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ORIGINAL RESEARCH ARTICLE

Emergence of *Cladosporium macrocarpum* disease in canola (*Brassica napus*)

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Abstract

New disease symptoms were observed on canola (*Brassica napus*) crops late in the 2019 and 2020 growing seasons in Western Australia. *Cladosporium macrocarpum* was isolated from infected material, and the fungus responsible for the symptoms was demonstrated by fulfilling Koch's postulates. One strain exhibited relatively high tolerance to prothioconazole and tebuconazole fungicides compared to other canola pathogens *Sclerotinia sclerotiorum* and *Leptosphaeria maculans*. In a 2019 field trial in a commercial canola crop, symptom incidence caused by *Cladosporium* was only significantly reduced by a double application of a foliar fungicide (active ingredients prothioconazole and tebuconazole) registered in canola for other diseases, while single applications gave no significant response. This new disease, caused by a ubiquitous fungal species, may be a consequence of changes to farm management strategies to reduce other fungal diseases of canola.

Keywords *Cladosporium brassicae* · Fungicide resistance · Rapeseed · Upper canopy infection

Introduction

Diseases impact the production of the oilseed crop canola (or rapeseed; *Brassica napus*) wherever it is cultivated around the world. In Australia, the most destructive disease is blackleg, caused primarily by the fungus *Leptosphaeria maculans* or a lesser extent by *Leptosphaeria biglobosa* (Van de Wouw et al. 2016b). Other diseases are also present in Australia of variable levels of severity depending on geographic location and seasonal conditions (Van de Wouw et al. 2016a). One trend has been the increase in diseases of the aerial parts of mature plants, such as Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* or upper canopy infection caused by *L. maculans* (Derbyshire and Denton-Giles 2016; Sprague et al. 2017).

In the 2019 and 2020 growing seasons, growers and agronomists in Western Australia (WA) noticed lesion symptoms on stems, branches and pods that were inconsistent with known canola diseases in Australia. The current study aimed to identify the cause.

Materials and methods

Fungal isolation and culturing

To isolate the causative organism on diseased canola, infected tissues were surface sterilized in diluted bleach (1.2% available chlorine; LabCo®) for 5 min, washed in sterile water, then excised with a scalpel and plated onto potato dextrose agar (PDA) supplemented with antibacterial chemicals cefotaxime (100 µg/ml) and chloramphenicol (30 µg/ml). Alternatively, diseased tissue was washed in sterile water to release spores, and then plated onto PDA supplemented with antibiotics. Emergent fungal colonies were subcultured onto the same medium by streaking spores with a metal loop, and isolating colonies derived from single spores.

One strain (UoM19-17) was tested for its ability to grow in the presence of two demethylation inhibitor (azole) fungicides, which target ergosterol biosynthesis, and are registered for use against Sclerotinia stem rot and commonly used to control upper canopy infection caused by *L. maculans*. The concentrations of tebuconazole and prothioconazole to

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inhibit growth on PDA were tested over a range of two-fold dilutions from 0.125 to 32 µg/ml. Colony diameters were measured and the data fit with a symmetrical sigmoidal model in MyCurveFit.com to estimate the effective concentration for inhibition of growth by 50% (EC_{50}).

For competition with other fungi, strain UoM19-17 was inoculated on PDA ~ 0.5 cm away from *L. maculans* strain M1 (alternative names for this strain are IBCN18 or D5).

Plant inoculations

The strains were cultured on PDA, and the surface scraped with a scalpel and placed in 0.5% Tween 20 solution, then spores filtered through miracloth. Drops of 10 µl containing 500 spores were placed at different intervals, from the base to below the start of flower production on stems of *B. napus* cv. Westar at the flowering stage of development and allowed to run downwards. Canola cotyledons (two-week old seedlings), and leaves and seed pods on mature plants were also inoculated. Wounding of the plant tissues was not used, and relative humidity was not altered. The negative control was 0.5% Tween 20 solution.

Strain UoM19-17 was used primarily for disease testing. Three weeks after inoculation of a stem with this strain, the wounded site was excised, surface sterilised as described above, and plated onto PDA containing antibiotics to reisolate the fungus.

Light and scanning electron microscopy

Spores were examined by light and scanning electron microscopy.

