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Screening for QoI resistance in UK populations of *Rhynchosporium secalis*

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This is the final report of an 18 month project (RD-2009-3645) which started in June 2009. The work was funded by a contract for £55,257 from HGCA.

CONTENTS

1. ABSTRACT

The development of strobilurin (QoI (quinone outside inhibitors)) fungicide resistance is now widespread in many fungal plant pathogens. However, this group of fungicides is still widely used to control *Rhynchosporium secalis* in winter and spring barley crops across the UK and have so far retained their efficacy. Resistance to this fungicide group in the majority of fungal pathogens is the result of a single point mutation found at either codon 143, 129 or 137 in the cytochrome *b* gene. During 2008, routine screening of the *R. secalis* populations by BASF discovered that some isolates in northern France had developed a mutation at codon 143.

This research examined the UK population of *R. secalis* over two growing seasons (2009-2010) for the occurrence of the mutations causing resistance to QoI fungicides with the combined use of molecular techniques and 96 well plate fungicide sensitivity assays During this project, a high throughput pyrosequencing assay was developed to examine populations directly from field samples. This coupled with the fungicide bioassays and a simple Polymerase Chain Reaction Random Amplified Length Polymorphism (PCR-RFLP), (a simple enzyme methodology that cuts DNA into small parts only if mutations causing fungicide resistance are present) indicated that the UK *R. secalis* population remained fully sensitive to QoI fungicides during the period of this project (2008-2010) with no declines in efficacy. However, this does not mean that resistance will not develop in the coming years and ongoing independent screening will be required to monitor the situation in the coming growing seasons.

2. SUMMARY

2.1. Introduction

Leaf blotch caused by *Rhynchosporium secalis* is the most economically important disease of barley in the United Kingdom. The estimate for barley production in the UK is 5.7 million tonnes, which has a value of £770 million at £135/tonne (Defra, 2011 based on the 5-year average data). The losses from disease in winter barley were estimated to be £5m and of this £2.57m resulted from that caused by *R. secalis* (Defra, 2011 data).

The development of strobilurin (QoI (quinone outside inhibitors)) fungicide resistance is now widespread in many fungal plant pathogens. However, this group of fungicides is still widely used to control *R. secalis* in winter and spring barley crops across the UK and have so far retained their efficacy. Resistance to this fungicide group in the majority of fungal pathogens is the result of a single point mutation found at either codon 143, 129 or 137 in the cytochrome *b* gene.

During 2008, routine screening of the *R. secalis* populations by BASF discovered that some isolates in northern France had developed a mutation at codon 143 (FRAC www.frac.info). The presence of this mutation was reported to cause *R. secalis* to become completely resistant to QoI fungicides. However, despite the development of the first field isolates of *R. secalis* resistant to QoI fungicides, the occurrence of the mutation was found at a low level and from only one location.

Once resistance develops in a crop it can be expected to develop quite rapidly thereafter, providing the resistance is stable. The risk to the UK from QoI fungicide resistance in *R. secalis* is considered to be high, due to the seed borne nature of this disease and the ability of the pathogen to develop stable resistance, thus putting further pressure on the sterol demethylation inhibitors (DMI) or azole groups, which have already been shown to be less effective than in the past (Oxley and Burnett, 2010).

Screening for fungicide resistance is not widely performed in the UK and a very limited amount is currently available in the public domain. Less than 12 isolates of *R. secalis* were tested by BASF for the whole of the UK in 2008, despite the widespread use of QoI fungicides in barley disease control. Therefore, this project was considered vital to assess independently the current situation of QoI resistance in *R. secalis*.

2.2. Materials and methods

Molecular-based techniques were used in this project to screen single spore isolates of the fungus from a wide variety of locations across the UK including the south-west of England, south-eastern England, East Anglia, Scotland and Northern Ireland. Single spore isolates are the strains of the pathogen grown from a single piece of the fungus, either a single spore or mycelium fragment, these individuals isolates are genetically distinct and uniform making them suitable to check for their resistance status. The molecular methods enabled accurate screening for single point mutations that can cause QoI fungicide resistance. In total 110 individual isolates had the target cytochrome *b* gene partially sequenced to check for the presence of any nucleotide changes and over 300 isolates were checked for the G143A mutation using a Polymerase Chain Reaction Random Amplified Length Polymorphism (PCR–RFLP). This simple test allows the screening of isolates for the presence of known mutations following the digestion with a restriction enzyme that only digests to the presence of the mutation causing QoI fungicide resistance. A similar number of field samples were also checked using the newly developed pyrosequencing assay. These techniques are all widely used in the agrochemical industry for screening for resistance.

This project also widely tested isolates of the fungus using a more traditional fungicide bioassay to calculate the half-maximal effective concentration EC50 values. These data were then compared to the resistant isolates (from northern France) which were used as a control in the bioassays.

2.3. Results

The range of EC_{50} values in the sensitive population was found to be between 0.007 to 0.35 µg/ml, whereas the resistant isolates consistently produced an EC_{50} value of around 6.4 µg/ml, showing that the occurrence of the G143A mutation had a dramatic effect on fungicide performance through a single change in the target cytochrome *b* gene changing a glycine to alanine at codon 143 (G143A). The combined screening using both conventional bioassays and a high throughput-sequencing assay has allowed many more samples to be tested during this project than originally proposed. However, the results of this study showed that no mutations causing QoI fungicide were detected in any UK samples during the project duration.

