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Temperature effects on three downy brome (*Bromus tectorum*) seed collections inoculated with the fungal pathogen *Pyrenophora semeniperda*

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Note

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Abstract

Downy brome (*Bromus tectorum* L., syn. cheatgrass) is a winter annual grass that invades North American cropping, forage, and rangeland systems. Control is often difficult to achieve, because *B. tectorum* has a large seedbank, which results in continuous propagule pressure. *Pyrenophora semeniperda* (Brittlebank and Adam) Shoemaker, a soilborne fungal pathogen, has been investigated as a biological control for *B. tectorum*, because it can kill seeds that remain in the seedbank, thereby reducing propagule pressure. Temperature influences *P. semeniperda* and has not been investigated in the context of seeds collected from different *B. tectorum* locations, that may vary in susceptibility to infection. We compared the effects of temperature (13, 17, 21, 25 C) and *B. tectorum* seed locations (range, crop, subalpine) with different mean seed weights on infection rates of *P. semeniperda* using a temperature-gradient table. Infection differed by seed location ($P < 0.001$) and temperature ($P < 0.001$), with lighter-weight seeds (i.e., range and subalpine) more susceptible to *P. semeniperda* infection. Infection increased as temperature increased and was higher at 21 C (66.7 ± 6.7%) and 25 C (73.3 ± 6.0%). Germination was affected by seed location ($P < 0.001$) and temperature ($P = 0.019$). Germination was highest for the crop seed location (45.4 ± 4.2%) and overall decreased at higher temperatures (21 and 25 C). Our results suggest that *B. tectorum* seeds from a crop location are less affected by *P. semeniperda* than those from range and subalpine locations. Moreover, this demonstrates a temperature-dependent effect on all populations.

Introduction

Downy brome (*Bromus tectorum* L., syn. cheatgrass) is a widespread invasive annual grass in western North America, where it infests millions of hectares of cropping, forage, and rangeland systems (Mack 1981; Rice 2005). The range of *B. tectorum* is expected to expand with increasing temperatures (Bradley 2009), compounding its invasion and impacts. As a winter annual, *B. tectorum* typically germinates and emerges in the fall, after it has lost primary dormancy as a result of warm summer temperatures (Hawkins et al. 2017). However, seeds will not germinate if conditions are unfavorable and instead may enter secondary dormancy, induced by low winter temperatures and moderate water stress, and germinate in the spring once precipitation arrives (Hawkins et al. 2017). This spring germination, combined with its fall germination, allows *B. tectorum* to outcompete perennial species for soil moisture (Kulmatiski et al. 2006).

Given the vast distribution of some invasive species, including *B. tectorum*, and the associated cost of control with herbicides or other methods, biological control is an attractive management option (Seastedt 2015). *Pyrenophora semeniperda* (Brittlebank and Adam) Shoemaker (Ascomycotina: Pyrenomycetes), a soilborne fungal pathogen, has been used for biological control of *B. tectorum* (Beckstead et al. 2007, 2010; Ehlert et al. 2014; Meyer et al. 2007, 2008a, 2008b, 2017). *Pyrenophora semeniperda* conidia in soil are carried via germination tubes to grass inflorescences, where they infect developing ovaries during anthesis (Medd et al. 2003). Infected mature host grass seeds disperse to the ground, where *P. semeniperda* remains as mycelium for the rest of the summer. The fungus prevents seeds from germinating by consuming starch resources within the ovary. *Pyrenophora semeniperda* kills the seeds by producing phytotoxic secondary metabolites such as cytochalasin B, which interferes with cytokinesis

Management Implications

Bromus tectorum (downy brome, cheatgrass) is an invasive winter annual grass that infests cropping, forage, and rangeland in North America. Common management techniques such as herbicides do not affect the vast seedbank associated with *B. tectorum* invasion, and control is often site specific due to environmental conditions or genetic variation of the target population that makes it difficult to manage large areas. *Pyrenophora semeniperda*, a soilborne fungal pathogen, has been investigated as a biological control for *B. tectorum*, as it can infect and kill seeds within the seedbank. Consequently, *B. tectorum* propagule pressure will be reduced, making it easier for land managers to control *B. tectorum* populations. However, *P. semeniperda* infection is affected by temperature and may also depend upon the source location of *B. tectorum* seeds. In our laboratory experiment, we exposed *B. tectorum* seed from three different locations (range, crop, subalpine) with different mean seed weights to *P. semeniperda* at varying temperatures. We found that seed location and temperature influenced infection and germination. Lightweight seeds from the range and subalpine seed locations were more susceptible to infection, and infection increased across temperatures. In contrast, lightweight seeds had lower germination, and germination was highest at lower temperatures. Overall, seed weight may act as a simple measure of susceptibility to *P. semeniperda*, thus allowing field practitioners a relatively easy way to assess whether *P. semeniperda* may contribute to their management strategies. Land managers should use a *P. semeniperda* strain that is endemic to the treatment area when temperatures are favorable for disease. Land managers interested in *P. semeniperda* should investigate how it interacts with other management techniques such as herbicides and revegetation.

