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Net blotch management in barley: Net form or spot form – does it matter?

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1. SUMMARY

1.1. Introduction

During 2018, we observed an unusual morphology of net blotch strains growing on agar plates, compared with strains isolated from other samples and in previous years. Using DNA-based molecular tools, we established that these isolates represented the spot form of net blotch (*Pyrenophora teres* f. *maculata* (Ptm)). This form differs from the net form of net blotch (*P. teres* f. *teres* (Ptt)) regarding symptom development. They are considered genetically distinct species. The use of healthy seeds and resistant cultivars can help manage this barley disease, although reliable control often requires seed treatments and foliar fungicides. At present, there is a lack of information about whether the net blotch form(s) present affect the optimum management strategy.

1.2. Objectives

The first objective aimed to determine whether the two net blotch fungi have different levels of fungicide sensitivity. Quinone outside inhibitor (QoI), succinate dehydrogenase inhibitor (SDHI) and azole resistance have been reported for net blotch on barley. The second objective aimed to develop DNA PCR-based tools to detect, identify and quantify both species in leaves and in seeds. Visual assessment to distinguish both diseases is difficult, especially in mixed infections. However, it is important to understand which species is present, because cultivars can have different R-genes for both diseases.

1.3. Key results

Six barley leaf samples with blotches, from five sites in 2018 – Cornwall (cv Propino), Dorset (cultivar unknown), Hampshire (cv Propino), Norfolk (cv Flagon) and Yorkshire (cv Tower) – were examined in this study.

Ptm was isolated from the early and late season sample from Yorkshire (cv Tower), whereas Ptt was isolated from all the other samples. Other fungi that were simultaneously isolated from net-blotch infected leaves included *Alternaria infectoria* and *Microdochium phragmitis*.

Using reference isolates to check for specificity, a multiplex PCR targeting fungal ITS and *P. teres* species-specific markers was developed to identify fungal isolates as Ptt, Ptm or as another fungal species. A second multiplex PCR targeting barley and the *P. teres* species-specific markers was used to screen archived barley grain samples (1852 to 2018) from the Hoosfield long-term spring barley experiment at Rothamsted for presence of Ptt and Ptm. Ptt was detected for the first time in 1890, with 21 positive reactions since this time. Ptm was only detected in four samples (1982, 1995, 2001 and 2012), with both species only once simultaneously present in 2012.

The Ptm population sampled in Yorkshire was more sensitive to azole and SDHI fungicides, relative to the Ptt populations sampled in four UK regions. Mutations leading to amino acid substitutions SdhD-G138V or SdhD-D145G were detected in SDHI-insensitive Ptm isolates, growing at 1.0 ppm of fluxapyroxad as highest concentration. Amino acid substitution SdhC-R64K was found in all Ptm isolates, including old reference strains which were never exposed to SDHI fungicides and is not likely to affect SDHI binding. The most sensitive Ptm strain did not carry any Sdh mutations known to affect SDHI sensitivity and was only able to grow at 0.1 ppm fluxapyroxad as highest concentration. Mutations SdhC-S135R and SdhD-H134R, known to affect SDHI binding, were detected in the majority of the SDHI-insensitive Ptt strains that were able to grow in the presence of 10 ppm of fluxapyroxad as highest concentration. The most SDHI-insensitive Ptt strain showed partial growth at 100 ppm fluxapyroxad and carried SdhC-S135R in combination with D-G138V. The majority of Ptm strains were able to grow at 1.0 ppm epoxiconazole, with a few showing partial growth at 10 or 100 ppm. The most sensitive Ptm isolate was only able to grow at 0.1ppm epoxiconazole as highest concentration. In contrast, the majority of Ptt strains showed some partial growth at 100 ppm of epoxiconazole, the highest concentration tested.

1.4. Conclusions and recommendation for further research

1.4.1. Net blotch disease diagnosis and epidemics

Archived grain samples show that the spot form is not common, compared with the net form. However, the spot form has recently become a problem of epidemic proportions in several important barley-producing regions worldwide. With the spot form detected in some UK samples, it could become more prevalent. Regarding improving net blotch disease diagnosis and the identification of factors that drive epidemics, we recommend the following research initiatives:

- Continued exploration of archived samples of barley leaves and grains (from the long-term Hoosfield experiment) to identify which factors drive net blotch epidemics
- Screening seed lots for presence of Ptm and Ptt when setting up variety trials, as part of efforts to assess host resistance
- Development of quantitative PCR assays, to quantify Ptm and Ptt disease levels on grains and leaves
- Development of rapid tests for detection of both forms of net blotch (and ramularia). Based on Loop Mediated Isothermal Amplification (LAMP), these can use species-specific DNA sequences. LAMP tests, typically carried out on location within 30 minutes, can assist training and help staff visually assess difficult-to-diagnose diseases in Recommended List (RL) trials, especially when mixed infections are present.

1.4.2. Fungicide sensitivity

Mycelium plug-based fungicide sensitivity assays show Ptt is less sensitive to azole and SDHI fungicides, compared to Ptm. Due to irregular radial growth, results for Ptm were more variable. Highly SDHI-insensitive isolates of Ptt carried SdhC-S135R or SdhD-H134R, while the least sensitive isolate carried a combination of SdhC-S135R and SdhD-G138V. SDHI-insensitive Ptm isolates carried SdhD-G138V or SdhD-D145G. Regarding fungicide sensitivity monitoring and understanding fungicide sensitivity shifts, we recommend the following research initiatives:

- Using high-throughput microtitre plate-based tests of spores to improve the sensitivity and accuracy of fungicide sensitivity testing for Ptt and Ptm
- Monitoring of SDHI sensitivity shifts in Ptt and Ptm populations, especially following the emergence of novel SDHI-resistant genotypes
- Conducting comparisons of the sensitivities of old reference strains with current populations to establish if the azole sensitivity in UK populations of Ptt and Ptm has shifted
- Using molecular approaches to elucidate the azole resistance mechanisms in UK populations of Ptt and Ptm
- Improving prediction of fungicide resistance development and identification of novel fungicide resistant alleles, through spore trapping in combination with NGS of PCR amplicons targeting fungicide resistance markers

2. TECHNICAL REPORT

2.1. INTRODUCTION

During early and late season isolation of net blotch strains from a winter barley Fungicide Performance trial in Yorkshire in 2018 we noticed an unusual morphology of net blotch strains growing on agar plates in comparison with strains isolated from other samples and in previous years. Using *Pyrenophora graminea* specific primers we established that these strains were not the causal agent of barley leaf stripe. Barley leaf stripe was once common in the UK but is rare in the UK since the mid-1920s after the introduction of resistant varieties and effective seed treatments, in particular (Fitt *et al.*, 2012). Partial ribosomal DNA internal transcribed spacer (ITS) sequence analysis confirmed our findings when these strains were identified as the spot form type of net blotch rather than the net form type which is common in the UK. The fungi causing the spot (*P. teres* f. *maculata* (Ptm)) and net form (*P. teres* f. *teres* (Ptt) of net blotch are genetically distinct species (hybridisation events are extremely rare in the field). Inoculum sources are infected stubble, volunteer plants and grains while alternative hosts like barley grass (*Hordeum murinum* ssp. *leporinum*) might also play a role in disease transmission and genetic differentiation (Brown *et al.*, 1993; Liu *et al.*, 2011; Linde & Smith, 2019). Both pathogens are known to produce both sexual and asexual spores which can be dispersed by air and rain splash, respectively. Disease

management is based on use of healthy seeds and resistant cultivars, but seed treatments and foliar fungicides are often needed for a reliable disease control.

