

Direct and indirect effects of plant litter on a seed–pathogen interaction in *Bromus tectorum* seed banks

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Abstract

The naturally occurring fungal seed pathogen, *Pyrenophora semeniperda*, reduces the seed bank of *Bromus tectorum* but the role of plant litter in this seed–pathogen interaction is unexplored. To investigate the direct and indirect effects of litter on this interaction, we first collected field seed-bank samples from low and high *Bromus* litter patches. From these data, we explored the relationship between litter depth, seed-bank density and seed mortality from *P. semeniperda*. Second, we manipulated the fungal stages (conidial spores and mycelium) in/on the litter through sterilization techniques, to measure the direct effect of litter on seed death. Third, for indirect effects, we manipulated litter levels and held seed density and inoculum constant to determine whether *Bromus* litter could modify the seed zone microsites to favour disease. We found that seed-bank samples from high-litter patches contained higher field-killed seed densities compared with low-litter patches, although the percent difference of disease between litter patch types varied among sites and years (e.g. 80% to 46%). In testing the direct effects of litter on the seed–pathogen interaction, we found that litter can act as a direct inoculum source for the pathogen in the early summer but decreases in disease transmission by the following spring when the litter naturally is in contact with seeds. Investigating indirect effects, we found four times as many pathogen-killed seeds in high-litter treatments as compared with low-litter treatments when inoculum loads and seed densities were held constant. In addition, we found that litter influences the seed–pathogen interaction through density-dependent disease transmission. Our findings demonstrate the ecological importance of litter in semi-arid environments as it influences disease levels of a seed pathogen by direct and indirect means.

Keywords: cheatgrass, density-dependent disease, *Drechslera campanulata*, inoculum source, microbial interaction, *Pyrenophora semeniperda*, shrub–steppe

Introduction

Plant litter plays important, but often overlooked, roles in many processes at the soil–litter interface. At this interface, litter influences a range of processes from nutrient cycling (Frey *et al.*, 2003) to plant establishment (Facelli and Pickett, 1991). Many plant seed banks physically occur at the soil–litter interface. Seeds are mixed into the top layer of soil and are also suspended in the litter directly above the soil. Litter may impact seed banks as a direct physical barrier to germination (Hamrick and Lee, 1987) or indirectly by modification of microenvironments affecting germination and establishment (Goldberg and Werner, 1983; Facelli and Pickett, 1991). Litter can also impact species interactions (i.e. predation, competition and parasitism) within the seed bank. For example, plant litter in central North American grasslands negatively affects seed removal at the soil–litter interface by rodents but not by ants (Reed *et al.*, 2006). Presence of litter may indirectly reduce competition in the seed bank. For example, in forests of eastern North America, plant litter reduced density and biomass of the dominant herb *Seteria faberii*, resulting in increased biomass of weaker seedling competitors (Facelli, 1994). Litter may also impact plant–pathogen species interactions. Seed pathogens have been shown to reduce seed banks in a range of natural ecosystems (Crist and Friese, 1993; Dalling *et al.*, 1998; Schafer and Kotanen, 2004) and these pathogens may be influenced by the presence of litter (Facelli *et al.*, 1999).

Given the importance of seed pathogens in mediating seed-bank dynamics at the soil–litter interface, coupled with the strong direct and indirect influences of plant litter, it is likely that plant litter will have important effects on seed–pathogen interactions. If plant litter facilitates seed pathogen diseases, then

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greater levels of seed death will occur in seed banks with higher litter density than in those with lower or no litter density. To address this hypothesis, we studied a seed–pathogen system that involved the invasive annual weed cheatgrass (*Bromus tectorum* L.) and the seed pathogen *Pyrenophora semeniperda* ((Brittlebank and Adam) Shoemaker (anamorph *Drechslera campanulata* (Lév.) B. Sutton)), in the semi-arid shrub–steppe community of western North America.

Following the introduction of *B. tectorum* in the late 19th century, it quickly reached dominance within perennial grasslands and shrublands of the western United States and British Columbia (Mack, 1981), and continues to expand its range today (e.g. Colorado Plateau; Floyd *et al.*, 2006). *B. tectorum* generates prodigious numbers of seeds and large quantities of litter (Kelrick, 1991). Seeds drop throughout the summer, creating a dense litter–soil seed bank as seeds become intermixed with the soil and litter from dead stems and leaves of the parent plants. Within this seed bank, large numbers of these seeds can become infected by the generalist fungal pathogen *P. semeniperda* (Meyer *et al.*, 2007), which occurs naturally in Eurasia (Stewart *et al.*, 2009), United States, Canada, Argentina, Australia, New Zealand and South Africa (Medd *et al.*, 2003). Infections of host seeds can occur either aerially during anthesis or from direct contact with other infected seeds. Infected seeds produce black, conidia-laden stromata that can infect other seeds in the seed bank (Beckstead *et al.*, 2007). Leaf ring spotting has been observed in some grasses, leading to the suggestion that the fungus may be systemic, with mycelium growing throughout the host (Medd *et al.*, 2003) and potentially overwintering in the dead plant material. Due to its efficacy against the invasive *B. tectorum* within the seed bank, *P. semeniperda* has been proposed as a potential biological control agent (Meyer *et al.*, 2007), but important questions still remain regarding the role of plant litter in the life cycle of this pathogen and its hosts.

