

Battling net blotch in barley – when co-innovation goes to work in WA’s south – resistance detection and in-field solutions

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Key messages

- Results from the first year of a three-year project between CCDM researchers and Western Australian farmers indicates barley net blotch Group 3 fungicide resistance is present in more locations in the south-west of WA than previous reports.
- Reduced sensitivity to Group 3 fungicides is now very common across the southern WA wheatbelt
- Working together, growers and researchers are developing an up-to-date snapshot of net blotch issues and solutions to control the disease.

Aims

A pilot project to directly engage and work with growers to find local, regionally relevant solutions to manage fungicide resistant pathogens on barley. The project focuses on spot form net blotch (SFNB) caused by *Pyrenophora teres f. maculata* (Ptm) and net form net blotch (NFNB) caused by *Pyrenophora teres f. teres* (Ptt).

Introduction

Resistance to Group 3 fungicides in NFNB was first detected in WA in 2013 (Mair et al 2016). In 2017, Group 3 resistance in SFNB was reported in WA (Mair et al 2019). Regionally relevant empirical data on the current fungicide resistance situation and available farmer management practices, are required to optimise integrated disease management (IDM) strategies in WA. The Centre for Crop and Disease Management (CCDM) Farmer Cohort project directly engages and works with growers and agronomists to find local, regionally relevant solutions to manage fungicide resistant pathogens on barley. This co-innovation approach will engage farmers in the sharing of data and knowledge in a yearly cycle, over a period of three years.

- Year 1. Farmers engaged to participate in the cohort and send in barley leaf samples with disease lesions, along with their management information. The samples are analysed using phenotypic and genotypic laboratory techniques to detect and quantify fungicide resistance. Farmers receive the results from the first year, along with current management recommendations. CCDM uses the data to inform fungicide-management research activity in the second year, including on-farm field trials, and continued optimisation of the analytical techniques.
- Year 2. The cycle in year 1 repeats, with additional empirical data derived from on-farm field trials and glasshouse trials contributing to fungicide management recommendations.

- Year 3. The cycle repeats.

Here we present the results from the analysis of the samples received from the Farmer Cohort project participants. Genetic analyses were undertaken to determine the presence of Ptt and Ptm in the samples received. In addition to this, the presence of F489L DMI fungicide resistance mutation was also determined. For the high rainfall sample group, a phenotypic assessment of fungicide resistance was carried out. The pathogen, mutation and isolation resistance data were mapped for the barley leaf samples for the first year of the Farmer Cohort.

Method

Request for samples and sample processing

CCDM initiated an extensive communications campaign at the start of the 2019 barley season, and 500 sampling kits were distributed to agronomists and farmers in the south-west Western Australian wheatbelt. The kits contained a flyer, five sample envelopes, and a return express post envelope. A total of 330 samples were received. Samples were assigned a cohort number on receipt, and the information from the farmer was recorded. Sample quality and leaf disease symptoms were visually assessed, and the leaves were counted and photographed. Depending on leaf number, a maximum of 10 leaves were selected for composite sample analysis, with a minimum of 20% of original leaves reserved for further tests. Leaves in the composite sample were scored for per cent leaf area lesions, and then oven dried (37°C). The per cent leaf area lesions were averaged for reporting to the farmer, including the percentile within the cohort sample set.

Composite sample preparation, DNA extraction and Ptm and Ptt PCR analysis

The oven-dried leaves were ground for two intervals of 15min at frequency 25Hz in a Mixer Mill MM400 (Retsch GmbH, Haan, Germany) to produce a homogenised, fine, dry powder. DNA was extracted from 20mg sub-samples of the composite sample using the Biosprint 15 DNA plant kit (QIAGEN, Hilden, Germany) and the KingFisher™ mL Purification System (Thermo Fisher Scientific, Waltham, MA, USA). Separate PCR analysis were run to detect the presence of Ptm or Ptt in the composite sample DNA, using species-specific primers (Kejal Dodhia, pers. comm.,). Screening for both Ptm and Ptt was required due to co-infection observed on the samples, and difficulty distinguishing the pathogens. Ptm, Ptt and negative controls were included in each reaction run. Amplification products were resolved on 1.5% agarose gels stained with SybrSafe (Life Technologies, ThermoFisher/Invitrogen, Australia) and visualized under UV light. The samples were scored and categorised for presence of both pathogens, presence of either Ptt or Ptm, or neither pathogen.

