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Evidence for seed transmission and symptomless growth of *Ramularia collo-cygni* in barley (*Hordeum vulgare*)

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Ramularia collo-cygni (Rcc) is becoming an increasing problem for barley growers across Europe. However, the life cycle of the pathogen is only slowly being elucidated. In this study, Rcc DNA was detected in a number of harvested seed samples from 1999 to 2010, with mean levels peaking in winter barley samples in 2009. A number of experiments were carried out to determine whether the pathogen could move from barley seed to seedlings, and also from seed through the developing plant and into the subsequent generation of seed, both in controlled experiments and in field trials. Results from testing of seed indicated that the fungus is widespread at the end of the growing season in harvested grain samples and can be transmitted to developing plants from infected seed stock. Examination of infected seedlings did not reveal the presence of spores but fungal structures were found within the leaf. The location of the fungus within seed was examined, with Rcc DNA found in both embryo and non-embryo tissue. The implications for barley production of the pathogen being seedborne are discussed.

Keywords: controlled environment, field experiment, pathogen movement, PCR, ramularia leaf spot, seed infection

Introduction

Ramularia collo-cygni (Rcc) is an increasingly important late season pathogen of barley (*Hordeum vulgare*) in continental Europe, the UK, Ireland and also South America (Sachs *et al.*, 1998; Pinnschmidt & Hovmøller, 2003; Oxley & Havis, 2004; M. Scandiani, Laboratorio Agrícola Río Paraná, Ruiz Moreno 225 (2930) San Pedro, Argentina, personal communication) and is the major biotic factor in ramularia leaf spot (RLS). Symptoms are most commonly observed on foliage after flowering in the crop. Initial signs of infection are small brown to blackish spots, 1–2 mm long. The spots develop a chlorotic halo and eventually neighbouring lesions may coalesce to form a larger necrotic region. The subsequent loss of green leaf area leads to deleterious effects on yield quantity and quality (Oxley & Havis, 2004; Hughes *et al.*, 2013). The fungus was first identified in barley crops in northern Italy in the late 19th century (Cavara, 1893). By the time of the detailed monograph of the pathogen (Sutton & Waller, 1988), the fungus had been identified in Germany and also in North America. In addition to being present on *Hordeum* spp. the fungus has also been detected on other grass species e.g. *Triticum secalim*, *Phalaris arundinacae*, *Festuca kingii*, *Lolium multiflorum* and *Lolium perenne* (Sprague, 1950) and is capable of infecting the model grass *Brachypodium distachyon* (Peraldi *et al.*, 2013).

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The infection process from airborne spores has been studied using scanning electron microscopy (Stabentheiner *et al.*, 2009). After spore germination on the leaf surface, hyphae enter the leaf via stomata. Conidiophores, with the distinctive swan neck structure, emerge from stomata but late in the plant life cycle condiophores can emerge from between plant epidermal cells. Experiments using a mycelial inoculation of green fluorescent protein (gfp)-transformed Rcc showed a similar pattern on infection and colonization of the plant (Thirugnanasambandam *et al.*, 2011). An aggregation of fungus was observed in the substomatal cavity prior to conidiophore emergence.

The development of molecular-based diagnostic tests for Rcc have allowed more detailed studies of the ecology, aetiology and epidemiology of this poorly understood pathogen (Havis *et al.*, 2006a; Frei *et al.*, 2007). Visual crop assessments and diagnostic analysis have indicated that the pathogen moves up the plant during the growing season and also infects awns and ears (Oxley & Havis, 2004).

The aims of these studies were (i) to determine if Rcc from infected seed could be transmitted to newly sown crops from infected seed asymptomatically; and (ii) to determine the location of the pathogen in the seed and its subsequent movement.