Litter may affect seed mortality by fungal pathogens through direct and indirect mechanisms. Directly, litter could act as an inoculum source for the pathogen and consequently directly kill seeds in the soil–litter seed bank. Medd *et al.* (2003) hypothesized that plant litter could serve as a secondary infection source in the life cycle of *P. semeniperda*. Although the fungal mycelium within plant litter has not been shown to produce ascospores and/or conidia, latent mycelium within infected tissues could grow and infect adjacent seeds suspended in the litter (L. Poston and J. Beckstead, unpublished data; *P. semeniperda* was isolated from 3-week-old leaf tissues of *B. tectorum* seedlings infected with *P. semeniperda*). Crop and weed plant debris has been reported as an important source of inoculum for some agricultural diseases (Latorre and Jones, 1979; Cortesi *et al.*, 1997), but little work has

addressed the possibility of direct litter infection in natural systems.

Litter may also affect the seed pathogen–*Bromus* interaction indirectly by altering microsite conditions. Plant litter alters seed-bank conditions by increasing soil moisture/water availability (Fowler, 1986), decreasing levels of incoming radiation (Facelli and Pickett, 1991), and reducing temperature fluctuations (Weaver and Rowland, 1952). Compared to bare ground, *B. tectorum* litter can reduce average maximum daily soil surface temperatures in the spring from 28°C to 19°C, insulate average minimum temperatures from 0°C up to 5°C, and significantly stabilize relative humidity over the diurnal range (Evans and Young, 1970). The environmental conditions described under *B. tectorum* litter align more closely with the optimal sporulation parameters of *P. semeniperda* (see Campbell *et al.*, 2003) than those recorded on adjacent bare ground. *B. tectorum* litter may provide the ideal suitable environment for disease proliferation in the seed bank at the soil–litter interface.

This study aims to explore how *B. tectorum* litter affects *B. tectorum* seed mortality by *P. semeniperda*. The effects of litter on the *B. tectorum* seed–pathogen interaction were first investigated with a field seed-bank study, involving the collection of seed-bank samples from low and high *B. tectorum* litter patches at three study sites. Using a combination of field studies and lab experiments, we aimed to explore the direct and indirect effects of litter on this seed–pathogen interaction, namely: (1) does *B. tectorum* litter directly determine seed disease in the seed bank via transmission of *P. semeniperda*; and (2) does *B. tectorum* litter indirectly affect the seed–pathogen interaction by altering the seed microsite that modifies the disease relationship?

Methods

Field seed-bank study

To investigate the effects of litter on the cheatgrass seed–pathogen interaction, soil-core seed-bank samples (for details, see Beckstead *et al.*, 2007) were obtained from three cheatgrass monocultures in the western United States: upper Saddle Mountain, Hanford Reach National Monument, Franklin County, Washington [46°47'N, 119°27'W; elevation 582 m above sea level (asl)]; Spokane, Spokane County, Washington (47°37.204'N, 117°18.513'W, elevation 871 m asl) and Whiterocks, Tooele County, Utah (40°N, 109°W; elevation 1839 m asl). Samples (6 cm diameter × 4 cm depth) were taken in two types of cheatgrass-litter patches, low litter (bare ground to <1 cm litter) and high litter (approximately 2–3 cm of litter) in May prior to the current year's seed dispersal. Forty samples were taken per litter treatment at each of three sites and

repeated across 2 years (2009 and 2010). Samples were screened to separate seeds from soil and litter material and then processed to quantify any apparently viable (intact) seeds and also any field-killed seeds with protruding stromata of *P. semeniperda*. Apparently viable seeds were then incubated for 2 weeks at 20°C and 24-h photoperiod by placing seeds on germination blotters (Anchor Paper, St. Paul, Minnesota, USA) saturated with water within a Petri dish (100 × 15 mm). Petri dishes were stacked randomly in plastic bags closed with wire ties to retard water loss. Seeds that developed stromata during incubation were considered to have been infected and killed in the field; these values were included in the total field-killed densities. The viable seed-bank densities consisted of seeds that germinated during incubation as well as dormant but viable seeds (determined by a cut test; Ooi *et al.*, 2004). Only 6% of the apparently viable seeds were dormant. At the Spokane site *Bromus arvensis* (field/Japanese brome; previously *Bromus japonica*), was intermixed with *B. tectorum*. Although *B. arvensis* occurred in low frequencies relative to *B. tectorum* (1% of the seed bank), it was involved in the disease interaction and was included in the seed-bank analysis.

