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Identification of potential fungal biocontrol agents for the management of ripgut brome

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Abstract Ripgut brome (*Bromus diandrus*) is a significant weed in arable systems with cereals being negatively impacted through yield reduction and contamination of harvested grain. There are limited herbicide options for its control, and reduction of the weed seed bank is a key part of its management. Ripgut brome seeds were incubated in three soils known to harbour a range of plant pathogens, and a range of fungi were subsequently isolated from ungerminated seeds. The most prevalent putative fungal pathogens isolated were *Fusarium* spp. and *Marasmius* sp. Selected isolates were cultured on artificial media and tested for their ability to inhibit germination of ripgut brome seeds in a series of *in vitro* assays, with many isolates completely inhibiting germination. When isolates were tested for their activity against non-target arable species in plant assays, three of five *Fusarium* spp. isolates significantly inhibited germination of oats and barley while a *Marasmius* sp. isolate had no effect on germination of these species. Further plant assays using four *Marasmius* sp. isolates against a wider range of plant species (oats, barley, ryegrass, brome, cocksfoot, and prairie grass) indicated some variability between *Marasmius* sp. isolates, with two isolates slightly inhibiting germination of some plant species, while two isolates (W14, W17) had no adverse effects. Sequencing of the ITS region of the *Marasmius* sp. isolates indicated that they were closely related but distinct from *M. graminum,* which is known to have some phytopathogenic activity and *M. oreades* which has not yet been reported as a plant pathogen. Further investigation of the *Marasmius* isolates as potential biocontrol agents for ripgut brome seed is warranted.

Keywords seed bank, weed management, arable, seed pathogen, *Marasmius* sp., *Fusarium* sp.

INTRODUCTION

Brome grasses, especially ripgut brome (*Bromus diandrus*), are significant weeds in New Zealand arable systems, with cereals being particularly affected through yield reduction and contamination of harvested cereal grain. In wheat, ripgut brome has been shown to be extremely competitive, with 100 ripgut brome plants/m^2 estimated to reduce the grain yield by 30% (Gill et al. 1987). Ripgut brome is tolerant of a wide range of climatic conditions and thrives in a broad range of soil types, leading to its widespread occurrence in cultivated crops, pastures, along roadsides and in waste ground (FAR 2021). In addition, its growth form is like winter-sown cereal crops which makes selective control difficult to achieve with herbicides. An integrated management approach for ripgut brome is needed to ensure good control because of limited herbicide options.

Management of ripgut brome is challenging as this weed has prolific seed production and seed dispersal, which ensures survival and seedling establishment at different crop planting dates. Thus, a key part of managing ripgut brome is reducing the soil seed bank. This weed has a very large seed with the ability to germinate quickly and emerge from deep (up to 20 cm) within the soil. Seed can remain viable for at least two years when current environmental conditions are unsuitable for germination.

Environmental concerns around weed management have driven interest in alternatives to chemical herbicides, in particular the use of deleterious rhizobacteria and fungi that colonise the surfaces of weed plant roots and suppress weed growth (Kremer & Kennedy 1996). The application of microorganisms for the management of weeds is not a new idea with many (mostly fungal) microbial control agents investigated over several decades. Some promising candidates have passed through pre-commercial field evaluations, to receive regulatory approval and commercial release. Proposed benefits to this strategy include reduced environmental impacts, increased target specificity, reduced

development costs compared to conventional herbicides and the identification of novel herbicidal mechanisms (Harding & Ralzada 2015).

Pathogens of *Bromus* spp. have been reported previously by several authors. For example, the bacterium *Pseudomonas fluorescens* D7 reduced downy brome (*Bromus tectorum*) growth by 40% while slightly stimulating winter wheat (*Triticum aestivum*) (Kennedy et al. 1991) and did not impact on non-target plant species (Kennedy et al. 2001). *Pseudomonas trivialis* X33d initially isolated from the rhizosphere of durum wheat seedlings was shown to inhibit emergence and seedling growth of great brome (*B. diandrus*). Great brome plants inoculated with X33d and co-seeded with durum wheat showed reduced root biomass and altered root architecture (shorter, less branched roots) while some growth promotion of durum wheat was observed suggesting the competitive ability of great brome towards durum wheat was reduced (Mejri et al. 2010). Previous work in New Zealand has identified some plant pathogens (five fungi and one bacterium) associated with ripgut brome but after more than a decade of research overseas with two of the most promising of these (seed pathogen *Pyrenophera semeniperda* and a smut *Ustilago bullata*), it was concluded that neither pathogen was an ideal candidate (Barton 2022).

To date, most weed biocontrol agents have been targeted at the growing plant, rather than at ungerminated seeds (Evans et al. 2001) but seed pathogenic microorganisms can have a major negative impact on seed survival in soil (Dalling et al. 2011). Multiple seed burial studies involving both fungicide-treated and untreated seeds (reviewed in Wagner & Mitschunas 2008) provide clear evidence for the importance of seed-borne and soil-borne saprophytic and pathogenic fungi as a seed mortality factor. Early studies demonstrated the potential of this approach for controlling ripgut brome through the application of various strains of *Fusarium* spp. in either laboratory or glasshouse studies against *Orobanche cumana* (Thomas et al. 1999), *Abutilon theophrasti* (Kremer & Shulte 1989), and *Striga hermonthica* (Abbasher & Sauerborn 1992). Ideally the microbial control agent would have a high degree of specificity to the target weed, thereby limiting the non-target impacts on beneficial plant species and potential for unintended spread as they may be unable to survive in the absence of their intended host. Testing for non-target effects is, therefore, a crucial early step in screening of candidate microbial control agents.