2.4. Conclusions

The mutation causing resistance has not been found in the UK, during the lifetime of this project. This is probably either because there are very low numbers of resistant strains in the UK or because currently the mutations are not present. However, the data generated do show some background variation in the EC_{50} values generated. This means that the efficacy of this fungicide group is variable and may suggest that resistance could occur in the future, if a single point mutation could arise. However, the EC_{50} values that have been generated for this project are all within the expected range for full field control. While the outcomes of this project are positive for the continued use of QoI fungicides, it should be noted that resistance has been shown to occur in a natural population and the reasons that this resistance has not spread are currently not clear. Therefore, the future of QoI fungicides for the widespread control of *R. secalis* may be in doubt in the future as the selection pressure of fungicide use could continue to drive the evolution towards a fully resistant population.

3. TECHNICAL DETAIL

3.1. Introduction

Leaf blotch infections caused by *Rhynchosporium secalis* are common in winter and spring barley grown in the whole of the UK (Zhan *et al*., 2008), but disease is especially severe in the west of England and in Scotland due to increased humidity. The pathogen is also known to infect plants other than barley including rye, triticale and several wild grasses (Caldwell 1937). However, in recent years research has identified that the fungus that infects grasses can be a different at the sequence level and has therefore been reclassified (Zaffarano *et al.,* 2006, and 2008). The levels of disease in barley crops can alter from year to year, with the cost of losses in winter barley estimated at £2.57 million due to *R. secalis* infection alone in fungicide treated crops (Defra data 2011).

Initial infection can result from infected barley stubble and/or crop debris on the soil (Stedman, 1977; Fitt *et al.,* 1986) and most commonly occurs when successive barley crops have been grown in the same field. The pathogen can also infect seed; this inoculum source has been known for a number of years following the original work of Skoropad, (1959). In the recent years Fountaine *et al.,* (2010) investigated the role of seed infection as a primary inoculum source for leaf blotch in winter barley crops. This research demonstrated that this source of inoculum has probably been overlooked because of the long latency of the disease. It appears that *R. secalis* can be transmitted from seeds, but may produce no symptoms in the plant for several months, and currently almost none of the commercial seed treatments used by growers are active against *R. secalis* infection contained in the seed (Havis *et al.,* unpublished data). This has very important implications for the spread of new more aggressive races of *R. secalis* strains from one location to another.

Fungicides are mainly used in Europe to control leaf blotch and other principal fungal diseases of barley, due to the high level of disease pressure compared to other areas of the world, where cultivar resistance is primarily used to reduce disease occurrence. Fungicide resistance has been found to have developed against many of the groups used to control this disease. During the 1970s and 80s, *R. secalis* was controlled by the use of two groups of fungicides: the methyl benzimidazole carbamates (MBCs) and the sterol demethylation inhibiting fungicides (DMIs). Both of these fungicide groups were successfully used to control *R. secalis* either alone or in a mixture up until the early 1990s, after which MBC fungicide resistance was first detected (Kendall *et al*., 1994). However, since the 1980s there has also been a gradual shift in the sensitivity of some of the older DMI chemistry such as epoxiconazole (Cooke *et al.,* 2004).

During 1996 a newer group of fungicides was introduced to control cereal diseases and this group belonged to the Quinone outside Inhibitors (QoIs). These were found to be highly effective against a wide number of diseases affecting barley crops and were widely used. This fungicide group has a single mode of action, which blocks the electron transport chain, thereby inhibiting respiration (Bartlett *et al.,* 2002). However, during 1998 the first cases of resistance occurred in the powdery mildew (*Blumeria graminis*) fungus on both wheat and barley crops. The cause of this resistance was the result of single point mutation found in the cytochrome *b* gene changing one amino acid from glycine to alanine at codon 143 (G143A). This change in the amino acids causes a high level of resistance to QoI fungicides. Other mutations can also occur but these do not cause a high level of resistance; the most common of these partial resistance mutations is the F129L, which causes a change from phenylalanine to leucine. An example of a barley pathogen containing this mutation is *Pyrenophora teres* (net blotch). Another mutation that also causes moderate levels of resistance is the mutation G137R that causes a change from glycine to arginine, however, this mutation has only been found at very low levels in *Pyrenophora tritici-repentis* causing tan spot. Based on the current knowledge, G143A and F129L or G137A mutations display difference resistance factors (RFs) (RF = Effective doses $_{50}$ (resistant strain)/Effective dose $_{50}$ (sensitive wild type strain)), ranging from 5 to 15 for F129L and G137A and usually greater than 100 for G143A (www.FRAC.info).

The effective concentration for control of a pathogen is often quoted in EC_{50} values (half-maximal effective concentration), the concentration of a fungicide that induces a response halfway between the baseline and the maximum after some specified exposure time. It is commonly used as a measure of fungicides potency.

The development of QoI fungicide resistance in *Rhynchosporium secalis* was first reported in France during the growing season ending in 2008 by BASF at a Fungicide Resistance Action Committee (FRAC) meeting (www.frac.info). They reported that a few individual isolates of *R. secalis* with the mutation at the codon 143 were detected following routine screening causing these isolates to have a high level of QoI fungicide resistance. Usually when resistance develops in a population, it will then be normal for the selection pressure to shift to the individuals that have the selective advantage. Therefore, since the lifecycle and epidemiology of *R. secalis* is now more clearly understood, it was concluded that the spread of QoI fungicide resistance from France may occur via seed through the international seed trade.

The aim of this project was to screen *R. secalis* single spore isolates and barley plant and seed samples for the presence of the mutations in the fungal cytochrome *b* gene conferring resistance to QoI fungicides. This study was carried out using a combination of both fungicide sensitivity assays and molecular techniques to detect and quantify levels of single nucleotide polymorphisms (SNP).

