Rothamsted Repository Download

A - Papers appearing in refereed journals

King, K. M., Barr, L., Bousquet, L., Glaab, A., Canning, G., Ritchie, F., Kildea, S., Fraaije, B. A. and West, J. S. 2024. Evolution of decreased sensitivity to azole fungicides in western European populations of Plenodomus lingam (cause of Phoma leaf spot / stem canker on oilseed rape). *Plant Pathology.* pp. 1-16. https://doi.org/10.1111/ppa.13897

Received: 8 December 2023 | Accepted: 29 February 2024

DOI: 10.1111/ppa.13897

Plant Pathology WILEY

ORIGINAL ARTICLE

Evolution of decreased sensitivity to azole fungicides in western European populations of *Plenodomus lingam* (Phoma stem canker on oilseed rape)

Kevin M. King[1] | Leo Barr[1] | Louise Bousquet[2] | Anna Glaab[3] | Gail Canning[1] | Faye Ritchie[4] | Steven Kildea[5] | Bart A. Fraaije[6] | Jonathan S. West[1]

[1]Rothamsted Research, Harpenden, UK

[2]BASF plc, Stockport, UK

[3]Agricultural Center, Limburgerhof, Germany

[4]ADAS Boxworth, Boxworth, UK

[5]Teagasc, Crops Research Centre, Oak Park, Ireland

[6]Wageningen University & Research, Biointeractions and Plant Health, Wageningen, Netherlands

Correspondence
Kevin M. King, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK.
Email: kevin.king@rothamsted.ac.uk

Funding information
Biotechnology and Biological Sciences Research Council, Grant/Award Number: BB/X011917/1; Growing Health Institute Strategic Programme, Grant/Award Number: BB/X010953/1 and BBS/E/RH/230003A; Resilient Farming Futures ISP, Grant/Award Number: BB/X010961/1 and BBS/E/RH/230004A

Abstract

Plenodomus lingam (*Leptosphaeria maculans*) and *P. biglobosus* (*L. biglobosa*) are fungi causing Phoma leaf spot/stem canker, an international damaging disease of oilseed rape (*Brassica napus*) and other brassicas. In Europe, fungicides used for disease management are mainly sterol 14α-demethylase (*CYP51*) inhibitors (DMIs/azoles); quinone-outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs) are also used. Decreased DMI sensitivity has emerged in Australian and eastern European *P. lingam* populations and is mediated by *CYP51* promoter inserts resulting in target site overexpression. In this study using in vitro sensitivity testing, we report decreased DMI (prothioconazole-desthio, mefentrifluconazole) sensitivity in modern western European *P. lingam* isolates (collected 2022–2023) compared to older baseline (1992–2005) isolates. Around 85% of modern western European *P. lingam* isolates collected, for which the *CYP51* promoter region was sequenced, carried a promoter insert, but target site alterations were not detected. Six different *CYP51* promoter inserts were identified, most commonly a 237 bp fragment of the *Sahana* transposable element. Inserts were associated with an approximately 3- to 10-fold decrease in sensitivity to the DMIs tested. In contrast to *P. lingam*, PCR screening revealed *CYP51* promoter inserts were absent in modern western European *P. biglobosus* isolates (2021–2023). Combined data indicate *P. lingam* isolates lacking an insert were similarly (or slightly more) sensitive to the DMIs tested for *P. biglobosus*, whereas those carrying an insert were slightly less sensitive than *P. biglobosus*. No evidence for substantive sensitivity shifts to the QoI (pyraclostrobin) or SDHI (boscalid) fungicides tested was obtained for either *Plenodomus* species.

KEYWORDS
azoles, *CYP51*, disease management, fungicide sensitivity, genotyping, promoter inserts

1 | INTRODUCTION

Oilseed rape (OSR) is an economically important crop in the United Kingdom and western Europe, with approximately 340,000 ha grown in the United Kingdom alone in 2023 (≥98% grown being winter OSR). Phoma leaf spot and stem canker (blackleg) has been shown to cause yield losses of 5%–50% in OSR crops in Australia, Canada and Europe (West et al., 2001). In the United Kingdom alone, Phoma is estimated to cause about £100 million in OSR yield losses per annum (www.ahdb.co.uk), and such financial losses occur despite extensive application of costly fungicides. In the United Kingdom in 2022, the most applied fungicides used on OSR crops were the sterol demethylation inhibitors (DMIs; FRAC group 3) prothioconazole and/or tebuconazole, with the total area treated being about 536,000 ha; the total area treated with the quinone-outside inhibitor (QoI; FRAC group 11) azoxystrobin was about 137,000 ha (Ridley et al., 2023); the succinate dehydrogenase inhibitors (SDHI; FRAC group 7) are less commonly used.

To protect OSR against Phoma, it is essential to consider pathogen population biology given that two closely related fungi are primarily responsible: *Plenodomus lingam* (syn. *Leptosphaeria maculans*) and *P. biglobosus* (syn. *L. biglobosa*) (Mendes-Pereira et al., 2003). Both *Plenodomus* spp. have a global geographic distribution, excluding China, in which only *P. biglobosus* occurs (Liu et al., 2014). A third species, *P. dezfulensis*, that is very closely related to *P. biglobosus*, has recently been described, but so far appears of only limited economic importance, having been identified on only a few OSR leaves at a single site in Iran (Safi et al., 2021). *P. lingam* is typically considered a more damaging pathogen than *P. biglobosus*, being associated with larger foliar lesions and damaging basal stem cankers (Fitt et al., 2006). There is evidence that *P. lingam* should be considered an invasive pathogen, having spread in recent decades to countries like Canada and Poland where previously only *P. biglobosus* was known to be resident (Fitt et al., 2006). Therefore, much research has focused on *P. lingam*, including incorporation of host genetic resistance into OSR cultivars that is effective only against *P. lingam*. Recent work has shown that *P. biglobosus* is becoming an increasingly prevalent pathogen on UK OSR crops, and Huang et al. (2014) suggested that this is due to control strategies being targeted exclusively to *P. lingam* to the detriment of disease caused by *P. biglobosus*.

As fungicides remain a key method of Phoma control, the potential that one or both *Plenodomus* species may become less sensitive to fungicides is a major concern. Past research has shown no or minor differences in sensitivity to DMIs between the two species isolated from OSR crops (Eckert et al., 2010; Huang et al., 2011; Sewell et al., 2017) and that, overall, both pathogens remained sensitive to this mode of action (MOA). However, this changed in 2015, when DMI resistance was detected in about 15% of the Australian *P. lingam* populations (Van de Wouw et al., 2017). Decreased DMI sensitivity has commonly been linked to target site mutations in *CYP51* (a gene coding for cytochrome P450 sterol 14α-demethylase, essential for biosynthesis of fungal sterols and target protein of DMIs) in numerous other ascomycete crop pathogens (Carter et al., 2014; Cools et al., 2012). However, to date, no such target site mutations have been identified through sequencing of the coding region of the *CYP51* gene in *P. lingam* (Huang et al., 2011; Van de Wouw et al., 2021). Instead, work by Yang et al. (2020) revealed that inserts in the *CYP51* promoter region were present in these isolates that were less sensitive to DMIs. These inserts were linked to *CYP51* overexpression and decreased DMI sensitivity, representing the first confirmed reports elucidating the underlying molecular mechanism in *P. lingam*.

Recent work has identified 11 different *CYP51* promoter inserts (denoted by the position of the insert relative to the gene start codon, followed by the size of the insert) in Australian isolates (described in detail by Scanlan et al., 2023). These inserts range in size from as little as approximately 150 bp to as large as approximately 6 kb. The different inserts are often derived from transposable elements (TE) such as the *Zolly* TE and *Sahana* TE, although other inserts have also been identified with no similarity to known *CYP51* or TE sequences and appear to originate from sequences present in multiple copies throughout the *P. lingam* genome. Other variants have also been identified that have so far proved impossible to sequence and assemble, while others have so far only been predicted bioinformatically (−132, com268:16; −79, ins 211). Fajemisin et al. (2022) reported that 24% of *P. lingam* isolates tested from the Czech Republic were resistant to DMIs and, although not sequenced, identified *CYP51* promoter inserts of approximately 100–300 bp present only in DMI-resistant isolates. Whether or not these inserts show homology to any of the known 11 reported insert variants present in the Australian *P. lingam* isolates remains unclear.

The extensive application in the United Kingdom of DMI fungicides for OSR disease control is likely to have imposed a strong selection pressure favouring genotypes with decreased fungicide sensitivity. Work is required to investigate whether decreased sensitivity to DMI fungicides has emerged in western European *P. lingam* populations, and if so, to explore the molecular mechanisms associated with such resistance (e.g., *CYP51* target site mutations and/or promoter inserts). There is also a need to determine whether western European *P. biglobosus* populations have become less sensitive to DMI fungicides, as this might contribute to the increased importance of this pathogen species in Phoma outbreaks (Huang et al., 2014).

This study reports the results of in vitro phenotyping of newly collected isolates of *P. lingam* (collected 2022–2023) and *P. biglobosus* (collected 2021–2023) from western Europe to the two DMIs prothioconazole-desthio and mefentrifluconazole, the QoI pyraclostrobin and the SDHI boscalid. Baseline reference isolates of *P. lingam* (collected 1992–2005) were included to establish whether any potential shifts in sensitivity are occurring. If DMI-insensitive phenotypes were found in *P. lingam* and *P. biglobosus* isolates, potential molecular mechanisms associated with any changes in DMI sensitivity, namely *CYP51* target site mutations and/or upstream promoter inserts, were investigated. The results of this work are reported alongside those of Scanlan et al. (2024) who independently investigated sensitivity to the DMI tebuconazole in international populations of *P. lingam* and explored the underpinning molecular mechanisms using a genome-wide association studies (GWAS)-based approach.

2 | MATERIALS AND METHODS

2.1 | Fungal isolation and storage

Baseline *P. lingam* isolates (collected 1992–2005) used in this study were already maintained on potato dextrose agar (PDA; Formedium) slopes at 4°C at Rothamsted Research, United Kingdom (Table 1). New isolates of *P. lingam* (2022–2023) and *P. biglobosus* (2021–2023) were obtained from leaf samples exhibiting Phoma leaf spot symptoms collected throughout western Europe (France, Germany, Ireland and the United Kingdom) (Tables 1 and 2). For fungal isolation, leaf segments including the lesion margin were surface sterilized in 10% (vol/vol) sodium hypochlorite solution for 30 s, rinsed twice with sterile distilled water (SDW) and placed on sterile tissue paper in a laminar flow hood until dry. Segments were subsequently transferred to PDA plates and incubated at 18°C in the dark for 5–10 days. After incubation single hyphal strands were selected under a stereomicroscope using a sterile pipette tip and transferred to new PDA plates to grow as purified cultures. Final cultures were stored at 4°C on PDA slopes for short/medium-term storage, and as mycelial plugs in 100% glycerol at −80°C for long-term storage.

