APPENDIX A

PYROSEQUENCING ANALYSIS

Biotage

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1. Assay 1 - I381V region

1.1. Original sequence

GAAGAGACGAGAGTCCAAAACGGGCGAACATGAGGAAGACATGATCCACAACTTGATGCAGTGCAAATACAAGGACG
GCAATGCCATTCCCGACAAGGAGATTGCTCATATGATGATTGCGCTGCTCATGGCCGGCCAGCACTCTTCATCTGCG
ACCGAGTCCTGGATCACTCTCCGCCTCGCATCCCGCCCCGACATCCAAGACGAACTCCTCCAAGAACAAAAGGATAT
GCTCGGTGTGAACGCCGACGGCAGTATCAAGGAGCTCACATACGCCAACCTCTCGAAACTCACCCTCCTCAATCAAG
TCGTCAAAGAAACCCTTCGTATTCACGC/GTCCAA/GTCC*ACTCCATTCTGCGCA*AGGTCAAGTCTCCCATGCCCAT
CGAAGGTACGGCATACGTCATTCCAACCACCCACACCTTCTGGCCGCTCCGGGCACAACGAGCCGCATGGACGAGCA
CTTTCCCGACTGCCTCCATTGGGAGCCGCAT

1.2. Primers

→	Forw PCR	Biotin-5´-GTCGTCAAAGAAACCCTTCGTATT
←	Rev PCR	5´-GACGTATGCCGTACCTTCGAT
←	Seq rev	5´-*TGCGCAGAATGGAGT*

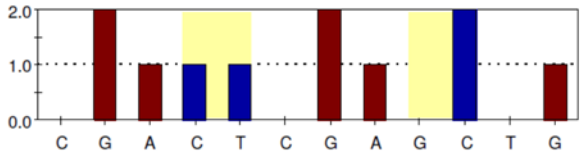
1.3. Pyrosequencing assay

Sequence to analyze in Pyrosequencing software: GGAC/TTGGAC/GCGTGA

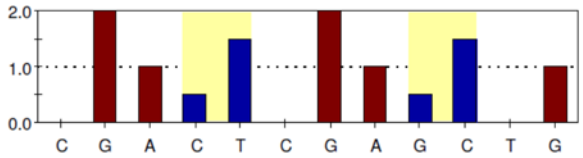
Position 1: A/G analyzed in reverse orientation.
Position 2: C/G analyzed in reverse orientation.

1.4. Selected theoretical outcomes

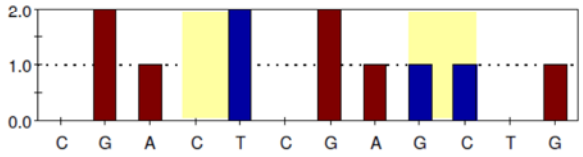
Pos 1: C/C Pos 2: C/C



Pos 1: C/T Pos 2: C/G

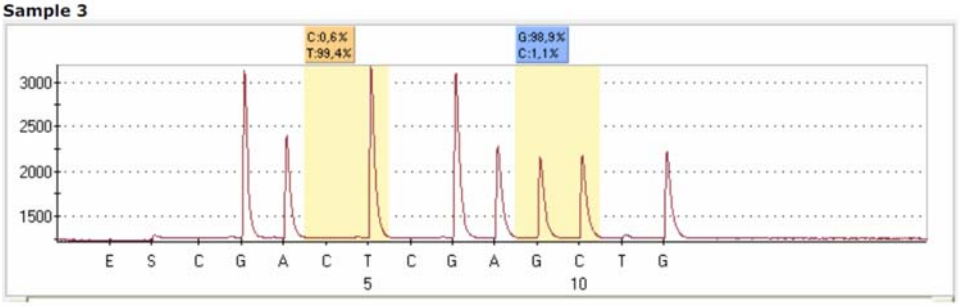


Pos 1: T/T Pos 2: G/G

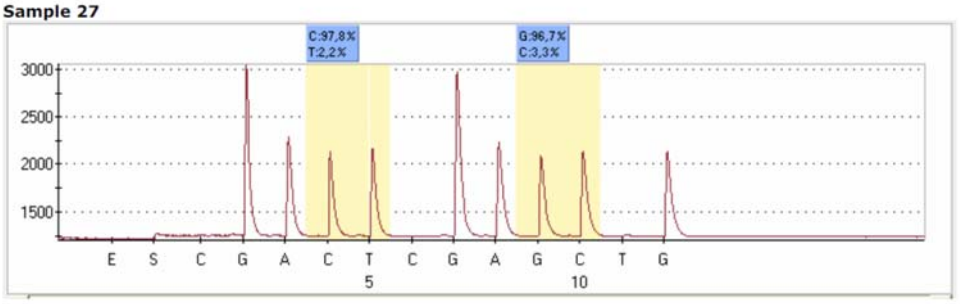


1.5. Results - representative pyrograms

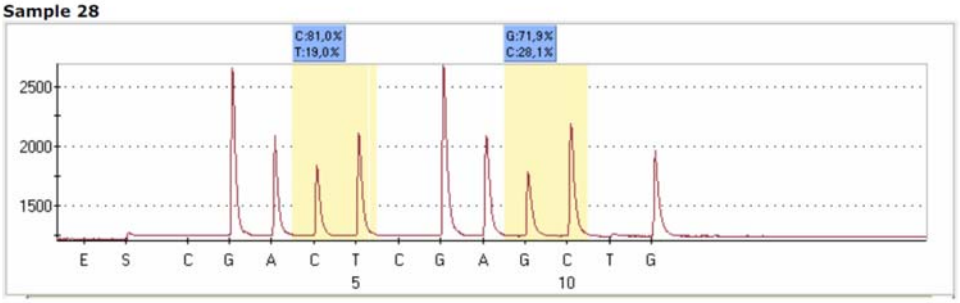
Sample 3



Sample 27



Sample 28



NOTE: The "Check" quality assessment (the values are marked yellow instead of blue) in the first position in sample 3 is due to "Uncertain allele frequency determination. Signal within uncertain signal to noise range." The software can not distinguish between a true peak and noise from the background.