Seed-bank data were analysed using analysis of variance (ANOVA) for a completely randomized design with year, site and litter patch type as fixed effects (JMP, Version 8.0.1, SAS Institute Inc., Cary, North Carolina, USA). The response variables, field-killed seeds and total seed-bank density, were log transformed to improve homogeneity of variance prior to analysis. Means separations from the LSMEANS statement were examined for each main effect and interactions that were significant ($P < 0.05$) in ANOVA.

To examine the effects of litter on seed disease independent of seed-bank density, we calculated the proportion of seeds killed by *P. semeniperda* from the total seed-bank density. Our prediction is that the proportion of seeds killed would be higher in the high-litter treatment, independent of the total seed density. The ANOVA was identical to the analysis above, with the exception that the proportion of seeds killed was transformed with an arcsine transformation.

Direct litter-to-disease experiment

To quantify the direct effects of litter on disease, cheatgrass plant litter was harvested from cheatgrass monocultures from three sites and repeated at three seasonal time intervals. The sites were identical to the field seed-bank study above (Saddle Mountain, Washington; Spokane, Washington; and Whiterocks, Utah), with the exception of the Saddle Mountain, Washington site, which was at a lower elevation on the mountain (46°45'N, 119°28'W, elevation 282 m asl). Standing, dead, above-ground biomass of cheatgrass

(hereafter referred to as litter) was collected, rather than dead plants lying on the ground, reducing soil fungal contamination. The experiment was repeated in time to capture temporal variability of any direct fungal disease transmission, which involved early summer litter (late May 2009), late summer litter (August 2009) and the following early spring litter (February 2010). Given that the bulk of carryover litter on the ground is composed primarily of cheatgrass stems, all seeds and leaves were removed and discarded. In addition, all plants infected with the smut fungus *Ustilago bullata* Berk. were discarded.

To isolate the fungal stage from the litter, the litter was split equally into three treatments. The control consisted of litter fully sterilized by autoclaving for 80 min at 121°C to kill any fungal spores or mycelium. The second treatment was surface-sterilized litter (sequential immersion in 70% ethanol, 10% bleach and deionized water for 1 min each) to remove any fungal conidia on the surface of the litter but to retain mycelium within the litter. The final treatment was unsterilized litter, which retained fungal conidia on the surface and mycelium within the litter. Aseptic technique was followed to limit contamination of materials following treatments. Airborne conidial spore contamination from *P. semeniperda* was a possibility, especially in the early summer, due to concurrent experiments in the same lab space, although measures were taken to limit this impact.

Twenty-five healthy, dormant cheatgrass seeds (stored at –18°C), which are highly susceptible to *P. semeniperda* seed death (Beckstead *et al.*, 2007), were placed in Petri dishes (100 × 15 mm) on moist germination blotters (Anchor Paper). Seeds were surface sterilized (1 min in 70% ethanol, 1 min in 0.0525% sodium hypochlorite) and safranin dyed for easy retrieval prior to placing in the Petri dish. Aseptic technique was followed to limit contamination of seeds. Although surface sterilization should remove the majority of *P. semeniperda* on the surface, there is the possibility of *P. semeniperda* infection within the seed from floral infection. However, floral infection occurs infrequently in the Intermountain West region (Meyer *et al.*, 2008) and is not expected to influence the seed infection rate in this study. For each treatment–location combination, 1 g of litter was placed on top of the seeds in the Petri dish. The number of replicated Petri dishes varied from 10 to 15, based on the availability of litter; most treatment combinations had 15 replicates, with the exception of Whiterock early summer ($n = 10$) and Whiterock late summer ($n = 12$). The dishes were sealed with Parafilm® and stacked randomly in plastic bags and incubated at 20°C and 24-h photoperiod for 4 weeks. The dishes were watered as needed, to saturation about once every 3 d.

At the end of the 4 weeks, the total number of seeds killed by *P. semeniperda*, indicated by fungal stromata

on ungerminated seeds, was tallied and the percentage of seeds killed by *P. semeniperda* was calculated. The total number of viable seeds was established, determined by a cut test on all ungerminated seeds at the end of the incubation period. Each repeated temporal trial of the experiment was analysed independently due to the vastly different environmental conditions the litter experienced in the field. Data were transformed with an arcsine square-root function to improve homogeneity of variance and analysed with a two-way ANOVA (site and litter treatment variables; JMP). Means separations from an LSMEANS statement were examined for each main effect and first-order interaction that was significant ($P < 0.05$) in the ANOVA.