Ptm and Ptt pathogen map, isohyets and designation of sample annual rainfall group

GPS northings and eastings were taken directly from the sample envelope or inferred from the information provided by the farmer. The 14-year annual Australian rainfall data from 2006 to 2018 (Bureau of Meteorology, Australia), was averaged in R (R Core Team, 2018), contoured as 250, 350, 450, 550 and 650 isohyets, using the `rasterToContour` function (Hijmans, 2019), and saved as a shapefile for ArcGIS. Additionally, average annual rainfall was derived from this data for each farmer sample location using the `extract` function. A map of rainfall isohyets, and Ptm and Ptt detected in the farmer samples, was made using ArcGIS Desktop 10.6.

qPCR analysis of composite sample DNA for CYP51A F489L mutation

The primers and probes used for the qPCR analysis were previously developed at CCDM. Composite sample DNA from the 330 Farmer Cohort samples were screened for the presence or absence of the CYP51A F489L mutation. Ptm isolates SG1 (wildtype) and 17FRG089 (resistant), and Ptt isolates 9254 (wildtype) and Ko103 (resistant) were used for the standard curves. Isolates were plated on V8-PDA, and genomic DNA extracted from the harvested mycelia by following the protocol of Mair et al (2016). The DNA was quantified using a fluorometer and standard isolate concentrations of 1.25, 0.125, 0.0125,

and 0.00125ng/μL were made by serial dilution to give 5000, 500, 50, and 5pg/reaction. Three replicates of the four standards at the four concentrations were run together with three replicates of 110 samples and six 'no template' controls on a 384 well plate.

A 10μL qPCR reaction volume contained 5μL reaction buffer (SensiFast Probe No-Rox Kit – Bioline), 0.3μL each of forward and reverse primer (10μM), 0.2μL each of Probes 1 and 2 (10μM, Sigma), 2μL of sample DNA, and 2μL of H₂O (Kejal Dodhia, pers comm). The reaction was run on a 384-well spectrofluorometric thermal cycler (BIO-RAD CFX384 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA, USA), with an initial denaturation at 95°C for 3min, followed by 40 cycles of 95°C for 15s, and 66°C for 60s. Fluorescence data from the qPCR machine were retrieved during the annealing step of every Cq. Threshold fluorescence was set automatically by the instrument manager system (CFX Manager Version 3.1.1517.0823) before carrying out the assay. A log-linear standard curve was generated by plotting the logarithms of known concentrations of fungal DNA against the Cq values, and the reaction efficiencies were calculated for each curve.

Samples that gave positive fluorescence before no-template controls, and with average Cq below 33 (in the range of the standard isolate concentrations) were concluded to be positive for the mutation, or the wildtype, for the relevant probe. If the sample only had amplification with the HEX probe, then it was categorised as "Wildtype", and if the sample had any amplification with the FAM probe (FAM only or FAM plus HEX), it was categorised as "F489L mutation". Samples with no amplification with either probe were categorised as "not detected". If the result was not clear-cut, the sample was categorised as "inconclusive".

Tebuconazole phenotypic test of high rainfall farmer samples isolations; Ptm and Ptt PCR analysis

A sub-set of high rainfall farmer samples (16-year average annual rainfall >450mm) was derived from the annual rainfall sample data. Standard isolates of Ptm and Ptt are summarised in Table 1. Four to eight lesions of each sample were excised and sterilised, as described by Mair et al (2016). The lesions were allowed to air dry before plating on PDA media amended with ampicillin (100μg/mL), streptomycin (100μg/mL), and neomycin (50μg/mL). After four to seven days of incubation in the dark, colonies with morphotypes that were possibly Ptm or Ptt, were selected for tebuconazole resistance screening.

