Research Article

Received: 6 July 2022 Revised: 27 September 2022 Accepted article published: 9 October 2022 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.7219

Azole fungicide sensitivity and molecular mechanisms of reduced sensitivity in Irish *Pyrenopeziza brassicae* populations

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Abstract

BACKGROUND: Light leaf spot, caused by *Pyrenopeziza brassicae*, is amongst the most damaging diseases of winter oilseed rape (*Brassica napus*), and currently the sterol 14α-demethylase (CYP51) inhibitors (azoles) represent the main class of fungicides used to control light leaf spot development. However, a shift in sensitivity to azole fungicides in *P. brassicae* populations has been observed in different European countries, including Ireland.

RESULTS: To assess the sensitivity status of Irish *P. brassicae* populations to azole fungicides, three collections of *P. brassicae* from 2018–2020 were tested *in vitro* against tebuconazole and prothioconazole-desthio, and the *PbCYP51* gene targeted by this class of fungicides was genotyped in different isolates. A change in sensitivity to azole fungicides was observed and differences in sensitivity to tebuconazole between Irish populations were present. There were two substitutions within *PbCYP51* (G460S and S508T) and inserts of different sizes in its promoter region. The presence of the G460S/S508T double mutant was reported for the first time, and the diversity in insert size was greater than previously known. Compared to wild type isolates, those carrying G460S or S508T were less sensitive to both fungicides and, where inserts were also identified, they further reduced sensitivity to azole fungicides.

CONCLUSIONS: The results of this study suggest that azole fungicides are still very effective in controlling light leaf spot in Ireland. However, using azole fungicides in mixtures of fungicides with different modes of action is recommended.

Supporting information may be found in the online version of this article.

Keywords: azole fungicides; disease control; fungicide sensitivity; light leaf spot; oilseed rape; *Pyrenopeziza brassicae*

1 INTRODUCTION

Oilseed rape (*Brassica napus* L.) (OSR) is the second most important source of vegetable oil worldwide[1,2] and the most cultivated oilseed crop in Europe.[3–5] Despite this, according to the Central Statistics Office of Ireland,[6] the area sown with this crop is relatively small in Ireland (9000–10 000 ha), but production has increased in the last 10 years.[7] Irish weather conditions are ideal for the development and spread of fungal pathogens, and increase in OSR production had led to severe disease outbreaks.[8,9]

This is the case for light leaf spot (LLS), caused by *Pyrenopeziza brassicae*, one of the most important diseases of OSR in the UK and Northern Europe[10] and widespread globally, with records in various countries in Europe, Oceania and Northern America.[11] The disease has recently overtaken phoma stem canker and become the primary disease of OSR in the UK, with severe epidemics causing annual yield losses up to £160 M per year.[12,13] The pathogen is capable of infecting the leaves, flowers, stems and pods during the cropping season,[14–16] and due to its mixed reproduction and its polycyclic life cycle,[17,18] the pathogen has a high evolutionary potential.[11] Although LLS has been present in Ireland for a long time[19–21] and Irish OSR growing conditions are favorable for its development, there is no detailed information on the impact of this disease on OSR in Ireland.

Current strategies to control LLS consist of a combination of crop management practices, host resistance and fungicide applications.[13,22] However, cultural practices alone cannot control epidemics sufficiently,[12] and cultivar resistance is often not enough to prevent the disease in conducive environments.[10] Therefore,

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LLS control often relies on the use of fungicides,[13] with the most widely used and effective class of fungicides being the demethylation inhibitors (azoles). These fungicides interfere with the fungal cytochrome P450 sterol 14α-demethylase (*CYP51*), which is involved in the biosynthesis of ergosterol.[23] Reduced sensitivity to other classes of fungicides previously used is already widespread[24] and the severity of LLS epidemics has increased progressively across England.[25] Thus there are concerns regarding the potential spread of less sensitive *P. brassicae* isolates that could cause a decline in the efficacy of azole fungicides.[26,27] Although the risk of development of reduced sensitivity for this class is medium,[28] changes in sensitivity to azole fungicides may have occurred since the early 1990s[29] and Scottish *P. brassicae* isolates with reduced sensitivity to tebuconazole have been reported since 2003.[30]

Carter *et al.*[26] characterized *P. brassicae* isolates with reduced sensitivity to azole fungicides using a combination of classical genetics and candidate gene approaches and identified the main mechanisms of reduced sensitivity to azole fungicides, related to changes in the *PbCYP51* gene. Two non-synonymous substitutions in the coding region lead to the amino acid substitutions G460S and S508T; S508T is equivalent to the *Zymoseptoria tritici CYP51* substitution S524T that is found only in combination with other changes and reduces the sensitivity to all azole fungicides[31]; and G460S is equivalent to G464S in *Candida albicans*, which reduces the binding affinity for azoles and the enzymatic activity.[32] Sequence duplications representing three insert sizes (46 bp, 151 bp and 233 bp) were also identified in the predicted regulatory regions of *PbCYP51*. However, the wild type variants were predominant in the collection analyzed by Carter *et al.*[26] and the populations were still very sensitive to fungicides.

More recently, King *et al.*[33] investigated the *in vitro* azole sensitivity status of *P. brassicae* populations from six European countries, including Ireland, and discovered that the populations were broadly similar in sensitivity to azole fungicides, but when compared to an older reference UK isolate their sensitivity was reduced. The two non-synonymous substitutions previously identified were present in the collections, G460S at high frequency and S508T at low frequency, and further variation in promoter insert sizes was identified (44/46 bp, 151 bp, 210 bp, 302 bp).[33] Combinations between the substitutions and promoter inserts were also widespread in the European populations and, based on the sensitivity screening, the least sensitive group of *P. brassicae* isolates was those carrying G460S substitution in combination with one of the different promoter inserts, a combination also identified in the limited Irish populations analyzed.

Although reduced azole fungicide sensitivity has already been reported for Irish *P. brassicae* isolates, it is not clear whether the reduced azole fungicide sensitivity is widespread in Ireland. Therefore, there is a need to confirm that previous results are representative for the wider Irish populations. Moreover, the two Irish populations tested in the study by King *et al.*[33] were assessed only using prochloraz and tebuconazole, while, in Ireland, the most important azole fungicide used contains prothioconazole. Additionally, reports show that for this fungicide prothioconazole-desthio is in fact the primary active form that is inhibiting the activity of *CYP51* in fungi,[34,35] and the sensitivity status of *P. brassicae* to prothioconazole-desthio was not assessed. To fill these gaps in knowledge, the sensitivity of *P. brassicae* isolates from throughout the main Irish OSR growing regions to the azole fungicides prothioconazole-desthio (PDZ) and tebuconazole (TBZ) was determined using an *in vitro* assay, and the mechanisms of associated reduced sensitivity were investigated molecularly.