Objectives

The first objective of the project is to determine if the two net blotch fungi have different levels of fungicide sensitivity. Quinone outside inhibitor (QoI), succinate dehydrogenase inhibitor (SDHI) and azole resistance have been reported for net blotch on barley (Semar *et al*., 2007; Mair *et al*., 2016; Rehfus *et al*., 2016) but it is not clear if both net blotch species are equally affected and if different disease management strategies are required for both species. Isolates will be screened for their sensitivity towards epoxiconazole and fluxapyroxad. A selection of isolates will be further screened for presence of target-site mutations conferring resistance to SDHI fungicides as mutations have recently been reported in UK field populations of *P. teres*. The second objective is to develop PCR-based tools to detect/quantify both species because PCR assays for Ptt and Ptm reported by Leisova *et al*. (2006) have shown to be not totally reliable when we previously evaluated them with different *Pyrenophora* species in our lab. DNA-based PCR tools can be used by breeders to identify/quantify disease levels of both pathogens in leaves and seeds. Visual assessment to distinguish both diseases is difficult, especially in mixed infections, but important, because cultivars can have different R-genes for both diseases (Akhavan et al., 2017).

2.2. MATERIALS AND METHODS

2.2.1. *Pyrenophora teres* reference strains and field samples

All the *Pyrenophora* strains used in this study are listed in Table 1. Six field samples (50 leaves each) were received from five locations in 2018: samples S1, cv. Tower, early season, and S2, cv. Tower, late season from untreated crop from ADAS, location High Mowthorpe (Yorkshire), S3, cv. Propino, late season from Hamilton Farm, Alresford (Hampshire), S4, cv. Propino, late season from Hutchinsons, location Truro (Cornwall), S5, cultivar not known, late season treated with T2 ear spray of Siltra Xpro (a.i. bixafen and prothioconazole) at 0.5 L/ha and Bravo (a.i. chlorothalonil) at 1.0 L/ha from Velcourt, location ACF Drax Farm, Wareham (Dorset) and S6, cv. Flagon from NIAB, location Morley (Norfolk). We also tested grain DNA samples of the Hoosfield long-term continuous spring barley experiment at Rothamsted for presence of *P. teres* using end-point multiplex PCR. All grain samples representing different cultivars are listed in Table 2. Short-straw cultivars starting with Julia were grown from 1970 onwards.

Table 1. *Pyrenophora* reference strains

Strains[1]	Species	Location	Host	Year
H120/2[a]	*Pyrenophora graminea*	Hungary	barley	2006
H157[a]	*Pyrenophora graminea*	Hungary	barley	2007
H172[a]	*Pyrenophora graminea*	Hungary	barley	2007
H290[a]	*Pyrenophora graminea*	Hungary	barley	2008
H311/1[a]	*Pyrenophora graminea*	Hungary	barley	2008
H160[a]	*Pyrenophora teres f. maculata*	Hungary	barley	2007
H170[a]	*Pyrenophora teres f. maculata*	Hungary	barley	2007
H297/2[a]	*Pyrenophora teres f. maculata*	Hungary	barley	2008
H301[a]	*Pyrenophora teres f. maculata*	Hungary	barley	2008
H364[a]	*Pyrenophora teres f. maculata*	Hungary	wheat	2007
H307/3[a]	*Pyrenophora teres f. maculata*	Hungary	barley	2008
NSW4/10[a]	*Pyrenophora teres f. maculata*	Australia	barley	1998
H196[a]	*Pyrenophora teres f. teres*	Hungary	barley	2007
H237[a]	*Pyrenophora teres f. teres*	Hungary	wheat	2006
H270[a]	*Pyrenophora teres f. teres*	Hungary	wheat	2007
H284[a]	*Pyrenophora teres f. teres*	Hungary	barley	2008
H337[a]	*Pyrenophora teres f. teres*	Hungary	barley	2008
H339/1[a]	*Pyrenophora teres f. teres*	Hungary	barley	2008
Pg98-1[b]	*Pyrenophora graminea*			Before 2008
Ptr 3609[b]	*Pyrenophora tritici-repentis*			Before 2008
Ptt 1669[c]	*Pyrenophora teres f. teres*	Germany	barley	2012
Ptt 1687[c]	*Pyrenophora teres f. teres*		barley	Before 2014
Ptt 1688[c]	*Pyrenophora teres f. teres*		barley	Before 2014
Ptt B0003	*Pyrenophora teres f. teres*			Before 2008
Pg 43499	*Pyrenophora graminea*?			Before 2008
Ptr 1246	*Pyrenophora tritici-repentis*			Before 2008
Ptt NB22	*Pyrenophora teres f. teres*	Norfolk, UK	barley	2017
Ptt NB29	*Pyrenophora teres f. teres*	Norfolk, UK	barley	2017
Ptt NB32	*Pyrenophora teres f. teres*	Norfolk, UK	barley	2017

[1]Strains were kindly provided by (a) Dr József Bakonyi (Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary), (b) Dr. Kerry Maguire (NIAB, Cambridge, UK) and (c) Dr. Gerd Stammler (BASF, Limburgerhof, Germany)

Table 2. Cultivars grown at the Hoosfield long-term continuous spring barley experiment at Rothamsted (1852-2018)

Cultivar[1]	**Growing years**
Chevalier	1852-1880
Archer's Stiff Straw	1881-1890, 1898-1901, 1904-1916
Carter's Paris Prize	1891-1897
Hallet's Pedigree Chevalier	1902-1903
Plumage Archer	1917-1966
Spratt Archer[2]	1927, 1929-1932
Maris Badger[3]	1964-1969
Julia	1970-1980
Georgie	1981-1983
Triumph	1984-1991
Alexis	1992-1995
Cooper	1996-1999
Optic	2000-2007
Tipple	2008-2015
Irina	2016-

[1]Fallow in 1912, 1933, 1943 and 1967; [2]Alternative strips of Spratt Archer grown for comparison with Plumage Archer; [3]Plots were split to test four N rates

2.2.2. Isolation and fungicide sensitivity testing of *P. teres* strains

Lesions with net blotch alike symptoms were cut from leaves with a small border of green leaf area around them. Leaf fragments were then surface sterilized with 70% ethanol for 2 min and 10 % sodium hypochlorite for 5 min, and rinsed in sterile distilled water for 1 min. Sterilized leaf fragments were then stapled with symptoms up onto round 9-cm filter paper disks and, after wetting the paper, incubated in sealed petri-dishes at 21°C in the dark for 48 h. Mycelium forming hyphae were picked up with watchmaker tweezers under the microscope and transferred onto Yeast Peptone Dextrose (YPB) (ForMedium) agar supplemented with 15 % (v/v) V8 juice (YPD-V8) and chloramphenicol at 0.1 mg/ml (prepared from 50 mg/ml ethanol stocks). After incubation in the dark at 21°C for seven days, colonies were selected for further characterization and/or storage at -80°C using mycelium agar plugs in 15 % (v/v) DMSO or in 80 % (v/v) glycerol.

