Research Article

Received: 26 March 2025 Revised: 3 July 2025 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.70263

Sensitivity of Irish *Pyrenopeziza brassicae* populations to methyl benzimidazole carbamate (MBC), quinone outside inhibitor (QoI) and succinate dehydrogenase inhibitor (SDHI) fungicides

Diana E Bucur,[a,b] Yong-Ju Huang,[b] Bruce DL Fitt[b] and Steven Kildea[a*]

Abstract

BACKGROUND: Light leaf spot, caused by *Pyrenopeziza brassicae*, is an economically damaging disease of winter oilseed rape in north-western Europe. Disease control relies upon the use of foliar fungicides, with the azoles the main class of fungicides being used. Changes in the sensitivity to azole fungicides have been reported for *Pyrenopeziza brassicae* populations across Europe. Therefore, there is a need to investigate the use of fungicides having alternative modes of action for control of this disease, although methyl benzimidazole carbamate (MBC) fungicides are no longer approved for use in the European Union (EU). Little information is available on the sensitivity of *Pyrenopeziza brassicae* to fungicides with alternative modes of action, with only a small number of Irish *Pyrenopeziza brassicae* isolates previously screened against such fungicides. This study investigated the sensitivity of three collections of Irish *Pyrenopeziza brassicae* isolates (representative collection, 2019 collection and 2020 collection) to MBC, quinone outside inhibitor (QoI) and succinate dehydrogenase inhibitor (SDHI) fungicides.

RESULTS: Different levels of sensitivity of *Pyrenopeziza brassicae* populations to the MBC, QoI and SDHI fungicides were detected. Three phenotypes of sensitivity (sensitive, moderately insensitive, insensitive) to MBC were identified, with the sensitive phenotype still predominant in Ireland. No differences in sensitivity to QoI and SDHI fungicides were found and no *cytb* mutations associated with reduced sensitivity to QoI fungicides were detected by sequencing.

CONCLUSION: The results suggest that, despite different levels of sensitivity to MBC fungicides, no sensitivity shifts to QoI or SDHI fungicides were identified in Irish *Pyrenopeziza brassicae* populations. However, continuous fungicide sensitivity monitoring and integrated management strategies using fungicides with different modes of action are necessary to sustain long-term effective control of *Pyrenopeziza brassicae*.

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

Keywords: disease control; fungicide sensitivity; light leaf spot; oilseed rape

1 INTRODUCTION

Light leaf spot (LLS), caused by the fungal pathogen *Pyrenopeziza brassicae* (anamorph *Cylindrosporium concentricum*), is one of the most damaging diseases of winter oilseed rape (OSR) (*Brassica napus* spp.) in northern Europe.[1–3] Severity of LLS epidemics has increased progressively in the last 15 years as the pathogen started to evolve and it has replaced phoma stem canker as the main disease causing yield loss in winter OSR in the United Kingdom (UK).[2,4] Moreover, a second, phylogenetically distinct lineage of *Pyrenopeziza brassicae* has been reported in North America.[5] LLS severity varies from one growing season to another and between different regions, and even from one OSR crop to another,[6] making *Pyrenopeziza brassicae* a difficult pathogen to control. This is related to a long symptomless period after initial infection under field conditions,[1] which can lead to poor timing of fungicide applications. Additionally, under certain

* *Correspondence to: S Kildea, Department of Crop Science, Teagasc Crops Environment and Land Use Programme, Oak Park Crop Research Centre, Carlow, R93 XE12, Ireland, E-mail: stephen.kildea@teagasc.ie*

a *Department of Crop Science, Teagasc Crops Environment and Land Use Programme, Carlow, Ireland*

b *Centre for Agriculture, Food and Environmental Management Research, School of Health, Medicine and Life Sciences, University of Hertfordshire, Hertfordshire, UK*

circumstances, fungicides may be ineffective even when used at the optimum time, because of reduced sensitivity of some *Pyrenopeziza brassicae* populations to certain fungicide groups.[7,8] The main chemical group used to control *Pyrenopeziza brassicae* is the demethylation inhibitor (azole – Fungicide Resistance Action Committee (FRAC) Group 3) fungicides, which are widely used.[4] Other classes of fungicides, including the quinone outside inhibitors (QoIs – FRAC Group 11) and succinate dehydrogenase inhibitors (SDHIs – FRAC Group 7), are used for control of other diseases in OSR, whilst the methyl benzimidazole carbamates (MBCs – FRAC Group 1) have previously been used, often in combination with an azole fungicide.[4,9]

The MBC fungicides were the first penetrant fungicides introduced in the 1960s and the first systemic compounds with a single-site mode of action used in agriculture.[10,11] They inhibit fungal activity by binding to β-tubulin, which is essential in the formation of microtubules that play an important role in eukaryotic cellular processes, such as chromosome segregation, cell division, generation and maintenance of cell shape, intracellular transport and cell motility.[12,13] Insensitivity to the MBC fungicides is widespread amongst numerous plant pathogens,[14] with alterations at the amino acid positions 167, 198, 200 and 240 in the β-tubulin identified in different fungal species, with differing effects on sensitivity.[11] Although no MBC fungicides are currently registered for use in the European Union (EU), they were previously widely used on OSR with reports of identified efficacy loss towards LLS.[7,15] As reported for *Pyrenopeziza brassicae* by Carter *et al.*[7] and subsequently by King *et al.*,[8] the *β-tubulin* mutation E198A confers considerable reductions in sensitivity to all MBC fungicides tested *in vitro*, suggesting this mutation is the most likely cause for the loss in efficacy. The mutation L240F is found less frequently and results in moderate reduction of sensitivity to the MBC fungicides, whilst E198G and F200Y mutations are rarely detected.[7] Interestingly, when studying the sensitivity of different European populations of *Pyrenopeziza brassicae* to MBC fungicides, King *et al.*[8] reported that the two Irish populations investigated contained the highest proportion of isolates sensitive to this class of fungicides, although MBC insensitive Irish isolates were also reported for the first time in their study.

The QoI fungicides inhibit fungal respiration by binding to the cytochrome bc1 at the quinone outside site.[10] They were introduced on the market in 1996.[16] Due to the specific nature of their mode of action, they are considered to be at high risk of insenstivity development.[17] Soon after their introduction, insensitivity was reported for a number of economically important plant pathogens, with the mutation G143A in the cytochrome *b* of the respective pathogens identified as the cause in most instances.[18] Although they are currently not registered for specific LLS control, they are often applied to OSR for control of other diseases (e.g., sclerotinia stem rot); therefore, *Pyrenopeziza brassicae* may be exposed to this group of fungicides. To date, insensitivity has not been reported amongst *Pyrenopeziza brassicae* populations.[8]

As with QoI fungicides, the SDHI fungicides act by disrupting pathogen respiration, although at a different site, the succinate dehydrogenase (SDH, complex II) enzyme.[10,19] This group includes two generations of fungicides[10]; the first generation were mostly used in the past as a seed treatment to control basidiomycete pathogens.[19–21] The second generation, collectively named SDHI fungicides[20,22] has a broader spectrum of control.[19,20] Amongst the European *Pyrenopeziza brassicae* collections tested by King *et al.*,[8] all were considered sensitive to the representative SDHI fungicide, penthiopyrad. Equally, although a number of SDHI fungicides are registered for use in OSR, they are primarily for control of other diseases on OSR rather than LLS. However, as with the QoI fungicides, *Pyrenopeziza brassicae* is likely to be exposed to the SDHI fungicides due to their applications to OSR against other pathogens.