2.2 | In vitro screening of fungicide sensitivity of *Plenodomus* spp. isolates

Testing of fungicide sensitivity was similar to that described by King et al. (2021), but with some differences. Fifty-five *P. lingam* isolates collected in 2022 and 2023 and five baseline *P. lingam* isolates collected between 1992 and 2005 were subcultured from PDA slope stocks onto V8 juice (Campbell Foods UK Ltd) agar plates (400 mL distilled water, 100 mL V8 juice, 1 g calcium carbonate, 7.5 g agar; autoclaved at 121°C 15 min; Dhingra & Sinclair, 1985) and incubated at 18°C in the dark for 7 days. After this time, cultures were transferred to near-UV light and incubated at 18°C for a further 12 days. After this time, 1 mL of SDW was pipetted onto the colony surface of each plated isolate and conidia dislodged via gentle agitation with a sterile L-shaped spreader. Resulting conidial suspensions were filtered through a single layer of sterile Miracloth to remove mycelial debris. The final conidial suspensions were quantified using a Neubauer improved haemocytometer and maintained at 4°C for up to 24 h prior to use.

Sabouraud dextrose broth (SAB, 2× concentrate; Millipore, Sigma Aldrich) was prepared, amended with various concentrations of fungicide and pipetted in 100 µL aliquots into 96-well microtitre plates. The fungicides tested and their final concentrations (after the addition of the 100 µL conidial suspension into each well) in 1× SAB (containing 0.2% vol/vol dimethyl sulphoxide [DMSO]) were (1) prothioconazole-desthio: 20, 6.67, 2.22, 0.741, 0.247, 0.082, 0.027, 0.009, 0.003, 0.0010, 0.00034, 0 µg/mL; (2) mefentrifluconazole: 30, 7.5, 1.88, 0.469, 0.117, 0.029, 0.007, 0.0018, 0.0005, 0.0001, 0.00003, 0 µg/mL; (3) pyraclostrobin: 30, 10, 3.33, 1.11, 0.37, 0.12, 0.04, 0.013, 0.004, 0.0015, 0.0005, 0 µg/mL; and (4) boscalid: 90, 30, 10, 3.33, 1.11, 0.37, 0.12, 0.04, 0.013, 0.004, 0.0015, 0 µg/mL. For each isolate tested, 100 µL aliquots of conidial suspension (prepared using SDW to 100,000 conidia/mL) were added to all 12 wells of each row containing liquid medium with serially diluted concentrations of fungicide (with well 12 containing no fungicide). All isolate × fungicide combinations were tested in duplicate as technical replicates.

Microtitre plates were sealed in a polyethylene bag to prevent water loss via evaporation and incubated at 18°C in the dark. After 10 days, fungal growth was measured by optical density (OD) at 630 nm using a FLUOstar Optima microplate reader (BMG Labtech) in well-scanning mode, with four reading points per well. For each isolate × fungicide combination, the 50% effective concentration (EC_{50}) was

TABLE 1 Details of the 60 *Plenodomus lingam* isolates screened for fungicide sensitivity.

ID	Country	Location	Date collected	No. of isolates
BASELINE 92-05	Australia	No data	2000, 2004	2
	Germany	No data	1992, 2005	2
	United States	North Dakota	2000	1
FRA 22	France	Lens	2022	3
GER 22/23	Germany	Grambow	2022	3
	Germany	Holstein	2023	5
	Germany	Niedersachsen	2022	1
IRE 23	Ireland	Carlow	2023	9
UK 22/23	United Kingdom	Cambridgeshire	2022	1
	United Kingdom	Essex	2023	5
	United Kingdom	Herefordshire	2022	10
	United Kingdom	Norfolk	2023	6
	United Kingdom	Suffolk	2023	9
	United Kingdom	Surrey	2022	2
	United Kingdom	Yorkshire	2022	1

Note: All isolates collected from oilseed rape (OSR, *Brassica napus*), apart from the single isolate from Cambridgeshire, United Kingdom, in 2022 that was collected from purple sprouting broccoli (*Brassica oleracea*).

TABLE 2 The 34 isolates of *Plenodomus biglobosus* (plus a single isolate of *P. dezfulensis*) collected primarily from oilseed rape (*Brassica napus*) and screened for *CYP51* promoter inserts using PCR primers KK2F/KK2R.

Isolate code(s)	Year	Region	Country
Modern *P. biglobosus* (2021–2023)			
22FRA/B/02, 22FRA/B/05, 22FRA/B/06, 22FRA/B/07, 22FRA/B/08, 22FRA/M/06	2022	Pas-de-Calais (Lens)	France
22THRIP02, 22THRIP04, 22BLACK01	2022	Cambridgeshire	United Kingdom
23ESXLB01	2023	Essex	United Kingdom
23JAME/LB/03, 23JAME/LB/04	2023	Lincolnshire	United Kingdom
23DERE/LB/01	2023	Norfolk	United Kingdom
21WAS7-1	2021	Northern Ireland	United Kingdom
23NEWMKTLB02	2023	Suffolk	United Kingdom
21WAS1-2	2021	Southern England	United Kingdom
21WAS8-4	2021	West Midlands	United Kingdom
Historical *P. biglobosus* (1996–2007)			
AUT 03–20, AUT1-15	2002	Austria	Austria
99–27	1999	Saskatchewan	Canada
05 70	2005	Saskatchewan	Canada
gui 2b3	1999	Guizhou	China
hl 11-2	2005	Hailar	China
hfa2a1-4	2006	Hefei	China
wh 9-1-2	2005	Wuhan	China
ARMC30	2006	Armailles	France
VERB31	2006	Vergognes	France
GR2 E 1-2, GR2-D 6-2	2006	Grabow	Poland
IGR-D 28-3	2007	Poznan	Poland
PL 2002-3	2007	Poznan	Poland
UK4, UK26	1996/7	Hertfordshire	United Kingdom
Leaf C-3	2007	Hertfordshire	United Kingdom
P. dezfulensis [a]			
SCUA-Ahm-S41	2019	Dezful	Iran

Note: The nine modern isolates screened for fungicide sensitivity are underlined.
[a]Isolate of the recently described *P. dezfulensis*, a species closely related to *P. biglobosus* (Safi et al., 2021).

determined according to a dose–response relationship curve-fitting option of the Optima software. The mean EC_{50} value for each of the two technical replicates was calculated. Due to fungicide insolubility issues affecting OD measurements, the wells containing the highest concentrations of boscalid (90 μg/mL) was excluded from analyses.

2.3 | DNA extraction and *Plenodomus* species-specific multiplex PCR

Genomic DNA was typically extracted from frozen *Plenodomus* conidial suspensions using a MasterPure Yeast DNA kit (Epicentre) according to the manufacturer's instructions. The species identities of all isolates as either *P. lingam* or *P. biglobosus* (and the utility of extracted DNA for use in PCR) were confirmed using species-specific multiplex PCR (Liu et al., 2006). PCR was carried out in 25 μL reaction volumes, each containing 12.5 μL RedTaq ReadyMix (2× concentrate), 0.1 μL each of three primers (100 μM stocks) [LMacF: 5′-CTTGCCCACCAATTGGATCCCCTA-3′; LbigF: 5′-ATCAGGGGATTG GTGTCAGCAGTTGA-3′; and LmacR: 5′-GCAAAATGTGCTGCGCTCC AGG-3′], 10.2 μL of PCR-grade water and 2 μL of genomic DNA (typically 5–10 ng). Reaction conditions were as follows: 35 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min; a final elongation step of 72°C for 5 min; and a final hold at 4°C. Amplicons were resolved and identified via electrophoresis on 1.2% agarose gels; *P. lingam* and *P. biglobosus* isolates yielded amplicons of 331 and 444 bp, respectively. A single isolate of *P. dezfulensis* (SCUA-Ahm-S41) was also included (Safi et al., 2021).

2.4 | PCR screening and sequencing of *P. lingam* isolates *CYP51* promoter inserts

The upstream (promoter) region of the *CYP51* gene was PCR amplified for 34 randomly selected modern *P. lingam* isolates (Table 3;

TABLE 3 Details of 34 *Plenodomus lingam* isolates collected from oilseed rape (*Brassica napus*) in 2022–2023 characterized for *CYP51* promoter inserts by sequencing (in vitro fungicide sensitivity data were available for 31 of these).

