Characterisation of Ramularia collo-cygni laboratory mutants resistant to Succinate Dehydrogenase Inhibitors

Characterisation of *Ramularia collo-cygni* laboratory mutants resistant to Succinate Dehydrogenase Inhibitors

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Abstract

BACKGROUND: *Ramularia collo-cygni* (*Rcc*) is responsible for Ramularia leaf spot (RLS), a foliar disease of barley contributing to serious economic losses. Protection against the disease has been almost exclusively based on fungicide applications, including Succinate Dehydrogenase Inhibitors (SDHIs). In 2015 the first field isolates of *Rcc* with reduced sensitivity to SDHIs were recorded in some European countries. ~~This~~ In this study we established baseline sensitivity of *Rcc* to SDHIs in the UK and characterised mutations correlating with resistance to SDHIs in UV-generated mutants.

RESULTS: Five SDHI resistant isolates were generated by UV mutagenesis. In four of these mutants a single amino acid change in a target succinate dehydrogenase (Sdh) protein was associated with decrease in sensitivity to SDHIs. Three of these mutations were stably inherited in the absence of SDHI fungicide and resistant isolates did not demonstrate a fitness penalty. There were no detectable declines in sensitivity in field populations in years 2010-2012 in the UK.

CONCLUSIONS: SDHIs remain effective in controlling *Rcc* in the UK. ~~however~~ However given that the first isolates of *Rcc* with reduced sensitivity appeared ~~in 2015~~ in other European countries in 2015, robust anti-resistance strategies need to be implemented ~~in~~ to maintain effective disease control.

1 Introduction

Ramularia collo-cygni (*Rcc*) is the causal agent of Ramularia leaf spot (RLS), a major barley disease in the UK.[1] It can cause yield losses of up to 1 t ha^{-1}, corresponding to around 18% of average yield in the UK.[2] Although there

is an increasing interest in breeding for host resistance, there currently are no lines of barley fully resistant to RLS, although varieties differ in their level of susceptibility to the pathogen.[1,3-6] Therefore protection against RLS remains based on foliar fungicide applications. *Ramularia collo-cygni* has already developed resistance to Quinone outside Inhibitors (QoIs), a fungicide class which initially provided good control of the disease.[7-9] Currently RLS is controlled by a wide range of fungicides comprising Succinate Dehydrogenase Inhibitors (SDHIs), Demethylation Inhibitors (DMIs) and a multisite inhibitor chlorothalonil.[1] Declines in field efficacy to both SDHIs and DMIs have been detected for several plant pathogens.[10-13] Equally concerning are new directives introduced by the European Commission on pesticide registration (Regulation (EC) No 1107/2009). These directives may restrict future use of some of the DMIs and chlorothalonil[14], leading to increasing concern about the provision of effective plant protection in the near future.

SDHIs are rapidly becoming one of the most important fungicide groups for plant protection with resistance to other fungicide classes reported in many crop pathogens.[15] They were initially introduced in 1966 as two active ingredients carboxin and oxycarboxin that showed a good spectrum of activity against a range of basidiomycete pathogenic fungi. [16] Modern SDHIs are broad-spectrum products, with 19 different active ingredients available, used both as foliar applications and seed treatments (FRAC MOA Poster 2016 (www.frac.info)). The current generation of SDHIs was introduced in 2005 for use on cereals in the UK (CRD (https://secure.pesticides.gov.uk/pestreg/)) and are now a mainstay in disease control programmes. In the 2014 growing

season 77% of winter barley and 40% of spring barley received SDHI treatment (summing up all reported actives), and the use of some ingredients such as bixafen increased by 94%, fluopyram by 382% and fluxapyroxad by 173% as compared to the 2012 growing season (all crops surveyed).[17] The extensive use of SDHIs in plant protection combined with the availability of products containing individual SDHI active ingredients has raised concerns over the evolution of pathogen resistance to SDHIs. Straight SDHI products do not provide anti-resistance strategy 'in the can' and whether cereals growers obey the label guidelines on their proper use, using effective mixing partners at the proper dosage remains uncertain.

SDHIs are inhibitors of the mitochondrial respiratory complex II (succinate dehydrogenase, Sdh, EC 1.3.5.1). The target protein of SDHI fungicides, Sdh, consists of four subunits, labelled A-D and it is responsible for oxidising succinate to fumarate and reducing ubiquinone to ubiquinol in the mitochondrial electron transport chain and citric acid cycle.[18-24] SDHIs inhibit fungal respiration by blocking the ubiquinone binding site, which is formed by residues of subunits B (SdhB), C (SdhC) and D (SdhD).[19,22,23,25,26] Sdh subunit A (SdhA) is not involved in forming the ubiquinone binding pocket, and no resistance mutations in this subunit have been described.[15,19] Single amino acid substitutions in SdhB, SdhC and SdhD have been shown to confer resistance to SDHI fungicides. Replacement of the highly conserved histidine residue in the third cysteine rich cluster [3Fe-4S] of SdhB has been linked with reduced sensitivity in lab mutants of *Zymoseptoria tritici* (B: H267Y/L/F/N/Q),[27-29] lab and field isolates of *Botrytis cinerea* (B: H272Y/R/L),[13,30-32] and field