3.2. Materials and methods

3.2.1. Isolation of *Rhynchosporium secalis* from leaves

Samples were selected from years 2008, 2009 and 2010 and from various locations across the UK from the south-west of England, south-eastern England, East Anglia, Scotland and Northern Ireland. These samples were variously collected from infected fields, either from trials or sent directly to SAC from growers. The isolations were made from leaves with *R. secalis* symptoms. Information on sample location, collection date, cultivar and fungicide applications was also noted. The leaves from the field were dried for at least 24 hours in paper before attempting isolation. Each lesion was cut from the leaves leaving a small amount of green leaf area around it. Lesions were then surface sterilised for 2 minutes in 70 % (w/v) ethanol followed by five minutes in 10 % (w/v) sodium hypochlorite and then rinsed in sterile distilled water for a minute. The lesions were then dried on tissue paper before being transferred to CDM agar plates (Czapek Dox agar, 0.5 % mycological peptone (Oxoid, Basingstoke, UK), 50 μg/ml kanamycin, 10 μg /ml streptomycin). Four sterilised lesions were placed on the surface of each agar plate and incubated for three days at 17°C. After this period, the fungal hyphae were visible in small clumps. Using a dissecting microscope and a sterile needle the single hyphae were then transferred and cultured on CDM agar plates the resulting colonies were regarded as single spore isolates. In addition, some multi spore isolates were also obtained, with the only difference in the isolation procedure being that multiple hyphae were transferred from the leaves and cultured on CDM agar plates. The multi spore isolates were used in the PCR-RFLP and pyrosequencing only and not in the fungicide sensitivity assays.

3.2.2. Isolation of total DNA

Plant or fungal material was placed in a pestle and mortar and ground to a powder in liquid nitrogen. To this powder, DNA extraction buffer was added. This consisted of 0.1 % (v/v) β-mercaptoethanol, 250 mM NaCl, 200 mM Tris-HCl, 25 mM EDTA, 2 % (w/v) SDS, 2 % (w/v) polyvinylpyrrolidone and 5 mM 1, 10-phenanthroline monohydrate; pH 8.0. Enough extraction buffer was added to produce a pouring consistency, the amount used varied according to the sample. An aliquot of 800 μl of sample/extraction buffer was transferred to a 1 ml tube and stored in the –20°C freezer until all the samples were ready to be further processed. Samples were then incubated for 30 mins at 70°C in a water bath, then 800 μl of ice-cold ammonium acetate (7.5 M) was added and the suspension kept on ice for 30 mins. The suspension was centrifuged for 10 mins at 14,000 rpm and the supernatant transferred to a fresh 2 ml tube containing 600 μl of ice-cold isopropanol. The tubes were inverted several times to mix and stored at room temperature for 15 mins. The tubes were again centrifuged at 14,000 rpm for 10 min and the supernatant removed,

leaving a DNA pellet. The pellet was washed by adding 600 μl of 70 % ethanol, centrifuging at 14,000 rpm for 10 min and then removing the ethanol. The pellet was allowed to dry in a fume cupboard for about 10 mins and re-suspended in 100 μl of sterile distilled water.

3.2.3. Amplification and sequencing of the cytochrome *b* gene

The cytochrome *b* gene was amplified from a selection of *R. secalis* isolates from across the UK, with the addition of two resistant isolates supplied by BASF. An initial DNA fragment of the cytochrome *b* gene (759 bp) was amplified using the primer set RSCBF3 and CBR3 (Table 1) using the following standard PCR in a Biometra T3 thermocycler (Biotron GmbH, Gottingen, Germany) with 1.25 units of Red Hot DNA polymerase (ABgene, Epsom, UK) using 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0, 0.01 % Tween 20, 1.5 mM $MgCl_2$, containing 125 μM of each dNTP, 0.5 μM primers and 1 ng template DNA in a final volume of 100 μl. The conditions to amplify part of the cytochrome *b* gene from *R. secalis* were: 94°C for 2 min 30 s, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min and 30 s and a final extension at 72°C for 8 min 30 s. PCR products were separated on a gel red stained (Biotium, CA, USA) 1.3 % (w/v), agarose gels run in Tris-borate-EDTA buffer (TBE: 89 mM Tris base, 89 mM Boric acid, 2 mM EDTA, pH 8.0) and exposed to UV light to visualise DNA fragments. Following amplification, these fragments were purified with the High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany) and the PCR products ligated directly into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA). Plasmids were transformed into *Escherichia coli* JM109 cells (Promega) according to the standard protocol by Sambrook *et al*. (1989). Plasmid DNA was extracted using the RPM Kit (Bio101 Inc, Carlsbad, California, USA). The nucleotide sequence was then determined in a dideoxy chain termination method reaction (Sanger *et al*., 1977; Rao *et al*., 2001) at Durham University sequence centre.

Table 1. Oligonuceotides tested for amplifying the initial fragments of the cytochrome *b* gene. Degenerate bases R= A + G, K= G + T, W= A + T.

Primer Designation	**Sequence (5'-3')**
Forward primer	
RsCBF3 (degenerate)	TTTARKTATACAAATWRTAACAGG
Reverse primer	
CBR3	CCTAATAATTTATTAGGTATAGATCTTA

3.2.4. PCR-RFLP

DNA extracted from single and multi spore isolates of *R. secalis* were used for this simple test for the G143A mutation following the procedure described by Torriani *et al.,* (2009). DNA concentration was initially measured on the Nano drop spectrophotometer (Thermo-Scientific Ltd) and diluted to 2.5 ng/µl. The PCR amplifications were carried out under the following conditions: initial denaturations at 96^{o}C for 2 min, followed by 35 cycles of 96^{o}C for 1 min, 50^{o}C for 1 min, and 72^{o}C for 1 min, with a final extension at 72^{o}C for 5 min. PCR products were separated on gel red stained (Biotium, CA, USA) 1.3 % (w/v) agarose gels run in Tris-borate-EDTA buffer (TBE: 89 mM Tris base, 89 mM Boric acid, 2 mM EDTA, pH 8.0) and exposed to UV light to visualise DNA fragments. A portion of each PCR product (10 µl) was digested using 1U *Fnu4*HI (New England Biolabs, England) for 4 h at 37^{o}C. This restriction enzyme specifically cuts at the mutated sequence found at codon 143 (GCT), but will not cut in the presence of the wild type sequence (GGT). The digests were then visualised on a 1% agarose gel with 1x TRIS-borate-EDTA. Each time this procedure was carried out for UK *R. secalis* samples, DNA from the resistant isolates collected from France was always run as a control.

