects the presence of stomatal  
53 pores (Stabentheiner *et al.*, 2009). Following stomatal penetration, *Rcc* establishes an

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

59 The process that triggers the transition of *Rcc* from asymptomatic to the symptom causing  
60 phase remains poorly understood. Host genetic factors (McGrann *et al.*, 2014; 2015a; 2015b)  
61 and environmental stimuli (Brown & Makepeace, 2009; Makepeace *et al.*, 2008; Peraldi *et*  
62 *al.*, 2014) appear to play important roles in the expression of RLS. The appearance of RLS  
63 symptoms is typically observed on plants late in the growing season, usually after the ear  
64 emergence (Schützendübel *et al.*, 2008; Walters *et al.*, 2008). Once the necrotic lesions  
65 appear, the remainder of the leaf becomes chlorotic and then necrotic, usually starting from  
66 the tip and leaf margins (Huss, 2004). These small, pale to medium brown pepper spots are  
67 usually surrounded by a yellow halo (Salamati & Reitan, 2006). The numerous local  
68 infections of the leaf tissue that usually occur during mass sporulation can often coalesce to  
69 form larger necrotic areas. Periods of high leaf surface wetness are a key environmental  
70 factor that induces the rapid sporulation of the pathogen (Sutton & Waller, 1988; Huss, 2004;  
71 Havis *et al.*, 2012). Detailed descriptions of *Rcc* colonisation during the transition to  
72 disease have not been described. Although Sutton and Waller (1988) first suggested that  
73 once inside the leaf, *Rcc* grows intercellularly, forming branched hyphae which colonise the  
74 mesophyll tissue, no evidence was presented to support this statement. Stabentheiner *et al.*  
75 (2009) showed the presence of fungal hyphae in the mesophyll layer of naturally infected  
76 samples from the field. However, it was not confirmed that these hyphae were specifically  
77 from *Rcc*. As such the biological events resulting in the change from endophytic to  
78 necrotrophic growth remains undetermined. Besides barley, *Rcc* has been isolated from other

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

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

97

98

## 99 **Materials and methods**

### 100 **Fungal isolates and inoculum preparation**

101 Two *Rcc* field isolates collected from naturally infected leaves of the spring barley cv.  
102 Braemar and two transgenic isolates were used in this study. The field isolates originated  
103 from Scotland, isolate B1, and Denmark, isolate DK05Rcc001. Transgenic *Rcc* isolates 8B9  
104 (*Rcc*-8B9-GFP) and Stratego (*Rcc*-ST-DsRed) expressing GFP and DsRed fluorescent  
105 proteins, respectively, have been previously described (Thirugnanasambandam *et al.*, 2011).  
106 Fungal cultures were maintained on clarified V8 juice agar (10 mM CaCO<sub>3</sub> in 20 % (v/v) V8  
107 juice, 1.5 % agar) at 15° C in the dark. Inoculum was prepared from mycelial fragments of  
108 *Rcc* isolates from two-week old spread-plates by scraping the colony surface with a sterile  
109 spatula, and then filtering through sterile glass wool in the neck of a sterile glass funnel. The  
110 mycelium harvested from a single spread plate was diluted in 5 mL sterile distilled water  
111 prior to inoculation.

## 112 **Plant material**

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

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

125 **Detached leaf assay**

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

138

139 **Whole plant inoculation assay**

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

148

149 **Confocal laser scanning microscopy (CLSM) conditions**

150 Plant material inoculated with transgenic *Rcc* isolates was examined using, a Leica SP2  
151 CLSM (Leica Ltd, London, UK) on a DM6000 microscope fitted with a FI/RH filter block  
152 (excitation filter BP 490/15, dichroic mirror 500, emission filter BP 525/20; excitation filter  
153 BP 560/25, dichroic mirror 580, emission filter BP 605/30) and Leica water-dipping lenses  
154 (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 /  
155 0.90 W U-V-1). GFP fluorescence was imaged at the excitation wavelength of 488 nm and  
156 emission was collected at 500–530 nm.