Current information on the sensitivity of Irish *Pyrenopeziza brassicae* populations to MBC, QoI and SDHI fungicides is very limited, with Irish *Pyrenopeziza brassicae* isolates collected from only two fields included in the study of King *et al.*[8] As the importance of the LLS disease continues to increase, it is important to ensure the efficacy of key fungicides used for its control (e.g., azoles) is maintained. Part of such anti-insensitivity strategies is to mix the azole fungicides with fungicides with different modes of action, such as the QoI or SDHI fungicides. As both groups of fungicides are currently applied to OSR, to implement the anti-insensitivity strategies, there is a need to monitor the sensitivity of *Pyrenopeziza brassicae* populations to the QoI and SDHI fungicides. This study reports *in vitro* sensitivity screening of wider Irish *Pyrenopeziza brassicae* populations collected over two growing seasons to pyraclostrobin (QoI) and to penthiopyrad (SDHI). Additionally, we have also screened the populations for their sensitivity to an MBC fungicide to provide a more detailed extension to the findings of King *et al.*[8]

2 MATERIALS AND METHODS

2.1 Collections of *Pyrenopeziza brassicae* isolates

Three collections of *Pyrenopeziza brassicae* isolates obtained from winter OSR crops grown across Ireland during March in the 2018–2019 and 2019–2020 growing seasons were used in this study. One collection was established from OSR crops (cv. Phoenix) located in three main regions (Carlow, Cork and Louth) where OSR is grown most intensively in Ireland (referred to as 'representative collection', with 107 *Pyrenopeziza brassicae* isolates). Two additional collections of *Pyrenopeziza brassicae* isolates were obtained from commercial OSR crops throughout Ireland, one sampled during the 2018–2019 growing season (referred to as '2019 collection', with 192 *Pyrenopeziza brassicae* isolates), and the other one sampled during the 2019–2020 growing season (referred to as '2020 collection', with 321 *Pyrenopeziza brassicae* isolates). Details of the three collections and their isolations were previously described in Bucur *et al.*[3]

2.2 Fungicide sensitivity assay *in vitro*

2.2.1 *Microtitre plate assay*

Sensitivity of the Irish *Pyrenopeziza brassicae* isolates from the representative collection and 2020 collection to the MBC (representative collection only), QoI and SDHI fungicides was determined using a microtitre plate assay as described by Bucur *et al.*[3] Briefly, *Pyrenopeziza brassicae* isolates were grown from 30% glycerol stocks stored at −80 °C on malt extract agar (MEA) 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, PA, USA) and were incubated for 21 days at 18 °C in dark conditions. Once grown, 1 mL of sterile distilled water was added onto each plate and the colonies were gently scraped using a sterile T-shaped spreader before the spore and mycelial suspension was pipetted into a 2 mL Eppendorf tube on ice. After 10 min of incubation on ice, the upper parts of the suspensions were transferred into new tubes and spores were quantified using KOVA® Glasstic® Slides (Kova International Inc., Garden Grove, CA, USA) and subsequently adjusted to 25 000

spores mL^{-1}. Flat bottomed microtitre plates (Sarsted AG & Co., Nümbrecht, Germany) were used in the experiment and were filled with 200 µL fungicide and conidial spore suspension mixture, of which 100 µL of 2 × potato dextrose broth (PDB) was amended with a three-fold dilution series of carbendazim (MBC), pyraclostrobin (QoI) or penthiopyrad (SDHI) (Sigma-Aldrich, St Louis, MO, USA) from stock solutions in dimethyl sulphate (DMSO) and 100 µL conidial suspension (25 000 spores mL^{-1}) in sterile distilled water. This way, the 96-well plates were filled with final concentrations of fungicides, ranging from 30 µg mL^{-1} to 0.0005 µg mL^{-1} for pyraclostrobin and from 90 µg mL^{-1} to 0.0015 µg mL^{-1} for penthiopyrad and carbendazim. Unlike King *et al.*,[8] who excluded the highest pyraclostrobin (30 µg mL^{-1}) and carbendazim (90 µg mL^{-1}) concentrations as insolubility affected optical-density readings, we observed no turbidity at any concentration; therefore, all concentrations were retained in our half-maximal effective concentration (or the fungicide concentration that inhibits 50% growth of *Pyrenopeziza brassicae*) (EC_{50}) calculations. Isolates were tested in batches of 30 isolates, with seven isolates and a negative control tested in each 96-well plate. Five reference isolates were included for each batch to confirm reproducibility (Table 1). Two technical replicates were prepared for all the isolates tested. Plates were sealed with parafilm, wrapped in cling film to prevent evaporation and incubated in the dark at 18 °C.

2.2.2 Data analysis

Fungal growth was assessed after 12 days of incubation as a measure of light absorbance at 630 nm using a Synergy-HT plate reader and Gen5™ microplate software (Bio-Tek Instruments, Inc., Winooski, VT, USA) in well-scanning mode. For each isolate–fungicide combination, the absorbance of the negative control was subtracted. The EC_{50} value was calculated for each isolate using a dose–response relationship, using the R package *drc*.[23] The data obtained were fitted using the 4-parameter log-logistic model that uses EC_{50} as a parameter (LL.4). For each isolate, the EC_{50} values were extracted from the model and the average EC_{50} between the two technical replicates was calculated. Furthermore, the isolates tested were grouped by population and their EC_{50} values were plotted against the EC_{50} values obtained for the reference isolates. The non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) test was used to identify if significant differences between populations exist using the kruska.test function from the R package *rstatix*.[24] Where significant differences were detected, subsequent *post hoc* Dunn's multiple comparison tests were done to identify where the differences are present, using kwAllPairsDunnTest function from *PMCMRplus* package, implemented with the 'holm' method.[25]

To further assess the sensitivity of the *Pyrenopeziza brassicae* isolates to carbendazim (MBC fungicide), a cluster analysis was done on the $logEC_{50}$ values obtained for each isolate. Initially to ensure robustness, potential outliers were identified and removed using the interquartile range (IQR) method. Model-based clustering was done using the Mclust function from *mclust* package in R. This package evaluates various Gaussian mixture models to determine the best fit for the data, balancing model complexity with fit. The optimal number of distinct sensitivity groups was determined by evaluating the Bayesian information criterion (BIC). Additionally, a silhouette analysis was done using the *cluster*[26] and *factoextra*[27] packages to validate the cohesion and separation of the clusters identified, providing further confidence in the clustering results. To enhance the visualisation of cluster distributions, a cumulative frequency plot was generated using *ggplot2* package.[28] All statistical analyses and visualisations were done using R (R version 4.3.2 (2023-10-31 ucrt)) and Rstudio version 1.2.5001 and 2023.12.0+369 'Ocean Storm', ensuring reproducibility and methodological consistency.

2.3 Molecular screening for the presence of mutations in *Pyrenopeziza brassicae* associated with changes in sensitivity to MBC and QoI fungicides

2.3.1 DNA extraction

Fungal mycelium grown for 3 weeks on MEA plates layered with cellulose discs was freeze-dried for 48 h, ground using a tissue lyser and the DNA was extracted from all isolates included in the three collections using the Promega Wizard Genomic DNA Purification Kit (Madison, WI, USA), following the manufacturer's guidance. The DNA obtained was quantified by absorbance using a

Table 1. Information about *Pyrenopeziza brassicae* reference isolates used in fungicide sensitivity screening.