						EC_{50} value (μg/mL)[a]			
Isolate code	Year	Site	Locality	Country	Insert details	Prothioconazole-desthio	Mefentrifluconazole	Pyraclostrobin	Boscalid
22FRA/M/02	2022	ND	Lens	France	0	0.003	0.000	0.116	0.009
22FRA/M/05	2022	ND	Lens	France	0	0.002	0.000	0.088	0.008
23ESXLM04	2023	St Ostwyth	Essex	United Kingdom	0	0.012	0.004	0.070	0.028
23ESXLM05	2023	St Ostwyth	Essex	United Kingdom	0	0.006	0.000	0.087	0.004
23DERELM02	2023	Dereham	Norfolk	United Kingdom	0	0.004	0.000	0.102	0.032
23ROSELB03	2023	Rosemaund	Herefordshire	United Kingdom	−104, ins168	0.016	0.001	0.083	0.008
23ROSELM03	2023	Rosemaund	Herefordshire	United Kingdom	−124, ins187	0.014	0.000	0.071	0.042
22LAD04	2022	Ladenthim	Grambow	Germany	−104, ins203	0.031	0.003	0.316	0.016
22LAD03	2022	Ladenthim	Grambow	Germany	−99, ins237	0.028	0.008	0.140	0.026
23GERSH02	2023	Schleswig	Holstein	Germany	−99, ins237	0.018	0.010	0.091	0.009
23GERSH03	2023	Schleswig	Holstein	Germany	−99, ins237	0.018	0.010	0.078	0.019
23GERSH04	2023	Schleswig	Holstein	Germany	−99, ins237	0.016	0.005	0.086	0.005
23GERSH05	2023	Schleswig	Holstein	Germany	−99, ins237	0.016	0.005	0.085	0.020
23GERSH06	2023	Schleswig	Holstein	Germany	−99, ins237	0.020	0.006	0.071	0.019
22MACK05	2022	Mackendorf	Niedersachsen	Germany	−99, ins237	0.026	0.003	0.260	0.022
IRE/A/02	2023	Field A	Carlow	Ireland	−99, ins237	0.027	0.002	0.335	0.032
IRE/B/04	2023	Field B	Carlow	Ireland	−99, ins237	0.034	0.004	0.215	0.044
23LAOISLM01	2023	Laois	Carlow	Ireland	−99, ins237	0.022	0.004	0.140	0.012
23LAOISLB01	2023	Laois	Carlow	Ireland	−99, ins237	0.013	0.001	0.124	0.010
23LAOISLB04	2023	Laois	Carlow	Ireland	−99, ins237	ND	ND	ND	ND
23ESXLM06	2023	St Ostwyth	Essex	United Kingdom	−99, ins237	0.013	0.002	0.071	0.008
23ROSELB01	2023	Rosemaund	Herefordshire	United Kingdom	−99, ins237	0.010	0.000	0.070	0.075
23DERELM01	2023	Dereham	Norfolk	United Kingdom	−99, ins237	0.016	0.002	0.260	0.048
23DERELM03	2023	Dereham	Norfolk	United Kingdom	−99, ins237	0.012	0.000	0.159	0.009
23NWMKTLM04	2023	Newmarket	Suffolk	United Kingdom	−99, ins237	0.025	0.004	0.106	0.020
23NWMKTLM05	2023	Newmarket	Suffolk	United Kingdom	−99, ins237	0.026	0.007	0.121	0.024
23NWMKTLM07	2023	Newmarket	Suffolk	United Kingdom	−99, ins237	0.011	0.005	0.062	0.032
23CLAYLM07	2023	Claydon	Suffolk	United Kingdom	−99, ins237	0.047	0.008	0.331	0.036
23SUFFLM04	2023	Great Barton	Suffolk	United Kingdom	−99, ins237	0.021	0.006	0.087	0.028
22SURREY02	2022	Horne	Surrey	United Kingdom	−99, ins237	0.013	0.002	0.092	0.007

(Continues)

TABLE 3 (Continued)

Isolate code	Year	Site	Locality	Country	Insert details	EC_{50} value (μg/mL)[a]			
						Prothioconazole-desthio	Mefentrifluconazole	Pyraclostrobin	Boscalid
23NWMKTLM08	2023	Newmarket	Suffolk	United Kingdom	−382, ins360	0.012	0.004	0.102	0.005
23ROSELB08	2023	Rosemaund	Herefordshire	United Kingdom	−382, ins360	ND	ND	ND	ND
22SURREY01	2022	Horne	Surrey	United Kingdom	−80, ins736	0.049	0.007	0.253	0.004
22HERTS02	2022	Harpenden	Hertfordshire	United Kingdom	−80, ins736	ND	ND	ND	ND

[a]Rounded values shown; values of <0.0005 are rounded to 0.000, for clarity.

collected from OSR crops in 2022–2023) using primers EPS1/EPS6 (Yang et al., 2020). They were also amplified for the five baseline older *P. lingam* isolates collected between 1992 and 2005 (Table 1). PCR was carried out in 12.5 μL volumes, containing 6.25 μL RedTaq ReadyMix (2× concentrate), 0.1 μL each of primers EPS1 and EPS6 (100 μM stocks) [EPS1: 5′-AGCACCCATGGACCACGG-3′; EPS6: 5′-CAGGATAAAGGAGGCGAAG-3′], 4.05 μL of PCR-grade water and 2 μL of genomic DNA (typically 5–10 ng). Reaction conditions were as follows: 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; a final elongation step of 72°C for 5 min; and a final hold at 4°C. PCR amplicons were visualized by agarose gel electrophoresis to confirm a single amplicon, purified using a MinElute PCR purification kit (Qiagen) and sent to MWG Eurofins (Germany) for bidirectional sequencing using both PCR primers. Sequences were aligned and analysed using Geneious software v. 8.1 (Biomatters). Any *CYP51* promoter inserts present were described, as is convention (Scanlan et al., 2023), on the position of the insert relative to the gene start codon, followed by the size of the insert. Note that inserts included a few base pairs of sequence duplicated from the *CYP51* promoter region, and the positioning of the inserts relative to the start codon were based on the downstream positioning of this repeated sequence.

2.5 | Sequencing of *CYP51* gene coding region for *P. lingam* isolates

The *CYP51* gene coding region was amplified and sequenced for five modern European *P. lingam* isolates (22HERTS02, 22LAD04, 22MACK05, 23NWMKTLM08, 23ROSELB03; Table 3). These five isolates were selected as each contained a differently sized *CYP51* promoter insert (see results). PCR was carried out in 20 μL volumes, containing 2 μL 10× PfuUltra II reaction buffer, 0.2 μL dNTP mix (25 mM stock), 0.06 μL each of primers PL_KK_CYP51_F and PL_KK_CYP51_R (each 100 μM stocks) [PL_KK_CYP51_F: 5′-CTCGCTCACACGACAAGACT-3′; PL_KK_CYP51_R: 5′-ACTCGACCTTCTCCCTCCTC-3′], 16.28 μL PCR-grade water, 0.4 μL PfuUltra II Fusion HS DNA polymerase (Agilent Technologies Inc.) and 1 μL of genomic DNA (5 ng). Reaction conditions were as follows: 35 cycles of 95°C for 20 s, 62°C for 20 s, 72°C for 1 min; a final elongation step of 72°C for 3 min; and a final hold at 4°C. Amplicons were bidirectionally sequenced using primers PL_KK_CYP51_F/PL_KK_CYP51_R and processed as described above, before being compared to the reference *CYP51 P. lingam* GenBank accession OP344492.

2.6 | Statistical analysis

Data were first processed in MS Excel, where initial exploratory data analysis and graph production was carried out. Subsequent statistical analyses were done using GraphPad Prism software package v. 8.4.2.

2.6.1 | In vitro fungicide sensitivity: Baseline (1992–2005) versus modern (2022–2023) *P. lingam* isolates

Initial analyses of EC_{50} values data suggested some violations of assumption of normality (Kolmogorov–Smirnov tests) and equal variance (Levene test) (data not shown). Thus, nonparametric Kruskal–Wallis one-way analysis of variance (ANOVA) tests were used to explore whether significant differences could be identified between the five *P. lingam* populations tested (BASELINE 92-05 [*N* = 5], FRA 22 [*N* = 3], GER 22/23 [*N* = 9], IRE 23 [*N* = 9] and UK 22/23 [*N* = 34]). If significant differences were identified, subsequent post hoc Dunn's multiple comparisons tests were carried out to pinpoint between which of the groups these differences occurred. Note that, on a few occasions, data were not obtained for a given isolate × fungicide combination due to difficulties in interpretation of dose–response curves (see descriptive statistics in results for exact numbers).

2.6.2 | Differences in proportions of European *P. lingam* isolates collected in 2022–2023 with and without *CYP51* promoter inserts

The proportions of 34 randomly selected European *P. lingam* isolates with/without promoter inserts (based on PCR amplification using primers EPS1/EPS6 and bidirectional sequencing) were expressed as percentages. Tallies were calculated for different *CYP51* insert variants for *P. lingam* isolates collected from the United Kingdom, Ireland, Germany and France.

2.6.3 | In vitro fungicide sensitivity: *P. lingam* isolates (all collected 2022–2023) with inserts (*N* = 26) and without inserts (*N* = 5), and *P. biglobosus* isolates (*N* = 10–13; collected 2021–2023)

For these analyses, the presence of promoter inserts in *P. lingam* was determined as a binary plus/minus based on PCR amplification using primers EPS1/EPS6 and agarose gel electrophoresis. Initial analyses of EC_{50} values obtained for each of the different groups suggested that some violated the assumption of normality (Kolmogorov–Smirnov tests) and equal variance (Levene test) (data not shown). Thus, nonparametric Kruskal–Wallis ANOVA tests were used to investigate whether significant differences could be identified between three groups of recently collected (2022–2023) isolates (*P. lingam* with inserts, *P. lingam* without inserts and *P. biglobosus*). If significant differences were identified, subsequent post hoc Dunn's multiple comparisons tests were carried out to pinpoint between which of the three groups these differences occurred. Note that, on a few occasions, data were not obtained for a given isolate × fungicide combination due to difficulties in interpretation of dose–response curves (see descriptive statistics in results for exact numbers).

2.6.4 | In vitro fungicide sensitivity: Differences between 36 *P. lingam* isolates with (*N* = 26; collected 2022–2023) and without (*N* = 10; five collected 1992–2005, five collected 2022–2023) *CYP51* promoter inserts (all determined by sequencing)

We used *t* tests to compare if statistical differences in EC_{50} values to different fungicides occurred between isolates with/without *CYP51* inserts. Initial Levene tests were carried out to evaluate whether the assumption of homogeneity of variance was violated; if not violated, a Student's *t* test was used, while if violated a Welch's *t* test was used instead.

2.6.5 | Cross resistance for *P. lingam* (*N* = 55) and *P. biglobosus* (*N* = 9) to four different fungicides

The strength, direction and statistical significance of pairwise correlations of EC_{50} values obtained for isolates between each of the four different fungicides tested (prothioconazole-desthio, mefentrifluconazole, pyraclostrobin and boscalid) were evaluated using Pearson correlation coefficient tests.

2.6.6 | In vitro DMI fungicide sensitivity: Differences between 36 *P. lingam* isolates (31 collected 2022–2023, 5 collected 1992–2005) with different *CYP51* promoter inserts (confirmed by sequencing)

Given the small samples sizes involved, sometimes only a single representative of a given promoter insert type was obtained, hence statistical analyses for differences in EC_{50} values between isolates with the six promoter inserts variants identified in the present study were not possible. Thus, descriptive data values (means and standard deviations) only were calculated. Resistance factors (RFs) were calculated for the prothioconazole-desthio and mefentrifluconazole data sets as the ratio of the mean EC_{50} for a given *CYP51* promoter insert variant divided by the mean EC_{50} of isolates with no *CYP51* promoter insert.