Hyphal strands were scraped from the colonies, suspended in 150μL of sterile H₂O, and gently mixed using a micro pestle. The sample and standard isolate hyphal suspensions were plated as three replicates each on different concentrations of tebuconazole PDA medium. The plates were stored in the dark at room temperature, and colony growth was scored after seven to eight days post inoculation (dpi). Mycelium growth beyond the initial inoculum and into the agar for at least two of the three replicates was scored as positive. The threshold tebuconazole limit of growth of the isolation was determined. Isolation DNA from the 0μg/mL of tebuconazole PDA plate, was obtained using a fast extraction method (Kejal Dodhia, pers. comm.) for PCR analysis. Separate PCR analysis was used to detect the presence of Ptm or Ptt in the sample DNA, using species specific primers (Poudel et al 2017) and the same protocol and scoring described for the composite samples.

ITS and GAPDH sequence analyses for various morphotypes isolated from the phenotypic test

Due to difficulties in differentiating colonies of Ptt and Ptm based on morphological characteristics, colonies with similar morphologies were divided into groups of morphotypes. Of the colonies grown during the phenotypic test, 32 were selected as morphotypes. To identify the associated fungal species to genus level and to obtain a generic idea of fungal species causing barley leaf spots, DNA of the selected isolates was extracted using the same fast DNA extraction technique from above. An ITS (internal transcribed spacer) region of the DNA was amplified using the primer pair ITS1 (forward) and ITS4 (reverse) (White et al 1990). To confirm the results obtained by ITS gene sequencing and to determine these species to species level using a more informative gene (Aryawansa et al 2014), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), gpd-1 (forward) and gpd-2 (reverse), were

included (Berbee et al 1996). A pictorial guide of all the morphotypes was provided based on the ITS and GAPDH gene analysis to aid differentiation of Ptm and Ptt colonies from other fungal species.

Table 1. Standard isolates used in tebuconazole phenotypic test

Pathogen	Isolate Name	DMI phenotype	Resistance description
Ptm	U7	WT	Sensitive
Ptm	16FRG073	MR1	Reduced sensitive
Ptm	19FRG195	MR2	Reduced sensitive
Ptm	17FRG089	HR	Resistant
Ptt	9254	WT	Sensitive
Ptt	Ko103	MR	Reduced sensitive
Ptt	18FRG038	HR1	Resistant
Ptt	18FRG040	HR2	Resistant

Maps of qPCR CYP51A F489L mutation result and Phenotypic Test results

The ArcGIS basemap from the Ptm and Ptt pathogen map was used to generate a map of the qPCR analysis results, showing the geographical distribution of the CYP51A F489L mutation (ArcGIS DeskTop 10.6.). The points were mapped as “F489L mutation”, “wildtype”, “inconclusive”, and “not detected”. Phenotypic test results were mapped in R (R Core Team, 2018), using ggplot2 (Wickham, 2016) and Simple Features (Pebesma, 2018).

Results

Sample description and mapped distribution of Ptm and Ptt in farmer cohort samples in south-west WA

Three hundred and thirty samples were submitted from 172 farmers between 5 July and 2 October 2019. The sample area covered a region extending from Cadoux in the north to Boyup Brook in the south-west and east to Boyatup. There were 14 barley varieties plus two unknowns (Figure 1) and the sample size varied from five to 50 leaves. Average sample leaf area lesions ranged from two to 67%.

Of the 330 paddock samples received, the composite sample DNA analysis confirmed 153 were positive for both Ptm and Ptt, 165 were positive for Ptm only, four were positive for Ptt only, and eight samples were negative for both Ptm and Ptt. The distribution of Ptm and Ptt is shown on the map in Figure 2. There were 163 samples in the high rainfall group (average annual rainfall >450mm for the period 2006 to 2018), and 167 samples in the low rainfall group.