(Masi et al. 2014a, 2014b). Consequently, *P. semeniperda* infection has been linked to how quickly host seeds capitalize on their seed reserves (Beckstead et al. 2007). Seed embryos that germinate quickly can effectively outcompete *P. semeniperda* for starch resources within the ovary, while slow-germinating and dormant seeds are more susceptible to mortality. Thus, *P. semeniperda* infection may be dictated by seed weight, as heavier seeds will have more starch resources within the ovary.

Pyrenophora semeniperda infection varies by temperature (Campbell et al. 2003; Campbell and Medd 2003). The rate of *P. semeniperda* sporulation was highest at 23/19 C (12-h light/12-h dark photoperiods) in a laboratory setting (Campbell et al. 2003).

Hydrothermal time models have been used to understand the effects of temperature and water potential on the disease development of *P. semeniperda* (Barth et al. 2015) as well as seed dormancy status and germination potential of *B. tectorum* (Hawkins et al. 2017; Meyer and Allen 2009). Differences among *B. tectorum* populations have been considered in previous *P. semeniperda* work, including studies on water potential and dormancy (Finch et al. 2013). Compared with light-seeded species, heavy-seeded species are less vulnerable to fungal pathogens (Crist and Friese 1993), have higher germination and emergence, and produce larger and more vigorous seedlings (Black 1956; Harper and Obeid 1967; Schaal 1980). Seed weight as it relates to available seed reserves may influence *P. semeniperda* infection on *B. tectorum*. Thus, our objective was to gain a preliminary understanding of the effect of *P. semeniperda* on three different *B. tectorum* populations with different mean seed weights across varying temperatures.

Materials and Methods

Bromus tectorum Seed Locations

Bromus tectorum seeds were collected in August 2015 from three locations (range, crop, subalpine) that had established *B. tectorum* infestations (Table 1). The rangeland location was 26 km northwest of Dillon, MT. The cropping location was at Montana State University's Arthur H. Post Agronomy Research Farm, 8.8 km west of Bozeman, MT. The subalpine location was 10.4 km northwest of Norris, MT. To determine differences in mean seed weight across locations, three samples of 100 seeds from each seed location were dried (50 C for 72 h) (ISTA 2018) and weighed to the nearest 0.01 g.

Pyrenophora semeniperda Inoculum Preparation

An isolate of *P. semeniperda* was obtained from a sagebrush steppe system 17.4 km southwest of Nephi, UT (39.613524°N, 112.002022°W) (S Meyer, personal communication) in the form of air-dried conidia. Air-dried conidia were placed on petri dishes containing modified alphacel medium (Stewart 2009). Four 40-W cool-white and four 40-W black light fluorescent tubes were positioned 40 cm above the petri dishes to provide a 12-h near-visible ultraviolet (320 to 420 nm) photoperiod, and the petri dishes were maintained at room temperature (20 C). The petri dishes were checked for contaminants every 2 to 3 d, and an X-Acto® knife dipped in ethanol and flame sterilized was used to remove bacterial contaminants. After 12 d, conidia were harvested from petri dishes by rinsing the surfaces with 5 ml of sterile deionized water and gently scraping them with a rubber-tipped glass stirring rod. Additional water was used, as needed, and the conidia suspension was poured into a sterile glass jar. A hemocytometer was used to quantify conidia concentration, and sterile deionized water was added until a 5,000 conidia ml^{-1} concentration was achieved for the inoculum (Beckstead et al. 2007). Inoculum was stored at 5 C until seed inoculation, approximately 60 d after preparation.