3.2.5. Pyrosequencing assay

A set of primers for a pyrosequencing assay was designed using the Pyrosequencing Assay design software (Version 2.0, Qiagen, Germany). These primers are shown in Table 2; this table also shows the forward primers labelled with biotin. The PCR reaction were carried out in a Biometra T3 thermocycler (Biotron GmbH, Gottingen, Germany) with 1.25 units of Red Hot DNA polymerase (ABgene, Epsom, UK) using 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0, 0.01 % Tween 20, 1.5 mM $MgCl_2$, containing 125 µM of each dNTP, 0.5 µM primers and 20 ng of template DNA in a final volume of 50 µl. The PCR conditions were: 95^{o}C 2 min, followed by 40 cycles at 95^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 30 secs, with a final DNA extension at 72^{o}C for 5 mins. The resulting PCR products were run on an agarose gel to show the DNA amplification. The PCR products were then immobilized to Sepharose beads by adding 2 µl of Streptavidin Sepharose HP beads (GE Healthcare) to 40 µl of binding buffer (Qiagen) and mixed with 15 µl of PCR sample and 25 µl of Milli-Q sterile water in small PCR tubes (200 µl) tubes at room temperature for 5-10 minutes at 1400 rpm. The following step was to wash the beads containing the captured DNA products using 70% v/v ethanol for 5 seconds, followed by a denaturation step for 10 seconds and a neutralisation steps with washing buffer as recommended by the manufactures Qiagen. The beads were then released into a PyroMark Q24 plate with 0.3 µM sequencing primer (Table 2) in 25 µl annealing buffer in each well. The plate was then transferred to a heat block for at 2 minutes and then cooled to room temperature for at least 5 minutes. The plates were then processed in the Pyromark Q24 machine according to the manufactures instructions using the PyroMark Gold Q24 Reagent kit (Qiagen).

Table 2. Primers for use in the pyrosequencing assay carried out on the Pyromark Q24.

Primer Designation	Sequence (5'-3')
Forward Primer	
Biotinylated forward primer*	*TGTGCTTCCTTCCTTATGGACAGATG
Reverse Primer	
RsPyroR1	AAAAACCCCCTCAGATAAACTCA
Sequencing primer	
RsPyroSeq1(Reverse)	GATTAGTTATAACTGTGGCA

3.2.6. Fungicide sensitivity testing

This procedure was carried out for all the samples of *R. secalis* directly isolated from leaves so that the EC_{50} values could be calculated from each isolate from a range of different locations. This was performed using the MARS software (BMG Labtech) which automatically calculated the EC_{50} values using the sigmoidal curves using the three replicate data samples. Once isolated the fungus was first grown on CDM agar for maximum of two weeks, the cultures should not be too old as this will dramatically affect spore production. The actively growing cultures were then transferred to Glucose – Gelatine media (Glucose 4 g, Gelatine 4 g, KH2PO4 1.7 g, MgSO4 0.75 g and 1 litre of ionised water). The upper growth of actively growing *R. secalis* isolates growing on agar plates were transferred into small Bijou bottles containing 7 ml of sterile GG media. The bottles were then stored in the fridge for seven days to allow for spore production to occur. A 96 flat well plate was setup with GG media amended with fungicides. To each of the wells a volume of 180 µl amended media was added, the fungicide concentrations used were 10, 1, 0.1, 0.01 and 0.001 ppm respectively with two blanks containing media alone. To each of these wells 20 µl of spores were added to the three replicates of each isolate. The plate setup is shown in Figure 1. The plates were then placed on the 96 well plate reader (BMG Labtech FLUO star omega, Offenburg, Germany)) and the absorbance measured for each well at 492 nm. The plates were then covered with a sterile sealing strip and placed on a plate shaker set at 100 rpm and covered in the dark at room temperature. The plates were then read on the plate reader after 7 and 14 days. The results of the absorbance are then calculated to produce an EC_{50} value for each of the isolates.

	1	2	3	4	5	6	7	8	9	10	11	12
	Isolate 1			**Isolate 2**			**Isolate 3**			**Isolate 4**		
A	Blank			Blank			Blank			Blank		
B	10 ppm fungicide			10 ppm fungicide			10 ppm fungicide			10 ppm fungicide		
C	1 ppm fungicide			1 ppm fungicide			1 ppm fungicide			1 ppm fungicide		
D	0.1 ppm fungicide			0.1 ppm fungicide			0.1 ppm fungicide			0.1 ppm fungicide		
E	0.01 ppm fungicide			0.01 ppm fungicide			0.01 ppm fungicide			0.01 ppm fungicide		
F	0.001 ppm fungicide			0.001 ppm fungicide			0.001 ppm fungicide			0.001 ppm fungicide		
G	0 ppm fungicide			0 ppm fungicide			0 ppm fungicide			0 ppm fungicide		
H	Blank			Blank			Blank			Blank		

Figure 1. The 96 well plate setup for the fungicide sensitivity assays.

