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Investigating a potential new variant of *Zymoseptoria tritici*, causal agent of septoria leaf blotch, and implications for UK winter wheat varieties

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1. Abstract

Septoria leaf blotch is the most economically damaging disease of UK winter wheat [1]. It is caused by the ascomycete fungus *Zymoseptoria tritici* (teleomorph *Mycosphaerella graminicola*, and formerly known as *Septoria tritici*) (*Zt*) [2]. Control of the disease relies on a combination of agronomy approaches, host resistance and a heavy reliance on fungicides.

Prior to recent successes on the Recommended List (RL), such as the varieties KWS Extase and LG Sundance, the majority of varieties were only moderately resistant, with ratings of 5 or 6 (where 1 = susceptible and 9 = resistant). Until December 2015, the variety Cougar was the exception to the rule, with a rating of 7. Introduced to the RL in 2013, Cougar had consistently shown high levels of resistance. Therefore, it was unusual to see moderate levels of disease on this variety in 2015. This project built on preliminary findings (that isolates collected in 2015 were different to historical isolates) and sought to:

1. Phenotype new isolates on seedlings and adult plants of Cougar and other RL varieties.
2. Sequence new isolates to:
    a) Establish potential differences between the new isolates that caused disease on Cougar and historic isolates.
    b) Initiate comparative genetic studies between Z. tritici (and/or closely related species) to shed light on potential differences in virulence in the new isolates.
    c) Identify polymorphisms between isolates to develop resources for genotyping, pathotyping and subsequent diversity analysis.
3. Test new isolates for resistance to different fungicides.

Seedling and adult plant tests, using both isolates classed as ‘Cougar’ (virulent on Cougar) and ‘non-Cougar’ (avirulent on Cougar) types, showed that the only variety consistently susceptible to the Cougar isolates was Cougar itself. This demonstrated that the risk posed to other varieties, such as LG Sundance, from these isolates is no higher than for any other isolate. This was confirmed by genotyping, where the different categories of isolates were unable to be distinguished from one another.

Fungicide sensitivity testing also confirmed a low risk from these isolates, with no unexpected mutations present.

These are positive results for growers, who seek to deploy cultivar resistance as part of integrated disease management. For researchers, this system enables further exploration of the host-pathogen interaction to understand why virulence to resistant varieties arises and how to prevent it.

2. Introduction

Septoria leaf blotch is the most economically damaging disease affecting UK winter wheat [1]. It is caused by the ascomycete fungus *Zymoseptoria tritici* (teleomorph *Mycosphaerella graminicola*, and formerly known as Septoria tritici) (*Zt*) [2]. Control of the disease currently relies on a combination of fungicidal inputs, agronomy approaches and host resistance, with heavy reliance on fungicides. The use of fungicides is complicated by the constantly evolving pathogen population, with the identification of isolates resistant to quinone outside inhibitors (QoIs) [3] and azoles [4]. Although resistance to azole fungicides is well documented, they do still form part of current control programmes due to residual activity and are often used in combination with succinate dehydrogenase inhibitors (SDHIs) [5], along with the preventative treatment of the multi-site inhibitor chlorothalonil. Insensitivity to SDHIs has recently been reported [6], [7], and chlorothalonil is due to be withdrawn in November 2019 and this highlights the importance of research into other disease management strategies.

Varietal resistance to *Zt* is under-researched in comparison to resistance to other diseases such as the cereal rusts. Resistance can be either near complete to specific isolates, following a gene-for-gene relationship, or it can be partial, with resistance to multiple isolates [8]. There are currently 20 mapped genes for resistance to *Zt*, termed *Stb* genes, of which 12 are isolate specific [8]. Most of the *Stb* genes identified to date operate at the seedling and adult plant stage, with the exception of *Stb17,* which is the only true adult plant resistance gene [9]. In UK wheat germplasm, the most commonly used resistance gene is *Stb6*, an isolate-specific resistance gene found in varieties such as Claire and Hereward [10], [11]. This resistance gene and the corresponding pathogen avirulence gene have recently been cloned [12], [13]. Prior to recent successes on the Recommended List such as the varieties KWS Extase and LG Sundance, the majority of varieties were only moderately resistant, with ratings of 5 or 6 (where 1 = susceptible and 9 = resistant). Until December 2015, the winter wheat variety Cougar was the exception to the rule and was the only variety with a resistance rating of 7. Introduced in 2013 to the Recommended List, this variety had consistently shown high levels of resistance, and it was therefore unusual to see moderate levels of disease on this variety in 2015. Levels of Septoria leaf blotch were on the whole generally quite low in 2015 compared to previous years (www.cropmonitor.co.uk). Despite these low levels, there were several sightings of higher than expected levels of disease on Cougar in different parts of the UK, and it was from these infected leaves that NIAB and the breeder of Cougar, RAGT Seeds isolated spores of *Zt*. Preliminary experiments were carried out by NIAB and RAGT to compare the isolates collected with older UK isolates. The results from these experiments confirmed that there was a difference in the reaction of Cougar to the different isolates, with pycnidia being produced only when isolates collected from Cougar were used. In both experiments, Solstice, as the susceptible check, produced pycnidia regardless of the isolate used.

Routine monitoring of the *Zt* population in the UK is not undertaken with respect to virulence profile due to the rare occurrence of such changes. Although this change in the UK population is unusual, a small number of similar changes have been reported before worldwide. The variety Gene was released in the USA 1992, but within the space of a 2-3 year period isolates were identified that were able to overcome the resistance in this variety [14]. More recently, a reduction in the effectiveness of the resistance in the variety Foote was also reported in the same region [15]. It is therefore entirely possible that some of the UK isolates have evolved to overcome the resistance in Cougar.

The reports of *Zymoseptoria tritici* on Cougar prompted a need for further investigation of the isolates collected in the 2015 field season. A project was initiated to further characterise these new isolates and compare them to historical isolates. This project built on preliminary findings that the isolates collected in 2015 were different to other historical isolates and sought to:

1. Phenotype the new isolates on seedlings and adult plants of Cougar and other Recommended List varieties.
2. Sequence the new isolates using the Illumina Hiseq 2500 platform in order to:
    a) Establish potential differences between Cougar isolate and historic isolates.
    b) Initiate comparative genetic studies between *Z. tritici* (and/or closely related species) to shed light on potential differences in virulence in the new isolate.
    c) Identify polymorphisms between isolates to begin to develop resources for genotyping/pathotyping and subsequent diversity analysis.
3. Test the new isolates for resistance to different fungicides.

3. Materials and methods

3.1. Collection and classification of isolates

3.1.1. Trap nurseries

To examine the prevalence of these new isolates, trap nurseries were set up at breeder trial sites across the UK (Table 1). Plot sizes ranged from 0.5 – 1m^2, depending on the host company. A panel of varieties was chosen in order to provide a diverse range of host resistance in order to capture different *Zt* isolates (Table 2). Samples were received between May and July in each of 2016 and 2017. From each sample received, three isolates of *Zt* were obtained and stored for further use in the project.

Table 1: Locations of the trap nurseries planted in 2015 and 2016 for the 2016 and 2017 seasons respectively.

Host	**Location**
KWS	Sherborne, Dorset
KWS	Glenrothes, Fife
KWS	Thriplow, Cambridgeshire
DSV	Wardington, Oxfordshire
RAGT	Ickleton, Essex
Elsoms	Spalding, Lincolnshire
Limagrain	Woolpit, Suffolk
Syngenta	Glenrothes, Fife
Syngenta	Sutton Bridge, Lincolnshire
Syngenta	Whittlesford, Cambridgeshire

Table 2: Varieties chosen for inclusion in the trap nurseries.

Variety	Reason for Inclusion
Cougar	Susceptible to new isolates, previously resistant
Solstice	Susceptible control
Gallant	Susceptible control
KWS Santiago	Susceptible control
Solace	Grown in the West for good Septoria resistance, related to Cougar
KWS Siskin	Rated 6.7 in 2015
Graham	Rated 6.6 in 2015
LG Sundance	Candidate, rated 7 in 2015
Moulton	Candidate, rated 7 in 2015
Marston	Candidate, rated 7 in 2015
Exsept	Previously resistant, potential combination of multiple partial resistance
Istabraq	Reduction in rating in previous years (6 to 5)
Revelation	Included at the suggestion of RAGT based on their experiments
Shamrock	Varied in susceptibility at different sites in early 2000s
Stigg	Previously resistant variety, likely to carry major gene resistance
Longbow	Standard susceptible control variety, carries *Stb15*, rated 3 in 1980s
Pastiche	High partial resistance, rated 7 in mid-1990s
Tonic	Carries *Stb9*
Avalon	Susceptible and carries *Stb15*
Cadenza	Moderately resistant and carries *Stb6*

3.1.2. Classification of isolates

Isolates collected in 3.1.1 were tested under growth room conditions to confirm their inability/ability to cause disease on Cougar. Seedlings of the varieties Gallant and Cougar grown to growth stage (GS) 13 were inoculated with isolates and symptoms were assessed 2-3 weeks later. In each test, each variety was sown as 1 replicate in 9cm x 9cm pots (4 seeds/pot). The plants were grown under controlled conditions (22/ 12^{o}C, 16h light/ 8h dark) in the growth rooms and randomised. The plants were inoculated at GS13, approximately three weeks after sowing, with a spore suspension of the respective isolate. Each plant received 10 ml of water with spore concentration 5 x 10^5 spores /ml of the *Zymoseptoria tritici* isolate by spray inoculation. The plants were covered immediately with plastic sheets and left for at least 72 hours. The first assessment of the percentage of the necrotic area with pycnidia was carried out at 28 dpi, followed by a second one at 35 dpi. Isolates were classed as virulent on Cougar ("Cougar" type) if disease levels were above 10% leaf area infected.

