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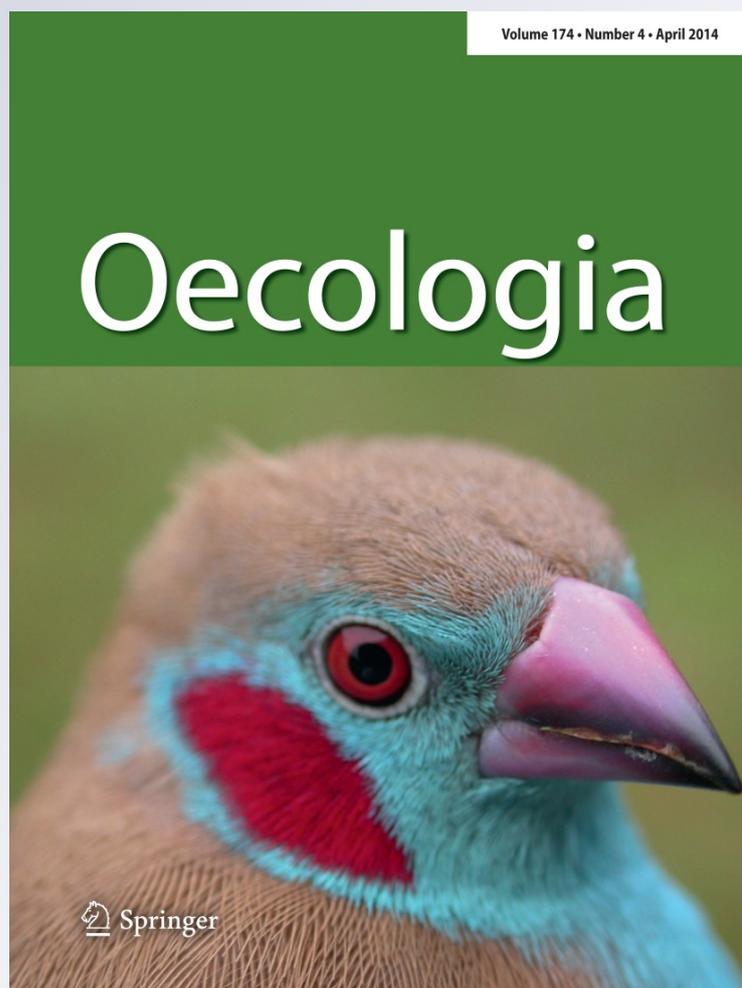
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# Indirect effects of an invasive annual grass on seed fates of two native perennial grass species

Susan E. Meyer · Katherine T. Merrill · Phil S. Allen · Julie Beckstead · Anna S. Norte

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**Abstract** Invasive plants exhibit both direct and indirect negative effects on recruitment of natives following invasion. We examined indirect effects of the invader *Bromus tectorum* (cheatgrass) on seed fates of two native grass species, *Elymus elymoides* and *Pseudoroegneria spicata*, by removing *B. tectorum* and by adding inoculum of the shared seed pathogen *Pyrenophora semeniperda* in factorial experiments at xeric and mesic field sites. We also included a supplemental watering treatment to increase emergence and also the potential for pathogen escape. We recorded emergence and survival of native seedlings and also determined the fate of unemerged seeds. At the xeric site, *Pyrenophora*-caused mortality was high (34 %), and effects of other pathogens and failed emergence of germinants were smaller. Cheatgrass removal negatively affected both emergence (35 vs. 25 %) and spring survival (69 vs. 42 %). *Pyrenophora*-caused seed mortality increased with inoculum augmentation for both species (22 vs. 47 % overall), but emergence was negatively impacted only for *P. spicata* (20 vs. 34 %). At the mesic site, *Pyrenophora*-caused mortality was low (6 %). Cheatgrass removal doubled emergence (26 vs. 14 %). Seed

mortality increased significantly with inoculum augmentation for *P. spicata* (12 vs. 5 %) but not *E. elymoides*, while emergence was not significantly affected in either species. A large fraction of seeds produced germinants that failed to emerge (37 %), while another large fraction (35 %) was killed by other pathogens. We conclude that facilitation by cheatgrass at the xeric site but interference at the mesic site was probably mediated through litter effects that could be ameliorative or suppressive. Apparent competition between cheatgrass and native grasses could occur through *Pyrenophora*, especially in a xeric environment, but effects were weak or absent at emergence. This was probably because *Pyrenophora* attacks the same slow-germinating fraction that is subject to pre-emergence mortality from other causes, including attack by other pathogens such as *Fusarium*.

**Keywords** Apparent competition · *Bromus tectorum* · Facilitation · Seed pathogen · Seedling recruitment

## Introduction

A hallmark of invasive plant species of high ecological impact is the ability to interfere with native species recruitment following disturbance and invasion. Invaders create this interference through both direct and indirect negative impacts that can operate at different life stages and through different mechanisms. The most obvious invader impacts operate directly through competition for limiting resources. It has become increasingly clear, however, that many of the processes that favor exotic plant invaders over native species are mediated through complex interactions with organisms at multiple trophic levels (Chaneton and Bonsall 2000; White et al. 2006). These can include interactions through

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herbivores (Orrock and Witter 2010), seed predators (Orrock et al. 2008), fungal soil mutualists (Stinson et al. 2006), and plant pathogens (Malmstrom et al. 2005, 2006). These interactions often fall under the concept of 'apparent competition,' where one species negatively impacts another through its effect on the numerical abundance or foraging behavior of a common enemy (Holt and Lawton 1994). Studies of apparent competition between invasives and natives have sometimes demonstrated major impacts on the outcome of interspecific interactions. For example, it has recently been shown that the mass invasion of California native perennial grasslands by a suite of Mediterranean annual species was driven at least in part by apparent competition mediated through a common enemy, the cereal yellow dwarf virus (Borer et al. 2007). Another example of apparent competition in this system is mediated through seed predators that increase in abundance in response to the high seed production of introduced annuals, then remove a majority of the more preferred seeds of already seed-limited perennial grasses (Orrock et al. 2008). These studies of California exotic annual grasslands raise the question of whether similar processes might be operating in exotic annual grasslands of the Great Basin, where the dominant invasive species is *Bromus tectorum* (cheatgrass).

In the case of the California grasslands, the native perennials have been shown to be competitive with exotic annuals in the absence of apparent competition (Seabloom et al. 2003; Corbin and D'Antonio 2004). In contrast, there are numerous studies showing the competitive superiority of cheatgrass over seedlings of native perennial grasses, at least under specific study conditions (e.g., Francis and Pyke 1996; Rafferty and Young 2002; Humphrey and Schupp 2004; Vasquez et al. 2008). Efforts to seed native or even exotic forage grasses into cheatgrass monocultures without some form of control usually fail, suggesting that the problem is not simply one of seed limitation (Monsen 1994). It is possible, however, that these direct competitive effects interact with indirect effects to further exacerbate the recruitment problems of native species in cheatgrass stands. For example, we have evidence that apparent competition with cheatgrass mediated through grasshopper herbivory can limit seed production in the native grass *Elymus elymoides* (Beckstead et al. 2008).