2 MATERIALS AND METHODS

2.1 Pyrenopeziza brassicae isolate collections

Three collections of *P. brassicae* were established from winter OSR crops grown across Ireland in 2018–2020 (Supporting Information, Fig. S1). These are the three collections: a representative collection for Ireland named 'representative collection', an extensive collection for the 2018-2019 cropping season named '2019 collection', and an extensive collection for the 2019-2020 cropping season, named '2020 collection'. The representative collection consists of *P. brassicae* isolates obtained from OSR cv. Phoenix grown in the three main regions where OSR is grown intensively in Ireland, sampled in March 2019, by walking in a 'W'-shaped pattern and collecting OSR leaves presenting characteristic symptoms of LLS every 10 m. The 2019 collection and 2020 collection were obtained from different commercial OSR crops in different locations in Ireland, that were sampled in the early spring of 2018–2019 and 2019–2020 cropping seasons by walking in a 'W'-shaped pattern and collecting a random OSR leaf regardless of the presence or absence of LLS symptoms every 10 m. The leaf samples were incubated at 4 °C for 3–4 days to promote *P. brassicae* sporulation. Where present, single conidiomata were isolated from each leaf and inoculated using a sterile needle onto 1% malt extract agar (MEA) media (Merck, Germany) amended with 50 mg L^{-1} of ampicillin sodium (Apollo Scientific Limited, Stockport, UK) and 100 mg L^{-1} of streptomycin sulphate (Fisher BioReagents, Pittsburgh, USA). The plates were incubated for 21 days at 18 °C and single spore colonies were obtained for each isolate by subculturing individual colonies obtained from asexual spore suspensions in sterile distilled water on fresh MEA media. The single spore isolates were stored in 30% glycerol stocks at −80 °C.

The representative collection of *P. brassicae* isolates obtained for Ireland comprised 107 single spore isolates, of which 27 were from Carlow, 58 from Cork and 22 from Louth. The 2019 collection of *P. brassicae* isolates comprised 192 isolates obtained from 17 commercial crops in Ireland (Table 1), the number of isolates per location ranging from one (site 19.15) to 30 (site 19.11). The 2020 collection of *P. brassicae* isolates comprised 321 isolates obtained from 18 commercial OSR crops (Table 1), the number of isolates per location ranging from one (site 20.01) to 42 (site 20.17).

2.2 *In vitro* sensitivity to tebuconazole and prothioconazole-desthio fungicides

The sensitivity of the isolates from the representative collection and 2020 collection to azole fungicides tebuconazole (TBZ) and prothioconazole-desthio (PDZ) was determined using a microtiter plate assay described by Carter *et al.*[26] The isolates were grown from conidial stock solutions, and spore suspensions were obtained by adding 1 mL of sterile distilled water (SDW) into each media plate, gently scraping the fungal colony using a sterile T-shaped spreader and pipetting the spore mixture obtained into a 2 mL Eppendorf tube using a wide-bore pipette tip. The spore suspensions were quantified using KOVA® Glasstic® Slides (Kova International, Inc., Garden Grove, USA) and adjusted to 25 000 spores mL^{-1}.

Flat bottomed microtiter plates (Sarsted AG & Co., Germany) filled with of 2x PDB (100 µL) and the adjusted conidial

Table 1. Isolates of *Pyrenopeziza brassicae* (Pb) obtained from commercial OSR crops for Ireland for 2019 collection (2018–2019 cropping season) and 2020 collection (2019–2020 cropping season)

2019 collection				2020 collection			
Code	Location	OSR cultivar	No. Pb isolates	Code	Location	OSR cultivar	No. Pb isolates
19.04	Knockbeg, Laois	Anastasia	30	20.01	Cork	Anastasia	1
19.05	Goresbridge, Kilkenny	DK Extrovert	3	20.02	Cork	Impression	5
19.06	Midleton, Cork	Phoenix CL	18	20.03	Wexford	Aquilla	24
19.07	Cappoquin, Waterford	Dariot	10	20.04	Cork	Anastasia	22
19.08	Carlow	Phoenix CL	5	20.05	Wexford	Aquilla	30
19.09	Wexford	Alizze	6	20.06	Wexford	N/A	29
19.10	Wexford	DK Expansion	13	20.07	Wexford	DK Expansion	23
19.11	Belgooly, Cork	Phoenix CL	30	20.08	Carlow	Aquilla	19
19.12	Arklow, Wicklow	Phoenix CL	24	20.09	Carlow	Aquilla	39
19.13	Kildare	PT 234	9	20.10	Wexford	DK Expansion	11
19.14	Kildare	DK Exclaim	10	20.11	Kilkenny	DK Expansion	17
19.15	Castlebelligham, Louth	Alizze	1	20.12	Kildare	Aquilla	17
19.16	Togher, Louth	DK Exclaim	3	20.13	Kildare	Elgar	2
19.17	Dunleer, Louth	Picto	3	20.14	Offaly	DK Exclaim	24
19.18	Ballough, Dublin	Picto	12	20.15	Meath	DK Exstar	4
19.19	Swords, Dublin	PT 256	5	20.16	Tipperary	Ergo	4
19.20	Navan, Meath	PT 256	10	20.17	Tipperary	DK Expansion	42
				20.18	Louth	DK Exclaim	10
Total			*192*				*323*

suspension (100 µL) were amended with three-fold dilution series of TBZ or two-fold dilution series of PDZ (Merck, Germany) to give final concentrations ranging from 30 µg mL^{-1} to 0.0005 µg mL^{-1} for TBZ and from 20 µg mL^{-1} to 0.0195 µg mL^{-1} for PDZ.

Seven isolates and a negative control were tested on each plate, and all the plates were prepared in duplicate. Five reference isolates with known azole sensitivity phenotypes[26] were used for each experimental batch to confirm reproducibility. The reference isolates were classified as most sensitive (*PbCYP51* wild type), reduced sensitivity (*PbCYP51* substitutions G460S or S508T) or least sensitive (*PbCYP51* substitutions G460S/S508T with inserts in the *PbCYP51* regulatory region) (Table 2).

The inoculated plates were sealed with parafilm and cling film to prevent evaporation and incubated in the dark at 18 °C. After 12 days, fungal growth was assessed by measuring light absorbance at 630 nm using a Synergy-HT plate reader and Gen5™ microplate software (BioTek Instruments, Inc., USA) in well-scanning mode. The absorbance for the negative control containing only PDB media and water was subtracted from the absorbance measured for each isolate-fungicide combination and converted into percentage inhibition relative to untreated control, and the average of the two replicates was calculated for each sample. The effective concentration reducing fungal growth by 50% (EC_{50} value) was calculated for each isolate using a dose–response relationship, using the *drc* package[36] in RStudio (2021.09.0 + 351 'Ghost Orchid' Release for Windows).

The data obtained were fitted using different dose–response models with EC_{50} as a parameter and logistic and log-logistic models (LL.4, LL2.4, W1.4, W2.4) were compared, with the 4-parameter log-logistic model (LL.4) being selected to provide the best fit for the data. The EC_{50} values for each isolate were extracted from the model and plotted for each population against the EC_{50} values obtained for the reference isolates using the *ggplot2* package.[37] Differences between the populations included in each collection were determined using the non-parametric ***K***ruskal-Wallis one-way ANOVA test using kruskal.test function

Table 2. *Pyrenopeziza brassicae* reference isolates used in fungicide sensitivity screening with their EC_{50} values for tebuconazole and prothioconazole

Isolate	Year	Location	Host crop	*PbCYP51* genotype	Tebuconazole		Prothioconazole	
					EC_{50}* (µg mL^{-1})	RF†	EC_{50} (µg mL^{-1})	RF
PbFr002	1995	Le Rheu, France	*B. napus*	Wild type	0.04	-	0.14	-
E3A	2007	Hertfordshire, UK	*B. napus*	Wild type	0.03	1	0.34	4
WC4	2007	Hertfordshire, UK	*B. oleracea*	S508T	0.49	21	3.06	38
8CAB	2011	East Lothian, UK	*B. oleracea*	G460S	0.59	25	3	37
I3	2011	East Lothian, UK	*B. oleracea*	S508T/151 bp	1.19	50	5.54	69