						Carbendazim		Tebuconazole		Prothioconazole	
Isolate	Year	Location	Host crop	*PbCYP51* genotype	*β-Tubulin* genotype	EC_{50}[†] (µg mL^{-1})	RF[‡]	EC_{50} (µg mL^{-1})	RF	EC_{50} (µg mL^{-1})	RF
PbFr002	1995	Le Rheu, France	*Brassica napus*	Wild-type	Wild-type	—	—	0.04	—	0.14	—
E3A	2007	Hertfordshire, UK	*Brassica napus*	Wild-type	Wild-type	—	—	0.03	1	0.34	4
WC4	2007	Hertfordshire, UK	*Brassica oleracea*	S508T	L240F	0.2	14	0.49	21	3.06	38
8CAB	2011	East Lothian, UK	*Brassica oleracea*	G460S	L240F	0.0	11	0.59	25	3	37
I3	2011	East Lothian, UK	*Brassica oleracea*	S508T/151 bp	E198A	>1.6	>398	1.19	50	5.54	69

Note: *PbCYP51* and *β-tubulin* genotypes include mutations with effect on the sensitivity to the class of fungicides that targets the respective gene.
[†] Half-maximal effective concentration (EC_{50}) values 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.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

ultraviolet (UV)-visible NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), by fluorescence using the Qubit® fluorometer from Invitrogen (Carlsbad, CA, USA) and by agarose gel electrophoresis.

2.3.2 *Detection of alterations within the β-*tubulin *gene related to changes in sensitivity to MBC fungicides*

A 865 bp fragment of the *β-tubulin* gene including codons 198 and 240 was amplified using *Taq* DNA polymerase with ThermoPol Buffer (New England BioLabs Inc., Ipswich, MA, USA) and the primer pair PZtubF1/PZtubR1, in a reaction with an annealing temperature of 52 °C and a final extension step of 5 min. The 865 bp *β-tubulin* amplicon from 59 *Pyrenopeziza brassicae* isolates was sent for Sanger sequencing (LGC Genomics, Berlin, Germany) to investigate the presence of mutations. Additionally, for the entire collection of *Pyrenopeziza brassicae* isolates, the presence of E198A and L240F mutations within the *β-tubulin* gene associated with insensitivity to the MBC fungicides was determined using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) as detailed by Carter *et al.*[7] Briefly, approximately 60 ng of the PCR product was purified using Wizard® SV Gel and PCR Clean-Up System from Promega and digested with 1 unit of *BsmAI* enzyme (GTCTCN) (New England BioLabs Inc.) and 1 × NE buffer 4. Following the digestion, the samples were incubated for 2 h at 55 °C. The restriction fragments that resulted were separated in a 3% (*w/v*) electrophoresis gel and visualised under UV-light using an ENDURO™ GDS Gel Documentation System for Electrophoresis (Labnet International, Inc., Edison, NJ, USA).

2.3.3 *Detection of alterations within the mitochondrial* cytb *gene related to changes in sensitivity to QoI fungicides*

To determine the molecular mechanisms involved in changes in sensitivity to QoI fungicides, the *cytb* gene targeted by this class of fungicides was amplified and the presence of mutations related to insensitivity previously identified was investigated. The primers cytb-fw (5′- GAGCACCTAGAACATTGGTATGA-3′) and cytb-rev (5′-ACCTGACCCTGCACTATCATG-3′) were designed to amplify a 1390 bp fragment of the mitochondrial *cytb* gene of *Pyrenopeziza brassicae*, the gene targeted by the QoI fungicides, based on a reference sequence generated by King *et al.* (GenBank accession MZ334482).[8] The *cytb* fragment was amplified using Q5 polymerase and 5 × Buffer Q5 (New England BioLabs Inc.) in a final reaction volume of 20 μL that contained 1 × Buffer Q5, 200 μM dNTPs, 0.5 μM of each primer, 0.02 U μL^{-1} Q5 HF polymerase and 1 × of Q5 High GC Enhancer. The reaction conditions were an initial denaturation step of 30 s at 98 °C, followed by 35 cycles at 98 °C for 10 s denaturation, 30 s of annealing at 64 °C and 45 s of extension at 72 °C, with a final extension step of 2 min at 72 °C. The PCR product was visualised on a 1% agarose gel under UV-light using an ENDURO™ GDS Gel Documentation System for Electrophoresis (Labnet International, Inc.) and 96 isolates that presented a clean amplification band, of which 54 isolates were from the 2019 collection and the remaining 42 isolates from the 2020 collection, were purified and sent to LGC Genomics for Sanger sequencing using the reverse primer cytb-rev.

2.3.4 *Molecular data analysis*

After the digestion of the purified 865 bp fragment of the *β-tubulin* gene with *BsmAI*, three types of band patterns were obtained, and the isolates were grouped as sensitive, moderately insensitive or insensitive to MBC fungicides, based on the size and number of bands obtained.[7] These were related to three phenotypes of sensitivity described by Carter *et al.*[7] and obtained based on the EC_{50} values, as follows: isolates with EC_{50} values of less than 1 μg mL^{-1} were classified as sensitive to carbendazim, isolates with EC_{50} values of between 1 and 40 μg mL^{-1} were classified as moderately insensitive to carbendazim, while isolates with EC_{50} values greater than 40 μg mL^{-1} were considered insensitive to carbendazim. The isolates presenting five bands (461, 200, 104, 69 and 31 bp) were considered sensitive with no mutations present (EC_{50} < 1 μg mL^{-1}). The isolates with only four bands were considered as insensitive (EC_{50} > 40 μg mL^{-1}) as the presence of mutation E198A led to the loss of the 104 bp band (565, 200, 69 and 31 bp), or moderately insensitive (EC_{50} 1–40 μg mL^{-1}), the presence of mutation L240F leading to the loss of the 69 bp band but not of the 100/104 bp band (461, 200, 104 and 100 bp). Based on these differences, the frequencies of the three groups were calculated for each collection.

For the mitochondrial gene *cytb*, the sequences obtained were aligned and subsequently translated using MEGA X software[29] and SnapGene software, version 6.0.2[30] and the presence of the mutation G143A related with reduced sensitivity to QoI fungicides and potential introns within the coding region of the gene were identified. Similarly, the sequences obtained for the *β-tubulin* gene were aligned, translated and used to investigate the presence of additional potential mutations.

3 RESULTS

3.1 Fungicide sensitivity

3.1.1 *Carbendazim (MBC fungicide)*

The sensitivity (EC_{50} value) of the five reference isolates to carbendazim ranged between 0.06 μg mL^{-1} (PbFr002) and 10 μg mL^{-1} (I3). For comparison, among the 107 *Pyrenopeziza brassicae* isolates tested, the EC_{50} values obtained ranged from 0.00013 μg mL^{-1} (an isolate from Cork) to 86.8 μg mL^{-1} (an isolate from Carlow) (Fig. 1). The resistance factors (RFs, ratio between the least sensitive isolate for each population and the wild-type reference isolate PbFr002) obtained for the three locations were 3737 for Louth, 411 for Carlow and 161 for Cork, respectively. No differences in sensitivity were detected between the three locations screened ($P = 0.53$, df = 7).