In the present investigation, we examine the possibility that a generalist seed bank pathogen mediates apparent competition between cheatgrass and native grasses. The transition from seed to germinant is probably the narrowest bottleneck in the life history of most plants, and also one of the most poorly understood. While a large body of research has addressed post-dispersal seed predation, studies to directly determine the fate of seeds once incorporated into the seed bank are rare in the ecological literature, and seed pathogens in natural systems have received remarkably

little explicit attention (Chambers and MacMahon 1994; Gilbert 2002; but see Crist and Friese 1993; Schafer and Kotanen 2004). A more common approach to pathogens that operate at the seed stage is the 'black box' approach, where levels of pathogenic fungi are manipulated through fungicide treatments and their effects on seeds determined indirectly (e.g., Leishman et al. 2000; Blaney and Kotanen 2001; Schafer and Kotanen 2003; Clark and Wilson 2003; Orrock et al. 2012). These types of studies have rarely revealed a major effect of seed bank pathogens.

Our studies of the generalist ascomycete seed bank pathogen *Pyrenophora semeniperda* have shown that this organism has much more impact on seed banks than has been shown in the earlier studies cited above. This pathogen, which can be identified macroscopically, often occurs at extremely high densities in cheatgrass seed banks, on the order of thousands to tens of thousands of killed seeds per square meter (Meyer et al. 2007). In a growth chamber study using seed-zone cores collected from the field, we found that this pathogen caused higher mortality on seeds of some native grass species when the seeds were planted into seed bank cores from cheatgrass-dominated areas than when planted into cores from their own seed banks, where the pathogen is rare (Beckstead et al. 2010). This suggested that apparent competition due to spillover of this pathogen from cheatgrass onto native grass seeds could be a mechanism limiting successful native grass recruitment into cheatgrass-dominated areas. Testing this hypothesis in a field setting was a principal goal of the research project described here.

We carried out a manipulative field experiment using a precision seeding technology that enabled us to follow the fate of individual seeds. We focused on the impact of cheatgrass on seed fate for *Elymus elymoides* (squirrel-tail) and *Pseudoroegneria spicata* (bluebunch wheatgrass) from the time of planting (incorporation into the transient seed bank) in September through seedling establishment the following spring. We manipulated levels of three variables in factorial combination at each of two sites, a mesic Palouse prairie site in central Washington, USA and a xeric salt desert site in west central Utah, USA. First, we removed the direct effects of cheatgrass on half the plots by applying glyphosate herbicide prior to seed production the spring preceding experimental installation. We manipulated levels of *P. semeniperda* through inoculum addition, and increased the probability of rapid emergence and pathogen escape with a supplemental water treatment. We tracked native grass emergence and establishment through spring, then retrieved unemerged seeds to determine their fate. By experimentally uncoupling the direct effect of cheatgrass from the indirect effect mediated through the pathogen, we aimed to test the relative importance of pathogen-mediated mortality vs. cheatgrass direct effects

in determining levels of emergence and establishment success for the native grasses.

## Materials and methods

### Field experiments with native grass seeds

Parallel field experiments were conducted at two locations: a xeric site at Whiterocks, located in Skull Valley, Utah (40°19.680'N 112°46.680'W, elevation 1,446 m, average annual precipitation 199 mm), and a mesic site at Packer Creek, located southeast of Sprague, Washington (47°5.981'N 117°49.862'W elevation 550 m, average annual precipitation 356 mm). Both sites were vegetated with near-monocultures of cheatgrass. The experiment had a split plot design with cheatgrass removal as the main plot and a factorial combination of two inoculation treatments, two watering treatments, and two seeded native grasses as the subplots, resulting in 16 treatment combinations and ten blocks, for a total of 160 plots (0.093 m<sup>-2</sup>) per site. The inoculum manipulation included *P. semeniperda* inoculum augmentation (45 g per plot) vs. the naturally occurring inoculum level. A third manipulation intended to reduce disease levels using a fungicide was ineffective, and the 80 additional plots with this treatment at each site were dropped from the analysis.

Cheatgrass removal was achieved by aerial spray application of glyphosate at the manufacturer's recommended rate in mid-April 2009, at a time when cheatgrass emergence was complete and plants were actively growing, but no seeds had yet been produced.

Bulk inoculum for field use was produced by seeding conidia of a representative pathogen strain (WRK0 isolated from the xeric Whiterocks site) into potato dextrose broth (PDB) that had been autoclaved in large-batch (10-l) glass fermenters. Mycelium was grown for 2–3 days at ca. 22 °C in aerated submerged culture. The mycelial mass was centrifuged to remove the spent medium, then combined during mixing with sufficient fresh PDB to saturate the sterilized inert carrier (calcined montmorillonite clay). The resulting material was dried, pressed through a no. 16 sieve, and weighed into vials for hand application in the field.

Water was applied to the supplemental water plots on days 0, 7 and 14 after sowing. Each plot received the equivalent of three 2.5-cm rainfall events, each applied over a 20- to 30-min period by punching holes in water-filled plastic Ziploc bags suspended over the plots on wire frames. This rate allowed the water to infiltrate without running off.

Both native grass species used in this study are susceptible to *P. semeniperda* (Beckstead et al. 2010). The xeric site supported *E. elymoides* in the past but is generally too dry

to support *P. spicata*, which is common on nearby upland sites. The mesic site was likely dominated by *P. spicata* prior to cheatgrass conversion, as this species is a dominant on similar sites throughout the Palouse region and is found as a remnant species near the study plots.

*Elymus elymoides* seeds were collected near Sunshine Canyon in Rush Valley, Utah, while *P. spicata* seeds were collected in Rock Canyon near Provo, Utah. Seeds were collected in summer 2009 and allowed to dry-after-ripen at room temperature, then hand-selected to ensure high fill. Viability percentage for each seedlot was near 100 %. Seeds were glued to bamboo toothpicks with Elmer's Washable School Glue (Elmer's Products, Columbus, OH) (Leger et al. 2009). Toothpicks with attached seeds were inserted into the mineral soil until the body of the seed (i.e., the carypsis with associated lemma and palea) was completely covered, leaving the awn tip and most of the toothpick above the soil surface. Toothpicks were arranged into a grid pattern 6.35 cm apart in four rows of five seeds ( $n = 20$  per plot). The experiment was installed in September 2009.

Emergence and survival of each individual seedling were recorded approximately every 2 weeks throughout the fall and spring. Emerged seedlings were marked by placing colored paperclips around both the new seedling and its attached toothpick. Each time plots were read, previously emerged seedlings were scored as dead or alive. Fall data collection was terminated once nighttime temperatures consistently dropped below freezing. The last dates for scoring of fall emergence were 3 December (xeric site) and 8 November (mesic site). Data collection began again the following spring once emergence was observed (11 March at the xeric site, 4 April at the mesic site) and stopped once emergence had ended due to drying soils (24 March at the xeric site; 19 May at the mesic site). Unemerged seeds were then collected by retrieving their toothpicks from the soil.

Unemerged seeds were scored as germinated (presence of a remnant radicle) or killed by *P. semeniperda* (protrusion of black stromata). Seeds that did not fall into these categories were incubated at 20 °C for 2 weeks between two water-saturated germination blotters (Anchor Paper, St. Paul, MN) within 10-cm plastic Petri dishes. At the end of 2 weeks seeds were scored as germinated, killed by *P. semeniperda*, viable as determined by a cut test to establish the presence of an intact embryo (Ooi et al. 2004), or potentially killed by other pathogens.