While several pathogens of *Bromus* spp. have been identified, the use of microorganisms to manage the seed bank in soil has not been explored despite the clear importance of the soil seed bank in sustaining populations of these intransigent weeds. The aim of this study was to begin to explore the potential for microbial control of the ripgut brome seed bank through the identification of soil-borne pathogens specific to this weed. As part of a wider study, potential pathogens were also isolated from seeds of various species of vulpia hair grass (*Vulpia* spp.), which are a problem in New Zealand crops of small seeds, for example perennial ryegrass, where they contaminate seed and reduce seed yield.

MATERIALS AND METHODS

Isolation of potential seed pathogens from soil

Fungi and bacteria with potential to suppress germination of ripgut brome and vulpia hair grass seeds were isolated from soil collected from under dairy pasture at Clifden (Southland, South Island), Wallacetown (Southland, South Island) and Hamilton (Waikato, North Island), New Zealand. Site details, soil physicochemical properties and sampling methods have been reported previously by Dignam et al. (2021) who found that growth of ryegrass and clover mixtures was significantly inhibited by the presence of a range of soil-borne plant pathogens. Soil was sieved to 4-mm diameter and stored at 4°C until used. Soil moisture content was adjusted and maintained at 75% of maximum water holding capacity as described by Wakelin et al. (2016). Soil (160 g) was weighed into plastic pots (diameter 10 cm, depth 7 cm).

Ripgut brome seed was collected from the field in 2020 and supplied to AgResearch by the Foundation for Arable Research (FAR), Canterbury, New Zealand. Vulpia hair grass seed was collected by AgResearch, Ruakura, New Zealand. Before use in experiments, germination tests were carried out to confirm seed viability and health. Seeds were rinsed three times for 5 min in sterile distilled water and air dried on sterile filter paper in a laminar-flow cabinet. For the three different soils, a 50 g sample of soil was mixed with ten ripgut brome seeds and secured in a mesh bag (80 x 80 mm of nylon mesh). One mesh bag was placed on the soil surface of each pot and covered with 10 mm of the corresponding soil. The same process was followed using vulpia hair grass seed (50 seeds per bag buried under a thin layer (approximately 2 mm) of soil). Six replicate pots of each seed and soil combination were set up.

Pots were placed in a growth room (16 h light at 22°C, 8 h dark at ambient temperature) for 4 weeks. Germination was recorded after 4 weeks when the seed bags were retrieved from the six replicate pots. Seeds were hand sorted from the soil using sterilised forceps and containers. Seeds were visually categorised into: a) intact seed; b) germinated seed; c) microbial-mediated decay; d) germinated seed fatally damaged; or e) seed damaged physically or by predation, as described in Chee-Sanford and Fu (2010). All ungerminated seeds collected at 4 weeks were examined by Plant Diagnostics Ltd, Christchurch, New Zealand for the presence of plant pathogenic fungi, oomycetes and bacteria by plating individual seeds on a general growth medium and transferring cultures onto a range of selective and semi-selective agars. Isolates were returned to AgResearch where the cultures were transferred on to potato dextrose agar (PDA, Oxoid Ltd, United Kingdom) and 3-mm disks of freshly growing mycelium were transferred into sterile 10% glycerol for long term storage at -80°C.

Identification of putative pathogens/candidate biocontrol isolates

Isolates were identified to genus or species level at Plant Diagnostics Ltd using standard morphological methods (Singleton et al. 1992) and classified as putative pathogens or

saprophytes. Following identification, isolates were checked against the Ministry for Primary Industries (MPI) unwanted organisms list (MPI 2020), and any species present on this list were not used in further experimentation.

Molecular identification of *Marasmius* sp. isolates

Pure cultures of identified isolates were subcultured into 100 mL potato dextrose broth (Oxoid) in 250 mL conical flasks and incubated in a rotary shaker at 150 rpm at 25°C for 14 d to produce mycelial biomass. The mycelial biomass was filtered and washed in sterile distilled water using a Buchner funnel and vacuum through a filter paper (Whatmann 114) and frozen at -80°C.

The Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research, USA) was used to extract DNA from 100 mg of mycelium following the manufacturer's instructions. For identification to genus level, the ITS1-5.8S-ITS2 region was amplified using primers ITS5 and ITS4 (White et al. 1990). The polymerase chain reaction (PCR) was performed in 25 µL volumes using 1µL of genomic DNA, 1 x Bioline MyTaq reaction buffer (containing 1 mM dNTPs, 3 mM $MgCl_2$, stabilizers and enhancers), 0.2 µM of each primer and 1 U MyTaq™ DNA Polymerase (Bioline Pty. Ltd, Australia). The PCR thermal cycling programmes were performed with an initial denaturing step at 95°C for 3 min, then 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s, and a final extension step of 72°C for 10 min. The PCR was validated by agarose electrophoresis and then PCR products were cleaned with Monarch® PCR & DNA Cleanup Kit (New England BioLabs, United Kingdom) following the manufacturer's instructions. The final product was sent to Macrogen (Korea) for sequencing in both directions. Using the Geneious Prime 2021.1.1 software (Biomatters, Auckland, New Zealand), consensus sequences of each sample were produced and ambiguous sites were edited manually before the sequences were exported for phylogenetic analysis.