Infected plant samples were rinsed with ethanol or bleach solution then incubated overnight with a wet paper towel, and transferred with sticky tape into lactic glycerol and a coverslip. Spores were examined with a Leica DM3000 compound microscope. Alternatively, spores were washed from actively growing cultures, filtered through miracloth, concentrated by centrifugation, and then mounted on a glass slide and examined with a Leica DM6000 compound microscope.

Strain UoM19-17 was cultured on nitrocellulose membrane placed on PDA, then transferred to a stub, sputter coated with gold using a Quorum 150 T ES plus machine, and examined in a Hitachi TM4000Plus scanning electron microscope.

DNA sequence generation and analysis

The strains were cultured in potato dextrose broth, then freeze-dried mycelia disrupted with glass beads and genomic DNA extracted (Pitkin et al. 1996). The internal transcribed spacer (ITS) regions were amplified with primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White et al. 1990), and the amplicons sequenced, as this information is a common approach for the efficient identification of fungal species (Schoch et al. 2012).

To generate additional DNA markers, a low level of coverage of the genome of strain UoM19-17 was obtained as 150 nucleotide paired-end reads using an Illumina NovaSeq 6000 instrument from genomic DNA processed by the Murdoch Children's Research Institute (Melbourne, Victoria). Reads were analysed using Geneious version 8.1.9 (Kearse et al. 2012) by alignment and reiterative assemblies to four gene regions used most commonly for *Cladosporium* phylogenetic comparisons (i.e. fragments of genes encoding actin, translation elongation factor 1, histone H3 and calmodulin). Sequences were submitted to GenBank, with the accessions provided in Table 1.

DNA sequences from strains in the *Cladosporium herbarum* species complex were obtained from GenBank (as in Table 2). *Cladosporium versiforme* was used in initial alignments and analyses, but subsequently excluded due to the availability of sequences for only two gene regions, and evidence that the sequences of the Western Australian strains did not match this species. The four strains of *C. macrocarpum* and *C. herbarum* were selected to represent nucleotide diversity within these species, as defined by Schubert et al. (2007). The new and previously obtained sequences were aligned using ClustalW, the alignments adjusted manually, then concatenated and phylogenetic trees produced in MEGA 7 (Kumar et al. 2016).

The *TEF1* region features multiple polymorphisms that distinguish *C. macrocarpum* and *C. herbarum*, and therefore a region of this gene was amplified with primers MAI0760 (5´-GCACATCCGAACACAATGAC-3´) and MAI0761 (5´-CTTGACACCGAGAGTGTAGG-3´) from the four other strains and sequencing using Sanger chemistry (GenBank accessions are provided in Table 1).

Field trials

The first focus on disease development was at Alma (near Northampton, Western Australia) in 2019 in a field trial conducted in a commercial canola crop designed to explore the management of foliar diseases. The paddock of Pioneer® 43Y23 RR was sown by the grower at 1.4 kg/ha with an emergence of an average of 30 plants/m^2. Trial plots were 20 m × 1.8 m, with a buffer area of crop in between plots, and the trial had a randomized complete block design. Treatments in the trial were a range of timings of application of a foliar fungicide registered in canola at the time for Sclerotinia stem rot. Prosaro® (prothioconazole 210 g/L, tebuconazole 210 g/L) at 450 mL/ha was

Table 1 Details of five *C. macrocarpum* strains isolated from *B. napus* in this study

Name	Origin	Plant material	Culture collection designations	GenBank DNA sequence accessions
UoM19-17	Alma, WA, November 2019	Stem (Pioneer® 43Y23 RR)	VPRI 44040; CBS 147892; WAC 14428	MW646553 (actin), MW646550 (calmodulin), MW646552 (histone H3), MW629802 (ITS), MW646551 (translation elongation factor),
UoM20-4	Greenough, WA, September 2020	Pod (Pacific Seeds® Hyola® 404 RR)	VPRI 44041; CBS 147893; WAC 14429	MW646554 (translation elongation factor)
UoM20-5	Mingenew, WA, September 2020	Pod (unknown; observed on multiple entries in the GRDC National Variety Trials™)	VPRI 44042; CBS 147894; WAC 14430	MW646556 (translation elongation factor)
UoM20-10	Wittenoom Hills, WA, October 2020	Stem (Nuseed® HyTTec® Trophy)	VPRI 44043; CBS 147895; WAC 14431	MW646555 (translation elongation factor)
UoM21-1	Dale, WA, September, 2020	Pod (Nuseed® ATR Bonito)	VPRI 44096; CBS 147896; WAC 14432	MW881222 (translation elongation factor)