Indirect litter-to-disease experiment

To quantify the indirect effects of cheatgrass litter on seed death by *P. semeniperda*, we designed artificial litter–seed banks that varied litter levels. The experiment was repeated at two time intervals, hereafter trials. There were three litter treatments, bare ground (no litter), low litter (1 cm) and a high litter treatment (2.5 cm). The litter was collected from cheatgrass monocultures from lower Saddle Mountain, Washington. The litter was saturated in deionized water and then sterilized in the autoclave for 80 min at 121°C to kill any fungal conidia and mycelium.

The artificial seed banks were created by planting 12 dormant cheatgrass seeds in each of 24 plots (8.9 cm square standard flower pots) filled with sterile 2:2:1 sandy loam:peat moss:surface (calcinated clay) mix for each of the three litter treatments, resulting in a total of 24 pots and 288 seeds per trial. Litter treatments were applied to each pot; bare ground (no litter added), low litter (approximately 1 cm; 0.6 g dry litter) and high litter (approximately 2.5 cm; 2.3 g dry litter). Inoculum of *P. semeniperda* (strain isolated from Whiterocks, Utah; strain no. 0) was applied equally (0.40 g) to all pots in a mixed form of mycelium–conidia–clay carrier (Agsorb Products, Chicago, Illinois, USA). Dormant cheatgrass seeds (collected at Saddle Mountain, Hanford, Washington) were planted into each pot. The seeds were dyed with safranin, glued to toothpicks using Elmer's glue and inserted into each pot. This method allowed for the full recovery of the seeds and to control the vertical placement of the seeds in the litter–seed bank. The pots were placed randomly into a grid design of trenches at a field site in Spokane, Washington (47°37'59.47"N, 117°22'38.68"W, elevation 715 m asl), allowing them to experience autumn conditions for 5 weeks. The repeated trials were separated by 2 weeks and experienced similar rainfall (6.12 cm for Trial 1 and 6.55 cm for Trial 2). In the second trial only one seed out of 288 was killed

by the fungus and thus this trial was excluded from the analysis.

Disease incidence was assessed by counting the number of seeds killed by *P. semeniperda* (seeds that did not germinate and developed the distinctive stromata of *P. semeniperda*). Data were transformed with a square root transformation and analysed using one-way ANOVA (JMP). To compare the different litter treatments the Tukey–Kramer Honestly Significant Difference (HSD) test was performed.

Results

Field seed-bank study

We initially predicted that disease would be higher in seed banks from high-litter patches compared with low-litter patches, indicating that litter facilitates the seed pathogen disease. Our results show that field-killed seed densities were significantly higher in high-litter patches compared with low-litter patches; however, the magnitude of the pattern is variable for a given location and a given year (Table 1 and Fig. 1a and b). The percent difference in field-killed seeds between the high- and low-litter patches varied from a high of 80% (Whiterocks 2009) to a low of 46% (Spokane 2010). In general, 2009 had higher differences between the high- and low-litter patches for all three sites in comparison to 2010 (Fig. 1a and b), but within a given year the site with the largest effect of litter treatment varied.

The total viable seed density was significantly different among seed-bank samples collected from the two litter patch types (Table 1 and Fig. 1c and d). In general, the high-litter patches had significantly more seeds (usually twice as many) compared with the low-litter patches, with the exception of Spokane in 2010. It is likely that high-litter patches with high seed density resulted in greater disease levels due to the higher host density, facilitating the seed pathogen disease.

In addition, we found that litter facilitates the seed pathogen disease even when seed density is accounted for, as in the proportional killed seed data (Table 1 and Fig. 1e and f). High-litter patches had significantly greater proportions of seeds killed (15% increase) compared with the low-litter patches, and this pattern was consistent across sites during most years. These results suggest that some of the litter facilitation of seed disease is independent of seed density and possibly due to microsite modification by the litter during the seed-infection process.

Direct litter-to-disease experiment

We hypothesized that conidial spores on the outside of the litter or fungal mycelium within the plant were the

Table 1. Results of three-way ANOVA on seed-bank measures of field-killed seed density by *Pyrenophora semeniperda*, total viable seed density, and the proportion of seeds of *Bromus* sp. killed. The three factors analysed are patch type (low litter and high litter), field site (Saddle Mountain, Washington; Spokane, Washington; and Whiterocks, Utah) and year (2009 and 2010) ($n = 40$). Seed-bank samples from Saddle Mountain and Whiterocks contained only *Bromus tectorum* seeds, whereas Spokane contained a small amount of *B. arvensis* intermixed with *B. tectorum* (see Methods)