isolates of *Alternaria alternata* (B: H277Y/R).[33] Field resistance to SDHIs had not been commonly detected in cereal pathogens, with the exception of loss of sensitivity mutants to carboxin seed treatment reported for *Ustilago nuda*[34] but recently examples of mutations conferring reduced sensitivity in isolates of *Z. tritici*[11] and *Pyrenophora teres*[Rehfus et al. 2016] pathogens of wheat and barley, respectively, have been reported. Moreover in 2015 the first isolates of *Rcc* showing strong decrease in sensitivity to SDHIs in bioassays, carrying a point mutation in the *SdhC* gene C: H142R and C: H149R were detected in Germany. An aAdditional mutation C: N83S, conferring a low ~~resistant~~ resistance factor *in vitro,* was reported in single isolates originating from Germany, Ireland and Slovenia (FRAC 2015 SDHI Working Group (www.frac.info)). This brings a concern about SDHIs field performance in the coming years and the long-term effective protection against RLS.

Given that the first isolates with decreased sensitivity to SDHIs in lab assays have evolved recently in *Rcc* ~~recently~~ (FRAC 2015 SDHI Working Group (www.frac.info)), it is important to obtain the baseline data to which subsequent testing ~~could~~ can be compared ~~to~~ and ~~investigate~~ to investigate possible consequences that resistant population could have on SDHI's field performance. This study ~~reported~~ reports the current level of sensitivity to SDHI fungicides in *Rcc* in the UK and ~~explored~~ explores the molecular basis of SDHI resistance in UV-induced mutants. Possible mutations in the target *Sdh* gene related to the resistance phenotype were examined at the nucleotide and protein level and fitness tests were conducted to see whether resistance mutations conferred any fitness penalty.

2 Experimental Methods

2.1 *In vitro* sensitivity testing of SDHI-resistant UV mutants and field isolates

In total 62 isolates sampled from barley in the UK in 2010 (n =7), 2011 (n =18) and 2012 (n =37) were tested in fungicide inhibition assays. Samples collected in 2010 originated from untreated plots in spring barley fungicide performance trial at Bush Estate, Scotland. Samples in 2011 originated from both untreated and treated plots of spring barley fungicide performance trial at Bush Estate, commercial fields in West Sussex, England and random plots in field trials at Lanark, Scotland. In 2012 samples were collected from a spring barley pathology SDHI *Ramularia* trial, including both untreated and treated plots. Single spore cultures of *Rcc* were isolated from leaves using a slight modification of the method described by Frei,[35] excluding leaf incubation prior to conidia isolation and using a fine sterile needle instead of a sterile blade. All of the *Rcc* isolates were maintained on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) media amended with streptomycin 5 µg ml^{-1} and/or kanamycin 50 µg ml^{-1}, in a growth cabinet (Sanyo Incubator, MIR-254, Osaka, Japan), in the dark, at 15°C.

Fungal cultures for inhibition assay were cultivated in alkyl ester (AE) broth[27] in 250 ml Erlenmeyer flask containing 150 ml of media. Each flask was inoculated with 150 µl of homogenised mycelium and cultured for 10-12 days in the dark at 16°C with shaking at 120 rpm. Subsequently 5 ml of each culture was homogenised for two minutes at 24000 rpm using an Ultra-Turrax T25 basic homogenizer (IKA®-Werke, GmbH&Co.KG, Staufen, Germany) with reusable plastic blades (T25 S18D, IKA®-Werke). The suspension was

vortexed for an additional minute and filtered through sterile nylon filters with a pore size of 100 µm (Millipore, Darmstadt, Germany). Five SDHI fungicides: isopyrazam, bixafen, boscalid, fluopyram and carboxin (Sigma-Aldrich, Saint Louis, USA) were used in the assay. Each test was performed in a 96 well plate, with three replicates per isolate. To each well 100 µl of mycelial suspension and 100 µl of media containing fungicide at a range of concentrations were added. The final concentration of fungal fragments in the assay was $2.5x10^3$ pieces of mycelium ml^{-1}. Final concentrations of isopyrazam, bixafen, boscalid and fluopyram for field isolates were 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0 mg $litre^{-1}$ and for carboxin were 50, 10, 5, 1, 0.5, 0.1, 0.05, 0 mg $litre^{-1}$. For SDHI-resistant mutants the same range of concentrations plus one additional higher concentration of each fungicide was used. This additional concentration was 50mg $litre^{-1}$ for isopyrazam, bixafen, boscalid, fluopyram and 100 mg $litre^{-1}$ for carboxin. All the mycelium and fungicide dilutions were made in AE broth. Fungicide stocks were prepared in DMSO. The final highest concentration of DMSO in wells was equal to 1% v/v when the highest concentration of carboxin was used (50 mg $litre^{-1}$) and 0.2% v/v for the highest concentration of the remaining four SDHI fungicides (10 mg $litre^{-1}$). Plates were incubated in the dark for seven days at 16°C shaking at 120 rpm (Gallenkamp, cooled orbital incubator, Weiss Technik Konigswinter, Germany). OD_{400} measurements, with 20 flashes per well, were taken at day zero and day seven on a spectrophotometer FLUOstar Omega (BMG Labtech, Offenburg, Germany). Data were analysed using MARS Data Analysis

Software (BMG Labtech). EC_{50} values were calculated from the 4-parameter fit of the standard curve.