2.7 | PCR screening of *P. biglobosus* isolates for possible *CYP51* promoter inserts

A BLAST search of the *P. lingam CYP51* gene (GenBank AY142146) against the NCBI nucleotide database identified the corresponding sequence in related *P. biglobosus* (GenBank FO905635, 81.2% identity). Subsequently, primers were designed to target an 805 bp fragment of the predicted upstream promoter region (KK2F: 5′-CGGGGAGTTCATCGAGATGG-3′/KK2R: 5′-CAGGACCACGGCAATTAGGA-3′). Primer KK2R was targeted within the predicted *P. biglobosus CYP51* coding region at 114 bp downstream of the predicted

gene start codon, with primer KK2F targeted 691 bp upstream of the predicted gene start codon. The PCR was carried out in 25 μL volumes, each containing 12.5 μL RedTaq ReadyMix (2 × concentrate), 0.2 μL each of both primers (100 μM stocks), 10.1 μL of PCR-grade water and 2 μL of genomic DNA (10 ng). Reaction conditions were as follows: 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min; a final elongation step of 72°C for 5 min; and a final hold at 4°C. Amplicons were resolved on 2% agarose gels. Full details of the 17 modern *P. biglobosus* (2021–2023), 17 baseline *P. biglobosus* (1996–2007; from the OREGIN culture collection maintained at Rothamsted Research, United Kingdom), and the single *P. dezfulensis*, isolates screened using primers KK2F/R are given in Table 2.

3 | RESULTS

3.1 | In vitro fungicide sensitivity

3.1.1 | Baseline (1992–2005) versus modern (2022–2023) *P. lingam* isolates

Screening for prothioconazole-desthio sensitivity showed that the five groups of *P. lingam* isolates tested (older baseline reference isolates, plus modern isolates grouped by geographic origin from France [FRA], Germany [GER], Ireland [IRE] and the United Kingdom [UK]; Table 1), when ranked from lowest to highest median EC_{50} values with interquartile range (IQR) in parentheses were: BASELINE 92-05 ($N = 5$), EC_{50} 0.002 (0.006) μg/mL; FRA 22 ($N = 3$), EC_{50} 0.004 (0) μg/mL; UK 22/23, ($N = 34$), EC_{50} 0.014 (0.011) μg/mL; IRE 23, ($N = 9$), EC_{50} 0.015 (0.015) μg/mL; and GER 22/23, ($N = 9$), EC_{50} 0.020 (0.010) μg/mL (Figure 1a). Kruskal–Wallis test one-way ANOVA results suggested significant differences in prothioconazole-desthio sensitivity existed between one or more pairs of the five groups ($H[4,60] = 23.19$, $p < 0.01$). Post hoc comparisons using Dunn's multiple comparisons test showed that significant differences occurred between less sensitive BASELINE 92-05 versus UK 22/23, IRE 23 and GER 22/23 (all $p < 0.01$) isolates. Calculated RFs, determined by dividing median EC_{50} values for a particular group by the mean of BASELINE 92-05, showed 7-fold (UK 22/23), 7.5-fold (IRE 23) and 10-fold (GER 22/23) decreases in sensitivity. Significant differences were also detected between less sensitive FRA 22 versus UK 22/23, IRE 23 and GER 22/23 (all at least $p < 0.05$). Differences that were approaching statistical significance ($p = 0.06$) were also identified between more sensitive UK 22/23 versus less sensitive GER 22/23.

Screening for mefentrifluconazole sensitivity showed that the five groups of *P. lingam* isolates tested, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were: BASELINE 92-05 ($N = 5$), EC_{50} 0.000006 (0) μg/mL; FRA 22 ($N = 3$), EC_{50} 0.00002 (0) μg/mL; IRE 23 ($N = 9$), EC_{50} 0.0010 (0.0028) μg/mL; UK 22/23 ($N = 34$), EC_{50} 0.0019 (0.0040) μg/mL; and GER 22/23 ($N = 9$) EC_{50} 0.0055 (0.0055) μg/mL (Figure 1b). Kruskal–Wallis test one-way ANOVA results suggested significant differences in mefentrifluconazole sensitivity existed between one or more pairs of the five groups ($H[4,60] = 25.48$, $p < 0.01$). Post hoc comparisons using Dunn's multiple comparisons test showed that significant differences occurred between more sensitive BASELINE 92-05 versus less sensitive UK 22/23, IRE 23 and GER 22/23 (all $p < 0.01$) isolates. Significant differences were also identified between more sensitive FRA 22 versus less sensitive UK 22/23 ($p < 0.05$) and GER 22/23 ($p < 0.01$); differences approaching statistical significance were also evident when more sensitive FRA 22 was compared to less sensitive IRE 23 ($p = 0.06$). Lastly, significant differences were also identified between more sensitive UK 22/23 and IRE 23 versus less sensitive GER 22/23 (both $p < 0.05$).

Screening for pyraclostrobin sensitivity showed that the five groups of *P. lingam* isolates tested, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were: BASELINE 92-05 ($N = 5$), EC_{50} 0.051 (0.168) μg/mL; GER 22/23 ($N = 9$), EC_{50} 0.091 (0.129) μg/mL; UK 22/23 ($N = 34$), EC_{50} 0.098 (0.082) μg/mL; FRA 22 ($N = 3$), EC_{50} 0.104 (0.028) μg/mL; and IRE 23 ($N = 9$), EC_{50} 0.140 (0.082) μg/mL (Figure 1c). Kruskal–Wallis test one-way ANOVA results suggested no significant differences in pyraclostrobin sensitivity existed between one or more pairs of the five groups ($H[4,60] = 4.282$, $p = 0.369$).

Lastly, screening for boscalid sensitivity showed that the five groups of *P. lingam* isolates tested, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were: FRA 22 ($N = 3$), EC_{50} 0.0079 (0.0010) μg/mL; IRE 23 ($N = 9$), EC_{50} 0.0118 (0.0240) μg/mL; GER 22/23 ($N = 9$), EC_{50} 0.0187 (0.0087) μg/mL; UK 22/23 ($N = 34$), EC_{50} 0.0191 (0.0208) μg/mL; and BASELINE 92-05 ($N = 5$), EC_{50} 0.0264 (0.0206) μg/mL (Figure 1d). Kruskal–Wallis test one-way ANOVA results suggested no significant differences in boscalid sensitivity existed between one or more pairs of the five groups ($H[4,60] = 4.554$, $p = 0.336$).

3.1.2 | *P. lingam* isolates (with/without *CYP51* inserts) versus recent *P. biglobosus* isolates (all collected 2022–2023)

Results described in this section are shown graphically in Figure S1. Screening for prothioconazole-desthio sensitivity showed that the three groups, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were 0.004 (0.006) μg/mL (*P. lingam* without inserts, $N = 5$), 0.011 (0.014) μg/mL (*P. biglobosus*, $N = 13$) and 0.018 (0.013) μg/mL (*P. lingam* with inserts, $N = 26$). Kruskal–Wallis test one-way ANOVA results suggested significant differences in prothioconazole-desthio sensitivity existed between one or more pairs of the three groups ($H[2,44] = 13.42$, $p < 0.01$). Post hoc comparisons using Dunn's multiple comparisons test showed that significant differences occurred between *P. biglobosus* versus *P. lingam* without inserts ($p < 0.05$) and *P. lingam* with inserts versus *P. lingam* without inserts ($p < 0.01$), but not quite between *P. biglobosus* versus *P. lingam* with inserts (approaching statistical significance, $p = 0.056$).

Screening for mefentrifluconazole sensitivity showed that the three groups, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were 0.00002 (0.002) μg/mL (*P. lingam*