2.2.3. *In vitro* fungicide sensitivity testing of *Pyrenophora teres* field strains

In vitro sensitivity assays were carried with square mycelium agar plugs (5 mm by 5 mm) harvested with sterile plastic culturing loops from seven-day old colonies grown on YPD-V8 agar plates. The plugs were transferred onto YPD-V8 agar plates amended with no fungicides (control)

and formulated fluxapyroxad and epoxiconazole at 0.01, 0.1, 1.0, 10 and 100 ppm. Plugs were placed upside down in the centre of the plate to allow contact with the substrate. The plates were sealed with parafilm tape and incubated at 21°C in the dark. After seven days, mycelium growth was determined by measuring the colony diameter at two perpendicular directions and the fungicide sensitivity calculated.

2.2.4. DNA extractions and quantification

DNA was extracted directly from mycelium scraped off YDP-V8 agar plates in liquid nitrogen using a pestle and a mortar. To each crushed sample, DNA extraction buffer consisting of 40 µl 1% (v/v) β-mercaptoethanol, 400 µl TEN buffer (500 mM NaCl, 400 mM Tris-HCl, 50 mM EDTA, 5 mM 1,10-phenanthroline monohydrate, 2 % (w/v) polyvinylpyrrolidone; pH 8.0) and 400 µl 2% (w/v) SDS was added until the mixture could be poured. After incubating the mixture for 30 min at 70°C, 400 µl ice-cold ammonium acetate (7.5 M) was mixed with the heat-treated sample and the total suspension kept on ice for 30 min. After centrifugation at 10,000 rpm for 10 min, an equal volume of cold (-20°C) isopropanol was added to the supernatant and the extract shaken at room temperature for 15 min. After centrifugation at 6,000 rpm for 5 min, DNA pellets were washed with ice-cold 70% (v/v) ethanol, centrifuged again and dissolved in 500 µl sterile distilled water. The DNA concentration of each sample was measured via nanodrop spectrophotometer and diluted to the required concentration using 1 x Tris-EDTA (TE) buffer.

2.2.5. PCR and sequencing of *P. teres* DNA targets

All primer sets with their DNA targets, including product sizes, that were used in this study are listed in Table 3.

Table 3. PCR primer sets and corresponding targets used in this study

Target	**Primers (5'-3' sequence)[1]**	**Annealing temperature (°C)**	**Amplicon size (bp)**
P. teres **f.** ***teres***	Ptt1F:	64	70
(Ptt) or ***P. teres***	GGATGATGACCTCGCCAGAT		
f. ***maculata***	Ptt1R:	60	110
(Ptm) specific	GCGATGGTATGTTCTGCGAA		
PCR markers	Ptt2F:	60	130
Ptt1-6 and	AACACTCTGAACGTGGTTGC		
Ptm7-12	Ptt2R:	62	150
	TTCAGTTGTAAGCTGCGTGG		
	Ptt3F:	60	173
ITS	CCTCGTCCTAAGTTGACTCGA		
	Ptt3R:	60	188
	TTACACGGGTTCCCTCCATC		
	Ptt4F: CGTCCCGCCGAAATTTTGTA	60	140
	Ptt4R:		
	CAAGGACTTACGCGCTCAAA	60	161
	Ptt5F:		
	GCATTGTCTAGCACTCGTCG	64	201
	Ptt5R:		
	CGCGGACTCAGAAGACATTG	60	220
	Ptt6F:		
	TCAGAATACTCCGCGGACTC	58	260
	Ptt6R:		
	GTCCGCATTGTCTAGCACTC	60	279
	Ptm7F:		
	GTAGAGGCTGTAGGAGATGTGATT		
	Ptm7R:		
	CATGGCAAATTGTTCGTAATCCTG		
	Ptm8F:		
	ACGCTAAGACCACCTCGTTT		
	Ptm8R:		
	TCGACCAGAGAGAGCACAAA		
	Ptm9F:		
	AATGCTCAATTCTGGTGGCG		
	Ptm9R:		

	TGTTCGAGTGCAAACTTGGG Ptm10F: TGCTGTGGACTTAGACGAGG Ptm10R: TGGGGATCCTTGACCAACTC Ptm11F: GATTAGACCATTACCACACTAGCG Ptm11R: ACCACCACATCTTTCCTACTAACT Ptm12F: CTAACCAAAGAACTTCACAGACGA Ptm12R: CCTTATTAGCCAATTCCATGTCGA		
ITS region	ITS1F: TCCGTAGGTGAACCTGCGG ITS4R: TCCTCCGCTTATTGATATGC	55	~650
P. graminea	PG2F: CTTCTTAGCGGGGCTACCGTC PG2R: ACCGACTCGGGAAAAGAGCA	65	435
ToxA region	ToxAF1: ATCTTGTTGCCAGCGATAGCTGA ToxAR1: GCATCCTTCACTCCCTAATAGGC	60	704
SdhB **SdhC** **SdhD** **SdhD**	KES1825: CATAACCGAGGAAGCTTGAGTG KES1837: CAAACACAACTCGCAATTAACGC KES1827: ATCACCCAACACCACCATCG KES1828: ATGTTGCAAACTTCAATCGTACCC KES1833: CGATCCTTCAACCCACCTCCGA KES1834: ACCCGCTTATGCATGCCACAG	62 64 64	1200 850 750
Barley	HORD91F:	62	91

	GTACATGGATCATCGTTGCATAA		
	HORD91R: CCTGGTCGAATCACACATCA	60	150
	HORD150F: GTGCACCATGATGTGTGATTC	60	399
	HORD150R: CGGATAGACTTACGATTCCAGTT		
	BarleyF2: GCTACTCTCGGACGAATTTTC		
	BarleyR2: GCCATAGACTAGAGCTACCTTTG		

[1] The following primer sets have been published: *P. teres* markers (Poudel *et al*., 2017), ITS primers (White et al., 1990), *P. graminea* (Taylor *et al*., 2001), KES primers (Rehfus *et al*., 2016), HORD primers (Gabriadze *et al*., 2015) and barley primers (Fountaine *et al*., 2007)

End-point PCR was carried out using GoTaq DNA polymerase (Promega) while Easy A High-Fidelity Cloning Enzyme (Agilent Technologies) was used when PCR products needed to be sequenced using a dideoxy chain termination method. Standard end-point PCR reactions were carried out in volumes of 25 µl, consisting of 2 µl of DNA sample (20 ng of genomic DNA), 5 µl of 5X GoTaq buffer, 0.125 µl of each primer (100 µM), 0.5 µl of dNTP solution (10 mM of each dNTP), 1.5 µl of $MgCl_2$ (25 mM) and 0.1 µl GoTaq DNA polymerase (5 U $µl^{-1}$) and 15.65 µl of sterile distilled water, using a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) under the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C or other annealing temperature for 20 s, 72°C for 1 minute with a final extension at 72°C for 9 minutes.

