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ORIGINAL RESEARCH ARTICLE

Endophytic genera in californian thistle (*Cirsium arvense* (L.) Scop.)

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Abstract

Cirsium arvense is an important weed in temperate areas, causing yield losses in pasture and cropping systems. Endophytes may affect fungal biocontrol agents deployed to control *C. arvense*. This was the first study sampling leaves, stems, and roots of *C. arvense* multiple times in one growing season to determine which endophytic genera were associated with this plant species. Eighty-eight endophytic genera were isolated by culture methods and identified with molecular markers. Sixty-five of these have not previously been reported for *C. arvense*. This study was the first to document many genera belonging to the orders Pleosporales, Hypocreales, and Diaporthales that have not previously been identified in association with *C. arvense*. In addition, this study isolated more Leotiomycetes and Helothiales than previous studies on *C. arvense* endophytes. Information on endophytic genera in *C. arvense* will aid our understanding of biotic factors influencing fungal biocontrol agents and may improve effectiveness of biocontrol agents.

Keywords Biocontrol · Biological control · Californian thistle · Canada thistle · *Cirsium arvense* · Creeping thistle · Endophytes

Introduction

Cirsium arvense L. (Scop.), commonly referred to as Californian thistle, is considered a major weed in temperate areas. It thrives in disturbed or bare ground and therefore is problematic on arable and pastoral farmlands. *C. arvense* can form large contiguous patches in grazing lands and outcompetes more desirable vegetation (Burns et al. 2013). Livestock avoids grazing this weed due to spines on the leaf margin. The weed is extremely competitive in low-growing vegetation, where its presence leads to yield losses in pastures (Tiley 2010) as well as economically important crops, such as oats, barley, wheat, peas, and beans (Kazinczi et al. 2001). *C. arvense* is commonly controlled by herbicides, but application of these on steep slopes is problematic (Cripps et al. 2019). Fungal biocontrol is an alternative option, which would be long-lasting and a sustainable way of controlling *C. arvense*. The main fungi considered as fungal biocontrol agents for *C. arvense* are *Sclerotinia sclerotiorum* and *Puccinia punctiformis*. *S. sclerotiorum* would be utilised as an inundative biocontrol agent, with at least annual application required, whereas *P. punctiformis* would be a classical biocontrol agent, which would be introduced to an area once and thereafter self-perpetuate. Both biocontrol agents currently perform inconsistently (Berner et al. 2013; Bourdôt et al. 2006).

Endophytes inhabit plant tissues without causing disease (Delaye et al. 2013). They are generally opportunists, which infect plants when the opportunity arises e.g. when the plant is wounded or stressed (Hardoim et al. 2015; Prell and Day 2000). The effect on their host can be positive, neutral, or negative depending on the relationship they established with their host (Rodriguez et al. 2009; Zhou et al. 2018). If the relationship is mutualistic, the host provides metabolites to the endophyte and the endophyte provides a service to the plant, for example protection against pathogens, or improved nutrient acquisition (Basu and Kumar 2020; Khare et al. 2018). Endophytes can alter outcomes of a host-pathogen interaction by inducing resistance in the host or interacting with a pathogen directly (Grabka et al. 2022). Endophytes may thus affect efficacy of biocontrol agents (Dodd et al. 2010).

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Endophytes within a plant can be identified with culture based or metabarcoding methods. Culturing is most cost effective but is unable to identify unculturable species. Metabarcoding can include unculturable species, provided sequences with taxonomic identification are available, which is not always the case (Durán et al. 2021).

To gain insights into endophytic communities within *C. arvense*, leaves, roots, and stem sections were excised and plated in winter spring, early- and mid-summer and autumn from July 2021 to April 2022. The culture method was chosen as it was cost-effective and would be sufficient to identify the majority of fungal genera. This study summarises data from three separate endophyte studies and provides invaluable information on endophyte populations of *C. arvense*. It increases our understanding of factors affecting performance of biocontrol agents.

Materials and methods

Sample collection

From one site near the arboretum at Lincoln University campus (S43°38'45.82" E172°27'34.76") in New Zealand (F1 site), *C. arvense* shoots and roots were harvested at five time points: July 2021, October 2021, November 2021, February 2022, and April 2022. Six *C. arvense* shoots were collected in July 2021, five in October 2021, five in November 2021, three in February 2022, and three in April 2022. From each shoot, three leaves, three stem-, and three root-pieces were collected from the following locations within a shoot: upper, middle, and lower part. Adventitious root tissue was taken from the subterranean part of the shoot, arising from root buds emanating from the lateral roots. Additionally, one leaf from six individual shoots was collected in February 2022, and two leaves from the same six individual shoots in March 2022 at the F1 site. At a fence line along the field research centre (P1), four additional shoots were collected in October 2021, of which three leaf- and root-pieces were collected. These shoots were in rosette form; hence a random selection of leaves was taken. Further, from 16 February to 20 April 2022, six shoots were collected at fortnightly intervals from a paddock of a farm (McMillan farm) at Days Road in the Lincoln area (S43°38'49.69" E172°27'59.42") and one leaf from the middle area of the shoots was taken for endophyte culturing.

Sample processing

All plant samples were surface sterilised by soaking for 1 min in 96% ethanol followed by 5 min in 10% bleach (0.53% available chlorine) and finally 1 min in 96% ethanol. Plant samples were subsequently rinsed twice with sterile water and air-dried on a paper tissue within a laminar flow hood. A leaf imprint was made on potato dextrose agar (PDA; Difco) by pressing a surface sterilised leaf onto the agar before excising sections from the leaf. This was done to determine whether all epiphytic fungi were successfully removed from the surface sterilisation procedure. From each leaf, 2 mm^2 sections were excised and 16 of these sections were placed on a PDA plate. A total of four PDA plates with leaf sections were made for each sample, thus a total of 64 leaf sections were plated. These four plates, plus the leaf imprint plate, were incubated for 7 days at 20°C with a 12-hr photoperiod. After 7 days, the presence of microorganisms was visually observed, and fungi were isolated by removing a 5 mm by 5 mm piece of agar with actively growing mycelium and plated onto new PDA plates.