applied in 100 L/ha water volume with a boom spray at 0.5 m above crop height. Fungicide treatments were applied at: 20% bloom stage of crop flower development (single application), 50% bloom stage (single application), and at both 20% and 50% bloom stages (i.e. a double application of fungicides). There were four replicates of each treatment, and eight replicates of the untreated plots. Disease incidence was assessed in the trial during pod ripening just prior to crop maturity by assessing forty plants (ten random plants at four sites) per plot for presence of the dark lesions on the plants. Symptom severity was not scored. At maturity, plots were harvested using a small plot harvester, yield per plot recorded and a 1 kg sample of grain from each plot retained to assess grain quality measures (percentage moisture, oil, and 1000 grain weight). Yield, oil content and 1000 grain weight were standardized to 6% moisture content. Results were analysed with Genstat 19th Edition statistical software (VSN Intl. Ltd).

A similar field trial was conducted at Dale (south east of Perth, Western Australia) in 2020 in a commercial canola crop of Nuseed® ATR Bonito. Treatments in the trial were plus and minus fungicide application, with Prosaro® applied at 450 mL/ha at 20% bloom. Fungicide application methods, replicates, harvest methods, grain and data analysis were the same as at the Alma site. The plot sizes at Dale were reduced to 15 m in length and disease assessments performed on twenty plants (five plants at four sites) per plot during pod ripening for the presence of pod damage (no stem or branch lesions were noted at the site).

Table 2 Sequences used in phylogenetic analyses obtained in this study or from GenBank as generated by Schubert et al. (2007) and Bensch et al. (2015). The ITS sequences, commonly used in constructing phylogenetic trees, were excluded from comparisons due to lack of sequence variation among these species and strains

Species	Strain	Actin	Translation elongation factor	Histone H3	Calmodulin
Cladosporium macrocarpum	UoM19-17	MW646553	MW646551	MW646552	MW646550
Cladosporium macrocarpum	CBS 121623	EF679529.1	EF679453.1	EF679684.1	EF679606.1
Cladosporium macrocarpum	CBS 299.67	EF679526.1	EF679450.1	EF679680.1	EF679602.1
Cladosporium macrocarpum	CPC 11817	EF679527.1	EF679451.1	EF679681.1	EF679603.1
Cladosporium macrocarpum	CPC 12054	EF679528.1	EF679452.1	EF679682.1	EF679604.1
Cladosporium herbarum	CBS 121621	EF679516.1	EF679440.1	EF679670.1	EF679592.1
Cladosporium herbarum	CPC 12178	EF679517.1	EF679441.1	EF679671.1	EF679593.1
Cladosporium herbarum	CPC 11603	EF679514.1	EF679438.1	EF679668.1	EF679590.1
Cladosporium herbarum	CBS 111.82	EF679510.1	EF679433.1	EF679663.1	EF679586.1
Cladosporium versiforme	CPC 19053	KT600613.1	KT600515.1	n/a	n/a
Cladosporium variabile	CBS 121635	EF679557.1	EF679481.1	EF679711.1	EF679633.1
Cladosporium variabile	CBS 121636	EF679556.1	EF679480.1	EF679710.1	EF679632.1
Cladosporium iridis	CBS 138.40	EF679523.1	EF679447.1	EF679677.1	EF679599.1

