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**Citation for published version:**

Fontaine, JM & Fraaije, BA 2009, 'Development of QoI resistant alleles in populations of *Ramularia collo-cygni*' Paper presented at The second European *Ramularia* Workshop, Edinburgh, United Kingdom, 7/04/09 - 8/04/09, pp. 123.

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## **Development of QoI resistant alleles in populations of *Ramularia collo-cygni*.**

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### **Summary**

*Ramularia collo-cygni* first appeared as a major pathogen of barley in Scotland during 1998 and was found to be easily controlled by the fungicide azoxystrobin (Amistar). This type of fungicide was therefore recommended for *Ramularia* leaf spot disease control. However, during 2002 there was a sharp decline in the efficacy of azoxystrobin, when used to control *Ramularia* leaf spot. It was therefore assumed that fungicide resistance had developed, although it was not confirmed by either bioassay or sequencing. This study showed that mutations causing QoI fungicide resistance were present in almost all recently obtained *R. collo-cygni* isolates from across Europe.

**Key words:** *Ramularia collo-cygni*, Strobilurin, QoI, Fungicide resistance.

### **Introduction**

*Ramularia* leaf spot first appeared in Scottish crops around ten years ago and was found to be most effectively controlled by azoxystrobin (Amistar, Syngenta) (Oxley *et al.* 2002). However this situation changed rapidly around 2002 when the control achieved by this fungicide group sharply declined. It was therefore assumed that fungicide resistance had developed. However, no further Scottish investigations were carried out to prove this until October 2007.

Strobilurin and other QoI fungicides were first introduced into UK agriculture in 1996 and subsequently became the largest selling fungicide for use in arable crops. However in 1998 QoI field resistance was found for the first time in wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*). A single point mutation was observed at codon 143 resulting in the replacement of a glycine (ggt) by an alanine (gct). The mutation found at codon 143 is widely known to cause complete fungicide resistance and has been found in a range of plant pathogens, such as *Mycosphaerella graminicola* and *Mycosphaerella fijiensis* both of which are closely related to *Ramularia* according to the genetic study by Crous *et al.*, (2001).

Therefore, this study was carried out to investigate if the decline of efficacy in QoI fungicides was the direct result of these or other mutations. In addition to this the Rothamsted Hoosfield spring barley archive started in 1852 was used, to see if *Ramularia* was present in England during this time period and if so when did QoI fungicide resistance first develop there. These studies aim to understand, when, where and how fungicide resistance developed and it is hoped that the information gained will give us an insight to the evolutionary potential of this pathogen. Using this information may allow us to design future spray programmes that can slow or prevent the development of future fungicide resistance problems.

## Material and methods

### *Fungicide sensitivity testing*

These were carried out in 24 well plates with each well containing 15 ml of potato dextrose agar and SHAM (an alternative oxidase inhibitor). Each of the well was then amended with a different concentration of technical grade azoxystrobin (Syngenta, Bracknell, UK). The concentrations of fungicide used in this study ranged from 0.0064 to 100 ppm. The plates were then incubated at 16 °C in the dark for 7 days before assessing the degree of growth for each individual isolate.

### *DNA extraction and sequencing*

DNA was isolated using the method described in Fountaine *et al.* (2007) from both fungal and plant material. The total DNA was then quantified and diluted to a working stock solution of 5 ng/µl. A 675 bp fragment was initially amplified from the target cytochrome *b* gene using primers CBF1 and CBR3 previously used in studies of both *Blumeria graminis* and *Mycosphaerella graminicola* (Fraaije *et al.*, 2002 & 2005). Following PCR and cloning, the plasmids were then sequenced at the University of Durham sequence centre. The sequences were then aligned using ClusterW ([www.ebi.ac.uk](http://www.ebi.ac.uk)) before designing specific primers for the cytochrome *b* gene (Fountaine & Fraaije, manuscript under preparation)

### *Real-time PCR and PCR-RFLP*

Using newly designed specific primers (Fountaine & Fraaije, manuscript under preparation) for the detection of Ramularia, both a PCR-RFLP and real-time PCR assay using SYBR green were carried out. Combining these two experiments enabled us to both accurately quantify the levels of Ramularia from leaf samples, but also to show which samples contained mutations for QoI fungicide resistance. The quantitative real-time PCR was performed in a Stratagene MX3000p using SYBR green with standard PCR protocols. The samples that showed positive PCR products for Ramularia were then all treated using the digestion enzyme *AluI* (Roche diagnostics), which 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 results of the digestion experiment were then run on an agarose gel stained with Gel Red (Biotium Ltd) to show the DNA samples under UV light

## Results

The results of the bioassays showed the sample B1 isolated from Denmark in 2005 was fully sensitive to QoI fungicides. However, all isolates from 2007 from both Scotland and Denmark were completely resistant.

The results from both sequencing and fungicide sensitivity assays fully complemented each other for all of the isolates tested to date. The results of the sequencing data shows that a mutation was present in the cytochrome *b* gene located at codon 143 changing glycine (ggt) to alanine (gct) in all of the resistant isolates. This is the same mutation present in all highly QoI resistant plant pathogens. No other mutations were seen in all of the sequences, this included the lesser resistance mutation found at codon 129 in some plant pathogen, such as Net blotch.