Resistance factors (RFs) were calculated as: RF = (EC_{50} value of mutant)/(EC_{50} of parental isolate). The classification of resistance levels was based on Leroux *et al.*[13] However it was calibrated separately for each fungicide using the RFs for the least sensitive isolates from the UK field population. Resistance factors <0.5 were considered as hypersensitive. Normal sensitivity for isopyrazam and bixafen was in the range ≥0.5<2, ≥0.5<5.5 for boscalid, ≥0.5<7.5 for fluopyram and ≥0.5<3.0 for carboxin. Weak resistance for isopyrazam and bixafen was classified as ≥2<10, for boscalid ≥5.5<10, for fluopyram ≥7.5<10 and for carboxin ≥3<10. Resistance factors ≥10<100 were considered as moderate resistance and ≥100 as high resistance.

2.2 Generation of SDHI-resistant UV mutants of *Rcc*

Mutants were developed using fungal mycelium fragments because we failed to reliably generate *Rcc* spores *in vitro*.[36,37] *Rcc* isolate DK05Rcc001ss2 (DK05) was used as the parental isolate for UV mutant generation. It was isolated in Denmark in 2005 prior to the commercial launch of SDHIs (Lise Nistrup Jorgensen pers. com.), from spring barley variety Braemar and was sensitive to QoIs. The genome and transcriptome of this particular isolate ~~were~~ have been sequenced[McGrann et al. 2016] (genome browser: http://ramularia.org/jbrowse). Isopyrazam was chosen as the selection agent to isolate resistant mutants. To define the minimum inhibitory concentration (MIC), isopyrazam in concentrations ranging between 0.0001 and 20 mg litre-

^{1} was added to AE plates.[27] Each plate was then inoculated with 1.5x10^{4} mycelial fragments and cultivated in a phytotron in the dark at 15°C for 18 days. The MIC of isoparazam was the lowest concentration for which growth of wild type isolate was not observed after 18 days.

Selection for SDHI resistance was performed in AE agar amended with 0.05 mg litre^{-1} (MIC) and 0.1 mg litre^{-1} (2x MIC) of isopyrazam. Isolate DK05 was cultivated in AE broth at 16°C in the dark whilst shaking at 120 rpm for seven days. The culture was homogenised, filtered and adjusted to a final concentration of 10^{5} pieces of mycelium ml^{-1}. Pieces of mycelium were counted in Improved Neubauer C-Chip Disposable haemocytometers (Digital Bio, Seoul, Korea) under the compound microscope using a 40x objective (Leica, PL Fluotar 40x/0.70). Each isopyrazam amended agar plate was inoculated with 1.5x10^{4} of mycelial fragments and exposed to UV energy between 12000 and 23000 µJ cm^{-2} in an UV Stratalinker 2400 (Stratagen, San Diego, USA) leading to between approximately 20% and 50% survival. Immediately after UV treatment, Petri dishes were sealed with parafilm and transferred to the dark to avoid the activation of DNA repair systems in the treated mycelial fragments. Samples were incubated for at least 18 days in the dark at 15°C and; any colonies growing on agar after this period were collected (between 22-33 days after UV treatment).

2.3 Characterisation of the *Sdh* gene

Prior to DNA extraction, fungal material was freeze dried overnight and tissue lysed (Tissue Lyser LT, Qiagen, Hilden, Germany). DNA extraction was performed using an Illustra Nucleon PhytoPure Genomic DNA Extraction Kit

according to the manufacturer's guidelines (GE Healthcare Life Sciences, Little Chalfont, UK). Primers for amplifying subunits B, C and D of *Rcc* (SdhC/D_Rcc_Final, Table 4~~Table 4~~) were designed using data from the *Rcc* genome sequence[McGrann et al. 2016] (genome browser: http://ramularia.org/jbrowse). Full *SdhB*, *SdhC* and *SdhD* sequences can be found in GenBank database under accession numbers: KU758973, KU758974, KU758975 respectively. PCR reactions were performed in the following thermocycler: GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). The PCRs were carried out using Go Taq® Green Master Mix (Promega, Madison, USA). The PCR mix comprised 1x Master Mix, 0.2 µM of each primer, 6.25 ng of DNA and sterile distilled water (SDW) up to final volume of 25 µl. To confirm the position of mutations, additional amplification of the final *Sdh* subunits was performed using the FastStart High Fidelity PCR System (Roche, Mannheim, Germany), containing proofreading polymerase. The PCR mix comprised 1x buffer, 0.4 µM of each primer, 200 µM of each dNTPs, 2.5 mM of $MgCl_2$, 2.5 U per reaction of an enzyme blend, 25 ng of DNA and SDW up to 50 µl. Thermocycler conditions included an initial denaturation at 95°C for two minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 30 seconds, extension at 72°C for one minute and a final extension at 72°C for ten minutes. After sequencing, all of the DNA fragments were analysed using Sequence Scanner Software v1.0 (Applied Biosystems).