Source of variation	df	Field-killed seed per dm ²		Viable seed density per dm ²		Proportion of seeds killed	
		F-ratio	P	F-ratio	P	F-ratio	P
Patch type	1	182.15	0.0001	84.38	0.0001	5.97	0.0149
Site	2	109.22	0.0001	147.07	0.0001	38.68	0.0001
Year	1	40.57	0.0001	71.09	0.0001	6.06	0.0143
Site × Year	2	10.36	0.0001	2.23	0.1084	3.01	0.0505
Site × Patch type	2	8.36	0.0003	9.49	0.0001	0.35	0.7043
Year × Patch type	1	2.45	0.1180	7.25	0.0074	0.69	0.4062
Site × Year × Patch type	2	1.20	0.3012	1.13	0.3239	2.05	0.1301

only possible means of litter acting as a direct inoculum source for seed death by *P. semeniperda*. The results show that in early summer the litter can act as a direct inoculum source for the pathogen and we found a significant litter treatment effect (Fig. 2a: $df = 2$, $F = 17.93$, $P < 0.0001$). In early summer, high levels of seed death occurred for both the surface-sterilized litter treatment (removal of conidia, but not mycelium) and unsterilized litter treatment (presence of both conidia and mycelium), but not for the autoclaved litter treatment (significance determined by LSMEANS difference Tukey–Kramer HSD). Seed mortality was 64% higher in the surface-sterilized treatment and 75% higher in the unsterilized treatment than in the autoclaved treatment. Although the significant litter treatment effect was consistent across the temporal scale (Fig. 2b: late summer: $df = 2$, $F = 48.80$, $P < 0.0001$; Fig. 2c: early spring: $df = 2$, $F = 6.46$, $P = 0.002$), the high levels of mycelial transmission (via surface-sterilized litter treatment) decreased in importance over time. In the late summer the difference between the autoclaved and surface-sterilized treatments was 79% and between the autoclaved and unsterilized was 88%. There was almost no difference between the autoclaved and surface sterilized treatments by the following early spring, but there was a 52% difference between the autoclaved and the unsterilized treatments. In addition, the proportion of seeds killed by direct litter transmission decreased from a high of 18% mortality in early summer to a high of only 5% in the following spring.

In early and late summer, there was no significant difference in seed death among the three sites (Fig. 2: early summer, site main effect: $df = 2$, $F = 0.70$, $P = 0.50$; and late summer, site main effect: $df = 2$, $F = 1.40$, $P = 0.25$), but in the following early spring there was a significant difference among the three sites ($df = 2$, $F = 7.31$, $P = 0.001$). The magnitude of seed

death derived from litter decreased over time (Fig. 2). The interaction effects of site × litter treatment were not significant for early summer ($df = 4$, $F = 0.09$, $P = 0.61$) or early spring ($df = 4$, $F = 1.90$, $P = 0.1152$) but were significant for late summer ($df = 4$, $F = 5.23$, $P = 0.0007$).

Indirect litter-to-disease experiment

We predicted that cheatgrass litter could indirectly affect the ability of *P. semeniperda* to kill cheatgrass seeds in artificial litter seed banks. Our data showed a significant difference in seed death among litter levels (Fig. 3: $df = 2$, $F = 5.38$, $P = 0.01$). Overall, the seed death rates were relatively low, less than 10%. The proportion of seeds killed was highest in the high-litter treatments and decreased by 75% in the low-litter treatment and by 100% in the bare ground.

Discussion

B. tectorum litter affects seed mortality within the seed bank caused by the pathogen, *P. semeniperda*. In all three experiments (field seed-bank study, direct litter-to-disease experiment and indirect litter-to-disease experiment) high-litter treatments resulted in higher seed death compared with bare soil or low-litter treatments. *Bromus* litter has the potential to directly affect seed disease in the seed bank via transmission of the pathogen in early summer, but the influence decreases in the autumn and subsequent spring when the litter is naturally in contact with seeds in the seed bank. Moreover, *Bromus* litter also indirectly increases seed disease by altering conditions conducive for disease transmission.