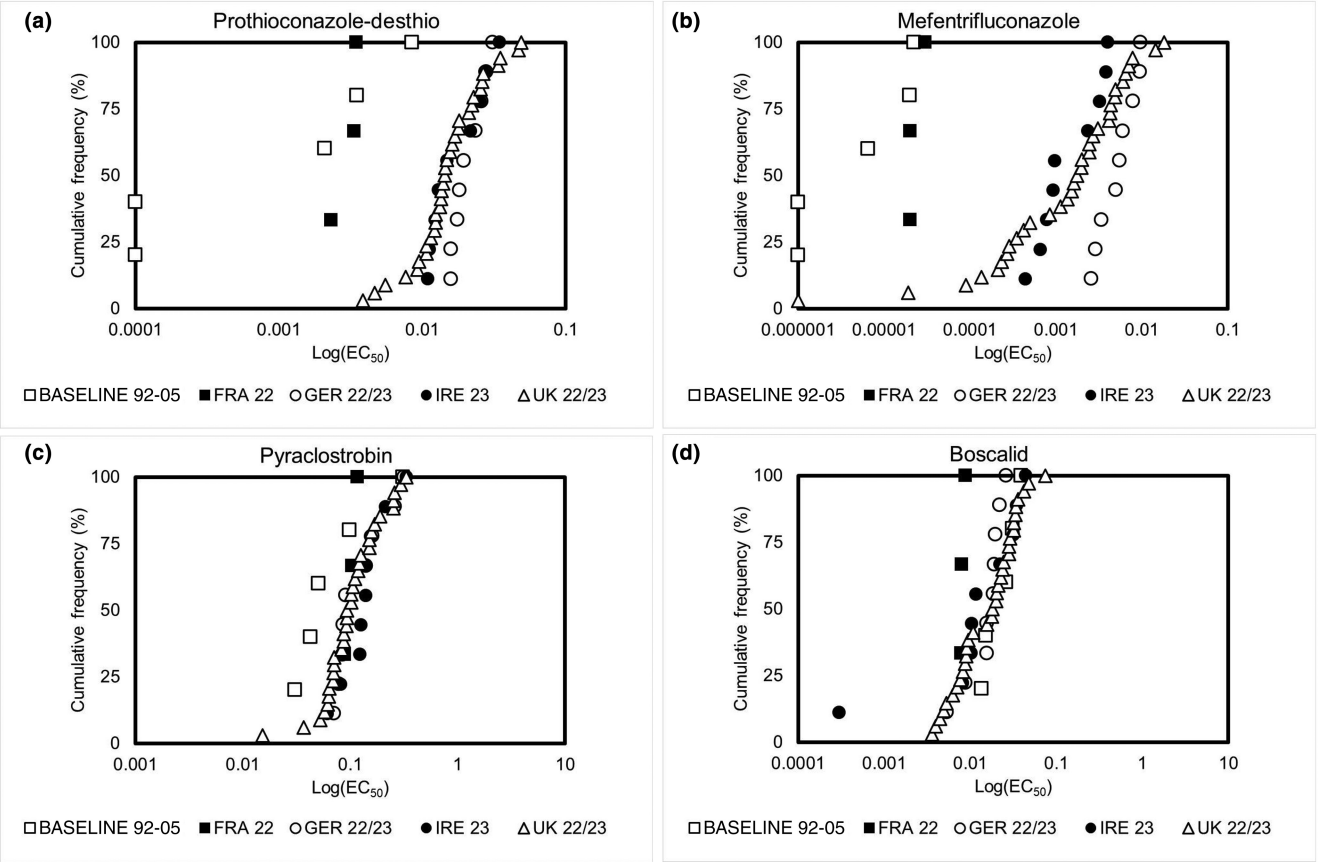

FIGURE 1 In vitro sensitivity of *Plenodomus lingam* to (a) prothioconazole-desthio, (b) mefentrifluconazole, (c) pyraclostrobin and (d) boscalid. Isolates are ranked according to increasing EC_{50} values (cumulative). Populations tested for each fungicide were collected from 1992 to 2005 (BASELINE 92-05) or from 2022 to 2023 from France (FRA 22), Germany (GER 22/23), Ireland (IRE 23) and the United Kingdom (UK 22/23) (see Table 1 for full details including numbers of isolates tested). Each data point shown represents the mean of two technical replicates. For a small number of highly sensitive isolates screened against prothioconazole-desthio and mefentrifluconazole, data points (two or three isolates each) are shown at the lowest point on the *x*-axis, for clarity.

without inserts, $N = 5$), 0.00009 (0.003) μg/mL (*P. biglobosus*, $N = 13$) and 0.004 (0.004) μg/mL (*P. lingam* with inserts, $N = 26$). Kruskal–Wallis test one-way ANOVA results suggested significant differences in mefentrifluconazole sensitivity existed between one or more pairs of the three groups ($H[2,44] = 11.85$, $p < 0.01$). Post hoc comparisons using Dunn's multiple comparisons test showed that significant differences occurred between *P. biglobosus* versus *P. lingam* with inserts ($p < 0.01$) and *P. lingam* with inserts versus *P. lingam* without inserts ($p < 0.01$), but not between *P. biglobosus* versus *P. lingam* without inserts ($p = 0.34$).

Screening for pyraclostrobin sensitivity showed that the three groups, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were: 0.088 (0.031) μg/mL (*P. lingam* without inserts, $N = 5$), 0.104 (0.132) μg/mL (*P. lingam* with inserts, $N = 26$) and 0.117 (0.052) μg/mL (*P. biglobosus*, $N = 10$). Kruskal–Wallis test one-way ANOVA results suggested no significant differences in pyraclostrobin sensitivity existed between one or more pairs of the three groups ($H[2,44] = 1.88$, $p = 0.391$).

Screening for boscalid sensitivity showed that the three groups, when ranked from lowest to highest median EC_{50} values with IQR in parentheses were: 0.003 (0.011) μg/mL (*P. biglobosus*, $N = 10$), 0.009 (0.024) μg/mL (*P. lingam* without inserts, $N = 5$) and 0.019 (0.023) μg/mL (*P. lingam* with inserts, $N = 26$). Kruskal–Wallis test one-way ANOVA results suggested significant differences in boscalid sensitivity existed between one or more pairs of the three groups ($H[2,40] = 6.92$, $p < 0.05$). Post hoc comparisons using Dunn's multiple comparisons test showed that relatively small but statistically significant differences occurred between *P. biglobosus* versus less sensitive *P. lingam* with inserts ($p < 0.05$), but neither between *P. biglobosus* versus *P. lingam* without inserts ($p = 0.218$) nor *P. lingam* with inserts versus *P. lingam* without inserts ($p = 0.499$).

3.1.3 | Differences between 36 *P. lingam* isolates with/without *CYP51* promoter inserts (determined by sequencing)

Significant differences of in vitro sensitivity to the DMI prothioconazole-desthio were found between more sensitive isolates that lacked *CYP51* promoter inserts (M = 0.004 μg/mL, *SD* = 0.004 μg/mL, $N = 10$) versus less sensitive isolates that possessed an insert (M = 0.021 μg/mL, *SD* = 0.010 μg/mL, $N = 26$)

(Welch $t(df = 34) = 7.34$; $p < 0.001$). Significant differences in sensitivity to the DMI mefentrifluconazole were also detected between more sensitive isolates lacking such an insert (M = 0.0004 μg/mL, *SD* = 0.001 μg/mL, *N* = 10) versus less sensitive isolates that carried such an insert (M = 0.004 μg/mL, *SD* = 0.002 μg/mL, *N* = 26) (Welch $t(df = 31) = 5.38$; $p < 0.001$). The differences of RFs of different promoter variants compared to those with no promoter insert are given in Table 4 and discussed further below. No significant differences in sensitivity to the QoI pyraclostrobin were identified between isolates lacking (M = 0.100 μg/mL, *SD* = 0.079 μg/mL, *N* = 10) or carrying (M = 0.146 μg/mL, *SD* = 0.089 μg/mL, *N* = 26) a promoter insert ($t(df = 34) = 1.44$; $p = 0.15$). Lastly, no significant differences in sensitivity to the SDHI boscalid were identified between isolates lacking (M = 0.021 μg/mL, *SD* = 0.020 μg/mL, *N* = 10) or possessing (M = 0.022 μg/mL, *SD* = 0.017 μg/mL, *N* = 26) a promoter insert ($t(df = 34) = 0.30$; $p = 0.77$).

3.2 | Cross resistance for *P. lingam* and *P. biglobosus* to fungicides with different MOAs

Possible cross resistance between the four fungicides tested in this study that represented three different MOAs (DMIs: prothioconazole-desthio and mefentrifluconazole; QoI: pyraclostrobin; SDHI: boscalid) were determined for 55 *P. lingam* isolates collected 2022–2023 (Table 1) and nine *P. biglobosus* isolates collected 2021–2023 (Table 2). Cross resistance plots for all four fungicides screened against *P. lingam* are shown in Figure 2. Positive cross resistance that was moderately strong and statistically significant was identified between the two DMIs tested for both *P. lingam* ($r = 0.61$, $p < 0.01$) and *P. biglobosus* ($r = 0.68$, $p < 0.05$). With one exception, no other statistically significant relationships were identified for either species between any of the other fungicide combinations tested ($p > 0.05$ in all cases). The single exception identified was a statistically significant but only weakly positive relationship for *P. lingam* between the QoI pyraclostrobin and the DMI prothioconazole-desthio ($r = 0.42$, $p < 0.01$), although, as previously noted, not for the DMI mefentrifluconazole ($r = -0.04$, $p = 0.78$). Although not statistically significant, it is noted that, for *P. biglobosus*, a very weak small positive relationship was observed between pyraclostrobin and prothioconazole-desthio ($r = 0.20$, $p = 0.60$) and a weak relationship was evident between pyraclostrobin versus mefentrifluconazole ($r = 0.35$, $p = 0.35$).

3.3 | Characterization of *CYP51* promoter inserts in European *P. lingam* isolates

Screening of 34 randomly selected modern *P. lingam* isolates from 2022 to 2023 from Europe, targeting the sequence of the *CYP51* promoter, revealed that the majority (29/34, 85.3%) carried a promoter insert (Table 5, Figure 3). Only a minority (5/34, 14.7%) were wild type, that is, carrying no promoter insert. The most common insert identified was 237 bp (22/34, 64.7%), and was identified in isolates from Germany, Ireland and the United Kingdom, showing a widespread geographic distribution throughout Europe. None of the five older baseline *P. lingam* isolates collected between 1992 and 2005 used in this study (Table 1) carried a *CYP51* promoter insert.

Inspection of sequences of the six *CYP51* promoter inserts identified from European *P. lingam* isolates revealed that five exhibited homology to the DNA sequence of the *Sahana* transposable element. Compared to the *Sahana* TE sequence (5834 bp) reported in the previously described Australian reference isolate 19BL148, the similarities were approximately 92.7% (736 bp insert, UK isolate 22HERTS02; GenBank accession: OR961468), approximately 97.4% (360 bp insert, UK isolate 23NWMKTLM08; GenBank accession: OR961467), approximately 97.5% (203 bp insert, German isolate 22LAD04; GenBank accession: OR961465), approximately 98.1% (168 bp insert, UK isolate 23ROSELB03; GenBank accession: OR961463) and approximately 99.1% (237 bp insert, German isolate 22MACK05; GenBank accession:

TABLE 4 Details of 36 *Plenodomus lingam* isolates (31 modern isolates collected 2022–2023, five baseline isolates collected 1992–2005) for which different *CYP51* promoter variants were confirmed by sequencing and their corresponding EC_{50} values to two DMI fungicides.

		Prothioconazole-desthio			Mefentrifluconazole		
Insert variant[a]	*N*	Mean EC_{50} (μg/mL)[b]	*SD*	RF[c]	Mean EC_{50} (μg/mL)	*SD*	RF
0	10	0.004	0.004	–	0.0004	0.001	–
−104, ins168	1	0.016	–	3.9	0.001	–	3.1
−124, ins187	1	0.014	–	3.4	0.0002	–	0.5
−104, ins203	1	0.031	–	7.5	0.003	–	5.9
−99, ins237	21	0.021	0.009	5.0	0.005	0.003	10.1
−382, ins360	1	0.012	–	2.9	0.004	–	9.4
−80, ins736	1	0.049	–	11.8	0.007	–	14.7

Abbreviation: RF, resistance factor.

[a]Given as the position of the insert (downstream orientation) relative to the *CYP51* start codon and size of the insert in bp.

[b]EC_{50} values calculated as the mean of two technical replicates for each isolate × fungicide combination.

[c]For each fungicide, RF was calculated as the ratio of the mean EC_{50} for a given *CYP51* promoter insert variant divided by the mean EC_{50} of isolates with no *CYP51* promoter insert.