Experimental Design

The experiment was conducted on a thermo-gradient table at Montana State University that had six parallel aluminum plates (101.6-cm long by 15.2-cm wide by 1.0-cm thick). Temperature was controlled with a 200-W fluid-loop chiller and a 260-W fluid heater at opposite ends of the table, resulting in a 19 C temperature gradient. The experiment consisted of two *P. semeniperda* treatments (inoculated, noninoculated [control]), three *B. tectorum* seed locations (range, crop, subalpine), and four table temperatures (13.0, 17.2, 20.9, and 24.6 C [± 0.1 C]). All treatments were replicated six times in a randomized block design. Due to the size of the thermo-gradient table, three replicates (1 to 3) were conducted from October 23, 2015, to November 22, 2015 (30 d), and another three replicates (4 to 6) were conducted from January 21, 2016, to February 20, 2016 (30 d).

Pyrenophora semeniperda Inoculum Application and Data Collection

Before inoculation, all *B. tectorum* seeds were surface sterilized by submerging them for 60 s in 70% ethanol, 60 s in 10% bleach, 60 s in 70% ethanol, and rinsing with sterile deionized water for

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Table 1. Information for the range, crop, and subalpine *Bromus tectorum* seed locations used in this study.

Seed location	Description	Elevation m	Coordinates	Soil[a]	Precipitation[a] mm	Mean annual temperature[b] C
Range	Hogsback Ranch	1,444	45.7570°N, 111.7953°W	Nuley clay loam (fine-loamy, mixed, superactive, frigid Calcidic Argiustolls); 0–18 cm clay loam; 18–38 cm clay loam	337	7.4
Crop	Montana State University Arthur H. Post Agronomy Research Farm	1,432	45.6775°N, 111.1560°W	Amsterdam–Quagle silt loam (fine-silty, mixed, superactive, frigid Typic Haplustolls); 0–20 cm silt loam; 20–38 cm silt loam	410	4.7
Subalpine[c]	Preacher Creek in Tobacco Root Mountains[c]	1,770	45.5950°N, 111.8310°W	Branham Rock Outcrop complex (coarse-loamy, mixed, superactive Ustic Haplocryolls); 0–10 cm coarse sandy loam; 10–30 cm gravelly coarse sandy loam	450	5.2

[a] USDA-NRCS 2016.
[b] WRCC 2016.
[c] Information on subalpine seed location obtained from Seipel et al. (2016).

30 s (Stewart 2009). *Bromus tectorum* seed inoculation with *P. semeniperda* was conducted by placing five seeds in a petri dish (35 by 11 mm) with 1 ml inoculum, and control seeds received an equivalent amount of sterile deionized water. All seeds were placed on a shaker table for 14 h at 50 rpm to allow for adsorption of *P. semeniperda* inoculum.

Five inoculated and five control *B. tectorum* seeds from each seed location were placed in individual petri dishes containing autoclaved filter paper (32-mm diameter) that was wetted with 1 ml of sterile deionized water. Petri dishes were sealed with Parafilm® (Bemis, Oshkosh, WI) to prevent the filter paper from drying out. Filter paper was rewetted with 1 ml of sterile deionized water as needed. *Pyrenophora semeniperda* infection and *B. tectorum* germination were recorded at day 30. Infection was based on the presence of *P. semeniperda* stromata (i.e., black finger-like appendages) emerging from the seed, and seeds were considered germinated if a radicle greater than 1 mm was present.

Statistical Analysis

Three ANOVAs were performed. First, an ANOVA was conducted to evaluate whether seed weight differed among *B. tectorum* seed locations, with seed location (range, crop, subalpine) as the model predictor and seed weight as the response variable. Second, an ANOVA was performed to evaluate whether *P. semeniperda* infection at day 30 was influenced by *B. tectorum* seed location and temperature, using a subset of data of only the inoculated treatment, as there was no infection present in the control. *Bromus tectorum* seed location, temperature, and their interaction were predictor variables, and the response variable was infection counts, which were logit transformed to meet assumptions of normality. Replicates were included as the error term. Third, an ANOVA was performed to evaluate whether *B. tectorum* germination at day 30 was affected by seed location, temperature, *P. semeniperda*, and their interactions. The response variable was germination counts, which were logit transformed to meet assumptions of normality. Replicates were included as the error term. All analyses were performed using R software (R Core Team 2017). When significant models were found, means were separated using least squared means (LSD) ($\alpha < 0.05$).