Standard Easy A High-Fidelity Cloning Enzyme-based PCR reactions (25 µl) consisted of 2 µl of DNA sample (20 ng of genomic DNA), 2.5 µl of 10X Easy-A reaction buffer, 0.125 µl of each primer (100 µM), 0.5 µl of dNTP solution (10 mM of each dNTP), 0.25 µl Easy A High-Fidelity Cloning Enzyme (5 U $µl^{-1}$) and 19.5 µl of sterile distilled water. Reaction conditions on the Biometra thermal cycler were: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 s, 60°C or other annealing temperature for 20 s, 72°C for 2 minutes with a final extension at 72°C for 10 minutes. Presence of PCR products was confirmed on ethidium bromide-stained 1.3 % (w/v) agarose gels run in 1X Tris-borate-EDTA buffer and exposed to UV light to visualise DNA fragments. PCR products were purified and directly sequenced with PCR primers by Eurofins Genomics using a dideoxy chain termination method reaction. Sequences were analysed and aligned using Geneious software version 10.0 (Biomatters, New Zealand).

2.3. RESULTS

2.3.1. Isolation of *Pyrenophora teres* strains

Strains with different morphological characteristics were isolated. Two main types were identified (Figure 1).

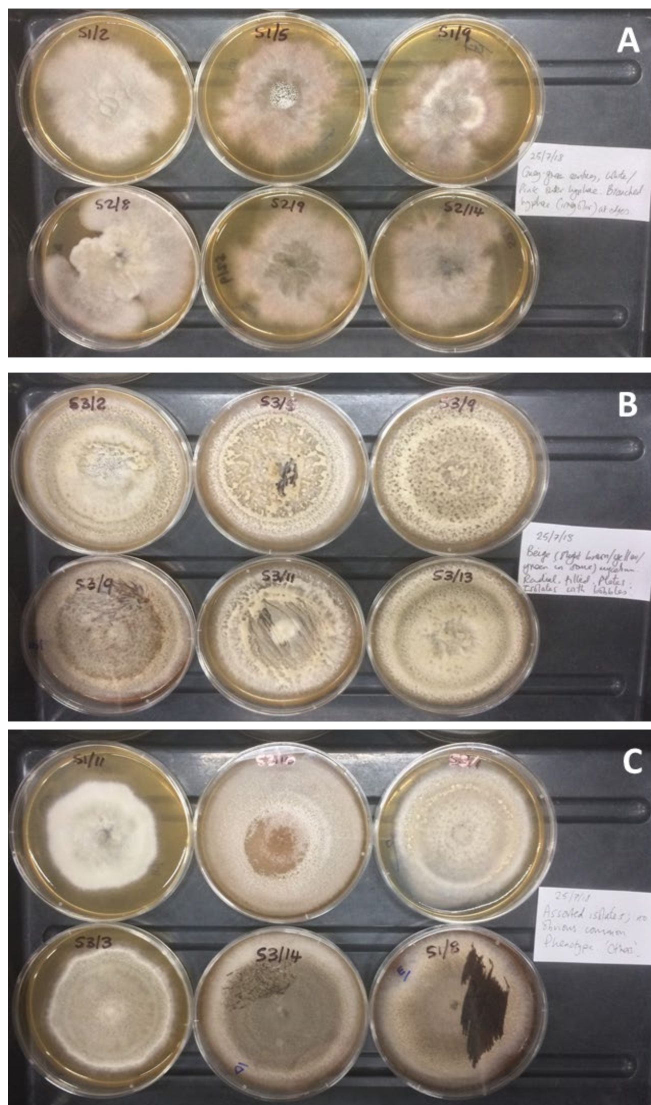

Figure 1. Net blotch alike isolates with different morphologies growing on YPD-V8 agar. *Pyrenophora teres* f. *maculata* isolates in panel A, *P. teres* f. *teres* isolates in panel B and *P. teres* f. *teres* isolates in panel C, with exception of isolate S1/11 (*A. infectoria* with white mycelium) and S1/8 (*Pyrenophora teres* f. *maculata*). Note: Some mycelium material of isolate S1/8 has been scraped off from the plate.

The first type had a grey/green centre with white/pink outer hyphae growing forming an irregular overall shape on YPD-V8 plates and was later identified as *Pyrenophora teres* f. *maculata* (Ptm) using DNA sequence analysis (Fig. 1A). The second type consisted of hyphae forming a beige to greenish circular shape with bobbles and was later identified as *Pyrenophora teres* f. *teres* (Ptt) using DNA sequence analysis (Fig. 1B). In addition, several other fungi (e.g. *Alternaria infectoria*)

and *P. teres* isolates with different morphology regarding shape and colour were also present (Fig. 1C). The growth rate of Ptt and *A. infectoria* isolates was approximately two times faster than Ptm isolates on YPD-V8 agar at 21°C in the dark.

All strains isolated and tested so far from samples 1 and 2 (cv. Tower, location High Mowthorpe, Yorkshire) were Ptm, whereas only Ptt strains were isolated from all other locations. The differences in symptoms caused by Ptm and Ptt are visualised in Figure 2, with dark-brown circular to elliptical lesions (spot form) and dark-brown narrow netted lesions (net form), respectively.

Figure 2. Leaf fragments with net blotch symptoms. Left, sample (cv. Tower) from Yorkshire (location 1) from which only Ptm was isolated; middle, sample (cv. Propino) from Hampshire (location 3) with stripe-like net blotch symptoms from which only Ptt was isolated; right, old sample with more typical net form net blotch symptoms.

2.3.2. Azole and SDHI fungicide sensitivity testing of *Pyrenophora teres*

Results of the fungicide sensitivity testing with 56 isolates are shown in Figures 3 and 4. Due to the irregular shape and hence large variation in colony diameter at two perpendicular directions, Ptm isolates present in samples 1 and 2 often produced higher than expected radial growth (>120 %) in comparison with untreated at the two lowest fungicide concentrations tested.

Several levels of SDHI sensitivity were detected for Ptm isolates (Fig. 3). Six out of the 19 isolates tested were only able to grow in the presence of 0.1 ppm of fluxapyroxad as the highest concentration, 12 isolates were growing at 1.0 ppm while only one isolate (S1/6) was able to grow at 10 ppm.