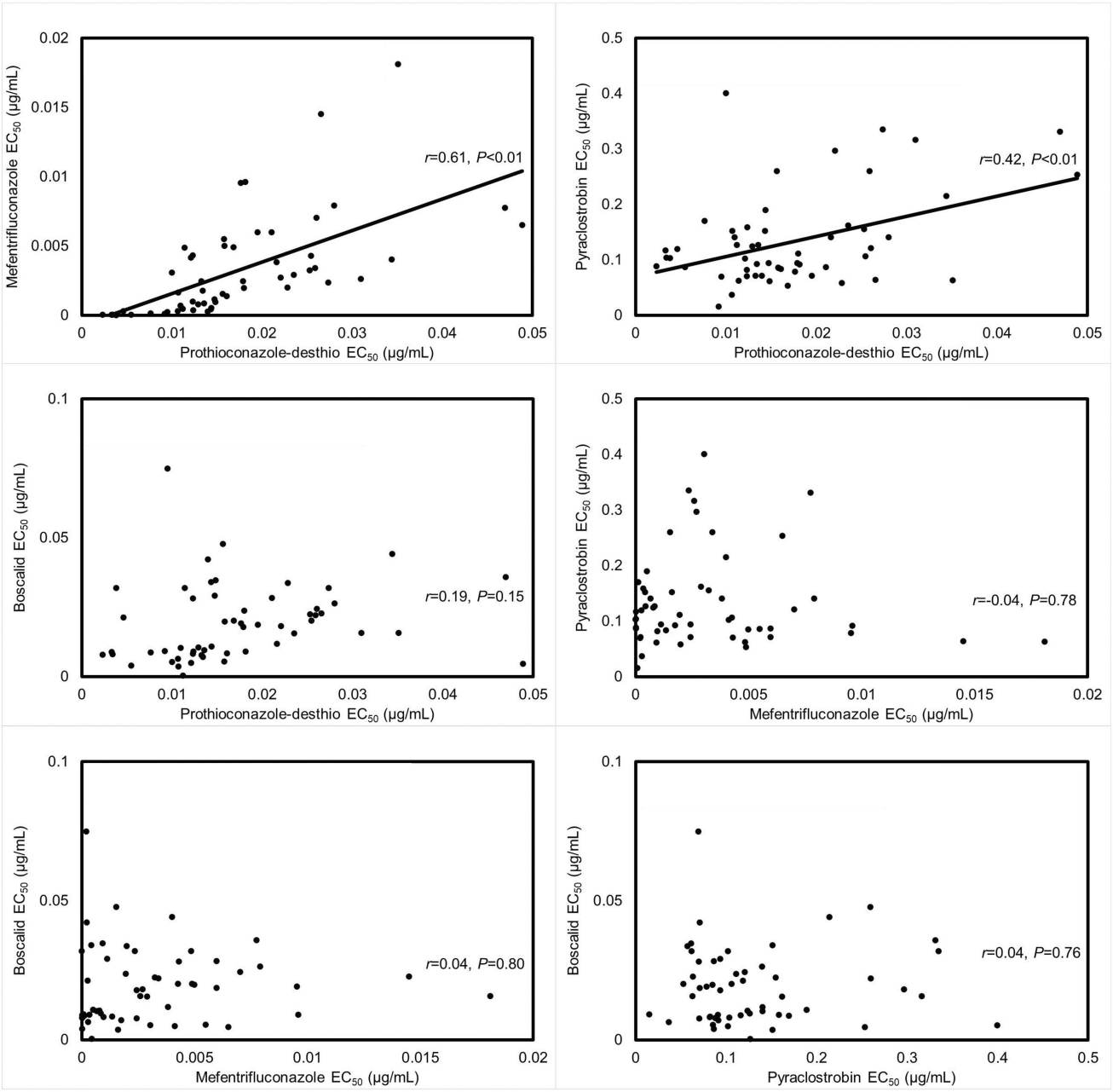

FIGURE 2 Cross resistance plots of four different fungicides screened in vitro against 55 western European isolates of *Plenodomus lingam* collected from oilseed rape in 2022–2023. The strength, direction and statistical significance (Pearson correlation coefficient tests) of pairwise correlations of EC_{50} values obtained for isolates between each of the four different fungicides tested (prothioconazole-desthio, mefentrifluconazole, pyraclostrobin and boscalid) are indicated.

OR961466). The 237 bp insert showed 100% identity to the same-sized insert identified previously in Australian isolate 20BL196 (−99, ins237). The final variant identified, a 187 bp insert found for a single UK isolate (23ROSELM03; GenBank accession: OR961464), was distinct with no identity to the *Sahana* TE, and instead sharing approximately 98.4% identity to a previously identified variant identified from Australian isolate 20BL200 (GenBank accession: OP344490); multiple copies of this 187 bp sequence sharing 90%–100% identity are distributed throughout the *P. lingam* genome.

Comparisons of in vitro sensitivity to DMI fungicides of 36 *P. lingam* isolates with different *CYP51* promoter inserts (confirmed by sequencing) revealed that for prothioconazole-desthio, RFs of isolates with an insert of 168/187/203/237/360 bp were all broadly similarly sensitive (approximately 3- to 7-fold decrease; Table 4). The single isolate with the larger 736 bp insert was, however, the least sensitive (RF about 12). For the DMI mefentrifluconazole, similar overall results were obtained, with the exception of a single isolate with a 187 bp insert that was found to be more sensitive (with a RF about 0.5). Isolates with an insert of 168/203/237/360 bp all exhibited RFs of approximately 3- to 10-fold decrease; the single isolate with the larger 736 bp insert was again the least sensitive (RF about 15-fold decrease).

TABLE 5 Variants of *CYP51* promoter inserts, as determined by sequencing, and their proportion in 34 European *Plenodomus lingam* isolates (collected 2022–2023) from four countries.

		CYP51 promoter insert variant						
Country	***N***	**No insert**	**−104, ins168[a]**	**−124, ins187**	**−104, ins203**	**−99, ins237**	**−382, ins360**	**−80, ins736**
France	2	2	–	–	–	–	–	–
Germany	8	–	–	–	1	7	–	–
Ireland	5	–	–	–	–	5	–	–
United Kingdom	19	3	1	1	–	10	2	2
Total	34	5	1	1	1	22	2	2

Note: The number of isolates of each genotype are indicated.

[a]Given as the position of the insert (downstream orientation) relative to the *CYP51* start codon and size of the insert in bp.

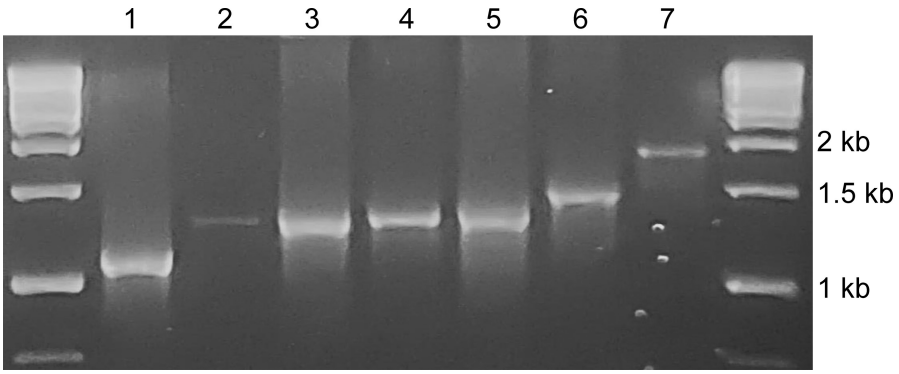

FIGURE 3 Six *CYP51* promoter inserts present in different European isolates of *Plenodomus lingam* collected from oilseed rape crops in 2022–2023. The *CYP51* promoter region was amplified using PCR primers EPS1F/EPS6R. Full details of isolates tested are given in Table 3. Representative isolates shown for lanes 1–7 are (1) 23ESXLM04 (no insert, i.e., wild type), (2) 23ROSELB03 (−104, ins168 bp), (3) 23ROSELM03 (−124, ins 187 bp), (4) 22LAD04 (−104, ins203 bp), (5) 22GERSH03 (−99, ins237 bp), (6) 23NWMKTLM08 (−382, ins360 bp) and (7) 22HERT02 (−80, ins736 bp), where insert variants are given as the position of the insert (downstream orientation) relative to the *CYP51* start codon and size of the insert in bp. The no-template water control was blank (data not shown). Molecular marker sizes are indicated to the right.

3.4 | *CYP51* gene coding region for *P. lingam* isolates

An identical 1581 bp sequence of high quality covering most of the *CYP51* gene coding region was obtained (using primers PL_KK_CYP51_F/PL_KK_CYP51_R) for all five 2022–2023 *P. lingam* isolates screened (22HERTS02, 23NWMKTLM08, 22LAD04, 23ROSELB03, 22MACK05; Table 3); this sequence had 100% identity to a sequence in GenBank (Australian isolate 21BL211; GenBank OP344492; position spanned 804–2384). High quality consensus sequence was obtained that encoded 509 amino acids (out of 527 total) of *CYP51*; no target site mutations that would have led to *CYP51* amino acid alterations were detected.

3.5 | PCR screening of *P. biglobosus* isolates for possible *CYP51* promoter inserts

For all *P. biglobosus* isolates screened, a similarly sized amplicon of about 805 bp was obtained using primers KK2F/R targeted to the *CYP51* promoter region (Figure 4). This amplicon was predicted to include 691 bp of sequence upstream of the predicted *CYP51* start codon. Testing included a mixture of older isolates (1996–2007) collected from different geographic locations throughout the world, and modern isolates (2021–2023) collected from throughout the United Kingdom and from France. Sequencing of representative isolates found no promoter inserts upstream of *CYP51* in *P. biglobosus* (data not shown). The amplified product from one representative isolate (23DERELB01) was sequenced, and the partial product obtained (786 bp) showed 100% identity to the *CYP51* region, predicted by BLAST analyses (see methods), of the *P. biglobosus* sequence (GenBank accession FO905635, positions 23038–23823). Lastly, a similarly sized amplicon of about 805 bp was obtained for the single isolate of *P. dezfulensis* tested, indicating the absence of *CYP51* promoter inserts in this recently described species, but more isolates of this species need to be tested to draw any conclusions about its level of resistance to DMIs.