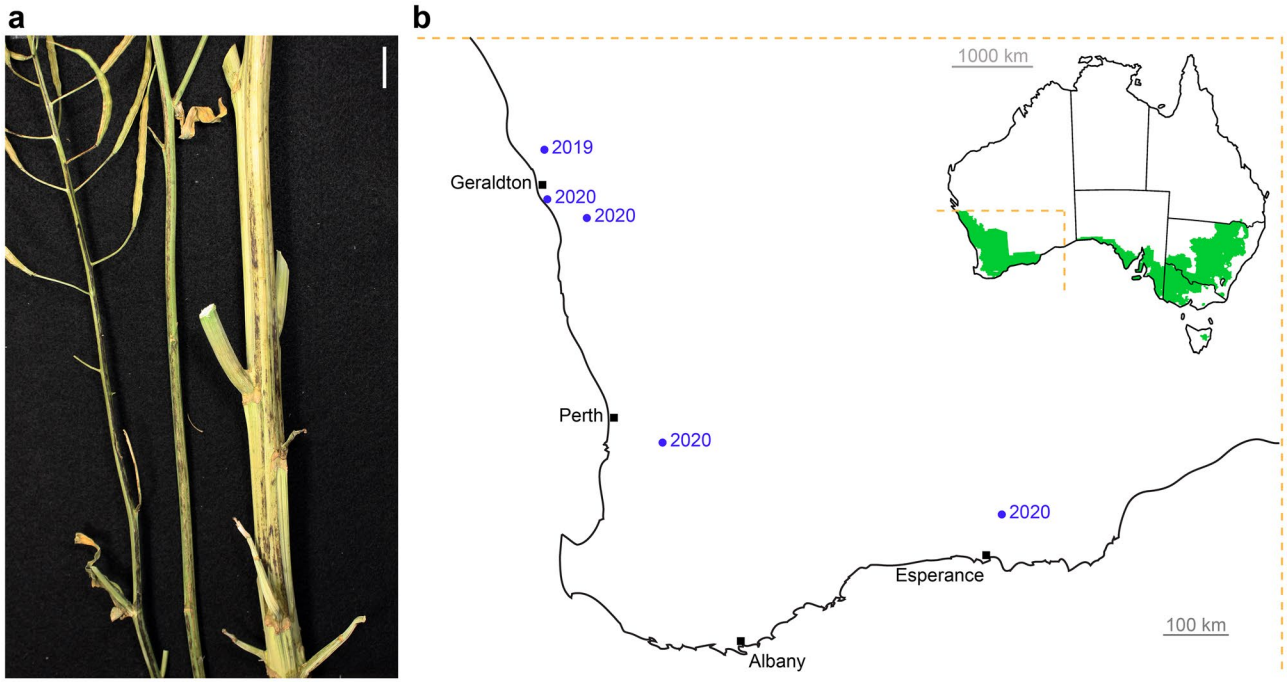

Fig. 1 Canola (*Brassica napus*) disease symptoms and locations of strain isolation in Western Australia. **a** Symptoms across different plant stages, from the top of the plant on the left to the bottom on the right, observed on plants collected from Wittenoom Hills in 2020. Scale bar = 2 cm. **b** Map of southern Western Australia and sites and years from which strains were obtained, relative to the rest of Australia (inset; shaded regions indicate canola cultivation areas as defined by production over 500 tonnes between 2010–2014, redrawn from the Australian Bureau of Statistics)

Results and discussion

In 2019 and 2020 dark lesions were observed on the main stems, branches and pods of *B. napus* crops in Western Australia that did not appear to be due to *L. maculans* or other common pathogenic fungi of canola (Fig. 1a). The disease symptoms were found in multiple canola varieties at several locations across the canola growing area of Western Australia, with these locations spaced over more than 900 km apart. Symptoms were observed in November, 2019 at Alma (north of Geraldton), and in September–October, 2020 at Greenough and Mingenew (south of Geraldton), Dale (south

