Ramularia collo-cygni: a new pathogen spreading in barley fields in Estonia

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Ramularia leaf spot (RLS) caused by the fungus *Ramularia collo-cygni (Rcc)* is affecting barley fields throughout temperate regions worldwide. The first finding of RLS in Estonia was reported on spring barley in 2012 and since then the area of RLS infection has been widening in Estonia. This work has been carried out to monitor the natural infection of *Rcc* in two winter barley cultivars and to follow artificial fungal infection by a PCR-based assay. Using our approach, we could detect presence of the fungal pathogen in barley leaves before the appearance of disease symptoms at early growth stages. Response of two tested cultivars to *Rcc* infection in the field conditions was different, showing genotype-environment interaction in the development and spreading of *Rcc.* In harvested grain samples at the end of growing season no *Rcc* infection was detected. The role of external inoculum, *Rcc* conidia transmitted from various grasses (*Poaceae*) growing next to crop fields, is discussed. These results provide further insight into the epidemiology of *Rcc.*

Key words: Ramularia leaf spot, pathogen, winter barley

Introduction

The fungus *Ramularia collo-cygni* (*Rcc*) is the biotic agent involved in Ramularia leaf spot (RLS) disease. In the 80s, RLS received scientific attention as a new serious disease affecting barley throughout Europe, South-America and New Zealand (Sachs et al. 1998, Walters et al. 2008). The first case of RLS in Estonia was detected in 2012 on spring barley (Sooväli et al. 2014). As an important barley pathogen, *Rcc* has also been reported to colonize rye, oats, wheat and many other grass species (Huss 2004, Kaczmarek et al. 2016) and causes severe risks not only at global but also at local level, especially in the case of small farms. The increased incidence of RLS is connected with shorter crop rotations and long-term cultivation of barley (Havis et al. 2015). Moreover, it is often difficult to distinguish RLS from physiological leaf spotting, further frustrating efforts to detect the pathogen at early stage of plant infection.

Current *Rcc* control methods rely on foliar fungicides applied at booting growth stage, before leaf spots appear (Oxley and Havis 2009). The control of pathogen by seed treatment has been examined but no effective control on RLS was achieved (Havis and Oxley 2006). It has been recognized that though barley cultivars varied in susceptibility to the disease, there is still a demand for improved resistance in genotypes (Havis et al. 2015). The use of resistant cereal cultivars and crop rotation can lower the number of fungicide treatments per season which may have preventative effect on fungicide resistance development. Rotation is more likely to be effective if the entire field is rotated out of susceptible crops rather than just the section previously planted to the crop.

The fungus is thought to have a latent endophytic phase in plants before becoming pathogenic (Salamati and Reitan 2006, Kaczmarek et al. 2016). The process that triggers the transition of *Rcc* from symptomless to the symptom-causing phase is poorly understood (Salamati and Reitan 2006, McGrann et al. 2015). Symptoms of *Rcc* infection become visible on older senescing (lower) leaves after ear emergence, suggesting a possible link between appearance of RLS symptoms and host plant developmental stage (Schützendübel et al. 2008, Havis et al. 2015). Publication of the entire genome sequence of *Rcc* isolate DK05 Rcc001 (McGrann et al. 2016) has made it a model for the experimental characterization of the molecular dynamics of *Ramularia*-host interactions.

There are several possible inoculum sources for *Rcc* infection, which include airborne conidia released and spreading from non-host to host plants (Heuser and Zimmer 2002, Salamati and Reitan 2006, Havis et al. 2014, Peraldi et al. 2014, Kaczmarek et al. 2016). Salamati and Reitan (2006) reported that in addition to airborne spores *Rcc* produces a second type of spore known as asteromella which develop late in the season on straw. These structures have also been reported from *in vitro* cultures (Kaczmarek et al. 2013). Frei et al. (2007) showed that *Rcc* overwinters on winter barley and therefore can be considered as an infection source for spring barley. The crop debris left in the field can also be one possible source of inoculum (Salamati and Reitan 2006). In addition, seed

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transmission is considered as a possible inoculation source with regard to epidemiology and is perceived as a challenge for disease control and quarantine (Havis and Oxley 2006, Matusinsky et al. 2011).