Model-based clustering of the $logEC_{50}$ values identified three distinct sensitivity groups among the *Pyrenopeziza brassicae* isolates exposed to carbendazim, namely clusters I to III (Fig. 2(A)). The optimal clustering model, determined by the highest BIC value, successfully differentiated the isolates based on their sensitivity levels (Supporting Information Fig. S1). Sensitivity group I comprised the most sensitive isolates with mean $logEC_{50}$ values ranging from −3.88 to −2.62 (EC_{50} < 0.002 μg mL^{-1}), accounting for 22.4% of the total isolates. Group II ranged from −2.28 to −0.757 (EC_{50} 0.005–0.175 μg mL^{-1}), constituting 51.4% of the isolates, while group III ranged from −0.55 to 1.85 (EC_{50} 0.28–65.8 μg mL^{-1}) accounting for 26.2% of the isolates. The RFs obtained for each cluster, determined from back-transformed adjusted mean EC_{50} values of carbendazim-sensitive isolates using group I as the reference (RF = 1) were: group I RF = 1, group II RF ≈ 66.7, group III RF ≈ 4730. Silhouette analysis corroborated the robustness of the clustering, with average silhouette widths exceeding 0.71, indicating well-defined and cohesive clusters (Fig. S2).

Population-wise distribution analysis revealed distinct patterns across the regions studied: in Carlow population ($n = 27$), group

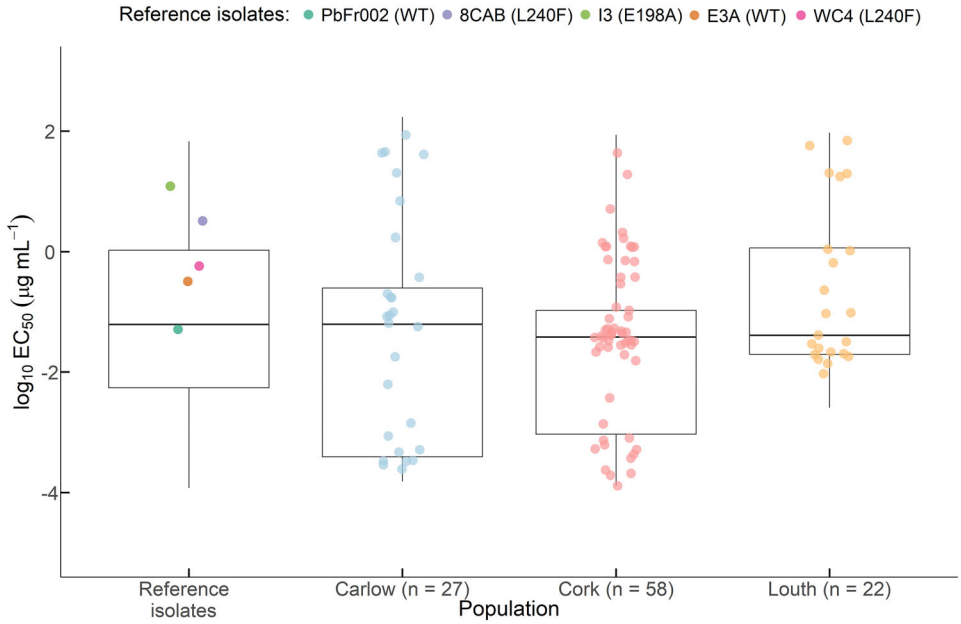

Figure 1. Sensitivity to carbendazim (MBC fungicide) of the representative collection of Irish *Pyrenopeziza brassicae* isolates, as EC_{50} values. The isolates are ranked based on their increasing EC_{50} values as cumulative frequency (%). The populations sampled are shown on the *x*-axis with the number of isolates (*n*) for each population. Each data point represents the mean of two technical replicates. The values for the five reference isolates (PbFr002, E3A – wild-type sensitive isolates, WC4 – moderately insensitive L240F mutation, 8CAB – moderately insensitive L240F mutation 240F, I3 – insensitive E198A mutation), calculated as average EC_{50} values obtained for each batch tested.

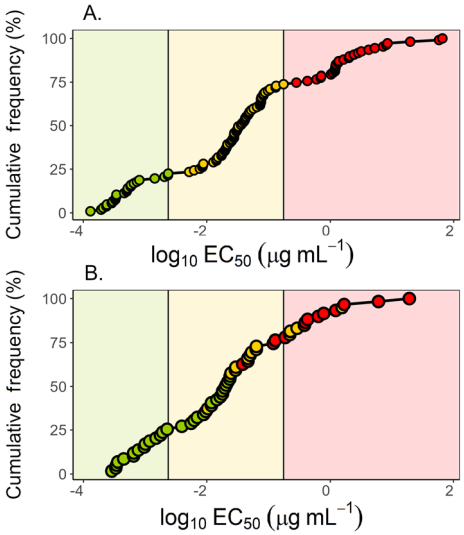

Figure 2. Cumulative frequencies (%) of isolates with different sensitivity levels to carbendazim (MBC fungicide) in the Irish representative collection of *Pyrenopeziza brassicae* isolates. (A) Cluster analysis of the $logEC_{50}$ values obtained from *in vitro* sensitivity screening. The isolates are grouped based on their $logEC_{50}$ values as group I (EC_{50} < 0.0024 µg mL^{-1}) – green; group II (EC_{50} 0.005–0.18 µg mL^{-1}) – yellow; group III (EC_{50} > 0.25 µg mL^{-1}) – red. (B) Phenotypes obtained from the enzymatic digestion and sequencing of the *β-tubulin* gene. The isolates are grouped based on the mutations present within their *β-tubulin* gene: sensitive – wild-type isolates (green), moderately insensitive – isolates with L240F (yellow), insensitive – isolates with E198A mutation (red). The sensitivity rating for each group is defined by the clustering analysis.

I accounted for 37% of isolates, group II for 37%, group III for 25.9%. The Louth population (*n* = 58) showed a greater proportion of isolates in group II (63.6%) and group III (36.4%), with no isolates assigned to group I. Finally, the Cork population (*n* = 22) predominantly consisted of isolates from group II (53.4%), while group III accounted for 22.4% of the isolates and group I accounted for 24.1% of the fungal isolates.

3.1.2 *Pyraclostrobin (QoI fungicide)*

The sensitivity (EC_{50} value) of the five reference isolates to pyraclostrobin ranged between 0.0011 µg mL^{-1} (PbFr002) and 0.01 µg mL^{-1} (I3), while the sensitivity of the Irish isolates tested (*n* = 421) as EC_{50} values ranged from 0.00002 µg mL^{-1} (an isolate from Louth) to 0.26 µg mL^{-1} (an isolate from Carlow) for the representative collection, and from 0.0002 µg mL^{-1} (an isolate from population 6) to 0.43 µg mL^{-1} (an isolate from population 12) (Fig. 3). The RF (ratio between least sensitive and wild-type reference isolate) obtained were 151 for the representative collection and 255 for the 2020 collection. No significant differences were observed between different locations in either of the collections analysed (representative collection: $P = 0.57$, df = 7; 2020 collection: $P = 0.07$, df = 20), based on the Kruskal–Wallis non-parametric test.

