Exposure to Low Water Potentials and Seed Dormancy Favour the Fungus in the *Pyrenophora semeniperda-Bromus tectorum* Pathosystem

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Abstract

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In semi-arid regions of the United States, *Pyrenophora semeniperda* kills seeds of the winter annual *Bromus tectorum*. We report on pathosystem outcomes under manipulated water potential and temperature environments commonly observed within semi-arid environments for dormant and non-dormant seeds. We propose a range of outcomes for infected seeds. During summer, seeds remain dormant and are killed across a range of water potentials. During autumn, seeds survive by rapidly germinating or are killed if radicle emergence is delayed by intermittent hydration. In winter/spring, secondarily dormant seeds can be killed by the fungus. The only likely scenarios where seeds escape death include absence of infection (autumn, spring, or following autumn, germination) or infection in autumn when seeds germinate rapidly.

Keywords: competitive outcomes; desiccation tolerance; hydration; germination; infection; seed fungi

Bromus tectorum L. is an annual grass native to Eurasia. This plant is invasive in many parts of North America, particularly in semi-arid habitats of the Western United States. *Bromus tectorum* is a prolific seed producer (Smith *et al.* 2008). Seeds ripen in early summer and exhibit dormancy (primary dormancy) upon maturation. This dormancy is lost through dry storage (after-ripening) and seed populations become non-dormant by the onset of autumn. Seeds can then germinate in response to autumn rains, postpone germination until winter or spring (i.e. become secondarily dormant), or carry over across years in the soil seed bank.

Pyrenophora semeniperda (anamorph *Drechlera campanulata*) is an ascomycete seed pathogen with multiple hosts and is common and widespread throughout the United States, Canada, Argentina, Australia, New Zealand, South Africa (Medd *et al.* 2003), and Eurasia (Stewart *et al.* 2009). While relatively little is known about seed bank pathogens in natural systems (Beckstead *et al.* 2010), some knowledge exists regarding the seed bank pathogen *P. semeniperda*. *Pyrenophora semeniperda* is a major cause of *in situ* seed mortality in *B. tectorum* (Meyer *et al.* 2007; Allen & Meyer 2013). Following infection, death due to the pathogen is directly related to the speed of seed germination; rapidly germinating seeds escape while slow germinating seeds are killed (Beckstead *et al.* 2007; Finch *et al.* 2013). Conditions that inhibit germination (e.g., seed dormancy, unfavourable temperatures or insufficient water) should favour the fungus as long as disease development is not similarly inhibited by these same conditions.

Because interactions between *P. semeniperda* and *B. tectorum* occur in environments characterised by intermittent and often unpredictable precipitation, it is critical to study this pathosystem in the context of wide fluctuations in water availability. *Bromus tectorum* seeds have been studied over a

wide range of water potentials (< –400 to 0 MPa), resulting in several ecologically relevant findings. For example, the level of primary dormancy decreased in response to water stress imposed during seed maturation (Meyer & Allen 1999), while loss of primary dormancy through dry after-ripening was progressively inhibited at water potentials below –150 MPa (Bair *et al.* 2006). The inhibitory effects of low water potentials on *B. tectorum* seed germination have also been characterised in the context of hydrothermal studies, leading to successful predictive models for germination under both laboratory and field conditions (e.g. Christensen *et al.* 1996; Bauer *et al.* 1998; Meyer & Allen 2009).

The overall aim of this study was to characterise the *P. semeniperda*–*B. tectorum* pathosystem under a variety of hydric environments previously shown to be important to *B. tectorum* seeds. We conducted experiments and collected data on competitive outcomes (i.e. seed death or seed escape) in answering each of the following six questions: (1) How long does it take for inoculated seeds to be killed across a range of water potentials? (2) How does seed dormancy influence competitive outcomes? (3) Under what constant water potential environments will seeds be killed by the fungus and, conversely, under what conditions will seeds escape? (4) How does time in free water followed by drying at different water potentials influence competitive outcomes? (5) Following infection in hydrated seeds, does *P. semeniperda* exhibit desiccation tolerance similar to the desiccation tolerance of hydrated *B. tectorum* seeds? (6) Does amount of time spent at low (germination-inhibiting) water potentials influence competitive outcomes in this pathosystem? We review results from our published study (Finch *et al.* 2013) along with results of recently completed experiments to address these questions.

