Impact of azole resistance on strategies for disease management

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Rothamsted Research
Azole fungicides

In medicine (first introduced 1973)
- Topical and systemic
  - Sporanox
  - Micinazole

In agriculture (first introduced 1973)
- Cereals
  - Prochloraz
  - Tebuconazole
  - Epoxiconazole
- OSR
  - Metconazole
  - Difenoconazole
- Soybean
  - Tetraconazole
  - Propiconazole

Mechanisms of resistance to azoles

1. Alteration of the target CYP51 enzyme * *
2. Enhanced active efflux * Often combined (human pathogens?)
3. Elevation of intracellular CYP51 levels * Often combined (plant pathogens?)
### Azole resistance in European arable crops

<table>
<thead>
<tr>
<th>Organism Crop Resistance phenotype</th>
<th>Mechanism(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP51 mutation</td>
</tr>
<tr>
<td><strong>Powdery mildew</strong>&lt;br&gt;Barley Triadimenol R* Propiconazole R</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Powdery mildew</strong>&lt;br&gt;Wheat Triadimenol R Propiconazole R</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Septoria leaf blotch</strong>&lt;br&gt;Wheat Varying levels of R to azoles</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Rhynchosporium</strong>&lt;br&gt;Barley Propiconazole R Tebuconazole R Epoxiconazole DS*</td>
<td>-</td>
</tr>
<tr>
<td><strong>Brown rust</strong>&lt;br&gt;Wheat Epoxiconazole DS</td>
<td>✓</td>
</tr>
<tr>
<td><strong>Stem canker</strong>&lt;br&gt;OSR No shift?</td>
<td>-</td>
</tr>
<tr>
<td><strong>Light leaf spot</strong>&lt;br&gt;OSR Flusilazole DS Prothioconazole DS</td>
<td>?</td>
</tr>
</tbody>
</table>

b R - resistance, DS - decreased sensitivity

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**Mycosphaerella graminicola** (Septoria leaf blotch)

- In the absence of fungicide, yield loss can be up to 3 t.ha⁻¹ (25%). Economic cost > £200 per hectare (UK total > £200 million)

- After QoI resistance emerged in 2002, control of *M. graminicola* relied on azoles. Erosion of efficacy observed since the mid 2000s

- New generation of Succinate Dehydrogenase Inhibitors (SDHIs) recently introduced in mixture with azoles
Rothamsted populations – Untreated early season

Molecular model of Sterol 14α-demethylase

- Essential enzyme in the sterol biosynthesis pathway
- Is a cytochrome P450 (CYP51)
- Protein target forazole fungicides

M. graminicola CYP51 (MgCYP51; Mullins et al., 2011, PLoS ONE)
Position of MgCYP51 alterations

- D107V (1992)
- Y137F (1992)
- ΔY459/G460 (1999)
- Y461H (2001)
- S188N (1993)
- N513K (1993)
- D134G (2006)
- S524T (2001)
- H303Y (2011)
- I381V (2001)
- ΔY459/S460 (1999)
- Y463H (2001)

* First detection in Rothamsted isolate collection

Effect of Y137F on triadimenol docking

Y137F-Triadimenol
### Characterisation of less sensitive isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>CYP51 variant</th>
<th>Epoxi</th>
<th>Prochl</th>
<th>Tebu</th>
<th>Prothio-dethio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference EC50 values in μg/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=4 Wild-type</td>
<td></td>
<td>0.0029</td>
<td>0.0164</td>
<td>0.072</td>
<td>0.0014</td>
</tr>
<tr>
<td><strong>1994-2008</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=5 Y137F</td>
<td></td>
<td>6.6</td>
<td>5.1</td>
<td>1.8</td>
<td>nd</td>
</tr>
<tr>
<td>n=5 Y137F &amp; S524T</td>
<td></td>
<td>39.5</td>
<td>32.7</td>
<td>12.3</td>
<td>nd</td>
</tr>
<tr>
<td>n=11 LS05, V136A &amp; Y461H</td>
<td></td>
<td>54.9</td>
<td>25.1</td>
<td>4.8</td>
<td>8.8</td>
</tr>
<tr>
<td>n=35 LS05, I381V &amp; Y461H</td>
<td></td>
<td>81.4</td>
<td>35.6</td>
<td>39.0</td>
<td></td>
</tr>
<tr>
<td>n=4 LS05, S188N, I381V, Δ &amp; NS13K*</td>
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<td>86.3</td>
<td>32.1</td>
<td>28.1</td>
<td>14.7</td>
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<tr>
<td>n=47 LS05, S188N, A379G, I381V, Δ &amp; NS13K</td>
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<td>148</td>
<td>1.2</td>
<td>82.2</td>
<td>20.6</td>
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<tr>
<td><strong>2009-12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>n=19 LS05, S188N, I381V, Δ &amp; NS13K**</td>
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<td>389</td>
<td>17.4</td>
<td>235</td>
<td>92</td>
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<tr>
<td>n=6 LS05, V136A, Y461S &amp; S524T</td>
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<td>206</td>
<td>61.7</td>
<td>5.4</td>
<td>46.4</td>
</tr>
<tr>
<td>n=2 V136C, I381V, Y461H &amp; S524T</td>
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<td>1111</td>
<td>14.5</td>
<td>110</td>
<td>93.2</td>
</tr>
<tr>
<td>n=1 LS05, D134G, V136A, Y461S &amp; S524T</td>
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<td>209</td>
<td>12.4</td>
<td>6.5</td>
<td>112</td>
</tr>
<tr>
<td>n=35 LS05, D134G, V136A, I381V &amp; Y461H</td>
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<td>196</td>
<td>11.2</td>
<td>5.0</td>
<td>102</td>
</tr>
<tr>
<td>n=6 LS05, V136A, I381V, Y461H &amp; S524T</td>
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<td>537</td>
<td>19.9</td>
<td>21.9</td>
<td>167</td>
</tr>
<tr>
<td>n=1 LS05, S188N, A379G, I381V, Δ, NS13K &amp; S524T</td>
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<td>477</td>
<td>4.3</td>
<td>77.1</td>
<td>81.3</td>
</tr>
<tr>
<td>n=1 LS05, S188N, H303Y, A379G, I381V, Δ &amp; NS13K</td>
<td></td>
<td>376</td>
<td>2.3</td>
<td>61.8</td>
<td>109</td>
</tr>
<tr>
<td>n=6 LS05, D134G, V136A, Y461H &amp; S524T</td>
<td></td>
<td>809</td>
<td>18.2</td>
<td>11.0</td>
<td>336</td>
</tr>
<tr>
<td>n=6 LS05, V136A, S188N, A379G, I381V, Δ &amp; S524T</td>
<td></td>
<td>999</td>
<td>11.9</td>
<td>20.6</td>
<td>418</td>
</tr>
<tr>
<td>n=4 LS05, V136C, S188N, A379G, I381V, Δ &amp; S524T</td>
<td></td>
<td>1486</td>
<td>3.3</td>
<td>282</td>
<td>162</td>
</tr>
<tr>
<td>n=3 LS05, V136A, S188N, A379G, I381V, Δ, NS13K &amp; S524T</td>
<td></td>
<td>996</td>
<td>8.1</td>
<td>22.5</td>
<td>610</td>
</tr>
</tbody>
</table>