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

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

167 **Light microscopy conditions**

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



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

### 175 **Aniline blue staining**

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

184

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

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

194

### 195 **Results**

196 **Symptomless infection characteristics**

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

202 Similarly to the related plant pathogens, *Z. tritici* and *Pseudocercospora fijiensis* (syn.  
203 *Mycosphaerella fijiensis*), *Rcc* gained entry into the host tissue by direct penetration of open  
204 stomatal pores. Development of a morphologically distinct structure, a stomatopodium, was  
205 observed at the hyphal tip prior to stomatal penetration (Fig 1a). Similar stomatopodium,  
206 known to occur in *P. fijiensis*, has not been reported previously in *Rcc* and appeared  
207 spherical or cylindrical in shape and somewhat swollen with the diameter of approximately 4  
208 - 5  $\mu\text{m}$  which was much thicker than leaf surface colonising hyphae. Stomatopodia could also  
209 develop as side branches of the epiphytic hyphae which facilitated penetration of stomatal  
210 pores (Fig 1b). Following entry to the substomatal cavity, stomatopodia started branching  
211 becoming multibranched, thick conidiogenous basal aggregates by 7 dpi (Fig 1c). As the  
212 fungus developed, an increasing number of stomata with conidiogenous aggregates were  
213 observed (Fig 1d). Characteristic swan necked *Rcc* conidiophores rising from mycelial  
214 aggregates developed from 14 dpi onwards (Fig 1e). The mesophyll layer is then colonised  
215 by thick hyphal extensions. At the edge of the colonised area the fungus was able to colonise  
216 more of the mesophyll layer after entering the leaf via stomata (Fig 1f). Throughout this  
217 initial development leaves remained asymptomatic with typical RLS not observed until  
218 approximately 4 weeks post inoculation.

219 **Transition of the fungal life style and symptomatic phase**

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

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

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

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

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

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

258 In all examined plant material, the number of substomatal aggregates appeared higher for  
259 *Rcc*-8B9-GFP than for *Rcc*-ST-DsRed. Numbers of stomatal aggregates were counted as an  
260 indication of successful infection for both isolates across ten previously collected low  
261 magnification images of infection development at 7 dpi. T-test analysis showed that there was  
262 significant difference between the numbers of the observed basal aggregates per leaf analysed  
263 with *Rcc*-8B9-GFP producing significantly more aggregates than *Rcc*-ST-DsRed ( $P =$   
264 0.004721; mean values 5.3 and 2.8, for *Rcc*-8B9-GFP and *Rcc*-ST-DsRed infected samples,  
265 respectively).

### 266 **Analysis of naturally infected leaf samples by light microscopy**

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

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

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

294

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

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

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

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

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

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

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

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

## 337 **Discussion**

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

346 Recent scanning electron microscopy (SEM) examinations of naturally infected leaves from  
347 the field have provided an initial insight into *Rcc* development on barley (Stabentheiner *et al.*,  
348 2009) but successful transformation of the fungus with fluorescent marker tags has facilitated  
349 studies of asymptomatic infection on barley (Thirugnanasambandam *et al.*, 2011). Studies on  
350 *Rcc* are challenging due to its sparse or even lack of sporulation *in vitro* (Sutton & Waller,  
351 1988).

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



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

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

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

399 It has been proposed that *Rcc* is an opportunistic saprophyte that is able to recognise and  
400 respond to a stress response in the host, be it the switch from vegetative to reproductive phase  
401 (Schutzendübel *et al.*, 2008), exposure to extreme environmental stress (Brown & Makepeace  
402 *et al.*, 2009; Makepeace *et al.*, 2008; Peraldi *et al.*, 2014), or altered host stress and cell death  
403 regulation pathways (McGrann *et al.*, 2014; 2015a; 2015b) by becoming a necrotrophic  
404 pathogen. These characteristics are typical of plant endophytes that can adapt rapidly to the  
405 growth habit and internal environment of the host that they have colonised (Schulze & Boyle,  
406 2005). Seed-borne transmission of *Rcc* (Havis *et al.*, 2014) together with asymptomatic  
407 sporulation, seen here and in previous work (Thirugnanasambandam *et al.*, 2011) supports the  
408 classification of *Rcc* as an endophyte. This suggests that *Rcc* inoculum may spread within a  
409 barley crop during the growing season without apparent symptoms, with disease only  
410 occurring under specific host and environmental conditions.