*EC_{50} are means of two independent replicates calculated on a log_{10}-scale and back-transformed.
† Resistance Factor (RF) values were calculated as the fold change in EC_{50} compared to the mean EC_{50} value of wild type isolate PbFr002.

from the R *rstatix* package.[38] Where significant differences were identified, subsequent post-hoc Dunn's multiple comparison tests were used to identify significant differences between the populations within the collection using the kwAllPairsDunnTest function from *PMCMRplus* package, implemented with 'holm' method.[39]

To determine if a pattern of cross-sensitivity is present for the two fungicides tested, the Spearman correlation coefficients rho were calculated and plotted for the 2018–2019 and 2019–2020 cropping seasons using the cor.test and ggscatter functions from ggpubr package[40] developed under devtools package[41] in R.[42]

2.3 Molecular mechanisms associated with reduced azole sensitivity within Irish *P. brassicae* populations

2.3.1 DNA extraction and quantification

DNA was extracted from freeze-dried fungal mycelium using the Wizard Genomic DNA Purification Kit from Promega (Madison, Wisconsin, USA), following the manufacturer's guidance. The quantity and quality of the DNA were measured by absorbance using a UV–VIS NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA), using the fluorescence method with a Qubit® fluorometer from Invitrogen (California, USA) and by agarose gel electrophoresis.

2.3.2 Detection of PbCYP51 *upstream insertions associated with changes in sensitivity to azole fungicides*

For all isolates, the presence of inserts in their *PbCYP51* regulator region was determined by PCR using the primer pair CYP51upstreamF/CYP51upstreamR,[26] with minor modifications. Reactions were done using Taq DNA polymerase with ThermoPol Buffer (New England BioLabs Inc., Massachusetts, USA) in a reaction volume of 20 µL, containing 1 × buffer, 200 µM dNTPs, and 0.025 units of the polymerase. The PCR conditions were 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, followed by a final extension of 5 min at 72 °C and a final hold at 10 °C. The PCR product obtained was migrated by electrophoresis on a 2% agarose gel and visualized under UV-light using an ENDURO™ GDS Gel Documentation System for Electrophoresis (Labnet International, Inc., New Jersey, USA). Due to challenges in discriminating between the inserts of either 44 bp and 46 bp or 210 bp and 233 bp, these inserts were grouped as either 44/46 bp or > 210 bp. To confirm the sizes of these inserts, PCR products from a selection of 96 isolates (48 from 2019 collection and 48 from 2020 collection, with 12 isolates from each insert size group) were sent for Sanger sequencing using the primer CYP51upstreamR (LGC Genomics, Berlin, Germany).

2.3.3 Detection of substitutions within the coding region of PbCYP51 *associated with changes in sensitivity to azole fungicides*

To screen for substitutions in *PbCYP51* for the 2019 and 2020 collections, the entire gene was amplified with the primer pairs PbCYP51F and PbCYP51R,[26] using Q5 polymerase and 5× Buffer Q5 (New England BioLabs Inc., USA), in a final reaction volume of 50 µL (1× Buffer Q5, 200 µM dNTPs, 0.5 µM Forward/Reverse primer, 0.02 U μL^{-1} Q5 HF polymerase and 1× of Q5 High GC Enhancer). The reaction conditions were 98 °C for 30 s initial denaturation, followed by 35 cycles at 98 °C for 10 s denaturation, 68 °C for 30 s annealing and 72 °C for 2 min extension, followed by a final extension step of 2 min at 72 °C and a final hold at 10 °C. The PCR product was visualized on a 1% agarose gel under UV-light using an ENDURO™ GDS Gel Documentation System for Electrophoresis (Labnet International, Inc., USA) and samples presenting clean bands were sent to LGC Genomics for Sanger sequencing using the primers CF2 and CR1 or PbCYP51R.[26] Sequences obtained were aligned to the sequence of the whole *PbCYP51* gene for isolate 18CAR04[33] using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms[43] and SnapGene software v6.0.2 (from Insightful Science).

For the isolates included in the representative collection, the presence of two substitutions in the coding region of *PbCYP51* was determined by amplifying a 1244 bp fraction of the gene encompassing the codons 460 and 508, using the primer pair CYP51expressionF1/CYP51R.[26] The PCR reaction mix and conditions used were as described, with an annealing temperature of 56 °C and an extension time of 1 min 30 s. Subsequent RFLP digest reactions were done to detect G460S using two units of *TspRI* (CASTG) (New England BioLabs Inc., USA). Approximately 60 ng of the purified PCR product were mixed with the enzyme and 1 × cutsmart NEBuffer 4 in a final volume of 10 µL, and the mix was incubated for 2 h at 65 °C. To detect the substitution S508T, approximately 60 ng of the purified PCR product were digested with 1 unit of *BssSI* (CACGAG) and 1 × cutsmart NEBuffer 3 in a total volume of 10 µL, the mix being incubated for 2 h at 37 °C. The digested products obtained were separated on a 1.5% (w/v) agarose gel and exposed to UV light to visualize DNA fragments.

2.4 Determining the sensitivity to azole fungicides based on the *PbCYP51* genotypes

The variants obtained for the different types of inserts identified in the regulatory region of *PbCYP51* combined with the substitutions observed within the coding region of the gene were combined to obtain different genotypes for the *PbCYP51* gene. The results from sensitivity screening for the two azole fungicides were visualized for the genotypes obtained using *ggplot2* package[37] and used to detect if significant differences are present between the different *PbCYP51* genotypes obtained by running the non-parametric Kruskal-Wallis, alternative to the one-way ANOVA using kruskal.test function from *rstatix* package.[38] Further, the impact that the presence of different inserts and substitutions have on the sensitivity to TBZ and PDZ was determined and visualized using conditional inference trees, constructed using the ctree function from the *partykit* package[44,45] in Rstudio. The predictors used were the different genotypes obtained for *PbCYP51*, with each type of alteration present: no insert, 44/46 bp insert, 151 bp insert, >210 bp insert, G460S, no G460S, S508T and no S508T, while the response variable was either TBZ or PDZ. The distribution of the test was computed using Bonferroni, the value given by mincriterion that the criterion needs to exceed before a split is implemented was 0.95, the minsplit (minimum sum of weights in a node before considering a split) was set to 40 and minbucket (the minimum sum of weights in a node before considering a split) was 20. Variables of importance were calculated for TBZ and PDZ using the R package *randomForest*[46] in Rstudio. Random forest regressions[47] were calculated using the randomForest function, using the arguments for the number of variables randomly sampled at each split (mtry) set to 1, number of trees (ntree) set to 500 and importance of predictors assessed (importance) set as TRUE. The varImpPlot function was used to plot the mean decrease in accuracy (%IncMSE) for each variable. Further, pirateplots were generated using the package *yarrr*[48] to visualize the impact of the alterations identified on TBZ and PDZ sensitivity.

The frequencies of the genotypes obtained for each location in the 2019 and 2020 collections were visualized using *ggplot2* package[37] in Rstudio.

3 RESULTS

3.1 *In vitro* sensitivity to the azole fungicides tebuconazole and prothioconazole-desthio

The sensitivity to TBZ (EC_{50} value) of the five reference isolates ranged between 0.03 μg mL^{-1} (PbFr002 – wild type) and 0.60 μg mL^{-1} (I3 - S508T/151 bp). All three populations from the representative collection and 16 populations from the 2020 collection had increased EC_{50} values compared to the wild type reference isolates. Although some of the isolates had extremely small EC_{50} values, the interquartile range for all the groups included values similar to the EC_{50} value obtained for 8CAB (G460S) (0.57 μg mL^{-1}) and I3 (S508T + 151 bp) (0.60 μg mL^{-1}), the least sensitive reference isolate (Fig. 1). However, 11 populations from the 2020 collection had median EC_{50} values greater than the least sensitive reference isolate (I3). The resistance factors (ratio between the least sensitive and wild type reference isolates) obtained for the two collections were 67.57 for the representative collection and 239.98 for the 2020 collection.