Materials and methods

Monitoring Rcc in seed

Seeds for experiments were sourced from unsprayed plots in SRUC trials and additional spring barley seed samples

Table 1 Detection of *Ramularia collo-cygni* (Rcc) DNA in harvested grain samples. (a) Qualitative identification in seed samples from 1999 to 2005; (b) quantitative detection from 2007 onwards

(a)

Year	Country of origin	Crop	No. of varieties	No. of seed samples	Positive test results
1999	Scotland	Spring barley	7	7	7
2004	Scotland	Winter barley	1	1	1
2004	Scotland	Spring barley	16	40	40
2005	Scotland	Spring barley	16	50	39

(b)

Year	Country of origin	Crop	No. of varieties	No. of seed samples	Annual mean Rcc DNA (pg ± SE)
2007	England	Winter barley	7	19	1·99 ± 0·11
2007	England	Spring barley	14	25	7·54 ± 2·04
2007	Scotland	Spring barley	10	34	11·66 ± 2·22
2008	Scotland	Winter barley	6	8	5·60 ± 2·00
2008	Scotland	Spring barley	17	44	19·38 ± 5·40
2009	Scotland	Winter barley	10	16	25·89 ± 17·08
2009	Scotland	Spring barley	12	34	10·09 ± 3·96
2010	Scotland	Winter barley	9	20	0·86 ± 0·37
2010	Scotland	Spring barley	10	30	5·76 ± 1·94
2011	Scotland	Winter barley	5	15	4·48 ± 3·03
2011	England	Winter barley	2	3	14·02 ± 5·04
2011	Scotland	Spring barley	4	8	2·80 ± 1·64
2011	England	Spring barley	10	26	4·69 ± 1·80

were provided by Dr Valerie Cockerell (Official Seed Testing Station for Scotland, Science and Advice for Scottish Agriculture [SASA]). Seeds were either untreated or Raxil-treated (20 g L^{-1} tebuconazole + 20 g L^{-1} triazoxide, applied at 150 mL per 100 kg seed).

Seed sample preparation for DNA extraction followed the method of Lee *et al.* (2001). For each sample, 100 seeds were ground to a fine powder in a food processor (Kenwood) for 5 min and then DNA was extracted using the REDExtract-N-Amp kit (Sigma-Aldrich) and a nested PCR reaction carried out as described in Havis *et al.* (2004) (Table 1a).

Rcc DNA in seed samples was quantified using a recently developed quantitative PCR (qPCR) assay (Taylor *et al.*, 2010). Seed samples were milled as above but DNA was extracted using the CTAB extraction method (Aldrich & Cullis, 1993).

Transmission of Rcc to developing plants

Controlled environment experiments

To study the potential transmission of Rcc from seed to seedling and subsequently to developing plants, 18-cm pots were filled with Levington F2 compost, and sown with nine seeds each. Pots were put in a controlled environment chamber at 18°C with a 16 h photoperiod. After emergence, the plants were thinned to six per pot.

For the first experiment, leaf samples were harvested when three leaves had unfurled, and were frozen at −20°C prior to testing with nested PCR. For DNA extraction, two leaf discs were taken arbitrarily from the harvested leaves using a 1 cm hole punch and each sample was tested three times.

Leaf material from seed samples that gave a positive PCR result was also examined microscopically for the presence of fungal spores or mycelial growth. To examine for fungal growth, tissue was cleared of chlorophyll using the acetiethanol method from Ryan & Claire (1974). Leaves were placed in a Petri dish on filter paper soaked with 1:1 v/v solution of glacial acetic acid and absolute ethanol, and the plates exposed to daylight for 4 days. The leaves were removed and added to new Petri dishes containing filter paper soaked in distilled water, and left for a further 3 days. Then leaves were stained with trypan blue lactophenol stain for a range of times from 30 s to 30 min and mounted on a microscope slide in clear lactophenol under a coverslip sealed with nail varnish.

In the second experiment, leaf layers were harvested after ear emergence (about growth stage (GS) 75) and any lesions found on leaves visually inspected for Rcc conidiophores under a microscope, prior to freezing at −20°C. The frozen leaves were ground under liquid nitrogen until they were a fine powder. Three 0·1 g samples were weighed out and DNA extracted using the Illustra Phytopure DNA extraction kit (GE Healthcare). Samples were tested for the presence of Rcc using the previously mentioned qPCR assay (Taylor *et al.*, 2010; Table 3).