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

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

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

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

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

#### 450 **Acknowledgements**

451 We thank BASF for the generous funding of Maciej Kaczmarek's PhD studentship. We are  
452 indebted to staff at the James Hutton Institute for provision of confocal microscopy and plant  
453 contained growth facilities. Transgenic *R. collo-cygni* cultures were held under the HSE  
454 Licence GM250/08.1 We are grateful for financial support for this work in part from the  
455 Rural and Environment Science and Analytical Services (RESAS) Division of the Scottish  
456 Government (2011–2016) under its Environmental Change and Food, Land and People  
457 Research Programmes.

#### 458 **References**

459 AHDB 2015. HGCA Recommended Lists 2015/16 for cereals and oilseeds. Agriculture and  
460 Horticulture Development Board, Stoneleigh, UK.

461 Balint-Kurti PJ, May GD, Churchill ACL, 2001. Development of a transformation system  
462 for *Mycosphaerella* pathogens of banana: a tool for the study of host/pathogen  
463 Interactions. *FEMS Microbiology Letters* **195**, 9-15

464 Brown JKM, Makepeace JC, 2009. The effect of genetic variation in barley on responses to  
465 *Ramularia collo-cygni*. *Aspects of Applied Biology* **92**:43–47.

466 Cavara F, 1893. Über einige parasitische Pilze auf dem Getreide. *Zeitschrift für*  
467 *Pflanzenkrankheiten* **3**, 16-26.

468 Churchill AC, 2011. *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana:  
469 progress towards understanding pathogen biology and detection, disease development, and  
470 the challenges of control. *Molecular Plant Pathology*. **12(4)**:307-28.

471 Cromey MC, Harvey IC, Sheridan JE, Grbavac N, 2004. Occurrence, Importance and  
472 Control of *Ramularia collo-cygni* in New Zealand. *Proceedings of the Second International*  
473 *Workshop on Barley Leaf Blights*. 7-11 April 2002, ICARDA, Aleppo, Syria: 337-342.

474 Fraaije BA, Lovell DJ, Rohel EA, Hollomon DW, 1999. Rapid detection and diagnosis of  
475 *Septoria tritici* epidemics in wheat using a polymerase chain reaction PicoGreen assay.  
476 *Journal of Applied Microbiology* **86**, 701-708.

477 Frei P, 2004 *Ramularia collo-cygni*: Cultivation, Storage, and Artificial Infection of Barley  
478 and Weed Grasses under Controlled Conditions. *Proceedings of the Second International*  
479 *Workshop on Barley Leaf Blights*. 7-11 April 2002, ICARDA, Aleppo, Syria: 351-354.

480 Frei P, Gindro K, Richter H, Schürch S, 2007. Direct-PCR detection and epidemiology of  
481 *Ramularia collo-cygni* associated with barley necrotic leaf spots. *Journal of Phytopathology*  
482 **155**: 281–288.

483 Goodwin SB, Ben M'Barek S, Dhillon B, Wittenberg AHJ, Crane CF, *et al.* (2011) Finished  
484 Genome of the Fungal Wheat Pathogen *Mycosphaerella graminicola* Reveals Dispensome  
485 Structure, Chromosome Plasticity, and Stealth Pathogenesis. *PLoS Genetics* **7(6)**: e1002070,  
486 doi:10.1371/journal.pgen.1002070

487 Havis ND, Oxley SJP, Piper SR, Langrell SRH, 2006. Rapid nested PCR-based detection of  
488 *Ramularia collo-cygni* direct from barley. *FEMS Microbiology Letters* **256**: 217-223.

489 Havis ND, Oxley SJP, Burnett F, Hughes G, 2012. Epidemiology of *Ramularia collo-cygni*.  
490 *Proceedings Crop Protection in Northern Britain*: 119–124.

491 Havis ND, Nyman M, Oxley SJP, 2014. Evidence for seed transmission and symptomless  
492 growth of *Ramularia collo-cygni* in barley (*Hordeum vulgare*). *Plant Pathology* **63**: 929–936.

493 Havis ND, Brown JKM, Clemente G, Frei P, Jedryczka M, Kaczmarek J, Kaczmarek M,  
494 Matusinsky P, McGrann GRD, Pereyra S, Piotrowska M, Sghyer H, Tellier A, Hess M,  
495 2015. *Ramularia collo-cygni*—an emerging pathogen of barley crops. *Phytopathology* **105**,  
496 895-904.