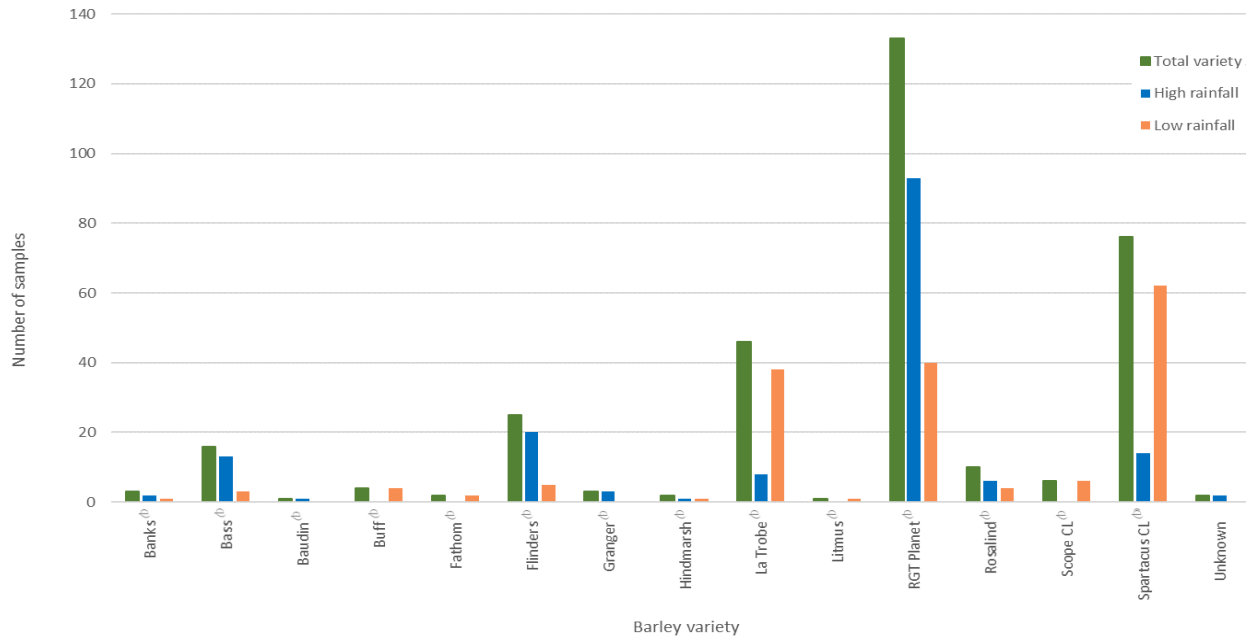


Figure 1. 2019 Farmer cohort barley varieties, showing the proportions of high (>450 mm average annual) and low (<450 mm average annual) rainfall samples.

qPCR results and map of CYP51A F489L mutation in farmer cohort samples

The qPCR analysis of the 2019 Farmer Cohort found 51 samples with the *CYP51A* F489L mutation, 231 samples were wildtype, 26 samples were inconclusive and 22 samples were negative for both the mutation and the wildtype of *CYP51A*. The map of the qPCR results and sample locations is shown in Figure 3.

Phenotypic Test of High Rainfall sample lesions and map of fungicide resistance

There were 163 high rainfall samples in the Farmer Cohort, derived from 99 farmers. Ten farmers submitted both a high and a low rainfall sample. Isolations were achieved for 122 samples. From these samples there were 318 isolations screened using the phenotypic test. ITS sequencing results provided a good understanding of all the fungi recovered from different lesions on infected barley leaves. Of the 32 morphotypes, two were identified as *Stemphylium* spp. and eight as *Alternaria* spp. One species was identified as *Arthrinium* sp. The remaining 22 isolates were identified as Ptm (50%) and both Ptm and Ptt (50%). GAPDH sequencing results provided more robust differentiation of Ptm and Ptt with 45% of absolute Ptm, 18% of absolute Ptt and 33% both Ptm and Ptt identifications. One isolate was identified as *Pyrenophora tritici-repentis* (Ptr) in GAPDH gene sequencing. *Alternaria* and *Stemphylium* species which were identified by ITS gene sequencing were not included in the GAPDH sequencing.