### *B. tectorum* seed bank study

To measure the impact of treatments in the seeding experiment on the *B. tectorum* seed bank, we sampled in early June 2010, before dispersal of current-year seeds, from half of the plots (all *P. spicata* plots, 80 plots at each site). Samples were obtained with a steel can (6 cm diameter × 4 cm

high), then sifted through screens in the laboratory to retrieve seeds (see Meyer et al. 2007 for details). *Bromus tectorum* seeds with visible fungal stromata were quantified. Apparently viable seeds were incubated as described earlier and scored for germination and disease. At the end of the 2-week incubation period all remaining seeds were cut to determine viability as previously described.

#### Packer Creek follow-up study

The category of unemerged native grass seeds classified as potentially killed by other pathogens was examined experimentally at the mesic Packer Creek study site in a follow-up study installed in late September 2011. Seeds of *E. elymoides* and *P. spicata* glued on toothpicks as described earlier were planted into a cheatgrass-dominated area. Two litter treatments, naturally high (2–5 cm depth) and naturally low (<1 cm depth), were included, with ten 20-seed plots of each species in each litter treatment. One week after planting, the plots received 2.5 cm of supplemental water applied as described earlier, and emergence was scored 4 weeks later (5 November 2011). Unemerged seeds were retrieved with their toothpicks, surface sterilized, and cultured on potato dextrose agar. Fungal isolates from the seeds were obtained in pure culture and identified using molecular-genetic characterization of the internal transcribed spacer 1 and 2 region of ribosomal DNA (White et al. 1990). The only known seed pathogens recovered were members of the genus *Fusarium*. The *Fusarium* isolates were subjected to further genetic analysis using primers for the translation elongation factor gene (Geiser et al. 2004) and were determined to belong to an undescribed species closely related to *Fusarium tricinctum* and *Fusarium negundis*. We selected three isolates for pathogenicity testing on seeds of the native grass species. The *Fusarium* cultures were produced on PDA and induced to sporulate by wounding, and liquid inoculum adjusted to 250,000 spores ml<sup>-1</sup> was applied to groups of 50 surface-sterilized host seeds to test the pathogenicity of the strains. One strain, LP03, was applied at a slightly lower concentration (187,500 spores ml<sup>-1</sup>). We also included a water stress treatment to determine whether the *Fusarium* strains would be more pathogenic on native seeds under water stress conditions, as has been demonstrated for these pathogens on cheatgrass seeds (S. E. Meyer, unpublished data). For the water stress treatment, seeds were incubated on blotters in Petri dishes for 7 days in a polyethylene glycol solution at -1.5 MPa prior to transfer to free water for 14 days. The -1.5 MPa treatment suppressed germination of otherwise non-dormant seeds (Finch et al. 2012). For the no water stress treatment, inoculated seeds were incubated directly in free water for 21 days. The pathogenicity experiment had a completely randomized design: 4 replicates × 3

isolates × 2 seed species × 2 water stress conditions plus uninoculated controls, for a total of 64 experimental units. At 21 days, seeds were scored as germinated or killed by *Fusarium* (white mycelial tufts at the radicle end). Remaining seeds were scored for viability with a cut test. The experiment was repeated; data represent means calculated from both repeats. The second repeat used a different lot of *E. elymoides* seeds and 100 seeds per replication, but was otherwise similar to the first.

#### Statistical analysis

Data from the seeding experiments at Whiterocks and Packer Creek were analyzed using generalized linear mixed models for binomial data with SAS Proc GLIMMIX (SAS 9.3, 2010). Fixed effects included cheatgrass removal, native species planted, watering treatment, and inoculum treatment; block and block × cheatgrass removal treatment were the random effects. Response variables described in events/trials format included emerged seedlings/total planted seeds and seedlings surviving through spring/emerged seedlings. The fate of seeds that did not produce emerged seedlings was included as numbers of seeds out of total planted seeds that: (1) germinated but failed to emerge, (2) were killed by *P. semeniperda*, (3) were viable and ungerminated at the time of retrieval, (4) were apparently killed by other pathogens. Because the data for unemerged seeds were obtained only from seeds that did not produce seedlings and also remained attached to their toothpicks at the end of the experiment, sample size within experimental units was highly variable (1–18 toothpicks per plot, mean 6.6 at Whiterocks, 6.9 at Packer Creek). However, it was possible to calculate the binomial variables based on seeds retrieved for each experimental unit. Numbers of unemerged seeds in the different categories were analyzed relative to the total seeds planted rather than relative to unemerged seeds [e.g., 50 % of unemerged seeds × (100 - 30 % emergence) = 35 % of total seeds]. Due to unavoidable experimental error, four plots at Whiterocks were lost from the data set and survival data could not be obtained from an additional 18 plots. At Packer Creek three plots were lost and survival data could not be obtained from an additional 15 plots.

We used correlation analysis to explore the relationship between *P. semeniperda*-killed seed fraction and emergence fraction in the inoculum augmentation and natural inoculum treatments on a plot by plot basis for each seeded native species at each of the two sites.

Densities of pathogen-killed and viable cheatgrass seeds in seed bank samples were examined using mixed model ANOVA (SAS MIXED procedure, SAS version 9.3, 2010) for each study site. Density (count) variables were log transformed prior to analysis.

Results of the Packer Creek follow-up field study were analyzed using SAS Proc GLIMMIX for binomial data as a completely randomized design with emerged seedlings/planted seeds as the response variable. The pathogenicity tests were analyzed using SAS Proc GLIMMIX with strain, water stress, and seed species as fixed effects and repeat as the random effect. The response variable was *Fusarium*-killed seeds/total seeds.

## Results

### Field experiments with native grass seeds

#### Seedling emergence and survival

Seedling emergence averaged 30 % at the xeric Whiterocks site and 20 % at the mesic Packer Creek site. Total emergence was significantly higher for *E. elymoides* than for *P. spicata* at the xeric site (33 vs. 27 %; Fig. 1d; species main effect; Table 1), whereas at the mesic site there was no significant emergence difference between species (*E. elymoides* 18 %, *P. spicata* 22 %). At the xeric site, emergence averaged higher in the intact cheatgrass plots than in the removal plots (Fig. 1a), but this effect was due entirely to reduced emergence of *P. spicata* in the removal plots (35 % in intact vs. 19 % in removal; Fig. 2a; cheatgrass × species interaction; Table 1). Emergence of *E. elymoides* was unaffected by the removal treatment. At the mesic site, emergence was similarly low for both species in the intact plots, and increased more with removal for *P. spicata* (13 % in intact vs. 30 % in removal) than for *E. elymoides* (14 % in intact vs. 23 % in removal; cheatgrass × species interaction; Fig. 2b; Table 2).

The results were similar for the inoculum augmentation treatment at the xeric site, which resulted in reduced emergence overall (Fig. 1b). This effect was again due to the response of *P. spicata* (34 % in unaugmented vs. 20 % in augmented) rather than that of *E. elymoides* (33 % in unaugmented vs. 32 % in augmented; Fig. 2b; inoculation treatment × species interaction; Table 1). At the mesic site, inoculum augmentation had a small but significant negative effect on emergence, but the effect did not vary by species (inoculum augmentation main effect; Fig. 2b; Table 2).