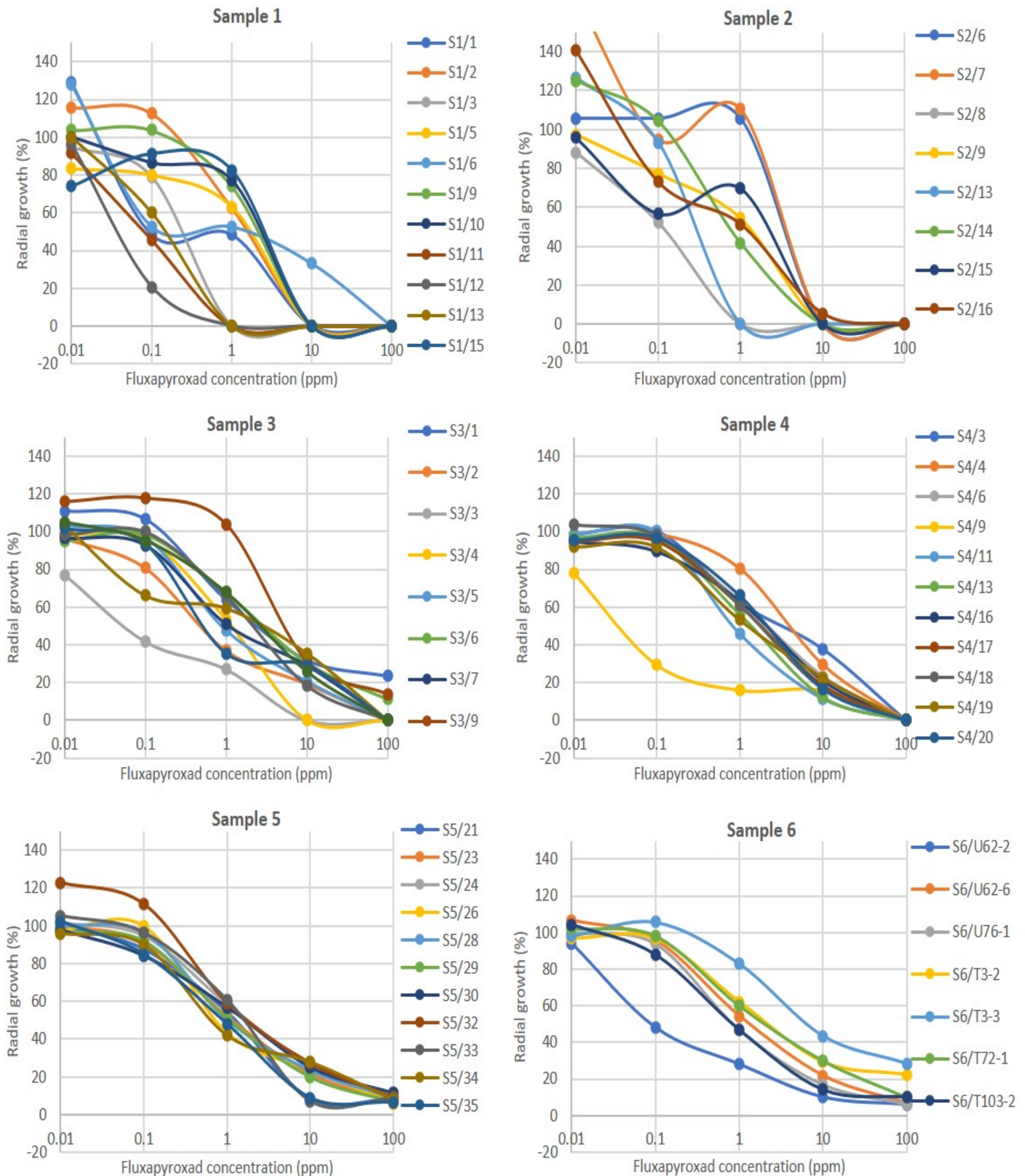

Figure 3. Fluxapyroxad sensitivity testing of *P. teres* strains originating from samples 1 to 6. Samples 1 and 2 contained Ptm strains whereas Ptt strains were mostly present in samples 3 to 6. Strains S6/T3-2, S6/T3-3 and S6/T72-1 were identified as *A. infectoria* at a later stage using ITS sequence analysis.

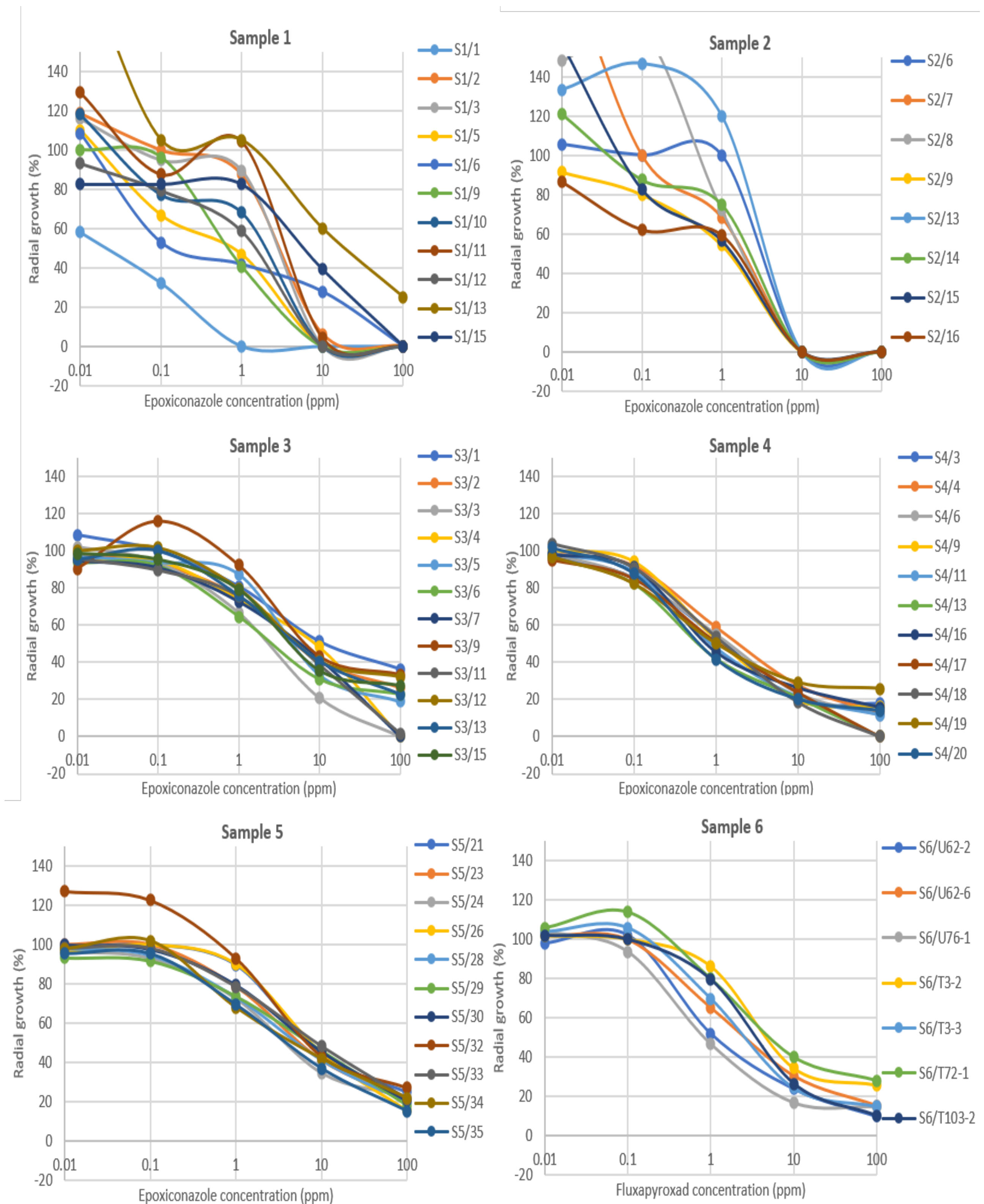

Figure 4. Epoxiconazole sensitivity testing of *P. teres* strains originating from samples 1 to 6. Samples 1 and 2 contained Ptm strains whereas Ptt strains were mostly present in samples 3 to 6. Strains S6/T3-2, S6/T3-3 and S6/T72-1 were identified as *A. infectoria* at a later stage using ITS sequence analysis.