The sequences were compared with the current National Center for Biotechnology Information (NCBI) nucleotide collection (nr/nt) (30/9/2022) using BLASTn (http://blast.ncbi.nlm.nih.gov) for the identification of ITS1-5.8S-ITS2 region in closely related strains. Sequences with high similarity (90-100% identity and e-value of 0.0) were downloaded for inclusion in the phylogenetic analysis. Sequences were aligned using the MUSCLE algorithm in Geneious Prime (Kearse et al. 2012) and trimmed to equal lengths of 500 nt. A neighbour-joining dendrogram of the ITS1-5.8S-ITS2 region was constructed using the Jukes-Cantor method (Jukes & Cantor 1969) and MEGA11 software (Tamura et al. 2021). The similarity in DNA sequences between the isolates was compared using 1000 replicates for bootstrap analysis.

Inhibition of germination of ripgut brome seeds by putative seed pathogens

Some potential biocontrol pathogens were isolated from *Vulpia* spp. but difficulties were encountered with low and inconsistent germination rates of seed. As limited resources were available, the decision was made to focus on ripgut brome for the remainder of this study.

Putative biocontrol pathogens of ripgut brome or *Vulpia* spp. (30 isolates; labelled W1 – W30; Table 1) were cultured on PDA in darkness at 20°C for 7 d. Isolates were then sub-cultured on to 1/5 PDA in sterile clear plant tissue culture tubs (Alto; 98 mm diameter, 250 mL volume) by placing 3-mm fungal mycelial disks (excised from the edge of an actively growing culture) onto the agar surface. Control tubs containing 1/5 PDA were inoculated with 3-mm disks of sterile PDA. Isolates were incubated at 20°C in the dark until fungal growth was established across the surface of the agar (~ 7 d). Washed ripgut brome seeds were placed on the surface of the agar (5 seeds per tub, 4 replicate tubs per putative pathogen). The tubs were placed in a growth room and incubated as described above. Germination was recorded every 3 d for 21 d.

Plant specificity of candidate biocontrol isolates

Non-target plant species of significance to the pasture and arable sectors were selected and seeds were provided by Grasslanz Technology Ltd, New Zealand and the New Zealand Institute for Plant and Food Research Ltd. Species included barley (*Hordeum vulgare*), oats (*Avena sativa*), brome grass (*Bromus* sp.), prairie grass (*Bromus catharticus*), cocksfoot (*Dactylis glomerata*) and ryegrass (*Lolium perenne*). Potential effects of candidate ripgut brome seed pathogens on these non-target plant seeds were tested in three independent experiments to ensure the isolates had no inhibitory effect on the non-target plants.

In the first experiment, six fungal isolates were randomly selected from the 30 isolates that inhibited ripgut brome or vulpia hair grass seed in the preliminary screening (Table 1) and tested against barley, oats and ripgut brome. The six isolates were *Fusarium culmorum* W4 and W21, *Fusarium* sp. W9 and W24, *F. graminearum* complex W7, and *Marasmius* sp. W15. Two replicate tubs were set up for each plant species, with each tub containing five seeds. The other plant species were not assessed owing to very low germination rates.

In the second experiment (Experiment 2), germination of barley, oats, ryegrass, cocksfoot and ripgut brome seeds was monitored in the presence of four isolates of *Marasmius* sp. (W14 – W17), which were inoculated into three replicate tubs for each plant species.

The third experiment (Experiment 3) followed the same design as Experiment 2, with the exception that preliminary germination tests were conducted to assess seed viability and to improve germination rates; seeds of brome, prairie grass and cocksfoot seeds were rubbed to remove husks before use. All experiments included untreated non-target plant seeds as control treatments. Ripgut brome was also re-tested in all experiments.

Statistical analyses

Germination rates of seeds in each of the three experiments were compared between the potential pathogens and untreated control, using a generalised linear model (GLM) with binomial distributions through a logit link function. The GLM analysis was applied to data from each experiment separately from other experiments, and, to each plant

Table 1: Fungal isolates recovered from ungerminated ripgut brome or vulpia hair grass seeds from different sites. Selected isolates have been tested on their effect (average germination rates %) on ripgut brome seed in *in vitro* screening assays. Each isolate was screened at least twice in two separate assays. Culture collection numbers shown in bold were used in Experiment 1; culture collection numbers shown in italics were used in Experiments 2 & 3.

