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

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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 CaCO₃ 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⁻¹ 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⁻²s⁻¹.

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 μm 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 μm , compared to epiphytic hyphae with an
225 average diameter of 1.2 μm . 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). Stomatapodia 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.

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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