There was a significant interaction between removal and inoculum augmentation at the xeric site, where emergence in the intact treatment without inoculum addition was higher than in any other treatment combination (41 vs. 24–28 %; Fig. 2c; cheatgrass treatment × inoculum treatment interaction; Table 1). This interaction was not observed at the mesic site.

The supplemental water treatment substantially increased emergence at the xeric site, where there was no

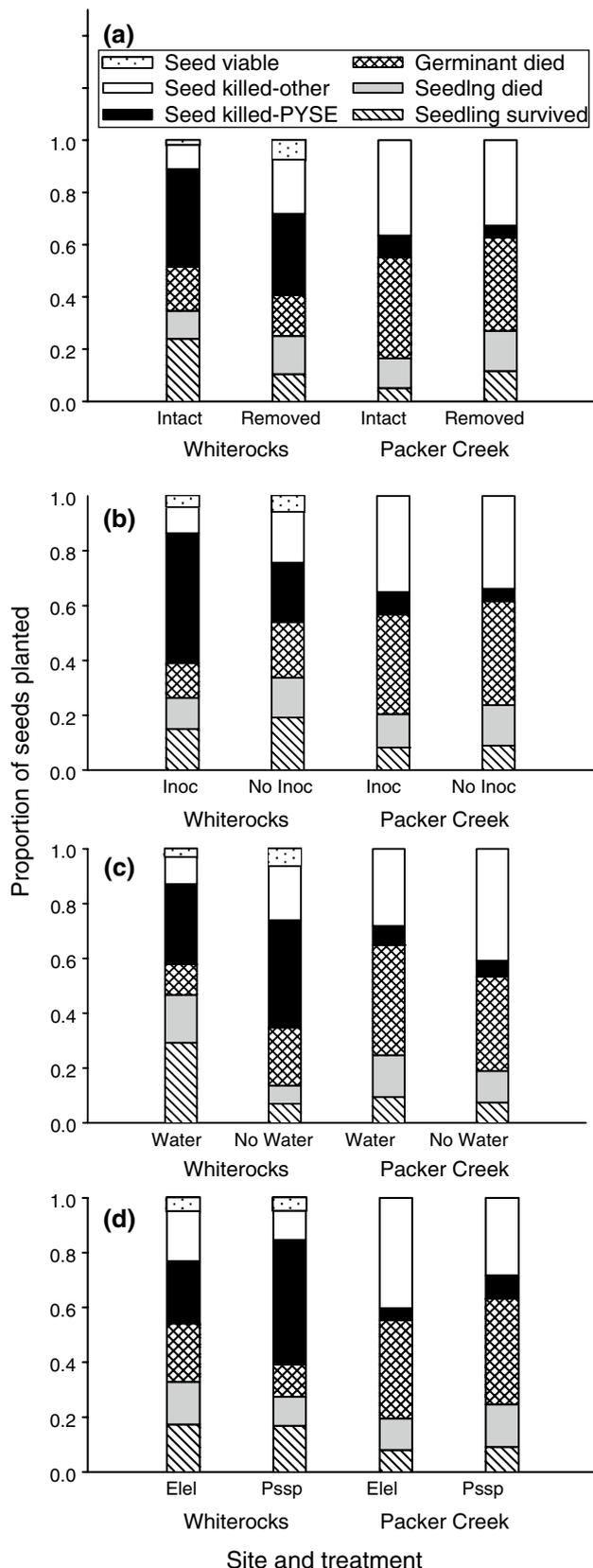
autumn emergence without supplemental water due to an exceptionally dry autumn (14 % in unwatered vs. 47 % in watered; Fig. 1c; water main effect; Table 1). Supplemental water also increased emergence significantly at the mesic site, though early natural precipitation resulted in fall emergence on both unwatered and watered plots (17 % in unwatered vs. 23 % in watered; Fig. 1c; water main effect; Table 2). At the xeric site, supplemental water increased emergence significantly more in the removal treatment (9–41 %) than in the intact treatment (19–53 %; water × cheatgrass interaction; Table 1).

Survival of emerged seedlings until the end of spring was higher in the intact treatment than in the removal treatment at the xeric site (69 vs. 42 %; Fig. 1a; cheatgrass main effect; Table 1). It was also significantly higher in the water treatment than in the no water treatment (63 vs. 50 % Fig. 1c, water main effect; Table 1). Water and removal interacted significantly, with a greater increase in survival with watering in the intact treatment (58–81 %) than in the removal treatment (35–45 %, cheatgrass × water interaction; Table 1). Inoculum augmentation had no effect on survival, and the two species were not significantly different. At the mesic site, survival was significantly lower in the intact cheatgrass treatment than in the removal treatment (28 % in intact vs. 41 % in removal; Fig. 1a; cheatgrass main effect; Table 2). Watering significantly increased survival in the intact plots (25–31 %) but decreased survival in the removal plots (47–36 %, cheatgrass × water interaction; Table 2).

#### Fate of unemerged seeds

Mortality of unemerged seeds as a percentage of total seeds due to the pathogen *P. semeniperda* was dramatically higher at the xeric site (34 %) than at the mesic site (6 %). Mean seed mortality was 22 % even without augmentation at the xeric site, whereas at the mesic site it was only 4 %. Mortality levels were higher for *P. spicata* than for *E. elymoides* at both sites (45 vs. 23 % at the xeric site, 9 vs. 4 % at the mesic site; Fig. 1d; species main effect; Tables 1, 2). Inoculum augmentation increased *Pyrenophora*-caused seed mortality to 47 % at the xeric site and to 8 % at the mesic site (Fig. 1b, inoculum main effect; Tables 1, 2). At the xeric site, *P. spicata* showed a significantly greater increase in *Pyrenophora*-caused seed mortality than *E. elymoides* with inoculum augmentation (28–62 % for *P. spicata* and 15–31 % for *E. elymoides*; Fig. 3; species × inoculum interaction; Table 1). At the mesic site, only *P. spicata* showed an increase in mortality with inoculum augmentation (5–12 %; Fig. 3; species × inoculum interaction; Table 2).

The supplemental water treatment significantly reduced *Pyrenophora*-caused mortality at the xeric site (29 % in



**Fig. 1** Fraction in each seed fate category at two sites (Whiterocks and Packer Creek) in different treatments: **a** cheatgrass removal, **b** inoculum (*Inoc*) augmentation, **c** supplemental watering, and **d** planted species [*Elymus elymoides* (*Elel*), *Pseudoroegneria spicata* (*Pssp*)]. See Tables 1, 2 for statistics. *PYSE* *Pyrenophora semiperda*

watered vs. 39 % in unwatered; Fig. 1c; water main effect; Table 1) but had no effect at the mesic site. *Pyrenophora*-killed seed proportions were significantly lower in the cheatgrass removal than in the intact treatment at both sites (4 vs. 8 % at the mesic site, 31 vs. 38 % at the xeric site; Fig. 1a; cheatgrass main effect; Tables 1, 2).

Correlations between *Pyrenophora*-caused seed mortality and emergence were not significant for either species at either site under the scenario of natural inoculum load, and were significant only for *P. spicata* under the scenario of inoculum augmentation (Table 3). The negative relationship for *P. spicata* was strongest at the xeric site, where inoculum augmentation also had a measurable negative impact on emergence, but was also significant at the mesic site, where there was no significant effect of inoculum augmentation on emergence.