Conventional identification techniques of *Rcc* are based on the microscopic identification of conidiophores on the leaf surface. However, conidiogenesis represents a relatively late stage of the infection process. Studies using scanning electron microscopy of naturally infected leaves (Stabentheiner et al. 2009) and fluorescently labelled transgenic *Rcc* isolates (Thirugnanasambandam et al. 2011) have provided valuable insights into the infection process by *Rcc*. To gain a better understanding of the distribution of this pathogen as well as its early detection, different molecular bioanalytical tools based on PCR have been developed for the detection of *Rcc* in plant tissues (Havis et al. 2006, Frei et al. 2007, Taylor et al. 2010, Matusinsky et al. 2011, Havis et al. 2015).

An accurate diagnostic test for *Rcc* in winter barley, together with the correct timing of fungicide application in the fields, should help to control the infection. The main objectives of the study were to find reliable *Rcc* detection methods in the early phase of infection and to reveal possible transfer routes of *Rcc*.

Materials and methods

Barley sampling

In field experiments winter barley cultivars (cv.) Fridericus and Cinderella were arranged with four replicates in a randomized design 10m² plots at Viljandi Variety Testing Centre in Estonia during the growing seasons of 2014/2015 and 2015/2016. In both years the preceding crop was clover. Both trials were seeded into a ploughed fields at optimal time in the first week of September. Before sowing sertified seeds were treated with Baytan Trio fungicide (fluopyram 5 g l^{-1}, fluoxastrobin 25 g l^{-1}, triadimenol 150 g l^{-1}) at a rate 2 l t^{-1}. In both years mineral fertilizer Yara Mila 7-20-28 300 kg ha^{-1} before sowing, additional fertilizers ASN 100 kg ha^{-1} and Axan 230 kg ha^{-1} were applied in spring.

The leaves were taken from 10 plants randomly selected places on each plot on every 7th day during the test period from tillering growth stage (GS 27) (Zadoks et al. 1974) to hard dough stage (GS 87), in total, from 7 time points. Leaves samples were stored at –20 °C in a freezer until the trial period was finished.

In three separate experiments the grain subsamples of the barley cultivars (100 g of grain per sample) were collected at full ripening stage (GS 91) and stored at 14% humidity till DNA isolation.

Local weather conditions

The weather conditions in the autumn of 2014 were characterised by average temperature of 8.9 °C and the sum of precipitation of 80 mm in September-October. The spring of 2015 was cool and dry: May-July average temperature 13.6 °C (long-term average 14.7 °C) and precipitation 170 mm (long-term average 196 mm), July being the warmest month of the period (average temperature 16.1 °C). The weather conditions in the autumn of 2015 with average temperature in September-October of 8.7 °C and the sum of precipitation of 70 mm were similar to that of autumn 2014. The spring of 2016 was warmer and rainy: in May-July average temperature 15.8 °C, was above the same period of 2015 (average temperature 17.6 °C). Precipitation 190 mm was similar of long-term average but exceeded the same period of 2015 by 20 mm.

DNA extraction and spike test

The *Rcc* isolate RCC2 (Sooväli et al. 2014) was maintained on potato dextrose agar (PDA) (Conda, Spain) in 9 cm Petri dishes incubated at 17 °C for 10–12 days in the dark. Mycelium from this culture was harvested and finely grounded for DNA extraction. Total genomic DNA was isolated from barley leaves (a composite sample of 10 g leaf material) and seeds (a composite sample of 100 seeds) by crushing the samples frozen in liquid nitrogen to fine powder using a pestle and a mortar.

In case of each sample, DNA from 100 mg of plant material and 50 mg of RCC2 mycelium was extracted with a DNeasy Pant Mini Kit (Qiagen, Gmbh, D-40724 Hilden, Germany) according to the manufacturer's instructions. DNA from barley seeds was also extracted on the NucliSENS® easyMAG® DNA/RNA (Biomerieux, UK) extraction platform according to the manufacturer's instructions. DNA concentration and purity were measured at 260 and 280 nm on a Nanodrop.