Krustal-Wallis one-way ANOVA suggested significant differences existed between one or more pairs of the 16 populations from the 2020 collection ($P = 0.0376$) and between the two collections analyzed ($P = 3.935e-07$). Subsequent *post hoc* comparison tests using Dunn's multiple comparison tests showed significant differences between population 8 and populations 5, 7, 12 and 17, between population 9 and populations 12 and 17, between population 12 and populations 10 and 14, and lastly between population 14 and populations 4, 5, 6, 7, 11 and 17 ($P < 0.05$).

Sensitivity of the five reference isolates used towards PDZ ranged between 0.008 and 0.029 μg mL^{-1}, with PbFr002 (wild type) the most sensitive and I3 (S508T/151 bp) the least sensitive (Fig. 2). For both the representative and 2020 collections, a broad range in sensitivity was observed, with resistance factors (ratio between least sensitive and wild type reference isolates) of 16.77 for the representative collection and 46.66 for the 2020 collection (Fig. 2). No differences in sensitivity were observed between the collections ($P = 0.056$) or within each collection (2019: $P = 0.619$; 2020: $P = 0.357$).

The Spearman rank correlation was calculated for the two fungicides used, to identify if cross-sensitivity between PDZ and TBZ existed, and the rho values (0.69 for the 2019 collection and 0.46 for the 2020 collection) indicated a significant positive correlation between the fungicides (Fig. 3).

3.2 Molecular mechanisms associated with reduced azole sensitivity within Irish *P. brassicae* populations

3.2.1 *Detection of* PbCYP51 *upstream insertions associated with changes in sensitivity to azole fungicides*

Based on the sequencing data obtained for the promoter region of *PbCYP51* in the 96 isolates sequenced, 11 different inserts were identified: 44 bp, 46 bp, 59 bp, 67 bp, 151 bp, 175 bp, 210 bp, 233 bp, 302 bp, 453 bp and 466 bp (Supporting Information, Table S1 and Fig. S2). Three of these inserts (46 bp, 151 bp and 233 bp) were previously identified by Carter *et al.*[26] and King *et al.*[33] detected an additional three inserts (44 bp, 210 bp and 302 bp); except for the 44 bp and 67 bp inserts, all contained an identical 46 bp repeat sequence. The 67 bp insert contained a repetitive conserved sequence of 34 bp that was also present in all the inserts >67 bp. Additionally, a sequence of 38 bp, which had been previously described as part of an 82 bp sequence characteristic for 210 and 233 bp inserts, was also identified in the 175 bp insert, with the 82 bp insert also present twice in the 466 bp insert.

As some of the insert sizes were difficult to differentiate following electrophoresis, they were grouped into four distinctive classes based upon their size: (I) no insert; (II) 44/46 bp (including the 59 and 67 bp inserts); (III) 151 bp (including 175 bp); (IV) >210 bp (210–466 bp). Based upon this classification, the most frequent group of inserts identified was those >210 bp (27% of the isolates), followed closely by 44/46 bp sequence

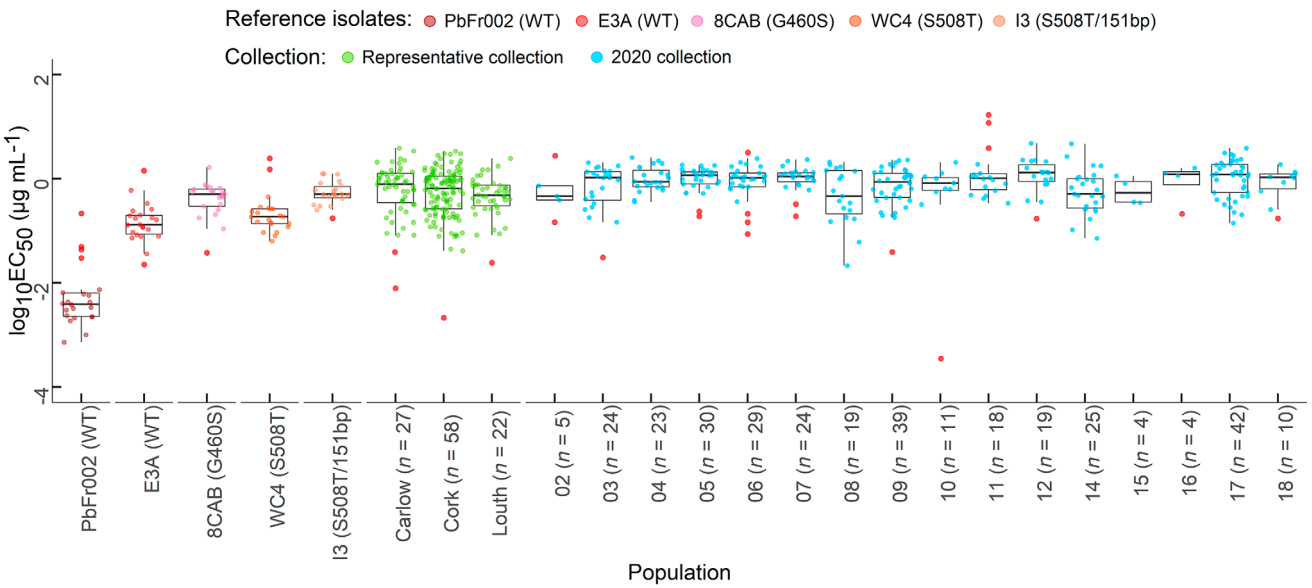

Figure 1. Sensitivity to tebuconazole of the representative collection and 2020 collection of Irish *Pyrenopeziza brassicae* isolates, as EC_{50} values. The isolates are ranked based on their increasing EC_{50} value (log_{10}-transformed). The populations tested are shown in different colors and the number of isolates (*n*) for each population is shown. Sample isolate locations are given in Supporting Information, Fig. S1. Each data point represents the mean of two technical replicates and outliers are presented as red data points. The reference isolates, with EC_{50} values calculated as averages for values obtained in each batch tested, are presented in different shades of red. PbFr002, E3A – wild type isolates, WC4 – S508T, 8CAB – G460S, I3 – S508T + 151 bp. Information on these five reference isolates is given in Table 2.