Previous fluxapyroxad sensitivity testing of Ptt isolates from Norfolk in 2016 showed that sensitive strains carrying no Sdh mutations were only able to grow at the lowest concentrations of 0.1 and 0.01 ppm, the majority of isolates carrying SdhD-H134R were able to grow on 1 ppm, whereas highly insensitive strains carrying SdhC-S135R or SdhC-H134R were able to grow at 10 ppm. The SDHI sensitivity profiles of most Ptt isolates originating from four different locations in this study were similar to the highly insensitive isolates carrying SdhC-S135R or SdhC-H134R, approximately 20 % radial growth was observed at 10 ppm fluxapyroxad, with exception of isolates S3/3 and S3/4 that were fully controlled at this rate and isolate S4/9 which grew weakly at 0.1, 1.0 and 10 ppm. Three Ptt isolates (S3/1, S3/6 and S3/9) and two *A. infectoria* isolates (S6/T3-2 and S6/T3-3) were least sensitive with some growth, approximately 20 % radial growth, recorded at 100 ppm.

Regarding epoxiconazole, only one Ptm isolate (S1/1) was growing at 0.1 ppm as the highest concentration, with 15 isolates growing at 1.0 ppm as the highest concentration and only three isolates (S1/6, S1/10 and S1/13) able to grow at 10 ppm (30-60 % radial growth). Only isolate S1/13 showed some growth (20 % radial growth) at 100 ppm.

The epoxiconazole sensitivity was similar for all Ptt isolates, all were growing at 10 ppm (20-50 % radial growth) and the majority also showing growth at 100 ppm, albeit at lower a radial growth rate between 10 and 40%.

2.3.3. PCR-based identification of *Pyrenophora teres* strains

A set of ten different isolates was initially tested with the *P. teres* f. *teres* and *P. teres* f. *maculata* specific markers reported by Poudel *et al*. (2017) (Table 4). To check if isolates were either *P. tritici-repentis* (Ptr) or *P. graminea* (Pg), primer sets ToxAF/ToxAR and PG2F/PG2R (see Table 3), respectively, were also used. A set of twelve isolates which included Ptm H301, Ptm S1/6, Ptt H284, Ptt S5/21, Ptt NB17, Ptr 1246, Ptr 3609 and S6/T3-3, Pg H290, Pg H120/2 and Pg 43499 was tested. ToxA has only been found in *Parastagonospora nodorum*, *P. tritici-repentis* and *Bipolaris sorokiniana* (Friesen *et al*., 2006; McDonald *et al*., 2019) and, as expected, only isolates Ptr 3609 and Ptr 1246 tested positive using primer set ToxAF/ToxAR. For PG2F/PG2R, a weak PCR amplification was obtained for Ptm H301 while strong positive reactions were observed for Pg H290, Pg H120/2 and, unexpectedly, Ptr 3609.

Table 4. Identification of *Pyrenophora* isolates using Ptt and Ptm specific PCR markers. Isolates displayed vertically and PCR markers (see Table 3) horizontally.

	Ptt1	Ptt2	Ptt3	Ptt4	Ptt5	Ptt6	Ptm7	Ptm8	Ptm9	Ptm10	Ptm11	Ptm12
Ptm H301	-	ns	+	-	-	-	+	+	+	+	+	+
Pg H290	+/-	ns	+/-	-	-	-	+	+	-	-	-	-
Ptt H284	+/-	+	+	+	+	+	-	-	-	-	-	-
Ptr 1246	-	ns	-	-	-	-	-	-	-	-	-	-
Pg 43499	-	nt	-	-	-	-	-	-	-	-	-	-
Ptt S5/21	+/-	+	+	+	+	+	-	-	-	-	-	-
S6/T3-3	-	ns	-	-	-	ns	-	-	-	-	-	-
Ptt NB17	+/-	nt	+/-	+	+	+	-	-	-	-	-	-
Ptm S1/6	-	ns	-	+/-	-	-	+	+	+	+	+	-
Ptm S2/13	-	ns	-	+	-	-	+	+	+	+	+	-
Control	-	-	-	-	-	-	-	-	-	-	-	-

nt, not tested; +, strong PCR amplification; +/-, weak PCR amplification; -, no PCR product; ns, non-specific PCR product(s), larger than expected size

A nucleotide BLAST search (National Center for Biotechnology Information (NCBI)) of the ITS1/ITS4 amplified PCR product for isolate S6/T3-3 revealed a very close match (99% without gaps) with *A. infectoria* with only one out of 574 bp different (position 522, G for A) (Fig. 5).

Based on the results shown in Table 4, with promising results for primer pairs/sets Ptt5F/Ptt5R, Ptm9F/Ptt9R, Ptt10F/Ptt10R and Ptt11F/Ptt11R regarding species-specific detection, a multiplex PCR was developed using three primer sets ITS1/ITS4, Ptt5F/Ptt5R and Ptm11F/Ptm11R simultaneously. Final reactions with 1 to 10 ng fungal template DNA were carried out in 10 µl volumes using the GoTaq DNA polymerase conditions as described before with an annealing temperature of 55°C. All fungal strains tested produced a ITS1/ITS4 amplicon of approximately 600 bp as expected, being a *Pyrenophora* species or not, while the identification of *P. teres* f. *teres* and *P. teres* f. *maculata* was confirmed with the additional presence of 173 bp (Ptt5F/Ptt5R) or 260 bp (Ptm11F/Ptm11R) PCR product, respectively (see Fig. 6). All Ptm and Ptt isolates listed in Table 1, as well other putative Ptm and Ptt strains isolated from samples 1 to 6 based on morphology, were

correctly identified by amplification of two products (not all results shown). All *P. graminae* and *P. tritici-repentis* isolates tested, as well as isolates not being *Pyrenophora* sp., including *A. infectoria* (S6/T3-2, S6/T3-3 and S6/T72-1) and *Microdochium phragmitis* (NB17-P10, isolated from infected barley leaves at Rothamsted in 2017), produced only single ITS1/ITS4 amplified products of approximately 600 bp.

Alternaria infectoria isolate IM-HT-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: MK684063.1 **Length:** 599 **Number of Matches:** 1