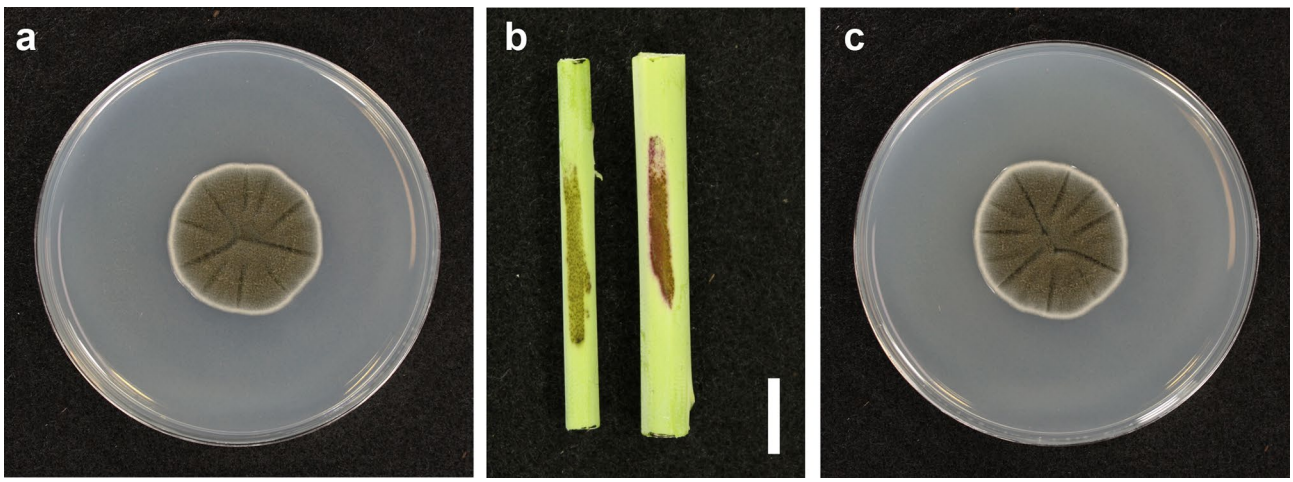

Fig. 2 Isolation of *Cladosporium macrocarpum* and reproduction of disease symptoms. **a** Colony of strain UoM19-17 growing on potato dextrose agar. **b** Lesions on *B. napus* cv. Westar stems at 12 (left) and 26 (right) days after inoculation. Scale bar = 1 cm. **c** Colony from a strain re-isolated from an inoculated plant, fulfilling Koch's postulates. Petri dishes are 6 cm in diameter; 14 days growth

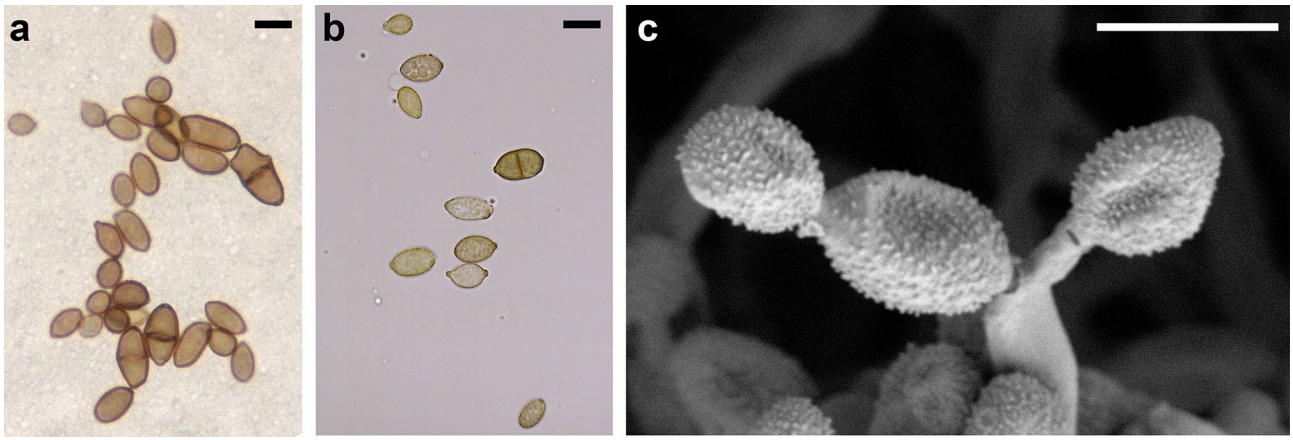

Fig. 3 Morphology of conidia of *C. macrocarpum*. Light microscopy images of conidia, illustrating variation in spore size and morphology: **a** directly from a field sample from Dale, and **b** from *in vitro* culture of strain UoM19-17. Panel **c** shows spore ultrastructure as resolved by scanning electron microscopy for UoM19-17. Scale bars = 10 µm

east of Perth) and Wittenoom Hills (north of Esperance) (Fig. 1b, Table 1).

Diseased materials were surface sterilized or spores liberated, then plated onto PDA to isolate potential pathogens. In addition to species in the genus *Alternaria*, which are commonly found in Australian canola fields (Al-Lami et al. 2019), one uncommon fungus was isolated from diseased material from five locations (Fig. 1b, Fig. 2a). Details of the origins of the five strains isolated by this approach are provided in Table 1.

The ability of the fungal isolates to cause disease was tested by inoculating *B. napus* cv. Westar grown in a glasshouse. Lesion development was clearest on stems, in which dark lesions appeared within eight days (Fig. 2b). The diseased material arising after inoculation with strain UoM19-17 was excised, surface sterilised, and the fungus re-isolated onto PDA, showing the same colony form and spores as strain UoM19-17 (Fig. 2c), and thus fulfilling Koch's postulates that the strain causes the symptoms. The UoM19-17 strain was also tested on stems of wheat (*Triticum aestivum* cv. Gladius) and Indian mustard (*Brassica juncea* cv. Aurea), where no lesions developed.