Isolate	Site of isolation	Isolated from	Genus	Description of genus/ species (based on literature)	Assigned Culture collection code and number	Germination Rate (mean %)
1	Hamilton *	*Bromus diandrus*	*Alternaria* sp.	Unknown pathogenicity		
2	Hamilton *	*Bromus diandrus*	*Alternaria* sp.			
3	Hamilton *	*Bromus diandrus*	*Alternaria* sp.			
4	Hamilton *	*Bromus diandrus*	*Alternaria* sp.			
5	Hamilton *	*Bromus diandrus*	*Chaetomium* sp.	Saprophyte		
6	Hamilton *	*Bromus diandrus*	*Epicoccum* sp.	Saprophyte		
7	Hamilton *	*Bromus diandrus*	*Epicoccum* sp.			
8	Hamilton *	*Bromus diandrus*	*Epicoccum* sp.			
9	Hamilton *	*Bromus diandrus*	*Fusarium oxysporum*	Broad spectrum pathogen, including of grass spp.		
10	Hamilton *	*Bromus diandrus*	*Fusarium oxysporum*			
11	Hamilton *	*Bromus diandrus*	*Fusarium* sp..	Broad spectrum pathogen, including of grass spp.		
12	Hamilton *	*Bromus diandrus*	*Marasmius* sp.	Common in pastures, unknown as pathogen	*W14*	0
13	Hamilton *	*Bromus diandrus*	*Marasmius* sp.		***W15***	0
14	Hamilton *	*Bromus diandrus*	*Marasmius* sp.		*W16*	0
15	Hamilton *	*Bromus diandrus*	*Marasmius* sp.		*W17*	0
16	Hamilton *	*Vulpia* spp.	*Alternaria* sp.	Unknown pathogenicity		
17	Hamilton *	*Vulpia* spp.	*Chaetomium* sp.	Saprophyte		
18	Hamilton *	*Vulpia* spp.	*Fusarium culmorum*	Grass pathogen	W6	10
19	Hamilton *	*Vulpia* spp.	*Fusarium culmorum*		W19	30
20	Hamilton *	*Vulpia* spp.	*Fusarium* sp.	Broad spectrum pathogen, including of grass spp.	**W24**	10
21	Hamilton *	*Vulpia* spp.	*Fusarium* sp.		W25	0
22	Hamilton *	*Vulpia* spp.	*Pythium* sp.	Broad spectrum pathogen, including of grass spp.	W30	90
23	Hamilton *	*Vulpia* spp.	*Pythium* sp.		W31	40
24	Hamilton *	*Vulpia* spp.	*Pythium* sp.			
25	Wallacetown **	*Vulpia* spp.	*Alternaria* sp.	Unknown pathogenicity		
26	Wallacetown **	*Vulpia* spp.	*Alternaria* sp.			
27	Wallacetown **	*Vulpia* spp.	*Fusarium culmorum*	Grass pathogen	W18	0
28	Wallacetown **	*Vulpia* spp.	*Fusarium culmorum*		W21	0
29	Wallacetown **	*Vulpia* spp.	*Pythium* sp.	Broad spectrum pathogen, including of grass spp.	W29	90
30	Wallacetown **	*Vulpia* spp.	*Pythium* sp.			
31	Wallacetown **	*Vulpia* spp.	*Stemphylium* sp.	Weak pathogen or saprophyte		
32	Wallacetown **	*Vulpia* spp.	*Stemphylium* sp.			
33	Wallacetown **	*Vulpia* spp.	*Trichoderma* sp.	Saprophyte		
34	Clifden **	*Bromus diandrus*	*Alternaria alternata*	Likely secondary pathogen	W10	100
35	Clifden **	*Bromus diandrus*	*Alternaria alternata*		W11	90

Table 1: *continued*

Isolate	Site of isolation	Isolated from	Genus	Description of genus/ species (based on literature)	Assigned Culture collection code and number	Germination Rate (mean %)
36	Clifden **	*Bromus diandrus*	*Alternaria infectoria*	Weak pathogen or saprophyte		
37	Clifden **	*Bromus diandrus*	*Alternaria infectoria*			
38	Clifden **	*Bromus diandrus*	*Alternaria* sp.	Unknown pathogenicity		
39	Clifden **	*Bromus diandrus*	*Botrytis cinerea*	Likely secondary pathogen	W23	70
40	Clifden **	*Bromus diandrus*	*Fusarium culmorum*	Grass pathogen		
41	Clifden **	*Bromus diandrus*	*Fusarium* sp.	Broad spectrum pathogen, including of grass spp.	**W9**	0
42	Clifden **	*Bromus diandrus*	*Pythium* sp.	Broad spectrum pathogen, including of grass spp.		
43	Clifden **	*Bromus diandrus*	*Rhizoctonia solani*	Broad spectrum pathogen, including of grass spp.	W1	50
44	Clifden **	*Bromus diandrus*	*Rhizoctonia solani*		W2	80
45	Clifden **	*Bromus diandrus*	*Rhizoctonia solani*		W3	80
46	Clifden **	*Bromus diandrus*	*Sordaria* sp.	Saprophyte		
47	Clifden **	*Bromus diandrus*	*Stemphylium* sp.	Weak pathogen or saprophyte		
48	Clifden **	*Vulpia* spp.	*Alternaria* sp.	Unknown pathogenicity		
49	Clifden **	*Vulpia* spp.	*Alternaria* sp.			
50	Clifden **	*Vulpia* spp.	*Alternaria* sp.			
51	Clifden **	*Vulpia* spp.	*Fusarium culmorum*	Grass pathogen	**W4**	0
52	Clifden **	*Vulpia* spp.	*Fusarium culmorum*		W5	25
53	Clifden **	*Vulpia* spp.	*Fusarium culmorum*		W20	10
54	Clifden **	*Vulpia* spp.	*Fusarium culmorum*			
55	Clifden **	*Vulpia* spp.	*Fusarium graminearum*	Grass pathogen	**W7**	5
56	Clifden **	*Vulpia* spp.	*Fusarium graminearum*		W8	50
57	Clifden **	*Vulpia* spp.	*Fusarium oxysporum*	Broad spectrum pathogen, including of grass spp.		
58	Clifden **	*Vulpia* spp.	*Fusarium* sp.	Broad spectrum pathogen, including of grass spp.	W22	10
59	Clifden **	*Vulpia* spp.	*Fusarium* sp.		W26	0
60	Clifden **	*Vulpia* spp.	*Fusarium* sp.		W27	0
61	Clifden **	*Vulpia* spp.	*Fusarium* sp.		W28	10
62	Clifden **	*Vulpia* spp.	*Pythium* sp.	Broad spectrum pathogen, including of grass spp.		
63	Clifden **	*Vulpia* spp.	*Stemphylium* sp.	Weak pathogen or saprophyte	W12	100
64	Clifden **	*Vulpia* spp.	*Trichoderma* sp.	Saprophyte		