Culture purification and morphotyping

After four weeks, fungal plates were morphotyped by visual and microscopic examination. Visual examination was done by examining the top and bottom of the culture and comparing mycelial colour and appearance. Plates, which macroscopically appeared similar, were examined with a stereomicroscope. Observations of mycelium and the presence/absence of spores or sporoma were undertaken to determine similarity between cultures. When applicable, a slide was made to examine microscopic structures with a compound microscope. If cultures were found identical by these examinations, they were assigned to the same morphotype and if they differed, they would be assigned a unique morphotype. For each morphotype, a plate was prepared for single spore isolation to grow a pure culture. For this, within a Class II biosafety cabinet, a sporoma or hyphal material was placed in 500 mL of Millipore water. This was vortexed for 30 s and 20 µL was streaked, with a sterile inoculation loop, onto a PDA plate. One plate for each morphotype was placed in an incubator at 20°C with a 12-hr photoperiod. After two days of incubation, using a dissecting microscope, a single spore or a hyphal tip was located. The spore or hyphal tip was subsequently removed from the streak plate with a sterile needle and placed onto a new PDA plate. These plates were placed in an incubator at 20°C and a 12-hr photoperiod. This resulted in pure cultures of the morphotypes. After growing in pure culture for 2 weeks, plates were morphotyped for a second time, with any adjustments to morphotype allocation undertaken. For each morphotype, one representative was chosen for molecular analysis.

PCR

The following primer pairs were used for the internal transcribe spacer (ITS) region: ITS1F (5' CTTGGTCATTTAG AGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATA TGC 3') (Gardes and Bruns 1993; White et al. 1990). The intron of the beta tubulin gene was amplified using: Bt2a (5′ GGTAACCAAATCGGTGCTGCTTTC 3') and a part of the exon of this gene was amplified using the Bt2b primer (5′ ACCCTCAGTGTAGTGACCCTTGGC 3') (Glass and Donaldson 1995; Hubka and Kolarik 2012).

PCR was performed in a total reaction volume of 20 µL containing: 2 µL of template DNA (10 ng/µL), 10 µL of Dream Taq Green Master Mix (ThermoFisher Scientific), 1 µL of each primer (10 µM; Integrated DNA Technologies), and 6 µL of ultra-pure water (ThermoFisher Scientific). A non-template control was included in each run. Amplification was performed in a ProFlexTM PCR thermal cycler (ThermoFisher Scientific). The following conditions for the ITSF and ITS4 primer set: initial denaturation at 95°C for 3 min; followed by 30 cycles at 95°C for 1 min, 53°C for 30 s and 72°C for 1 min and a final extension for 10 min at 72°C. For the Beta tubulin primer pair cycling parameters were: initial denaturation at 95°C for 5 min; followed by 35 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension for 10 min at 72°C.

PCR products were subjected to electrophoresis on a 1% agarose gel (Meridian Bioscience) cast with 1x GelRed (Dnature). Electrophoresis was undertaken in 0.5 x TBE buffer (45 mM Tris, 1 mM NaEDTA, pH 8.3) at 90 V for 30 min. The product size was visualised with the aid of a 1 kb plus DNA ladder (ThermoFisher Scientific) under UV illumination using a UVIreader (Uvitec). The expected product size for the ITS1F and ITS4 primer pair was 600–700 base pairs (bp) l (Fujita et al. 2001), and the Bt2 primer pair 723–808 bp (Rezaei-Matehkolaei et al. 2014).

Sequencing

The resulting PCR products were sent to BioProtection Aotearoa for sequencing. A Thermo Scientific™ Hitachi 3500xL sequencer (ThermoFisher Scientific) was used for sequencing. Forward and reverse sequences were aligned using the software package Geneious Prime (Dotmatics) and the resultant consensus sequence was compared to other sequences in the NCBI database using a Basic Local Alignment Search tool (BLAST) (NCBI 2023).

Results

From July 2021 to April 2022, 32 *C. arvense* shoots were sampled for this study at two sites at Lincoln University. Further, 30 shoots were sampled at a nearby farm at Days Road. Of these shoots, 234 samples were taken, and 14,976 sub-samples (2 mm x 2 mm) were plated onto PDA plates. A total of 1774 isolates belonging to 88 fungal genera were cultured from *C. arvense* shoots for this study (Fig. 1). As the primers used for identification were not species-specific, results will be presented at the genus level. Genera which have currently not been submitted to GenBank are indicated with an astrix (*).

Genera belonging to the phylum Ascomycota were most commonly isolated (1698 isolates), with 75 genera (85% of the total number of genera) from this phylum being cultured (Fig. 2).

Of the Ascomycota, the class Sordariomycetes and Dothiodeomycetes were predominantly isolated. Of the 75 genera isolated, 29 genera (39% of Ascomycota) belonged to the class Sordariomycetes and these were: *Acremonium, Apiognomonia, Arthrinium*, Beauveria, Chaetomium, Clonostachys, Colletotrichum, Coniochaeta, Cryptodiaporthe*, Cytospora*, Dactylonectria, Diaporthe, Fusarium, Gliocladiopsis, Humicola, Ilyonectria, Microdochium, Myxospora, Nigrospora, Phaeoacremonium, Plectosphaerella, Podospora, Remersonia, Sarocladium, Schizothecium, Sordaria, Thelonectria, Trichoderma*, and *Verticillium* (Figs. 3 and 4). Hypocreales was the largest order within the Sordariomycetes, with the following 11 orders being represented: *Acremonium, Beauveria, Clonostachys, Dactylonectria, Fusarium, Gliocladiopsis, Ilyonectria, Myxospora, Sarocladium, Thelonectria*, and *Trichoderma*.