The ability of the *Rcc* PCR primer Rc3/Rc5 to identify and amplify its target in the presence of a high concentration of nontarget (plant) DNA was analyzed in spiking studies. Varying amounts of *Rcc* DNA (500–0.005 pg) were spiked into a fixed amount of plant DNA (100 ng) and the mixture was used as a template in the PCR assay.

Standard PCR

Several PCR primers pairs for preliminary study of qualitative detection of *Rcc* in samples of barley were tested (data not shown). Primers pair Rc3 and Rc5 (Frei et al. 2007) were proved to be the most specific with a PCR product of 348 bp amplified for all *Rcc* infected plant samples tested on the conventional PCR. PCR was carried out in a Mastercycler nexus GX2 (Eppendorf AG, Hamburg) in 25 µl reaction mixture containing 1X Taq polymerase buffer (20 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.5 µl) 0.2 mM each dNTP (ThermoFisher Scientific, USA), 250 nM primers Rc3 and Rc5, 0.6 U Taq DNA polymerase (ThermoFisher Scientific, USA), and approximately 100 ng of genomic DNA. The optimized PCR temperature programme consisted of Taq activation at 94 °C for 4 min and 35 cycles of amplification (denaturation at 94 °C for 45 s, primer annealing at 60 °C for 45 s and extension at 72 °C for 45 s). Appropriate positive (*Rcc* DNA) and negative controls (sterile distilled water [SDW]) were included in all experiments PCR products were separated in ethidium bromide-stained 1.5% agarose gels run in 1X Tris-Acetate-EDTA buffer and exposed to UV-light to visualize the DNA fragments (Sambrook et al. 1989). Nucleotide sequencing reactions were performed by the dideoxynucleotide method of Sanger (Sanger et al. 1977). DNA and protein sequence homology searches were done in the GenBank, EMBL and SWISS-PROT databases by using the University of Wisconsin Genetic Computer Group programs BLASTN, BLASTX and FASTEMBL.

In vitro inoculation of barley plants

To study systemic growth and spreading of *Rcc* under the controlled environment the *Rcc* seeds of the barley cv. Fridericus from growing season 2014/2015 were used. The seeds were pre-germinated on watered sterile filter paper in Petri dishes at 20 °C for 2 days. The seedlings were planted in Ø 20 cm pots (4 plants per pot) filled with mixture of sterilized soil, turf substrate and sand (2:2:1) and propagated in environmental chamber at 18 °C applying a 16 h photoperiod. Plants were fertilized 10 days after sowing with a solution containing $(NH_4)_2SO_4$ (0.6 g kg^{-1} soil), K_2SO_4 (0.3 g kg^{-1} soil), KH_2PO_4 (0.2 g kg^{-1} soil), $MgSO_4$ (0.5 g kg^{-1} soil). After inoculation the plants were kept in the dark for 48 h and covered with plastic bags to achieve relative humidity close to 100%. After this treatment the 16 h photoperiod was restored. In all experiments, ten pots per treatment were inoculated and five pots were inoculated with water to serve as controls.

Ramularia collo-cygni isolate RCC2 was maintained on PDA at 17 °C. For inoculation, a 0.5 cm^2 section of agar covered with fungus was transferred into 50 ml of potato broth (Salamati and Reitan 2006) and grown at 17 °C under constant agitation at 170 rpm for 14 days in the dark. To prepare the inoculum for plant trials, the fungal mycelium was passed through cheesecloth and a drop of Tween 20 was added per 20 ml of suspension. The obtained suspension was used to infect the leaf F-3 (3rd leaf under the flag leaf) at the beginning of tillering (GS 19–20) by spraying it evenly until runoff. A control set of plants was sprayed with water. From infected and control plants sampling involved the collection of six to eight plants randomly and separation of leaf layers at medium milk stage (GS 75). The experiment was performed twice.