Results and Discussion

Bromus tectorum Seed Weight

Seed weight differed among *B. tectorum* seed locations ($P < 0.01$). The subalpine seed location had the lowest 100 seed weight (mean ± SE, 191.7 ± 4.3 mg), while the crop seed location had the highest seed weight (299.3 ± 2.2 mg). The range seed location had an intermediate seed weight (224.3 ± 1.5 mg).

Pyrenophora semeniperda Infection

Differences in *P. semeniperda* infection rates varied by seed location ($P < 0.001$; Table 2) and temperature ($P < 0.001$; Table 2). Averaged across temperatures, the heavier *B. tectorum* seeds from the crop seed location were less susceptible to *P. semeniperda* infection (40.0 ± 4.8%). The range and subalpine seed locations had higher and similar infection rates (65.0 ± 4.4% and 69.2 ± 6.1%, respectively). Averaged across seed locations, infection rates increased from 13 C (41.1 ± 4.7%) to 17 C (51.1 ± 6.5%) and was higher at 25 C (73.3 ± 6.0%). The infection rate at 21 C was intermediate (66.7 ± 6.7%).

Our results indicate that differences in *P. semeniperda* infection rates among *B. tectorum* seed locations did not vary by temperature ($P = 0.274$; Table 2). Instead, seed from different locations responded similarly across temperatures, and infection across seed locations was highest at 21 and 25 C (intermediate but relatively warm soil temperatures of 70 and 77 F).

Our experimental design did not allow us to isolate genetic, environmental, and/or physiological effects or their interactions on the response of *B. tectorum* seed from different locations to temperature and *P. semeniperda*. Despite this, we found that *B. tectorum* seed weight was correlated with *P. semeniperda* infection. Heavier seeds (i.e., from the crop seed location) were less susceptible to *P. semeniperda* infection compared with lightweight seeds (i.e., from the range and subalpine seed locations). These results suggest that differences in intraspecific seed weights across populations could have consequences for *P. semeniperda* infection and its application for biological control of *B. tectorum*. Heavier *B. tectorum* seed weight improved survival and performance in a field study conducted by Leger et al. (2009), who also concluded

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Table 2. P-values from an ANOVA to test for the effects of *Bromus tectorum* seed location, temperature, and replicates on seed infection by *Pyrenophora semeniperda*.

Parameter	df	P
Seed location	2, 55	<0.001
Temperature	3, 55	<0.001
Seed location × temperature	6, 55	0.274
Replicates	5, 55	0.680

Table 3. P-values from an ANOVA to test for the effects of *Bromus tectorum* seed location, temperature, *Pyrenophora semeniperda*, and replicates on seed germination.

Parameter	df	P
Seed location	2, 115	<0.001
Temperature	3, 115	0.019
P. semeniperda	1, 115	0.056
Seed Location × temperature	6, 115	0.720
Seed Location × *P. semeniperda*	2, 115	0.702
P. semeniperda × temperature	3, 115	0.144
Seed Location × *P. semeniperda* × temperature	6, 115	0.242
Replicates	5, 115	0.047

that seed size was inherited. Thus, heavier *B. tectorum* seeds may be more resilient to the phytotoxic secondary metabolites produced by *P. semeniperda* (Masi et al. 2014a, 2014b). Our results, combined with this previous research, suggest that seed weight may act as a simple measure of *B. tectorum* seed susceptibility to *P. semeniperda*, thus allowing field practitioners a relatively easy way to assess whether *P. semeniperda* may contribute to their site-specific management strategies.

Our results demonstrate that temperature influences susceptibility of *B. tectorum* seeds to *P. semeniperda* infection. The temperature optima of *P. semeniperda* and *B. tectorum* may overlap (Elton 2001) in such a way that *B. tectorum* exists in a stressed state and *P. semeniperda* can flourish. The experimental temperatures we tested (13 to 25 C) are within the range of soil temperatures *B. tectorum* experiences during the fall months in Montana, such as September and October, when soil temperatures in 2016 ranged from 7 to 24 C (Western Regional Climate Center, Central Agricultural Research Center station, Moccasin, MT). *Bromus tectorum* seeds generally germinate on or near the soil surface (<2 cm) in the fall months, and fewer seedlings emerge as burial depth increases from 2 to 10 cm (Hulbert 1955). *Pyrenophora semeniperda* infection would be felt by dormant seeds at similar soil depth throughout the winter and into the spring. Seed burial greater than 2 cm is likely to occur in cropping systems that use tillage. Consequently, *B. tectorum* seeds in cropping systems would experience lower soil temperatures and lower *P. semeniperda* infection. Using *P. semeniperda* for *B. tectorum* control may be possible, provided further research pinpoints site-specific environmental conditions that allow *P. semeniperda* to flourish and gain control of *B. tectorum* infestations. Understanding interactions between abiotic (temperature) and biotic (*B. tectorum* seed location) factors could give field practitioners the opportunity to align *B. tectorum* management strategies like herbicide applications with *P. semeniperda* outbreaks to maximize *B. tectorum* control.