1.6. Results - Quantification

Well	Sample	Position 1 Result		Position 2 Result	
A1	1				
A2	2				
A3	3	C : 0,6%	T : 99,4%	G : 98,9%	C : 1,1%
A4	4	C : 1,0%	T : 99,0%	G : 99,9%	C : 0,1%
A5	5	C : 0,9%	T : 99,1%	G : 99,6%	C : 0,4%
A6	6	C : 0,9%	T : 99,1%	G : 99,2%	C : 0,8%
A7	7	C : 0,0%	T : 100,0%	G : 99,4%	C : 0,6%
A8	8	C : 0,8%	T : 99,2%	G : 100,0%	C : 0,0%
A9	9	C : 0,0%	T : 100,0%	G : 100,0%	C : 0,0%
A10	10	C : 0,9%	T : 99,1%	G : 99,8%	C : 0,2%
A11	11	C : 0,8%	T : 99,2%	G : 98,8%	C : 1,2%
A12	12	C : 0,9%	T : 99,1%	G : 100,0%	C : 0,0%
B1	13	C : 1,0%	T : 99,0%	G : 99,6%	C : 0,4%
B2	14	C : 13,5%	T : 86,5%	G : 98,7%	C : 1,3%
B3	15	C : 0,7%	T : 99,3%	G : 97,9%	C : 2,1%
B4	16	C : 0,9%	T : 99,1%	G : 98,9%	C : 1,1%
B5	17	C : 0,9%	T : 99,1%	G : 98,8%	C : 1,2%
B6	18	C : 0,8%	T : 99,2%	G : 99,3%	C : 0,7%
B7	19	C : 0,8%	T : 99,2%	G : 98,6%	C : 1,4%
B8	20	C : 0,0%	T : 100,0%	G : 100,0%	C : 0,0%
B9	21	C : 0,7%	T : 99,3%	G : 99,2%	C : 0,8%
B10	22	C : 0,7%	T : 99,3%	G : 100,0%	C : 0,0%
B11	23	C : 20,1%	T : 79,9%	G : 98,4%	C : 1,6%
B12	24	C : 6,6%	T : 93,4%	G : 100,0%	C : 0,0%
C1	25	C : 57,5%	T : 42,5%	G : 99,6%	C : 0,4%
C2	26	C : 66,7%	T : 33,3%	G : 71,1%	C : 28,9%
C3	27	C : 97,8%	T : 2,2%	G : 96,7%	C : 3,3%
C4	28	C : 81,0%	T : 19,0%	G : 71,9%	C : 28,1%
C5	29	C : 66,2%	T : 33,8%	G : 66,3%	C : 33,7%
C6	30	C : 74,2%	T : 25,8%	G : 55,2%	C : 44,8%
C7	31	C : 55,2%	T : 44,8%	G : 82,4%	C : 17,6%
C8	32	C : 96,6%	T : 3,4%	G : 38,1%	C : 61,9%
C9	33	C : 35,8%	T : 64,2%	G : 97,0%	C : 3,0%
C10	34	C : 56,3%	T : 43,7%	G : 70,5%	C : 29,5%
C11	35	C : 49,5%	T : 50,5%	G : 78,1%	C : 21,9%
C12	36	C : 83,1%	T : 16,9%	G : 61,2%	C : 38,8%
D1	37	C : 94,3%	T : 5,7%	G : 23,6%	C : 76,4%
D2	38	C : 79,7%	T : 20,3%	G : 68,8%	C : 31,2%
D3	39	C : 99,4%	T : 0,6%	G : 29,7%	C : 70,3%
D4	40	C : 24,4%	T : 75,6%	G : 96,5%	C : 3,5%
D5	41	C : 1,2%	T : 98,8%	G : 99,6%	C : 0,4%
D6	42	C : 0,0%	T : 100,0%	G : 99,9%	C : 0,1%
D7	43	C : 100,0%	T : 0,0%	G : 98,2%	C : 1,8%
D8	44	C : 100,0%	T : 0,0%	G : 99,5%	C : 0,5%
D9	45	C : 100,0%	T : 0,0%	G : 1,5%	C : 98,5%
D10	46	C : 100,0%	T : 0,0%	G : 98,4%	C : 1,6%
D11	47	C : 100,0%	T : 0,0%	G : 98,1%	C : 1,9%
D12	48	C : 0,8%	T : 99,2%	G : 99,6%	C : 0,4%

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2. Assay 2 – Deletion region