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2.4 Fitness tests on SDHI-resistant UV mutants

To verify the stability of mutations, mutants were sub-cultured six times on AE agar, not amended with SDHI fungicide and antibiotics. Plates were incubated in the dark at 15°C for seven to ten days between subculture steps. In addition the stability of mutants retrieved from long term storage in 0.25% v/v PDB was verified. Growth of mutants was verified *in vitro* on agar plates in the dark at 15°C. Cultures of the parental isolate DK05 and mutants were cultivated on AE agar without antibiotics and fungicides for three to four weeks. From each isolate an 8mm plug was excised and transferred into the center of a fresh AE agar plate. Five replicates for each culture were prepared. The growth of a colony was measured using an electronic digital caliper after two and four weeks, in four directions, excluding the mycelium plug.

For the detached leaf assay, barley plants of cultivar Optic were cultivated in pots in a Micro Clima Plant Growth Chamber MC1000E (Snijders Scientific, Tilburg, Netherlands) for up to four weeks under the following conditions: 16 hours light at 20°C, 80% humidity (day) and 8 hours dark at 16°C, 90% humidity (night). A detached leaf assay was performed using a modified method described by Thirugnanasambandam *et al*.[36] after Newton *et al*.[38] F-1 and F-2 leaves were used in the experiment. The assay was divided into two parts: untreated control and leaves sprayed with 1 mg litre^{-1} of isopyrazam. Fungicide solutions were prepared in AE broth and, control material was sprayed with AE broth not containing fungicide. Sections around 4 cm long were cut and placed with the abaxial part downwards on 0.5% (w/v) water agar (Oxoid), amended with 1 mM benzimidazole (Sigma-Aldrich). Up to

six leaves were placed into push fit polystyrene boxes of dimensions 79x47x22 mm (Steward-Solutions, Croydon, UK).

Rcc inoculum was prepared from two week old AE agar plates, cultured at 15°C in the dark. SDW (0.5-1 ml) was added to the plate and mycelium was scraped from the colony surface and centrifuged for three minutes at 4000 rpm. It was washed three times with SDW and finally diluted in 1.5 ml of SDW. Each leaf was inoculated in two places with 10 µl of mycelial suspension as described by Thirugnanasambandam *et al*.[36] Both of the drops were inoculated on the same, adaxial part of the leaf, approximately 1.5-2 cm apart, avoiding the midrib. Boxes were incubated in the phytotron under 12 hours dimmed light and 12 hours dark at 15°C, in high humidity conditions to promote fungal growth. Fungal hyphae were stained dark blue with Aniline Blue:ethanol (50:50 v/v) and the infection process was observed under the compound microscope (DM RBE Research Microscope, Leica, Wetzlar, Germany) using 10x, 20x and 40x objectives (PL Fluotar 10x/0.30, 20x/0.50, 40x/0.70) up to 29 days post inoculation (dpi). Images were acquired using a CMEX DC.5000 5Mpix camera (Euromex, Arnhem, Netherlands) and edited using ImageJ[39] and Adobe Photoshop® CS5 (Adobe Systems, San Jose, USA) softwares. The experiment was repeated twice.

2.5 Statistical analysis

Statistical analysis was performed in Minitab v16 (Minitab Inc., State College, USA). One way ANOVA was used to examine differences between the group means in *in vitro* fungicide sensitivity assay. If significant differences between group means were indicated, Tukey's pairwise comparisons were

conducted. Pearson product moment correlation coefficients (r) were used to verify the cross resistance patterns between SDHI fungicides. A correlation of ≤ 0.35 was categorised as weak, 0.36 to 0.67 as moderate, 0.68 to 1.00 as strong, with correlation coefficients ≥ 0.90 described as very strong.[40]

3 Results

3.1 Baseline sensitivity of *Rcc* populations to SDHIs

A fungicide inhibition assay was used to screen 62 UK isolates collected in 2010, 2011 and 2012, for sensitivity to five SDHI fungicides: isopyrazam, bixafen, boscalid, fluopyram and carboxin (Table 1). Isopyrazam and bixafen most effectively inhibited *Rcc* growth, with mean EC_{50} values of 0.019 mg litre^{-1} and 0.015 mg litre^{-1} respectively. Boscalid (EC_{50} = 0.137 mg litre^{-1}) and fluopyram (EC_{50} = 0.151 mg litre^{-1}) also showed good control of the pathogen *in vitro*. Carboxin (EC_{50} = 1.120 mg litre^{-1}) was the least effective fungicide. There were no significant differences between years in sensitivity of *Rcc* populations (Table 1~~Table 1~~) to isopyrazam (*P* =0.216), bixafen (*P* =0.216), boscalid (*P* =0.262), fluopyram (*P* =0.110) or carboxin (*P* =0.079).