MATERIAL AND METHODS

Bromus tectorum seeds for all studies were collected from a wild population at the Brigham Young University Research Farm (Spanish Fork, Utah, USA). Prior to use, seeds were cleaned by hand and either stored in a –10°C freezer to preserve primary dormancy or allowed to after-ripen under laboratory conditions until seeds became non-dormant. The *Pyrenophora semeniperda* inoculum originated as a moderately virulent strain collected from Whiterocks, Utah, USA, and was produced as described by Meyer *et al.* (2010). Prior to imbibition, seeds were inoculated with a 1:100 (w/w) spore:talc mixture by placing seeds and an excess of inoculum in a test tube vial and shaking for 30 seconds.

Scanning electron microscopy (SEM) was used to visually assess the timing of *P. semeniperda* spore germination and mycelial penetration through the wall of the caryopsis, as well as to observe disease development. Following inoculation and an appropriate incubation time period, samples were chemically fixed with 2% glutaraldehyde solution buffered with sodium cacodylate to pH 7.3, followed by 1% osmium (OsO_4) solution buffered with sodium cacodylate to pH 7.3, then dehydrated with a series of acetone solutions. Samples were critically point dried and coated with gold/palladium before evaluation with the electron microscope.

For all experiments, inoculated seeds (either two replications of 50 seeds or four replications of 25 seeds) were imbibed in covered Petri dishes on the surface of two blue germination blotters (Anchor Paper, St. Paul, USA) that had been saturated to excess with water (i.e., free water, 0 MPa) or solutions of Polyethylene glycol 6000 (Michel & Kaufmann 1972) to achieve controlled low water potentials (–0.5 to –2 MPa). Where lower water potentials were needed (–4 to –150 MPa), seeds were placed in sealed containers above saturated salt solutions as described by Allen *et al.* (1992). A variety of continuous hydration or hydration-dehydration-rehydration treatment combinations were used in this study. In some cases, temperature was included as a treatment variable. Specific details are indicated in the results and discussion section.

RESULTS AND DISCUSSION

Additional research using scanning electron microscopy (SEM) has indicated that on inoculated seeds incubated at 20°C (near-optimum temperature for both seed germination and disease development), visible spore germination occurred within 6 h and mycelial penetration into the seed occurred within 24 h (not shown). Fungal stromata (evidence of seed death) appeared beginning on day 11 (Figure 1A), by which time the endosperm had largely been depleted by the fungus (Figure 1B).