3.2. Phenotyping of isolates

To evaluate the risk posed by these new isolates, a subset of isolates classified in 3.1.2 were tested on a panel of Recommended List varieties and controls at both the seedling and adult plant

stages. Five isolates of the “Cougar” type and five “non-Cougar” isolates were tested from each of the years of the project, so in total 10 “Cougar” isolates were tested and 10 “non-Cougar” (avirulent on Cougar) isolates were tested. Isolates were selected based on geographical location and host variety to ensure as much diversity as possible was captured.

3.2.1. Seedling Variety Tests

In each seedling variety test the pathogenicity of two *Zymoseptoria tritici* isolates, one “Cougar” type and one “non-Cougar” type, was tested. For each isolate 2 replicates of 60 lines of winter wheat were sown in 9cm x 9cm pots (4 seeds/pot), put under controlled conditions (22/ 12^{o}C, 16h light/ 8h dark) in the growth rooms and randomised. The plants were inoculated as described in 3.1.2. The plants were covered immediately with plastic sheets and left for at least 72 hours. The first assessment of the percentage of the necrotic area with pycnidia was carried out at 28 dpi, followed by a second one at 35 dpi. Least significant difference values were calculated using GenStat and were based on the differences across the entire data set of 20 isolates.

3.2.2. Adult Plant Variety Tests

The pathogenicity of two *Zymoseptoria tritici* isolates, one “Cougar” type and one “non-Cougar” type, was also tested in each adult plant test. 12 plants of 60 winter wheat varieties were sown in 96 cells multitrays and put under controlled conditions (22/ 12^{o}C, 16h light/ 8h dark) in the growth rooms for 10 days, followed by vernalisation for 8 weeks. The plants were acclimatised for 1 week before being transplanted into 20cm pots (3 plants/ pot) and randomised. For each variety 4 pots were allocated, 2 of which were to be inoculated with the “Cougar” type isolate and the other 2 with the “non-Cougar” type isolate. At the flag leaf stage, the plants were sprayed with spore suspension at 5 x10^{5} spores /ml in water of the respective isolate. The plants were covered immediately with plastic sheets and left for at least 72 hours. The first assessment of the percentage of the necrotic area with pycnidia was carried out at 28 dpi, followed by two more at 35 and 42 dpi. Least significant difference values were calculated using GenStat and were based on the differences across the entire data set of 20 isolates.

3.3. Genotyping of isolates

3.3.1. DNA extraction/sequencing of select new/historic Zt isolates

DNA was extracted from isolates selected in Table 5 along with the internationally-used control isolate IPO323 using a Ultrapure DNA kit according to the manufacturer’s protocol (Qiagen, UK). DNA quality was assessed on a Qubit (Applied Biosystems, UK) and submitted to an external service provider for sequencing (Centre for genomic research, University of Liverpool). Libraries for sequencing were prepared using a Truseq PCR free kit (Illumina, UK) to generate 150 bp paired-end reads with 350 bp inserts with barcoded adaptors included to enable multiplexing on a single

sequencing lane. The libraries were sequenced on a Hiseq4000 (Illumina, UK) to obtain a minimum of 20 x coverage for calling single nucleotide polymorphisms (SNPs).

3.3.2. Mapping sequencing reads to reference accession (MG2/IPO323) for identifying SNPs for genotyping purposes

Raw sequencing reads were trimmed to remove any adaptor sequences using cutadapt v. 1.2.1 [16], and the trimmed reads were then aligned to the reference genome assembly IPO323 v. MG2 [17] with bwa-mem v. 0.7.12. Alignments were deduplicated before further processing. SNPs, insertions and deletions were called using freebayes v. 1.2.0 [18]. Genic regions were annotated using snpEff v. 4.3.T [19] based on annotation MG2.40. Coverage statistics for each chromosome were produced using samtools depth v. 1.9 [20] and plotted with R v. 3.4, library igraph.

3.3.3. Perform Phylogenetic analysis to assess diversity of new/historic Zt isolates

SNPs from genic regions were used to conduct a phylogenetic analysis; neighbour-joining trees were estimated in R v. 3.4 using the ape (Analysis of Phylogenetics and Evolution) package.

3.4. Fungicide sensitivity tests

3.4.1. Phenotyping

In vitro fungicide sensitivity assays were carried out as previously described [21] using sabouraud dextrose broth (SDB; Oxoid Basingstoke, UK), spores at 2.5×10^4 spores ml^{-1}, an untreated control and 11 different fungicide concentrations (Table 3). The triazoles tebuconazole, epoxiconazole, prothioconazole-desthio (most active metabolite of prothioconazole [22]) and the imidazole prochloraz are azoles and inhibit sterol demethylation. Bixafen is a succinate dehydrogenase inhibitor (SDHI) fungicide whereas fentin chloride insensitivity is linked to efflux pump overexpression. For the 2016 isolates growth in the presence of 5 ppm azoxystrobin, a quinone outside inhibitor (QoI), was also measured. Strains IPO323 and NT321.17 were included in the tests, representing a sensitive (IPO323) and insensitive phenotype (NT321.17) to azole, QoI and SDHI fungicides.

One hundred μl of spore suspension (10^5 conidia ml^{-1}) of *Z. tritici* isolates was added to each well. Plates were incubated for 4 days at 23°C, and growth measured at 630 nm using a Fluostar Optima microplate reader (BMG Labtech GmbH). Fungicide sensitivities were determined as 50% effective concentration (EC_{50}) using a dose-response relationship according the BMG Labtech Optima Software.

Table 3: Fungicide concentrations (ppm) used for *in vitro* fungicide sensitivity testing.

Tebuconazole	Epoxiconazole	Prothio-desthio	Prochloraz	Bixafen	Fentin chloride
7.500E+01	7.500E+01	2.000E+01	1.500E+01	1.500E+01	1.000E+01
2.727E+01	2.000E+01	6.667E+00	3.000E+00	7.500E+00	3.333E+00
9.917E+00	5.333E+00	2.222E+00	6.000E-01	3.750E+00	1.111E+00
3.606E+00	1.422E+00	7.407E-01	1.200E-01	1.875E+00	3.704E-01
1.311E+00	3.793E-01	2.469E-01	2.400E-02	9.375E-01	1.235E-01
4.769E-01	1.011E-01	8.230E-02	4.800E-03	2.344E-01	4.115E-02
1.734E-01	2.697E-02	2.743E-02	9.600E-04	5.859E-02	1.372E-02
6.306E-02	7.192E-03	9.145E-03	1.920E-04	1.465E-02	4.572E-03
2.293E-02	1.918E-03	3.048E-03	3.840E-05	3.662E-03	1.524E-03
8.338E-03	5.114E-04	1.016E-03	7.680E-06	9.155E-04	5.081E-04
3.032E-03	1.364E-04	3.387E-04	1.536E-06	2.289E-04	1.694E-04

3.4.2. Sequencing of fungicide target encoding genes

For a selection of strains, the fungicide target encoding genes for azoles (sterol 14α-demethylase (*CYP51*)) and SDHIs (succinate dehydrogenase subunits B, C and D (*SdhB*, *SdhC*, *SdhD*)), were sequenced to check for target-site mutations. For this, DNA extractions and PCR reactions were carried out with Red Hot *Taq* DNA Polymerase (Thermo Scientific), Phusion High Fidelity Polymerase (Finnzymes Oy) or Easy-A high fidelity PCR cloning enzyme (Agilent) kits and cycling programmes as described previously [23] using the PCR primer sets and conditions listed in Table 4. PCR products were sequenced using the PCR amplification primers; except for *CYP51* where a third primer, 51S1 (5'-AGAAGTTCGCATCGAC-3'), was also used in addition to the two PCR primers to cover the whole area of the gene where key mutations have been reported.

Table 4: PCR primer sequences, amplification targets and reaction conditions

Primer sets (forward and reverse primers) and sequences (5'-3')[1]		Target	Size[2] (bp)	PCR kit and annealing temperature
51F1: TTCTCCCGGAACATTGACAT	51R1: TGCATACCCACACCAATTCT	*CYP51*	~1958	Phusion, 60°C
SdhBF: TAAACACTCCACGCCTCACG	SdhBR: GTCTTCCGTCGATTTCGAGAC	*SdhB*	1270	Phusion, 63°C
SdhCF: CTACAARAAMGCCAAMCCCAAC	SdhCR: ATGTTGGCACAGAAGCTCAC	*SdhC*	~749	Easy-A, 57°C
SdhDF: CGGGAATAACCAACCTCACT	SdhDR: CCTCACTCCTCCAAACCGTA	*SdhD*	840	Phusion, 57°C

[1]Primer SdhBF designed by Dubos et al. [24], primers SdhDF and SdhDR developed by Dooley et al [25];
[2]PCR reactions can result in different sizes due to isolate-dependent insert length differences for both targets.

4. Results

4.1. Identification and classification of isolates

Samples of wheat showing symptoms of Septoria leaf blotch were collected from the trap nurseries established at the breeder partner sites in each of the 2016 and 2017 field seasons. Isolates were obtained from these samples and classified according to their virulence to the varieties Cougar and Gallant.