Isolates were selected according to following criteria: not an unwanted organism, not saprophytic, culturable under standard conditions
* Indicates collection sites within Waikato (North:South and East:West: −38.578852:−37.77133 and 175.7413232:176.230559) (Dignam et al. 2021)
** Indicates collection sites within Southland (North:South and East:West: −46.57536:−45.43365 and 167.71716:168.86184) (Dignam et al. 2021)

species within each experiment independently from other plant species. Therefore, each GLM consisted of only a single treatment factor, which represented the tested isolates and untreated control treatment. All GLM analyses were carried out with statistical software Minitab version 16.2 (https://www.minitab.com).

RESULTS

Isolation of fungi from seeds and preliminary screening

Ungerminated and/or decaying seed was obtained from all three sites. On average, 10% of ripgut brome seeds and 30% of vulpia hair grass seeds failed to germinate. Also, 20% of the ungerminated vulpia hair grass seeds were unrecoverable.

A total of 64 isolates from 11 fungal genera were recovered from ungerminated seed and/or seed that appeared to have microbial-mediated decay. It was common for more than one, and up to three, fungal taxa to be isolated from a single seed. No bacterial pathogens were recovered. The genera of fungi recovered from seeds varied between sites and type of seed. The most prevalent soil-borne fungal taxa detected on ungerminated and decayed seed were *Fusarium* spp., which were recovered from both ripgut brome and vulpia hair grass seeds. Identified species included *F. culmorum, F. graminearum* and *F. oxysporum.* Isolates of *Fusarium* spp. can be either pathogenic or saprophytic, so 16 *Fusarium* isolates were retained for screening for their ability to inhibit ripgut brome seed germination (Table 1). A single unidentified *Marasmius* species was isolated from only one site and only from ripgut brome seeds. Various *Alternaria* species were also detected; these are likely to be weakly pathogenic or saprophytic. Three isolates belonging to the genus *Pythium* were also recovered and tested further. The remaining isolates were provisionally identified as members of the genera *Chaetomium, Epicoccum, Sordaria* and *Trichoderma*; these genera are generally considered as saprophytic and were discounted from further study.

The ability of the 30 selected isolates to inhibit germination of ripgut brome seed varied widely (Table 1). Four isolates of *Marasmius* sp. completely inhibited germination, as did four isolates of *Fusarium* sp., three isolates of *F. culmorum,* while one isolate of *F. graminearum* resulted in an average germination rate of only 5%. Treatment with 10 additional isolates of *Fusarium* sp. resulted in germination rates ranging between 10-100%. Isolates from other genera generally considered as plant pathogens - *Alternaria alternata*, *Rhizoctonia solani*, *Botrytis cinerea, Pythium* sp., *Stemphylium* sp. - did not substantially reduce germination.

Host range of candidate biocontrol isolates

Plant specificity of six candidate biocontrol isolates was evaluated by measuring their impact on germination of two crop species (oats and barley) and the weed species ripgut brome (Table 2). Germination of untreated seed was only 40% for oats and 60% for barley and ripgut brome. Even so, five isolates completely inhibited germination of ripgut brome seed, with isolate *F. graminearum* W7 allowing only 10% germination, which was consistent with the preliminary screening results. Unfortunately, all isolates, except *Marasmius* sp. W15 significantly reduced gemination of desirable oats and barley seeds. *Fusarium culmorum* isolates W4 and W21 prevented all germination in both oats and barley. There were differences in the inhibitory effects of *F. graminearum* W7, *Fusarium* sp. W9 and *Fusarium* sp. W24 between oats and barley (Table 2) but the germination rates were unacceptable. *Marasmius* sp. W15 was the only isolate where the germination of ripgut brome was reduced without a reduction in the crop species barley and oats. In fact, the germination rates for barley and oats seed treated with *Marasmius* sp. W15 were actually higher than for untreated seed. Based on this positive result, *Marasmius* sp. W15 and an additional three *Marasmius* sp. isolates (W14, W16 and W17) were evaluated against a wider range of non-target plant species in Experiment 2 (Figure 1). None of the *Marasmius* sp. isolates significantly impacted on germination of barley or ryegrass, while one of the four isolates, W17, reduced germination of oats and cocksfoot seeds. Germination of ripgut brome was significantly reduced by all *Marasmius* isolates, with germination rates (%) ±SE of 6.7±6.7, 0, 0, 13.3±9.1 for W14 – 17, respectively, compared with 73.3±11.8 % for untreated seed.

In Experiment 3 (Figure 2), testing against six non-target species, *Marasmius* sp. isolates had no adverse effects on germination of barley, ryegrass, cocksfoot, and prairie grass. One isolate (W16) inhibited germination of oats

Table 2 Percentage germination (±SE) of ripgut brome, oats and barley seeds treated with candidate biocontrol isolates after 21 days in Experiment 1.

