Fungicide resistance: discoveries in barley net blotches pave way to better understanding the resistance mechanisms

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Key messages

- Misuse of fungicides and poor disease management practices have an impact on everybody.
- Overuse of fungicides with the same mode of action will speed up fungicide resistance.
- We can limit the development of fungicide resistance by using the lowest doses that give good control, appropriate MOA rotations and employing IDM practices including crop rotation, stubble management and selection of resistant cultivars.
- Fast (and cheap) monitoring of pathogen populations for fungicide resistance is central for the sustainable chemical management of diseases.

Aims

Fungicides have been in the forefront of control of fungal pathogens of humans, animals and plants for nearly 40 years. The direct consequence of the undeniable success of fungicides in controlling diseases has been the rise of fungicide resistance due to continuous exposure of fungal populations to these compounds. Fungicide resistance is now common around the world and has become a serious problem in agricultural systems.

The widespread adoption of the use of multiple fungicide treatments in Australian agriculture did not begin in earnest until about 15 years ago. One consequence was that a small number of actives from a single mode of action (MOA) group – the DMIs or Group 3 fungicides - dominated the market.

It is widely accepted that the risk of fungicide resistance is greatest in pathogens with short latent periods, with high levels of virulence against prevalent cultivars and when fungicides with a single MOA are used. Under this scenario it is not surprising to find in Australia several diseases of grain crops with resistance to fungicides, especially from the groups 3 (DMI) and 11 (QoI/strobilurins). So far six cases of fungicide resistance and four cases of reduced sensitivity (resistance does not reach the level of field failure) have been identified in Australia since 2012 (table 1).

Table 1. Fungicide resistance cases identified in Australia during the period 2012 – 2017.

Disease	Fungicide Group	Detected in WA
Barley powdery mildew[a]	Group 3 (DMI)	√
Wheat powdery mildew[a]	Groups 3* and 11 (QoI)	-
Barley net-form of net blotch[a]	Group 3*	√
Barley spot-form of net blotch[b]	Group 3	√
Canola blackleg[a]	Groups 2 (MAP-Kinase) and 3*	√
Wheat septoria leaf blotch[a]	Group 3*	-
Chocolate spot[a]	Group 1 (MBC)	-
Ascochyta blight[a]	Group 1	-

[a]Identified between 2012-2016; [b]Identified in 2017; *Reduced sensitivity that does not reach the level of field failure

The 2009-2012 epidemic of highly virulent and tebuconazole-resistant barley powdery mildew (Bgh) in WA was estimated to have caused at least an average of $100M loses p.a. In addition, we have recently discovered a mutation (F489L) present in the target gene for DMI fungicides (Cyp51) in net form net blotch in some areas of WA that confers resistance to DMIs (Mair et al., 2016). The lesson learnt from these episodes is the need to be prepared in order to avoid and/or mitigate similar situations in the future. Here we present the most recent discoveries of fungicide resistance in spot form net blotch and use the data obtained

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from the analysis of different resistance mechanisms to address the development of new and more effective anti-resistance management strategies.

Method

Fungal samples and in vitro fungicide resistance analysis

One hundred and twelve samples of spot form of net blotch (SFNB) were collected by a combination of especially designed bait trials and a network of collaborators from farms and trials during the 2016 and 2017 growing seasons. Bait trials sown with the SFNB susceptible varieties Stirling and Planet were designed to work as a fungicide resistance early warning system. Plots of 2m × 4m were sprayed with either 1× or 2× the maximum registered dose of fungicides from the groups 3 (DMI), 11 (QoI) and 7 (SDHI), at growth stages GS31 and GS39. Treatments were replicated three times. Leaf samples from bait trials were collected seven days following the second spray application.

Samples were processed in the laboratory and 20 SFNB pure fungal isolates established following the isolation procedure described by Mair et al. (2016). A set of fungicide discriminatory concentrations was established based on previous analysis of their sensitivity baselines to different fungicides (tebuconazole = 10µg/mL; epoxiconazole = 5µg/mL; boscalid = 10µg/mL; Azoxystrobin = 5µg/mL). Isolates able to grow above those concentrations were considered to be resistant *in vitro*. Growth of the isolates at these discriminatory doses does not necessarily imply field failure. Further studies are needed.

Molecular analysis of known fungicide resistance

Isolates able to grow above discriminatory concentrations were subjected to digital Polymerase Chain Reaction (dPCR) analysis for the detection of the fungicide resistance mutation F489L. In addition to the isolate analysis, samples from field surveys are currently being investigated for the presence of resistance using this same methodology.