Based on morphology, 15 of the 318 isolations were discounted and 303 isolations were screened by PCR to detect the presence of Ptm or Ptt. Ultimately, 210 sample isolations were designated as Ptm, or Ptt, or both Ptm and Ptt. There were 21 isolations categorized as sensitive, 161 reduced sensitive, and 28 resistant. The map of DMI fungicide threshold of the isolations is shown in Figure 4.

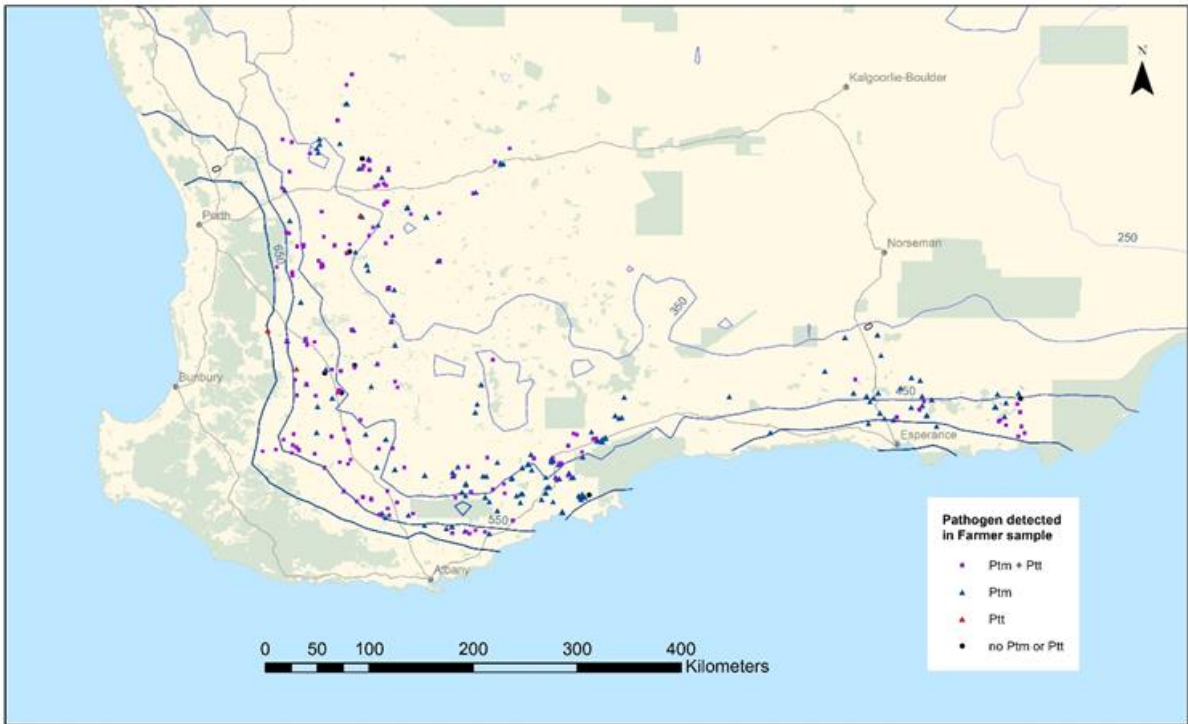


Figure 2. Map showing the distribution of Ptm and Ptt in the 330 Farmer Cohort samples (2019) in south-west Western Australia. 153 samples were positive for both Ptm and Ptt, 165 samples were positive for Ptm only, four samples were positive for Ptt only, and eight samples were negative for both Ptm and Ptt.

