WEED RESEARCH An International Journal of Weed Biology, Ecology and Vegetation Management

DOI: 10.1111/wre.12094

Does *Fusarium*-caused seed mortality contribute to *Bromus tectorum* stand failure in the Great Basin?

S E MEYER*, J-L FRANKE†, O W BAUGHMAN‡, J BECKSTEAD§ & B GEARY†

**USFS Rocky Mountain Research Station, Shrub Sciences Laboratory, Provo, UT, USA, †Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT, USA, ‡Department of Natural Resources and Environmental Science, University of Nevada, Reno, NV, USA, and §Department of Biology, Gonzaga University, Spokane, WA, USA*

Received 4 November 2013
Revised version accepted 16 April 2014
Subject Editor: Karen Bailey, AAFC, Canada

Summary

Bromus tectorum (cheatgrass, downy brome) is an important invader in western North America, dominating millions of hectares of former semi-arid shrubland. Stand failure or 'die-off' is relatively common in monocultures of this annual grass. The study reported here investigated whether soil-borne pathogens could be causal agents in die-offs. Soils from two die-off areas and adjacent *B. tectorum* stands were used in a glasshouse experiment with sterilised and non-sterilised treatments. Soil sterilisation did not increase emergence, which averaged 80% in both die-off and non-die-off soils. Seedling biomass was higher in die-off soils, probably due to increased nitrogen availability. *Fusarium* was isolated from 80% of killed seeds in non-sterilised soil treatments. In pathogenicity tests with 16 *Fusarium* isolates, host seeds incubated under water stress (−1.5MPa for 1 week prior to transfer to free water) suffered over twice the mortality of seeds incubated directly in free water (25–83% with water stress vs. 5–43% without water stress). These results suggest that soil-borne *Fusarium* could play a role in *B. tectorum* stand failure in the field, but that low water stress conditions in the glasshouse experiment were not conducive to high levels of disease. Pathogenic *Fusarium* isolates were obtained from seeds planted in both die-off and non-die-off soils, suggesting that microenvironmental factors that affect levels of water stress might be as important as relative abundance of soil-borne pathogens in mediating spatial patterns of disease incidence in the field.

Keywords: cheatgrass, downy brome, die-off, seed rot, semi-arid shrubland, soil-borne pathogen, water stress.

MEYER SE, FRANKE J-L, BAUGHMAN OW, BECKSTEAD J & GEARY B (2014). Does *Fusarium*-caused seed mortality contribute to *Bromus tectorum* stand failure in the Great Basin?. *Weed Research* **54**, 511–519.

Introduction

The invasive annual grass *Bromus tectorum* L. (cheatgrass, downy brome) was introduced into the western United States from Eurasia. A winter annual that produces fine-textured fuel that dries early in the summer, *B. tectorum* has been a major driver of the increase in the size and frequency of wildfires throughout the region (Brooks *et al.*, 2004). These fires repeatedly create new openings for *B. tectorum* perpetuation and spread, leading to the millions of hectares of former shrubland that are now occupied by near monocultures of this grass (Knapp, 1996).

Bromus tectorum is known to be subject to attack by pathogens that prevent seed production or cause dormant seed mortality (Meyer *et al.*, 2007, 2008, 2010), but pathogens that can cause high mortality of non-dormant seeds are poorly studied. The phenomenon of

Correspondence: S E Meyer, USFS Rocky Mountain Research Station Shrub Sciences Laboratory, 735 North 500 East, Provo UT 84606, USA. Tel: (+1) 801 356 5122; Fax: (+1) 801 375 6968; E-mail: semeyer@xmission.com

B. tectorum stand failure or 'die-off' on a large scale has recently come to the attention of land managers in several parts of the Great Basin (Donald Major, Bureau of Land Management, Boise, Idaho, personal communication; Baughman & Meyer, 2013). These die-offs are especially conspicuous in spring, when barren areas covered with grey-brown litter from previous years contrast sharply with the bright green of established stands (Fig. 1). The affected areas suffer decreased forage production and are exposed to increased risk of soil erosion, but they may also present opportunities for restoration. Understanding the management implications of these die-offs requires a mechanistic explanation of their causes as well as their consequences.

Field seedbank studies revealed that the seed pathogen *Pyrenophora semeniperda* was unlikely to be a *B. tectorum* die-off causal agent, although it could affect the rate of post-die-off stand recovery through its impacts on the persistent seedbank (Baughman & Meyer, 2013). Integration of herbicide management with *P. semeniperda* shows potential for long-term control of *B. tectorum* (Ehlert *et al.*, 2014). In the present investigation, the objective was to determine whether stand failure could potentially be caused by other soil-borne pathogens. This objective was approached through a series of studies, beginning with die-off site characterisation and a glasshouse seeding experiment using die-off field soils, followed by isolation of fungal organisms from killed seeds from the glasshouse experiment, identification of potential pathogens and pathogenicity trials on *B. tectorum* seeds. The research addressed the following hypotheses: (i) Soils from die-off areas contain soil-borne pathogens that can cause *B. tectorum* emergence failure, (ii) *Fusarium* is an important soil-borne pathogen in *B. tectorum* communities, (iii) *Fusarium* isolates from killed seeds recovered from die-off soils will be capable of causing *B. tectorum* seed mortality and (iv) *Fusarium*-caused seed mortality will vary as a function of isolate and whether the seeds are under water stress and unable to germinate during infection.