Characterisation of fungicide resistance in SFNB

SFNB isolates that grew above Group 3 discriminatory doses were subjected to further molecular characterisation. The Group 3 fungicide target site gene was sequenced in order to determine if mutations were associated with the resistance levels detected.

In addition, the expression level of target gene of the resistant isolates was measured to compare the level with the wild types. Sensitive isolate U7 and resistant 16FRG073 were cultured in Fries2 liquid media amended with tebuconazole at a concentration that inhibits the growth of each isolate by 50 per cent, also known as effective concentration 50 (EC_{50}; Mair et al., 2016).

A dPCR detection methodology was developed for the detection of mutations in SFNB resistant to some Group 3 fungicides.

Results

Discovery of resistance to Group 3 fungicides (DMI) in SFNB

The analysis of the samples collected during the 2016 and 2017 (analysis still in progress) growing seasons has revealed the existence of two Group 3 SFNB resistant populations in the Southern region of WA. 20 SFNB isolates selected from populations collected from across Australia were tested in 2016. One isolate (5%) was resistant to Group 3 fungicides. A similar trend was found for 2017 with 35 SFNB isolates analysed and only one resistant (2,85%) detected.

Case 1: Esperance, WA

The analysis of a sample collected near Esperance in late 2016 using a discriminatory concentration test showed high levels of resistance to the Group 3 fungicide tebuconazole. Further *in vitro* characterization of isolates from this sample revealed the existence of an isolate named 16FRG073 that showed high levels of resistance to tebuconazole and epoxiconazole, (resistance factors RF = 8.3 and 7.7, respectively) and lower to prothioconazole (RF = 5.1) (table 2).

Table 2. Effective concentration 50 (EC_{50}) and resistance factors of Group 3 resistant isolate 16FRG073 (Esperance), sensitive isolate U7 and the mean of 20 sensitive SFNB isolates collected between 1996 – 2013. Cultures were grown at different concentration ranges of the fungicides tebuconazole, epoxiconazole and prothioconazole.

Isolates	Tebuconazole	Epoxiconazole	Prothioconazole
Mean (1996 – 2013)	0.31	0.17	0.07
U7 (wild type)	0.24	0.32	0.09
16FRG073	2.57	1.29	0.34
Resistance Factor	8.3	7.7	5.1

The analysis of the fungicide target site did not identify any mutations that could be associated with resistance. However, when the genetic region that regulates the expression of the target was investigated, an insertion (piece of DNA 'inserted' into region) was observed in the resistant isolate compared to the wild type. Similar insertions have been previously correlated with increased levels of the target in other plant fungal pathogens (Ishii and Hollomon, 2015; figure 1).

The expression of the Group 3 target in the unamended control cultures was >2500 higher in 16FRG073 than in U7 (p=.001, Mann-Whitney U=72). Expression of the Group 3 target in the cultures under tebuconazole EC_{50} treatment was 22-fold higher in 16FRG073 than in U7 ($p < .001$, U=576). These results reveal two important elements of this resistance; i) the resistant isolate has a higher expression of the fungicide target (i.e. more target available for the fungicide molecules which require more molecules of the fungicide to inhibit fungal growth) even in the absence of the fungicide, and ii) the presence of the fungicide increases further the expression of the target.