Table 2 Seedling leaf layers tested for the presence of *Ramularia collo-cygni* DNA using nested PCR diagnostic (positive/total)

Crop	No. of samples	Leaf 1	Leaf 2	Leaf 3
Spring barley	8	8/8	8/8	8/8
Winter barley	2	2/2	2/2	2/2

Table 3 *Ramularia collo-cygni* DNA levels and visual fungal structures in spring barley leaf layers from controlled environment experiment

Crop/leaf layer	Ears	Flag	F−1	F−2	F−3	F−4
Mean ± SE (fg)[a]	4·8 ± 2·3	1·8 ± 0·6	6·6 ± 3·5	7·4 ± 2·0	19·3 ± 9·2	119·2 ± 77·7
Fungal structures	0	5/7	6/7	6/7	4/7	5/7

DNA levels analysed by ANOVA, 30 degrees of freedom.
[a]$P < 0{\cdot}001$.

Field experiments

Untreated spring barley seeds from three varieties (cultivars Cocktail, Decanter and Optic) were sown in tussocks at Drumalbin Farm, Lanark, UK in 2008 and 2009. Plants were harvested at fortnightly intervals in 2008 and weekly in 2009. Untreated winter barley seeds (cvs Saffron and Retriever) were sown in tussocks in autumn 2009 at the same site and sampled on a monthly basis. Sampling involved the collection of 10 plants randomly sampled from tussocks and the separation of leaf layers. Leaves were assessed for visual symptoms and then pooled prior to grinding in liquid nitrogen. Samples (1 g) were then used for DNA extraction using the Illustra Phytopure DNA extraction kit. Rcc DNA levels were quantified using the qPCR assay.

Monitoring of Rcc spores in the environment

A Burkard 7-day spore sampler was set up at Drumalbin Farm, Lanark. To sample from the environment, this machine draws air through a small aperture and over coated Mellinex tape. After 7 days the tape was removed and divided into segments that corresponded to 24 h periods. These were then halved lengthways and stored at −20°C. DNA was extracted from the tape using the method described in Fountaine *et al.* (2007). Rcc DNA levels were quantified using the qPCR assay.

Location of Rcc fungus in the seed

In three separate experiments, barley seeds were pre-germinated and dissected into embryo and non-embryo parts (embryo and endosperm). DNA was extracted by Illustra Phytopure in the first experiment and in the latter two by the method described by Fraaije *et al.* (1999) and Fountaine *et al.* (2007). The extracted DNA was quantified as described previously. In addition, the embryo/endosperm samples were analysed with the more sensitive nested PCR (minimum detection level 0·5 fg; Havis *et al.*, 2006a) and run on a 1·5% agarose gel containing GelRed (Biotium Inc) to visualize the DNA.

Statistical analysis

Results were collated and ANOVA carried out using GenStat v. 11.1, (VSN International Ltd).

Results

Monitoring Rcc in seed

DNA of Rcc was detected in a number of varieties harvested in 1999 (Table 1a), when RLS was only just starting to be observed as a problem in Scottish fields (Oxley *et al.*, 2002). The proportion of seed samples providing positive test results increased from 2004 onwards. Quantification of fungal DNA in samples from 2007 onwards indicates that levels varied from season to season. The highest mean recorded levels across winter and spring barley were in 2009 (25·89 and 10·09 pg respectively; Table 1b).