	Germination (%)		
Isolate	**Ripgut brome**	**Oats**	**Barley**
F. culmorum W4	0 ± 0	0 ± 0	0 ± 0
F. culmorum W21	0 ± 0	0 ± 0	0 ± 0
F. graminearum W7	10.0 ± 10.0	20.0 ± 13.3	0 ± 0
Fusarium sp. W9	0 ± 0	30.0 ± 15.3	20.0 ± 13.3
Fusarium sp. W24	0 ± 0	0 ± 0	20.0 ± 13.3
Marasmius sp. W15	0 ± 0	70.0 ± 15.3	100 ± 0
Untreated	60.0 ± 16.3	40.0 ± 16.3	60.0 ± 16.3

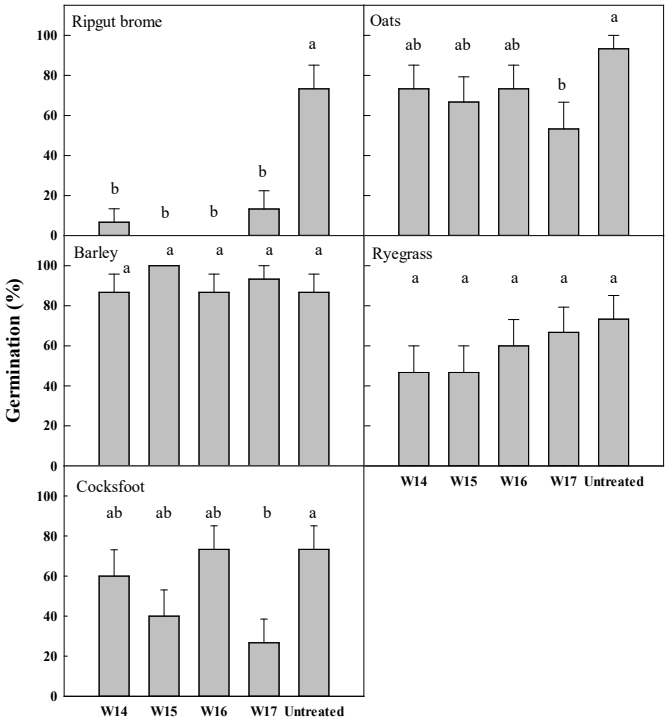

Figure 1 Germination (%) of ripgut brome seeds and seeds of four non-target plant species (oats, barley, ryegrass and cocksfoot) treated with four *Marasmius* sp. isolates (W14-W17) after 21 days in Experiment 2. Error bars are SE (%). Alphabetical letters show statistically significant differences at 5% significance levels, where isolates that share a common letter do not differ significantly. Alphabetical letters are comparable only within each plant species and are not comparable across the different plant species tested.

but for ryegrass and brome, some *Marasmius* sp. isolates resulted in higher germination rates than were recorded for the untreated control treatments. Germination rates (%) (±SE) of ripgut brome were also significantly less than the untreated control (90.0±6.9) in this assay but were higher than in Experiment 2, with 30.0±10.5, 50.0±11.5, 30.0±10.5, 60.0±11.2 for W14 – 17, respectively.

Molecular identification of *Marasmius* isolates

Nucleotide alignment of the ITS1-5.8S-ITS2 sequences of the four *Marasmius* sp. isolates W14 – W17 identified nucleotide similarity between the four isolates at >99%, indicating that the four isolates are likely of the same origin. Nucleotide alignment using BLASTn of 500 nt of AGR W14 – W17 consensus sequence against the NCBI database confirmed the AGR W14 – 17 isolates to be from the Marasmiaceae family (Figure 3). Isolates W14 – 17 are most closely related to two strains of *M. graminum* and one strain of *M. oreades*, forming a clade with *M. roforotula*, *M. nigrobrunneus, M. brunneoarantiacus*, which is distinct to other strains currently labelled *M. oreades*.

DISCUSSION

Fusarium spp. isolates were frequently recovered from ungerminated and decaying seed. *Fusarium* is arguably the most common genus of soil-borne pathogenic fungi with isolates causing damping off and root and stem rots on a wide range of plants (Booth 1971) and less commonly as seed pathogens (Thomas et al. 1999). The use of *Fusarium* spp. isolates as mycoherbicides has been investigated previously. *Fusarium oxysporum* has been shown to suppress emergence of sunflower broom rape (*Orobanche cumana*; Thomas et al. 1999) and striga (*Striga hermonthica*; Abbasher and Sauerborn 1992) under controlled conditions, and *F. tumidum* reduced gorse seedling emergence and growth (Yamoah et al. 2006). Pearson et al. (2016) identified three *Fusarium* spp. isolates active against ragwort (*Jacobaea vulgaris*) that did not cause disease in six non-target pasture grass species but attacked two clover species, demonstrating the difficulties of identifying isolates with sufficient specificity to allow their use as biocontrol agents. In one of the few field evaluations of *Fusarium* spp. isolates for weed management, *Fusarium oxysporum* f. sp. *strigae* applied to soil for management of striga resulted in significantly increased maize yields of smallholder farmers in Kenya (Nzioki et al. 2016), demonstrating proof-of-concept of this approach.