Morphological features of the colonies, hyphae and spores were consistent with a species of *Cladosporium* (Fig. 2a, Fig. 3). Strains produced colonies with dark brown pigments, including the production of asexual spores, but no sexual structures were observed. Under light microscopy, the conidia formed in alternations off hyphae, sometimes as short chains. The spores varied in size, and were usually, but not always, without a septum, and when formed, were primarily uniseptate (Fig. 3a, b). Strain UoM19-17 was examined by scanning electron microscopy (Fig. 3c). This level of resolution revealed that the spores of the strain were muricate, in contrast to undecorated hyphae, and consistent in appearance with strains in the *C. herbarum* species complex.

Identification of *Cladosporium* strains to the species level is challenging when there are few morphological differences between species, such as those within the *C. herbarum* species complex (Bensch et al. 2012, 2015; David 1997), and

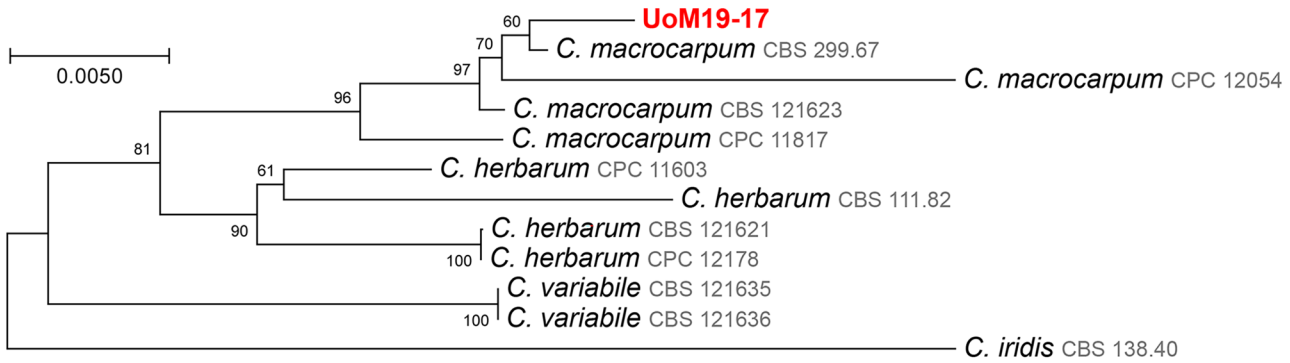

Fig. 4 Phylogenetic analysis of sequences from *C. macrocarpum* strain UoM19-17 compared with other *Cladosporium* species, using *C. iridis* as the outgroup. Sequences from four gene regions (Table 2) were concantenated, aligned and compared using neighbour-joining. Numbers indicate bootstrap support (% from 100 reiterations)

Table 3 *Cladosporium* symptom incidence in fungicide efficacy field trials (for other canola diseases) in Alma (2019) and Dale (2020). Disease incidence was assessed in the trial during pod ripening just prior to crop maturity. Severity was not scored. Average Alma yield across all treatments at the site was 0.4 t/ha, oil 42%, and 1000 grain weight was 2.8 g. Average yield across the Dale site was 1.87 t/ha. Numbers followed by different letters are significantly different ($P < 0.05$)

Fungicide Treatment	Incidence Alma (%)	Incidence Dale (%)
Untreated	28.75[a]	30.83
Prosaro® @ 450 mL/ha at 20% bloom	20.50[a]	33.75
Prosaro® @ 450 mL/ha at 20 & 50% bloom	**13.50[b]**	n/a
Prosaro® @ 450 mL/ha at 50% bloom	23.50[a]	n/a
Statistics		
LSD 5% (Untreated compared to others)	10.37	11.08
LSD 5% (Treated compared to each other)	11.97	n/a

hence DNA sequence information was obtained to refine species identification. The ITS sequences for the five strains were identical. When compared by BLAST to the GenBank nr database, the top hits (100% identity) from the strains were all to species in the *C. herbarum* species complex. This complex includes a number of distinctive species that cannot be resolved with ITS sequences (Schubert et al. 2007).