Thirty-three genera (44% of Ascomycota) belonged to the class Dothideomycetes. These were Alternaria, Amycosphaerella, Ascochyta*, Aureobasidium, Bipolaris, Boeremia, Cladosporium, Curvularia, Didymella, Epicoccum, Exserohilum, Leptosphaeria, Neoascochyta, Neodidymelliopsis, Neofusicoccum, Neosetophoma, Ophiobolus*, Paradendryphiella, Paraphoma, Parastagonospora, Phaeobotryosphaeria, Phaeosphaeria, Phoma*, Plenodomus*, Preussia, Pseudogymnoascus, Pseudopithomyces, Pyrenophora, Septoria, Septoriella, Setophoma, Stagonospora, and Stemphylium (Fig. 5). Of the class Dothideomycetes, 25 genera belonged to the order Pleosporales (76% of Dothodeomycetes). These were: Alternaria, Ascochyta*, Bipolaris, Curvularia, Didymella, Epicoccum, Exserohilum, Leptosphaeria, Neoascochyta, Neodidymelliopsis, Neosetophoma, Ophiobolus*, Paradendryphiella, Paraphoma, Parastagonospora, Phaeosphaeria, Phoma*, Plenodomus*, Preussia, Pseudopithomyces, Pyrenophora, Septoriella, Setophoma, Stagonospora, and Stemphylium.

Fig. 1 Heatmap of endophytic genera found in samples from *Cirsium arvense* plants in the Lincoln (New Zealand) area. Genera which were more often isolated are displayed in darker shades of green, whereas genera which were isolated fewer times are displayed in lighter shades of green

Other classes recovered from *C. arvense* plants were Eurotiomycetes (4 genera; 5% of Ascomycota) which included *Aspergillus, Penicillago, Penicillium*, and *Talaromyces*, and Letiomycetes (7 genera; 9% of Ascomycota), which were the genera *Botrytis, Cadophora, Hyaloscypha*, Mycochaetophora, Neofabraea, Pezicula*, and *Tricellula*. All previously stated 7 genera in the class Letiomycetes belong to the order Helotiales.

Ascomycota genera, which were most isolated in this study, included: *Alternaria* (398 isolates), *Stemphylium* (122 isolates), *Epicoccum* (146 isolates), *Cladosporium* (127 isolates), *Diaporthe* (114 isolates), *Fusarium* (86 isolates), and *Colletotrichum* (60 isolates). Genera for which only one isolate was obtained included *Apiognomonia, Beauveria, Cryptodiaporthe*, Cytospora*, Gliocladiopsis, Hyaloscypha*, Leptospharia, Mycochaetophora, Myxospora, Neofabraea, Neosetophoma, Paraphoma, Penicillago, Penicillium,*

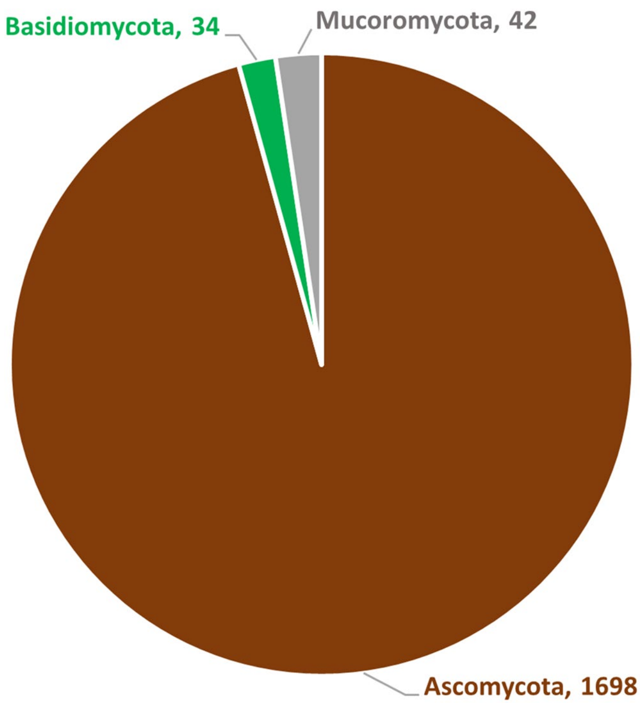


Fig. 2 Number of isolates obtained for each division in this study

Phomopsis, Plenodomus, Setophoma, Stagonospora, Thelonectria*, and *Xylaria**.

The remaining isolated genera belonged to the phyla Mucoromycota (6 genera) and Basidiomycota (7 genera). Of the Mucoromycota, three genera belonged to the order Mucorales (*Absidia* (1 isolate), *Gongronella* (1 isolate), and *Mucor* (28 isolates)), two to the order Mortierellales (*Linnemannia* (4 isolates), and *Mortierella* (2 isolates)), and only *Umbelopsis* (6 isolates) to the order Umbelopsidales (Fig. 6).

Of the Basidiomycota, only one genus was recovered for each of the following six orders: Agaricales (*Coprinellus* (2 isolates)), Cystofilobasidiales (*Itersonilia* (1 isolate)), Polyporales (*Trametes* (5 isolates)), Russulales (*Peniophora**(4 isolates)), and Trichosporonales (*Apiotrichum* (7 isolates) (Fig. 7). The two orders for Cantharellales were *Ceratobasidium* (14 isolates) and *Sistotrema* (1 isolate).

In total, 65 of the 88 endophytic genera isolated from *C. arvense* in this study were never reported as endophytes of *C. arvense* (Table 1).

Discussion

This study has recorded the fungal endophytes associated with *C. arvense* during one growing season, giving a better understanding of organisms in various plant tissues of this noxious weed. Endophytes were cultured from leaves, stems, and roots of *C. arvense* plants in the Lincoln area of New Zealand. There were 88 genera cultured from plant tissue, of which 65 have not previously been reported for *C. arvense.*

Ascomycota were most frequently isolated from *C. arvense* plants sampled for this study. Comparatively,