3.1.3 *Penthiopyrad (SDHI fungicide)*

The sensitivity (EC_{50} value) of the five reference *Pyrenopeziza brassicae* isolates to penthiopyrad ranged from 0.01 µg mL^{-1} (PbFr002) to 0.082 µg mL^{-1} (I3), while the sensitivity of the two Irish collections of *Pyrenopeziza brassicae* ranged between 0.0002 and 0.68 µg mL^{-1}, with RFs of 31 for the representative collection and 84 for 2020 collection (Fig. 4). No significant differences were observed either for the representative collection ($P = 0.72$, df = 7) or the 2020 collection ($P = 0.2$, df = 21), based on the non-parametric Kruskal–Wallis test.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

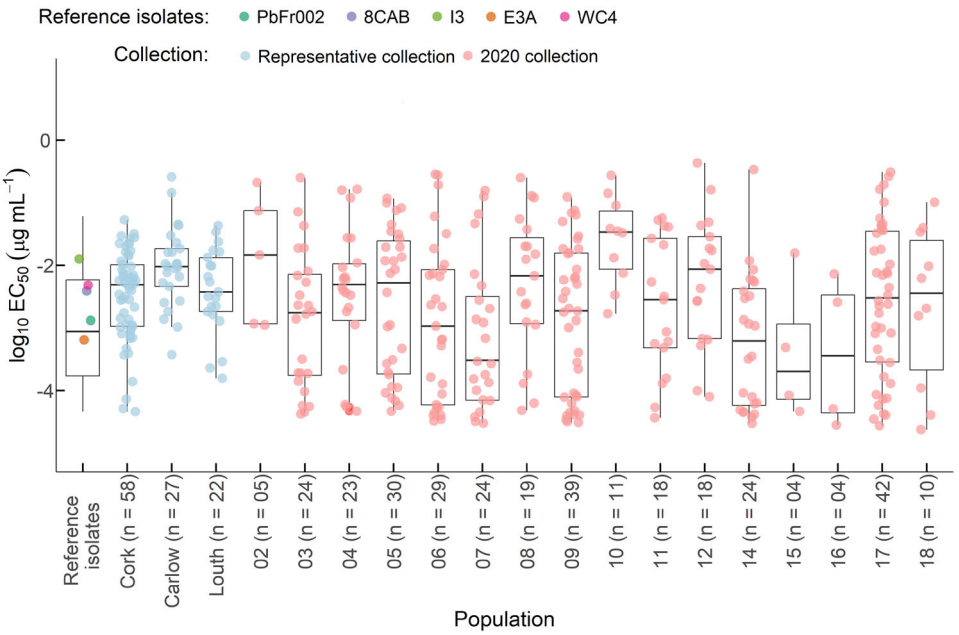

Figure 3. Sensitivity to pyraclostrobin (QoI fungicide) of the representative collection and 2020 collection of Irish *Pyrenopeziza brassicae* isolates, as EC_{50} values. The isolates are ranked based on their increasing EC_{50} values as cumulative frequency (%). The number of isolates (*n*) for each population are shown in brackets. Each data point represents the mean of two technical replicates. The values for the five reference isolates (PbFr002, E3A, WC4, 8CAB, I3 – no previous information on sensitivity to QoI fungicides for the reference isolates available), calculated as average EC_{50} values obtained for each batch tested.

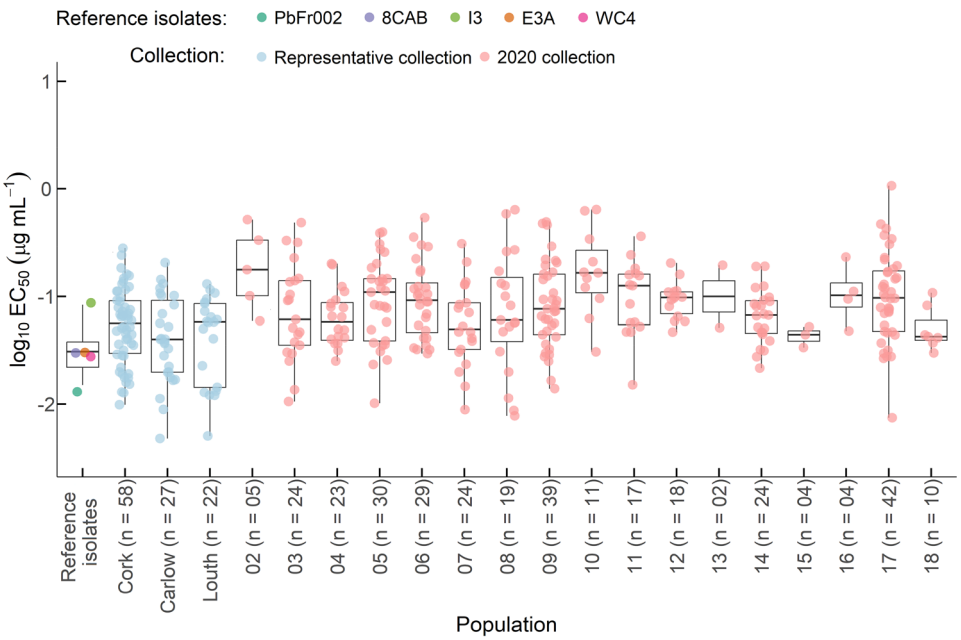

Figure 4. Sensitivity to penthiopyrad (SDHI fungicide) of the representative collection and 2020 collection of Irish *Pyrenopeziza brassicae* isolates, as EC_{50} values (log_{10}-transformed). The isolates are ranked based on their increasing EC_{50} values. The number of isolates (*n*) for each population are shown in brackets. Each data point represents the mean of two technical replicates. The values for the five reference isolates (PbFr002, E3A, WC4, 8CAB, I3 – no previous information on sensitivity to SDHI fungicides for the reference isolates available), calculated as average EC_{50} values obtained for each batch tested.

3.2 Molecular screening for the presence of mutations in *Pyrenopeziza brassicae* associated with changes in sensitivity to MBC and QoI fungicides

*3.2.1 Detection of alterations within the β-*tubulin *gene related to changes in sensitivity to MBC fungicides*

Following the sequencing of the 865 bp *β-tubulin* fragment from 59 *Pyrenopeziza brassicae* isolates, the presence of E198A and L240F mutations was confirmed, with no additional mutations being detected. E198A was detected in 23.7% of the isolates sequenced, while L240F was present in 25.4% of the fungal isolates sequenced. More than half (50.8%) of the sequenced isolates had no alterations present (Fig. 2(B)).

The band patterns obtained for the 865 bp *β-tubulin* fragments after digestion with *BsmAI* were similar to those described by

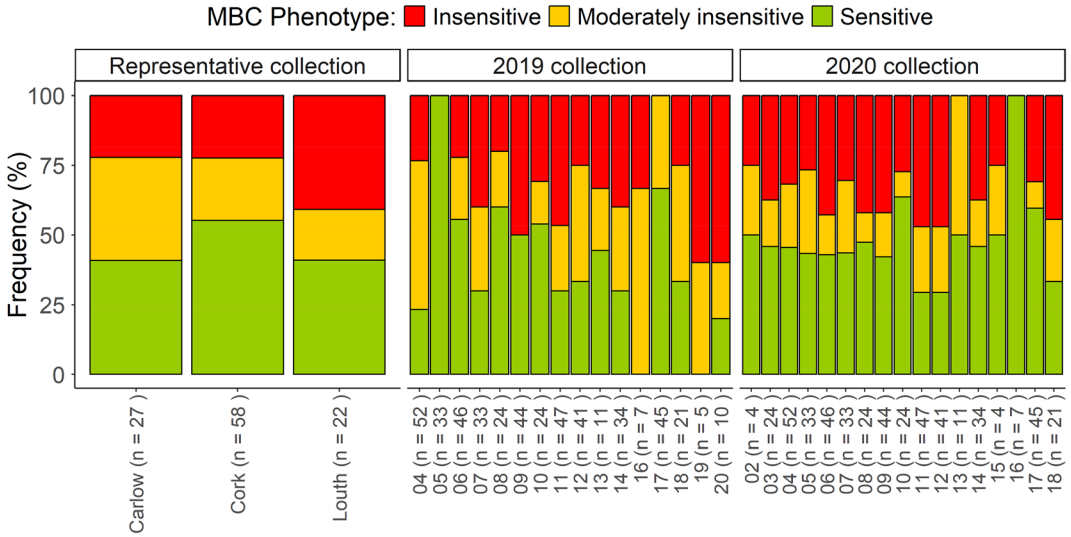

Figure 5. Frequency (%) of *Pyrenopeziza brassicae* isolates with different carbendazim (MBC fungicide) sensitivity phenotypes in the Irish representative collection, 2019 collection and 2020 collection. Phenotypes were assigned based on *β-tubulin* genotype and confirmed by EC_{50} clustering: insensitive – isolates with E198A mutation (red), moderately insensitive – isolates with L240F mutation (yellow), sensitive – wild-type isolates (green).