Rcc DNA was present in DNA extracted from all of the emerging leaves of plants sown from infected seed and grown in a controlled environment chamber

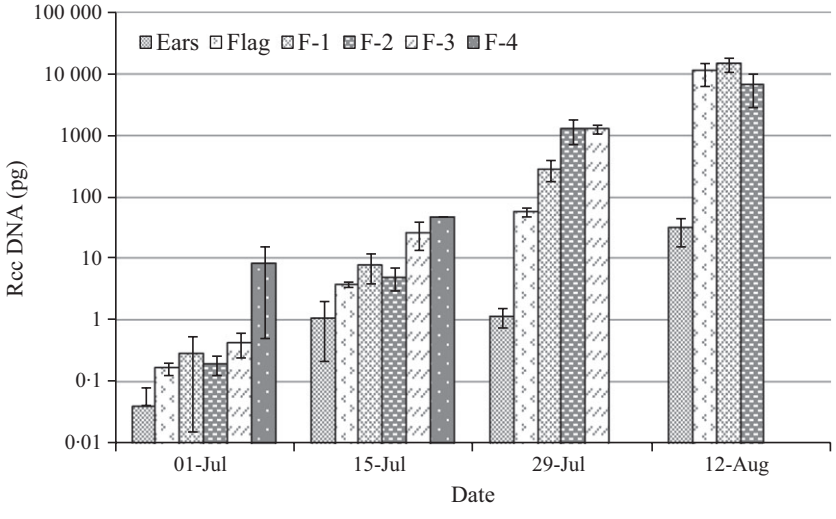

Figure 1 *Ramularia collo-cygni* DNA levels in spring barley leaf layers – Lanark 2008. Bars indicate standard errors. Significant differences were observed between leaf layers on 15 and 29 July ($P < 0{\cdot}05$). Mean disease levels at last sampling date in top three leaf layers = 17%.

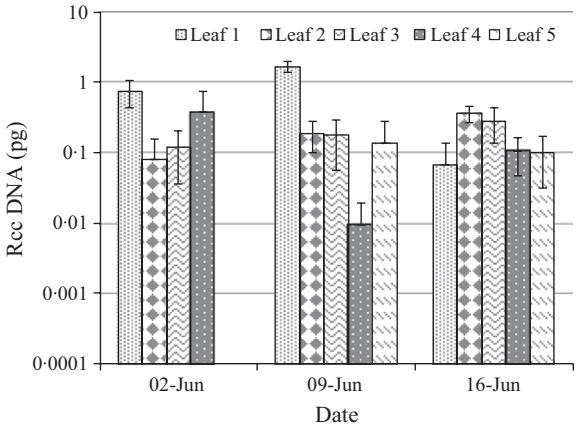

Figure 2 Early season *Ramularia collo-cygni* DNA in emerging leaves – Lanark 2009. Bars indicate standard errors. No significant differences were observed between leaf layers at sampling dates.

(Table 2). The presence of two fungicidal compounds in a seed dressing on the winter barley had not stopped the movement of the fungus from infected seed to emerging seedlings and developing plants.

The microscopic examination of cleared leaf tissue did not show any conidiophores or mycelia on the surface of the leaf. However, longer staining of the leaf segments indicated the presence of hyphal structures in the sub-stomatal cavity.

Movement of fungus from infected seed

In the controlled environment experiment, fungal DNA was detected in all leaves, even in the absence of visual foliar symptoms (Table 2). Rcc DNA could be detected at picogram (pg) levels in the initial leaves and then femtogram (fg) levels in ears and upper leaf layers, even in the absence of microscopic structures (Table 3). Rcc DNA levels were found to be higher in the lower leaves of the plant. The controlled environment experiments had no external inoculum to affect the results.

The field experiments were open to the influence of external inoculum. Results from winter and spring barley leaf samples showed that colonization of the host plant by Rcc began at the lower leaves and then moved up the plant during the growing season (Figs 1–3). Rcc DNA levels reached 10 000 pg in the F−3 leaf layer of spring barley in both years (Figs 1 & 2). A linear correlation was observed between visual symptoms in the 2008 trial and Rcc DNA levels ($R = 0{\cdot}894$, d.f. = 16, $P < 0{\cdot}001$; data not shown). A linear correlation was also observed between the two variables in the 2009 trial ($R = 0{\cdot}696$, d.f. = 73, $P < 0{\cdot}05$; data not shown). This relationship between Rcc DNA and visual symptoms was also shown in the results from Taylor *et al.* (2010). Results from the spore sampler at the site indicated that Rcc DNA levels in the environment did not increase significantly until late June and early July (Figs 4 & 5). Rcc DNA levels were lower in winter barley in 2009–10 but the general pattern of higher levels lower in the canopy was repeated (Fig. 6). However, the rapid senescence of lower leaves in the winter barley made DNA extraction and Rcc quantification more difficult at the June sampling date. A major spore release event occurred in November 2009 (870 pg) but thereafter levels remained below 100 pg for 2010 (Fig. 7).