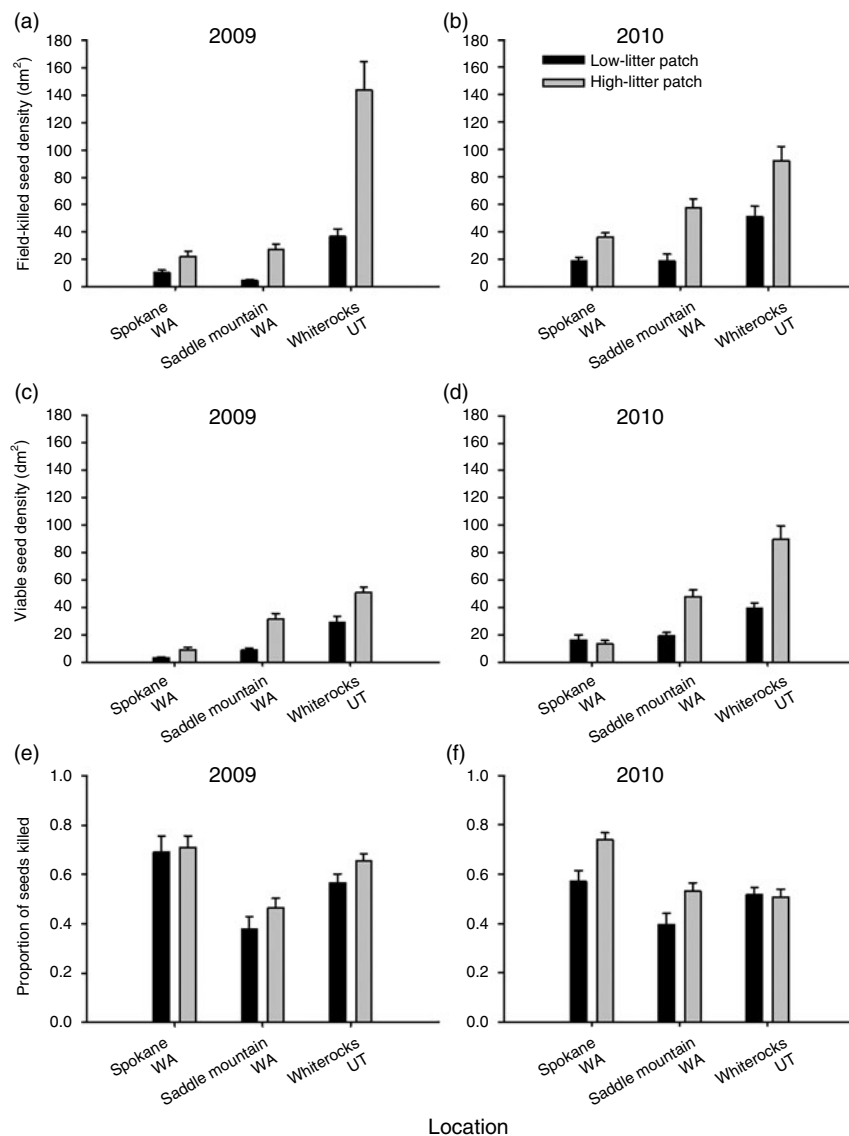


Figure 1. Seed-bank measures of field-killed seed density (i.e. recently killed by *Pyrenophora semeniperda* in the field and exhibiting stromata) in 2009 and 2010 (a, b), total viable seed density (i.e. living, free of disease) in 2009 and 2010 (c, d), and the proportion of seeds killed (field-killed seed density divided by total viable seed density) in 2009 and 2010 (e, f) for *Bromus* sp. in each of two litter patches (low-litter patch and high-litter patch) for three site locations (Spokane, Washington; Saddle Mountain, Washington; and Whiterocks, Utah; mean +1 SE; $n = 40$). Seed-bank samples from Saddle Mountain and Whiterocks contained only *Bromus tectorum* seeds, whereas Spokane contained a small amount of *B. arvensis* intermixed with *B. tectorum* (see Methods).

Field seed-bank study

We found an increase in pathogen field-killed seed densities in high-litter patches compared with low-litter patches, although the magnitude of disease varied with site and year. These results support our initial hypothesis that plant litter can facilitate seed pathogen disease at the soil–litter interface. Facelli *et al.* (1999) found greater seedling mortality by fungal pathogens in treatments with litter than treatments without litter. In the same study, the researchers found the effects of litter on seedling disease to vary

with soil water content (Facelli *et al.*, 1999). The subtle differences in water content at the soil–litter interface (Goldberg and Werner, 1983) are likely to be of importance in determining the magnitude of disease, which we found to vary among sites and between years. Our findings are correlative in nature and do not indicate which mechanisms (i.e. direct versus indirect effects of litter on disease, or accumulation of seeds) are creating the pattern. However, our results from the proportion of seeds killed in the seed bank showed that high-litter patches had on average a 15% increase in the proportion of seeds killed compared with the