Other causes of pre-emergence mortality had strong impacts, especially at the mesic site. Germinants that failed to emerge at the mesic site accounted for over a third of the planted seeds, and this high failure rate was positively associated with the supplemental water treatment (34 % without water vs. 40 % with water, water main effect; Table 2). At the xeric site, the failed germinant fraction averaged only 16 % and was almost twice as high in the unwatered treatment (21 % in watered vs. 11 % in unwatered; Fig. 1c; water main effect; Table 1).

The failed germinant fraction was significantly higher for *E. elymoides* than for *P. spicata* at the xeric site (21 vs. 12 %; Fig. 1d; species main effect; Table 1) but not at the mesic site. The two species also responded differently to the combination of cheatgrass removal and watering treatments at the xeric site (species × cheatgrass × water interaction; Table 1). In the removal treatment, watering decreased failed emergence similarly for both species, whereas in the intact treatment, watering caused a much larger decrease in failed emergence for *P. spicata* than for *E. elymoides* (Fig. 4). At the mesic site, there was a small decrease in failed emergence in the inoculum augmentation treatment for *P. spicata* (42 vs. 36 %) but not for *E. elymoides* (34 vs. 38 %, species × inoculum interaction; Table 2).

Pre-emergence mortality potentially due to other pathogens also played a larger role at the mesic site (35 %) than

**Table 1** Results of ANOVA of treatment effects on various demographic parameters of native grass species at the xeric Whiterocks study site

	Emergence		Survival		PYSE mortality		Failed emergence		Other mortality	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>P</i>	<i>P</i>
Cheatgrass removal (cheat.)	15.33	0.0035	14.49	0.0042	5.42	0.045	0.27	0.62	20.53	0.0014
Supplemental water (water)	351.71	<0.0001	4.85	0.030	41.60	<0.0001	54.31	<0.0001	49.30	<0.0001
Inoculum addition (inoc.)	14.32	0.0002	0.08	0.78	193.71	<0.0001	21.43	<0.0001	9.39	0.0027
Seeded species (species)	27.34	<0.0001	0.67	0.41	161.55	<0.0001	58.38	<0.0001	7.54	0.0070
Cheat. × water	5.34	0.022	5.06	0.027	0.26	0.61	1.67	0.20	7.09	0.0089
Cheat. × inoc.	5.50	0.021	0.01	0.94	1.41	0.24	0.21	0.65	4.12	0.045
Water × inoc.	0.04	0.84	3.80	0.054	0.17	0.68	1.87	0.17	4.05	0.047
Cheat. × species	13.77	0.0003	0.04	0.85	1.21	0.27	17.93	<0.0001	0.08	0.78
Water × species	3.72	0.056	3.88	0.052	0.31	0.58	7.10	0.0087	7.36	0.0077
Inoc. × species	18.04	<0.0001	0.65	0.42	8.32	0.0046	2.16	0.14	5.74	0.018
Cheat. × water × inoc.	0.87	0.35	0.06	0.81	8.47	0.0043	3.81	0.053	2.47	0.12
Cheat. × water × species	0.12	0.72	0.22	0.64	2.69	0.10	6.82	0.010	2.85	0.094
Cheat. × inoc. × species	0.08	0.77	0.33	0.5693	0.46	0.50	2.33	0.13	6.41	0.013
Water × inoc. × species	0.18	0.67	0.37	0.55	3.57	0.061	6.30	0.013	6.18	0.014
Cheat. × water × inoc. × species	0.00	0.96	3.06	0.083	5.08	0.026	1.77	0.19	10.93	0.0013

Analysis was performed using the SAS GLIMMIX procedure for binomial data. Model *df* = 1 for all effects and response variables, error *df* for cheatgrass main effect = 9 for all response variables, error *df* for other main effects and interactions = 100 for survival, 122 for other response variables

#### PYSE *Pyrenophora semeniperda*

at the xeric site (14 %). *E. elymoides* was more negatively impacted than *P. spicata* at both sites (18 % mortality for *E. elymoides* vs. 10 % for *P. spicata* at the xeric site, 40 % mortality for *E. elymoides* vs. 28 % for *P. spicata* at the mesic site; Fig. 1d; species main effect; Tables 1, 2). In addition, watering significantly decreased mortality due to other pathogens at both sites (9 % water vs. 19 % no water at the xeric site, 28 % water vs. 41 % no water at the mesic site; Fig. 1c; water main effect; Tables 1, 2). At the xeric site, mortality due to other pathogens was also significantly higher in the cheatgrass removal than in the intact treatment (20 vs. 10 %; Fig. 1a; cheatgrass main effect) and higher in the uninoculated than the inoculated treatment (19 vs. 10 %; Fig. 1b, inoculum main effect; Table 1). Cheatgrass removal and inoculum augmentation had no significant effect on pre-emergence mortality potentially due to other pathogens at the mesic site. At both sites, a complex interplay among the main effect factors resulted in significant four-way interactions, but examination of these interactions did not reveal any biologically interpretable pattern (Tables 1, 2).

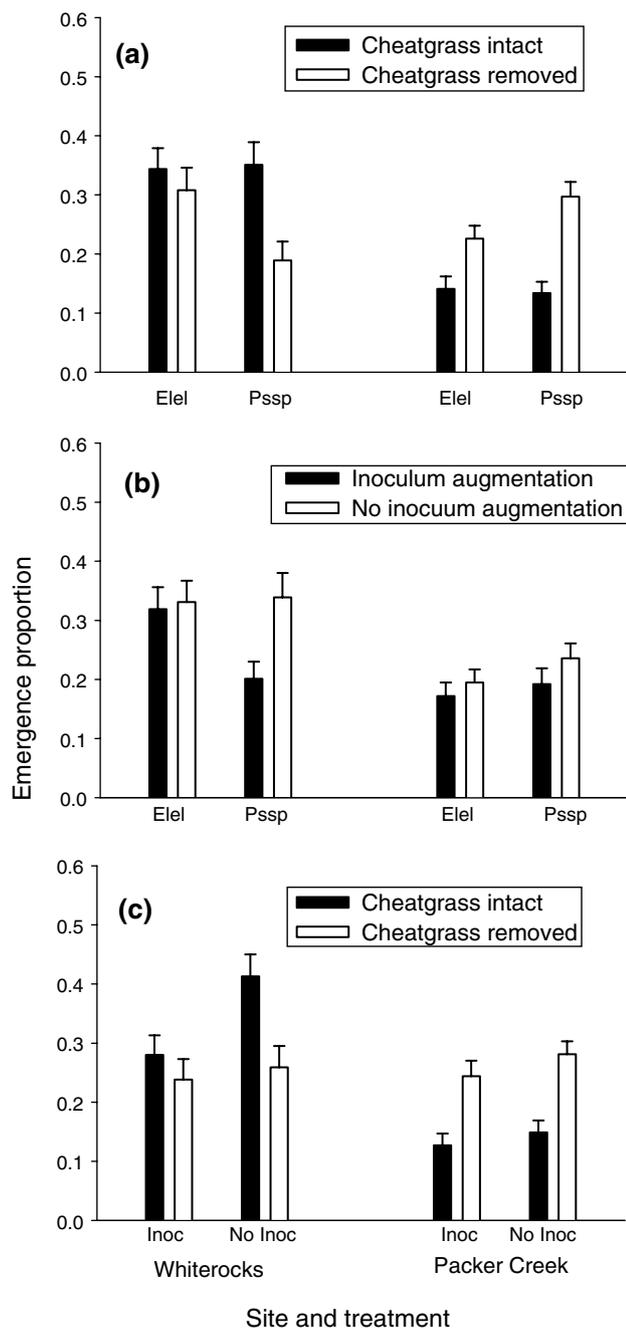
We observed viable native grass seeds remaining at the end of spring only at the xeric site, where an average of 5 % of the seeds was present as a potential carryover seed bank. This percentage was higher in the no water treatment than in the supplemental water treatment (6 vs. 3 %; Fig. 1c) and higher in the cheatgrass removal than the intact

treatment (7 vs. 2 %; Fig. 1a). There was no difference in viable seed percentage between species or in response to inoculum augmentation.