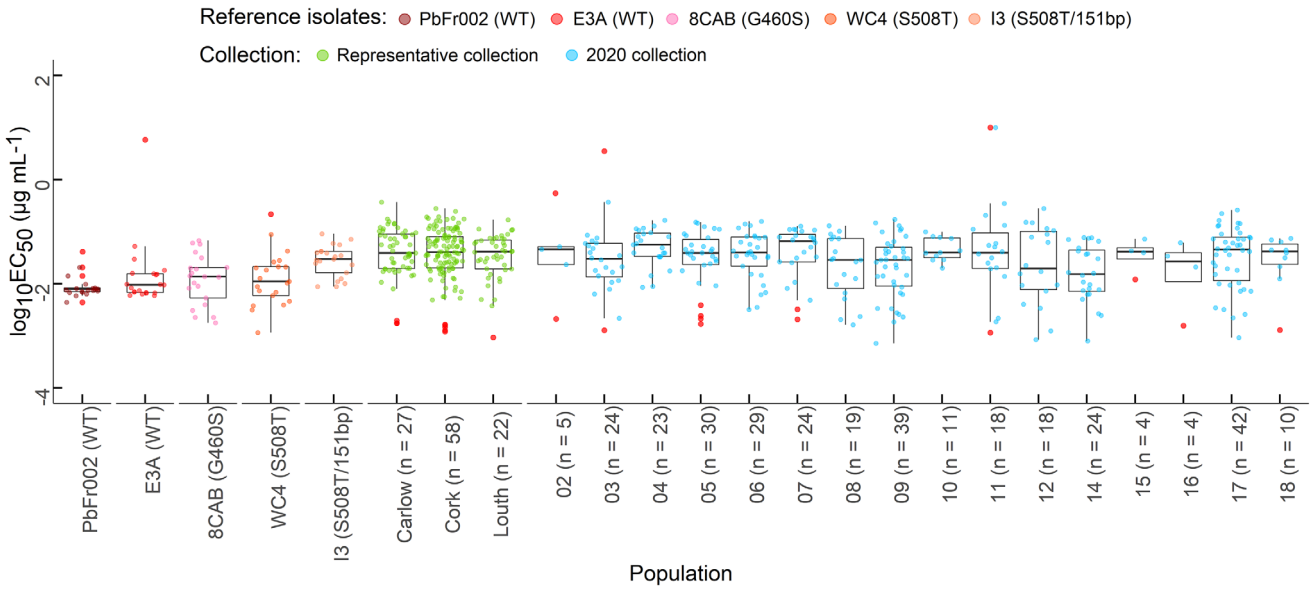

Figure 2. Sensitivity to prothioconazole-desthio of Irish *Pyrenopeziza brassicae* isolates from the representative collection and 2020 collection. The isolates are ranked based on their increasing EC_{50} value (log_{10}-transformed). The populations tested are shown in different colors and the number of isolates (*n*) for each population is shown. Sample isolate locations are given in Supporting Information, Fig. S1. Each data point represents the mean of two technical replicates and the outliers are presented as red data points. The reference isolates, calculated as averages for values obtained in each batch tested, are presented in different shades of red. PbFr002, E3A – wild type isolates, WC4 – S508T, 8CAB – G460S, I3 – S508T + 151 bp. Information on these five reference isolates is given in Table 2.

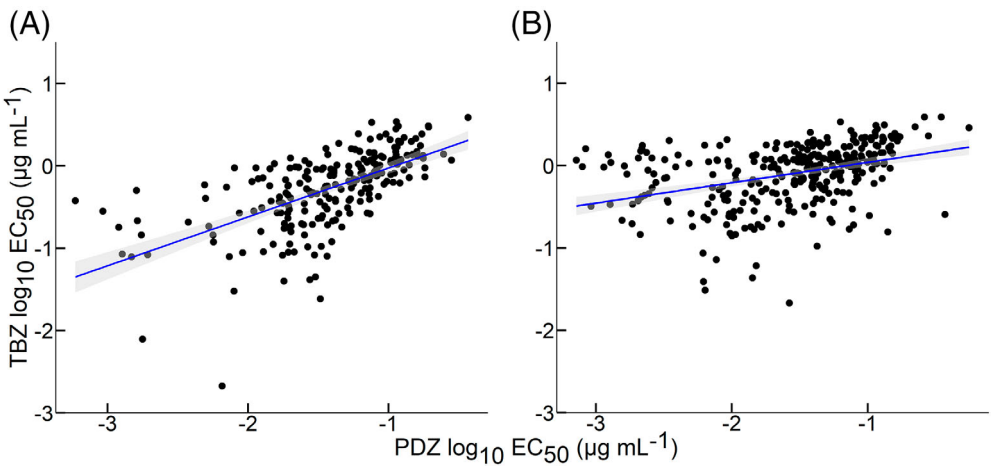

Figure 3. Spearman Rank correlation between prothioconazole-desthio (PDZ) and tebuconazole (TBZ) EC_{50} values for the 2019 (A) and 2020 (B) *Pyrenopeziza brassicae* collections. Spearman correlation coefficients Rho (R) obtained for the two collections were 0.69 (2019) and 0.46 (2020), $P < 2.2e\text{-}16$.

(26.7% of the isolates) and the 151 bp insert (present in 26.5% of the isolates). No differences were observed between the groups in the frequencies of these inserts (Table 3).

3.2.2 *Detection of substitutions within the coding region of* PbCYP51 *associated with changes in sensitivity to azole fungicides*

Only two substitutions in *PbCYP51* were identified amongst the 480 isolates sequenced, both non-synonymous, leading to the amino acid substitutions G460S and S508T, which have been previously described by Carter *et al.*[26] and King *et al.*.[33] The G460S substitution was the most prevalent, present in 78.7% of the isolates analyzed, while S508T was present in 12.4% of the isolates. The two substitutions were identified together in only three isolates (0.48% of the isolates), one from each collection.

By combining the inserts from the *PbCYP51* regulatory region with *PbCYP51* substitutions, a total of 14 *PbCYP51* genotypes were identified (Table 3). Only seven wild type isolates (neither insert or substitution) were detected. The majority of the isolates with the substitution G460S also had an insert in the *PbCYP51* regulatory region, with >210 bp inserts the most abundant (30.4%), followed by 44/46 bp inserts (27.3%) and 151 bp inserts (24.2%). Isolates with S508T also predominantly had a *PbCYP51* promoter insert, 44/46 bp being the most common group (33.8%), followed by 151 bp insert (20.8%) and > 210 bp inserts (10.4%). Amongst the three isolates with

Table 3. Frequencies of *PbCYP51* genotypes within the three collections of Irish *Pyrenopeziza brassicae* isolates

	Population sampled			
PbCYP51 genotype	Representative collection*	2019 collection	2020 collection	Total no. isolates genotyped
Wild type (WT)	1	4	2	7
44/46 bp insert	2	1	1	4
151 bp insert	8	9	12	29
>210 bp insert	4	3	5	12
G460S	21	27	40	88
G460S + 44/46 bp	21	43	69	133
G460S + 151 bp	21	29	68	118
G460S + >210 bp	17	38	93	148
S508T	6	12	9	27
S508T + 44/46 bp	1	12	13	26
S508T + 151 bp	3	7	6	16
S508T + >210 bp	1	2	5	8
G460S + S508T + 44/46 bp	1	1	0	2
G460S + S508T + 151 bp	0	0	1	1
Total no. isolates genotyped (sequenced)	*107*	*188*	*324*	*619*

*Isolates genotyped by RFLP.