3.3. Results

3.3.1. Sequencing of QoI fungicide resistant isolates of *Rhynchosporium secalis*

Sequence data were obtained from two resistant and two sensitive isolates of *R. secalis*. The data in Figure 2 show the occurrence of a point mutation at codon 143 changing a glycine to alanine at codon 143 (G143A). This mutation causes a high level of resistance affecting the fungicide performance in the field. The isolates with the mutation at codon 143 were all supplied from BASF and came from a single location in northern France in the 2008 growing season. Isolates collected from around the UK were also screened to check for the occurrence of the G143A mutation and others such as F129L and G137A, but none were observed in all the 110 isolates that were checked. These individual isolates were selected from years 2008, 2009 and 2010 from locations from across the UK (a few of these samples are shown in Figure 3). The sequence data obtained were uniform with no variation due to location; this confirms the results obtained from Torriani *et al.,* (2009) who also observed no polymorphisms in the sequence data.

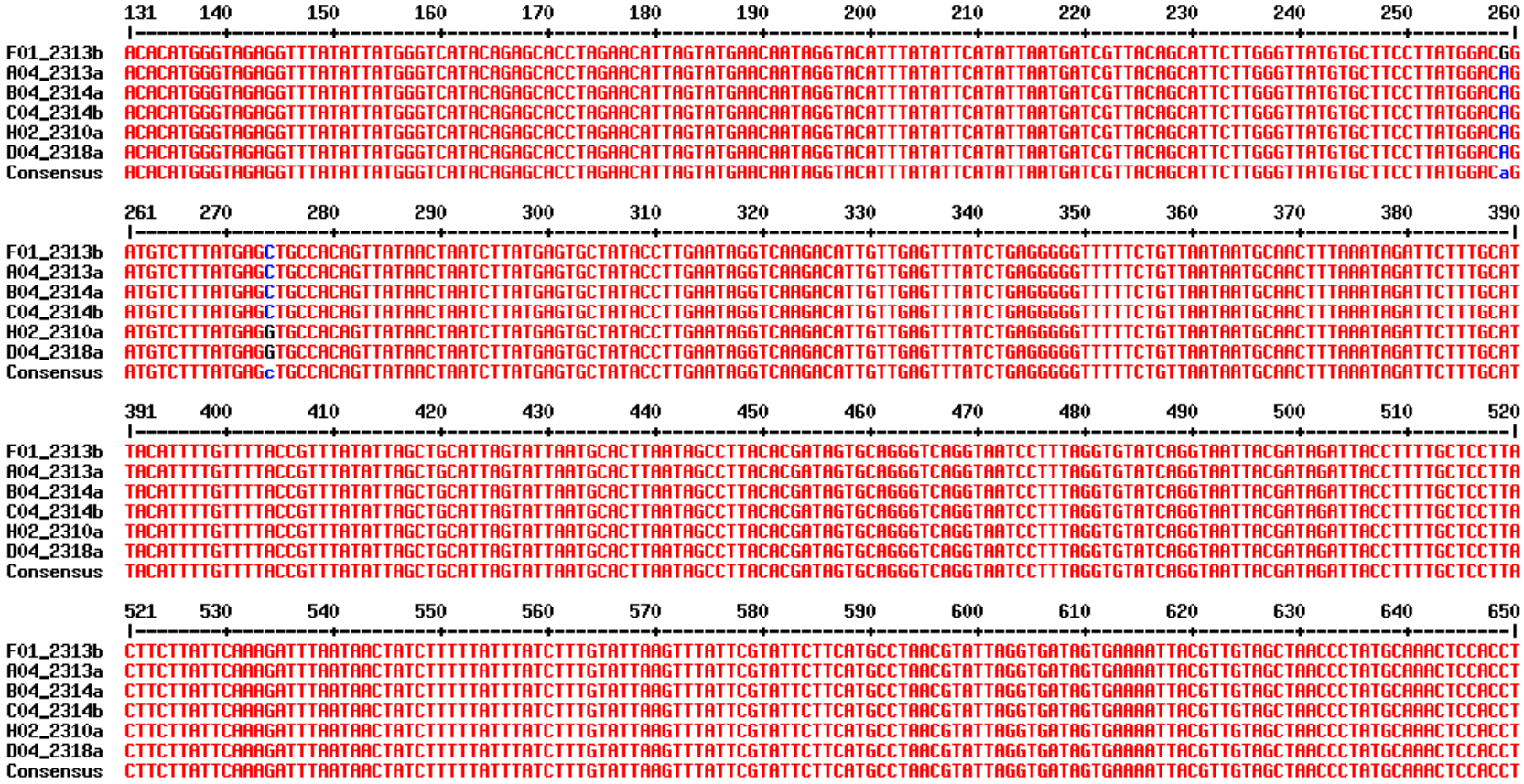

Figure 2. A partial sequence of the cytochrome *b* gene, the blue oval shows that point mutation at codon 143 changing a G to C in four samples from northern France and two sensitive isolates from the same location which show the sensitive sequence.