Range 1: 18 to 591 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1055 bits(571)	0.0	573/574(99%)	0/574(0%)	Plus/Minus

```
Query  1    TTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAAGGTTGaaaa  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  591  TTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAAGGTTGAAAA  532

Query  61   aaaGGCTTGCTGGACGCTGACCTTGGCTGGCAAAGAGCGCGACTTGTGCTGCGCTCCGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  531  AAAGGCTTGCTGGACGCTGACCTTGGCTGGCAAAGAGCGCGACTTGTGCTGCGCTCCGAA  472

Query  121  ACCAGTAGGCCGGCTGCCAATGACTTTAAGGCGAGTCTCCAGCGAACTGGAGACAAAAGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471  ACCAGTAGGCCGGCTGCCAATGACTTTAAGGCGAGTCTCCAGCGAACTGGAGACAAAAGA  412

Query  181  CGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  411  CGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGG  352

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Query  301  CACTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT  360
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Query  421  TGGTCCTGAGGGCGGGCGAGCCCACCCAGGAAACAAGAAGTACGCAAAAGACACGGGTGA  480
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Query  481  ATAATTCAGCAGGGCCGGCCCCGCCGCGCACGCCGTGAAGCGGTGCCCAGCGGGGGGTGT  540
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Sbjct  111  ATAATTCAGCAGGGCCGGCCCCGCCGCGCACGCCGTGAAGCAGTGCCCAGCGGGGGGTGT  52

Query  541  CCAGCCCGCCTTGTTATTGTGTAATGATCCCTCC  574
            ||||||||||||||||||||||||||||||||||
Sbjct  51   CCAGCCCGCCTTGTTATTGTGTAATGATCCCTCC  18
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Figure 5. Alignment of ITS fragment of isolate S6/T3-3 with *A. infectoria* isolate IM-HT-2 after NCBI nucleotide BLAST search

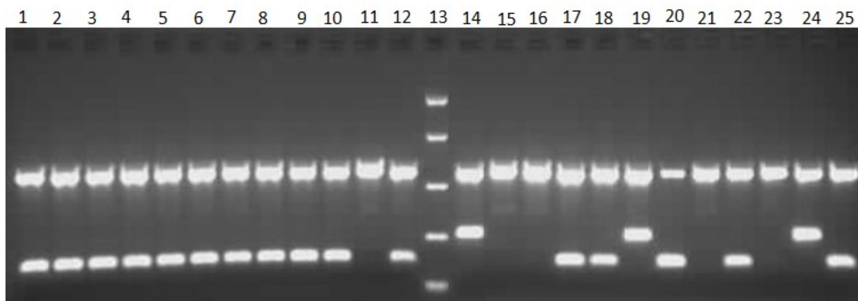

Figure 6. Identification of *Pyrenophora teres* spot and net form isolates using primer sets ITS1/ITS4, Ptt5f/Ptt5R and Ptm11F/Ptm11R in multiplex PCR. DNA markers of 100, 250, 500, 1000 and 2000 bp in lane 13. Isolates (left to right): 1, Ptt S5/23; 2, Ptt S5/24; 3, Ptt S5/26; 4, Ptt S5/28; 5, Ptt S5/29; 6, Ptt S5/30; 7, Ptt S5/32; 8, Ptt S5/34; 9, S6/T103-2; 10, Ptt S6/U76-1; 11, S6/T3-2; 12, Ptt S5/21; 14, Ptm S1/6; 15, S6/T3-3; 16, NB17-P10; 17, Ptt B0003; 18, Ptt NB17; 19, Ptm S1/2; 20, Ptt S3/4; 21, Ptr 1246; 22, Ptt H284; 23, Pg H290; 24, Ptm H301 and 25, Ptt H284.

In addition, a multiplex PCR for simultaneous detection of Ptt and Ptm in barley plant tissues was also developed. This assay used primer sets barleyF2/barleyR2, Ptt5F/Ptt5R, Ptm11F/Ptm11R and had similar reaction conditions, except an annealing temperature of 60^{o}C. The multiplex PCR assay was tested on archived grain samples from the Hoosfield long-term spring barley experiment (see Fig. 7 for a selection of samples). The only year that both Ptm and Ptt were detected was 2012.

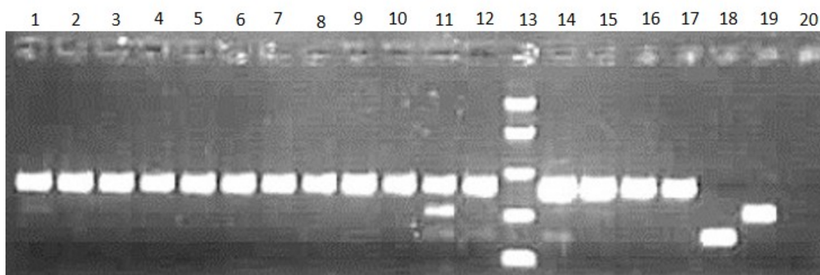

Figure 7. Detection of *Pyrenophora teres* in Hoosfield barley grain archive samples using multiplex PCR with primer sets barleyF2/barleyR2, Ptt5F/Ptt5R and Ptm11F/Ptm11R. DNA markers of 100, 250, 500, 1000 and 2000 bp in lane 13. Samples (left to right): 1, 2001; 2, 2002; 3, 2003; 4, 2004; 5, 2005; 6, 2006; 7, 2008; 8, 2009; 9, 2010; 10, 2011; 11, 2012; 12, 2013; 14, 2014; 15, 2016; 16, 2017; 17, 2018; 18, Ptm H284 (control); 19, Ptm S1/6 (control) and 20, SDW control.

Ptt with 21 positive samples was detected much more often than Ptm with only four positive samples in the Hoosfield barley grain archive. Ptt was detected as early as 1890 with further detection throughout the time span of the archive. Ptm was only sporadically detected, also more recently, with positive reactions for the 1982, 1995, 2001 and 2012 grain samples. Only three leaf samples were tested from the Hoosfield archive (2004-2006), with positive detection of Ptt in both 2005 (faint product) and 2006 (bright product).

2.3.4. Characterisation of SDHI-resistant *P. teres* isolates

The Sdh subunits B, C and D were amplified and sequenced from a set of *Pyrenophora teres* isolates using primer sets KES1825/KES1837, KES1827/KES1828 and KES1833/KES1834, respectively. The Sdh sequences of isolates Ptt 1669, Ptt 1687, Ptt 1688, NB22, NB29 and NB32 were already previously characterised but included as references in sequence alignments. The mutations resulting in non-synonymous amino acid substitutions are summarised in Table 5.

Several alterations in Sdh intron or coding sequences, not resulting in synonymous substitutions, were also observed. Several of these, mostly located in SdhC and to a lesser extent SdhD, were species-specific. Amino acid substitution C-R64K was found in all Ptm isolates, including the Australian reference strain NSW4/10 that was isolated in 1998, well before the introduction of second generation SDHI fungicides on the cereal market. Ptm isolate S2/13 was more sensitive to fluxapyroxad then isolates S1/2 and S1/6, the least sensitive isolate (see Fig. 3), carrying C-D145G and C-G138V, respectively. Most Ptt strains isolated and further characterised in this study, showed moderate levels of fluxapyroxad insensitivity (some growth at 10 ppm) and carried C-S135R, a mutation together with C-G79R and C-H134R regarded to confer the highest level of insensitivity to different SDHI fungicides in in vitro testing (Rehfus et al., 2016). Isolate S3/4 showed a lower level of fluxapyroxad insensitivity (growth at 1 ppm) and carried D-G138V, a mutation also found in Ptm and reported to be associated with low resistance factors (FRAC SDHI working Group 2020 meeting). Isolate S3/9 was least sensitive (some growth at 100 ppm) and carried simultaneously two key mutations in different Sdh subunits, resulting in C-S135R and D-G138V, respectively.

Table 5. Sequencing of SdhB, C and D subunits from a selection of *P. graminea*, *P. teres* f. *maculata* and *P. teres* f. *teres* isolates. Key Sdh amino acid substitutions reported to affect the binding of SDHI fungicides and impacting on the sensitivity are in bold.

Isolate	SdhB	SdhC	SdhD
Pg H120/2	A6T, P52S	none	V40L
Pg H157	A6T, P52S	none	V40L
Pg H172	A6T, P52S	none	V40L
Pg H290	A6T, P52S	none	V40L
Ptm H160	none	**R64K**	nt
Ptm H170	none	**R64K**	none
Ptm H301	none	**R64K**	V40L
Ptm NSW4/10	none	**R64K**	V40L
Ptm S1/2	none	**R64K**	V40L, **D145G**
Ptm S1/6	A6T, P52S, K311M	**R64K**	**G138V**
Ptm S2/13	A6T, P52S, K311L	**R64K**	none
Ptt 1669	none	**G79R**	none
Ptt 1687	**H277Y**	none	none
Ptt 1688	none	**N75S**	none
Ptt S3/4	K311L	none	**G138V**
Ptt S3/9	none	**S135R**	**G138V**
Ptt S5/21	none	**S135R**	none
Ptt S5/23	none	**S135R**	none
Ptt S5/24	none	**S135R**	none
Ptt S5/26	none	**S135R**	none
Ptt S5/28	none	**S135R**	none
Ptt S5/29	none	nt	**H134R**
Ptt S5/30	none	**S135R**	none
Ptt S5/32	none	**S135R**	none
Ptt S5/34	none	**S135R**	none
Ptt S6/T103-2	none	**N75S**	none
Ptt S6/U62-2	none	nt	S125F
Ptt S6/U62-6	none	**S135R**	none
Ptt S6/U76-1	none	**S135R**	nt
NB22	none	**H134R**	none
NB29	none	**G79R**	none
NB32	none	**S135R**	none

nt, not tested, no sequences available

2.4. DISCUSSION AND FUTURE RESEARCH