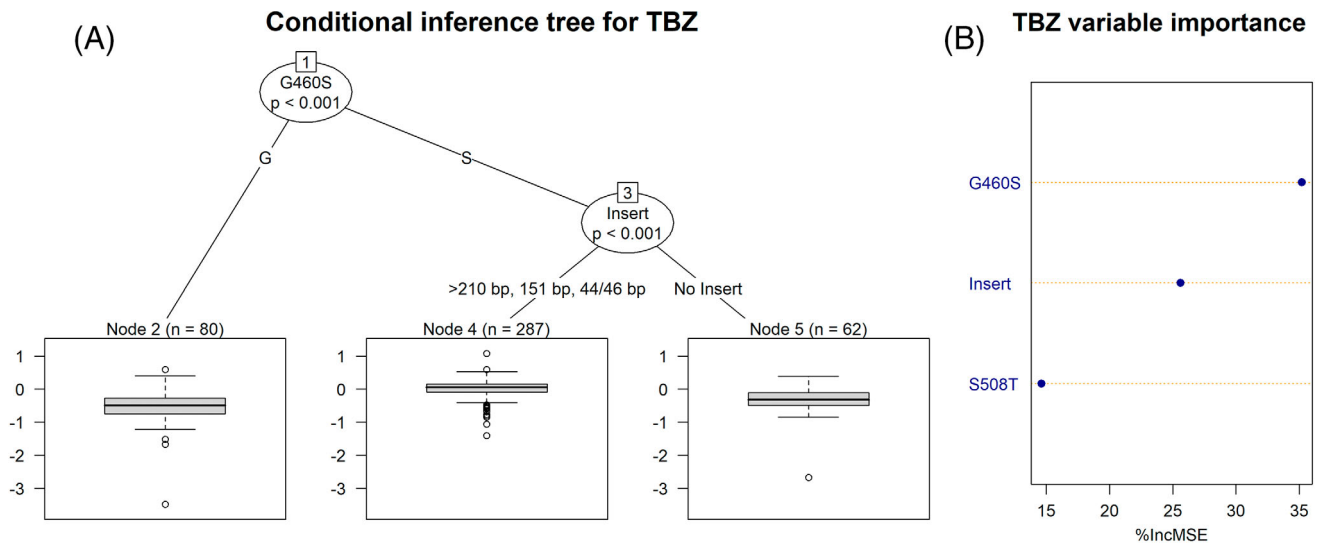

Figure 4. (A) Conditional inference tree with the tebuconazole (TBZ) $\log_{10}$-EC_{50} (μg mL^{-1}) as response variable and amino acid substitutions and inserts as predictors. The box plots show TBZ $\log_{10}$-EC_{50} values (μg mL^{-1}) for samples within each node (n = number of isolates). *PbCYP51* alterations are shown on the tree. (B) Variable importance plot showing how model accuracy decreases as variables are omitted.

both substitutions, two had the 44/46 bp insert and the remaining isolate had a 151 bp insert.

3.3 Determining the sensitivity to azole fungicides based on the *PbCYP51* genotypes

Isolates with the G460S substitution had a four-fold reduction in sensitivity to TBZ and a six-fold reduction in sensitivity to PDZ compared to the wild type isolates when combined with a 151 bp insert, while when in combination with the 210 bp insert, a five-fold reduction in sensitivity to TBZ and a four-fold reduction in sensitivity to PDZ was observed (Supporting Information, Table S2). For S508T, the greatest reduction in sensitivity was for the isolates carrying S508T + 151 bp: three-fold reduction in sensitivity to TBZ and four-fold reduction in sensitivity to PDZ, compared to one-fold reduction in sensitivity to TBZ and two-fold reduction in sensitivity to PDZ for the isolates with S508T only (Supporting Information, Table S2). However, the sensitivity of the *P. brassicae* isolates to TBZ was less than their sensitivity to PDZ, and a wide range of isolates were even more sensitive to PDZ than the wild type *P. brassicae* isolates used as reference isolates (Supporting Information, Fig. S3).

Conditional inference trees were generated to determine relationships between the substitutions and different inserts and their impact on sensitivity to TBZ and PDZ. For TBZ, these identified the G460S substitution as a significant contributor to the reduced sensitivity observed ($P < 0.001$), and this was greatest

for isolates with a regulatory insert (Figs 4(A) and 5). For PDZ, both the 151 bp and > 210 bp inserts were identified as the dominant factors reducing sensitivity, while the presence of the substitution G460S only had a moderate effect on sensitivity in these isolates. For isolates with either a 44/46 bp insert or no insert, only subtle differences were observed between them in sensitivity to PDZ, irrespective of the presence of either G460S or S508T (Figs 6(A) and 7). Random forests were used to determine what percentage of the variance in the sensitivity is explained by the alterations detected by the conditional inference trees. For TBZ, 26.13% of the variance in sensitivity could be explained by the alterations identified (Fig. 4(B)), while for PDZ, the alterations explained only 9.88% of the variance (Fig. 6(B)).

The only difference in sensitivity between different populations was observed for the 2020 collection of *P. brassicae* isolates for sensitivity to TBZ, and the frequency of the substitutions and inserts was identified for each location of this collection, as these results might be caused by different proportions of these alterations present in different populations. The two populations that were different from the other populations were those from location 8 and location 14, and these two locations differed in the frequency of the S508T substitution, either alone or in combination with an insert (Fig. 8). For the population from location 8, differences were also observed for the combinations G460S + 44/46 bp insert and G460S + 151 bp insert, whose frequencies were less (approximatively 5% and 10%) compared to the other populations. Moreover, for this population the overall frequency of combinations of G460S was less, close to 50% of the total alterations. Similarly, for population 14, the proportion of alterations including the G460S substitution was even less, with a reduced proportion of the G460S substitution without inserts, while approximatively 25% of the isolates in this population had the

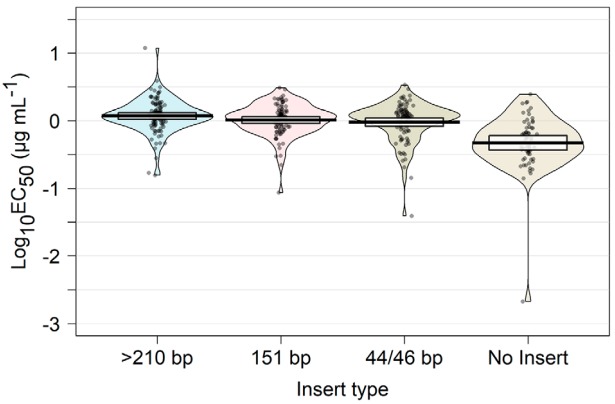

Figure 5. Plot (PiratePlot) showing tebuconazole (TBZ) $\log_{10}$-EC_{50} (µg mL^{-1}) raw data points, density plot, central tendency, and 95% confidence interval for the different groups of inserts (>210 bp, 151 bp, 44/46 bp or no insert) observed for the *Pyrenopeziza brassicae* isolates carrying the G460S substitution.

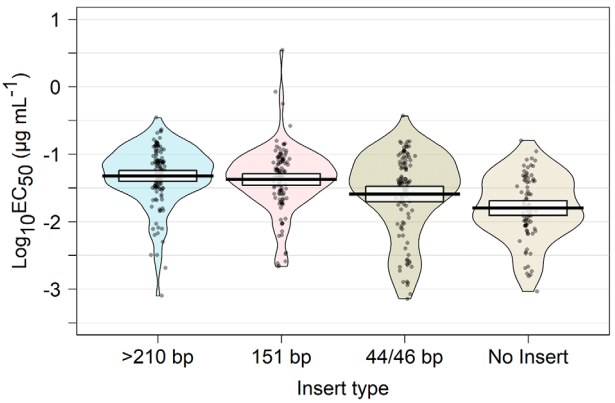

Figure 7. Plot (PiratePlot) showing prothioconazole-desthio (PDZ) $\log_{10}$-EC_{50} (µg mL^{-1}) raw data points, density plot, central tendency, and 95% confidence interval for the different groups of inserts (>210 bp, 151 bp, 44/46 bp or no insert) observed for the *Pyrenopeziza brassicae* isolates regardless of the substitutions present.