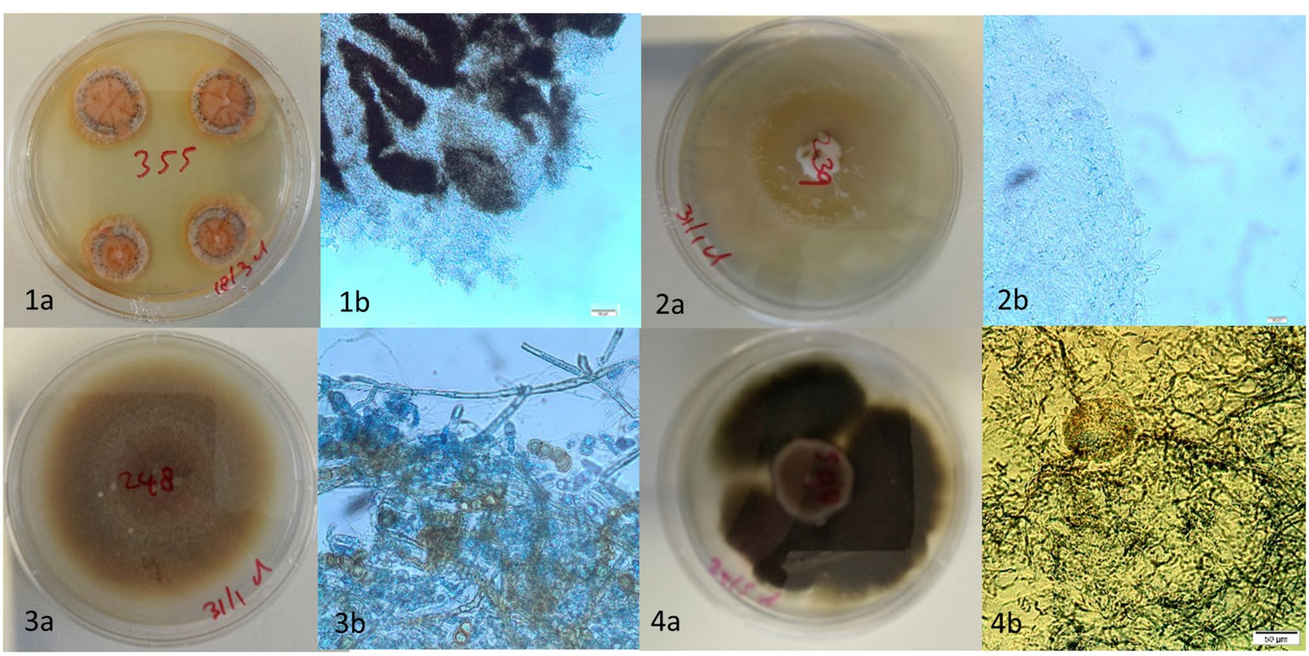


Fig. 3 A selection of *C. arvense* endophytic Sordariomycetes isolated from *Cirsium arvense* for the first time and cultured on PDA within the current study: *Coniochaeta* culture **1a** and sporoma **1b**, *Dactylonectria* culture **2a** and mycelium **2b**, *Ilyonectria* culture **3a** and chlamydospores **3b**, and *Schizothecium* culture **4a** and mycelium **4b**

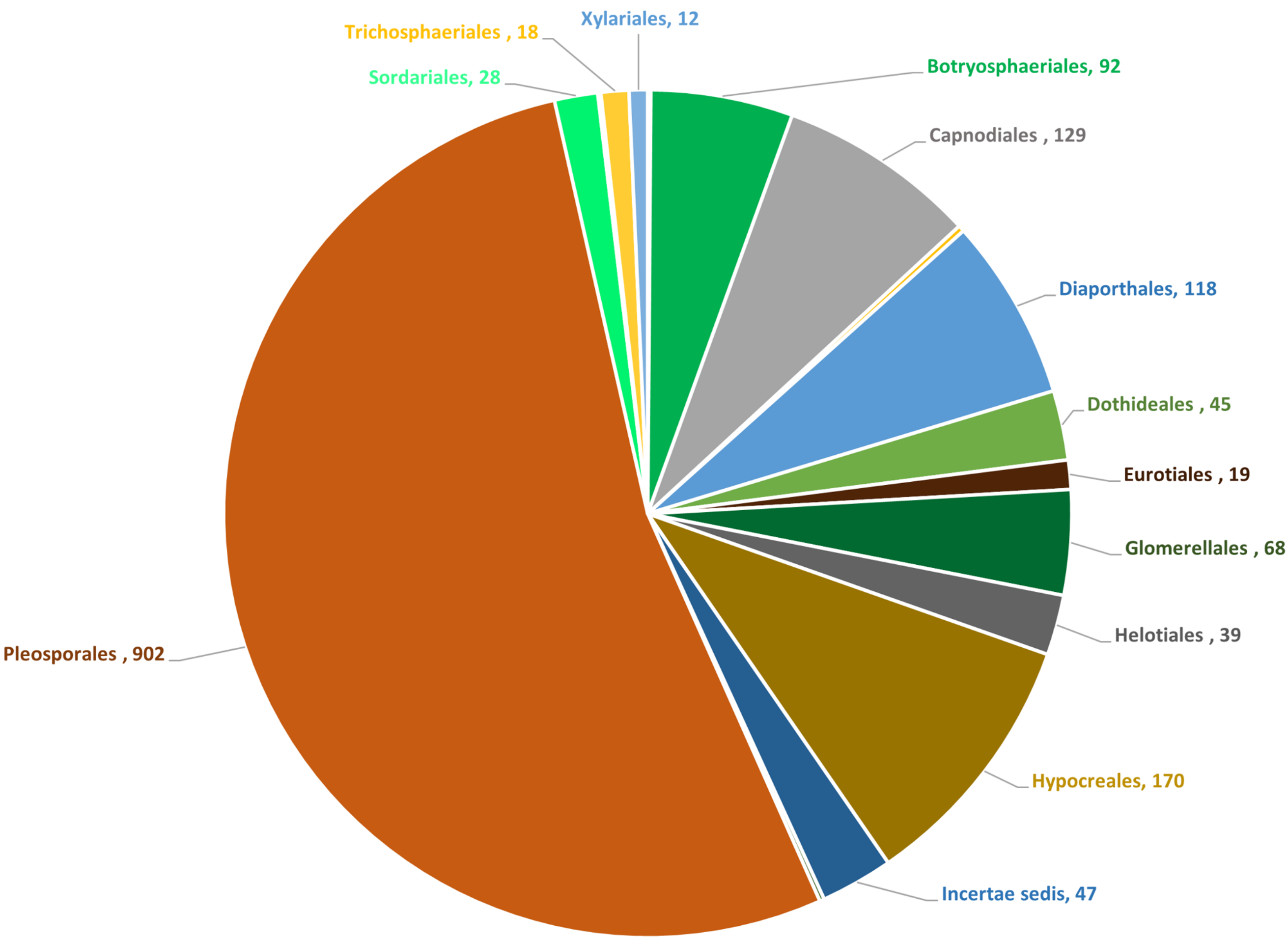


Fig. 4 Orders of Ascomycota isolated in this study. Numbers follow order equal isolate number. Note: All orders which were isolated less than ten times have been removed. Incertae cedis were isolates of which the order is undetermined. These were of the genera *Plectosphaerella* (26), *Arthrinium* (6), and *Pseudogymnoascus* (15)