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3.2 Identification of the target mutations conferring resistance to SDHIs

Twenty two *Rcc* colonies with putative resistance to SDHIs were isolated after UV mutagenesis (Table S 1). In total 112.5 mycelial fragments were plated out, resulting in overall mutation frequency (collected colonies/total no of plated mycelial fragments) of 2x10^{-5}. These included five isolates (designated Mut1, Mut2, Mut7, Mut8 and Mut11) showing a notable decrease in sensitivity to SDHI fungicides and 17 false positives. False positives were initially isolated from agar plates after UV mutagenesis, however in further testing did not show a decrease in sensitivity to SDHIs *in vitro* and as a consequence were eliminated from the analysis. UV treatments to create verified SDHI-resistant mutants used energy inputs of 18000 µJ cm^{-2} and 22000 µJ cm^{-2}, corresponding to around 20% of colony survival (Figure S 1,

Figure S 2). Mutants were successfully selected both using the MIC (four isolates) and 2x MIC (one isolate) of isopyrazam.

Sequencing of the *Rcc* Sdh subunit genes revealed that in Mut2 there were neither nucleotide nor amino acid changes in genes *SdhB, SdhC* or *SdhD*. However the four remaining isolates each possessed a single nucleotide mutation positioned either in locus *SdhB* (Figure 1, Mut1, Mut11) or locus *SdhC* (Figure 1, Mut7, Mut8).The single nucleotide mutation in *SdhB* of isolate Mut1 conferred an amino acid change from serine (tca) to leucine (tta) at position 217 (B: S217L). In isolate Mut11 the single nucleotide mutation in *SdhB* conferred a change from asparagine (aac) to isoleucine (atc) at position 224 (B: N224I). Both of these mutations are positioned in a region of subunit B that is conserved across the species (Table 2, Figure 1). Mutation of ~~a~~ two distinct nucleotides in the same codon in *SdhC* was observed in Mut7 and Mut8. In the case of Mut7 the wild type histidine (cat) residue at position 142 was substituted with arginine (cgt) (C: H142R), while in the case of isolate Mut8 it was substituted with glutamine (caa) (C: H142Q). As was the case above, this particular residue of histidine in SdhC at position 142 (C: H142) is highly conserved across the species (Table 2, Figure 1).

3.3 Assessment of SDHI resistance associated with mutations

Mut7 (C: H142R) was highly resistant to boscalid fungicide (resistance factor (RF) =1114), and moderately resistant to four other SDHI active ingredients, with high RFs for bixafen (RF =55.31), isopyrazam (RF =44.10), carboxin (RF =32.80) and fluopyram (RF =16.77), compared to the parental isolate DK05 (RF =1) and the least sensitive isolate from the UK field

population (Table 3). Mut8 (C: H142Q) similarly showed the same high level of resistance to boscalid (RF =1114) and moderate resistance to carboxin (RF =19.19), bixafen (RF =10.69) and fluopyram (RF =15.91). However we observed no differences in sensitivity to isopyrazam (RF =0.688) as compared to the parental isolate and the least sensitive isolate from the UK field population (Table 3).

In the case of Mut11 (B: N224I) a moderate level of resistance was observed to most of the tested SDHI fungicides (RF =37.28 for boscalid, RF =12.65 for bixafen, RF =21.82 for fluopyram and RF =13.23 for carboxin), with the exception of isopyrazam to which weak resistance was found (RF =6.758). In contrast, for Mut1 (B: S217L) a moderate resistance was shown only in the case of fluopyram (RF =49.90). For isopyrazam (RF =9.239), boscalid (RF =8.999) and bixafen (RF =2.246) only weak resistance was detected and for carboxin (RF =1.481) there were no changes in sensitivity as compared to the parental isolate and the UK field population (Table 3).

Mut2, which had no detectable mutations in SdhB, C or D, showed moderate level of resistance to isopyrazam (RF =31.55) and bixafen (RF =24.51). For the remaining three active ingredients no differences in sensitivity were observed compared to the DK05 and the UK field population (boscalid RF =1.532, fluopyram RF =1.177, carboxin RF =2.752), (Table 3).

We observed ~~a~~ very strong cross resistance (r =0.901, *P* =0.037) only between isopyrazam and bixafen (Figure S 3). For the remainder of the fungicides the correlations in sensitivity to different fungicides were not significant (*P* >0.05).

3.4 Fitness tests on SDHI-resistant mutants of *Rcc*

1.1.1 Culture characteristics

There were no morphological differences between the mutants and DK05 when grown on agar plates (data not shown). There was no difference in the AE broth liquid culture growth phenotype of the Mut1, 7, 8 and 11 compared to the wild type isolates. However Mut2 liquid cultures had a much darker colouration than wild type (Figure S 4). At this time it is unclear if other uncharacterised mutations are responsible for this aberrant phenotype in Mut2.

1.1.2 Stability of mutations

Most of the mutations correlating with resistance to SDHIs in *Rcc* were stable, with the exception of Mut8 (C: H142Q) in which the target mutation was not detected after the subculturing process. In long term storage, we noted a mixture of resistant and wild type alleles during the sequencing.