The results of the PCR-RFLP showed that the mutation at codon 143 could be easily detected using this simple digestion with the results from Scotland and Denmark showing that all 2007 isolates were completely digested, indicating that only the mutated DNA sequence was present. The primers designed for this work were also used in the development of a real-time PCR system which enabled us to quantify the amount of Ramularia infection from leaves and then subsequently carry out a PCR-RFLP digestion to determine if a mutation was found in the population of Ramularia. The results obtained from the Rothamsted Hoosfield spring barley

archive showed that low levels of the pathogen could be detected during the last century; however the levels of this now important disease first significantly increased in the last 10 to twelve years. This was carried out in both leaf and seed material, with a similar results pattern for both with figure 1 showing the leaf material. The results obtained from the PCR-RFLP digestion also revealed that the development of complete QoI resistance in the Rothamsted archive was first detected in 2002, and from then on the population remains fully resistant. Further to this work, samples from both Norfolk in the UK and Northern France were also examined for the development of QoI fungicide resistance and the results obtained from the PCR-RFLP showed that mutated DNA was present in both locations at close to 100 % in all the leaf samples.

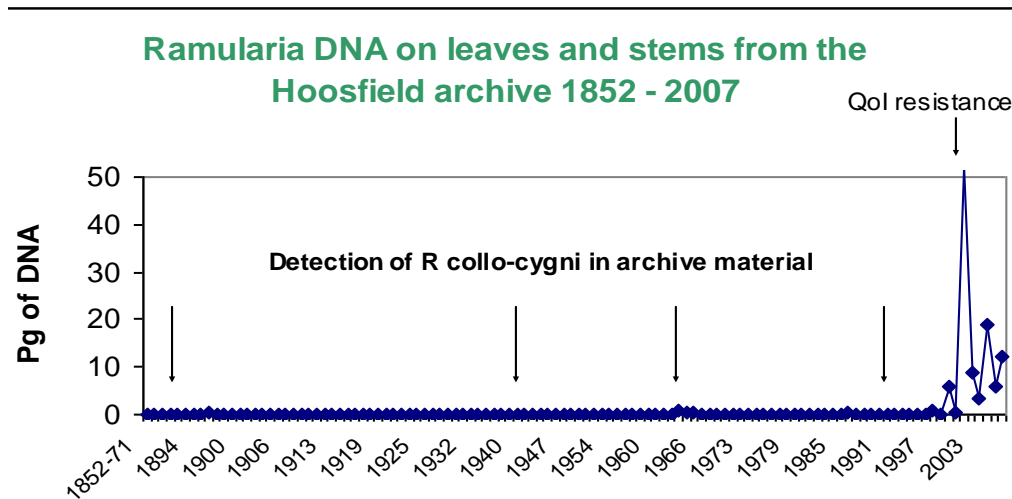


Figure 1. *Ramularia* DNA levels detected by real-time PCR over the time course of the Hoosfield spring barley experiment from 1852-2007. The arrows indicate the detection of *Ramularia* DNA at small levels and the large peak in 2002 when QoI resistance was detected for the first time in the UK.

## Discussion

The results obtained from this study show that the development of QoI fungicide resistance in *Ramularia collo-cygni* is widespread and therefore expected to be found in nearly all regions of the UK and many parts of Europe.

The results of the fungicide sensitivity bioassay show that all isolates containing only the wild type sequence were found to be fully sensitive to azoxystrobin at levels as low as 0.032 ppm. However, the majority of isolates collect from Scotland and Denmark in 2007 were found to grow in the presence of 4 ppm of QoI fungicide, but at higher concentrations of azoxystrobin growth was inhibited. All isolates that showed resistance in the bioassay contained the mutated sequence (gct) in the cytochrome *b* gene with the sensitive isolates containing only the wild-type sequence (ggt). This amino acid substitution is the same mutation that has occurs in all other highly resistant cereal pathogens, such as wheat and barley powdery mildew and *M. graminicola*.

The results obtained from the Rothamsted archive experiments also shown that this disease could be detected in barley in England during the 19<sup>th</sup> century. However the levels of the

pathogen do not seem to have been significantly high. The first description of *R. collo-cygni* was made by Cavara in 1893 in Northern Italy. The results obtained in this study indicate that *Ramularia* was already present in the UK at this point but was still only a minor pathogen. The data obtained from real-time PCR does support the evidence from Scottish trial data that *Ramularia* became an important disease from around 1998. It also indicates that *Ramularia* levels were increasing throughout the wider UK. The question that now needs to be answered is what changed during this period to cause such an increase in this pathogen. Interestingly, the archive data also shows that in 2002, the year that a sharp decline in efficacy to QoI fungicide was seen, a large peak of *Ramularia* DNA was also observed (Figure 1).

In summary, this work has shown that this pathogen is able to develop fungicide resistance quickly and this has led to several important questions for future research in this area. Firstly are all the fungicide groups currently in use working as effectively as we assume? Secondly, with the introduction of new fungicide groups such as complex II inhibitors will these remain effective for long or will resistance develop quickly? We also need to establish if the increasing importance of *Ramularia* is due to changes in agricultural practices or other factors, such as climate change?

### Acknowledgments

This work is financially supported by the Scottish Government Rural and Environment Research and Analysis Directorate Work package 1.4 (Barley Pathology) and the Home-Grown Cereals Authority (HGCA).

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