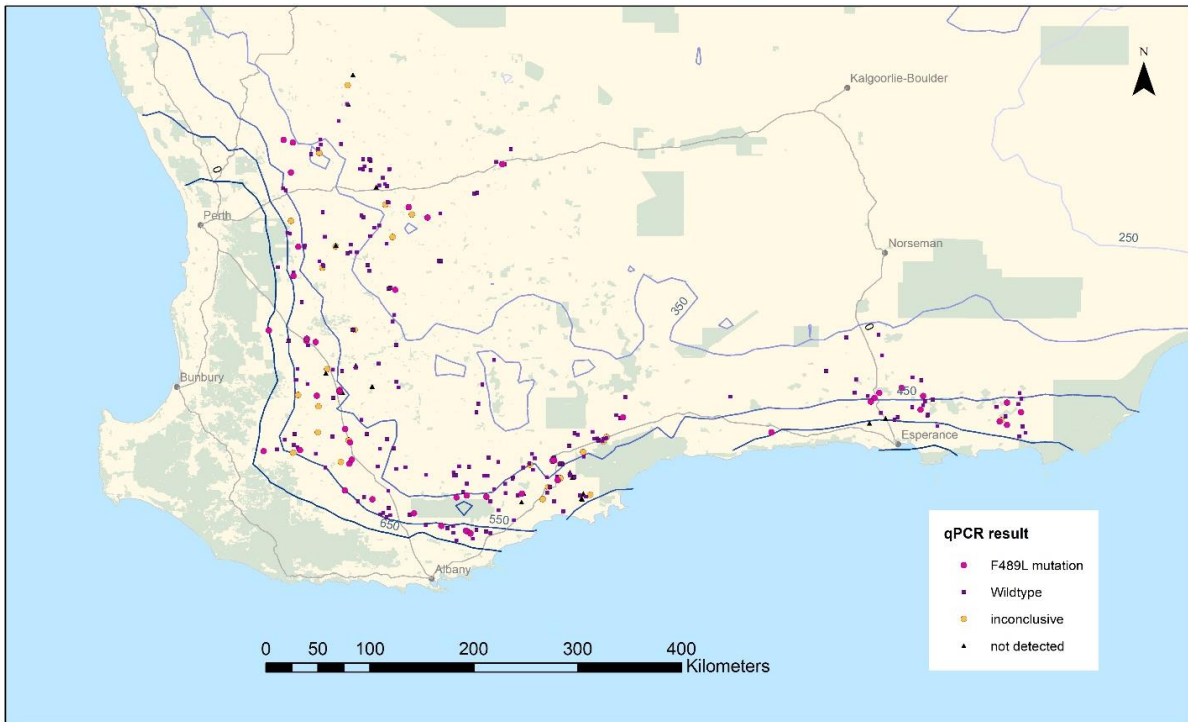


Figure 3. Map of south-west Western Australia showing the location of samples with qPCR analysis results of the *CYP51A* F489L mutation. There were 51 samples with the mutation, 231 samples with the wildtype, 26 samples were inconclusive, and 22 samples were negative for the mutation and the wildtype.