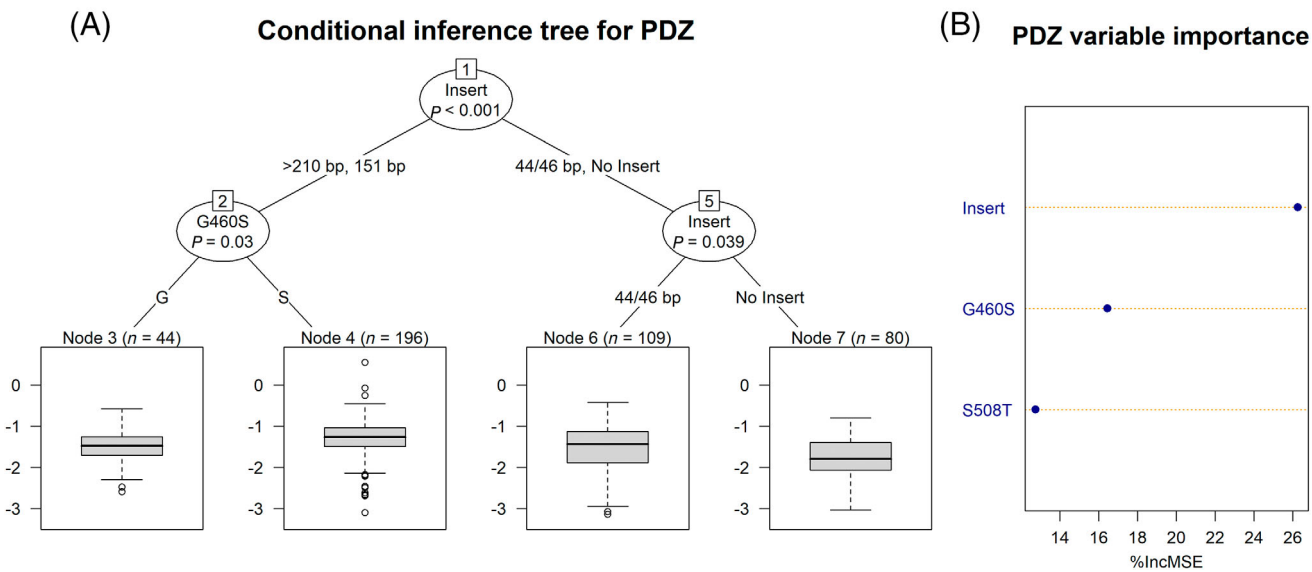

Figure 6. (A) Conditional inference tree with the prothioconazole-desthio (PDZ) $\log_{10}$-EC_{50} (µg mL^{-1}) as response variable and alterations within *PbCYP51* as predictors. The box plots show PDZ $\log_{10}$-EC_{50} values (µg mL^{-1}) for samples within each node (n = number of isolates), *PbCYP51* alterations are shown on the tree. (B) Variable importance plot showing how model accuracy decreases as variables are omitted.

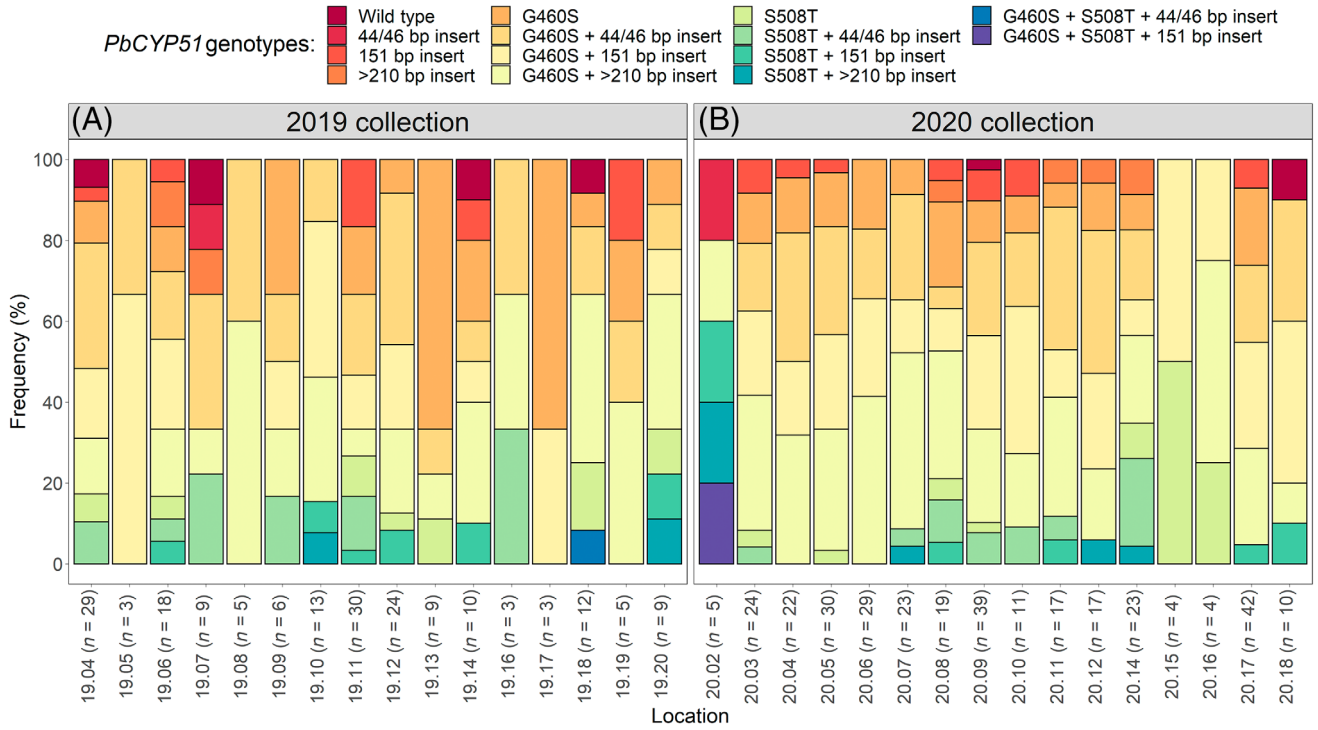

Figure 8. The frequency of inserts and substitutions at different locations in the extensive collections of *Pyrenopeziza brassicae* from commercial oilseed rape crops for 2019 (A) and 2020 (B). Fourteen genotypes for *PbCYP51* were identified for these collections, and the frequencies of these genotypes at the 16 locations included in each of the collections are presented as stacked bar charts. The number of isolates for each location (*n*) varied, from three (populations 19.05, 19.16 and 19.17) to 42 (population 20.17). See Supporting Information, Fig. S1 for locations of populations.

S508T + 44/46 bp insert combination, the greatest in the whole 2020 collection.

4 DISCUSSION

In this study, three collections of *P. brassicae* isolates facilitated an in-depth investigation of the sensitivity status of the Irish *P. brassicae* population to the azole fungicides TBZ and PDZ. Our results report for the first time the double substitution G460S/S508T in *P. brassicae* isolates. Furthermore, a wider range of regulatory insert sizes was observed compared to previous studies. These findings suggest that *P. brassicae* has a high evolutionary potential to develop fungicide insensitivity or fungicide resistance, which will make it more challenging in future to control using fungicides. In terms of sensitivity, the results reported show that a broad range of sensitivity to TBZ exists in the population studied, in agreement with King *et al.*,[33] who included a small collection of Irish *P. brassicae* isolates in their study. Additionally, we confirm a similarly broad range of sensitivity to PDZ exists in the Irish population. PDZ is a metabolite of prothioconazole, which is widely used for control of LLS throughout north-western Europe. This is the first report of sensitivity of any European *P. brassicae* populations to PDZ and can serve as a reference point for future monitoring studies.