1.1.3 *In vitro* plate growth assay

Significant differences in growth on agar plates between DK05, Mut1, Mut7, Mut8 and Mut11 were indicated at both time points, after 14 days (P <0.001) and 28 days (P <0.001), (Figure 2). After 14 days we observed significantly faster growth than for the wild type for Mut7 (C: H142R) and Mut11 (B: N224I), (P <0.05); after 28 days faster growth was observed only for Mut11 (B: N224I), (P <0.05), (Figure 2). The test was performed separately for Mut2 because it failed to grow in the first experiment. In the case of Mut2 we detected no significant differences in growth on agar plates compared to the wild type after 14 days (P =0.532) and 28 days (P =0.916), (Figure 3).

1.1.4 In planta leaf assay

The infection process was examined for two mutants, Mut11 (B: N224I) and Mut7 (C: H142R) and compared to the development of parental isolate DK05. Colonisation of untreated barley leaves by both *Rcc* mutant isolates and isolate DK05 occurred in a very similar manner, typical of the infection process described previously.[36] *Rcc* infection of barley began with formation of an extensive hyphal network on the leaf surface (Figure 4a) and entry through stomatal pores. Sporulation occurred from 8 dpi onwards from distinctive swan neck conidiophores on top of which spores developed (Figure 4b). Disease symptoms, initial pepper-like spots expanding to small, brown to blackish necrotic lesions, on the untreated leaf segments were observed macroscopically from 25 dpi for all three isolates used in this study (data not shown). This showed that Mut11 and Mut7 could infect the host plant barley, reproduce successfully by generating spores and complete their life cycle, further suggesting that there was no measurable fitness penalty associated with the target mutations conferring resistance to SDHIs.

Infection by isolate Mut11 and Mut7 was not affected by foliar isopyrazam application at a concentration of 1 mg litre^{-1}. Both mutants were able to form an epiphytic hyphal network on the leaf surface and penetrate multiple stomata (Figure 4d). Conidiophores developed, resulting in abundant sporulation, both in stomata and on the leaf surface (Figure 4e). The progressing infection of both mutants led to red discolouration of the guard and surrounding epidermal cells (Figure 4g), followed by rapid development of the typical RLS symptoms from 28 dpi by both Mut7 (Figure 4h) and M11 (Figure 4i). In contrast the

growth of the parental isolate DK05, was clearly inhibited after treatment with isopyrazam (Figure 4f). Some hyphae attempted to colonise the leaf surface. However there was a lack of highly branched and controlled epiphytic growth of hyphae, no subsequent infection of stomata, and no disease development was observed (results not shown).

4 Discussion and conclusions

This study has shown that ~~presents the baseline sensitivity of~~ *Rcc* populations ~~to SDHIs~~ in the UK presently remain sensitive to SDHIs. This is~~,~~ ~~which is~~ of high relevance given that in 2015 the first field isolates with decreased sensitivity to SDHIs in lab assays ~~have been~~were reported in some European countries ~~in 2015~~ (FRAC 2015 SDHI Working Group (www.frac.info)). Furthermore using UV induced mutants we have characterised mutations correlating with resistance to SDHIs in *Rcc* and assessed some of ~~its~~ their fitness parameters, giving an insight into the possible behaviour of a resistant population.

Two mutants Mut7 (C: H142R) and Mut8 (C: H142Q) generated by UV mutagenesis carry a mutation of the highly conserved histidine residue in SdhC and are highly resistant to boscalid. The exact same mutation as in the case of Mut7 (C: H142R) was reported in *Rcc* isolates showing strong decrease in sensitivity to SDHIs in *in vitro* assays in Germany (FRAC 2015 SDHI Working Group (www.frac.info)). Additionally the replacement of this particular amino acid in SdhC has previously been linked with resistance to SDHIs in both *A. alternata* (C: H134R)[41] and *P. teres* (C: H134R)Refhus et al. 2016 field isolates, as well as in a laboratory mutant of *Z. tritici* (C: H145R).[28] Histidine residue C: H142 was not predicted to be involved directly in ubiquinone binding and reduction in the *Z. tritci* Sdh protein model.[28,29] However, this histidine residue has been shown to ligate with heme b and its polar propionate side chains form an integral part of the ubiquinone binding pocket, explaining the loss of sensitivity to SDHIs in mutants carrying variant

C: H145R.[28] In the light of this evidence we suggest that histidine C: H142 acts as a ligand for heme b in *Rcc*, and this explains the reduced sensitivity to inhibitors of mitochondrial respiratory complex II in mutant *Rcc* isolates carrying variant C: H142R/Q.