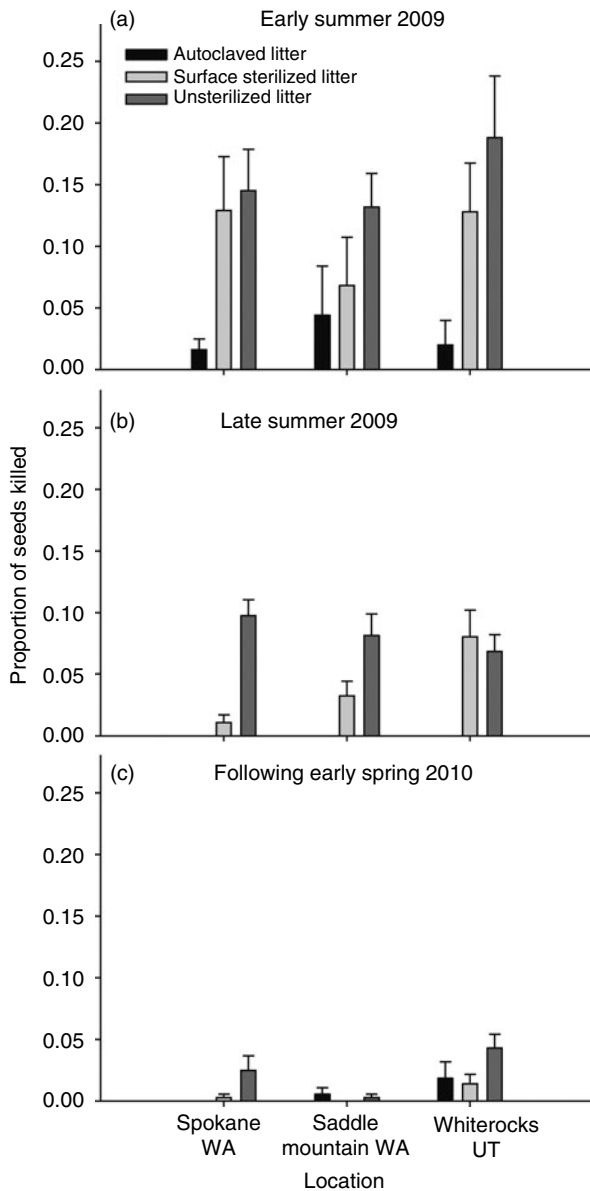


Figure 2. Proportion of *Bromus tectorum* seeds killed by *Pyrenophora semeniperda* in early summer 2009 (a), late summer 2009 (b), and the following early spring 2010 (c) in each of three sterilization treatments (mean +1 SE; $n = 15$, for exceptions see Methods). Sterilization treatments included: autoclaved litter (reduction of conidia on the surface of litter and reduction of mycelium within litter tissues), surface sterilized litter (reduction of conidia on the surface of litter but retained mycelium within litter tissues) and unsterilized litter (retained conidia on litter surface and also retained mycelium within litter tissues).

low-litter patches. These results suggest that litter facilitation of disease is partially independent of seed density and potentially due to microsite modification by the litter during the seed-infection process.

An important possible mechanism for the litter–disease pattern is that litter may affect the seed–pathogen interaction indirectly by altering seed

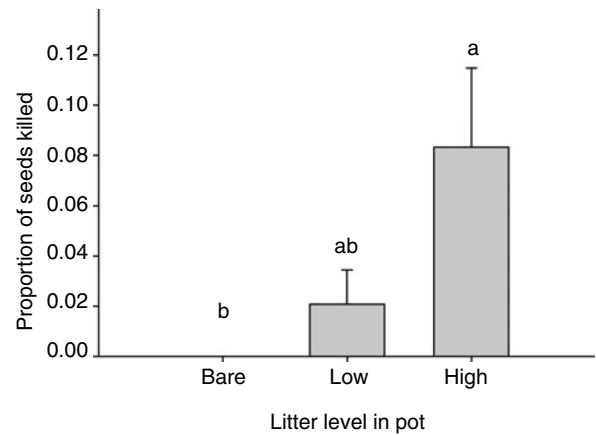


Figure 3. Proportion of *Bromus tectorum* seeds killed by *Pyrenophora semeniperda* in pots placed in the field with equal inoculum levels and equal seed densities containing three litter levels (bare, low and high litter; mean +1 SE; $n = 8$). Different letters represent significant differences ($P < 0.05$) among treatments, by the Tukey–Kramer HSD multiple comparison test.

densities that are fundamental to density-dependent diseases. Pathogen/parasite transmission often shows some level of positive density dependence (Gilbert, 2002). Beckstead *et al.* (2010) found that the high abundance of *B. tectorum* seed densities increased the incidence of disease by *P. semeniperda*. In other species litter has been shown to increase seed densities, especially in arid systems (Chambers and MacMahon, 1994; Rotundo and Aguiar, 2005), and could therefore facilitate indirectly the seed pathogen–*Bromus* interaction. Supporting the density-dependent disease transmission hypothesis, our results showed that total seed density was two times higher in high-litter patches compared with low-litter patches. This high seed density in high-litter patches could result from litter preventing secondary dispersal of seeds or the build-up of seeds that do not germinate. In addition, the high biomass of plants during the prior season also produces large quantities of seeds and subsequent high amounts of plant litter. Either way, within the high-litter patches there are more hosts for the pathogen to feed on, leading to positive density-dependent frequency for *Bromus* seed infection (i.e. increasing disease with increasing host seed densities associated with litter accumulation). Our support for this density-dependent disease relationship with litter is based on sampled field data. Future experiments that manipulate seed density are needed to test this hypothesis fully.