**CYP51 over-expressing strains (Cools et al., 2012, Pest Manag. Sci.)**

### Conclusions – *M. graminicola*

- Studies of azole resistance in plant pathogenic fungi have identified the molecular mechanisms associated with less sensitive or resistant phenotypes.

- Alteration of *M. graminicola* CYP51 (MgCYP51) is the primary determinant of reduced azole sensitivity. Additional mechanisms operate in some isolates (e.g. constitutive *MgCYP51* over-expression).

- Changes in the target protein affect all azoles, but some mutations affect certain azoles more than others – mixtures?

- Evolution continues. New combinations of changes are still emerging.

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*Characterisation of less sensitive isolates*
Reduced fungicide sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*
Fungicides and control of Light Leaf Spot

- MBCs and azoles are the fungicides of choice for *P. brassicae*
- In the UK carbendazim and prothioconazole were the most commonly applied active ingredients in 2011
- Resistance to MBCs is now widespread in UK *P. brassicae* populations (Carter et al 2013 Pest Manag Sci 2013; 69: 1040–1048)

### P. brassicae azole sensitivity

<table>
<thead>
<tr>
<th>Pre-2000 isolates</th>
<th>Recent isolates (2008-2011)</th>
</tr>
</thead>
</table>

20-fold
CYP51 substitutions in *P. brassicae* isolates

**Tebuconazole EC$_{50}$ (µg.ml$^{-1}$)**

- **No CYP51 changes**
- **S508T (S524T in MgCYP51)**
- **G460S (G476S in MgCYP51)**

**Pre-2000 isolates**
- UK29
- UK68
- UK59
- UK05
- E2C
- E2E
- B9X53
- WC3
- C3E
- C3D
- UK82
- E4B
- WC0
- I3

**Recent isolates (2008-2011)**

**Induced overexpression of PbCYP51**

- Treatment: EC$_{50}$ tebuconazole after 72 hours growth
**Summary of *P. brassicae* CYP51 modifications**

- All isolates with tebuconazole sensitivities of > 0.1µg/ml have associated CYP51 changes
- Least sensitive isolate has accumulated CYP51 changes

**Summary Chichester (Sussex) 2013**

After treatment: (prothioconazole and flusilazole)
Conclusions – *P. brassicae*

- *P. brassicae* is one of the most damaging diseases of OSR in the UK
- Resistance to MBC fungicides is widespread and reduced sensitivity to azoles has evolved
- Reduced azole sensitivity correlates with increased induced CYP51 expression and CYP51 mutations, although other genetic factors contribute to the final phenotype
- Knowledge of azole resistance in *M. graminicola* can inform studies of *P. brassicae* (e.g. evolution of the S508T/G460S variant could compromise disease control)

‘Improved tools to rationalise and support stewardship programmes for SDHI fungicides to control cereal diseases in the UK’

Modelling effects of fungicide mixtures on selection for resistance
Background: SDHI-azole mixtures

Prochloraz selects for the V136A mutation
Tebuconazole selects against the V136A mutation

Hypotheses

- Mixture benefit does not depend on reducing the dose of the azole component
- Increasing SDHI dose will reduce selection for azole resistant mutants
- There will be an additional benefit from reducing dose of azole component

Experimental design

Winter wheat cv Consort

T1 and T2 treatment with tebuconazole or prochloraz in mixture with different doses of SDHI (Isopyrazam)

Septoria population then sampled in July and frequency of mutations determined
SDHI LINK Conclusions

• Model predictions were good

• Mixtures with an SDHI reduced selection for mutations affecting azole sensitivity

• If converse is true, robust doses of azoles in mixtures should reduce the risk of resistance to SDHIs

• Resistance to SDHIs in Septoria. One mutation of little effect (SdhcT79N) found in France in 2012 – monitoring continues

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