4.1.1. 2016

In 2016, 84 samples were collected from across the trap nurseries. Additional samples were also received from external trials, such as demonstration trials. The samples came from 15 counties and 43 varieties (Figure 1). There were 11 samples from Cougar and 21% of the isolates tested were classed as virulent on Cougar (Figure 2) and therefore termed “Cougar” isolates. The Cougar isolates from 2016 were mainly found on samples from the variety Cougar, although one isolate was obtained from a sample each of Amplify, KWS Santiago, KWS Siskin and Solace. The collection of isolates from varieties other than Cougar highlight that it is possible for these new isolates to be found on varieties currently grown in the UK, even in the absence of Cougar. Cougar isolates were found across the UK, with an approximately equal distribution, although this is based on only a limited number of samples.

Figure 1: Location of samples collected in 2016.

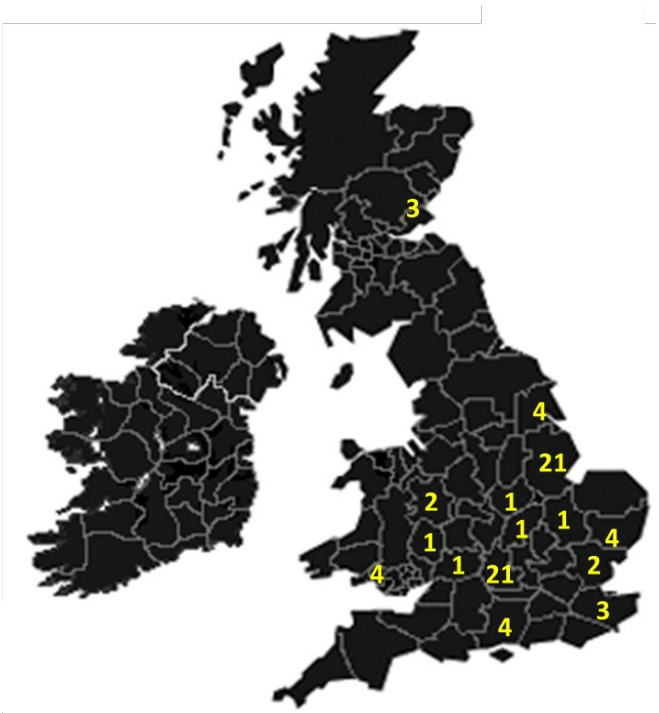
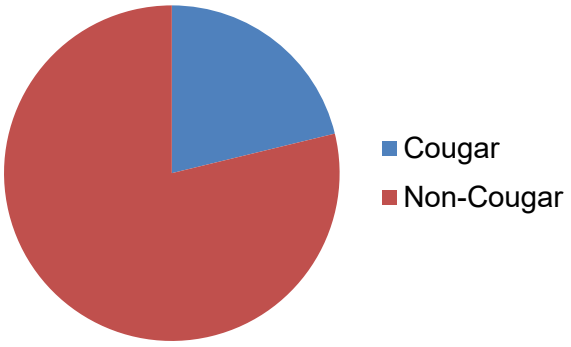
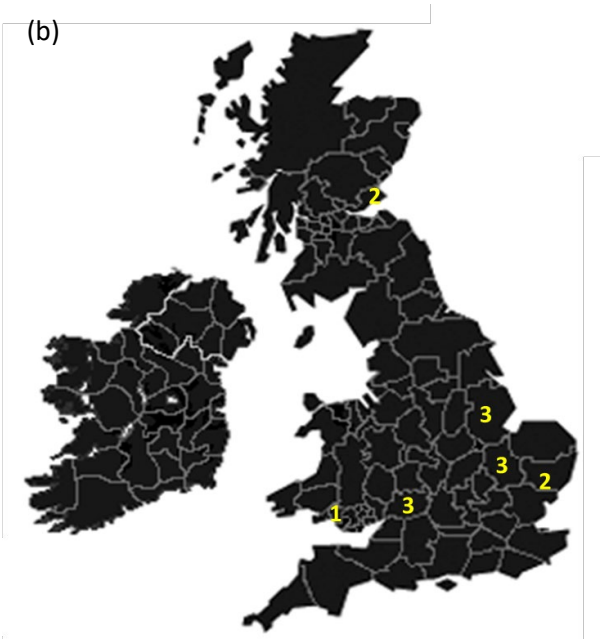


Figure 2: Classification of isolates collected in 2016 given as a percentage (a) and the locations of Cougar isolates collected in 2016 (b).

(a)



(b)



4.1.2. 2017

In 2017, 85 samples were collected from 11 counties and 27 varieties (Figure 3). There were 9 samples from Cougar and 27% of the isolates tested were classed as virulent on Cougar (Figure 4). The Cougar isolates from 2017 were mainly found on samples from the variety Cougar, although one isolate was obtained from a sample of Longbow. As in 2016, Cougar samples were found across the UK (Figure 4).

Figure 3: Location of samples collected in 2017.

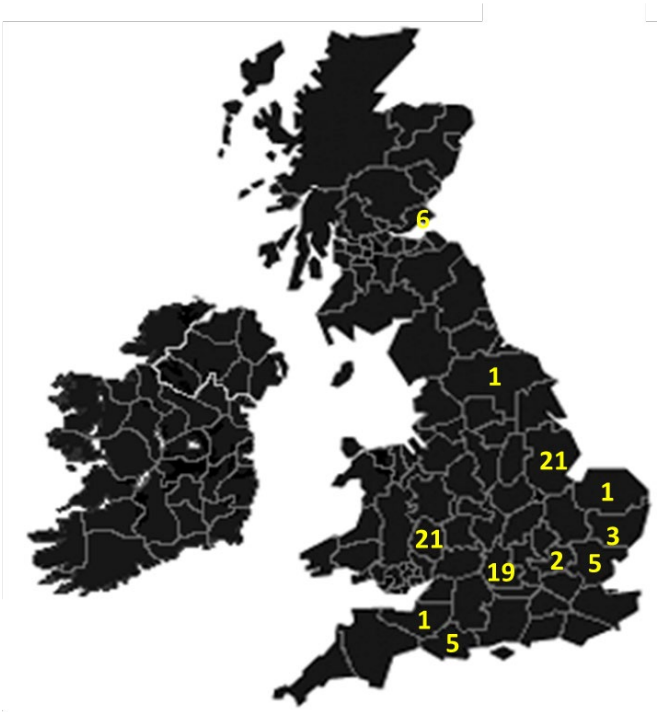
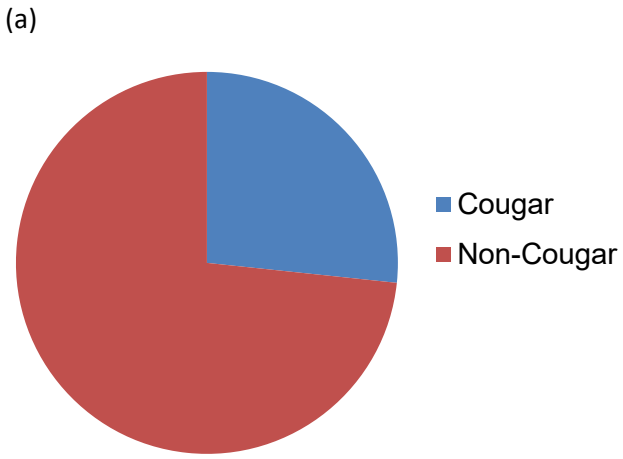
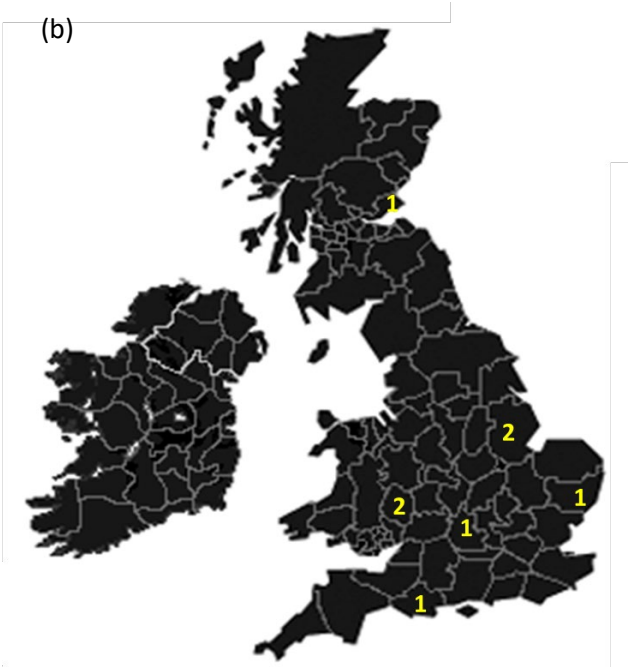


Figure 4: Classification of isolates collected in 2017 given as a percentage (a) and the locations of Cougar isolates collected in 2017 (b).





4.2. Phenotyping of Isolates

In order to assess the risk posed by the new Cougar isolates to other resistant varieties on the 2018/19 Recommended List, five isolates of each type (Cougar and non-Cougar) were chosen from 2016 and 2017 (Table 5).

Table 5: Isolates selected for further testing in the seedling and adult plant tests.