4 | DISCUSSION

In comparison to a collection of baseline isolates (BASELINE 92-05), statistically significant 6- to 7-fold reductions of in vitro sensitivity to the DMI fungicide prothioconazole-desthio were

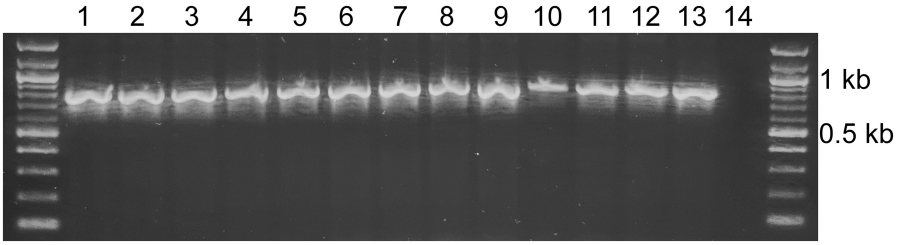

FIGURE 4 Amplification of the *CYP51* promoter region of six older (1996–2007) and seven modern (2022–2023) isolates of *Plenodomus biglobosus* (and a single isolate of the related *P. dezfulensis*) reveal no evidence for promoter inserts. Amplification of the *CYP51* promoter region used PCR primers KK2F/KK2R. Full details of isolates tested are given in Table 2. Representative isolates shown for lanes 1–13 are (1) 05-70, (2) gui 2b3, (3) VERB31, (4) ARMC39, (5) PL2002-3, (6) UK4, (7) 22FRA/B/05, (8) 22FRA/B/08, (9) 22BLACK01, (10) 23JAME/LB/04, (11) 23DERE/LB/01, (12) 23NWMKT LB02 and (13) SCUA-Ahm-S41. Lane 14 was a no-template water control. Molecular marker sizes are indicated to the right.

measured for *P. lingam* populations sampled from OSR in Germany (GER 22/23), Ireland (IRE 22) and the United Kingdom (UK 22/23), although not in the French (FRA 22) population, which remained similarly sensitive but only contained three isolates. Decreased sensitivity to the DMI mefentrifluconazole was also evident and was statistically significant for the GER 22/23 but not for the IRE 22 and UK22/23 populations. Positive cross resistance was found between both fungicides, as could be expected given their similar MOA targeting CYP51. Past research found no evidence for substantive resistance to DMI fungicides in western European pathogen populations, including the United Kingdom (Huang et al., 2011). Thus, the present study represents the first confirmation, alongside the results of Scanlan et al. (2024), of decreased sensitivity to DMIs evolving in western European *P. lingam* pathogen populations. Although we acknowledge that the five older international *P. lingam* isolates (collected 1992–2005, including two from Europe), all of which lacked *CYP51* promoter inserts, used to establish baseline sensitivity may not be entirely representative of the European *P. lingam* population at that time, it is noted that all five gave broadly similar results in sensitivity testing. The emergence of decreased DMI sensitivity in this geographic region is not surprising given the strong selective pressure imposed on pathogen populations there through extensive application of this chemistry to OSR crops. For example, there was an area of about 536,000 ha of formulation treated (prothioconazole and/or tebuconazole) in the United Kingdom in 2022 (Ridley et al., 2023). These findings are congruent with the identification of isolates with decreased DMI sensitivity in *P. lingam* populations recently sampled in Europe (Fajemisin et al., 2022; Scanlan et al., 2024) and Australia (Van de Wouw et al., 2017, 2021).

Sequencing of the *CYP51* coding region of five representative *P. lingam* isolates (each carrying a different upstream *CYP51* promoter insert) revealed all exhibited 100% identity to sequences previously deposited into GenBank (e.g., OP344492); thus, there is no evidence for target site mutations leading to *CYP51* amino acid alterations. The reason for the lack of *CYP51* coding region variation in *P. lingam* reported in the present and other studies (Huang et al., 2011; Scanlan et al., 2023, 2024) remains unclear, but may, in part, be due to the monocyclic nature of this disease; in contrast, multiple CYP51 target protein alterations have evolved in populations of *Zymoseptoria tritici* and *Pyrenopeziza brassicae* that cause polycyclic diseases in wheat and brassicas, respectively (Carter et al., 2014; Cools & Fraaije, 2008). However, amplification of the promoter region upstream of *CYP51* in the present study revealed that *P. lingam* isolates with decreased DMI sensitivity are all associated with the presence of variably sized promoter inserts, ranging from around 200 to 800 bp in length, that are absent in sensitive isolates. Identical *CYP51* promoter inserts have previously been shown to be linked to increased *CYP51* expression and decreased DMI sensitivity in Australian isolates of *P. lingam* (Yang et al., 2020). Our study of the *CYP51* promoter region revealed six different insert variants (168, 187, 203, 237, 360 and 736 bp) in *P. lingam* isolates collected from OSR crops in Germany, Ireland and the United Kingdom in 2022–2023. Isolates carrying these inserts (−104, ins168; −124, ins187; −104, ins203; −99, ins237; and −382, ins360) were about 3- to 7.4-fold less sensitive to prothioconazole-desthio and about 3- to 10-fold less sensitive to mefentrifluconazole in comparison with isolates lacking an insert. One isolate carrying a longer 736 bp insert (−80, ins736) showed the highest reduction in DMI sensitivity, approximately 12-fold to prothioconazole-desthio and 15-fold to mefentifluconazole in comparison to the sensitivity of baseline isolates. Further studies on phenotype-to-genotype relationships with a larger number of *P. lingam* isolates are needed to determine whether the variously sized inserts identified are associated with different levels of decreased DMI sensitivity.

The RFs for the European isolates carrying *CYP51* promoter inserts are broadly similar to the 2.4- to 5.1-fold increase in RFs described for Australian isolates with inserts (Van de Wouw et al., 2021). They are also consistent with those described (typically often RFs of about 6–12) for other economically important ascomycete fungal crop pathogens (e.g., *Z. tritici*, *P. brassicae*; Carter et al., 2014; Cools et al., 2012; King et al., 2021), for which insertions in the *CYP51* promoter region have previously been linked to decreased DMI sensitivity. It remains unclear to what extent the reduction of in vitro DMI sensitivity levels identified for *P. lingam*, associated with *CYP51* overexpression, might impact on the efficacy of DMI fungicides to control Phoma leaf spot and stem canker in

the field. Other studies on Septoria tritici blotch have been able to quantify the crop yield losses associated with target site (amino acid) alterations caused by mutations in the *CYP51* coding region, but such RFs are typically much larger than those identified for *P. lingam* and the contribution of an overexpression of efflux pump might also contribute to this (Young et al., 2021).

Based on our data set of 34 randomly selected *P. lingam* isolates collected from western Europe in 2022–2023, the majority (85.3%) carried a promoter insert (−104, ins168; −124, ins187; −104, ins203; −99, ins237; −382, ins360; −80, ins736), while isolates lacking a promoter insert were uncommon. Specifically, the majority of the *P. lingam* isolates screened from Germany (8/8, 100%), Ireland (5/5, 100%) and the United Kingdom (16/19, 84.2%) all carried a promoter insert. These data are broadly consistent with those of Scanlan et al. (2024) who also report that the majority of modern German (collected 2018–2020; 18/19, 94.7%) and Czech Republic (collected 2017, 2019–2020, 7/8, 87.5%) *P. lingam* isolates also carried an insert. In contrast, neither of the two French isolates sequenced in the present study (collected 2022) nor the 14 French isolates of Scanlan et al. (2024) (collected 2012–2013, 2017–2019) carried a promoter insert. It is possible that the variation in *CYP51* promoter insert frequency between *P. lingam* populations from different geographic locations might be due to differences in DMI use and consequent selective pressure imposed on the pathogen populations resident there.

Most of the *CYP51* promoter inserts identified in the western European *P. lingam* populations examined (e.g., −104, ins168; −124, ins187; −104, ins203; −382, ins360 and −80, ins736) were found rarely, being present in one or two isolates at a specific geographic location. However, a 237 bp *CYP51* promoter insert variant (−99, ins237) was detected in the majority of isolates (64.7%). This variant of the *CYP51* promoter insert had a widespread geographic distribution, being detected at multiple localities in Germany (Grambow, Holstein and Niedersachsen), Ireland (Carlow), and throughout England in the United Kingdom (Essex, Herefordshire, Norfolk, Suffolk and Surrey). This variant (−99, ins237) is identical to a variant previously reported in Australian isolates (e.g., 20BL196; also −99, ins237), and is based on a partial fragment of the *Sahana* TE (Van de Wouw et al., 2021). Work by Scanlan et al. (2024) identified only the 237 bp insert variant in the modern German and Czech *P. lingam* populations tested. It is unclear at present whether this variant might have emerged independently in Australian and European *P. lingam* populations, and/or whether introduction events between the continents might have occurred. Work by Scanlan et al. (2023), based on application of deep-amplicon sequencing-based approaches to populations of ascospores, estimates that this 237 bp variant of the promoter insert has a frequency of >10% in some of the Australian populations, and is the predominant insert variant identified in some populations there. The other variants of *CYP51* promoter insert that were identified less frequently in western European isolates tested in this study (168, 203, 360 and 736 bp) are also all associated with partial *Sahana* inserts. This contrasts markedly with Australian *P. lingam* populations, were several variants of *CYP51* promoter insert are derived from large fragments of the *Zolly* TE (Van de Wouw et al., 2021).

Compared to *P. lingam*, less research has been undertaken on *P. biglobosus*. In this study, no *CYP51* promoter inserts were detected in the screening of 34 baseline and modern *P. biglobosus* isolates collected from a broad geographic range (along with a single isolate of the recently described and closely related *P. dezfulensis*; Safi et al., 2021). The presence of target site alterations was not investigated further for *P. biglobosus*, as no isolate showed a significant level of DMI insensitivity (RF ≥ 5) that is commonly associated with this resistance mechanism. The in vitro fungicide sensitivity screening suggested only minor differences in sensitivity between less-sensitive *P. biglobosus* isolates versus more-sensitive *P. lingam* isolates (isolates without *CYP51* promoter inserts). These differences were statistically significant for prothioconazole-desthio ($p < 0.01$) but not for mefentrifluconazole ($p = 0.34$). Conversely, there were differences between *P. biglobosus* isolates and less-sensitive *P. lingam* isolates (isolates with *CYP51* promoter inserts), these differences being borderline statistically significant for prothioconazole-desthio ($p = 0.056$) and statistically significant for mefentrifluconazole ($p < 0.01$). Overall, these results are consistent with past studies showing that *P. lingam* isolates (older isolates probably lacking an insert) were slightly more sensitive (Eckert et al., 2010), or similarly sensitive (Sewell et al., 2017), to DMI fungicides than *P. biglobosus*. It is likely that any minor intrinsic differences in DMI sensitivity between *Plenodomus* spp., due to differential interactions of DMI with slightly different CYP51 proteins, are likely to be masked by the presence of *CYP51* promoter inserts associated with decreased sensitivity in *P. lingam*. Given the combined results that (1) *P. lingam* isolates with inserts appear more common than those without inserts in recent pathogen populations collected from Germany, Ireland and the United Kingdom, and (2) *P. lingam* with inserts appear slightly less sensitive to DMI fungicides, it is possible that application of DMI fungicides to crops may influence the overall *Plenodomus* spp. population structure with stronger selection for *P. lingam*. This contrasts with reports of the increased importance of *P. biglobosus* in Phoma disease outbreaks (Huang et al., 2014), but other factors such as resistance breeding providing improved control of *P. lingam* in comparison with *P. biglobosus* might be responsible for this and needs further study. Population biology of *Plenodomus* spp. needs further consideration in OSR resistance breeding trials: for instance, in the UK recommended list trials, Phoma stem canker ratings are reported for OSR cultivars yet no distinction between the causal pathogen(s) are made.