#### *B. tectorum* seed bank study

Cheatgrass removal decreased the density of both *P. semeniperda*-killed and viable seeds in the cheatgrass seed bank at both study sites, not a surprising result given the lack of seed input from previous-year seed rain in the removal treatment. At the xeric site, this was the only significant treatment effect for densities of both killed seeds (79 seeds  $\text{dm}^{-2}$  in intact vs. 27 seeds  $\text{dm}^{-2}$  in removal; cheatgrass main effect,  $F_{1,18} = 15.41$ ,  $P = 0.0010$ ) and viable seeds (14 seeds  $\text{dm}^{-2}$  in intact vs. 2 seeds  $\text{dm}^{-2}$  in removal; cheatgrass main effect,  $F_{1,18} = 37.55$ ,  $P < 0.0001$ ). Inoculum augmentation had no effect on the density of killed seeds, which was already very high at natural inoculum loads, especially in intact cheatgrass.

At the mesic site, cheatgrass removal decreased *Pyrenophora*-killed seed density from 25 to 14 seeds  $\text{dm}^{-2}$  (cheatgrass main effect,  $F_{1,18} = 9.69$ ,  $P > 0.0060$ ) and viable seed density from 7 to 1 seed  $\text{dm}^{-2}$  (cheatgrass main effect,  $F_{1,18} = 23.89$ ,  $P < 0.0001$ ). Inoculum addition significantly increased killed seed density (24 vs. 15 seeds  $\text{dm}^{-2}$ ; inoculum main effect,  $F_{1,45} = 10.02$ ,  $P = 0.0028$ ).



**Fig. 2** Proportion of seedlings emerging at Whiterocks and Packer Creek in response to interaction treatments: **a** planted species  $\times$  cheatgrass removal, **b** planted species  $\times$  inoculum augmentation and **c** the cheatgrass removal  $\times$  inoculum augmentation. Bars are means  $\pm$  SE. See Tables 1, 2 for statistics. See Fig. 1 for abbreviations

#### Packer Creek follow-up emergence study

Emergence was again low at Packer Creek in the follow-up study of autumn 2011, averaging 40 % of planted seeds, in spite of near-ideal conditions for emergence. There was

no significant difference between species (42 % for *E. elymoides*, 38 % for *P. spicata*). Emergence was significantly reduced, however, when seeds were planted into high-litter vs. low-litter areas (36 vs. 45 %;  $F_{1,36} = 7.16$ ,  $P = 0.0111$ ). Post-emergence seed retrieval revealed that all unemerged seeds had been killed by other pathogens, primarily of the genus *Fusarium*.

When three *Fusarium* isolates obtained from field-killed seeds were inoculated onto seeds of the two native grass species in laboratory pathogenicity trials, moderate to high levels of seed mortality were observed (Fig. 5). All main effects and interactions were significant ( $P < 0.0004$ ). *E. elymoides* was generally more resistant to *Fusarium* than *P. spicata*, primarily because of its superior ability to escape death when incubated directly in water. High mortality levels were observed for both species under water stress (water stress  $\times$  species interaction  $F_{1,111} = 31.14$ ,  $P < 0.0001$ ). Seed mortality levels in the laboratory pathogenicity trials were only slightly higher overall than those observed in the follow-up field experiment (70 % in the laboratory trials vs. 60 % in the field experiment), but both were considerably higher than mortality due to unknown pathogens in the original field experiment at Packer Creek (35 %).

#### Discussion

We know from previous research that natural levels of the pathogen *P. semeniperda* are orders of magnitude higher in cheatgrass seed banks than in native grass seed banks (Meyer et al. 2007; Beckstead et al. 2010). To demonstrate that apparent competition from cheatgrass occurs through this shared seed pathogen in our current experiment, we needed to show that *Pyrenophora*-caused disease had a negative impact on native grass fitness independent of other effects of cheatgrass. The evidence we produced for apparent competition was mixed, and depended on site and species as well as on the criterion used to define a negative fitness effect. At the xeric site, *Pyrenophora*-caused seed mortality overall was relatively high (mean 22 %) even at natural inoculum loads, while at the mesic site it was low (4 %). Inoculum augmentation significantly increased seed mortality for both species at the xeric site, and for *P. spicata* at the mesic site. This shows that the pathogen reduced the size of the transient native grass seed bank even at natural loads, and that increasing the inoculum load significantly increased this negative effect, especially at the xeric site. In terms of negative impact through seed bank diminution, these results show that apparent competition could be occurring.

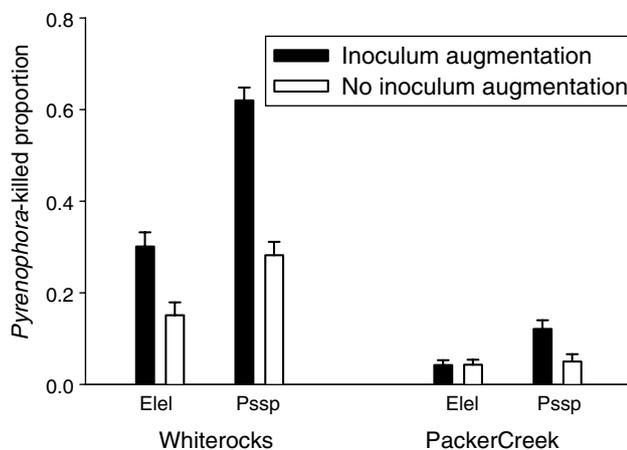
Emergence success is a more readily observable measure of fitness than seed mortality, and the impact of the pathogen on this fitness measure was much less clear.