Isolate Code	Year	Type	Host Variety	Location
Zt014C	2016	Cougar	Cougar	Cambridgeshire
Zt018A	2016	Cougar	Solace	Bridgend
Zt020A	2016	Cougar	Cougar	Gloucestershire
Zt049A	2016	Cougar	Amplify	Lincolnshire
Zt062A	2016	Cougar	Cougar	Suffolk
Ztr(ii)2015	2016	Non-Cougar	Unknown	Unknown
Zt001B	2016	Non-Cougar	Marston	Oxfordshire
Zt009A	2016	Non-Cougar	KWS Santiago	Fife
Zt044C	2016	Non-Cougar	Stratosphere	Lincolnshire
Zt069C	2016	Non-Cougar	KWS Cashel	Essex
Zt110A	2017	Cougar	Longbow	Herefordshire
Zt143A	2017	Cougar	Cougar	Lincolnshire
Zt159A	2017	Cougar	Cougar	Dorset
Zt161A	2017	Cougar	Cougar	Kinross-shire
Zt168A	2017	Cougar	Cougar	Suffolk
Zt100A	2017	Non-Cougar	Stigg	Herefordshire
Zt142A	2017	Non-Cougar	Istabraq	Lincolnshire
Zt158A	2017	Non-Cougar	Gallant	Dorset
Zt165A	2017	Non-Cougar	Graham	Fife
Zt153A	2017	Non-Cougar	Solstice	Essex

4.2.1. Seedling variety tests

The isolates selected were tested on a panel of varieties that consisted of the control varieties (Table 2) and the varieties in RL trials in either 2016 or 2017. This also included some candidate varieties for each of the years (Table 6 and Table 7). Isolate Zt110A had to substituted for another isolate Zt113A for these tests due to poor sporulation in the multiplication of spores. Good levels of disease were seen in most tests, however three tests showed only moderate-low levels of disease. These tests used the isolates Zt001B, Zt020A, Zt009A, Zt018A, Zt044C and Zt049A (Table 6), the reasons behind these low disease scores are unclear.

The susceptible varieties Gallant, KWS Santiago, Solstice and Viscount generally achieved levels of disease around 55-60% leaf area infected at the final disease assessment point. These varieties were not universally susceptible to all isolates however, for example Gallant was not the most susceptible variety when challenged with the Cougar isolate Zt161A. Seed problems led to low or absent assessments on the control variety Longbow in the 2016 isolate tests.

The variety Cougar reacted as expected, showing clear susceptibility to all of the Cougar isolates and resistance to all of the non-Cougar isolates. The related variety Solace showed general susceptibility to all of the Cougar isolates and resistance to the non-Cougar isolates, although deviation from the expected disease levels were seen. The reactions of the other varieties were variable and there appeared to be no consistent reaction to one group of isolates, i.e. the Cougar or non-Cougar isolates.

From the control varieties, Avalon (susceptible control, carries *Stb15*) was susceptible to all isolates and Pastiche (high partial resistance) was moderately resistant to most isolates although this did vary between tests. Stigg (high resistance, possible major gene) was generally resistant to all isolates, although did exhibit more disease symptoms when tested with Zt113A (Cougar) and Zt158A (non-Cougar). Cadenza (moderately resistant, carries *Stb6*) was more susceptible than expected suggesting that these isolates also carry virulence to *Stb6*. Similarly, Exsept (multiple partial resistance genes) was more susceptible than expected.

Varieties currently rated with high resistance on the 2018/19 RL such as LG Sundance (rated 7.9), Dunston (6.7), Freiston (6.7), Graham (6.9) and Marston (resistant candidate) generally maintained their high disease resistance status. LG Sundance showed very low levels of disease to most isolates, with the exception of Zt161A (Cougar) and Zt165A (non-Cougar). The same variability was seen for Dunston, Freiston, Graham and Marston and in all cases this did not appear to be influenced by whether the isolate was Cougar or non-Cougar type.

Table 6: Reactions of wheat varieties at the seedling stage to the selected Cougar and non-Cougar isolates identified in 2016. Highlighting has been applied to show the range of reactions with green indicating low disease and red indicating high disease. * = missing data. LSD = 8.082

Variety	2018/19 RL rating or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2016 Isolates					Non-Cougar, 2016 Isolates				
		Zt014C	Zt018A	Zt020A	Zt049A	Zt062A	Ztr(ii)2015	Zt001B	Zt009A	Zt044C	Zt069C
Avalon	Susceptible, Stb15	38	10	2	23	45	70	13	8	25	45
Belgrade	6.1	1	13	1	7	40	1	2	1	23	30
Bennington	6.3	2	3	1	6	23	1	1	3	30	8
Britannia	5.3	2	13	0	7	12	1	7	12	28	4
Cadenza	Moderately resistant, Stb6	50	45	0	38	55	13	33	9	28	45
Claire	5.3	28	13	2	13	38	50	7	6	25	45
Cordiale	4.8	35	18	0	33	*	13	7	10	33	30
Costello	6.1	1	4	0	9	12	2	2	3	8	*
Cougar		45	15	5	20	55	0	0	0	1	0
Crusoe	6.5	55	9	13	20	55	30	9	6	15	50
Dickens	4.7	45	15	0	20	33	7	7	6	25	45
Dunston	6.7	1	1	0	14	55	1	2	1	23	50
Elation	4.3	*	*	*	*	*	*	*	*	*	*
Elicit	6	*	*	*	*	*	*	*	*	*	*
Evolution	5.4	35	18	2	15	40	30	8	5	18	43
Exsept	Multiple partial resistant	45	12	12	20	55	10	8	5	20	28
Freiston	6.7	20	19	1	12	35	0	23	9	20	8
Gallant	Susceptible Control	63	23	1	18	50	40	23	8	28	55
Gleam	6.4	*	*	*	*	*	*	*	*	*	*
Grafton	5.3	60	23	0	25	50	10	4	12	28	45
Graham	6.9	2	4	0	13	28	8	2	5	9	20
Hardwicke		3	6	0	4	25	2	3	8	18	18
Istabraq		50	20	5	20	53	60	9	8	18	45

Variety	RL rating or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2016 Isolates					Non-Cougar, 2016 Isolates				
		Zt014C	Zt018A	Zt020A	Zt049A	Zt062A	Ztr(ii)2015	Zt001B	Zt009A	Zt044C	Zt069C
JB Diego	5.2	23	9	1	15	60	55	3	4	15	50
KWS Barrel	4.5	65	20	0	14	50	5	10	7	23	50
KWS Basset	5.1	9	5	0	7	30	1	2	8	25	5
KWS Crispin	5.8	7	2	2	5	12	2	2	1	9	4
KWS Jackal	4.9	*	*	*	*	*	*	*	*	*	*
KWS Kerrin	5	18	33	0	13	50	3	0	13	15	33
KWS Lili	5.9	12	5	2	15	30	4	4	4	14	23
KWS Luther		*	*	*	*	*	*	*	*	*	*
KWS Santiago	4.3	45	30	0	28	60	38	25	23	23	50
KWS Silverstone	4.6	40	33	7	18	45	40	11	12	15	50
KWS Siskin	6.7	35	7	1	13	30	6	8	2	15	23
KWS Trinity	5.5	50	9	25	12	25	12	20	5	10	25
KWS Zyatt	6.4	1	8	0	6	40	0	1	2	20	28
Leeds	4.6	50	18	0	13	48	15	5	10	28	33
LG Bletchley		10	9	1	18	30	1	7	12	25	23
LG Cassidy		48	38	4	35	60	50	33	12	30	55
LG Generation		*	*	*	*	*	*	*	*	*	*
LG Motown	5.7	23	15	2	10	28	3	23	7	23	30
LG Sundance	7.9	2	5	0	2	13	0	0	1	10	1
Longbow	Susceptible control, Stb15	*	*	*	*	*	*	*	*	*	*
Marlowe		50	3	0	7	18	1	1	4	28	20
Marston		5	3	9	7	28	0	7	1	5	9
Moulton	6.5	1	0	0	5	25	1	1	3	12	15
Myriad	5.6	40	15	1	12	50	4	2	5	5	40
Pastiche	High partial resistance	30	5	2	23	28	13	1	7	23	23
Reflection	5.4	3	8	0	8	28	2	2	8	18	13

Variety	RL rating or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2016 Isolates					Non-Cougar, 2016 Isolates				
		Zt014C	Zt018A	Zt020A	Zt049A	Zt062A	Ztr(ii)2015	Zt001B	Zt009A	Zt044C	Zt069C
Pastiche	High partial resistance	30	5	2	23	28	13	1	7	23	23
Reflection	5.4	3	8	0	8	28	2	2	8	18	13
Relay	6.4	45	15	8	20	43	35	8	6	28	48
Revelation	6.3	2	3	0	8	23	1	5	15	12	18
RGT Conversion	5.4	4	9	0	7	18	5	0	13	9	20
RGT Gravity	5	*	*	*	*	*	*	*	*	*	*
RGT Illustrious	6.1	45	13	1	12	55	4	1	1	18	55
RGT Knightsbridge		43	7	0	12	50	10	7	3	14	33
RGT Universe		*	*	*	*	*	*	*	*	*	*
RGT Westminster		48	20	3	10	28	5	7	7	18	38
Savello	5.3	10	9	0	14	33	0	30	9	23	23
Scout	5.7	23	15	7	18	55	10	3	9	18	43
Shabras	6.2	1	5	1	1	45	3	1	2	25	18
Shamrock		23	18	5	18	50	28	45	10	28	50
Skyfall	5.9	9	6	1	7	30	3	13	1	15	23
Solace		50	8	15	20	35	2	0	0	5	4
Solstice	Susceptible Control	65	40	23	18	50	35	33	9	9	45
Spyder		14	2	3	9	35	2	4	0	3	13
Stigg	5.7	2	4	2	5	15	4	2	1	13	10
Stratosphere		35	13	0	7	20	3	20	7	23	15
Tonic	Stb9	70	25	0	20	58	50	33	5	28	40
Verso		*	*	*	*	*	*	*	*	*	*
Viscount	4.8	65	6	0	11	35	5	10	4	18	40
Zulu	5.2	63	23	45	25	40	40	30	9	28	33
Max		70	45	45	38	60	70	45	23	33	55
Min		1	0	0	0	0	0	0	0	0	0