497 Huss H, 2004. The biology of *Ramularia collo-cygni*. Meeting the Challenges of Barley  
498 Blights. *Proceedings of the Second International Workshop on Barley Leaf Blights*,  
499 (ICARDA), Aleppo, Syria, April 2002: 321–328.

500 Kema GHJ, Yu D, Rijkenberg FHJ, Shaw MW, Baayen R P, 1996. Histology of the  
501 pathogenesis of *Mycosphaerella graminicola* in wheat. *Phytopathology* **86**:777-786.

502 Keon J, Antoniw J, Carzaniga R, Deller S, Ward JL, Baker JM, Beale MH, Hammond-  
503 Kosack KE, Rudd JJ. (2007) Transcriptional adaptation of *Mycosphaerella graminicola* to  
504 programmed cell death (PCD) of its susceptible wheat host. *Molecular Plant Microbe*  
505 *Interactions* **20**: 178–193

506 McGrann GRD, Stavrinides A, Russell J, Corbitt M, Booth A, Chartrain L, Thomas WTB,  
507 Brown JKM. 2014. A trade-off between *mlo* resistance to powdery mildew and increased  
508 susceptibility of barley to a newly important disease, *Ramularia* leaf spot. *Journal of*  
509 *Experimental Botany* **65**:1025–1037.

510 McGrann GRD, Steed A, Burt C, Nicholson P, Brown JKM, 2015a. Differential effects of  
511 lesion mimic mutants in barley on disease development by facultative pathogens. *Journal of*  
512 *Experimental Botany*. <http://dx.doi.org/10.1093/jxb/erv154>

513 McGrann GRD, Steed A, Burt C, Goddard R, LaChaux C, Bansal A, Corbitt M, Gorniak K,  
514 Nicholson P, Brown JKM, 2015b. Contribution of the drought tolerance-related *Stress-*  
515 *responsive NAC 1* transcription factor to resistance of barley to *Ramularia* leaf spot.  
516 *Molecular Plant Pathology* **16**, 201–209.

517 Makepeace JC, Oxley SJP, Havis ND, Hackett R, Burke J I, Brown JKM, 2007.  
518 Associations between fungal and abiotic leaf spotting and the presence of *mlo* alleles in  
519 barley. *Plant Pathology*. **56**:934–942.

520 Makepeace, JC, Havis, N D, Burke JI, Oxley SJP, Brown, JKM, 2008. A method of  
521 inoculating barley seedlings with *Ramularia collo-cygni*. *Plant Pathology*. **57**:991–999.

522 Newton AC, Searle J, Guy DC, Hackett CA, Cooke DEL, 2001. Variability in pathotype,  
523 aggressiveness, RAPD profile, and rDNA ITS1 sequences of UK isolates of *Rhynchosporium*  
524 *secalis*. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz-Journal of Plant Diseases and*  
525 *Protection* **108**: 446-458.

526 Nyman M, Havis ND, Oxley SJP, 2009. Importance of seed-borne infection of *Ramularia*  
527 *collo-cygni*. Proceedings of the Second European *Ramularia* Workshop. *Aspects in Applied*  
528 *Biology* **92**: 91-96.

529 Oxley SJP, Havis ND, 2004. Development of *Ramularia collo-cygni* on spring barley and its  
530 impact on yield. *Proceedings Crop Protection in Northern Britain 2004*, 147-152.

531 Oxley SJP, Havis ND, 2009. Understanding *Ramularia collo-cygni* in the past, present and  
532 future. *Aspects of Applied Biology*. 92:141–146

533 Palmer CL, Skinner W, 2002. *Mycosphaerella graminicola*: latent infection, crop devastation  
534 and genomics. *Molecular Plant Pathology*, 2002 Mar **1;3(2)**:63-70.

535 Peraldi A, Griffe LL, Burt C, McGrann GRD, Nicholson P, 2014. *Brachypodium distachyon*  
536 exhibits compatible interactions with *Oculimacula* spp. and *Ramularia collo-cygni*, providing  
537 the first pathosystem model to study eyespot and *ramularia* leaf spot diseases. *Plant*  
538 *Pathology*. **63**:544–562.

539 Piotrowska MJ, Ennos RA, Fountaine JM, Burnett FJ, Kaczmarek M, Hoebe PN, 2016.  
540 Development and use of microsatellite markers to study diversity, reproduction and  
541 population genetic structure of the cereal pathogen *Ramularia collo-cygni*. *Fungal Genetics*  
542 *and Biology*. **87**: 64-71.