Carter *et al.*[7] and correlated with the three phenotypes of sensitivity obtained based on the EC_{50} values (Figs 2 and S3) in proportion of 60%, which suggests the presence of additional mechanisms of insensitivity. *Pyrenopeziza brassicae* isolates carrying the substitutions E198A or L240F were present at all locations in the representative collection and almost all locations in the 2019 and 2020 collections, with the exception of populations 16, 17 and 19 from the 2019 collection and populations 13 and 16 from the 2020 collection (Fig. 5). For the populations where one of the phenotypes was absent, usually it was the insensitive phenotype. This was different for populations 16 and 19 in the 2019 collection, where all the *Pyrenopeziza brassicae* isolates screened were either insensitive or moderately insensitive.

In all collections, wild-type strains dominated, with frequencies ranging from 38% (2019 collection) to 49% (representative collection). Based on the frequencies of the two mutations known to confer different levels of sensitivity to MBC fungicides, the three collections investigated were not significantly different ($P = 0.95$) (Fig. 5).

3.2.2 *Detection of alterations within the mitochondrial* cytb *gene related to changes in sensitivity to QoI fungicides*

A 1390 bp fragment of the *cytb* gene was successfully obtained and sequenced for 96 *Pyrenopeziza brassicae* isolates, of which 54 isolates were from the 2019 collection and the remaining 42 isolates from the 2020 collection. However, the amplification failed for 27% of the fungal isolates, suggesting that additional alterations are present in the sequence, interfering with the attachment of forward primer from the amino acid positions 107–114 or reverse primer attaching on the amino acid sequence in position 556–563, or both. No alterations present in other pathogens and known to cause changes in sensitivity to QoI fungicides[31] were identified in any of the isolates successfully sequenced. As with King *et al.*,[8] an intron of 1072 bp was present for all the sequences, 78 bp downstream from the G143 codon, although the role of this intron insensitivity was not confirmed.

4 DISCUSSION

Although we detected insensitivity to MBC fungicides in the collections, the overall frequencies of insensitivity were small, similar to those found in the Irish populations tested by King *et al.*,[8] in which the Irish population was more sensitive when compared to the other European populations tested. In our study, approximately 27% of isolates were carbendazim-insensitive, a frequency close to the approximately 24% reported in Poland,[32] suggesting that low-level MBC resistance is now detectable in several European regions, even though Ireland remains more sensitive than many countries surveyed by King *et al.*[8] The model-based clustering of the $logEC_{50}$ values showed the presence of three distinct sensitivity groups (sensitive, moderately insensitive, insensitive) among the *Pyrenopeziza brassicae* isolates in their sensitivity to carbendazim, supporting the results of Carter *et al.*,[7] which categorised insensitive isolates into two distinct groups: moderately insensitive (EC_{50} values ranging 1–40 mL^{-1} or $logEC_{50}$ of 0 to 0.5) and insensitive ($EC_{50} > 40$ µg mL^{-1} or $logEC_{50} > 1.6$). The molecular screening of the *β-tubulin* gene using the PCR-RFLP assay described by Carter *et al.*[7] confirmed these categories observed for the *in vitro* sensitivity screening, with no mutations in the sensitive isolates and mutations E198A or L240F in those isolates deemed insensitive or moderately insensitive, respectively.

The relative sensitivity to MBC fungicides among Irish collections tested here and those non-Irish collections of King *et al.*[8] may reflect differences in cropping and fungicides usage histories between the countries. As Lucas *et al.*[33] have suggested for eyespot (*Oculimacula yallundae*), the presence of insensitive isolates in high proportions in crops could be a result of a single discriminatory concentration of fungicide used that led to selection. In Ireland, the intensification of OSR production and fungicide usage started relatively recently, with the area cultivated increasing from 1792 ha in 2004 to 17 282 ha in 2014[34]; this probably contributed to the lower historical selection for MBC insensitivity. In contrast, in the UK the MBC fungicides were used extensively up until the early 1990s when a subsequent loss in efficacy led to reductions in usage.[7,15,35] Since the application of MBC fungicides has not

been permitted within the EU or the UK since 2014 for carbendazim and 2002 for benomyl,[36,37] the selection for MBC insensitivity has probably decreased, potentially leading to a reversion in sensitivity in populations where insensitive strains may have been selected previously.

Fitness penalties associated with MBC insensitivity might explain the preserved sensitivity in the Irish isolates. Another plausible explanation is that as the expansion of OSR in Ireland has been relatively recent, the exposure of Irish *Pyrenopeziza brassicae* populations to MBCs has been limited, thereby reducing selection for insensitivity. In the absence of MBC fungicides, sensitive strains, which in several other plant pathogens are often more fit than insensitive strains, may outcompete insensitive strains.[33] This may potentially explain the greater frequencies of sensitive strains observed in Ireland compared to regions with a longer history of MBC fungicide use on OSR.

However, despite the general sensitivity to carbendazim (MBC fungicide), significant variability within the sensitive group of *Pyrenopeziza brassicae* isolates was observed. This variation could be influenced by several factors, notably additional genetic mutations. For instance, mutations at the *β-tubulin* codon position E198 not covered by the PCR-RFLP assay used, such as E198K, and E198G, are commonly reported in other studies and are known to confer varying levels of insensitivity to MBC fungicides. Carter *et al.*[7] and a review by Hawkins and Fraaije[11] also noted other *β-tubulin* substitutions including H6Y, F167Y, E198Q/K/G and F200Y, that have been reported to confer variations in MBC insensitivity levels. However, sequencing of an 865 bp *β-tubulin* fragment from 59 isolates confirmed only the presence of E198A and L240F mutations, with 23.73% of the isolates carrying E198A and 25.42% containing L240F. No additional mutations were detected, and more than 50% of the sequenced isolates type had no alterations at the key amino acids known to confer insensitivity. These sequencing results corroborate the PCR-RFLP findings and highlight the predominance of these two mutations in conferring insensitivity within the populations studied. However, unlike in the study of Carter *et al.*,[7] the definition and relationship between each specific mutation and the sensitivity cluster is less clear. Whilst the isolates carrying E198A are all within cluster III (insensitive), of the 30 isolates distributed in cluster II (moderately insensitive), only 11 carried the L240F mutation. Therefore, they cannot fully explain the variation in sensitivity to this class of fungicide, with additional potential insensitivity mechanisms contributing to the reductions in sensitivity observed. This observed variance highlights the limitations of molecular data, emphasising the need to combine phenotypic and molecular data in assessing the sensitivity level of *Pyrenopeziza brassicae* to this class of fungicides. Notably, the recently described North American lineage 2 of *Pyrenopeziza brassicae* is fully sensitive to MBC fungicides,[5] reinforcing the importance of regional monitoring.