Fig. 1 Eden Valley study site near Winnemucca, Nevada, in late spring 2009, at the time of soil sampling for the glasshouse study, showing the sharp boundary between the die-off area and the intact *Bromus tectorum* stand.

Materials and methods

Sample collection and preparation

Two die-off sites, in Skull Valley, Utah, and Eden Valley, Nevada, were chosen for soil collection (Table 1, Fig. 1). Two types of samples were collected at each site. Seedbed cores were collected by pressing a steel ring 8 cm in diameter by 2.5 cm deep into the soil surface. Samples were kept in the rings within plastic Petri dishes, preserving the seedbed and upper soil structure. Deep cores were collected similarly, but with a steel ring 5 cm deep so as to include deeper soil as well as surface soil. Deep core samples were stored in small paper bags, so that the soil structure was not preserved. At each site, 60 samples of each core type were collected within each condition (die-off and non-die-off control), for a total of 240 samples per site. All samples were stored air-dry (*c.* 8% moisture content). For the sterilisation treatment, half of the samples in each core type x site x condition group (240 samples total) were moist-sterilised in an autoclave at 121°C and 140 kPa for two back-to-back sessions of 45 min each. Remaining samples were not sterilised.

Soils were also collected for physicochemical characterisation from die-off and non-die-off (control) areas at each site (Table 2). Samples represented the first 15 cm of the soil profile below the litter layer. These were analysed using standard protocols to obtain texture, pH, salinity, sodium adsorption ratio, organic matter content, calcium carbonate equivalent, nitrate nitrogen, soluble potassium and plant available (Olsen) phosphorus (Van Reeuwijk, 2002). Soils

Table 1 Die-off study site location and habitat information

	Skull Valley study site	Eden Valley study site
GPS co-ordinates	40°23′51.08″N, 112°56′54.15″W	41°10′14.71″N, 117°24′49.30″W
Elevation	1593 m	1546 m
County	Tooele County	Humboldt County
State	Utah	Nevada
Potential plant community	Shadscale (*Atriplex confertifolia*)	Sagebrush (*Artemisia tridentata*)
Exposure/slope	ESE *c.* 28°	SE *c.* 18°

Table 2 Soil properties (0–15 cm) for field soils used in the 2009 glasshouse die-off study

	Skull Valley site				Eden Valley site			
	Control		Die-off		Control		Die-off	
Soil properties	Mean	SE	Mean	SE	Mean	SE	Mean	SE
P (Olsen.) ppm†	19.1	1.9	21.5	1.7	26.1	2.3	24.5	2.5
Nitrate-N ppm††	3.1	0.35	14.5	1.21	2.4	0.23	18.3	0.72
pH†	7.55	0.02	7.36	0.13	6.31	0.13	6.22	0.18
EC dS/m (salinity)†	0.89	0.04	0.87	0.03	0.45	0.02	0.625	0.041
$CaCO_3$ equiv %†	29.0	0.53	30.6	0.33	1.21	0.20	1.14	0.28
Clay (%)†	24.9	0.67	22.8	0.42	17.2	1.07	18.2	1.10
Silt (%)†	43.1	0.75	33.1	0.50	52.3	1.16	48.1	2.16

Only properties that showed significant differences between sites or between control and die-off soils are shown. Properties followed by †were significantly different ($P < 0.05$) between sites, while properties followed by ††were significantly different ($P < 0.05$) between die-off and control soils across both sites. Sample size was 10 per condition for the Skull Valley site and five for the Eden Valley site.

variables were statistically examined using analysis of variance (SAS Proc GLM) for a completely randomised design, with site and condition (die-off vs. control) as fixed main effects.

Glasshouse experiment

The experiment was set up in a completely randomised design with 15 replications and repeated twice in time. Each trial (repetition) included a total of 2880 seeds and 240 experimental units (pots). Each field soil sample was placed in a 1200 cm^3 pot on the surface of 600 cm^3 of sterilised sand. The seedbed cores were placed intact within sample rings on the surface of the sand, whereas the soil/litter material from the deeper cores was spread evenly over the surface.

Twelve non-dormant *B. tectorum* seeds (collected near Spanish Fork, Utah, in 2008) were planted into each pot to test for the effects of soil pathogens on seedling emergence, survival and growth (Beckstead & Parker, 2003). Seeds were surface-sterilised (1 min 70% ethanol, 1 min 0.05% sodium hypochlorite), rinsed and dyed with a non-toxic safranin stain, then planted vertically with the red-dyed awns visible above the soil surface. Pots were watered to field capacity and randomly redistributed every 2 days in a temperature-controlled natural-sunlight summer glasshouse environment at *c.* 24°C. All non-dyed seeds that emerged from the natural seedbank in non-sterilised soil were removed to avoid confounding of emergence estimates. The number of safranin-dyed seeds that emerged was scored seven times over 18 days in each trial. At 18 days, surviving seedlings were counted and above-ground seedling tissue was harvested, dried at 60°C for 24 h and weighed. Mean per-seedling biomass was then calculated for each pot.