This study showed that the spot form of net blotch caused by *P. teres* f. *maculata* was present in a barley trial at High Mowthorpe in North Yorkshire. Only *P. teres* f. *teres* was isolated from the other samples originating from four different locations. The symptoms of the spot form are different from the net form and can be further confused with symptoms of other pathogens like *Ramularia collo-cygni* (Ramularia leaf spot), especially when multiple diseases are present. We did not detect *P. graminea* but *Microdochium phragmitis* (previous studies) and *Alternaria infectoria* was simultaneously isolated from net blotch infected barley leaves. *P. graminea* (barley leaf stripe) was once common in the UK but is rare in the UK since the mid-1920s after the introduction of resistant varieties and effective seed treatments, in particular (Fitt et al., 2012). *Microdochium phragmitis* has been described as an endophyte on common reed (Ernst *et al*., 2011) while *Alternaria infectoria* has been associated with black (sooty) head mold of wheat and barley (Poursafar *et al*., 2018).

Using archived grain samples from Hoosfield the long-term spring barley experiment we established that Ptm is only sporadically present over time with positive samples in 1982, 1995, 2001 and 2012 using end-point PCR. Of the two pathogens, Ptm has only recently become a problem of epidemic proportions in several important barley-producing regions worldwide and less is known about the pathogen biology and host interaction (Liu *et al*., 2011). Further research is needed to establish potential links with weather and agronomic practises. Cultivar Tower might be more susceptible to Ptm than other cultivars. However, Ptm was detected on different cultivars in the archived grain samples and seedborne infection can also not be ruled out as Ptm has also been recently found on cv Tower in Ireland and might be from the same seed batch (Dr. Steven Kildea, personal communication).

The fungicide sensitivity testing using mycelium plugs appeared to be less reliable for Ptm than Ptt due to its irregular and slower growth on agar plates (Fig. 1). Both species have developed insensitivity to SDHI fungicides with most Ptm isolates growing in the presence of 1.0 ppm of fluxapyroxad and Ptt isolates growing at 10 ppm as the highest concentrations. Isolates without Sdh mutations affecting SDHI binding (‘wild-type strains’) are able to grow on concentrations up to 0.1 ppm, whereas isolates with key mutations are able to grow at 1.0, 10 and, sometimes, at 100 ppm, albeit very slowly. Mutations B-H277Y, D-124E, D-G138V and D-D145G have been associated with low resistance factors in *in vitro* assays, whereas moderate resistance factors have been reported for C-K49E, C-R64K, C-N75S, C-G79R, C-H134R, C-S135R and D-H134R (FRAC SDHI Working Group, 2020 Annual Meeting). Mutations C-G79R, C-H134R and C-S135R were most frequently detected in Europe in 2019. Isolates carrying these Sdh variants were also least sensitive in greenhouse experiments (Rehfus et al., 2016). We detected C-R64K in all seven Ptm isolates tested, including an old reference strain from Australia isolated in 1998, and isolate S2/13

carrying C-R64K was sensitive to fluxapyroxad showing no growth at 1.0 ppm. Isolates S1/2 and S1/6 were less sensitive to fluxapyroxad but also carried D-D145G and D-G138V, respectively. We conclude that it is likely that C-R64K has no impact on SDHI binding, together with mutations like B-A6T, B-P52S and D-V40L that were also detected in all *P. graminea* reference isolates. Interestingly, we also detected D-G138V in one Ptt isolate, S3/9, where it was simultaneously found with C-S135R. This strain was least sensitive to fluxapyroxad, with partial growth observed at 100 ppm. Most Ptt isolates that we characterised grew in the presence of 10 ppm of fluxapyroxad and carried C-S135R, with one isolate carrying D-H134R. D-G138V was also detected in isolate S3/4 but this strain was only growing in the presence of up to 1.0 ppm of fluxapyroxad. There were differences in locations regarding the presence of mutations, but this can also be driven by factors like host, fungicide selection pressure and origin of primary/secondary inoculum.

Regarding epoxiconazole sensitivity, only one Ptm isolate was sensitive, able to grow only in the presence of 0.1 ppm of epoxiconazole, with 15 growing at concentrations of up to 1.0 ppm and only 3 showing some growth at 10 ppm. All Ptt isolates were able to grow in the presence of 10 ppm with some showing some growth at 100 ppm. Differences in growth rate between Ptm and Ptt might explain the difference but it is not known if Ptt has been intrinsically less sensitive to azoles than Ptm or has been more exposed to fungicides due to its higher prevalence. Lower sensitivities to azoles have been found in *Pyrenophora teres* isolates from France and Germany since 2017 (FRAC SBI Working Group, 2020 Annual Meeting). Recently, both a CYP51A mutation and over-expression of different CYP51 paralogs reported have been reported as acquired resistance mechanisms in Australian isolates of Ptt and Ptm (Mair *et al*., 2016 & 2019). More azole sensitivity testing of *P. teres* isolates, including old reference strains, and molecular characterisation together with genotyping of archived samples is needed to establish if and when shifts in sensitivity have occurred in UK field populations of Ptt and Ptm and if the evolution is still ongoing. An *in vitro* microtiter plate-based assay using spores rather than mycelium plugs on agar plates as recently described for *P. teres* isolates (Mair *et al*., 2019). Introducing this assay will improve the accuracy and speed of establishing base-line sensitivities and detecting fungicide sensitivity shifts in field populations and enable implementation of anti-resistance strategies and measures before resistance is further spread and established in the field.

With regard to identification of both diseases, spot and net form of net blotch, the markers evaluated in this study (Ptt5 and Ptm11) and genetic variation in SdhC can be further explored to develop rapid on-the-spot detection of both diseases. Isothermal DNA based amplification tools such as LAMP (Loop-Mediated Isothermal Amplification) are becoming more affordable and can produce test results within 30 minutes of taking a sample (King *et al*., 2017). When assays are successfully developed for Ptt, Ptr and Ramularia, this tool can assist in training of staff visually

assessing diseases in Recommended List trials and also be used directly by breeders if disease symptoms are different to recognise due to mixed infections.

2.5. Acknowledgements

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3. PROJECT OUTPUTS

Fraaije BA (2019) 'SDHI sensitivity status of UK *Zymoseptoria tritici* and *Pyrenophora teres* field populations' presentation at 19th International Reinhardsbrunn Symposium Modern Fungicides and Antifungal Compounds, Friedrichroda, Germany (8 April 2019)