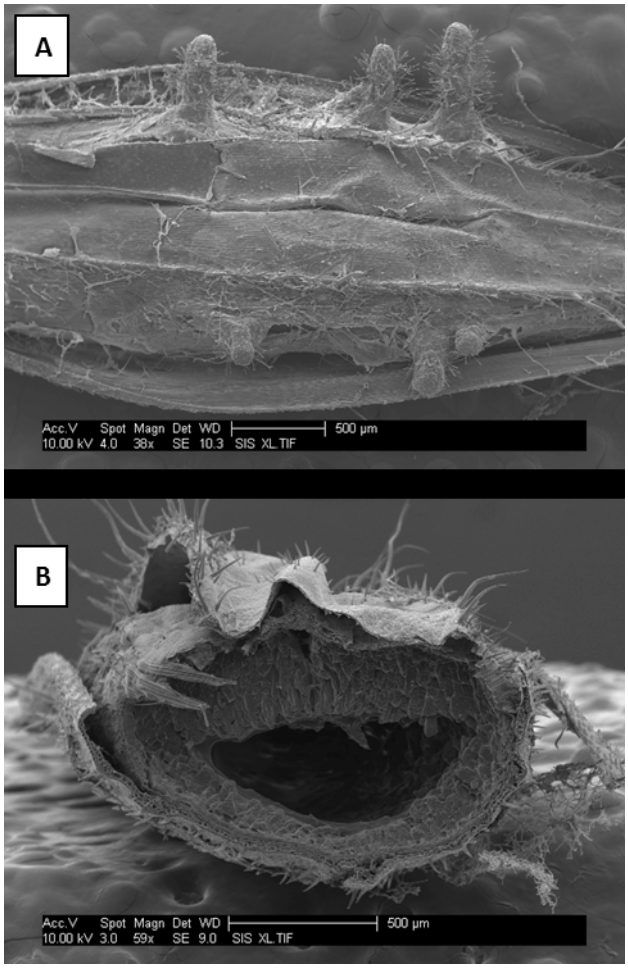

Figure 1. SEM micrographs of dormant *Bromus tectorum* seeds killed by *Pyrenophora semeniperda*. (A) Emergence of finger-like stromata is evidence of seed death. With continuous hydration of inoculated seeds at 20°C, stromata were observed beginning 11 days following inoculation; (B) The endosperm was consumed during disease development, leaving an open cavity period

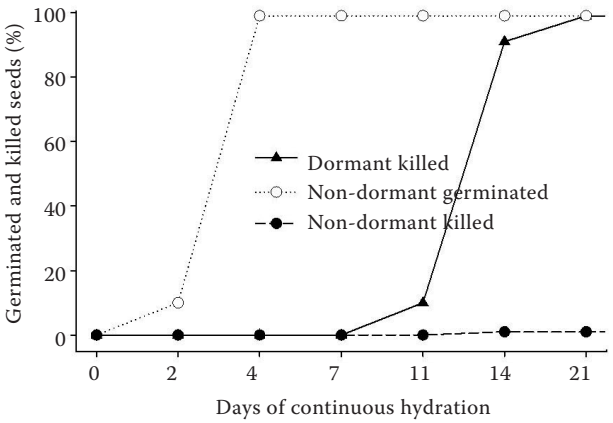

Figure 2. Outcomes for dormant and non-dormant seeds following inoculation with *Pyrenophora semeniperda* and incubation at 20°C in water. Non-dormant seeds germinated in 2–4 days and escaped mortality. All dormant seeds were killed period

All SEM work was carried out with dormant seeds, as most non-dormant seeds germinated within 2–4 days (Figure 2).

Nearly all dormant seeds were killed by *P. semeniperda* when continuously hydrated at pretreatment water potentials ranging from 0 to –2 MPa (Figures 2 and 3). The only exception was when seeds were hydrated at 5°C, where less than 10% of dormant seeds were killed (Finch *et al.* 2013). For non-dormant seeds, incubation at water potentials below –0.5 MPa resulted in a greater fraction of killed seeds (Figure 3). Rapid stromatal production, shortly before or shortly following transfer to water, indicates that disease development occurred during incubation at the two lowest water potentials (–1.5 and –2 MPa). Prior to transfer to water, disease development at less negative water potentials was markedly reduced. Less than 15% of non-dormant seeds were killed with incubation at –1 MPa, and no seeds were killed at –0.5 MPa.

Results in Figure 3 are for non-dormant seeds incubated for four weeks at low water potential pretreatments (20°C) followed by transfer to water. With shorter durations at low water potentials, fewer non-dormant seeds were killed as a progressively greater fraction escaped through germination (Finch *et al.* 2013). Similarly, reducing the incubation temperature resulted in progressively lower seed mortality, until at 5°C no non-dormant seeds were killed by the fungus with any duration of low water potential pretreatment (Finch *et al.* 2013).

These results indicate that the question of how long it takes for *P. semeniperda* to kill non-dormant *B. tectorum* seeds is clearly relative to the specific treatment conditions applied. It may take several weeks. However, because seed germination in semi-arid habitats may be associated with one to several periods of insufficient soil moisture to complete radicle emergence (Meyer & Allen 2009), it is plausible that non-dormant seeds in *B. tectorum* seed banks may suffer significant mortality in the field (Allen & Meyer 2013).