2.1. Sequence

CAATCAAGTCGTCAAAGAAACCCTTCGTATTCACGCTCCAATCCACTCCATTCTGCGCAAGGTCAAGTCT
CCCATGCCCATCGAAGGTACGGCATACGTCATTCCAACCACCCACACTCTTCTGGCCGCTCCGGGCACAA
CGAGCCGCATGGACGAGCACTTTCCCGACTGCCTCCATTGGGAGCCGCATCGATGGGACGAGAGCCCGTC
CGAGAAATACAAGCACCTGTCCCCGACGACTGCCCTAGGAAGCATCGCCGAGGAGAAAGAAGACT[T/G][ATGGCT/C]
ACGGCCT[G/T]GTAAGCAAGGGCGCGGCGTCGCCATACTTACCCTTTGGTGCGGGACGACACAGATGTATCGGCGAGC
AATTCGCGTACGTGCAATTGCAGACCATTACAGCGACGATGGTTCGCGATTTCAAGTTTTACAATGTGGATGGCAGC
GACAACGTGGTGGGTACGGATTACAGCAGTTTGTTCAGCCGGCCGCTGTCGCCGGCAGTAGTAAAGTGGGAGAGGAG
GGAGGAGAAGGAGGAGAAGAAC

2.2. Primers

➘	Forw PCR	5´-CTAGGAAGCATCGCCGAGGA
⬅	Rev PCR	Biotin-5´-TGTCGTCCCGCACCAAAG
➡	Seq forw	5´-*TCGCCGAGGAGAAAGAA*

2.3. Pyrosequencing assay

Sequence to analyze in Pyrosequencing software: GACKA[TGG]C[Y][AC]GGCCT

Variants
A; stCTRL2001 and ST1CYP51
B; stopus2
C; stfoli3, stopus1 and stflul
D; stfoli1

Variant	Sequence																									
A	G	A	C	T	A	T	G	G	C	T	A	C	G	G	C	C	T	G	G	T	A	A	G	C	A	A	G	G
B	G	A	C	G	A	T	G	G	C	T	A	C	G	G	C	C	T	G	G	T	A	A	G	C	A	A	G	G
C	G	A	C	T	A	T	G	G	C	C	A	C	G	G	C	C	T	G	G	T	A	A	G	C	A	A	G	G
D	G	A	C	T	A				C				G	G	C	C	T	T	G	T	A	A	G	C	A	A	G	G

The percentage given for each variant was estimated as follow:
Variant A: % T in [Y] comes from both variant A and B. Since the amount of variant B is given from % G in K, the percentage of variant A can be calculated from % T in [Y] minus % G in K.
Variant B: % G in K
Variant C: % C in [Y]
Variant D: Mean value for % deletion in [Y] and [AC]. % deletion in [TGG] was excluded from the calculation since these values seemed to differ more than % deletion in in [Y] and [AC].

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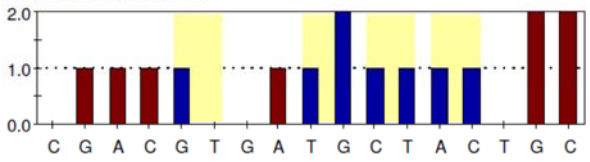
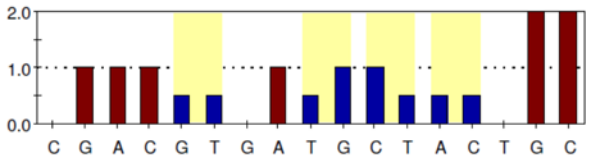
2.4. Selected theoretical outcomes