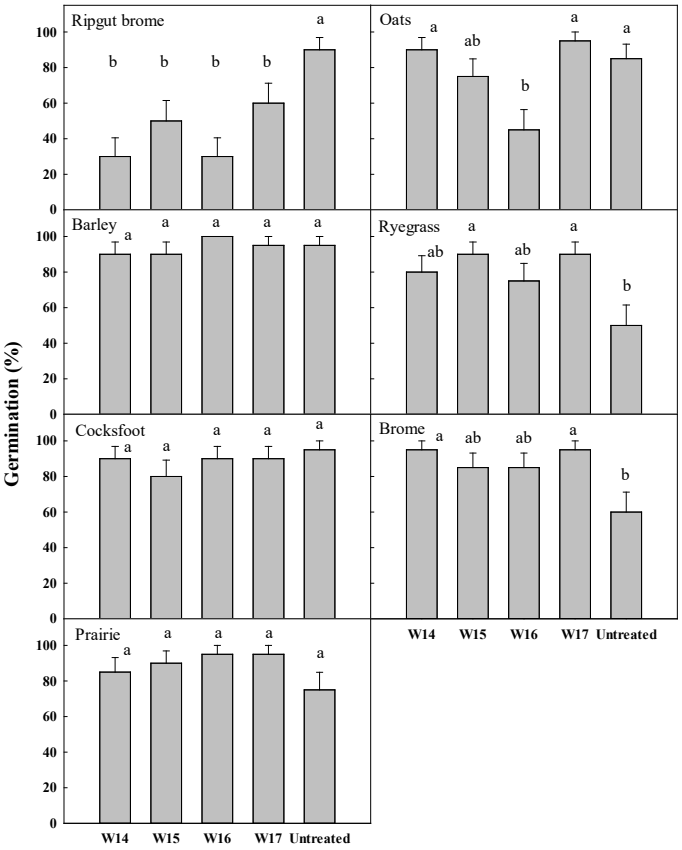

Figure 2 Germination (%) of ripgut brome seeds and seeds of six non-target plant species (oats, barley, ryegrass, cocksfoot, brome and prairie) treated with four *Marasmius* sp. isolates (W14-W17) after 21 days in Experiment 3. Error bars are SE (%). Alphabetical letters show statistically significant differences at 5% significance levels, where isolates that share a common letter do not differ significantly. Alphabetical letters are comparable only within each plant species and are not comparable across the different plant species tested.