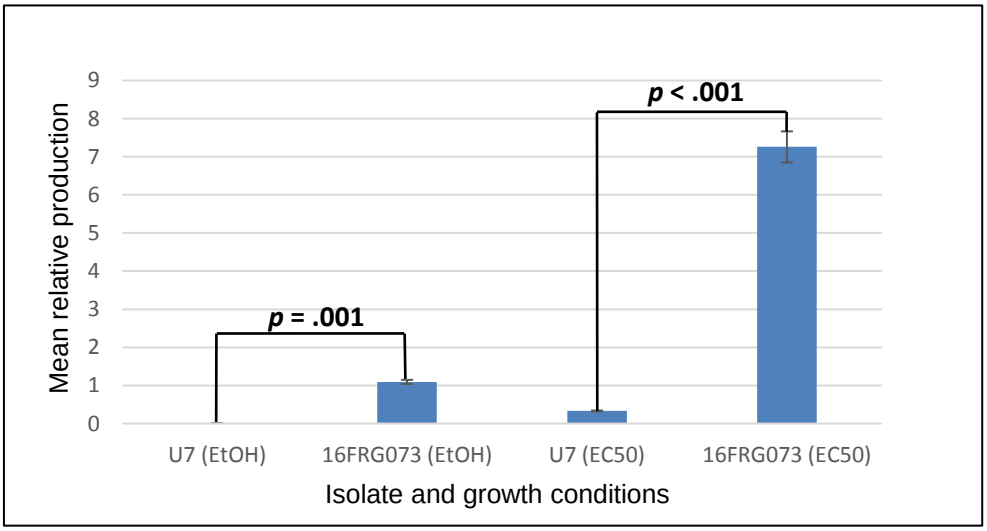

Figure 1. Normalized expression of the Group 3 fungicide target in SFNB sensitive isolate U7 and resistant isolate 16FRG073. EtOH = no fungicide; EC50 = specific concentration of tebuconazole that inhibits growth of each isolate by 50 per cent.

The implications of this type of resistance for the management of SFNB are important since a higher production of the fungicide target has the potential to affect all actives within the Group 3 chemistry, which will be perceived as a progressive decline of the fungicide activity. This means that the overuse of Group 3 fungicides for the control of SFNB may contribute to the increase of resistance through time thus possibly jeopardizing the current effectiveness of existing Group 3 compounds such as prothioconazole, and propiconazole, which have good activity against current resistant populations.

Case 2: South Stirling, WA

In late 2017, researchers from the Department of Primary Industries and Regional Development (DPIRD) collected SFNB samples from a crop in South Stirling with significant levels of disease after five consecutive fungicide applications (tebuconazole as seed dressing at 400mL/100kg seed, propiconazole at 325 mL/ha at Z25, cyproconazole + azoxystrobin at 400 mL/ha at Z31, epoxiconazole at 250 mL/ha at Z39 and propiconazole at 500 mL/ha at Z52). The *in vitro* analysis of the samples using a discriminatory concentration test showed the same pattern found in the case of the samples collected from Esperance (table 3).

Table 3. Fungicide sensitivity profile of Group 3 resistant isolates 16FRG073 (Esperance) and 17FRG089 (South Stirling), and sensitive isolate U7 when grown on agar plates amended with specific fungicide discriminatory concentrations.

Isolate	No Fungicide (Control)			Tebuconazole (10µg/mL)			Epoxiconazole (5µg/mL)			Boscalid (10µg/mL)			Azoxystrobin (5µg/mL)		
16FRG073	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
17FRG089	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
U7	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

+ = Growth on agar plate; - = No growth. Scored at 48 hrs pi (boscalid and azoxystrobin); 96 hrs pi (tebuconazole and epoxiconazole).

The analysis of the genetic region that regulates the expression of the Group 3 fungicide target indicated that there was an insertion very similar to that found in the isolates collected from Esperance. Although the analysis is not complete yet, it is expected that the level of target expression in the South Stirling samples containing this gene alteration will be similar to that of the resistant samples collected from Esperance

To our surprise, the analysis of the fungicide target revealed a mutation, F489L, which was previously reported to occur in NFNB isolates resistant to Group 3 fungicides (Mair et al., 2016). It is still unclear as to whether this mutation has originated independently in both species due to fungicide selection pressure or it has been transferred from NFNB to SFNB during a hybridisation event (McLean et al., 2014).

What have we learnt from the analysis of SFNB resistance?

The characterization of SFNB resistance to Group 3 fungicides has improved our understanding on how fungicide resistance develops under fungicide selection pressure. While in NFNB there seems to exist only one mechanism of resistance to Group 3 fungicides that has spread across the WA wheatbelt, in the case of SFNB the resistance found is due to two different mechanisms: an insertion in the genetic region that control expression of the target, and the combination of a similar insertion and a mutation in the target of the fungicide. The presence of the two different insertions in SFNB increases the expression of the fungicide target with the result that more molecules of fungicides are required to inhibit fungal growth. The second mechanism found, the mutation F489L, contributes to modifying the binding affinity of the fungicide target hence making the interaction between the target and the fungicides less effective (calculation of binding affinities conducted by collaborators Jonathan Mullins & Samuel West of the Institute of Life Science, Swansea University, UK). The combination of these two mechanisms provides SFNB with a clear advantage when the majority of fungicides used for its control are from the Group 3 chemistry.

However, there is also some good news. Thanks to the understanding that we now have on the mechanisms underlying this resistance, we can now develop new tools for the fast, accurate and affordable detection of resistance in field samples. Based on the knowledge already available on mutation F489L (Mair et al., 2016) and the findings reported here, we have been able to develop a digital PCR test that accurately detects and quantifies resistance in SFNB (figure 2). This test uses highly specific molecular probes that detect the presence of the genetic changes associated with resistance directly in the infected plant material. This means that there is no need to purify samples in the lab to obtain pure fungal cultures, which substantially reduces turnaround time and cost of analysis. In addition to this, the presence of the resistant pathogen can be accurately quantified as shown in table 4. The advantage of this technique is that it provides growers with the possibility of adjusting their spraying regimes shortly after a disease sample has been submitted for analysis.