Mutation of the serine residue found in Mut1 (B: S217L) has so far only been correlated with resistance to SDHIs in a laboratory mutant of *Z. tritici* (B: S218F)[28] and has not to date been found for any field resistant pathogen. The mutation we detected in *Rcc* Mut11 (B: N224I) has been reported to confer SDHI resistance in both artificially induced mutants and in naturally occurring fungal isolates. The *Z. tritici* laboratory mutant carrying the corresponding asparagine mutation (B: N225I) exhibits reduced SDHI sensitivity,[28] while *B. cinerea* laboratory mutants[44] and field isolates[13,30] carrying the equivalent mutation (B: N230I) are resistant to SDHIs. Substitution of the same asparagine residue, this time by threonine, was described recently for SDHI resistant field isolates of *Z. tritici* (B: N225T), (FRAC 2014 SDHI Working Group (www.frac.info)). Although these particular amino acid positions in SdhB in the *Z. tritici* Sdh model were not predicted to be involved in forming the ubiquinone binding pocket, they were positioned in the vicinity of key residues involved in SDHI binding.[28,29] Both of the mutations in Mut1 and Mut11 could have a long-distance effect on the architecture of the ubiquinone binding pocket, which then explains the sensitivity loss they cause towards inhibitors of mitochondrial respiratory complex II as proposed by Scalliet *et al*.[28] None of the substitutions corresponded to a replacement of a conserved histidine residue (B: H266 in *Rcc*) in a third cysteine rich cluster [3Fe-4S] of SdhB, found

to be responsible for resistant development to SDHIs in lab mutants of *Z. tritici* (B: H267Y/L/F/N/Q).[27-29] However, given the limited number of mutants generated in this study, it cannot be excluded that such mutations could develop and contribute to SDHIs efficacy loss in the field.

In the case of Mut2 we observed no amino acid changes in the target Sdh enzyme which could be linked with the resistance to some of the SDHIs, isopyrazam and bixafen. Although alterations of the target gene are the most common mechanism responsible for sensitivity loss towards SDHIs among plant pathogenic fungi,[15,19] they are only one of the possible known mechanisms conferring resistance to such fungicides. SDHI-resistant isolates of *Corynespora cassiicola*,[48] *A. alternata*[49] and *B. cinerea*[13] have been reported with no sequence mutation of the Sdh subunit genes. ~~For the~~At present ~~moment~~ there is no evidence as to~~of~~ which of these mechanisms could be responsible for the resistance patterns in Mut2 and further work is needed to investigate this phenomenon. However given that Mut2 was only resistant to some of the SDHIs tested, overexpression of the target gene or its multiple copies seem to be the two most likely possibilities.

We noted positive cross resistance profiles in this study only between isopyrazam and bixafen; for the remaining SDHI active ingredients tested we observed a lack of cross resistance. Although FRAC classifies inhibitors of mitochondrial respiratory complex II as cross resistant[15], recent studies have demonstrated a lack of cross resistance between newer SDHIs. [19,28,29,44,50,51] In *Rcc* SDHI- resistant mutants the resistance profiles varied notably between the mutated isolates and were strongly associated with the particular position

of amino acids. For example Mut7 (C: H142R) was highly resistant to boscalid but moderately resistant to the other four tested SDHIs. Mut11 (B: N224I) was moderately resistant to all SDHIs tested, with the exception of isopyrazam to which weak resistance was observed. This suggests that different mutations could differently influence the affinity of SDHIs to the target site, explaining the limited positive cross resistance among mutated isolates. Additionally it cannot be ruled out that additional mutations, outside the *Sdh* gene, ~~incurred~~ occurred as a consequence of UV mutagenesis studies, and have an impact on the sensitivity profiles of *Rcc* UV mutants.

Commented [MP1]: I had to change this section. Assuming that Mut8 might have been picked up as mixed colony our previus discussion in that point was irrelevant and too speculative

No measurable fitness penalty associated with resistance to SDHIs was observed in terms of radial colony growth on agar plates in any of the mutated isolates. Additionally *in planta* assays performed for two isolates, Mut7 (C: H142R) and Mut11 (B: N224I), indicated that both of the mutants were able to colonise the leaf and effectively reproduce in untreated barley leaves as well as barley leaves treated with isopyrazam. These results are consistent with previous studies on SDHI-resistant mutants of *Z. tritici*, which were able to colonise the leaf, cause symptoms and produce spores, despite the impaired enzyme activity due to mutation.[28,29] Although the concentration of isopyrazam used in this study of 1mg litre^{-1} may not give a good measure of fitness in the presence of commercial rates of fungicide application, it provides a good estimation of fitness in the presence of rates sufficient to eliminate non-resistant genotypes. Thus the extrapolation of this data to field conditions should be taken with caution. More fitness tests on a wider variety of traits, especially on recently emerged reduced sensitivity field isolates of *Rcc,* should

be performed to fully understand the behaviour of resistant population and their influence on SDHIs field performance. Additionally it will be important to verify if the recorded field mutations reappear in the following seasons, and if ~~yes~~ so, in what frequency. In this study most of the mutations were stable in the absence of fungicide, except Mut8 (C: H142Q). Mut8 was either undergoing the process of reversion to the wild type haplotype at the *SdhC* gene or was originally picked up as a mixed colony of a wild type and a mutant which could explain differences in its sensitivity profiles as compared to Mut7 (C: H142R). This suggests that mutations responsible for SDHI resistance in *Rcc* ~~could~~ can be stable, a possibility which needs further verification.