Mucoromycota and Basidiomycota were isolated less frequently. Ascomycota and Basidiomycota are major divisions within the fungal kingdom with Basidiomycota more commonly found in woody tissue than in leaves (Rodriguez et al. 2009). Agaricales, Polyporales, and Cantharellales are the most isolated Basidiomycota (Rashmi et al. 2019). In the current study, we isolated the aforementioned three orders plus the orders Russulales, Cystofilobasiales, and Trichosphaeriales. Mucoromycota are plant pathogens, saprophytes, arbuscular mycorrhizae, and root endophytes. They assist the plant in nutrient acquisition as well as induce resistance to pathogens (Frąc et al. 2018; Ozimek 2021). In the current study, six genera of Mucoromycota were found, belonging to the orders Mucorales, Mortierellales, and Umbelopsidales. No previous record of associations between Mucoromycota and *C. arvense* have been published (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012) making these findings novel.

Ascomycota and Mucoromycota are generally faster growing in culture than Basidiomycota, which may cause underrepresentation of Basidiomycota in culture-based research (Sun and Guo 2012; Tang et al. 2018). Usually only a small fraction of isolated endophytes will be Basidiomycota (Gardes and Bruns 1993; Martin et al. 2015). This was true for this current research with only seven genera of Basidiomycota isolated. Additionally, Basidiomycota often appear morphologically similar, which may lead to incorrect morphotype grouping and a subsequent underestimation of the true number. As in the current study, only one representative of each morphotype was sequenced and subsequently the identity of this one representative was assigned to all isolates of this morphotype. As such, morphologically similar genera may have been grouped together. Thus, more Basidiomycota may have been found if additional isolates of each morphotype were sequenced. In addition, to ascertain a more comprehensive genus identification for each of the morphotypes, further gene regions need to be undertaken (Reller et al. 2007).

In endophyte studies, genera belonging to Ascomycota are predominantly isolated. In this study 75 genera of the 88

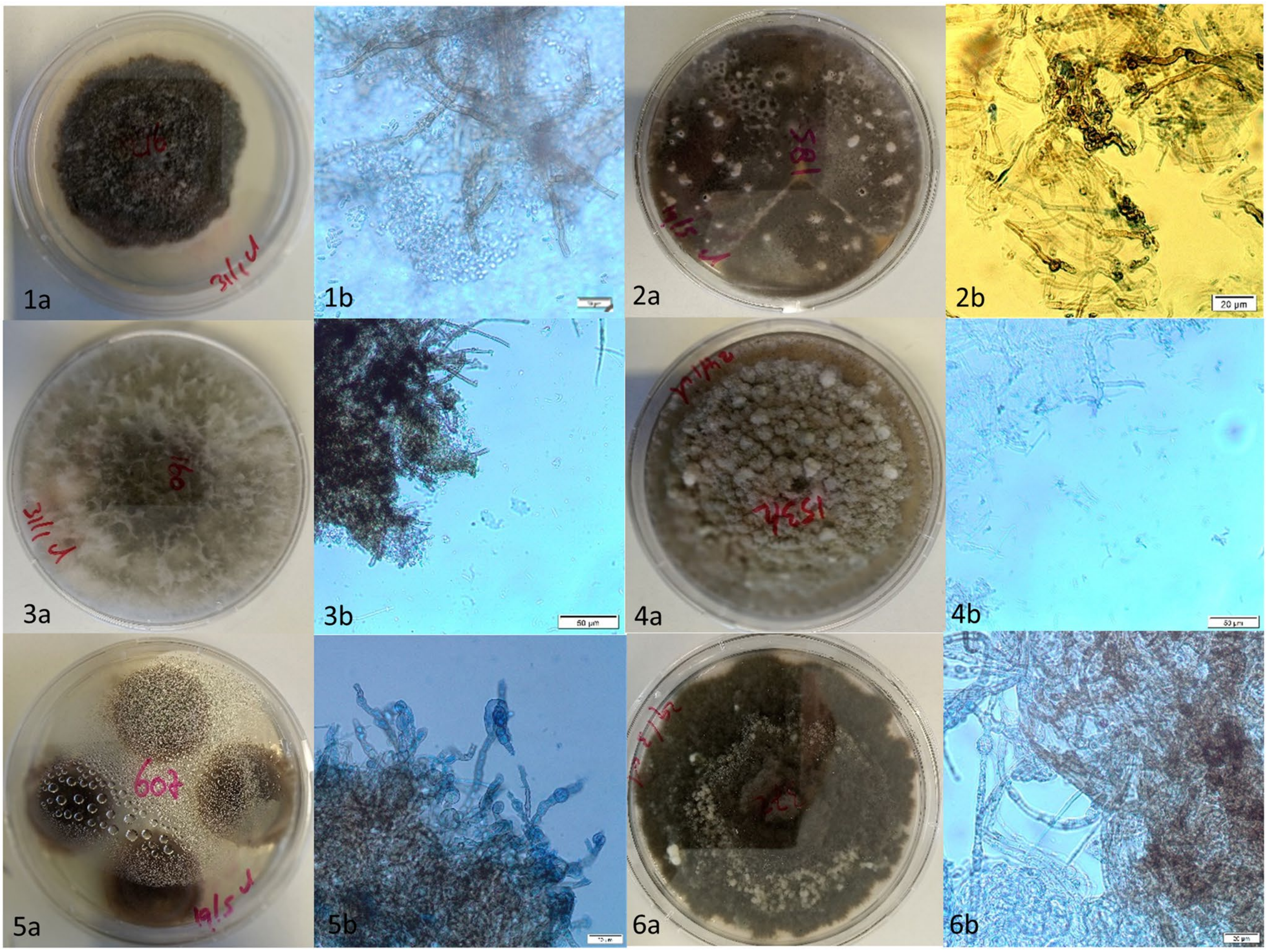


Fig. 5 A selection of *C. arvense* endophytic Dothideomycetes isolated from *C. arvense* for the first time and cultured on PDA within the current study: *Boeremia* culture **1a** and conidia **1b**, *Exserohilum* culture **2a** and mycelium **2b**, *Neofusicoccum* culture **3a** and conidioma **3b**, *Paradendryphiella* culture **4a** and mycelium **4b**, *Phaeosphaeria* culture **5a** and conidiophore **5b**, and *Pyrenophora* culture **6a** and immature chlamydospore **6b**

genera endophytically recovered were Ascomycota. For the division Ascomycota, multiple previous studies found that classes Sordariomycetes and Dothideomycetes were most abundant, followed by Letiomycetes and Eurotiomycetes (Oita et al. 2021; Rashmi et al. 2019; Rim et al. 2021). In the current study, Dothideomycetes and Sordariomycetes were most frequently isolated, followed by Letiomycetes and Eurotiomycetes.