**Table 2** Results of ANOVA of treatment effects on various demographic parameters of native grass species at the mesic Packer Creek study site

	Emergence		Survival		PYSE mortality		Failed emergence		Other mortality	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Cheatgrass removal (cheat.)	12.56	0.0063	6.38	0.032	5.61	0.042	0.19	0.68	1.46	0.26
Supplemental water (water)	17.70	<0.0001	0.02	0.89	1.83	0.18	12.41	0.0006	67.09	<0.0001
Inoculum addition (inoc.)	3.92	0.050	1.28	0.26	11.03	0.0012	1.03	0.31	0.03	0.87
Seeded species (species)	2.95	0.088	0.26	0.61	13.97	0.0003	3.34	0.070	38.90	<0.0001
Cheat. × water	1.68	0.20	5.20	0.025	2.52	0.11	0.95	0.33	1.91	0.17
Cheat. × inoc.	0.00	0.99	0.16	0.69	4.81	0.03	0.06	0.81	0.64	0.43
Water × inoc.	0.08	0.78	0.86	0.36	1.03	0.31	3.22	0.075	4.01	0.048
Cheat. × species	6.58	0.012	1.28	0.26	0.19	0.67	0.20	0.66	3.24	0.074
Water × species	0.77	0.38	0.04	0.84	1.95	0.16	0.12	0.73	0.03	0.87
Inoc. × species	0.35	0.56	0.04	0.85	6.87	0.0099	8.01	0.0054	1.01	0.32
Cheat. × water × inoc.	0.95	0.33	0.00	0.95	1.53	0.22	1.19	0.28	4.21	0.042
Cheat. × water × species	3.09	0.081	0.00	0.99	1.84	0.18	0.01	0.91	3.56	0.062
Cheat. × inoc. × species	0.71	0.40	1.66	0.20	0.44	0.51	2.12	0.15	3.78	0.054
Water × inoc. × species	0.01	0.94	0.04	0.85	0.01	0.91	1.11	0.29	0.48	0.49
Cheat. × water × inoc. × species	1.47	0.23	0.01	0.92	0.99	0.32	2.52	0.12	8.20	0.0049

Analysis was performed using the SAS GLIMMIX procedure for binomial data. Model *df* = 1 for all effects and response variables, error *df* for cheatgrass main effect = 9 for all response variables, error *df* for other main effects and interactions = 108 for survival, 123 for other response variables

PYSE *Pyrenophora semeniperda*



**Fig. 3** Proportion of planted seeds at Whiterocks and Packer Creek killed by *Pyrenophora semeniperda* prior to emergence, as a function of planted species and inoculum augmentation. Bars are means + SE. See Tables 1, 2 for statistics. See Fig. 1 for abbreviations

There was no evidence that the presence of the pathogen resulted in reduced emergence for *E. elymoides* at either site. Inoculum augmentation did not reduce emergence, and there was no significant negative correlation on a per plot basis between seed mortality and emergence success for this species at either natural or augmented inoculum levels. For *P. spicata*, inoculum augmentation significantly reduced emergence at the xeric site but not at the mesic site. This was the only clear case of a negative impact of

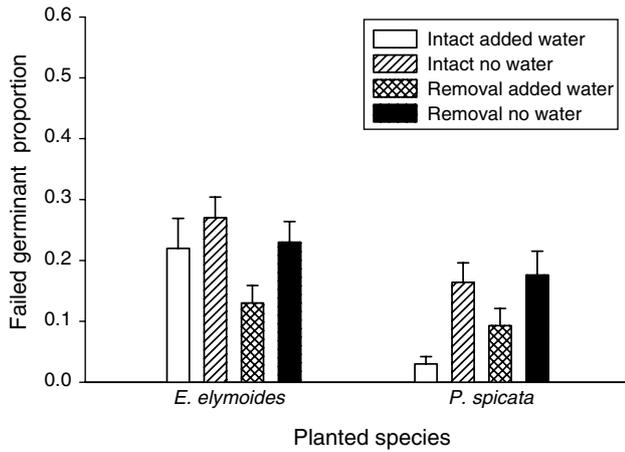
the pathogen on emergence. Seed mortality and emergence success for *P. spicata* were significantly negatively correlated in the inoculum augmentation treatment but not in the natural inoculum treatment at both sites. The study generally showed that *P. spicata* is more susceptible to this pathogen than *E. elymoides*, but that it was strongly negatively impacted only at the xeric site, where natural pathogen loads were high. Inoculum augmentation had no effect on seedling survival, indicating that this pathogen has its primary impact at the seed stage. Even though many pathogen-infected seeds probably germinated and emerged, these apparently did not suffer subsequent fitness costs as seedlings, a result similar to that found for this pathogen on wheat seeds (Campbell and Medd 2003).

Inoculum augmentation also had contrasting effects on the cheatgrass seed bank at the two sites. At the mesic site, where *Pyrenophora*-killed cheatgrass seed densities were relatively low, augmentation increased killed seed density from 15 to 24 seeds  $\text{dm}^{-2}$ , whereas at the xeric site, where natural levels were more than three times higher, augmentation had no effect. We hypothesize that the cheatgrass population at the xeric site has been subjected to selection by this pathogen, so that host genotypes in the carryover seed bank possess high general resistance to pathogen attack (S. E. Meyer, unpublished data). At the mesic site, where disease levels are low, this selection has apparently not occurred.

The contrasting effect of cheatgrass removal at the two sites made interpretation of the evidence for apparent competition more difficult. At the mesic site, removal had a

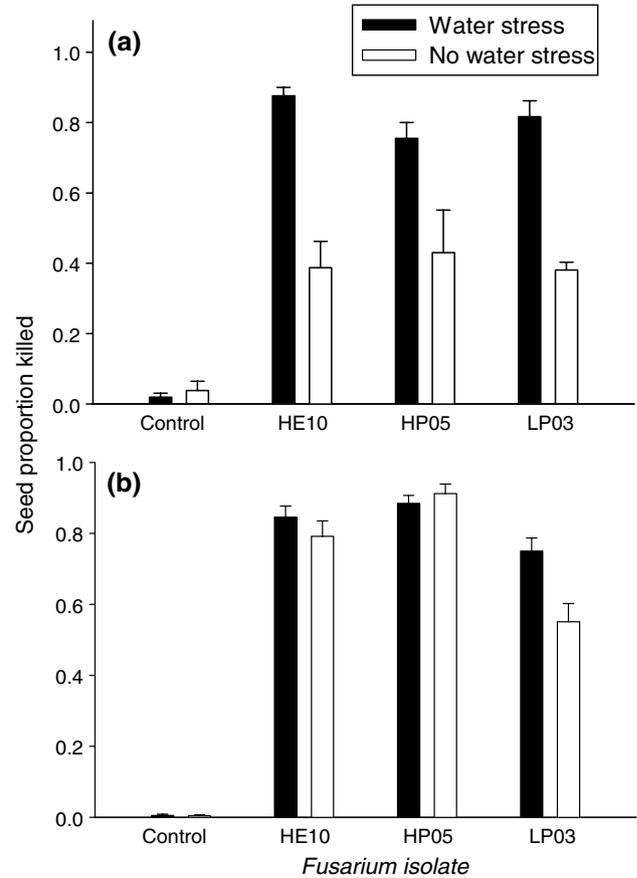
**Table 3** Correlations between *Pyrenophora semeniperda*-caused seed mortality and emergence percentage of *Elymus elymoides* and *Pseudoroegneria spicata* for Whiterocks (xeric) and Packer Creek (mesic), under two inoculum scenarios: natural inoculum level vs. inoculum augmentation