Many seeds tolerate periods of imbibition followed by drying. Indeed, desiccation tolerance of imbibed seeds provides the foundation for the commercial agricultural practice of seed priming

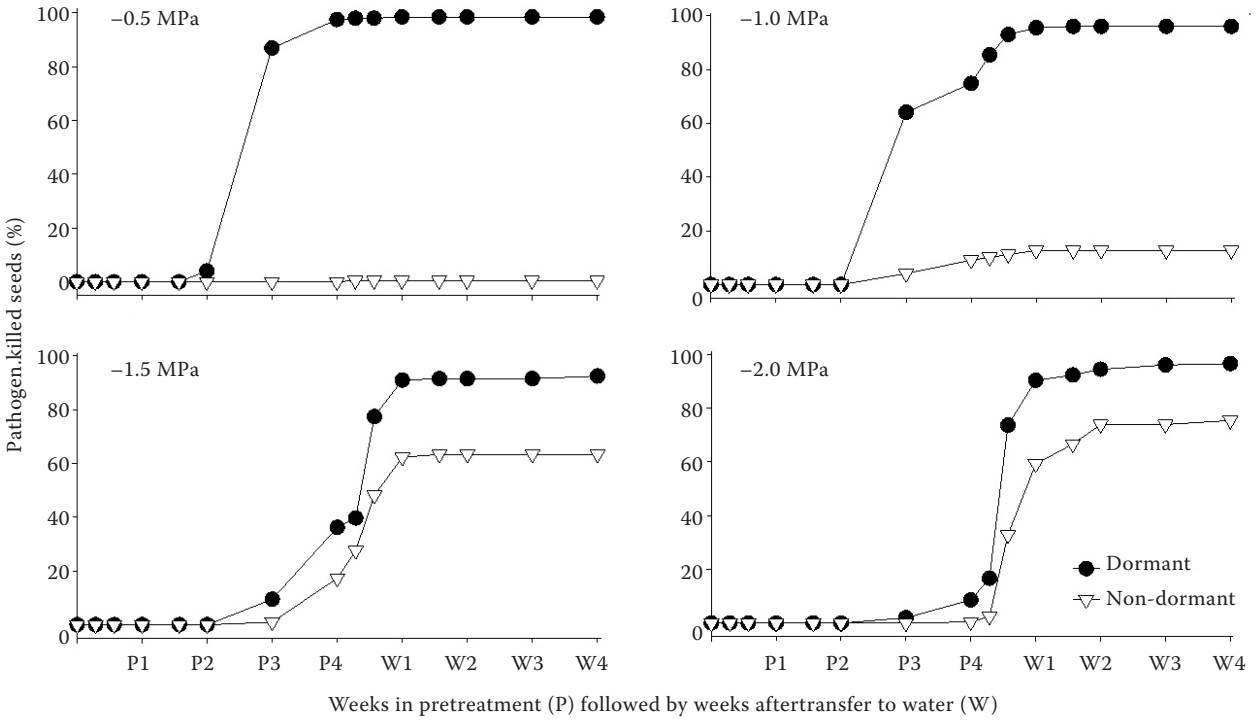

Figure 3. Mortality of *Bromus tectorum* seeds from a single population incubated in the presence of *Pyrenophora semeniperda* at 20°C. Seeds were either dormant or non-dormant as indicated, and were subjected to the low water potential pretreatments indicated for four weeks before transfer to water period

(i.e., hydrating seeds until shortly before radicle emergence occurs and then drying them in order to promote accelerated germination) (Taylor *et al.* 1998), and seeds may accumulate progress toward germination over a series of "hydration-dehydration" cycles (e.g., Allen *et al.* 1993). Mycelium of *P. semeniperda* is certainly desiccation tolerant; mycelial cultures can readily be resuscitated even after extended periods in the air-dry state (Meyer unpublished data). However, we have not previously investigated how dehydration influences outcomes in the *P. semeniperda–B. tectorum* pathosystem.

We therefore subjected inoculated non-dormant seeds to hydration in free water (0 MPa) for short (i.e., 8 h, at which point the phase of rapid water uptake characterised by physical imbibition was just completed) or long (i.e., 24 h, until just prior to radicle emergence) periods before drying (–4 to –150 MPa for 1–21 days) followed by rehydration. In this experiment, the longer imbibition period prior to drying resulted in increased seed mortality (Figure 4). When seeds were imbibed for only 8 h, high mortality occurred only at –4 MPa. With this treatment, drying for a period of 14 or 21 days resulted in near complete seed mortality. Drying at lower water potentials resulted in near 0% (–40 and –150 MPa) to 50% (–10 MPa for 14 days) mortality. In contrast, seeds hydrated for 24 h showed a trend toward increased mortality with increased drying duration at all low water potentials.