Fungal location within seed

Results from the qPCR were inconclusive in describing a pattern of fungal distribution in the seed (data not shown). A gel image of the nested PCR test indicated the presence of the pathogen in both seed components of nearly all of the varieties (summarized in Table 4). Only

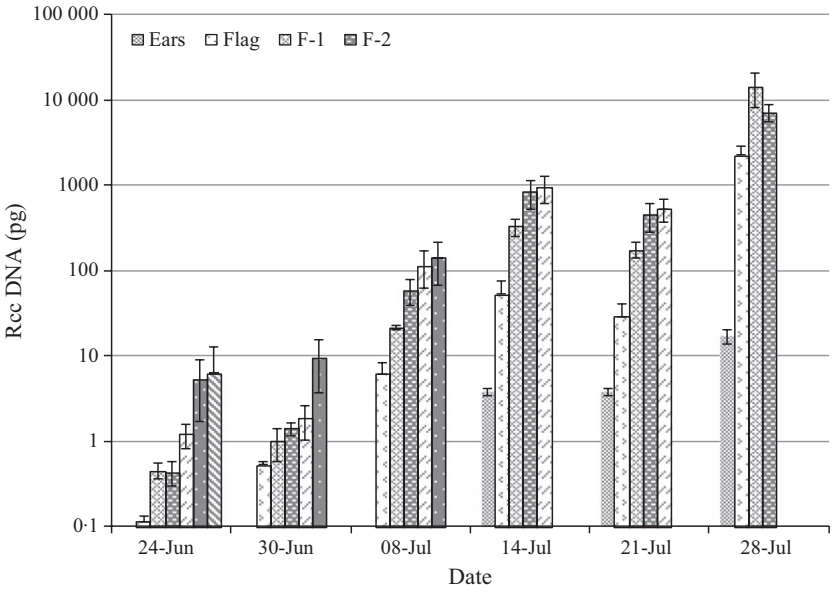

Figure 3 Late season *Ramularia collo-cygni* DNA in leaf layers – Lanark 2009. Bars indicate standard errors. Significant differences were observed between leaf layers on 14, 21 and 28 July ($P < 0{\cdot}05$). Mean disease levels at last sampling date in top three leaf layers =14·7%.

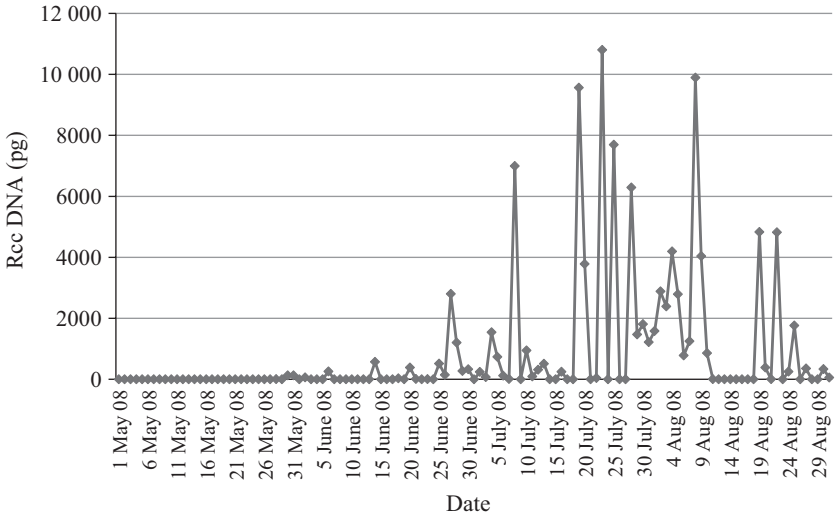

Figure 4 *Ramularia collo-cygni* DNA levels on spore tapes – Lanark 2008.