Dothideomycetes is the largest class within the division Ascomycota with many being saprotrophs, epiphytes, and endophytes (Hyde et al. 2013). Current study found 25 genera of Dothiodeomycetes not previously known to be associated with *C. arvense*. These included: *Amycosphaerella, Ascochyta, Bipolaris, Boeremia, Didymella, Exserohilum, Leptosphaeria, Neoascochyta, Neodidymelliopsis, Neofusicoccum, Neosetophoma, Ophiobolus, Paradendryphiella, Paraphoma, Parastagonospora, Phaeobotryosphaeria, Phaeospharia, Plenodomus, Pseudogymnoascus, Pseudopithomyces, Pyrenophora, Septoria, Septoriella, Setophoma*, and *Stagonospora.* In previous studies on *C. arvense*, 15 genera of Dothideomycetes were identified. These included: *Alternaria, Aureobasidium, Botryospharia, Cladosporium, Curvularia, Davidiella, Drechslera, Epicoccum, Eudarluca, Lewia, Periconiella, Phoma, Pithomyces, Preussia*, *Pyrenochaeta*, and *Stemphylium* (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012). The current study found eight genera of the class Dothiomycetes in common with previously mentioned studies on *C. arvense* endophytes. These included: *Alternaria, Aureobasidium, Cladosporium, Curvularia, Epicoccum, Phoma*, *Preussia*, and *Stemphylium*. 37% of the genera obtained in this study were Dothideomycetes. Thus, this study showed that associations between *C. arvense* and Dothideomycetes are very common. As this study found 25 not previously reported endophytic Dothideomycetes in *C. arvense* tissues in addition to the 15 previously reported genera, this study

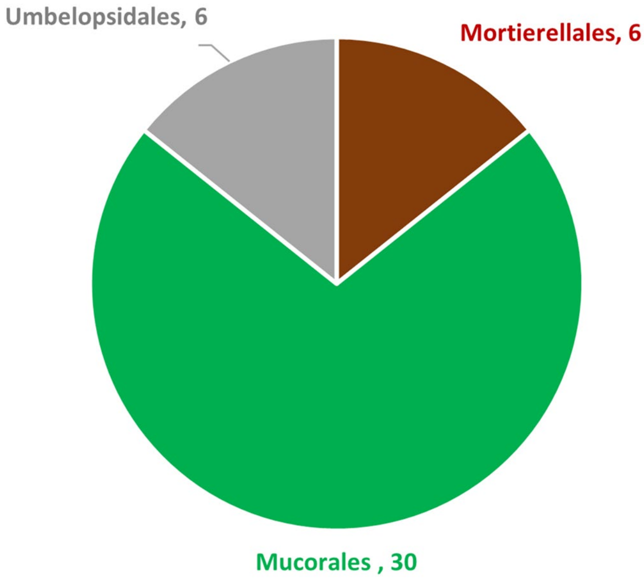


Fig. 6 Orders of the division Mucoromycota and the number of isolates obtained in this study. Numbers follow order indicate isolate number

has contributed significantly to our knowledge of these associations.

Sordariomycetes are the second largest class in the division Ascomycota. Many are saprophytes and some are known as endophytes or pathogens of plants (Lee et al. 2019). The current study found 18 genera of the class Sordariomycetes not previously known to be associated with *C. arvense*. These included: *Apiognomonia, Beauveria, Coniochaeta, Cryptodiaporthe, Cytospora, Dactylonectria, Gliocladiopsis, Humicola, Ilyonectria, Microdochium, Myxospora, Phaeoacremonium, Remersonia, Rosellinia, Sarocladium, Schizothecium, Thelonectria, and Xylaria*. In previous studies on *C. arvense*, 21 genera of Sordariomycetes were identified.

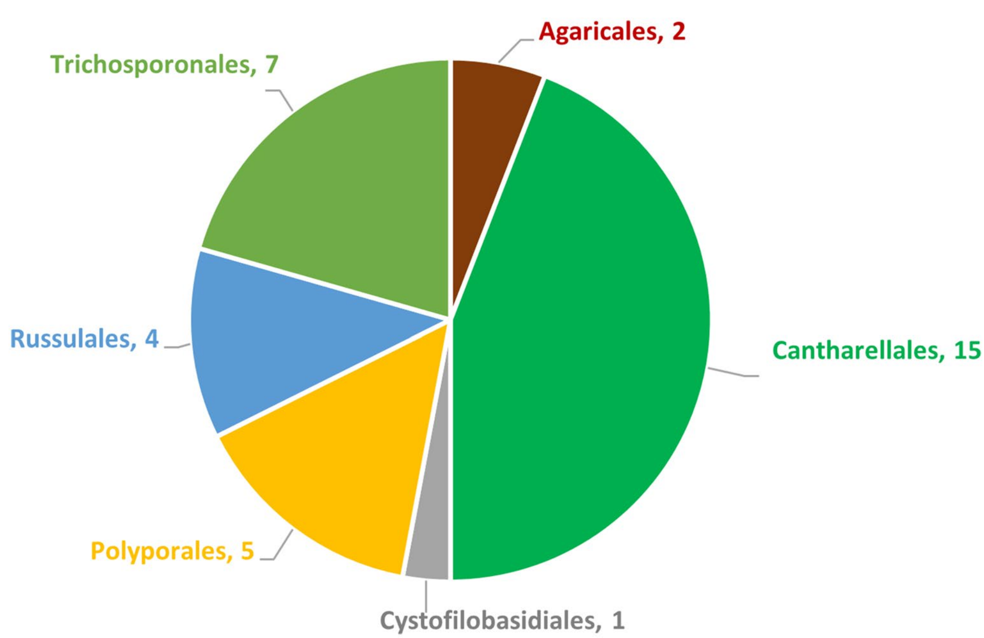
Table 1 Genera of *C. arvense* endophytes not previously reported in other published studies (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012)