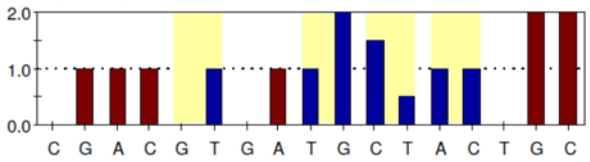
G/G TGG/TGG T/T AC/AC



G/T TGG/--- T/- AC/--

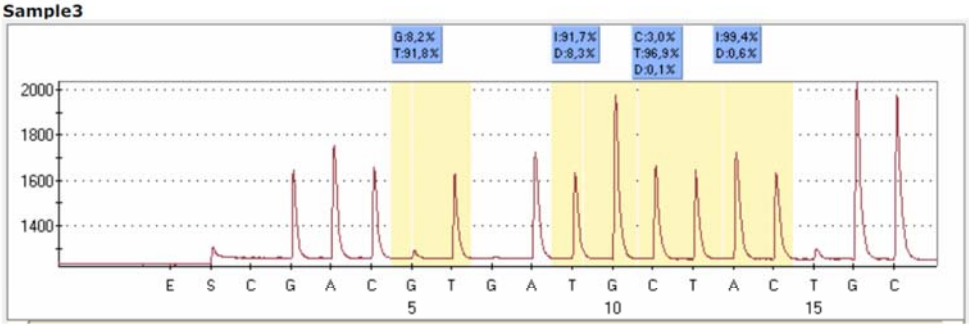


T/T TGG/TGG T/C AC/AC



2.5. Results – Representative pyrograms

Sample3



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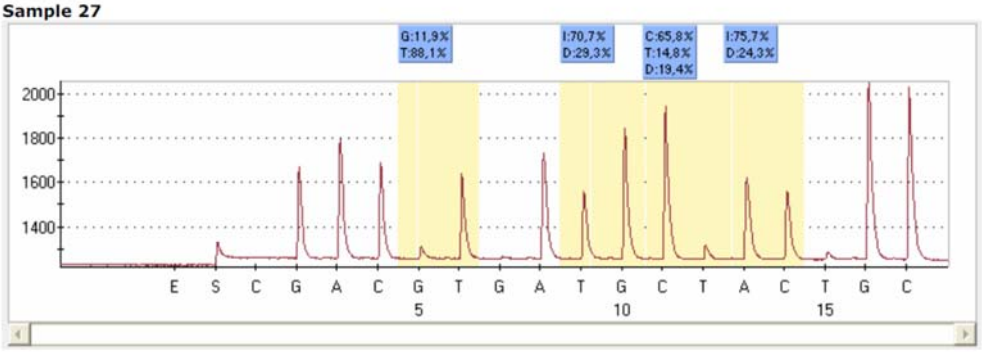
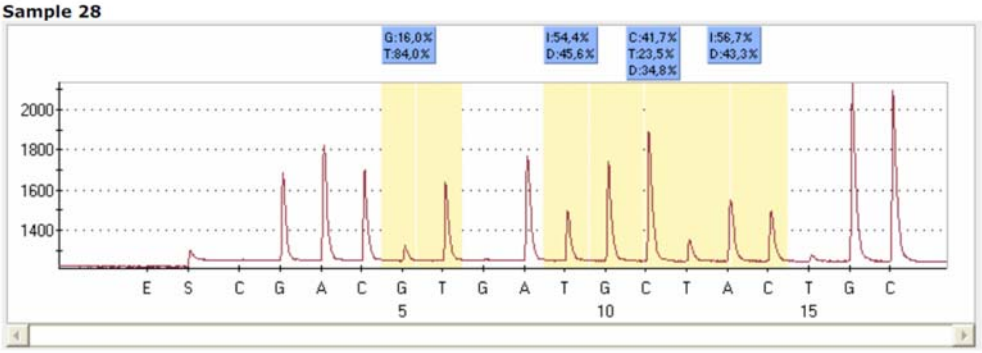
Sample 27



Sample 28



NOTE: "D" in the pyrograms is percentage of deletion. The mean value for [Y] and [AC] is indicated as the percentage of the sample variant D. The value for [TGG] is not used since it is based on three base incorporations and therefore less reliable. The [TGG] value also deviated slightly from the other two.

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2.6. Results – Quantification

For calculations of the percentage of each sample variant, please see under paragraph 2.3 Pyrosequencing assays.

Well	Sample	% Variant A	% Variant B	%Variant C	%Variant D
A1	1				
A2	2				
A3	3	88,7	8,2	3,0	0,4
A4	4	86,3	7,8	1,4	4,7
A5	5	87,8	8,5	0,9	2,4
A6	6	91,0	6,8	1,8	0,7
A7	7	87,7	9,8	0,2	3,2
A8	8	90,2	8,6	1,1	1,9
A9	9	86,7	7,8	4,4	3,2
A10	10	87,0	9,3	1,7	3,2
A11	11	90,4	7,4	2,1	1,8
A12	12	86,4	6,7	3,6	3,4
B1	13	91,3	7,1	1,0	1,7
B2	14	87,0	8,2	4,7	1,6
B3	15	76,8	8,9	0,0	7,2
B4	16	72,5	7,0	6,4	7,1
B5	17	89,1	8,7	0,0	1,1
B6	18	89,0	6,8	4,1	0,6
B7	19	84,3	11,5	4,1	3,1
B8	20	27,2	13,6	27,3	29,9
B9	21	22,5	16,5	35,9	27,4
B10	22	47,1	7,9	0,0	22,5
B11	23	38,6	14,8	25,0	26,9
B12	24	5,6	36,4	56,1	4,2
C1	25	19,4	26,7	23,8	31,5
C2	26	9,2	12,0	44,6	35,6
C3	27	2,9	11,9	65,8	21,9
C4	28	7,5	16,0	41,7	39,1
C5	29	20,6	12,4	40,6	32,7
C6	30	17,6	8,2	29,2	48,3
C7	31	21,1	13,9	49,0	22,7
C8	32	5,9	19,3	27,1	50,5
C9	33	36,5	22,8	37,9	9,3
C10	34	19,1	18,3	31,6	34,6
C11	35	38,4	9,7	32,1	25,8
C12	36	2,2	19,7	34,8	45,6
D1	37	2,4	11,4	17,1	72,8
D2	38	2,6	14,4	43,9	38,9
D3	39	1,1	11,0	21,5	67,7
D4	40	6,1	11,7	55,2	29,1
D5	41	85,9	8,4	3,9	2,7
D6	42	85,7	6,9	3,7	5,6
D7	43	8,8	8,1	4,1	80,1
D8	44	0,6	8,2	79,1	13,6
D9	45	0,2	7,6	8,8	81,7
D10	46	-3,3	98,1	4,8	1,4
D11	47	-7,7	98,4	3,9	4,3
D12	48	9,9	6,2	0,0	42,0

Biotage

2.7. Comments to results

It is important to note that this is a very difficult quantification assay and the values given for each sample variant should be taken cautiously.

- It should be noted that the lowest value given for sample variant B is around 6%. This could be due either to the presence of the B variant in all samples or simply to background in the assay.
- Sample 48 is a fifth variant (E) with the sequence GACTATG**A**CT. Sample 22 is a mix of the sample variant A, and the variant E with the sequence GACTATG**R**CT. The analysis for these two samples are given the quality assessment "Failed" by the software since the sequence variants are not found in the sequence to analyze and the peak pattern can not be recognized by the software.
- Samples 21, 23 and 29-35 seem to contain an additional sample variant. It is very difficult to get the correct sequence from the current analysis but sample 35 indicates that at least this sample might contain small amounts of a variant with the sequence GACTATGGCT**C**CGG. These samples get the quality assessment "Check" by the software since the reference pattern deviates slightly from any possible.
- Samples 46 and 47 show negative values for variant A. The value for variant A is calculated by subtracting the percentage of the B variant (% G in K) from the % T in [Y] which represents both variants A and B. The % T is measured in a very complicated region and might therefore not be fully reliable.
- The percentage given for sample variant D is calculated from the mean value for % deletion in [Y] and [AC]. In sample 15 and 16 these two values differ more than for other samples (14,3% and 0% for sample 15 and 0% and 14,1% for sample 16) which might indicate the presence of a sequence not fully corresponding to the given sequence to analyze.