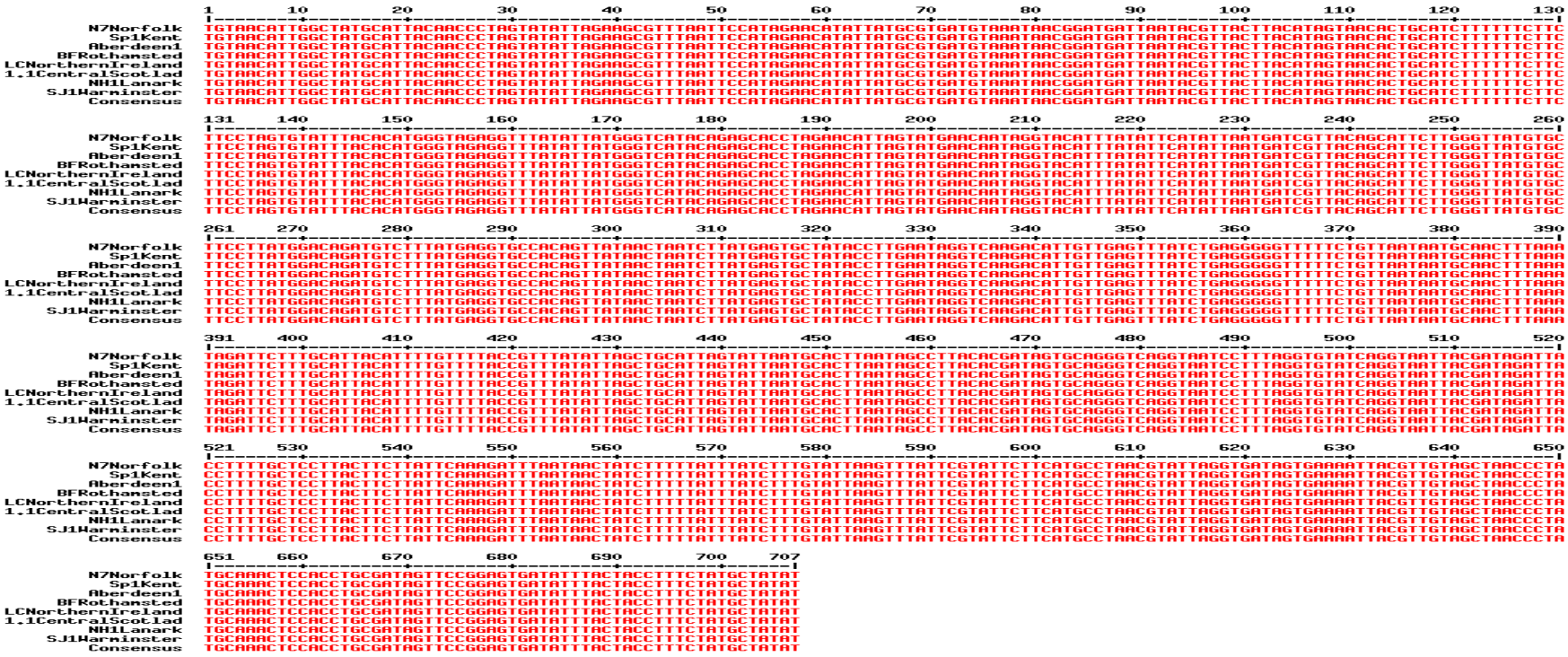

Figure 3. A partial sequence of the cytochrome *b* gene from a range of UK isolate, showing the high conserved nature of this gene across locations. No mutations are seen in codon 143 indicated by the blue oval.

3.3.2. PCR-RFLP results

The data obtained from nearly 300 isolates of *R. secalis* confirmed that the G143A mutation was not detected in any of the samples collected from the UK. The PCR samples were always tested at the same time as a positive control, to show that digestion of the PCR products was able to take place. This positive control used DNA from the resistant isolate collected from northern France by BASF. The gel picture shown in Figure 4 clearly shows a 100 bp marker and next to this a PCR fragment which has been cut into two fragments at 418 bp and 234 bp, this is sensitive sample, has no mutation at codon 143, and therefore will not be cut in to three fragments. However, the next lane shows a PCR which is cut into three distinct fragments, at 274, 234 and 144 bp respectively and is highly resistant. The original size of the PCR product used for the PCR RFLP was 652 bp and the number of base pairs adds up to this when put together. The digestions in the rest of the gel picture show that no mutations at the G143A could be detected. In total nearly 300 samples from across the UK were tested using the PCR-RFLP. This test is a simple test that can be performed on isolates should any results from a bioassay suggest or dramatic shift in efficacy be observed in the field. **However, in this study no resistance could be detected originating from the UK.**