Genus	Genus	Genus	Genus
Amycosphaerella	*Exserohilum*	*Neofabraea*	*Pyrenophora*
Apiognomonia	*Gliocladiopsis*	*Neofusicoccum*	*Remersonia*
Apiotrichum	*Gongronella*	*Neosetophoma*	*Rosellinia**
*Ascochyta**	*Humicola*	*Ophiobolus**	*Sarocladium*
Beauveria	*Hyaloscypha*	*Paradendryphiella*	*Schizothecium*
Bipolaris	*Ilyonectria*	*Paraphoma*	*Septoria*
Biscogniauxia	*Itersonilia*	*Parastagonospora*	*Septoriella*
Boeremia	*Leptosphaeria*	*Penicillago*	*Setophoma*
Botrytis	*Linnemannia*	*Peniophora**	*Sistrotrema*
Cadophora	*Microdochium*	*Pezicula*	*Sordaria*
Ceratobasidium	*Mortierella*	*Phaeoacremonium*	*Stagonospora*
Coniochaeta	*Mucor*	*Phaeobotryosphaeria*	*Talaromyces*
Coprinellus	*Mycochaetophora*	*Phaeosphaeria*	*Thelonectria*
*Cryptodiaporthe**	*Myxospora*	*Plenodomus**	*Trametes*
*Cytospora**	*Neoascochyta*	*Pseudogymnoascus*	*Tricellula*
Dactylonectria	*Neodidymelliopsis*	*Pseudopithomyces**	*Umbelopsis*
Didymella			

*Genera for which sequences are currently not submitted to Genbank

These included: *Acremoniella, Acremonium, Arthrinium, Bionectria, Chaetomium, Clonostachys, Colletotrichum, Cylindrocarpon, Fusarium, Geniculosporium, Gliomastrix, Hypocrea, Neonectria, Nigrospora, Pithoascus, Plectosphaerella, Sordaria, Stachybotrys, Trichoderma*, and

Fig. 7 Orders of the division Basidiomycota and the number of isolates obtained in this study. Numbers follow order indicate isolate number



Trichothecium (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012). The current study found a total 29 Sordariomycetes and had 11 genera of Sordariomycetes in common with previously mentioned studies on *C. arvense* endophytes. These included: *Acremonium, Arthrinium, Chaetomium, Clonostachys, Colletotrichum, Diaporthe, Fusarium, Nigrospora, Plectosphaerella, Sordaria*, and *Trichoderma*. These 29 genera of Sordariomycetes made up 33% of the genera found in this study. Thus, this study showed that *C. arvense* commonly assocites with Soradariomycetes. This study has identified 18 endophytic Sordariomycetes not previously reported in *C. arvense* tissues in addition to the 21 previously reported genera. As such, this study has contributed significantly to our knowledge of these associations.

Letiomycetes were less frequently isolated in this study than the Dothideomycetes and the Sordariomycetes. This class includes plant pathogens, mycorrhizal species, dark septate endophytes and aquatic hyphomycetes (conidial moulds) (Johnston et al. 2019). The Leotiomycete *Sclerotinia* has been previously identified in studies on *C. arvense* endophytes (Dodd et al. 2010). In the current study, the genus *Sclerotinia* was not found, but this study did find seven other genera of Leotiomycetes. These included: *Botrytis, Cadophora, Hyaloscypha, Mycochaetophora, Neofabraea, Pezicula*, and *Tricellula*.

Eurotiomycetes were another less frequently isolated class in this study with only four genera being found. They can be endophytes, ectomycorrhiza, endophytes or saprotrophs. Many Eurotiomycetes produce mycotoxic secondary metabolites which shape their interactions with other microorganisms as well as with herbivores and plants (Pfliegler et al. 2020; Prieto et al. 2021). In previous studies on *C. arvense* endophytes, seven genera of the class Eurotiomycetes have been identified. These included: *Aspergillus, Exophilia, Penicillium, Phialophora*, and *Rhinocladiella* (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012). In the current study, the genera *Aspergillus* and *Penicillium* were also found. The current study also found two genera which were not previously known to be associated with *C. arvense: Penicillago* and *Talaromyces*.

In the current study, Pleosporales (902), Hypocreales (170), Capnodiales (129), and Diaporthales (118) were frequently isolated. In weed endophyte research, Pleosporales are commonly found in great abundance, followed by Capnodiales, Helothiales, Hypocreales and Xylariales (Triolet et al. 2022). The order Pleosporales is the largest order of Dothideomycetes, currently including 41 families. They can have epiphytic, endophytic, saprophytic, and parasitic lifestyles and occur in diverse habitats (Hyde et al. 2013; Zhang et al. 2012). In the current study, this was the most isolated order with 902 isolates being cultured. Nineteen genera of the order Pleosporales which were not previously known to be associated with *C. arvense* were identified in current study. These included: *Ascochyta, Bipolaris, Didymella, Exserohilum, Leptosphaeria, Neoascochyta, Neodidymelliopsis, Neosetophoma, Ophiobolus, Paradendryphiella, Paraphoma, Parastagonospora, Phaeosphaeria, Plenodomus, Pseudopithomyces, Pyrenophora, Septoriella, Setophoma*, and *Stagonospora.* In other studies on *C. arvense* endophytes, ten genera of the order Pleosporales were identified. These included: *Alternaria, Curvularia, Drechslera, Epicoccum, Eudarluca, Lewia, Phoma, Pithomyces, Preussia*, *Pyrenochaeta*, and *Stemphylium* (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012). The current study had the following genera of the order Pleosporales in common with the aforementioned studies: *Alternaria, Curvularia, Epicoccum, Phoma, Preussia*, and *Stemphylium*. This study has found 19 not previously reported endophytic Pleosporales in *C. arvense* tissues in addition to the 10 previously reported genera. Capnodiales is an order of the Dothideomycetes which is often found in weed endophyte research (Triolet et al. 2022). The main representative of this order is the genus *Cladosporium*, which was found in all studies on *C. arvense* endophytes (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012). In the current study, the genus *Cladosporium* was also found in great abundance (127 isolates).