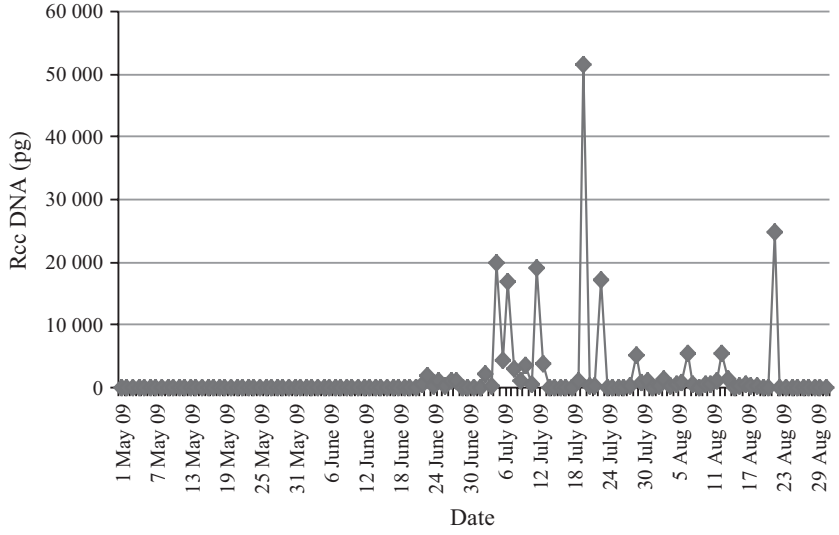

Figure 5 *Ramularia collo-cygni* DNA from spore sampler – Lanark 2009.

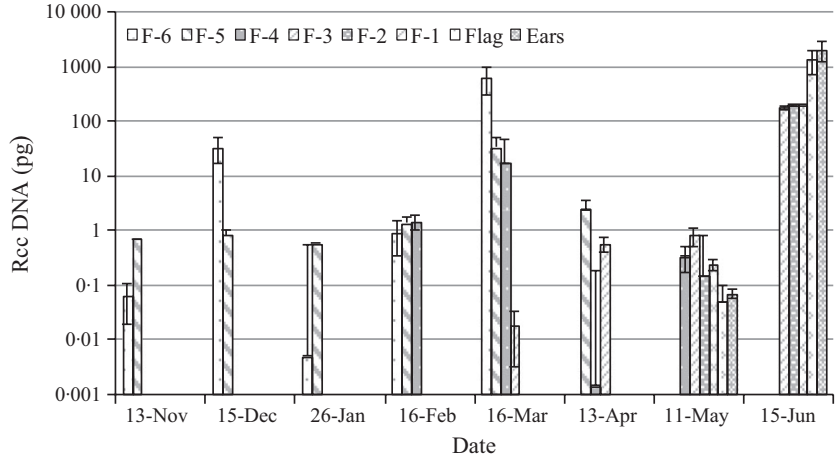

Figure 6 *Ramularia collo-cygni* DNA in winter barley leaf layers – Lanark 2009–2010. Bars indicate standard errors. Significant differences were observed between leaf layers on 13 November ($P < 0{\cdot}01$). Mean disease levels at last sampling date in top three leaf layers = 2·5%.