Results

Specificity and sensitivity of the primers

The ability of the primer pair Rc3 / Rc5 to amplify a 348 bp DNA fragment from *Rcc* DNA (Frei et al. 2007) was tested on purified DNA of *Ramularia collo-cygni* isolate RCC2 as well as on *Rcc* - spiked barley DNA. Serial ten-fold dilutions of the DNA isolated from RCC2 were tested in the PCR protocol (Fig. 1a). The PCR method could amplify a DNA fragment of 348 bp with content of RCC2 DNA as low as 0.005 pg. To determine the impact of plant DNA background in the field samples on the PCR assay performance, *Rcc* DNA ranging from 5 ng to 0.00005 pg was spiked into 100 ng of barley DNA. The lowest amount of *Rcc* DNA yielding a visible Rc3 / Rc5 PCR product on agarose gel was 0.005 pg in spiked samples (Fig. 1b).

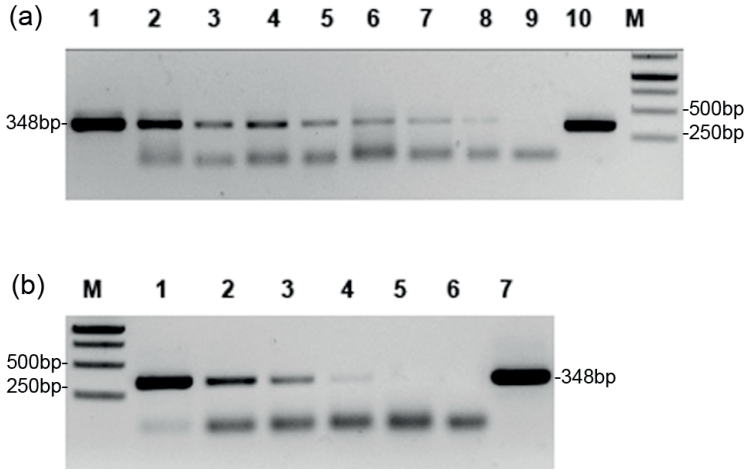

Fig. 1. Sensitivity of the Rc3/Rc5 primer set on *Rcc* strain RCC2. (a) 10-fold serial dilutions of purified *Rcc*. Lanes: 1. 50 ng; 2. 5 ng; 3. 0.5 ng; 4. 50 pg; 5. 5 pg; 6. 0.5 pg; 7. 0.05 pg; 8. 0.005 pg; 9. negative control (SDW); 10. positive control (100 pg of *Rcc* DNA); M. 1.0 kbp DNA ladder Plus (Thermo Scientific). (b) Spiking 100 ng of barley DNA with varying amounts of *Rcc* DNA: Lanes: 1. 5 ng; 2. 50 pg; 3. 0.5 pg; 4. 0.005 pg; 5. 0.00005 pg; 6. negative control (SDW); 7. positive control (100 pg of *Rcc* DNA)

Detection of *Rcc* in field samples of winter barley

In the 2014/15 growing season on Viljandi Variety Testing Centre fields two winter barley cultivars - Fridericus and Cinderella were grown. Necrotic spots or signs of senescence resembling *Rcc* infection were detected in June at flowering stage (GS 61, week 24) on some lower F-3 and F-4 leaves. A 348 bp DNA fragment characteristic to *Rcc* was amplified from extracts isolated from the leaves with necrotic spots of cv. Fridericus and Cinderella (Fig. 2, lanes 4–6 and 10–12 respectively). In healthy barley plants grown in the controlled environment chamber and used as a negative control, the *Rcc* specific 348 bp PCR fragment was not detected, cv. Fridericus and Cinderella (Fig. 2, lanes 1–3 and 7–9 respectively).