2.8. Conclusion

Several different sequence variants combinations were clearly detected by this assay design. In addition, at least one, but probably several other sequence variants were detected. Some of the samples were difficult to interpret, most likely due to sample variants deviating from the predefined sequences. By running a second Pyrosequencing reaction and using another sequence to analyze and dispensation order, several of these could probably also be correctly quantified.

3. Pyrosequencing™ analysis

Sample preparation was carried out using the Vacuum Prep Tool according to standard procedures. 5 µl PCR product was immobilized to 2 µl Streptavidin Sepharose™ HP beads (GE Healthcare, formerly Amersham Biosciences) followed by annealing to 4 pmol sequencing primer for 2′ at 80°C.
The PSQ HSA System was used with the dispensation orders assigned for the assay as stated in this report. All equipment was handled according to standard procedures. The analysis criteria were those specified by factory settings.

APPENDIX B

Rothamsted Research Press Release, 15 October 2009

Sensitivity of Septoria tritici to azole fungicides

There have been ongoing concerns about the potential loss of activity of azole (triazole and imidazole) fungicides in controlling Septoria leaf blotch. These have been heightened by recent reports about the emergence of new, more resistant strains of Septoria in 2009.

This update is based on independent research being done as part of a Defra sponsored LINK project coordinated by Rothamsted and involving a consortium of partners (see below for details).

The key findings of the project to date are:

- The decline in efficacy of azole fungicides against Septoria since the 1990s has been associated with changes in the fungal protein targeted by these fungicides.
- These changes have been cumulative and therefore the shifts in efficacy have been gradual rather than sudden.
- Although all azoles affect the same target in the fungus, not all azoles have been equally affected by these changes. Activity of some older azoles has been compromised, while others, such as epoxiconazole and prothioconazole, have been much less affected.
- The most effective azoles have therefore continued to provide good control of Septoria when used at recommended rates.
- Investigations into the efficacy of azole mixtures and/or alternations for control of the evolving Septoria population have demonstrated the value of diversity within the azole class (see HGCA Fungicide Performance in Wheat 2008 trial results) .
- During 2009, laboratory tests on artificial media have detected some Septoria strains that appear to be less sensitive to most azole fungicides tested. These new strains have been reported in Ireland, France, and in a few samples from England.
- Glasshouse tests with these strains on plants showed no differences in efficacy of control by azoles when compared with strains from previous years.
- The altered laboratory sensitivity of these strains has not yet been correlated with changes in the fungal target protein, and work is underway to identify the resistance mechanism.

- While plant tests and field trials suggest that control of Septoria has not been compromised, it will be important to monitor field populations of Septoria over the coming season to see if any further changes occur, that might affect disease control. This and additional research to understand the implications of the shifts in sensitivity will be done under a further programme sponsored by industry, Defra and CRD.
- At present, advice for control of Septoria in 2010 remains unchanged (see FRAG at www.pesticides.gov.uk/rags_home.asp), but any developments likely to affect advice will be distributed via the FRAG website and press releases as necessary.

The Defra-funded Sustainable Arable Link project (LK0976) involves Rothamsted Research, Scottish Agricultural Colleges, ADAS and Velcourt as research partners, and HGCA, BASF, Bayer Crop Science, Du Pont, Syngenta and Velcourt as sponsors. Additional funding has recently been provided by the Chemicals Regulation Directorate (CRD) to support more detailed analysis of new Septoria strains with reduced sensitivity.

Contacts:

Dr Bart Fraaije, Head of the Fungicide Research Group at Rothamsted Research (bart.fraaije@bbsrc.ac.uk)

For further information, please contact the Rothamsted Research Press Office Tel: 01582 763133 ext 2260 or email Dr Adélia de Paula (adelia.depaula@bbsrc.ac.uk).

Rothamsted Research, an Institute of the BBSRC, is based in Hertfordshire and is one of the oldest and largest agricultural research institutes in the country.

APPENDIX C

RRA Newsletter
January 2010

Septoria Resistance and Azole use 2010

Bill Clark, Bart Fraaije, John Lucas, Hans Cools

Introduction

Despite the excitement about new races of brown and yellow rust in recent seasons *Septoria tritici* remains the most damaging disease in UK wheat crops. The control of this disease depends on the continued use of azole fungicides. Without the azoles we would struggle to control Septoria in the UK. Chlorothalonil (Bravo) still plays an important role but has virtually no eradicant activity and the new SDHI (succinate-dehydrogenase inhibitors) fungicides such as bixafen and isopyrazam, although having some eradicant activity, will need to be used in mixture with azoles. So what are the current issues with resistance in *Septoria tritici* and what impact will it have on future azole use?