Four other gene region sequences were obtained from strain UoM19-17, aligned with those from other *Cladosporium* strains and used to construct phylogenetic trees. The phylogenies consistently placed strain UoM19-17 within *C. macrocarpum* (Fig. 4). As an example of this affinity, comparison with five gene regions revealed that strain UoM19-17 had only a single nucleotide difference to the same regions of *C. macrocarpum* strain CBS 299.67, which was isolated from wheat in Turkey. The *TEF1* region was then amplified and sequenced from the other four strains, showing identical sequences for strains UoM20-4, UoM20-10 and UoM21-1, and one single nucleotide polymorphism for UoM20-5. These sequences support the identification of the other four strains as being *C. macrocarpum*, and thereby the wide distribution of this species on canola in Western Australia.

The analysis of the strains isolated from diseased canola is consistent with an identification as *C. macrocarpum*, both from morphological features and similarity in DNA sequences. An alternative designation could be *C. brassicae*. However, *C. brassicae* has been rarely studied beyond original diseased material, in that strains are not available in culture collections or DNA sequences in GenBank. As the *C. brassicae* species epithet implies, the species is associated with causing disease on *Brassica* plants, however, it is similar in morphology to *C. macrocarpum* (Bensch et al. 2012). Further, strains of *C. macrocarpum* have been associated with *Brassica* for over two centuries: for example, Schubert et al. 2007 synonymised *Dematium herbarum* β [equivalent to var.] *brassicae*, found on the stems and leaves of *Brassica* species (Persoon, 1801), with *C. macrocarpum*. *Cladosporium* species, including *C. macrocarpum*, occur on species in the *Brassicaceae* family, including *B. napus* in Canada (Clear and Patrick 1995; Corlett 1988), while in Australia *C. macrocarpum* has been isolated from *Brassicaceae* species in Victoria and wheat in Western Australia (J. Edwards and V. Lanoiselet, pers. commun.).

In a 2019 field trial at Alma, Western Australia, incidence of these new disease symptoms were noticeable, whereas no other diseases were observed at the site. The symptoms on pods and upper stem at crop maturity were significantly reduced ($P < 0.05$) by a double application of commercial fungicide applied at the 20% and 50% bloom stages

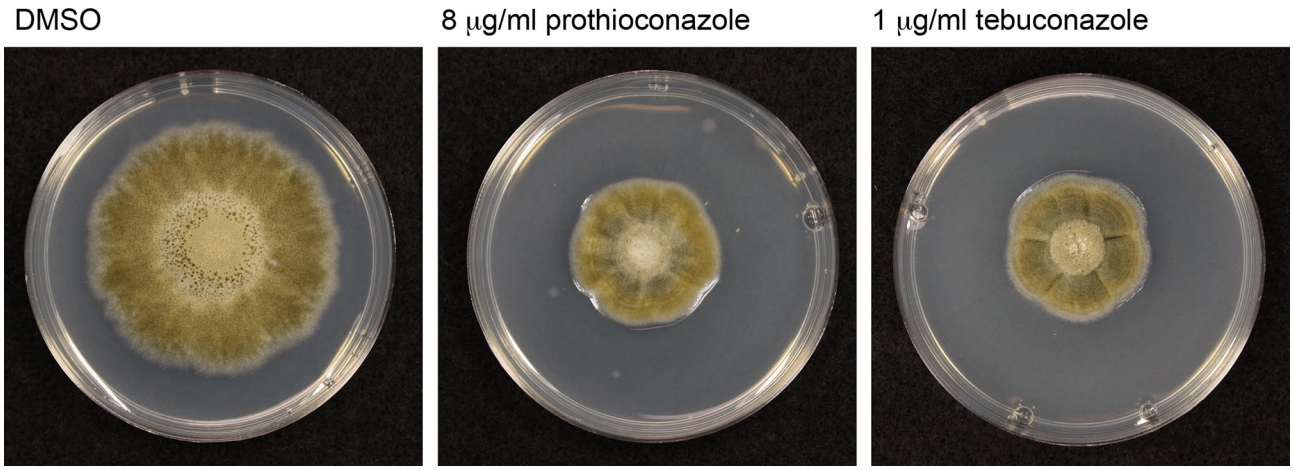

Fig. 5 Inhibition of approximately 50% radial growth of *C. macrocarpum* strain UoM19-17 by two azole-class fungicides, compared to the solvent (dimethyl sulfoxide; DMSO), that are used to control other fungal diseases of canola. 14 days growth on potato dextrose agar; Petri dishes are 6 cm in diameter

of canola development, whereas the single applications at those stages had no significant effect (Table 3). However, no significant yield or grain quality benefit was seen in the trial. This may reflect that the 2019 growing season was extremely dry throughout the WA grain belt and therefore canola yields were low. Average yield across all treatments at the site was 0.4 t/ha. Average grain moisture was 6.7% but yield and grain quality results were standardised to 6% moisture. Average grain quality results were 42% oil, and 2.8 g for 1000 grain weight. At a similar field trial conducted at Dale, Western Australia, in 2020 a single application of the same commercial fungicide applied at 20% bloom stage of canola development had no significant effect on *Cladosporium* disease incidence (Table 3), but was in the presence of Sclerotinia stem rot, blackleg canker and upper canopy blackleg diseases (data not presented). Average yield across the site at Dale was 1.87 t/ha.