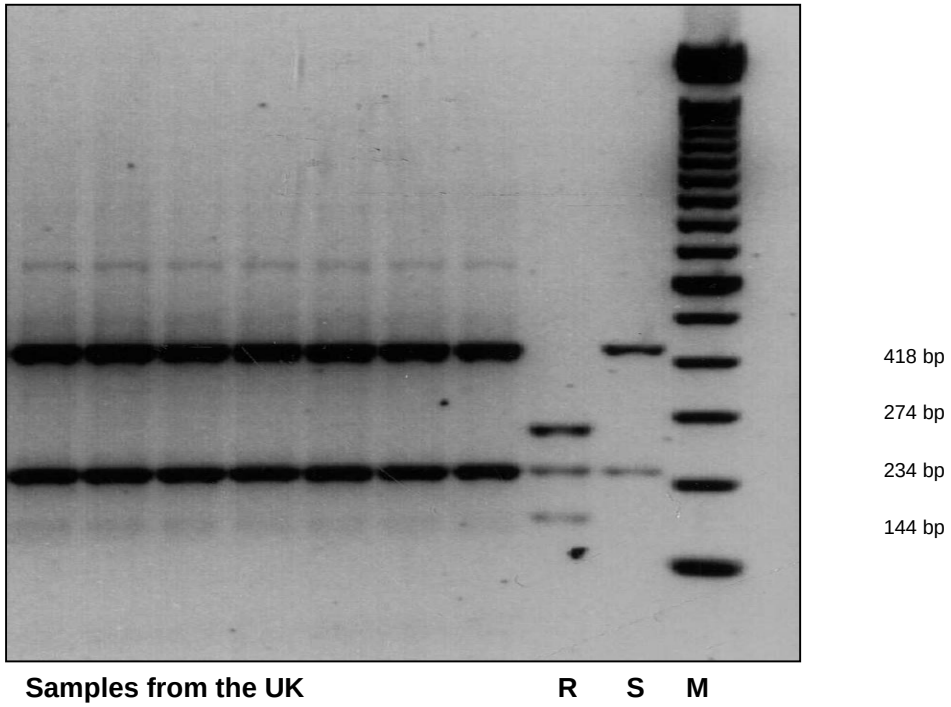

Figure 4. PCR RFLP digestion following the treatment with the restriction enzyme *Fnu*4HI. **R** indicates the PCR products of the DNA from the resistance isolate collected from northern France with three fragments. **S** indicates the sensitive isolate showing only two PCR fragment showing that no mutation was at codon 143. The samples from the UK also show that mutation was not present in these samples. **M** indicates the 100 bp marker.

3.3.3. Pyrosequencing

The use of pyrosequencing has been developed as part of this project as it will allow for the detection of mutations at codon 143 in populations of leaf samples collected from around the UK. This type of assay is now being routinely used in the agrochemical industry to detect small mutations in fungicide target genes. This assay allows for DNA to be used directly from leaves containing *R. secalis* by directly sequencing a small part of the cytochrome *b* gene. The assay has been extensively tested and only amplifies *R. secalis* samples without the detection of other fungi, which may be found in the leaf. Figures 5 and 6 show the output that is obtained for the sensitive and resistant isolates of *R. secalis*. The assay was also tested by mixing both a sensitive and resistant isolates together and extracting DNA so as to be able to detect the proportion of both alleles in a population. The assay detection can detect 10 % resistance alleles in a 90 % sensitive population, and an example of one of these mixing experiments is shown in Figure 7. This assay is now fully working and can be used a screening method if efficacy issues should arise in the future. Screening for the G143A mutation using this technique was extensively used for samples sent in by farmers and advisors following the SAC farmer open evenings during 2009 and 2010. Samples were also screened from a wide range of locations across the UK and from existing HGCA trials. The development of this assay has allowed a greater number of samples to be screened than originally proposed for this research; however, at present the levels of resistance appear to be at lower levels than the assay detection threshold or are not yet present in the UK.

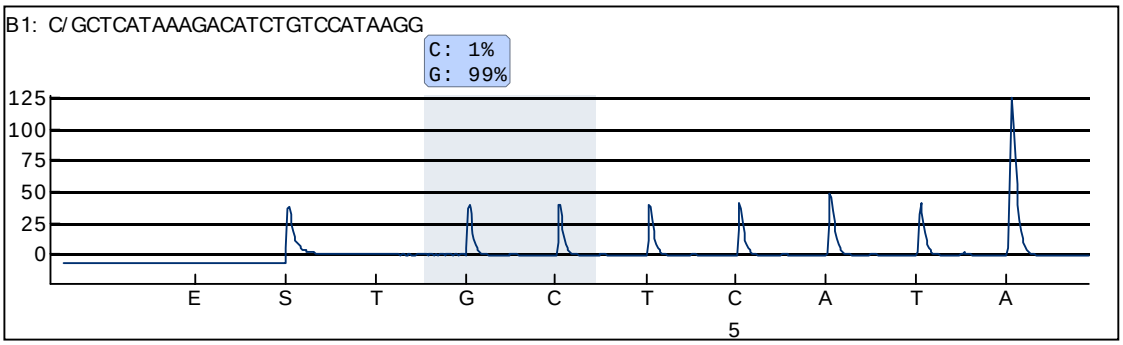

Figure 5. Pyrogram for a resistant type mutated isolate of *R. secalis*, which is fully resistant to QoI fungicides. Note the sequencing primer for this assay is on the reverse sequence.

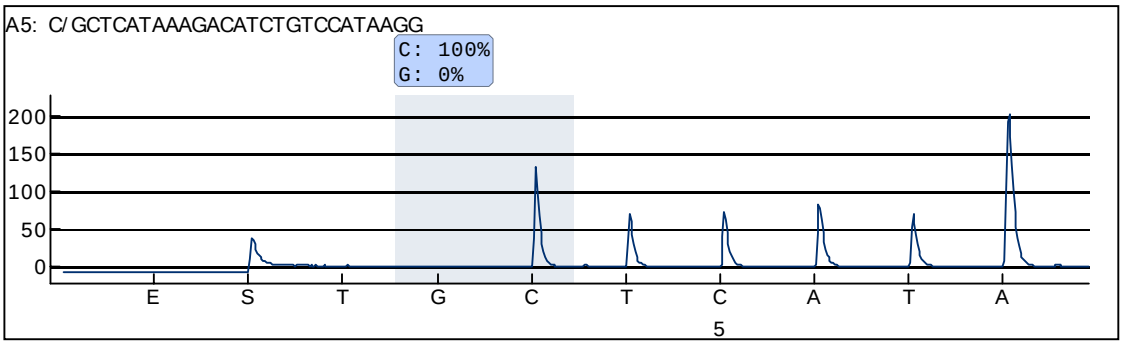

Figure 6. Pyrogram for a wild type non-mutated isolate of *R. secalis*, which is fully sensitive to QoI fungicides. Note the sequencing primer for this assay is on the reverse sequence.