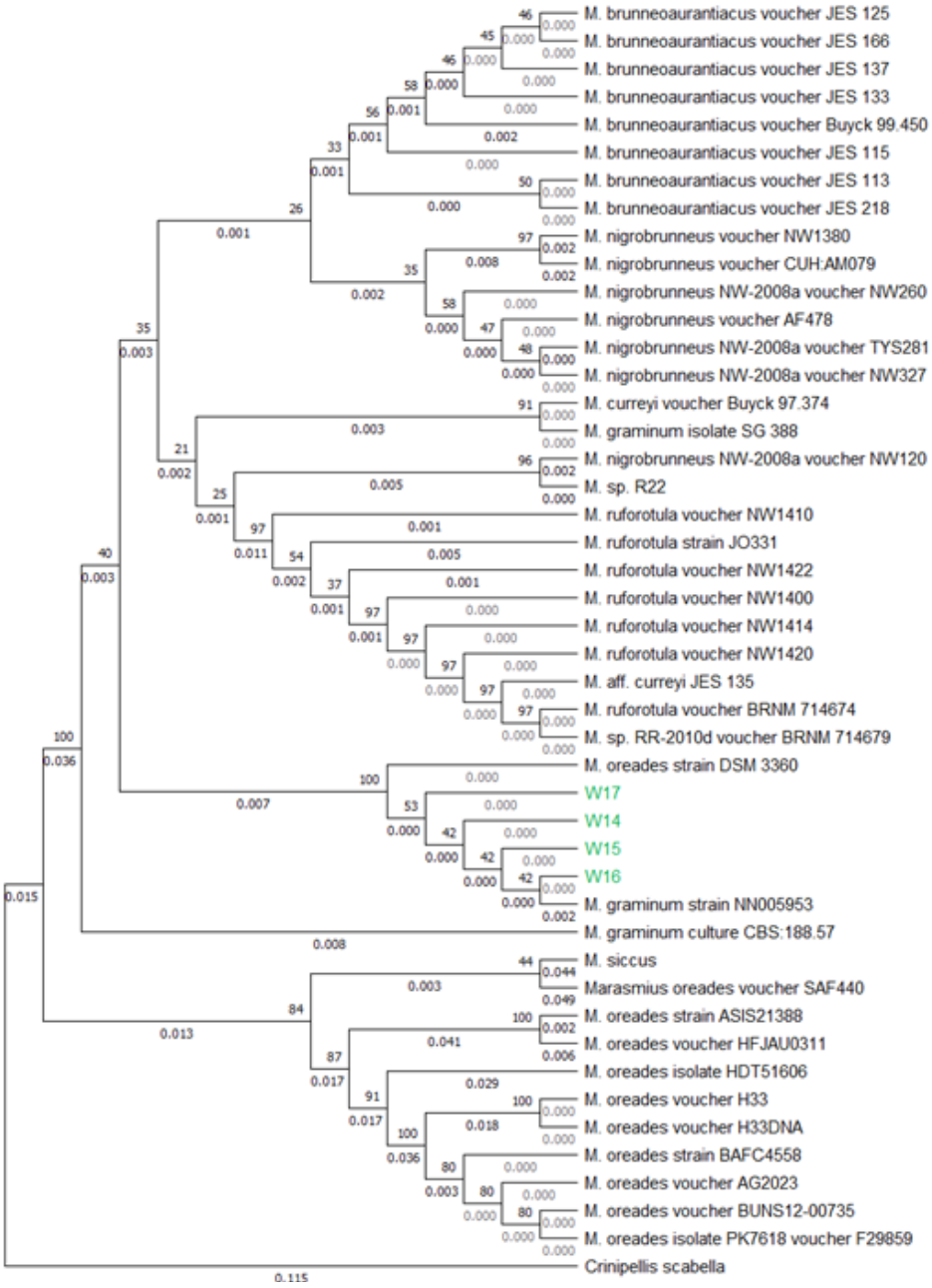

Figure 3 Phylogenetic tree was constructed in MEGA11 (Tamura et al. 2021) with sequences from the closest hits NCBI BLAST retrieved sequences that showed highest similarity to W14 – 17 consensus sequence. The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The optimal tree is shown and isolates of interest W14-W17 are highlighted in green. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felenstein 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor 1969) and are in the units of the number of base substitutions per site indicated below branches. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The ITS1-5.8S-ITS2 region of *Crinipellis scabella* was used as an outgroup.

There was wide variability in the ability of *Fusarium* spp. isolates to inhibit ripgut brome seed germination. Some isolates completely prevented germination in this study so it is possible that some *Fusarium* spp. isolates are primary pathogens of ripgut brome, but further testing would be needed to confirm this. However, as *Fusarium* spp. are well known as plant pathogens, we took the decision to focus on *Marasmius* sp. isolates given their lack of effect on key non-target plant species.

The genus *Marasmius* is comprised of over 600 species, some of which display phytopathogenicity, e.g. *M. graminum, M. palmivores* and *M. pueraria* (Sridhar et al. 2022). The *Marasmius* genus is sectioned into *Marasmius* sect. *Globulares, Leveilleani, Marasmius, Neosessiles* and *Sicci* (Oliveira et al. 2020). Further phylogenetic analysis by Oliveria et al. (2020) using ntITS data grouped the *Marasmius* sect. *Marasmius* into four clades: sect. *Marasmius* subsect. *Marasmius* (MM), *Marasmius horrigulus* (MH), sect.

Marasmius subsect. *Sicciformes* 1 (MS-1) and sect. *Marasmius* subsect. *Sicciformes* 2 (MS-2). Based on the ITS sequencing of W14 – 17, our isolates fall into the MS-2 clade group A. The ITS sequencing showed that W14 – 17 are closely related to *M. graminum* NN00593, strains of which cause seedblight in rice, several bean species, corn, and squash (Gaire et al. 2021). Our isolates are also closely related to *M. oreades* DSM 3360. *Marasmius oreades* is commonly known as the fairy-ring fungus, because of its circular, underground mycelia that produces characteristic rings of mushrooms in pasture (Hiltunen et al. 2021). *Marasmius oreades* is generally regarded as a saprophytic species and has rarely been recorded as a plant pathogen to date. However, our phylogenetic analysis suggests the possibility that *M. oreades* DSM 3360 may be misclassified and, therefore, our W14 – 17 isolates may not be of *M. oreades* origin. The identified taxonomic subgroup MS-2 of *Marasmius* sp., in which the W14 – 17 isolates cluster, is poorly described and needs further characterisation through multigene phylogeny. To confirm species level identification and the genetic differences between W14 – 17 isolates (which may impact their activity against ripgut brome seed), whole genome sequencing will be required in future studies.

Assay results (Experiment 3) suggested that some *Marasmius* sp. isolates enhanced germination of ryegrass and brome. Increased growth of grasses has been reported in association with the presence of *Marasmius oreades* in soil, thought to result from the recycling of plant nutrients, particularly the solubilisation of nitrogen and phosphorus (Gramss et al. 2005). Further work is needed to validate the possible growth enhancement observed in this experiment.

There was some variability between the assays in terms of the extent of inhibition of germination of ripgut brome. Assays were run under standard conditions so this variability could be related to a loss of virulence during the storage of the cultures. This is unlikely as cultures were not stored for long periods between assays, but this may require investigation if subsequent experimentation indicated ongoing variability. Regardless of this variability, the *Marasmius* sp. isolates show promise for management of the ripgut brome seed bank. Experiments to date have been conducted under artificial conditions and further work will be needed to replicate these promising results in soil. To date, attempts to culture the isolates on substrates amenable to evaluation under more natural conditions have been unsuccessful and work is needed to understand the specific conditions that will allow production of sufficient biomass needed to allow larger scale evaluation.

It is vital that safety and specificity of any biocontrol agent is thoroughly evaluated before any organism is released (Barton 2004). Ripgut brome is a challenging target for biocontrol as there are useful grasses in the same genus as well as in closely related genera. Therefore, further work is needed to test the isolates against a wider range of non-target species to allow a true assessment of its potential for development as a biological control agent of ripgut brome. Should sufficient specificity be demonstrated, detailed investigations into its mode of action would be needed to determine how best to utilise this capacity for weed suppression.

CONCLUSIONS

Isolates of *Marasmius* sp. that prevent the germination of ripgut brome seed in *in vitro* assays have been recovered from New Zealand soils. As the isolates do not have adverse effects on several key non-target arable species, further investigation of *Marasmius* sp. isolates as potential biocontrol agents for the management of the ripgut brome seed bank is warranted as few tools exist for the management of this intractable weed species capable of prolific seed production and dispersal. Next steps include testing the activity of isolates against a wider range of economically important non-target species such as ryegrass and clovers, and optimisation of culture methods to allow evaluation of activity in soil.

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