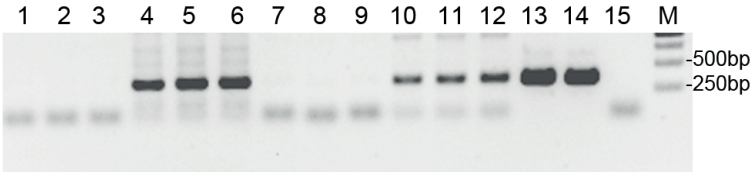

Fig. 2. *Rcc*-specific Primers Rc3 and Rc5 were used for amplification of 348 bp DNA fragment. Lanes: 1–6 cv. Fridericus; 7–12 cv. Cinderella; 13–14 positive control (100 pg of *Rcc* DNA); 15. negative control (SDW). Marker - M 1.0 kbp DNA ladder Plus (Thermo Scientific)

To verify whether the disclosed pathogenic isolates really belong to *Rcc* species, the PCR fragments amplified from both winter barley cultivars were sequenced and the sequences had 98% identity with that of *Ramularia collo-cygni* (*Rcc* accession number http://www.ebi.ac.uk/ena/data/view/PRJEB11432).

Though the *Rcc* DNA was detected in the leaves of both barley cultivars (GS 83, week 25), the seeds of winter barley harvested in 2015 were not contaminated with *Rcc* (Fig. 3). In the growing season of 2015/2016, the monitoring of *Rcc* infection was started in May 2016 at stem elongation stage (GS 32, week 19) and carried on at weekly intervals until the beginning of July 2016. On both barley cultivars, the *Rcc* colonization started at lower leaves and then moved upward (Fig. 4). The first traces of *Rcc* DNA were found at inflorescence emerged stage (GS 57–58, week 22) in the case of cv. Fridericus and only at early dough stage (GS 83, week 25) in the case of cv. Cinderella (Fig. 4), which is respectively 28 and 8 days prior estimation of visible leaf symptoms at hard dough stage (GS 87) of both cultivars. The PCR analysis of seeds harvested from both cultivars in 2016 were not contaminated with *Rcc* (data not shown).

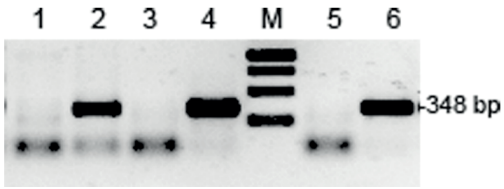

Fig. 3. PCR analysis of *Rcc* DNA in infected winter barley (cv. Fridericus –lanes 1–2 and cv. Cinderella, lanes 3–4) seeds and leaves Lanes: 1. seeds of cv. Fridericus, 2. leaves (GS 83, week 25) of cv. Fridericus, 3. seeds of cv. Cinderella, 4. leaves (GS 83, week 25) of cv. Cinderella, M. 1.0 kbp DNA ladder Plus (Thermo Scientific), 5. negative control (SDW), 6. positive control (100 pg of *Rcc* DNA)

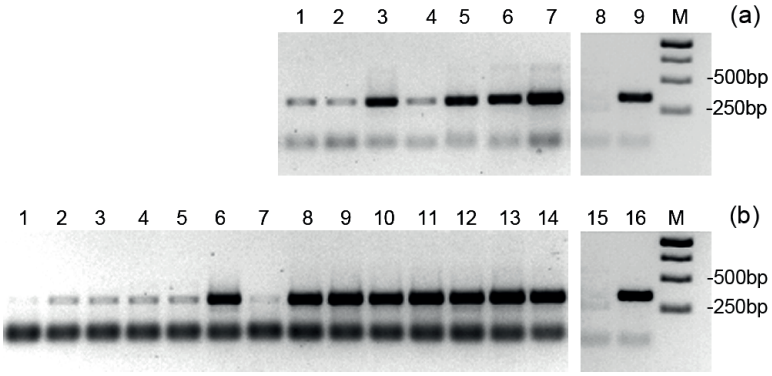

Fig. 4. Transmission of the fungus in winter barley. *Rcc* DNA detected by PCR in F-1, F-2, and F-3 leaves at different growth stages. (a) cv. Cinderella: 1–3. GS 83 of F-1, F-2, F-3 leaves; 4–6. GS 85 of F-1, F-2, F-3 leaves; 7. GS 87 of F-1 leaf; 8. negative control (SDW); 9. positive control (100 pg of *Rcc* DNA). (b) cv. Fridericus: 1–3. GS 57–58 of F-1, F-2, F-3 leaves; 4–6. GS 73 of F-1, F-2, F-3 leaves; 7–9. GS 75 of F-1, F-2, F-3 leaves; 10–12. GS 83 of F-1, F-2, F-3 leaves; 13–14. GS 85 of F-1, F-2 leaves; 15. negative control (SDW); 16. positive control (100 pg of *Rcc* DNA); M. 1.0 kbp DNA ladder Plus (Thermo Scientific)