Table 7: Reactions of wheat varieties at the seedling stage to the selected Cougar and non-Cougar isolates identified in 2017. Highlighting has been applied to show the range of reactions with green indicating low disease and red indicating high disease. * = missing data. LSD = 8.082

Variety	2018/19 RL rating or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2017 Isolates					Non-Cougar, 2017 Isolates				
		Zt113A	Zt143A	Zt159A	Zt161A	Zt168A	Zt100A	Zt142A	Zt153A	Zt158A	Zt165A
Avalon	Susceptible, Stb15	50	70	48	37	55	53	63	55	33	47
Belgrade	6.1	23	43	23	22	43	9	43	42	7	17
Bennington	6.3	10	33	17	31	30	23	43	33	10	27
Britannia	5.3	*		*	*		*			*	*
Cadenza	Moderately resistant, Stb6	63	11	50	43	45	50	60	50	35	44
Claire	5.3	*		*	*		*			*	*
Cordiale	4.8	53	53	48	36	59	55	58	58	48	15
Costello	6.1	28	18	35	36	48	18	33	42	35	34
Cougar		55	70	47	43	70	1	3	5	2	2
Crusoe	6.5	55	55	33	33	40	50	45	33	33	15
Dickens	4.7	50	35	35	41	43	53	55	50	28	40
Dunston	6.7	15	20	8	27	44	25	40	30	15	22
Elation	4.3	40	35	40	53	48	55	53	39	30	58
Elicit	6	25	20	27	44	53	60	16	48	35	9
Evolution	5.4	25	53	33	44	37	50	55	43	20	6
Exsept	Multiple partial resistant	50	70	40	47	53	58	58	33	31	52
Freiston	6.7	35	15	35	23	43	48	23	33	28	21
Gallant	Susceptible Control	60	60	49	32	55	53	73	43	43	38
Gleam	6.4	48	30	27	50	42	50	78	42	23	58
Grafton	5.3	55	45	33	35	45	55	35	42	38	32
Graham	6.9	30	63	30	38	47	35	35	18	33	43
Hardwicke		40	38	16	35	40	48	15	30	19	14
Istabraq		53	65	55	53	38	55	65	60	50	47

Variety	RL rating or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2017 Isolates					Non-Cougar, 2017 Isolates				
		Zt113A	Zt143A	Zt159A	Zt161A	Zt168A	Zt100A	Zt142A	Zt153A	Zt158A	Zt165A
JB Diego	5.2	45	55	38	48	50	43	63	44	39	36
KWS Barrel	4.5	53	48	35	43	53	58	78	58	38	45
KWS Basset	5.1	33	23	38	35	45	58	45	31	14	38
KWS Crispin	5.8	33	30	40	30	38	35	23	32	35	45
KWS Jackal	4.9	55	68	58	60	45	60	70	44	47	48
KWS Kerrin	5.0	50	63	33	42	53	58	73	38	48	45
KWS Lili	5.9	40	28	48	38	52	43	43	53	42	35
KWS Luther		33	35	35	40	40	60	55	36	28	43
KWS Santiago	4.3	53	68	58	53	60	58	70	53	45	45
KWS Silverstone	4.6	40	38	23	50	38	53	65	41	20	41
KWS Siskin	6.7	55	33	48	29	43	45	38	43	38	45
KWS Trinity	5.5	25	45	28	35	50	48	45	23	19	30
KWS Zyatt	6.4	20	35	27	37	42	17	18	30	20	20
Leeds	4.6	43	65	48	66	48	55	63	34	45	53
LG Bletchley		*		*	*		*			*	*
LG Cassidy		*		*	*		*			*	*
LG Generation		55	48	43	43	49	48	48	34	30	39
LG Motown	5.7	30	30	53	25	43	55	28	46	48	*
LG Sundance	7.9	10	27	23	38	27	15	20	20	16	49
Longbow	Susceptible control, Stb15	63	70	63	66	65	58	80	53	70	55
Marlowe		*		*	*		*			*	*
Marston		33	30	16	48	38	23	50	42	21	45
Moulton	6.5	11	20	13	13	39	5	9	38	8	25
Myriad	5.6	53	68	63	40	55	40	68	57	20	40
Pastiche	High partial resistance	33	20	28	43	49	53	53	53	20	49
Reflection	5.4	18	25	35	26	28	50	9	28	18	27

Variety	RL rating or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2017 Isolates					Non-Cougar, 2017 Isolates				
		Zt113A	Zt143A	Zt159A	Zt161A	Zt168A	Zt100A	Zt142A	Zt153A	Zt158A	Zt165A
Relay	6.4	*		*	*		*			*	*
Revelation	6.3	8	28	20	45	40	35	38	47	15	40
RGT Conversion	5.4	*		*	*		*			*	*
RGT Gravity	5.0	60	65	45	52	55	55	58	53	45	44
RGT Illustrious	6.1	38	58	43	53	68	43	58	52	29	29
RGT Knightsbridge		*		*	*		*			*	*
RGT Universe		35	40	23	47	50	58	28	39	25	46
RGT Westminster		*		*	*		*			*	*
Savello	5.3	50	15	23	40	45	55	28	37	28	45
Scout	5.7	*		*	*		*			*	*
Shabras	6.2	35	15	23	35	38	8	35	30	25	29
Shamrock		50	65	38	38	38	45	70	43	25	72
Skyfall	5.9	13	25	35	19	43	43	28	38	23	30
Solace		50	63	23	38	48	14	15	35	28	22
Solstice	Susceptible Control	60	70	50	50	63	65	70	63	30	50
Spyder		23	33	20	33	43	33	18	17	10	18
Stigg	5.7	33	30	17	4	22	28	45	17	30	4
Stratosphere		*		*	*		*			*	*
Tonic	Stb9	60	63	27	35	58	58	30	43	35	26
Verso		33	45	15	47	40	40	55	33	13	42
Viscount	4.8	45	38	48	45	48	53	45	15	45	39
Zulu	5.2	55	70	50	33	57	55	73	45	32	33
Max		63	70	63	66	70	65	80	63	70	72
Min		8	11	8	4	22	1	3	5	2	2

4.2.2. Adult plant variety tests

The same isolates and varieties were also examined at the adult plant stage under glasshouse conditions (Table 8 and Table 9) with the exception of Zt113A (as discussed above). As with the seedling tests, good levels of disease were seen in the adult plant tests. Two tests using four of the 2016 isolates were unfortunately unable to be completed due to vernalisation problems (isolates Zt018A, Zt020A, Zt001B, Zt009A).

The susceptible varieties Gallant, KWS Santiago, Solstice and Viscount generally achieved levels of disease with values ranging from 6-82% leaf area infected at the last disease assessment point. Similar to the seedling tests, these varieties were not universally susceptible to all isolates. The susceptible control Longbow was able to be included in the adult plant tests and was universally susceptible to all isolates tested.

The variety Cougar reacted as expected, showing clear susceptibility to almost all of the Cougar isolates and resistance to all of the non-Cougar isolates. There was one exception: disease levels were lower than expected on Zt110A (Cougar). The related variety Solace showed a more variable reaction with no obvious trend associated to whether the isolates were Cougar or non-Cougar. The reactions of the other varieties were variable and there appeared to be no consistent reaction to one group of isolates, i.e. the Cougar or non-Cougar isolates.

From the control varieties, Avalon (susceptible control, carries *Stb15*) was susceptible to most isolates and Pastiche (high partial resistance) was resistant to most isolates highlighting a difference in performance between the seedling and adult plant growth stages. A similar situation was seen for Stigg (high resistance, possible major gene) was generally resistant to all isolates, compared to the more modest levels of resistance seen at the seedling stage. Cadenza (moderately resistant, carries *Stb6*) was very susceptible at the adult plant stage, perhaps more so than at the seedling stage, providing further confirmation that these isolates carry virulence to *Stb6*. Exsept (multiple partial resistance genes) was more resistant at the adult plant stage and exhibited the expected partial resistance phenotype.

Varieties currently rated with high resistance on the 2018/19 RL such as LG Sundance (rated 7.9), Dunston (6.7), Moulton and Marston (resistant candidate) were resistant to all of the isolates under investigation. The varieties Freiston, Graham (6.9) and KWS Siskin (6.7) were generally resistant to most isolates and similar to the seedling tests, any susceptibility seen was not influenced by whether the isolate was Cougar or non-Cougar type. Other varieties under evaluation performed as expected from their RL ratings, with the exception of Crusoe. Crusoe showed disease levels of between 8 and 55%. Its current rating is 6.5 and other varieties with similar ratings generally

Table 8: Reactions of wheat varieties at the adult plant stage to the selected Cougar and non-Cougar isolates identified in 2016. Highlighting has been applied to show the range of reactions with green indicating low disease and red indicating high disease. * = missing data. LSD = 9.146