Hypocreales and Diaporthales are larges orders of Sordariomycetes. They consist of endophytes, saprophytes, and plant- and animal- pathogens (Rica et al. 2006; Senanayake et al. 2017; Zeng et al. 2020). In previous studies on *C. arvense* endophytes, the following 12 genera of the order Hypocreales were identified: *Acremoniella, Acremonium Bionectria, Clonostachys, Cylindrocarpon, Fusarium, Gliomastrix, Hypocrea, Neonectria, Stachybotrys, Trichoderma*, and *Trichothecium* (Dodd et al. 2010; Eschen et al. 2010; Gange et al. 2007; Wearn et al. 2012). The current study had four genera in common with these previous studies. These included: *Acremonium, Clonostachys, Fusarium*, and *Trichoderma*. In the current study, seven genera of Hypocreales were found which were not previously known to be associated with *C. arvense*. These included: *Beauveria, Dactylonectria, Gliocladiopsis, Ilyonectria, Myxospora, Sarocladium*, and *Thelonectria*. In previous studies on *C. arvense* endophytes, only one genus of the order Diaporthales was identified, which was the genus *Diaporthe*, also identified in the current study. In addition, three other genera of the order Diaporthales were found. These included: *Apiognomonia, Cryptodiaporthe*, and *Cytospora.*

Helothiales is the largest order in the Leotiomycetes, currently including 13 families (Wang et al. 2006). The current study identified seven genera of this order. These included: *Botrytis, Cadophora, Hyaloscypha, Mycochaetophora,* *Neofabraea, Pezicula*, and *Tricellula*. In previous studies on *C. arvense* endophytes, only one study found a genus in this order, which was the genus *Sclerotinia* (Dodd et al. 2010). Thus, the current study found more Helothiales than previous studies on *C. arvense* endophytes. This study has additionally identified many genera belonging to the orders Pleosporales, Hypocreales, Diaporthales and Helothiales that have not previously been identified in association with *C. arvense.* This is the first study to document these associations.

This was the first study of *C. arvense* endophytes sampling leaves, stems, and roots of *C. arvense* over one growing season and 65 not previously reported genera for *C. arvense* have been found in current study. Possibly, the sampling over the season and taking three tissue types into consideration has contributed to the large number of new genera found compared to the above-mentioned studies, suggesting that variable climatic conditions, plant age and physiology may influence survival and or expression of endophytes.

Effects of endophytic species on biocontrol agents are on the spectrum of antagonism to synergism (Kurose et al. 2012). Hence, it is important to understand effects key endophytic species have on functioning of biocontrol agents. Endophytic mutualists may have implications for weed biocontrol. They may enhance the plant's resistance to a biocontrol agent, or interact directly with the biocontrol (Currie et al. 2019). Mechanisms of direct interaction could be antibiosis, competition, or mycoparasitism (Den Breeyen et al. 2022). The current study found four Eurotiomycetes (*Aspergillus, Penicillium, Penicillago*, and *Talaromyces*), which are known to produce mycotoxic metabolites (Prieto et al. 2021). The genus *Trichoderma* was also found in the current study and can be an antagonist of pathogens by mycoparasitism as well as by antibiosis (Guzmán-Guzmán et al. 2023). Some endophytic members of the genera *Beauvaria, Diaporthe, Colletotrichum*, and *Fusarium* are also known to inhibit pathogen growth (Grabka et al. 2022). The aforementioned genera are examples of endophytic genera found in current study, which may aid *C. arvense* in its defence against biocontrol agents. It is likely, however, that more of the endophytic genera found in this study could have a mutualistic relationship with *C. arvense*. Eluding the interaction of these plant mutualists with plants and biocontrol agents would aid our understanding of success or failure of biocontrol agents.

Endophytes can also have a synergistic relationship with pathogens used for biocontrol. On barley and wheat, synergistic effects were found between members of the *Septoria* genus and *Puccinia* pathogens (Junior et al. 2014; Garin et al. 2018, van der Wal et al. 1970). Another endophytic genus found in current study which may be interesting in this regard would be *Phoma*, as *Phoma destructiva* and *P. punctiformis* are known to have a synergistic effect with more severe disease being present for both pathogens when applied simultaneously on *C. arvense* (Kluth et al. 2005). Future research could focus on elucidating relationships of endophytes found in the current study with biocontrol agents, with a focus on endophytes which may enhance the effect of biocontrol agents. These could potentially be used to augment endophyte communities to increase the effectiveness of a biocontrol agent. Endophytes themselves could be potential biocontrol agents. Many genera in the division Ascomycota which contain pathogens have been isolated in this study. *Alternaria, Botrytis, Ilyonectria, Pezicula, Phaeobotryosphaeria, Pyrenophora, and Thelonectria* are some examples of these. The pathogenicity of these isolates on *C. Arvense* could be explored in a future study.

Conclusions

This study contributed to current knowledge of *C. arvense* endophytes. Sixty-five endophytic genera not previously known to be associated with *C. arvense* were ascertained. Seventy-five of the 88 genera found belonged to the division Ascomycota. From this study, it was evident that *C. arvense* commonly associates with Dothideomycetes and Sordariomycetes. The order Pleosporales was most commonly isolated, followed by Hypocreales, Capnodiales, and Diaporthales. This study was the first study to document many genera belonging to the orders Pleosporales, Hypocreales, and Diaporthales that have not previously been identified in association with *C. arvense*. In addition, this study found more Leotiomycetes and Helothiales than previous studies on *C. arvense* endophytes. This study was a significant addition to current knowledge on endophytic genera in *C. arvense*, which enhances our understanding of biotic factors that may influence fungal biocontrol agents. This knowledge may be used in future studies to further explore the relationships between these fungi and biocontrol agents. Ultimately, this could help to solve problems around the inconsistent performance of biocontrol agents.

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Declarations

Conflict of interest No conflict of interest.

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