Pyrenophora semeniperda spores require approximately 6–8 h of hydration in free water to germinate, and mycelium had not penetrated the seed by the time drying was initiated in the 8-h imbibition treatment. Still, infection and disease development continued when short imbibition was followed by drying at –4 MPa. Taken together with results from previous experiments, this shows that *P. semeniperda* is capable of disease development at water potentials from 0 to –4 MPa. These lower water potentials are above the range of water potentials where agriculturally important storage fungi operate (Jayaraman *et al.* 2011).

Based on our results, we have proposed the range of likely outcomes for *B. tectorum* seeds that become infected by *P. semeniperda* at different times during the year (Finch *et al.* 2013). If it rains, seeds can become infected during summer, autumn, or later. With summer infection, seeds are still in a state of primary dormancy and are likely killed across a range of water potentials. This is true whether seeds are continuously

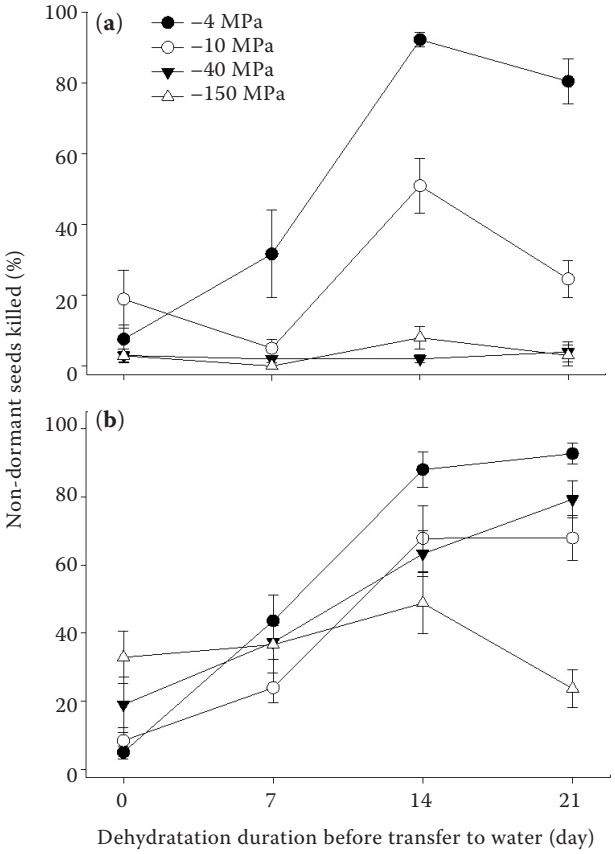

Figure 4. Mortality of non-dormant *Bromus tectorum* seeds inoculated with *Pyrenophora semeniperda* and subjected to imbibition-dehydration-rehydration treatments. Seeds were imbibed for (**a**) 8 h or (**b**) 24 h as indicated, dehydrated at one of four water potentials for 1–21 days, then rehydrated for 28 days. Temperature was 20°C throughout. Error bars indicate the standard error of the mean

hydrated (unlikely during summer in semi-arid ecosystems) or subjected to wetting and drying soils. With autumn infection, seeds escape death through rapid germination or are killed if radicle emergence is delayed by intermittent hydration. In late autumn through early spring, seeds may become secondarily dormant (Finch *et al.* 2013; Hawkins *et al.* 2013). These seeds are also highly vulnerable to the fungus.

In conclusion, there are only a few scenarios where seeds escape death caused by the fungus. The first escape scenario occurs at low inoculum loads, which our field data suggest has a highly likelihood of occurrence (Allen & Meyer 2013). The second escape scenario occurs when previously non-infected seeds become infected under conditions of adequate moisture for complete germination. In this case they escape death through germination that occurs faster than disease development. *Pyrenophora semeniperda* is ideally suited to infect *B. tectorum* seeds in semi-arid habitats. Both primary and secondary dormancy render seeds vulnerable to attack during the year. In addition, the ability for *P. semeniperda* to infect and carry out disease development across a range of water potentials, especially low or variable water potentials that inhibit rapid germination of even non-dormant seeds, provides a competitive advantage under many environments that are likely to occur in the field.

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