Azole resistance

The term 'resistance' is very emotive - resistance occurs when a pathogen becomes so insensitive to a fungicide that the field performance of the fungicide is impaired. Resistance can arise rapidly and completely so that disease control is lost suddenly (as happened with MBCs in eyespot and strobilurin resistance in mildew). Alternatively, resistance develops gradually so that the pathogen becomes progressively less sensitive – which is what happened with the azoles and *Septoria tritici* in the late 1990s to early 2000s. We saw a decline in activity of many (but not all) of the azoles, even at full label doses. Epoxiconazole (Opus) and prothioconazole (Proline) remained highly active against the disease when used at robust doses in the field. The situation seemed to have stabilised and no further decline in activity was seen for a number of years. While this change in activity was seen in the field with single products used alone, in commercial farm situations disease control was generally good as products were used in mixtures and sequences with other non-azole fungicides such as chlorothalonil and boscalid. This is still the case today, even though laboratory tests frequently report reduced sensitivity on a regular basis.

Laboratory testing of isolates

Laboratory testing can reveal a number of things about fungal strains isolated from the field. They can tell us about the genetic make-up of the isolate and how it has changed (mutated) by comparison with sensitive "wild-type" isolates from many years ago. Growth tests on laboratory media with different doses of fungicide can show how sensitive that isolate is compared with the wild type. Today, in UK fields it is very difficult to find any 'wild type' *Septoria tritici* – because such isolates are very sensitive to fungicides and have been removed from the population by frequent fungicide use. The isolates that remain are less sensitive than the wild type – often by a large factor – up to 200 times less sensitive in lab tests, but these isolates are not 'resistant' in practical terms as they are still well-controlled in the field by robust fungicide programmes using recommended label doses.

The relative sensitivity of the Septoria population in any field can be measured by isolating samples from infected leaves and testing them in lab culture as described above.

ROTHAMSTED RESEARCH ASSOCIATION

The sensitivity of individual isolates is usually expressed as an EC50 value (the concentration of fungicide needed to reduce growth by 50%). The range of sensitivities in any collection of isolates can then be plotted as a "cumulative frequency" curve that gives an overall impression of the distribution of EC50 values in a particular population. In all of the following figures, the further to the left the isolates are the more 'sensitive' they are, the further to the right the more 'resistant' they are.

Figure 1 shows the range of sensitivities of *Septoria tritici* isolates collected from New Zealand, France, Germany and England in 2008. The range of sensitivity in a population is shown as a series of EC50 (sensitivity) values. In New Zealand disease pressure is low and historically, fungicide use has been limited – so Septoria isolates are still very sensitive and have low EC50 values. English isolates are about 100-200 times less sensitive (more 'resistant') than New Zealand isolates – but they are currently still well-controlled in field trials. Thus, while laboratory measurements of EC50 values provide useful information on the baseline sensitivity of a field population, they do not necessarily correlate directly with the field performance of fungicides. Nonetheless, the trends revealed by such comparisons may be a cause for concern, as if the curves continue to shift towards the less sensitive end of the range, ultimately they will begin to impact on field performance, as has already occurred with the less effective azoles.

Laboratory testing of isolates is therefore useful to measure changes in sensitivity that could indicate possible field performance changes in the future.

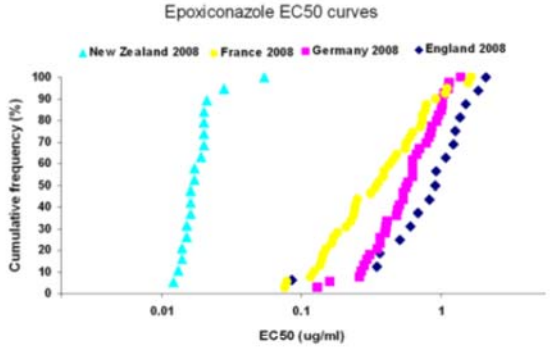

Figure 1. Range of sensitivities of isolates from New Zealand, France, Germany and England (from 2008) to epoxiconazole

Figure 2 shows the sensitivity of UK (Rothamsted) and Irish (Carlow) isolates to epoxiconazole, tebuconazole and prothioconazole tested at Rothamsted. Rothamsted isolates showed a shift in sensitivity from 2003 to 2009 but no isolates outside the normal range of EC50 values were found. Rothamsted isolates in 2003 (R2003) were more sensitive to epoxiconazole and tebuconazole than 2009 isolates from Rothamsted (R2009) and Carlow (C2009), indicated by shifts in EC50 values. The 2009 Carlow isolates were more sensitive to tebuconazole than the 2009 Rothamsted isolates.