Mixed model analysis of variance (SAS Proc Mixed) was used to analyse the glasshouse experimental data set. Site, treatment (die-off vs. non-die-off soil), sterilization and sample type (seedbank vs. deep core) were considered fixed effects, while trial was the random effect. Soils from two sites were included in the study to broaden the scope of inference beyond that possible from an experimental study with a single soil, but these two sites were not intended to be a representative random sample of die-off sites in general. For this reason, site was considered to be a fixed effect in the analysis, and inferences about die-offs were limited appropriately. Response variables included emergence proportion (arcsine-square root-transformed) and above-ground biomass per seedling (log-transformed). Least squares means comparisons were made for significant interactions.

Isolation and identification of potential pathogens

To identify potential soil-borne pathogens, one killed safranin-dyed seed was selected from each of four randomly selected pots from each of the following treatment combinations in the first glasshouse experimental trial: two sites x sterilised vs. non-sterilised treatments x die-off vs. control x seedbed vs. deep soil, for a total of 64 seeds. Seeds were rinsed, surface-sterilised and plated individually onto malt extract agar. Following grow-out, one or more apparently different fungi from each plate were transferred to PDA (potato dextrose agar) by single-sporing, hyphal tipping or mycelial plug transfer. A total of 68 pure cultures were obtained using this protocol, many of which appeared to be similar, based on superficial cultural attributes (e.g. colony colour, surface texture). The pure cultures were air-dried (*c.* 8% moisture content) in Petri dishes under sterile conditions and stored at room temperature (*c.* 22°C) for 18 months. For molecular-genetic analyses, the dried cultures were rehydrated and cultured on PDA plates and increased in liquid culture in PDB (potato dextrose broth), and the resulting

mycelial mass was allowed to air-dry. Cultures were also maintained in actively growing condition on PDA for possible use in pathogenicity studies.

DNA was extracted from the dried mycelium of each of the 68 isolates using the ZR fungal/bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. The ITS1 and ITS2 regions and the intervening 5.8 s coding region of the ribosomal RNA gene were amplified as a single unit (White *et al.*, 1990). Presence of a single PCR product was confirmed with agarose gel electrophoresis, and the product was purified using a Promega Wizard SV Gel and PCR Clean-Up system (Promega Corporation, Fitchburg, Wisconsin) then sequenced with the BigDye Terminator Kit (Perkin-Elmer). Sequenced fragments were detected and analysed on an ABI 3730xl DNA analyzer and aligned with the GENEIOUS 5.4 program (Biomatters Ltd., Auckland, New Zealand (available from http://www.geneious.com/). GENBANK was used to blast search each ITS sequence and make an identification to genus.

Based on its numerical dominance (40 of 68 isolates) in the collection obtained from the glasshouse study, *Fusarium* was identified as a probable key pathogen. Sixteen of the 40 *Fusarium* isolates were selected for further study. There was approximately equal representation of isolates from Eden Valley and Skull Valley and from die-off and control soils. All isolates came from non-sterilised treatments.

A more specific primer set was used for *Fusarium* species identifications, targeting the TEF (translation elongation factor) gene region (Park *et al.*, 2011). The isolates that had been reserved on PDA plates were grown in shaker culture (170 RPM) for 1–2 weeks at 25°C (12 h:12 h photoperiod) in ½ strength PDB to produce mycelium for DNA extraction. The cultures were strained from the broth using surface-sterilised tulle material. The mycelium was then exposed to two freeze–thaw cycles to maximise DNA yield. Between 200 and 600 mg (wet weight) of fungal tissue was used for extraction using the ZR fungal/bacterial DNA Miniprep Kit following the manufacturer's protocol. Final elutions were performed with 2 × 50 μL elution buffer producing a final DNA concentration of 50–150 μL. The TEF region was amplified as a single unit and sequenced as described earlier for ITS sequencing. Aligned sequences were blast searched against GENBANK and the FUSARIUM-ID database (Park *et al.*, 2011).

Pathogenicity testing

The objectives of pathogenicity testing were to determine: (i) whether *Fusarium* isolates were pathogenic on non-dormant *B. tectorum* seeds, (ii) whether mortality was increased when seeds were incubated under water stress that suppressed germination following inoculation and (iii) whether the isolates differed in their response to the presence or absence of water stress during infection.

The isolates were subcultured onto 10 plates of 1/3 strength PDA and incubated at room temperature for 2 weeks, so that mycelial mats *c.* 3.5 cm in diameter were produced. The mycelium in each dish was then wounded by scraping with a sterile rubber policeman (scraper) to induce spore production (Campbell *et al.*, 2003). The cultures were incubated for another 2 weeks to ensure adequate spore production for the target load of 250000 spores mL^{-1}. The subculturing and inoculum production procedure were performed separately for each repetition of the pathogenicity test, which was performed twice in time. Each repetition had the same factorial design with isolate and water potential during the first week of incubation (0 or −1.5 MPa) as the main factors and with four replicates of 50 seeds for each treatment combination, for a total of 128 experimental units (Petri dishes) and 6400 seeds in each repetition. Non-inoculated controls were also included for each water potential treatment in each repetition. *Bromus tectorum* seeds (collected near Spanish Fork, Utah, in 2009) were cleaned, surface-sterilised as described earlier and counted into sterile vials in groups of 50 for inoculation. Spores were harvested from the culture dishes by pipetting 5 mL of autoclaved double-deionised water into each of the 10 dishes for each isolate. The back of a sterile metal weighing spatula was used to gently knock the spores free, and the mixture was decanted. Spore densities were quantified for each isolate using a hemocytometer. Each spore suspension was then diluted to a concentration of *c.* 250 000 spores mL^{-1}. The seeds were inoculated by pipetting 2–5 mL of inoculum suspension into each vial and soaking for 1 min. Control seeds were mock-inoculated with sterile water.