PCR analysis on *in vitro* infected plants

In the controlled environment chamber experiment, cv. Fridericus seeds of growing season 2014/2015 which were found to be free of *Rcc* contamination (Fig. 3) were used. *Rcc* DNA was detected in all leaves at medium milk stage (GS 75), the fungus was present on flag-leaf, F-1, F-2 and F-3 leaves (Fig. 5a) although necrotic spots were not observed (data not shown). At GS 13, 25 and 75 *Rcc* DNA was not detected in control barley plants (Fig. 5b). Our findings show a correlation between the accumulations of *Rcc* DNA in naturally infected plants in the field (Fig. 4b) and artificially inoculated controlled environment chamber grown plants (Fig. 5a).

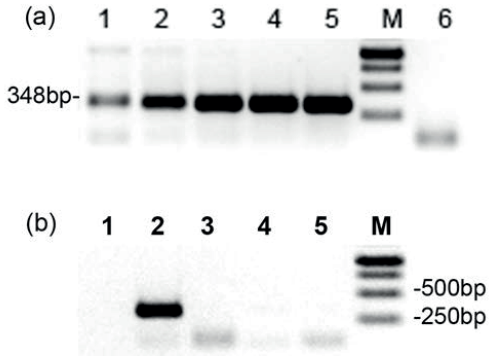

Fig. 5. Detection of *Rcc* DNA in barley cv. Fridericus plants (seeded from the 2015 harvest) in controlled environment. (a) Artificially infected barley at GS 75 in leaves: 1. flag leaf; 2. F-1; 3. F-2; .4 F-3; 5. positive control (100 pg of *Rcc* DNA); M. 1.0 kb DNA ladder Plus (Thermo Scientific); ,6. negative control (SDW). (b) Not-infected barley: 1. negative control (SDW); 2. positive control (100 pg of *Rcc* DNA); 3. GS 13; 4. GS 25; 5. GS 75; M. 1.0 kb DNA ladder Plus (Thermo Scientific)

Discussion

Since the first detection of *Rcc* in spring barley in Estonia in 2012 (Sooväli et al. 2014), the pathogen has rapidly spread in winter barley (Mäe and Sooväli 2018). Accordingly, the urgent need for methods for timely crop protection emerged. The objective of our study was to achieve early detection of *Rcc* infection in local fields and to identify possible sources of *Rcc* infection in winter barley.

During recent years, rapid development of molecular and bioanalytical PCR-based tools have enabled detection of *Rcc* in plant tissues (Taylor et al. 2010, Matusinsky et al. 2011, Havis et al. 2015). Although all primer sets tested appeared to function after optimizing the PCR cycle, primers pair Rc3/Rc5 was proved to be specific with a PCR product of 348 bp (Frei et al. 2007) and was selected because of amplification selectivity and intensity. This set of primers could specifically amplify as low as 0.005 pg of *Rcc* DNA (Fig. 1a) even in complex samples containing the barley genomic DNA (Fig. 1b). Although our experiments had lower sensitivity of a direct PCR method compared to that showed by Frei et al. (2007), we were certainly able to detect the fungal DNA in barley leaves before the disease symptoms became visible. The fungus was present in winter barley already in the beginning of flowering stage (GS 61) before the appearance of the disease symptoms (Fig. 2).