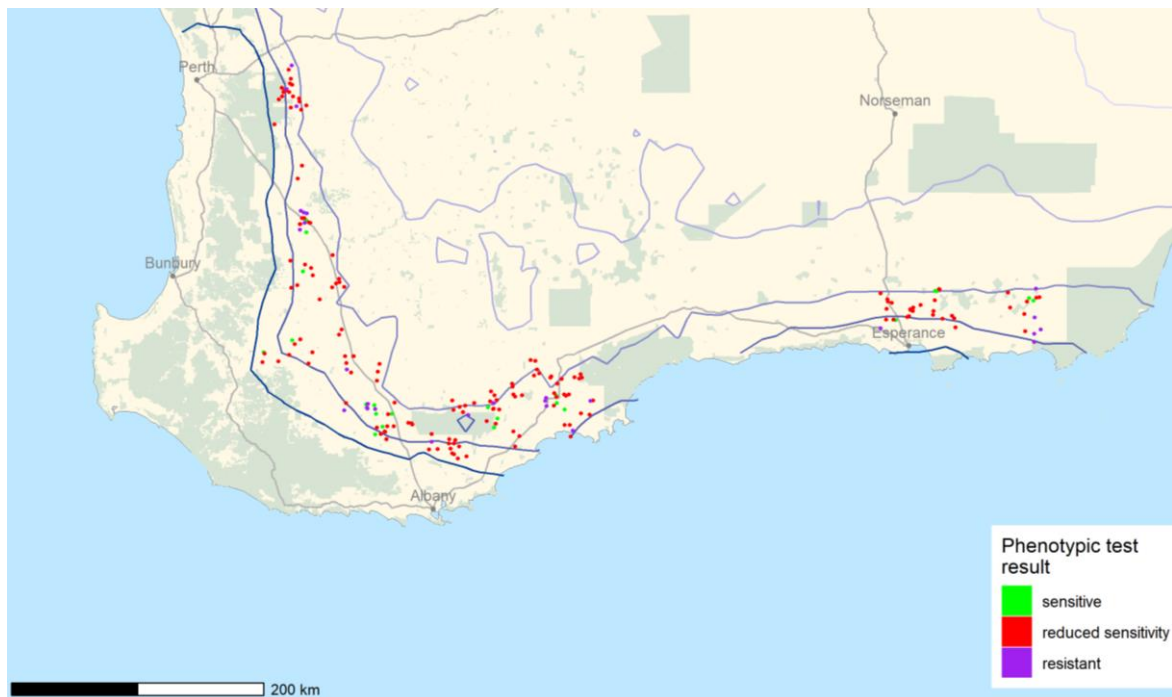


Figure 4. Phenotypic test results of the high rainfall 2019 Farmer Cohort lesion isolations from south-west Western Australian barley samples. There are 210 isolations on the map, with 21 isolations categorized as sensitive, 161 as reduced sensitive and 28 as resistant.









Conclusion

Two years ago (2018), SFNB fungicide resistance was limited to the Esperance and South Stirling areas, but is now found in more locations in south-west WA. In addition, reduced sensitivity to DMI fungicides is now very common across the southern WA wheatbelt.

Farmers have been provided with their 2019 sample results and management recommendations for the 2020 barley growing season. The growers were advised that the results were limited to the analysis of one sample per paddock and resistance reports might not necessarily correlate with fungicide field failure. Farmers with paddocks that received a resistant or a reduced sensitivity result need to be cautious with their fungicide management. Fungicides containing tebuconazole, propiconazole, epoxiconazole and prothioconazole might still work at controlling net blotch, but not as well as they would on a sensitive population. Management options are still available, however reduced sensitivity populations are at greater risk of becoming fungicide resistant if these actives are not used with caution. The summary of fungicide management advice to farmers is shown in Table 2.

The co-innovation cycle continues in 2020 with requests for farmer samples and management information, and on-farm field trials.

Table 2. Summary of management advice provided to Farmer Cohort participants

Active ingredient from Group 3	Fungicide resistant mutation <u>not present</u>	Fungicide resistant mutation <u>present</u>	Examples of common brand names of foliar fungicides registered for SFNB and NFNB
Tebuconazole			Veritas [®] , Custodia [®]
Propiconazole			Topnotch [®] , Aurora [®] 250, Bumper [®] , Cracker [®] , Detour [®] , Procon [®] , Propi [®] 250, Propicol [®] , Propicure [®] , Prestige [®] , Petulant [®] 250EC, Propeller [®] 250, Picaro [®] 250EC, Pace [®] , Restore [®] , Tilt [®] 250, Propiconazole 250, Propicon [®] 250, Slipstream [®] , Throttle [®] 500, Prop [®] 500, Propiconazole 500
Prothioconazole			Aviator Xpro [®] , Prosaro [®]
Epoxiconazole			Radial [®] , Serial [®] 150 EC, Tazer [®] Xpert, Avior Gold, Epoxiconazole 500, Soprano [®] 500 1, Opera [®] , Opus [®] 125

Green = active is fully effective on disease

Yellow = active's performance is lower than what you should expect when treating a fully sensitive population.

Red = active is not effective any longer and control failure is to be expected

**Note- Obviously, the frequency at which resistance is found in the paddock is what ultimately determines the performance of the active.*

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