Compared to the MBC fungicides, for QoI fungicides (pyraclostrobin), lower RFs were observed amongst the Irish populations. None of the isolates for which the *cytb* was partially sequenced had the mutations F129L, G137R or G143A, which have previously been identified as conferring insensitivity to the QoI fungicides in several plant pathogens.[16,38] The continued absence of G143A may reflect a severe fitness cost: Deising *et al.* suggest that in the related species *Rhynchosporium commune*, codon 143 lies at an exon–intron boundary, and the G143A change abolishes proper splicing, producing a non-functional cytochrome-*b* protein and rendering mutants non-viable.[39] However, Phelan *et al.* confirmed this hypothesis, confirming the presence of G143A in a small proportion (2–18%) of *R. commune* isolates from 2013 to 2014 they tested.[40] Nonetheless, an intron at the border of G143A is indeed present in some species, such as certain *Puccinia* and *Phyrenophora* species.[31,38] As with King *et al.*,[8] a 1072 bp intron was present at 78 bp downstream from the G143 codon. Unlike the self-splicing intron located immediately after the codon G143 in other species, this intron is unlikely to prevent the emergence of the G143A mutation in *Pyrenopeziza brassicae*,[41] yet any G143A mutants that did arise might still suffer respiratory fitness penalties and be selected against in the field. As the *cytb* amplification was not possible for some of the Irish isolates tested, it suggests some variability in the regions of the primers used for amplification. As there was no association between those that failed amplification and sensitivity, it is unlikely that this variability affects QoI sensitivity. The small differences in sensitivity that were observed may result from differences in alternative oxidase (AOX) activity amongst the isolates. AOX allows the fungus to bypass the blocked electron transport chain, maintaining energy production even when the primary target of QoI fungicides, cytochrome b, is inhibited.[42,43] Therefore, isolates with higher AOX activity would show reduced sensitivity to QoIs since they were able to grow despite use of the fungicides at commercial rates, which potentially could lead to reductions in the overall effectiveness of QoI fungicides.[44] To date, this mechanism only contributes to QoI insensitivity in a limited number of plant pathogens, most notably *Plasmopara viticola*, the fungal pathogen causing grapevine downy mildew.[45,46] As the differences in sensitivity observed in this study were small, it is unlikely that they would influence the efficacy of QoI fungicides when applied at commercial rates.

Based on the low resistance factors observed towards penthiopyrad (SDHI fungicide), our results suggest that the Irish *Pyrenopeziza brassicae* population is still fully sensitive to the SDHI fungicides. The levels of sensitivity observed are consistent with those of King *et al.*,[8] who reported that all eight European populations of *Pyrenopeziza brassicae*, including two from Ireland, were broadly similar in terms of penthiopyrad sensitivity. Due to their excellent efficiency in controlling LLS in vegetable brassicas,[8,47] the use of the SDHI fungicides in OSR for control of LLS is expected to increase with time. As with the MBC and QoI fungicides, the SDHI fungicides are single site fungicides, and experiences with comparable pathogens, such as the wheat pathogen *Zymoseptoria tritici*, suggest that a wide number of potential mutations could arise across the succinate dehydrogenase subunits B, C and D, with different effects on sensitivity to the different SDHI fungicides.[22,48] For this reason, future sensitivity monitoring studies should include additional SDHI fungicides.

Overall, the results of this study suggest that majority of the *Pyrenopeziza brassicae* Irish population remains sensitive to all three classes of fungicides tested, with all isolates sensitive to the QoI and SDHI fungicides. As LLS is amongst the most economically destructive diseases of OSR in western Europe, it is encouraging that no indications of insensitivity to either QoI or SDHI fungicides have been detected in this study. Even though fungicides belonging to these two groups are not widely used for LLS control, they are used extensively for control of sclerotina stem rot during flowering in OSR; thus, it can be expected that *Pyrenopeziza brassicae* has been exposed to both groups of fungicides. Field observations in Ireland are consistent with our laboratory findings: QoI- and SDHI-based programmes continue to deliver reliable control of LLS and other foliar diseases in commercial OSR crops, and no loss of efficacy has yet been reported. However, precautions are

needed to manage potential insensitivity in pathogens which may not be the intended target of the fungicide applications. Such precautions may include the mixing of fungicides with different modes of action. With a broad spectrum of activity, the azole fungicides are undoubtedly the ideal fungicide group to mix with either the QoI or SDHI fungicides. As the Irish *Pyrenopeziza brassicae* population has already shown some reductions in sensitivity to the azole fungicides, caution and careful management of doses of each fungicide partner is required to ensure that both sufficient control and insensitivity management is achieved.[49] Modelling by Evans *et al.*[49] predicted that future UK climates could lessen LLS pressure if current fungicide efficacy is maintained through resistance management; our baseline sensitivity data suggest current fungicides should indeed remain effective under those scenarios.

Consequently, developing integrated pest management (IPM) strategies that combine chemical controls with cultural practices such as crop rotation and resistant cultivars should provide a sustainable approach to managing fungicide insensitivity. However, the broader ecological and economic impacts of these findings must be considered. Fungicide insensitivity not only affects crop yields and quality but also has implications for biodiversity and the ecological balance, as fungicides can impact non-target organisms. Therefore, maintaining a delicate balance between effective disease control and minimising environmental impact is crucial. This balance requires ongoing research into fungicide insensitivity mechanisms, monitoring of insensitivity trends, and adaptation of fungicide use strategies to ensure both agricultural productivity and environmental sustainability are maintained.

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 Kevin King from Rothamsted Research for valuable discussions and for providing historical *Pyrenopeziza brassicae* isolates used as references in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors report no conflict of interest.