Recent works have shown a seed-borne stage of the fungal life cycle, supporting the assumption of fungus spreading from season to season (Nyman et al. 2009, Matusinsky et al. 2011, Havis et al. 2014). Yet, our results showed that grain harvested in 2015 was not contaminated with *Rcc* (Fig. 3) although it originated from the fields where the infected barley plants of cv. Cinderella and Fridericus were detected. *Rcc* DNA was also not detected in grain harvested in 2016 from the infected barley plants of cv. Cinderella and Fridericus. It can be inferred that the contamination of plants is not the decisive factor for the subsequent spreading of pathogen to seeds. Supposedly testing more different winter barley cultivars and growth conditions could lead to different infecting patterns of the pathogen. The possible reason that affected the spreading and maturation of spores of *Rcc* from May to July in 2015 were the lower average temperature and precipitation. Correlation between the spore release and ambient climatic/environmental conditions has been reported also by Havis et al. (2015). The seeds from harvested winter barley (both cv. Fridericus and Cinderella) in 2015 were used for seeding the fields in the next 2015/2016 growing season. Also, the same batch of seeds of cv. Fridericus was used to grow barley under controlled conditions and no pathogen was found at any growth stages studied (GS 13, GS 25 and GS 75 on Fig. 5b). This proves that the fungus did not move during the growing season 2014/2015 from infected barley leaves to grain. Therefore the infections found during the next 2015/2016 growing season in the fields in both cultivars were probably externally obtained (Fig. 4).

On the contrary, Havis et al. (Havis and Oxley 2006, Havis et al. 2014) demonstrated *Rcc* transmission by seeds although the colonization depended on the presence of an inoculum source, susceptibility of the crop and was largely determined by local environmental conditions (Havis and Oxley 2006, Matusinsky et al. 2011). Our data indicate that comparatively warm and dry weather during late growth stages in July 2015 in winter barley fields did not favour *Rcc* spore germination and point to *Rcc* spreading in the fields of the winter barley via external inoculum. Conidia can be considered as a source of external inoculum in plant infection in the field.

According to Zamani-Noor et al. (2009) conidia of *Rcc* can travel long distances with the help of wind, rainfall or even snow. A possible external inoculum source for *Rcc* infection could be grasses (*Poaceae*) growing next to the crop fields (Heuser and Zimmer 2002). Rcc is shown to infect the model grass Brachypodium distachyon (Peraldi et al. 2014) and pass infection from grasses and weeds into the crops (Kaczmarek et al. 2016). Similar track of transmission has been described also for the wheat pathogen *Zymoseptoria pseudotritici* (Stukenbrock and Bataillon 2012). The infection of barley through external sources was also reported by Nyman et al. (2009) supporting our hypothesis of external origin of the cause of RLS disease under dry environmental conditions.

Different discovery times of *Rcc* infection symptoms were detected for the 2 cultivars studied, just as Havis et al. (2012) demonstrated different susceptibility of cultivars to RLS disease. Some specific genotype-environment interactions in the development and spreading of *Rcc* on different winter barley cultivars (Miethbauer et al. 2003, Oxley et al. 2008, Marik et al. 2011) could explain the differences between cv. Fridericus and Cinderella in the progression of *Rcc* infection.

As the initial source and time of infection have not been clearly identified, the spreading of *Rcc* was analysed in artificially infected cv. Fridericus winter barley in controlled environment. In inoculated plants grown from *Rcc*-free seeds the fungus was detected in emerging leaf layers and moved from the lower to the upper leaves of the plant

similarly to barley plants infected in the field confirming vertical movement of the pathogen (Fig. 5a). Nyman et al. (2009) got similar results with artificially inoculated plants grown from *Rcc*-free seed.

In summary, the impact and role of external inoculum on distribution of RLS was shown by detecting *Rcc* DNA on asymptomatic leaves of winter barley plant sown from uninfected seeds. It is possible that under specific climate and environmental conditions only external sources of infection could be a major threat to barley production. In future studies it is intended to further investigate the other possible sources of inoculum for this pathogen, including other graminaceous hosts. Ryegrass and black-grass could be a major inoculum source for *Rcc*, as it can often be seen growing next to crop fields. Therefore, this issue, together with the climatic conditions that affect the spreading of *Rcc* on barley, should be a priority for continuing research.

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