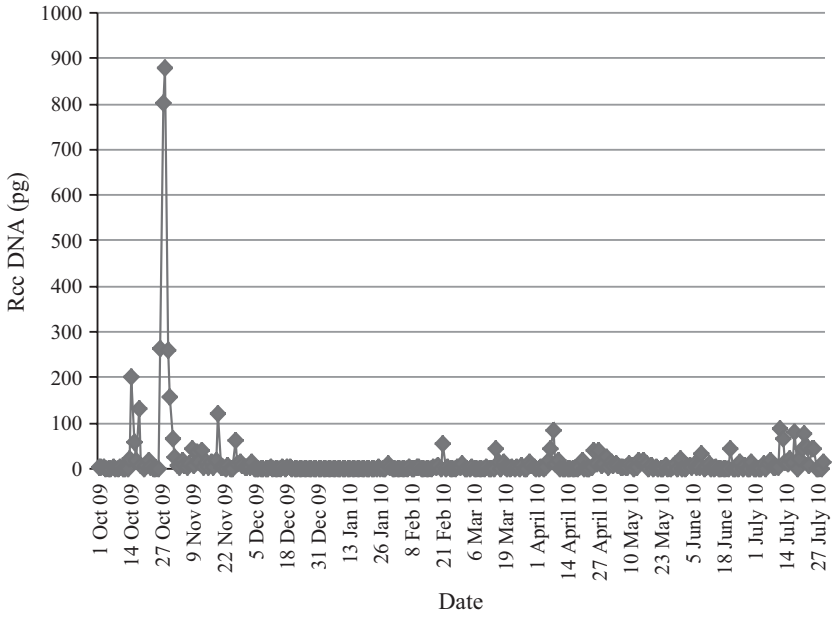

Figure 7 *Ramularia collo-cygni* DNA from spore sampler – Lanark 2009–2010.

Table 4 Qualitative testing for *Ramularia collo-cygni* DNA in seed fractions of spring barley. Minimum detection level was 0·5 fg

Cultivar	Seed fraction	Endosperm	Embryo
Decanter	Untreated	+	+
Decanter	Treated	+	+
Optic	Treated	+	+
Optic	Untreated	+	+
Westminster	Treated	+	+
Westminster	Untreated	+	+
Forensic	Treated	+	+
Concerto	Treated	–	+
Cropton	Treated	+	+
Berlioz	Treated	+	+

the endosperm fraction from cv. Concerto gave a negative result.

Discussion

As previously stated the aims of these studies were two-fold: (i) to determine if Rcc from infected seed could be transmitted to newly sown crops from infected seed asymptomatically; and (ii) to determine the location of the pathogen in the seed.

The life cycle of *Ramularia collo-cygni* is poorly understood. Preliminary schemes have the fungus overwintering on winter crops, volunteers and secondary hosts prior to infecting spring crops (Sachs, 2002). The infected spring crops then act as a source of inoculum later in the growing season for the next season's winter crops.

A number of pathogens of barley are known to be seedborne e.g. *Rhynchosporium commune*, *Pyrenophora teres*, *Pseudoseptoria stomaticola* (Murray *et al.*, 1998). Anecdotal evidence from Austria indicated that there could be a seedborne stage in the life cycle of Rcc (H. Huss, Institut für Biologische Landwirtschaft, Versuchstation, Lambach-Stadl, Austria, personal communication). Recent reports from the Czech Republic have also indicated that Rcc DNA can be detected in harvested grain (Matusinsky *et al.*, 2011). However, the DNA levels reported were much lower than the figures recorded for barley seed harvested in the UK (Table 1b). Analysis of seed tested in earlier projects in this laboratory indicated higher mean levels in Scotland compared to England in 2007 (32·7 pg compared to 6·2 pg; Oxley & Havis, 2007). Barley varieties were scored on an annual basis for RLS symptom expression. No relationship was found between fungal DNA levels in seed and proposed resistance ratings (data not shown). The movement of Rcc from infected seed to developing plant has been reported previously (Havis *et al.*, 2006a,b; Frei, 2009) and other studies indicated that the fungus moves up the crop leaf layers during the growing season (Salamati & Reitan, 2002; Oxley & Havis, 2004) but the initial infection source has not been clearly identified. However, this is the first report of the fungus being able to move within the plant and colonize the emerging leaf layers during its growth stages in the absence of external inoculum. The pattern of fungal movement corresponds well with the findings of symptom formation in the field (Huss *et al.*, 2006; Stabentheiner *et al.*, 2009). These new findings indicate the potential for continual colonization by the pathogen and so the difficulty in controlling the spread of the fungus via vertical transmission. Many fungi have a symptomless phase during colonization of their host (Saikonnen *et al.*, 2004). Indeed, another seedborne barley pathogen, *R. commune*, has been reported to grow symptomlessly in plants prior to symptom expression (Zhan *et al.*, 2008). The controlled environment testing of barley plants grown from infected seed demonstrated