It is unknown why *Cladosporium* disease emerged to prominence starting in 2019. One hypothesis is that the species has been present at a low level in canola or other plant species and that relatively recent changes in farming to control diseases caused by *L. maculans* or *S. sclerotiorum,* such as fungicide applications during flowering [reviewed by (Van de Wouw et al. 2021)], may have altered the prevalence of fungal species in canola fields. Thus, the UoM19-17 strain was tested for its ability to grow in the presence of two demethylation inhibitor (azole) fungicides, which target ergosterol biosynthesis, and are registered in Australia for use against Sclerotinia stem rot and commonly used to control upper canopy infection caused by *L. maculans*. The values for the inhibition of growth by 50% (effective concentration; EC_{50}) were 9.96 µg/ml for prothioconazole and 1.32 µg/ml for tebuconazole (as illustrated in Fig. 5). These values, particularly for prothioconazole, are higher than observed for *S. sclerotiorum* (Dalili et al. 2015) or *L. maculans* (Eckert et al. 2010; Yang et al. 2020). Analysis of the sequence of the *C. macrocarpum erg11/cyp51* homolog that encodes the enzyme that is targeted by the azole fungicides, as assembled from genome sequencing reads (GenBank accession MW646549), did not reveal any overt features that would account for the high level of tolerance to these antifungals, and is consistent with the field trial data where a reduction in disease incidence was only obtained from a double application of these fungicides.

The release of fungicides that provide protection to canola later in the growing season against blackleg disease and sclerotinia stem rot could have inadvertently created a plant environment relatively free of species that may previously have outcompeted *C. macrocarpum*. This hypothesis is further supported by co-culturing *C. macrocarpum* and *L. maculans*, wherein the blackleg pathogen inhibited *C. macrocarpum* growth (Fig. 6).

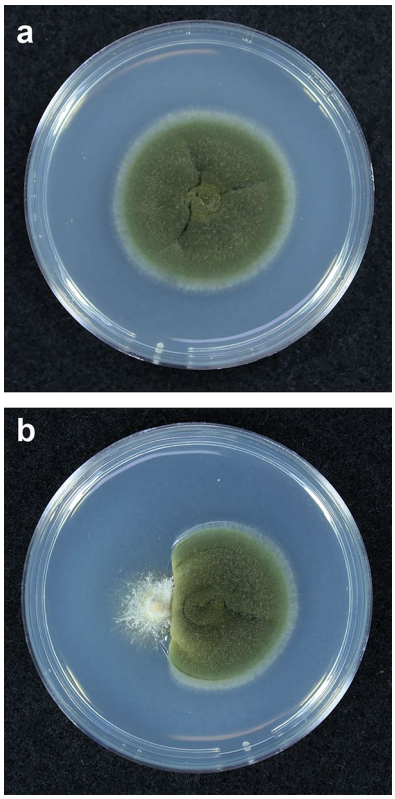

Fig. 6 Inhibition of *C. marcocarpum* by the blackleg fungus *L. maculans*. **a** *C. macrocarpum* strain UoM19-17 by itself, or **b** strain UoM19-17 inoculated adjacent to *L. maculans*. 8 days growth on potato dextrose agar; Petri dishes are 6 cm in diameter

Future research could explore further if infection by *C. macrocarpum* results in a reduction in canola seed yield or oil quality. If *C. macrocarpum* were to be a cause of decline in productivity or quality then control measures could be established, such as reducing or avoiding inoculum, exploring genetic resistance in *B. napus* cultivars, or the application of fungicides in classes other than demethylation inhibitors.

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Declarations

Conflict of interest statement Through ARC linkage project LP170100548 A.I. receives funding for research from Syngenta Australia. Any use of registered trade names is for the purpose of research transparency, and constitutes neither an endorsement nor a recommendation against products or services by the authors, their institutions, or their funding bodies.

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