Bromus tectorum Germination

Differences in germination rates varied by seed location ($P < 0.001$; Table 3) and temperature ($P = 0.019$; Table 3). Averaged across temperature and *P. semeniperda* treatment, the seed from the range location had the lowest germination rate (5.8 ± 1.9%), increasing for seed from the subalpine and crop locations (28.8 ± 3.8% and 45.4 ± 4.2%, respectively). Unlike infection, germination was similar and lowest at 21 C (22.8 ± 4.4%) and 25 C (19.4 ± 4.6%) and higher at 13 C (30.6 ± 5.0%) and 17 C (33.9 ± 4.8%), averaged across seed location and *P. semeniperda* treatment. Inoculation with *P. semeniperda* had a marginal effect on germination ($P = 0.056$; Table 3), decreasing germination (23.1 ± 3.0%) compared with the noninoculated control (30.3 ± 3.6%). Replicates had a small effect on germination ($P = 0.047$; Table 3), with low germination in Replicate 4 (15.8 ± 4.8%) compared with Replicates 1 and 3 (33.3 ± 7.1% and 35.0 ± 6.6%, respectively)—all other replicates had intermediate levels of germination.

Germination differed by seed location and was particularly low for seed from the range location. Lower *B. tectorum* germination is not unheard of under laboratory and greenhouse conditions when investigating contrasting habitats. Recently harvested *B. tectorum* seeds incubated at varying temperatures had 10% to 90% germination across seeds collected from three semiarid habitats (Allen et al. 1995). Slow germination was also a characteristic among recently harvested *B. tectorum* seeds from five populations (Beckstead et al. 1996). These studies and Allen and Meyer (2002) demonstrate that *B. tectorum* germination increases under laboratory and greenhouse conditions as time in storage (20 C) increases. Again, our experimental design did not allow us to isolate genetic, environmental, and physiological effects or their interactions on the response of *B. tectorum* from three different locations to temperature and *P. semeniperda*. However, these studies suggest that germination responses in *B. tectorum* habitats (i.e., seed locations) might have both genetic and environmental components (Beckstead et al. 1996).

Temperature is a significant factor in the rate of *P. semeniperda* growth, sporulation, and aggressiveness and the rate of *B. tectorum* seed germination after being inoculated with *P. semeniperda*. In our experiment, temperature influenced *P. semeniperda* infection and *B. tectorum* germination. Laboratory research has found an optimal temperature for *P. semeniperda* growth to be 23 C (Campbell et al. 2003), but the temperature at which *P. semeniperda* loses efficacy has not been reported. *Bromus tectorum* can germinate at just above freezing (5 C) (Evans and Young 1972), but germination is inhibited above 30 C (Harris 1976). Thus, testing more temperatures below 25 C may further clarify the relationship between *P. semeniperda* and *B. tectorum*.

The development of alternative management strategies for *B. tectorum* that incorporate biological control with *P. semeniperda* has been underway for some time (Beckstead et al. 2007, 2010; Ehlert 2013; Ehlert et al. 2014; Meyer et al. 2007, 2008a, 2008b). Further advancement is needed as climate change affects *B. tectorum* distribution (Bradley 2009; Bradley et al. 2010; Concilio et al. 2013; Taylor et al. 2014; Zelikova et al. 2013) and the efficacy of current control methods (Hellmann et al. 2008). Our results indicate that *P. semeniperda* infection increased and *B. tectorum* germination decreased with increasing temperature, and *B. tectorum* seed from different locations experienced varying levels of infection and germination. However, our conclusions are limited because of the single inoculum source and lack of replication across seed locations. Despite these limitations, this study provides further insight into temperature optima of *P. semeniperda* infection and consequent effects on *B. tectorum* and supplies

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information on how a simple measure (i.e., seed weight) can serve as a starting point for elucidating whether or not a location infested with *B. tectorum* is likely to experience *P. semeniperda* infection.

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