that the fungus was transferred from seed to emerging leaf tissue. Microscopic examination of the leaf revealed no spores or mycelia present on the leaf surface. The presence of fungal hyphae in the substomatal cavity points to the fungus having successfully invaded the leaf tissue during plant development. Previous scanning electron microscope work in this laboratory and Austria has shown the presence of a network of fungal structures within the leaves of *R. collo-cygni* infected plants (Oxley *et al.*, 2009; Stabentheiner *et al.*, 2009). Specifically, a complex network of hyphae was seen growing between the mesophyll cells on the inner epidermis and this congregated near stomata. Although it cannot be confirmed that the fungus observed in controlled environment experiments was Rcc, the fungal pattern appears similar to infected samples from the field and no external inoculum was able to infect the plants during the experiment.

Results from field experiments indicate that Rcc does not have a polycyclic pattern of spore production like *R. commune* (Stedman, 1980). Minor spore release events can be detected at points in the growing season but the major spore release events occur post-flowering in spring barley crops. In the spring barley experiments reported here, Rcc DNA multiplied rapidly in the lower canopy in both years (Figs 1–3). In 2009 this increase was shown to take place when no spores were being released (Fig. 5). Seedborne pathogens have been shown to be responsible for the initiation of disease in *Stagonospora nodorum* and *Pyricularia oryzae* (Shah *et al.*, 1995; Manandhar *et al.*, 1998). The results in the current study point to a similar situation with *R. collo-cygni*. Matusinsky *et al.* (2011) carried out experiments using infected seed at two sites in the Czech Republic and found no relationship between low Rcc DNA seed infection and disease levels. However, previous work in Scotland with infected seed has indicated that crops will develop disease symptoms at geographically separate sites but that the major influence on RLS severity is local environmental conditions (Havis *et al.*, 2006a).

Molecular-based diagnostic techniques have been used previously to determine the location of fungal material in seed e.g. mycelia from the loose smut fungus, *Ustilago nuda* have been found within the non-embryo half of barley seed (Eibel *et al.*, 2005). The results from these current studies show that Rcc is also situated within the seed. Distribution of the fungus seems to be throughout the whole seed, both the embryo and non-embryo parts. These results confirm the report from Matusinsky *et al.* (2011) that Rcc DNA could be detected in the lemma of seed, with lower amounts found in the embryo and pericarp. Rcc DNA was not detected in the endosperm in the studies of Matusinsky *et al.* (2011), but these current studies with nested PCR, and recent experiments gfp-transformed isolate (Thirugnanasambandam *et al.*, 2011), indicate colonization of the endosperm can occur (Table 4; M. Kaczmarek, SRUC/University of Edinburgh, Edinburgh, UK, unpublished data). Thus, the fungus seems capable of extensive colonization of seed and the presence of a significant seedborne stage for this pathogen has implications for all barley growers. Rcc may be present in a majority of crops prior to symptom expression. Protectant late season fungicide applications still represent the most effective way of checking the development of the fungus and also its movement onto emerging ears. Previous work has shown that untreated ears can become infected by Rcc as early as GS 61 (Oxley & Havis, 2004). Further work is also required to examine the effectiveness of seed treatments in controlling transmission of the fungus.

The presence of a seedborne stage in the fungal life cycle would help explain the transfer of the fungus from season to season. Symptomless growth and a potential endophytic stage in the fungal life cycle suggest that categorization of Rcc as a necrotrophic pathogen may not be accurate. The quantification of the influence of seedborne infection on final disease severity requires further experimentation. However, recent work shows the pathogen seems to colonize the plant more effectively when a combination of seed infection and external inoculum occurs (Nyman, 2009).

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