Site	Species	Treatment	<i>r</i>	<i>n</i>	<i>P</i>
Whiterocks	<i>E. elymoides</i>	Inoculum augmentation	−0.301	37	0.070
Whiterocks	<i>E. elymoides</i>	Natural inoculum level	−0.239	39	0.14
Whiterocks	<i>P. spicata</i>	Inoculum augmentation	−0.652	40	<0.0001
Whiterocks	<i>P. spicata</i>	Natural inoculum level	−0.286	40	0.074
Packer Creek	<i>E. elymoides</i>	Inoculum augmentation	−0.004	38	0.98
Packer Creek	<i>E. elymoides</i>	Natural inoculum level	0.198	39	0.22
Packer Creek	<i>P. spicata</i>	Inoculum augmentation	−0.384	40	0.014
Packer Creek	<i>P. spicata</i>	Natural inoculum level	0.159	40	0.33



**Fig. 4** Proportion of seeds of *Elymus elymoides* and *Pseudoroegneria spicata* sown at Whiterocks that germinated but failed to emerge in different treatments. Bars are means + SE. See Table 1 for statistics

positive effect on emergence overall, while at the xeric site it had a negative effect that was significant for *P. spicata* but not *E. elymoides*. Intact cheatgrass thus tended to have an ameliorative effect on native seedling emergence at the xeric site, but a suppressive effect at the mesic site. Facilitative effects of invasive species on native species are rarely considered but are sometimes reported to occur (Rodriguez 2006). In this case the most likely explanation lies in the difference in litter dynamics at the two sites. We did not quantify litter depth when the study was installed, but it was obvious that the litter was much deeper and heavier at the mesic site than at the xeric site. At the mesic site, the toothpicks were completely invisible under the litter in the intact cheatgrass treatment, while at the xeric site the toothpicks were easily visible above the litter, yielding approximate estimates for litter depth of >4 cm at Packer Creek and 1–2 cm at Whiterocks. The glyphosate treatment the previous spring resulted in an almost complete lack of litter on the cheatgrass removal plots at the time of experimental installation at both sites (<1 cm). Rapid dissolution of the litter following cheatgrass control has been documented in earlier studies (Smith et al. 2008).



**Fig. 5** Pathogen-caused seed mortality for seeds of **a** *Elymus elymoides* and **b** *Pseudoroegneria spicata* inoculated with three *Fusarium* isolates (HE10, HP05, LP03) from field-killed seeds and subjected to two water stress treatments. Bars are means + SE. See text for statistics

We hypothesize that the facilitative effect of litter at the xeric site was due to creation of a more favorable moisture regime for seedling emergence and survival, particularly for *P. spicata*, which was only marginally adapted to the site. This interpretation was supported by the strong positive effect of supplemental water on emergence and survival at this site. Other studies have reported positive effects of moderate amounts of litter on grass seedling establishment

in xeric environments and have concluded that the positive effect was a result of improved moisture relations (Evans and Young 1970; Fowler 1986). At the mesic site, the negative effect of intact cheatgrass was probably also indirect, and was likely due to light limitation from the thick layer of litter that seedlings had to penetrate in order to emerge (Facelli and Pickett 1991). The negative effects of direct competition from cheatgrass were not yet evident at the time the experiment ended in spring, because deeper soil moisture needed for survival of established seedlings had apparently not yet become limiting.

There was a trend for higher levels of *Pyrenophora*-caused native grass seed mortality in intact cheatgrass than in removal plots. There were also much higher densities of both killed and viable cheatgrass seeds in the intact cheatgrass treatment at both sites. Heavier litter has been positively associated with higher levels of disease in this pathosystem, both because of the association with higher cheatgrass seed production (as in this study) and because the microclimate under litter is more favorable for disease development (Beckstead et al. 2012). We thus obtained the rather counterintuitive result that the same conditions in intact cheatgrass that increased *P. spicata* seedling emergence at the xeric site also tended to favor *Pyrenophora*-caused disease development. There was also a significant decrease in seed mortality in response to supplemental watering at the xeric site, probably because native seeds were more likely to escape through emergence in this treatment.

Failed emergence of germinated seeds followed quite different patterns at the two sites. The most likely cause of high germinant mortality at the mesic site was an early hard frost that happened within a week of the first germination-triggering rainfall event. Increased failed emergence in the supplemental water treatment could have been a result of increased early germination, increasing the numbers of new germinants susceptible to the frost event. At the xeric site the failed germinant fraction was almost twice as high in the unwatered treatment, suggesting that it may have been related to premature drying prior to emergence. The role of water limitation at the xeric site is also highlighted by the fact that ungerminated viable seeds remained at the end of spring only at this site. Seeds were apparently more likely to remain ungerminated in treatments where they were more likely to have dried quickly, namely in the unwatered treatment and in the cheatgrass removal treatment, where the ameliorative effect of litter on moisture relations was reduced.

One reason that *Pyrenophora*-caused seed mortality and emergence success were not tightly coupled in this study was that other factors made major contributions to pre-emergence mortality, especially at the mesic site, and this tended to weaken the apparent relationship between

*Pyrenophora*-caused seed mortality and emergence. It appears that there is a fraction of seeds susceptible to mortality from multiple causes, while the remainder are more likely to escape mortality, regardless of the cause, and successfully emerge. The most likely difference between these two fractions is germination rate, i.e., rapidly germinating seeds are probably more likely to escape mortality from multiple causes than slowly germinating seeds (e.g., Beckstead et al. 2007). There may be scenarios where the conservative, slowly germinating seeds have a selective advantage, for example, in the case of the early season hard frost at the mesic site, which apparently killed early germinating seeds. However, the slowly germinating seeds at this site were highly susceptible to mortality from another cause, most likely *Fusarium*, as demonstrated in our follow-up study. The rather small fraction of seeds with intermediate germination rates were probably the ones that emerged successfully under this scenario.

The low levels of *P. semeniperda*-caused disease at the mesic site, even in the face of the addition of a massive amount of inoculum, could be due to the fact that predictable late autumn precipitation tends to trigger complete germination and thus to decrease the density of vulnerable slow-germinating seeds at this site (Beckstead et al. 2010). We rarely see high levels of *P. semeniperda* in cheatgrass seed banks at mesic sites (Meyer et al. 2008; Meyer unpublished data). Seed pathogens of the genus *Fusarium*, in contrast, are capable of causing seed mortality of relatively rapidly germinating seeds (S. E. Meyer, unpublished data), and were probably a major cause of mortality at the mesic site. They may also be important seed pathogens at xeric sites if weather and litter scenarios are conducive to their success (S. E. Meyer, unpublished data). Low levels of *P. semeniperda* at the mesic site could be due in part to interference competition from *Fusarium*, which is known to produce toxic compounds that inhibit the growth of competing fungi (Massey et al. 2004; J. Beckstead and T. Davis, unpublished data). *Fusarium* species may therefore represent another common enemy mediating apparent competition between cheatgrass and native species through impacts at the seed stage, as well as changing the disease dynamic through competitive interactions with other soil pathogens.

In summary, this study demonstrated that pathogen-caused seed mortality can be high for native grasses seeded into cheatgrass monocultures, and that the indirect effect of this heavy pathogen load could interact with direct competitive effects to limit the ability of these native grass species to recruit into cheatgrass-dominated sites. We also showed that cheatgrass indirect effects through litter dynamics may be either suppressive or ameliorative. The methodology used here, namely retrieval and examination of individual unemerged seeds, has the potential to add greatly to our knowledge of pathogen impacts on seeds in soil, and could

reveal a whole suite of organisms important in the multi-trophic interactions that mediate relationships between native and invasive plants.

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