SUPPORTING INFORMATION

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

REFERENCES

1 Boys EF, Roques SE, Ashby AM, Evans N, Latunde-Dada AO, Thomas JE *et al.*, Resistance to infection by stealth: *Brassica napus* (winter oilseed rape) and *Pyrenopeziza brassicae* (light leaf spot). *Eur J Plant Pathol* **118**:307–321 (2007).
2 Karandeni Dewage CS, Klöppel CA, Stotz HU and Fitt BDL, Host–pathogen interactions in relation to management of light leaf spot disease (caused by Pyrenopeziza brassicae) on brassica species. *Crop Pasture Sci* **69**:9–19 (2018).
3 Bucur DE, Huang Y j, Fitt BDL and Kildea S, Azole fungicide sensitivity and molecular mechanisms of reduced sensitivity in Irish Pyrenopeziza brassicae populations. *Pest Manag Sci* **80**:2393–2404 (2022).
4 Ritchie F, Fraaije BA, King KM, Boor T, Walker P and Paveley N, Maximising the effective life of fungicides to control oilseed rape diseases through improved resistance management. AHDB Project Report No. 622 (2020).
5 Carmody SM, King KM, Ocamb CM, Fraaije BA, West JS and du Toit LJ, A phylogenetically distinct lineage of Pyrenopeziza brassicae associated with chlorotic leaf spot of Brassicaceae in North America. *Plant Pathol* **69**:518–537 (2020).
6 Calderon C, Ward E, Freeman J, Foster SJ and McCartney HA, Detection of airborne inoculum of Leptosphaeria maculans and Pyrenopeziza brassicae in oilseed rape crops by polymerase chain reaction (PCR) assays. *Plant Pathol* **51**:303–310 (2002).
7 Carter HE, Cools HJ, West JS, Shaw MW and Fraaije BA, Detection and molecular characterisation of Pyrenopeziza brassicae isolates resistant to methyl benzimidazole carbamates. *Pest Manag Sci* **69**:1040–1048 (2013).
8 King KM, Bucur DE, Ritchie F, Hawkins NJ, Kaczmarek AM, Duan Y *et al.*, Fungicide resistance status and chemical control options for the brassica pathogen Pyrenopeziza brassicae. *Plant Pathol* **70**:2086–2103 (2021).
9 Ritchie F, Walker P, Burnett F, Knight S and Harries C, Fungicides for light leaf spot control in winter oilseed rape. AHDB Cereals & Oilseeds (2019).
10 Beckerman JL, Detection of fungicide resistance, in *Fungicides - Showcases of Integrated Plant Disease Management from around the World*. InTech, Rijeka, Croatia, p. 13 (2013).
11 Hawkins NJ and Fraaije BA, Predicting resistance by mutagenesis: lessons from 45 years of MBC resistance. *Front Microbiol* **7**:1814 (2016) (NOV).
12 Luck JE and Gillings MR, Rapid identification of benomyl resistant strains of Botrytis cinerea using the polymerase chain reaction. *Mycol Res* **99**:1483–1488 (1995).
13 Einax E and Voigt K, Oligonucleotide primers for the universal amplification of β-tubulin genes facilitate phylogenetic analyses in the regnum fungi. *Org Divers Evol* **3**:185–194 (2003).
14 Fungicide Action Committee (FRAC), *List of First Confirmed Cases of Plant Pathogenic Organisms Resistant To Disease Control Agents*. CropLife International, Brussels, Belgium, p. 76 (2020).
15 Burnett FJ, Light leaf spot (Pyrenopeziza brassicae) in oilseed rape: extent of triazole fungicide resistance in Scotland; Fungicide strategies. HGCA Project Report No. OS63 LIGHT. Edinburgh (2003).
16 Gisi U, Sierotzki H, Cook A and McCaffery HA, Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag Sci* **58**:859–867 (2002).
17 FRAC, FRAC Code List 2019: Fungal control agents sorted by cross resistance pattern and mode of action (including FRAC Code numbering) (2019).
18 Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M and Parr-Dobrzanski B, The strobilurin fungicides. *Pest Manag Sci* **58**:649–662 (2002).
19 Scalliet G, Bowler J, Luksch T, Kirchhofer-Allan L, Steinhauer D, Ward K *et al.*, Mutagenesis and functional studies with succinate dehydrogenase inhibitors in the wheat pathogen Mycosphaerella graminicola. *PLoS One* **7**:e35429 (2012).
20 Sierotzki H and Scalliet G, A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* **103**:880–887 (2013).
21 Rehfus A, Strobel D, Bryson R and Stammler G, Mutations in sdh genes in field isolates of Zymoseptoria tritici and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathol* **67**:175–180 (2018).
22 Fraaije BA, Bayon C, Atkins S, Cools HJ, Lucas JA and Fraaije MW, Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Mol Plant Pathol* **13**:263–275 (2012).
23 Ritz C, Baty F, Streibig JC and Gerhard D, Dose-response analysis using R. *PLoS One* **10**:1–13 (2015).

24 Kassambara A, Package 'rstatix': Pipe-Friendly Framework for Basic Statistical Tests (2021). Report No.: R package version 0.7.0.
25 Pohlert T, PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended. R package version 1.9.4 (2022).
26 Maechler M, Rousseeuw P, Struyf A, Hubert M, Studer M and Hornik Cluster Analysis Basics and Extensions. Vienna, Austria: R Foundation for Statistical Computing; R package version 2.1.0. (2019). Available from: https://CRAN.R-project.org/package=cluster.
27 Kassambara A and Mundt F, Package 'factoextra'. Extract and Visualize the Results of Multivariate Data Analyses. Sthda (2016).
28 Wickham H, *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York, New York. ISBN: 978-3-319-24277-4, (2016).
29 Kumar S, Stecher G, Li M, Knyaz C and Tamura K, MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* **35**:1547–1549 (2018).
30 SnapGene software, version 6.0.2. [Internet]. Insightful Science (2022). Available from: https://www.snapgene.com.
31 Sierotzki H, Frey R, Wullschleger J, Palermo S, Karlin S, Godwin J *et al.*, Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Manag Sci* **63**:225–233 (2007).
32 Karolewski Z and Kosiada T, Sensitivity of *Pyrenopeziza brassicae* (light leaf spot) populations to fungicides recommended for the fungus control. *Phytopathologia* **56**:59–65 (2010).
33 Lucas JA, Hawkins NJ and Fraaije BA, The Evolution of Fungicide Resistance, in *Advances in Applied Microbiology*, Vol. **90**. Academic Press, Waltham, MA, USA, pp. 29–92 (2015).
34 Central Statistics Office Ireland, Area, Yield and Production of Crops [Internet]. CSO statistical release (2021). [cited 2021 Dec 2]. Available from: https://www.cso.ie/en/releasesandpublications/er/aypc/areayieldandproductionofcrops2019/.
35 Cheah LH, Corbin JB and Hartill WFT, Control of light leaf spot of brassicas (Pyrenopeziza brassicae Sutton & Rawlinson) with fungicides. *N Z J Agric Res* **24**:391–395 (1981).
36 European Food Safety Authority, Conclusion on the peer review of the pesticide risk assessment of the active substance carbendazim. *EFSA J* **9**:1962 (2011).
37 European Commission, Commission implementing regulation (EU) No 540/2011 of 25 May 2011 implementing regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the list of approved active substances. *Off J Eur Union* **54**:1–186 (2011).
38 Grasso V, Palermo S, Sierotzki H, Garibaldi A and Gisi U, Cytochromeb gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag Sci* **62**:465–472 (2006).
39 Deising HB, Reimann S and Pascholati SF, Mechanisms and significance of fungicide resistance. *Braz J Microbiol* **39**:286–295 (2008).
40 Phelan S, Barthe M, Tobie C and Kildea S, Detection of the cytochrome b mutation G143A in Irish Rhynchosporium commune populations using targeted 454 sequencing. *Pest Manag Sci* **73**:1154–1160 (2016). https://doi.org/10.1002/ps.4434.
41 Fisher N, Meunier B and Biagini GA, The cytochrome bc1 complex as an antipathogenic target. *FEBS Lett* **594**:2935–2952 (2020).
42 Fisher N, Meunier B and Biagini GA, The cytochrome bc1 complex as an antipathogenic target. *FEBS Lett* **594**:2935–2952 (2020).
43 Aguirre J, Ríos-Momberg M, Hewitt D and Hansberg W, Reactive oxygen species and development in microbial eukaryotes. *Trends Microbiol* **13**:111–118 (2005).
44 Wood PM and Hollomon DW, A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. *Pest Manag Sci* **59**:499–511 (2003).
45 Toffolatti SL and Vercesi A, QoI resistance in Plasmopara viticola in Italy: evolution and management strategies, in *Fungicide Resistance in Crop Protection: Risk and Management*. CABI, Wallingford UK, pp. 172–183 (2012).
46 Fontaine S, Remuson F, Caddoux L and Barrès B, Investigation of the sensitivity of Plasmopara viticola to amisulbrom and ametoctradin in French vineyards using bioassays and molecular tools. *Pest Manag Sci* **75**:2115–2123 (2019).
47 Kennedy R and Wakeham AJ, Impact of fungicide resistance on light leaf spot control on vegetable brassicas in Scotland. *Asp Appl Biol* **78**:51–58 (2006).
48 Dooley H, Shaw MW, Mehenni-Ciz J, Spink J and Kildea S, Detection of Zymoseptoria tritici SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD -R47W substitutions. *Pest Manag Sci* **72**:2203–2207 (2016).
49 Evans N, Butterworth MH, Baierl A, Semenov MA, West JS, Barnes A *et al.*, The impact of climate change on disease constraints on production of oilseed rape. *Food Secur* **2**:143–156 (2010).