The water stress (negative water potential) treatment was achieved using a polyethylene glycol 8000 (PEG) solution at −1.5 MPa, verified with a water activity meter (Michel & Kaufman, 1972). The PEG was autoclaved to ensure sterility; water potential was measured after autoclaving and was not altered. For the water stress treatment, seeds were incubated for 7 days in the PEG solution then transferred to water for 14 days. For the no water stress treatment, seeds were incubated for the entire 21 days in water.

To set up the experiment, water or PEG solution was added to labelled Petri dishes by soaking two blue germination blotters (Anchor Paper, St. Paul MN, USA) in the appropriate liquid and transferring them to the dishes. Fifty inoculated seeds were then spread out into each dish. The dishes were stacked, bagged

and placed in an incubator at 25°C with 12 h:12 h photoperiod. On days 2, 4, 7, 9, 11, 14, 17 and 21, seeds that were killed or had germinated were counted and removed. Seed death was indicated by the development of a conspicuous white tuft of mycelium at the radicle end (Fig. 2). A preliminary test showed that seeds that manifested these mycelial tufts never germinated and were clearly dead. All seeds were either germinated or killed.

Mixed model ANOVA (SAS Proc Mixed) was used to analyse the results from the pathogenicity experiments. Pathogen isolate and water stress treatment were considered fixed effects, and repetition in time was considered a random effect. The response variable was *Fusarium*-killed seed proportion (arcsine-square root-transformed). To determine seed mortality levels and water stress treatment effects in the absence of added inoculum, non-inoculated control treatments were analysed independently using mixed model ANOVA with water stress treatment as a fixed effect and repetition as the random effect as described above.

A third mixed model ANOVA that included site and soil condition (control vs. die-off) as independent variables was carried out to determine whether there were significant differences in seed mortality between sets of isolates from each site or condition. For this analysis, isolate was considered a random variable nested within site and condition.

Results

Site characteristics

The two study sites were separated by *c*. 500 km. Records indicate that the vegetation present before conversion to *B. tectorum* monoculture at Eden Valley was dominated by Wyoming big sagebrush (*Artemisia tridentata* Nutt. ssp. *Wyomingensis)*, whereas the Skull Valley site was salt desert shrubland dominated by shadscale (*Atriplex confertifolia* Torr. & Frém.; Table 1). Soils at the two sites were also very different (Table 2). The soil at Eden Valley was non-calcareous, relatively light textured, slightly acidic and high in phosphorus, probably because it was derived largely from igneous materials. The soil at Skull Valley was developed in alluvial material largely derived from limestone and consequently was strongly calcareous, with a slightly alkaline pH and a significantly higher clay content than the Eden Valley soil. Both sites had low salinity and sodicity, although Skull Valley soil had slightly but significantly higher salinity than Eden Valley soil. The only significant difference between die-off and control soils was in nitrate-N, which averaged 7.6 times higher in die-off soils at Eden Valley and 4.7 times higher in die-off soils at Skull Valley. Site by die-off condition interactions were not significant.

Fig. 2 Seeds of *Bromus tectorum* killed by *Fusarium* isolate Skull D5 in the laboratory pathogenicity study, showing the conspicuous white mycelial tufts that form at the radicle end of killed seeds.

Glasshouse experiment

Overall emergence in the glasshouse experiment (80%) was relatively low for *B. tectorum*, and only two treatment main effects were significant. The Eden Valley soil averaged 6% higher emergence than the Skull Valley soil (83 vs. 77%; d.f. = 1, 440; $F = 15.30$; $P < 0.001$), and the seedbed ring samples averaged 3% higher emergence than the deep core samples (82 vs. 79%; d.f. = 1, 440; $F = 6.37$; $P = 0.012$). None of the interactions were significant. Soil sterilisation had no effect on seedling emergence, and there was no difference in emergence between die-off and control soils. No evidence for the presence of a soil-borne pathogen affecting seedling emergence differentially in non-sterilised die-off soils was found.

Seedling biomass averaged 31% higher overall in die-off soils than in control soils (0.0068 vs. 0.0053 g; d.f. = 1, 439; $F = 41.02$; $P < 0.001$). This effect was especially dramatic in the Eden Valley soil, where seedlings in die-off soil averaged 60% greater biomass than in control soil (Fig. 3). The biomass increase was only 9% overall in the Skull Valley die-off soil (site x die-off treatment interaction: d.f. = 1, 439; $F = 12.74$; $P < 0.001$).