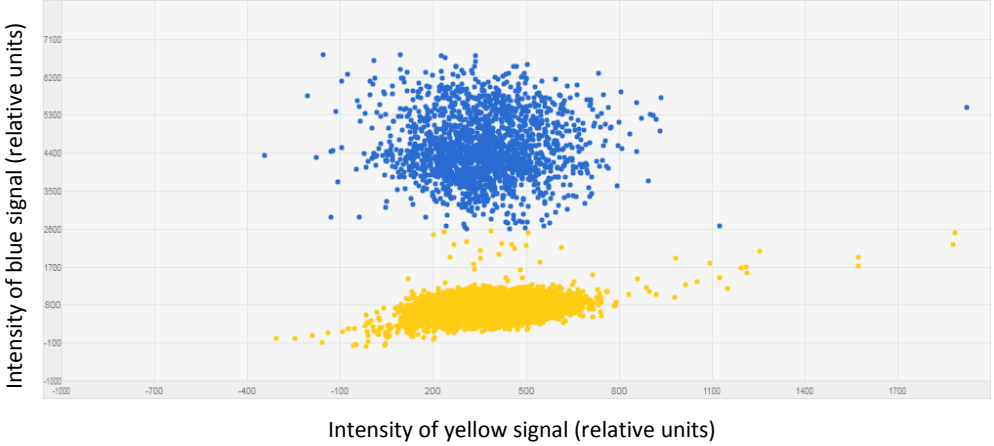

Figure 2. Scatter plots of a field sample depicting the detection of one of the two insertions in the genetic region that controls the Group 3 fungicide target expression. Individual isolates with the insertion (resistant) are represented by blue signals, while sensitive isolates without the insertion are represented by yellow signals.

Table 4. Digital PCR analysis of SFNB samples collected in the Esperance region (WA) indicating percentage of resistant isolates in each of the samples analysed. Lower detection threshold was established at 0.1%.

Sample	Wild type control	B003	B013	B002	B010	B012	B008	EDRS	Mutant control
% Resistant	<0.1%	<0.1%	<0.1%	<0.1%	0.349%	0.591%	1.973%	23.728%	99.991%

Conclusion

Many growers will experience a disappointing result with a fungicide spray at some stage. This could be due to application problems – the conditions were wrong for spraying, or the product was not appropriate for the diseases. Or it could be due to the build-up of resistance in the disease population. The key issue is how growers respond to such a situation of failed disease control. Spraying again with a different fungicide may control the disease or it may make the resistance problem worse – especially if the second fungicide application is the same MOA. Sometimes just changing the fungicide product does not help because actives used in different products are often the same. For this reason it is very important to clearly identify the active being used and the MOA it belongs to (commonly Groups 3 (DMI), 7 (SDHI) and 11 (QoI)). Understanding how fungicide resistance occurs will allow us to develop specific and rapid tests to detect resistance in the field thus enabling timely adjustment to spray programs, therefore minimizing the impact and delaying the worst effects.

Since resistance to some Group 3 fungicides was found in NFNB in 2013, there has been a debate around the development of resistance in the other net blotch disease, SFNB. It has been now confirmed that resistance to Group 3 fungicides has developed in the south of WA and that there are two different mechanisms responsible for this. Growers need to be cautious about SFNB control and implement adequate integrated disease management strategies to minimise the ongoing selection of SFNB resistant populations. Being a stubble borne disease, rotating crops or managing stubble are paramount for reducing disease carryover and selecting disease resistant varieties will reduce disease severity. However, these measures will not be very effective unless care is given to the choice of fungicide. Any spray program heavily dependent upon Group 3 fungicides will increase the selection of the resistant populations. The introduction of seed dressing, in-furrow and foliar products containing fungicide mixtures from different chemical groups (Groups 3, 7 and 11) in combination with the removal of tebuconazole and epoxiconazole from the control programs in those areas were resistance is found, will provide the best opportunity to limit the spread of the resistance in SFNB and its emergence in other barley growing regions of Australia.

Key Words

Fungicide resistance, fungal disease, mutations, DMI, SDHI, resistance mechanism, monitoring, net form of net blotch, spot form of net blotch, barley powdery mildew, wheat powdery mildew.

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