Amongst the isolate collection from 2020, differences in sensitivity to TBZ observed depended on sampling location, suggesting that sensitivity differences exist within the wider Irish population. These differences were related to proportions of different *PbCYP51* genotypes (combination of *PbCYP51* substitution and/or promoter insert) present in the different crops sampled, possibly reflecting histories of fungicide usage. Although TBZ is not typically used for LLS control, it is used as a means of growth regulation in OSR[49,50] in late autumn and early spring, which coincides with the initiation of LLS epidemics; thus, *P. brassicae* was probably directly exposed to the fungicide. The azole metconazole has also been extensively used for growth regulation[51,52] and Carter *et al.* (2014) have previously demonstrated the cross-resistance between it and TBZ,[26] so exposure to this fungicide may have affected the sensitivity of the populations to TBZ. However, similar differences between locations were not observed for PDZ, which was unexpected, given the moderate degree of cross-sensitivity identified in the isolates.

The detection of positive cross-sensitivity between TBZ and PDZ identified in both collections screened *in vitro* (representative and 2020 collection) is an important finding. Although such cross-sensitivity would be expected since both fungicides target the same protein, differences have emerged between TBZ and PDZ in other economically important plant pathogens, including *Zymoseptoria tritici*.[53] Unlike *ZtCYP51*, where numerous non-synonymous substitutions are currently detectable in European populations, and multiple combinations were identified in the same isolate,[54] only two non-synonymous substitutions were identified in *PbCYP51* for the *P. brassicae* isolates sequenced in the present study. Both substitutions, G460S and S508T, have previously been identified by Carter *et al.*[26] and King *et al.*,[33] but these two substitutions have never been found in the same *P. brassicae* isolate in previous studies. Inserts in the upstream regulatory promoter region of *PbCYP51* were also identified by these authors, and in the present study, the diversity in the size with which these inserts are present was greater than previously reported. While Carter *et al.*[26] reported only three such promoter inserts in 2014 (46 bp, 151 bp and 233 bp) and King *et al.* identified three

additional insert sizes in 2021 (44 bp, 210 bp and 302 bp),[33] we report 11 different size inserts, of which five were detected for the first time (59 bp, 67 bp, 175 bp, 453 bp and 466 bp). Carter *et al.* (2014),[26] by using a combination of heterologous expression of altered *PbCYP51* in *Saccharomyces cerevisiae* and analysis of *in vitro* crosses of isolates carrying different *PbCYP51* promoter region inserts confirmed that both mechanisms (*PbCYP51* substitution and promoter inserts) confer reduced sensitivity to azole fungicides. As highlighted by Carter *et al.* (2014),[26] S508T is equivalent to the substitution S524T in *Z. tritici*, and Cools *et al.*[55] reported that S524T in *Z. tritici* affects all azole fungicides. Indeed, in *Z. tritici*, the differences in cross-sensitivity observed between the azole fungicides are probably due to specific substitutions at positions 136 and 381,[56] the equivalents of which have not yet been detected in *P. brassicae*. Unlike Carter *et al.*,[26] S508T was not identified through the conditional inference trees as a significant contributor to the observed reductions in sensitivity to either PDZ or TBZ in the isolates tested. This may reflect the relatively small number of isolates without either G460S or S508T in the collection or the possibility, as suggested by Carter *et al.* (2014), of an additional reduced sensitivity mechanism in the *P. brassicae* population that limits the possibility of detecting or demonstrating the effect this substitution may have on the sensitivity of the isolates carrying it. The possibility of additional mechanisms of reduced sensitivity in *P. brassicae* populations is also suggested by our results from the random forest analysis, which showed that a small percentage of the variance can be explained by the alterations identified within *PbCYP51*. Therefore, this requires further investigation.

The substitution G460S was the most predominant of the two substitutions found in *PbCYP51*, with >78% of the isolates tested with the substitution. This frequency is similar to those in the UK[13] and wider European collections (King *et al.*,).[33] Carter *et al.*[26] highlighted that the equivalent substitution in *C. albicans*, G464S, is implicated in reduced sensitivity to clinical azole fungicides by directly affecting the azole binding.[32] In our study, the presence of this substitution had a significant negative effect on the sensitivity to TBZ, but its impact on PDZ was less apparent, possibly suggesting that even though cross-sensitivity is present between the two fungicides, the impact of the substitution is greater towards TBZ. However, the presence of this substitution had a greater impact on the sensitivity to both fungicides compared to S508T, and this confirms the results of King *et al.*,[33] who found that isolates carrying G460S were less sensitive than isolates with S508T. Additionally, for both fungicides, the presence of an insert, and in particular the larger groups of inserts (151 bp and > 210 bp), further reduced sensitivity. Again, this is similar to what King *et al.* reported for the European collections of *P. brassicae*,[33] with the least sensitive isolates identified by the authors carrying G460S and a promoter insert, particularly one of the larger inserts (151 bp or > 210 bp). Unlike King *et al.*, a much larger diversity of inserts was detected in the present study, with a total of 11 different inserts identified. It should be noted that this diversity was identified only following sequencing of the regulatory region in a selection of isolates. It is likely that further diversity will be identified amongst the collections if they are sequenced.

Three *P. brassicae* isolates were identified with both the G460S and S508T substitutions. Although neither Carter *et al.* (2014)[26] nor King *et al.* (2021)[33] found isolates carrying both G460S and S508T substitutions, Carter *et al.*[26] did suggest their existence; using *Saccharomyces cerevisiae* YUG37::erg11 transformants expressing this variant, Carter *et al.* found that this combination had a significant impact on all azoles they tested. Our results also show that sensitivity to both PDZ and TBZ was decreased considerably when the two substitutions were present together. It can be expected that, as azole fungicides continue to be applied, strains with this combination of substitutions will be selected.

Our results confirmed that the diversity of Irish *P. brassicae* population in relation to azole fungicides is evolving and has an effect on the sensitivity tested *in vitro*, which highlights the importance of continually monitoring for reduced sensitivity to azole fungicides in *P. brassicae* populations. As we observed a greater genetic diversity compared with previous studies and accumulation of substitutions, together with the fact that azole fungicides continue to be widely used in OSR for disease control and canopy management, erosion of sensitivity will occur in the Irish *P. brassicae* populations. However, cultivar resistance to *P. brassicae* is still important and equally PDZ, which is widely used, continues to provide control including against a strain with reduced sensitivity. Nevertheless, we highlight the need to understand LLS epidemic development to ensure fungicide applications are made preventatively in field conditions, and as additional fungicides with different modes of action are increasingly used for LLS control, further monitoring of sensitivity changes amongst the Irish *P. brassicae* population to these fungicides is recommended.

ACKNOWLEDGEMENTS

This work was supported by Teagasc Agriculture and Food Authority of Ireland, under its Walsh Scholarships Scheme, grant number/project number 0154. YJH and BDLF were also funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC, M028348/1 and P00489X/1), the Innovate UK (102100 and 102641), AHDB Cereals & Oilseeds (RD-2140021105) and the UK Department for Environment, Food and Rural Affairs (Defra, CH0110). The authors are grateful to the team working on the OptiBC project in Teagasc for help with field sampling and to Dr Stephen Byrne for his input on the conditional inference trees and random forests. Open access funding provided by IReL.

CONFLICT OF INTEREST

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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