The pattern of biomass response to soil sterilisation was also different between the two sites (site x die-off treatment x sterilisation interaction: d.f. = 1, 439; $F = 7.13$; $P = 0.008$). In the Eden Valley soil, sterilisation increased seedling biomass in both control and die-off soils, indicating the presence of a negative biotic effect, possibly from a pathogen, in both soil conditions (Fig. 3). In contrast, sterilisation significantly

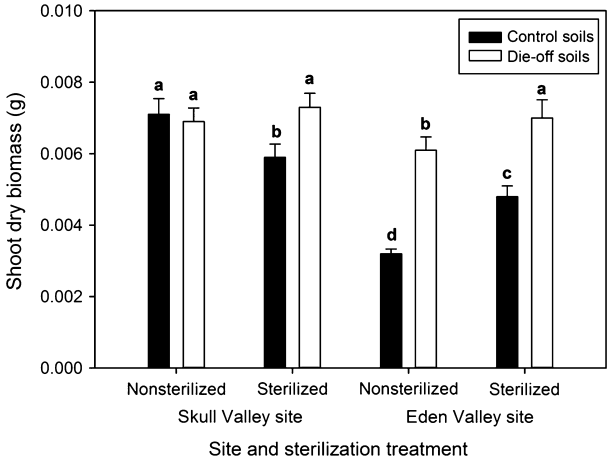

Fig. 3 Significant interaction in the glasshouse experiment for site (Eden vs. Skull) x condition (die-off vs. control soils) x treatment (sterilised vs. non-sterilised) for the response variable seedling above-ground dry biomass. Values represent means + SE from combined analysis with both repetitions (n = 30). Bars labelled with the same letter are not significantly different at $P < 0.05$ according to an LSMEANS least significant difference test carried out in conjunction with mixed model ANOVA.

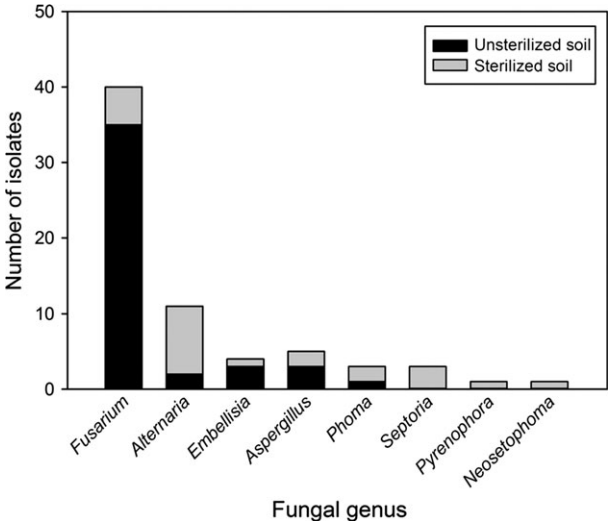

Fig. 4 Frequency histogram showing the number of isolates in each of eight genera recovered from *Bromus tectorum* killed seeds in sterilised and non-sterilised soil treatments after completion of the glasshouse study. Total number of isolates = 68.

decreased seedling biomass in Skull Valley control soils but had no significant effect in die-off soils, suggesting that it destroyed a microbe that could indirectly increase biomass in the control soil. Sterilised die-off soils at Eden Valley produced seedlings with biomass not significantly lower than seedling biomass in the Skull Valley non-sterilised die-off, non-sterilised control and sterilised die-off treatments.

The soil seedbed vs. deep core samples also produced differential biomass responses, with deep samples showing a 31% reduction in biomass relative to the seedbed samples (0.0052 vs. 0.0068 ***g***; sample type main effect: d.f. = 1, 439; $F = 47.76$, $P < 0.001$). Sample type also interacted significantly with site (sample type x site interaction: d.f. = 1, 439; $F = 7.08$; $P = 0.008$). The decrease in biomass in the deep core samples relative to seedbed samples was much greater in the Eden Valley soil (48%) than in the Skull Valley soil (17%).

Potential pathogens

The 68 isolates obtained from the glasshouse experiment belonged to eight fungal genera (Fig. 4). A majority (65%) was obtained from the non-sterilised treatment. *Fusarium* was by far the most frequently encountered genus, accounting for 59% of the total and 80% of the isolates obtained from non-sterile soil. The next most common genus was *Alternaria*, which accounted for 14% of the total. Nine of eleven *Alternaria* isolates were obtained from sterilised soil, suggesting that they were either seed-borne or glasshouse contaminants. The genera *Embellisia, Aspergillus, Phoma* and *Septoria* each comprised from 4 to 7% of the isolates, while *Pyrenophora* and *Neosetophoma* were encountered a single time and only in the sterilised treatment.

The *Fusarium* isolates selected for use in the pathogenicity trials were determined to belong primarily to the *F. tricinctum* species group, but some were more closely related to unnamed strains present in the FUSARIUM- ID database (Park *et al*., 2011) and may represent one or more undescribed species (Table 3).