543 Salamati S, Reitan L, 2006. *Ramularia collo-cygni* on spring barley, an overview of its  
544 biology and epidemiology. *Proceedings of the First European Ramularia Workshop*:7–23.

545 Schulze B, Boyle C, 2005. The endophytic continuum. *Mycological Research*, 109(6), 661-686  
546 Schützendübel A, Stadler M, Wallner D, von Tiedemann A, 2008. A hypothesis on  
547 physiological alterations during plant ontogenesis governing susceptibility of winter barley to  
548 *Ramularia* leaf spot. *Plant Pathology*. **57**:518–526.

549 Sprague R, 1950. *Diseases of Cereals and Grasses in North America*. New York: Ronald  
550 Press



551 Stabentheiner E, Minihofer T, Huss H, 2009. Infection of barley by *Ramularia collo-cygni*:  
552 Scanning electron microscopic investigations. *Mycopathologia* **168**: 135-143.

553 Stukenbrock EH, Banke S, Javan-Nikkhah J, McDonald BA, 2007. Origin and Domestication  
554 of the Fungal Wheat Pathogen *Mycosphaerella graminicola* via Sympatric Speciation .  
555 *Molecular Biology and Evolution* **24 (2)**. 398-411.

556 Stukenbrock EH, Bataillon T (2012) A Population Genomics Perspective on the Emergence  
557 and Adaptation of New Plant Pathogens in Agro-Ecosystems. PLoS Pathog 8(9): e1002893.  
558 doi:10.1371/journal.ppat.1002893

559 Sutton B, Waller J, 1988. Taxonomy of *Ophiocladium hordei* causing leaf lesions on Triticale  
560 and other Gramineae. *Transactions of the British Mycological Society* **90**: 55–61.

561 Thirugnanasambandam A, Wright KM, Havis N, Whisson SC, Newton AC, 2011a.  
562 *Agrobacterium*-mediated transformation of the barley pathogen *Ramularia collo-cygni* with  
563 fluorescent marker tags and live tissue imaging of infection development. *Plant Pathology*  
564 **60**: 929–937.

565 Taylor JMG, Paterson LJ, Havis ND, 2010. A quantitative real-time PCR assay for detection  
566 of *Ramularia collo-cygni* in barley (*Hordeum vulgare*). *Letters in Applied Microbiology* **50**:  
567 493-499.

568 Walters DR, Havis ND, Oxley SJP, 2008. *Ramularia collo-cygni*: the biology of an emerging  
569 pathogen of barley. *FEMS Microbiology Letters* **279**: 1-7.

570 Zadoks J C, Chang T T, Konzak C F, 1974. A Decimal Code for the Growth Stages of  
571 Cereals. *Weed Research*. **14**:415–421.

572

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) ( $\pm$ S.E.)	Range Rcc DNA range (pgrams)
2009	Central Scotland	Winter wheat (untreated)	35	0.49 ( $\pm$ 0.33)	Cassius (0.68 pg) – Claire (0.002pg)
2010	Central Scotland	Winter wheat (Full fungicide programme)	35	5.14 ( $\pm$ 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
<i>Hordeum vulgare</i>	<i>H. vulgare</i> , or barley is <i>Rcc</i> 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.	+	+	+	+	+	+
<i>Hordeum vulgare var. nudum</i>	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.	+	+	+	+	+	+
<i>Triticum aestivum cv. emerald</i>	A reported host of <i>Rcc</i> (Huss, 2004) and a major crop.	Colonisation progressed in a very similar manner to <i>H. vulgare</i> .	+	+	+	+	+	+
<i>Lolium multiflorum</i>	Common name annual ryegrass. Previously identified as a host of <i>Rcc</i> (Sprague, 1950).	Hyphal growth on the surface disorganised but some infection of stomata observed.	-	-	-	-	-	-
<i>Alopecurus myosuroides</i>	A major weed of crops found in Europe.	Growth of hyphae towards stomata and evidence of potential sporulation.	+	-	+	+	+	+
<i>Dactylis glomerata</i>	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. Stomatopodium 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	2009	Variety	2010	2010
	Rcc DNA (pgrams)	S.E.		Rcc DNA (pgrams)	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