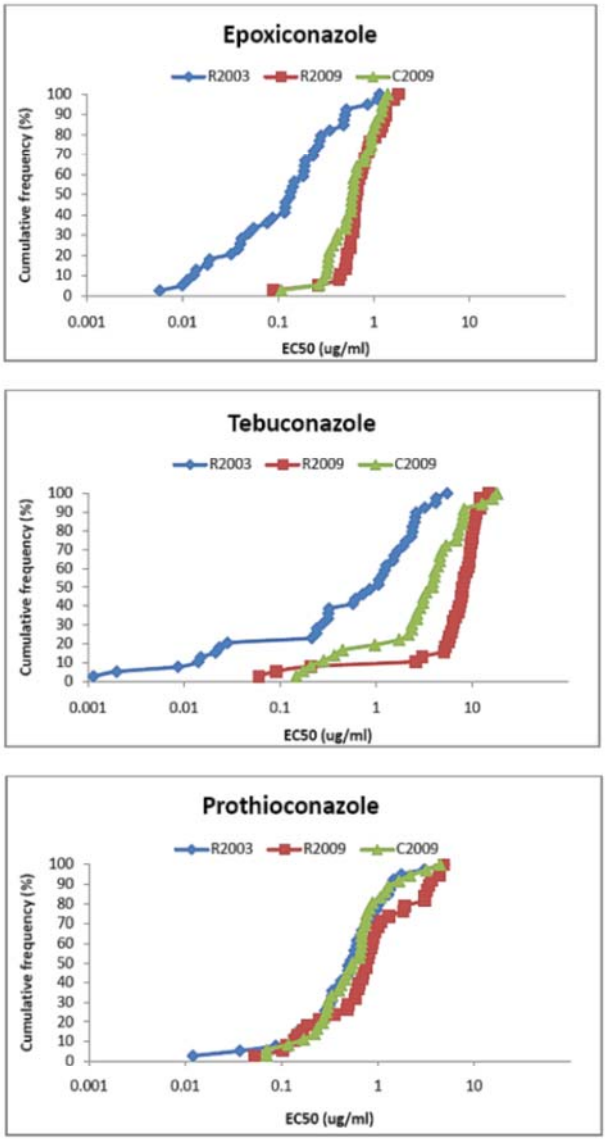

Figure 2. Range of sensitivities of Rothamsted isolates (R2003 and R2009) and Irish isolates (C2009) from 2009 to epoxiconazole, tebuconazole and prothioconazole

Recent mutations and fungicide performance

All azoles have the same mode of action inhibiting a key step in sterol biosynthesis, sterol 14α-demethylase (CYP51). See figure 3 below.

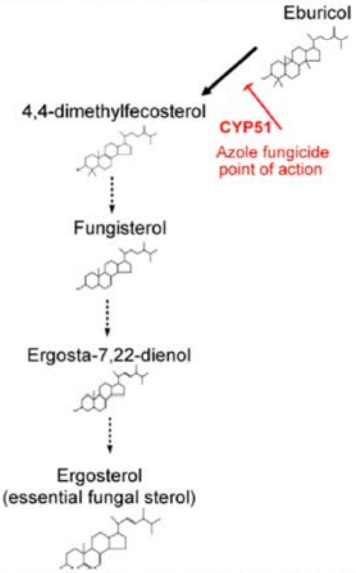

Figure 3. Key steps in ergosterol biosynthesis showing CYP51 position in sequence.

Consequently it was assumed that if resistance occurred in a pathogen then all azoles would be equally affected. This is now known not to be true. The Rothamsted-led LINK project , alongside a considerable amount of other independent research done at Rothamsted, has identified a large number of mutations in the azole target site protein (CYP51) in *Septoria tritici*, some of which clearly affect the sensitivity of *Septoria tritici* to particular azole fungicides, while others have no measurable impact on sensitivity. The target site for azoles in *Septoria tritici* is very complex and mutations at or near the target site can have very subtle effects—some directly affect the way in which the fungicide 'fits' into the site (e.g. I381V). Others, further away from the target site (e.g. V136A), can affect the folding of the molecule, again affecting how the fungicide gets into the target. Some mutations can affect the lipophilic nature of the binding site - affecting how strongly the fungicide binds to the site. Consequently, combinations of mutations may have significant effects on fungicide performance or little effect.

The Rothamsted Broadbalk archive of wheat crop samples collected since 1843 has allowed us to look back over 150 years of wheat cultivation and see when *Septoria tritici* contained in the leaf samples began to show these mutations.

For most of the period, the sensitive wild type *Septoria tritici* completely dominated the population, and no mutations in the azole target site can be detected.

Following the introduction and use of azole fungicides, the population began to change, with mutations beginning to appear in the 1990s. At first, individual mutations occurred, but this has been followed by an accumulation of changes, particularly since around 2000. By this time the wild-type could no longer be detected in the archive samples.

Unlike the situation with MBC or strobilurin fungicides, where a single mutation with a major effect on sensitivity explained the emergence of resistance, and the breakdown of control in the field, with the azoles the story is more complex. The picture is one of gradual evolution, with the azole target site protein becoming modified over time in response to selection by fungicides.

No single change is completely responsible for the observed shift in sensitivity, although some mutations have had a greater impact than others. The best example is the I381V mutation that first occurred in 2000 and quickly increased in frequency thereafter. This change had a serious effect on the performance of tebuconazole, but not on most other azoles. Hence it became clear that the various mutations affected fungicide performance differently, while some appeared not to affect field performance at all.

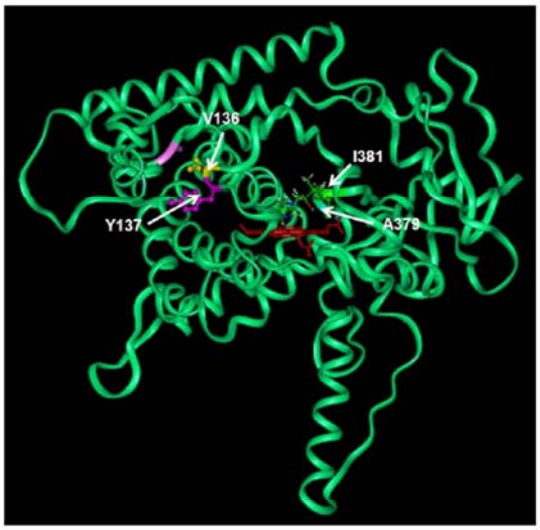

Figure 4. Some amino acid codon positions in the target site (CYP51) of azole fungicides where changes linked with resistance in *Septoria tritici* have been found.