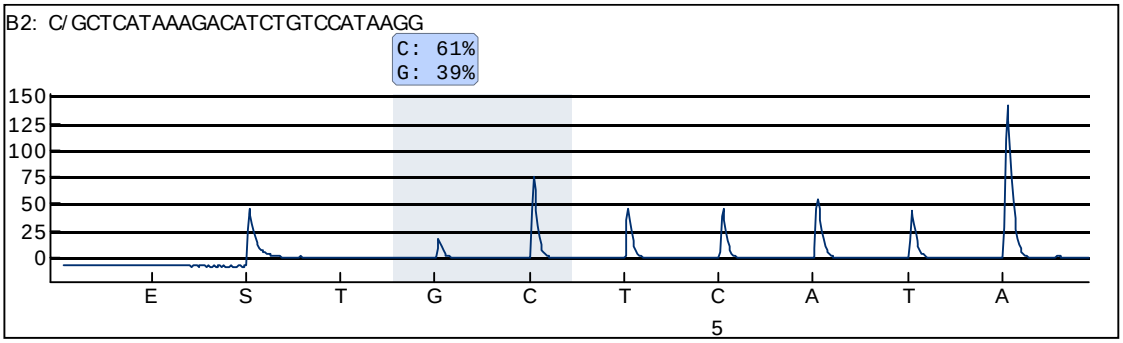

Figure 7. Pyrogram showing allele detection from a mix of both resistant and sensitive *R. secalis* DNA, this sample shows the results of a mixture of one third resistant DNA mixed with two thirds of sensitive DNA.

3.3.4. Fungicide sensitivity results

In addition to molecular based assay this study also used a standard 96 well plate bioassay test, and calculated the half maximal effective concentration (EC_{50}) value for single and mixed spore isolates of *R. secalis* from across the UK. The resistant isolates from northern France showed higher EC_{50} values than those observed in all isolates tested from across the UK. However, the EC_{50} values obtained from this wide range of isolates do show that a variable range of values, demonstrating the natural variation that can occur in all isolates. The values obtained from a selection of these isolates are shown in Table 3.

Table 3. Shows the range of EC_{50} generated from isolates collected from across the UK (all of these isolates are fully sensitive and do not contain a mutation at codon 143). In addition, this table also shows the EC_{50} values obtained from two resistant isolates collected by BASF in northern France.

Isolate name	Location	Year of collection	Isolated by	EC_{50} value in µg/ml
C240	Central Scotland	2008	SAC	0.001
C17A	Lanark, Scotland	2010	SAC	0.03
C86b	Bush, Edinburgh	2009	SAC	0.12
SP1-11-3	Belfast, NI	2008	SAC	0.036
N-3	Dereham, Norfolk	2010	SAC	0.013
N-7.1	Morley, Norfolk	2009	SAC	0.001
SJ1	Warminster, Wiltshire	2009	SAC	0.007
S1.5	Auchencairn, Scotland	2009	SAC	0.35
S1.5	Dumfriesshire, Scotland	2010	SAC	0.032
2313	Northern France	2008	BASF	6.43
2314	Northern France	2008	BASF	6.32

3.4. Discussion

The results of this survey of the UK *R. secalis* population show that although QoI resistance can develop in this plant pathogen it is not currently found in the UK at detectable levels that would have an economical impact on growers. This is an important finding for UK growers as it means that QoI fungicide resistance will not have an impact on disease control at this current time.

The methods used in this project have been shown to accurately detect small amounts of mutated DNA in the presence of non-mutated wild type alleles and as a result, these will be important tools for the future detection of a mutation at codon 143 should it develop in the UK in the coming years.

The isolates that developed in northern France also appear to have declined and have not been detected in the field since 2008 (Gerd Stammler (BASF) personal communication). The reason the resistant isolate in France has not been detected either elsewhere in France or in the UK is not currently clear and further investigation may be needed to understand if these isolates have a fitness cost due to a mutation at codon 143. However, this has not been observed in other plant pathogens affecting cereal crops such as that of *Ramularia collo-cygni* in barley (Fountaine and Fraaije, 2009).

As seed infection is part of the life cycle in *R. secalis,* it will be important to screen population from around the world for the importation of resistance into the UK. This can now be done as result of this project and independent screening will become necessary to inform growers in the coming years.

The results of this project have shown that the UK has a QoI sensitive population. However, the results also show that the populations of *R. secalis* do have a wide range of EC_{50} values to QoI fungicides and this may be due to activity of the Alternative Oxidase (AOX) pathway, which may be more active in some isolates than in others. The AOX pathway is found in some fungi and allows a fungicide treated pathogen to grow by activating by using an alternative biochemical pathway. However, this alternative can only allow for up to 40 % of normal activity and therefore this type of growth does not have any impact on these fungicides when used at commercial rates (Miguez *et al.,* 2003).Further investigations are needed to understand the role of this pathway in *R. secalis*. Unpublished work carried out in Japan by Fountaine and Ishii has demonstrated that AOX gene expression levels in QoI resistant isolates of *Botrytis cinerea* were expressed at slightly higher levels than those found in sensitive isolates. Therefore, the levels of AOX gene expression in *R. secalis* may be different in isolates which have gone on to develop QoI fungicide resistance and the development could be due to the oxidative damage that can occur due to the increased use of this pathway in a fungi (Hideo Ishii, personal communication).

The main message that has emerged from this project is that *R. secalis* has been shown to have the ability to develop high levels of resistance to QoI fungicides with the development of the G143A mutation. This fungus has previously developed resistance to MBC fungicides and has been shown to develop quite large shifts in sensitivity to the DMI fungicide group. Therefore, despite the recent introduction of the new SDHI fungicide group, the overall number of active groups controlling this disease continues to decline. This means that in future there maybe more pressure on the QoI fungicide group and this may mean greater selection pressure that could be to the advantage of resistant isolates that may develop resistance even with a slight fitness penalty to its growth. Therefore, continued monitoring will be needed of this pathogen into the future.

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