The results of our study show that western European isolates of *P. lingam* and *P. biglobosus* have remained sensitive to the QoI pyraclostrobin, indicating that this MOA continues to be an important tool in Phoma disease management. No statistically significant differences were found between baseline (1992–2005) and modern (2022–2023) *P. lingam* isolates. Interestingly, positive cross resistance (albeit with only weak strength) was detected for *P. lingam* isolates between the QoI pyraclostrobin and the DMI prothioconazole-desthio ($r = 0.42$), although not mefentrifluconazole ($r = -0.04$). These findings provide support to other reports of positive cross resistance, and the hypothesis proposed by Van de Wouw et al. (2021) that pre-existing, widespread resistance to DMI fungicides may have allowed for the

evolution of resistance to Veritas (a formulated fungicide mixture containing tebuconazole [DMI] and azoxystrobin [QoI]) by selecting for a polymorphism associated with low levels of insensitivity to both fungicides. Multidrug resistance associated with overexpression of efflux pumps has been found in other plant pathogens, such as *Z. tritici* (Omrane et al., 2017), but further work is required to identify the genetic basis of this low level of insensitivity towards multiple fungicides in *P. lingam* isolates.

Finally, no evidence was found in this study for decreased sensitivity to the SDHI boscalid in western European isolates of *P. lingam* and *P. biglobosus* isolates, thus supporting the use of this MOA for effective control of Phoma. Proactive monitoring for any future emergence of decreased sensitivity to SDHIs in *P. lingam* and *P. biglobosus* populations is needed, given that resistance has already evolved to this MOA in other phytopathogenic fungi (Sierotzki & Scalliet, 2013). It is essential that strategies for resistance management are communicated to growers, and the use of different MOAs in mixtures and alternation throughout the fungicide programme is implemented, to slow selection for fungicide-insensitive strains.

ACKNOWLEDGEMENTS

This project was supported by joint funding from BASF Plc. and the Biotechnology and Biological Sciences Research Council (BBSRC; project BB/X011917/1). We also acknowledge funding from the UK BBSRC, via the Growing Health Institute Strategic Programme (BB/X010953/1; BBS/E/RH/230003A) and Resilient Farming Futures ISP (BB/X010961/1; BBS/E/RH/230004A). The authors thank Jack Scanlan, Alexander Idnurm and Angela Van de Wouw (University of Melbourne, Australia) for communication of their unpublished results to us.

DATA AVAILABILITY STATEMENT

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

ORCID

Kevin M. King https://orcid.org/0000-0002-9928-1353
Gail Canning https://orcid.org/0000-0001-5242-3812
Faye Ritchie https://orcid.org/0000-0001-9642-1712
Steven Kildea https://orcid.org/0000-0001-8240-6343
Jonathan S. West https://orcid.org/0000-0002-5211-2405

REFERENCES

Carter, H.E., Fraaije, B.A., West, J.S., Kelly, S.L., Mehl, A., Shaw, M.W. et al. (2014) Alterations in predicted regulatory and coding regions of the sterol 14α-demethylase gene (*CYP51*) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*. *Molecular Plant Pathology*, 15, 513–522.

Cools, H.J., Bayon, C., Atkins, S., Lucas, J.A. & Fraaije, B.A. (2012) Overexpression of the sterol 14α-demethylase gene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science*, 68, 1034–1040.

Cools, H.J. & Fraaije, B.A. (2008) Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Management Science*, 64, 681–684.

Dhingra, O.D. & Sinclair, J.B. (1985) *Basic plant pathology methods*. Boca Raton, FL: CRC Press, Inc.

Eckert, M.R., Rossall, S., Selley, A. & Fitt, B.D.L. (2010) Effects of fungicides on in vitro spore germination and mycelial growth of the phytopathogens *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker of oilseed rape). *Pest Management Science*, 66, 396–405.

Fajemisin, O., Mazáková, J. & Ryšánek, P. (2022) Emergence of fungicide sensitivity in *Leptosphaeria maculans* isolates collected from the Czech Republic to DMI fungicides. *Agriculture*, 12, 237.

Fitt, B.D.L., Brun, H., Barbetti, M.J. & Rimmer, S.R. (2006) World-wide importance of Phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology*, 114, 3–15.

Huang, Y.J., Hood, J.R., Eckert, M.R., Stonard, J.F., Cools, H.J., King, G.J. et al. (2011) Effects of fungicide on growth of *Leptosphaeria maculans* and *L. biglobosa* in relation to development of phoma stem canker on oilseed rape (*Brassica napus*). *Plant Pathology*, 60, 607–620.

Huang, Y.J., Karandeni-Dewage, C.S. & Fitt, B.D.L. (2014) Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK. *Aspects of Applied Biology*, 127, 117–122.

King, K.M., Bucur, D.E., Ritchie, F., Hawkins, N.J., Kaczmarek, A.M., Duan, Y. et al. (2021) Fungicide resistance status and chemical control options for the brassica pathogen *Pyrenopeziza brassicae*. *Plant Pathology*, 70, 2086–2103.

Liu, S.Y., Liu, Z., Fitt, B.D.L., Evans, N., Foster, S.J., Huang, Y.J. et al. (2006) Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathology*, 55, 401–412.

Liu, Z., Latunde-Dada, A.O., Hall, A.M. & Fitt, B.D.L. (2014) Phoma stem canker disease on oilseed rape (*Brassica napus*) in China is caused by *Leptosphaeria biglobosa* 'brassicae'. *European Journal of Plant Pathology*, 140, 841–857.

Mendes-Pereira, E., Balesdent, M.-H., Brun, H. & Rouxel, T. (2003) Molecular phylogeny of the *Leptosphaeria maculans–L. biglobosa* species complex. *Mycological Research*, 107, 1287–1304.

Omrane, S., Audéon, C., Ignace, A., Duplaix, C., Aouini, L., Kema, G. et al. (2017) Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *mSphere*, 2, e00393-17.

Ridley, L., Parrish, G., Chantry, T., Richmond, A., MacArthur, R. & Garthwaite, D. (2023) *Arable crops in the United Kingdom 2022: pesticide usage survey report 309*. Sand Hutton: Fera.

Safi, A., Mehrabi-Koushki, M. & Farokhinejad, R. (2021) *Plenodomus dezfulensis* sp. nov. causing leaf spot of rapeseed in Iran. *Phytotaxa*, 523, 141–154.

Scanlan, J.L., Idnurn, A. & Van de Wouw, A.P. (2024) Genome-wide mapping in an international isolate collection identifies a widespread *erg11/CYP51* promoter insertion associated with fungicide resistance in the canola blackleg pathogen *Leptosphaeria maculans*. *Plant Pathology*. https://doi.org/10.1111/ppa.13898

Scanlan, J.L., Mitchell, A.C., Marcroft, S.J., Forsyth, L.M., Idnurm, A. & Van de Wouw, A.P. (2023) Deep amplicon sequencing reveals extensive allelic diversity in the *erg11/CYP51* promoter and allows multi-population DMI fungicide resistance monitoring in the canola pathogen *Leptosphaeria maculans*. *Fungal Genetics and Biology*, 168, 103814.

Sewell, T.R., Hawkins, N.J., Stotz, H.U., Huang, Y.J., Kelly, S.L., Kelly, D.E. et al. (2017) Azole sensitivity in *Leptosphaeria* pathogens of oilseed rape: the role of lanosterol 14α-demethylase. *Scientific Reports*, 7, 15849.

Sierotzki, H. & Scalliet, G. (2013) A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology*, 103, 880–887.

Van de Wouw, A.P., Elliott, V.L., Chang, S., López-Ruiz, F.J., Marcroft, S.J. & Idnurm, A. (2017) Identification of isolates of the plant

pathogen *Leptosphaeria maculans* with resistance to the triazole fungicide fluquinconazole using a novel in planta assay. *PLoS One*, 12, e0188106.

Van de Wouw, A.P., Scanlan, J.L., Marcroft, S.J., Smith, A.J., Sheedy, E.M., Perndt, N.W. et al. (2021) Fungicide sensitivity and resistance in the blackleg fungus, *Leptosphaeria maculans*, across canola growing regions in Australia. *Crop and Pasture Science*, 72, 994–1007.

West, J.S., Kharbanda, P.D., Barbetti, M.J. & Fitt, B.D.L. (2001) Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology*, 50, 10–27.

Yang, Y., Marcroft, S.J., Forsyth, L.M., Zhao, J., Li, Z. & Van de Wouw, A.P. (2020) Sterol demethylation inhibitor fungicide resistance in *Leptosphaeria maculans* is caused by modifications in the regulatory region of *ERG11*. *Plant Disease*, 104, 1280–1290.

Young, C., Boor, T., Corkley, I., Fraaije, B., Clark, B., Havis, N. et al. (2021) *Managing resistance evolving concurrently against two or more modes of action to extend the effective life of new fungicides*. AHDB project report 637. Available from: https://projectblue.blob.core.windows.net/media/Default/Research%20Papers/Cereals%20and%20Oilseed/2021/PR637%20final%20project%20report.pdf [Accessed 1st March 2024].

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: King, K.M., Barr, L., Bousquet, L., Glaab, A., Canning, G., Ritchie, F. et al. (2024) Evolution of decreased sensitivity to azole fungicides in western European populations of *Plenodomus lingam* (Phoma stem canker on oilseed rape). *Plant Pathology*, 00, 1–16. Available from: https://doi.org/10.1111/ppa.13897