Direct litter-to-disease experiment

We found that *Bromus* litter may directly determine seed disease in the seed bank via transmission of *P. semeniperda*, both via conidia on the litter surface and

mycelium embedded within the plant tissues. Most other studies on spore infection related to plant litter have had systems where the spores are produced within the plant litter itself (e.g. Davidson *et al.*, 2005). Distinctly, in our system the conidial spores are produced only on the infected seeds (Beckstead *et al.*, 2007), but the spores can adhere to the litter surface and then be a mechanism of disease transmission, as indicated by the data in this paper. Our data do not support Medd's hypothesis that mycelium within plant tissues is an important inoculum source (Medd *et al.*, 2003). We found pathogen-caused seed mortality from mycelium infection to be present but rather low from spring-collected plant litter, ranging from 1 to 4%. However, spring is the season when plant litter is most likely to have the greatest contact with the seed bank as a result of winter storms pushing the plant litter from a vertical position to a horizontal position. These results suggest that fungal mycelium within litter does not likely play an important role in the seed death of *B. tectorum* seed banks. However, if early summer litter infected with fungal mycelium is matted down to the soil surface and seeds are still germinating, then direct disease transmission from fungal mycelium within the litter could occur.

The evidence of high mycelial infection from litter collected in early summer and mid-summer does indicate that the fungus can live systemically within plant tissues as the plant is senescing. Likewise, *P. semeniperda* has been isolated from 3-week-old leaf tissues of *B. tectorum* seedlings infected with *P. semeniperda* (L. Poston and J. Beckstead, unpublished data) and also from reproductive *B. tectorum* individuals collected from the field (G. Newcomb, unpublished data). Studies have shown that some pathogens can be commensal endophytes under certain conditions and pathogens under others (Moricca and Ragazzi, 2008). Whether or not *P. semeniperda* behaves as a pathogen while in *B. tectorum* leaf or stem tissue, or whether its role is endophytic, will require further investigation.

Indirect litter-to-disease experiment

Our data provide evidence for positive indirect litter effects on seed disease caused by *P. semeniperda*. Specifically, we found pathogen-killed seeds to be four times higher in high-litter treatments compared with low-litter treatments in our artificially created seed-bank experiment. When seed density and inoculum loads are controlled, the indirect effects are most likely related to seed-bank microenvironments. Likewise, our results from the field seed-bank experiment, which examined the effects of litter on seed disease independent of seed density, also pointed towards indirect effects involving seed-bank microsite modification by litter. Evans and Young (1970) found that the

accumulation of litter at the soil–litter interface in arid systems with *Bromus* spp. increased moisture retention and reduced temperature fluctuations. Although most studies have focused on the indirect litter effects of seed germination and seedling survival (Hamrick and Lee, 1987; Facelli and Peckett, 1991), it is likely that these same altered microenvironments are at play in the disease–host–environment triangle of the *P. semeniperda*–*B. tectorum* seed interaction. Growth of *P. semeniperda* is sensitive to moisture, temperature and light (Campbell *et al.*, 2003), thus indicating that litter could definitely influence disease by this organism. Although we found positive, small, indirect effects of litter on seed disease, our findings were not consistent across the two repeated trials of the indirect litter-to-disease experiment (no disease was monitored in the second trial; see Methods for description). It is likely that the subtle localized changes in seed-bank microsite created by litter could be overwhelmed by the larger scale microsite and weather conditions. For example, our second trial was located adjacent to a large tree, which could have shielded the pots from adequate rainfall. Or the timing of dew events and rain events could have held the pathogen infection at a latent mycelial stage in the second trial, such that disease, evidenced by stromata formation, was not observed. Future studies should examine directly how litter modifies the microenvironment to complement the data presented here.

Alternatively, the positive indirect effect of litter on seed disease may be the result of leachate from litter stimulating fungal growth and seed infection. If litter provides a chemical cue needed for pathogen conidial germination, then pots with higher litter additions will experience greater disease than plots with lower litter additions. Unfortunately, there is little plant litter data supporting this potential mechanism. For example, Boserup and Reader (1995) found that litter leachate in a prairie system reduced seedling emergence instead of enhancing emergence. Likewise, litter leachate effects on fungi are commonly negative (Nilsen *et al.*, 1999; Piotrowski *et al.*, 2008) not positive as hypothesized.

In conclusion, our findings indicate that plant litter is an important factor affecting pathogen-caused seed mortality in seed banks at the soil–litter interface. We have shown that indirect effects of litter can operate with density-dependent disease relations and also that significant, but small, indirect effects of litter on disease can occur through alteration of the seed microenvironment. In general, seed banks are dynamic, both temporally and spatially. Seed pathogens have the potential to reduce seed banks dramatically, such that an understanding of factors that affect the seed pathogen–seed bank interaction will help us to better understand this early life-stage interaction.

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