Pathogenicity tests

Pathogenicity testing clearly showed that *Fusarium* isolates from the glasshouse experiment were pathogenic on *B. tectorum* seeds and could cause seed mortality (Fig. 5). Overall mortality levels were lower in the second repetition (48.5 vs. 31.2% mean mortality), but patterns were similar (data not shown). Incubating inoculated seeds under water stress for 7 days prior to transfer to pure water caused a dramatic increase in mortality (water stress main effect: d.f. = 1, 223, $F = 181.6$, $P < 0.001$; water stress treatment mean mortality 55.2% vs. no water stress treatment mean mortality 24.4%). Non-inoculated control seeds showed low levels of *Fusarium*-caused mortality overall (3.2%, probably due to laboratory contamination). The water stress main effect was significant (d.f. = 1, 61, $F = 35.79$, $P < 0.001$), with higher *Fusarium*-caused mortality on non-inoculated seeds in the water stress

Table 3 Species identifications of *Fusarium* isolates included in the study based on BLAST searches on the TEF gene sequence in the FUSARIUM-ID database at: http://isolate.fusariumdb.org/index.php

Isolate	Match (%)	Closest relative in database
Eden C1	98.14	*Fusarium* species FD_01317_EF-1a
Eden C2	96.72	*Fusarium* species FD_01317_EF-1a
Eden C3	98.38	*Fusarium tricinctum* species complex
Eden D1	98.38	*Fusarium tricinctum* species complex
Eden D2	99.69	*Fusarium* species FD_01861_EF-1a
Eden D3	98.38	*Fusarium tricinctum* species complex
Eden D4	86.65	*Fusarium* species FD_01317_EF-1a
Skull C1	99.11	*Fusarium tricinctum* species complex
Skull C2	99.11	*Fusarium tricinctum* species complex
Skull C3	98.97	*Fusarium tricinctum* species complex
Skull D1	—	—
Skull D2	98.97	*Fusarium tricinctum* species complex
Skull D3	98.38	*Fusarium tricinctum* species complex
Skull D4	98.82	*Fusarium tricinctum* species complex
Skull D5	100	*Fusarium* species FD_01861_EF-1a
Skull D6	99.11	*Fusarium tricinctum* species complex

treatment (6.0%) than in the no water stress treatment (0.4%).

There were also large differences among isolates in the ability to kill *B. tectorum* seeds, with mean mortality varying from 14.7 to 53% (isolate main effect: d.f. = 15, 223, $F = 6.33$, $P < 0.001$). Mean seed mortality varied from 24.7 to 83.4% in the water stress treatment and from 4.7 to 43% in the no water stress treatment (Fig. 5A,B). The isolate by water stress treatment interaction was also significant (d.f. = 15, 223, $F = 2.60$, $P = 0.001$). Seed mortality levels across isolates in the water stress and no water stress treatments were generally parallel and significantly positively correlated ($r = +0.794$, d.f. = 12, $P = 0.007$), but only when two conspicuous exceptions (Eden D1 and Eden C1) were excluded. The two excluded isolates were the most virulent isolates in the water stress treatment (76.9% and 83.4% respectively; Fig. 5A) but were among the less virulent without water stress (Fig. 5B).

There was no evidence that isolates from Eden Valley vs. Skull Valley or from die-off vs. control soils exhibited differential virulence. There were no significant differences in seed mortality caused by groups of isolates from the two sites or from die-off vs. control soils (data not shown).

Discussion

This study of the role of soil-borne pathogens in *B. tectorum* stand failure produced mixed results. There was no direct evidence that soil-borne pathogens in die-off soils could reduce seedling emergence, but seed-pathogenic *Fusarium* isolates were readily retrieved from killed seeds in the non-sterilised soil treatment. Glasshouse conditions differed markedly from those likely to be experienced by *B. tectorum* seeds in the field, as soil moisture was continuously

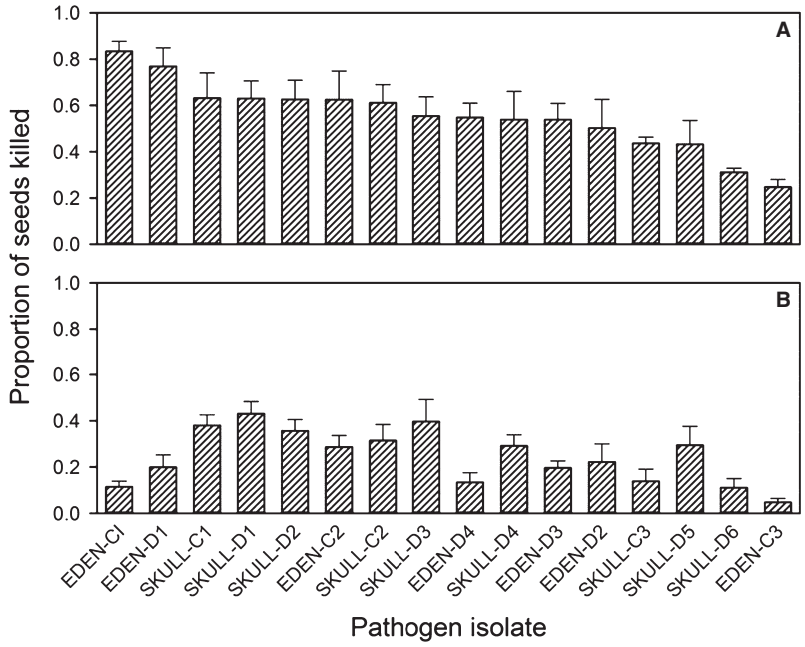

Fig. 5 Mean mortality of *Bromus tectorum* seeds in laboratory pathogenicity trials with 16 *Fusarium* isolates: (A) high water stress treatment ($n = 8$) and (B) low water stress treatment ($n = 8$). Isolates are ranked according to mean mortality in the high water stress treatment. Values represent means + SE from combined analysis with both repetitions. Isolate labels indicate site (Eden or Skull) and soil condition (C = control soil with *B. tectorum* stand, D = die-off soil) of origin.