Variety	RL Rating 2018/19 or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2016 Isolates					Non-Cougar, 2016 Isolates				
		Zt014C	Zt018A	Zt020A	Zt049C	Zt062A	Ztr(ii) 2015	Zt001B	Zt009A	Zt044C	Zt069C
Avalon	Susceptible, *Stb15*	13	*	*	25	18	30	*	*	13	33
Belgrade		1	*	*	2	9	3	*	*	2	3
Bennington	6.3	0	*	*	50	28	1	*	*	0	13
Britannia		5	*	*	2	10	6	*	*	28	35
Cadenza	Moderately resistant, *Stb6*	35	*	*	55	25	30	*	*	55	30
Claire		9	*	*	15	13	4	*	*	9	25
Cordiale		2	*	*	17	35	40	*	*	35	18
Costello	6.1	0	*	*	19	35	20	*	*	5	20
Cougar		12	*	*	40	25	0	*	*	1	2
Crusoe	6.5	3	*	*	28	35	33	*	*	33	28
Dickens		5	*	*	23	15	1	*	*	25	15
Dunston	6.7	0	*	*	0	0	10	*	*	0	3
Elation	4.3	*	*	*	*	*	*	*	*	*	*
Elicit	6.0	*	*	*	*	*	*	*	*	*	*
Evolution	5.4	4	*	*	9	9	30	*	*	23	15
Exsept	Multiple partial resistant	1	*	*	18	10	1	*	*	13	23
Freiston		1	*	*	50	13	1	*	*	1	12
Gallant	Susceptible Control	7	*	*	43	18	50	*	*	55	40
Gleam	6.4	*	*	*	*	*	*	*	*	*	*
Grafton		3	*	*	20	23	2	*	*	20	33
Graham	6.9	0	*	*	13	35	12	*	*	4	8
Hardwicke		10	*	*	8	25	25	*	*	18	50
Istabraq		15	*	*	15	30	6	*	*	10	38
JB Diego	5.2	37	*	*	30	18	50	*	*	38	25

Variety	RL Rating 2018/19 or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2016 Isolates					Non-Cougar, 2016 Isolates				
		Zt014C	Zt018A	Zt020A	Zt049C	Zt062A	Ztr(ii) 2015	Zt001B	Zt009A	Zt044C	Zt069C
KWS Barrel	4.5	40	*	*	45	25	10	*	*	50	25
KWS Basset	5.1	25	*	*	9	18	3	*	*	45	28
KWS Crispin	5.8	1	*	*	7	30	3	*	*	8	28
KWS Jackal	4.9	*	*	*	*	*	*	*	*	*	*
KWS Kerrin	5.0	12	*	*	12	9	2	*	*	15	13
KWS Lili	5.9	0	*	*	4	5	8	*	*	13	8
KWS Luther		*	*	*	*	*	*	*	*	*	*
KWS Santiago		18	*	*	38	15	20	*	*	50	30
KWS Silverstone		5	*	*	30	15	50	*	*	33	30
KWS Siskin	6.7	2	*	*	3	10	3	*	*	5	13
KWS Trinity	5.5	2	*	*	4	25	1	*	*	15	18
KWS Zyatt	6.4	0	*	*	0	7	0	*	*	3	4
Leeds	4.6	20	*	*	35	35	10	*	*	25	25
LG Bletchley		18	*	*	18	18	15	*	*	40	43
LG Cassidy		9	*	*	30	23	38	*	*	18	30
LG Generation		*	*	*	*	*	*	*	*	*	*
LG Motown	5.7	1	*	*	8	18	2	*	*	23	23
LG Sundance	7.9	1	*	*	3	0	0	*	*	12	0
Longbow	Susceptible control, *Stb15*	47	*	*	*	*	70	*	*	*	*
Marlowe		22	*	*	15	9	2	*	*	30	15
Marston		0	*	*	0	15	2	*	*	2	7
Moulton		0	*	*	50	4	5	*	*	3	3
Myriad	5.6	10	*	*	25	20	2	*	*	18	30
Pastiche	High partial resistance	1	*	*	8	8	32	*	*	25	10
Reflection		3	*	*	4	20	20	*	*	8	18
Relay		3	*	*	18	30	35	*	*	15	9
Revelation	6.3	1	*	*	1	4	4	*	*	25	9

Variety	RL Rating 2018/19 or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2016 Isolates					Non-Cougar, 2016 Isolates				
		Zt014C	Zt018A	Zt020A	Zt049C	Zt062A	Ztr(ii) 2015	Zt001B	Zt009A	Zt044C	Zt069C
RGT Conversion		0	*	*	4	9	45	*	*	28	9
RGT Gravity	5.0	*	*	*	*	*	*	*	*	*	*
RGT Illustrious	6.1	3	*	*	20	18	4	*	*	18	20
RGT Knighstbridge		10	*	*	28	30	15	*	*	28	18
RGT Universe		*	*	*	*	*	*	*	*	*	*
RGT Westminster		12	*	*	23	25	17	*	*	23	20
Savello		25	*	*	12	5	2	*	*	15	15
Scout		10	*	*	15	15	25	*	*	35	23
Shabras	6.2	1	*	*	0	0	2	*	*	0	3
Shamrock		0	*	*	18	20	23	*	*	15	20
Skyfall	5.9	1	*	*	15	23	3	*	*	25	30
Solace		20	*	*	50	3	1	*	*	7	4
Solstice	Susceptible Control	5	*	*	40	28	20	*	*	38	33
Spyder		0	*	*	2	5	2	*	*	0	5
Stigg	High resistance, major gene	8	*	*	2	13	13	*	*	5	13
Stratosphere		4	*	*	33	15	13	*	*	60	45
Tonic	*Stb9*	12	*	*	60	33	37	*	*	40	50
Verso		*	*	*	*	*	*	*	*	*	*
Viscount	4.8	25	*	*	23	20	2	*	*	40	38
Zulu	5.2	17	*	*	20	20	5	*	*	30	25
Max		47	*	*	60	35	70	*	*	60	50
Min		0	*	*	0	0	0	*	*	0	0

Table 9: Reactions of wheat varieties at the adult plant stage to the selected Cougar and non-Cougar isolates identified in 2017. Highlighting has been applied to show the range of reactions with green indicating low disease and red indicating high disease. * = missing data. LSD = 9.146

Variety	RL Rating 2018/19 or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2017 Isolates					Non-Cougar, 2017 Isolates				
		Zt110A	Zt143A	Zt159A	Zt161A	Zt168A	Zt100A	Zt142A	Zt158A	Zt153A	Zt165A
Avalon	Susceptible, *Stb15*	37	35	28	13	42	12	19	37	29	10
Belgrade		5	2	3	5	7	2	4	11	5	11
Bennington	6.3	8	6	4	7	3	5	14	6	4	3
Britannia		*	*	*	*	*	*	*	*	*	*
Cadenza	Moderately resistant, *Stb6*	48	62	63	18	57	48	93	53	69	21
Claire		*	*	*	*	*	*	*	*	*	*
Cordiale		45	53	72	8	38	18	67	49	53	6
Costello	6.1	4	3	5	3	21	2	8	3	12	6
Cougar		4	55	73	14	24	2	2	8	7	2
Crusoe	6.5	44	38	49	14	29	33	38	55	14	8
Dickens		27	34	48	9	16	7	50	34	13	7
Dunston	6.7	9	3	3	6	5	5	5	3	4	6
Elation	4.3	14	28	55	7	9	19	54	33	12	3
Elicit	6.0	6	18	24	5	5	8	45	12	5	5
Evolution	5.4	13	25	19	8	6	12	40	38	17	8
Exsept	Multiple partial resistant	3	17	22	14	19	6	18	6	5	5
Freiston		20	2	2	6	2	4	5	4	4	3
Gallant	Susceptible Control	40	30	60	12	41	62	79	34	53	10
Gleam	6.4	4	5	3	7	9	3	5	5	25	4
Grafton		40	23	59	8	32	39	77	44	32	4
Graham	6.9	6	20	6	5	13	5	38	3	5	5
Hardwicke		15	23	9	5	15	35	54	37	35	7
Istabraq		37	20	37	6	39	20	53	24	30	4
JB Diego	5.2	22	24	38	7	16	18	59	30	16	5

Variety	RL Rating 2018/19 or reason for inclusion	Percentage Leaf Area With Pycnidia									
		Cougar, 2017 Isolates					Non-Cougar, 2017 Isolates				
		Zt110A	Zt143A	Zt159A	Zt161A	Zt168A	Zt100A	Zt142A	Zt158A	Zt153A	Zt165A
KWS Barrel	4.5	34	29	61	10	44	38	65	42	40	13
KWS Basset	5.1	32	9	27	4	5	18	59	6	7	2
KWS Crispin	5.8	6	6	11	9	5	5	20	14	24	5
KWS Jackal	4.9	24	47	50	10	41	7	59	45	33	8
KWS Kerrin	5.0	7	14	6	6	20	5	38	4	10	3
KWS Lili	5.9	4	11	3	7	10	5	10	2	16	4
KWS Luther		10	16	12	9	5	23	48	13	18	6
KWS Santiago		29	39	68	6	20	28	69	72	29	11
KWS Silverstone		33	37	60	11	33	11	63	39	32	11
KWS Siskin	6.7	5	4	8	8	11	2	3	3	14	8
KWS Trinity	5.5	3	14	5	9	17	4	16	16	24	5
KWS Zyatt	6.4	6	2	4	4	5	4	4	5	3	3
Leeds	4.6	25	34	53	13	16	20	49	15	8	14
LG Bletchley		*	*	*	*	*	*	*	*	*	*
LG Cassidy		*	*	*	*	*	*	*	*	*	*
LG Generation		13	47	30	13	18	4	70	52	28	9
LG Motown	5.7	25	10	48	9	17	6	13	22	29	6
LG Sundance	7.9	6	2	3	4	4	9	4	4	4	4
Longbow	Susceptible control, *Stb15*	59	73	84	33	49	69	78	74	63	26
Marlowe		*	*	*	*	*	*	*	*	*	*
Marston		4	2	3	3	2	1	6	6	2	3
Moulton		9	3	5	4	7	3	8	3	6	6
Myriad	5.6	25	44	52	7	21	13	60	16	25	6
Pastiche	High partial resistance	6	1	3	13	5	10	6	4	6	6
Reflection		6	8	3	7	6	33	9	3	7	4
Relay		*	*	*	*	*	*	*	*	*	*
Revelation	6.3	13	5	7	4	4	19	16	4	3	3
RGT Conversion		*	*	*	*	*	*	*	*	*	*