New mutations

The analysis of the Broadbalk archive samples confirmed that the Septoria population has been changing over the past 20 years. But the process has not yet stopped. There are new mutations in Septoria being regularly found from year to year. At present there are more than 20 different combinations of changes and the number continues to increase. During 2009 several new mutations, or combinations of mutations, have been reported. The Broadbalk archive allows us to look back in time and see if a new mutation is indeed 'new' or whether we just haven't detected it before. The S524T mutation has recently been reported as a new change linked to a further reduction in sensitivity to azoles. However, when Broadbalk samples were analysed it was discovered that this mutation was already present in 1999. This finding was confirmed with the detection of S524T in the CYP51 protein of a Rothamsted strain isolated in 2001. In the UK Septoria population S524T is not considered important in affecting field performance of any azole fungicides.

Azoles and mutation selection

Research carried out on a range of azoles has demonstrated that certain azoles have a definite selection for certain mutations whilst others exert very little selection. This led to the theory that individual azoles could be used alone, in mixture or in alternating programmes to manipulate the mutations present in the Septoria population. The theory being that by devising such programmes, disease control could be improved. Figure 5 summarises the selection pressure exerted by a range of azoles on a few of the major mutations. Clearly tebuconazole, metconazole and difenoconazole select strongly for the I381V mutation and yet tebuconazole is the only one of these azoles that has been significantly affected in terms of field performance. Prochloraz clearly has a strong positive selection for V136A. This can, as a consequence, reduce the proportion of I381V in the population (making some fungicides such as tebuconazole more effective). Other azoles have only weak selection pressure for specific mutations. Thus, tebuconazole, metconazole difenoconazole and prochloraz could potentially be suitable candidates for mixing and alternation strategies. Other combinations are unlikely to have a significant impact on the Septoria population structure or field performance.

	CYP51 Mutation selection pressure		
Azoles	**V136A**	**I381V**	**A379G**
Tebuconazole		++++	+
Metconazole		++++	+
Difenoconazole		++++	++
Prochloraz	+++		-
Flutriafol	++		+
Cyproconazole	+		
Flusilazole			
Fluquinconazole			+
Epoxiconazole		+	+
Prothioconazole	+		

Figure 5. Selection pressure exerted by a range of current azoles on main CYP51 mutations

Prochloraz – an unusual azole

Prochloraz is an imidazole, in a different chemical class to the majority of azole fungicides used in agriculture. Figure 6 shows the sensitivity range of Rothamsted isolates of *Septoria tritici* to prochloraz. In this case Rothamsted isolates are now more sensitive to prochloraz than they were in 2003 and also in comparison with the 2009 population of Carlow. This is the reverse of what we have found with other azoles, particularly for tebuconazole. Sensitivity to prochloraz tends to be associated with high frequencies of mutations that have reduced sensitivity to other azoles. So, while sensitivity to some azoles has decreased, it has increased in others. Thus, each azole must be considered individually in terms of how it may be affected by certain mutations. This may also partly explain why some mixtures of azoles give better disease control than might be expected.

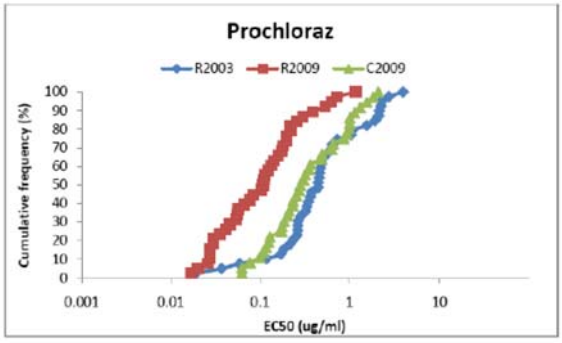

Figure 6. Range of sensitivities of Rothamsted isolates (2003 and 2009) and Irish isolates from 2009 to prochloraz.

Mixing of Azoles – an anti-resistance strategy for *Septoria tritici*?

We have seen how some mutations have affected field performance while others have not. Thus it is clear that the complex of mutations now present in UK Septoria populations does not affect all azoles in the same way. This leads to the debate about the merits of mixing azoles as an anti-resistance strategy. Because the mutations affect azoles in different ways it is possible that mixtures of azoles could help slow down further shifts in sensitivity. However, the number and combinations of mutations are very dynamic so it is difficult to predict future performance of single and multiple azole products. For example, there are now strains carrying both V136A and I381V, so at the moment it is difficult to give robust advice on anti-resistance strategies. Often azole mixtures such as Prosaro (tebuconazole + prothioconazole) and Brutus (epoxiconazole + metconazole) give improved performance over their component fungicides. Some of this is simply due to extra 'loading' of azole in the product, some is probably due to improved formulation but some may well be due to complementarity between the two azole components. This is very difficult to demonstrate. Recent HGCA-funded experiments on fungicide performance with Brutus suggest that dose-for-dose it is more effective than epoxiconazole alone. This would not be predicted from the activity of the two components of the mixture, suggesting other mechanisms may be involved.

The Future of azoles

Azoles remain the foundation of all cereal fungicide programmes. Although the shift in sensitivity to azoles in *Septoria tritici* appears to have stabilised over the last few years it is quite possible that new CYP51 variants or other resistance mechanisms may evolve that could shift sensitivity again, so continued vigilance and use of anti-resistance strategies should be employed. Except when used as seed-treatments, azoles are normally used in mixtures. It is difficult to limit their use as they are key components throughout the spray programme, so they should be used in mixture whenever practicable. Furthermore, with shifts in sensitivity field performance often begins to be affected when low doses are used. Higher doses are more effective, more versatile in terms of timing and persistence and allow flexibility in follow-up sprays.

Further information

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