maintained at or near field capacity. These non-limiting water conditions were apparently not conducive to epidemic levels of disease development, because they favoured very rapid seed germination and placed seed-attacking pathogens at a disadvantage (Finch *et al.*, 2013). Emergence failure in the non-sterilised glasshouse soil treatment (20%) was quite similar to *Fusarium*-caused mortality at low water stress in the pathogenicity trials (24%). This suggests that mortality from *Fusarium*-caused seed rot disease could potentially reach epidemic levels under water stress conditions in the field. Water stress associated with intermittent small precipitation events early in the season is a common feature of the soil environment of *B. tectorum* monocultures (Meyer & Allen, 2009).

The 80% emergence observed on average in the glasshouse experiment was much lower than observed in past experiments with *B. tectorum* under glasshouse conditions, which often approached 100%, but there was no difference between sterilised and non-sterilised treatments. This suggests the possible presence of a seed-borne pathogen in the sterilised treatment. Many killed seeds from sterilised soil yielded isolates of *Alternaria*, a genus that includes known seed pathogens (Schafer & Kotanen, 2004). Seed-borne *Alternaria* has been observed to kill *B. tectorum* seeds in previous germination trials (Meyer SE, unpublished data) and could have confounded the results of the current experiment by causing mortality in sterilised soil. Some of the other genera isolated from seeds in non-sterilised soil in the glasshouse experiment may also be pathogens that could potentially attack seeds (e.g. *Phoma, Embellisia*). These need to be examined experimentally along with *Alternaria* in pathogenicity trials to determine their possible role in the die-off phenomenon.

There were significant treatment effects on seedling biomass in the glasshouse experiment. Surprisingly, seedlings showed increased biomass in die-off soils relative to control soils. This was most likely due to increased available nitrate-N in die-off soils as a result of the fallow condition caused by the die-off (Table 2). This biomass effect was much larger in soil from the Eden Valley site, which also had higher nitrate-N in the die-off soil (Fig. 3). The greater difference in biomass between surface and deep soil treatments in the Eden Valley soil was probably also due to a nitrate-N effect, with a greater difference between the surface and deep soils in the soil with higher nitrate-N.

There was also evidence for a negative biotic effect on biomass in the Eden Valley soil, possibly due to a pathogen present in both soil conditions, as sterilisation significantly increased seedling biomass in both control and die-off soils. A different pattern was seen in the Skull Valley soil, where sterilisation decreased seedling biomass in the control soil but not in the die-off soil. A possible explanation for this is that sterilisation eliminated a microbe associated with increased N availability, so that the effect was observed only in the more N-limited control soil (Nannipieri & Eldor, 2009).

This study demonstrated that members of the genus *Fusarium* are potentially important soil-borne pathogens in semi-arid wildlands of the Great Basin. *Fusarium* isolates obtained from seeds planted in non-sterilised field soils belonged primarily to the *F. tricinctum* species group (Table 3). This species group is reported primarily as a pathogen of grasses, including cereal grains (Harrow *et al.*, 2010). It is most closely related to *F. avenaceum* based on recent phylogenetic work with multiple gene genealogies (Watanabe *et al.*, 2011). *Fusarium avenaceum* has a wide host range but is an important pathogen of cereal grains (Desjardins, 2003). The distribution of *Fusarium* species in the soils of semi-arid wildlands is largely unknown, but the prevalence of the same species at two widely separated locations (Table 3) suggests that the *Fusarium* community in these soils might be relatively simple, at least at the species level.

There was ample evidence that *Fusarium* from the soils of semi-arid wildlands are pathogenic on *B. tectorum* seeds and capable of causing seed mortality. The isolates varied considerably in their ability to kill seeds, even at high inoculum loads and under conditions favourable for infection, and could represent a continuum from primarily saprophytic to fully pathogenic life histories. Pathogenic *Fusarium* isolates were ubiquitous in soils of both *B. tectorum* die-off areas and intact stands, suggesting that environmental conditions such as localised water stress may be as important in mediating levels of disease as the presence of disease-causing organisms.

The hypothesis that incubation under water stress prior to transfer to water would increase seed mortality was also fully supported, with over twice the mortality in the water stress treatment relative to the no water stress treatment. *Fusarium* can remain active at water potentials far below those that permit seed germination (Brownell & Schneider, 1985; Woods & Duniway, 1986). Isolates in our study differed in their environmental tolerance as measured by their differential responses to the water stress treatment. The two most virulent isolates under water stress conditions were only moderately virulent in the absence of water stress. Most of the other isolates showed similar proportional increases in seed mortality under water stress conditions.

The case for *Fusarium* as a primary causal agent in *B. tectorum* stand failure in the Great Basin is still not fully resolved. The next step will be experimental

manipulation of pathogen inoculum loads and water stress levels in conjunction with controlled seeding to determine whether this pathogen can cause significant levels of seed mortality in the field**.**

Acknowledgements

This research was funded by the Joint Fire Sciences Program (Grant 2007-1-3-10 to SEM and JB) and through support to SEM, JB and BG from the USDI Bureau of Land Management Integrated Cheatgrass Die-off Project. There are no conflict of interest issues to report. Special thanks to Don Major and Mike Pellant of the Bureau of Land Management, Idaho State Office for their interest in this work. Thanks to Phil Allen for the soils data, Suzette Clement for culture work, Desiree Lara for ITS identifications, Todd Fineman, Lindsay Poston, Trevor Davis and Lauren Miller for glasshouse help, and Travis Poh and Sam Saunders for help with the pathogenicity study.