Variety	RL Rating 2018/19	Percentage Leaf Area With Pycnidia									
		Cougar, 2017 Isolates					Non-Cougar, 2017 Isolates				
		Zt110A	Zt143A	Zt159A	Zt161A	Zt168A	Zt100A	Zt142A	Zt158A	Zt153A	Zt165A
RGT Gravity	5.0	38	53	85	11	41	29	73	37	38	8
RGT Illustrious	6.1	25	23	35	18	26	16	39	25	18	17
RGT Knighstbridge		*	*	*	*	*	*	*	*	*	*
RGT Universe		29	24	22	6	9	40	53	29	13	4
RGT Westminster		*	*	*	*	*	*	*	*	*	*
Savello		15	7	12	5	16	12	52	3	20	5
Scout		*	*	*	*	*	*	*	*	*	*
Shabras	6.2	8	2	1	4	4	4	3	2	2	5
Shamrock		20	12	30	9	44	8	36	43	39	8
Skyfall	5.9	45	11	54	8	13	26	49	38	12	8
Solace		2	25	14	6	18	2	17	9	19	5
Solstice	Susceptible Control	40	54	68	24	23	26	72	47	30	20
Spyder		3	2	17	5	3	2	10	2	6	3
Stigg	High resistance, major gene	4	7	27	3	6	2	3	4	7	5
Stratosphere		*	*	*	*	*	*	*	*	*	*
Tonic	*Stb9*	33	43	37	16	45	63	61	32	40	23
Verso		19	4	9	22	23	8	50	14	29	23
Viscount	4.8	17	49	66	10	30	24	82	10	23	6
Zulu	5.2	14	33	57	8	23	12	54	29	21	4
Max		59	73	85	33	57	69	93	74	69	26
Min		2	1	1	3	2	1	2	2	2	2

showed much lower levels of disease. For example, KWS Zyatt (6.4) and Shabras (6.2) both had disease levels below 10% across all of the tests.

4.2.3. Correlation between seedling and adult plant data

Visual inspection of the data suggested that there was good agreement between the seedling and adult plant test results. The correlation between the data sets was 0.659 and significant at the p = 0.01 level. As highlighted above, some varieties were more resistant at the adult plant stage, perhaps explaining the remaining variation in this correlation.

4.3. Genotyping and phylogenetic analysis of 2016-2017 isolates

DNA sequencing (Illumina Hiseq4000, 150 bp paired end reads) of 20 *Zt* isolates from the collection was utilised to establish whether the isolates were genetically distinct from other known reference isolates, or if there were detectable differences between the Cougar and non-Cougar isolates,. In the original proposal we had planned to use RNA-seq, however in the intervening period capability was developed and DNA sequencing gave better value for money. Average sequencing coverage varied between 32-fold and 63-fold, providing data of suitable quality for accurately identifying genetic polymorphisms between isolates. SNP variant -calling with freebayes between the panel-isolates and the control isolate IPO323 yielded 3012991 filtered variants of which 2680385 were single-nucleotide polymorphisms (SNPs), 662378 multi nucleotide polymorphisms (MNPs) and 89618 variants were insertions or deletions (Indels).

The first comparison to plot the coverage distribution across the chromosomes of the different isolates revealed complete loss of one or multiple accessory chromosomes in all but four isolates (Zt001 B, Zt020 A, Zt100A, Zt165A). Other isolates had parts of chromosomes missing: Zt062 A, Zt018 A and Zt049 A had entirely or partially lost chromosome 18, Zt044 C lost chromosomes 15 and 17, Zt069 C lost chromosomes 18 and 21, STR2015 lost 15 and 21, Zt032 A lost 15, 17 and 18 and NIAB's culture of the reference isolate IPO323 lacked chromosomes 14 and 18. From the sequenced isolates collected in 2017 Zt110A lost chromosome 15, Zt142A lost parts of 14 and 18 and the entire chromosome 16, Zt 143 lost chromosomes 16 and 18, Zt 161A lost chromsomes 16 and 17 and Zt 159 lost chromosome 15 and the majority of chromosome 18.The presence or absence of accessory chromosomes appeared to be independent of the Cougar virulence phenotype. Structural rearrangements were commonly observed when comparing isolates to the IPO323 reference, as were large regions of gene-containing sequences, which although absent in the reference, could be observed in sequence obtained for other international *Zt* isolates.

Looking at more detail at the variation between isolates using the SNP data, neighbour-joining analysis was carried out, comparing polymorphism data from 20 *Zt* isolates from the collection (Figure 5). Clustering of isolates did not accurately distinguish between Cougar and non-Cougar

types although some cougar type isolates did group together, indicating a similar marker profile in certain cases. This result was not unexpected and indicated that the isolates used this analysis are derived from a genetically diverse population, reflecting the polycyclic, sexual habit of the pathogen. Addition of a greater number of contrasting Cougar/Non-Cougar isolates would likely aid the selection of more rare variants that would facilitate greater resolution. Despite not identifying any polymorphisms linked to genes conferring virulence on Cougar, our data highlights that the allele(s) responsible are widely distributed across the U.K. *Zt* populations and were likely to be present before the release of cv. Cougar, as opposed to this being a new breakdown in host-resistance.

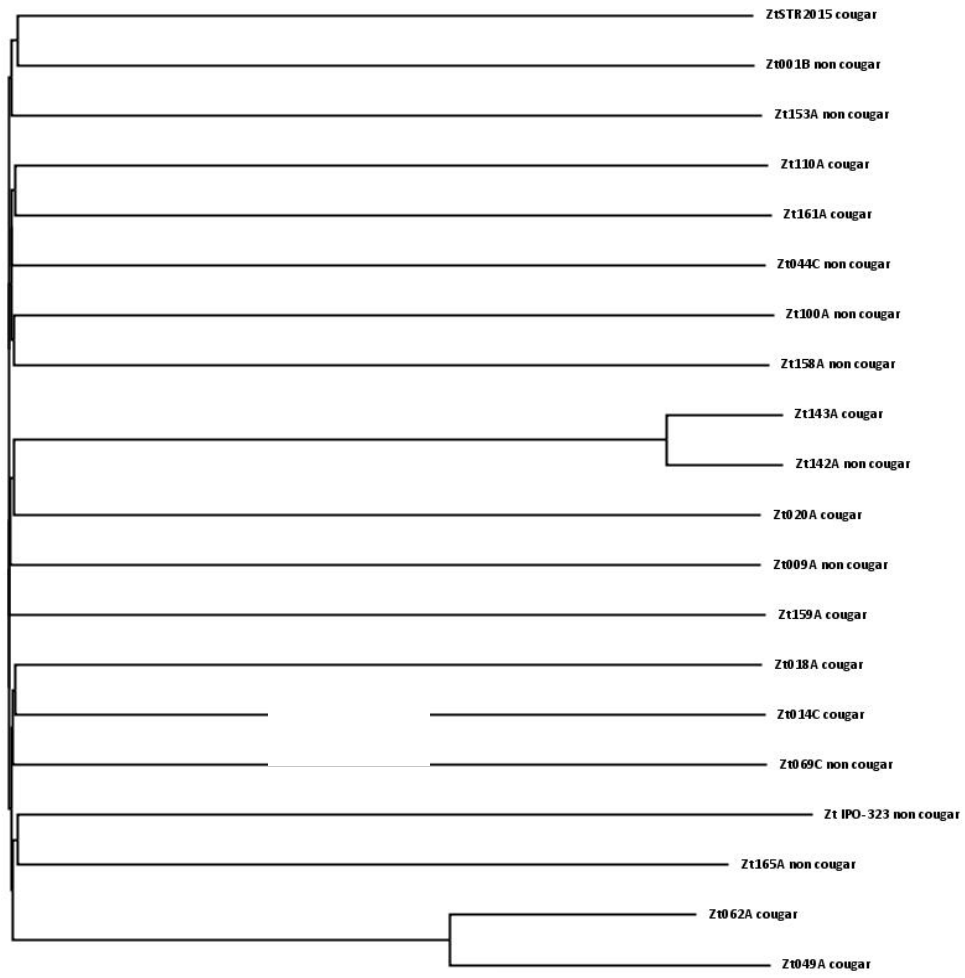


Figure 5: Neighbour-joining tree based on genic SNPs in 20 *Z. tritici* isolates (2016 isolates coded ‘Zt-0**’, whereas 2017 isolates ‘Zt-1**’).

4.4. Fungicide sensitivity testing and genotyping

Results of the fungicide sensitivity testing are presented in Table 10. In addition, NT321.17 and all 2016 strains tested were able to grow in the presence of 5 ppm azoxystrobin whereas IPO323 showed no growth. As expected, strain NT321.17 was highly insensitive to most fungicides tested. This strain carries cytochrome *b* G143A, which is linked to QoI resistance [26], and has a complex CYP51 variant, [L50S, V136C, S188N, A379G, I381V, Δ, N513K, S524T] which reduces azole binding. In addition, this strain overexpresses the efflux pump MgMFS1 due to a 519-bp promoter insert [27] which affects the sensitivity to QoIs, azoles, SDHIs and fentin chloride. Strain IPO323 has no alterations in cytochrome b and CYP51 and does not overexpress MgMFS1. A wide range of fungicide sensitivities to azoles and bixafen was measured for both the Cougar and non-Cougar type strains with no obvious pattern to distinguish them. In comparison with 2016 strains, strains isolated in 2017 showed higher levels of insensitivity to epoxiconazole, prothio-desthio and bixafen.