This study presents baseline sensitivity of *Rcc* populations to SDHIs in the UK and ~~analysis~~ analyses the possible behaviour of a resistant population using UV mutants. Further UV mutagenesis studies on *Rcc* population are required to verify the possibility of ~~the~~ other mutations occurring. These should then be incorporated into a molecular monitoring assay, together with any mutations occurring in the field conditions, allowing detection of any amino acid changes associated with resistance at the field level. At the same time it is important to carry on *in vitro* sensitivity testing in order to detect any possible non-target site mechanisms of resistance, which could have been missed ~~i~~due to reliance ~~f relying only~~ on molecular screening tests alone. *Ramularia collo-cygni* has been exposed to SDHI fungicides since 2005 in the UK and recent population genetic studies have suggested that it has a high potential for evolutionary adaptation.[9,55,56] Based on the current evidence and the recent reports of *Rcc* isolates with decreased sensitivity to SDHIs in some European

countries (FRAC 2015 SDHI Working Group (www.frac.info)), we conclude that the risk of resistance development in *Rcc* to SDHIs in the UK is high and robust anti-resistance strategies should be implemented in order to prolong SDHIs' effective life span. The long term effective control of RLS in barley requires integrated management systems, which cannot be based exclusively on fungicide applications but should include a combination of chemical control and resistant varieties, a strategy that needs to be revised and implemented in a relatively short time.

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7 Tables

Table 1 Mean EC_{50} values (mg $litre^{-1}$) of UK populations of *Ramularia collo-cygni* tested for five SDHI fungicides.

Number of *Rcc* isolates tested from UK					
2010	7				
2011	18				
2012	37				
Total	62				
Mean EC_{50} values in each year					
	Isopyrazam	Bixafen	Boscalid	Fluopyram	Carboxin
2010	0.028	0.016	0.079	0.106	0.959
2011	0.018	0.013	0.127	0.128	0.891
2012	0.019	0.016	0.152	0.172	1.252
Over a period of 3 years	0.019	0.015	0.137	0.151	1.120
Range of EC_{50} values for UK population over 3 years					
Min.	<0.001	<0.001	0.006	0.024	0.113
Max.	0.056	0.056	0.475	0.551	3.981

Table 2 Overview of amino acid and nucleotide substitutions in *Sdh* subunits genes responsible for resistance to SDHIs in *Ramularia collo-cygni*.

Mutant	AA changes in the subunits B and C	Corresponding codon change	Mutations described at the same position for other plant pathogens
Mut1	B: S217L	t**c**a>t**t**a	*Z. tritici*: B: S218F (lab mutant)[28]
Mut11	B: N224I	a**a**c>a**t**c	*Z. tritici*: B: N225I (lab mutant)[28] *Z. tritici*: B: N225T (field isolate)[a] *B. cinerea*: B: N230I (field isolate)[13,30]
Mut7	C: H142R	c**a**t>c**g**t	*Rcc:* C: H142R (field isolate)[b] *Z.tritici*: C: H145R (lab mutant)[28] *A. alternata*: C: H134R (field isolate)[41] *P. teres*: C: H134R (field isolate)Refhus et al.2016
Mut8	C: H142Q	ca**t**>ca**a**	As above
Mut2	no AA changes	na	na

[a] FRAC 2014
[b] FRAC 2015

Table 3 Resistance factors of five SDHI-resistant *Ramularia collo-cygni* mutants.

	EC_{50} values of parental isolate and UK population (mg litre^{-1})				
	Isopyrazam	Bixafen	Boscalid	Fluopyram	Carboxin
DK05	0.044	0.036	0.090	0.075	1.489
UK pop. range[a]	<0.001-0.056	<0.001-0.056	0.006- 0.475	0.024-0.551	0.113-3.981
	RFs (EC_{50} of mutant/ EC_{50} of parental isolate)				
DK05	1.000	1.000	1.000	1.000	1.000
Mut1 B: S217L	9.239	2.246	8.999	49.90	1.481
Mut11 B: N224I	6.758	12.65	37.28	21.82	13.23
Mut7 C: H142R	44.10	55.31	1114	16.77	32.80
Mut8 C: H142Q	0.688	10.69	1114	15.91	19.19
Mut2 No AA subst.	31.55	24.51	1.532	1.177	2.752
Highest EC_{50} from UK pop.	1.274	1.560	5.294	7.320	2.673

[a] UK population range = 62 isolates

Table 4 Primer sets used to amplify *Ramularia collo-cygni SdhB*, *SdhC* and *SdhD*.

Subunit	Primer name	Primer sequence 5'-3'
SdhB	SdhB_Rcc_Final_F	CAAATCACACACCATCCAGT
	SdhB_Rcc_Final_R	CCAGCCCTCTTTACATCCTC
SdhC	SdhC_Rcc_Final_F	CACTCCAGCAAACCACGACC
	SdhC_Rcc_Final_R	TAAAGCAGTTCTGTTGCTCT
SdhD	SdhD_Rcc_Final_F	TTCCACCACAACACCACCCACC
	SdhD_Rcc_Final_R	TCATCTCATCACCACACCCT