References

Baughman OW & Meyer SE (2013) Is *Pyrenophora semeniperda* the cause of downy brome (*Bromus tectorum*) die-offs? *Invasive Plant Science and Management* **6**, 105–111.

Beckstead J & Parker IM (2003) Invasiveness of *Ammophila arenaria*: release from soil-borne pathogens? *Ecology* **84**, 2824–2831.

Brooks ML, D'Antonio CM, Richardson DM *et al*. (2004) Effects of invasive alien plants on fire regimes. *BioScience* **54**, 677–688.

Brownell KH & Schneider RW (1985) Roles of matric and osmotic components of water potential and their interaction with temperature in the growth of *Fusarium oxysporum* in synthetic media and soil. *Phytopathology* **75**, 53–57.

Campbell MA, Medd RW & Brown JF (2003) Optimizing conditions for growth and sporulation of *Pyrenophora semeniperda*. *Plant Pathology* **52**, 448–454.

Desjardins AE (2003) *Gibberella* from A(venaceae) to Z(eae). *Annual Review of Phytopathology* **41**, 177–198.

Ehlert KA, Mangold JM & Engel RE (2014) Integrating the herbicide imazapic and the fungal pathogen *Pyrenophora semeniperda* to control *Bromus tectorum*. *Weed Research* **54**, doi: 10.1111/wre.12089.

Finch H, Allen PS & Meyer SE (2013) Environmental factors influencing *Pyrenophora semeniperda*-caused seed mortality in *Bromus tectorum*. *Seed Science Research* **23**, 57–66.

Harrow SA, Farrokhi-Nejad R, Pitman AR *et al*. (2010) Characterization of New Zealand *Fusarium* populations using a polyphasic approach differentiates the *F. avenaceum*/*F. acuminatum*/*F. tricinctum* species complex in cereal and grassland systems. *Fungal Biology* **114**, 293–311.

Knapp PA (1996) Cheatgrass (*Bromus tectorum* L.) dominance in the Great Basin Desert: history, persistence, and influences to human activities. *Global Environmental Change* **6**, 37–52.

Meyer SE & Allen PS (2009) Predicting seed dormancy loss and germination timing for *Bromus tectorum* in a semi-arid environment using hydrothermal time models. *Seed Science Research* **19**, 225–239.

Meyer SE, Quinney D, Nelson DL & Weaver J (2007) Impact of the pathogen *Pyrenophora semeniperda* on *Bromus tectorum* seed bank dynamics in North American cold deserts. *Weed Research*, **47**, 54–62.

Meyer SE, Nelson DL, Clement S & Beckstead J (2008) Cheatgrass (*Bromus tectorum*) biocontrol using indigenous fungal pathogens. In: *Shrublands Under Fire: Disturbance and Recovery in a Changing World; 6–8 June 2006; Cedar City, Utah* (eds SG Kitchen, RL Pendleton, T Monaco & J Vernon), 61–67. USDA Forest Service, Rocky Mountain Research Station, Fort Collins, Colorado.

Meyer SE, Nelson DL, Clement S & Ramakrishnan AP (2010) Ecological genetics of the *Bromus tectorum* (Poaceae): *Ustilago bullata* (Ustilaginaceae) pathosystem: a role for frequency-dependent selection? *American Journal of Botany* **97**, 1304–1312.

Michel BE & Kaufman MR (1972) The osmotic potential of polyethylene glycol 6000. *Plant Physiology* **51**, 914–916.

Nannipieri P & Eldor P (2009) The chemical and functional characterization of soil N and its biotic components. *Soil Biology and Biochemistry* **41**, 2357–2369.

Park B, Park J, Cheong KC *et al*. (2011) Cyber infrastructure for *Fusarium*: three integrated platforms supporting strain identification, phylogenetics, comparative genomics and knowledge sharing. *Nucleic Acids Research*, **39** (Database Supplement), D640–D644.

Schafer M & Kotanen PM (2004) Impacts of naturally-occurring soil fungi on seeds of meadow plants. *Plant Ecology* **175**, 19–35.

Van Reeuwijk LP (ed) (2002) *Procedures for Soil Analysis*. 6th edition. Technical Paper 9. International Soil Reference and Information Center, Wageningen, the Netherlands.

Watanabe M, Yonezawa T, Lee K *et al*. (2011) Molecular phylogeny of the higher and lower taxonomy of the *Fusarium* genus and differences in the evolutionary histories of multiple genes. *BMC Evolutionary Biology* **11**, 322.

White TJ, Bruns T, Lee S & Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. (eds MA Innis, DH Gelfand, JJ Sninsky & TJ White). Academic Press, New York.

Woods DM & Duniway JM (1986) Some effects of water potential on growth, turgor, and respiration of *Phytophthora cryptogea* and *Fusarium moniliforme*. *Phytopathology* **76**, 1248–1254.