Table 10: Fungicide sensitivity profiles (EC_{50} values in µg ml^{-1}) of *Zymoseptoria tritici* strains. Control strains (grey), Cougar type (blue) and non-Cougar strains (white).

Isolate	Tebuconazole	Epoxiconazole	Prothio-desthio	Prochloraz	Bixafen	Fentin chloride
2016						
Zt049A	12.5	0.838	0.141	0.194	0.112	0.182
Zt044C	19.4	1.23	0.135	0.249	0.0554	0.117
Zt069C	>25	0.763	0.0872	0.169	0.0462	0.184
Zt062A	0.473	0.616	0.142	0.121	0.137	0.151
Zt001B	0.967	0.00185	0.0030	0.0017	0.0757	0.189
Zt020A	0.436	0.632	0.112	0.127	0.0388	0.216
Zt014C	19.3	0.947	0.124	0.187	0.608	0.349
Ztr(ii)2015	7.28	3.50	0.198	0.195	0.0954	0.421
IPO323	0.00907	0.0001	0.0013	0.0003	0.0278	0.162
NT321.17	>25	5.78	0.337	0.890	1.35	1.21
2017						
Zt100A	0.704	0.949	0.216	0.122	0.052	0.104
Zt110A	0.895	0.620	0.105	0.226	0.199	0.098
Zt142A	>25	6.43	0.258	0.167	0.232	0.183
Zt143A	9.79	0.554	0.045	0.179	0.558	0.124
Zt153A	1.40	1.90	0.336	0.108	0.672	0.064
Zt158A	0.845	1.82	0.380	0.415	0.0182	0.063
Zt159A	0.188	0.433	0.046	0.444	0.789	0.095
Zt161A	4.75	6.24	0.737	1.55	0.283	0.291
Zt165A	1.25	2.12	0.289	0.298	0.025	0.064
Zt168A	8.10	2.13	0.097	0.115	0.035	0.055
IPO323	0.024	0.0004	0.0012	0.0002	0.013	0.0621
NT321.17	>25	6.05	0.282	0.603	0.749	0.580

Note: Values in red indicate a highly insensitive phenotype

The *CYP51* and *Sdh* genes were amplified and sequenced from a selection of 11 strains (Table 11). The CYP51 overexpressing variant [L50S, S188N, I381V, Δ, N513K↑] was found in three

Cougar strains. This variant was first detected in 2009 [28] and is since 2015 the most frequently occurring CYP51 variant in UK populations (https://cereals.ahdb.org.uk/media/1431368/3713-120817-annual-project-report-apr-2018.pdf). In comparison with other variants, this variant is highly insensitive to tebuconazole. CYP51 variant [L50S, D134G, V136A, I381V, Y461H], found twice in this study, was most common in the UK during 2013-2014 with moderate levels of insensitivity to epoxiconazole and prothioconazole but sensitive to tebuconazole. More complex variants with S524T have emerged since 2017 and show the highest levels of insensitivity to epoxiconazole and prothioconazole. A good example is strain Zt142A carrying variant [L50S, V136C, S188N, A379G, I381V, Δ, S524T] and highly insensitive to epoxiconazole and prothioconazole.

Table 11: *CYP51*, *SdhB*, *SdhC* and *SdhD* sequence analysis of a selection of *Z. tritici* strains

Isolate	Year	Type	CYP51[1]	Key Sdh mutations[2]
Zt014C	2016	Cougar	L50S, S188N, I381V, Δ, N513K↑	nd
Zt049A	2016	Cougar	L50S, S188N, I381V, Δ, N513K↑	nd
Zt062A	2016	Cougar	L50S, D134G, V136A, I381V, Y461H	nd
Zt110A	2017	Cougar	L50S, D134G, V136A, I381V, Y461H	-
Zt143A	2017	Cougar	L50S, S188N, I381V, Δ, N513K↑	C-N86S
Zt159A	2017	Cougar	L50S, V136A, S188N, Δ, S524T	C-R151T
Zt168A	2017	Cougar	L50S, V136C, S188N, I381V, Y461H, S524T	-
Zt100A	2017	Non-Cougar	L50S, D87A, D134G, V136A, I381V, Y461H	-
Zt142A	2017	Non-Cougar	L50S, V136C, S188N, A379G, I381V, Δ, S524T	-
Zt158A	2017	Non-Cougar	L50S, D134G, V136A, I381V, Y461H, S524T	-
Zt165A	2017	Non-Cougar	L50S, D134G, V136A, I381V, Y461S, S524T	-

[1]Arrow indicates CYP51 overexpression due to presence of 120 bp promoter insert
[2]Mutations that have been reported to be linked to SDHI insensitivity

Regarding Sdh mutations conferring resistance to SDHI fungicides, the SDHI insensitivity of stains Zt143A and Zt159A was caused by mutations in the SdhC (C-N86S and C-R151T). SDHI insensitive strains with Sdh alterations (e.g. B-T268I, C-T79N, C-W80S, C-N86S, C-R151M/S/T, C-H152R) were for the first time detected in UK populations during late season in 2015 and have been spreading since (https://cereals.ahdb.org.uk/media/1431365/3713-120817-annual-project-report-apr-2017.pdf). Strain Zt153A is also likely to carry a Sdh alteration while the bixafen insensitivity in strain Zt014C is most likely due to altered efflux pump activity because of the fentin chloride insensitivity.

5. Discussion

5.1. Identification and classification of isolates

At the start of this project in 2016, Cougar had just been removed from the RL and areas grown with this variety were likely to drop significantly. Nevertheless, isolates carrying virulence for Cougar were found in both years of the project to a moderately high extent. The project set out to establish how frequently and widely the isolates were found and the results of this project show that they are found moderately frequently and they are widely dispersed. The project also sought to establish the risks to other varieties and the identification of Cougar isolates on varieties other than Cougar in 2016 was slightly concerning. In 2017 this reduced and Cougar isolates were only found on Cougar and the susceptible control Longbow. It is therefore likely that these isolates will only be found at low frequency in the future as Cougar is no longer grown. This does of course assume that the only difference between the Cougar and non-Cougar isolates is virulence for Cougar and that there are no other fitness benefits in these isolates.

5.2. Varietal performance

To assess the risk of these Cougar isolates to currently grown varieties, seedling and adult plant tests were conducted using Cougar and non-Cougar isolates on a panel of RL varieties and control varieties. The tests confirmed that the distinction between the isolates into Cougar and non-Cougar groups was valid, with Cougar being notably more susceptible when challenged with the Cougar isolates than the non-Cougar isolates at both growth stages. This provides an explanation for what was seen in the field during the 2015 field season and is similar to that seen by others when resistance has been overcome [14].

The risk to other varieties appeared to be a lot lower however. There were no other varieties that were consistently more susceptible to the Cougar isolates and most varieties performed as expected. The resistance in varieties such as LG Sundance, Moulton, Freiston and Dunston continues to provide adequate protection from Septoria leaf blotch and stakeholders should continue to use varieties such as these as part of an integrated disease management programme. There were occasions when some varieties performed worse than expected, such as Crusoe. Although disease levels were high in these tests, it is possible that the growth under glasshouse conditions may have inhibited the resistance mechanisms in this variety leading to this susceptible phenotype. The results from these tests show a worst case scenario and re-iterate the need for continued monitoring of crops throughout the season.

The relationship between seedling and adult plant resistance was briefly explored in this study and highlighted that although most varieties generally perform similarly at both growth stages, there were notable exceptions where varieties appeared to have adult plant-only resistance. For example, both Exsept and Pastiche were included as resistant controls and at the seedling stage

did not appear especially resistant. At the adult plant stage however, disease levels were much lower and more in line with expectations.

5.3. Genotyping

Z. tritici is known to be a genetically diverse, sexually reproducing organism, with high levels of variation even at the field level. Results from the isolate sequencing revealed a large number of polymorphisms within the genes of the individuals that were tested confirming the expected high level of diversity. The clustering of the isolates based on differences in SNPs within these gene coding regions, however, was observed not to demonstrate any significant correlation with geographic origin or on reaction type on Cougar. This indicated that the specific set of SNPs tested were in this case limited in their ability to distinguish between virulence profiles, highlighting the difficulty of using solely genotypic information to distinguish between pathotypes in only a few isolates. The accuracy of sequenced based genotyping approach could potentially be improved by sequencing a greater number of *Z. tritici* isolates from the population to increase likelihood of identifying genetic markers that are capable of discriminating between pathotypes, through screening for genetic differences in additional targets, and conducting additional phylogenetic analyses. Further work is required to fully evaluate the efficacy of pathogenomics strategies for conducting molecular pathotyping of *Z. tritici.*

5.4. Fungicide sensitivity testing and genotyping

As expected from a sexually recombining population, a large diversity of phenotypes and genotypes were identified in the fungicide sensitivity testing and corresponding genotyping. Similar to the whole genome genotyping (4.3), there were no obvious differences between the Cougar and non-Cougar strains. In both groups, insensitivity was detected for azoles, QoIs and SDHIs. Based on the evidence here it is difficult to conclude whether the virulence for Cougar emerged prior to accumulation of fungicide insensitivity mutations.

5.5. Conclusions

The work undertaken in this project has demonstrated that although the Cougar isolates were of importance in 2015 when Cougar was being grown, the risk posed by these isolates is no greater than with any other *Z. tritici* isolate found in the field. Genotyping of isolates and fungicide sensitivity testing also showed that aside from the virulence for Cougar, these isolates look similar to other *Z. tritici* isolates, again confirming the low risk posed by this change.

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